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Summary

Worldwide, metal-contaminated soils pose a serious threat to environmental health. High metal concentrations are toxic to most organisms. From soil, metals can leach into the groundwater or accumulate in plants and thus in multiple food chains. The remediation of metal-contaminated soils is urgent. Vast areas with diffuse metal contamination cannot be remediated using conventional techniques (*e.g.* excavation). In this work, we focus on the natural ability of plants and their associated microorganisms to remove trace elements from soils by concentrating them in the harvestable plant parts (*i.e.* phytoextraction). The main constraints limiting full-scale application of this technique using economically attractive high-biomass accumulator plants are low metal availability in the soil and low metal uptake, translocation, accumulation and tolerance of plants. It is known that plant-associated bacteria with metal-mobilizing and plant growth-promoting properties and a natural capacity to cope with trace elements can be exploited to increase plant biomass and metal tolerance on the one hand and metal uptake on the other hand. The enrichment of such bacteria in high-biomass accumulator plant species might result in a more efficient phytoextraction of trace elements.

Since we are interested in using the oil-producing energy crop *Brassica napus* L. (rapeseed) as phytoextractor, a sampling and isolation workflow was developed to accomplish a representative genotypic and phenotypic characterization of the cultivable bacterial communities associated with field-grown rapeseed (**Chapter 3**). Reliable data were obtained using 3 root, stem or leaf replicates. Correspondence analysis can be used to test the variability between replicates and their correlation with the mean. Based on these correlation coefficients, the representativeness of the mean dataset can be estimated.

In order to find interesting rapeseed-associated bacteria to improve Cd phytoextraction efficiency, cultivable bacterial strains associated with field-grown rapeseed (soil, rhizosphere and roots) from a trace elements (Cd, Zn and Pb) contaminated field and a non-contaminated control field were genotypically and phenotypically characterized using the optimized workflow (**Chapter 4**). To elucidate possible effects of site-specific conditions and seasons on cultivable bacterial communities, isolations were performed in December and June

(**Chapter 5**). Root communities were similar between fields and seasons, whereas bulk and rhizosphere soil communities were susceptible to changing seasonal and field conditions. Bacterial diversity and Cd tolerance were found to be higher in June; the highest percentages of Cd-tolerant strains were consistently found at the field site contaminated with Cd, Zn and Pb. Furthermore, we observed the presence of some dominating strains associated with rapeseed*,* which are hardly affected by external factors and might be transferred from one generation to the next via seeds. Strains that are present during the whole growing season are considered to be the most promising to improve plant growth and Cd uptake.

From all isolated bacterial strains, 63 were selected based on their *in vitro* screening for plant growth-promoting capacity (*i.e.* phosphate solubilization, nitrogen fixation and production of siderophores, organic acids, indole-3-acetic acid, acetoin and 1-aminocyclopropane-1-carboxylate deaminase) and Cd tolerance (**Chapter 6**). Strains with the potential to improve plant growth and/or Cd uptake were tested *in planta* using agar plates. The best performing strains on agar were selected for inoculation experiments on sand and field soil. In the end, the remaining 5 strains (4 *Pseudomonas* sp. and 1 *Variovorax* sp.), all isolated from roots or rhizosphere of rapeseed grown at the contaminated field, were tested *in planta* in the field (*i.e*. *in situ*).

During the field inoculation experiment (**Chapter 7**), 4 out of the 5 strains significantly increased root dry weight. None of the strains affected metal uptake by *B. napus* in the field, although one of them significantly increased metal availability by decreasing soil pH. The seed endophyte (UH1), originating from the contaminated field, although showing highly promising *in vitro* and *in planta* characteristics, did not increase Cd phytoextraction efficiency in the field.

These results demonstrate that a thorough *in vitro* and *in planta* screening of bacterial strains is no guarantee for a successful application in a more complex field situation. In an optimized experimental set-up for the selection of rapeseed-associated bacterial strains, the *in vitro* screening should be extended with bacterial colonization properties and the bacterial ability to decrease soil pH. In this way, a more representative first selection is allowed and therefore rendering the screening experiments using agar plates redundant. On sand, the

most relevant bacterial performance results will be obtained while working with appropriate nutrient concentrations. Before inoculation in the field, strains should ideally be tested on potted field soil exposed to fluctuations in environmental conditions since they can considerably influence the metabolic activity of plant roots, the native plant-associated bacterial communities and the inoculated strain(s). Differences in soil temperature between growth chamber/greenhouse and field might be an important factor responsible for the observed discrepancies between the inoculation effects in the growth chamber on potted field soil and in the field.

Despite the need for optimization efforts during the inoculation experiments, the obtained data demonstrate that bacterial-assisted phytoextraction using rapeseed on moderately Cd-contaminated soils is promising. A big challenge still is to find the optimal conditions for bacterial performance.

Samenvatting

Metaalverontreinigde bodems vormen een wereldwijd milieuprobleem, aangezien verhoogde concentraties aan metalen zoals cadmium (Cd) toxisch zijn voor de meeste organismen. Metalen kunnen vanuit de bodem uitlogen naar het grondwater of accumuleren in planten en zo in verschillende voedselketens terechtkomen. Het remediëren van metaalverontreinigde bodems dringt zich op. Nochtans kunnen uitgestrekte gebieden die diffuus verontreinigd zijn met metalen niet geremedieerd worden met behulp van conventionele technieken zoals afgraving. Daarom ligt in dit werk de focus op het gebruik van planten en hun geassocieerde micro-organismen om metalen uit de bodem te extraheren en vervolgens te accumuleren in de oogstbare plantendelen (*i.e.* fyto-extractie). De voornaamste beperkingen die de grootschalige toepassing van deze techniek met behulp van hoge biomassa producerende accumulatoren in de weg staan, zijn de beperkte beschikbaarheid van metalen in de bodem, alsook een lage metaalopname, translocatie, accumulatie en tolerantie van planten. Plant-geassocieerde bacteriën die in staat zijn om metalen te mobiliseren en plantengroei te bevorderen, kunnen aangewend worden om biomassaproductie en metaalopname door de plant te verhogen. De verrijking van deze bacteriën kan resulteren in een efficiëntere fyto-extractie van metaalverontreinigde bodems.

In **Hoofdstuk 3** werd de staalname- en isolatieprocedure geoptimaliseerd om een representatieve genotypische en fenotypische karakterisatie te bekomen van de cultiveerbare bacteriële gemeenschappen geassocieerd met koolzaad in het veld. *Brassica napus* L. (koolzaad), een olie-producerend energiegewas, kan immers aangewend worden als fyto-extractor plant. Betrouwbare data werden bekomen met 3 herhalingen voor zowel wortel-, stengel- als bladstalen. Correspondentie analyse werd gebruikt om de variabiliteit tussen de herhalingen en hun correlatie met de gemiddelden na te gaan. Gebaseerd op de bekomen correlatiecoëfficiënten kan worden nagegaan hoe representatief de gemiddelde dataset is.

Om koolzaad-geassocieerde bacteriën te detecteren die in staat zijn om de fyto-extractie efficiëntie van Cd te verbeteren, werden stammen geïsoleerd uit

bodem-, rhizosfeer- en wortelstalen van koolzaad gegroeid op een niet-gecontamineerd controleveld en een verontreinigd veld met verhoogde concentraties aan Cd, zink (Zn) en lood (Pb). Deze stammen werden vervolgens genotypisch en fenotypisch gekarakteriseerd (**Hoofdstuk 4**). Om mogelijke effecten gerelateerd aan het veld en het seizoen op de cultiveerbare bacteriële gemeenschappen te bestuderen, werden zowel in december als in juni isolaties uitgevoerd (**Hoofdstuk 5**). De bacteriële gemeenschappen in de wortel waren gelijkaardig tussen velden en seizoenen, terwijl gemeenschappen in bodemstalen (bulk en rhizosfeer) gevoelig waren aan veranderingen qua seizoen en veldcondities. De bacteriële diversiteit en Cd-tolerantie was hoger in juni, terwijl de hoogste percentages aan Cd-tolerante stammen consistent werden terug gevonden in stalen afkomstig van het metaalverontreinigde veld. Verder werden dominante stammen, die nagenoeg niet beïnvloed werden door externe factoren, geobserveerd. Deze stammen worden verondersteld doorgegeven te worden van de ene naar de andere generatie via de zaden. Stammen die gedurende het volledige groeiseizoen aanwezig zijn, lijken het meest veelbelovend om de groei en Cd-opname van planten te verbeteren.

Uit alle geïsoleerde bacteriële stammen werden 63 stammen geselecteerd gebaseerd op hun *in vitro* karakteristieken met betrekking tot het stimuleren van plantengroei (*i.e.* fosfaatsolubilisatie, stikstoffixatie en de productie van sideroforen, organische zuren, indol-3-azijnzuur, acetoine en 1-aminocyclopropaan-1-carboxylaat deaminase) en Cd-tolerantie (**Hoofdstuk 6**). Deze stammen, die dus mogelijk in staat zijn om de groei en Cd-opname van planten te verbeteren, werden *in planta* getest op agarplaten. De beste stammen werden vervolgens geïnoculeerd op zand en bodem afkomstig van het metaalverontreinigd veld. Tenslotte werden de overblijvende 5 stammen (4 *Pseudomonas* soorten en 1 *Variovorax* soort), allen afkomstig van het gecontamineerde veld uit de wortels of de rhizosfeer van koolzaad, getest *in planta* in het veld (*i.e. in situ*).

Tijdens het inoculatie-experiment in het veld (**Hoofdstuk 7**) konden 4 van de 5 stammen het droog gewicht van de wortels verhogen. Geen enkele stam was in staat om de metaalopname door koolzaad te beïnvloeden. Nochtans kon één stam de metaalbeschikbaarheid in de bodem significant verhogen door de

bodem pH te verlagen. De zaadendofyt (UH1), afkomstig van het gecontamineerde veld en met veelbelovende *in vitro* en *in planta* eigenschappen, kon de Cd fyto-extractie efficiëntie in het veld echter niet verhogen.

De bekomen resultaten tonen aan dat een uitgebreide *in vitro* en *in planta* screening van bacteriële stammen geen garantie biedt voor een succesvolle toepassing in een meer complexe veldsituatie. In een geoptimaliseerde experimentele set-up voor de selectie van koolzaad-geassocieerde bacteriële stammen, moet de *in vitro* screening uitgebreid worden met bacteriële kolonisatie-eigenschappen en de bacteriële mogelijkheid de bodem pH te verlagen. Op deze manier kan een meer representatieve eerste selectie gemaakt worden, zodat het screenen op agarplaten overbodig wordt. Op zand zullen de meest relevante bacteriële effecten bekomen worden wanneer gewerkt wordt met geschikte nutriëntconcentraties. Alvorens te inoculeren in het veld zouden stammen idealiter getest moeten worden in een pot-experiment met bodem afkomstig van het veld en blootgesteld aan veranderende omgevingsfactoren. Deze schommelingen kunnen de metabole activiteit van de plantenwortels alsook de plant-geassocieerde bacteriële gemeenschappen en de geïnoculeerde stam(men) beïnvloeden. De verschillen in bodemtemperatuur tussen de groeikamer/serre en het veld zijn waarschijnlijk verantwoordelijk voor de geobserveerde verschillen tussen de effecten van inoculatie in de groeikamer met bodem afkomstig van het veld en deze in het veld zelf.

Ook al moeten inoculatie-experimenten geoptimaliseerd worden, toch tonen de bekomen data aan dat de aanrijking van veelbelovende plant-geassocieerde bacteriën tijdens Cd-fytoextractie met koolzaad een interessante piste is. Een grote uitdaging ligt in het optimaliseren van de condities die de prestaties van bacteriën maximaliseren.

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Chapter 1

Introduction

As a consequence of both increasing population and industrial technology, humanity has created a situation where many life forms, including humans, are increasingly at risk.

Problem outline

Since mankind is faced with the drawbacks of industrialization, society is looking for strategies to reduce environmental pollution and to remediate **historical pollution**. Mineral oil (38%), trace elements (37%) and polycyclic aromatic hydrocarbons (13%) are the most occuring soil contaminants in Europe (EEA, 2007). In this review, we focus on the natural ability of plants and their associated microorganisms to stabilize or remove trace elements from soils. In contrast to organic contaminants, trace elements cannot be degraded (phytotransformation) to non-hazardous compounds and must either be stabilized in the soil to make them less available (*i.e.* phytostabilization); extracted, transported, and accumulated in plant tissues (*i.e.* phytoextraction); or in specific cases (*e.g.* Hg, Se) transformed into volatile forms (*i.e.* phytovolatilization) (Pilon-Smits, 2005).

Considering the sites in the northeastern part of Belgium that are moderately contaminated with **Cd**, **phytoextraction** might be an effective green remediation technique. Although hyperaccumulator plants might seem the most obvious approach to realize a fast phytoextraction, high biomass-producing plants can provide important additional advantages, *e.g.* the produced biomass can be used as a bio-energy source making the long-term remediation process economically more attractive. Anyway, determining the right balance between high levels of metal uptake and high productivity remains a major challenge. Moreover, the most appropriate **remediation plant** depends strongly on the characteristics of the contaminated soil (Vangronsveld *et al*., 2009).

In the case of phytoremediation of metal-contaminated soils and (ground)water with high biomass-producing plants, low metal availability in the substrate and low metal uptake, translocation, accumulation and tolerance of plants are the major **constraints** still limiting full-scale application of phytoextraction. To improve the efficiency of phytoextraction, **plant-associated bacteria** with metal mobilizing and plant growth-promoting (PGP) properties and a natural capacity to cope with trace elements could be exploited (Weyens *et al*., 2009).

In this PhD, the use of ***Brassica napus*** **L.**, an oil-producing energy crop, with the help of its associated bacteria to phytoextract Cd is investigated.

Historical pollution

In this work, the metal-contaminated Campine region, a cross-border area in the northeastern part of Flanders (Belgium) and the southeastern part of the Netherlands (Belgium part see figure 1.1), is considered. At the end of the 19th century the metallurgic industry was attracted to this area by a combination of high quality transport infrastructure, low population densities and high unemployment rates. Until the mid 1970ies, Zn was refined at several locations in this area using a pyrometallurgical process. During this process Zn ores were heated up to 1400°C leading to volatilization of metals like Cd and Pb. Since they were not captured, their atmospheric deposition resulted in a moderately (historically) contaminated area of 700 km^2 in both Belgium and the Netherlands (Vangronsveld *et al*., 1995; Hogervorst *et al*., 2007). Electrolytic processes resulted in a drop of annual Cd emissions from 125,000 kg in 1950 to 130 kg in 1980. Since metals cannot be degraded and hence persist in the environment, metal contamination is one of the most severe environmental problems (Rajkumar *et al*., 2009).

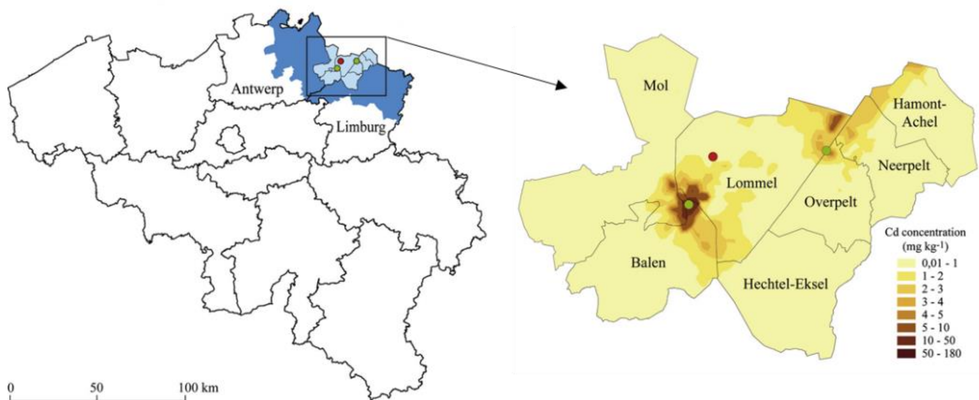

Figure 1.1 Location of the Belgian Campine region (shaded in dark blue) and the 7 most suffering municipalities.

Until 2003, excavation significantly lowered the contamination level around the dismantled (1904-1974) Lommel Maatheide smelter (red dot in figure 1.1). The highest Cd concentrations are found in the vicinity of the 2 still active non-ferro smelters in Balen and Overpelt (green dots in figure 1.1). The 7 municipalities surrounding these Zn smelters in the Belgian part of the Campine region suffer

the most from the contamination (Schreurs *et al.*, 2011); Balen and Mol in the province of Antwerp and Hamont-Achtel, Hechtel-Eksel, Lommel, Neerpelt and Overpelt in the province of Limburg. These municipalities cover an area of 494 km^2, counting more than 147,000 inhabitants. Soil contamination in the 7 municipalities is mainly diffuse and moderate (1-5 mg Cd kg^1).
Large areas of this contaminated region are in agricultural use (Schreurs *et al*., 2011) and soils are characterized by a sandy texture and relatively low values of pH and organic matter (De Temmerman *et al*., 2003; Kirkham, 2006). These soil characteristics favour an enhanced uptake of these metals in crops and leaching to the groundwater, resulting in food and fodder crops that often exceed European and Belgian legal *threshold* values for Cd (Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002; Commission Regulation no. 1881/2006; Meers *et al*., 2010; Ruttens *et al*., 2011). This imposes a serious threat on the profitability of the farming industry (McGrath *et al.*, 2001; Römkens *et al*., 2007; Thewys *et al*., 2010) and led the Public Waste Agency of Flanders (OVAM) to decide that these soils need proper management.

Cadmium (Cd)

The main contaminants from historical pollution in the Campine region are Cd, Zn and Pb. Cadmium poses the greatest threat since it is relatively mobile in soil and highly toxic even at low concentrations. Lead is like Cd a non-essential potentially toxic metal, but much less available in the soil (table 1.1 from Grispen *et al.*, 2006). Zinc is an essential metal and only toxic at high concentrations.

Table 1.1 Total and extractable Cd, Zn and Pb concentrations (mg kg^{-1} dry soil) in moderate contaminated Campine soils according to 4 replicates (mean ± standard error).

		Moderate contaminated Campine soils
Total soil	Cd	5.5 ± 0.3
	Zn	390 ± 72
	Pb	167 ± 5
1M HNH_4 extractable soil	Cd	3.1 ± 0.1
	Zn	43 ± 6
	Pb	1.0 ± 0.1
H_2O extractable soil	Cd	0.22 ± 0.01
	Zn	2.1 ± 0.4
	Pb	0.8 ± 0.3

An increasing awareness of the critical role that soil resources play in promoting a sustainable environment and economic development, led to the elaboration of the European Thematic Strategy for Soil Protection, adopted by the European Commission in September 2006 (COM(2006) 231). In case of trace element-contaminated agricultural soils, there is only one set of guidance or critical levels that apply to all the countries of the EU, those defined in Annex 1A of Council Directive 86/278/EEC. Clean-up values for Cd, Zn and Pb are respectively 2, 600 and 200 mg kg^{-1} dry soil. Taking this into consideration, soil standards are only exceeded for Cd in the Campine region (Ruttens *et al*., 2008). Close to the zinc smelters soils contain more than 10 mg Cd kg^{-1}, while background concentrations are below 0.5 mg Cd kg^{-1} dry soil (Koopmans *et al*., 2008).
Cadmium in its elemental form is a soft, silver-white metal belonging to group II B of the Periodic Table (atomic number: 48 / relative atomic mass: 112.41). Characterized by a $4d^{10}5S^2$ electron configuration, its most common oxidation state is Cd(II). Cadmium has a density of 8.65 g cm^{-3}, melts at 321.07 °C and boils at 767 °C. In nature, Cd is most often present as complex oxides, sulphides, and carbonates in zinc, lead, and copper ores in concentrations of 200 - 14,000 mg kg^{-1} (ATSDR, 1999). Although, it is a relatively rare element with an average concentration of 0.1 - 0.2 mg kg^{-1} earth's crust (EC, 2001).
The major natural sources of Cd emission to air are volcanoes, airborne soil particles, sea pray, biogenic material and forest fires. The weathering of rocks releases Cd to soils and aquatic systems and plays a significant role in the global Cd cycle. The main anthropogenic Cd sources are associated with mining activities and metallurgical, paint, coating and electroplating industries (UNEP, 2010). Also the use of fertilizers containing phosphates from mineral products, waste treatment, power plants and transport have a clear contribution (Strincone *et al*., 2013). Various human and natural activities have been and are still polluting extensive areas throughout the world (Khan *et al*., 2000). Above all, mining and smelting activities are largely the cause of numerous metal-contaminated ecosystems (Smith *et al*., 1996; Herawati *et al*., 2000).

Effects of Cd on humans

Dietary intake (next to intake via inhalation and drinking-water) is the main source of Cd exposure in the general population, providing over 90 % of the

total intake in non-smokers (WHO/UNECE, 2006). Cadmium absorbed via the lungs or the gastrointestinal tract is stored mainly in liver and kidneys, leading to nephrotoxicity and osteoporosis later in life (Schoeters *et al*., 2006). Since the US Environmental Protection Agency (EPA) has determined that Cd is a probable human carcinogen by inhalation (ATSDR, 1999), the International Agency for Research on Cancer (IARC) classifies Cd in Group 1: carcinogenic to humans (IARC, 1993).

Much of the information that has become available since publication of Environmental Health Criteria 134 (WHO, 1992) on the effects of long-term exposure to low doses of Cd on human health is from a cross-sectional, population-based epidemiological study conducted between 1985 and 1989 in Belgium, known as the Cadmibel (Cadmium in Belgium) study (Lauwerys *et al*., 1990). It was concluded that Cd may induce renal tubular dysfunction and may affect Cd, Zn and Cu homeostasis (Lauwerys *et al*., 1990; Staessen *et al*., 1994). Also calcium metabolism is gradually affected, as Cd accumulates in the body (Staessen *et al*., 1991). Indeed, even occupational exposure to Cd is associated with lower bone mineral density promoting skeletal demineralisation (Staessen *et al*., 1999; Schutte *et al*., 2008; Nawrot *et al*., 2010).

Starting from 1985, Nawrot *et al*. (2008) monitored blood cadmium (BCd) (until 2003), 24-h urinary cadmium (UCd) (until 1996), and mortality (until 2007). The risks ($p \leq 0.04$) associated with a doubling of baseline UCd were 20% and 44% for total and non-cardiovascular mortality, and 25% and 33% for a doubling of BCd. Earlier, Nawrot *et al*. (2006) proved the association between environmental exposure to Cd and overall cancer risk using the 24-h urinary excretion as a biomarker of lifetime exposure. Lung cancer was proportional with soil Cd concentration. Although the molecular mechanisms of Cd-induced carcinogenesis are not yet understood, several factors may contribute to it, including perturbance of mitogenic signalling, the DNA repair mechanism and apoptotic resistance, as well as effects on E-cadherin and its role in cell-cell adhesion, especially in epidermal cells (Prozialeck *et al*., 2003; Goyer *et al*., 2004).

Hogervorst *et al.* (2007) investigated house dust as a possible route of environmental exposure to Cd and Pb. A two-fold increase in the metal loading rate in house dust was associated with increases in blood cadmium, 24-h urinary

cadmium, and blood lead, independent of the vegetable index and other covariates. An increased Cd body burden is associated with lower aortic pulse wave velocity, lower pulse pressure throughout the arterial system, and higher femoral distensibility (Schutte *et al*., 2008).

These studies concluded that historical pollution from non-ferrous smelters continues to present a serious health hazard and that one should not underestimate the importance of the recent epidemiological evidence on Cd toxicity as to its medical and public health implications (Nawrot *et al*., 2010). In the Campine region, the inhalation of house dust is next to dietary intake an important exposure route and should be incorporated in the assessment of health risks.

Effects of Cd on plants

Cadmium is taken up by plant roots in an active and/or passive way (Greger and Lindberg, 1986; Hart *et al*., 1998; Lindberg *et al*., 2004). Although most Cd ions bind to the cell wall, some Cd^{2+} enters the cytosol by KCa channels (Perfus-Barbeoch *et al*., 2002; Lindberg *et al*., 2004). Once taken up by the plant roots, some metal ions are transported to the shoot and can be stored in the apoplast of epidermal cells, mesophyll cells, or trichomes. The transport of trace elements from the apoplast to the cytosol and eventually to the vacuole (where they will not damage the vital cellular processes) is considered as a common mechanism for contaminant tolerance and accumulation in plants (Memon and Schroder, 2009) and is driven by transporter proteins (Marschner, 1995). Complexes of trace elements and metal chelating molecules such as metallothioneins and phytochelatins (Verbruggen *et al*., 2009a,b) are actively shuttled into the vacuole or out of the cell by ABC-type cassette transporters (Salt and Rauser, 1995; Lu *et al*., 1997; Jasinski *et al*., 2003). For sequestering the Cd^{2+} ion from the cytosol Cd^{2+}/H^{+}-antiporters are used (Salt and Wagner, 1993; Hall, 2002; Lindberg *et al*., 2007).

Despite the presence of detoxification pathways (Schat *et al*., 2000; Clemens, 2001; Sarret *et al*., 2002; Morel *et al*., 2009), elevated levels of trace elements can cause detrimental effects on plants at cellular and whole plant level (Barcelo *et al*., 1988; Shaw *et al*., 2004; Cherian *et al*., 2007; Llamas *et al*., 2008). At whole plant level, trace element stress causes chlorosis, necrosis, turgor loss,

and closure of stomata and affect normal growth and even can lead to plant death depending on the level of stress (Foy *et al*., 1978; Bingham *et al*., 1986; Vangronsveld and Clijsters, 1994; Pandey and Sharma, 2002; Perfus-Barbeoch *et al*., 2002; Rahman *et al*., 2005). Trace elements above threshold levels rapidly cause increased activity of *NADPH* oxidase, which can decrease cell wall extensibility, affect lipid peroxidation, modify calcium channels, and lead to oxidative burst and generation of H_2O_2 (Cuypers *et al*., 2000; Smeets *et al*., 2008; Cuypers *et al*., 2010). Increased H_2O_2 production can generate reactive oxygen species (ROS); increase cell wall rigidity, jasmonate, and ethylene levels; and affect photosystems (Maksymiec and Baszynski, 1999; Vassilev *et al*., 2004; Cho and Seo, 2005; Maksymiec *et al*., 2005; Maksymiec and Krupa 2006).

To counteract the deleterious effects of signaling molecules, plants' defensive systems are activated and produce various defense metabolites such as ascorbate, glutathione, and antioxidative enzymes (*e.g.* superoxide dismutase, ascorbate peroxidase, catalase); stress proteins; and other secondary metabolites (Inze and Van Montagu, 1995; Noctor and Foyer, 1998; Sandalio *et al*., 2001; Arrigoni and De Tullio, 2002; Tung *et al*., 2007; Marquez-Garcia and Cordoba, 2009).

Effects of Cd on microorganisms

Trace metals affect microbial growth and survival, community diversity and structure, enzymatic activity, and microbial-mediated edaphic processes (C and N mineralization, decomposition) (Baath, 1989; Roane and Kellogg, 1996). Cadmium negatively influences biodiversity and the activity (*e.g.* soil respiration) of microbial communities (Doelman, 1985; Giller *et al*., 1998; McLaughlin *et al*., 1999; Sandaa *et al*., 1999; Kamnev *et al*., 2005; Liao *et al*., 2005; Akerblom *et al*., 2007; Bamborough and Cummings, 2009). Soil metal contamination may lead to a reduction of total microbial biomass (Brookes and McGrath, 1984; Fliessbach *et al*., 1994); a decrease in the number of specific populations (Chaudri *et al*., 1993) and shifts in the microbial community structure (Frostegård *et al*., 1993, 1996; Gray and Smith, 2005; Akerblom *et al*., 2007). A frequently observed shift in the microbial community structure due to metal stress was that Gram-positive bacteria increased and Gram-negative

bacteria decreased (Frostegard *et al*., 1993; Pennanen *et al*., 1996; Baath *et al*., 2005; Akerblom *et al*., 2007). Despite elevated metal concentrations in ultramafic soils, highly diverse microbial communities and numerous metal-resistant bacterial strains have been isolated from these soils (Mengoni *et al*., 2001; Abou- Shanab *et al*., 2003; Amir and Pineau, 2003a; Amir and Pineau, 2003b; Pal *et al*., 2004; Sessitsch *et al*., 2013).

Remediation of (historical) soil pollution

The detrimental effects resulting from Cd contamination, which were worrying numerous researchers already decades ago (Buchet *et al*., 1980; Chang *et al*., 1980; Lauwerys *et al*., 1980; Roels *et al*., 1980; Roels *et al*., 1981), resulted in an action plan (see Flemisch "Action Plan Cadmium" (2006)). BeNeKempen developed management and remediation strategies for the pollution of toxic metals in the Campine region, where the soil Cd contents still are and the past Cd emissions and discharges were the highest in Flanders. In total 42 actions were undertaken in Flanders of which 18 in the Campine region (table 1.2). Next to control, monitoring and contaminant removal actions, several awareness and sensitization actions were applied. The 2 most interesting actions concerning an immediate plant-based restoration at the different smelter sites are actions 10 and 11.

The process of phytostabilisation and phytoextraction offers significantly more benefits than civil engineering based conventional technologies (techniques such as leaching of pollutants, solidification/stabilisation, size selection and pyrometallurgical processes, electrokinetical treatment, chemical oxidation/reduction of pollutant, excavation) (Sekhar *et al*., 2005; Fischerova *et al*., 2006). Phytoremediation technologies help to prevent landscape destruction and enhance activity and diversity of soil microorganisms to maintain healthy ecosystems (Wenzel *et al*., 1999; Lombi *et al*., 2000; Mulligan *et al*., 2001; Barcelo and Poschenrieder, 2003; McGrath and Zhao, 2003).

Table 1.2 Action plans in the area of the (former) zinc smelters in the Belgian Campine region.

Action	Description
1	Additional control in the context of IPPC in Balen en Overpelt
2	Additional control in the context of the self-monitoring program of Nyrstar
3	Monitoring metal concentrations in the air at the measuring stations of the VMM
4	The follow-up of the remediation project of Nyrstar (implementation and timing)
5	Acceleration of the zinc ash removal in residential areas as a precaution
6	Zinc ash removal in Lommel at the historically heavily loaded residential district
7	Tackle public roads made of zinc ash in consultation with the local government
8	Evaluation of the procedure associated with earthmoving
9	Starting a dust monitoring program in Overpelt at the residential district
10	Greening of wastelands
11	The cultivation of energy crops in consultation with farming organizations
12	Additional control on the wastewater management of Nyrstar Overpelt
13	Making additional resources available for sediment removal from water bodies
14	Analysis of soil samples at a reduced cost for inhabitants
15	Professional farmers receive additional cultivation advice
16	Additional sensitization: do not use well water for irrigation purposes
17	Start-up of new biomonitoring research
18	Nyrstar evaluates the scientific feasibility of a study of cancer incidence

In 1990, the first effective environmentally friendly restoration approach in the Campine region was applied by Vangronsveld *et al*.; 3 ha of the heavily impacted former smelter site were treated with a combination of compost and beringite, a modified aluminosilicate that originated from the fluidized bed burning of coalmine slag (Vangronsveld *et al*., 1995). The use of metal-tolerant grass cultivars resulted in a rapid and effective revegetation of the bare area of Maatheide (red dot in figure 1.1). Moreover, metal availability in soils substantially decreased resulting in the development of a healthy vegetation, while soil organisms (*e.g.* bacteria, nematodes) in the treated plots rose to normal levels (Vangronsveld *et al*., 1993; Vangronsveld *et al*., 1996; Vangronsveld *et al*., 2000; Bouwman *et al*., 2001). Direct revegetation of the area was not possible, mainly because of the high metal concentrations in the soil. The metal-immobilizing effect of beringite (5%) remained stable allowing the further (generative and vegetative) development of the vegetation and subsequently the humus layer, improving the overall success of vegetation establishment (Vangronsveld *et al*., 1996). Revegetation is expected to result in a supplementary metal-immobilizing capacity of the upper soil layer: lateral wind erosion is eliminated, and percolation of metals from metal-polluted

substrate to the groundwater is highly reduced (Vangronsveld *et al*., 1991; Bleeker *et al*., 2003; Kucharski *et al*., 2005).
The combination of amendments and/or compost soil treatment with the application of metal-tolerant plant cultivars might be necessary only on the most contaminated soils close to the zinc smelters (5-50 mg Cd per kg dry soil, see figure 1.1). For the large portion of slightly to moderate contaminated soils in the Campine region (1-5 mg Cd per kg dry soil, see figure 1.1) that are mainly in agricultural use (Ruttens *et al*., 2010; Witters *et al*., 2011), metal-immobilizing techniques (*i.e.* (assisted) phytostabilisation)) can be substituted by remediation techniques which remove the metals from the soil (*i.e.* phytoextraction) (Vangronsveld *et al*., 2009).

Phytoextraction

In case of phytoextraction, plants remove trace elements from the soil and concentrate them in harvestable above-ground parts (Kumar *et al*., 1995; Salt *et al*., 1998). It involves the uptake of contaminants from the soil and further translocation and accumulation mainly in shoot tissues. Subsequently, the plant biomass is harvested and processed (*e.g.* incineration, fermentation, pyrolysis) to remove the contaminants permanently from the soil (Vangronsveld *et al*., 2009; Witters *et al*., 2009).
In first instance, phytoextraction refers to the ability of hyperaccumulator plants to extract metals from soil and transport them to the above-ground parts, which are able to accumulate concentrations up to 100-fold greater than those normally found in non-accumulator species (mg kg^{-1}; >10,000 (Mn or Zn), >1000 (Cu, Co, Cr, Ni, Pb) or >100 (Cd)) (Baker and Brooks, 1989; Chaney *et al*., 1997; Salt *et al*., 1998; Baker *et al*., 2000; McGrath and Zhao, 2003). Examples of natural metal hyperaccumulator plant species are *Alyssum*, *Thlaspi* and *Berkheya* (McGrath *et al*., 2001).
However, hyperaccumulator plants generally do not produce sufficient biomass to allow rapid and efficient remediation (Brooks, 1994; Khan *et al*., 2000; Puschenreiter *et al*., 2001). An ideal plant for trace element phytoextraction should possess the following characteristics: (a) tolerance to the trace element concentrations accumulated, (b) fast growth and highly effective trace element-accumulating biomass, (c) accumulation of trace elements in the above-ground

parts, and (d) easy to harvest (Vangronsveld *et al*., 2009). More recently, some genotypes of high biomass crops (such as *Nicotiana*, *Salix*, *Populus* or *Brassica*) which tolerate increased concentrations of trace metals and show sufficient metal accumulation have been proposed as viable alternatives for hyperaccumulators in phytoextraction technologies (Pulford and Watson, 2003; Quartacci *et al*., 2003, 2005, 2006; Dickinson and Pulford, 2005; Meers *et al*., 2007; Unterbrunner *et al*., 2007; Kuffner *et al*., 2008). When metal excluder bioenergy or industrial crops like maize are used instead of metal accumulators, the remediation aspect is demoted to a secondary objective with sustainable risk-based land use as first objective (Meers *et al*., 2005; Zhang and Banks, 2006). In this case, the term 'phytoattenuation' is more applicably (Meers *et al*., 2010).

Anyway, to effectively clean up metal-contaminated sites, an adequate plant yield and high metal concentrations in the harvestable tissues, resulting from an efficient transfer of metals from the roots to shoots, must be combined (Rajkumar *et al.*, 2009). So, phytoextractor plants must have mechanisms for tolerating or accumulating metals in their rhizosphere and tissues (Hayes *et al*., 2003). If the produced biomass can be valorised into an alternative income, then the main drawback of metal phytoextraction, namely the long remediation period required, may become invalid. In this regard, a number of research projects based on the cultivation of industrial non-food crops are initiated in the Campine region (Meers *et al*., 2007; Van Ginneken *et al*., 2007; Vangronsveld *et al*., 2009; Witters *et al*., 2009).

Remediation plant

Among many fast-growing, high biomass, metal-tolerant and accumulating plant species suitable for phytoextraction, the *Brassicaceae* family have received considerable attention (Kumar *et al*., 1995; Saxena *et al*., 1999; Prasad and Freitas, 2003). Indian mustard (*Brassica juncea*) was reported to be one of the most promising, non-hyperaccumulating plant species for extracting toxic metals from contaminated soils. However, other species of the *Brassica* genus, such as *B. campestris, B. carinata, B. napus, B. nigra, B. oleracea* and *B. rapa*, have also been studied (Kumar *et al*., 1995; Marchiol *et al*., 2004; Meers *et al*., 2005; Gisbert *et al*., 2006) based on their capacity to uptake and accumulate heavy

metals in amounts higher than those of other plant species (Kumar *et al*., 1995). The use of energy and/or bio-diesel crops (*e.g. Brassica napus*) would give contaminated soil an economic value and minimize remediation costs (Kos *et al*., 2003). Energy crop cultivation is expected to be the prevalent form of biomass production for reaching renewable energy targets set by the US Congress and European Union in order to mitigate climate change and enhance energy security (Panoutsou, 2009). Applying phytoremediation on marginal soils may be an effective and sustainable way to produce biomass without increasing pressure on clean agricultural soils. In the meanwhile, the soils are being remediated.

Schreurs *et al*. (2011) examined the Campine region in Belgium and illustrated that more than 2000 ha is suitable for phytoextraction and estimated the biomass potential (19,067 Mg $year^{-1}$) and remediation time (42 years) with willow. Short rotation coppice (SRC) with willow produced the best results regarding maximal soil Cd reclamation compared to other tested plant species like poplar, maize and rapeseed (Ruttens *et al*., 2008; Witters *et al*., 2009; Van Slycken *et al*., 2013). Even in comparison with the Cd hyperaccumulator *Thlaspi caerulescens*, Salix spp. extracted 2 times more Cd per hectare while shoot Cd concentrations were 10 times lower (Hammer and Keller, 2002; Lewandowski *et al*., 2006).

Although poplar and willow are the most likely crops to be used in the Campine region (Schröder *et al*., 2008), more conventional crops are being proposed in this study aiming the acceptance by the farmers. Instead of using metal excluder crops like maize (Meers *et al*., 2010; Thewys *et al*., 2010; Van Slycken *et al*., 2013), rapeseed is further evaluated as a possible phytoextractor crop suitable for Cd phytoextraction in the Campine region (Rossi *et al*., 2002). Rapeseed can be grown every 3 to 4 years to avoid lower maize biomass yields as a result of the lack of a strict rotation scheme (Schreurs *et al*., 2011) and to guarantee an accelerated phytoextraction process since rapeseed is considered as a natural metal-accumulator.

Constraints

Several factors are still limiting the metal remediation process with high biomass-producing metal-accumulators, thereby restricting its widespread application. The most important limitations are low metal availability in the soil

and low metal uptake, translocation, accumulation and tolerance by plants (Kumar *et al*., 1995; Burd *et al*., 2000; Kayser *et al*., 2000; Quartacci *et al*., 2006; Li *et al*., 2007; Vangronsveld *et al*., 2009; Weyens *et al*., 2009).

Metal availability

The low amount of metals extracted by plants from the soil as well as the tardiness of their extraction is mainly the consequence of the low metal availability. Metal bioavailability can be defined as the fraction of the total metal content of the soil that can interact with a biological target (Geebelen *et al*., 2003) and is often lower than 1% of the total metal content in soil (Whiting *et al*., 2001; Braud *et al*., 2006). A large proportion of many metals are adsorbed or occluded by carbonates, organic matters, Fe-Mn oxides and primary or secondary minerals (Garbisu and Alkorta, 2001); their bioavailability can be influenced by soil characteristics such as pH, cation exchange capacity (CEC) and organic matter (Kayser *et al*., 2001). Proportions of bioavailable and unavailable contaminants in soils are often at equilibrium, but any change in environmental factors (such as pH, oxygen, climate, hydrology and biology, mineral and organic content, colloids, and weathering due to dissolution–precipitation, oxidation–reduction, complexation–dissociation, and adsorption–desorption) can affect the labile contaminant pools. Taking into account biotic interactions makes the situation even more complicated. Important plant-induced factors influencing the solubilisation of metals could be: (1) root-induced changes in pH of the rhizosphere (2) increased reducing capacity of the roots and (3) quantity and composition of root exudates. Plants can either stabilize contaminants by adsorbing and precipitating them in the root zone or alter their chemical form by changing the soil environment (*e.g.* elemental concentrations, pH, *p*CO2, *p*O2, redox potential and organic ligand concentrations, and microbial biomass) (Marschner, 1995; Puschenreiter *et al*., 2005; Do Nascimento and Xing, 2006; Martinez-Alcala *et al*., 2009). For pH-dependent trace elements such as Cd, Ni, Zn, and As, relevant relationships exist between concentrations in the labile pool and in the crops (Baltrenaite and Butkus 2007; Memon and Schröder 2009). The challenge is to manipulate contaminant labile pools in the rhizosphere durig the period of root uptake in such a manner that plant uptake and physiology are not adversely affected and

to avoid contaminant migration into other environmental compartments (Nowack *et al*., 2006).

Metal phytotoxicty

Besides low metal bioavailability, metal phytotoxicity is often a limiting factor for metal phytoextraction. Soon after the idea of using non-hyperaccumulating high biomass plants to remediate soils, the problem of phytotoxicity emerged. Exposure to excess Zn, Cd, and other toxic metals, especially on marginal land, leads to the formation of reactive oxygen species (oxidative stress) and the activation of antioxidative defence mechanisms (Semane *et al*., 2003; Smeets *et al*., 2005) which might result in an affected biomass production, nutrient uptake, homeostasis and in chlorosis, especially in sensitive plants (Sanita di Toppi and Gabrielli, 1999). Therefore, phytoextraction can only be considered for low to moderately contaminated soils (Rulkens *et al*., 1998).

Along with metal toxicity, there are often additional factors limiting plant growth and thus reducing metal uptake in contaminated soils including arid conditions, a lack of soil structure, low water supply and nutrient deficiency. Plants require quite critical nutritional conditions (*e.g.* water, N, P, K and oxygen, etc.) and proper soil characteristics (*e.g.* soil texture, pH, salinity, etc.) to maintain normal growth and complete their life cycle. Therefore, improvement of plant growth under stressing growth conditions is critical for optimal performance of phytoremediation. Elevated levels of trace metals, however, lead to impaired metabolic activity and result in reduced plant growth. The concentrations of target pollutants and the presence of other toxins must be within the limits of plant tolerance.

Genetically engineered solutions

In order to apply phytoextraction using metal accumulating high biomass-producing plants, the challenge lies in the improvement of metal tolerance and uptake as well as biomass production on these unfavorable substrates.

Using genetic engineering technologies, it is possible to transfer appropriate resistance genes or hyperaccumulation traits into high biomass plants. Similarly, transfer and overexpression of genes from other organisms than plants can improve remediation (Rugh *et al*., 1998; Kärenlampi *et al*., 2000; Kramer and

Chardonnens, 2001). Genetically engineered plants are predominantly useful if the first objective is remediation (*i.e.* during phytoextraction instead of phytoattenuation) and thus more often applied to accumulator plants in order to achieve properties of hyperaccumulator plants in terms of tolerance and accumulation capacities while preserving relative high plant biomasses (Kärenlampi *et al*., 2000; Banuelos *et al*., 2005; Farwell *et al*., 2006; Doty *et al*., 2007; Viktorova *et al*., 2014). However, genetically engineered organisms, expected to greatly contribute to trace element phytoextraction, are not allowed to apply in nature since the European Union and several other countries are still reluctant to their introduction (Dunwell 1999; Kärenlampi *et al*., 2000; Clemens *et al*., 2002).

Next to the genetic engineering approach, growth of accumulator plants can be promoted by the use of fertilizers, especially on marginal lands. In order to maximise metal uptake by the plants, soil metal bioavailability can be increased by several synthetic chelators such as EDTA (Blaylock *et al*., 1997; Huang *et al*., 1997; Vassil *et al*., 1998; Cooper *et al*., 1999; Grčman *et al*., 2001; Puschenreiter *et al*., 2001; Shen *et al*., 2002; Thayalakumaran *et al*., 2003; Evangelou *et al*., 2007). However, the application of expensive fertilizers and chelators in chemically assisted phytoextraction may lead to environmental risks (Kayser *et al*., 2000; Sun *et al*., 2001; Wu *et al*., 2004; Quartacci *et al*., 2006; Tandy *et al*., 2006; Meers *et al*., 2008). As mentioned previously, phosphate fertilizers are a source of Cd contamination and many metal immobilizing chelators are persistent (Lombi *et al*., 2001) and toxic for plants, microorganisms (biomass, diversity and activity) and nematodes (Lasat, 2002; McGrath *et al*., 2002; Römkens *et al*., 2002; McGrath and Zhao, 2003; Bouwman *et al*., 2005; Evangelou *et al*., 2007). Even in unpolluted control soil, some amendments may show undesirable side effects like matrix effects (*e.g.* zeolites with high sodium content destroying soil structure) or immobilization of essential nutrients (Mn, Mg, etc.) (Vangronsveld *et al*., 2009). Moreover, they can cause groundwater pollution by uncontrolled metal dissolution and leaching (Shen *et al*., 2002; Chen *et al*., 2004). Indeed, it has been suggested that plants cannot extract high amount of metals in a short period (Barona *et al*., 2001).

Another, more interesting strategy to improve the efficiency of phytoextraction is by exploiting plant-associated microbes (Weyens *et al*., 2009). Many

researchers are exploring the possibilities of enhancing the biomass production and the metal tolerance and uptake of metal-accumulating plants using metal-tolerant and plant growth-promoting bacteria (PGPB) or mycorrhizal fungi as bioinoculants (Belimov *et al*., 2005; Abou-Shanab *et al*., 2006; Sheng and Xia, 2006; Rajkumar *et al*., 2009; Ma *et al*., 2011; Luo *et al*., 2012). Since like many *Brassicaceae*, rapeseed is a non-mycorrhizal plant species, we'll further focus on the role of plant-associated bacteria.

Plant-associated bacteria

Plants live in close relationship with microorganisms (*e.g.* bacteria and fungi) that can support nutrient uptake, increase resistance against pathogens and enhance plant growth (Mathesius, 2009). Plant-associated bacteria include endophytic, phyllosphere and rhizosphere bacteria.

Endophytic bacteria colonize the internal tissues of the plant without causing symptoms of infection or negative effects on their host (Schulz and Boyle, 2006). Endophytes are considered to primarily gain entry into plants via the roots and to subsequently colonize the root intercellular space, aerenchym and cortical tissues (Pan *et al*., 1997; Germaine *et al*., 2004; Taghavi *et al*., 2009; Weyens *et al*., 2011). Since few bacteria enter the vascular system (Mahaffee *et al*., 1997; Quadt-Hallmann *et al*., 1997), the endophytic concentration generally is highest in the roots, followed by the stem and the leaves (Compant *et al*., 2010; Weyens *et al*., 2011). Rhizosphere bacteria live on the root surface in close relationship with the plant; they thrive on root exudates that are believed to have a major influence on bacterial diversity (Lemanceau *et al*., 1995). An interesting group of bacteria within the rhizosphere community that exerts beneficial effects on plant growth was first defined by Kloepper and Schroth (1978) and termed as plant growth-promoting rhizobacteria (PGPR). As rhizosphere bacteria live in the immediate vicinity of the root below-ground, phylloshere bacteria can be found in the external regions of the above-ground plant parts including leaves, stems, blossoms and fruits (Redford *et al*., 2010).

In comparison with rhizosphere and phyllosphere bacteria, which reside in the external regions of the below-ground and above-ground plant parts respectively, endophytic bacteria are likely to interact more closely with their host, face less competition for nutrients and are more protected from adverse changes in the

environment (Reinhold-Hurek and Hurek, 1998; Beattie, 2007). In addition, endophytic bacteria can be transferred to consecutive generations via seeds (Mastretta *et al*., 2009; Remans *et al*., 2012), by colonization of meristems (Pirttilâ *et al*., 2000), by transfer through gametes (Madmony *et al*., 2005) or through direct vascular connections from the maternal plant (Block *et al*., 1998). In these very close plant-bacteria interactions, plants provide nutrients and residency for bacteria, which in exchange can directly or indirectly improve plant growth and health (for review see Mastretta *et al*. (2006) and Weyens *et al*. (2009)). These bacterial mechanisms to promote plant growth could be exploited to improve the yields of food, feed and bioenergy crops (Haberl *et al*., 2010). Next to their growth promoting traits, plant-associated bacteria are known to be able to cope with contaminants present in their environment. A better understanding of both mechanisms can contribute to a more sustainable growth of biomass crops for biofuel production and feedstocks for industrial processes on trace element-contaminated land (Rajkumar *et al*., 2009; Weyens *et al*., 2009a,b). Furthermore, plant-associated bacteria that are equipped with the appropriate characteristics can be enriched by inoculation, leading to an improved phytoextraction efficiency (van der Lelie *et al*., 2000; Kuffner *et al*., 2008, 2010; Sessitsch and Puschenreiter, 2008; Sheng *et al*., 2008; Weyens *et al*., 2009a,b; Glick, 2010). Studying the composition of bacterial communities living in a naturally contaminated environment (Diaz-Ravina and Baath, 1996) and especially the interactions between endophytes and hyperaccumulator plants attracted the attention of several investigators (Lodewyckx *et al*., 2002; Idris *et al*., 2004) due to biotechnological applications for bioremediation.

Plant-associated bacteria can affect metal uptake by plants by 2 complementary means, recently reviewed in Sessitsch *et al*. (2013): (1) **enhancement of the mobility of metals** in soil and sediment, aiming higher metal concentrations in plants, by producing biosurfactants (Herman *et al*., 1995; Mulligan *et al*., 2001; Sheng *et al*., 2008), siderophores (Diels *et al*., 1999; Dubbin and Louise Ander, 2003) and organic acids (Francis and Dodge, 1988; Cantafio *et al*., 1996; Di Simine *et al*., 1998; Kalinowski *et al*., 2000) and/or (2) **enhancement of the tolerance and biomass of plants** by plant growth promotion (Zhuang *et al*., 2007). Through natural enrichment of bacteria having these properties,

investigators hope to circumvent the limitations of phytoextraction that are listed above.

Enhancement of the mobility of metals

Soil and rhizosphere microorganisms can affect metal availability to the plant (Burd *et al*., 2000; Abou-Shanab *et al*., 2003; Abou-Shanab *et al*., 2006; Braud *et al*., 2006). Sorbed, precipitated and occluded trace elements can be solubilized by acidification and redox-changes or through chelation and ligand-induced dissolution (Gadd, 2004; Sessitsch *et al*., 2013). To date two groups of bacterially produced natural chelators are known. These are carboxylic acid anions and siderophores. Microbial siderophores, mainly localized in the rhizosphere (Bossier *et al*., 1988), enhance the Fe(III) mobility and also various other cations (Höfte *et al*., 1993; Diels *et al*., 2002; Saravanan *et al*., 2007; Braud *et al*., 2009) improving the uptake of trace elements by plants (van der Lelie, 1998; van der Lelie *et al*., 1999). Also nitrogen fixing and phosphorus-solubilizing bacterial strains can increase extractable metal fractions by decreasing the pH value (Sheng and Xia, 2006), probably by excreting low molecular weight organic acids (Chen *et al*., 2006). The production of these bacterial chelators is in tight equilibrium with the plant's activity, meaning that trace element mobilization only takes place when plants are active and by consequence can take up the elements (Kidd *et al*., 2007). This may prevent uncontrolled trace element dissolution and leaching which constitutes a major risk for food chain and groundwater contamination (Adriano *et al*., 2004). Since a significant part of endophytic bacteria originate from the rhizosphere (Idris *et al*., 2004; Ryan *et al*., 2008; Sheng *et al*., 2008), also facultative endophytes can contribute to enhanced trace element availability and uptake (Madhaiyan *et al*., 2007; Kuffner *et al*., 2010) when living in the rhizosphere.

Plant growth-promoting rhizobacteria such as *Agrobacterium, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Serratia, Pseudomonas and Rhizobium* (O'Sullivan and O'Gara, 1992; Höflich *et al*., 1994; Carlot *et al*., 2002; Glick, 2003) are particularly interesting since they increase both the rate of metals accumulated by plants and the plant biomass.

Enhancement of the tolerance and biomass of plants

Plant growth-promotion

Rhizobacteria as well as endophytes can affect plant's nutrition and resistance (Glick, 1995; Belimov *et al*., 2004). Plant-associated bacteria can improve plant growth and development in 2 ways: (1) indirectly by preventing the growth and/or activity of plant pathogens through competition for space (*e.g.* binding sites on the root) and nutrients (*e.g.* depletion of iron from the rhizosphere), production of antibiotics, antibiosis, induction of plant defence mechanisms (*i.e.* induced systemic resistance (ISR)) and through production of fungal cell wall lysing enzymes (*i.e.* hydrolytic enzymes) and inhibition of pathogen-produced enzymes or toxins (Glick, 1995; Glick *et al*., 2007), and (2) directly by increasing nutrient and water uptake by means of plant growth regulators like auxins, gibberelins and cytokinins and other molecules such as siderophores, specific enzymes (*e.g.* 1-amino-cyclopropane-1-carboxylate (ACC) deaminase) (Burd *et al*., 1998; Belimov *et al*., 2005; Reed *et al*., 2005; Safronova *et al*., 2006), organic acids (involved in the fixation of atmospheric nitrogen and the mobilization of unavailable nutrients such as phosphorus and other mineral nutrient, or in plant protection against metal toxicity) (Kloepper *et al*., 1980; O'Sullivan and O'Gara, 1992; Pattern and Glick, 1996).

Since many plant growth-promoting bacteria (PGPB) possess several of these traits, a bacterium may utilize different traits at various times during the life cycle of the plant. Typically, PGPB have little or no measurable effect on plant growth when the plants are cultivated under optimal and stress-free conditions (Glick *et al*., 2007). The mechanisms most often raised to explain the various direct effects of PGPB on plants are related to the production of phytohormones especially auxins (*e.g.* indole-3-acetic acid (IAA)) (Brown, 1974; Patten and Glick, 1996). One of the main effects of bacterial IAA production is the enhancement of lateral and adventitious rooting leading to improved mineral and nutrient uptake and root exudation that in turn stimulates bacterial proliferation on the roots (Dobbelaere *et al*., 1999; Lambrecht *et al*., 2000; Steenhoudt and Vanderleyden, 2000). Low levels of IAA promote primary root elongation while high levels stimulate lateral root formation but inhibit the primary root growth (Xie *et al*., 1996). Too high levels of IAA can even inhibit plant growth (Dubeikovsky *et al.,* 1993; Malik and Sindhu, 2011). Nevertheless,

most PGPB synthesizing IAA may contribute to reduce the deleterious effects of environmental stresses (Lindberg *et al*., 1985; Frankenberger and Arshad, 1995).

Furthermore, it became clear that a number of PGPB also contain the enzyme ACC deaminase (Jacobson *et al*., 1994; Glick *et al*., 1995; Burd *et al*., 1998; Kaneko *et al*., 2000; Belimov *et al*., 2001, 2005; Babalola *et al*., 2003; Ma *et al*., 2003; Ghosh *et al*., 2003; Dey *et al*., 2004; Mayak *et al*., 2004; Hontzeas *et al*., 2005; Madhaiyan *et al*., 2006; Shaharoona *et al*., 2006; Blaha *et al*., 2006; Nadeem *et al*., 2007; zhaoRodriguez *et al*., 2008). This enzyme has no known function in bacteria but antagonizes ethylene synthesis in plants by cleaving the ethylene precursor ACC in ammoniac and α-ketobutyrate (Glick *et al*., 1998). Several ACC deaminase-producing strains were reported to increase plant biomass by reducing metal toxicity (Burd *et al*., 1998; Borgmann, 2000; Glick, 2003).

As toxic metals inhibit the uptake of nutrients, which play an important role in metal detoxification, the potential of bacteria to increase nutrient assimilation by plants is crucial in reaching optimal plant growth in metal-contaminated soils (Belimov *et al*., 2001; Belimov *et al*., 2004; Zaidi *et al*., 2006). Nitrogen, phosphorus and iron are often limiting factors for plant growth because they are largely present in for plants inaccessible forms. Bacteria possessing the enzyme nitrogenase can catalyse the reduction of atmospheric nitrogen to ammonia, that can be (next to nitrate) incorporated into organic molecules by plants. In order to make phosphorus accessible to plants, phosphate solubilizing and mineralizing bacteria are of great importance as they can render phosphorus in its monobasic ($H_2PO_4^-$) or dibasic ($H_2PO_4^{2-}$) soluble forms (Glass, 1989) by secreting organic acids and/or phosphatases (Kim *et al*., 1998). The presence of organic acids in the soil often results in a pH decline stimulating nutrient availability (Ström, 1997). The insoluble form of iron (*i.e.* Fe^{3+}) can bind to siderophores (that like organic acids can be produced by plants and bacteria). The Fe^{3+}-siderophore complexes can facilitate iron uptake by rendering it in its preferred form, Fe^{2+}. So, plant-associated bacteria that function as biofertilizers play in addition to IAA- and ACC deaminase-producing strains an important role as plant growth-promoting agents, especially in metal-contaminated environments.

Metal-sequestration

During phytoextraction, it is observed that endophytes possessing certain efflux-based systems can contribute to bacterial, but also plant's trace element resistance and involve post-efflux sequestration of trace elements (*i.e.* the prevention of extruded trace element ions from re-entering the cell by precipitation, chelation, or by binding to exopolymers) (Diels *et al*., 1995; Salt *et al*., 1999). Focussing on the remediation of Cd-contaminated soils, the CZC and CZR efflux mechanisms are of special interest since they allow Cd ions to be precipitated onto the bacterial cell wall (Nies *et al*., 1995; Hassan *et al*., 1999). As a consequence of the cation/H^+ antiporter (*i.e.* part of the efflux mechanisms), pH increases at the cell membrane. In this alkaline environment, carbon dioxide, produced by the cellular respiration, is transformed into carbonates and bicarbonates. These (bi)carbonates precipitate with the excreted metal cations onto the bacterial cell wall, preventing metal ions from re-entering the bacterial cell (Diels *et al.*, 1995). Endophytes equipped with metal-resistance/sequestration systems and able to produce natural metal chelators may contribute to trace element detoxification in plants resulting in lowered trace element phytotoxicity and an increased trace element translocation to the above-ground plant parts (Lodewyckx *et al*., 2001; Weyens *et al*., 2009d).

Rhizosphere bacteria that can synthesize the enzyme ACC deaminase may lower metal toxicity by reducing the plant stress hormone ethylene produced by the plant in repsons to toxic metals (Gora and Clijsters, 1989; Weckx *et al*., 1993; Schellingen *et al*., 2014). Moreover, it has been shown that genetically modified plants expressing bacterial ACC deaminase accumulated higher amounts of Co, Cu, Mg, Ni, Pb, and Zn than non-transgenic plants (Grichko *et al*., 2000). Also the most common plant growth hormone auxin indole-3-acetic acid (IAA), synthesized by rhizobacteria, has been shown to be indirectly involved in the metal uptake (Lopez *et al*., 2005; Zaidi *et al*., 2006) since it can promote root elongation (Belimov *et al*., 2005) and root dry weight (Sheng and Xia, 2006).

Endophytes versus rhizosphere bacteria

Many reports demonstrated to the role of rhizospheric bacteria in phytoremediation (Gentry *et al*., 2004; Thompson *et al*., 2005; Rajkumar *et al*., 2006; Lebeau *et al*., 2008), but endophytes offer several advantages over

rhizospheric bacteria. For instance, colonization of the plant rhizosphere with PGPR is often problematic since they lose competitiveness over endemic bacteria. In contrast, endophytic bacteria may derive significant competitive advantages since they can be better controlled and are better protected living in the internal tissues of the plant (Hallmann *et al*., 1997; Newman and Reynolds, 2005; Zhuang *et al*., 2007). In addition, re-inoculation of endophytic bacteria does not affect the indigenous endophyte population in plants (Conn and Franco, 2004) in comparison with rhizosphere bacteria (Duponnois *et al*., 2006). Furthermore, endophytes of the Ni hyperaccumulator *Thlaspi goesingense* were tolerant to higher Ni concentrations than the rhizospheric isolates (Idris *et al*., 2004). Even isolates from shoot and root can display different tolerances, suggesting that different microbial communities exist in different compartments of the plant (Lodewyckx *et al*., 2002).

Conclusions

The last few years there is a growing interest in the role of plant-associated microorganisms in trace element bioavailability and uptake by plants. For example, an increasing number of studies in the literature suggest that the accumulation of metals by (hyper)accumulating plants is influenced by their microflora (Schlegel *et al*., 1991; Delorme *et al*., 2001; Mengoni *et al*., 2001; Whiting *et al*., 2001; Lodewyckx *et al*., 2002; Abou-Shanab *et al*., 2006; Cloutier-Hurteau *et al*., 2008; Becerra-Castro *et al*., 2009; Becerra-Castro *et al*., 2011). This microflora might also have important functions in relation to plant growth under these adverse conditions. Microorganisms (bacteria and mycorrhiza) can actively contribute to change the trace element speciation and assist the plant in biomass production and overcoming phytotoxicity (van der Lelie *et al*., 1999; Mastretta *et al*., 2006; Sessitsch *et al*., 2013).

Allthough enrichment of plant-associated bacteria, equipped with the appropriate traits, might speed up the phytoextraction process, remediation time remains longer than what is generally called ‘reasonable’ (5-10 years) (Robinson *et al*., 1998; Baker *et al*., 2000; Blaylock and Huang, 2000; Khan *et al*., 2000; Dickinson and Pulford, 2005). This time constraint may become less important if phytoextraction can be combined with revenue earning operations (Vassilev *et al*., 2004; Vangronsveld *et al*., 2009; Meers *et al*., 2010). Producing

viable products of economical value (*e.g.* renewable energy) may help sustain long-term application of field phytoremediation (Sas-Nowosielska *et al*., 2004; Banuelos, 2006).

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Chapter 2

Objectives

From the introduction **(Chapter 1)** it became clear that plant-associated bacteria can be exploited to increase biomass and metal tolerance on the one hand and metal uptake on the other hand of plants growing on metal-contaminated soil. The enrichment of such bacteria in high-biomass accumulator plant species can result in a more efficient phytoextraction of trace elements. Using fast growing plants for bacterial-assisted phytoextraction can make the remediation process more efficient in the field, especially when economically valuable crops are used. Our model plant throughout the study was *Brassica napus* L. (rapeseed), an oil-producing and relatively high biomass producing metal accumulating crop.

Since we are interested in the enrichment of promising *B. napus*-associated bacterial strains able to improve Cd phytoextraction on a historically contaminated field, we first developed and verified a sampling and isolation workflow to accomplish a representative genotypic and phenotypic characterization of the cultivable bacterial communities associated with *B. napus* grown in the field **(Chapter 3)**. The optimized workflow was subsequently used to characterise the bacterial communities associated with *B. napus* grown on a trace element-contaminated and a non-contaminated field in order to make a genotypic and phenotypic comparison between the fields **(Chapter 4)**. In addition to these site-specific effects, we investigated the possible seasonal effects on these cultivable bacterial communities **(Chapter 5)**. Therefore additional isolations are performed in December, as chapter 2 contains the bacterial information concerning isolations from the bulk soil, rhizosphere soil and roots in June. Finally, the most promising bacterial strains, based on their *in vitro* screening for plant growth-promoting capacity and Cd tolerance (see phenotypic characteristics in chapter 2 and 3), were tested *in planta* using 3 different experimental set-ups with increasing complexity and decreasing controllability aiming to get step by step closer to the real field conditions. Plants were first grown on vertical agar plates, subsequently on sand and finally on

field soil under controlled conditions **(Chapter 6)**. Ultimately, the best-performing strains *in planta* were selected to use for an inoculation experiment in the field **(Chapter 7)**. The *in situ* effects of inoculation on plant growth and Cd uptake were evaluated.

CHAPTER 3

Characterization of the cultivable bacterial populations associated with field-grown *Brassica napus* L.: an evaluation of sampling and isolation protocols

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Abstract

Plant-associated bacteria are intensively investigated concerning their characteristics for plant growth promotion, biocontrol mechanisms and enhanced phytoremediation efficiency. To obtain endophytes, different sampling and isolation protocols are used although their representativeness is not always clearly demonstrated. The objective of this study was to acquire representative pictures of the cultivable bacterial root, stem and leaf communities for all *Brassica napus* L. individuals growing on the same field.

For each plant organ genotypic identifications of the endophytic communities were performed using 3 replicates. Root replicates were composed of 3 total root systems, whereas stem and leaf replicates needed to consist of 6 independent plant parts in order to be representative. Greater variations between replicates were found when considering phenotypic characteristics. Correspondence analysis revealed reliable phenotypic results for roots and even shoots, but less reliable ones for leaves. Furthermore, realistic Shannon-Wiener biodiversity indices were calculated for all 3 organs and showed similar Evenness factors.

Additionally, it was striking that all replicates and thus the whole plant contained *Pseudomonas* and *Bacillus* strains although above- and below-ground plant tissues differed in most dominant bacterial genera and characteristics.

Introduction

Associations between plants and microorganisms are diverse and complex and have been the subject of considerable research. Plant-associated bacteria have been described to possess plant growth promotion capacities (Lugtenberg and Kamilova, 2009), biocontrol mechanisms (Compant *et al*., 2005) and also natural capacities to cope with contaminants (Weyens *et al*., 2009a). A profound understanding of these beneficial characteristics can be exploited for agronomic and phytoremediation purposes (Zhang *et al*., 2007; Singh *et al*., 2011).

Plant growth promoting bacteria (PGPB) can be utilized as biofertilizers for agricultural crops and to increase biomass production during phytoremediation or production of biofuel crops on marginal land (Vessey, 2003; Lucy *et al*., 2004; Spaepen *et al*., 2009). Furthermore, contaminant degrading/resistant bacteria can enhance phytoremediation efficiency and reduce the levels of agrochemicals in food crops (Weyens *et al*., 2009a; Ning *et al*., 2010). Moreover, PGPB mediate a wide range of biocontrol mechanisms including competition for ecological niches, production of inhibitory allelochemicals and induction of systemic resistance in host plants to a broad spectrum of pathogens and abiotic stresses (Bloemberg and Lugtenberg, 2001; do Vale Barreto Figueiredo *et al*., 2011). As a consequence, much lower amounts of agrochemicals will be needed for crop protection diminishing negative effects associated with pesticide use (i.e. development of pathogen resistance and non-target environmental impacts).

Since bacteria living inside the plant (endophytes), in the rhizosphere and in the bulk soil can improve the efficiency and hence also the applicability of phytoremediation processes, researchers are isolating and characterising bacterial populations associated with plant species of interest. Multiple reviews already listed published studies on bacterial effects on phytoremediation. Glick (2010) reported soil bacteria that facilitate phytoremediation while Zhang *et al*. (2007) and Ma *et al*. (2011) reviewed new advances in plant growth promoting rhizobacteria (PGPR) for bioremediation. Rajkumar *et al.* (2009) presented a list of endophytic bacteria with potential to enhance heavy metal phytoextraction. Endophytes with the potential to degrade organic pollutants can reduce both toxicity for and evapotranspiration of volatile contaminants from plants (Barac *et al*., 2004; Taghavi *et al*., 2005; Weyens *et al*., 2010a). In case of mixed

contaminations, endophytes with the appropriate characteristics can diminish phytotoxicity and evapotranspiration of organic molecules and in the meanwhile increase metal uptake (Weyens *et al*., 2010b; Marmiroli *et al*., 2011; Weyens *et al*., 2011; Doni *et al*., 2012).

Since the final aim of these studies is to improve efficiency of phytoremediation by inoculation of beneficial strains, only cultivable plant-associated bacteria are considered. A first critical step in the characterisation of cultivable bacterial communities associated with a certain plant species, is the sampling set-up followed by the isolation procedure. However, many different protocols have been reported for the characterization of plant-associated bacterial populations (Kuklinsky-Sobral *et al*., 2005; Park *et al*., 2005; Barzanti *et al*., 2007; Becerra-Castro *et al*., 2011; Trivedi *et al*., 2011). Also fungal endophyte researchers do not count with a standardized collection protocol (Gamboa *et al*., 2003; Gazis *et al*., 2011), which should be desirable to be aware of the errors introduced by insufficient sampling. Anyway, sample representativeness cannot be generalized along different habitats because of their heterogeneity (Cao *et al*., 2002). Sample coverage should always be estimated as a way of evaluating result accuracy.

We focus on the sampling and isolation protocol for bacterial endophytes associated with *Brassica napus* L. (rapeseed) (table 3.1). Rapeseed is a high biomass oil-producing crop with a good tolerance to Cd and Zn (Marchiol *et al*., 2004) and consequently a plant of interest in multiple bacterial-assisted metal phytoextraction studies and applications (Sheng and Xia, 2006; Dell'Amico *et al*., 2008; Sheng *et al*., 2008). Moreover, valorization of the oil produced from the seeds of this species may allow financial return during phytoextraction.

Table 3.1 illustrates that most studies are based on a tentative sampling instead of using a statistically justified amount of biologically independent replicates. Furthermore, in most cases 1 (mixed) plant sample is plated several times to ensure 1 consistent dilution series for further research. The use of different procedures hinders comparison of outcomes between studies.

Table 3.1 Different sampling and isolation protocols for the isolation of root endophytes associated with *B. napus*.

Sampling set-up	Surface sterilisation	Washing steps	Dilution buffer	Plating medium	Isolation	Reference
3 mixed samples each containing 2 independent roots	95% v/v ethanol (1') 0.1% w/v acidified $HgCl_2$ (1')	10x tap H_2O	1/10 (w/v) PBS	0.3% (w/v) TSB	50% of all colonies growing on 1 plate of a selected dilution (4 plates per dilution)	Germida *et al.* (1998)
4 mixed samples each containing 4 independent roots	1.05% v/v NaClO in PBS (10')	4x PBS	PBS	0.3% (w/v) TSB	50 colonies growing on 1 plate of a selected dilution (3 plates per dilution)	Siciliano and Germida (1999)
3 root samples	thoroughly rinsed in distilled H_2O		PBS	diluted 1% (w/v) TSB	all colonies growing on 1 plate of a selected dilution (2 plates per dilution)	Granér *et al.* (2003)
3 root samples	0.9% NaCl (15')		0.9% NaCl	nutrient broth agar	all different morphotypes growing on 3 plates of a selected dilution	Larcher *et al.* (2008)
root samples	75% (v/v) ethanol (2')	3x tap H_2O	distilled H_2O	sucrose-minimal SLP (with 20 mg l^{-1} Pb)	all different Pb-resistant morphotypes	Sheng *et* al. (2008)
3 root samples	75% (v/v) ethanol (2')	3x tap H_2O	distilled H_2O	1/5 LB (with 0.08 mM Cu)	100 Cu-resistant isolates	Zhuang *et al.* (2011)

The duration of surface sterilisation is presented between brackets (x' = x min). Washing solutions, buffers and media were sterilised before use. PBS: phosphate buffer saline; TSB: trypticase soy broth; SLP: salts low phosphate; LB: Luria-Bertani's. Studies represented in light grey rows are population studies, the ones in white rows are studies interested in specific root endophytes capable of the direct inhibition of a wilt pathogen (Granér *et al*., 2003) or metal-resistant (Sheng *et al*., 2008; Zhuang *et al*., 2011).

Bacteria were isolated from 1 or multiple plant samples containing 1 or more independent plant parts. All sterilised and washed samples were crushed in a buffer solution and serially diluted. Dilutions were plated only once or in more replicates on different media. Germida *et al.* (1998) and Siciliano and Germida (1999) continued to work with the plates containing 30-300 colonies and thus selected just 1 dilution. Subsequently, they numbered each bacterial colony growing on the selected plates and investigated only a portion of these colonies in more detail. Granér *et al.* (2003) and Larcher *et al.* (2008) collected all (morphologically) different bacterial strains from the selected plates. Sheng *et al.* (2008) and Zhuang *et al.* (2011) only characterised bacteria tolerant to Pb and Cu while the other studies concentrated on the total cultivable bacterial communities associated with *B. napus*, whether or not capable to directly inhibit the wilt pathogen *Verticillium longisporum* (as it was the case for Granér *et al*.

2003). Also, identification procedures differ between the studies mentioned in table 3.1; the more recent ones use 16S rDNA analysis instead of tentative FAME profile analysis (Haack *et al*., 1994).
It is obvious that there exists a high interest in plant-associated bacteria, but until now little attention has been paid to the performance of sampling and isolation procedures. Comparative studies to identify which protocols deliver the most reliable information about bacterial communities associated with plants are scarce (Hughes *et al*., 2001). In addition, the basic principles of statistical analysis and its importance are often ignored (Prosser, 2010). Therefore, the primary aim of this study was to develop and verify a sampling (i) and isolation (ii) workflow that can accomplish a representative genotypic and phenotypic characterisation of cultivable bacteria.
(i) In order to determine the number of replicates required to sufficiently cover the biological variation between bacterial communities associated with different *B. napus* plants growing on the same field site, we compared 3 replicates. To ensure an acceptable workload during the isolation and characterisation process, no additional replicates were added but the amount of individual plants used to make up a replicate was changed. Also from a statistical point of view, increasing the number of replicates is not needed.
(ii) Preliminary isolation experiments based on the procedure used for Oak and Ash (Weyens *et al.*, 2009b) showed that a less rigorous procedure is necessary for *B. napus* plant organs.
Most attention has been given to the sampling workflow, as we based the isolation technique on former successful experiments with bacterial endophytes (Mastretta *et al*., 2009; Kabagale *et al*., 2010) and soil bacteria (Thijs *et al*., 2014). Nevertheless, we have to notice that various culture media and incubation temperatures can result in different microbial numbers (Vieira and Nahas, 2005; Sun *et al*., 2006) and can lead to phenotypical divergences between populations (Sørheim *et al*., 1989). In order to isolate a wide variety of plant-associated bacteria, we used 1/10 strength rich (869) medium (Mergeay *et al*., 1985) containing various carbon and nitrogen sources as well as other essential nutrients for microbial growth. Indeed, diverse low-nutrient media improve the recovery of bacteria from natural samples (Bussman *et al*. 2001). Next to the isolation medium, also each type of strain selection, either random

or morphologically based, affects the final results. We combined both strategies by including 5 randomly selected strains from a group of morphologically similar bacterial colonies.

Results and Discussion

In order to find out if 1 replicate (composed from parts of various individual *B. napus* plants) can be sufficient to cover the variation between bacterial communities associated with individual plants growing on the same field plot, we prepared 3 replicates per plant organ (root, stem and leaf). Each replicate was composed of 3 independent plant parts. Appendix 3.1a presents the data concerning bacterial diversity and abundance in replicates 1, 2 and 3 of the studied plant organs (root (R), stem (S) and leaf (L)). Data were statistically analysed using a principal component analysis (PCA) related ordination technique based on chi-square distances, i.e. correspondence analysis (CA). Correlations between replicates and organs are represented in appendix 3.1b. All replicate bacterial communities from the root were highly correlated with the mean bacterial community calculated for the root (correlation coefficients (CC) higher than 0.80). Higher variations were found between the stem and especially the leaf replicates, resulting in low correlations with their mean bacterial community. In fact this discrepancy between roots and aerial parts is not surprising since for roots entire organs were processed and for aerial organs only segments could be used for isolation.

Based on these results, we concluded that more independent plant parts were needed to obtain a representative view on the microbial diversity present in the above-ground plant parts. We decided to increase the amount of plants used to compose 1 replicate instead of expanding the number of replicates, since the workload is proportional with each additional replicate. Root data were retained, whereas stem and leaf isolations were repeated using 6 independent plant parts per replicate. These data concerning bacterial diversity and abundance in replicates 1, 2 and 3 of the studied plant organs (root (R), stem (S) and leaf (L)) are pooled in appendix 3.2. A clearly lower and acceptable variability between stem and leaf replicates was observed and therefore we continued with these data. Figure 3.1 presents the mean diversity and abundance of the cultivable bacterial genera isolated from *B. napus* roots, stems and leaves (diagrams

Rmean, Smean and Lmean respectively). Genera isolated from each root, stem and leaf replicate are schematically shown in subfigures R, S and L of figure 3.1. Per replicate, all purified bacterial strains with their genotypic and phenotypic characteristics are listed in appendix 3.3.

Genotypic characterisation

The 3 replicates of the root-associated bacterial community (shown in appendix 3.2 as diagrams R1, R2 and R3) are similar in terms of dominant genera, but differences in their abundance were observed. The genera *Variovorax*, *Pseudomonas*, *Bacillus*, *Caulobacter*, *Pantoea* and *Labrys* were isolated from all replicates (figure 3.1R) and are also dominating genera in diagram Rmean (figure 3.1). About 94 % of this mean diagram is composed of the bacterial genera that are common between the 3 root replicates (underlined). Like for the root replicates, bacterial stem community replicates S1, S2 and S3 share dominant genera, but again their abundances were different (appendix 3.2). Genera *Frigoribacterium*, *Massilia*, *Pseudomonas*, *Aeromicrobium* and *Bacillus* were isolated from all stem replicates (figure 3.1S) and together made up 90.7 % of diagram Smean (figure 3.1). In the leaves, genera *Frigoribacterium*, *Massilia, Pseudomonas* and *Bacillus* isolated from the 3 biological replicates (figure 3.1L) represent about 90 % of the mean diagram (figure 3.1). The mean diagrams take into account the differences in abundance and by taking the mean, a more accurate picture on the diversity and evenness of the cultivable endophytes is obtained than when considering the replicates themselves. Taken together the genotypic results of all studied plant organs, it is reasonable to assume that a representative sample is obtained when more than 90 % of the genera in the mean diagram were isolated from each replicate. Most likely these genera are the dominant ones in the mean diagram that contains average abundances.

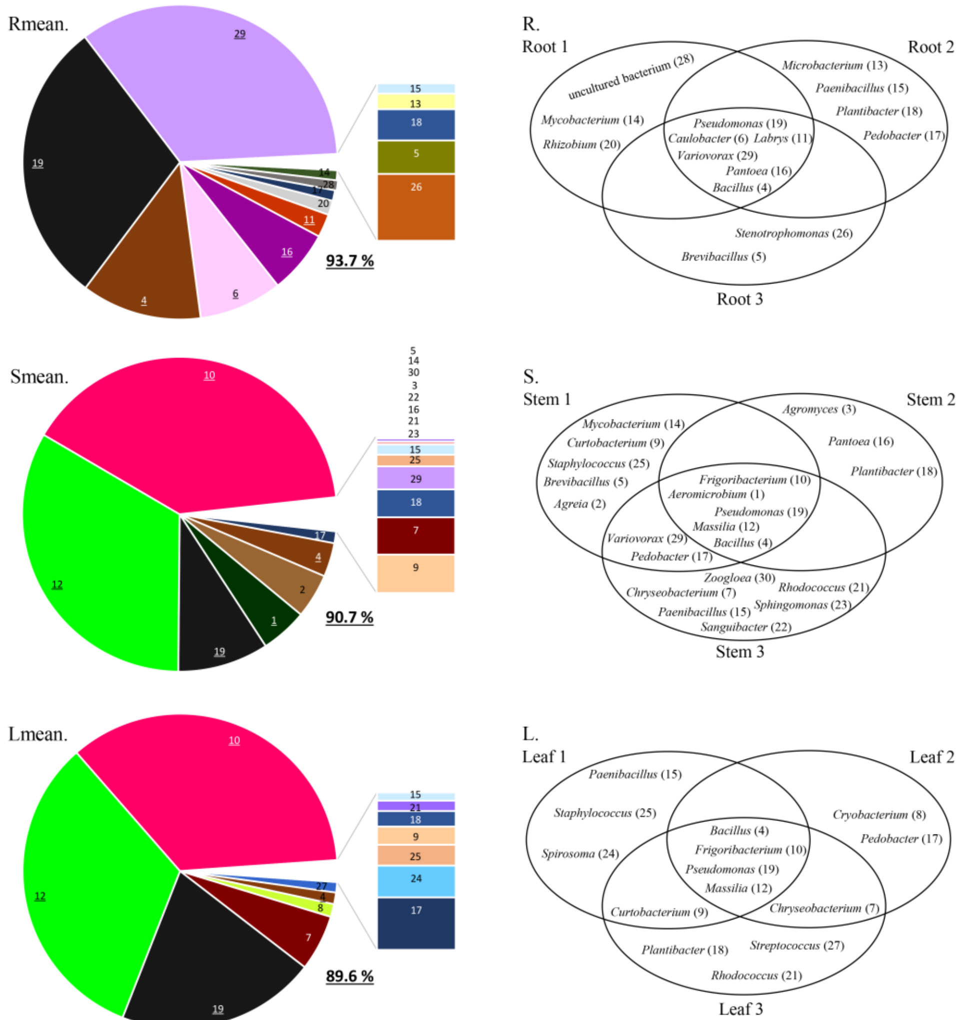


Figure 3.1 Diversity and abundance of cultivable bacterial genera isolated from field-grown *B. napus*. Diagrams Rmean, Smean and Lmean summarise the mean abundance of each genus isolated from the 3 root, stem and leaf replicates respectively (see appendix 3.2 and 3.3). Each root replicate was composed of roots from 3 independent plants, whereas stem and leaf replicates consisted of 6 independent plant parts. Diagrams R, S and L are schematic representations of bacterial genera as appearing within the 3 root, stem and leaf replicates. Each colour (number) represents a bacterial genus. Pie fragment sizes reflect the mean relative abundance, expressed in percentages, of the total number of cultivable bacterial isolates belonging to a specific genus per gram fresh weight. Details for bacterial genera with mean abundances lower than 1 % are shown separately next to the pie diagram.

Moreover, the CA in figure 3.2a shows that all replicate bacterial communities are highly correlated with the mean bacterial community per plant organ (correlation coefficients (CC) higher than 0.80).

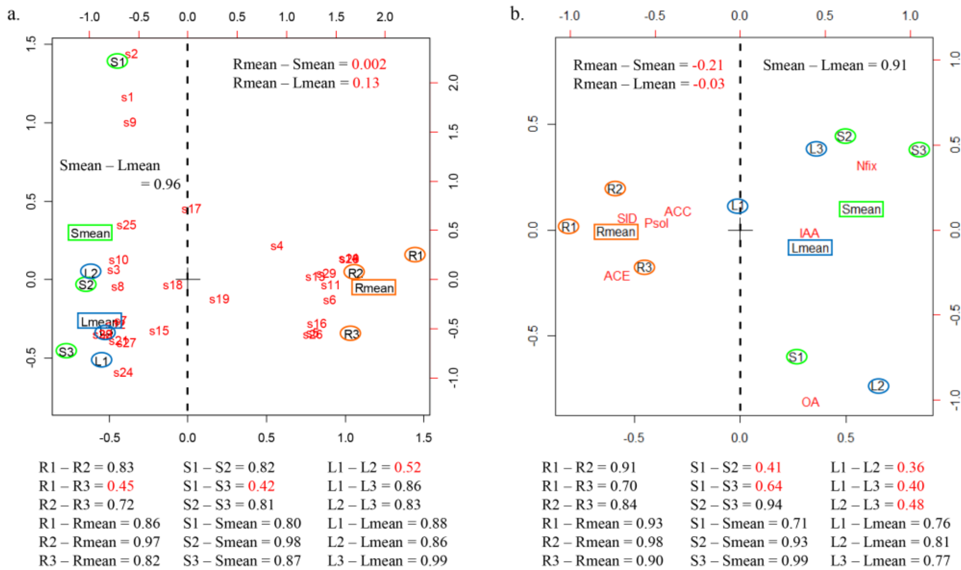


Figure 3.2 Correspondence analysis based on the genotypic (a) and phenotypic (b) data of the bacterial communities isolated from field-grown *B. napus.* Replicate (1, 2 and 3) and mean bacterial communities isolated from the roots (R), stems (S) and leaves (L) are plotted in black. Each red s-number (a) represents an isolated bacterial genus (see figure 3.1). Bacterial characteristics Psol, Nfix, SID, OA, ACC, IAA and ACE are also plotted in red (b) and represent the bacterial capability to solubilise phosphorus, fixate nitrogen and produce siderophores, organic acids, 1-aminocyclopropane-1-carboxylate deaminase, indole-3-acetic acid and acetoin respectively. Correlation coefficients are present per plant organ to indicate the correlation between the bacterial communities of the 3 replicates and their correlation with the mean bacterial community. Correlations between mean communities are also represented.

This observation also indicates that the mean root, stem and leaf bacterial communities of *B. napus* are representative for all individual plants growing on the same field. Nevertheless, it was necessary to combine the 3 replicates per plant organ since not all replicates were highly correlated (CC > 0.70). Although all replicates were sharing similar dominating genera per plant organ, the correlation coefficients between replicates R1 and R3, S1 and S3 and L1 and L2 were low (red CC in figure 3.2a). Dominant genera in these replicates showed

different abundances, suggesting that the combination of more than one replicate would be better to encompass the variation between bacterial endophytic communities of *B. napus* individuals growing on the same field.

Table 3.2 Shannon-Wiener indices (diversity and evenness) of cultivable bacterial communities isolated from field-grown *B. napus.*

	$H' = -\sum p_i(\mathit{ln}p_i)$	**$E = H'/H'max$**
Root 1	1.7138	0.7800
Root 2	1.2642	0.5490
Root 3	1.5950	0.7670
Root	**1.7595**	**0.6497**
Stem 1	1.796968	0.723153
Stem 2	1.197415	0.575835
Stem 3	1.051384	0.409904
Stem	**1.593764**	**0.523486**
Leaf 1	1.157157	0.556475
Leaf 2	1.340849	0.68906
Leaf 3	1.536711	0.699387
Leaf	**1.504976**	**0.57027**

Indices of the bacterial communities isolated from the root, stem and leaf replicates as well as the indices of the combined bacterial root, stem and leaf communities (marked in bold) are represented. The indices represent a measure for biodiversity and are calculated using the formula $H' = -\sum p_i(\mathit{ln}p_i) = -[p_1(\mathit{ln}p_1) + p_2(\mathit{ln}p_2) + p_3(\mathit{ln}p_3) + ... + p_R(\mathit{ln}p_R)]$, H' is the Shannon-Wiener index and p_i is the proportion of individuals belonging to the ith genus in the dataset of interest with R number of genera. Evenness (E) is an index that makes H' values comparable between communities (E = H'/H'max (the maximal potential evenness between genera = *ln*S)). Each root replicate was composed of roots from 3 independent plants, whereas stem and leaf replicates consisted of 6 independent plant parts.

A sufficiently large sampling is not only crucial to cover genotypic variation but also to produce a representative picture of the biodiversity since the presence of genera and their abundance can differ between replicates. In accordance with our previous results, the biodiversity indices in table 3.2 indicate that 1 replicate is not sufficient to calculate a representative Shannon-Wiener biodiversity index (H'). This index is taking into account both the genus richness and their relative abundance. However, it is tricky to compare H' between communities because

indices can be similar although relative abundances and richness of genera are different. Evenness (E) is an index that makes H' values comparable between communities by taking into account the number of genera found within the communities (S). Evenness is calculated by dividing H' by H' max (the maximal potential evenness between genera = *ln*S) and ranges from 0, where most genera are rare and just a few are abundant, to 1, where H' max equals H'. The most representative indices (H' and E) can be derived from the mean endophytic communities and are marked bold in table 3.2.

Based on these findings, we can be confident that 3 replicates are sufficient to representatively describe the genotypic characteristics of the endophytic communities associated with *B. napus*. Nevertheless, it should be noted that more individual plants are needed to make up replicates for stems and leaves (minimum 6 instead of 3 for the roots). In contrast to the roots that were entirely processed, only segments from stems and leaves were used, which impose the hypothesis that different bacterial strains are not always homogenously spread inside plant organs (justified by Fisher *et al*. (1992)).

Above- and below-ground differences

Table 3.2 illustrates that the mean bacterial communities have similar evenness factors which means that the biodiversity indices can be compared. Biodiversity decreases from root to shoot (see also the number of dominant genera in the mean diagrams of figure 3.1). In the root, 6 genera accounted for 90 % of the mean diagram while this was 5 for the stem and 4 for the leaf. The latter might also be due to the fact that more cultivable bacteria could be isolated from roots than stems and leaves (figure 3.3). It indeed is known that endophytes may spread systemically inside the plant (Compant *et al*., 2010), whereby bacterial density and diversity decrease from the rhizosphere to the aerial parts (Fisher *et al*., 1992), which was also observed in our study (figure 3.3, table 3.2). This suggests that the root should be the principal organ for bacterial entry from the environment in the plant. The 2 mean cultivable bacterial communities from stems and leaves were highly correlated (CC: 0.96), whereas the mean community of the root (Rmean) was different from those of the aboveground plant parts (CC Smean: 0.002 and CC Lmean: 0.13) (figure 3.2a).

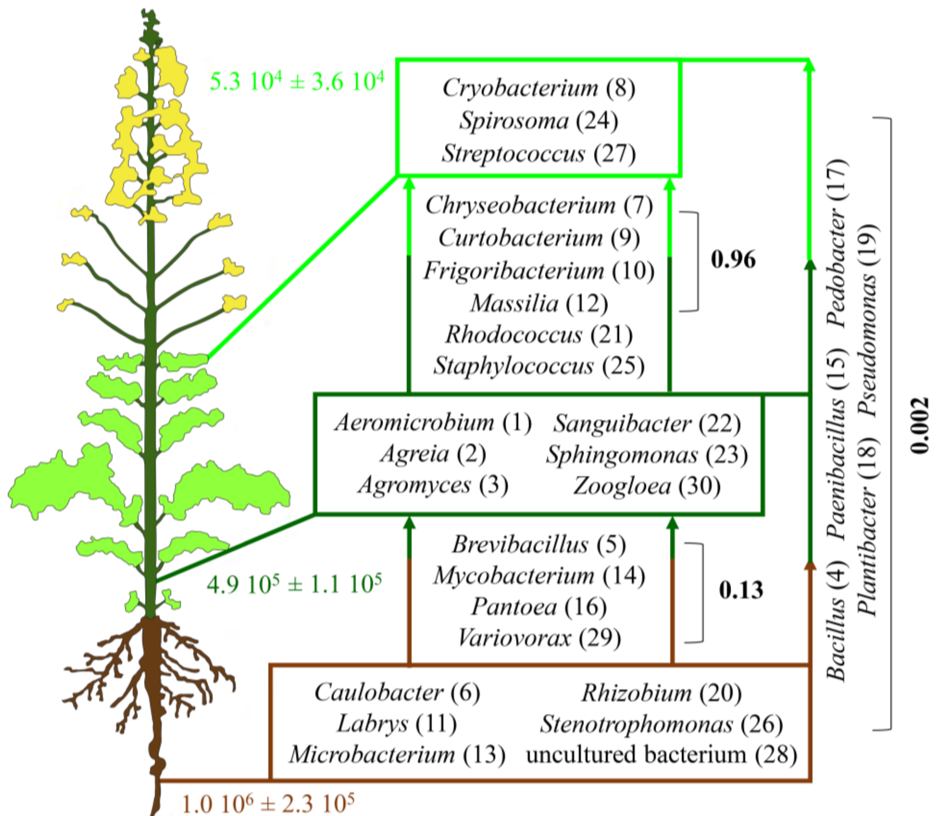


Figure 3.3 Diversity of cultivable bacterial genera associated with roots, stems and leaves of field-grown *B. napus.* Bacterial genera in the brown frame were only isolated from the roots, in the dark green frame only from the stems and in the light green frame only from the leaves. Genera between 2 frames were found in both adjacent plant organs. *Bacillus, Paenibacillus, Pedobacter, Plantibacter* and *Pseudomonas* species were occurring throughout the entire plant. The relation between plant organs is indicated with correlation coefficients and mean total numbers of colony forming units (cfu) of 3 replicates calculated per gram fresh weight root, stem and leaf are represented ± standard error.

Obvious differences in the 'habitat conditions' in respectively above-ground and below-ground plant organs might explain the differences observed between the leaf/stem and root bacterial communities. For example, Lindow and Brandl (2003) reported that pigmented bacteria dominate leaf surfaces whereas common root colonizers such as *Rhizobium* and *Azospirillum* fail to establish on/in leaves. Stout (1960) raised that despite the close physical proximity of the leaf/stem and soil bacterial communities each preserves its integrity and its distinctive taxonomic and physiological character. Izumi *et al.* (2008) formulated

a similar conclusion concerning the diversity of endophytic bacteria residing in root, stem and leaf tissues of coniferous and deciduous tree species. Using cultivation-dependent and -independent methods, significantly different bacterial communities below-ground (roots and rhizosphere) and above-ground (leaves and stems) were observed in the host trees.

Figure 3.3 demonstrates that some bacterial genera associated with *B. napus* are typically occurring in roots, stems or leaves while other genera do not show specific habitat preference and appear in more than 1 of the investigated organs. *Bacillus, Paenibacillus, Pedobacter, Plantibacter* and *Pseudomonas* species were isolated from all organs. Examples of most dominating species that are exclusively present in the roots, shoots and leaves are respectively *Caulobacter* and *Labrys*, *Aeromicrobium* and *Agreia*, and *Cryobacterium* and *Streptococcus*.

Phenotypic characterisation

Phenotypic data were used to verify the correspondence between the replicate bacterial communities per organ and their calculated mean community (figure 3.2b). The correspondence analysis shows that all replicate bacterial communities are highly correlated with the mean bacterial community per plant organ by means of correlation coefficients (CC) higher than 0.70. These results assume that the mean phenotypic data of the root, stem and leaf bacterial communities of *B. napus* are representative for all individual plants growing on the same field. For these data, more than for the genotypic data, it was necessary to combine the 3 replicates per plant organ since not all replicates were highly correlated (CC > 0.70). Mainly the phenotypic characteristics of the bacteria living in the aboveground plant parts are variable (red CC in figure 3.2b), suggesting that the combination of all replicates is better to encompass the phenotypic variation between bacterial endophytic communities of *B. napus* individuals growing on the same field.

Table 3.3 Phenotypic characterisation of cultivable bacterial communities isolated from field-grown *B. napus.*

	SID	OA	ACC	IAA	ACE	P sol	N_2 fix
Root 1	73.8	3.8	10.8	15.6	21.7	44.2	5.5
Root 2	93.0	1.3	32.5	35.3	6.5	50.2	15.2
Root 3	75.6	26.8	56.1	58.3	22.9	56.0	5.5
Root Mean	**80.8**	**10.6**	**33.1**	**36.4**	**17.0**	**50.1**	**8.7**
Root Error	*6.1*	*8.1*	*13.1*	*12.3*	*5.3*	*3.4*	*3.2*
Stem 1	16.2	30.4	5.5	41.4	9.3	8.4	16.7
Stem 2	11.1	1.3	9.6	37.0	1.2	10.9	43.3
Stem 3	5.4	4.7	4.5	89.4	0.6	5.0	71.3
Stem Mean	**10.9**	**12.1**	**6.5**	**56.0**	**3.7**	**8.1**	**43.7**
Stem Error	*3.1*	*9.2*	*1.6*	*16.8*	*2.8*	*1.7*	*15.7*
Leaf 1	25.4	5.7	54.6	70.0	0.7	11.5	14.1
Leaf 2	6.0	52.9	0.0	74.2	1.5	12.7	28.9
Leaf 3	20.5	3.1	9.9	51.1	0.8	26.3	55.7
Leaf Mean	**17.3**	**20.6**	**21.5**	**65.1**	**1.0**	**16.9**	**32.9**
Leaf Error	*5.8*	*16.2*	*16.8*	*7.1*	*0.3*	*4.8*	*12.2*

Data were obtained by calculating total numbers of cultivable bacterial strains per gram fresh weight and per replicate that were capable of phosphorus solubilisation (P sol), nitrogen fixation (N_2 fix) and the production of siderophores (SID), organic acids (OA), 1-aminocyclopropane-1-carboxylate deaminase (ACC), indole-3-acetic acid (IAA) and acetoin (expressed as percentages). Mean relative abundances of positive testing strains, expressed as percentages with standard error, are shown per plant organ (root, stem and leaf). Each root replicate was composed of roots from 3 independent plants, whereas stem and leaf replicates consisted of 6 independent plant parts.

Table 3.3 summarises the plant growth stimulating characteristics, such as nitrogen fixation, phosphorus solubilisation, and the production of siderophores, organic acids, 1-aminocyclopropane-1-carboxylate (ACC) deaminase and phytohormones (i.e. indol-3-acetic acid (IAA) and acetoin) (Compant *et al*., 2005; Weyens *et al*., 2009a) of the bacterial communities isolated in this study. Indeed, many endophytes are members of common soil bacterial genera (Lodewyckx *et al*., 2002), well known for their beneficial effects on overall plant growth and establishment (Joshi and Bhatt, 2011).

More than half of the isolated endophytic strains per organ from *B. napus* can produce siderophores while only a low percentage of endophytes can produce organic acids (table 3.3). The statistical principal component based test (figure 3.2b) suggests that bacteria occurring in the roots often are able to produce

siderophores whereas the production of organic acids seems more common for endophytes from the aboveground plant parts. Both characteristics may contribute to an increased nutrient uptake. A high occurrence of siderophore producing at the root level is not surprising, since, in function of phytoextraction, high levels of siderophores at root level might lead to an increased uptake of (toxic) metals (Weyens *et al*., 2009a; Li and Wong, 2010; Rajkumar *et al*., 2010). Furthermore, about 40 % of the isolated endophytes possess the capacity to solubilise phosphorus and/or produce the plant hormone IAA (table 3.3). Phosphorus solubilizing bacteria were predominantly present in the roots (were phosphorous uptake takes place) while IAA producing bacteria were mainly found in the aboveground plant parts (figure 3.2b). The bacterial production of acetoin (a volatile plant growth regulator) was low throughout the plant but highest in the roots (table 3.3 and figure 3.2b). One fourth of the endophytic cultivable strains were able to produce ACC deaminase. This ACC deaminase can decrease stress-induced ethylene release in plants and consequently improve plant growth during stress conditions like for example exposure to toxic metals (Arshad *et al*., 2007) and other forms of abiotic and biotic stress (Gamalero and Glick, 2012). The numbers of these potential stress reducing bacteria were the highest in the roots (figure 3.2b). This might be related to the fact that ACC is produced in the roots and transported to the shoot where it is converted into ethylene (Else and Jackson, 1998). Indeed, under metal or drought stress, ACC transported in the xylem sap might result in an increased production of ethylene in the aerial parts (Mertens *et al*., 1999; Sobeih *et al*., 2004). By consequence, a high rate of deamination of ACC at the root level will result in a reduced transport of this compound to the aerial parts, leading to a reduced stress ethylene production. The trait of nitrogen fixation was expressed in one fifth of the isolated strains and especially in the shoot of *B. napus* plants; this is statistically supported in figure 3.2b.

Conclusions

An important conclusion from this study is that the use of 3 replicates is necessary to obtain a representative genotypic picture of the cultivable endophytic bacteria associated with root, stem and leaves of field-grown *B. napus*. Although figure 3.1 and appendix 3.2 suggest that only 1 replicate could

be sufficient (since most of the replicates are similar to the mean diagram), the statistical analysis in figure 3.2a clearly indicates that more replicates are necessary to obtain a representative picture, especially with regard to the abundances of the different genera. Also the diversity data in table 3.2 confirm the necessity of replicates to acquire relevant diversity and evenness indices.

We propose the use of 3 replicates since the genera isolated from all root, stem and leaf replicates made up at least 90 % of the mean diagrams for each plant organ (figure 3.1). Such a mean diagram takes into account the differences in abundance and by taking the mean of 3 replicates a more representative picture is obtained of the diversity and evenness of the cultivable endophytes than from each separate replicate (table 3.2). A further increase in replicates would lead to an enhanced workload and most likely will produce very similar genotypic results. Also from a statistical point of view, 3 replicates are sufficient to work with. In order to make replicates more representative, extra independent plants can be included. To compose a root replicate, 3 complete root systems were used, whereas for the stem and leaf replicates only fragments of the plant organ were collected per considered plant. The fact that more individual plants were needed to compose representative stem and leaf samples supports our hypothesis that different bacterial strains are not homogenously spread inside plant organs.

Despite the variation between replicates concerning phenotypic characteristics (table 3.3), some remarkable tendencies could be observed when the data were plotted in a correspondence analysis using the genotypic (figure 3.2a) and phenotypic (figure 3.2b) characteristics of the bacterial communities. Mean bacterial communities isolated from stems and leaves were highly correlated based on the genotypic as well on the phenotypic data. The characteristics of the bacterial community associated with the roots of *B. napus* plants obviously differed from those in the aboveground plant parts.

In this study, we chose to test sample representativeness with a correspondence analysis (CA) since 3 replicates render non-interpretable accumulation curves (Gotelli and Colwell, 2001; Hughes and Hellmann, 2005). Estimating sample coverage was also difficult to apply on our dataset since very high amounts of colony forming units per gram fresh weight (FW) were counted (see appendix 3.3). Full coverage was already reached by counting fewer colonies than

replicates contained. Furthermore, we want to highlight the importance of standardized sampling protocols although we have to be aware of the heterogeneity of habitats that make generalizations inappropriate. That is why we insist to always check replicate variability with the help of correspondence analysis.

The future objective of our study is to select a strain or consortium from these cultivable communities that can be inoculated to enhance metal uptake and/or biomass production and thus phytoextraction by rapeseed. In this respect it might be relevant to concentrate on *Bacillus* and *Pseudomonas* strains since they seem able to colonize all plant organs (figure 3.3) of most individual *B. napus* plants (figure 3.1).

Experimental Procedures

Experimental set-up

In order to isolate the cultivable bacterial community associated with *B. napus*, plants were sampled after the flowering stage (June 2010) in the field. Sampling was performed on a light loamy field in Alken (Belgium) with an organic matter content of 1.9 % and a potential soil pH of 5.8, subdivided into 3 subplots (figure 3.4).

Three plants (1 plant from each subplot, standing approximately 2 m apart from each other in a row) were combined to prepare root replicates. Sampling was repeated 3 times using each time 3 other plants of circa 1.3 m high (1 per subplot) in order to obtain 3 replicates (biological replicates). Three stem and leaf replicates were obtained in the same way, but using 2 plants from each plot (for replicate 1 plants 1 and 1', for replicate 2 plants 2 and 2' and for replicate 3 plants 3 and 3'). In this way each stem and leaf replicate contained 6 independent plant parts. Root samples were submerged in sterile Falcon tubes (50 ml) filled with 20 ml sterile 10 mM $MgSO_4$ to keep the tissue hydrated. Stem and leaf samples were stored in closed Falcon tubes (50 ml).

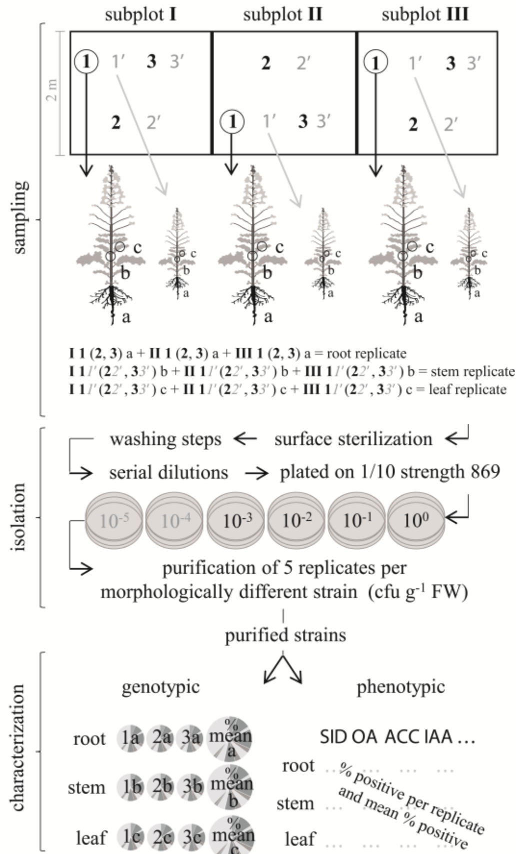


Figure 3.4 Workflow with sampling, isolation and characterisation procedures. Sampling started with subdividing the field into 3 subplots (I, II and III). Subsequently, 3 individual *B. napus* plants (± 1.3 m high) per subplot (plant 1, 2 and 3) were selected randomly. Plants with a specific number made up 1 root, stem and leaf replicate. In this way 3 root, stem and leaf replicates were obtained. For stem and leaf samples, 3 additional independent plant parts were added to each replicate (plants with number 1′ were used to complete stem and leaf mixed sample 1). After isolation all plated dilutions were considered and per morphologically different bacterial strain 5 replicates were purified. All colony forming units (cfu) per morphologically different strain were counted per gram fresh weight (g FW). Purified strains were subjected to 16S rDNA analysis and plant growth promoting capacity tests (solubilisation of phosphates (P sol), nitrogen fixation (N_2 fix) and production of siderophores (SID), organic acids (OA), indol-3-acetic acid (IAA), acetoin (ACE) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase). Percentages of genotypically different bacterial strains were calculated per replicate in order to acquire 3 independent community diagrams per plant organ (root, stem and leaf). Also means were calculated to obtain detailed diagrams. The phenotypic characterisation table consists of percentages of positive strains per replicate and mean percentages of positive strains per plant organ.

Isolation of B. napus-associated bacteria

Approximately entire roots were used for bacterial isolation, while only 3 stem segments per plant were harvested. Segments were 3 cm long and coming from the lower, middle and upper part of the plant. Leaf samples consisted of the central part of 6 leaves positioned in the middle of the plants. Sampling was performed within a period of 1 month, just before harvest in July.

Before isolation of the cultivable endophytic bacteria from pieces of roots, stems and leaves of *B. napus*, the tissue was surface sterilised for 1 min in a 1 % (roots) and a 0.1 % (stems and leaves) active chloride solution supplemented with 1 droplet Tween 80 (Merck) per 100 ml solution. Sterilised tissues were subsequently rinsed 3 times for 1 min in sterile distilled water. The third rinsing water was plated on 896 solid medium (Mergeay *et al*., 1985) and checked after a 7 days incubation period at 30°C. Surface sterilisation was considered to be successful in case no bacterial colonies were observed. After drying the plant tissue (3 g) on sterile paper filters, it was macerated during 1 min in 10 ml sterile 10 mM $MgSO_4$ using a Polytron PR1200 mixer (Kinematica A6). The obtained mixtures were used to make serial dilutions (roots up to 10^{-5}, stems up to 10^{-4} and leaves up to 10^{-3}) that were plated on 1/10 strength 869 solid medium. All plated samples were incubated for 7 days at 30°C; after this incubation period colony forming units (cfu) were counted and calculated per gram plant fresh weight. Morphologically different colonies were purified using 5 replicates and subsequently stored at -70°C in a glycerol solution (15 % (w:v) glycerol; 0.85 % (w/v) NaCl) for further analysis. This complete isolation procedure was performed on 3 biologically independent replicates per plant organ (figure 3.4). All purified strains were subjected to 16S rDNA (genotypical) and phenotypical analysis. Percentages of genotypic/phenotypic different bacterial strains were calculated per replicate, means per plant organ were calculated to produce detailed composite diagrams and tables.

Genotypic characterisation

Total genomic DNA was extracted from all purified morphologically different bacterial strains using the DNeasy 96 Blood & Tissue Kit (Qiagen, Valencia, CA, USA) following the protocol for gram positive bacteria (Croes *et al*., 2013). The quantity and quality of the extracted DNA were determined using a Nanodrop

ND-1000 spectrophotometer (Isogen Life Science). Polymerase chain reaction (PCR) amplification of the 16S rRNA genes was performed on aliquots of the extracted DNA using the universal primers, 16S-1392-R (5'-ACGGGCGGTGTGTRC-3') and 16S-27-F (5'-AGAGTTTGATCCTGGCTCAG-3') (Weyens *et al*., 2009b).

For amplified 16S rDNA restriction analysis (ARDRA), 20 µl of the PCR products were digested with the HpyCH4IV enzyme and visualized by gel electrophoresis (Weyens *et al*., 2009b). Bacterial strains with the same ARDRA patterns were manually grouped. The PCR products of 1 representative strain per group were purified according to the QIAquick 96 PCR Purification Kit (Qiagen, Valencia, CA, USA). Subsequently, purified 16S rRNA genes were sequenced by Macrogen (Korea) under BigDye^{TM} terminator cycling conditions with an Automatic Sequencer 3730XL. After constructing the consensus sequences with Staden Package, sequence match at the Ribosome Database Project II (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) was used for nearest neighbour and species identification (Maidak *et al*., 2001). Sequences were online aligned using Clustal Omega and used for constructing a neighbour-joining tree, using default settings (Sievers and Higgins, 2014).

Phenotypic characterisation

All isolated and purified bacterial strains were screened for their potential plant growth promoting characteristics (solubilisation of phosphates, nitrogen fixation and production of siderophores, organic acids, indol-3-acetic acid (IAA), acetoin and 1-aminocyclopropane-1-carboxylate (ACC) deaminase). Bacterial strains were grown in 869 medium and subsequently washed twice with 10 mM $MgSO_4$ to obtain bacterial suspensions which were used for the screening tests. Mixtures without bacterial cell suspension were used as controls.

Solubilisation of phosphates

National Botanical Research Institute's phosphate (NBRIP) growth medium was used for screening phosphate-solubilizing microorganisms (Nautiyal, 1999) (per liter: 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ and 15 g agar (pH = 7.0)). Aliquots of 50 µl of the bacterial suspensions were inoculated in holes (Ø: 0.5 cm) made in the solid

medium and incubated for 7 days at 30°C. Bacterial strains capable of producing a halo/clear zone around the hole, owing to the production of organic/inorganic acids, were considered positive.

Nitrogen fixation

Bacterial strains were tested for nitrogenase activity during an incubation period of 5 days at 30°C in a semi-solid malate-sucrose medium (LMM) modified from Döbereiner (1989) (per liter: 10 g sucrose, 5 g L-malic acid, 0.2 g $MgSO_4.H_2O$, 0.01 g $FeCl_3$, 0.1 g NaCl, 0.02 g $CaCl_2.2H_2O$, 0.1 g K_2HPO_4, 0.4 g KH_2PO_4, 0.002 g $Na_2MoO_4.H_2O$). An amount of 3 ml bromothymol blue (0.5 g dissolved in 53 ml 95 % EtOH and 47 ml distilled water) per liter medium was added as a pH indicator, pH was adjusted to 7.2 with 1 N NaOH and 1.75 g agar per liter was added. Anaerobic growth conditions were established in sterile closed Eppendorf tubes. Bacterial nitrogenase activity was inspected visually as a colour change of the growth medium from blue to yellow which indicates the acidification of sugars (Nabti *et al*., 2007). Strains that were not capable of growing on LMM medium with nitrogen source (per liter: 0.12 g NH_4Cl) were considered as not detectable (nd).

Production of siderophores, organic acids, IAA, acetoin and ACC deaminase

Siderophore secretion by strains was qualitatively detected by the "universal" colorimetrical method of Schwyn and Neilands (1987). Bacterial strains were inoculated in 800 µl selective 284 medium with addition of a carbon mix and 0, 0.25 and 3 µM iron (Fe), as we were interested in the bacterial production of siderophores during deficient (0 µM Fe) and optimal/realistic Fe conditions in the rhizosphere (0.25 µM). The last condition with an excess of Fe (3 µM Fe) served as a blank. After an incubation period of 5 days at 30°C, 100 µl of a chrome azurol S (CAS) shuttle solution (per ml: 150 µl 10 mM hexadecyl-trimethyl-ammonium bromide (HDTMA), 375 µl 10 mM HCl, 37.5 µl 0.1 mM $FeCl_3$, 187.5 µl 2 mM CAS, 750 µl 0.02 mM piperazine and 250 µl 40 mM 5-sulphosalicidic acid) was added as a colorimetric indicator. Bacterial strains were considered as positive for siderophore production when a change in the dye colour from blue to orange was observed.

Strains were tested for **organic acid production** according to the method of Cunningham and Kuiack (1992). Bacterial strains were inoculated in 800 µl sucrose tryptone (ST) medium. After 5 days of growth at 30°C, 100 µl alizarine red S 0.1 % was added as a colorimetric indicator. Positive strains induced a change in the dye colour from pink to yellow.

Bacterial **IAA production** capacity was tested in 1 ml 1/10 strength 869 medium supplemented with 0.5 g l^{-1} tryptophan. After an incubation period of 5 days at 30°C, growth media were centrifuged for 30 min at 4000 rpm and 0.5 ml supernatant was added to 1 ml Salkowski reagent (Gordon and Weber, 1951). Strains capable to produce IAA induced a colour change of the Salkowski reagent from yellow to pink.

To detect bacterial strains that utilize the butylene glycol pathway and **produce acetoin**, washed bacterial cells were inoculated in Methyl Red-Voges Proskauer (MRVP) medium containing per liter 17 g MRVP medium (Sigma-Aldrich) (Romick and Fleming, 1998). After an incubation period of 2 days at 30°C, 100 µl supernatant was added to 10 µl L-arginine (10 mg ml^{-1}), which solution was supplemented with 10 µl 5 % (w/v) α-naphtol and 25 µl 40 % KOH. A pink-to-red colour development during an aerobic vortex period of 30 min indicated a positive test.

The **ACC deaminase activity** was determined by monitoring the amount of α-ketobutyrate generated by the enzymatic hydrolysis of ACC (Saleh and Glick, 2001). Washed bacterial pellets were resuspended in 1 ml salts minimal (SM) medium (Belimov *et al.*, 2005) with 10 mM ACC as sole nitrogen source. Subsequently, bacterial cells were incubated for 3 days at 30°C, centrifuged (as indicated above), resuspended in 0.5 ml Tris-HCl buffer (pH 8.5) and disrupted by addition of 20 µl of toluene and vigorously vortexing. An amount of 100 µl broken cell suspension was added to 10 µl 0.5 M ACC and 100 µl 0.1 M Tris-HCl buffer (pH 8.5), and incubated for 45 min at 30°C. Possible ACC deaminase activity was stopped by adding 0.5 ml 0.56 N HCl. Subsequently, a mixture of 400 µl 0.56 N HCl, 150 µl 0.2 % 2.4-dinitrophenylhydrazine in 2 N HCl and 500 µl supernatant, obtained by centrifugation of the previous mixture (as indicated above), was incubated during 45 min at 30°C. Bacterial strains which induce a colour change from yellow to brown after addition of 1 ml 2 N NaOH were considered ACC deaminase producing (Belimov *et al.*, 2005).

Strains not able to grow in the test media were considered as not detectable (nd).

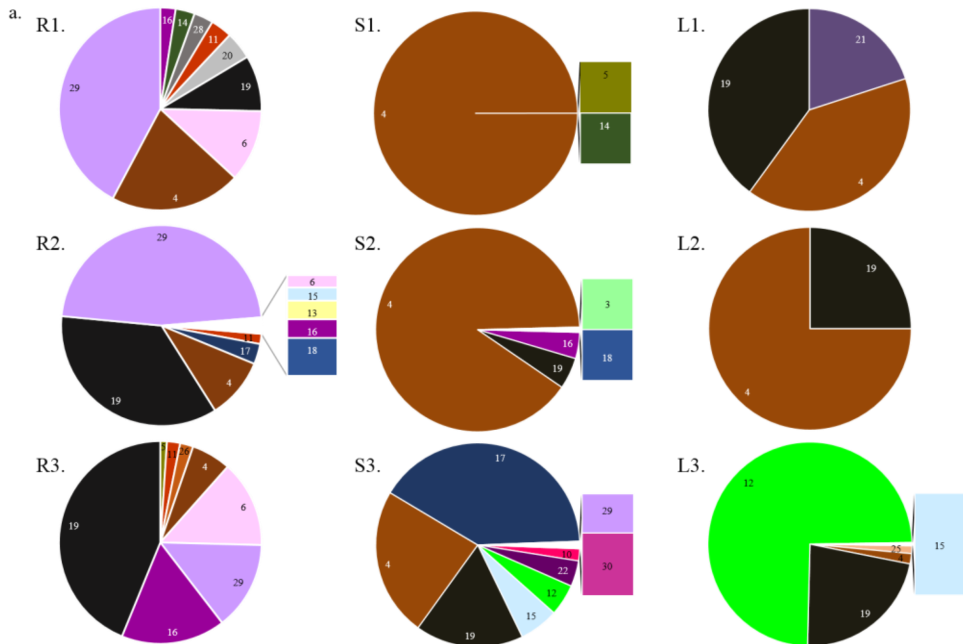
Statistical Analysis

Percentages of genotypic different bacterial strains and phenotypic different bacterial strains were calculated per replicate and means were calculated from the 3 replicates per plant organ (root, stem and leaf). The combined data per organ created a comprehensive picture of the cultivable bacterial communities and their growth promoting capacities but were not appropriate for univariate statistical analysis. Genotypic en phenotypic information of all replicates was analysed using correspondence analysis (CA). By applying this principal component analysis (PCA) related ordination technique based on chi-square distances, correlations between replicates and their mean could be found.

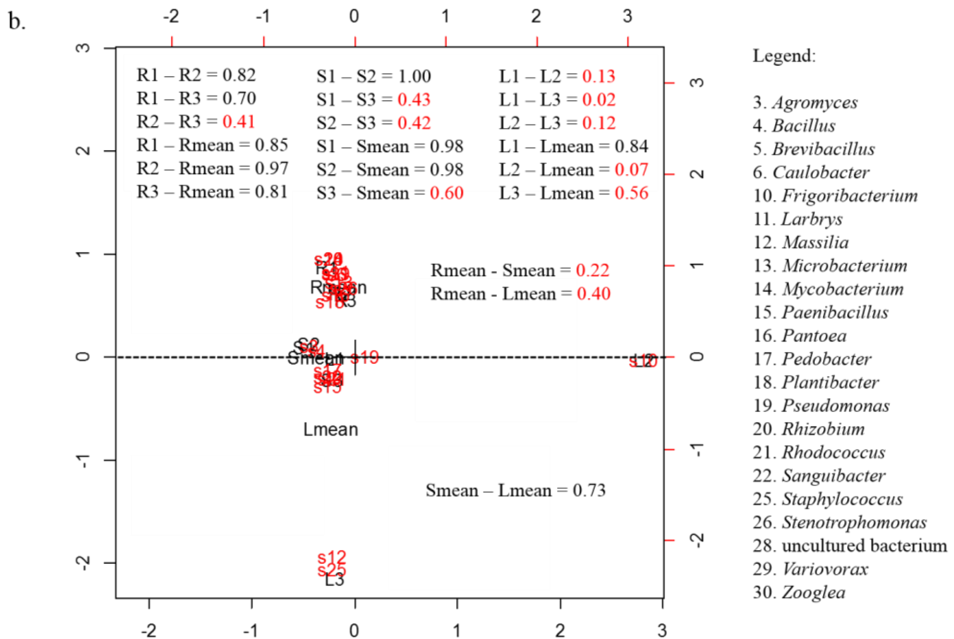
Supporting Information

Appendix 3.1

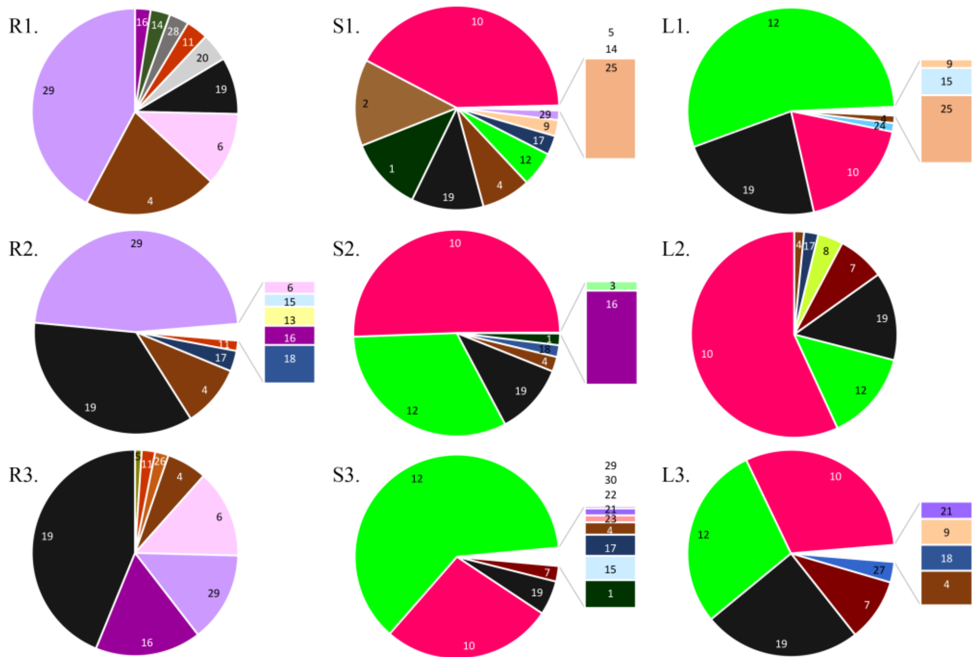
(a) Diversity and abundance of cultivable bacterial genera isolated from 3 root, stem and leaf replicates, each containing 3 independent plant parts of field-grown *B. napus*. Diagrams R (1, 2 and 3), S (1, 2 and 3) and L (1, 2 and 3) represent bacterial diversity and abundance in root, stem and leaf replicates 1, 2 and 3 respectively. Each colour (number) represents a bacterial genus (see legend appendix 3.1b). Pie fragment sizes reflect the relative abundance, expressed in percentages, of the total number of cultivable bacterial isolates belonging to a specific genus per gram fresh weight. Details for bacterial genera with abundances lower than 1 % are shown separately next to the pie diagram.



(b) Correspondence analysis based on the genotypic data of the isolated bacterial communities (isolated from 3 root, stem and leaf replicates, each containing 3 independent plant parts of field-grown *B. napus*). Replicate (1, 2 and 3) and mean bacterial communities isolated from the roots (R), stems (S) and leaves (L) of field-grown *B. napus* are plotted in black. Each red s-number represents an isolated bacterial genus (see legend). Correlation coefficients are present per plant organ to indicate the correlation between the bacterial communities of the 3 replicates and their correlation with the mean bacterial community. Correlations between mean communities are also represented.



Appendix 3.2 Diversity and abundance of cultivable bacterial genera isolated from 3 root, stem and leaf replicates of field-grown *B. napus*. Diagrams R (1, 2 and 3), S (1, 2 and 3) and L (1, 2 and 3) represent bacterial diversity and abundance in root, stem and leaf replicates 1, 2 and 3 respectively. Each root replicate was composed of roots from 3 independent plants, whereas stem and leaf replicates consisted of 6 independent plant parts. Each colour (number) represents a bacterial genus (see figure 3.1). Pie fragment sizes reflect the relative abundance, expressed in percentages, of the total number of cultivable bacterial isolates belonging to a specific genus per gram fresh weight. Details for bacterial genera with abundances lower than 1 % are shown separately next to the pie diagram.



Appendix 3.3 Detailed characterisation of all purified strains isolated from field-grown *B. napus* plant tissues (root, stem, leaf). The presence of each strain is shown as relative abundances, expressed in percentages, of the total number of colony forming units per gram fresh weight (cfu gFW^{-1}). Strains are identified to the genus level, their accession numbers as well as their presence in the 1st, 2nd or 3rd replicate (repl) are displayed (each root replicate was composed of roots from 3 independent plants, whereas stem and leaf replicates consisted of 6 independent plant parts). Their potential plant growth promoting (PGP) characteristics are indicated by + when positive and by ++(+) in case of a strong positive test. Bacterial strains testing negative for a phenotypic test were labeled by a – symbol and those not applicable for the test by 'not detected' (nd). Strains were screened for the capacity to solubilise phosphorus (P sol), fixate nitrogen (N_2 fix) and produce siderophores (SID), organic acids (OA), ACC deaminase (ACC), indole-3-acetic acid (IAA) and acetoin (ACE).

ROOT											
repl.	cfu g FW^{-1}	%	identification	accession	SID	OA	ACC	IAA	ACE	P sol	N_2 fix
1	3258	0.31	*Bacillus*	HG942090	++	-	-	-	+++	-	-
1	3258	0.31	*Bacillus*	HG942091	-	-	-	+	-	-	+++
1	3258	0.31	*Bacillus*	HG942095	+	-	-	-	+++	-	-
1	14480	1.36	*Bacillus*	HG942095	-	nd	-	-	nd	-	-
1	32580	3.06	*Bacillus*	HG942095	+	+	-	-	+++	-	-
1	97739	9.18	*Bacillus*	HG942095	+	-	-	-	+++	+	-
1	3258	0.31	*Bacillus*	HG942097	+	-	+	-	+++	+++	++
1	32580	3.06	*Bacillus*	HG942097	+	-	-	-	+++	+	-
1	32580	3.06	*Bacillus*	HG942097	+	-	-	-	+++	++	-
1	3258	0.31	*Caulobacter*	HG942105	-	-	-	-	-	-	-
1	3258	0.31	*Caulobacter*	HG942105	-	-	-	-	-	++	-
1	3258	0.31	*Caulobacter*	HG942105	-	-	-	-	-	+++	-
1	3258	0.31	*Caulobacter*	HG942105	-	-	-	+	++	-	-
1	6516	0.61	*Caulobacter*	HG942105	-	nd	-	-	-	-	-
1	6516	0.61	*Caulobacter*	HG942105	-	nd	-	-	-	++	-
1	7819	0.73	*Caulobacter*	HG942105	-	-	-	-	-	-	-
1	7819	0.73	*Caulobacter*	HG942105	-	-	-	-	-	-	-
1	7819	0.73	*Caulobacter*	HG942105	-	-	-	+	-	++	-
1	7819	0.73	*Caulobacter*	HG942105	-	-	-	-	+++	-	-
1	32580	3.06	*Caulobacter*	HG942105	-	-	-	-	-	-	-
1	32580	3.06	*Caulobacter*	HG942105	-	-	-	-	nd	-	-
1	3910	0.37	*Labrys*	HG942114	-	nd	nd	++	nd	-	-
1	32580	3.06	*Labrys*	HG942114	-	nd	nd	-	nd	nd	-
1	32580	3.06	*Mycobacterium*	HG942122	+	-	-	-	-	-	-
1	26064	2.45	*Pantoea*	HG942128	++	-	+	++	-	-	-
1	5701	0.54	*Pseudomonas*	HG942136	+	-	-	-	-	+	-
1	19548	1.84	*Pseudomonas*	HG942137	+	-	+	+	-	+	-

1	19548	1.84	*Pseudomonas*	HG942137	+	-	+	+	-	+	+++
1	3258	0.31	*Pseudomonas*	HG942146	+	-	-	-	-	+	+++
1	3910	0.37	*Pseudomonas*	HG942146	+	+	+	+	-	+	-
1	6516	0.61	*Pseudomonas*	HG942146	+	-	+	++	-	-	-
1	6516	0.61	*Pseudomonas*	HG942146	+	-	+	+	-	++	-
1	3258	0.31	*Pseudomonas*	HG942148	+	-	+	++	-	-	-
1	3258	0.31	*Pseudomonas*	HG942148	+	-	+	++	-	+	-
1	3258	0.31	*Pseudomonas*	HG942148	+	-	+	++	-	+++	-
1	5701	0.54	*Pseudomonas*	HG942148	+	-	+	++	-	+++	-
1	14480	1.36	*Pseudomonas*	HG942148	+	-	+	-	++	+++	-
1	14480	1.36	*Rhizobium*	HG942151	-	-	-	-	-	-	+
1	32580	3.06	*Rhizobium*	HG942151	+	-	-	++	-	+	-
1	3910	0.37	uncultured bact	HG942165	+	+	-	-	-	++	-
1	14480	1.36	uncultured bact	HG942165	-	-	-	-	-	+	-
1	14480	1.36	uncultured bact	HG942164	-	nd	nd	+	nd	nd	-
1	3910	0.37	*Variovorax*	HG942167	+	-	-	-	-	-	-
1	14480	1.36	*Variovorax*	HG942168	+	-	-	-	-	++	++
1	3258	0.31	*Variovorax*	HG942169	+	-	-	-	-	-	-
1	3258	0.31	*Variovorax*	HG942169	+	-	-	-	-	-	-
1	3258	0.31	*Variovorax*	HG942169	+	-	-	+	-	-	-
1	3258	0.31	*Variovorax*	HG942169	+	-	-	-	-	+++	-
1	3258	0.31	*Variovorax*	HG942169	+	-	-	-	-	+++	-
1	3258	0.31	*Variovorax*	HG942169	+	-	-	-	-	+++	-
1	5213	0.49	*Variovorax*	HG942169	+	-	-	-	-	-	-
1	5213	0.49	*Variovorax*	HG942169	+	-	-	-	-	-	-
1	5701	0.54	*Variovorax*	HG942169	+	-	-	-	-	+	-
1	5701	0.54	*Variovorax*	HG942169	+	-	-	-	-	+	-
1	6516	0.61	*Variovorax*	HG942169	+	-	-	-	-	-	-
1	6516	0.61	*Variovorax*	HG942169	+	-	-	-	-	-	-
1	6516	0.61	*Variovorax*	HG942169	+	-	-	-	-	-	-
1	6516	0.61	*Variovorax*	HG942169	+	-	-	-	-	-	-
1	6516	0.61	*Variovorax*	HG942169	+	-	-	-	-	++	-
1	14480	1.36	*Variovorax*	HG942169	+	-	-	-	-	-	-
1	14480	1.36	*Variovorax*	HG942169	+	-	-	-	-	+++	-
1	15638	1.47	*Variovorax*	HG942169	+	-	-	-	-	-	-
1	26064	2.45	*Variovorax*	HG942169	-	-	-	-	-	-	-
1	26064	2.45	*Variovorax*	HG942169	+	-	-	-	-	++	-
1	32580	3.06	*Variovorax*	HG942169	-	-	-	-	-	-	-
1	32580	3.06	*Variovorax*	HG942169	+	-	-	-	-	-	-
1	32580	3.06	*Variovorax*	HG942169	+	-	-	-	-	-	-
1	32580	3.06	*Variovorax*	HG942169	+	-	-	-	-	-	-
1	32580	3.06	*Variovorax*	HG942169	+	-	-	-	-	-	-
1	32580	3.06	*Variovorax*	HG942169	+	-	-	-	-	++	-
1	32580	3.06	*Variovorax*	HG942169	+	-	-	-	-	+++	-

1	32580	3.06	*Variovorax*	HG942169	+	-	-	-	-	-	-
2	94153	6.55	*Bacillus*	HG942095	+	-	-	-	+++	+	-
2	47077	3.27	*Bacillus*	HG942097	++	-	-	-	-	-	-
2	4708	0.33	*Caulobacter*	HG942105	-	-	-	-	-	+	-
2	4708	0.33	*Labrys*	HG942114	-	nd	-	++	nd	-	-
2	18831	1.31	*Labrys*	HG942114	-	nd	-	++	nd	-	-
2	7061	0.49	*Microbacterium*	HG942119	-	+	-	+	-	+++	-
2	4708	0.33	*Paenibacillus*	HG942127	-	-	-	+	-	++	-
2	7061	0.49	*Pantoea*	HG942128	+	-	+	+	-	-	-
2	47077	3.27	*Pedobacter*	HG942131	+	-	-	-	-	-	-
2	7061	0.49	*Plantibacter*	HG942132	-	-	+	+	-	-	-
2	7061	0.49	*Plantibacter*	HG942132	+	+	+	+	-	++	-
2	6277	0.44	*Pseudomonas*	HG942137	++	-	+	+	-	+	-
2	211844	14.73	*Pseudomonas*	HG942137	+	-	+	+	-	+	-
2	211844	14.73	*Pseudomonas*	HG942137	+	-	+	+	-	+	+++
2	4708	0.33	*Pseudomonas*	HG942144	+	-	+	++	-	+	-
2	4708	0.33	*Pseudomonas*	HG942144	+	-	-	-	-	+	-
2	4708	0.33	*Pseudomonas*	HG942144	++	-	+	+	-	+	-
2	7061	0.49	*Pseudomonas*	HG942144	-	-	+	+	-	++	-
2	7061	0.49	*Pseudomonas*	HG942144	+	-	-	-	-	++	+
2	47077	3.27	*Pseudomonas*	HG942147	-	-	-	-	-	+++	-
2	4708	0.33	*Pseudomonas*	HG942148	+	+	-	++	-	++	-
2	263629	18.33	*Variovorax*	HG942168	++	-	-	-	-	-	-
2	4708	0.33	*Variovorax*	HG942169	+	-	-	-	-	+	-
2	4708	0.33	*Variovorax*	HG942169	+	-	-	-	-	++	-
2	6277	0.44	*Variovorax*	HG942169	+	-	-	-	-	+	-
2	6277	0.44	*Variovorax*	HG942169	+	-	-	-	-	+	-
2	7061	0.49	*Variovorax*	HG942169	+	-	-	-	-	++	-
2	47077	3.27	*Variovorax*	HG942169	++	-	-	-	-	-	-
2	65907	4.58	*Variovorax*	HG942169	+	-	-	-	-	+	-
2	273044	18.99	*Variovorax*	HG942169	++	-	-	-	-	-	-
3	3303	0.53	*Bacillus*	HG942090	+	-	-	-	+++	-	-
3	33033	5.26	*Bacillus*	HG942090	+	-	-	-	+++	-	-
3	3303	0.53	*Bacillus*	HG942095	+	+	-	-	+++	+	-
3	3303	0.53	*Brevibacillus*	HG942103	+	-	-	-	-	-	-
3	3303	0.53	*Brevibacillus*	HG942103	++	nd	-	+	-	-	++
3	3303	0.53	*Caulobacter*	HG942105	+	-	-	-	-	-	-
3	3303	0.53	*Caulobacter*	HG942105	-	-	-	-	-	+	-
3	3303	0.53	*Caulobacter*	HG942105	-	-	-	-	nd	-	-
3	6276	1.00	*Caulobacter*	HG942105	-	-	-	-	-	-	-
3	6276	1.00	*Caulobacter*	HG942105	+	-	-	++	-	-	-
3	6276	1.00	*Caulobacter*	HG942105	-	-	-	-	-	+	-
3	6276	1.00	*Caulobacter*	HG942105	-	-	-	-	-	+	-

3	6276	1.00	*Caulobacter*	HG942105	-	-	-	-	-	+	-
3	6276	1.00	*Caulobacter*	HG942105	-	-	-	-	-	+	-
3	6276	1.00	*Caulobacter*	HG942105	-	-	-	-	-	+	-
3	33033	5.26	*Caulobacter*	HG942105	-	+	-	-	-	+	-
3	13213	2.10	*Labrys*	HG942114	-	-	+	+	-	-	-
3	3303	0.53	*Pantoea*	HG942128	+	-	-	+	+++	-	-
3	101080	16.10	*Pantoea*	HG942128	+	-	+	++	+	-	-
3	33033	5.26	*Pseudomonas*	HG942136	-	-	-	-	-	-	-
3	9910	1.58	*Pseudomonas*	HG942137	+	-	+	++	-	-	-
3	11231	1.79	*Pseudomonas*	HG942137	+	-	-	++	-	+	-
3	31381	5.00	*Pseudomonas*	HG942137	+	-	+	+	-	+	-
3	31381	5.00	*Pseudomonas*	HG942137	+	-	+	+	-	+	+++
3	3303	0.53	*Pseudomonas*	HG942140	+	-	+	-	-	-	-
3	33033	5.26	*Pseudomonas*	HG942146	+	+	+	++	-	++	-
3	99098	15.78	*Pseudomonas*	HG942146	+	+	+	++	-	+	-
3	13213	2.10	*Pseudomonas*	HG942147	-	nd	+	+	-	+	-
3	3303	0.53	*Pseudomonas*	HG942148	+	-	+	++	-	+++	-
3	6276	1.00	*Pseudomonas*	HG942148	+	-	-	++	-	-	-
3	13213	2.10	*Stenotrophomonas*	HG942162	-	-	+	-	-	-	-
3	3303	0.53	*Variovorax*	HG942169	-	-	-	-	-	-	-
3	3303	0.53	*Variovorax*	HG942169	+	-	-	-	-	-	-
3	3303	0.53	*Variovorax*	HG942169	+	-	-	-	-	-	-
3	3964	0.63	*Variovorax*	HG942169	+	-	-	-	-	-	-
3	3964	0.63	*Variovorax*	HG942169	+	-	-	-	-	-	-
3	6276	1.00	*Variovorax*	HG942169	+	-	-	-	-	+	-
3	7928	1.26	*Variovorax*	HG942169	+	-	-	-	-	+	-
3	13213	2.10	*Variovorax*	HG942169	+	-	-	-	-	-	-
3	13213	2.10	*Variovorax*	HG942169	+	-	-	-	-	+	-
3	30390	4.84	*Variovorax*	HG942169	++	-	-	-	-	+	-
				STEM							
repl.	cfu g FW^{-1}	%	identification	accession	SID	OA	ACC	IAA	ACE	P sol	N_2 fix
1	7096	1.02	*Aeromicrobium*	HG942086	-	nd	-	nd	-	-	-
1	7096	1.02	*Aeromicrobium*	HG942086	nd	nd	-	-	nd	-	-
1	22617	3.24	*Aeromicrobium*	HG942086	nd	nd	-	nd	nd	-	-
1	22617	3.24	*Aeromicrobium*	HG942086	-	nd	-	nd	nd	-	-
1	22617	3.24	*Aeromicrobium*	HG942086	-	nd	-	nd	nd	-	-
1	5322	0.76	*Agreia*	HG942087	nd	nd	-	nd	-	-	-
1	5322	0.76	*Agreia*	HG942087	nd	+	-	-	-	-	-
1	5322	0.76	*Agreia*	HG942087	nd	+	-	-	-	-	-
1	5322	0.76	*Agreia*	HG942087	nd	nd	-	nd	nd	-	-
1	14901	2.13	*Agreia*	HG942087	nd	-	-	nd	nd	-	-
1	14901	2.13	*Agreia*	HG942087	nd	nd	-	nd	nd	-	-
1	14901	2.13	*Agreia*	HG942087	nd	nd	-	nd	nd	-	-

1	14901	2.13	*Agreia*	HG942087	nd	nd	-	nd	nd	-	-
1	14901	2.13	*Agreia*	HG942087	nd	nd	nd	nd	nd	-	-
1	43748	6.26	*Bacillus*	HG942090	+	-	-	-	+++	-	-
1	10587	1.52	*Bacillus*	HG942095	+	-	-	-	+++	-	-
1	44	0.01	*Brevibacillus*	HG942102	-	-	-	+	-	-	+
1	8869	1.27	*Curtobacterium*	HG942109	nd	-	-	nd	-	-	-
1	8869	1.27	*Curtobacterium*	HG942109	-	-	-	-	-	-	-
1	5322	0.76	*Frigoribacterium*	HG942113	nd	-	nd	+	nd	-	-
1	7096	1.02	*Frigoribacterium*	HG942113	nd	+	-	+	-	-	-
1	8869	1.27	*Frigoribacterium*	HG942113	-	+	-	-	nd	-	-
1	21287	3.05	*Frigoribacterium*	HG942113	nd	-	-	+	nd	-	-
1	25012	3.58	*Frigoribacterium*	HG942113	nd	-	-	+	-	-	-
1	25012	3.58	*Frigoribacterium*	HG942113	nd	+	-	+	-	-	-
1	25012	3.58	*Frigoribacterium*	HG942113	nd	+	nd	+	-	-	-
1	25012	3.58	*Frigoribacterium*	HG942113	nd	+	-	+	-	-	-
1	25012	3.58	*Frigoribacterium*	HG942113	nd	+	-	+	-	-	-
1	25012	3.58	*Frigoribacterium*	HG942113	nd	+	nd	+	-	-	-
1	25012	3.58	*Frigoribacterium*	HG942113	nd	+	-	+	nd	-	-
1	25012	3.58	*Frigoribacterium*	HG942113	nd	+	-	+	nd	-	-
1	25012	3.58	*Frigoribacterium*	HG942113	nd	-	-	+	nd	-	-
1	25012	3.58	*Frigoribacterium*	HG942113	-	-	-	+	-	-	-
1	5322	0.76	*Massilia*	HG942117	-	-	-	-	-	-	+
1	5322	0.76	*Massilia*	HG942117	-	-	-	-	-	-	+
1	5322	0.76	*Massilia*	HG942117	-	-	-	-	-	-	+
1	22617	3.24	*Massilia*	HG942117	-	-	-	-	-	-	+
1	44	0.01	*Mycobacterium*	HG942122	-	-	-	-	-	-	-
1	21287	3.05	*Pedobacter*	HG942130	-	-	-	-	nd	-	-
1	7096	1.02	*Pseudomonas*	HG942141	-	-	++	-	-	-	+
1	7096	1.02	*Pseudomonas*	HG942141	nd	-	-	-	nd	-	-
1	7096	1.02	*Pseudomonas*	HG942141	-	-	+++	-	-	-	+
1	13304	1.91	*Pseudomonas*	HG942143	+	-	++	-	-	+	+
1	13304	1.91	*Pseudomonas*	HG942143	+	-	-	-	-	+	+
1	13304	1.91	*Pseudomonas*	HG942143	+	-	-	-	-	+	+
1	13304	1.91	*Pseudomonas*	HG942143	+	-	-	-	-	+	+
1	5322	0.76	*Pseudomonas*	HG942149	+	-	-	+	-	+	+
1	5322	0.76	*Staphylococcus*	HG942157	nd	+++	++	-	+	-	-
1	5322	0.76	*Variovorax*	HG942166	nd	+++	++	-	+	-	-
1	5322	0.76	*Variovorax*	HG942166	-	-	-	-	nd	-	+
2	2923	0.92	*Aeromicrobium*	HG942086	-	-	-	-	nd	-	-
2	2923	0.92	*Aeromicrobium*	HG942086	-	-	-	+	nd	-	-
2	34	0.01	*Agromyces*	HG942088	-	-	-	-	nd	-	-
2	34	0.01	*Bacillus*	HG942095	+	++	-	-	+++	-	+++
2	508	0.16	*Bacillus*	HG942095	+	-	-	-	+++	-	-

2	3385	1.07	*Bacillus*	HG942095	+	nd	-	-	+++	++	-
2	3385	1.07	*Bacillus*	HG942095	+	nd	-	-	nd	-	-
2	34	0.01	*Bacillus*	HG942097	+	-	-	+	+++	+	-
2	17832	5.61	*Frigoribacterium*	HG942113	nd	-	-	-	-	-	-
2	17832	5.61	*Frigoribacterium*	HG942113	nd	-	-	-	nd	-	-
2	17832	5.61	*Frigoribacterium*	HG942113	nd	-	-	-	-	-	-
2	17832	5.61	*Frigoribacterium*	HG942113	nd	-	-	-	-	-	-
2	17832	5.61	*Frigoribacterium*	HG942113	nd	-	-	-	nd	-	-
2	17832	5.61	*Frigoribacterium*	HG942113	nd	-	-	-	-	-	-
2	17832	5.61	*Frigoribacterium*	HG942113	nd	-	-	-	-	-	-
2	17832	5.61	*Frigoribacterium*	HG942113	nd	-	-	-	-	-	-
2	17832	5.61	*Frigoribacterium*	HG942113	nd	-	-	-	-	-	-
2	11368	3.58	*Massilia*	HG942117	-	-	+	++	nd	-	+
2	11368	3.58	*Massilia*	HG942117	-	-	-	++	nd	-	+
2	11368	3.58	*Massilia*	HG942117	-	-	-	+	nd	-	+
2	11368	3.58	*Massilia*	HG942117	-	-	-	++	nd	-	+
2	11368	3.58	*Massilia*	HG942117	-	-	-	++	nd	-	+
2	11368	3.58	*Massilia*	HG942117	-	-	-	+	nd	-	+
2	11368	3.58	*Massilia*	HG942117	-	-	-	++	nd	-	+
2	11368	3.58	*Massilia*	HG942117	-	-	-	++	nd	-	+
2	11368	3.58	*Massilia*	HG942117	-	-	-	++	nd	-	+
2	339	0.11	*Pantoea*	HG942128	+	+	+	++	-	-	-
2	34	0.01	*Plantibacter*	HG942132	-	-	-	++	-	+	-
2	2923	0.92	*Plantibacter*	HG942134	-	nd	+	-	-	-	-
2	2923	0.92	*Plantibacter*	HG942134	-	-	-	-	-	-	-
2	3898	1.23	*Pseudomonas*	HG942141	+	-	++	-	-	+	+
2	3898	1.23	*Pseudomonas*	HG942141	+	-	-	-	-	+	+
2	3898	1.23	*Pseudomonas*	HG942141	+	-	-	-	-	+	+
2	3898	1.23	*Pseudomonas*	HG942141	+	-	++	-	-	+	+
2	3898	1.23	*Pseudomonas*	HG942141	-	-	+++	-	-	-	+
2	3898	1.23	*Pseudomonas*	HG942141	+	-	-	-	-	+	+
2	3898	1.23	*Pseudomonas*	HG942143	-	-	-	++	-	+	+
2	3898	1.23	*Pseudomonas*	HG942143	+	+	++	+	-	+	+
2	3898	1.23	*Pseudomonas*	HG942143	+	-	-	+	-	+	+
2	102	0.03	*Pseudomonas*	HG942145	++	-	-	-	-	-	-
2	102	0.03	*Pseudomonas*	HG942145	++	-	+	++	-	-	-
2	102	0.03	*Pseudomonas*	HG942145	+	-	+	++	-	-	-
2	102	0.03	*Pseudomonas*	HG942145	++	-	+	++	-	-	++
3	907	0.20	*Aeromicrobium*	HG942086	nd	+	++	-	-	-	++
3	2720	0.59	*Aeromicrobium*	HG942086	nd	-	nd	-	nd	-	-
3	307	0.07	*Bacillus*	HG942090	+	++	-	-	+++	-	-
3	184	0.04	*Bacillus*	HG942095	+	++	-	-	+++	++	-
3	184	0.04	*Bacillus*	HG942095	+	nd	-	-	+++	++	-

3	307	0.07	*Bacillus*	HG942095	+	-	-	+	+++	-	-
3	615	0.13	*Bacillus*	HG942095	+	-	-	-	++	-	-
3	816	0.18	*Chryseobacterium*	HG942107	-	-	-	++	-	-	+
3	816	0.18	*Chryseobacterium*	HG942107	-	-	-	-	-	-	++
3	816	0.18	*Chryseobacterium*	HG942107	nd	-	-	-	-	-	++
3	816	0.18	*Chryseobacterium*	HG942107	-	-	-	-	-	-	++
3	2720	0.59	*Chryseobacterium*	HG942107	-	-	-	-	nd	-	-
3	2720	0.59	*Chryseobacterium*	HG942107	-	-	-	-	nd	-	-
3	2720	0.59	*Chryseobacterium*	HG942107	-	-	-	+	nd	-	++
3	31	0.01	*Frigoribacterium*	HG942112	+	-	-	-	nd	-	-
3	98	0.02	*Frigoribacterium*	HG942112	-	++	-	+	-	-	-
3	453	0.10	*Frigoribacterium*	HG942113	-	-	nd	++	-	-	-
3	2720	0.59	*Frigoribacterium*	HG942113	-	-	+	-	-	-	-
3	2720	0.59	*Frigoribacterium*	HG942113	-	-	nd	-	nd	-	-
3	8159	1.76	*Frigoribacterium*	HG942113	nd	-	-	++	-	-	-
3	8159	1.76	*Frigoribacterium*	HG942113	nd	-	-	++	-	-	-
3	8159	1.76	*Frigoribacterium*	HG942113	nd	-	-	++	-	-	-
3	8159	1.76	*Frigoribacterium*	HG942113	-	-	-	++	-	-	-
3	8159	1.76	*Frigoribacterium*	HG942113	nd	-	-	++	-	-	-
3	8159	1.76	*Frigoribacterium*	HG942113	nd	-	-	++	-	-	-
3	8159	1.76	*Frigoribacterium*	HG942113	nd	-	-	++	-	-	-
3	8897	1.92	*Frigoribacterium*	HG942113	nd	-	-	++	nd	-	-
3	8897	1.92	*Frigoribacterium*	HG942113	-	-	-	++	nd	-	-
3	8897	1.92	*Frigoribacterium*	HG942113	nd	-	-	++	-	-	-
3	8897	1.92	*Frigoribacterium*	HG942113	nd	-	-	++	-	-	-
3	8897	1.92	*Frigoribacterium*	HG942113	-	-	-	++	nd	-	+
3	8897	1.92	*Frigoribacterium*	HG942113	-	-	-	++	-	-	-
3	8897	1.92	*Frigoribacterium*	HG942113	-	-	-	++	-	-	-
3	31	0.01	*Massilia*	HG942116	-	-	nd	+	-	nd	-
3	307	0.07	*Massilia*	HG942116	+	-	-	-	+++	-	-
3	1305	0.28	*Massilia*	HG942117	-	-	-	-	nd	-	-
3	1305	0.28	*Massilia*	HG942117	-	-	-	++	-	-	+
3	1305	0.28	*Massilia*	HG942117	-	-	-	++	-	-	+
3	1305	0.28	*Massilia*	HG942117	-	-	-	++	-	-	+
3	1305	0.28	*Massilia*	HG942117	-	-	-	++	-	-	+
3	1305	0.28	*Massilia*	HG942117	-	-	-	++	-	-	+
3	1305	0.28	*Massilia*	HG942117	-	-	++	++	-	-	+
3	1305	0.28	*Massilia*	HG942117	-	-	-	++	-	-	+
3	1305	0.28	*Massilia*	HG942117	-	-	-	++	-	-	+
3	1305	0.28	*Massilia*	HG942117	-	-	-	++	-	-	+
3	2266	0.49	*Massilia*	HG942117	-	-	++	++	nd	-	+
3	3626	0.78	*Massilia*	HG942117	-	-	+	++	-	-	+
3	3626	0.78	*Massilia*	HG942117	-	-	-	++	-	-	+
3	3626	0.78	*Massilia*	HG942117	-	-	-	++	-	-	+

3	26108	5.64	*Massilia*	HG942117	-	-	-	++	nd	-	+
3	26108	5.64	*Massilia*	HG942117	-	-	-	++	nd	-	+
3	26108	5.64	*Massilia*	HG942117	-	-	-	++	nd	-	+
3	26108	5.64	*Massilia*	HG942117	-	-	-	++	nd	-	+
3	26108	5.64	*Massilia*	HG942117	-	-	-	++	-	-	+
3	26108	5.64	*Massilia*	HG942117	-	-	-	++	nd	-	+
3	26108	5.64	*Massilia*	HG942117	-	-	-	++	nd	-	+
3	26108	5.64	*Massilia*	HG942117	-	-	-	++	nd	-	+
3	26108	5.64	*Massilia*	HG942117	-	-	-	++	-	-	+
3	26108	5.64	*Massilia*	HG942117	-	-	-	++	nd	-	+
3	680	0.15	*Paenibacillus*	HG942127	-	+	-	-	-	-	++
3	680	0.15	*Paenibacillus*	HG942127	-	+	-	-	-	-	++
3	680	0.15	*Paenibacillus*	HG942127	+	++	-	-	-	+	+
3	680	0.15	*Paenibacillus*	HG942127	-	+	-	-	-	-	++
3	115	0.02	*Paenibacillus*	HG942127	-	+	nd	++	+++	+++	-
3	307	0.07	*Paenibacillus*	HG942127	-	++	-	-	+++	-	-
3	2767	0.60	*Pedobacter*	HG942129	-	-	-	+	-	-	-
3	272	0.06	*Pseudomonas*	HG942141	+	-	+	-	-	+	+
3	907	0.20	*Pseudomonas*	HG942143	+	-	++	-	-	+	+
3	1118	0.24	*Pseudomonas*	HG942143	+	+++	+	-	-	+	+
3	1118	0.24	*Pseudomonas*	HG942143	+	+	-	-	-	+	+
3	1118	0.24	*Pseudomonas*	HG942143	+	+++	-	-	-	+	+
3	1118	0.24	*Pseudomonas*	HG942143	+	+	-	-	-	+	+
3	1118	0.24	*Pseudomonas*	HG942143	+	+	-	-	-	+	+
3	1118	0.24	*Pseudomonas*	HG942143	+	+	-	-	-	+	+
3	1118	0.24	*Pseudomonas*	HG942143	+	-	-	-	-	+	+
3	1118	0.24	*Pseudomonas*	HG942143	+	+++	-	-	-	+	+
3	1118	0.24	*Pseudomonas*	HG942143	+	+++	-	-	-	+	+
3	2266	0.49	*Pseudomonas*	HG942143	+	-	++	-	-	+	+
3	2266	0.49	*Pseudomonas*	HG942143	+	+++	nd	-	-	+	+
3	2720	0.59	*Pseudomonas*	HG942143	+	+	++	-	-	+	+
3	2720	0.59	*Pseudomonas*	HG942143	+	+	-	-	-	+	+
3	2720	0.59	*Pseudomonas*	HG942141	-	-	++	-	-	-	+
3	215	0.05	*Pseudomonas*	HG942142	++	-	-	++	-	-	-
3	215	0.05	*Pseudomonas*	HG942142	++	-	-	++	-	++	-
3	115	0.02	*Pseudomonas*	HG942148	+	+	-	++	-	-	-
3	307	0.07	*Pseudomonas*	HG942148	+	-	-	++	-	+	-
3	307	0.07	*Pseudomonas*	HG942148	+	-	-	++	-	+	-
3	453	0.10	*Rhodococcus*	HG942152	-	-	-	-	nd	-	-
3	453	0.10	*Rhodococcus*	HG942152	-	-	-	-	-	-	+
3	277	0.06	*Sanguibacter*	HG942154	-	++	-	-	+++	-	-
3	907	0.20	*Sphingomonas*	HG942155	-	-	nd	-	nd	-	+
3	31	0.01	*Variovorax*	HG942169	-	-	-	++	-	-	+++

3	49	0.01	*Zoogloea*	HG942170	-	++	-	-	+++	+	-
				LEAF							
repl.	cfu g FW^{-1}	%	identification	accession	SID	OA	ACC	IAA	ACE	P sol	N_2 fix
1	190	0.15	*Bacillus*	HG942095	+	-	-	-	+++	+	-
1	634	0.51	*Bacillus*	HG942095	+	+	-	-	+++	-	-
1	634	0.51	*Bacillus*	HG942097	+	-	-	++	nd	+	++
1	140	0.11	*Curtobacterium*	HG942109	nd	+	nd	-	-	-	-
1	140	0.11	*Frigoribacterium*	HG942113	nd	-	-	-	-	-	-
1	140	0.11	*Frigoribacterium*	HG942113	-	+	-	-	nd	-	-
1	140	0.11	*Frigoribacterium*	HG942113	nd	+	nd	-	-	-	-
1	280	0.23	*Frigoribacterium*	HG942113	nd	+	nd	+	nd	-	-
1	280	0.23	*Frigoribacterium*	HG942113	nd	-	-	-	nd	-	-
1	322	0.26	*Frigoribacterium*	HG942113	nd	+	-	-	nd	-	-
1	322	0.26	*Frigoribacterium*	HG942113	nd	-	-	-	nd	-	-
1	322	0.26	*Frigoribacterium*	HG942113	nd	+	-	-	-	-	-
1	322	0.26	*Frigoribacterium*	HG942113	-	+	nd	-	nd	-	-
1	322	0.26	*Frigoribacterium*	HG942113	nd	+	nd	-	nd	-	-
1	322	0.26	*Frigoribacterium*	HG942113	nd	+	nd	-	nd	-	-
1	322	0.26	*Frigoribacterium*	HG942113	nd	+	nd	-	-	-	-
1	322	0.26	*Frigoribacterium*	HG942113	-	-	-	-	nd	-	-
1	322	0.26	*Frigoribacterium*	HG942113	-	-	nd	-	-	-	-
1	322	0.26	*Frigoribacterium*	HG942113	-	-	nd	-	nd	-	-
1	491	0.39	*Frigoribacterium*	HG942113	-	-	nd	-	nd	-	-
1	491	0.39	*Frigoribacterium*	HG942113	-	-	-	-	-	-	-
1	491	0.39	*Frigoribacterium*	HG942113	-	+	nd	-	nd	-	-
1	491	0.39	*Frigoribacterium*	HG942113	nd	+	nd	-	-	-	-
1	491	0.39	*Frigoribacterium*	HG942113	-	+	-	-	nd	-	-
1	491	0.39	*Frigoribacterium*	HG942113	nd	+	-	-	nd	-	-
1	491	0.39	*Frigoribacterium*	HG942113	-	+	nd	-	nd	-	-
1	491	0.39	*Frigoribacterium*	HG942113	-	+	nd	-	nd	-	-
1	491	0.39	*Frigoribacterium*	HG942113	-	+	nd	-	-	-	-
1	491	0.39	*Frigoribacterium*	HG942113	-	-	-	-	nd	-	-
1	491	0.39	*Frigoribacterium*	HG942113	-	-	nd	-	nd	-	-
1	491	0.39	*Frigoribacterium*	HG942113	-	-	-	-	nd	-	-
1	2523	2.03	*Frigoribacterium*	HG942113	-	-	nd	-	-	-	-
1	2523	2.03	*Frigoribacterium*	HG942113	-	-	-	-	nd	-	-
1	2523	2.03	*Frigoribacterium*	HG942113	-	-	-	-	nd	-	-
1	2523	2.03	*Frigoribacterium*	HG942113	nd	-	-	-	nd	-	-
1	2523	2.03	*Frigoribacterium*	HG942113	nd	-	-	-	nd	-	-
1	101	0.08	*Massilia*	HG942116	-	-	-	+	-	+	-
1	405	0.33	*Massilia*	HG942116	-	-	nd	++	-	++	-
1	2534	2.04	*Massilia*	HG942116	-	-	-	-	-	-	-
1	63355	50.91	*Massilia*	HG942116	-	nd	+	+	nd	-	-

1	140	0.11	*Massilia*	HG942117	-	-	++	+	-	-	+
1	140	0.11	*Massilia*	HG942117	-	-	+++	++	-	-	+
1	140	0.11	*Massilia*	HG942117	-	-	-	+	-	-	+
1	140	0.11	*Massilia*	HG942117	-	-	-	++	-	-	+
1	1402	1.13	*Massilia*	HG942118	-	-	-	+	nd	-	+
1	443	0.36	*Paenibacillus*	HG942127	+	-	-	-	-	+	-
1	140	0.11	*Pseudomonas*	HG942143	+	-	++	-	-	-	+
1	280	0.23	*Pseudomonas*	HG942143	+	-	nd	-	-	-	+
1	407	0.33	*Pseudomonas*	HG942143	+	-	+++	-	-	+	+
1	407	0.33	*Pseudomonas*	HG942143	+	-	-	-	-	-	+
1	407	0.33	*Pseudomonas*	HG942143	+	-	-	-	-	+	+
1	407	0.33	*Pseudomonas*	HG942143	+	-	-	-	-	-	+
1	407	0.33	*Pseudomonas*	HG942143	+	-	-	-	-	+	+
1	407	0.33	*Pseudomonas*	HG942143	+	-	-	-	-	-	+
1	407	0.33	*Pseudomonas*	HG942143	+	-	-	-	-	+	+
1	407	0.33	*Pseudomonas*	HG942143	+	-	-	-	-	-	+
1	407	0.33	*Pseudomonas*	HG942143	+	-	-	-	-	-	+
1	407	0.33	*Pseudomonas*	HG942143	+	++	-	-	-	-	+
1	1402	1.13	*Pseudomonas*	HG942143	+	-	-	-	-	+	+
1	1402	1.13	*Pseudomonas*	HG942143	+	-	-	-	-	+	+
1	1402	1.13	*Pseudomonas*	HG942143	+	-	++	-	-	+	+
1	63	0.05	*Pseudomonas*	HG942146	-	-	-	++	-	+	-
1	127	0.10	*Pseudomonas*	HG942146	+	-	-	++	-	++	-
1	279	0.22	*Pseudomonas*	HG942146	+	-	-	++	-	+	-
1	63	0.05	*Pseudomonas*	HG942148	+	-	-	++	-	+	-
1	190	0.15	*Pseudomonas*	HG942148	+	-	-	++	-	-	-
1	190	0.15	*Pseudomonas*	HG942148	+	-	-	++	-	+	-
1	279	0.22	*Pseudomonas*	HG942148	+	-	-	++	-	+	-
1	443	0.36	*Pseudomonas*	HG942148	+	-	-	++	-	-	-
1	558	0.45	*Pseudomonas*	HG942148	+	-	-	++	-	+	-
1	634	0.51	*Pseudomonas*	HG942148	+	-	-	+	-	-	-
1	634	0.51	*Pseudomonas*	HG942148	+	-	-	+	-	+	-
1	634	0.51	*Pseudomonas*	HG942148	+	-	-	+	-	+	-
1	634	0.51	*Pseudomonas*	HG942148	+	-	-	++	-	+	-
1	634	0.51	*Pseudomonas*	HG942148	+	-	-	+	-	++	-
1	634	0.51	*Pseudomonas*	HG942148	+	-	-	++	-	-	++
1	634	0.51	*Pseudomonas*	HG942148	+	-	-	+	-	+	++
1	634	0.51	*Pseudomonas*	HG942148	+	-	-	+	-	+	++
1	634	0.51	*Pseudomonas*	HG942148	+	-	+	++	-	+	++
1	634	0.51	*Pseudomonas*	HG942148	+	-	-	++	-	-	+++
1	634	0.51	*Pseudomonas*	HG942148	++	-	+	++	-	-	+++
1	634	0.51	*Pseudomonas*	HG942148	+	-	-	++	-	-	+
1	634	0.51	*Pseudomonas*	HG942148	+	-	-	++	-	+	++
1	4720	3.79	*Pseudomonas*	HG942148	+	-	-	+	-	-	-

1	4720	3.79	*Pseudomonas*	HG942148	+	-	-	+	-	-	-
1	140	0.11	*Spirosoma*	HG942156	-	-	nd	-	nd	-	-
1	140	0.11	*Spirosoma*	HG942156	+	-	-	-	-	+	+
1	1402	1.13	*Spirosoma*	HG942156	-	-	-	-	nd	-	-
1	634	0.51	*Staphylococcus*	HG942159	+	-	+	-	-	-	+++
1	190	0.15	*Staphylococcus*	HG942160	++	-	+	+	-	-	++
1	279	0.22	*Staphylococcus*	HG942160	++	-	+	++	-	-	+
2	244	1.54	*Bacillus*	HG942095	+	-	-	-	+++	+	-
2	294	1.86	*Chryseobacterium*	HG942107	nd	-	-	-	nd	-	-
2	294	1.86	*Chryseobacterium*	HG942107	nd	-	-	-	nd	-	-
2	294	1.86	*Chryseobacterium*	HG942107	nd	-	-	-	nd	-	-
2	294	1.86	*Chryseobacterium*	HG942107	nd	-	-	-	nd	-	-
2	628	3.96	*Cryobacterium*	HG942108	-	-	nd	-	-	-	-
2	628	3.96	*Frigoribacterium*	HG942113	-	+	-	-	nd	-	-
2	628	3.96	*Frigoribacterium*	HG942113	-	nd	-	nd	nd	-	-
2	777	4.90	*Frigoribacterium*	HG942113	-	+	nd	+	nd	-	-
2	777	4.90	*Frigoribacterium*	HG942113	-	+	-	+	nd	-	-
2	777	4.90	*Frigoribacterium*	HG942113	-	+	-	+	nd	-	-
2	777	4.90	*Frigoribacterium*	HG942113	-	+	-	+	-	-	-
2	777	4.90	*Frigoribacterium*	HG942113	-	+	-	+	nd	-	-
2	777	4.90	*Frigoribacterium*	HG942113	-	+	-	+	-	-	-
2	777	4.90	*Frigoribacterium*	HG942113	-	+	-	+	nd	-	-
2	777	4.90	*Frigoribacterium*	HG942113	-	+	-	+	nd	-	-
2	777	4.90	*Frigoribacterium*	HG942113	-	+	-	+	nd	-	-
2	777	4.90	*Frigoribacterium*	HG942113	-	+	-	+	nd	-	-
2	373	2.35	*Massilia*	HG942117	-	-	-	++	nd	-	+
2	373	2.35	*Massilia*	HG942117	-	-	-	+	nd	-	+
2	373	2.35	*Massilia*	HG942117	-	-	-	+	nd	-	+
2	373	2.35	*Massilia*	HG942117	-	-	-	+	nd	-	+
2	373	2.35	*Massilia*	HG942117	-	-	-	+	nd	-	+
2	373	2.35	*Massilia*	HG942117	-	-	-	+	nd	-	+
2	118	0.74	*Pedobacter*	HG942130	-	-	-	-	-	-	-
2	118	0.74	*Pedobacter*	HG942130	-	-	-	-	-	-	+
2	118	0.74	*Pedobacter*	HG942130	-	-	-	++	nd	-	+
2	236	1.48	*Pseudomonas*	HG942141	+	-	-	-	-	+	+
2	236	1.48	*Pseudomonas*	HG942141	+	-	-	+	-	+	+
2	236	1.48	*Pseudomonas*	HG942141	+	-	-	-	-	+	+
2	353	2.23	*Pseudomonas*	HG942143	-	-	-	+	-	+	+
2	353	2.23	*Pseudomonas*	HG942143	-	-	-	+	-	+	+
2	353	2.23	*Pseudomonas*	HG942143	-	-	-	+	-	-	+
2	353	2.23	*Pseudomonas*	HG942143	-	-	-	+	-	+	+
2	81	0.51	*Pseudomonas*	HG942147	-	-	nd	-	nd	-	-
3	84	0.44	*Bacillus*	HG942095	+	-	-	-	+++	-	-

3	84	0.44	*Bacillus*	HG942100	+	+	-	-	-	-	+++
3	63	0.33	*Chryseobacterium*	HG942106	-	-	-	-	nd	-	++
3	63	0.33	*Chryseobacterium*	HG942106	-	-	-	++	nd	-	+
3	63	0.33	*Chryseobacterium*	HG942106	-	-	-	+	nd	-	-
3	106	0.55	*Chryseobacterium*	HG942107	-	+	-	-	nd	-	++
3	106	0.55	*Chryseobacterium*	HG942107	-	+	-	-	nd	-	++
3	106	0.55	*Chryseobacterium*	HG942107	-	+	-	-	nd	-	++
3	691	3.60	*Chryseobacterium*	HG942107	nd	-	-	-	nd	-	-
3	691	3.60	*Chryseobacterium*	HG942107	nd	-	-	-	nd	+	-
3	63	0.33	*Curtobacterium*	HG942110	-	-	-	-	-	-	-
3	63	0.33	*Curtobacterium*	HG942110	-	+	-	-	-	-	-
3	63	0.33	*Frigoribacterium*	HG942113	-	-	nd	-	-	-	-
3	63	0.33	*Frigoribacterium*	HG942113	-	-	-	-	-	-	-
3	63	0.33	*Frigoribacterium*	HG942113	-	-	-	-	-	-	-
3	63	0.33	*Frigoribacterium*	HG942113	nd	-	-	-	nd	-	-
3	63	0.33	*Frigoribacterium*	HG942113	nd	-	nd	-	-	-	-
3	63	0.33	*Frigoribacterium*	HG942113	-	-	-	++	-	-	+
3	691	3.60	*Frigoribacterium*	HG942113	nd	nd	nd	nd	nd	-	-
3	691	3.60	*Frigoribacterium*	HG942113	nd	nd	nd	nd	nd	-	-
3	691	3.60	*Frigoribacterium*	HG942113	nd	nd	-	nd	nd	-	-
3	691	3.60	*Frigoribacterium*	HG942113	-	-	-	-	nd	-	-
3	691	3.60	*Frigoribacterium*	HG942113	nd	nd	-	nd	nd	-	-
3	691	3.60	*Frigoribacterium*	HG942113	nd	-	-	nd	nd	-	-
3	691	3.60	*Frigoribacterium*	HG942113	nd	nd	nd	nd	nd	-	-
3	691	3.60	*Frigoribacterium*	HG942113	nd	nd	-	nd	nd	-	-
3	615	3.21	*Massilia*	HG942117	-	-	-	+	-	-	+
3	615	3.21	*Massilia*	HG942117	-	-	-	++	-	-	+
3	615	3.21	*Massilia*	HG942117	-	-	-	++	-	-	+
3	615	3.21	*Massilia*	HG942117	-	-	-	++	-	-	+
3	615	3.21	*Massilia*	HG942117	-	-	+	++	nd	-	+
3	615	3.21	*Massilia*	HG942117	-	-	-	++	-	-	+
3	615	3.21	*Massilia*	HG942117	-	-	-	++	nd	-	+
3	615	3.21	*Massilia*	HG942117	-	-	-	++	-	-	+
3	615	3.21	*Massilia*	HG942117	-	-	-	++	nd	-	+
3	63	0.33	*Plantibacter*	HG942133	nd	nd	-	-	-	-	-
3	63	0.33	*Plantibacter*	HG942133	nd	nd	-	-	-	-	-
3	63	0.33	*Pseudomonas*	HG942138	+	+++	+++	-	-	-	+
3	178	0.93	*Pseudomonas*	HG942138	-	-	++	++	-	+	+
3	178	0.93	*Pseudomonas*	HG942138	-	-	-	++	-	+	+
3	178	0.93	*Pseudomonas*	HG942138	-	-	-	++	-	+	+
3	178	0.93	*Pseudomonas*	HG942138	-	-	-	++	-	+	+
3	178	0.93	*Pseudomonas*	HG942138	-	-	-	++	-	+	+
3	63	0.33	*Pseudomonas*	HG942141	+	++	+	+++	+	++	+
3	63	0.33	*Pseudomonas*	HG942141	+	-	++	-	-	+	+

3	222	1.16	*Pseudomonas*	HG942141	+	-	++	-	-	-	+
3	222	1.16	*Pseudomonas*	HG942143	+	-	++	-	-	+	+
3	304	1.59	*Pseudomonas*	HG942143	+	-	+++	+	-	++	+
3	304	1.59	*Pseudomonas*	HG942143	+	-	-	+	-	+	+
3	304	1.59	*Pseudomonas*	HG942143	+	-	-	+	-	+	+
3	304	1.59	*Pseudomonas*	HG942143	+	-	-	+	-	+	+
3	304	1.59	*Pseudomonas*	HG942143	+	-	-	+	-	+	+
3	304	1.59	*Pseudomonas*	HG942143	+	-	-	+	-	+	+
3	304	1.59	*Pseudomonas*	HG942143	+	-	-	+	-	+	+
3	304	1.59	*Pseudomonas*	HG942143	+	-	-	+	-	+	+
3	304	1.59	*Pseudomonas*	HG942143	+	-	-	+	-	+	+
3	304	1.59	*Pseudomonas*	HG942143	+	-	-	+	-	++	+
3	84	0.44	*Pseudomonas*	HG942148	-	-	+	-	-	-	-
3	84	0.44	*Pseudomonas*	HG942148	-	-	+	++	-	-	-
3	84	0.44	*Rhodococcus*	HG942153	+	-	-	-	nd	+	-
3	615	3.21	*Streptococcus*	HG942163	nd	nd	-	nd	nd	-	-

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Chapter 4

Bacterial communities associated with *Brassica napus* L. grown on trace element-contaminated and non-contaminated fields: a genotypic and phenotypic comparison

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Abstract

Cultivable bacterial strains associated with field-grown *Brassica napus* L. (soil, rhizosphere and roots) from a trace elements (Cd, Zn and Pb) contaminated field and a non-contaminated control field were characterised genotypically and phenotypically. Correspondence analysis of the genotypic data revealed a correlation between soil and rhizosphere communities isolated from the same field, indicating that local conditions play a more important role in influencing the composition of (rhizosphere) soil bacterial communities than root exudates. In contrast, endophytic communities of roots showed a correlation between fields, suggesting that plants on the two fields contain similar obligate endophytes derived from a common seed endophytic community and/or can select bacteria from the rhizosphere. The latter seemed not very likely since, despite the presence of several potential endophytic taxa in the rhizosphere, no significant correlation was found between root and rhizosphere communities. The majority of Cd/Zn tolerant strains capable of phosphorus solubilisation, nitrogen fixation, indol-3-acetic acid production and showing 1-aminocyclopropane-1-carboxylate deaminase capacity were found in the rhizosphere and roots of plants growing on the contaminated field.

Introduction

In our industrialized world, high trace element (TE) concentrations pose a serious concern. Due to atmospheric deposition from four zinc ore smelters in the Dutch-Belgian border region, soils in the Campine region got enriched with cadmium (Cd), zinc (Zn) and lead (Pb) (Sonke *et al*., 2002). Close to the zinc smelters soils contain 3 to 10 mg Cd per kg dry soil, while at 30 km, background concentrations below 0.5 mg Cd per kg dry soil are found (Koopmans *et al*., 2008). A large portion of this diffusely contaminated region is in agricultural use (Ruttens *et al*., 2010; Witters *et al*., 2011). Increased Cd levels in fodder plants grown on these soils, can lead to increased Cd levels in cattle and hence in the human food chain (Römkens *et al*., 2007). Since Cd is potentially cytotoxic, mutagenic and carcinogenic, farmers are encouraged to remediate their land to ultimately prevent bioaccumulation of toxic metals in food products (Lim and Schoenung, 2010).

An often suggested remediation strategy for vast areas with diffuse trace element (TE)-contamination is the use of plants and their associated microorganisms to extract TEs from soils and accumulate them in harvestable plant parts (phytoextraction) (Vangronsveld *et al*., 2009). However, low TE availability, uptake, translocation, accumulation and tolerance of plants are still limiting full scale application of phytoextraction, (Vangronsveld *et al*., 2009; Weyens *et al*., 2009a). To improve the efficiency of phytoextraction, plant-associated microorganisms with plant growth promoting (PGP) properties and a natural capacity to cope with TE could be exploited (Weyens *et al*., 2009b). PGP bacteria can stimulate root development (Weyens *et al*., 2011) resulting in an enhanced soil volume explored by roots. Most of the published reviews on PGP bacteria focus on their direct PGP effects in soil (Glick, 2010), rhizosphere (Zhuang *et al*., 2007; Khan *et al*., 2009; Ma *et al*., 2011) and plant (Rajkumar *et al*., 2009; Ma *et al*., 2011). Direct PGP effects can be achieved by the production of phytohormones, 1-aminocyclopropane-1-carboxylate (ACC) deaminase and siderophores, by nitrogen fixation and phosphates solubilisation. Plant-associated bacteria that produce siderophores and/or organic acids can also enhance TE availability in soils and by consequence their uptake by plants (Li and Wong, 2010; Rajkumar *et al*., 2010). To assist their host plants to cope with these increased amounts of TEs, endophytic bacteria equipped with a TE

sequestration system are of special interest since they can reduce phytotoxicity and increase TE translocation to aerial plant parts (Lodewyckx *et al*., 2001; Sessitsch and Puschenreiter, 2008). Exploiting these bacterial skills, plants with higher biomass and increased tolerance to TEs can be obtained, eventually resulting in a more efficient phytoextraction.

We investigate *Brassica napus* L. (rapeseed) as a candidate phytoextraction crop because it combines high biomass production with a good tolerance to Cd and Zn (Marchiol *et al*., 2004). At the same time it is a valorisable oil producing crop (Vangronsveld *et al*., 2009), mainly used in food applications and biofuel production (Grispen *et al*., 2006). Combining phytoextraction and biofuel production sounds economically attractive, especially since oil prices are increasing and environmental standards are high (Stephenson *et al*., 2008). Moreover, oil and seed meal with acceptable Cd and Zn concentrations could be used to enrich fodder with carbohydrates, proteins and phytosterols (Gül and Şeker, 2006; Iqbal *et al*., 2008). Combining the phytoextraction and economic potentials of *B. napus* could become the decisive factor for a successful remediation in diffusely contaminated areas, like the Campine region, especially when rapeseed-associated bacteria could enhance Cd phytoextraction efficiency.

Since the natural habitat is considered as an interesting model for the evolution of TE tolerant PGP microorganisms (Ma *et al*., 2009), we characterized the cultivable bacterial communities associated with bulk soil, rhizosphere soil and roots of *B. napus* grown on a Cd, Zn and Pb-contaminated field in Lommel (Belgium; trace element field (TE-F)) and a non-contaminated field in Alken (Belgium; control field (CO-F)). The main objectives of this study were to extend our knowledge on the poorly known bacterial communities associated with *B. napus* and to identify PGP, Cd tolerant and Cd solubilising bacteria which might increase biomass production and Cd uptake by rapeseed growing under the unfavourable environmental conditions occurring on contaminated fields.

Results

Isolation of B. napus-associated bacteria

Bacteria were isolated from bulk soil, rhizosphere soil and roots of *B. napus*, grown on an uncontaminated control soil (CO-F) and a contaminated soil (TE-F) (table 4.1).

Table 4.1 Mean total numbers of colony-forming units (cfu) per gram fresh weight of the compartments (COMPT) bulk soil (BS), rhizosphere soil (RS) and *B. napus* root tissue (R) isolated on the control field (CO-F) and the contaminated field (TE-F).

field	COMPT	cfu g^{-1} fresh weight
CO-F	BS	$99.7\ 10^5 \pm 51.8\ 10^5$ (6)
	RS	$23.7\ 10^8 \pm 21.2\ 10^8$ (17)
	R	$10.4\ 10^5 \pm 23.4\ 10^4$ (15)
TE-F	BS	$20.5\ 10^6 \pm 22.4\ 10^5$ (20)
	RS	$78.1\ 10^7 \pm 30.8\ 10^7$ (25)
	R	$22.4\ 10^5 \pm 19.8\ 10^5$ (18)

Values are mean ± standard error of 3 biological independent replicates. Numbers of different bacterial genera are marked between parentheses.

For both fields, the number of cultivable strains recovered from the bulk soil was lower compared to the rhizosphere soil and higher compared to the roots. More cultivable strains were isolated from contaminated bulk soil and roots compared to the control ones, whereas more cultivable strains inhabited the control rhizosphere soil. At both fields, the number of different bacterial genera was higher in the rhizosphere soil than in bulk soil and root.

Genotypic characterisation

After isolation and purification, 5 purified replicates of all morphologically different bacterial strains isolated from bulk soil, rhizosphere soil and roots of *B. napus* were characterised by amplified 16S rDNA restriction analysis (ARDRA) using the restriction enzyme HpyCH4IV. One representative member of all strains with identical fingerprints was sequenced for identification by means of Sequence Match at the Ribosomal Database Project II. All (except *Chryseobacterium* DQ337589) strains have a sequence match score higher than 0.900, which indicates a confident identification to the genus level (appendix 4.1). Also the neighbour-joining tree clustered strains belonging to the same genus together, confirming the results of the 16S rRNA genes based identification procedure (appendix 4.2). The identification resulted in 37 different

bacterial genera recovered from the contaminated field and 29 from the control field (figure 4.1).

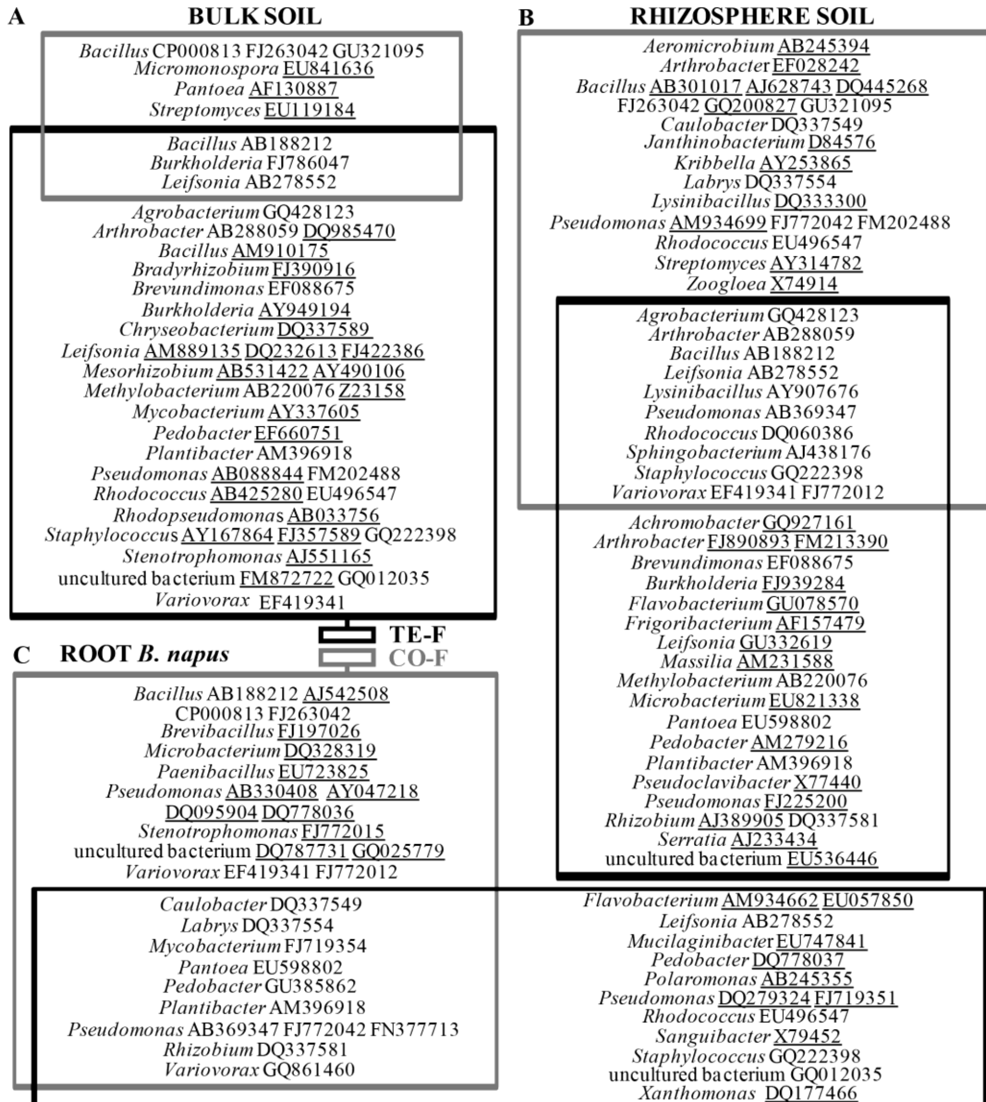


Figure 4.1 Diversity of cultivable bacterial strains isolated from bulk soil (A), rhizosphere soil (B) and *B. napus* root samples (C) taken at the control field (CO-F) and the contaminated field (TE-F). Bacterial strains present in the intersections were found at both fields, underlined strains were exclusively found in that specific compartment. Behind each bacterial genus, different accession numbers are represented (see appendix 4.1 for abundances).

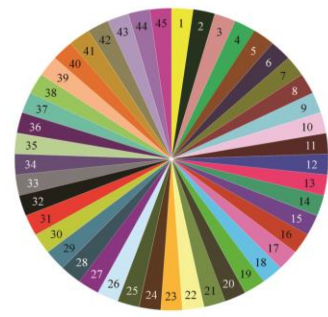
Eighteen of the identified genera were found at both fields. To visualize the diversity and abundance of cultivable *B. napus*-associated bacteria, pie diagrams were prepared (figure 4.2). Each colour (number) represents a bacterial genus; subdivided colours represent genera with different accession numbers. The relative abundance of each genus was expressed as a percentage of the total number of cultivable isolates per gram fresh weight bulk soil, rhizosphere soil and roots (table 4.1).

The cultivable soil bacteria at the control field were dominated by the genera *Micromonospora* (50%), *Bacillus* (38.7%) and *Leifsonia* (5.8%) and at the contaminated field by *Arthrobacter* (26.9%), *Leifsonia* (15.7%), *Staphylococcus* (10.0%), *Burkholderia* (7.4%), *Variovorax* (6.4%) and *Rhodopseudomonas* (5.6%). The major part of the cultivable rhizosphere bacteria at the control field consisted of *Bacillus* (43.2%), *Pseudomonas* (19.3%), *Variovorax* (13.7%) and *Staphylococcus* (8.3%) and at the contaminated field of *Leifsonia* (29.3%), *Staphylococcus* (22.7%), *Plantibacter* (12.3%), *Serratia* (6.9%) and *Microbacterium* (6.0%). *Variovorax* (34.5%), *Pseudomonas* (29.4%), *Bacillus* (12.4%), *Caulobacter* (8.6%) and *Pantoea* (6.5%) dominated the cultivable root endophytes at the control field, while at the contaminated field it were *Pseudomonas* (33.0%), *Rhizobium* (16.1%), *Caulobacter* (14.9%), *Pedobacter* (14.7%) and *Variovorax* (7.8%) (figure 4.2).

The number of genotypically different bacterial strains occurring in the same compartment at both fields (see intersections) increases from bulk soil to rhizosphere soil and roots (figure 4.1). Bacterial strains present at both fields in the bulk soil were *Bacillus* (AB188212), *Burkholderia* (FJ786047) and *Leifsonia* (AB278552); in the rhizosphere it were *Agrobacterium* (GQ428123), *Arthrobacter* (AB288059), *Bacillus* (AB188212), *Leifsonia* (AB278552), *Lysinibacillus* (AY907676), *Pseudomonas* (AB369347), *Rhodococcus* (DQ060386), *Sphingobacterium* (AJ438176), *Staphylococcus* (GQ222398) and *Variovorax* (EF419341, FJ772012); and in the roots it were *Caulobacter* (DQ337549), *Labrys* (DQ337554), *Mycobacterium* (FJ719354), *Pantoea* (EU598802), *Pedobacter* (GU385862), *Plantibacter* (AM396918), *Pseudomonas* (AB369347, FJ772042, FN377713), *Rhizobium* (DQ337581) and *Variovorax* (GQ861460).

CO-F	TE-F

Legend:



1 *Achromobacter*
2 *Aeromicrobium*
3 *Agrobacterium*
4 *Arthrobacter*
5 *Bacillus*
6 *Bradyrhizobium*
7 *Brevibacillus*
8 *Brevundimonas*
9 *Burkholderia*
10 *Caulobacter*
11 *Chryseobacterium*
12 *Flavobacterium*
13 *Frigoribacterium*
14 *Janthinobacterium*
15 *Kribella*
16 *Labrys*
17 *Leifsonia*
18 *Lysinibacillus*
19 *Massilia*
20 *Mesorhizobium*
21 *Methylobacterium*
22 *Microbacterium*
23 *Micromonospora*
24 *Mucilaginibacter*
25 *Mycobacterium*
26 *Paenibacillus*
27 *Pantoea*
28 *Pedobacter*
29 *Plantibacter*
30 *Polaromonas*
31 *Pseudoclavibacter*
32 *Pseudomonas*
33 *Rhizobium*
34 *Rhodococcus*
35 *Rhodopseudomonas*
36 *Sanguibacter*
37 *Serratia*
38 *Sphingobacterium*
39 *Staphylococcus*
40 *Stenotrophomonas*
41 *Streptomyces*
42 uncultured bacterium
43 *Variovorax*
44 *Xanthomonas*
45 *Zoogloea*

Figure 4.2 Diversity and abundance of cultivable bacterial strains isolated from bulk soil, rhizosphere soil and root samples taken at the control field (CO-F) (A, C and E respectively) and the contaminated field (TE-F) (B, D and F respectively). Each colour (number) represents a bacterial genus, subdivided colours represent bacterial genera with different accession numbers. Pie fragments indicate the relative abundance, expressed in percentages (see appendix 4.1), of the total number of cultivable bacteria isolates per gram fresh weight that are present in the bulk soil, rhizosphere soil and inside the roots of *B. napus.* Bacterial strains with abundances lower than 1% (percentage shown between parentheses) are shown separately next to the pie diagram. Bacterial genera which are marked bold in the legend were found at both fields.

Bacterial strains exclusively occurring in one of the investigated compartments at the control respectively the contaminated field are underlined in figure 4.1. From the strains shown in the intersection (= occurring at both fields) of figure 4.1C, only *Mycobacterium* (FJ719354), *Pedobacter* (GU385862), *Pseudomonas* (FN377713) and *Variovorax* (GQ861460) were exclusively found in the roots. *Pantoea* (EU598802), *Plantibacter* (AM396918) and *Rhizobium* (DQ337581) were isolated from roots at both fields; at the control field they were not found in bulk neither rhizosphere soil, like at the contaminated field *Caulobacter* (DQ337549), *Labrys* (DQ337554) and *Pseudomonas* (FJ772042) did not occur in other compartments. Other root endophytes were also found in bulk and/or rhizosphere soil (CO-F: *Bacillus* (AB188212), *Bacillus* (FJ263042); TE-F: *Leifsonia* (AB278552), *Plantibacter* (AM396918), *Staphylococcus* (GQ222398)). Bacterial strains restricted to the bulk and rhizosphere soil at the control field were *Bacillus* (GU321095) and *Leifsonia* (AB278552) and at the contaminated field *Agrobacterium* (GQ428123), *Arthrobacter* (AB288059), *Bacillus* (AB188212), *Brevundimonas* (EF088675), *Methylobacterium* (AB220076) and *Variovorax* (EF419341).

Based on the correspondence analysis (CA) of these data (figure 4.3), we can conclude that the bacterial communities isolated from bulk and rhizosphere soil at both fields are correlated within field (correlation coefficients: 0.48 (TE-F) and 0.50 (CO-F)). Also root bacterial communities at both fields are correlated (correlation coefficient: 0.64).

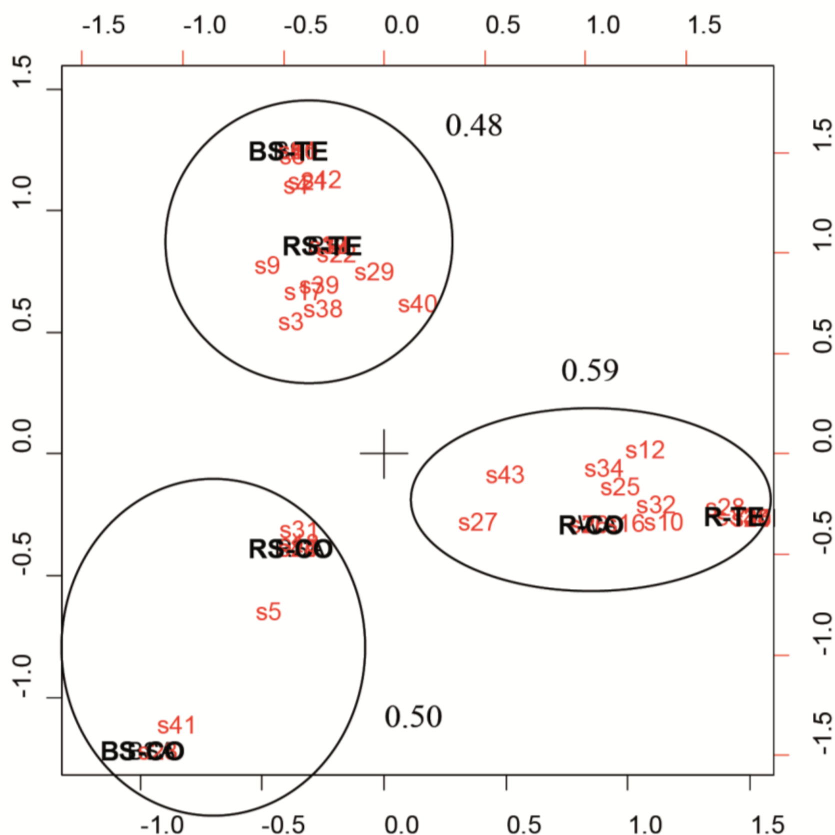


Figure 4.3 Correspondence analysis of bacterial communities isolated from bulk soil, rhizosphere soil and *B. napus* root samples taken at the control field and the contaminated field. Each number (s1-s45) represents an isolated bacterial genus, the connection between genera and numbers can be found in the legend of figure 4.2. Clustered compartments point out the correlation between the bacterial communities found in the bulk soil, rhizosphere soil and roots collected at the control field (BS-CO, RS-CO and R-CO respectively) and the contaminated field (BS-TE, RS-TE and R-TE respectively). Correlation coefficients of clustered compartments are indicated.

Phenotypic characterisation

Trace element (TE) tolerance

All purified isolates were screened for their TE tolerance (table 4.2A). Percentages of strains from the rhizosphere and from the roots tolerant to 0.8 mM Cd were similar at both fields. At the contaminated field, higher percentages of bulk soil bacteria were tolerant to 0.8 and 1.6 mM Cd, also the rhizosphere soil and the roots contained much higher percentages of strains tolerant to 1.6

mM Cd compared to the control field. The highest percentages of Cd tolerant strains at the contaminated field were found in the roots, while at the control field highest Cd tolerance was occurring in the rhizosphere and roots. Contaminated bulk and rhizosphere soil contained higher percentages of Zn tolerant strains for all tested Zn concentrations (0.6, 1.0 and 2.5 mM Zn). Roots from both fields harboured similar percentages of 0.6 mM Zn tolerant bacteria, while percentages of root strains tolerant to 1.0 and 2.5 mM Zn were higher at the contaminated field. The highest percentages of Zn tolerant strains at the contaminated field were found in the rhizosphere soil, while at the control field this was in the roots.

Table 4.2 Phenotypic characterisation of all purified bulk soil, rhizosphere soil and *B. napus* root isolates collected at the control field (BS-CO, RS-CO and R-CO respectively) and the contaminated field (BS-TE, RS-TE and R-TE respectively).

A	**BS-CO**	**BS-TE**	**RS-CO**	**RS-TE**	**R-CO**	**R-TE**
Cd (0.8 mM)	1.8	10.8	14.5	13.1	28.1	22.7
Cd (1.6 mM)	1.8	9.2	3.0	12.5	2.2	14.7
Zn (0.6 mM)	25.8	95.3	43.1	99.2	66.8	52.2
Zn (1.0 mM)	10.5	61.0	2.4	71.5	17.6	32.8
Zn (2.5 mM)	8.8	58.0	1.1	65.5	16.7	32.6
B	**BS-CO**	**BS-TE**	**RS-CO**	**RS-TE**	**R-CO**	**R-TE**
SID	21.2	52.6	79.1	26.3	80.8	41.5
OA	6.0	4.9	39.8	23.6	10.6	19.3
ACC	0.0	39.2	18.3	37.1	33.1	37.3
IAA	26.8	32.9	31.7	36.4	36.4	61.5
acetoin	17.3	2.8	49.2	8.6	17.0	2.5
P sol	11.6	44.3	19.6	58.1	50.1	49.2
N_2 fix	2.0	3.7	6.2	30.4	8.7	10.8

Data are shown as relative abundances, expressed in percentages, of the total number of cultivable bacterial isolates per gram fresh weight bulk soil (BS), rhizosphere soil (RS) and roots (R) at both fields which were tolerant to different concentrations of Cd (0.8 and 1.6 mM) and Zn (0.6, 1.0 and 2.5 mM) (A); and were capable of phosphorus solubilisation (P sol), nitrogen fixation (N_2 fix) and the production of siderophores (SID), organic acids (OA), ACC deaminase (ACC), indole-3-acetic acid (IAA) and acetoin (B).

Plant growth promoting (PGP) characteristics

All strains were screened for their potential PGP characteristics (table 4.2B). The percentage of siderophore producing strains in the bulk soil was more than 2 times higher at the contaminated field, while in the rhizosphere and roots more siderophore producing strains were present at the control field. At both fields similar low percentages of organic acid producing strains were isolated from the bulk soil, while rhizosphere soil and root samples from the control field contained higher respectively lower percentages of these bacteria compared to the contaminated field. All compartments at the contaminated field contained higher percentages of ACC deaminase producing strains as compared to the control field. Moreover similar percentages were found in all compartments at the contaminated field while percentages increased from bulk soil to the root at the control field. Phosphorus solubilisation capacity showed a similar distribution pattern. The relative abundance of indol-3-acetic acid (IAA) producing strains was similar in the bulk and rhizosphere soil from both fields and in the control roots, while proportionally twice as much IAA producing strains were isolated from roots at the contaminated field. The percentages of acetoin producing bacterial strains at the control field were higher in all studied compartments; at the contaminated field this was the case for nitrogen fixating strains.

The highest percentages of strains able of solubilising phosphorus, fixating nitrogen and producing siderophores, ACC deaminase and IAA at the control field were found in the roots, while proportionally the most organic acid and acetoin producing strains were detected in the rhizosphere soil. At the contaminated field, percentages of siderophore and ACC deaminase producing strains were highest in the bulk soil, while percentages of phosphorus solubilising, nitrogen fixating and organic acid/acetoin producing strains were proportionally highest in the rhizosphere soil. The most IAA producing strains at the contaminated field were found inside the roots.

Trace element (TE) concentrations in soils and plants

Total TE concentrations were determined in bulk soil and plant parts (root, stem, leaf and seed) (table 4.3). In addition, plant available TE concentrations in bulk soil were estimated using a $Ca(NO_3)_2$ selective extraction.

Table 4.3 Soil and plant trace element (TE) concentrations; $Ca(NO_3)_2$-extractable (*extr*) essential (Zn, Cu, Fe) and non-essential (Cd, Pb) TE concentrations [mg (kg dry weight)$^{-1}$] measured in bulk soil and total essential and non-essential TE concentrations [mg (kg dry weight)$^{-1}$] in bulk soil and *B. napus* plants (root, stem, leaf and seed) from the control field (CO-F) and the contaminated field (TE-F).

	CO-F											
	bulk soil *(extr)*		bulk soil *(total)*		root		stem		leaf		seed	
	mean	error	mean	error	mean	error	mean	error	mean	error	mean	error
Cd	0.15	0.0073	0.50	0.00	0.29	0.054	0.22	0.021	0.40	0.012	nd	nd
Zn	4.6	0.023	89	4.6	33	7.4	22	3.6	77	4.0	39	0.89
Pb	nd	nd	25	0.85	nd	nd	nd	nd	nd	nd	nd	nd
Cu	0.20	0.0073	15	0.41	3.7	1.1	nd	nd	nd	nd	9.5	4.8
Fe	1.8	0.98	9141	223	354*	97	27	3.7	88	10	47	1.4
	TE-F											
	bulk soil *(extr)*		bulk soil *(total)*		root		stem		leaf		seed	
	mean	error	mean	error	mean	error	mean	error	mean	error	mean	error
Cd	1.0****	0.0033	5.1****	0.088	3.1***	0.32	4.6***	0.31	7.2**	0.45	0.88	0.081
Zn	78****	0.48	277****	6.7	490***	35	472****	27	863***	70	96****	1.5
Pb	0.38	0.010	199****	2.7	8.5	0.63	nd	nd	3.4	0.20	nd	nd
Cu	0.18	0.0050	27****	0.23	3.2	0.074	nd	nd	3.6	0.22	4.8	0.27
Fe	0.58	0.035	2209****	64	43	2.6	18	0.28	89	6.3	76****	3.2

Values are mean ± standard error of 3 biological independent replicates. Trace element concentrations in soil and plant compartments were compared between fields (significance levels: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$). Trace element contents which were too high or too low to be detected are indicated by 'saturated' (sat) and 'not detected' (nd) respectively.

Amounts of both total and $Ca(NO_3)_2$-extractable Cd, Zn and Pb in the bulk soil were significantly higher at the contaminated field as compared to the control field, like also the Cd and Zn concentrations measured in the roots, stems, leaves and seeds. Lead concentrations in plant parts were below detection limit while total and $Ca(NO_3)_2$-extractable Pb concentrations in the soil at the contaminated field were significantly higher than at the control field. Plants grown at the contaminated field contained significantly more Cd and Zn in their leaves compared to the roots and stems, their seeds accumulated acceptable Cd

concentrations (< 1.14 mg Cd kg^{-1}) according to the European standards for animal feed (appendix 4.3). At both fields, plants contained adequate tissue levels of Ca, K, Mg, Na and Zn (data not shown). The Cu and Fe concentrations in plant tissues were just below the prescribed levels for adequate growth but are not expected to be limiting. Total bulk soil Cu and Fe concentrations were significantly higher at the contaminated field whereas the $Ca(NO_3)_2$-extractable concentrations were similar at both fields. When plant parts were compared between fields, total Fe concentrations were significantly higher in roots from the control and seeds from the contaminated field.

Discussion

Agricultural soils in the Campine region are diffusely contaminated with Cd, Zn and Pb (table 4.3). Since the contamination is due to past atmospheric deposition of these TEs from Zn-smelters it is concentrated in the upper soil layer (Sonke *et al*., 2002). Therefore, accumulating plant species developing their root system in this soil layer might be interesting candidates for phytoextraction in this region. An ideal plant for TE phytoextraction should produce high biomass and should take up and translocate to its shoots a significant part of the TEs of concern (Kärenlampi *et al*., 2000). Since the calculated time periods for phytoextraction of toxic TEs are long, it is necessary that the selected crop can be valorised (Vassilev *et al*., 2004; Vangronsveld *et al.,* 2009; Meers *et al*. 2010).

Brassica napus, a high biomass oil delivering crop**,** has a good potential to meet most of these criteria. Indeed, roots of *B. napus*, are abundantly developing in the upper soil layer (Marchiol *et al*., 2004). Moreover, rapeseed translocates assimilated TEs to its leaves at the end of the growing season (table 4.3 and appendix 4.3). In case rapeseed can be used as a crop for phytoextraction of toxic TEs, farmers can make profit out of the cultivation by valorising the seeds (biofuel vs. fodder) and other plant parts (biogas). Rapeseed oil can be utilized as alternative fuel whereas intact seeds can be used as animal fodder. Seeds of plants grown on the contaminated soils contained acceptable Cd and Pb concentrations (< 1.14 mg Cd kg^{-1} and < 11.4 mg Pb kg^{-1}) according to the European standards for animal feed, unlike the other plant parts harvested on the contaminated field (table 4.3 and Grispen *et al*., 2006). Fermentation

processes can be adopted to reduce the quantity of contaminated biomass while producing biogas (Van Ginneken *et al*., 2007).

However, the amounts of toxic TE that can be extracted by *B. napus* are still much too low to allow significant reductions of these TE contents in the soil in realistic periods of time (Vangronsveld *et al*., 2009). To improve the applicability and efficiency of phytoextraction, plant-associated bacteria could be exploited to enhance biomass production and to increase TE availability, uptake, translocation and tolerance of plants (Weyens *et al*., 2009a, b). Therefore, we investigated the diversity of cultivable bacteria associated with *B. napus* from plants grown on a non-contaminated (control) and a contaminated field as well as the characteristics of the isolated bacterial communities that might contribute to improve biomass production and TE uptake and translocation. Approximately 500 morphologically different bacterial strains were isolated from bulk soil, rhizosphere soil and roots of *B. napus* at both fields and identified based on 16S rDNA sequencing (figure 4.1 and 4.2). All (except *Chryseobacterium* DQ337589) strains showed a sequence match score higher than 0.900, which indicates a confident identification to the genus level (appendix 4.1).

The higher amount of cultivable bacteria in the rhizosphere than in bulk soil (table 4.1) can be explained by the 'rhizosphere effect' (Rouatt *et al*., 1960). Growth and activity of soil microorganisms are mainly limited by organic carbon (Demoling *et al*., 2007). Poor decomposability of soil organic matter in contrast with easily decomposable root exudates results in higher microbial density/diversity in the rhizosphere (Soderberg and Bååth, 1998). The numbers of cultivable rhizosphere bacteria and root endophytes are in accordance with literature (Benizri *et al*., 2001; Hallmann, 2001); bacterial density/diversity decreased from the rhizosphere to the roots (table 4.1 and Fisher *et al*. 1992). The rapeseed-associated bacterial populations that we characterised were dominated by *Bacillus*, *Pseudomonas*, *Variovorax*, *Leifsonia*, *Micromonospora*, *Staphylococcus*, *Arthrobacter* and *Caulobacter* (figure 4.2). Some of these genera were already reported in earlier studies on rapeseed-associated populations (see below), while others are mentioned for the first time in our study. This might be due to (1) differences in isolation protocols (Siciliano and Germida, 1999), growth media and identification procedures (Germida and

Theoret, 1997; Kaiser *et al*., 2001) and (2) the environmental growing conditions of the plants (Lemanceau *et al*., 1995; Song, 1999).

Germida *et al*. (1998) and Siciliano and Germida (1999) were the first to investigate bacterial communities associated with field-grown *B. napus* using fatty acid methyl ester (FAME) profiles, a tentatively identification method (Haack *et al*., 1994). They concluded that the rhizosphere and root interior were colonized mainly by the genera *Bacillus*, *Flavobacterium*, *Micrococcus*, *Rathayibacter*, *Pseudomonas*, *Variovorax* and *Arthrobacter*. Larcher *et al*. (2008) isolated similar genera from the rhizosphere (*Serratia*, *Stenotrophomonas*, *Microbacterium*, *Paenibacillus*, *Arthrobacter*, *Variovorax* and *Pseudomonas*) and roots (*Serratia*, *Pseudomonas*, *Stenotrophomonas* and *Microbacterium*) of field-grown *B. napus*. Additionally, Kaiser *et al*. (2001) and Granér *et al*. (2003) demonstrated that greenhouse and field-grown *B. napus* showed corresponding genera in the rhizosphere, root, stem and/or seed, including *Agrobacterium*, *Paenibacillus*, *Bacillus*, *Pseudomonas*, *Chryseobacterium*, *Pantoa*, *Caulobacter*, *Variovorax*, *Stenotrophomonas*, *Arthrobacter*, *Microbacterium*, *Streptomyces* and *Staphylococus*.

Common bacterial genera identified during our research and not yet mentioned in earlier work on *B. napus* are *Burkholderia*, *Labrys*, *Leifsonia*, *Lysinibacillus*, *Micromonospora*, *Mycobacterium*, *Pedobacter*, *Plantibacter*, *Rhizobium*, *Rhodopseudomonas* and *Sphingobacterium* (figure 4.2). Many bacterial strains appeared in both soil compartments (figure 4.1) and based on the correspondence analysis of the genotypical information (figure 4.3), we conclude that at both fields the rhizosphere communities correlate well with the bulk soil communities. This observation can be explained by the fact that bacterial rhizosphere colonization is driven by the production of root exudates (Lugtenberg and Dekkers, 1999) to which soil microorganisms are chemo-attracted (Lugtenberg and Kamilova, 2009; Compant *et al*., 2010). Consequently, root exudates control rhizosphere populations like Grayston *et al*. (1998) postulated, but also field-specific soil factors play a significant role since rhizosphere communities at both fields were not identical. Accordingly, Lundberg *et al*. (2012) and Bulgarelli *et al*. (2012) reported that soil type defines the composition of bacterial rhizosphere and root communities of *Arabidopsis thaliana* plants.

A second remarkable observation was that endophytic root communities from both fields were similar (figure 4.3). Lundberg *et al*. (2012) as well as Bulgarelli *et al*. (2012) observed that the host plant determined to a limited extent the bacterial ribotype profiles in roots. We suggest that plants grown from the same seed stock at different fields possess similar obligate endophytes originating from their common seed endophytic community. This statement is based on the exclusive presence of several strains in the roots of plants grown at both fields including *Mycobacterium* (FJ719354), *Pedobacter* (GU385862), *Pseudomonas* (FN377713) and *Variovorax* (GQ861460) (figure 4.1). Other strains exclusively present in the roots at the control field respectively the contaminated field may also be considered as potential seed endophytes since we hypothesize that some of them flourish more than others depending on the local environmental conditions. Root endophytes also isolated from the bulk and/or rhizosphere soil assent with Kobayashi (2000) who mentioned that many endophytic taxa also occur in the rhizosphere. However, in our study, no significant correlation was found between root endophytic and rhizosphere communities within the same field, despite the fact that 8 root endophytic strains at the control field and 6 at the contaminated field were also detected in the rhizosphere soil (figure 4.1). Based on these genotypical data, we presume that plants are capable of favouring the dominance of some specific seed endophytes as obligate endophytes and that the isolated facultative endophytes systemically colonized the inside of the plant via the rhizosphere soil (*cfr.* Compant *et al*., 2010). Probably, most of these facultative endophytes are selected from the soil by plant root exudates that have pronounced selective and promoting effects on specific soil microbial populations (Hartmann *et al*., 2009).

All isolated strains were tested for their Cd and Zn tolerance. The highest numbers of strains tolerant to 1.6 mM Cd and 2.5 mM Zn originated from the contaminated field (table 4.2A). Most likely, the significantly higher Cd and Zn concentrations in bulk soil and roots, compared to the background concentrations at the control field (table 4.3), caused a selective pressure in favour of Cd and Zn tolerant bacteria at the contaminated field. Moreover, it seems that in this case Zn exerts a higher selective pressure than Cd on bacterial communities since multiple bacterial strains isolated at the contaminated field tolerate the highest Zn concentration, while strains isolated

from the control field could hardly survive (table 4.2A). Chemical similarity between Cd and Zn and their association in the environment can lead to interactions between these 2 ions (McKenna *et al*., 1993), resulting in a lowering of Cd toxicity (Wajda *et al*., 1989). Further, the uptake and translocation of Zn by plants is higher than Cd (Shrivastava and Shing, 1989) since Cd is a non-essential ion and toxic at a lower concentration than Zn (Chakravarty and Shrivastava, 1994).

A high number of bacterial strains isolated from the control rhizosphere and roots could produce siderophores (table 4.2B). This might explain why the Fe-content in roots of plants grown on the control field was about 8 times higher, while the $Ca(NO_3)_2$-extractable Fe concentrations in the soils from both fields were not significantly different (table 4.3). Iron deficiency (< 100 mg kg^{-1}) was noticed in all parts of plant from the contaminated field; this might inhibit chlorophyll synthesis and chloroplast development and increase ethylene production in plant tissues, eventually leading to decreased remediation efficiency (Glick, 2003). Bacterial siderophore production can promote plant growth, especially in case of iron deficiency by sequestering Fe in siderophore-Fe complexes which plants can use as Fe source (Wang *et al*., 1993). In addition siderophores may also enhance Cd availability since Cd can also be sequestered by siderophores (Rajkumar *et al*., 2010). At both fields the highest amounts of organic acid producing bacteria were found in the rhizosphere (table 4.2B). Plant roots exude organic acids into the rhizosphere for the mobilisation of poorly soluble nutrients in the soil (Ström *et al*., 2002). An increased acidity in the rhizosphere will also increase TE solubility and eventually phytoextraction potential (Li and Wong, 2010).

At the contaminated field, more bacterial strains showed potential for phosphorus solubilisation, nitrogen fixation and production of ACC deaminase and IAA (table 4.2B). This might be an indication for bacterial selection by plants during stress conditions. Increased availability of nutrients, bacterial production of plant growth hormones and breakdown of the immediate precursor of the plant stress hormone ethylene can be crucial for plant survival in adverse conditions. In contrast, more acetoin producing bacteria were found at the control field (table 4.2B). Higher amounts of organic matter may favour the

activity of fermentative bacteria using acetoin as an external energy store (Xiao and Xu, 2007).

This phenotypic information suggests that the TE contamination generates a selective pressure in favour of Cd/Zn tolerant, phosphorus solubilising, nitrogen fixating and ACC deaminase/IAA producing bacteria since their amounts are consistently higher in all studied compartments at the contaminated field (table 4.2).

In conclusion, genotypic and phenotypic characteristics of rapeseed-associated bacterial populations can be affected by environmental conditions (*e.g.* soil contamination) as well as by their host plant (*i.e.* selection from the rhizosphere/bulk soil and present seed endophytes). The environmental conditions at the contaminated field seem inductive for the occurrence of rapeseed-associated bacteria with potential to enhance Cd phytoextraction. Enriching the rhizophere with these PGP, siderophore and/or organic acid producing bacteria might enhance TE uptake while endophytes equipped with a TE sequestration system might reduce Cd phytotoxicity. In future inoculation experiments, the *in planta* potential of promising strains to enhance phytoextraction efficiency will be tested (Sheng and Xia, 2006; Dell'Amico *et al*., 2008; Sheng *et al*., 2008). Once the most appropriate plant-associated bacteria will be identified, they can be exploited to accelerate the TE extraction process, adjusting the high biomass producing *B. napus* into a reasonable Cd phytoextractor that at the same time can be economically valorised.

Experimental Procedures

Sampling

In order to isolate the cultivable bacteria associated with field-grown *B. napus*, soils and plants were sampled after the flowering stage (June 2010). Sampling was performed on a TE (Cd, Zn and Pb)-contaminated former maize field in Lommel (TE-F; see Ruttens *et al*., 2010) and on a non-contaminated field in Alken (Belgium) (CO-F). On both fields the sampling area was subdivided into 3 subareas. One plant, with its surrounding rhizosphere soil and bulk soil, from each subarea (3 in total) made up a mixed bulk soil, rhizosphere soil, root, stem and leaf sample. Sampling was repeated 3 times using each time 3 other plants

(1 per subarea). Bulk soil was sampled at a depth of 30 cm. Roots were stored in sterile Falcon tubes containing 20 ml sterile 10 mM $MgSO_4$.

Isolation of B. napus-associated bacteria

All cultivable bacterial strains were isolated from bulk soil, rhizosphere soil and roots according to Weyens *et al*. (2009c), but using less active chloride solution (1%) and time (1 min) during root surface sterilisation. All plated samples were incubated for 7 days at 30°C and colony forming units (cfu) were counted and calculated per gram soil or fresh plant weight. Morphologically different strains were purified using 5 replicates and subsequently stored at -70°C in a glycerol solution (15% (w:v) glycerol; 0.85% (w:v) NaCl).

Genotypic characterisation

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 was performed on aliquots of the extracted DNA using the universal primers, 16S-prokaryotic-R (5'- ACGGGCGGTGTGTRC-3') and 16S-prokaryotic-F (5'-AGAGTTTGATCCTGGCTCAG-3') as described previously by Weyens *et al*. (2009c).

For amplified 16S rDNA restriction analysis (ARDRA), 20 µl of the PCR products were digested with the HpyCH4IV enzyme and visualized by gel electrophoresis as described by Weyens *et al*. (2009c). Bacterial strains from bulk and rhizosphere soil with the same ARDRA patterns were grouped; strains isolated from plant tissue were grouped separately. The 16S rDNA PCR products of 1 representative strain per group were purified according to the QIAquick 96 PCR Purification Kit (Qiagen, Valencia, CA, USA). Subsequently, purified 16S rRNA genes were sent for sequencing by Macrogen (Korea) with an Automatic Sequencer 3730XL. Consensus sequences, sequence matches and sequence alignments used for constructing a neighbour-joining tree to verify identification were obtained as in Weyens *et al*. (2009c).

Phenotypic characterisation

All purified bacterial isolates were screened for TE tolerance (Cd and Zn) and potential PGP characteristics (phosphate solubilisation, nitrogen fixation and production of siderophores, organic acids, IAA, acetoin and ACC-deaminase). Before screening, strains were grown in 869 medium (Mergeay *et al*., 1985) and subsequently washed twice with sterile 10 mM $MgSO_4$. Strains, not able to grow in the test media (pH 7) during incubation (5 (liquid media) to 7 days (solid media) at 30°C), were considered as not detectable (nd). Media without cell suspension were used as controls.

Trace element (TE) tolerance

All isolates were plated on selective 284 medium with a carbon mix and 0.0, 0.8 and 1.6 mM Cd ($CdSO_4$) or 0.0, 0.6, 1 and 2.5 mM Zn ($ZnSO_4$), tolerance was rated visually (Weyens *et al*., 2009c).

Plant growth promoting (PGP) characteristics

National Botanical Research Institute's phosphate growth solid medium was used for screening **phosphate-solubilizing microorganisms** (Nautiyal, 1999), 50 µl aliquots of washed strains were inoculated in holes (Ø: 0.5 cm). Strains capable of producing a clear zone were considered positive. Bacterial **nitrogenase activity** was tested in a semi-solid malate-sucrose medium modified from Döbereiner (1989) (Xie *et al*., 2006). Three ml bromothymol blue per liter medium was used as a pH indicator (Nabti *et al*., 2007). Anaerobic nitrogenase activity was visually rated as a colour change from blue to yellow which indicated the acidification of sugars and therefore growth. **Siderophore secretion** was qualitatively evaluated by the "universal" colorimetrical method of Schwyn and Neilands (1987) after inoculating strains in 800 µl selective 284 medium with a carbon mix and 0, 0.25 and 3 µM Fe (respectively deficient, optimal and oversupply Fe conditions). Bacterial **organic acid production** was detected according to the colorimetric method of Cunningham and Kuiack (1992) after inoculating strains in 800 µl sucrose tryptone medium. Bacterial **IAA production** capacity was tested in 1 ml 1/10 869 medium with 0.5 g l^{-1} tryptophan. After incubation, a colorimetric reaction was induced to find positive strains (Gordon and Weber, 1951). To detect strains that utilize the butylene

glycol pathway and **produce acetoin**, strains were inoculated in Methyl Red-Voges Proskauer (MRVP) medium containing per liter 17 g MRVP medium (Sigma-Aldrich). After 48h of incubation, a colorimetric reaction was induced according to Romick and Fleming (1998), in order to observe positive strains. **ACC deaminase activity** was evaluated by a slight modified protocol according to Belimov *et al*. (2005). Washed bacterial pellets were resuspended in 1 ml salts minimal medium with 10 mM ACC as sole nitrogen source. After 3 days at 30°C, bacterial cells were resuspended in 0.1 ml Tris-HCl buffer (pH 8.5) and disrupted by 15 µl of toluene. Subsequently, 15 µl 0.5 M ACC and 100 µl 0.1 M Tris-HCl buffer (pH 8.5) were added to induce ACC deaminase activity, which was stopped by adding 0.5 ml 0.56 N HCl. An aliquot of the supernatant was used as described in Belimov *et al*. (2005) to check the presence of ACC deaminase visually.

Trace element (Na, Mg, K, Fe, Cu, Zn, Cd, Pb) (TE) concentrations in soils and plants

The plant available fractions of TEs present in the bulk soil were estimated using 0.1 M $Ca(NO_3)_2$ extraction (Mench *et al*., 1994). Total soil TE contents were determined by *aqua regia* digestion (Van Ranst *et al*., 1999). To measure total TE concentrations in plant organs (root, stem, leaf and seed), samples collected in the field were treated as described by Weyens *et al*. (2010). Trace element concentrations were determined using inductively coupled plasma optical emission spectrometry (ICP-OES). All mixed soil and plant samples were tested in triplicate.

Statistical Analysis

Percentages of genotypic and phenotypic different strains per mixed sample and their mean percentages per compartment were calculated but not appropriate for ANOVA analysis. Genotypic information was subjected to correspondence analysis (CA), a principal component analysis related ordination technique based on chi-square distances, illustrating correlations between compartments. Trace element concentrations were statistically compared between both fields using 1 way ANOVA, Cd and Zn contents measured in different plant parts within both fields using 2 way ANOVA and *post hoc* multiple comparison testing (Tukey

Kramer). Transformations were applied when necessary to approximate normality and/or homoscedasticity. In case normality could not be reached, data were analysed using Kruskal-Wallis multiple comparisons test.

Supporting Information

Appendix 4.1 Detailed characterisation of all purified bulk soil, rhizosphere soil and *B. napus* root isolates collected at the control field (BS-CO, RS-CO and R-CO respectively) and the contaminated field (BS-TE, RS-TE and R-TE respectively). The presence of each strain is shown as relative abundances, expressed in percentages, of the total number of colony forming units (cfu) per gram fresh weight (gFW^{-1}) bulk soil (BS), rhizosphere soil (RS) or roots (R). Strains are identified to the genus level (ID), their accession (acc) numbers as well as their presence in the 1st, 2nd or 3rd replicate (R) are displayed. Their potential plant growth promoting (PGP) characteristics are indicated by + when positive and by ++(+) in case of a strong positive test. Bacterial strains testing negative for a phenotypic test were labeled by a – symbol and those not applicable for the test by 'not detected' (nd). The PGP characteristics tested were Cd (0.8 and 1.6 mM) and Zn (0.6, 1.0 and 2.5 mM) tolerance and the capacity to solubilise phosphorus (P sol), fixate nitrogen (N_2 fix) and produce siderophores (SID), organic acids (OA), ACC deaminase (ACC), indole-3-acetic acid (IAA) and acetoin (ACE).

BS-CO																
R	cfu	%	ID	acc	Cd	Cd	Zn	Zn	Zn	SID	OA	ACC	IAA	ACE	P sol	N_2 fix
1	4783773	100.00	*Micromonospora*	EU841636	-	-	-	-	-	-	nd	nd	-	nd	nd	-
3	531237	3.51	*Bacillus*	AB188212	-	-	+	+	+	++	nd	-	-	+++	-	-
3	531237	3.51	*Bacillus*	AB188212	-	-	-	-	-	-	-	-	-	-	++	-
3	53124	0.35	*Bacillus*	AB188212	-	-	-	-	-	++	-	-	-	+++	-	-
3	53124	0.35	*Bacillus*	AB188212	-	-	-	-	-	+	-	-	-	+++	-	-
3	212495	1.40	*Bacillus*	AB188212	-	-	-	-	-	++	-	-	-	+++	-	-
3	53124	0.35	*Bacillus*	AB188212	-	-	-	-	-	++	-	-	-	+++	-	-
3	531237	3.51	*Bacillus*	CP000813	-	-	+	+	-	+	+	-	-	+++	+++	-
3	531237	3.51	*Bacillus*	FJ263042	-	-	+	-	-	+	-	nd	-	++	-	-
3	1062473	7.01	*Bacillus*	FJ263042	-	-	++	-	-	+	-	-	-	+++	-	-
3	531237	3.51	*Bacillus*	FJ263042	-	-	++	+	+	+	+	-	-	+++	++	-
3	607128	4.01	*Bacillus*	FJ263042	-	-	+	-	-	+	nd	-	-	+++	++	-
3	607128	4.01	*Bacillus*	FJ263042	-	-	+	-	-	-	nd	-	-	nd	-	-
3	371866	2.45	*Bacillus*	FJ263042	-	-	+	+	+	+	-	-	-	+++	-	-
3	53124	0.35	*Bacillus*	FJ263042	-	-	++	+	+	+	+	-	-	+++	+++	-
3	53124	0.35	*Bacillus*	FJ263042	-	-	+	+	+	+	+	-	-	+++	+++	-
3	53124	0.35	*Bacillus*	FJ263042	-	-	+	+	+	+	+	-	-	-	-	-
3	5312367	35.07	*Bacillus*	FJ263042	-	-	-	-	-	-	-	-	+	-	-	-
3	531237	3.51	*Bacillus*	GU321095	-	-	-	-	-	+	++	-	-	-	-	-
3	53124	0.35	*Bacillus*	GU321095	-	-	-	-	-	+	+	-	-	-	-	-
3	531237	3.51	*Burkholderia*	FJ786047	-	-	-	-	-	+	-	-	+	nd	-	-
3	531237	3.51	*Leifsonia*	AB278552	-	-	++	+	+	-	-	-	+	-	-	-
3	607128	4.01	*Leifsonia*	AB278552	-	-	++	-	-	-	-	-	+	-	-	-
3	607128	4.01	*Leifsonia*	AB278552	-	-	+	-	-	-	-	-	+	-	++	-
3	607128	4.01	*Pantoea*	AF130887	-	-	+	-	-	+	nd	-	-	+++	+++	++
3	531237	3.51	*Streptomyces*	EU119184	++	+	++	++	+	-	-	-	+	nd	-	-
BS-TE																
R	cfu	%	ID	acc	Cd	Cd	Zn	Zn	Zn	SID	OA	ACC	IAA	ACE	P sol	N_2 fix
1	377287	1.60	*Agrobacterium*	GQ428123	-	-	+	-	-	+	-	nd	+	nd	nd	-
1	754575	3.20	*Arthrobacter*	AB288059	++	+	++	++	++	+	+	-	+	-	-	-
1	3772873	16.01	*Arthrobacter*	AB288059	-	-	++	++	++	-	-	-	+	-	+	-
1	377287	1.60	*Arthrobacter*	DQ985470	-	-	+	-	-	-	-	-	+	-	+	-
1	377287	1.60	*Bacillus*	AB188212	-	-	++	-	-	+	-	-	-	-	-	-
1	377287	1.60	*Bradyrhizobium*	FJ390916	-	-	-	-	-	-	nd	nd	-	nd	nd	-
1	37729	0.16	*Brevundimonas*	EF088675	-	-	+	+	+	-	nd	-	-	nd	-	-

1	377287	1.60	*Brevundimonas*	EF088675	-	-	++	++	++	+	-	+	-	-	-	-
1	3772873	16.01	*Burkholderia*	AY949194	-	-	+	-	-	+	-	-	-	-	-	-
1	377287	1.60	*Burkholderia*	AY949194	-	-	+	-	-	-	nd	nd	nd	nd	nd	-
1	377287	1.60	*Burkholderia*	FJ786047	-	-	++	-	-	-	-	-	-	nd	nd	nd
1	377287	1.60	*Leifsonia*	AB278552	-	-	++	+	+	-	-	-	+	nd	-	-
1	377287	1.60	*Leifsonia*	AB278552	+	-	++	-	-	-	-	-	-	-	-	-
1	377287	1.60	*Leifsonia*	AM889135	-	-	++	++	++	-	-	-	+	-	+	+
1	754575	3.20	*Leifsonia*	FJ422386	-	-	+	+	-	-	-	-	+	-	++	-
1	377287	1.60	*Leifsonia*	FJ422386	-	-	-	-	-	-	-	+	-	-	++	-
1	754575	3.20	*Mesorhizobium*	AB531422	-	-	+	+	+	-	-	-	+	nd	+	-
1	471609	2.00	*Methylobacterium*	Z23158	-	-	++	-	-	+	nd	+	-	-	-	-
1	377287	1.60	*Pedobacter*	EF660751	-	-	++	+	+	-	-	-	-	-	-	-
1	377287	1.60	*Pseudomonas*	AB088844	++	+	++	-	-	+	-	+	+	-	++	-
1	37729	0.16	*Rhodococcus*	EU496547	++	-	++	-	-	+	-	-	-	-	-	-
1	377287	1.60	*Staphylococcus*	AY167864	-	-	+	+	+	+	-	+	-	-	++	-
1	377287	1.60	unc.bact	GQ012035	-	-	++	-	-	++	-	+	+	-	-	+
1	3772873	16.01	unc.bact	GQ012035	-	-	+	+	+	+	nd	+	-	-	-	-
1	3772873	16.01	*Variovorax*	EF419341	++	+	++	-	-	+	-	-	-	-	+	-
2	507924	2.33	*Arthrobacter*	AB288059	+	+	++	++	++	-	-	-	+	+++	+	-
2	50792	0.23	*Arthrobacter*	AB288059	+	+	++	+	+	-	-	-	+	-	++	+
2	507924	2.33	*Arthrobacter*	AB288059	-	-	+	-	-	-	-	-	++	-	-	-
2	507924	2.33	*Arthrobacter*	DQ985470	-	-	+	+	+	+	-	-	++	-	-	-
2	50792	0.23	*Bacillus*	AM910175	-	-	+	-	-	-	nd	-	+	-	-	-
2	507924	2.33	*Burkholderia*	FJ786047	-	-	+	-	-	++	-	-	-	-	-	-
2	50792	0.23	*Chryseobacterium*	DQ337589	-	-	+	+	+	-	-	-	+	-	-	-
2	672999	3.09	*Leifsonia*	AB278552	-	-	++	+	+	-	-	-	-	nd	+	-
2	507924	2.33	*Leifsonia*	AB278552	-	-	-	-	-	-	-	-	-	-	-	-
2	507924	2.33	*Leifsonia*	AB278552	-	-	+	+	+	-	+	-	-	-	-	-
2	507924	2.33	*Leifsonia*	AM889135	-	-	++	++	++	-	+	-	+	-	++	-
2	50792	0.23	*Leifsonia*	DQ232613	-	-	++	++	+	+	+	-	nd	-	-	-
2	336499	1.54	*Mesorhizobium*	AY490106	-	-	+	-	-	+	-	nd	+	-	-	-
2	507924	2.33	*Mesorhizobium*	AY490106	-	-	++	-	-	-	nd	nd	+	-	++	-
2	50792	0.23	*Methylobacterium*	AB220076	-	-	+	-	-	nd	-	nd	-	-	-	-
2	50792	0.23	*Methylobacterium*	Z23158	-	-	++	+	+	+	-	+	+	-	+	-
2	50792	0.23	*Plantibacter*	AM396918	-	-	++	++	++	nd	+	-	-	-	-	-
2	50792	0.23	*Pseudomonas*	FM202488	+	-	++	-	-	+	++	+	++	-	++	-
2	50792	0.23	*Rhodococcus*	AB425280	-	-	+	+	+	-	-	-	+	-	-	-
2	507924	2.33	*Rhodococcus*	EU496547	+	-	++	++	++	-	-	-	-	-	++	-
2	3628026	16.65	*Rhodopseudomonas*	AB033756	-	-	+	-	-	nd	-	nd	-	nd	nd	-
2	507924	2.33	*Staphylococcus*	FJ357589	-	-	+	+	+	+	-	+	+	-	+	-
2	50792	0.23	*Staphylococcus*	FJ357589	-	-	++	++	++	+	-	+	-	-	++	-
2	50792	0.23	*Staphylococcus*	FJ357589	-	-	++	++	++	+	-	+	-	-	-	-
2	243803	1.12	*Staphylococcus*	GQ222398	-	-	++	-	-	nd	-	nd	-	nd	nd	-
2	50792	0.23	*Staphylococcus*	GQ222398	-	-	++	-	-	+	nd	+	++	-	-	-
2	50792	0.23	*Staphylococcus*	GQ222398	-	-	++	-	-	-	-	-	+	nd	nd	-
2	50792	0.23	*Staphylococcus*	GQ222398	-	-	++	++	++	+	+	+	+	-	-	-
2	50792	0.23	*Staphylococcus*	GQ222398	-	-	++	++	++	++	++	+	+	-	-	-
2	5079236	23.31	*Staphylococcus*	GQ222398	-	-	++	+	+	++	-	+	-	-	+	-
2	725605	3.33	*Stenotrophomonas*	AJ551165	-	-	++	+	+	-	nd	+	-	+	++	-
2	507924	2.33	unc.bact	GQ012035	-	-	++	+	+	+	+	+	+	-	+	-
2	1149177	5.27	unc.bact	GQ012035	-	-	++	-	-	+	-	+	+	-	-	+
2	121902	0.56	unc.bact	GQ012035	-	-	++	-	-	+	-	+	-	-	-	-
2	121902	0.56	unc.bact	GQ012035	-	-	++	-	-	+	-	+	++	-	-	-
2	50792	0.23	unc.bact	GQ012035	-	-	++	+	+	+	-	+	-	-	-	-
2	336499	1.54	unc.bact	GQ012035	-	-	++	+	+	+	-	+	-	-	-	-
2	336499	1.54	unc.bact	GQ012035	-	-	++	+	+	+	-	+	+	-	+	-
2	336499	1.54	unc.bact	GQ012035	-	-	++	-	-	++	nd	+	-	-	-	-
2	507924	2.33	unc.bact	GQ012035	-	-	++	+	+	+	-	+	+	-	-	-
2	507924	2.33	unc.bact	GQ012035	-	-	++	+	+	+	nd	+	-	-	++	-
2	507924	2.33	unc.bact	GQ012035	-	-	++	+	+	+	+	+	+	-	-	+
2	725605	3.33	unc.bact	GQ012035	-	-	++	+	+	+	nd	+	++	-	-	-

2	50792	0.23	*Variovorax*	EF419341	-	-	++	-	-	+	-	-	-	-	-	-
3	43463	0.27	*Agrobacterium*	GQ428123	++	-	++	+	-	+	+	+	++	-	-	-
3	434631	2.70	*Agrobacterium*	GQ428123	-	-	-	-	-	-	-	-	-	-	-	-
3	760605	4.72	*Agrobacterium*	GQ428123	-	-	-	-	-	+	-	-	-	-	-	-
3	202828	1.26	*Arthrobacter*	AB288059	+	+	+	-	-	+	-	-	-	-	++	nd
3	202828	1.26	*Arthrobacter*	AB288059	-	-	-	-	-	-	-	-	-	-	-	-
3	202828	1.26	*Arthrobacter*	AB288059	-	-	++	++	++	+	-	-	+	-	-	nd
3	43463	0.27	*Arthrobacter*	AB288059	+	+	++	-	-	-	-	-	-	-	+	-
3	434631	2.70	*Arthrobacter*	AB288059	+	+	++	+	+	-	-	-	++	-	-	-
3	2607789	16.17	*Arthrobacter*	AB288059	-	-	++	+	+	-	-	-	-	-	-	-
3	434631	2.70	*Arthrobacter*	AB288059	-	-	++	+	+	-	-	-	-	-	-	-
3	4346314	26.95	*Arthrobacter*	AB288059	-	-	++	+	+	+	-	+	-	-	++	-
3	43463	0.27	*Brevundimonas*	EF088675	+	-	++	+	+	-	-	+	++	-	-	-
3	86926	0.54	*Burkholderia*	FJ786047	-	-	++	-	-	+	+	-	-	nd	nd	-
3	173853	1.08	*Leifsonia*	AB278552	-	-	++	-	-	+	nd	nd	-	nd	-	-
3	130389	0.81	*Leifsonia*	AB278552	-	-	++	++	+	-	-	+	+	-	++	-
3	2281815	14.15	*Leifsonia*	AB278552	-	-	+	-	-	-	-	-	+	-	-	nd
3	434631	2.70	*Leifsonia*	AB278552	-	-	+	+	+	-	-	+	+	-	+	-
3	43463	0.27	*Leifsonia*	AM889135	-	-	++	++	++	-	+	-	-	-	+	-
3	869263	5.39	*Leifsonia*	AM889135	-	-	+	+	-	-	nd	-	-	nd	-	-
3	434631	2.70	*Leifsonia*	DQ232613	-	-	++	+	+	-	-	+	-	-	++	-
3	86926	0.54	*Mycobacterium*	AY337605	-	-	+	+	+	+	-	-	-	-	-	nd
3	434631	2.70	*Plantibacter*	AM396918	-	-	++	++	++	-	-	-	+	-	++	-
3	43463	0.27	*Staphylococcus*	GQ222398	-	-	++	++	++	-	-	+	-	-	+	-
3	434631	2.70	unc.bact	FM872722	-	-	+	-	-	-	nd	-	-	++	-	-
3	434631	2.70	unc.bact	GQ012035	-	-	++	+	+	-	-	+	+	-	-	-
3	43463	0.27	*Variovorax*	EF419341	-	-	++	-	-	+	nd	+	++	-	-	-
3	434631	2.70	*Variovorax*	EF419341	-	-	++	+	+	-	-	-	-	-	nd	-
					RS-CO											
R	cfu	%	ID	acc	Cd	Cd	Zn	Zn	Zn	SID	OA	ACC	IAA	ACE	P sol	N_2 fix
1	10599378	5.90	*Aeromicrobium*	AB245394	-	-	-	-	-	-	nd	-	-	nd	+	-
1	6838308	3.81	*Agrobacterium*	GQ428123	-	-	-	-	-	-	-	-	+	-	++	-
1	10257462	5.71	*Bacillus*	AB188212	-	-	+	-	-	++	-	-	-	+++	-	-
1	341915	0.19	*Bacillus*	GU321095	-	-	-	-	-	+	++	-	-	-	-	++
1	683831	0.38	*Leifsonia*	AB278552	-	-	++	-	-	-	-	+	+	-	-	-
1	3419154	1.90	*Leifsonia*	AB278552	-	-	-	-	-	-	-	-	+	-	-	-
1	6838308	3.81	*Leifsonia*	AB278552	-	-	+	+	-	-	-	-	+	-	++	-
1	10599378	5.90	*Lysinibacillus*	AY907676	-	-	-	-	-	+	nd	-	-	+++	-	-
1	13676616	7.62	*Pseudomonas*	AB369347	-	-	-	-	-	+	-	+	++	-	-	-
1	6838308	3.81	*Pseudomonas*	AM934699	-	-	-	-	-	+	-	+	++	-	+++	-
1	3419154	1.90	*Rhodococcus*	EU496547	-	-	+	+	+	+	-	-	-	++	+++	-
1	3419154	1.90	*Staphylococcus*	GQ222398	-	-	-	-	-	-	nd	-	+	+++	-	-
1	34191541	19.05	*Staphylococcus*	GQ222398	-	-	-	-	-	+	-	nd	-	nd	nd	-
1	34191541	19.05	*Variovorax*	EF419341	+	-	-	-	-	+	-	-	-	-	+++	-
1	34191541	19.05	*Variovorax*	EF419341	-	-	-	-	-	-	-	nd	-	++	nd	-
2	5439217	1.70	*Agrobacterium*	GQ428123	-	-	-	-	-	-	+	-	++	-	-	-
2	2472371	0.77	*Agrobacterium*	GQ428123	-	-	-	-	-	-	nd	-	+	nd	nd	-
2	2472371	0.77	*Agrobacterium*	GQ428123	-	-	-	-	-	-	nd	-	++	nd	-	-
2	2472371	0.77	*Arthrobacter*	AB288059	-	-	-	-	-	+	-	-	+	-	-	-
2	2472371	0.77	*Arthrobacter*	AB288059	-	-	-	-	-	+	-	-	+	-	-	-
2	4944743	1.54	*Arthrobacter*	AB288059	-	-	-	-	-	+	-	-	+	-	-	-
2	4944743	1.54	*Arthrobacter*	AB288059	-	-	-	-	-	+	-	-	-	-	+++	-
2	2472371	0.77	*Bacillus*	AB188212	-	-	-	-	-	++	++	-	-	+++	-	-
2	24723713	7.72	*Bacillus*	AB188212	-	-	-	-	-	++	-	-	-	++	-	-
2	5439217	1.70	*Bacillus*	AB301017	-	-	-	-	-	++	-	-	-	+++	-	-
2	2472371	0.77	*Bacillus*	AJ628743	-	-	+	-	-	+	-	-	-	-	-	-
2	8900537	2.78	*Bacillus*	DQ445268	-	-	+	-	-	+	-	-	+	+++	+	-
2	7417114	2.32	*Bacillus*	GQ200827	-	-	-	-	-	+	+	-	+	-	nd	-
2	2472371	0.77	*Bacillus*	GQ200827	-	-	-	-	-	+	-	-	+	nd	+++	-
2	2472371	0.77	*Bacillus*	GQ200827	-	-	-	-	-	+	-	-	+	-	-	-
2	24723713	7.72	*Bacillus*	GQ200827	-	-	-	-	-	-	-	-	-	-	-	-

2	4944743	1.54	*Caulobacter*	DQ337549	-	-	-	-	-	-	nd	-	-	-	-	-
2	2472371	0.77	*Janthinobacterium*	D84576	+	+	++	++	++	+	-	+	+	+++	++	-
2	4944743	1.54	*Kribbella*	AY253865	-	-	+	-	-	-	nd	-	-	nd	nd	-
2	2966846	0.93	*Labrys*	DQ337554	-	-	-	-	-	nd	nd	-	++	nd	nd	nd
2	4944743	1.54	*Leifsonia*	AB278552	-	-	+	-	-	-	-	-	+	-	+++	-
2	4944743	1.54	*Leifsonia*	AB278552	-	-	-	-	-	-	-	-	-	-	-	-
2	2472371	0.77	*Lysinibacillus*	DQ333300	-	-	++	-	-	-	-	-	+	-	+++	+++
2	12361856	3.86	*Pseudomonas*	AB369347	+	+	+	-	-	++	-	-	+	-	++	+++
2	8653299	2.70	*Pseudomonas*	AB369347	+	+	-	-	-	+	-	-	++	-	-	-
2	2472371	0.77	*Pseudomonas*	AB369347	-	-	++	+	+	++	++	+	+	-	+++	++
2	2472371	0.77	*Pseudomonas*	AB369347	-	-	++	-	-	+	-	+	+	-	-	-
2	59336910	18.53	*Pseudomonas*	AM934699	-	-	-	-	-	+	-	+	++	-	-	-
2	5439217	1.70	*Pseudomonas*	AM934699	+	-	-	-	-	+	++	+	++	-	-	-
2	5439217	1.70	*Pseudomonas*	AM934699	+	-	-	-	-	+	++	+	++	-	-	++
2	5439217	1.70	*Pseudomonas*	AM934699	+	-	-	-	-	+	++	+	++	-	-	-
2	8653299	2.70	*Pseudomonas*	AM934699	+	-	-	-	-	+	-	+	++	-	-	++
2	8653299	2.70	*Pseudomonas*	AM934699	-	-	-	-	-	+	-	+	++	-	-	-
2	24723713	7.72	*Pseudomonas*	AM934699	+	-	-	-	-	+	++	+	++	-	-	++
2	2966846	0.93	*Pseudomonas*	FJ772042	-	-	-	-	-	-	-	+	-	+++	-	-
2	2472371	0.77	*Pseudomonas*	FM202488	++	++	+	-	-	++	-	+	-	-	++	+++
2	4944743	1.54	*Rhodococcus*	DQ060386	-	-	+	-	-	+	-	-	-	-	-	-
2	2472371	0.77	*Sphingobacterium*	AJ438176	-	-	++	-	-	+	-	-	+	-	+++	-
2	2472371	0.77	*Staphylococcus*	GQ222398	-	-	-	-	-	-	-	-	+	-	-	-
2	4944743	1.54	*Staphylococcus*	GQ222398	-	-	-	-	-	-	-	+	+	-	+++	-
2	4944743	1.54	*Staphylococcus*	GQ222398	-	-	-	-	-	+	-	-	+	-	+++	-
2	4944743	1.54	*Variovorax*	FJ772012	-	-	-	-	-	+	-	-	+	-	-	-
2	2472371	0.77	*Variovorax*	FJ772012	+	+	+	-	-	-	-	+	+	-	nd	-
2	2472371	0.77	*Variovorax*	FJ772012	-	-	-	-	-	+	-	-	-	-	-	-
2	7417114	2.32	*Zoogloea*	X74914	-	-	-	-	-	-	+	-	++	-	+	-
3	50842718	0.77	*Arthrobacter*	EF028242	-	-	++	-	-	-	-	-	++	nd	nd	-
3	508427	0.01	*Bacillus*	AB188212	-	-	-	-	-	++	-	-	-	+++	-	-
3	508427	0.01	*Bacillus*	AB188212	-	-	-	-	-	++	-	-	-	+++	-	-
3	508427	0.01	*Bacillus*	AB188212	-	-	-	-	-	++	-	-	-	++	-	-
3	508427	0.01	*Bacillus*	FJ263042	-	-	++	-	-	+	+	-	-	-	-	-
3	50842718	0.77	*Bacillus*	FJ263042	-	-	+	-	-	+	+	-	-	+	-	-
3	6406182475	96.87	*Bacillus*	GQ200827	-	-	++	-	-	+	+	-	-	+++	-	-
3	50842718	0.77	*Bacillus*	GQ200827	-	-	+	-	-	+	+	-	-	++	++	-
3	508427	0.01	*Bacillus*	GU321095	-	-	-	-	-	+	+	-	-	-	-	-
3	508427	0.01	*Pseudomonas*	FM202488	+	+	-	-	-	-	-	-	++	-	+++	-
3	508427	0.01	*Rhodococcus*	EU496547	-	-	++	-	-	+	-	-	-	-	-	-
3	50842718	0.77	*Streptomyces*	AY314782	-	-	++	-	-	+	-	-	-	nd	-	-
					RS-TE											
R	cfu	%	ID	acc	Cd	Cd	Zn	Zn	Zn	SID	OA	ACC	IAA	ACE	P sol	N_2 fix
1	3238604	0.24	*Achromobacter*	GQ927161	++	++	-	-	-	-	-	-	-	-	-	++
1	1619302	0.12	*Agrobacterium*	GQ428123	+	-	++	++	++	+	-	+	+	-	+++	++
1	2267023	0.17	*Arthrobacter*	AB288059	-	-	++	++	+	+	-	-	+	-	+	++
1	161930208	11.90	*Arthrobacter*	AB288059	+	+	++	-	-	+	++	-	+	-	-	-
1	3238604	0.24	*Arthrobacter*	FJ890893	-	-	-	-	-	+	nd	nd	-	nd	nd	-
1	2267023	0.17	*Arthrobacter*	FJ890893	-	-	++	-	-	-	nd	nd	-	nd	nd	-
1	1619302	0.12	*Arthrobacter*	FM213390	++	+	++	++	+	+	-	-	+	-	-	++
1	6801069	0.50	*Arthrobacter*	FM213390	-	-	+	+	+	-	-	-	+	-	-	++
1	2267023	0.17	*Arthrobacter*	FM213390	++	+	++	++	+	+	-	-	+	-	-	-
1	1619302	0.12	*Arthrobacter*	FM213390	+	-	++	++	++	+	-	-	+	-	+	-
1	1619302	0.12	*Arthrobacter*	FM213390	++	+	++	++	+	+	-	-	+	-	-	-
1	161930208	11.90	*Bacillus*	AB188212	-	-	+	-	-	+	-	-	-	-	-	++
1	1619302	0.12	*Brevundimonas*	EF088675	++	+	++	++	++	+	-	-	-	-	-	-
1	14573719	1.07	*Burkholderia*	FJ939284	-	-	++	+	+	+	-	-	-	-	+	++
1	2267023	0.17	*Burkholderia*	FJ939284	-	-	++	++	+	+	-	-	-	-	+	-
1	2428953	0.18	*Frigoribacterium*	AF157479	-	-	-	-	-	-	-	-	-	-	-	-
1	4318139	0.32	*Leifsonia*	AB278552	-	-	++	++	+	-	-	-	+	-	++	-
1	1619302	0.12	*Leifsonia*	AB278552	-	-	++	++	+	-	-	+	-	-	-	-

1	1619302	0.12	*Leifsonia*	AB278552	+	-	++	++	+	-	-	+	-	-	+	-
1	18621974	1.37	*Leifsonia*	AB278552	-	-	-	-	-	-	nd	-	-	nd	-	-
1	18621974	1.37	*Leifsonia*	GU332619	-	-	++	+	+	-	-	-	+	-	-	-
1	161930208	11.90	*Leifsonia*	GU332619	-	-	++	-	-	-	-	-	-	-	-	-
1	1619302	0.12	*Lysinibacillus*	AY907676	-	-	+	+	+	-	-	-	+	-	++	-
1	1619302	0.12	*Massilia*	AM231588	-	-	-	-	-	-	-	+	+	-	+++	-
1	19836450	1.46	*Microbacterium*	EU821338	+	+	+	+	+	-	-	+	-	-	-	-
1	19836450	1.46	*Microbacterium*	EU821338	+	+	+	+	+	+	-	+	+	-	-	-
1	19836450	1.46	*Microbacterium*	EU821338	-	-	+	+	+	-	-	+	+	-	-	-
1	19836450	1.46	*Microbacterium*	EU821338	-	-	+	+	+	-	-	+	+	-	-	-
1	3238604	0.24	*Microbacterium*	EU821338	+	-	++	++	++	+	-	+	-	-	+++	-
1	1619302	0.12	*Pedobacter*	AM279216	-	-	++	++	++	-	-	-	-	-	-	-
1	3238604	0.24	*Plantibacter*	AM396918	+	-	+	+	+	-	-	+	+	-	++	-
1	4857906	0.36	*Plantibacter*	AM396918	-	-	+	+	+	-	-	+	+	-	+	-
1	1619302	0.12	*Plantibacter*	AM396918	-	-	++	+	+	-	-	+	+	-	++	-
1	4534046	0.33	*Plantibacter*	AM396918	++	++	++	++	++	-	-	+	+	-	++	++
1	485790624	35.71	*Plantibacter*	AM396918	-	-	++	+	+	-	+	-	-	-	+	-
1	2428953	0.18	*Pseudoclavibacter*	X77440	-	-	++	-	-	-	-	-	-	-	++	-
1	72868594	5.36	*Pseudomonas*	AB369347	-	-	++	-	-	++	-	+	-	-	++	-
1	4318139	0.32	*Pseudomonas*	AB369347	+	-	+	+	+	++	-	-	-	-	++	-
1	2267023	0.17	*Pseudomonas*	AB369347	-	-	++	-	-	+	-	+	+	-	+	-
1	1619302	0.12	*Pseudomonas*	AB369347	-	-	+	+	+	++	-	+	-	-	-	-
1	1619302	0.12	*Pseudomonas*	FJ225200	+	+	+	-	-	++	-	+	-	-	+++	-
1	1619302	0.12	*Rhizobium*	AJ389905	++	-	++	++	++	+	-	+	+	-	+++	-
1	4318139	0.32	*Rhizobium*	DQ337581	-	-	++	++	++	-	-	-	+	-	++	-
1	1619302	0.12	*Sphingobacterium*	AJ438176	-	-	++	-	-	-	-	-	-	-	-	-
1	1619302	0.12	*Sphingobacterium*	AJ438176	-	-	++	+	+	-	-	-	-	-	+++	+++
1	18621974	1.37	*Sphingobacterium*	AJ438176	-	-	++	++	++	-	-	-	-	-	-	-
1	18621974	1.37	*Sphingobacterium*	AJ438176	-	-	++	++	++	-	-	-	-	-	+++	++
1	1619302	0.12	*Staphylococcus*	GQ222398	-	-	+	+	+	-	-	+	+	-	+++	-
1	18621974	1.37	*Staphylococcus*	GQ222398	-	-	+	-	-	-	-	-	+	-	-	-
1	16193021	1.19	unc.bact	EU536446	-	-	+	+	+	+	-	+	++	-	-	++
1	18621974	1.37	*Variovorax*	EF419341	-	-	+	-	-	-	-	-	-	-	nd	-
1	1619302	0.12	*Variovorax*	FJ772012	-	-	+	-	-	-	-	-	-	-	-	-
1	3238604	0.24	*Variovorax*	FJ772012	-	-	+	+	+	+	-	-	-	-	-	-
1	3238604	0.24	*Variovorax*	FJ772012	++	++	-	-	-	-	-	+	-	-	+	++
2	7340796	2.38	*Flavobacterium*	GU078570	-	-	++	++	++	-	-	-	-	-	++	-
2	36703982	11.89	*Leifsonia*	AB278552	-	-	++	+	+	-	-	-	-	-	++	++
2	36703982	11.89	*Leifsonia*	AB278552	-	-	++	+	+	+	+	-	-	-	+++	-
2	36703982	11.89	*Leifsonia*	GU332619	-	-	++	+	-	-	-	+	-	-	+++	-
2	3670398	1.19	*Methylobacterium*	AB220076	-	-	++	-	-	-	-	-	-	nd	++	-
2	36703982	11.89	*Microbacterium*	EU821338	-	-	++	++	++	-	-	+	+	-	+	-
2	3670398	1.19	*Pseudoclavibacter*	X77440	-	-	+	-	-	-	-	-	-	nd	+	-
2	367040	0.12	*Staphylococcus*	GQ222398	-	-	++	++	++	-	-	+	+	-	+	-
2	110111947	35.67	*Staphylococcus*	GQ222398	-	-	++	++	++	-	-	+	+	-	-	++
2	36703982	11.89	*Variovorax*	FJ772012	-	-	++	+	+	-	-	-	-	-	+++	-
3	3480016	0.52	*Leifsonia*	AB278552	-	-	++	+	-	-	++	-	+	-	-	++
3	3480016	0.52	*Leifsonia*	AB278552	-	-	++	++	-	-	+	-	+	-	+	-
3	34800160	5.15	*Leifsonia*	AB278552	-	-	++	++	+	-	++	-	+	-	++	-
3	174000800	25.77	*Leifsonia*	AB278552	-	-	++	-	-	-	-	-	-	-	+	-
3	34800160	5.15	*Leifsonia*	AB278552	-	-	++	+	-	-	-	+	-	-	-	-
3	34800160	5.15	*Pantoea*	EU598802	-	-	++	-	-	+	++	-	++	+++	++	-
3	3480016	0.52	*Pseudomonas*	AB369347	+	+	++	+	+	+	-	+	+	-	-	-
3	34800160	5.15	*Pseudomonas*	AB369347	-	-	++	+	+	+	-	+	-	-	-	-
3	3480016	0.52	*Rhodococcus*	DQ060386	+	-	++	+	+	-	-	-	-	-	-	-
3	139200640	20.62	*Serratia*	AJ233434	+	+	+	+	+	+	-	-	+	+++	-	-
3	174000800	25.77	*Staphylococcus*	GQ222398	-	-	++	+	+	-	-	+	-	-	++	+
3	34800160	5.15	*Staphylococcus*	GQ222398	-	-	++	-	-	-	-	-	+	-	-	-
					R-CO											
R	cfu	%	ID	acc	Cd	Cd	Zn	Zn	Zn	SID	OA	ACC	IAA	ACE	P sol	N_2 fix
1	3258	0.31	*Bacillus*	AB188212	-	-	-	-	-	++	-	-	-	+++	-	-

1	3258	0.31	*Bacillus*	AJ542508	+	+	+	-	-	-	-	-	+	-	-	+++
1	3258	0.31	*Bacillus*	CP000813	-	-	-	-	-	+	-	-	-	+++	-	-
1	14480	1.36	*Bacillus*	CP000813	-	-	++	+	-	-	nd	-	-	nd	-	-
1	97739	9.18	*Bacillus*	CP000813	-	-	++	+	+	+	-	-	-	+++	+	-
1	32580	3.06	*Bacillus*	CP000813	-	-	++	-	-	+	+	-	-	+++	-	-
1	3258	0.31	*Bacillus*	FJ263042	-	-	+	-	-	+	-	+	-	+++	+++	++
1	32580	3.06	*Bacillus*	FJ263042	-	-	++	-	-	+	-	-	-	+++	++	-
1	32580	3.06	*Bacillus*	FJ263042	-	-	++	-	-	+	-	-	-	+++	+	-
1	3258	0.31	*Caulobacter*	DQ337549	-	-	-	-	-	-	-	-	-	-	+++	-
1	3258	0.31	*Caulobacter*	DQ337549	-	-	-	-	-	-	-	-	+	++	-	-
1	3258	0.31	*Caulobacter*	DQ337549	-	-	++	+	+	-	-	-	-	-	-	-
1	7819	0.73	*Caulobacter*	DQ337549	-	-	++	-	-	-	-	-	-	+++	-	-
1	7819	0.73	*Caulobacter*	DQ337549	-	-	++	-	-	-	-	-	-	-	-	-
1	7819	0.73	*Caulobacter*	DQ337549	-	-	-	-	-	-	-	-	-	-	-	-
1	7819	0.73	*Caulobacter*	DQ337549	-	-	-	-	-	-	-	-	+	-	++	-
1	3258	0.31	*Caulobacter*	DQ337549	-	-	-	-	-	-	-	-	-	-	++	-
1	6516	0.61	*Caulobacter*	DQ337549	-	-	-	-	-	-	nd	-	-	-	++	-
1	6516	0.61	*Caulobacter*	DQ337549	-	-	+	-	-	-	nd	-	-	-	-	-
1	32580	3.06	*Caulobacter*	DQ337549	-	-	-	-	-	-	-	-	-	nd	-	-
1	32580	3.06	*Caulobacter*	DQ337549	-	-	-	-	-	-	-	-	-	-	-	-
1	3910	0.37	*Labrys*	DQ337554	-	-	++	-	-	-	nd	nd	++	nd	-	-
1	32580	3.06	*Labrys*	DQ337554	-	-	+	-	-	-	nd	nd	-	nd	nd	-
1	32580	3.06	*Mycobacterium*	FJ719354	-	-	-	-	-	+	-	-	-	-	-	-
1	26064	2.45	*Pantoea*	EU598802	-	-	-	-	-	++	-	+	++	-	-	-
1	5701	0.54	*Pseudomonas*	AB330408	-	-	+	-	-	+	-	-	-	-	+	-
1	19548	1.84	*Pseudomonas*	AB369347	+	-	++	-	-	+	-	+	+	-	+	-
1	19548	1.84	*Pseudomonas*	AB369347	++	-	++	-	-	+	-	+	+	-	+	+++
1	3910	0.37	*Pseudomonas*	DQ778036	++	-	++	-	-	+	+	+	+	-	+	-
1	6516	0.61	*Pseudomonas*	DQ778036	+	+	-	-	-	+	-	+	++	-	-	-
1	3258	0.31	*Pseudomonas*	DQ778036	++	+	++	-	-	+	-	-	-	-	+	+++
1	6516	0.61	*Pseudomonas*	DQ778036	++	+	++	-	-	+	-	+	+	-	++	-
1	3258	0.31	*Pseudomonas*	FN377713	++	-	++	-	-	+	-	+	++	-	+++	-
1	14480	1.36	*Pseudomonas*	FN377713	-	-	+	+	+	+	-	+	-	++	+++	-
1	3258	0.31	*Pseudomonas*	FN377713	++	-	+	+	+	+	-	+	++	-	-	-
1	3258	0.31	*Pseudomonas*	FN377713	-	-	-	-	-	+	-	+	++	-	+	-
1	5701	0.54	*Pseudomonas*	FN377713	-	-	+	-	-	+	-	+	++	-	+++	-
1	14480	1.36	*Rhizobium*	DQ337581	-	-	+	-	-	-	-	-	-	-	-	+
1	32580	3.06	*Rhizobium*	DQ337581	++	+	++	++	+	+	-	-	++	-	+	-
1	14480	1.36	unc.bact	DQ787731	-	-	-	-	-	-	nd	nd	+	nd	nd	-
1	3910	0.37	unc.bact	GQ025779	+	-	++	-	-	+	+	-	-	-	++	-
1	14480	1.36	unc.bact	GQ025779	+	-	+	+	+	-	-	-	-	-	+	-
1	3910	0.37	*Variovorax*	EF419341	++	-	++	++	++	+	-	-	-	-	-	-
1	14480	1.36	*Variovorax*	FJ772012	+	+	++	++	++	+	-	-	-	-	++	++
1	6516	0.61	*Variovorax*	GQ861460	-	-	++	++	++	+	-	-	-	-	-	-
1	3258	0.31	*Variovorax*	GQ861460	++	-	++	++	++	+	-	-	+	-	-	-
1	3258	0.31	*Variovorax*	GQ861460	+	-	++	++	++	+	-	-	-	-	+++	-
1	26064	2.45	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	++	-
1	32580	3.06	*Variovorax*	GQ861460	++	-	++	+	+	+	-	-	-	-	-	-
1	6516	0.61	*Variovorax*	GQ861460	-	-	++	-	-	+	-	-	-	-	-	-
1	26064	2.45	*Variovorax*	GQ861460	-	-	-	-	-	-	-	-	-	-	-	-
1	6516	0.61	*Variovorax*	GQ861460	-	-	+	-	-	+	-	-	-	-	-	-
1	14480	1.36	*Variovorax*	GQ861460	-	-	+	-	-	+	-	-	-	-	+++	-
1	14480	1.36	*Variovorax*	GQ861460	-	-	+	-	-	+	-	-	-	-	-	-
1	3258	0.31	*Variovorax*	GQ861460	-	-	+	-	-	+	-	-	-	-	-	-
1	15638	1.47	*Variovorax*	GQ861460	-	-	+	-	-	+	-	-	-	-	-	-
1	5213	0.49	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	-	-
1	5213	0.49	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	-	-
1	3258	0.31	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	+++	-
1	3258	0.31	*Variovorax*	GQ861460	-	-	++	-	-	+	-	-	-	-	+++	-
1	3258	0.31	*Variovorax*	GQ861460	+	-	++	+	+	+	-	-	-	-	-	-
1	6516	0.61	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	++	-
1	6516	0.61	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	-	-
1	5701	0.54	*Variovorax*	GQ861460	-	-	+	-	-	+	-	-	-	-	+	-
1	5701	0.54	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	+	-
1	32580	3.06	*Variovorax*	GQ861460	++	-	++	-	-	+	-	-	-	-	-	-
1	32580	3.06	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	-	-

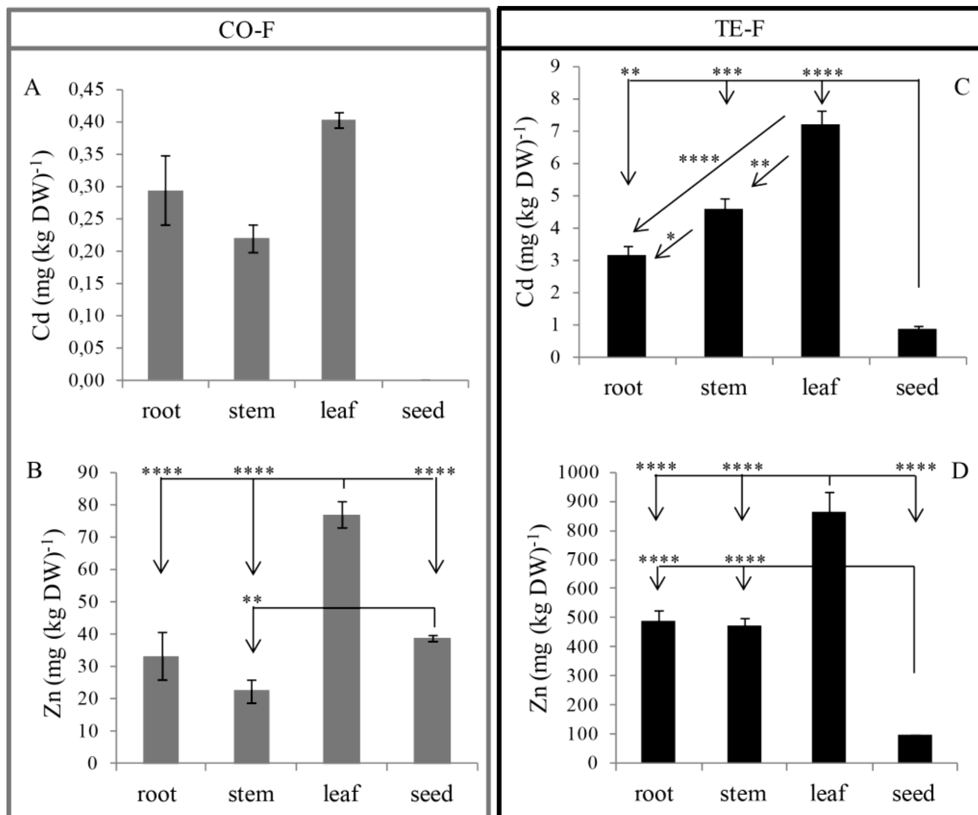
1	32580	3.06	*Variovorax*	GQ861460	-	-	+	-	-	-	-	-	-	-	-	-
1	32580	3.06	*Variovorax*	GQ861460	-	-	+	-	-	+	-	-	-	-	++	-
1	32580	3.06	*Variovorax*	GQ861460	-	-	+	-	-	+	-	-	-	-	-	-
1	32580	3.06	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	+++	-
1	32580	3.06	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	-	-
2	94153	6.55	*Bacillus*	CP000813	-	-	++	+	+	+	-	-	-	+++	+	-
2	47077	3.27	*Bacillus*	FJ263042	-	-	+	-	-	++	-	-	-	-	-	-
2	4708	0.33	*Caulobacter*	DQ337549	-	-	-	-	-	-	-	-	-	-	+	-
2	18831	1.31	*Labrys*	DQ337554	-	-	-	-	-	-	nd	-	++	nd	-	-
2	4708	0.33	*Labrys*	DQ337554	-	-	-	-	-	-	nd	-	++	nd	-	-
2	7061	0.49	*Microbacterium*	DQ328319	-	-	+	-	-	-	+	-	+	-	+++	-
2	4708	0.33	*Paenibacillus*	EU723825	-	-	+	-	-	-	-	-	+	-	++	-
2	7061	0.49	*Pantoea*	EU598802	++	-	+	-	-	+	-	+	+	-	-	-
2	47077	3.27	*Pedobacter*	GU385862	-	-	++	-	-	+	-	-	-	-	-	-
2	7061	0.49	*Plantibacter*	AM396918	-	-	-	-	-	-	-	+	+	-	-	-
2	7061	0.49	*Plantibacter*	AM396918	-	-	+	-	-	+	+	+	+	-	++	-
2	211844	14.73	*Pseudomonas*	AB369347	+	-	++	-	-	+	-	+	+	-	+	-
2	211844	14.73	*Pseudomonas*	AB369347	++	-	++	-	-	+	-	+	+	-	+	+++
2	6277	0.44	*Pseudomonas*	AB369347	++	-	++	-	-	++	-	+	+	-	+	-
2	4708	0.33	*Pseudomonas*	DQ095904	-	-	-	-	-	+	-	+	++	-	+	-
2	4708	0.33	*Pseudomonas*	DQ095904	++	-	++	-	-	++	-	+	+	-	+	-
2	4708	0.33	*Pseudomonas*	DQ095904	++	-	++	+	+	+	-	-	-	-	+	-
2	7061	0.49	*Pseudomonas*	DQ095904	-	-	+	+	+	-	-	+	+	-	++	-
2	7061	0.49	*Pseudomonas*	DQ095904	++	-	++	++	++	+	-	-	-	-	++	+
2	47077	3.27	*Pseudomonas*	FJ772042	-	-	-	-	-	-	-	-	-	-	+++	-
2	4708	0.33	*Pseudomonas*	FN377713	++	+	-	-	-	+	+	-	++	-	++	-
2	263629	18.33	*Variovorax*	FJ772012	-	-	++	-	-	++	-	-	-	-	-	-
2	4708	0.33	*Variovorax*	GQ861460	+	-	++	+	+	+	-	-	-	-	+	-
2	273044	18.99	*Variovorax*	GQ861460	+	-	++	+	+	++	-	-	-	-	-	-
2	6277	0.44	*Variovorax*	GQ861460	-	-	+	-	-	+	-	-	-	-	+	-
2	6277	0.44	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	+	-
2	7061	0.49	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	++	-
2	4708	0.33	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	++	-
2	47077	3.27	*Variovorax*	GQ861460	-	-	++	-	-	++	-	-	-	-	-	-
2	65907	4.58	*Variovorax*	GQ861460	-	-	+	-	-	+	-	-	-	-	+	-
3	3303	0.53	*Bacillus*	AB188212	-	-	-	-	-	+	-	-	-	+++	-	-
3	33033	5.26	*Bacillus*	AB188212	-	-	-	-	-	+	-	-	-	+++	-	-
3	3303	0.53	*Bacillus*	CP000813	-	-	++	-	-	+	+	-	-	+++	+	-
3	3303	0.53	*Brevibacillus*	FJ197026	-	-	-	-	-	++	nd	-	+	-	-	++
3	3303	0.53	*Brevibacillus*	FJ197026	-	-	++	-	-	+	-	-	-	-	-	-
3	6276	1.00	*Caulobacter*	DQ337549	-	-	-	-	-	-	-	-	-	-	+	-
3	6276	1.00	*Caulobacter*	DQ337549	-	-	++	-	-	-	-	-	-	-	+	-
3	6276	1.00	*Caulobacter*	DQ337549	-	-	-	-	-	-	-	-	-	-	+	-
3	6276	1.00	*Caulobacter*	DQ337549	-	-	-	-	-	-	-	-	-	-	+	-
3	6276	1.00	*Caulobacter*	DQ337549	-	-	-	-	-	-	-	-	-	-	-	-
3	6276	1.00	*Caulobacter*	DQ337549	-	-	-	-	-	+	-	-	++	-	-	-
3	6276	1.00	*Caulobacter*	DQ337549	-	-	-	-	-	-	-	-	-	-	+	-
3	3303	0.53	*Caulobacter*	DQ337549	-	-	-	-	-	-	-	-	-	-	+	-
3	3303	0.53	*Caulobacter*	DQ337549	-	-	+	-	-	-	-	-	-	nd	-	-
3	3303	0.53	*Caulobacter*	DQ337549	-	-	-	-	-	+	-	-	-	-	-	-
3	33033	5.26	*Caulobacter*	DQ337549	-	-	-	-	-	-	+	-	-	-	+	-
3	13213	2.10	*Labrys*	DQ337554	-	-	-	-	-	-	-	+	+	-	-	-
3	101080	16.10	*Pantoea*	EU598802	-	-	+	-	-	+	-	+	++	+	-	-
3	3303	0.53	*Pantoea*	EU598802	+	-	++	-	-	+	-	-	+	+++	-	-
3	33033	5.26	*Pseudomonas*	AB330408	-	-	-	-	-	-	-	-	-	-	-	-
3	11231	1.79	*Pseudomonas*	AB369347	-	-	-	-	-	+	-	-	++	-	+	-
3	31381	5.00	*Pseudomonas*	AB369347	+	-	++	-	-	+	-	+	+	-	+	-
3	31381	5.00	*Pseudomonas*	AB369347	++	-	++	-	-	+	-	+	+	-	+	+++
3	9910	1.58	*Pseudomonas*	AB369347	-	-	-	-	-	+	-	+	++	-	-	-
3	3303	0.53	*Pseudomonas*	AY047218	+	-	++	-	-	+	-	+	-	-	-	-
3	99098	15.78	*Pseudomonas*	DQ778036	-	-	-	-	-	+	+	+	++	-	+	-
3	33033	5.26	*Pseudomonas*	DQ778036	-	-	-	-	-	+	+	+	++	-	++	-
3	13213	2.10	*Pseudomonas*	FJ772042	-	-	+	-	-	-	nd	+	+	-	+	-
3	6276	1.00	*Pseudomonas*	FN377713	++	-	++	-	-	+	-	-	++	-	-	-
3	3303	0.53	*Pseudomonas*	FN377713	-	-	-	-	-	+	-	+	++	-	+++	-
3	13213	2.10	*Stenotrophomonas*	FJ772015	-	-	++	-	-	-	-	+	-	-	-	-

3	6276	1.00	*Variovorax*	GQ861460	+	-	++	+	+	+	-	-	-	-	+	-
3	30390	4.84	*Variovorax*	GQ861460	-	-	++	-	-	++	-	-	-	-	+	-
3	7928	1.26	*Variovorax*	GQ861460	-	-	+	+	-	+	-	-	-	-	+	-
3	3964	0.63	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	-	-
3	3964	0.63	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	-	-
3	3303	0.53	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	-	-
3	3303	0.53	*Variovorax*	GQ861460	-	-	+	-	-	+	-	-	-	-	-	-
3	13213	2.10	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	+	-
3	13213	2.10	*Variovorax*	GQ861460	-	-	-	-	-	+	-	-	-	-	-	-
3	3303	0.53	*Variovorax*	GQ861460	-	-	-	-	-	-	-	-	-	-	-	-
					R-TE											
R	cfu	%	ID	acc	Cd	Cd	Zn	Zn	Zn	SID	OA	ACC	IAA	ACE	P sol	N_2 fix
1	93738	1.51	*Flavobacterium*	EU057850	-	-	+	+	+	-	nd	-	++	nd	nd	-
1	9374	0.15	*Pedobacter*	DQ778037	-	-	++	++	++	-	-	-	-	-	-	-
1	9374	0.15	*Pedobacter*	DQ778037	-	-	++	++	++	-	-	-	-	-	-	-
1	93738	1.51	*Pedobacter*	DQ778037	-	-	++	++	++	-	-	-	-	-	+	+++
1	93738	1.51	*Pedobacter*	DQ778037	+	+	++	++	++	-	-	-	-	-	-	-
1	2343457	37.76	*Pseudomonas*	FN377713	-	-	-	-	-	+	-	+	++	-	-	-
1	2343457	37.76	*Pseudomonas*	FN377713	-	-	-	-	-	+	-	+	++	-	-	-
1	93738	1.51	*Pseudomonas*	FN377713	-	-	+	-	-	+	-	+	++	-	-	-
1	93738	1.51	*Rhizobium*	DQ337581	-	-	-	-	-	-	nd	-	++	nd	nd	-
1	524934	8.46	*Variovorax*	GQ861460	++	-	++	+	+	+	-	-	-	-	-	-
1	131234	2.11	*Variovorax*	GQ861460	+	+	++	+	+	+	-	-	+	-	-	-
1	93738	1.51	*Variovorax*	GQ861460	-	-	++	-	-	+	-	-	+	-	-	-
1	93738	1.51	*Variovorax*	GQ861460	++	+	++	-	-	+	-	-	-	-	-	-
1	93738	1.51	*Variovorax*	GQ861460	+	+	++	-	-	+	-	-	++	-	-	-
1	93738	1.51	*Variovorax*	GQ861460	++	+	++	-	-	+	-	-	+	-	+++	-
2	24719	18.18	*Caulobacter*	DQ337549	-	-	+	-	-	-	-	-	-	nd	++	-
2	6180	4.55	*Caulobacter*	DQ337549	+	+	++	-	-	-	+	-	-	nd	+++	-
2	6180	4.55	*Caulobacter*	DQ337549	-	-	+	+	+	-	-	-	-	nd	-	-
2	6180	4.55	*Caulobacter*	DQ337549	+	+	+	+	+	-	-	-	-	-	+++	-
2	6180	4.55	*Flavobacterium*	AM934662	+	+	++	+	+	-	-	-	-	-	+++	-
2	6180	4.55	*Pedobacter*	DQ778037	+	+	++	++	++	-	-	-	-	-	+++	+++
2	6180	4.55	*Pedobacter*	DQ778037	+	+	++	++	++	-	-	-	-	-	+++	-
2	6180	4.55	*Pedobacter*	GU385862	-	-	++	++	++	-	nd	nd	+	nd	nd	-
2	61797	45.45	*Rhizobium*	DQ337581	-	-	-	-	-	-	+	-	++	-	++	-
2	6180	4.55	*Rhodococcus*	EU496547	++	++	-	-	-	+	-	-	-	-	-	-
3	8673	2.26	*Caulobacter*	DQ337549	-	-	+	-	-	-	-	-	-	nd	++	-
3	1489	0.39	*Caulobacter*	DQ337549	-	-	++	-	-	-	-	-	-	-	+	-
3	2365	0.62	*Caulobacter*	DQ337549	-	-	+	-	-	-	-	-	-	-	+++	-
3	2365	0.62	*Caulobacter*	DQ337549	-	-	+	-	-	-	-	-	-	-	+	-
3	2365	0.62	*Caulobacter*	DQ337549	-	-	++	-	-	-	-	-	++	-	+	-
3	2190	0.57	*Caulobacter*	DQ337549	-	-	++	-	-	-	-	-	-	-	+++	-
3	14630	3.82	*Caulobacter*	DQ337549	-	-	+	-	-	-	-	-	-	-	+	-
3	7885	2.06	*Caulobacter*	DQ337549	-	-	++	++	++	-	-	-	-	-	-	-
3	7885	2.06	*Caulobacter*	DQ337549	-	-	+	+	+	-	-	-	-	-	+++	-
3	2190	0.57	*Labrys*	DQ337554	+	-	++	-	-	-	-	-	+	-	-	-
3	788	0.21	*Labrys*	DQ337554	++	-	++	-	-	+	-	+	++	-	-	-
3	14630	3.82	*Labrys*	DQ337554	-	-	+	-	-	-	-	+	-	nd	-	-
3	2190	0.57	*Leifsonia*	AB278552	-	-	++	++	++	+	-	+	+	-	-	+
3	2190	0.57	*Leifsonia*	AB278552	-	-	++	-	-	+	-	+	-	-	+	-
3	7885	2.06	*Mucilaginibacter*	EU747841	-	-	-	-	-	-	-	-	+	nd	-	-
3	7885	2.06	*Mycobacterium*	FJ719354	-	-	++	++	++	+	-	-	-	-	-	-
3	2365	0.62	*Pantoea*	EU598802	+	+	++	++	++	+	-	+	+	+++	-	+
3	1656	0.43	*Pantoea*	EU598802	+	-	-	-	-	+	++	nd	++	nd	+++	+++
3	7885	2.06	*Pedobacter*	DQ778037	-	-	++	++	++	+	-	-	+	-	++	-
3	2190	0.57	*Pedobacter*	DQ778037	-	-	++	++	++	-	-	+	-	-	+++	+
3	14630	3.82	*Pedobacter*	DQ778037	-	-	++	++	++	-	-	-	-	-	-	-
3	78846	20.59	*Pedobacter*	DQ778037	-	-	++	++	++	-	-	+	++	-	+++	+
3	1489	0.39	*Plantibacter*	AM396918	-	-	++	++	+	-	-	+	+	-	+++	-
3	4731	1.24	*Plantibacter*	AM396918	+	-	-	-	-	+	-	-	-	-	-	-

3	2365	0.62	*Plantibacter*	AM396918	-	-	++	++	++	-	-	+	+	-	+	-
3	7885	2.06	*Plantibacter*	AM396918	-	-	++	++	++	-	+	-	+	-	+++	-
3	14630	3.82	*Polaromonas*	AB245355	-	-	+	+	+	-	-	-	-	-	-	-
3	788	0.21	*Pseudomonas*	AB369347	+	+	++	-	-	+	-	+	+	+++	+++	-
3	2190	0.57	*Pseudomonas*	AB369347	+	-	++	-	-	++	-	+	++	-	+	+++
3	2478	0.65	*Pseudomonas*	DQ279324	+	+	++	+	+	+	-	+	+	-	++	++
3	2478	0.65	*Pseudomonas*	DQ279324	+	-	++	-	-	++	-	+	+	-	+++	-
3	2478	0.65	*Pseudomonas*	DQ279324	+	-	++	-	-	+	-	+	+	-	+++	-
3	2478	0.65	*Pseudomonas*	DQ279324	+	-	++	-	-	++	-	+	+	-	++	++
3	2478	0.65	*Pseudomonas*	DQ279324	+	-	++	-	-	+	-	+	+	-	+	++
3	2478	0.65	*Pseudomonas*	DQ279324	+	-	++	+	+	++	-	+	+	-	-	++
3	2478	0.65	*Pseudomonas*	FJ719351	-	-	-	-	-	+	-	-	+	-	+++	-
3	2365	0.62	*Pseudomonas*	FJ772042	-	-	++	-	-	-	-	-	-	-	-	-
3	788	0.21	*Pseudomonas*	FJ772042	-	-	++	+	+	-	-	+	+	-	+	-
3	788	0.21	*Pseudomonas*	FJ772042	-	-	++	-	-	-	-	+	+	-	-	-
3	14630	3.82	*Pseudomonas*	FJ772042	-	-	++	++	++	-	-	-	-	-	-	-
3	14630	3.82	*Pseudomonas*	FJ772042	-	-	++	-	-	-	-	-	-	-	-	-
3	11590	3.03	*Pseudomonas*	FN377713	-	-	-	-	-	+	-	-	++	+++	+++	-
3	1656	0.43	*Pseudomonas*	FN377713	+	-	-	+	+	+	-	-	++	+++	+++	++
3	1656	0.43	*Pseudomonas*	FN377713	-	-	-	-	-	+	-	-	++	+++	+++	-
3	7885	2.06	*Pseudomonas*	FN377713	+	+	++	+	+	+	++	-	++	-	++	-
3	7885	2.06	*Pseudomonas*	FN377713	+	+	+	-	-	+	++	-	++	+++	+++	-
3	788	0.21	*Rhizobium*	DQ337581	++	+	++	++	-	-	-	+	-	-	-	-
3	1577	0.41	*Rhizobium*	DQ337581	-	-	++	++	-	+	-	-	-	-	+++	-
3	2190	0.57	*Rhizobium*	DQ337581	-	-	-	-	-	+	-	-	++	-	++	-
3	2365	0.62	*Rhodococcus*	EU496547	++	-	++	+	+	-	-	-	-	-	+++	-
3	14630	3.82	*Rhodococcus*	EU496547	+	-	-	-	-	-	-	-	-	-	++	-
3	14630	3.82	*Rhodococcus*	EU496547	+	-	-	-	-	-	-	-	-	-	-	-
3	788	0.21	*Sanguibacter*	X79452	-	-	++	-	-	-	-	-	-	-	-	-
3	788	0.21	*Sanguibacter*	X79452	-	-	++	+	+	-	-	-	-	-	-	-
3	2365	0.62	*Staphylococcus*	GQ222398	-	-	++	+	+	-	-	+	-	-	-	-
3	1577	0.41	*Staphylococcus*	GQ222398	-	-	+	-	-	-	-	+	+	-	-	-
3	2190	0.57	unc.bact	GQ012035	-	-	++	++	++	-	-	+	+	-	+	-
3	1489	0.39	*Variovorax*	GQ861460	-	-	++	-	-	+	-	-	-	-	-	-
3	1489	0.39	*Variovorax*	GQ861460	+	+	++	+	+	+	+	-	-	-	-	-
3	1489	0.39	*Variovorax*	GQ861460	+	-	++	-	-	+	-	-	-	-	-	-
3	1489	0.39	*Variovorax*	GQ861460	+	+	++	-	-	-	-	-	-	-	++	-
3	1489	0.39	*Variovorax*	GQ861460	-	-	++	-	-	+	-	-	+	-	+	-
3	1489	0.39	*Variovorax*	GQ861460	-	-	++	-	-	+	-	-	-	-	-	-
3	1489	0.39	*Variovorax*	GQ861460	++	+	++	-	-	+	-	-	-	-	-	-
3	2365	0.62	*Variovorax*	GQ861460	-	-	+	+	+	-	++	-	+	++	-	-
3	2365	0.62	*Variovorax*	GQ861460	++	+	++	++	++	+	-	-	-	-	-	-
3	1577	0.41	*Variovorax*	GQ861460	++	++	++	++	++	+	-	-	-	-	-	-
3	3154	0.82	*Variovorax*	GQ861460	-	-	+	+	+	-	-	-	-	-	-	-
3	788	0.21	*Variovorax*	GQ861460	-	-	++	+	+	+	-	-	-	-	-	-
3	788	0.21	*Variovorax*	GQ861460	-	-	++	++	++	+	-	-	-	-	-	-
3	788	0.21	*Variovorax*	GQ861460	-	-	++	++	++	+	-	-	-	-	++	-
3	788	0.21	*Variovorax*	GQ861460	-	-	++	-	-	-	-	-	+	-	-	-
3	788	0.21	*Variovorax*	GQ861460	+	+	++	+	+	-	-	-	-	-	-	-
3	788	0.21	*Variovorax*	GQ861460	+	+	++	-	-	-	++	-	-	-	-	-
3	788	0.21	*Variovorax*	GQ861460	++	+	++	++	++	+	-	-	-	-	-	-
3	788	0.21	*Variovorax*	GQ861460	+	+	++	++	++	-	-	-	-	-	+++	-
3	2365	0.62	*Xanthomonas*	DQ177466	-	-	++	-	-	-	-	-	+	-	-	-

Appendix 4.2 The neighbour-joining tree of all strains with a different identification (accession number) clustered strains belonging to the same genus together. Sequences were aligned and used for constructing a neighbour-joining tree with PAUP*4.0b10, using default settings. In order to assess branch supports, bootstrap values were calculated with 2000 pseudoreplicates.

Appendix 4.3 Total Cd and Zn concentrations [mg (kg dry weight)$^{-1}$] in roots, stems, leaves and seeds of *B. napus* grown at the control field (CO-F) (A and B respectively) and the contaminated field (TE-F) (C and D respectively). Values are means ± standard error of 3 biological independent replicates (significance levels: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$).



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CHAPTER 5

Seasonal influences on bacterial communities associated with *Brassica napus* L. studied on a trace element-contaminated and a non-contaminated field

Abstract

In order to elucidate the possible effects of seasons and site specific conditions on cultivable bacterial communities, isolations were performed in December and June from bulk soil, rhizosphere soil and roots of *Brassica napus* L. growing on both a trace element-contaminated and a non-contaminated field. It appeared that bulk and rhizosphere soil communities were highly correlated but susceptible to changing seasonal and field conditions. In contrast, root endophytic communities seemed to be protected against various effects from outside and consisted of some conserved bacterial lineages.

For soil and root communities, bacterial diversity was higher in summer. At both fields, more bacterial strains tolerant to Cd were isolated in June while the highest percentages of Cd tolerant strains were consistently found at the field site contaminated with Cd, Zn and Pb.

All *B. napus* plants were persistently colonised by *Bacillus, Pseudomonas* and *Variovorax* species in the rhizosphere and by *Pseudomonas* strains in the roots. At the end of the growing season, high numbers of *Bacillus* and *Pseudomonas* strains were recovered from the shoots harvested at both fields, suggesting the transfer of essential endophytes to the seeds.

We observed the presence of some dominating strains associated with *B. napus,* which are hardly affected by factors from outside and seem to be transferred from one generation to the next via the seeds, indicating their importance in plant development and survival.

Introduction

Natural microbial communities are complex and mysterious entities (Lipson and Schmidt, 2004). Microbes exhibit a remarkably high genetic diversity compared with plant and animal species (Bouskill *et al*., 2011). Estimated numbers of bacterial species per gram soil vary between 2000 and 8.3 million (Gans *et al*., 2005; Schloss and Handelsman, 2006), and the significance of this phylogenetic and functional diversity, in terms of ecosystem functioning, has been difficult to understand. Bacterial community structure in multiple soil and plant biomes are recently being elucidated using culture-dependent (Kim *et al*., 2005; Park *et al*., 2005; Sharma *et al*., 2005; Braun *et al*., 2006; Wang *et al*., 2007) and culture-independent methods (Roesch *et al*., 2007; Acosta-Martínez *et al*., 2008; Redford *et al*., 2010; Uroz *et al*., 2010; Andrew *et al*., 2012; Lundberg *et al*., 2012; Rastogi *et al*., 2012; Russo *et al*., 2012).

The occurrence and distribution of bacteria in bulk soil and rhizosphere were reported to be influenced by many factors, including soil type and host plant species (Germida *et al*., 1998; Dunfield and Germida, 2003; Thirup *et al*., 2003; Garbeva *et al*., 2004; Berg *et al*., 2005; Costa *et al*., 2006; Houlden *et al*., 2008). Studies do not agree if soil type whether plant species have the greatest effect on soil community structure. In contrast, there exists a consensus that plants have a significant influence on rhizosphere community structure due to species specific and growth stage dependent differences in root exudation. Further, rhizodeposition is not homogenous in different root zones. Eilers *et al*. (2010) showed that inputs of compounds, present in root exudates, to soil are associated with shifts in bacterial community structure.

In addition, other factors might influence the composition of the microbial communities in the soil and rhizosphere, such as cropping practices and seasonal changes (Di Cello *et al*., 1997; Lupwayi *et al*., 1998; Grayston *et al*., 2001; Smalla *et al*., 2001; Dunfield and Germida, 2003). Buckley and Schmidt (2003) revealed significant changes at temporal scales relative to seasonal events, resulting in a specific soil moisture and temperature. Bouskill *et al*. (2011) documented the seasonal reoccurrence of specific lineages, identified by nitrification key functional genes. On the contrary, Meier *et al*. (2008) concluded that agricultural soil communities fluctuated very little during seasons but were

rather influenced by human interference, like field management (Roesch *et al*., 2007).
Summarized, soil type and cropping practices have a rather long lasting effect (over several years) on microbial community structure, while temporal changes occur at scales that are relevant to seasonal events. The observed seasonal variations in the activity and relative abundance of rhizosphere microbial communities are plant-dependent (Dunfield and Germida, 2003; Berg *et al*., 2005; Mougel *et al*., 2006). Plants can affect available soil carbon, temperature and water content. These same variables change seasonally, so plant control on microbial community composition may be modulated or overshadowed by annual climatic patterns (Waldrop and Firestone, 2006; Houlden *et al*., 2008).
In conclusion, the soil/rhizosphere ecosystem is exposed to fluctuations mainly connected with shifts in environmental conditions (temperature, humidity, etc.) and composition of root exudates, which have a marked influence on microbial communities. All of the cited studies leading to this conclusion investigated spatial and/or temporal effects on the genotypical structure of bacterial communities; data about the effects on phenotypical characteristics of communities are scarce. Since we are interested in the fluctuations of bacterial populations in soil, rhizosphere and roots of *Brassica napus* L. (rapeseed) grown on a non-contaminated control field (CO-F) and a field contaminated with trace elements (Cd, Zn and Pb) (TE-F), we studied the seasonal effects on both the genotypic and phenotypic structure of the associated bacterial communities. Our main focus is on the seasonal changes, but also differences in genotypic and phenotypic structure between fields are investigated. The results should point out if specific bacterial genotypes or phenotypes are dominating as a function of season or field. For this purpose, all cultivable bacteria associated with the 3 sampled compartments were characterized genotypically and phenotypically in December and June. We chose to study the bacterial communities associated with *B. napus* in winter, *i.e.* after rosette formation, and in summer during seed formation, *i.e.* after growth resumes in spring and the stem with the inflorescence is developing.
Seed endophytes were isolated from the seeds sown in September and the ones harvested in June in order to explore the importance of these endophytes during

the life cycle of rapeseed. Moreover, shoots were also examined for the presence of seed endophytes.

Experimental Procedures

Sampling

All cultivable bacterial strains associated with the bulk soil, rhizosphere soil and the organs of *B. napus* (root and shoot) were isolated at the rosette stage (December) and the flowering stage (June); seeds were harvested at the end of June. Sampling was performed on a trace elements (TE) (Cd, Zn and Pb) contaminated former maize field in Lommel (TE-F; see Ruttens *et al*., 2010) and on a non-contaminated field in Alken (Belgium) (CO-F).

To compose 3 mixed samples per studied compartment, both fields were subdivided into 3 subareas. One plant, with its surrounding rhizosphere soil and bulk soil, from each subarea (3 in total) made up a mixed bulk soil, rhizosphere soil, root, shoot and seed sample. Bulk soil was sampled at a depth of 30 cm. Roots were stored in sterile Falcon tubes containing 20 ml sterile 10 mM $MgSO_4$.

Isolation of B. napus-associated bacteria

Cultivable bacterial strains from all mixed bulk soil, rhizosphere soil and plant samples (30 mixed samples in total) were isolated according to Weyens *et al*. (2009), but optimized for rapeseed. For plant surface sterilisation a less concentrated chloride solution (1 % for roots and 0.1 % for shoots and seeds) and a reduced time (1 min) were applied. Also the crushing of plant material was less intensive (1 min). All plated samples were incubated for 7 days at 30°C and colony forming units (cfu) were counted and calculated per gram soil or fresh plant weight. Morphologically different strains were purified using 5 replicates and subsequently stored at -70°C in a glycerol solution (15% (w:v) glycerol; 0.85% (w:v) NaCl).

To isolate the seed endophytes, the acclimatization method described by Hahn *et al*. (2004) was used. Crushed solutions of sterilized seeds were added to minimal medium 284 (Weyens *et al*., 2009) with a carbon 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) and to IBM medium (Hahn *et al*., 2004). The 284 medium was stepwise enriched with 869 medium (Mergeay *et al*., 1985) and diluted 869

medium while the IBM medium was provided with NSY medium according to Hahn *et al*. (2004). After acclimatisation, aliquots of the enriched liquid media were plated on solid medium (869, 1/10 896 or NSY) for bacterial count. We used 3 different isolation media in order to isolate as many different seed genera as possible. Each medium was represented by 3 biological independent replicates.

Since our final goal (in future experiments) is to perform inoculation experiments, this study is based on culture-dependent methods. Making conclusions using this method appeared to be no problem according to Bodenhausen *et al.* (2013) and van Overbeek and van Elsas (2008).

Genotypic characterisation

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 was performed on aliquots of the extracted DNA using the universal primers, 16S-prokaryotic-R (5'- ACGGGCGGTGTGTRC-3') and 16S-prokaryotic-F (5'-AGAGTTTGATCCTGGCTCAG-3') as described previously by Weyens *et al*. (2009). For amplified 16S rDNA restriction analysis (ARDRA), 20 µl of the PCR products were digested with the HpyCH4IV enzyme and visualized by gel electrophoresis as described by Weyens *et al*. (2009). Bacterial strains from bulk and rhizosphere soil with the same ARDRA patterns were grouped; strains isolated from roots, shoots and seeds were grouped separately. The 16S rDNA PCR products of 1 representative strain per group were purified according to the QIAquick 96 PCR Purification Kit (Qiagen, Valencia, CA, USA). Subsequently, purified 16S rRNA genes were sent for sequencing by Macrogen (Korea) with an Automatic Sequencer 3730XL. Consensus sequences and sequence matches were obtained as described in Weyens *et al*. (2009).

Phenotypic characterisation

All purified bacterial strains from the bulk soil, rhizosphere soil, and roots were screened for trace element tolerance (Cd and Zn) since the 2 studied field sites strongly differ in their soil metal concentrations (table 5.1). In this context it is interesting to know whether there exists also a difference in the numbers of Cd

and Zn tolerant bacteria from both fields. Isolates were plated on selective 284 medium with a carbon mix and 0.0, 0.8 and 1.6 mM Cd ($CdSO_4$) or 0.0, 1 and 2.5 mM Zn ($ZnSO_4$). Tolerance was rated visually after an incubation period of 7 days at 30°C (Weyens *et al*., 2009). Before screening, strains were grown in 869 medium and subsequently washed twice with sterile 10 mM $MgSO_4$. Strains, not able to grow on the 284 agar medium (pH 7) without additional Cd and Zn sulphate, were considered as not detectable (nd). Mixtures without cell suspension were used as controls.

Soil properties

On the bulk soils, soil type, potential soil pH (pH (KCl)), soil organic matter content (OC) expressed in % and trace metal concentrations (Cd, Zn and Pb) in mg kg^{-1} dry soil were determined. Soils were oven-dried (48h at 65°C), sieved through a 2-mm sieve and stored until further use. Soil texture classification was done according to the USDA triangle while the potential soil pH was measured in a 1/2.5 (w/w) soil to 1 M KCl mixture. The OC was determined by a modified Walkley-Black method, 10 ml 1 N $K_2Cr_2O_7$ and 20 ml H_2SO_4 (concentrated) was added to 1 g soil to reduce C and Fe^{2+}. After 30 min 150 ml distilled water, 10 ml H_3PO_4 (concentrated) and 1 ml diphenylamine-indicator were added. H_3PO_4 became a complex with Fe^{3+} and the excess of $K_2Cr_2O_7$ was titrated with 1 N Mohr's Salt. The 0.1 M $Ca(NO_3)_2$-exchangable trace element fractions in the soil (Mench *et al*., 1994) and the pseudo-total (*aqua regia* digestion; Van Ranst *et al*., 1999) soil trace element contents were determined. Trace element concentrations in the extracts were measured using inductively coupled plasma optical emission spectrometry (ICP-OES). Quality of the analyses was verified by including blanks and soil standards.

Statistical Analysis

The obtained data (bacterial counts, biodiversity indices, percentages of Cd tolerant bacteria and soil metal concentrations) were analysed using a 3 way ANOVA. Mean data were calculated from 3 biologically independent replicates, which each consist of 3 independent samples. Transformations were applied when necessary to approximate normality and/or homoscedasticity. In case normality could not be reached, data were analysed using Kruskal-Wallis

multiple comparisons test. Genotypic information was subjected to correspondence analysis (CA), a principal component analysis related ordination technique based on chi-square distances, illustrating correlations between compartments.

Results

Characterization of both field sites

The 2 fields differ in soil type; while the control soil is light sandy-loam, the contaminated soil is composed of fine sand. Both soil types share a similar percentage of organic matter content (1.9 %) and soil pH(KCl) (+/- 5.9). Trace element concentrations (Cd, Zn and Pb) were significantly higher at the contaminated field (table 5.1).

Table 5.1 Soil metal concentrations at the control field (CO-F) and the trace element-contaminated field (TE-F).

Soil metal concentration		CO-F	TE-F
$Ca(NO_3)_2$	Cd	0.15 ± 0.0073	1.0 ± 0.0033
	Zn	4.6 ± 0.023	78 ± 0.48
	Pb	nd	0.38 ± 0.010
Aqua regia	Cd	0.50 ± 0.00	5.1 ± 0.088
	Zn	89 ± 4.6	277 ± 6.7
	Pb	25 ± 0.85	199 ± 2.7

Trace metal concentrations (Cd, Zn and Pb) in mg kg^{-1} dry soil. Potential plant available metal concentrations were estimated using a 0.1 M $Ca(NO_3)_2$-extraction. Pseudo-total trace elements were extracted using *aqua regia* digestion. Values on the metal concentrations are mean ± standard error of 3 biological independent replicates. nd: not detectable.

Characterization of bacterial communities

Since the root-rhizosphere interface is the nexus of a variety of associations and interactions between soil organisms and the host plant, we are most interested in the bacterial populations present in the soil, the rhizosphere and the roots. Each studied compartment was represented by 3 mixed samples, each containing 3 independent samples. Both, genotypic and phenotypic bacterial features, were investigated in December and June to obtain insight into the seasonal effects, and on a control and a contaminated field to elucidate the consequences of trace element stress.

In addition to the root-rhizosphere interface, also the seeds might be of high interest. It was suggested that seeds can serve as vectors to pass beneficial bacteria from the one generation to the next (Cankar *et al*., 2005; Truyens *et al*., 2014). A possible way of transferring bacteria to the seeds is through the shoot of the plant. In this regard, shoot and seed endophytes were also investigated.

Soil, rhizosphere and root bacterial communities

Isolation

Bacteria were isolated from bulk soil, rhizosphere soil and roots of *B. napus* plants grown on a non-contaminated control soil (CO-F) and a contaminated soil (TE-F) in December and June (figure 5.1).

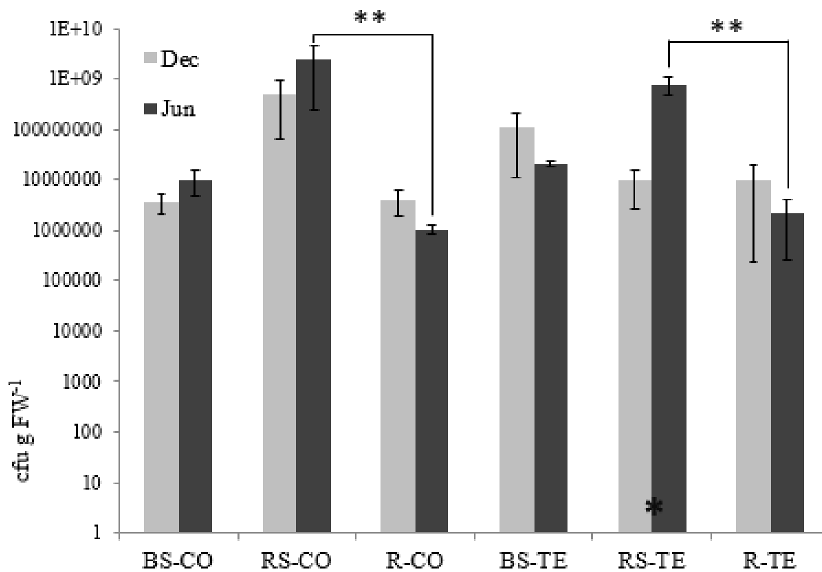


Figure 5.1 Mean total numbers of colony-forming units (cfu g FW^{-1}) isolated from the compartments bulk soil (BS), rhizosphere soil (RS) and root (R) collected at the control field (CO-F) (BS-CO, RS-CO and R-CO respectively) and the contaminated field (TE-F) (BS-TE, RS-TE and R-TE respectively) in December and June. Values are mean ± standard error of 3 biological independent replicates (significance level: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Each replicate consisted of 3 independent compartment samples (mixed samples).

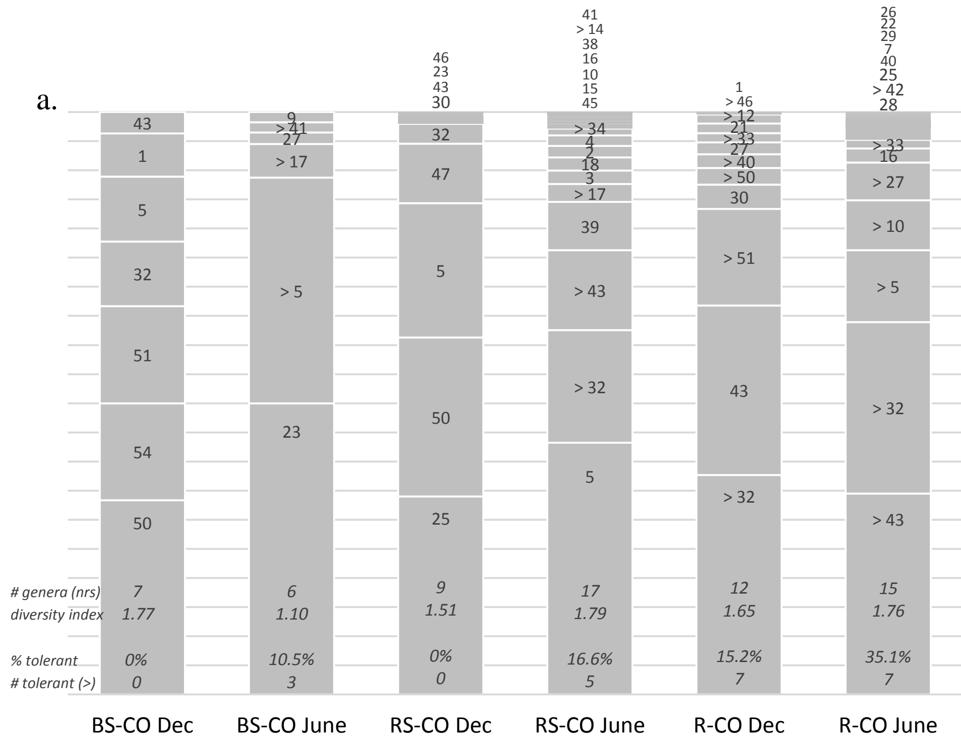
According to the 3 way ANOVA, there was no significant field effect on the amount of colony forming units (cfu) per gram fresh weight (g FW^{-1}). This means that similar results for a specific compartment were found regardless on which field the samples were taken. After removing the field parameter from our dataset, the seasonal effect became less important (*p*-value: from 0.08 to 0.12). However, bacterial counts significantly increased during summer in the rhizosphere soil at the contaminated field (*p*-value: 0.05). Furthermore, isolated numbers were compared between compartments within one field and season. In general, the quantities of cultivable strains recovered from the bulk soil are lower compared to the rhizosphere soil which in turn contained a higher (significant in June (*p*-values: CO-F = 0.005; TE-F = 0.003)) bacterial density than the root interior. Only at the contaminated field in December, bacterial communities isolated from the bulk soil, rhizosphere soil and root were similar in magnitude. No significant differences were found between the numbers of bacterial strains isolated from the bulk soil and root within fields and seasons.

Characterisation

The diversity and abundance of all isolated strains per compartment (bulk soil (BS), rhizosphere soil (RS) and root (R)) in a specific field (non-contaminated control field (CO-F) and trace element-contaminated field (TE-F)) and season (December (Dec) and June (Jun)) are presented in figure 5.2. Per condition all bacterial strains were grouped together in genera (table 5.2); the total amount of different genera and the Shannon-Wiener biodiversity index per condition were calculated (genotypic characterisation). Also at the bottom of each bar diagram, the percentage of tolerant members (to 0.8 mM Cd and/or 1 mM Zn) and the amount of different bacterial genera with tolerant members are shown (phenotypic characterisation).

Table 5.2 Bacterial genera isolated from soil, rhizosphere and roots of *B. napus* with their corresponding number. This legend is used in figures 5.2, 5.3 and 5.5.

1 *Achromobacter*	10 *Caulobacter*	19 *Massilia*	28 *Pedobacter*	37 *Serratia*	46 *Agromyces*
2 *Aeromicrobium*	11 *Chsyseobacterium*	20 *Mesorhizobium*	29 *Plantibacter*	38 *Sphingobacterium*	47 *Alcaligenes*
3 *Agrobacterium*	12 *Flavobacterium*	21 *Methylobacterium*	30 *Polaromonas*	39 *Staphylococcus*	48 *Chitinophaga*
4 *Arthrobacter*	13 *Frigoribacterium*	22 *Microbacterium*	31 *Pseudoclavibacter*	40 *Stenotrophomonas*	49 *Clavibacter*
5 *Bacillus*	14 *Janthinobacterium*	23 *Micromonospora*	32 *Pseudomonas*	41 *Streptomyces*	50 *Duganella*
6 *Bradyrhizobium*	15 *Kribella*	24 *Mucilaginibacter*	33 *Rhizobium*	42 uncultured bact.	51 *Luteibacter*
7 *Brevibacillus*	16 *Labrys*	25 *Mycobacterium*	34 *Rhodococcus*	43 *Variovorax*	52 *Mitsuaria*
8 *Brevundimonas*	17 *Leifsonia*	26 *Paenibacillus*	35 *Rhodopseudomonas*	44 *Xanthomonas*	53 *Sphingomonas*
9 *Burkholderia*	18 *Lysinibacillus*	27 *Pantoea*	36 *Sanguibacter*	45 *Zoogloea*	54 *Sphingopyxis*


a.
genera (nrs)
diversity index
% tolerant
# tolerant (>)
7 6 9 17 12 15
1.77 1.10 1.51 1.79 1.65 1.76
0% 10.5% 0% 16.6% 15.2% 35.1%
0 3 0 5 7 7
BS-CO Dec
BS-CO June
RS-CO Dec
RS-CO June
R-CO Dec
R-CO June

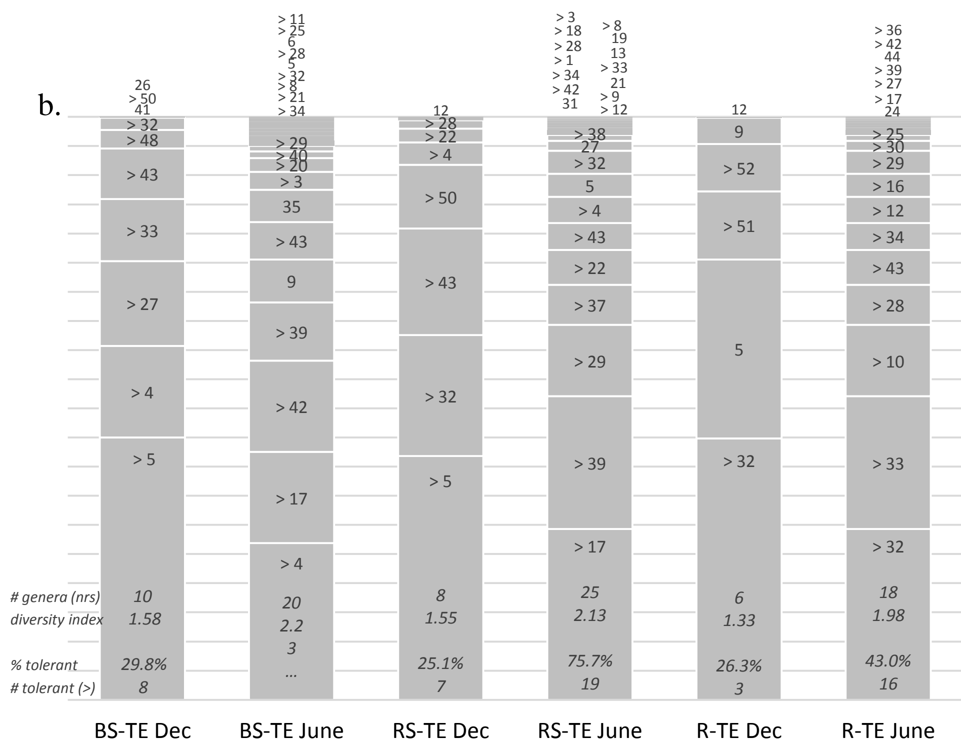

b.
genera (nrs)
diversity index
% tolerant
# tolerant (>)
10 20 8 25 6 18
1.58 2.2 1.55 2.13 1.33 1.98
29.8% 25.1% 75.7% 26.3% 43.0%
8 7 19 3 16
BS-TE Dec
BS-TE June
RS-TE Dec
RS-TE June
R-TE Dec
R-TE June

Figure 5.2 Diversity and abundance of cultivable bacterial strains isolated at (a) the control field (CO-F) and (b) the trace element-contaminated field (TE-F) in December and June from the bulk soil (BS), rhizosphere soil (RS) and roots (R) of *B. napus* plants*.* Each number represents a bacterial genus (see table 5.2); genera with members tolerant to 0.8 mM Cd and/or 1 mM Zn are indicated by arrows (>). Bar fragments represent the relative abundance expressed in percentages (see appendix 5.1) of the total number of cultivable bacteria isolates per gram fresh weight. Data are means of 3 replicates consisting of 3 independent compartment samples (mixed samples). At the base of each bar diagram, following parameters are found: total amount of different bacterial genera isolated, Shannon-Wiener biodiversity index, proportion of tolerant members and amount of different bacterial genera with tolerant members (to 0.8 mM Cd and/or 1 mM Zn). Genera positioned above the bar represent less than 1.10 %.

The first interesting observation is that higher numbers of different genera were isolated in June (except for the bulk soil at the CO-F). Also biodiversity indices were significantly higher in June (*p*-value season effect: 0.008). When percentages of Cd tolerant bacteria are compared within compartment and field, lower percentages were isolated in December (*p*-value season effect: 0.0004). Also the number of different tolerant genera was lower during winter (except for the root at the CO-F).

When comparing percentages Cd tolerant bacteria between fields at the same sampling moment for specific compartments, relatively more Cd tolerant bacteria were found at the contaminated field (*p*-value field effect: 0.00005). The same conclusion can be made for the different tolerant bacterial genera (except for the root in December). Biodiversity did not significantly differ between fields.

According to the 3-way ANOVA's (with as fixed factors compartment, field and season), tolerance characteristics and biodiversity indices did not significantly differ between compartments. More information on the tolerant strains (tolerant to 0.8 mM Cd and/or 1 mM Zn) is presented in appendix 5.1.

Special attention is attributed to the strains that are present throughout the year in rhizosphere soil and roots since such (tolerant) strain(s) might be the most promising for inoculation. In the contaminated rhizosphere soil, the bacterial genera *Arthrobacter*, *Flavobacterium, Microbacterium*, *Pedobacter*, *Bacillus*, *Pseudomonas* and *Variovorax* were present at the 2 sampling times. The latter 3 genera were also isolated from the control rhizosphere soil in December and June. The roots from the contaminated field were permanently colonised by

Flavobacterium and *Pseudomonas* and the roots from the control field by *Pantoea*, *Pseudomonas, Rhizobium*, *Stenotrophomonas* and *Variovorax*. *Pseudomonas* is the only bacterial genus present at each time in the rhizosphere and roots of *B. napus* at both fields.

The data from figure 5.2 were used to run a correspondence analysis (CA); a principal component analysis related ordination technique based on chi-square distances, illustrating correlations between compartments (figure 5.3).

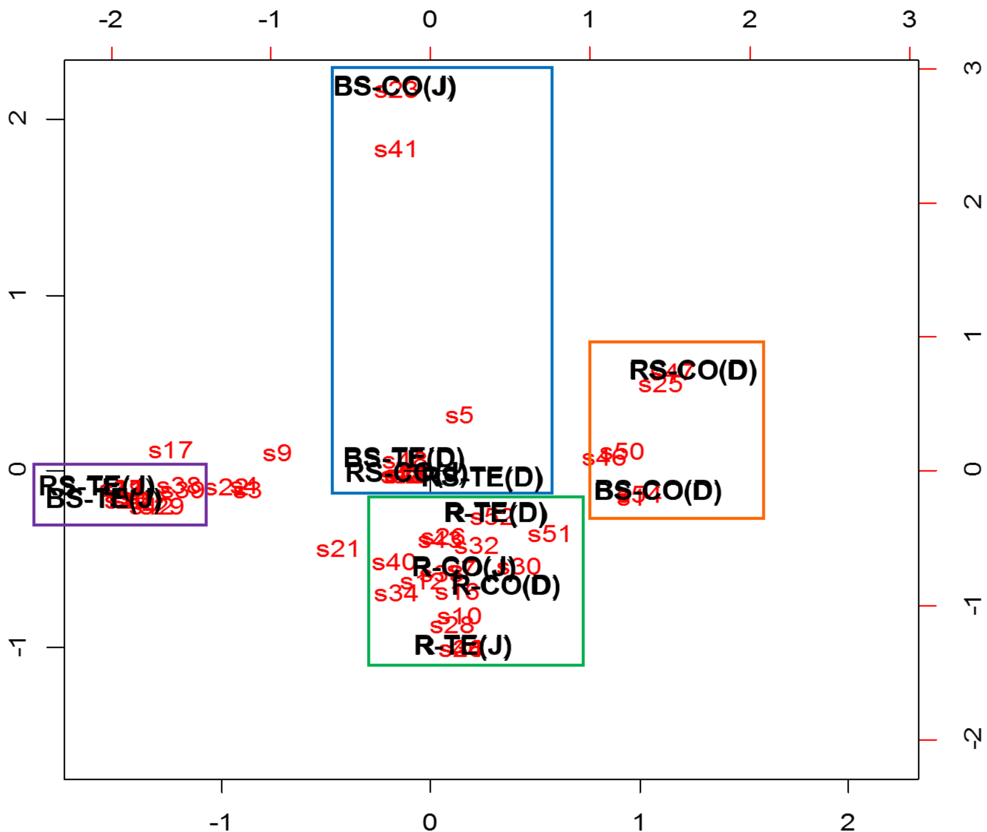


Figure 5.3 Correspondence analysis of bacterial communities isolated from bulk soil, rhizosphere soil and *B. napus* roots at the control and the contaminated field in December and June. Each s-number represents an isolated bacterial genus, the connection between genera and numbers can be found in table 5.2. Clustered compartments point out the correlation between the bacterial communities found in the bulk soil, rhizosphere soil and roots collected at the control field (BS-CO, RS-CO and R-CO respectively) and the contaminated field (BS-TE, RS-TE and R-TE respectively) in December (D) and June (J). Data are based on 3 replicates consisting of 3 independent compartment samples (mixed samples).

If relevant correlation coefficients (CC) above 0.45 are considered (appendix 5.2), following conclusions can be made regarding the genotypic similarities of the studied bulk soil, rhizosphere soil and root bacterial communities:

(1) All bulk soil and rhizosphere soil communities were correlated within one field, each season (CO-F(D) (orange square): *Bacillus*, *Duganella*, *Pseudomonas*, *Variovorax*; TE-F(D) (blue square): *Arthrobacter*, *Bacillus*, *Duganella, Pseudomonas*, *Variovorax*; CO-F(J) (blue square): *Bacillus*, *Leifsonia*, *Streptomyces*; TE-F(J) (purple square): *Agrobacterium*, *Arthrobacter*, *Bacillus*, *Brevundimonas*, *Burkholderia*, *Leifsonia*, *Methylobacterium*, *Pedobacter, Plantibacter*, *Pseudomonas*, *Rhodococcus*, *Staphylococcus*, uncultured bacterium, *Variovorax*).

(2) Soil communities differed largely between fields during December and June except for the rhizosphere soil in December.

(3) The rhizosphere soil population and root community were correlated during summer at the CO-F (*Bacillus*, *Caulobacter, Labrys, Pseudomonas*, *Variovorax*) and during winter at the TE-F (*Bacillus*, *Flavobacterium, Pseudomonas*).

(4) Remarkable are the correlations observed between all endophytic root communities, which were correlated between seasons and fields (green square in figure 5.3). Roots harvested in December share some common genera (*Flavobacterium, Luteibacter*, *Pseudomonas*) just like those harvested in June (*Caulobacter*, *Labrys*, *Mycobacterium*, *Pantoea*, *Pedobacter*, *Plantibacter*, *Pseudomonas*, *Rhizobium*, uncultured bacterium, *Variovorax*). Not only root populations within one season from both fields are similar, but also those isolated in December and June within one field (R-CO: *Pantoea*, *Pseudomonas*, *Rhizobium*, *Stenotrophomonas, Variovorax*; R-TE: *Flavobacterium, Pseudomonas*). Moreover, root communities isolated from *B. napus* plants growing in different fields and seasons correlate (R-CO(D) and R-TE(J): *Flavobacterium*, *Pantoea, Polaromonas*, *Pseudomonas*, *Rhizobium*, *Variovorax*; R-CO(J) and R-TE(D): *Bacillus, Pseudomonas*).

The general picture is that the bacterial communities isolated from the bulk and rhizosphere soil were similar at a specific time and place, and that they are unique (BS/RS-CO(D) ≠ BS/RS-CO(J) ≠ BS/RS-TE(D) ≠ BS/RS-CO(J)). In

contrast, bacterial root communities were not affected by changing seasons and soils.

Shoot and seed bacterial communities

Some bacterial genera seem dominantly associated with *B. napus* (figures 5.2 and 5.3). To verify if these genera that play an important role during the entire lifecycle of rapeseed are transferred from one generation to the next via the seeds, shoot and seed endophytes were isolated. Bacterial shoot communities were isolated in December and June at both fields (CO-F and TE-F), while seed endophytes were obtained from the seeds sown at both fields (SE-SOWN) and from the seeds harvested from both fields (SE-CO and SE-TE).

Appendix 5.3 contains the genotypic details concerning the shoot endophytes. Before considering the correlations between root, shoot and seed bacterial communities (figure 5.5), the data of the seed endophytes are discussed. The bacterial communities of the seeds sown (SE-SOWN) and the seeds harvested at both fields (SE-CO and SE-TE) are presented in figure 5.4. Appendix 5.4 contains further details on the seed endophytes.

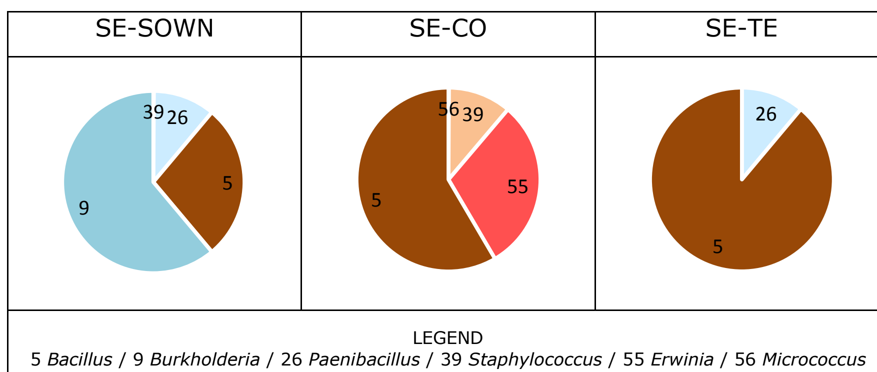


Figure 5.4 Diversity and abundance of cultivable bacterial strains isolated from *B. napus* seeds (SE). Bacteria living inside seeds sown at both fields (SE-SOWN) and harvested at the control field (SE-CO) and the trace element-contaminated field (SE-TE) were isolated using 3 different liquid growth media (284+869/10; 284+869; IBM+NSY) which were inoculated with the same amount crushed seed solution. Three replicates per medium were used and data shown are means per seed type using the data gathered from all 3 enrichment media (appendix 5.5). Pie fragments indicate relative abundances of bacterial genera, expressed in percentages of the total number of cultivable isolates per 100 µl culture medium. Each colour (number, see legend) represents a bacterial genus.

The originally sown seeds were mainly inhabited by *Bacillus, Burkholderia* and *Paenibacillus* species, the seeds harvested at the control field by *Bacillus, Erwinia* and *Staphylococcus* species and the seeds collected from the contaminated field by *Bacillus* and *Paenibacillus* species.
Genera of seed endophytes that were also present in rhizosphere soil and roots at one sampling moment at both fields or throughout the whole growing cycle at one field, received special attention (figures 5.2 and 5.4) since they can be supposed to be conserved bacterial strains. *Bacillus*, *Pseudomonas* and *Variovorax* strains were present throughout the growth cycle in the rhizosphere at both fields, *Pseudomonas* strains in the roots. The genus *Staphylococcus* appeared at both fields during summer in the rhizosphere. In conclusion, 2 out of the 6 genera that were isolated from seeds seemed also dominant in the rhizosphere of *B. napus*. These 2 genera, *Bacillus* and *Staphylococcus*, were also found in the shoots at both fields in December and/or June, as it was also the case for *Pseudomonas* and *Variovorax* strains (appendix 5.3). Their high presence in the shoots can be considered as an additional confirmation for their transfer to the inflorescence and thus the seeds and their significance as endophytes.

Figure 5.5 shows the correspondence analysis (CA) based on the genotypic data of the root, shoot and seed endophytes isolated from *B. napus* plants grown at the control (CO-F) and contaminated field (TE-F) in December (D) and June (J).
It appears that most root (R) and shoot (S) compartments are highly correlated (green square). Bacterial communities associated with compartments S-CO(J) and S-TE(J) (green arrows) tend to be more similar to bacterial seed communities. The 3 bacterial seed communities are grouped together in the CA plot, although the composition of the cultivable bacterial community of the harvested seeds is not exactly the same as the one from the sown seeds (correlation coefficients (CC), see appendix 5.6).
Bacterial genera highly related to compartments SE-SOWN, SE-CO and SE-TE are *Bacillus, Burkholderia, Erwinia,* and *Micrococcus*. Also genera *Frigoribacterium* and *Massilia* are situated in this part of the CA plot since compartment S-CO(J) shared a very high amount of *Bacillus* strains with the 3 seed compartments. Seed genera *Paenibacillus* and *Staphylococcus* were shared

with compartment S-TE(J), which also contained specific genera *Brevundimonas* and *Sanguibacter*. All the other bacterial genera were related to the root and shoot bacterial communities. Compartment S-CO(J) correlated to SE-SOWN, SE-CO and SE-TE (CC: 0.35, 0.81 and 0.92), and S-TE(J) to SE-SOWN and SE-TE (CC: 0.11 and 0.08). Appendix 5.6 contains all CC's linked to the CA in figure 5.5.

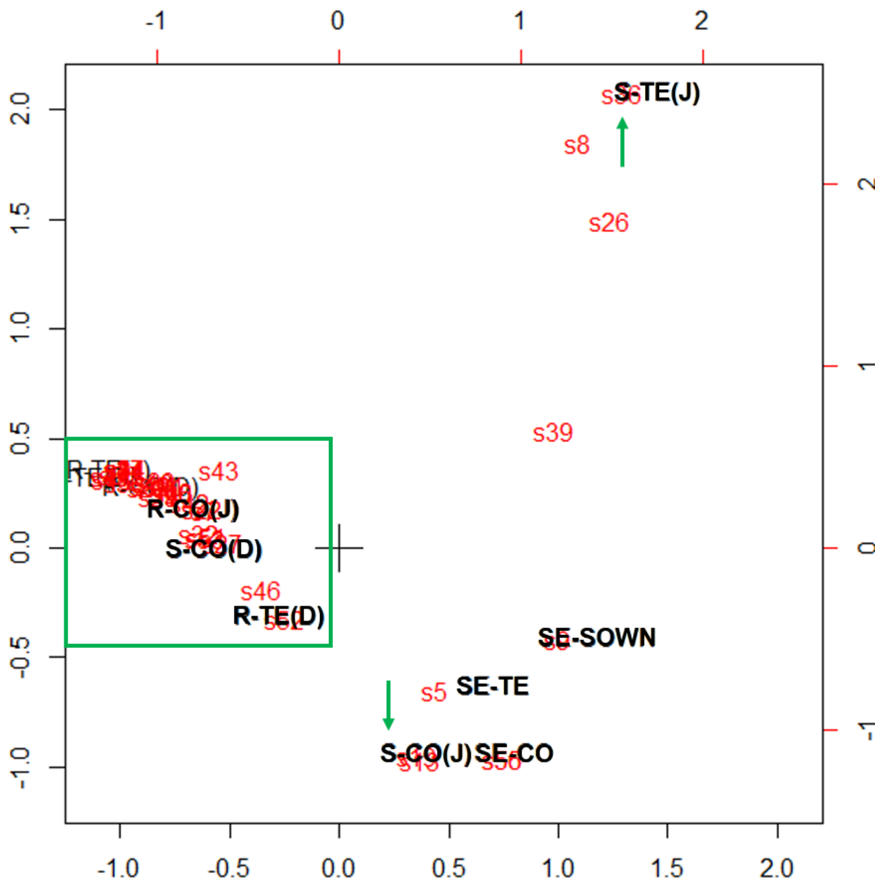


Figure 5.5 Correspondence analysis of bacterial communities isolated from *B. napus* roots, shoots and seeds. Clustered compartments point out the correlation between the bacterial communities found in the roots (R), shoots (S) and seeds (SE) collected at the control field (CO-F) and the contaminated field (TE-F) in December (D) and June (J). Data are based on 3 replicates consisting of 3 independent compartment samples (mixed samples). Each s-number represents an isolated bacterial genus, the connection between genera and numbers can be found in table 5.2 and figure 5.4.

Discussion

The main purpose of the present work was to explore the effects of seasonal changes on the cultivable bacteria in the bulk soil and especially those associated with the rhizosphere soil and roots of *B. napus* plants. This study was performed on 2 different fields in order to verify the obtained results and to investigate the effects of soil contaminants on bacterial communities.

Seasonal, field and compartment effects on bacterial counts

Seasonal variations, mainly due to changes in environmental conditions and the composition of root exudates, had little influence on the total amount of cultivable bacteria present in the bulk soil, rhizosphere soil and roots of *B. napus* (figure 5.1). Seasonal changes in soil bacterial communities were extensively studied (Rogers and Tate, 2001; Blume *et* al., 2002; Schadt *et al.*, 2003; Shishido *et al*., 2008) as well as those in the rhizosphere (Marschner *et al*., 2002; Houlden *et al*., 2008). Rogers and Tate (2001) and Blume *et al*. (2002) described that the size of the microbial biomass in soils was not significantly affected by seasonal variations. In Alpine soils microbial biomass was reported to be at its annual maximum during late winter when soils are frozen (Schadt *et al.*, 2003). Also Shishido *et al*. (2008) found a quantitative seasonal effect on soil microbial populations. Marschner *et al*. (2002) mentioned a decline in microbial biomass during the dry season in the rhizosphere of 2 tropical plants. Houlden *et al*. (2008) observed that cultivable bacterial and fungal rhizosphere community densities were stable in pea and wheat rhizospheres, with dynamic shifts observed in the sugar beet rhizosphere. Form the previous it is clear that bacterial (rhizosphere) soil communities do not show consistent changes in function of seasons. In our study, no significant seasonal effects on population size were found in the bulk soil and roots; only at the contaminated field (TE-F) bacterial counts significantly increased during summer in the rhizosphere soil (*p*-value ≤ 0.05) (figure 5.1). Here, as in other seasonal ecosystems, the general assumption is that soil microbes are less active during winter (Bardgett, 2005) when soil labile C from root exudates is generally lower than in summer (Guicharnaud *et al*., 2010).

Apart from the seasonal effect on bacterial counts, differences in colony forming units per gram fresh weight were found between compartments within one field

and season. The higher amount of cultivable bacteria in the rhizosphere than in bulk soil can be explained by the 'rhizosphere effect' (Rouatt and Katznelson, 1960). The general decrease in colony forming units from rhizosphere soil to root was significant in June. Compant *et al.* (2010) described the shifting of soil bacteria to the rhizosphere and subsequently the rhizoplane of their hosts. From the rhizoplane they can penetrate into plant roots from where they may move to the aerial plant parts, with a decreasing bacterial density in comparison to rhizosphere colonizing populations. The general decrease in colony forming units from rhizosphere soil to root was not significant in December, which may be due to a reduced plant activity and exudate secretion (Lipson *et al.*, 1999).
In contrast to the differences between compartments, no field effect was observed since similar numbers of bacteria were isolated from soil and root samples taken at both fields at the same moment (figure 5.1).

Seasonal, field and compartment effects on bacterial biodiversity indices and Cd tolerance

Diversity and phenotypic characteristics of the cultivable bacterial communities in the bulk soil, rhizosphere soil and roots of *B. napus* differed significantly between seasons (figure 5.2) suggesting that static bacterial population surveys might underestimate microbial diversity. Higher numbers and higher percentages of Cd tolerant genera were isolated in June, which indicates that populations isolated in December were less diverse and contained lower percentages of Cd tolerant bacteria. Multiple studies confirm temporal shifts in bacterial communities isolated from bulk and rhizosphere soils (Rogers and Tate, 2001; Blume *et al*., 2002; Marschner *et al*., 2002; Dunfield and Germida, 2003; Houlden *et al.*, 2008; Shishido *et al*., 2008; Tabuchi *et al*., 2008; Cavaglieri *et al*., 2009; Cruz-Martinez *et al*., 2009; Rasche *et al*., 2011). These studies found seasonal variability in bacterial diversity and relative activity. Recent results of Koranda *et al*. (2013) demonstrate that variation in resource availability as well as seasonality in temperate forest soils cause a seasonal variation in functional properties of soil microorganisms. Also bacterial communities isolated from plant tissues were influenced both by variations in temperature and different plant developmental stages (Mocali *et al*., 2003; de Campos *et al*., 2013).

Furthermore, no differences in bacterial diversity and tolerance were observed between studied compartments (BS, RS and R). Also bacterial diversity between fields was similar for specific compartments. Interesting to mention is that significantly higher percentages of Cd tolerant bacteria and different tolerant genera were isolated at the metal contaminated field (figure 5.2) (Croes *et al.*, 2013). Also Siciliano *et al*. (2001) reported that bacterial numbers containing specific phenotypes correlated with the presence and concentration of contaminants as a result of selective pressure.

Seasonal and field effects on bacterial community structure in soil and root

In order to obtain a more comprehensive view on the changes in bacterial community structure in function of season and field, genotypic data from the bulk soil, rhizosphere soil and roots of *B. napus* were included in a statistical correspondence analysis (CA) (figure 5.3).

At both, the control field (CO-F) as well as at the contaminated field (TE-F), bulk soil and rhizosphere soil bacterial communities were correlated in December and June but their structure differed between seasons. Dominating bacterial genera (> 20 %) present at the control field in winter were *Bacillus, Duganella* and *Mycobacterium*. *Bacillus* remained dominant in summer while *Duganella* and *Mycobacterium* were not detected, instead *Micromonospora* was prominently present in the soil. At the contaminated field in December the soil was dominated by the genera *Bacillus* and *Pseudomonas*. These 2 genera were suppressed by *Arthrobacter*, *Leifsonia* and *Staphylococcus* strains in summer. There indeed exists a rich literature describing seasonal dynamics of many components of the belowground community in a range of natural and farming ecosystems (Wardle 2002; Bardgett, 2005). Similar to our study, Schadt *et al.* (2003) and Tabuchi *et al.* (2008) found that soil microbial communities exhibited shifts in response to seasonal changes. Seasonal alterations in resource availability, which are driven by plants via belowground C allocation, nutrient uptake and litter fall, also influence soil microbial community composition (Lipson and Schmidt, 2004; Koranda *et al*., 2013). Moreover, significant variations depending on soil variables such as texture, pH and organic carbon

were demonstrated by Pastorelli *et al.* (2011). Soil bacterial communities in our study indeed also differed between the 2 test fields during December and June samplings (except for the rhizosphere soil in December). Other studies also reported that soil type influences the composition of bacterial communities (Lundberg *et al.*, 2012; Bulgarelli *et al*., 2012).

Changes in rhizosphere microbial communities in function of seasons were also reported for genetically modified rapeseed (Dunfield and Germida, 2003), maize (Cavaglieri *et al*., 2009), pea, wheat and sugar beet (Houlden *et al*., 2008). The extent of the diversity of microorganisms in soil is considered to be critical to the maintenance of soil health and quality, as a wide range of microorganisms is involved in soil functions (Garbeva *et* al., 2004).

Regarding the endophytic root communities, correlations were observed between seasons and fields (green square in figure 5.3). In December, roots at the control field were mainly colonized by *Bacillus* and *Pseudomonas* strains. *Pseudomonas* was also dominant in June, while *Rhizobium* strains took the place of *Bacillus* strains. At the contaminated field, *Pseudomonas* and *Variovorax* were dominant at both sampling moments.

de Campos *et al.* (2013) also investigated the diversity of root bacterial communities associated with the rosette and flowering phase of rapeseed (*B. napus* L.). Applying new generation sequencing technologies they illustrated how bacterial communities inside the roots changed with the growing stage of the plants. In both root samples the phylum Proteobacteria was dominating; *Pseudomonas* was the most common genus at the rosette stage and *Xanthomonas* at the flowering stage. In contrast to our findings, this study concluded with a switch in predominant bacteria in the different developmental stages of the plant, suggesting that the plant itself interferes with the associated microbial community. Hallmann *et al.* (1997) postulated that the endophytic bacterial community possesses a dynamic structure and is influenced by both biotic and abiotic factors, with the plant itself constituting one of the major influencing factors. Since endophytic bacteria rely on the nutritional supply provided by the plant, any parameter affecting the nutritional status of the plant will consequently affect the endophytic community. Only a few detailed studies considered to test the hypotheses that endophytic microbiota of plants grown

under controlled conditions in natural soils are sufficiently dependent on the host to remain consistent across different soil types and developmental stages. In line with our results, Lundberg *et al.* (2012) found that bacterial communities were strongly influenced by soil type although endophytic communities from different soils feature overlapping, low-complexity communities. These findings illustrate that the plant interior is buffered against various effects and consists of some conserved bacterial lineages.

Schlaeppi *et al*. (2014) investigated the diversity of the bacterial root microbiota in the *Brassicaceae* family and revealed a largely conserved and taxonomically narrow root microbiome, which comprises stable community members belonging to the *Actinomycetales*, *Burkholderiales* and *Flavobacteriales*. Since members of each of these bacterial genera are known to promote plant growth and plant health, Schlaeppi *et al.* hypothesized a standing reservoir of retrievable host services independent of environmental parameters and host species-specific niche adaptations. In our opinion, this reservoir might consist of seed endophytes as the seed is the only 'connection' between 2 plants similar in genotype growing at different soil types.

Permanent and/or overall bacterial colonizers of B. napus

In our study, all *B. napus* plants were at each sampling moment and field colonised by *Bacillus, Pseudomonas* and *Variovorax* (figure 5.2). Isolates of *Agrobacterium, Phyllobacterium, Pseudomonas* and *Variovorax* were previously described as belonging to the most efficient plant growth-promoting bacteria associated with the rhizoplane and endorhizosphere of *B. napus* (Bertrand *et al*., 2001). *Pseudomonas* strains are also important in the rhizosphere of *A. thaliana* and *B. napus* grown on different soils (Achouak *et al*., 2000). Also Granér *et al*. (2003) isolated mainly *Pseudomonas* and *Bacillus* strains from *B. napus* seeds and postulated their beneficial effects on plants.

Our hypothesis is that the bacterial strains that are strongly (permanently and/or overall) associated with *B. napus* are transferred to the next generation via the seeds. Although, from the 3 genera that were associated with rapeseed at the 2 sampling moments on each field, only *Bacillus* was also isolated from the seeds (figure 5.4). The absence of *Pseudomonas* and *Variovorax* species in

our seeds might be due to the dominant growth of *Bacillus* species during the enrichment period (Hahn *et al*., 2004) before plating. Nevertheless, *Pseudomonas* and *Variovorax* strains (as well as *Bacillus* strains) were present in the shoots at both fields, which is an important prerequisite for transfer of those endophytes to the seeds. Moreover, bacterial communities associated with compartments S-CO(J) and S-TE(J) (green arrows in figure 5.5) tend to be more similar to bacterial seed communities.

Conclusion

In general, *Brassica napus*-associated bacterial communities were more diverse and Cd tolerant in June. Field effects, primarily based on elevated trace elements levels (table 5.1), do not have any influence on bacterial diversity indices but affect the presence of Cd tolerant strains.

Genotypically, the bacterial soil community structure differs in function of seasons and fields while bacterial endophyte root communities are highly related between seasons and fields. Furthermore, we have indications that seed endophytic communities remain stable over successive generations, suggesting the presence of some important endophytic strains.

According to our findings, the conclusion made in Croes *et al*. (2013) that genotypic and phenotypic characteristics of rapeseed-associated bacterial populations can be affected by environmental conditions (*e.g.* soil contamination) as well as by their host plant (*i.e.* selection from the rhizosphere/bulk soil and present seed endophytes) has gained evidence and can be expanded, as genotypic and phenotypic characteristics of rapeseed-associated bacterial populations can also be affected by seasonal variations.

This rather fundamental knowledge can be important for future applications regarding phytoremediation. Isolation of strains in June on a trace element-contaminated field provides the highest probability to find *Brassica napus*-associated bacteria with potential to enhance Cd phytoextraction. Indeed, the conditions at the contaminated field seem to promote the occurrence of rapeseed-associated bacteria with potential to enhance Cd phytoextraction. In June, the bacteria are more active and more adapted to the contamination. Further, for inoculation, strains that are present in winter as well as in summer seem more promising to improve plant growth and Cd uptake.

Supporting Information

Appendix 5.1 Detailed characterisation of all purified bulk soil, rhizosphere soil and *B. napus* root isolates collected at the control field (BS-CO, RS-CO and R-CO respectively) and the contaminated field (BS-TE, RS-TE and R-TE respectively) in December (D) and June (J). The presence of each strain is shown as relative abundances, expressed in percentages, of the total number of colony forming units per gram fresh weight (cfu gFW^{-1}) bulk soil (BS), rhizosphere soil (RS) or roots (R). Strains are identified to the genus level, their accession numbers as well as their presence in the 1st, 2nd or 3rd replicate (repl) are shown. Mean percentages were calculated based on the 3 replicates. Tolerance to Cd (0.8 and 1.6 mM) and Zn (0.6, 1.0 and 2.5 mM) is indicated by + when positive and by ++ in case of a strong positive test. Bacterial strains testing negative for tolerance were labeled by a – symbol and those not applicable for the test by 'not detected' (nd).

				BS-CO(D)					
repl	cfu gFW^{-1}	% repl	% mean	identification	accession	Cd (0.8 mM)	Cd (1.6 mM)	Zn (1 mM)	Zn (2.5 mM)
2	380228	11.11	3.70	*Achromobacter*	GQ359326	-	-	-	-
2	380228	11.11	3.70	*Achromobacter*	GQ359326	-	-	-	-
2	380228	11.11	3.70	*Bacillus*	AM934688	-	-	-	-
2	380228	11.11	3.70	*Bacillus*	AM934688	nd	nd	nd	nd
2	380228	11.11	3.70	*Bacillus*	AM934688	nd	nd	nd	nd
1	1141553	100	33.33	*Duganella*	GU332616	-	-	-	-
3	3278689	50	16.67	*Luteibacter*	AJ580498	-	-	-	-
2	380228	11.11	3.70	*Pseudomonas*	EF491969	-	-	-	-
2	380228	11.11	3.70	*Pseudomonas*	EF491969	-	-	-	-
2	380228	11.11	3.70	*Pseudomonas*	EF491969	-	-	-	-
3	3278689	50	16.67	*Sphingopyxis*	AF367204	-	-	-	-
2	380228	11.11	3.70	*Variovorax*	GQ861460	-	-	-	-
				BS-CO(J)					
repl	cfu gFW-1	% repl	% mean	identification	accession	Cd (0.8 mM)	Cd (1.6 mM)	Zn (1 mM)	Zn (2.5 mM)
3	53124	0.35	0.18	*Bacillus*	AB188212	-	-	-	-
3	53124	0.35	0.18	*Bacillus*	AB188212	-	-	-	-
3	53124	0.35	0.18	*Bacillus*	AB188212	-	-	-	-
3	212495	1.4	0.70	*Bacillus*	AB188212	-	-	-	-
3	531237	3.51	1.76	*Bacillus*	AB188212	-	-	+	+
3	531237	3.51	1.76	*Bacillus*	AB188212	-	-	-	-
3	531237	3.51	1.76	*Bacillus*	CP000813	-	-	+	-
3	53124	0.35	0.18	*Bacillus*	FJ263042	-	-	+	+
3	53124	0.35	0.18	*Bacillus*	FJ263042	-	-	+	+
3	53124	0.35	0.18	*Bacillus*	FJ263042	-	-	+	+
3	371866	2.45	1.23	*Bacillus*	FJ263042	-	-	+	+
3	531237	3.51	1.76	*Bacillus*	FJ263042	-	-	-	-
3	531237	3.51	1.76	*Bacillus*	FJ263042	-	-	+	+
3	607128	4.01	2.01	*Bacillus*	FJ263042	-	-	-	-
3	607128	4.01	2.01	*Bacillus*	FJ263042	-	-	-	-
3	1062473	7.01	3.51	*Bacillus*	FJ263042	-	-	-	-
3	5312367	35.07	17.54	*Bacillus*	FJ263042	-	-	-	-

3	53124	0.35	0.18	*Bacillus*	GU321095	-	-	-	-
3	531237	3.51	1.76	*Bacillus*	GU321095	-	-	-	-
3	531237	3.51	1.76	*Burkholderia*	FJ786047	-	-	-	-
3	531237	3.51	1.76	*Leifsonia*	AB278552	-	-	+	+
3	607128	4.01	2.01	*Leifsonia*	AB278552	-	-	-	-
3	607128	4.01	2.01	*Leifsonia*	AB278552	-	-	-	-
1	4783773	100	50.00	*Micromonospora*	EU841636	-	-	-	-
3	607128	4.01	2.01	*Pantoea*	AF130887	-	-	-	-
3	531237	3.51	1.76	*Streptomyces*	EU119184	++	+	++	+

RS-CO(D)

repl	cfu gFW-1	% repl	% mean	identification	accession	Cd (0.8 mM)	Cd (1.6 mM)	Zn (1 mM)	Zn (2.5 mM)
1	6257822	0.68	0.34	*Agromyces*	EF363711	-	-	-	-
1	62578223	6.8	3.40	*Alcaligenes*	AJ509012	-	-	-	-
1	62578223	6.8	3.40	*Alcaligenes*	AJ509012	nd	nd	nd	nd
1	62578223	6.8	3.40	*Alcaligenes*	AJ509012	-	-	-	-
1	6257822	0.68	0.34	*Bacillus*	AM934688	-	-	-	-
2	7012623	11.36	5.68	*Bacillus*	AM934688	-	-	-	-
2	7012623	11.36	5.68	*Bacillus*	AM934688	-	-	-	-
2	7012623	11.36	5.68	*Bacillus*	AM934688	-	-	-	-
2	7012623	11.36	5.68	*Bacillus*	AM934688	-	-	-	-
2	11220196	18.18	9.09	*Duganella*	GU332616	-	-	-	-
2	11220196	18.18	9.09	*Duganella*	GU332616	-	-	-	-
2	11220196	18.18	9.09	*Duganella*	GU332616	-	-	-	-
1	6257822	0.68	0.34	*Micromonospora*	AY221494	-	-	-	-
1	625782228	68.03	34.02	*Mycobacterium*	AF055332	-	-	-	-
1	6257822	0.68	0.34	*Polaromonas*	AB245355	-	-	-	-
1	6257822	0.68	0.34	*Polaromonas*	AB245355	nd	nd	nd	nd
1	6257822	0.68	0.34	*Polaromonas*	AB245355	-	-	-	-
1	62578223	6.8	3.40	*Pseudomonas*	EF491969	nd	nd	nd	nd
1	6257822	0.68	0.34	*Variovorax*	AB245358	-	-	-	-

RS-CO(J)

repl	cfu gFW-1	% repl	% mean	identification	accession	Cd (0.8 mM)	Cd (1.6 mM)	Zn (1 mM)	Zn (2.5 mM)
1	10599378	5.9	1.97	*Aeromicrobium*	AB245394	-	-	-	-
2	2472371	0.77	0.26	*Agrobacterium*	GQ428123	-	-	-	-
2	2472371	0.77	0.26	*Agrobacterium*	GQ428123	-	-	-	-
2	5439217	1.7	0.57	*Agrobacterium*	GQ428123	-	-	-	-
1	6838308	3.81	1.27	*Agrobacterium*	GQ428123	-	-	-	-
2	2472371	0.77	0.26	*Arthrobacter*	AB288059	-	-	-	-
2	2472371	0.77	0.26	*Arthrobacter*	AB288059	-	-	-	-
2	4944743	1.54	0.51	*Arthrobacter*	AB288059	-	-	-	-
2	4944743	1.54	0.51	*Arthrobacter*	AB288059	-	-	-	-
3	50842718	0.77	0.26	*Arthrobacter*	EF028242	-	-	-	-
3	508427	0.01	0.003	*Bacillus*	AB188212	-	-	-	-
3	508427	0.01	0.003	*Bacillus*	AB188212	-	-	-	-
3	508427	0.01	0.003	*Bacillus*	AB188212	-	-	-	-
2	2472371	0.77	0.26	*Bacillus*	AB188212	-	-	-	-
1	10257462	5.71	1.90	*Bacillus*	AB188212	-	-	-	-
2	24723713	7.72	2.57	*Bacillus*	AB188212	-	-	-	-
2	5439217	1.7	0.57	*Bacillus*	AB301017	-	-	-	-

2	2472371	0.77	0.26	*Bacillus*	AJ628743	-	-	-	-
2	8900537	2.78	0.93	*Bacillus*	DQ445268	-	-	-	-
3	508427	0.01	0.003	*Bacillus*	FJ263042	-	-	-	-
3	50842718	0.77	0.26	*Bacillus*	FJ263042	-	-	-	-
2	2472371	0.77	0.26	*Bacillus*	GQ200827	-	-	-	-
2	2472371	0.77	0.26	*Bacillus*	GQ200827	-	-	-	-
3	50842718	0.77	0.26	*Bacillus*	GQ200827	-	-	-	-
2	7417114	2.32	0.77	*Bacillus*	GQ200827	-	-	-	-
2	24723713	7.72	2.57	*Bacillus*	GQ200827	-	-	-	-
3	6406182475	96.87	32.29	*Bacillus*	GQ200827	-	-	-	-
3	508427	0.01	0.003	*Bacillus*	GU321095	-	-	-	-
1	341915	0.19	0.06	*Bacillus*	GU321095	-	-	-	-
2	4944743	1.54	0.51	*Caulobacter*	DQ337549	-	-	-	-
2	2472371	0.77	0.26	*Janthinobacterium*	D84576	+	+	++	++
2	4944743	1.54	0.51	*Kribbella*	AY253865	-	-	-	-
2	2966846	0.93	0.31	*Labrys*	DQ337554	-	-	-	-
1	683831	0.38	0.13	*Leifsonia*	AB278552	-	-	-	-
2	4944743	1.54	0.51	*Leifsonia*	AB278552	-	-	-	-
2	4944743	1.54	0.51	*Leifsonia*	AB278552	-	-	-	-
1	3419154	1.9	0.63	*Leifsonia*	AB278552	-	-	-	-
1	6838308	3.81	1.27	*Leifsonia*	AB278552	-	-	+	-
1	10599378	5.9	1.97	*Lysinibacillus*	AY907676	-	-	-	-
2	2472371	0.77	0.26	*Lysinibacillus*	DQ333300	-	-	-	-
2	2472371	0.77	0.26	*Pseudomonas*	AB369347	-	-	+	+
2	2472371	0.77	0.26	*Pseudomonas*	AB369347	-	-	-	-
2	8653299	2.7	0.90	*Pseudomonas*	AB369347	+	+	-	-
2	12361856	3.86	1.29	*Pseudomonas*	AB369347	+	+	-	-
1	13676616	7.62	2.54	*Pseudomonas*	AB369347	-	-	-	-
2	5439217	1.7	0.57	*Pseudomonas*	AM934699	+	-	-	-
2	5439217	1.7	0.57	*Pseudomonas*	AM934699	+	-	-	-
2	5439217	1.7	0.57	*Pseudomonas*	AM934699	+	-	-	-
2	8653299	2.7	0.90	*Pseudomonas*	AM934699	+	-	-	-
2	8653299	2.7	0.90	*Pseudomonas*	AM934699	-	-	-	-
1	6838308	3.81	1.27	*Pseudomonas*	AM934699	-	-	-	-
2	24723713	7.72	2.57	*Pseudomonas*	AM934699	+	-	-	-
2	59336910	18.53	6.18	*Pseudomonas*	AM934699	-	-	-	-
2	2966846	0.93	0.31	*Pseudomonas*	FJ772042	-	-	-	-
3	508427	0.01	0.003	*Pseudomonas*	FM202488	+	+	-	-
2	2472371	0.77	0.26	*Pseudomonas*	FM202488	++	++	-	-
2	4944743	1.54	0.51	*Rhodococcus*	DQ060386	-	-	-	-
3	508427	0.01	0.003	*Rhodococcus*	EU496547	-	-	-	-
1	3419154	1.9	0.63	*Rhodococcus*	EU496547	-	-	+	+
2	2472371	0.77	0.26	*Sphingobacterium*	AJ438176	-	-	-	-
2	2472371	0.77	0.26	*Staphylococcus*	GQ222398	-	-	-	-
2	4944743	1.54	0.51	*Staphylococcus*	GQ222398	-	-	-	-
2	4944743	1.54	0.51	*Staphylococcus*	GQ222398	-	-	-	-
1	3419154	1.9	0.63	*Staphylococcus*	GQ222398	-	-	-	-
1	34191541	19.05	6.35	*Staphylococcus*	GQ222398	-	-	-	-
3	50842718	0.77	0.26	*Streptomyces*	AY314782	-	-	-	-

repl	cfu gFW-1	% repl	% mean	identification	accession	Cd (0.8 mM)	Cd (1.6 mM)	Zn (1 mM)	Zn (2.5 mM)
1	34191541	19.05	6.35	*Variovorax*	EF419341	+	-	-	-
1	34191541	19.05	6.35	*Variovorax*	EF419341	-	-	-	-
2	2472371	0.77	0.26	*Variovorax*	FJ772012	+	+	-	-
2	2472371	0.77	0.26	*Variovorax*	FJ772012	-	-	-	-
2	4944743	1.54	0.51	*Variovorax*	FJ772012	-	-	-	-
2	7417114	2.32	0.77	*Zooglea*	X74914	-	-	-	-
					R-CO(D)				
repl	cfu gFW-1	% repl	% mean	identification	accession	Cd (0.8 mM)	Cd (1.6 mM)	Zn (1 mM)	Zn (2.5 mM)
3	52695	0.67	0.22	*Achromobacter*	GQ359326	-	-	-	-
3	52695	0.67	0.22	*Agromyces*	EF363711	-	-	++	-
1	6078	0.48	0.16	*Duganella*	GU332616	-	-	-	-
2	84667	3.37	1.12	*Duganella*	GU332616	nd	nd	nd	nd
1	60783	4.81	1.60	*Duganella*	GU332616	+	-	-	-
3	73774	0.93	0.31	*Flavobacterium*	DQ778318	-	-	-	-
3	73774	0.93	0.31	*Flavobacterium*	DQ778318	-	-	-	-
3	73774	0.93	0.31	*Flavobacterium*	DQ778318	-	-	-	-
3	73774	0.93	0.31	*Flavobacterium*	DQ778318	+	-	+	-
3	73774	0.93	0.31	*Flavobacterium*	DQ778318	++	++	-	-
2	8467	0.34	0.11	*Luteibacter*	AJ580498	nd	nd	nd	nd
2	8467	0.34	0.11	*Luteibacter*	AJ580498	nd	nd	nd	nd
1	6078	0.48	0.16	*Luteibacter*	AJ580498	-	-	-	-
1	6078	0.48	0.16	*Luteibacter*	AJ580498	-	-	-	-
1	6078	0.48	0.16	*Luteibacter*	AJ580498	-	-	-	-
1	6078	0.48	0.16	*Luteibacter*	AJ580498	-	-	-	-
1	6078	0.48	0.16	*Luteibacter*	AJ580498	-	-	-	-
1	6078	0.48	0.16	*Luteibacter*	AJ580498	nd	nd	nd	nd
1	6078	0.48	0.16	*Luteibacter*	AJ580498	nd	nd	nd	nd
1	6078	0.48	0.16	*Luteibacter*	AJ580498	nd	nd	nd	nd
1	6078	0.48	0.16	*Luteibacter*	AJ580498	nd	nd	nd	nd
1	6078	0.48	0.16	*Luteibacter*	AJ580498	-	-	-	-
1	6078	0.48	0.16	*Luteibacter*	AJ580498	-	-	+	-
1	6078	0.48	0.16	*Luteibacter*	AJ580498	-	-	+	-
1	6078	0.48	0.16	*Luteibacter*	AJ580498	-	-	+	-
1	6078	0.48	0.16	*Luteibacter*	AJ580498	+	+	-	-
1	10941	0.87	0.29	*Luteibacter*	AJ580498	-	-	-	-
1	10941	0.87	0.29	*Luteibacter*	AJ580498	-	-	-	-
1	10941	0.87	0.29	*Luteibacter*	AJ580498	nd	nd	nd	nd
1	10941	0.87	0.29	*Luteibacter*	AJ580498	-	-	-	-
1	10941	0.87	0.29	*Luteibacter*	AJ580498	nd	nd	nd	nd
3	189703	2.4	0.80	*Luteibacter*	AJ580498	-	-	-	-
3	189703	2.4	0.80	*Luteibacter*	AJ580498	-	-	-	-
3	189703	2.4	0.80	*Luteibacter*	AJ580498	-	-	-	-
3	189703	2.4	0.80	*Luteibacter*	AJ580498	-	-	-	-
3	189703	2.4	0.80	*Luteibacter*	AJ580498	+	-	-	-
2	84667	3.37	1.12	*Luteibacter*	AJ580498	-	-	-	-
3	468472	5.92	1.97	*Luteibacter*	AJ580498	nd	nd	nd	nd
3	658692	8.33	2.78	*Luteibacter*	AJ580498	-	-	-	-
3	658692	8.33	2.78	*Luteibacter*	AJ580498	-	-	-	-
1	60783	4.81	1.60	*Methylobacterium*	HQ005421	nd	nd	nd	nd

3	52695	0.67	0.22	*Pantoea*	DQ531643	-	-	-	-
3	52695	0.67	0.22	*Pantoea*	DQ531643	-	-	-	-
1	60783	4.81	1.60	*Pantoea*	DQ531643	-	-	-	-
3	52695	0.67	0.22	*Polaromonas*	AB245355	-	-	-	-
3	468472	5.92	1.97	*Polaromonas*	AB245355	-	-	-	-
3	468472	5.92	1.97	*Polaromonas*	AB245355	-	-	-	-
1	6078	0.48	0.16	*Pseudomonas*	AJ417068	-	-	-	-
1	6078	0.48	0.16	*Pseudomonas*	AJ417068	-	-	-	-
2	8467	0.34	0.11	*Pseudomonas*	EF491969	-	-	++	-
2	8467	0.34	0.11	*Pseudomonas*	EF491969	+	-	-	-
1	6078	0.48	0.16	*Pseudomonas*	EF491969	-	-	++	-
2	13547	0.54	0.18	*Pseudomonas*	EF491969	-	-	-	-
2	13547	0.54	0.18	*Pseudomonas*	EF491969	-	-	-	-
2	13547	0.54	0.18	*Pseudomonas*	EF491969	-	-	-	-
2	13547	0.54	0.18	*Pseudomonas*	EF491969	-	-	-	-
2	13547	0.54	0.18	*Pseudomonas*	EF491969	-	-	-	-
2	35560	1.41	0.47	*Pseudomonas*	EF491969	-	-	-	-
2	35560	1.41	0.47	*Pseudomonas*	EF491969	-	-	-	-
2	35560	1.41	0.47	*Pseudomonas*	EF491969	-	-	-	-
2	35560	1.41	0.47	*Pseudomonas*	EF491969	-	-	-	-
2	35560	1.41	0.47	*Pseudomonas*	EF491969	-	-	+	-
3	242399	3.06	1.02	*Pseudomonas*	EF491969	+	+	-	-
3	242399	3.06	1.02	*Pseudomonas*	EF491969	+	-	-	-
3	242399	3.06	1.02	*Pseudomonas*	EF491969	+	-	-	-
3	242399	3.06	1.02	*Pseudomonas*	EF491969	+	-	-	-
3	242399	3.06	1.02	*Pseudomonas*	EF491969	+	-	-	-
2	101600	4.04	1.35	*Pseudomonas*	EF491969	-	-	-	-
2	101600	4.04	1.35	*Pseudomonas*	EF491969	-	-	-	-
2	101600	4.04	1.35	*Pseudomonas*	EF491969	-	-	-	-
2	101600	4.04	1.35	*Pseudomonas*	EF491969	-	-	-	-
2	101600	4.04	1.35	*Pseudomonas*	EF491969	-	-	++	-
2	103294	4.11	1.37	*Pseudomonas*	EF491969	-	-	-	-
2	103294	4.11	1.37	*Pseudomonas*	EF491969	-	-	-	-
2	103294	4.11	1.37	*Pseudomonas*	EF491969	-	-	-	-
2	103294	4.11	1.37	*Pseudomonas*	EF491969	-	-	-	-
2	103294	4.11	1.37	*Pseudomonas*	EF491969	-	-	++	-
1	60783	4.81	1.60	*Pseudomonas*	EF491969	-	-	-	-
3	468472	5.92	1.97	*Pseudomonas*	EF491969	-	-	-	-
2	846668	33.67	11.22	*Pseudomonas*	EF491969	nd	nd	nd	nd
2	8467	0.34	0.11	*Pseudomonas*	EU853180	-	-	-	-
2	8467	0.34	0.11	*Pseudomonas*	EU853180	+	-	-	-
1	60783	4.81	1.60	*Rhizobium*	FJ405385	+	-	-	-
2	11853	0.47	0.16	*Stenotrophomonas*	GU186115	nd	nd	nd	nd
2	11853	0.47	0.16	*Stenotrophomonas*	GU186115	-	-	-	-
2	11853	0.47	0.16	*Stenotrophomonas*	GU186115	-	-	-	-
2	11853	0.47	0.16	*Stenotrophomonas*	GU186115	-	-	-	-
2	11853	0.47	0.16	*Stenotrophomonas*	GU186115	-	-	-	-
2	23707	0.94	0.31	*Stenotrophomonas*	GU186115	-	-	-	-
2	23707	0.94	0.31	*Stenotrophomonas*	GU186115	nd	nd	nd	nd

2	23707	0.94	0.31	*Stenotrophomonas*	GU186115	-	-	++	-
2	23707	0.94	0.31	*Stenotrophomonas*	GU186115	-	-	++	-
2	23707	0.94	0.31	*Stenotrophomonas*	GU186115	+	-	++	-
1	607829	48.08	16.03	*Variovorax*	GQ861460	nd	nd	nd	nd
3	52695	0.67	0.22	*Variovorax*	GQ861460	nd	nd	nd	nd
3	468472	5.92	1.97	*Variovorax*	GQ861460	-	-	-	-
3	468472	5.92	1.97	*Variovorax*	GQ861460	-	-	-	-
3	468472	5.92	1.97	*Variovorax*	GQ861460	-	-	-	-
3	468472	5.92	1.97	*Variovorax*	GQ861460	-	-	-	-
1	6078	0.48	0.16	*Variovorax*	HQ005421	-	-	-	-
1	60783	4.81	1.60	*Variovorax*	HQ005421	-	-	-	-
1	60783	4.81	1.60	*Variovorax*	HQ005421	-	-	-	-
1	60783	4.81	1.60	*Variovorax*	HQ005421	-	-	-	-

					R-CO(J)				
repl	cfu gFW-1	% repl	% mean	identification	accession	Cd (0.8 mM)	Cd (1.6 mM)	Zn (1 mM)	Zn (2.5 mM)
1	3258	0.31	0.10	*Bacillus*	AB188212	-	-	-	-
3	3303	0.53	0.18	*Bacillus*	AB188212	-	-	-	-
3	33033	5.26	1.75	*Bacillus*	AB188212	-	-	-	-
1	3258	0.31	0.10	*Bacillus*	AJ542508	+	+	-	-
1	3258	0.31	0.10	*Bacillus*	CP000813	-	-	-	-
3	3303	0.53	0.18	*Bacillus*	CP000813	-	-	-	-
1	14480	1.36	0.45	*Bacillus*	CP000813	-	-	+	-
1	32580	3.06	1.02	*Bacillus*	CP000813	-	-	-	-
2	94153	6.55	2.18	*Bacillus*	CP000813	-	-	+	+
1	97739	9.18	3.06	*Bacillus*	CP000813	-	-	+	+
1	3258	0.31	0.10	*Bacillus*	FJ263042	-	-	-	-
1	32580	3.06	1.02	*Bacillus*	FJ263042	-	-	-	-
1	32580	3.06	1.02	*Bacillus*	FJ263042	-	-	-	-
2	47077	3.27	1.09	*Bacillus*	FJ263042	-	-	-	-
3	3303	0.53	0.18	*Brevibacillus*	FJ197026	-	-	-	-
3	3303	0.53	0.18	*Brevibacillus*	FJ197026	-	-	-	-
1	3258	0.31	0.10	*Caulobacter*	DQ337549	-	-	-	-
1	3258	0.31	0.10	*Caulobacter*	DQ337549	-	-	-	-
1	3258	0.31	0.10	*Caulobacter*	DQ337549	-	-	+	+
1	3258	0.31	0.10	*Caulobacter*	DQ337549	-	-	-	-
2	4708	0.33	0.11	*Caulobacter*	DQ337549	-	-	-	-
3	3303	0.53	0.18	*Caulobacter*	DQ337549	-	-	-	-
3	3303	0.53	0.18	*Caulobacter*	DQ337549	-	-	-	-
3	3303	0.53	0.18	*Caulobacter*	DQ337549	-	-	-	-
1	6516	0.61	0.20	*Caulobacter*	DQ337549	-	-	-	-
1	6516	0.61	0.20	*Caulobacter*	DQ337549	-	-	-	-
1	7819	0.73	0.24	*Caulobacter*	DQ337549	-	-	-	-
1	7819	0.73	0.24	*Caulobacter*	DQ337549	-	-	-	-
1	7819	0.73	0.24	*Caulobacter*	DQ337549	-	-	-	-
1	7819	0.73	0.24	*Caulobacter*	DQ337549	-	-	-	-
3	6276	1	0.33	*Caulobacter*	DQ337549	-	-	-	-
3	6276	1	0.33	*Caulobacter*	DQ337549	-	-	-	-
3	6276	1	0.33	*Caulobacter*	DQ337549	-	-	-	-
3	6276	1	0.33	*Caulobacter*	DQ337549	-	-	-	-

3	6276	1	0.33	*Caulobacter*	DQ337549	-	-	-	-
3	6276	1	0.33	*Caulobacter*	DQ337549	-	-	-	-
3	6276	1	0.33	*Caulobacter*	DQ337549	-	-	-	-
1	32580	3.06	1.02	*Caulobacter*	DQ337549	-	-	-	-
1	32580	3.06	1.02	*Caulobacter*	DQ337549	-	-	-	-
3	33033	5.26	1.75	*Caulobacter*	DQ337549	-	-	-	-
2	4708	0.33	0.11	*Labrys*	DQ337554	-	-	-	-
1	3910	0.37	0.12	*Labrys*	DQ337554	-	-	-	-
2	18831	1.31	0.44	*Labrys*	DQ337554	-	-	-	-
3	13213	2.1	0.70	*Labrys*	DQ337554	-	-	-	-
1	32580	3.06	1.02	*Labrys*	DQ337554	-	-	-	-
2	7061	0.49	0.16	*Microbacterium*	DQ328319	-	-	-	-
1	32580	3.06	1.02	*Mycobacterium*	FJ719354	-	-	-	-
2	4708	0.33	0.11	*Paenibacillus*	EU723825	-	-	-	-
2	7061	0.49	0.16	*Pantoea*	EU598802	++	-	-	-
3	3303	0.53	0.18	*Pantoea*	EU598802	+	-	-	-
1	26064	2.45	0.82	*Pantoea*	EU598802	-	-	-	-
3	101080	16.1	5.37	*Pantoea*	EU598802	-	-	-	-
2	47077	3.27	1.09	*Pedobacter*	GU385862	-	-	-	-
2	7061	0.49	0.16	*Plantibacter*	AM396918	-	-	-	-
2	7061	0.49	0.16	*Plantibacter*	AM396918	-	-	-	-
1	5701	0.54	0.18	*Pseudomonas*	AB330408	-	-	-	-
3	33033	5.26	1.75	*Pseudomonas*	AB330408	-	-	-	-
2	6277	0.44	0.15	*Pseudomonas*	AB369347	++	-	-	-
3	9910	1.58	0.53	*Pseudomonas*	AB369347	-	-	-	-
3	11231	1.79	0.60	*Pseudomonas*	AB369347	-	-	-	-
1	19548	1.84	0.61	*Pseudomonas*	AB369347	+	-	-	-
1	19548	1.84	0.61	*Pseudomonas*	AB369347	++	-	-	-
3	31381	5	1.67	*Pseudomonas*	AB369347	+	-	-	-
3	31381	5	1.67	*Pseudomonas*	AB369347	++	-	-	-
2	211844	14.73	4.91	*Pseudomonas*	AB369347	+	-	-	-
2	211844	14.73	4.91	*Pseudomonas*	AB369347	++	-	-	-
3	3303	0.53	0.18	*Pseudomonas*	AY047218	+	-	-	-
2	4708	0.33	0.11	*Pseudomonas*	DQ095904	-	-	-	-
2	4708	0.33	0.11	*Pseudomonas*	DQ095904	++	-	-	-
2	4708	0.33	0.11	*Pseudomonas*	DQ095904	++	-	+	+
2	7061	0.49	0.16	*Pseudomonas*	DQ095904	-	-	+	+
2	7061	0.49	0.16	*Pseudomonas*	DQ095904	++	-	++	++
1	3258	0.31	0.10	*Pseudomonas*	DQ778036	++	+	-	-
1	3910	0.37	0.12	*Pseudomonas*	DQ778036	++	-	-	-
1	6516	0.61	0.20	*Pseudomonas*	DQ778036	+	+	-	-
1	6516	0.61	0.20	*Pseudomonas*	DQ778036	++	+	-	-
3	33033	5.26	1.75	*Pseudomonas*	DQ778036	-	-	-	-
3	99098	15.78	5.26	*Pseudomonas*	DQ778036	-	-	-	-
3	13213	2.1	0.70	*Pseudomonas*	FJ772042	-	-	-	-
2	47077	3.27	1.09	*Pseudomonas*	FJ772042	-	-	-	-
1	3258	0.31	0.10	*Pseudomonas*	FN377713	++	-	-	-
1	3258	0.31	0.10	*Pseudomonas*	FN377713	++	-	+	+
1	3258	0.31	0.10	*Pseudomonas*	FN377713	-	-	-	-

2	4708	0.33	0.11	*Pseudomonas*	FN377713	++	+	-	-
3	3303	0.53	0.18	*Pseudomonas*	FN377713	-	-	-	-
1	5701	0.54	0.18	*Pseudomonas*	FN377713	-	-	-	-
3	6276	1	0.33	*Pseudomonas*	FN377713	++	-	-	-
1	14480	1.36	0.45	*Pseudomonas*	FN377713	-	-	+	+
1	14480	1.36	0.45	*Rhizobium*	DQ337581	-	-	-	-
1	32580	3.06	1.02	*Rhizobium*	DQ337581	++	+	++	+
3	13213	2.1	0.70	*Stenotrophomonas*	FJ772015	-	-	-	-
1	14480	1.36	0.45	unc. bact.	DQ787731	-	-	-	-
1	3910	0.37	0.12	unc. bact.	GQ025779	+	-	-	-
1	14480	1.36	0.45	unc. bact.	GQ025779	+	-	+	+
1	3910	0.37	0.12	*Variovorax*	EF419341	++	-	++	++
1	14480	1.36	0.45	*Variovorax*	FJ772012	+	+	++	++
2	263629	18.33	6.11	*Variovorax*	FJ772012	-	-	-	-
1	3258	0.31	0.10	*Variovorax*	GQ861460	++	-	++	++
1	3258	0.31	0.10	*Variovorax*	GQ861460	+	-	++	++
1	3258	0.31	0.10	*Variovorax*	GQ861460	-	-	-	-
1	3258	0.31	0.10	*Variovorax*	GQ861460	-	-	-	-
1	3258	0.31	0.10	*Variovorax*	GQ861460	-	-	-	-
1	3258	0.31	0.10	*Variovorax*	GQ861460	+	-	+	+
2	4708	0.33	0.11	*Variovorax*	GQ861460	+	-	+	+
2	4708	0.33	0.11	*Variovorax*	GQ861460	-	-	-	-
2	6277	0.44	0.15	*Variovorax*	GQ861460	-	-	-	-
2	6277	0.44	0.15	*Variovorax*	GQ861460	-	-	-	-
1	5213	0.49	0.16	*Variovorax*	GQ861460	-	-	-	-
1	5213	0.49	0.16	*Variovorax*	GQ861460	-	-	-	-
2	7061	0.49	0.16	*Variovorax*	GQ861460	-	-	-	-
3	3303	0.53	0.18	*Variovorax*	GQ861460	-	-	-	-
3	3303	0.53	0.18	*Variovorax*	GQ861460	-	-	-	-
3	3303	0.53	0.18	*Variovorax*	GQ861460	-	-	-	-
1	5701	0.54	0.18	*Variovorax*	GQ861460	-	-	-	-
1	5701	0.54	0.18	*Variovorax*	GQ861460	-	-	-	-
1	6516	0.61	0.20	*Variovorax*	GQ861460	-	-	++	++
1	6516	0.61	0.20	*Variovorax*	GQ861460	-	-	-	-
1	6516	0.61	0.20	*Variovorax*	GQ861460	-	-	-	-
1	6516	0.61	0.20	*Variovorax*	GQ861460	-	-	-	-
1	6516	0.61	0.20	*Variovorax*	GQ861460	-	-	-	-
3	3964	0.63	0.21	*Variovorax*	GQ861460	-	-	-	-
3	3964	0.63	0.21	*Variovorax*	GQ861460	-	-	-	-
3	6276	1	0.33	*Variovorax*	GQ861460	+	-	+	+
3	7928	1.26	0.42	*Variovorax*	GQ861460	-	-	+	-
1	14480	1.36	0.45	*Variovorax*	GQ861460	-	-	-	-
1	14480	1.36	0.45	*Variovorax*	GQ861460	-	-	-	-
1	15638	1.47	0.49	*Variovorax*	GQ861460	-	-	-	-
3	13213	2.1	0.70	*Variovorax*	GQ861460	-	-	-	-
3	13213	2.1	0.70	*Variovorax*	GQ861460	-	-	-	-
1	26064	2.45	0.82	*Variovorax*	GQ861460	-	-	-	-
1	26064	2.45	0.82	*Variovorax*	GQ861460	-	-	-	-
1	32580	3.06	1.02	*Variovorax*	GQ861460	++	-	+	+

1	32580	3.06	1.02	*Variovorax*	GQ861460	++	-	-	-
1	32580	3.06	1.02	*Variovorax*	GQ861460	-	-	-	-
1	32580	3.06	1.02	*Variovorax*	GQ861460	-	-	-	-
1	32580	3.06	1.02	*Variovorax*	GQ861460	-	-	-	-
1	32580	3.06	1.02	*Variovorax*	GQ861460	-	-	-	-
1	32580	3.06	1.02	*Variovorax*	GQ861460	-	-	-	-
1	32580	3.06	1.02	*Variovorax*	GQ861460	-	-	-	-
2	47077	3.27	1.09	*Variovorax*	GQ861460	-	-	-	-
2	65907	4.58	1.53	*Variovorax*	GQ861460	-	-	-	-
3	30390	4.84	1.61	*Variovorax*	GQ861460	-	-	-	-
2	273044	18.99	6.33	*Variovorax*	GQ861460	+	-	+	+

BS-TE(D)

repl	cfu gFW-1	% repl	% mean	identification	accession	Cd (0.8 mM)	Cd (1.6 mM)	Zn (1 mM)	Zn (2.5 mM)
1	24452	3.18	1.06	*Arthrobacter*	D84573	-	-	-	-
1	24452	3.18	1.06	*Arthrobacter*	D84573	-	-	-	-
1	24452	3.18	1.06	*Arthrobacter*	D84573	-	-	-	-
1	24452	3.18	1.06	*Arthrobacter*	D84573	-	-	-	-
1	63576	8.28	2.76	*Arthrobacter*	D84573	-	-	-	-
1	63576	8.28	2.76	*Arthrobacter*	D84573	-	-	-	-
1	63576	8.28	2.76	*Arthrobacter*	D84573	-	-	-	-
1	63576	8.28	2.76	*Arthrobacter*	D84573	+	+	-	-
2	49759	0.23	0.08	*Arthrobacter*	DQ985470	-	-	-	-
2	49759	0.23	0.08	*Arthrobacter*	DQ985470	-	-	-	-
2	49759	0.23	0.08	*Arthrobacter*	DQ985470	-	-	-	-
2	49759	0.23	0.08	*Arthrobacter*	DQ985470	-	-	+	-
3	523903	0.17	0.06	*Arthrobacter*	FN673551	-	-	-	-
3	523903	0.17	0.06	*Arthrobacter*	FN673551	-	-	++	-
1	24452	3.18	1.06	*Bacillus*	AM934688	-	-	-	-
1	24452	3.18	1.06	*Bacillus*	AM934688	-	-	-	-
1	24452	3.18	1.06	*Bacillus*	AM934688	-	-	-	-
2	4975867	23.18	7.73	*Bacillus*	AM934688	-	-	-	-
2	4975867	23.18	7.73	*Bacillus*	AM934688	-	-	-	-
2	4975867	23.18	7.73	*Bacillus*	AM934688	-	-	-	-
2	4975867	23.18	7.73	*Bacillus*	AM934688	-	-	++	-
3	32743942	10.92	3.64	*Bacillus*	FJ859701	-	-	-	-
3	32743942	10.92	3.64	*Bacillus*	FJ859702	-	-	-	-
3	32743942	10.92	3.64	*Bacillus*	FJ859703	-	-	-	-
1	24452	3.18	1.06	*Chitinophaga*	CP001699	-	-	-	-
1	24452	3.18	1.06	*Chitinophaga*	CP001699	nd	nd	nd	nd
1	24452	3.18	1.06	*Chitinophaga*	CP001699	-	-	++	-
3	104781	0.03	0.01	*Duganella*	GU332616	-	-	-	-
3	104781	0.03	0.01	*Duganella*	GU332616	-	-	-	-
3	104781	0.03	0.01	*Duganella*	GU332616	+	-	-	-
3	104781	0.03	0.01	*Duganella*	GU332616	++	++	++	-
3	137525	0.05	0.02	*Paenibacillus*	EU179327	-	-	-	-
3	137525	0.05	0.02	*Paenibacillus*	EU179327	-	-	-	-
3	137525	0.05	0.02	*Paenibacillus*	EU179327	-	-	-	-
3	32743942	10.92	3.64	*Pantoea*	AF130887	-	-	-	-
3	32743942	10.92	3.64	*Pantoea*	AF130887	-	-	-	-

3	32743942	10.92	3.64	*Pantoea*	AF130887	-	-	-	-
3	32743942	10.92	3.64	*Pantoea*	AF130887	++	-	++	-
3	32744	0.01	0.003	*Pseudomonas*	AF456229	++	-	-	-
2	49759	0.23	0.08	*Pseudomonas*	AJ581999	nd	nd	nd	nd
2	49759	0.23	0.08	*Pseudomonas*	AJ581999	-	-	-	-
2	49759	0.23	0.08	*Pseudomonas*	AJ581999	-	-	-	-
2	49759	0.23	0.08	*Pseudomonas*	AJ581999	nd	nd	nd	nd
2	49759	0.23	0.08	*Pseudomonas*	AJ581999	-	-	-	-
2	49759	0.23	0.08	*Pseudomonas*	AJ581999	nd	nd	nd	nd
2	49759	0.23	0.08	*Pseudomonas*	AJ581999	nd	nd	nd	nd
2	49759	0.23	0.08	*Pseudomonas*	AJ581999	nd	nd	nd	nd
2	49759	0.23	0.08	*Pseudomonas*	AJ581999	-	-	+	-
2	49759	0.23	0.08	*Pseudomonas*	AJ581999	-	-	+	-
2	79614	0.37	0.12	*Pseudomonas*	AJ581999	-	-	-	-
2	79614	0.37	0.12	*Pseudomonas*	AJ581999	nd	nd	nd	nd
2	79614	0.37	0.12	*Pseudomonas*	AJ581999	-	-	-	-
2	79614	0.37	0.12	*Pseudomonas*	AJ581999	-	-	-	-
2	497587	2.32	0.77	*Pseudomonas*	AJ581999	nd	nd	nd	nd
1	244523	31.85	10.62	*Rhizobium*	FJ405385	-	-	+	+
2	49759	0.23	0.08	*Streptomyces*	EU144078	-	-	-	-
3	3274394	1.09	0.36	*Variovorax*	AB245358	-	-	-	-
3	32743942	10.92	3.64	*Variovorax*	AB245358	-	-	-	-
3	32743942	10.92	3.64	*Variovorax*	AB245358	-	-	+	-
1	24452	3.18	1.06	*Variovorax*	GQ861460	-	-	-	-
					BS-TE(J)				
repl	cfu gFW-1	% repl	% mean	identification	accession	Cd (0.8 mM)	Cd (1.6 mM)	Zn (1 mM)	Zn (2.5 mM)
3	43463	0.27	0.09	*Agrobacterium*	GQ428123	++	-	+	-
1	377287	1.6	0.53	*Agrobacterium*	GQ428123	-	-	-	-
3	434631	2.7	0.90	*Agrobacterium*	GQ428123	-	-	-	-
3	760605	4.72	1.57	*Agrobacterium*	GQ428123	-	-	-	-
2	50792	0.23	0.08	*Arthrobacter*	AB288059	+	+	+	+
3	43463	0.27	0.09	*Arthrobacter*	AB288059	+	+	-	-
3	202828	1.26	0.42	*Arthrobacter*	AB288059	+	+	-	-
3	202828	1.26	0.42	*Arthrobacter*	AB288059	-	-	-	-
3	202828	1.26	0.42	*Arthrobacter*	AB288059	-	-	++	++
2	507924	2.33	0.78	*Arthrobacter*	AB288059	+	+	++	++
2	507924	2.33	0.78	*Arthrobacter*	AB288059	-	-	-	-
3	434631	2.7	0.90	*Arthrobacter*	AB288059	+	+	+	+
3	434631	2.7	0.90	*Arthrobacter*	AB288059	-	-	+	+
1	754575	3.2	1.07	*Arthrobacter*	AB288059	++	+	++	++
1	3772873	16.01	5.34	*Arthrobacter*	AB288059	-	-	++	++
3	2607789	16.17	5.39	*Arthrobacter*	AB288059	-	-	+	+
3	4346314	26.95	8.98	*Arthrobacter*	AB288059	-	-	+	+
1	377287	1.6	0.53	*Arthrobacter*	DQ985470	-	-	-	-
2	507924	2.33	0.78	*Arthrobacter*	DQ985470	-	-	+	+
1	377287	1.6	0.53	*Bacillus*	AB188212	-	-	-	-
2	50792	0.23	0.08	*Bacillus*	AM910175	-	-	-	-
1	377287	1.6	0.53	*Bradyrhizobium*	FJ390916	-	-	-	-
1	37729	0.16	0.05	*Brevundimonas*	EF088675	-	-	+	+

3	43463	0.27	0.09	*Brevundimonas*	EF088675	+	-	+	+
1	377287	1.6	0.53	*Brevundimonas*	EF088675	-	-	++	++
1	377287	1.6	0.53	*Burkholderia*	AY949194	-	-	-	-
1	3772873	16.01	5.34	*Burkholderia*	AY949194	-	-	-	-
3	86926	0.54	0.18	*Burkholderia*	FJ786047	-	-	-	-
1	377287	1.6	0.53	*Burkholderia*	FJ786047	-	-	-	-
2	507924	2.33	0.78	*Burkholderia*	FJ786047	-	-	-	-
2	50792	0.23	0.08	*Chryseobacterium*	DQ337589	-	-	+	+
3	130389	0.81	0.27	*Leifsonia*	AB278552	-	-	++	+
3	173853	1.08	0.36	*Leifsonia*	AB278552	-	-	-	-
1	377287	1.6	0.53	*Leifsonia*	AB278552	-	-	+	+
1	377287	1.6	0.53	*Leifsonia*	AB278552	+	-	-	-
2	507924	2.33	0.78	*Leifsonia*	AB278552	-	-	-	-
2	507924	2.33	0.78	*Leifsonia*	AB278552	-	-	+	+
3	434631	2.7	0.90	*Leifsonia*	AB278552	-	-	+	+
2	672999	3.09	1.03	*Leifsonia*	AB278552	-	-	+	+
3	2281815	14.15	4.72	*Leifsonia*	AB278552	-	-	-	-
3	43463	0.27	0.09	*Leifsonia*	AM889135	-	-	++	++
1	377287	1.6	0.53	*Leifsonia*	AM889135	-	-	++	++
2	507924	2.33	0.78	*Leifsonia*	AM889135	-	-	++	++
3	869263	5.39	1.80	*Leifsonia*	AM889135	-	-	+	-
2	50792	0.23	0.08	*Leifsonia*	DQ232613	-	-	++	+
3	434631	2.7	0.90	*Leifsonia*	DQ232613	-	-	+	+
1	377287	1.6	0.53	*Leifsonia*	FJ422386	-	-	-	-
1	754575	3.2	1.07	*Leifsonia*	FJ422386	-	-	+	-
1	754575	3.2	1.07	*Mesorhizobium*	AB531422	-	-	+	+
2	336499	1.54	0.51	*Mesorhizobium*	AY490106	-	-	-	-
2	507924	2.33	0.78	*Mesorhizobium*	AY490106	-	-	-	-
2	50792	0.23	0.08	*Methylobacterium*	AB220076	-	-	-	-
2	50792	0.23	0.08	*Methylobacterium*	Z23158	-	-	+	+
1	471609	2	0.67	*Methylobacterium*	Z23158	-	-	-	-
3	86926	0.54	0.18	*Mycobacterium*	AY337605	-	-	+	+
1	377287	1.6	0.53	*Pedobacter*	EF660751	-	-	+	+
2	50792	0.23	0.08	*Plantibacter*	AM396918	-	-	++	++
3	434631	2.7	0.90	*Plantibacter*	AM396918	-	-	++	++
1	377287	1.6	0.53	*Pseudomonas*	AB088844	++	+	-	-
2	50792	0.23	0.08	*Pseudomonas*	FM202488	+	-	-	-
2	50792	0.23	0.08	*Rhodococcus*	AB425280	-	-	+	+
1	37729	0.16	0.05	*Rhodococcus*	EU496547	++	-	-	-
2	507924	2.33	0.78	*Rhodococcus*	EU496547	+	-	++	++
2	3628026	16.65	5.55	*Rhodopseudomonas*	AB033756	-	-	-	-
1	377287	1.6	0.53	*Staphylococcus*	AY167864	-	-	+	+
2	50792	0.23	0.08	*Staphylococcus*	FJ357589	-	-	++	++
2	50792	0.23	0.08	*Staphylococcus*	FJ357589	-	-	++	++
2	507924	2.33	0.78	*Staphylococcus*	FJ357589	-	-	+	+
2	50792	0.23	0.08	*Staphylococcus*	GQ222398	-	-	-	-
2	50792	0.23	0.08	*Staphylococcus*	GQ222398	-	-	-	-
2	50792	0.23	0.08	*Staphylococcus*	GQ222398	-	-	++	++
2	50792	0.23	0.08	*Staphylococcus*	GQ222398	-	-	++	++

3	43463	0.27	0.09	*Staphylococcus*	GQ222398	-	-	++	++
2	243803	1.12	0.37	*Staphylococcus*	GQ222398	-	-	-	-
2	5079236	23.31	7.77	*Staphylococcus*	GQ222398	-	-	+	+
2	725605	3.33	1.11	*Stenotrophomonas*	AJ551165	-	-	+	+
3	434631	2.7	0.90	unc. bact.	FM872722	-	-	-	-
2	50792	0.23	0.08	unc. bact.	GQ012035	-	-	+	+
2	121902	0.56	0.19	unc. bact.	GQ012035	-	-	-	-
2	121902	0.56	0.19	unc. bact.	GQ012035	-	-	-	-
2	336499	1.54	0.51	unc. bact.	GQ012035	-	-	+	+
2	336499	1.54	0.51	unc. bact.	GQ012035	-	-	+	+
2	336499	1.54	0.51	unc. bact.	GQ012035	-	-	-	-
1	377287	1.6	0.53	unc. bact.	GQ012035	-	-	-	-
2	507924	2.33	0.78	unc. bact.	GQ012035	-	-	+	+
2	507924	2.33	0.78	unc. bact.	GQ012035	-	-	+	+
2	507924	2.33	0.78	unc. bact.	GQ012035	-	-	+	+
2	507924	2.33	0.78	unc. bact.	GQ012035	-	-	+	+
3	434631	2.7	0.90	unc. bact.	GQ012035	-	-	+	+
2	725605	3.33	1.11	unc. bact.	GQ012035	-	-	+	+
2	1149177	5.27	1.76	unc. bact.	GQ012035	-	-	-	-
1	3772873	16.01	5.34	unc. bact.	GQ012035	-	-	+	+
2	50792	0.23	0.08	*Variovorax*	EF419341	-	-	-	-
3	43463	0.27	0.09	*Variovorax*	EF419341	-	-	-	-
3	434631	2.7	0.90	*Variovorax*	EF419341	-	-	+	+
1	3772873	16.01	5.34	*Variovorax*	EF419341	++	+	-	-

RS-TE(D)

repl	cfu gFW-1	% repl	% mean	identification	accession	Cd (0.8 mM)	Cd (1.6 mM)	Zn (1 mM)	Zn (2.5 mM)
1	410391	1.84	0.61	*Arthrobacter*	D84573	-	-	-	-
1	410391	1.84	0.61	*Arthrobacter*	D84573	-	-	-	-
1	410391	1.84	0.61	*Arthrobacter*	D84573	-	-	-	-
2	6330	1.19	0.40	*Arthrobacter*	DQ985470	-	-	-	-
2	6330	1.19	0.40	*Arthrobacter*	DQ985470	-	-	+	-
2	6330	1.19	0.40	*Arthrobacter*	DQ985470	+	-	-	-
2	6330	1.19	0.40	*Arthrobacter*	DQ985470	+	+	-	-
2	6330	1.19	0.40	*Arthrobacter*	DQ985470	++	+	-	-
1	4103911	18.38	6.13	*Bacillus*	AM934688	-	-	-	-
1	4103911	18.38	6.13	*Bacillus*	AM934688	-	-	-	-
1	4103911	18.38	6.13	*Bacillus*	AM934688	-	-	-	-
1	4103911	18.38	6.13	*Bacillus*	AM934688	-	-	-	-
1	4103911	18.38	6.13	*Bacillus*	AM934688	-	-	-	-
3	249203	4.79	1.60	*Bacillus*	AY919667	-	-	-	-
3	249203	4.79	1.60	*Bacillus*	AY919667	-	-	-	-
3	249203	4.79	1.60	*Bacillus*	AY919667	-	-	-	-
3	249203	4.79	1.60	*Bacillus*	AY919667	-	-	+	+
3	249203	4.79	1.60	*Bacillus*	FJ859701	-	-	-	-
3	249203	4.79	1.60	*Bacillus*	FJ859701	-	-	-	-
3	249203	4.79	1.60	*Bacillus*	FJ859701	-	-	-	-
1	41039	0.18	0.06	*Duganella*	GU332616	-	-	-	-
1	41039	0.18	0.06	*Duganella*	GU332616	-	-	-	-
1	41039	0.18	0.06	*Duganella*	GU332616	-	-	++	++

2	29217	5.49	1.83	*Duganella*	GU332616	-	-	-	-
2	29217	5.49	1.83	*Duganella*	GU332616	-	-	-	-
2	29217	5.49	1.83	*Duganella*	GU332616	-	-	-	-
2	29217	5.49	1.83	*Duganella*	GU332616	-	-	-	-
2	29217	5.49	1.83	*Duganella*	GU332616	-	-	++	-
3	249203	4.79	1.60	*Duganella*	D14256	-	-	-	-
1	410391	1.84	0.61	*Flavobacterium*	EU057850	-	-	-	-
2	5356	1.01	0.34	*Microbacterium*	AJ277840	-	-	++	++
2	5356	1.01	0.34	*Microbacterium*	AJ277840	+	-	++	-
2	6817	1.28	0.43	*Microbacterium*	AJ277840	-	-	++	-
2	6817	1.28	0.43	*Microbacterium*	AJ277840	-	-	++	-
2	6817	1.28	0.43	*Microbacterium*	AJ277840	+	-	-	-
2	6817	1.28	0.43	*Microbacterium*	AJ277840	+	+	++	+
3	54825	1.05	0.35	*Pedobacter*	HM224489	-	-	++	+
3	54825	1.05	0.35	*Pedobacter*	HM224489	-	-	++	++
3	54825	1.05	0.35	*Pedobacter*	HM224489	-	-	++	+
3	54825	1.05	0.35	*Pedobacter*	HM224489	-	-	++	++
1	4925	0.02	0.01	*Pseudomonas*	AF456229	-	-	-	-
1	4925	0.02	0.01	*Pseudomonas*	AF456229	-	-	+	-
1	4925	0.02	0.01	*Pseudomonas*	AF456229	-	-	+	-
1	4925	0.02	0.01	*Pseudomonas*	AF456229	-	-	+	-
1	4925	0.02	0.01	*Pseudomonas*	AF456229	-	-	++	-
1	1806	0.01	0.003	*Pseudomonas*	AF456229	-	-	-	-
1	1806	0.01	0.003	*Pseudomonas*	AF456229	-	-	-	-
1	1806	0.01	0.003	*Pseudomonas*	AF456229	-	-	-	-
1	1806	0.01	0.003	*Pseudomonas*	AF456229	-	-	+	-
1	1806	0.01	0.003	*Pseudomonas*	AF456229	+	-	-	-
3	249203	4.79	1.60	*Pseudomonas*	AF456229	-	-	-	-
3	249203	4.79	1.60	*Pseudomonas*	AF456229	nd	nd	nd	nd
3	2492026	47.89	15.96	*Pseudomonas*	AJ417074	-	-	-	-
1	985	0.001	0.0003	*Pseudomonas*	EF673038	-	-	-	-
1	985	0.001	0.0003	*Pseudomonas*	EF673038	-	-	-	-
1	985	0.001	0.0003	*Pseudomonas*	EF673038	-	-	-	-
1	985	0.001	0.0003	*Pseudomonas*	EF673038	++	++	++	-
1	985	0.001	0.0003	*Pseudomonas*	EF673038	++	-	++	-
2	24347	4.57	1.52	*Pseudomonas*	EU747696	-	-	+	-
1	1395	0.01	0.003	*Pseudomonas*	GU784939	-	-	++	++
1	1395	0.01	0.003	*Pseudomonas*	GU784939	-	-	++	++
1	1395	0.01	0.003	*Pseudomonas*	GU784939	-	-	++	++
1	1395	0.01	0.003	*Pseudomonas*	GU784939	-	-	++	++
1	1395	0.01	0.003	*Pseudomonas*	GU784939	-	-	++	++
2	58434	10.98	3.66	*Variovorax*	GQ861460	-	-	-	-
2	58434	10.98	3.66	*Variovorax*	GQ861460	+	-	-	-
2	58434	10.98	3.66	*Variovorax*	GQ861460	+	-	-	-
2	58434	10.98	3.66	*Variovorax*	GQ861460	++	-	-	-
2	58434	10.98	3.66	*Variovorax*	GQ861460	+++	-	+	-
					RS-TE(J)				
repl	cfu gFW-1	% repl	% mean	identification	accession	Cd (0.8 mM)	Cd (1.6 mM)	Zn (1 mM)	Zn (2.5 mM)
1	3238604	0.24	0.08	*Achromobacter*	GQ927161	++	++	-	-

1	1619302	0.12	0.04	*Agrobacterium*	GQ428123	+	-	++	++
1	2267023	0.17	0.06	*Arthrobacter*	AB288059	-	-	++	+
1	161930208	11.9	3.97	*Arthrobacter*	AB288059	+	+	-	-
1	2267023	0.17	0.06	*Arthrobacter*	FJ890893	-	-	-	-
1	3238604	0.24	0.08	*Arthrobacter*	FJ890893	-	-	-	-
1	1619302	0.12	0.04	*Arthrobacter*	FM213390	++	+	++	+
1	1619302	0.12	0.04	*Arthrobacter*	FM213390	+	-	++	++
1	1619302	0.12	0.04	*Arthrobacter*	FM213390	++	+	++	+
1	2267023	0.17	0.06	*Arthrobacter*	FM213390	++	+	++	+
1	6801069	0.5	0.17	*Arthrobacter*	FM213390	-	-	+	+
1	161930208	11.9	3.97	*Bacillus*	AB188212	-	-	-	-
1	1619302	0.12	0.04	*Brevundimonas*	EF088675	++	+	++	++
1	2267023	0.17	0.06	*Burkholderia*	FJ939284	-	-	++	+
1	14573719	1.07	0.36	*Burkholderia*	FJ939284	-	-	+	+
2	7340796	2.38	0.79	*Flavobacterium*	GU078570	-	-	++	++
1	2428953	0.18	0.06	*Frigoribacterium*	AF157479	-	-	-	-
1	1619302	0.12	0.04	*Leifsonia*	AB278552	-	-	++	+
1	1619302	0.12	0.04	*Leifsonia*	AB278552	+	-	++	+
1	4318139	0.32	0.11	*Leifsonia*	AB278552	-	-	++	+
3	3480016	0.52	0.17	*Leifsonia*	AB278552	-	-	+	-
3	3480016	0.52	0.17	*Leifsonia*	AB278552	-	-	++	-
1	18621974	1.37	0.46	*Leifsonia*	AB278552	-	-	-	-
3	34800160	5.15	1.72	*Leifsonia*	AB278552	-	-	++	+
3	34800160	5.15	1.72	*Leifsonia*	AB278552	-	-	+	-
2	36703982	11.89	3.96	*Leifsonia*	AB278552	-	-	+	+
2	36703982	11.89	3.96	*Leifsonia*	AB278552	-	-	+	+
3	174000800	25.77	8.59	*Leifsonia*	AB278552	-	-	-	-
1	18621974	1.37	0.46	*Leifsonia*	GU332619	-	-	+	+
2	36703982	11.89	3.96	*Leifsonia*	GU332619	-	-	+	-
1	161930208	11.9	3.97	*Leifsonia*	GU332619	-	-	-	-
1	1619302	0.12	0.04	*Lysinibacillus*	AY907676	-	-	+	+
1	1619302	0.12	0.04	*Massilia*	AM231588	-	-	-	-
2	3670398	1.19	0.40	*Methylobacterium*	AB220076	-	-	-	-
1	3238604	0.24	0.08	*Microbacterium*	EU821338	+	-	++	++
1	19836450	1.46	0.49	*Microbacterium*	EU821338	+	+	+	+
1	19836450	1.46	0.49	*Microbacterium*	EU821338	+	+	+	+
1	19836450	1.46	0.49	*Microbacterium*	EU821338	-	-	+	+
1	19836450	1.46	0.49	*Microbacterium*	EU821338	-	-	+	+
2	36703982	11.89	3.96	*Microbacterium*	EU821338	-	-	++	++
3	34800160	5.15	1.72	*Pantoea*	EU598802	-	-	-	-
1	1619302	0.12	0.04	*Pedobacter*	AM279216	-	-	++	++
1	1619302	0.12	0.04	*Plantibacter*	AM396918	-	-	+	+
1	3238604	0.24	0.08	*Plantibacter*	AM396918	+	-	+	+
1	4534046	0.33	0.11	*Plantibacter*	AM396918	++	++	++	++
1	4857906	0.36	0.12	*Plantibacter*	AM396918	-	-	+	+
1	485790624	35.71	11.90	*Plantibacter*	AM396918	-	-	+	+
1	2428953	0.18	0.06	*Pseudoclavibacter*	X77440	-	-	-	-
2	3670398	1.19	0.40	*Pseudoclavibacter*	X77440	-	-	-	-
1	1619302	0.12	0.04	*Pseudomonas*	AB369347	-	-	+	+

1	2267023	0.17	0.06	*Pseudomonas*	AB369347	-	-	-	-
1	4318139	0.32	0.11	*Pseudomonas*	AB369347	+	-	+	+
3	3480016	0.52	0.17	*Pseudomonas*	AB369347	+	+	+	+
3	34800160	5.15	1.72	*Pseudomonas*	AB369347	-	-	+	+
1	72868594	5.36	1.79	*Pseudomonas*	AB369347	-	-	-	-
1	1619302	0.12	0.04	*Pseudomonas*	FJ225200	+	+	-	-
1	1619302	0.12	0.04	*Rhizobium*	AJ389905	++	-	++	++
1	4318139	0.32	0.11	*Rhizobium*	DQ337581	-	-	++	++
3	3480016	0.52	0.17	*Rhodococcus*	DQ060386	+	-	+	+
3	139200640	20.62	6.87	*Serratia*	AJ233434	+	+	+	+
1	1619302	0.12	0.04	*Sphingobacterium*	AJ438176	-	-	-	-
1	1619302	0.12	0.04	*Sphingobacterium*	AJ438176	-	-	+	+
1	18621974	1.37	0.46	*Sphingobacterium*	AJ438176	-	-	++	++
1	18621974	1.37	0.46	*Sphingobacterium*	AJ438176	-	-	++	++
1	1619302	0.12	0.04	*Staphylococcus*	GQ222398	-	-	+	+
2	367040	0.12	0.04	*Staphylococcus*	GQ222398	-	-	++	++
1	18621974	1.37	0.46	*Staphylococcus*	GQ222398	-	-	-	-
3	34800160	5.15	1.72	*Staphylococcus*	GQ222398	-	-	-	-
3	174000800	25.77	8.59	*Staphylococcus*	GQ222398	-	-	+	+
2	110111947	35.67	11.89	*Staphylococcus*	GQ222398	-	-	++	++
1	16193021	1.19	0.40	unc. bact.	EU536446	-	-	+	+
1	18621974	1.37	0.46	*Variovorax*	EF419341	-	-	-	-
1	1619302	0.12	0.04	*Variovorax*	FJ772012	-	-	-	-
1	3238604	0.24	0.08	*Variovorax*	FJ772012	-	-	+	+
1	3238604	0.24	0.08	*Variovorax*	FJ772012	++	++	-	-
2	36703982	11.89	3.96	*Variovorax*	FJ772012	-	-	+	+
					R-TE(D)				
repl	cfu gFW-1	% repl	% mean	identification	accession	Cd (0.8 mM)	Cd (1.6 mM)	Zn (1 mM)	Zn (2.5 mM)
1	131978	0.46	0.15	*Bacillus*	AM934688	-	-	-	-
1	13197836	45.79	15.26	*Bacillus*	AM934688	-	-	-	-
1	13197836	45.79	15.26	*Bacillus*	AM934688	-	-	-	-
3	7291	2.7	0.90	*Burkholderia*	FJ786047	nd	nd	nd	nd
3	7291	2.7	0.90	*Burkholderia*	FJ786047	nd	nd	nd	nd
3	7291	2.7	0.90	*Burkholderia*	FJ786047	nd	nd	nd	nd
3	7291	2.7	0.90	*Burkholderia*	FJ786047	nd	nd	nd	nd
3	7291	2.7	0.90	*Burkholderia*	FJ786047	nd	nd	nd	nd
1	131978	0.46	0.15	*Flavobacterium*	DQ339596	-	-	-	-
3	7291	2.7	0.90	*Luteibacter*	AJ580498	-	-	-	-
3	7291	2.7	0.90	*Luteibacter*	AJ580498	nd	nd	nd	nd
3	16040	5.95	1.98	*Luteibacter*	AJ580498	-	-	+	++
3	16040	5.95	1.98	*Luteibacter*	AJ580498	-	-	++	+
3	16040	5.95	1.98	*Luteibacter*	AJ580498	-	-	++	++
3	16040	5.95	1.98	*Luteibacter*	AJ580498	-	-	++	++
3	16040	5.95	1.98	*Luteibacter*	AJ580498	-	-	++	++
3	13123	4.86	1.62	*Mitsuaria*	GU332617	-	-	-	-
3	13123	4.86	1.62	*Mitsuaria*	GU332617	-	-	-	-
3	13123	4.86	1.62	*Mitsuaria*	GU332617	-	-	-	-
3	13123	4.86	1.62	*Mitsuaria*	GU332617	+	-	-	-
3	13123	4.86	1.62	*Mitsuaria*	GU332617	+	-	-	-

2	873	0.44	0.15	*Pseudomonas*	CP000094	+	-	-	-
2	873	0.44	0.15	*Pseudomonas*	CP000094	+	-	-	-
2	873	0.44	0.15	*Pseudomonas*	CP000094	+	-	-	-
2	873	0.44	0.15	*Pseudomonas*	CP000094	+	-	++	-
2	873	0.44	0.15	*Pseudomonas*	CP000094	++	-	-	-
2	1572	0.8	0.27	*Pseudomonas*	CP000094	-	-	-	-
2	1572	0.8	0.27	*Pseudomonas*	CP000094	-	-	-	-
2	1572	0.8	0.27	*Pseudomonas*	CP000094	-	-	++	-
2	1572	0.8	0.27	*Pseudomonas*	CP000094	+	-	-	-
2	1572	0.8	0.27	*Pseudomonas*	CP000094	++	-	-	-
2	34922	17.78	5.93	*Pseudomonas*	CP000094	-	-	-	-
2	34922	17.78	5.93	*Pseudomonas*	CP000094	-	-	-	-
2	34922	17.78	5.93	*Pseudomonas*	CP000094	-	-	-	-
2	34922	17.78	5.93	*Pseudomonas*	CP000094	-	-	-	-
2	34922	17.78	5.93	*Pseudomonas*	CP000094	-	-	+	-
2	1921	0.98	0.33	*Pseudomonas*	EF102850	-	-	-	-
2	1921	0.98	0.33	*Pseudomonas*	EF102850	-	-	-	-
2	1921	0.98	0.33	*Pseudomonas*	EF102850	-	-	++	-
2	1921	0.98	0.33	*Pseudomonas*	EF102850	+	-	++	-
2	1921	0.98	0.33	*Pseudomonas*	EF102850	++	-	-	-
1	79187	0.27	0.09	*Pseudomonas*	EF491969	-	-	-	-
1	79187	0.27	0.09	*Pseudomonas*	EF491969	-	-	-	-
1	79187	0.27	0.09	*Pseudomonas*	EF491969	-	-	-	-
1	79187	0.27	0.09	*Pseudomonas*	EF491969	+	-	-	-
1	79187	0.27	0.09	*Pseudomonas*	EF491969	++	-	+	-
1	116141	0.4	0.13	*Pseudomonas*	EF491969	-	-	-	-
1	116141	0.4	0.13	*Pseudomonas*	EF491969	-	-	-	-
1	116141	0.4	0.13	*Pseudomonas*	EF491969	-	-	-	-
1	116141	0.4	0.13	*Pseudomonas*	EF491969	-	-	-	-
1	116141	0.4	0.13	*Pseudomonas*	EF491969	-	-	-	-
3	14582	5.41	1.80	*Pseudomonas*	EF491969	-	-	-	-
3	14582	5.41	1.80	*Pseudomonas*	EF491969	-	-	-	-
3	14582	5.41	1.80	*Pseudomonas*	EF491969	-	-	-	-
3	14582	5.41	1.80	*Pseudomonas*	EF491969	-	-	+	-
3	14582	5.41	1.80	*Pseudomonas*	EF491969	-	-	++	++
1	131978	0.46	0.15	*Pseudomonas*	GU784939	++	-	-	-
1	131978	0.46	0.15	*Pseudomonas*	GU784939	++	-	+	-
1	184770	0.64	0.21	*Pseudomonas*	GU784939	-	-	-	-
1	184770	0.64	0.21	*Pseudomonas*	GU784939	-	-	-	-
1	184770	0.64	0.21	*Pseudomonas*	GU784939	+	-	-	-
1	184770	0.64	0.21	*Pseudomonas*	GU784939	+	-	-	-
1	184770	0.64	0.21	*Pseudomonas*	GU784939	++	-	-	-
					R-TE(J)				
repl	cfu gFW-1	% repl	% mean	identification	accession	Cd (0.8 mM)	Cd (1.6 mM)	Zn (1 mM)	Zn (2.5 mM)
3	1489	0.39	0.13	*Caulobacter*	DQ337549	-	-	-	-
3	2190	0.57	0.19	*Caulobacter*	DQ337549	-	-	-	-
3	2365	0.62	0.21	*Caulobacter*	DQ337549	-	-	-	-
3	2365	0.62	0.21	*Caulobacter*	DQ337549	-	-	-	-
3	2365	0.62	0.21	*Caulobacter*	DQ337549	-	-	-	-

3	7885	2.06	0.69	*Caulobacter*	DQ337549	-	-	++	++
3	7885	2.06	0.69	*Caulobacter*	DQ337549	-	-	+	+
3	8673	2.26	0.75	*Caulobacter*	DQ337549	-	-	-	-
3	14630	3.82	1.27	*Caulobacter*	DQ337549	-	-	-	-
2	6180	4.55	1.52	*Caulobacter*	DQ337549	+	+	-	-
2	6180	4.55	1.52	*Caulobacter*	DQ337549	-	-	+	+
2	6180	4.55	1.52	*Caulobacter*	DQ337549	+	+	+	+
2	24719	18.18	6.06	*Caulobacter*	DQ337549	-	-	-	-
2	6180	4.55	1.52	*Flavobacterium*	AM934662	+	+	+	+
1	93738	1.51	0.50	*Flavobacterium*	EU057850	-	-	+	+
3	788	0.21	0.07	*Labrys*	DQ337554	++	-	-	-
3	2190	0.57	0.19	*Labrys*	DQ337554	+	-	-	-
3	14630	3.82	1.27	*Labrys*	DQ337554	-	-	-	-
3	2190	0.57	0.19	*Leifsonia*	AB278552	-	-	++	++
3	2190	0.57	0.19	*Leifsonia*	AB278552	-	-	-	-
3	7885	2.06	0.69	*Mucilaginibacter*	EU747841	-	-	-	-
3	7885	2.06	0.69	*Mycobacterium*	FJ719354	-	-	++	++
3	1656	0.43	0.14	*Pantoea*	EU598802	+	-	-	-
3	2365	0.62	0.21	*Pantoea*	EU598802	+	+	++	++
1	9374	0.15	0.05	*Pedobacter*	DQ778037	-	-	++	++
1	9374	0.15	0.05	*Pedobacter*	DQ778037	-	-	++	++
3	2190	0.57	0.19	*Pedobacter*	DQ778037	-	-	++	++
1	93738	1.51	0.50	*Pedobacter*	DQ778037	-	-	++	++
1	93738	1.51	0.50	*Pedobacter*	DQ778037	+	+	++	++
3	7885	2.06	0.69	*Pedobacter*	DQ778037	-	-	++	++
3	14630	3.82	1.27	*Pedobacter*	DQ778037	-	-	++	++
2	6180	4.55	1.52	*Pedobacter*	DQ778037	+	+	++	++
2	6180	4.55	1.52	*Pedobacter*	DQ778037	+	+	++	++
3	78846	20.59	6.86	*Pedobacter*	DQ778037	-	-	++	++
2	6180	4.55	1.52	*Pedobacter*	GU385862	-	-	++	++
3	1489	0.39	0.13	*Plantibacter*	AM396918	-	-	++	+
3	2365	0.62	0.21	*Plantibacter*	AM396918	-	-	++	++
3	4731	1.24	0.41	*Plantibacter*	AM396918	+	-	-	-
3	7885	2.06	0.69	*Plantibacter*	AM396918	-	-	++	++
3	14630	3.82	1.27	*Polaromonas*	AB245355	-	-	+	+
3	788	0.21	0.07	*Pseudomonas*	AB369347	+	+	-	-
3	2190	0.57	0.19	*Pseudomonas*	AB369347	+	-	-	-
3	2478	0.65	0.22	*Pseudomonas*	DQ279324	+	+	+	+
3	2478	0.65	0.22	*Pseudomonas*	DQ279324	+	-	-	-
3	2478	0.65	0.22	*Pseudomonas*	DQ279324	+	-	-	-
3	2478	0.65	0.22	*Pseudomonas*	DQ279324	+	-	-	-
3	2478	0.65	0.22	*Pseudomonas*	DQ279324	+	-	-	-
3	2478	0.65	0.22	*Pseudomonas*	DQ279324	+	-	+	+
3	2478	0.65	0.22	*Pseudomonas*	FJ719351	-	-	-	-
3	788	0.21	0.07	*Pseudomonas*	FJ772042	-	-	+	+
3	788	0.21	0.07	*Pseudomonas*	FJ772042	-	-	-	-
3	2365	0.62	0.21	*Pseudomonas*	FJ772042	-	-	-	-
3	14630	3.82	1.27	*Pseudomonas*	FJ772042	-	-	++	++
3	14630	3.82	1.27	*Pseudomonas*	FJ772042	-	-	-	-

3	1656	0.43	0.14	*Pseudomonas*	FN377713	+	-	+	+
3	1656	0.43	0.14	*Pseudomonas*	FN377713	-	-	-	-
1	93738	1.51	0.50	*Pseudomonas*	FN377713	-	-	-	-
3	7885	2.06	0.69	*Pseudomonas*	FN377713	+	+	+	+
3	7885	2.06	0.69	*Pseudomonas*	FN377713	+	+	-	-
3	11590	3.03	1.01	*Pseudomonas*	FN377713	-	-	-	-
1	2343457	37.76	12.59	*Pseudomonas*	FN377713	-	-	-	-
1	2343457	37.76	12.59	*Pseudomonas*	FN377713	-	-	-	-
3	788	0.21	0.07	*Rhizobium*	DQ337581	++	+	++	-
3	1577	0.41	0.14	*Rhizobium*	DQ337581	-	-	++	-
3	2190	0.57	0.19	*Rhizobium*	DQ337581	-	-	-	-
1	93738	1.51	0.50	*Rhizobium*	DQ337581	-	-	-	-
2	61797	45.45	15.15	*Rhizobium*	DQ337581	-	-	-	-
3	2365	0.62	0.21	*Rhodococcus*	EU496547	++	-	+	+
3	14630	3.82	1.27	*Rhodococcus*	EU496547	+	-	-	-
3	14630	3.82	1.27	*Rhodococcus*	EU496547	+	-	-	-
2	6180	4.55	1.52	*Rhodococcus*	EU496547	++	++	-	-
3	788	0.21	0.07	*Sanguibacter*	X79452	-	-	-	-
3	788	0.21	0.07	*Sanguibacter*	X79452	-	-	+	+
3	1577	0.41	0.14	*Staphylococcus*	GQ222398	-	-	-	-
3	2365	0.62	0.21	*Staphylococcus*	GQ222398	-	-	+	+
3	2190	0.57	0.19	unc. bact.	GQ012035	-	-	++	++
3	788	0.21	0.07	*Variovorax*	GQ861460	-	-	+	+
3	788	0.21	0.07	*Variovorax*	GQ861460	-	-	++	++
3	788	0.21	0.07	*Variovorax*	GQ861460	-	-	++	++
3	788	0.21	0.07	*Variovorax*	GQ861460	-	-	-	-
3	788	0.21	0.07	*Variovorax*	GQ861460	+	+	+	+
3	788	0.21	0.07	*Variovorax*	GQ861460	+	+	-	-
3	788	0.21	0.07	*Variovorax*	GQ861460	++	+	++	++
3	788	0.21	0.07	*Variovorax*	GQ861460	+	+	++	++
3	1489	0.39	0.13	*Variovorax*	GQ861460	-	-	-	-
3	1489	0.39	0.13	*Variovorax*	GQ861460	+	+	+	+
3	1489	0.39	0.13	*Variovorax*	GQ861460	+	-	-	-
3	1489	0.39	0.13	*Variovorax*	GQ861460	+	+	-	-
3	1489	0.39	0.13	*Variovorax*	GQ861460	-	-	-	-
3	1489	0.39	0.13	*Variovorax*	GQ861460	-	-	-	-
3	1489	0.39	0.13	*Variovorax*	GQ861460	++	+	-	-
3	1577	0.41	0.14	*Variovorax*	GQ861460	++	++	++	++
3	2365	0.62	0.21	*Variovorax*	GQ861460	-	-	+	+
3	2365	0.62	0.21	*Variovorax*	GQ861460	++	+	++	++
3	3154	0.82	0.27	*Variovorax*	GQ861460	-	-	+	+
1	93738	1.51	0.50	*Variovorax*	GQ861460	-	-	-	-
1	93738	1.51	0.50	*Variovorax*	GQ861460	++	+	-	-
1	93738	1.51	0.50	*Variovorax*	GQ861460	+	+	-	-
1	93738	1.51	0.50	*Variovorax*	GQ861460	++	+	-	-
1	131234	2.11	0.70	*Variovorax*	GQ861460	+	+	+	+
1	524934	8.46	2.82	*Variovorax*	GQ861460	++	-	+	+
3	2365	0.62	0.21	*Xanthomonas*	DQ177466	-	-	-	-

Appendix 5.2 Correlation coefficients (CC) between bulk soil, rhizosphere soil and *B. napus* root bacterial communities isolated at the control field (BS-CO, RS-CO and R-CO respectively) and the contaminated field (BS-TE, RS-TE and R-TE respectively) in December (D) and June (J). Genotypic information was subjected to correspondence analysis (CA), a principal component analysis related ordination technique based on chi-square distances. Compartment data are based on 3 replicates consisting of 3 independent samples (mixed samples).

	BS.CO.D.	BS.TE.D.	RS.CO.D.	RS.TE.D.	R.CO.D.	R.TE.D.
BS.CO.D.	1.00000000	0.17238624	0.49271491	0.44525344	0.34211656	0.36440352
BS.TE.D.	0.17238624	1.00000000	0.35339843	0.78725785	0.07574372	0.46921012
RS.CO.D.	0.49271491	0.35339843	1.00000000	0.47596816	0.01210604	0.25184142
RS.TE.D.	0.44525344	0.78725785	0.47596816	1.00000000	0.47572788	0.74554380
R.CO.D.	0.34211656	0.07574372	0.01210604	0.47572788	1.00000000	0.63317876
R.TE.D.	0.36440352	0.46921012	0.25184142	0.74554380	0.63317876	1.00000000
BS.CO.J.	0.09065323	0.50975413	0.23830568	0.46327868	-0.06201837	0.29612141
BS.TE.J.	-0.10294570	0.17682638	-0.09712263	0.03765606	0.01211302	-0.05811548
RS.CO.J.	0.27072716	0.80371370	0.37290953	0.94796117	0.39581220	0.75783333
RS.TE.J.	-0.05317211	0.06820484	-0.04572855	0.08926316	0.05039192	0.05067249
R.CO.J.	0.20393058	0.36099874	0.10635876	0.68230358	0.85566197	0.59516148
R.TE.J.	0.11715782	0.05790226	-0.02646013	0.32284389	0.65456616	0.57473954

	BS.CO.J.	BS.TE.J.	RS.CO.J.	RS.TE.J.	R.CO.J.	R.TE.J.
BS.CO.D.	0.09065323	-0.10294570	0.27072716	-0.05317210	0.20393058	0.11715781
BS.TE.D.	0.50975413	0.17682638	0.80371370	0.06820483	0.36099874	0.05790226
RS.CO.D.	0.23830568	-0.09712263	0.37290953	-0.04572854	0.10635876	-0.02646012
RS.TE.D.	0.46327868	0.03765606	0.94796117	0.08926316	0.68230358	0.32284388
R.CO.D.	-0.06201837	0.01211302	0.39581220	0.05039192	0.85566197	0.65456615
R.TE.D.	0.29612141	-0.05811548	0.75783333	0.05067249	0.59516148	0.57473954
BS.CO.J.	1.00000000	-0.02962687	0.50000324	0.05604348	0.10415188	-0.07437214
BS.TE.J.	-0.02962687	1.00000000	0.07052471	0.48821141	0.04382958	-0.06602354
RS.CO.J.	0.50000324	0.07052471	1.00000000	0.21657690	0.62068216	0.28747373
RS.TE.J.	0.05604348	0.48821141	0.21657691	1.00000000	0.08272192	0.00980507
R.CO.J.	0.10415188	0.04382958	0.62068216	0.08272191	1.00000000	0.64350164
R.TE.J.	-0.07437215	-0.06602355	0.28747373	0.00980507	0.64350165	1.00000000

Appendix 5.3 Detailed characterisation of all purified *B. napus* shoot isolates collected at the control field (S-CO) and the contaminated field (S-TE) in December (D) and June (J). The presence of each strain is shown as relative abundances, expressed in percentages, of the total number of colony forming units per gram fresh weight (cfu gFW^{-1}). Strains are identified to the genus level, their accession numbers as well as their presence in the 1st, 2nd or 3rd replicate (repl) are displayed. Mean percentages were calculated based on the 3 replicates.

S-CO(D)					
repl	cfu gFW^{-1}	% repl	% mean	identification	accession
2	272	0.98	0.33	*Bacillus*	AJ628745
2	288	1.04	0.35	*Bacillus*	AM934688
2	288	1.04	0.35	*Bacillus*	AM934688
2	288	1.04	0.35	*Bacillus*	AM934688
2	288	1.04	0.35	*Bacillus*	AM934688
2	288	1.04	0.35	*Bacillus*	AM934688
1	679	8.87	2.96	*Bacillus*	AM934688
1	679	8.87	2.96	*Bacillus*	AM934688
1	679	8.87	2.96	*Bacillus*	AM934688
1	679	8.87	2.96	*Bacillus*	AM934688
1	679	8.87	2.96	*Bacillus*	AM934688
2	185	0.67	0.22	*Brevundimonas*	EF088675
2	185	0.67	0.22	*Brevundimonas*	EF088675
2	185	0.67	0.22	*Brevundimonas*	EF088675
2	185	0.67	0.22	*Brevundimonas*	EF088675
2	185	0.67	0.22	*Brevundimonas*	EF088675
2	300	1.09	0.36	*Duganella*	GU332616
2	300	1.09	0.36	*Duganella*	GU332616
2	300	1.09	0.36	*Duganella*	GU332616
2	300	1.09	0.36	*Duganella*	GU332616
2	300	1.09	0.36	*Duganella*	GU332616
2	49	0.18	0.06	*Flavobacterium*	DQ339596
2	49	0.18	0.06	*Flavobacterium*	DQ339596
2	49	0.18	0.06	*Flavobacterium*	DQ339596
2	49	0.18	0.06	*Flavobacterium*	DQ339596
2	49	0.18	0.06	*Flavobacterium*	DQ339596
3	338	0.06	0.02	*Luteibacter*	AJ580498
3	338	0.06	0.02	*Luteibacter*	AJ580498
3	338	0.06	0.02	*Luteibacter*	AJ580498
3	338	0.06	0.02	*Luteibacter*	AJ580498
3	338	0.06	0.02	*Luteibacter*	AJ580498
2	76	0.28	0.09	*Massilia*	AM231588
2	76	0.28	0.09	*Massilia*	AM231588
2	76	0.28	0.09	*Massilia*	AM231588
2	76	0.28	0.09	*Massilia*	AM231588
2	76	0.28	0.09	*Massilia*	AM231588
3	34104	5.91	1.97	*Pantoea*	DQ531643
3	34104	5.91	1.97	*Pantoea*	DQ531643
3	34104	5.91	1.97	*Pantoea*	DQ531643

3	34104	5.91	1.97	*Pantoea*	DQ531643
3	34104	5.91	1.97	*Pantoea*	DQ531643
2	27	0.10	0.03	*Pantoea*	EU598802
2	2300	8.33	2.78	*Pseudomonas*	AB098591
2	2300	8.33	2.78	*Pseudomonas*	AB098591
2	2300	8.33	2.78	*Pseudomonas*	AB098591
2	2300	8.33	2.78	*Pseudomonas*	AB098591
2	2300	8.33	2.78	*Pseudomonas*	AB098591
2	167	0.60	0.20	*Pseudomonas*	AY043360
2	167	0.60	0.20	*Pseudomonas*	AY043360
1	358	4.68	1.56	*Pseudomonas*	EF491969
1	358	4.68	1.56	*Pseudomonas*	EF491969
1	358	4.68	1.56	*Pseudomonas*	EF491969
1	358	4.68	1.56	*Pseudomonas*	EF491969
1	358	4.68	1.56	*Pseudomonas*	EF491969
2	2200	7.96	2.65	*Pseudomonas*	EF491969
2	2200	7.96	2.65	*Pseudomonas*	EF491969
2	2200	7.96	2.65	*Pseudomonas*	EF491969
2	2200	7.96	2.65	*Pseudomonas*	EF491969
2	2200	7.96	2.65	*Pseudomonas*	EF491969
3	80925	14.02	4.67	*Pseudomonas*	EF491969
3	80925	14.02	4.67	*Pseudomonas*	EF491969
3	80925	14.02	4.67	*Pseudomonas*	EF491969
3	80925	14.02	4.67	*Pseudomonas*	EF491969
3	80925	14.02	4.67	*Pseudomonas*	EF491969
3	211	0.04	0.01	*Sphingomonas*	FM865678
3	211	0.04	0.01	*Sphingomonas*	FM865678
1	494	6.45	2.15	*Variovorax*	GQ861460
1	494	6.45	2.15	*Variovorax*	GQ861460
1	494	6.45	2.15	*Variovorax*	GQ861460
1	494	6.45	2.15	*Variovorax*	GQ861460
1	494	6.45	2.15	*Variovorax*	GQ861460
			S-CO(J)		
repl	cfu gFW^{-1}	% repl	% mean	identification	accession
2	17	0.40	0.13	*Agromyces*	AM410681
3	154	0.32	0.11	*Bacillus*	AB188212
1	218742	97.53	32.51	*Bacillus*	AB188212
1	42	0.02	0.01	*Bacillus*	CP000813
3	92	0.19	0.06	*Bacillus*	CP000813
3	92	0.19	0.06	*Bacillus*	CP000813
3	95	0.20	0.07	*Bacillus*	CP000813
3	154	0.32	0.11	*Bacillus*	CP000813
2	17	0.40	0.13	*Bacillus*	CP000813
3	308	0.64	0.21	*Bacillus*	CP000813
3	317	0.66	0.22	*Bacillus*	CP000813
1	5294	2.36	0.79	*Bacillus*	CP000813
2	122	2.88	0.96	*Bacillus*	CP000813
2	254	5.99	2.00	*Bacillus*	CP000813
2	1693	39.89	13.30	*Bacillus*	CP000813

2	1693	39.89	13.30	*Bacillus*	CP000813
2	17	0.40	0.13	*Bacillus*	FJ263042
3	317	0.66	0.22	*Bacillus*	FJ263042
1	42	0.02	0.01	*Bacillus*	GQ284474
1	22	0.01	0.003	*Brevibacillus*	AF378230
3	16	0.03	0.01	*Frigoribacterium*	AF157479
3	49	0.10	0.03	*Frigoribacterium*	AF157479
3	16	0.03	0.01	*Massilia*	AM231588
3	51	0.11	0.04	*Massilia*	AM231588
3	154	0.32	0.11	*Massilia*	AM231588
3	203	0.42	0.14	*Massilia*	AM231588
3	1267	2.64	0.88	*Massilia*	AM231588
3	31678	65.97	21.99	*Massilia*	AM231588
1	22	0.01	0.003	*Mycobacterium*	FJ719354
3	58	0.12	0.04	*Paenibacillus*	EU723825
3	154	0.32	0.11	*Paenibacillus*	EU723825
3	222	0.46	0.15	*Paenibacillus*	EU723825
2	170	3.99	1.33	*Pantoea*	EU598802
3	1384	2.88	0.96	*Pedobacter*	DQ778037
2	17	0.40	0.13	*Plantibacter*	AM396918
3	108	0.22	0.07	*Pseudomonas*	AY574907
3	108	0.22	0.07	*Pseudomonas*	AY574907
2	51	1.20	0.40	*Pseudomonas*	DQ146946
2	51	1.20	0.40	*Pseudomonas*	DQ146946
2	51	1.20	0.40	*Pseudomonas*	DQ146946
2	51	1.20	0.40	*Pseudomonas*	DQ146946
3	32	0.07	0.02	*Pseudomonas*	DQ778036
3	64	0.13	0.04	*Pseudomonas*	DQ778036
3	140	0.29	0.10	*Pseudomonas*	DQ778036
2	41	0.95	0.32	*Pseudomonas*	FJ772042
1	42	0.02	0.01	*Pseudomonas*	FN377713
1	42	0.02	0.01	*Pseudomonas*	FN377713
3	32	0.07	0.02	*Pseudomonas*	FN377713
3	58	0.12	0.04	*Pseudomonas*	FN377713
3	95	0.20	0.07	*Pseudomonas*	FN377713
3	95	0.20	0.07	*Pseudomonas*	FN377713
3	140	0.29	0.10	*Pseudomonas*	FN377713
3	154	0.32	0.11	*Pseudomonas*	FN377713
3	154	0.32	0.11	*Pseudomonas*	FN377713
3	222	0.46	0.15	*Pseudomonas*	FN377713
3	279	0.58	0.19	*Pseudomonas*	FN377713
3	317	0.66	0.22	*Pseudomonas*	FN377713
3	317	0.66	0.22	*Pseudomonas*	FN377713
3	317	0.66	0.22	*Pseudomonas*	FN377713
3	317	0.66	0.22	*Pseudomonas*	FN377713
3	317	0.66	0.22	*Pseudomonas*	FN377713
3	317	0.66	0.22	*Pseudomonas*	FN377713
3	317	0.66	0.22	*Pseudomonas*	FN377713
3	317	0.66	0.22	*Pseudomonas*	FN377713

3	317	0.66	0.22	*Pseudomonas*	FN377713
3	317	0.66	0.22	*Pseudomonas*	FN377713
3	317	0.66	0.22	*Pseudomonas*	FN377713
3	317	0.66	0.22	*Pseudomonas*	FN377713
3	317	0.66	0.22	*Pseudomonas*	FN377713
3	2360	4.91	1.64	*Pseudomonas*	FN377713
3	2360	4.91	1.64	*Pseudomonas*	FN377713
1	42	0.02	0.01	*Rhodococcus*	EU496547
3	139	0.29	0.10	*Sanguibacter*	X79452
3	317	0.66	0.22	*Staphylococcus*	AY167864
3	95	0.20	0.07	*Staphylococcus*	GQ222398
3	140	0.29	0.10	*Staphylococcus*	GQ222398
3	16	0.03	0.01	*Variovorax*	GQ861460
3	25	0.05	0.02	*Zooglea*	X74914

S-TE(D)

repl	cfu gFW^{-1}	% repl	% mean	identification	accession
2	16	0.65	0.22	*Bacillus*	EF443162
2	16	0.65	0.22	*Bacillus*	EF443162
2	16	0.65	0.22	*Bacillus*	FJ627946
2	16	0.65	0.22	*Clavibacter*	DQ339617
2	16	0.65	0.22	*Clavibacter*	DQ339617
2	16	0.65	0.22	*Clavibacter*	DQ339617
2	23	0.90	0.30	*Clavibacter*	DQ339617
2	23	0.90	0.30	*Clavibacter*	DQ339617
2	23	0.90	0.30	*Clavibacter*	DQ339617
2	23	0.90	0.30	*Clavibacter*	DQ339617
1	19	0.0002	0.0001	*Duganella*	GU332616
1	19	0.0002	0.0001	*Duganella*	GU332616
1	19	0.0002	0.0001	*Duganella*	GU332616
1	19	0.0002	0.0001	*Duganella*	GU332616
1	19	0.0002	0.0001	*Duganella*	GU332616
2	39	1.55	0.52	*Duganella*	GU332616
2	39	1.55	0.52	*Duganella*	GU332616
2	39	1.55	0.52	*Duganella*	GU332616
2	39	1.55	0.52	*Duganella*	GU332616
2	39	1.55	0.52	*Duganella*	GU332616
2	262	10.35	3.45	*Duganella*	GU332616
2	262	10.35	3.45	*Duganella*	GU332616
2	262	10.35	3.45	*Duganella*	GU332616
2	262	10.35	3.45	*Duganella*	GU332616
2	262	10.35	3.45	*Duganella*	GU332616
2	16	0.65	0.22	*Flavobacterium*	FJ786049
2	164	6.47	2.16	*Luteibacter*	AJ580498
3	4	0.01	0.005	*Pseudomonas*	AF456229
1	7	0.0001	0.00003	*Pseudomonas*	AY574912
1	7	0.0001	0.00003	*Pseudomonas*	AY574912
1	7	0.0001	0.00003	*Pseudomonas*	AY574912
1	7	0.0001	0.00003	*Pseudomonas*	AY574912
1	7	0.0001	0.00003	*Pseudomonas*	AY574912

1	7	0.0001	0.00003	*Pseudomonas*	AY574912
1	7	0.0001	0.00003	*Pseudomonas*	AY574912
3	4	0.01	0.005	*Pseudomonas*	CP000094
3	4	0.01	0.005	*Pseudomonas*	CP000094
3	8	0.03	0.01	*Pseudomonas*	CP000094
3	8	0.03	0.01	*Pseudomonas*	CP000094
3	8	0.03	0.01	*Pseudomonas*	CP000094
3	8	0.03	0.01	*Pseudomonas*	CP000094
3	8	0.03	0.01	*Pseudomonas*	CP000094
3	313	1.08	0.36	*Pseudomonas*	CP000094
3	313	1.08	0.36	*Pseudomonas*	CP000094
3	313	1.08	0.36	*Pseudomonas*	CP000094
3	313	1.08	0.36	*Pseudomonas*	CP000094
3	313	1.08	0.36	*Pseudomonas*	CP000094
2	69	2.72	0.91	*Pseudomonas*	CP000094
2	69	2.72	0.91	*Pseudomonas*	CP000094
2	69	2.72	0.91	*Pseudomonas*	CP000094
2	69	2.72	0.91	*Pseudomonas*	CP000094
2	69	2.72	0.91	*Pseudomonas*	CP000094
3	1007	3.47	1.16	*Pseudomonas*	CP000094
3	1007	3.47	1.16	*Pseudomonas*	CP000094
3	1007	3.47	1.16	*Pseudomonas*	CP000094
3	1007	3.47	1.16	*Pseudomonas*	CP000094
3	1007	3.47	1.16	*Pseudomonas*	CP000094
3	4473	15.40	5.13	*Pseudomonas*	CP000094
3	4473	15.40	5.13	*Pseudomonas*	CP000094
3	4473	15.40	5.13	*Pseudomonas*	CP000094
3	4473	15.40	5.13	*Pseudomonas*	CP000094
3	4473	15.40	5.13	*Pseudomonas*	CP000094
3	6	0.02	0.01	*Pseudomonas*	EF491969
3	6	0.02	0.01	*Pseudomonas*	EF491969
3	6	0.02	0.01	*Pseudomonas*	EF491969
3	6	0.02	0.01	*Pseudomonas*	EF491969
3	6	0.02	0.01	*Pseudomonas*	EF491969
2	16	0.65	0.22	*Pseudomonas*	EF491969
2	16	0.65	0.22	*Pseudomonas*	HQ420253
2	16	0.65	0.22	*Pseudomonas*	HQ420253
2	16	0.65	0.22	*Pseudomonas*	HQ420253
2	16	0.65	0.22	*Pseudomonas*	HQ420253
2	16	0.65	0.22	*Pseudomonas*	HQ420253
2	16	0.65	0.22	*Pseudomonas*	HQ420253
2	16	0.65	0.22	*Pseudomonas*	HQ420253
1	1350	0.01	0.005	*Rhizobium*	FJ405385
1	1808367	19.99	6.66	*Rhizobium*	FJ405385
1	1808367	19.99	6.66	*Rhizobium*	FJ405385
1	1808367	19.99	6.66	*Rhizobium*	FJ405385
1	1808367	19.99	6.66	*Rhizobium*	FJ405385
1	1808367	19.99	6.66	*Rhizobium*	FJ405385
1	1350	0.01	0.005	*Sphingomonas*	AJ009706

2	16	0.65	0.22	*Staphylococcus*	GQ222398
1	37	0.0004	0.0001	*Streptomyces*	EU144078
1	37	0.0004	0.0001	*Streptomyces*	EU144078
1	37	0.0004	0.0001	*Streptomyces*	EU144078
1	37	0.0004	0.0001	*Streptomyces*	EU144078
1	37	0.0004	0.0001	*Streptomyces*	EU144078
1	162	0.002	0.001	*Variovorax*	GQ861460
1	162	0.002	0.001	*Variovorax*	GQ861460
1	162	0.002	0.001	*Variovorax*	GQ861460
1	162	0.002	0.001	*Variovorax*	GQ861460
1	162	0.002	0.001	*Variovorax*	GQ861460
2	164	6.47	2.16	*Zooglea*	X74914
			S-TE(J)		
repl	cfu gFW^{-1}	% repl	% mean	identification	accession
3	29		6.84	*Brevundimonas*	EF088675
3	287		68.79	*Paenibacillus*	AM934687
3	29		6.84	*Sanguibacter*	X79452
3	45		10.68	*Staphylococcus*	AJ717376
3	29		6.84	*Variovorax*	GQ861460

Appendix 5.4 Detailed characterisation of all purified *B. napus* seed isolates from enrichment media. Bacteria living inside seeds sown at both fields (SE-SOWN) and harvested at the control field (SE-CO) and the trace element-contaminated field (SE-TE) were isolated using 3 different liquid growth media (284+869/10; 284+869; IBM+NSY) which were inoculated with the same amount crushed seed solution. Three replicates (repl) per medium were used. The presence of each strain is shown as relative abundances, expressed in percentages of the total number of cultivable isolates per 100 µl culture medium. Strains are identified to the genus level, their accession numbers as well as their presence in the 1st, 2nd or 3rd replicate (repl) are displayed. (Overall) mean percentages were calculated based on the 3 replicates.

SE-SOWN (284 + 1/10 869)						
repl	identification	accession	kve/100µl	% repl	% mean	% overall mean
1	*Burkholderia*	AY512825	52000	20.00	6.67	2.22
	Burkholderia	AY512825	52000	20.00	6.67	2.22
	Burkholderia	AY512825	52000	20.00	6.67	2.22
	Burkholderia	AY512825	52000	20.00	6.67	2.22
	Burkholderia	AY512825	52000	20.00	6.67	2.22
2	*Bacillus*	AM747225	1	0.88	0.29	0.10
	Paenibacillus	AB043866	20	17.52	5.84	1.95
	Paenibacillus	AB043866	20	17.52	5.84	1.95
	Paenibacillus	AB043866	20	17.52	5.84	1.95
	Paenibacillus	AB043866	20	17.52	5.84	1.95
	Paenibacillus	AB043866	20	17.52	5.84	1.95
	Paenibacillus	AB043866	3	2.30	0.77	0.26
	Paenibacillus	AB043866	3	2.30	0.77	0.26
	Paenibacillus	AB043866	3	2.30	0.77	0.26
	Paenibacillus	AB043866	3	2.30	0.77	0.26
	Paenibacillus	AB043866	3	2.30	0.77	0.26
3	*Bacillus*	EU849125	118667	19.82	6.61	2.20
	Bacillus	EU849125	118667	19.82	6.61	2.20
	Bacillus	EU849125	118667	19.82	6.61	2.20
	Bacillus	EU849125	118667	19.82	6.61	2.20
	Bacillus	EU849125	118667	19.82	6.61	2.20
	Staphylococcus	AB177642	1333	0.22	0.07	0.02
	Staphylococcus	AB177642	1333	0.22	0.07	0.02
	Staphylococcus	AB177642	1333	0.22	0.07	0.02
	Staphylococcus	AB177642	1333	0.22	0.07	0.02

SE-SOWN (284 + 869)						
repl	identification	accession	kve/100µl	% repl	% mean	% overall mean
2	*Burkholderia*	AY512825	204000	20.00	20.00	6.67
	Burkholderia	AY512825	204000	20.00	20.00	6.67
	Burkholderia	AY512825	204000	20.00	20.00	6.67
	Burkholderia	AY512825	204000	20.00	20.00	6.67
	Burkholderia	AY512825	204000	20.00	20.00	6.67

SE-SOWN (IBM + NSY)						
repl	identification	accession	kve/100µl	% repl	% mean	% overall mean
1	*Burkholderia*	AY512825	6400000	20.00	10.00	3.33
	Burkholderia	AY512825	6400000	20.00	10.00	3.33

	Burkholderia	AY512825	6400000	20.00	10.00	3.33
	Burkholderia	AY512825	6400000	20.00	10.00	3.33
	Burkholderia	AY512825	6400000	20.00	10.00	3.33
2	*Bacillus*	EU849125	7	24.14	12.07	4.02
	Bacillus	EU849125	3	8.97	4.48	1.49
	Bacillus	EU849125	3	8.97	4.48	1.49
	Bacillus	EU849125	3	8.97	4.48	1.49
	Bacillus	EU849125	3	8.97	4.48	1.49
	Bacillus	EU849125	3	8.97	4.48	1.49
	Bacillus	EU849125	1	3.45	1.72	0.57
	Bacillus	EU849125	1	3.45	1.72	0.57
	Bacillus	EU849125	1	3.45	1.72	0.57
	Bacillus	EU849125	1	3.45	1.72	0.57
	Bacillus	EU849125	1	3.45	1.72	0.57
	Bacillus	EU849125	1	3.45	1.72	0.57
	Bacillus	EU849125	1	3.45	1.72	0.57
	Bacillus	EU849125	1	3.45	1.72	0.57
	Bacillus	EU849125	1	3.45	1.72	0.57

			SE-CO (284 + 1/10 869)			
repl	identification	accession	kve/100µl	% repl	% mean	% overall mean
1	*Bacillus*	FN869038	36000000000	0.80	0.27	0.09
	Bacillus	FN869038	36000000000	0.80	0.27	0.09
	Bacillus	FN869038	36000000000	0.80	0.27	0.09
	Bacillus	FN869038	36000000000	0.80	0.27	0.09
	Bacillus	FN869038	36000000000	0.80	0.27	0.09
	Bacillus	FN869038	36000000000	0.80	0.27	0.09
	Bacillus	FN869038	36000000000	0.80	0.27	0.09
	Bacillus	FN869038	36000000000	0.80	0.27	0.09
	Bacillus	FN869038	36000000000	0.80	0.27	0.09
	Bacillus	FN869038	36000000000	0.80	0.27	0.09
	Bacillus	FN869038	10000000000	0.22	0.07	0.02
	Bacillus	FN869038	10000000000	0.22	0.07	0.02
	Bacillus	FN869038	10000000000	0.22	0.07	0.02
	Bacillus	FN869038	10000000000	0.22	0.07	0.02
	Bacillus	FN869038	10000000000	0.22	0.07	0.02
	Erwinia	AJ001190	730000000000	16.22	5.41	1.80
	Erwinia	AJ001190	730000000000	16.22	5.41	1.80
	Erwinia	AJ001190	730000000000	16.22	5.41	1.80
	Erwinia	AJ001190	730000000000	16.22	5.41	1.80
	Erwinia	AJ001190	730000000000	16.22	5.41	1.80
	Erwinia	AJ001190	40000000000	0.89	0.30	0.10
	Erwinia	AJ001190	40000000000	0.89	0.30	0.10
	Erwinia	AJ001190	40000000000	0.89	0.30	0.10
	Erwinia	AJ001190	40000000000	0.89	0.30	0.10
	Erwinia	AJ001190	40000000000	0.89	0.30	0.10
	Erwinia	AJ001190	40000000000	0.89	0.30	0.10
	Erwinia	AJ001190	40000000000	0.89	0.30	0.10
	Erwinia	AJ001190	40000000000	0.89	0.30	0.10
	Erwinia	AJ001190	40000000000	0.89	0.30	0.10

repl	identification	accession	kve/100µl	% repl	% mean	% overall mean
	Bacillus	FN869038	40000000000	0.89	0.30	0.10
	Erwinia	AJ001190	10000000000	0.22	0.07	0.02
	Bacillus	FN869038	10000000000	0.22	0.07	0.02
	Bacillus	FN869038	10000000000	0.22	0.07	0.02
	Bacillus	FN869038	10000000000	0.22	0.07	0.02
2	*Bacillus*	AM747225	398600000	20.00	6.67	2.22
	Bacillus	AM747225	398600000	20.00	6.67	2.22
	Bacillus	AM747225	398600000	20.00	6.67	2.22
	Bacillus	AM747225	398600000	20.00	6.67	2.22
	Bacillus	AM747225	398600000	20.00	6.67	2.22
3	*Staphylococcus*	AB177642	1	50.00	16.67	5.56
	Staphylococcus	AB177642	1	50.00	16.67	5.56

SE-CO (284 + 869)

repl	identification	accession	kve/100µl	% repl	% mean	% overall mean
1	*Erwinia*	AJ001190	7740000000000	99.87	33.29	11.10
	Erwinia	AJ001190	10000000000	0.13	0.04	0.01
2	*Bacillus*	AM747225	200000000000	100.00	33.33	11.11
3	*Bacillus*	FN869038	42000000000	20.00	6.67	2.22
	Bacillus	FN869038	42000000000	20.00	6.67	2.22
	Bacillus	FN869038	42000000000	20.00	6.67	2.22
	Bacillus	FN869038	42000000000	20.00	6.67	2.22
	Bacillus	FN869038	42000000000	20.00	6.67	2.22

SE-CO (IBM + NSY)

repl	identification	accession	kve/100µl	% repl	% mean	% overall mean
1	*Bacillus*	FN869038	70000000000	2.97	0.99	0.33
	Bacillus	FN869038	70000000000	2.97	0.99	0.33
	Bacillus	FN869038	70000000000	2.97	0.99	0.33
	Bacillus	FN869038	70000000000	2.97	0.99	0.33
	Bacillus	FN869038	70000000000	2.97	0.99	0.33
	Erwinia	AJ001190	10000000000	0.42	0.14	0.05
	Bacillus	FN869038	10000000000	0.42	0.14	0.05
	Bacillus	FN869038	10000000000	0.42	0.14	0.05
	Bacillus	FN869038	10000000000	0.42	0.14	0.05
	Erwinia	AJ001190	394000000000	16.69	5.56	1.85
	Erwinia	AJ001190	394000000000	16.69	5.56	1.85
	Erwinia	AJ001190	394000000000	16.69	5.56	1.85
	Erwinia	AJ001190	394000000000	16.69	5.56	1.85
	Erwinia	AJ001190	394000000000	16.69	5.56	1.85
	Micrococcus	AJ409096	3	0.00	0.00	0.00
	Micrococcus	AJ409096	3	0.00	0.00	0.00
	Micrococcus	AJ409096	3	0.00	0.00	0.00
	Micrococcus	AJ409096	3	0.00	0.00	0.00
	Micrococcus	AJ409096	3	0.00	0.00	0.00
2	*Bacillus*	FN869038	30000000000	3.85	1.28	0.43
	Bacillus	FN869038	30000000000	3.85	1.28	0.43
	Bacillus	FN869038	30000000000	3.85	1.28	0.43
	Bacillus	FN869038	30000000000	3.85	1.28	0.43
	Bacillus	FN869038	30000000000	3.85	1.28	0.43
	Bacillus	EU849125	50000000000	6.41	2.14	0.71

repl	identification	accession	kve/100µl	% repl	% mean	% overall mean
	Bacillus	EU849125	50000000000	6.41	2.14	0.71
	Bacillus	EU849125	50000000000	6.41	2.14	0.71
	Bacillus	EU849125	50000000000	6.41	2.14	0.71
	Bacillus	EU849125	50000000000	6.41	2.14	0.71
	Bacillus	EU849125	26000000000	3.33	1.11	0.37
	Bacillus	EU849125	26000000000	3.33	1.11	0.37
	Bacillus	EU849125	26000000000	3.33	1.11	0.37
	Bacillus	EU849125	26000000000	3.33	1.11	0.37
	Bacillus	EU849125	26000000000	3.33	1.11	0.37
	Bacillus	FN869038	10000000000	1.28	0.43	0.14
	Bacillus	FN869038	10000000000	1.28	0.43	0.14
	Bacillus	FN869038	10000000000	1.28	0.43	0.14
	Bacillus	FN869038	16000000000	2.05	0.68	0.23
	Bacillus	FN869038	16000000000	2.05	0.68	0.23
	Bacillus	FN869038	16000000000	2.05	0.68	0.23
	Bacillus	FN869038	16000000000	2.05	0.68	0.23
	Bacillus	FN869038	16000000000	2.05	0.68	0.23
	Bacillus	FN869038	10000000000	1.28	0.43	0.14
	Bacillus	FN869038	10000000000	1.28	0.43	0.14
	Bacillus	FN869038	10000000000	1.28	0.43	0.14
	Bacillus	EU849125	12000000000	1.54	0.51	0.17
	Bacillus	EU849125	12000000000	1.54	0.51	0.17
	Bacillus	EU849125	12000000000	1.54	0.51	0.17
	Bacillus	EU849125	12000000000	1.54	0.51	0.17
	Bacillus	EU849125	12000000000	1.54	0.51	0.17
	Bacillus	EU849125	10000000000	1.28	0.43	0.14
	Bacillus	EU849125	10000000000	1.28	0.43	0.14
	Bacillus	FN869038	10000000000	1.28	0.43	0.14
	Bacillus	FN869038	10000000000	1.28	0.43	0.14
	Bacillus	FN869038	10000000000	1.28	0.43	0.14
3	*Bacillus*	EU849125	256000000000	19.54	6.51	2.17
	Bacillus	EU849125	256000000000	19.54	6.51	2.17
	Bacillus	EU849125	256000000000	19.54	6.51	2.17
	Bacillus	EU849125	256000000000	19.54	6.51	2.17
	Bacillus	EU849125	256000000000	19.54	6.51	2.17
	Bacillus	EU849125	10000000000	0.76	0.25	0.08
	Bacillus	FN869038	10000000000	0.76	0.25	0.08
	Micrococcus	EU438932	10000000000	0.76	0.25	0.08

SE-TE (284 + 1/10 869)

repl	identification	accession	kve/100µl	% repl	% mean	% overall mean
1	*Bacillus*	AM747225	2800000	2.72	0.91	0.30
	Bacillus	AM747225	2800000	2.72	0.91	0.30
	Bacillus	AM747225	2800000	2.72	0.91	0.30
	Bacillus	AM747225	2800000	2.72	0.91	0.30
	Bacillus	AM747225	2800000	2.72	0.91	0.30
	Bacillus	AM747225	89000000	86.41	28.80	9.60
2	*Bacillus*	AM747225	170000000	100.00	33.33	11.11
3	*Bacillus*	FN869038	1333	16.67	5.56	1.85
	Bacillus	FN869038	1333	16.67	5.56	1.85

	Bacillus	FN869038	1333	16.67	5.56	1.85
	Bacillus	FN869038	1333	16.67	5.56	1.85
	Bacillus	FN869038	1333	16.67	5.56	1.85
	Bacillus	FN869038	1333	16.67	5.56	1.85

SE-TE (284 + 869)

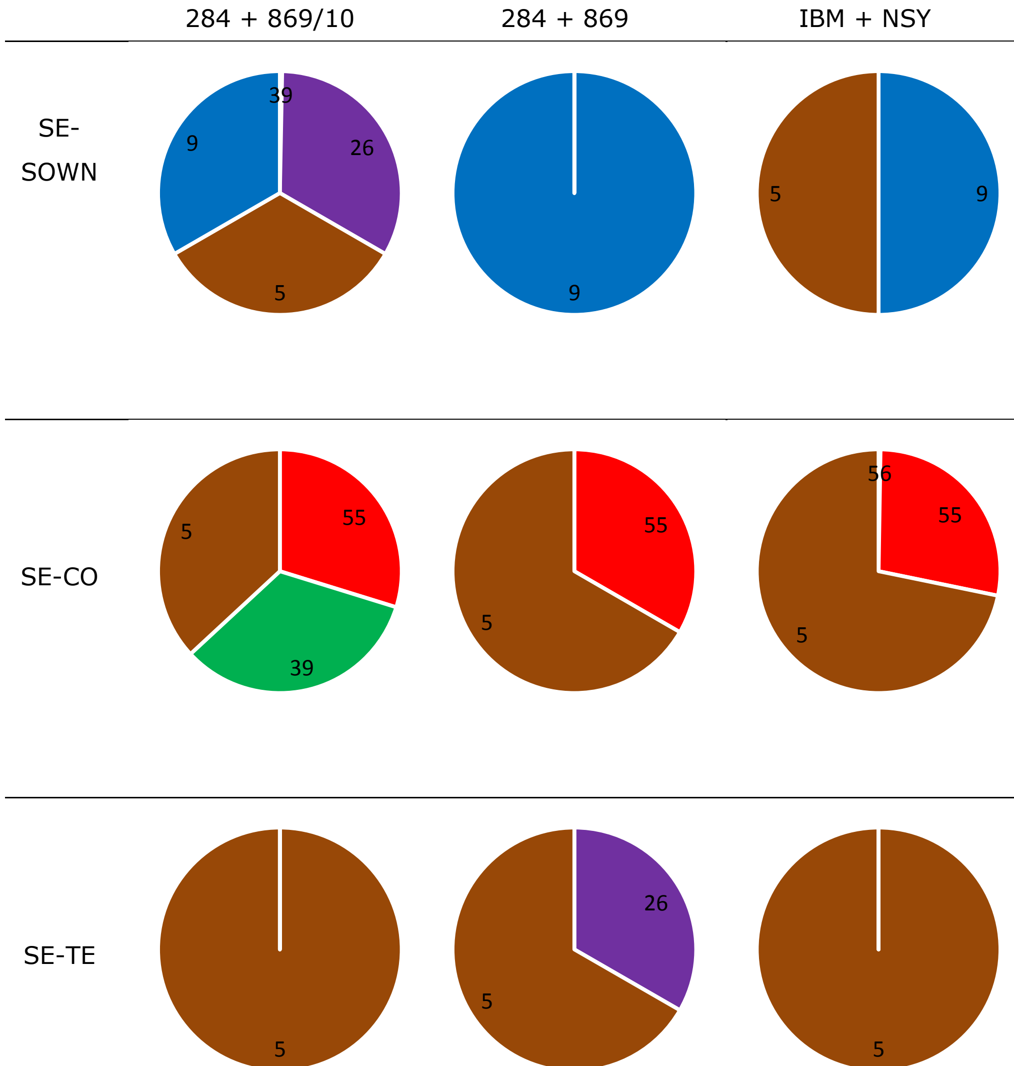
repl	identification	accession	kve/100µl	% repl	% mean	% overall mean
1	*Bacillus*	FN869038	220000000000	19.64	6.55	2.18
	Bacillus	FN869038	220000000000	19.64	6.55	2.18
	Bacillus	FN869038	220000000000	19.64	6.55	2.18
	Bacillus	FN869038	220000000000	19.64	6.55	2.18
	Bacillus	FN869038	220000000000	19.64	6.55	2.18
	Bacillus	FN869038	10000000000	0.89	0.30	0.10
	Bacillus	FN869038	10000000000	0.89	0.30	0.10
2	*Bacillus*	EU849125	46000000	100.00	33.33	11.11
3	*Bacillus*	FN869038	1	0.04	0.01	0.00
	Paenibacillus	AM162342	458	19.99	6.66	2.22
	Paenibacillus	AM162342	458	19.99	6.66	2.22
	Paenibacillus	AM162342	458	19.99	6.66	2.22
	Paenibacillus	AM162342	458	19.99	6.66	2.22
	Paenibacillus	AM162342	458	19.99	6.66	2.22

SE-TE (IBM + NSY)

repl	identification	accession	kve/100µl	% repl	% mean	% overall mean
1	*Bacillus*	EU849125	134000000000	7.32	2.44	0.81
	Bacillus	EU849125	134000000000	7.32	2.44	0.81
	Bacillus	EU849125	134000000000	7.32	2.44	0.81
	Bacillus	EU849125	134000000000	7.32	2.44	0.81
	Bacillus	EU849125	134000000000	7.32	2.44	0.81
	Bacillus	EU849125	93000000000	5.08	1.69	0.56
	Bacillus	EU849125	93000000000	5.08	1.69	0.56
	Bacillus	EU849125	93000000000	5.08	1.69	0.56
	Bacillus	EU849125	93000000000	5.08	1.69	0.56
	Bacillus	EU849125	93000000000	5.08	1.69	0.56
	Bacillus	EU849125	93000000000	5.08	1.69	0.56
	Bacillus	EU849125	93000000000	5.08	1.69	0.56
	Bacillus	EU849125	93000000000	5.08	1.69	0.56
	Bacillus	EU849125	93000000000	5.08	1.69	0.56
	Bacillus	EU849125	93000000000	5.08	1.69	0.56
	Bacillus	EU849125	16000000000	0.87	0.29	0.10
	Bacillus	EU849125	16000000000	0.87	0.29	0.10
	Bacillus	EU849125	16000000000	0.87	0.29	0.10
	Bacillus	EU849125	16000000000	0.87	0.29	0.10
	Bacillus	EU849125	16000000000	0.87	0.29	0.10
	Bacillus	EU849125	24000000000	1.31	0.44	0.15
	Bacillus	EU849125	24000000000	1.31	0.44	0.15
	Bacillus	EU849125	24000000000	1.31	0.44	0.15
	Bacillus	EU849125	24000000000	1.31	0.44	0.15
	Bacillus	EU849125	24000000000	1.31	0.44	0.15
	Bacillus	EU849125	10000000000	0.55	0.18	0.06
	Bacillus	EU849125	10000000000	0.55	0.18	0.06

	Bacillus	EU849125	10000000000	0.55	0.18	0.06
2	*Bacillus*	FN869038	52000000000	3.35	1.12	0.37
	Bacillus	FN869038	52000000000	3.35	1.12	0.37
	Bacillus	FN869038	52000000000	3.35	1.12	0.37
	Bacillus	FN869038	52000000000	3.35	1.12	0.37
	Bacillus	FN869038	52000000000	3.35	1.12	0.37
	Bacillus	FN869038	252000000000	16.26	5.42	1.81
	Bacillus	FN869038	252000000000	16.26	5.42	1.81
	Bacillus	FN869038	252000000000	16.26	5.42	1.81
	Bacillus	FN869038	252000000000	16.26	5.42	1.81
	Bacillus	FN869038	252000000000	16.26	5.42	1.81
	Bacillus	FN869038	10000000000	0.65	0.22	0.07
	Bacillus	FN869038	10000000000	0.65	0.22	0.07
	Bacillus	FN869038	10000000000	0.65	0.22	0.07
3	*Bacillus*	FN869038	34000000000	4.15	1.38	0.46
	Bacillus	FN869038	34000000000	4.15	1.38	0.46
	Bacillus	FN869038	34000000000	4.15	1.38	0.46
	Bacillus	FN869038	34000000000	4.15	1.38	0.46
	Bacillus	FN869038	34000000000	4.15	1.38	0.46
	Bacillus	FN869038	30000000000	3.66	1.22	0.41
	Bacillus	FN869038	30000000000	3.66	1.22	0.41
	Bacillus	FN869038	30000000000	3.66	1.22	0.41
	Bacillus	FN869038	30000000000	3.66	1.22	0.41
	Bacillus	FN869038	30000000000	3.66	1.22	0.41
	Bacillus	FN869038	44000000000	5.37	1.79	0.60
	Bacillus	FN869038	44000000000	5.37	1.79	0.60
	Bacillus	FN869038	44000000000	5.37	1.79	0.60
	Bacillus	FN869038	44000000000	5.37	1.79	0.60
	Bacillus	FN869038	44000000000	5.37	1.79	0.60
	Bacillus	FN869038	10000000000	1.22	0.41	0.14
	Bacillus	FN869038	10000000000	1.22	0.41	0.14
	Bacillus	FN869038	10000000000	1.22	0.41	0.14
	Bacillus	FN869038	10000000000	1.22	0.41	0.14
	Bacillus	FN869038	10000000000	1.22	0.41	0.14
	Bacillus	FN869038	10000000000	1.22	0.41	0.14
	Bacillus	FN869038	10000000000	1.22	0.41	0.14
	Bacillus	FN869038	20000000000	2.44	0.81	0.27
	Bacillus	FN869038	20000000000	2.44	0.81	0.27
	Bacillus	FN869038	20000000000	2.44	0.81	0.27
	Bacillus	FN869038	20000000000	2.44	0.81	0.27
	Bacillus	FN869038	10000000000	1.22	0.41	0.14
	Bacillus	FN869038	10000000000	1.22	0.41	0.14
	Bacillus	FN869038	10000000000	1.22	0.41	0.14
	Bacillus	FN869038	16666666667	2.03	0.68	0.23
	Bacillus	FN869038	16666666667	2.03	0.68	0.23
	Bacillus	FN869038	16666666667	2.03	0.68	0.23
	Bacillus	FN869038	16666666667	2.03	0.68	0.23
	Bacillus	FN869038	16666666667	2.03	0.68	0.23
	Bacillus	FJ937903	16666666667	2.03	0.68	0.23

Appendix 5.5 Diversity and abundance of cultivable bacterial strains isolated from *B. napus* seeds which were sown (SE-SOWN) and harvested in June 2010 at the control field (SE-CO) and the trace element-contaminated field (SE-TE). Each colour (number, see figure 5.4) represents a bacterial genus and pie fragments indicate the relative abundance, expressed in percentages of the total number of cultivable isolates per 100 µl culture medium (inoculated with the same amount crushed seed solution). Three different growth media were used to enrich the minimal medium which was present at inoculation (284+869/10; 284+869; IBM+NSY). Data are means of 3 replicates.



Appendix 5.6 Correlation coefficients (CC) between *B. napus* root (R), shoot (S) and seed (SE) bacterial communities isolated at the control field (CO-F) and the contaminated field (TE-F) in December (D) and June (J). Genotypic information was subjected to correspondence analysis (CA), a principal component analysis related ordination technique based on chi-square distances. Compartment data are based on at least 3 replicates consisting of 3 independent samples (mixed samples).

	R.CO.D.	R.CO.J.	R.TE.D.	R.TE.J.	S.CO.D.	S.CO.J.	S.TE.D.	S.TE.J.
R.CO.D.	1.00000000	0.852709795	0.62684842	0.64673293	0.78436354	0.03469715	0.54177451	-0.011653530
R.CO.J.	0.85270980	1.000000000	0.58784873	0.63480843	0.76393416	0.27207121	0.40217181	0.001780249
R.TE.D.	0.62684842	0.587848728	1.00000000	0.56636814	0.88229335	0.58958154	0.54719979	-0.063751976
R.TE.J.	0.64673293	0.634808426	0.56636814	1.00000000	0.72513927	0.03006330	0.74273973	-0.064528931
S.CO.D.	0.78436354	0.763934159	0.88229335	0.72513927	1.00000000	0.34461901	0.65636417	-0.037373090
S.CO.J.	0.03469715	0.272071210	0.58958154	0.03006330	0.34461901	1.00000000	0.04718626	-0.045967470
S.TE.D.	0.54177451	0.402171814	0.54719979	0.74273973	0.65636417	0.04718626	1.00000000	-0.064134870
S.TE.J.	-0.01165353	0.001780249	-0.06375198	-0.06452893	-0.03737309	-0.04596747	-0.06413487	1.000000000
SEED.TE	0.07014916	0.075858434	0.18861122	0.08484948	0.18522904	0.10792473	0.12268971	0.156011778
SE.SOWN	-0.07420192	0.035030698	0.24767463	-0.08876906	0.05696097	0.34809074	-0.06227880	0.114014034
SE.CO	-0.07565037	0.162612444	0.44147988	-0.08906527	0.18858720	0.80632929	-0.05709511	-0.026660323
SE.TE	-0.05570825	0.213120114	0.52040878	-0.06665426	0.23429674	0.92352963	-0.03837397	0.086481292

	SEED.TE	SE.SOWN	SE.CO	SE.TE
R.CO.D.	0.07014916	-0.07420192	-0.07565037	-0.05570825
R.CO.J.	0.07585843	0.03503070	0.16261244	0.21312011
R.TE.D.	0.18861122	0.24767463	0.44147988	0.52040878
R.TE.J.	0.08484948	-0.08876906	-0.08906527	-0.06665426
S.CO.D.	0.18522904	0.05696097	0.18858720	0.23429674
S.CO.J.	0.10792473	0.34809074	0.80632929	0.92352963
S.TE.D.	0.12268971	-0.06227880	-0.05709511	-0.03837397
S.TE.J.	0.15601178	0.11401403	-0.02666032	0.08648129
SEED.TE	1.00000000	0.02014769	0.06853044	0.13258088
SE.SOWN	0.02014769	1.00000000	0.32203125	0.40253789
SE.CO	0.06853044	0.32203125	1.00000000	0.86446406
SE.TE	0.13258088	0.40253789	0.86446406	1.00000000

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Chapter 6

Selection of promising *Brassica napus* L.-associated bacterial strains for improving Cd phytoextraction

Abstract

An experimental set-up is described for the selection of *Brassica napus* L.-associated bacterial strains that can improve Cd phytoextraction. This selection starts with an *in vitro* screening of a huge number of bacteria. The most promising bacteria were tested *in planta* in different experimental set-ups, which are step by step increasing in complexity, but also better mimicking real field conditions.

Brassica napus plants grown at a Zn-, Pb- and Cd-contaminated and a non-contaminated field were sampled for the isolation of bacteria from bulk soil, rhizosphere soil, roots and shoots. Out of 850 bacterial isolates, 63 strains were selected based on their *in vitro* phenotypic characteristics (Cd/Zn tolerance, phosphate solubilisation, nitrogen fixation and production of siderophores, organic acids, indole-3-acetic acid, acetoin and 1-aminocyclopropane-1-carboxylate deaminase). These strains, with the potential to improve growth and/or Cd uptake of plants, were inoculated in *B. napus* grown on agar medium in order to examine their *in planta* functioning. Bacteria that could enhance Cd phytoextraction on agar were tested in plants growing on sand and the best-performing strains on sand were used for the final experiments where plants were grown in field soil.

The last selection resulted in 5 strains (4 *Pseudomonas* sp. and 1 *Variovorax* sp.), all isolated at the contaminated field from the roots or rhizosphere of *B. napus*. The beneficial effects exhibited by these bacteria were related to their metal tolerance and plant growth-promoting efficiency, and indicate that their inoculation might significantly improve phytoextraction efficiency of metal-contaminated soils.

Introduction

The excessive presence of toxic metals in agricultural soils is of increasing concern due to food safety issues and potential health risks as well as detrimental effects on soil ecosystems (McLaughlin *et al*., 1999). Since, in contrast to organic contaminants, metals cannot be degraded to harmless products and hence persist in the environment indefinitely, metal contamination is one of the most severe environmental problems (Rajkumar *et al*., 2009). Contamination with metals can result from industrial (*e.g.* mining and smelting of metal ores), military and agricultural activities (Qu *et al*., 2011; Ali *et al*., 2013). Among toxic metals, cadmium (Cd) is relatively mobile in soils and is one of the most dangerous for organisms (Alloway, 1995). In plants, Cd inhibits root and shoot growth, affects nutrient uptake and homeostasis, changes chloroplast ultrastructure, initiates oxidative stress and is frequently accumulated by important crops consumed by animals and humans (Wang *et al*., 2009; Atafar *et al*., 2010; Gill *et al*., 2011). Soil contamination with Cd also negatively affects biodiversity and the activity of soil microbial communities (Liao *et al*., 2005). Therefore, the development of remediation strategies that can remove or neutralize its toxic effects in soils, sediments and wastewaters is urgent for environmental conservation and human health (Abou-Shanab *et al*., 2006).

Commonly used clean-up methods for soils involve excavation and/or chemical cleaning. These conventional technologies are generally too costly to be used to restore vast and diffusely contaminated sites (Vangronsveld *et al*., 1995, 1996, 2009). Moreover, they are harmful to the general soil properties (*i.e.* structure and organic matter) (Holden, 1989). Consequently, techniques with lower costs and a more environmental friendly nature are emerging and received increasing attention in the last decades (Kumar *et al*., 1995; Haque *et al*., 2008; Chehregani *et al*., 2009; Kotrba *et al*., 2009). Bioremediation is a relatively recent technology that utilizes the possibility of the *in situ* attenuation/elimination/transformation of hazardous materials exploiting particular biological processes. Among these, plant-assisted bioremediation, better known as phytoremediation has shown good results (Wenzel, 2009).

Phytoremediation, using plants to remove pollutants from the environment is considered as an aesthetically pleasing, cost-effective and eco-friendly technology (Vangronsveld *et al.,* 2009; Raziuddin *et al*., 2011; Shin *et al*.,

2012). This technique may be employed using different approaches including phytoextraction, phytovolatilization and phytostabilization (Chaney *et al*., 1997). In particular, phytoextraction refers to the ability of hyperaccumulator plants to uptake metals from soil and transport them to the above-ground parts, which are able to accumulate concentrations up to 100-fold greater than those normally found in non-accumulator species (Baker and Brooks, 1989; Baker *et al*., 2000; McGrath and Zhao, 2003). The effectiveness of the phytoextraction process, whereby metals are removed from soil, primarily depends on an adequate plant yield and high metal concentration in the above-ground tissues of the plant (Rajkumar *et al.*, 2009). Most hyperaccumulators are not suitable for phytoremediation field applications due to their slow growth and low-biomass production (Puschenreiter *et al*., 2001). Hence, recent research projects on phytoextraction have focused on high-biomass crop species and soil management practices to enhance the metal uptake of these species (Chen *et al.*, 2004). Among many fast-growing and high biomass-accumulating plant species suitable for phytoextraction, *Brassicaceae* have received considerable attention (Prasad and Freitas, 2003) based on their capacity to uptake and accumulate metals in amounts higher than those of many other plant species (Kumar *et al*., 1995). *Brassica juncea* is one of the most promising, non-hyperaccumulating species for extracting metals from contaminated soils, however other species of the *Brassica* genus, such as *B. campestris, B. carinata, B. napus, B. nigra, B. oleracea* and *B. rapa*, have also been studied (Kumar *et al*., 1995; Rossi *et al*., 2002; Marchiol *et al*., 2004; Meers *et al*., 2005; Gisbert *et al*., 2006; Szczygłowska *et al*., 2011; Bareen, 2012).

Brassica napus L. (rapeseed) deserves special attention since it is cultivated worldwide and one of the most common oil sources (Park *et al*., 2012). Rapeseed can be a very useful candidate for phytoextraction because of its fast growth, high above-ground biomass and high metal uptake potential (Vamerali *et al*., 2010). The capability of *B. napus* to accumulate Cd and translocate this element into the harvestable parts has been described by Rossi *et al*. (2002) and Grispen *et al*. (2006). These results strengthen the suggestion that *B. napus* may be effective for phytoremediation of Cd-polluted soils. However, the harvestable parts might be utilized only for industrial purposes and not for human or animal consumption. Since phytoextraction is a long-lasting process

(Felix, 1997; Vangronsveld *et al*., 2009), fields undergoing phytoremediation need to generate an economic benefit in order to achieve an economically viable and socially acceptable decontamination. The use of energy and/or bio-diesel crops (*e.g. B. napus*) as metal phytoextraction plants would provide the contaminated soil an economic value and in this way decrease remediation costs (Kos *et al*., 2003; Witters *et al*., 2012a,b).

Several factors still restrict widespread application of phytoextraction including (a) too low growth rate and small biomass of the plants, (b) phytotoxicity of metals, and (c) too limited metal uptake (Kumar *et al*., 1995; Burd *et al*., 2000; Kayser *et al*., 2000; Quartacci *et al*., 2006; Li *et al*., 2007). This has prompted scientists to explore alternative strategies to improve the efficiency of phytoremediation of metal-contaminated soils. Many researchers are exploring the possibilities of enhancing the biomass and metal uptake by plants using metal-tolerant and plant growth-promoting bacteria (PGPB) as bioinoculants (Abou-Shanab *et al*., 2006; Sheng and Xia, 2006; Rajkumar *et al*., 2009; Weyens *et al.,* 2009a,b; Ma *et al.*, 2011; Luo *et al.*, 2012). Bacteria can improve plant growth through various mechanisms including the production of plant growth-promoting substances such as indole-3-acetic acid (IAA), acetoin, siderophores, 1-aminocyclopropane-1-carboxylic (ACC) deaminase, or phosphate solubilisation and nitrogen fixation (Ryan *et al*., 2008; Weyens *et al.* 2009a). The IAA released by bacteria enhances plant growth directly by stimulating cell elongation or affecting cell division (Glick *et al*., 1998; Spaepen *et al*., 2007; Dodd *et al*., 2010; Hayat *et al*., 2010). Also other plant growth hormones produced by microorganisms can improve the growth of plants (Xie *et al*., 2006). Acetoin is used as an external energy storage by a number of fermentative bacteria and trigger plant growth promotion and induced systemic resistance in plants (Xiao and Xu, 2007; Farag *et al*., 2006; Rudrappa *et al*., 2010). Bacterial siderophores, which can solubilize and sequester iron from the soil by binding the unavailable form of Fe^{+3}, can make iron available for uptake by the plant roots, leading thereby to an improved plant growth (Burd *et al*., 2000; Rajkumar *et al*., 2010). Decreasing excess ethylene production by bacterial ACC deaminase activity is one of the major mechanisms in promoting plant growth (Glick *et al*., 1998; Hontzeas *et al*., 2004; Ma *et al*., 2011). Plant growth promotion can also be influenced by microorganisms through nutrient

mineralization (Koslowsky and Boerner, 1989; Adesemoye *et al*., 2009), solubilisation of mineral phosphate (Kloepper *et al*., 1989; Zaidi *et al*., 2006; Linu *et al*., 2009; Lugtenberg and Kamilova, 2009) and nitrogen fixation (Malik *et al*., 1997; Lucy *et al*., 2004).

Plants also benefit from bacteria that act out biocontrol of pathogenic organisms (Bashan & Holguin, 1998; Gamalero *et al*., 2002). There are several mechanisms by which this proceeds, including bacterial production of antibiotics and antifungal metabolites, competition between the beneficial bacteria and pathogens for nutrients and niches along the plant tissues, and induced systemic resistance where colonization of root tissues by local plant growth-promoting rhizobacteria (PGPR) leads to systemic plant defence signalling that deters pathogen action (Lugtenberg and Kamilova 2009).

Apart from a low plant biomass, a limited mobility of metals in soils is another main constraint for feasible phytoextraction of metals (Kayser *et al*., 2000; Garbisu and Alkorta, 2001; Chen *et al*., 2004). A large proportion of many metals is adsorbed or occluded by carbonates, organic matter, Fe–Mn oxides and primary or secondary minerals (Garbisu and Alkorta, 2001). Soil factors such as pH, cation exchange capacity, or organic matter content play an important role in successful soil remediation processes. Certain PGPR can alter metal availability to the plant by producing organic ligands, exudation of organic acids and complexation of cationic metals or desorption of anionic species via ligand exchange by siderophores (Gadd, 2004; Saravanan *et al*., 2007; Long *et al*., 2011; Yeh *et al*., 2012; Freitas *et al*., 2013). Thus bacteria, including metal-tolerant PGPR, can affect trace metal mobility and availability for possible plant uptake (Zhuang *et al*., 2007; Khan *et al*., 2009; Glick, 2010; de-Bashan *et al*., 2012), making phytoextraction more effective. Once taken up by the plant, toxic metals cause deleterious effects.

Metal-tolerant endophytes possess different tolerance mechanisms involving exclusion, active removal, biosorption, precipitation or bioaccumulation (Haferburg and Kothe, 2007; Harrison *et al*., 2007). These processes can influence the solubility and the availability of the metal to the plant, thus modifying the toxic effects of the metal (Lodewyckx *et al*., 2001). Metal-tolerant endophytic bacteria not only protect plants from metal toxicity but also enhance the metal accumulation in plant tissue with concurrent stimulation of plant

growth (Rajkumar *et al*., 2009). There exists also evidence that treatment with some plant growth hormones diminishes the inhibitory effect of Cd on plant growth (Moya *et al*., 1995; Wozny *et al*., 1995). Siderophores are another important metabolite released by the PGPB that indirectly alleviate metal toxicity by increasing the supply of iron to the plant (Burd *et al*., 2000). The presence of PGPB that produce ACC deaminase and IAA increases plant tolerance to contaminants (Reed and Glick 2005; Reed *et al*., 2005; Belimov *et al*., 2005; Burd *et al*., 1998; Mayak *et al*., 2004) and plant pathogens (Wang *et al*., 2000).

Many of the studies cited above show that inoculation of bacteria possessing these interesting traits (plant growth promotion and/or metal tolerance) have positive effects on plant growth and/or metal uptake. However, most of the experiments have been performed on laboratory scale under highly controlled conditions that are hardly comparable to field conditions.

In this study a large collection of thoroughly *in vitro* characterized rapeseed-associated bacteria (Croes *et al*., 2014) was used to make a selection of interesting bacteria, based on their potential to improve plant growth and Cd uptake. The *in planta* characteristics of these bacteria were first tested in *B. napus* seedlings on vertical agar plates (VAPs) containing Cd. The best-performing strains were further selected for inoculation experiments on sand spiked with Cd. Our final selection was inoculated on field soil and, like the other above mentioned *in planta* experiments, performed in a growth chamber. The effects of inoculation were evaluated based on plant growth, plant Cd uptake and $Ca(NO_3)_2$-exchangeable Cd concentrations in the sand/soil.

Experimental Procedures

Inoculation

Based on the *in vitro* phenotypic characteristics from a total of 850 *Brassica napus*-associated bacterial strains, isolated at a contaminated (Cd, Zn and Pb) and a non-contaminated field in June and December 2010 (Croes *et al*., 2013, 2014), a selection of 63 strains was made (see table 6.1). These strains, with potential to improve plant growth and Cd uptake, were used for inoculation.

Seeds of *B. napus* L. (rapeseed) were surface-sterilized (0.1% sodium hypochlorite; 1 drop Tween 80 per 100 ml) for 1 min, rinsed 3 times with sterile tap water, and placed on moist filter paper at 4°C for 2 nights in order to

synchronize germination. The next day, bacterial cultures were grown in 869 medium (Mergeay *et al*., 1985) at 30°C for 24 h, after which the bacterial cells were collected by centrifugation (for 30 min by 4000 rpm) and resuspended in sterile 10 mM $MgSO_4$ until a density of 10^9 cfu ml^{-1} was reached (OD_{660nm}=1). Surface-sterilized and homogenized seeds were imbibed for 1 h in a 10 times diluted bacterial suspension (10^8 cfu ml^{-1}) or in a sterile 10 mM $MgSO_4$ solution (control). Subsequently, soaked seeds were transferred to 1/10 strength 869 agar medium. Before sowing on sterile vertical agar plates (VAPs), non-sterile calibrated sand or field soil, seeds were incubated overnight in a strictly controlled LED-lighted growth chamber (12 h photoperiod, 22°C/18°C day/night, photosynthetic photon flux density of 200 µmol m^{-2} s^{-1}, 65 % relative humidity).
Since the reisolation of all inoculated bacteria was practically impossible for all tested strains, our inoculation protocol on VAPs and sand/soil, was based on preliminary inoculation experiments using strains of *Methylobacterium* (accession number AB220076) and *Pseudomonas* (accession number FN377713) (unpublished results). On VAPs, only inoculation of seeds was performed as described above. On sand and soil, inoculation of seeds was followed by a weekly addition of 20 ml bacterial suspension (in 10 mM $MgSO_4$) prepared to reach an end concentration of 10^8 cfu ml^{-1} per pot. This concentration lead to good reisolation yields of the inoculated strains and thus a successful colonization of the plants.

Experiments on VAPs

All selected strains were first tested *in planta* on VAPs, according to a modified protocol of Remans *et al*. (2012). Inoculated and non-inoculated *B. napus* seeds were sown in square petri dishes (21 x 21 cm) on 200 ml sterile Murashige and Skoog (MS) medium with 1% (w/v) plant agar (pH 5.7 - 5.8 with KOH) containing 250 µM Cd (as $3CdSO_4.8H_2O$) (Murashige and Skoog, 1962). Per condition, 3 agar plates with each 5 seedlings made up 15 replicates. The control condition with non-inoculated seeds contained at least one third replicates of the total amount of plants compared with this control group. After 10 days growth in the growth chamber, plates were scanned on a Canoscan 4400F (Canon) at 600 d.p.i. and primary root length was analysed using the Optimas 6.1 Image analysis program (Media Cybernetics). At the end of the

experiment, root and shoot weights were determined as well as their Cd uptake (see below).

Experiments on sand and soil

From the results of the experiments on VAPs, the 25 best-performing strains were selected based on their host plant Cd uptake and improvement of plant growth especially in the shoot. This selection of bacteria was further investigated (in triplicate) on sand spiked with 5 mg Cd kg^{-1}. Each pot contained 1 plant and 1300 g calibrated sand with 200 ml half strength Hoagland's nutrient solution (1/2 HL) (per liter distilled water 50 ml macro-elements, 500 µl micro-elements and 300 µl Fe-EDTA (macro-elements (g l^{-1}): 10.2 HNO_3, 7.08 $Ca(NO_3)_2.4H_2O$, 2.30 $NH_4H_2PO_4$, 4.9 $MgSO_4.7H_2O$; micro-elements (g l^{-1}): 2.86 H_3BO_3, 1.81 $MnCl_2.4H_2O$, 0.08 $CuSO_4.5H_2O$, 0.09 $H_2MoO_4.H_2O$, 0.22 $ZnSO_4.7H_2O$; Fe-EDTA (g l^{-1}): 5.00 EDTA-Na, 7.60 $FeSO_4.7H_2O$)).

The most promising bacteria on sand were screened on field soil (Lommel, Belgium) contaminated with Cd, Zn and Pb due to aerial deposition from zinc smelters (Ruttens *et al*., 2010). Seeds were sown in 1 kg soil saturated with tap water. As on sand, 3 biologically independent replicates were used per inoculated condition and non-inoculated plants (one third of the inoculated plants) grown on the same substrate served as controls. Plants were watered every 2 days.

After 4 weeks of growth on sand or soil, several phytotoxicity parameters were investigated. Plant (root and shoot) weight as well as the Cd concentration in the sand/soil ($Ca(NO_3)_2$-exchangeable fraction) and in the shoot were considered.

Evaluation of the effect of inoculation on plants grown on VAPs, sand and soil

All phytotoxicity parameters were calculated relative to corresponding non-inoculated plants; due to the high number of tested strains these parameters could not be collected in a single inoculation experiment. Statistics were performed separately within each set-up, control values were calculated combining the control data derived from the different set-ups.

Plant growth

At harvest, roots and shoots were separated and their weights were determined for 3 biologically independent replicates per inoculated condition. For the pot experiments all plants were sampled while for the plants grown on VAPs 3 mean values were obtained by calculating the mean root and shoot weight per square petri dish (each condition was represented by 3 dishes each containing 5 seedlings). For the VAPs experiments, root lengths were additionally measured (electronically) for 15 biologically independent replicates per inoculated condition.

Trace element concentrations in sand/soil and plants

During the harvest of plants grown in pots, plants were vigorously washed with tap water to remove surface adhered soil particles. Three root and shoot samples per inoculated condition were oven-dried (48 h at 65°C) and subsequently crushed to a fine powder. Root and shoot samples harvested from the VAPs experiments were all pooled per plate within one condition. Due to insufficient dry matter, in some cases all roots/shoots had to be pooled excluding statistical evaluation of some results obtained in the VAPs experiments. This was not considered as a problem, since the eventual effects on trace element concentrations had to be confirmed on sand and soil.

After drying, the samples were 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 4 h. Samples were then dissolved in HCl (37%) and diluted to a final volume of 5 ml (2% HCl). Cadmium concentrations (mg kg^{-1} dry weight) were determined using inductively coupled plasma optical emission spectrometry (ICP-OES).

Additionally, during the inoculation test on sand and soil, plant available fractions of trace elements present in the substrate were estimated using 0.1 M $Ca(NO_3)_2$ extraction (Mench *et al*., 1994). Trace element concentrations (mg kg^{-1} dry weight) were determined as described above. For each inoculated condition, 3 independent sand/soil samples were used.

Statistical analysis

All datasets were statistically analysed using one-way ANOVA and post hoc pair-wise comparison testing (Dunnett's two-tailed t-test) where all independent

groups are compared with the same control group. Since this control group acts for several independent groups, the group contained at least one-third plants of the total amount of plants compared with this control group. Transformations were applied when necessary to approximate normality and/or homoscedasticity. In case normality could not be reached, data were analysed using Kruskal-Wallis multiple comparisons test (non-parametric) and multiple comparisons were accomplished using Pairwise Wilcoxon rank sum test. The statistical analyses were performed in R 2.13.0.

Results

Inoculation experiments on VAPs

All 63 bacterial strains that were selected based on their *in vitro* phenotypic characteristics, were evaluated for their *in planta* ability to promote plant growth and Cd uptake on VAPs. An overview of the genotypic and phenotypic characteristics of each strain, together with the used codes, is provided in table 6.1. Below, these strain codes are used to refer to a specific strain. Twenty-three strains (see table 6.1b) had a negative or no effect on plant growth and Cd uptake on VAPs, and were therefore excluded from the results section. Root length, plant weight and Cd concentration in shoot and root, after inoculating the most promising 40 strains (table 6.1a), are presented in figures 6.1 (growth parameters) and 6.2 (Cd concentrations).

Vertical agar plate (VAP) experiments specifically aimed to investigate the effects of contaminant exposure on the development of the root system by determining length of primary and secondary roots and numbers of secondary roots. Strains 6a, 10b, 32a, 36b, 47a and 124b isolated from the rhizosphere soil (RS) and root (R) endophytic strains 10a and 62a significantly promoted primary root length during Cd exposure (figure 6.1a). Significantly increased root and shoot weights were observed after inoculation with rhizosphere strain 36b and root endophytic strains 10a and 21e (figure 6.1b,c). Rhizosphere strains 52a and 92c and root strain 49h significantly stimulated shoot growth (figure 6.1b), while rhizosphere strain 124b and root strains 23a and 33b significantly promoted root growth (figure 6.1c).

Table 6.1 Detailed characterisation of all selected bulk soil, rhizosphere soil and *B. napus* tissue isolates collected at a control field (CO) and a contaminated field (TE) in December (Dec) and June (Jun).

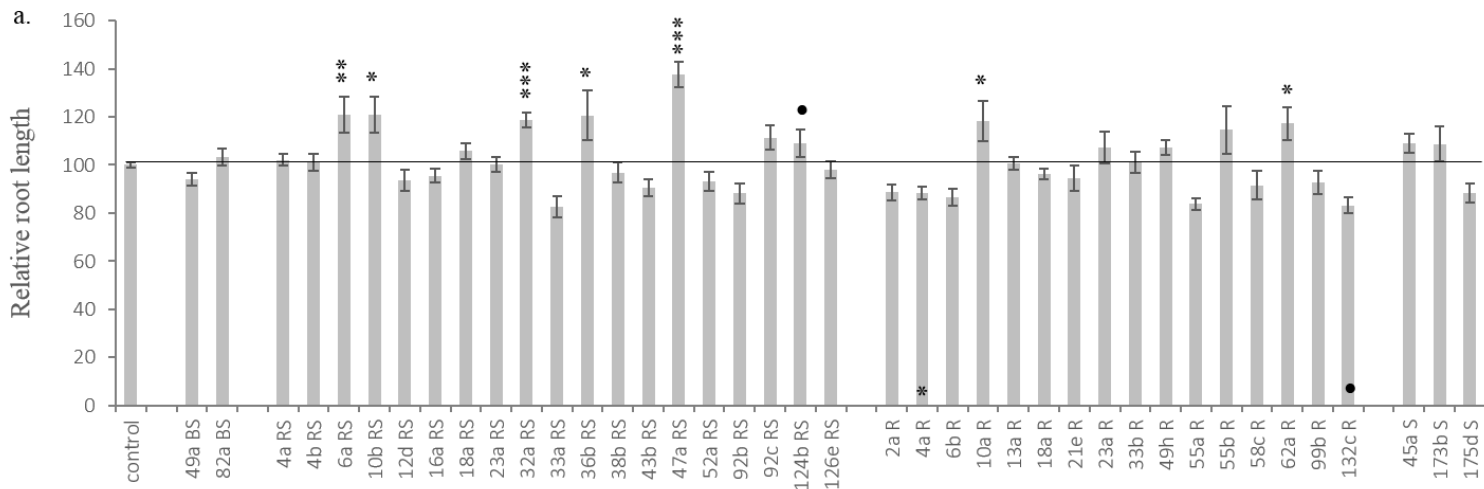
												Cd (mM)			Zn (mM)						
a.	**comp**	**code**		**field**	**season**	**cfu gFW^{-1}**	**identification**	**accession**	**SID**	**OA**	**IAA**	**0.4**	**0.8**	**1.6**	**0.6**	**1.0**	**2.5**	**ACC**	**P sol**	**N$_2$ fix**	**ACE**
	BS	49	a	TE	Jun	377287	*Pseudomonas*	AB088844	+	-	+	++	++	+	++	-	-	+	++	-	-
	BS	82	a	TE	Jun	50792	*Pseudomonas*	FM202488	+	++	++	++	+	-	++	-	-	+	++	-	-
	RS	4	a	TE	Jun	19836450	*Microbacterium*	EU821338	-	-	-	+	+	+	+	+	+	+	-	-	-
√	RS	4	b	TE	Jun	19836450	*Microbacterium*	EU821338	+	-	+	+	+	+	+	+	+	+	-	-	-
√	RS	6	a	CO	Jun	12361856	*Pseudomonas*	AB369347	++	-	+	++	+	+	+	-	-	-	++	+++	-
√	RS	10	b	TE	Jun	4534046	*Plantibacter*	AM396918	-	-	+	++	++	++	++	++	++	+	++	++	-
√	RS	12	d	CO	Jun	8653299	*Pseudomonas*	AB369347	+	-	++	+	+	+	-	-	-	-	-	-	-
	RS	16	a	CO	Jun	2472371	*Janthinobacterium*	D84576	+	-	+	++	+	+	++	++	++	+	++	--	+++
	RS	18	a	CO	Jun	2472371	*Pseudomonas*	AB369347	++	++	+	++	-	-	++	+	+	+	+++	++	-
	RS	23	a	CO	Jun	2472371	*Pseudomonas*	FM202488	++	-	-	++	++	++	+	-	-	+	++	+++	-
√	RS	32	a	TE	Jun	1619302	*Pseudomonas*	FJ225200	++	-	-	++	+	+	+	-	-	+	+++	-	-
	RS	33	a	TE	Jun	3238604	*Variovorax*	FJ772012	-	-	-	++	++	++	-	-	-	+	+	++	-
√	RS	36	b	TE	Jun	3480016	*Pseudomonas*	AB369347	+	-	+	++	+	+	++	+	+	+	-	-	-
√	RS	38	b	CO	Jun	508427	*Pseudomonas*	FM202488	-	-	++	++	+	+	-	-	-	-	+++	-	-
	RS	43	b	TE	Jun	161930208	*Arthrobacter*	AB288059	+	++	+	++	+	+	++	-	-	-	-	-	-
√	RS	47	a	TE	Jun	36703982	*Leifsonia*	AB278552	+	+	-	-	-	-	++	+	+	-	+++	-	-
√	RS	52	a	CO	Jun	50842718	*Bacillus*	GQ200827	+	+	-	-	-	-	+	-	-	-	++	-	++
√	RS	92	b	TE	Dec	985	*Pseudomonas*	EF673038	+	-	++	++	++	++	++	++	-	-	+	-	+
√	RS	92	c	TE	Dec	985	*Pseudomonas*	EF673038	+	+	+	+	++	++	++	-	-	-	-	nd	-
√	RS	124	b	TE	Dec	6817	*Microbacterium*	AJ277840	+	-	-	+	+	+	++	+	+	-	+++	++	++
√	RS	126	e	TE	Dec	58434	*Variovorax*	GQ861460	++	-	++	+	++	+++	+	-	-	-	++	+	-
	R	2	a	TE	Jun	2478	*Pseudomonas*	DQ279324	+	-	+	++	+	+	++	+	+	+	++	++	-
	R	4	a	TE	Jun	788	*Rhizobium*	DQ337581	-	-	-	++	++	+	++	++	-	+	-	-	-
√	R	6	b	CO	Jun	3258	*Bacillus*	AJ542508	-	-	+	++	+	+	+	-	-	-	-	+++	-
√	R	10	a	TE	Jun	788	*Pseudomonas*	AB369347	+	-	+	++	+	+	++	-	-	+	+++	-	+++
	R	13	a	TE	Jun	2365	*Pantoea*	EU598802	+	-	+	++	+	+	++	++	++	+	-	+	+++
	R	18	a	CO	Jun	6516	*Pseudomonas*	DQ778036	+	-	++	+	+	+	-	-	-	+	-	-	-
√	R	21	e	CO	Dec	73774	*Flavobacterium*	DQ778318	+	-	-	++	+	++	-	++	-	+	-	+	-
√	R	23	a	CO	Dec	242399	*Pseudomonas*	EF491969	++	-	+	+	++	+	-	+	-	++	+	-	-
√	R	33	b	CO	Jun	6516	*Pseudomonas*	DQ778036	+	-	+	++	++	+	++	-	-	+	++	-	-
√	R	49	h	CO	Jun	7061	*Plantibacter*	AM396918	+	+	+	-	-	-	+	-	-	+	++	-	-
√	R	55	a	TE	Jun	7885	*Pseudomonas*	FN377713	+	++	++	+	+	+	++	+	+	-	++	-	-
√	R	55	b	TE	Jun	7885	*Pseudomonas*	FN377713	+	++	++	+	+	+	+	-	-	-	+++	-	+++
	R	58	c	CO	Dec	23707	*Stenotrophomonas*	GU186115	++	+	+	+	++	+	++	-	-	+	+++	++	++
√	R	62	a	CO	Dec	6078	*Luteibacter*	AJ580498	+	-	-	++	-	+	-	+	-	++	+++	-	++
√	R	99	b	TE	Dec	79187	*Pseudomonas*	EF491969	+	-	++	+	+	+	-	-	-	++	-	++	-
	R	132	c	TE	Dec	1921	*Pseudomonas*	EF102850	+	++	-	++	+	+	++	-	-	++	++	-	-
√	S	45	a	CO	Dec	4600	*Pseudomonas*	AB098591	++	++	-	+	++	+	-	-	-	++	-	-	-
√	S	173	b	TE	Dec	6263	*Pseudomonas*	CP000094	-	-	-	++	++	+	++	-	-	+	+++	++	++
	S	175	d	TE	Dec	89465	*Pseudomonas*	CP000094	++	-	+	+	++	+	++	-	-	++	++	-	-

b.	comp	code		field	season	cfu gFW^{-1}	identification	accession	SID	OA	IAA	Cd (mM)			Zn (mM)			ACC	P sol	N_2 fix	ACE
												0.4	0.8	1.6	0.6	1.0	2.5				
	BS	3	c	TE	Jun	202828	*Arthrobacter*	AB288059	+	-	-	+	+	+	+	-	-	-	++	-	-
	BS	18	f	CO	Jun	53124	*Bacillus*	FJ263042	+	+	-	-	-	-	++	+	+	-	+++	-	+++
	BS	18	g	CO	Jun	53124	*Bacillus*	FJ263042	+	+	-	-	-	-	+	+	+	-	+++	-	+++
	BS	55	a	TE	Jun	507924	*Arthrobacter*	AB288059	-	-	+	++	+	+	++	++	++	-	+	-	+++
	BS	66	a	TE	Jun	434631	*Arthrobacter*	AB288059	-	-	++	+	+	+	++	+	+	-	-	-	-
	BS	81	a	TE	Jun	50792	*Arthrobacter*	AB288059	-	-	+	++	+	+	++	+	+	-	++	+	-
	BS	82	a	TE	Jun	50792	*Pseudomonas*	FM202488	+	++	++	++	+	-	++	-	-	+	++	-	-
	BS	155	d	TE	Dec	104781	*Duganella*	GU332616	-	-	-	+	++	++	++	++	-	+	+++	++	++
	RS	9	b	TE	Jun	1619302	*Arthrobacter*	FM213390	+	-	+	++	++	+	++	++	+	-	-	++	-
	RS	12	d	TE	Jun	2267023	*Arthrobacter*	FM213390	+	-	+	++	++	+	++	++	+	-	-	-	-
	RS	14	a	CO	Jun	2472371	*Variovorax*	FJ772012	-	-	+	++	+	+	+	-	-	+	nd	-	-
	RS	53	a	TE	Jun	139200640	*Serratia*	AJ233434	+	-	+	++	+	+	+	+	+	-	-	-	+++
	RS	55	b	TE	Jun	34800160	*Pantoea*	EU598802	+	++	++	+	-	-	++	-	-	-	++	-	+++
	RS	127	c	TE	Dec	6330	*Arthrobacter*	DQ985470	+	++	-	++	++	++	-	+	-	-	-	++	-
	R	18	a	CO	Jun	6516	*Pseudomonas*	DQ778036	+	-	++	+	+	+	-	-	-	+	-	-	-
	R	23	d	CO	Dec	242399	*Pseudomonas*	EF491969	+	-	++	+	-	+	-	-	-	+	-	nd	-
	R	28	b	TE	Jun	131234	*Variovorax*	GQ861460	+	-	+	++	+	+	++	+	+	-	-	-	-
	R	31	d	TE	Jun	93738	*Variovorax*	GQ861460	+	-	+	++	++	+	++	-	-	-	+++	-	-
	R	98	a	CO	Jun	33033	*Pseudomonas*	DQ778036	+	+	++	+	-	-	-	-	-	+	++	-	-
	R	131	a	TE	Dec	873	*Pseudomonas*	CP000094	+	-	-	++	++	+	-	-	-	+	-	++	++
	R	132	d	TE	Dec	1921	*Pseudomonas*	EF102850	++	++	+	++	++	++	-	-	-	++	++	nd	-
	R	134	e	TE	Dec	1572	*Pseudomonas*	CP000094	+	-	++	++	++	++	-	-	-	-	++	-	-
	S	136	c	TE	Dec	327	*Pseudomonas*	HQ420253	-	-	-	++	++	++	+	-	-	-	+++	+++	++

Strains are identified to the genus level, their accession numbers as well as their total numbers of colony forming units per gram fresh weight (cfu gFW^{-1}) bulk soil (BS), rhizosphere soil (RS), roots (R) or shoots (S) are displayed. Their potential plant growth-promoting (PGP) characteristics are indicated by + when positive and by ++(+) in case of a strong positive test. Bacterial strains testing negative for a phenotypic test were labeled by a – symbol and those not applicable for the test by 'not detected' (nd). Strains were screened for the capacity to solubilise phosphorus (P sol), fixate nitrogen (N_2 fix) and produce siderophores (SID), organic acids (OA), indole-3-acetic acid (IAA), ACC deaminase (ACC) and acetoin (ACE). Tolerance to Cd (0.4, 0.8 and 1.6 mM) and Zn (0.6, 1.0 and 2.5 mM) was also evaluated. Part b of the table consists of the bacterial strains with a negative effect on plant growth and Cd uptake on VAPs. From the strains represented in part a, only the ones with a checkmark (√) were selected for inoculation on sand.

10 **Figure 6.1** Root length (a), shoot weight (b) and root weight (c) of inoculated plants relative to corresponding non-
11 inoculated control plants during Cd exposure (250 μM) on VAPs (21 cm x 21 cm; MS medium) after 10 days of growth.
12 Inoculated bacteria were isolated from the bulk soil (BS), rhizosphere soil (RS), roots (R) and shoots (S) of *B. napus*. Values
13 are mean ± standard error of at least 3 biological independent replicas (significance level: • = $p < 0.1$; * = $p < 0.05$; ** =
14 $p < 0.01$; *** = $p < 0.001$). Variation within the control group is also shown.

15



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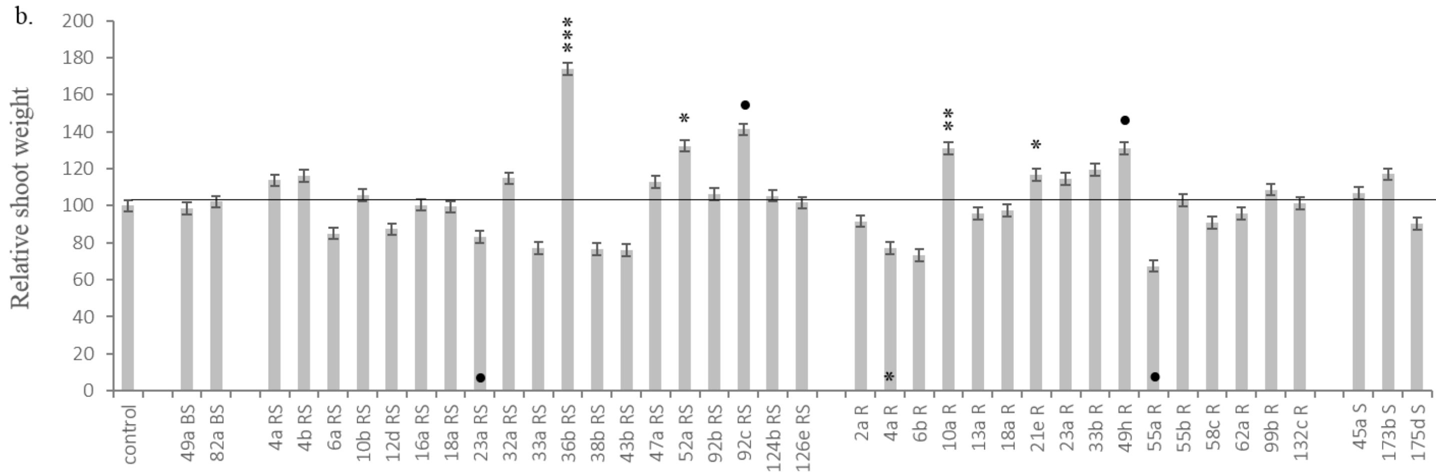

b.
Relative shoot weight
200
180
160
140
120
100
80
60
40
20
0
control
49a BS
82a BS
4a RS
4b RS
6a RS
10b RS
12d RS
16a RS
18a RS
23a RS
32a RS
33a RS
36b RS
38b RS
43b RS
47a RS
52a RS
92b RS
92c RS
124b RS
126e RS
2a R
4a R
6b R
10a R
13a R
18a R
21e R
23a R
33b R
49h R
55a R
55b R
58c R
62a R
99b R
132c R
45a S
173b S
175d S

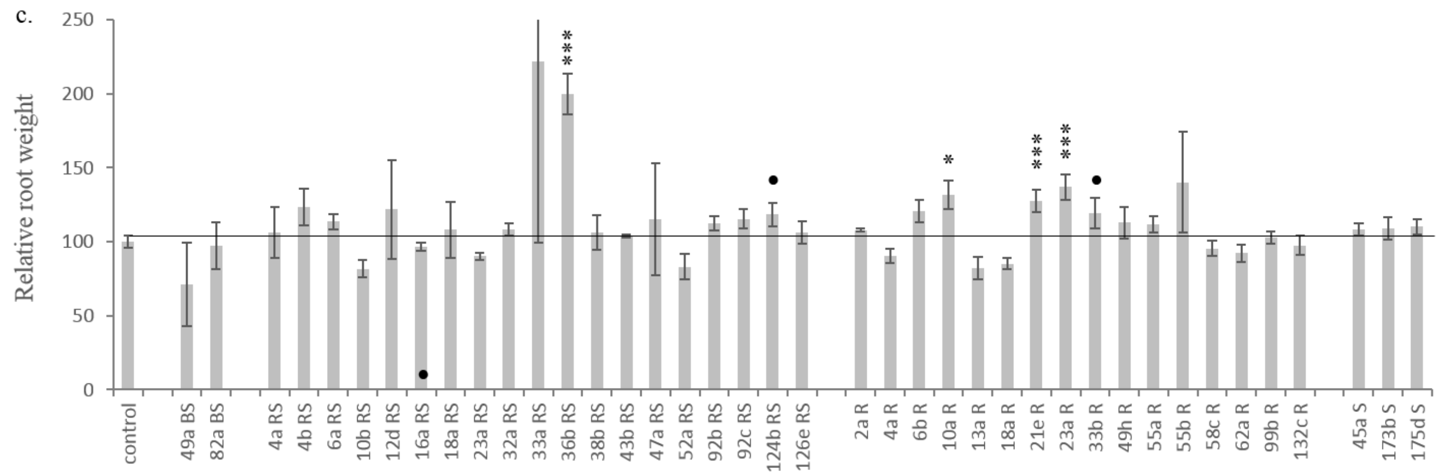
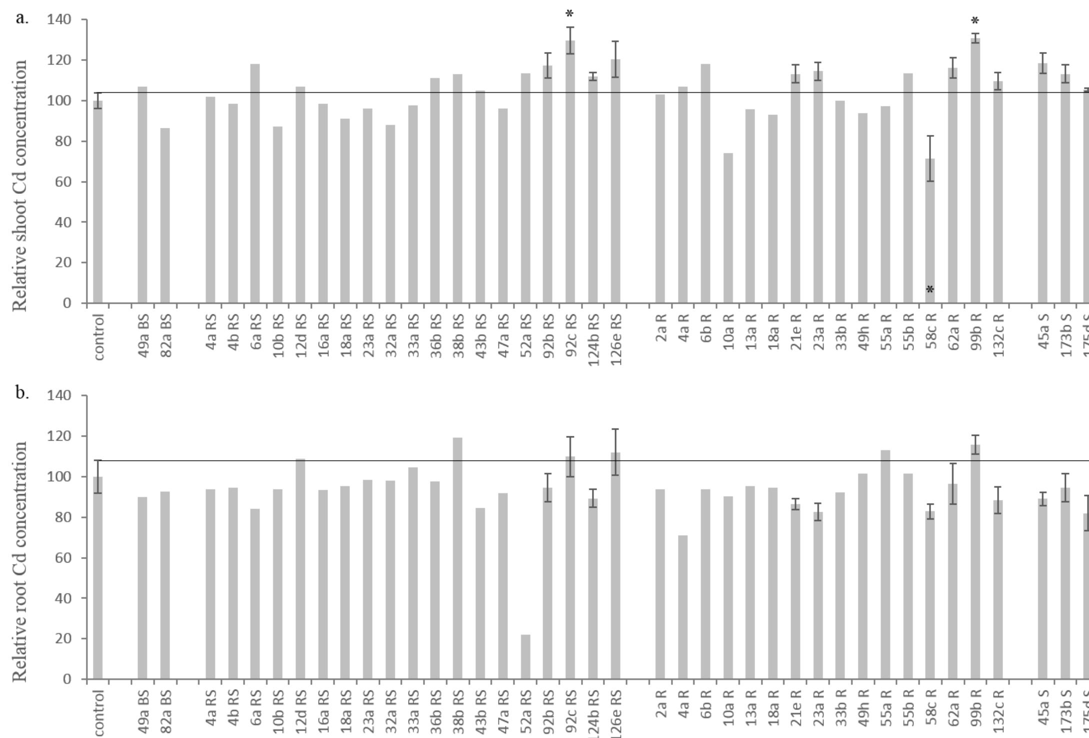

c.
Relative root weight
250
200
150
100
50
0
control
49a BS
82a BS
4a RS
4b RS
6a RS
10b RS
12d RS
16a RS
18a RS
23a RS
32a RS
33a RS
36b RS
38b RS
43b RS
47a RS
52a RS
92b RS
92c RS
124b RS
126e RS
2a R
4a R
6b R
10a R
13a R
18a R
21e R
23a R
33b R
49h R
55a R
55b R
58c R
62a R
99b R
132c R
45a S
173b S
175d S

Figure 6.2 Shoot (a) and root (b) Cd concentrations of inoculated plants relative to corresponding non-inoculated control plants during Cd exposure (250 µM) on VAPs (21 cm x 21 cm; MS medium) after 10 days of growth. Inoculated bacteria were isolated from the bulk soil (BS), rhizosphere soil (RS), roots (R) and shoots (S) of *B. napus*. Values with standard error are means of at least 3 biological independent replicas (significance level: ● = $p < 0.1$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Values without standard error are based on one pooled sample. The control group is based on a statistically justified amount of replicates, such as the standard error testifies.



Rhizosphere strains 6a, 36b, 38b, 52a, 92b, 92c, 124b and 126e, root strains 6b, 21e, 23a, 55b, 62a and 99b and the shoot endophytes 45a and 173b were of special interest because their inoculation lead to substantial increases in shoot Cd concentrations (significant for strains 92c and 99b) (figure 6.2a). Root Cd concentration showed a modest increase after inoculation of rhizosphere strain 38b and root endophytes 55a and 99b (figure 6.2b).

Strains without any positive effects on plant growth and/or Cd uptake were not further studied (BS: 49a, 82a; RS: 4a, 16a, 18a, 23a, 33a, 43b; R: 2a, 4a, 13a, 18a, 58c, 132c; S: 175d) (figures 6.1 and 6.2).

Strains 4b (RS), 12d (RS) and 55a (R) that slightly increased root and shoot weight (4b), root and shoot Cd concentrations (12d) and root weight and Cd concentration (55a) were also included in the selection.

Strains that can significantly promote root growth and slightly increase shoot Cd concentration (RS: 6a, 36b, 124b; R: 21e, 23a, 62a) might induce plants to explore a higher volume of soil and by consequence larger amounts of Cd can be assimilated and transported to the shoots. Rhizosphere strains 36b, 52a and 92c and roots strain 21e combined a stimulation of shoot growth and increased Cd concentration, which is promising in view of a more efficient phytoextraction. Likewise, strains 38b (RS) and 99b (R) that induced a substantiated increase in root and shoot Cd concentration can be mentioned. Strain 10a was of special interest since it promoted root length, root weight as well as shoot weight on VAPs.

To achieve an improved phytoextraction capacity it is important to inoculate strains that promote root development in function of an increased Cd uptake capacity and at the same time enhance shoot biomass in which more metals may be accumulated. Ultimately, only the shoots can be harvested; therefore an optimal metal translocation from roots to shoots is important. The most promising strains that could promote plant biomass and/or Cd uptake and translocation on VAPs were further investigated on sand, spiked with 5 mg Cd kg^{-1}.

Inoculation experiments on sand

Strains that were promising based on their capability to promote plant growth (figure 6.1) and/or Cd uptake (figure 6.2) during Cd exposure on VAPs were

selected (marked with a '√' in table 6.1) for further testing on sand. Their effects on weight (shoot and root) and Cd concentration (available concentration in soil and total concentration in shoot) of Cd-exposed plants on sand are presented in figures 6.3 and 6.4 respectively. Due to the fact that roots could not be recovered intact from the substrate, neither morphological parameters nor metal concentrations were determined. Instead the $Ca(NO_3)_2$-exchangeable fraction of Cd in the sand was examined.

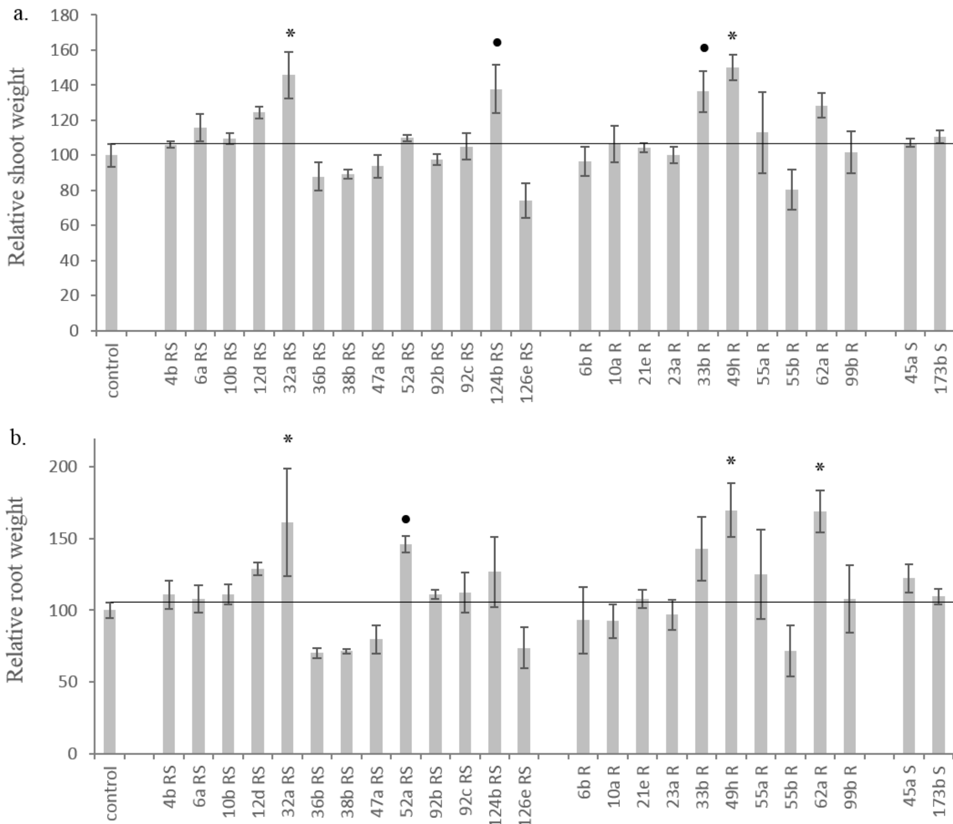


Figure 6.3 Shoot (a) and root weight (b) of inoculated plants relative to corresponding non-inoculated control plants during Cd exposure (5 mg Cd kg^{-1}) on sand (1/2 HL) after 4 weeks of growth. Inoculated bacteria were isolated from the rhizosphere soil (RS), roots (R) and shoots (S) of *B. napus.* Values are mean ± standard error of at least 3 biological independent replicas (significance level: • = $p < 0.1$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Variation within the control group is also shown.

Plant growth (root and shoot weight) was significantly enhanced after inoculation of bacterial strains 32a (RS) and 49h (R) (figure 6.3). Inoculation of

strains 124b (RS) and 33b (R) resulted in significantly larger shoots (figure 6.3a) and strains 52a (RS) and 62a (R) in significantly larger roots (figure 6.3b). Strains 36b, 38b, 47a, 55b and 126e had a negative effect on plant growth.

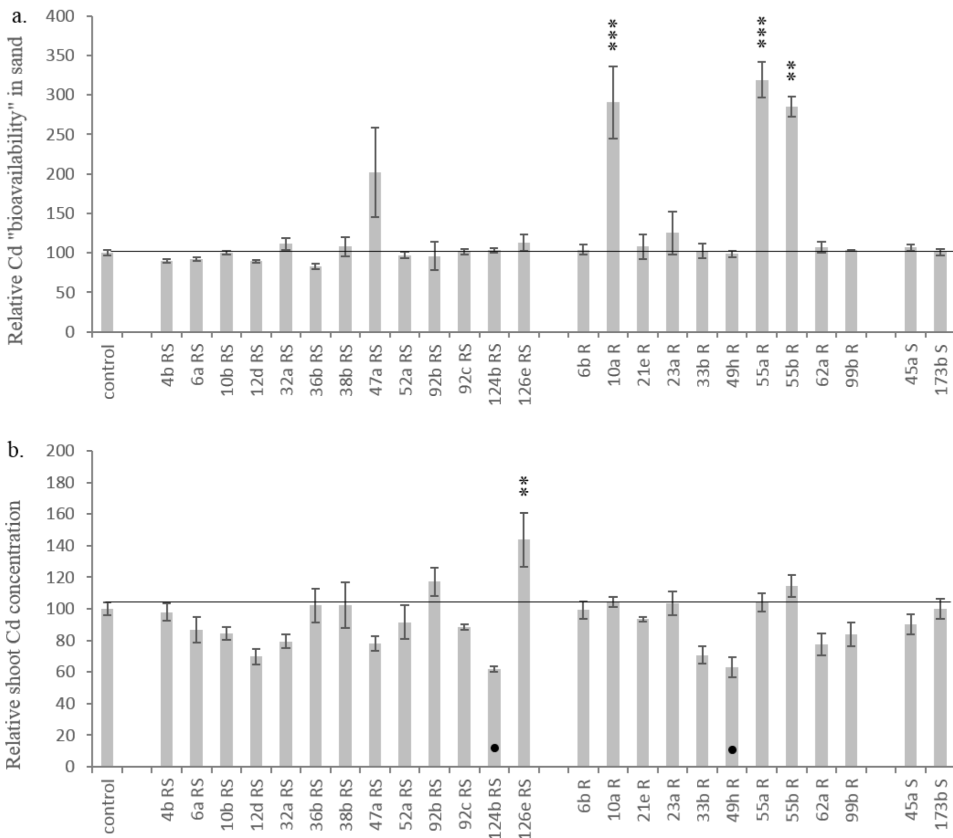


Figure 6.4 Calcium nitrate-exchangeable Cd concentrations in the sand (a) and shoot Cd concentrations (b) of inoculated plants relative to corresponding non-inoculated control plants during Cd exposure (5 mg Cd kg^{-1}) on sand (1/2 HL) after 4 weeks of growth. Inoculated bacteria were isolated from the rhizosphere soil (RS), roots (R) and shoots (S) of *B. napus*. Values are based mean ± standard error of at least 3 biological independent replicas (significance level: ● = $p < 0.1$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Variation within the control group is also shown.

The amount of $Ca(NO_3)_2$-exchangeable Cd in the sand was 3 times higher ($p < 0.01$) after inoculation of root endophytes 10a, 55a and 55b (figure 6.4a). Strains 32a and 47a had a similar (though not significant) effect. Higher shoot Cd concentrations (in comparison with the non-inoculated Cd-exposed control condition) were found in plants inoculated with strains 55b (R), 92b (R) and

126e (RS) (figure 6.4b). Although only the effect of the latter was significant, this strain had no effect on the $Ca(NO_3)_2$-exchangeable Cd. Plants with the lowest Cd concentrations in the shoots were inoculated with strains 12d, 32a, 33b, 47a, 49h, 62a and 124b (figure 6.4b).

Inoculation experiments on field soil

The best-performing strains from the former experiment were inoculated in plants grown on a trace element-contaminated soil from a former maize field in Lommel (Ruttens *et al*., 2010).

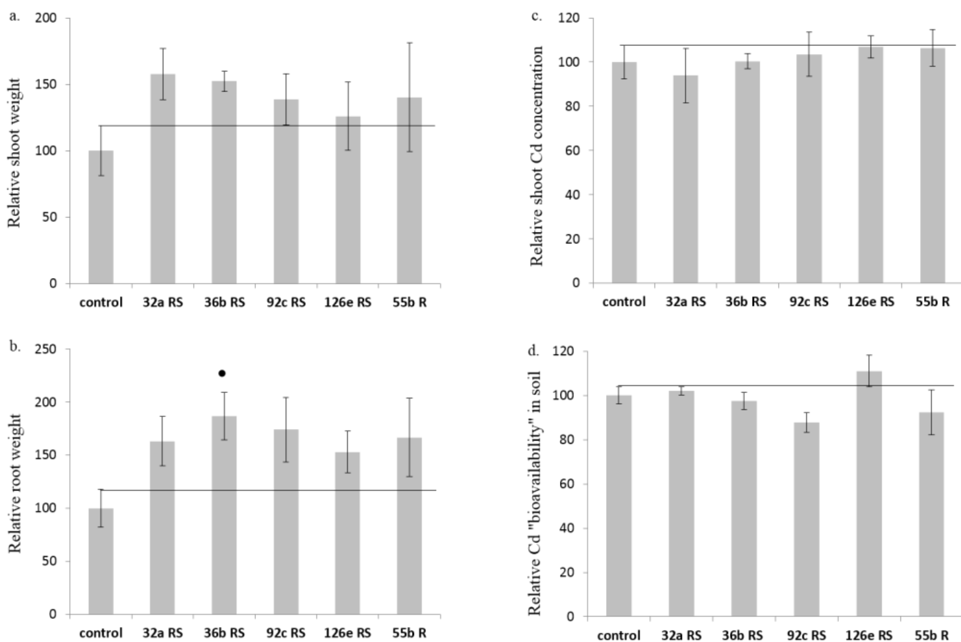


Figure 6.5 Shoot (a) and root weight (b), shoot Cd concentration (c) and $Ca(NO_3)_2$-exchangeable soil Cd concentration (d) of inoculated plants relative to corresponding non-inoculated control plants grown on field soil mainly contaminated with Cd and moistened with tap water after 4 weeks of growth. Inoculated bacteria were isolated from the rhizosphere soil (RS) and roots (R) of *B. napus.* Values are mean ± standard error of at least 3 biological independent replicas (significance level: ● = $p < 0.1$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Variation within the control group is also shown.

Although strains 32a (RS) and 49h (R) both had a significant positive effect on root and shoot weight on sand (figure 6.3), only strain 32a was tested on field soil since the shoots of plants inoculated with strain 49h showed significantly

reduced Cd concentrations (figure 6.4b). Strain 55b (R) was preferred over strains 10a (R) and 55a (R) to promote Cd solubility because it showed the least variable results on sand. Of all strains that were tested on sand, only strain 126e (RS) increased shoot Cd concentration and was therefore selected for inoculation on field soil.

Although strains 36b (RS) and 92c (RS) were not effective in the experiment on sand, they were adopted in this experiment because of their good performance on VAPs, respectively improving plant growth and Cd uptake.

Strain 36b was the best root growth promoter on field soil ($p = 0.0746$). All other strains (32a (RS), 92c (RS), 126e (RS and 55b (R)) only slightly increased root weight (figure 6.5b). None of the tested strains could significantly promote shoot growth, although strains 32a and 36b had a minor positive effect on shoot weight (figure 6.5a).

The tested strains did not increase the $Ca(NO_3)_2$-exchangeable fraction of Cd in the field soil neither Cd accumulation in the shoots (figure 6.5c,d). Strain 92c (RS) even slightly decreased the $Ca(NO_3)_2$-exchangeable fraction of Cd.

Discussion

In order to improve the Cd phytoextraction efficiency of the oil crop *B. napus*, a selection of bacteria with promising *in vitro* characteristics (table 6.1) was made in function of their capabilities to enhance plant growth and Cd uptake. Enriching the numbers of these beneficial bacteria, originally associated with the plant, is expected to result in better performing plants especially under stress conditions (in this case metal stress). The bacterial strains used in plant-assisted remediation should not only improve plant growth, but also easily colonize their host (Rajkumar *et al*., 2009).

In this work, an approach is presented to identify bacterial strains that are promising for use in field applications (figure 6.6). In a first step, bacterial strains were selected based on their *in vitro* features. Subsequently, their *in planta* performance was tested in different experimental set-ups step by step approaching field conditions.

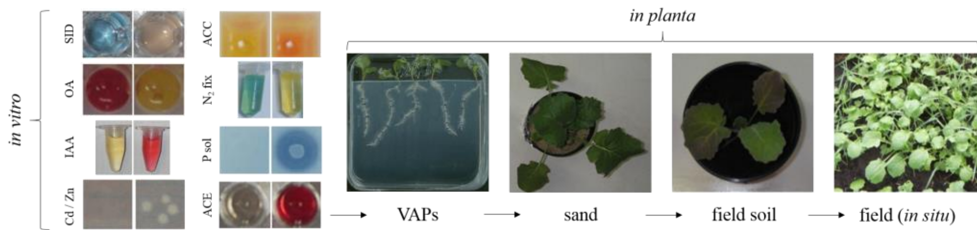


Figure 6.6 Bacterial selection strategy from isolation to reinoculation in the field. After isolation, bacterial strains were first selected based on their *in vitro* characteristics (phosphorus solubilization (P sol), nitrogen fixation (N_2 fix) and production of siderophores (SID), organic acids (OA), indole-3-acetic acid (IAA), ACC deaminase (ACC) and acetoin (ACE), and tolerance to Cd (0.4, 0.8 and 1.6 mM) and Zn (0.6, 1.0 and 2.5 mM)). Inoculation experiments on VAPs highlighted the best strains to test on sand, whereas the most promising strains on sand were tested on field soil. Finally, the reduced bacterial selection list was used in the field (*in situ*).

Inoculation experiments on VAPs

Bacterial performance in *B. napus* grown on MS-medium (250 µM Cd) was evaluated by investigating root length, plant weight and Cd uptake of inoculated and non-inoculated plants. Bacterial strains that lead to better growing plants with a higher Cd extraction potential than the control plants (marked with a '√' in table 6.1) were selected for inoculation in pot experiments on sand.

Plant growth promotion

Siderophore production and phosphate solubilisation

Under metal stress, plant's physiological activities are disturbed, growth and development are severely impeded, and resistance mechanisms are weakened (Cuypers *et al*., 2010; Ma *et al*., 2011). Further, their metal phytoextraction efficiency is strongly affected, and the potential of metal decontamination is proportionally impeded (Martin and Ruby, 2004). The exploitation of siderophore-producing (SPB) and phosphorus-solubilizing (PSB) bacteria, present in the rhizosphere, could be of particular importance as they can provide nutrients to plants, which could reduce the deleterious effects of the presence of toxic metals (Sinha and Mukherjee, 2008; Ahemad, 2014) and promote plant growth (Rajkumar *et al*., 2010; Misra *et al*., 2012). In the presence of Cd (250 µM) on VAPs, it seems important for *B. napus* to be associated with SPB and PSB in order to perform as good as control plants grown in the absence of Cd and

inoculum (40 % longer roots, 30 % higher root weight and 10 % higher shoot weight). Of all bacteria capable to significantly improve growth of Cd-exposed plants on VAPs (figure 6.1), only strain 10b (RS) did not produce siderophores while strains 21e (R) and 36b (RS) could not solubilize phosphorus *in vitro* (table 6.1a).

IAA and acetoin production

Plants demonstrating a significant growth promotion on VAPS during Cd exposure are predominantly the ones inoculated with bacterial strains showing IAA and acetoin production. These findings confirm former studies concerning IAA-producing bacteria, especially in the roots of *B. napus* exposed to metal stress (Sheng and Xia, 2006; Sheng *et* al., 2006; Sheng *et al*., 2008). Also the plant growth-promoting effect of acetoin, a volatile compound released by several PGPB, is mainly observed as a stimulated root formation (Ryu *et al*., 2003); this is probably because the bacterial acetoin-pathway, leading to the production of 2.3-butanediol, is usually triggered by low partial pressures of O_2, comparable to conditions in the soil around the root area (Ryu *et al*., 2004). Rhizobacterial volatiles also appear to trigger plant defenses thereby enhancing the capacity to mobilize cellular defense responses when plants are faced with herbivore/pathogen attacks (Farag *et al*., 2006; Choudhary *et al*., 2008). In this study, most of the plants with a stimulated biomass on VAPs were inoculated with IAA- and/or acetoin-producing bacteria (root: 10a, 23a, 33b, 36b, 124b; shoot: 10a, 36b, 49h, 52a, 92c). A larger root system makes it possible to extract more nutrients from the soil which logically should result in higher above-ground biomasses.

Organic acid production

Of all inoculants resulting in a significantly increased plant growth on VAPs (14 in total, see figure 6.1), only 3 could produce organic acids *in vitro* (table 6.1a). Remarkably, 2 of them (49h and 52a) significantly increased shoot weight during Cd exposure on VAPs (figure 6.1b). In a specific study, Parale *et al*. (2010) concluded that pyruvic acid effectively enhanced the production of bacoside-A in shoots of *Bacopa monniera* L. (Pennell) which in turn raised shoot weight. Generally, organic acids are involved in many processes going on in the

rhizosphere, including nutrient acquisition and metal detoxification, alleviation of anaerobic stress in roots and mineral weathering (Jones, 1998). Overexpression of malate dehydrogenase in transgenic alfalfa enhances organic acid synthesis and confers tolerance to aluminium (Tesfaye *et al*., 2001).

ACC deaminase production and Cd tolerance

Multiple studies confirmed a positive correlation between the *in vitro* ACC deaminase activity of plant-associated bacteria and their stimulating effect on root elongation, suggesting that the utilization of ACC is an important bacterial trait involved in root growth promotion (Belimov *et al*., 2005; Madhaiyan *et al*., 2006). Many studies observed that the regulation of ethylene synthesis was primarily concentrated in the inoculated roots (Jacobson *et al*., 1994; Glick *et al*., 1995; Li *et al*., 2000; Penrose *et al*., 2001; Ghosh *et al*., 2003; Shaharoona *et al*., 2006a, b). Bacterial ACC deaminase catalyses the cleavage of the immediate precursor of ethylene, ACC, which is secreted from the plant cell and taken up by bacteria in the rhizosphere. Thus, bacteria bound to the plant root or seed and expressing ACC deaminase act as a sink for plant ACC, thereby lowering the concentration of this metabolite within the plant (Penrose *et al*., 2001). The inoculation of Cd-tolerant bacteria can improve *B. napus* plant growth and Cd uptake (Sheng *et al*., 2006), but probably not only bacterial metal tolerance can render plants more suitable for phytoremediation. In most cases the inoculated metal-tolerant bacteria have several beneficial properties such as IAA, siderophores and ACC deaminase production (Dell'Amico *et al*., 2008; Sheng *et al*., 2008). Also in this study, these characteristics seemed most important for bacterial inoculants to promote simultaneous root and shoot weight of *B. napus* plants exposed to Cd stress on VAPs (10a, 21e, 36b (figure 6.1b,c and table 6.1a)).

Synopsis

Bacterial strains which significantly increased root length during the VAPs experiment were predominantly Cd-tolerant and mostly able to solubilize phosphorus and produce siderophores and ACC deaminase (RS: 6a, 10b, 32a, 47a; R: 62a (figure 6.1a and table 6.1a)). Strains capable of significantly increasing root weight showed the same features with in addition also IAA

production (R: 21e, 23a, 33b (figure 6.1c and table 6.1a)). Besides the above-mentioned properties, bacterial acetoin production became important when a significant positive effect on root length and weight was accomplished (RS: 36b, 124b; R: 10a (figure 6.1 and table 6.1a)). So, inoculated plants with significantly longer roots do not necessarily possess higher root weights and vice-versa. Anyway, a stimulated root development allows the plant to explore a higher soil volume from which more nutrients and (toxic) metals can be taken up. Inoculation of strains 10a (R), 21e (R) and 36b (RS) led to significantly increased root and shoot weights (figure 6.1b,c) whereas strains 49h (R), 52a (RS) and strain 92c (RS) only significantly enhanced shoot weights (figure 6.1b). Important bacterial characteristics for improving shoot weight seem to be phosphorus solubilisation and the production of siderophores, organic acids, IAA and acetion (table 6.1a).

Improved Cd uptake

Plant growth under adverse environmental conditions is correlated with population density of beneficial bacteria in the rhizosphere or roots (Ashraf *et al*., 2004). Whereas plant growth is easily promoted after bacterial applications, an improved metal uptake is more difficult to achieve by bacterial inoculation (Rajkumar *et al*., 2005; Wu *et al*., 2006; Dell'Amico *et al*., 2008). In this study on VAPs, the best strains to increase Cd accumulation in the root are 38b, 55a and 99b (figure 6.2b) and in the shoot 6a, 6b, 92c and 99b (figure 6.2a). All these metal uptake enhancing strains show increased tolerance to Cd and can produce IAA; only strains 92c and 99b that significantly increased Cd uptake in the shoots of *B. napus* plants grown on VAPs could additionally produce siderophores (table 6.1a). So, a higher metal uptake seems to be correlated with a more extended root system (Weyens *et al*., 2011), the presence of Cd-tolerant bacteria that can reduce Cd toxicity (Lodewyckx *et al*., 2001) and the operation of siderophores which can facilitate uptake and translocation of metals to the above-ground plant parts (Carrillo-Castaneda *et al*., 2003). Higher Cd concentrations in the plant can lead to impaired shoot weights (figure 6.1b: 6a, 6b, 38b, 55a); only inoculation of strain 92c resulted in a significantly increased shoot weight and Cd concentration (figure 6.1a,b).

Inoculation experiments on sand

In the VAP experiments, all selected bacteria were inoculated via the seeds. However, in preliminary experiments on sand we noticed that additional inoculations were needed; suggesting that inoculants are susceptible to the bacteria present in the sand. Experiments on sand were carried out in a non-sterile environment in contrast to the experiments on VAPs in which only the present seed endophytes could interact with the inoculant. Thus, the efficacy of a PGPB, even if it is a good colonizer, may be reduced by bacterial competition (Larcher *et al*., 2008). The initial amount of the PGPB compared to the indigenous bacteria and the number of applications is essential in order to secure successful inoculation (Larcher *et al*., 2008), especially on field soil and in the field. Additional inoculations were performed in the pots after seedlings emerged when lateral root formation was taking place because this results in tissue cracks that form the perfect entrances to the interior of the root (both cortex and stele). In this way, some bacteria are capable of colonizing the internal tissues of the plant (Zinniel *et al*., 2002), where competition is lower. For the selection of bacterial strains it is advisable to take the dominance and the colonisation efficiency of the cultivable strain into account (Belimov *et al*., 2005). Colonisation efficiency can be estimated *in vitro* by testing the bacteria on their ability to produce enzymes capable of degrading plant cell walls (*e.g.* protease, cellulose, pectinase); also mobility (flagella) and positive chemotaxis can be indicative for a high colonisation potential (Truyens *et al*., 2014). In future work, it would be interesting to include these *in vitro* colonisation efficiency tests to the phenotypical screening.

Plant growth promotion

Bacterial strains 36b and 47a, both with significant plant growth promoting effects on VAPs, exerted a negative effect on plant growth when inoculated on sand (figure 6.3). Additional to the differences *in vitro* and *in planta*, bacterial performance can even differ *in planta* depending on the experimental set-up. In this context, a systems biology approach to understand the synergistic interactions between plants and their beneficial bacteria represents an important field of research (Weyens *et al.*, 2010). The sequencing of the genomes of

model plant species and their associated beneficial bacteria will contribute to better understand these complex associations.

Bacterial inoculants can have inhibiting effects on plant growth (figure 6.3: 36b, 38b, 47a, 55b and 126e). Indeed, bacterial induced systemic responses expressed under pathogen-free conditions can have negative effects on plant fitness when plants suffer from a shortage of nutrients (Heil *et al*., 2000; Heil and Baldwin, 2002). Factors associated with detrimental effects of rhizobacteria on plant growth can also be related to the concentration of bacterial cells in the inoculum (cfu ml^{-1}) used and the presence or absence of certain nutrients in the bacterial suspension used for inoculation (Alström, 1987). Next to an overconcentration of the inoculum, high levels of IAA can inhibit plant growth (Dubeikovsky *et al., 1993*; Malik and Sindhu, 2011).

Improved Cd uptake

Strain 126e, isolated from the rhizosphere of *B. napus* plants grown on a Cd-contaminated field during winter, only increased Cd accumulation in the shoots of *B. napus* plants grown on sand, not on VAPs (figures 6.2a and 6.4b). Strain 126e is highly Cd-tolerant and produces siderophores and IAA (table 6.1a). In comparison with VAPs experiments, for inoculation experiments on sand, bacterial phosphorus solubilisation seemed more important for an increased metal uptake. Many studies indicate that phosphate solubilisation is a consequence of the decrease of pH due to the production of organic acids by bacteria (Ivanova *et al*., 2006). However, Kim *et al* (2002) could not establish a correlation between the acidic pH and the quantity of P_2O_5 liberated. Also in our study, bacterial phosphorus solubilisation could not be related to organic acid production (table 6.1a). No significant increase of $Ca(NO_3)_2$-extractable Cd was found in the sand after inoculation of strain 126e (figure 6.7a).

However, it is quite common that inoculated strains are capable to significantly increase the $Ca(NO_3)_2$-exchangeable fraction of Cd while having no effect on Cd uptake (Wu *et al*., 2006). Likewise, the Cd-tolerant phosphorus-solubilizing strains 10a, 55a and 55b capable of producing siderophores and IAA seem promising (figure 6.4a), but plants inoculated by these strains performed similar to the non-inoculated Cd-exposed control plants concerning their growth (figure 6.3) and Cd uptake (figure 6.4b). A higher availability of metals in the soil does

not automatically result in increased metal concentrations in plant tissues (Shenker *et al*., 2001; Wu *et al*., 2006; Cao *et al*., 2007; Doumett *et al*., 2008; Wenzel, 2009), although many bacteria have been reported to significantly increase the availability of metals in metal-polluted soils and enhance metal uptake by plants (Sheng *et al*., 2008; Kumar *et al*., 2009; Chen *et al*., 2010; He *et al*., 2013). The production of protons and organic acids by bacteria appears to be the most significant mechanism for metal mobilization (Saravanan *et al*., 2007). Soil acidification can also be accomplished by bacteria by releasing siderophores and solubilising phosphate (Abou-Shanab *et al*., 2005; Zaidi *et al*., 2006; Dimpka *et al*., 2009; Bruad *et al*., 2009; Jeong *et al*., 2012; Ren *et al*., 2013), although the presence of siderophores and phosphate can either promote or reduce heavy metal uptake depending on the combination of plant, bacterium and metal (Bolan *et al*., 2003; Ma *et al*., 2011).

Synopsis

Bacterial *in planta* characteristics differ between VAPs and sand; only plant growth-promoting strains 32a, 33b, 49h, 52a, 62a and 124b on VAPs could also significantly promote plant growth on sand (figure 6.3). For improved plant growth on spiked sand, the most interesting bacteria were the phosphorus-solubilizing Cd-tolerant ones that can produce siderophores and ACC deaminase (table 6.1a). To cope with Cd exposure on sand, Cd tolerance and siderophore production remain crucial while phosphorus solubilisation becomes more important. The production of plant growth hormones is still vital but not only depends on IAA but also on acetoin (table 6.1a).

Interesting strains

As it appears from our inoculation experiments, it is not evident to identify a bacterial strain with multiple characteristics that can improve Cd phytoextraction efficiency on sand. Indeed, the strains significantly improving plant weight, seems to lead to a lower Cd uptake than in the non-inoculated control plants (32a, 33b, 49h, 52a, 62a, 124b) (figures 6.3 and 6.4b). Possibly, better results could be obtained by inoculating bacterial consortia (Zaets *et al*., 2010; Panneerselvam *et al*., 2013).

Remarkable is that most of the selected strains (marked with a '√' in table 6.1) belong to the genus *Pseudomonas* followed by *Bacillus, Plantibacter* and *Microbacterium*. In a former study, we found that *Peudomonas*, *Bacillus*, *Plantibacter, Pedobacter* and *Pseudobacillus* species could thrive in the roots, stems and leaves of *B. napus* plants (Croes *et al*., 2014). *Pseudomonas* and *Bacillus* strains were of particular interest since they colonized all plant organs of most individual *B. napus* plants. The genus *Pseudomonas* includes bacteria with a broad range of functions and its members are known for their metabolic versatility (Gyamfi *et al*., 2002). Besides, pseudomonads are known as dominant colonizers of the root surface and major members of the *B. napus* root- and rhizosphere-associated microbial communities (Germida *et al*., 1998; Kaiser *et al.*, 2001; Misko & Germida, 2002; Siciliano & Germida, 1999). Several studies confirm that *Pseudomonas* strains possess a series of properties to increase the efficiency of phytoremediation (Sheng and Xia, 2006; Rajkumar and Freitas, 2008; Sheng *et al*., 2008; Long *et al*., 2011).

Inoculation experiments on field soil

The best-performing strains on sand (32a, 55b and 126e) as well as 2 remarkable strains from the VAPs experiment (36b and 92c) that did not perform well on sand were selected for inoculation experiments on field soil. The *in vitro* characteristics that most of these 5 selected strains share are increased Cd tolerance, and the capacities to solubilize inorganic phosphate and to produce IAA, siderophores and ACC deaminase. Dell'Amico *et al*. (2008) and Rodriguez *et al*. (2008) showed that bacteria possessing these characteristics might have the potential for plant growth promotion and increased metal uptake.

The expectations of the selected strains were not fulfilled on field soil (figures 6.5 and 6.6). The most obvious explanation is that the growth period of 4 weeks was too short for the inoculated bacteria to compete with the indigenous bacterial community of the field soil. We may assume that the slight increases in root weight might indicate the first positive effects of the inoculated bacteria in and around the roots of *B. napus*. Since for practical reasons the plants could only be grown for 4 weeks in the growth chamber, we decided to test these strains in a future field experiment.

After studying the effects of inoculation in the field, consortia can be composed in order to simultaneously increase metal uptake and plant biomass, especially in the above-ground plant parts. Even if the inoculated strains have no specific effect on the accumulation of Cd in the plant, an increased biomass will enhance the total Cd exported from the soil (Dell'Amico *et al*., 2008).

Conclusions

Our results show that the final effects of *in vitro* determined characteristics of bacterial strains in function of improving plant growth and Cd uptake can vary depending on the experimental set-up. Cadmium tolerance and siderophore production were common traits of strains capable of improving growth and Cd uptake of *B. napus* grown on VAPs and sand. The ability to solubilise phosphorus was shared between bacteria that could increase Cd phytoextraction efficiency on sand. Bacterial production of IAA seemed important for plant growth while ACC deaminase activity coincided with increased Cd uptake.

As a lot of bacteria share the above-mentioned characteristics, we suggest the incorporation of tests concerning efficiency of colonisation in order to improve the efficiency of the *in vitro* selection of strains. Besides, it is logical that good colonisers can exert their properties more efficiently. Since most of the bacterial strains can either improve plant biomass or plant Cd uptake, it can be useful to consider the inoculation of consortia after the first *in planta* screening.

Finally, we notice an unpredictable increase in complexity when shifting closer to field conditions, which means that we still not can guarantee the *in planta* performance of specific strains in the field until effective *in situ* inoculations are performed.

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CHAPTER 7

Bacterial-assisted phytoextraction potential of *Brassica napus* L. on a cadmium-contaminated field

Abstract

The associated bacterial community of *Brassica napus* L. (rapeseed) was enriched during a field inoculation experiment aiming to improve the Cd phytoextraction potential of rapeseed. In earlier work, all cultivable bacteria associated with rapeseed growing on the same Cd-contaminated field were isolated and characterized. The selection of the 6 strains used in this study was based on the results of previous (a) *in vitro* screening for plant growth-promoting potential and Cd tolerance and (b) inoculation experiments to evaluate the *in planta* effect on plant growth and Cd translocation. All selected strains had a positive effect on plant weight (especially root growth) when inoculated in pots on field soil. This effect was only significant for seed endophytic strain UH1, which was the only isolate slightly increasing Cd concentrations in the roots.

Despite the high expectations of strain UH1, no significant effects were observed *in situ*. However, 4 of the 5 remaining root/rhizosphere-associated strains significantly increased root dry weight. None of the strains affected metal uptake by *B. napus* in the field, although one of them significantly increased metal availability by decreasing soil pH.

These results demonstrate that even a thorough *in vitro* and *in planta* screening of bacterial strains is no guarantee for a successful application in a more complex field situation. Nevertheless, the obtained data indicate potential for bacterial-assisted phytoextraction of toxic metals. The challenge is to find the particular conditions wherein bacterial performance is maximal.

Introduction

From the past century, when industrial processes became prevalent, metals were spread into the environment. Also the use of fertilisers, pesticides and sewage sludge resulted in pollution with different metals. Environmental contamination with essential elements generally presents no major problems because they only become toxic at high concentrations. On the contrary, Cd ranks 7th on the priority list of hazardous substances established by the US Comprehensive Environmental Response, Compensation and Liability Act (CERCA; ATSDR, 2013) and is classified as a human carcinogen by the International Agency for Research on Cancer (Waalkes, 2000). In soils, excessive trace metal concentrations can lead to phytotoxicity (Kim and McBride, 2009), suppression of soil microbial processes (Kelly *et al*., 2003) and toxicant movement into local water sources (Simona *et al*., 2004). Cadmium bioaccumulation in the human food chain poses important health concerns (Nawrot *et al*., 2006, Cuypers *et al*., 2010; Gallego *et al*., 2012).

The use of plants to transport and concentrate metals from the soil into the harvestable parts, usually termed 'phytoextraction', is often presented as a valid alternative to traditional physicochemical remediation methods that do not provide acceptable solutions for the removal of metals from soils (Salt *et al*., 1998; Garbisu *et al*., 2002). Nonetheless, despite the firm establishment of phytoremediation technology in literature and in small-scale demonstrations, full-scale applications are currently limited to a small number of projects (Vangronsveld *et al*., 2009). The first field-based experiment using natural hyperaccumulator plants (*e.g. Thlaspi caerulescens*) was conducted in 1991-1992 in a sewage sludge-treated plot at Woburn, UK (McGrath *et al*., 1993). Saxena *et al*. (1999) found that under optimal growth conditions *T. caerulescens* could remove 125 kg Zn ha^{-1} year^{-1} and 2 kg Cd ha^{-1} year^{-1}. If the high trace element concentration accumulated by *T. caerulescens* is an advantage, its slow growth rate and low dry mass yield are the main limitations of an efficient phytoextraction procedure (Ernst, 1998; Assunçao *et al*., 2003).

The use of high biomass-producing non-hyperaccumulator plants for trace element phytoextraction is often mentioned to be more promising when they can guarantee a sufficient uptake and translocation of the trace elements of concern combined with an economic benefit (Vangronsveld *et al*., 2009; Witters *et al*.

2012a,b). Some *Salix* clones were found to contain up to 70 mg Cd kg^{-1} dry weight in leaves, which is close to Cd hyperaccumulation criteria of 100 mg kg^{-1} (Greger and Landberg, 1999). Nevertheless, it was calculated from field trials that it would take decades to remove Cd and Zn (Hammer *et al*., 2003; Vangronsveld *et al*., 2009). Anyway, willow under short rotation coppice on metal-contaminated agricultural soils seems economically feasible for phytomanagement if wood and energy prices remain high (Ruttens *et al.*, 2011; Van Slycken *et al*., 2013).

The success of phytoextraction depends on several factors including the extent of soil contamination, the availability and accessibility of contaminants for rhizosphere microorganisms and uptake into roots and the ability of the plants and their associated microorganisms to intercept, absorb and accumulate trace elements in shoots (Ernst, 2000). Ultimately, the potential for phytoextraction hinges on the interaction between soil, trace elements, plants and microorganisms (bacteria and mycorrhiza) to increase element availability in the soil and uptake by the plants (Vangronsveld *et al*., 2009). The complexity of these interactions is site-specific and requires a multidisciplinary approach (Alcantara *et al*., 2001; Lasat, 2002).

Plant-associated bacteria can increase plant availability of trace metals in the rhizosphere and subsequently metal extraction by plants because some of them show the ability to mobilize metals present in the soil by excreting organic acids, siderophores and other bacterial trace element chelators (Sessitsch *et al*., 2013). Bacterial strains can also enhance the absorptive properties of the roots by increasing root length, root surface area and numbers of root hairs. Furthermore, bacteria can promote metal translocation to the shoots via beneficial effects on plant growth, trace element complexation and alleviation of phytotoxicity. Overall, the inoculation of plant-associated beneficial microbes can enhance the phytoextraction efficiency directly by altering the metal accumulation in plant tissues and indirectly by promoting the shoot and root biomass production (Rajkumar *et al*., 2012).

Concerning phytoremediation of inorganic compounds, many studies confirmed the ability of high biomass-producing non-hyperaccumulator plants to reach an increased phytoextraction potential with the help of inoculated bacterial strains (Lodewyckx *et al*., 2001; Belimov *et al*., 2005; Rajkumar *et al*., 2005; Sheng

and Xia, 2006; Wu *et al*., 2006; Zaidi *et al*., 2006; Dell'Amico *et al*., 2008; Jiang *et al*., 2008; Rajkumar and Freitas, 2008; Sheng *et al*., 2008; Braud *et al*., 2009; Chen *et al*., 2010; Guo *et al.*, 2011; Jeong *et al*., 2012; Luo *et al*., 2012; He *et al*., 2013; Ren *et al*., 2013). Nevertheless, the method is not used widely to restore the thousands of contaminated areas.

Apart from limited metal availability, the lack of competitiveness of inoculated microbial strains in field conditions appears to be another major obstacle (Larcher *et al*., 2008). Furthermore, plant stress factors not present in laboratory and greenhouse studies can result in significant challenges for field studies; also the current methods of assessing phytoremediation may not be adequate to show decreasing contaminant concentrations (Gerhardt *et al*., 2009).

Although there have been encouraging results for bacterial-assisted phytoextraction in the field (Jankong *et al*., 2007; Yang *et al*., 2012; Brunetti *et al.*, 2012; Deng *et al*., 2012; Wang *et al*., 2013; Sprocati *et al*., 2014), many inconclusive and unsuccessful attempts exists due to the reasons mentioned above. It is obvious that there is a requirement for up-to-date information on successes and failures based on evidence from the field to further improve *in situ* phytoremediation technologies (Mench *et al*., 2010).

Our focus lies on the ability of *Brassica napus* L. (rapeseed), previously described as a high biomass energy crop with Cd accumulation capacity (Grispen *et al*., 2006), to remediate the Campine region which has been severely contaminated during the last century with heavy metals by the metal processing industry. In this region several field experiments are already conducted to evaluate the possibilities of cultivation of crops for energy purposes as a possible economic alternative for farming on historically contaminated soils (Ruttens *et al*., 2008). The phytoextraction potential of maize (*Zea mays* L.), rapeseed (*B. napus* L.), willow (*Salix* spp.) and poplar (*Populus* spp.) for this specific field are shown in table 7.1 (Thewys *et al*., 2010).

Although willow is the most likely crop to be used for phytoremediation of the historically contaminated soils in the Campine region (Voets *et al*., 2013), the use of energy maize was proposed by Thewys *et al.* (2010) aiming at acceptance of the farmers (maize being a conventional crop). To avoid lower maize biomass

yields as a result of the lack of a strict rotation scheme (Schreurs *et al*., 2011), we recommend using rapeseed as an alternative crop every 3 to 4 years.

Table 7.1 Extraction of Cd to reduce concentration in soil from 3.6 to 1.2 mg kg^{-1} for energy maize, rapeseed, willow and poplar (Thewys *et al*., 2010).

	Biomass production (ton DM ha^{-1})	Concentration Cd (mg kg^{-1} DM)	Cd removal (kg ha^{-1} $year^{-1}$)	Clean-up time (years)*
Energy maize	20	3	0.06	150
Rapeseed	8	6	0.05	188
Willow-twigs	8	24	0.19	94
Willow-leaves	2.4	60	0.14	
Willow-twigs+leaves			0.34	54
Poplar-twigs	8	11	0.09	205
Poplar-leaves	2.4	28	0.07	
Poplar-twigs+leaves			0.16	116

* Calculations based on 25 cm soil depth for energy maize and rapeseed; 50 cm soil depth for willow and poplar; a soil density of 1.5 ton m^{-3}; assuming linear extrapolation.

To our knowledge, a detailed study about the potential of plant-associated bacteria to improve Cd phytoextraction by *B. napus* in the field is lacking and therefore of high value in studying rapeseed as a possible phytoextractor. In former pot experiments on polluted Cd substrates (Croes *et al*., in preparation), we noticed a significantly increased Cd availability in the soil and a significant positive effect on *B. napus* plant growth and Cd uptake after bacterial inoculation. In this study, 6 selected strains (5 *Pseudomonas* strains and 1 *Variovorax* strain) were examined in the field.

Experimental Procedures

Field site description

The Campine region (51°12'41"N; 5°14'32"E) is a cross-border area in the north-eastern part of Belgium and the south-eastern part of the Netherlands. Until the seventies, atmospheric deposition from 4 non-ferro smelters contaminated an area of 700 km^2 in this region (of which 280 km^2 in Belgium) with Cd and Zn (Vangronsveld *et al*., 1995). *Brassica napus* was sown in September 2012 on a field plot of 70 m^2 (4 m x 17.5 m) situated on a

moderately contaminated soil in the Campine region (Lommel, Belgium) (figure 7.1).

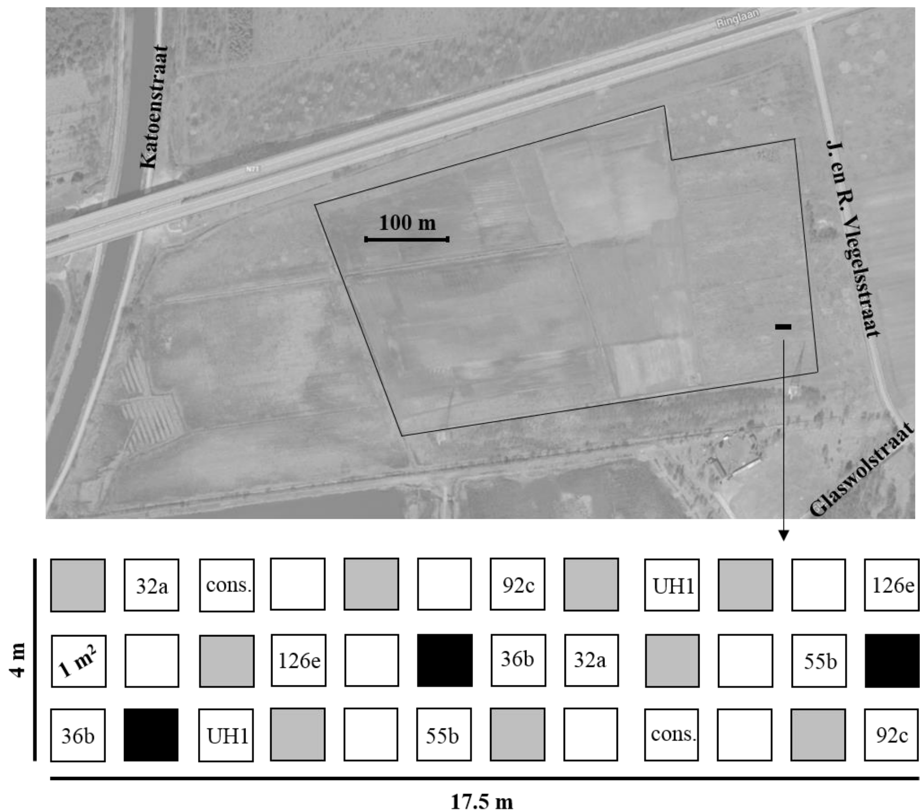

Figure 7.1 Satellite photo of the experimental field site in Lommel (Belgium) largely planted with maize, poplar and willow. The black rectangle depictures the 70 m^2 large field plot sown with *B. napus*. The detailed scheme shows the division in subplots. Each subplot was 1 m^2 and all bacterial conditions were represented twice. The grey subplots functioned as control plots and were provided with sterile 869 medium without bacterial cells during inoculation events. The plants growing in the buffer area between subplots and the black subplots were left unthreatened. The empty subplots were inoculated with bacteria other than those included in this manuscript.

Two weeks before sowing (50-80 plants per m^2), 3 cm of compost (based on horse and chicken manure), was applied. Before bacterial treatment, the field plot was subdivided in 36 subplots (1 m x 1 m) separated by a buffer zone (0.5 m). Each inoculum was applied on 2 randomly chosen subplots. The 9 grey

coloured subplots in figure 7.1 were used as controls and inoculated with sterile 869 medium during the inoculation events. The 3 black coloured subplots were free from treatments in order to analyse the effect of 869 medium wherein the inoculated bacteria were cultivated and applied. Control plants (+ 869 medium) and untreated plants (- 869 medium) had similar growth and Cd accumulation rates, also metal availability was not influenced by the addition of the medium. The empty subplots were inoculated with bacteria other than those included in this study.

Selection of B. napus-associated bacteria

In previous work, cultivable bacterial populations associated with rapeseed growing on this metal-contaminated field site were thoroughly characterized (Croes *et al*., 2013). All isolates were screened for their Cd (0.8 mM) and Zn (1 mM) tolerance and their potential plant growth-promoting characteristics. Subsequently, the best *in vitro* performing strains were extensively tested *in planta* on vertical agar plates (VAPs), sand and field soil in a strictly controlled LED-lighted growth chamber (Croes *et al*., in preparation). Based on these lab-scale *in planta* experiments a selection of 6 strains, with the potential to improve plant growth and Cd uptake during field conditions, was made (table 7.2). When the term "*in planta*" is used, the inoculation experiments under controlled conditions are considered, whereas the "*in situ"* inoculation experiment was performed in the field.

Inoculation

Seeds treated with fungicides and insecticides provided by SUET GmbH (Germany) were used. Since coated seeds cannot be surface-sterilized and inoculated, seeds were only stored at 4°C before sowing in the field. In previous work, inoculation procedures were already optimized for different experimental set ups in the lab (Croes *et al*., in preparation). However, to ensure a successful colonization in the field, inocula were provided 6 times: 2 times after the seedlings were emerging in September 2012 and 4 times after growth restart in 2013 (April 23th, May 14th, June 4th, June 25th). Bacterial strains were grown overnight in sterile Pyrex bottles with 869 medium at 30°C during vigorous shaking. Bacterial cells in exponential growth phase ($0.5 < OD_{660nm} < 1$) were

inoculated in the field (1 square meter plot) by diluting the medium (1 l) to 5 l with tap water in a watering can. Bacteria that were part of the consortium were pooled together after growth (just before inoculation on the field). Because the consortium consisted of 5 different bacterial strains, 200 ml of inoculum per strain was used per subplot. Between inoculations, materials were sterilized with bleaching water (15%) and subsequently rinsed 3 times with tap water. Each inoculum was applied on 2 plots, while the non-inoculated (control) condition (sterile 869 medium without bacteria) was represented by 9 plots.

Effect of inoculation on phytoextraction efficiency

After harvest in the field (July 2013), several parameters were investigated. To determine the effect of the inoculation on the metal availability, $Ca(NO_3)_2$-exchangeable and total metal concentrations, electrical conductivity and pH were analysed. Metal accumulation was evaluated by measuring metal concentrations in the roots and shoots and metal phytotoxicity by determining root biomasses. Shoot and leaf biomasses could not be determined in the field since many shoots were consumed by herbivores up to 10 cm from the ground.

Exchangeable and total metal concentrations

The exchangeable (available) fractions (mg kg^{-1} dry weight) of Cd, Zn, Fe, Mg and Mn in soil were estimated using 0.1 M $Ca(NO_3)_2$ extraction (soil solution ratio 1:5, 2 h shaking at 65 rpm, filtration through Whatman filter paper No. 40) (Mench *et al*., 1994) and inductively coupled plasma optical emission spectrometry (ICP-OES). Samples were oven-dried (48h at 65°C) and sieved through a 2-mm sieve. One mixed soil sample per subplot was used. To ensure metal homogeneity during the field experiment, total metal concentrations were measured in each plot. Total metal concentrations (*aqua regia*-extractable) were determined upon digestion of 500 mg air-dried soil in 4 ml aqua regia using a microwave (Milestone, 1200 MEGA) (Van Ranst *et al*., 1999).

Electrical conductivity and pH

All dried and sieved field soil samples were analysed for pH and electrical conductivity (EC). To determine actual soil pH (pH(H_2O)), 10 g was allowed to equilibrate in 25 ml of Millipore water (KCl to determine potential pH) during

vigorous stirring for 18h. After filtration through a paper filter (Whatman No. 1), the pH of the supernatant was measured using a standard pH meter (Radiometer, PHM82) and glass electrode (Hamilton, LiqPlast), calibrated using pH 4.0 and 7.0 standards. The electrical conductivity of the substrates was measured in the same filtrates using a conductimeter (Dionex DX-120).

Cd concentrations in roots and shoots

To remove present soil particles, plants were cleaned with a toothbrush. Since plant parts from the field could not be crushed as a result of their hard texture, the 2nd cm (measured from the ground level) of the root and stem of 5 independent plants made up 1 mixed root and shoot sample. Per plot, 3 mixed root and shoot samples were taken as replicates.

All samples were 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 4 h. Samples were then dissolved in HCl (37%) and diluted with Millipore water to a final volume of 5 ml (2% HCl). Cd, Zn, Fe, Mg and Mn concentrations (mg kg^{-1} dry weight) were determined using ICP-OES.

Plant growth

In the field, remaining plants were harvested on July 9th 2013 (2 weeks after the last inoculation event). In total 220 plants were collected at the 9 non-inoculated plots (sterile 869 medium), while an average of 50 plants were harvested per inoculated condition (2 plots). Also 50 plants were recovered from the 3 plots that were not provided with inoculum nor medium.

Statistical analysis

All datasets were statistically analysed using one-way ANOVA and post hoc pair-wise comparison testing (Dunnett’s two-tailed t-test) where all independent groups are compared with the same control group. Transformations were applied when necessary to approximate normality and/or homoscedasticity. In case normality could not be reached, data were analysed using Kruskal-Wallis multiple comparisons test (non-parametric) and multiple comparisons were accomplished using Pairwise Wilcoxon rank sum test. The statistical analyses were performed in R 2.13.0.

Results

Selection of B. napus-associated bacteria

The bacterial strains interesting to test in this field experiment were obtained after an intensive *in vitro* (Croes *et al*., 2013) and *in planta* (Croes *et al*., in preparation) selection procedure (table 7.2). All *in planta* characteristics were attained under controlled conditions in a LED-lighted growth chamber (12 h photoperiod, 22°C/18°C day/night, photosynthetic photon flux density of 200 µmol m^{-2} s^{-1}, 65 % relative humidity).

Table 7.2 Detailed characterisation of the selected *B. napus*-associated bacterial strains.

	Selected strains	32a	36b	92c	126e	55b	UH1
Strain information							
	compartment	rhizosphere	rhizosphere	rhizosphere	rhizosphere	root	seed
	identification	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Variovorax*	*Pseudomonas*	*Pseudomonas*
	accession n°	FJ225200	AB369347	EF673038	GQ861460	FN377713	AJ011504
***In vitro* characteristics**							
	Cd (0.8 mM)	+	+	++	++	+	++
	Zn (1 mM)	-	+	-	-	-	+
	SID	++	+	+	++	+	++
	OA	-	-	+	-	++	+
	ACC	+	+	-	-	-	+++
	IAA	-	+	+	++	++	++
	ACE	-	-	-	-	+++	-
	P sol	+++	-	-	++	+++	+
	N_2 fix	-	-	nd	+	-	++
***In planta* characteristics**							
VAPs	root weight	0	+++	+	0	+	/
	shoot weight	+	+++	++	0	0	/
	shoot Cd	(-)	(+)	+++	+	(+)	/
Sand	root weight	+++	-	0	-	-	+
	shoot weight	+++	0	0	-	-	+++
	shoot Cd	-	0	-	+++	+	---
Field soil	root weight	+	++	+	+	+	+++
	shoot weight	+	+	+	0	0	+++
	shoot Cd	0	0	0	0	0	+

Strains isolated from the rhizosphere, root or seed were identified to the genus level (with accession number). Isolates were screened for their capacity to solubilise phosphorus (P sol), fixate nitrogen (N_2 fix) and produce siderophores (SID), organic acids (OA), ACC deaminase (ACC), indole-3-acetic acid (IAA) and acetoin (ACE). Tolerance to Cd (0.8 mM) and Zn (1.0 mM) was also evaluated. Positive testing strains for these *in vitro* characteristics are indicated by + or ++(+) in case of a strong positive test; those testing negative are labeled by a – symbol. When the *in vitro* test was not applicable, this isindicated by nd (not detected). The *in planta* potential of bacteria on vertical agar plates (VAPs) and in pots on sand and field soil to increase phytoextraction efficiency by promoting plant biomass production and Cd accumulation in the shoot is evaluated by symbols --- (significant negative effect), - (negative effect), 0 (no effect), + (positive effect), ++ (significant positieve effect (0.05 < p-value < 0.10) and +++ (significant positive effect (p-value ≤ 0.05). In case of strains 32a, 36b, 92c, 126e and 55b *in vitro* results and *in planta* results that are shown are based on Croes *et al*. (2013) and Croes *et al*. (in prep). Results for strain UH1 are based on unpublished data.

The best-performing strain on VAPs considering plant growth promotion (36b) was included in the field experiment as well as the one capable of significantly improving Cd uptake and translocation on VAPs (92c). On sand, increased plant weight and Cd accumulation rate in the shoot were the factors to select strain 32a and 126e respectively. Although bacterial isolate 55b significantly increased Cd availability in sand (Croes *et al*., in preparation), it only slightly enhanced shoot Cd concentration after inoculation.

All selected strains associated with the rhizosphere or roots of *B. napus* promoted root growth on field soil. This effect was not significant but extended to the shoot for strains 32a, 36b and 92c. From the 6 selected bacterial strains, strain UH1 isolated from the seeds of *B. napus* significantly increased plant growth (root and shoot) on field soil (on sand only shoot weight was significantly increased after inoculation). Furthermore, the seed endophyte had the ability to slightly increase Cd concentration in the shoot of plants grown on field soil (this effect was significant in the roots (data not shown)).

All 6 strains were Cd-tolerant and capable to produce siderophores; only strain 32a could not produce indole-3-acetic acid (IAA). All strains that significantly promoted plant weight on VAPs, sand and/or field soil shared the capacity to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Isolates promoting Cd availability in the soil and/or Cd uptake and translocation by the plant had the ability to produce organic acids and/or solubilize phosphorus. The bacterial *in vitro* characteristics Zn tolerance, acetoin production and nitrogen fixation did not seem to have any direct effect on the *in planta* characteristics. Interesting to notice is that seed endophyte UH1 tested positive to all but one *in vitro* test, *i.e.* acetoin production. More interesting seed endophytes isolated from plants harvested at the contaminated field in Lommel (Belgium) are found in appendix 7.1.

Bacterial inoculation in the field

Effect of inoculation on metal availability

Total metal concentrations were determined in all field plots to ensure metal homogeneity. Table 7.3 contains all average (± standard error) total metal concentrations present in the field soil surrounding non-inoculated control plants. The inoculated soils contained similar total metal concentrations (data

not shown). Given this homogeneous spreading of the metals, eventual effects of inoculation on $Ca(NO_3)_2$-exchangeable metal fractions can be interpreted.

Table 7.3 Total soil metal (Cd, Zn, Fe, Mg and Mn) concentrations in the field.

	Cd	Zn	Fe	Mg	Mn
Mean	4.07	231.69	3029.49	317.87	84.04
Error	0.17	10.38	168.65	7.34	3.93

Values are mean ± standard error of at least 6 biological independent replicas.

Only strain 92c significantly affected metal availability: Cd, Zn and Fe became more accessible after inoculation (figure 7.2). This might result from the capability of strain 92c to lower soil pH(H_2O) from 6.61 ± 0.05 to 6.38 ± 0.06 (p-value: 0.122) and significantly lower soil pH(KCl) from 5.81 ± 0.04 to 5.45 ± 0.19 (p-value: 0.0143). All other bacteria did not have any effect on pH. Besides, not one inoculum had any effect on the conductivity of the substrate as compared to the average value measured in the surrounding soil of non-inoculated (control) plants (197.00 ± 12.43 mS cm^{-1}).

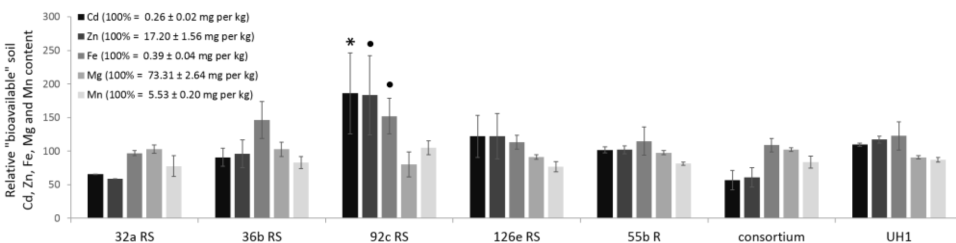

Figure 7.2 Calcium nitrate-exchangeable (available) Cd, Zn, Fe, Mg and Mn concentrations in the soil of inoculated plants relative to non-inoculated control plants (100%). One condition consisted of plants inoculated with strains 32a, 36b, 92c, 126e and 55b (consortium). All plants were grown at the contaminated field. Standard errors were calculated based on 2 biological independent replicas (significance level: • = $p < 0.1$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

Effect of inoculation on metal uptake by B. napus

Although strain 92c significantly increased soil metal availability, inoculated plants did not accumulate more metals (figure 7.3). Iron concentrations in the roots were even significantly lower. Also inoculation of bacterial strains 32a,

36b, 126e, 55b, UH1 and the consortium (32a + 36b + 92c + 126e + 55b) did not increase plant metal concentrations in the field. Despite the ability of seed endophytic strain UH1 to slightly increase Cd accumulation in the shoot (table 7.2) and significantly increase root Cd concentration of plants grown on field soil in pots (data not shown) no satisfying results were obtained after inoculation *in situ*.

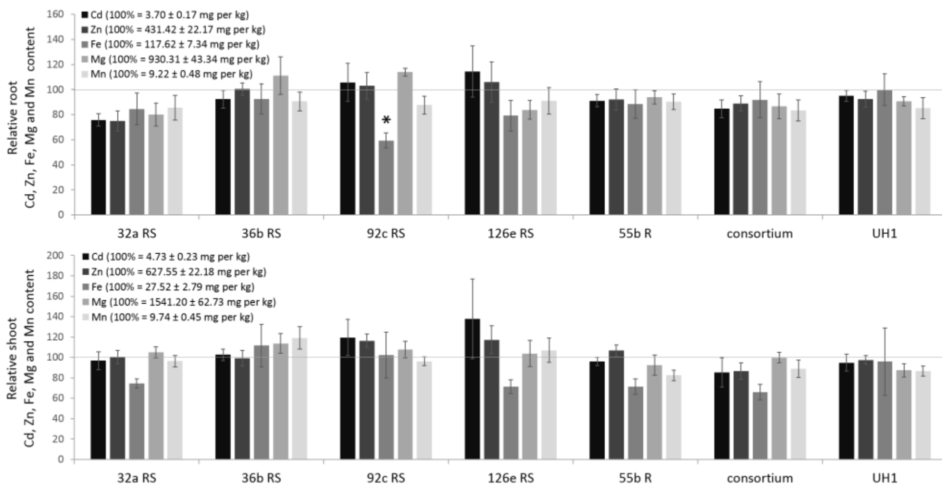

Figure 7.3 Total root (a) and shoot (b) Cd, Zn, Fe, Mg and Mn concentration of inoculated plants relative to non-inoculated control plants (100%). One condition consisted of plants inoculated with strains 32a, 36b, 92c, 126e and 55b (consortium). All plants were grown at the contaminated field. Standard errors were calculated based on 6 biological independent replicas (significance level: • = $p < 0.1$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

Hereby we should mention that the $Ca(NO_3)_2$-exchangeable Cd concentrations decreased from 0.91 ± 0.02 mg kg^{-1} on field soil (during *in planta* experiments) to 0.26 ± 0.02 mg kg^{-1} in the field (during *in situ* experiments, see figure 7.2). According to our earlier results it seems more likely for bacteria to exert an effect on Cd uptake and translocation when Cd is more available in the substrate (sand = 1.52 ± 0.05 mg $Ca(NO_3)_2$-exchangeable Cd kg^{-1}) (table 7.2). Cadmium concentrations in roots and shoots of plants grown on sand were respectively 996.91 ± 46.23 and 84.78 ± 3.54 mg kg^{-1} dry weight. Plants grown on potted field soil only accumulated 19.25 ± 2.04 mg Cd kg^{-1} dry weight and 6.62 ± 0.35 mg Cd kg^{-1} dry weight. In the field, accumulation rates were even lower, *i.e.*

3.70 ± 0.17 mg Cd kg^{-1} dry weight in the roots and 4.73 ± 0.23 mg Cd kg^{-1} dry weight in the shoots (figure 7.3).

Effect of inoculation on the growth of B. napus

Most of the inoculations resulted in plants with significantly higher dry root weights (except strains 126e and UH1). In case of inoculation with strain UH1, no inoculation effects on root growth were noticed *in situ* (figure 7.4). This was not expected as UH1 significantly promoted plant growth on sand and field soil under controlled condition.

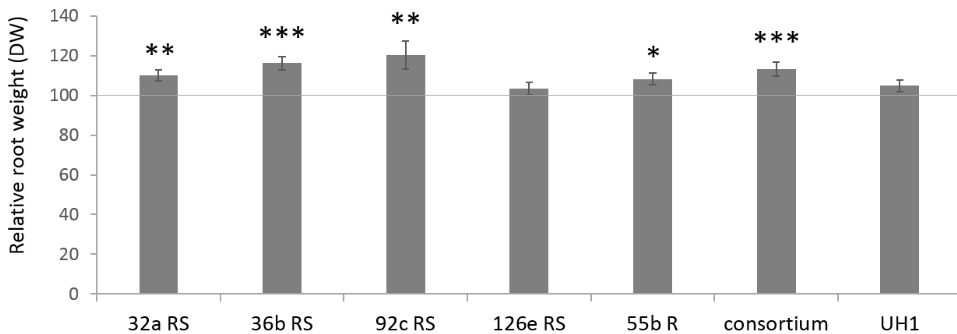

Figure 7.4 Dry root weight of inoculated plants relative to non-inoculated control plants (100% = 49.63 ± 0.71 mg). One condition consisted of plants inoculated with strains 32a, 36b, 92c, 126e and 55b (consortium). All plants were grown at the contaminated field. Standard errors were calculated based on more than 40 biological independent replicas (significance level: • = $p < 0.1$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

The average weight of dried roots from the field was only 49.63 ± 0.71 mg. Non-inoculated control plants grown for 4 weeks in pots on spiked sand had a fresh root weight of 6.45 ± 0.37 g and fresh shoot weight of 25.33 ± 0.68 g; the average fresh root weight on potted field soil was 2.62 ± 0.34 g while average fresh shoots weighted 3.42 ± 0.50 g.

Discussion

During the past decade, scientists experienced how various bacteria contribute to phytoremediation and demonstrated their efficacy in the laboratory. For organic contaminants, phytoremediation has even already been found to be effective in the field (Barac *et al*., 2009; Weyens et al., 2009). To efficiently

phytoremediate metal-contaminated soils, it is necessary to address the problem of low metal availability. Microbially produced iron chelating agents have been added several times to the soil in an attempt to increase metal availability (Ma *et al*., 2011). This strategy usually works in a small-scale laboratory trials but hardly in the field (Glick, 2010).

For bacterial-assisted phytoextraction to become effective, inoculated bacteria should not only solubilize soil metals but also be effective colonizers and plant growth-promoting agents (Sessitsch *et al*., 2013). Also very important is the ability of the inoculants to increase the metal tolerance and accumulation of the phytoextractor plants (Ma *et al*., 2011). Bacterial metabolites can alter the uptake of metals indirectly, through their effects on plant growth dynamics, and directly, through acidification, chelation, precipitation, immobilization and oxidation-reduction reactions in the rhizosphere (Smith and Read, 1997; Abou-Shanab *et al*., 2003). Endophytic bacteria that possess mechanisms to lower metal availability in the plant, will allow the plant to accumulate more metals before toxic effects will appear. Regardless of this range of bacterial possibilities to increase plant metal uptake, most inoculants have little influence on the metal concentrations in plant tissues (Wu *et al*., 2006; Wang *et al*., 2013). Also in this study, no major increase of Cd uptake after inoculation was found (table 7.2 and figure 7.3).

Effect of inoculation on metal availability

In this study, seed endophytic strain UH1, able to solubilize phosphorus and produce siderophores and organic acids (table 7.2), did not increase Cd availability during the pot (Croes *et al*., in preparation) and the field experiments (figure 7.2). The bacterial root endophyte 55b shared the same *in vitro* abilities to solubilize trace elements (SID, OA, P sol) and significantly increased $Ca(NO_3)_2$-exchangeable Cd fractions on sand (Croes *et al*., in preparation). Rhizospheric strain 92c, also a *Pseudomonas* sp., showed less promising *in vitro* characteristics but significantly increased $Ca(NO_3)_2$-extractable Cd, Zn and Fe in the field (figure 7.2).

From these results, it seems that the potential to solubilize phosphorus and produce siderophores and organic acids is not sufficient to significantly influence trace element speciation, mobility and availability in the field. However, under

controlled conditions, many studies found that microbial-assisted phytoextraction can lead to a significant enhancement of metal extraction primarily as a result of bacterial metal solubilisation (Abou-Shanab 2006; Sheng and Xia, 2006; Tiwari *et al*., 2013; Langella *et al*., 2014). In the field, it seems more important to effectuate a pH decrease in the (rhizosphere) soil in order to significantly increase trace element availability (Alloway, 1995). A decrease of soil pH might be attributed to the secretion of protons, amino acids and organic acids through metabolic activities of bacteria (van der Lelie *et al*., 1999 and Huang *et al*., 2002). From all investigated strains, only 92c could significantly decrease potential soil pH (pH(KCl)) in the field. So, it is obvious that the increased soil acidity resulting from the inoculation of strain 92c was related to the significantly higher extractability of Cd, Zn and Fe in these plots (figure 7.2).

It is obvious that more representative bacterial *in vitro* traits should be identified in order to efficiently identify metal solubilizing microbial strains. In this respect we suggest to measure the activation of poorly soluble Cd by bacteria in fluid medium as described by Sheng and Xia (2006). Bacterial strains that can effectuate a pH decrease and increase the water-soluble Cd concentration in the medium are probably interesting to include in future inoculation experiments.

Moreover, the discrepancies between bacterial *in vitro*, *in planta* and *in situ* capabilities should be considered. For example in the case of siderophore production, Cornu *et al.* (2014) found that 250 µmol pyoverdine per kg soil enhanced the mobility, the phytoavailability and the phytoextraction of Cu while the fate of Cd was not affected. As we know for siderophores (Rajkumar *et al*., 2010), also organic acids are able to solubilize “unavailable” forms of metals in the soil (Kalinowski *et al*., 2000; Li *et al*., 2010). But as for siderophores, their efficiency has in many cases been unsatisfactory (Evangelou *et al*., 2008). Organic acids may also inhibit metal uptake complexing the metals outside the root (Murphy *et al*., 1999). Bacterial siderophores and organic acids can reduce metal availability by chelating the metal ions (Tripathi *et al*., 2005; Dimkpa *et al*., 2009). Phosphate solubilizing bacteria (PSB) can solubilize the insoluble and biologically unavailable metals such as Ni (Becerra-Castro *et al*., 2011), Cu (Li and Ramakrishna, 2011) and Zn (He *et al*., 2013) but reduce Pb in pore water (Bolan *et al*., 2003). So, despite promising *in planta* phytoremediation studies

with bacteria capable of phosphorus solubilisation and the production of siderophores and organic acids (Rajkumar *et al*., 2010; Jeong *et al*., 2012; Becerra-Castro *et al*., 2013), complex interactions in the soil can alter *in vitro* based *in planta* expectations (Becerra-Castro *et al*., 2012; Cabello-Conejo *et al*., 2011; Luo *et al*., 2011). *In situ*, soil conditions and interactions with the native bacterial communities are even more complex than in potted soil under controlled conditions. Moreover, the lower soil temperatures in the field (12-14 °C) compared to the 22 °C in the growth chamber can lead to significant differences in metabolic activity of the plant roots and the inoculated bacteria and of course the native plant-associated bacterial communities. These differences in soil temperature might be an important reason for the discrepancies between the inoculation effects in the growth chamber on potted field soil and in the field.

Effect of inoculation on metal uptake by B. napus

An increased metal availability in the soil does not automatically result in an increased metal uptake by plants (Wu *et al*., 2006). Root endophytic strain 55b and rhizosphere strain 92c increased $Ca(NO_3)_2$-extractable Cd in sand and in the field respectively, but their inoculation did not lead to higher Cd concentrations in the plants (table 7.2 and figure 7.3). Plants inoculated with strain 92c even contained significantly less Fe in the roots than non-inoculated control plants (figure 7.3). In contrast, strain UH1 significantly increased Cd and Mn uptake in the roots and Mg accumulation in the shoots of plants grown in potted field soil (data not shown) while not having an effect on $Ca(NO_3)_2$-extractable metals in the soil (despite its interesting *in vitro* capabilities for metal solubilisation) (table 7.2).

Non-inoculated *B. napus* plants grown for 4 weeks in the growth chamber accumulated respectively 996.91 ± 46.23 and 84.78 ± 3.54 mg Cd per kg dry weight in roots and shoots on spiked sand; on field soil this was respectively 19.25 ± 2.04 and 6.62 ± 0.35 mg Cd per kg dry weight (Croes *et al*., in preparation). In the field, accumulation rates were the lowest (3.70 ± 0.17 and 4.73 ± 0.23 mg Cd per kg dry root and stem (figure 7.3)). Indeed, vegetative uptake can be affected by environmental conditions (Burken and Schnoor, 1996; Brunetti *et al*., 2011). Significantly lower soil temperatures in the field (12-14°C

instead of 22°C in the growth chamber) result in lower metabolic activities in the plant roots and around (*i.e.* rhizosphere organisms) the roots, which might affect plant growth and trace element uptake. The much higher metal availability in the spiked sand lead to remarkably higher metal accumulation rates compared to those on field soil. In the field, low metal availability resulted in low Cd uptake. Noteworthy is the fact that the remaining stems from herbivory showed higher accumulation rates than the roots (49.63 ± 0.71 mg dry weight). Therefore, it is possible that we have missed the effect of the bacteria in the field, since we have no idea of the metal accumulation rates in the leaves. From our previous study in the field (Croes *et al*., 2013) we know that towards the end of the growing season Cd, Zn and Fe are transported to the leaves.

An important bacterial *in vitro* characteristic for improving metal accumulation in the plant is metal tolerance. Therefore, all strains that were selected for inoculation in the field possessed increased Cd-tolerance (to 0.8 mM Cd, see table 7.2). During the pot and field experiments, only strain 126e could significantly increase Cd accumulation rates in the shoot on spiked sand (table 7.2), which suggests that also other bacterial mechanisms can promote metal accumulation by plants. To cope with an excess of toxic metals, different mechanisms have been reported in bacteria: enzymatic detoxification, intracellular sequestration, active efflux transport, extracellular sequestration, reduction in metal sensitivity of cellular targets and metal exclusion (Bruins, 2000). By binding (precipitating) metal ions onto their cell wall or by intra- and extracellular sequestration, the 'internal availability' of the toxic metals inside the plant decreases. Endophytic bacteria that possess mechanisms to lower the 'plant availability' of Cd inside the plant, will allow the plant to take up more Cd before toxic effects will appear. Focussing on remediation of Cd-contaminated soils, the CZC / CZR efflux mechanism is of special interest since it allow Cd ions to be precipitated onto the bacterial cell wall (Nies *et al*., 1989). It is also important that many soil bacteria are tolerant to toxic metals since soil contamination with Cd negatively affects microbial diversity in rhizosphere and bulk soil and inhibits several microbiological processes in the soil (Wuertz and Mergeay, 1997). Tolerant soil bacteria play a significant role in immobilization or mobilization of metals (Gadd, 1990).

Effect of inoculation on the growth of B. napus

Strain UH1 could produce high amounts of ACC deaminase (table 7.2), which regulates ethylene levels in plants by metabolizing its precursor ACC (Glick, 2005). Accelerated ethylene production in response to stress induced by contaminants is known to inhibit root growth and is considered as a major limitation in improving phytoremediation efficiency (Arshad *et al*., 2007). Plants inoculated with ACC deaminase-producing bacteria have lower ethylene levels resulting in a more extensive root system (Stearns *et al*., 2005; Safronova *et al*., 2006; Rodriguez *et al*., 2008; Zhang *et al*., 2011). Proliferation of roots in contaminated soil can lead to higher uptake of toxic metals (Grichko *et al*., 2000). Also in this study we noticed an improved plant growth after inoculation of the ACC deaminase-producing strain UH1, but only during the pot experiments (table 7.2).

The plant growth-promoting effect of strain UH1 was more pronounced on field soil than on sand; inoculated plants on field soil became almost twice as big and extracted approximately 20 % more Cd (data not shown). The reason for this is probably hidden in the fact that non-inoculated control plants on spiked sand produced much more fresh biomass than control plants grown on field soil for 4 weeks (sand: 6.45 ± 0.37 g root and 25.33 ± 0.68 g shoot; field soil: 2.62 ± 0.34 g root and 3.42 ± 0.50 g shoot), despite the higher $Ca(NO_3)_2$-exchangeable Cd concentrations in the sand (1.52 ± 0.05 mg kg^{-1} in sand and 0.91 ± 0.02 mg kg^{-1} in potted field soil). Indeed, the sand contains no organic matter to bind Cd; therefore, the major fraction of the total concentration of 5 mg Cd kg^{-1} remains in the soils solution and is by consequence available for the plants. Most likely, plants grown on potted field soil remained smaller primarily because of nutrient limitations (plants on sand received every other day 50 ml of ½ HL solution while plants on field soil were moistened with tap water). Therefore, we may conclude that bacterial effects are probably more distinct in nutrient-poor conditions. In order to obtain more relevant bacterial actions *in planta*, we suggest the use of field-based nutrient solutions during future inoculation experiments on artificial soil (in this case spiked sand).

Despite the promising *in vitro* and *in planta* characteristics of seed endophyte UH1 (table 7.2), no improvements on phytoextraction potential *in situ* were achieved (figure 7.2, 7.3 and 7.4). The capacity of strain UH1 to tolerate Cd and

Zn, to produce siderophores, organic acids, ACC deaminase and IAA and to solubilize phosphorus and fixate nitrogen could not render in the field. So, regardless the bacterial potential to survive in a with Cd and Zn contaminated environment, to solubilize trace elements and nutrients and to produce 2 highly plant growth-promoting factors (Glick and Stearns, 2011) (*i.e.* ACC deaminase (Arshad *et al*., 2007) and the plant growth hormone IAA (Tanimoto, 2005)), strain UH1 did not affect the phytoextraction efficiency of *B. napus* grown in a contaminated field.

As it was the case on sand with adding Hoagland solution, the nutrient concentration in the field was increased by adding 3 cm compost, which probably impaired bacterial effects. Moreover, $Ca(NO_3)_2$-exchangeable Cd, Zn and Fe concentrations in the field were remarkably lower than in the field soil (from exactly the same place) used in the pot experiments, while total concentrations were similar (table 7.3). The 'potentially available' Cd concentration to non-inoculated control plants in the field was 0.26 ± 0.02 mg kg^{-1} (figure 7.2), *i.e.* 70 % less Cd than in the collected field soil. Before adding compost, we did not expect such an impact on metal availability. The effect of the manure will surely have relieved the plant from extra stress concerning low nutrient availability, while the harsh conditions imposed by the weather and herbivores caused extra stress in the field. So, before repeating this field inoculation experiment, it is important to find out which conditions are ideal to test bacterial performance.

At last, 4 (32a, 36b, 92c and 55b) of the 6 selected strains for application in the field significantly increased dry root weight (figure 7.4) of which 32a significantly increased plant growth on sand and 36b in the field soil (root weight) (table 7.2). Also the consortium of rhizosphere strains 32a, 36b, 92c and 126e and root endophytic strain 55b resulted in a significant root growth promotion (figure 7.4). So despite all problems related to field inoculation experiments, we conclude with good prospects for future experiments certainly when high Cd availability rates are pursued.

Conclusions

Our bacterial selection procedure which is based on an *in vitro* characterization of plant-associated bacteria isolated from a contaminated soil, followed by *in*

planta evaluation towards the final *in situ* inoculation experiment, delivered us promising bacterial strains for future optimized field experiments with *B. napus* on Cd-contaminated soils. We learned that the addition of manure in the field can have a major impact on metal availability in the soil and probably diminishes bacterial capacities. It is clear that it is very important to know the exact conditions wherein bacterial performance can be at its maximum.

We conclude that based on a thorough understanding of the processes and interactions between plants, contaminants, soils and bacteria, bacterial-assisted phytoremediation can become of high interest for the remediation of large-scale metal contaminated sites. However, it may be the combination of engineering methods and enhanced phytoextraction that will provide the ultimate solution to clean up contaminated sites (Newman and Reynolds, 2005; Doty, 2008; Sessitsch *et al*., 2013). Anyway, even when beneficial bacteria can only stimulate plant growth and protect the plant from metal toxicity, a higher phytoextraction efficiency is obtained even without an increase metal availability and uptake.

Supporting Information

Appendix 7.1 Detailed characterisation of all purified *B. napus* seed isolates. Strains were collected from seeds harvested at the contaminated field. The presence of each strain is shown as relative abundances, expressed in percentages, of the total number of colony forming units per gram fresh weight (cfu gFW^{-1}). Strains are identified to the genus level, their accession numbers as well as their presence in the 1st, 2nd or 3rd replicate (repl) are displayed. Mean percentages were calculated based on 3 replicates. The potential plant growth-promoting characteristics of the strains are indicated by + when positive and by ++(+) in case of a strong positive test. Bacterial strains testing negative for a phenotypic test were labeled by a – symbol and those not applicable for the test by 'not detected' (nd). The plant growth-promoting characteristics tested were Cd (0.8 mM) and Zn (1 mM) tolerance and the capacity to solubilise phosphorus (P sol), fixate nitrogen (N_2 fix) and produce siderophores (SID), organic acids (OA), ACC deaminase (ACC), indole-3-acetic acid (IAA) and acetoin.

repl	cfu gFW^{-1}	% repl	% mean	identification	accession	Cd	Zn	SID	OA	ACC	IAA	ACE	P sol	N_2 fix
1	1000	0.06	0.03	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	++	nd
1	1000	0.06	0.03	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	++	nd
1	3000	0.18	0.09	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	-	nd
1	9000	0.55	0.275	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	-	-
1	10000	0.61	0.305	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	-	nd
1	10000	0.61	0.305	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	-	nd
1	17700	1.08	0.54	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	-	nd
1	20000	1.22	0.61	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	++	nd
1	35400	2.17	1.085	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	-	nd
1	40000	2.45	1.225	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	+	nd
1	40000	2.45	1.225	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	+	nd
2	1000	3.18	1.59	*Alcaligenes*	AJ509012	+	-	-	-	-	+	-	++	nd
1	53100	3.25	1.625	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	+	nd
1	56000	3.43	1.715	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	-	nd
1	70000	4.28	2.14	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	++	nd
1	89600	5.48	2.74	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	+	nd
1	200000	12.24	6.12	*Alcaligenes*	AJ509012	++	++	-	+	-	-	-	+	-
1	240000	14.69	7.345	*Alcaligenes*	AJ509012	++	++	-	-	-	-	-	++	nd
2	100	0.32	0.16	*Bacillus*	AJ717381	-	-	-	+	-	-	-	++	+
2	1000	3.18	1.59	*Bacillus*	AJ717381	-	-	-	+++	-	nd	-	++	++
2	1000	3.18	1.59	*Bacillus*	AJ717381	-	-	-	+	+	-	-	++	++
2	100	0.32	0.16	*Bacillus*	AJ920000	nd	nd	-	nd	-	nd	-	+	++
2	400	1.27	0.635	*Bacillus*	AJ920000	-	-	-	nd	-	nd	-	-	nd
2	100	0.32	0.16	*Bacillus*	JQ359106	-	-	-	nd	+	nd	-	-	++
2	100	0.32	0.16	*Bacillus*	JQ359106	-	-	-	-	-	-	-	+	+
2	100	0.32	0.16	*Bacillus*	JQ359106	-	-	-	nd	-	nd	-	-	nd
1	17700	1.08	0.54	*Bacillus*	JQ359106	-	-	-	-	+++	-	-	-	nd
2	1000	3.18	1.59	*Bacillus*	JQ359106	-	-	-	nd	+++	nd	-	-	nd
2	100	0.32	0.16	*Lysinibacillus*	AJ310083	-	-	-	-	++	-	+	+	+++
2	100	0.32	0.16	*Lysinibacillus*	AJ310083	-	-	+	++	-	-	++	+	-
2	1000	3.18	1.59	*Lysinibacillus*	AJ310083	-	-	+	+	-	-	+++	-	-
2	100	0.32	0.16	*Micrococcus*	AJ409096	-	-	-	+	+++	-	-	+	++
1	8600	0.53	0.265	*Micrococcus*	AJ409096	-	-	-	+	+++	-	-	-	++
1	8600	0.53	0.265	*Micrococcus*	AJ409096	-	++	-	+	+++	-	-	-	++
1	8600	0.53	0.265	*Micrococcus*	AJ409096	++	++	++	-	+++	++	-	++	++

1	10000	0.61	0.305	*Micrococcus*	AJ409096	-	++	-	-	+++	-	-	-	+++
1	11200	0.69	0.345	*Micrococcus*	AJ409096	-	++	-	-	+	-	-	-	-
1	12900	0.79	0.395	*Micrococcus*	AJ409096	-	-	-	-	+++	-	-	-	-
1	20000	1.22	0.61	*Micrococcus*	AJ409096	-	-	-	-	+	-	-	-	++
1	30000	1.84	0.92	*Micrococcus*	AJ409096	-	-	-	-	-	-	-	-	+++
1	40000	2.45	1.225	*Micrococcus*	AJ409096	-	-	-	+	+++	-	-	-	++
1	40000	2.45	1.225	*Micrococcus*	AJ409096	-	-	-	-	+++	-	-	-	nd
2	1000	3.18	1.59	*Micrococcus*	AJ409096	-	++	-	++	-	-	-	-	+
2	1000	3.18	1.59	*Micrococcus*	AJ409096	-	++	-	+++	-	-	-	+	nd
1	53100	3.25	1.625	*Micrococcus*	AJ409096	-	-	-	+	+++	-	-	-	++
1	53100	3.25	1.625	*Micrococcus*	AJ409096	-	-	-	+	+++	-	-	-	+
1	88500	5.42	2.71	*Micrococcus*	AJ409096	-	+	-	-	+++	-	-	-	++
1	200000	12.24	6.12	*Micrococcus*	AJ409096	-	++	-	-	+++	-	-	-	+++
2	100	0.32	0.16	*Paenibacillus*	AM162303	-	-	-	-	++	-	-	+	-
2	100	0.32	0.16	*Paenibacillus*	AY289507	-	-	-	-	-	nd	-	-	nd
2	100	0.32	0.16	*Paenibacillus*	AY289507	-	-	-	+	+++	nd	-	++	-
2	100	0.32	0.16	*Paenibacillus*	AY289507	-	-	-	+++	+++	nd	-	++	-
2	100	0.32	0.16	*Paenibacillus*	AY289507	-	-	-	+++	+++	nd	-	+	-
2	100	0.32	0.16	*Paenibacillus*	AY289507	-	-	-	+++	+++	nd	-	+	-
2	100	0.32	0.16	*Paenibacillus*	AY289507	-	-	-	+	-	-	-	+	-
1	9000	0.55	0.275	*Paenibacillus*	AY289507	-	-	-	++	+++	nd	-	++	+
2	200	0.64	0.32	*Paenibacillus*	AY289507	-	-	-	++	+++	nd	-	++	-
2	200	0.64	0.32	*Paenibacillus*	AY289507	-	-	-	++	-	+	-	+	-
2	300	0.96	0.48	*Paenibacillus*	AY289507	-	-	-	+	-	-	-	+	nd
1	17700	1.08	0.54	*Paenibacillus*	AY289507	++	-	-	-	-	-	-	-	nd
2	1000	3.18	1.59	*Paenibacillus*	AY289507	nd	nd	-	+	+++	-	-	+	-
2	1000	3.18	1.59	*Paenibacillus*	AY289507	-	-	-	-	-	-	-	++	-
2	1000	3.18	1.59	*Paenibacillus*	AY289507	-	-	-	-	-	+	-	-	-
2	1000	3.18	1.59	*Paenibacillus*	AY289507	-	-	-	-	-	-	-	++	+
2	100	0.32	0.16	*Pseudoclavibacter*	HM584267	-	-	-	-	-	-	-	-	nd
1	10000	0.61	0.305	*Pseudoclavibacter*	HM584267	++	++	-	-	-	-	-	-	nd
1	17700	1.08	0.54	*Pseudoclavibacter*	HM584267	-	-	-	-	-	-	-	-	nd
2	10000	31.85	15.925	*Pseudoclavibacter*	HM584267	-	+	-	++	+++	-	-	++	++
1	4000	0.24	0.12	*Pseudomonas*	AJ011504	++	+	+	-	+	++	-	++	+
2	100	0.32	0.16	*Pseudomonas*	AJ011504	+	-	-	-	+	+	-	+++	nd
2	100	0.32	0.16	*Pseudomonas*	AJ011504	-	-	-	-	-	+	-	++	nd
1	9000	0.55	0.275	*Pseudomonas*	AJ011504	++	+	++	+	-	++	-	++	+
2	200	0.64	0.32	*Pseudomonas*	AJ011504	-	-	-	-	+	+	-	++	nd
2	200	0.64	0.32	*Pseudomonas*	AJ011504	+	-	-	-	-	+	-	++	nd
1	22400	1.37	0.685	*Pseudomonas*	AJ011504	++	+	++	+	+++	++	-	+	++
1	22400	1.37	0.685	*Pseudomonas*	AJ011504	++	+	++	+	+++	++	-	+	++
1	22400	1.37	0.685	*Pseudomonas*	AJ011504	++	++	-	-	-	-	-	++	nd
2	1000	3.18	1.59	*Pseudomonas*	AJ011504	-	-	-	-	-	+	-	++	nd
2	3000	9.55	4.775	*Pseudomonas*	AJ011504	-	-	-	-	-	+	-	++	nd
1	1000	0.06	0.03	*Rhizobium*	AF025853	+	-	-	-	++	+	-	++	-
2	2000	6.37	3.185	*Rhizobium*	AF025853	-	-	-	-	-	-	-	+	-
2	100	0.32	0.16	*Staphylococcus*	HM355630	-	-	-	++	-	+	-	-	nd
2	1000	3.18	1.59	*Staphylococcus*	HM355630	-	-	-	+++	+++	-	-	-	nd

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CHAPTER 8

General discussion, conclusions and perspectives

To remediate trace element-contaminated soils, high biomass plants accumulating these elements in their above-ground plant parts are of high interest (Vangronsveld *et al*., 2009). A major challenge is to obtain high accumulation rates without significant reduction of plant biomass. A sustainable strategy to increase phytoextraction efficiency is to exploit microorganisms (*i.e.* bacteria and fungi) associated with plants of interest (Weyens *et al*., 2009). In this work, we investigated *Brassica napus* L. (rapeseed) as a possible phytoextractor plant for Cd-contaminated soils. In addition, the potential of its associated bacteria to increase phytoextraction efficiency is explored. As it is known from literature, bacteria with the most interesting characteristics to improve phytoextraction efficiency should to be isolated from a naturally contaminated environment (Diaz-Ravina and Baath, 1996; Lodewyckx *et al*., 2002; Idris *et al*., 2004).

First of all, it was of critical importance to optimize a sampling and isolation procedure for the cultivable rapeseed-associated bacteria isolated in the field (Hughes *et al*., 2001) (chapter 3 (figure 3.4)). To obtain a reliable characterization (genotypic and phenotypic) of the cultivable root endophytic community, the data of 3 independent replicates, each consisting of 3 root systems, should be combined. In case of stem and leaf endophytic populations, each replicate had to be enlarged with plant parts of 3 additional plants (appendix 3.1 versus 3.2). These optimized procedures for sampling and isolation are applied in the next chapters (4 and 5) to determine contaminant-induced and seasonal effects on bacterial populations of rapeseed. In chapter 4, rapeseed was grown in non-contaminated (CO) and a Cd-contaminated (TE) field in order to study the effects of metal stress on the characteristics of the rapeseed-associated bacterial communities. In addition to these site-specific effects, we investigated the possible seasonal effects on these cultivable bacterial communities (chapter 5). Figure 8.1 summarizes the major genotypic results gathered from these two chapters.

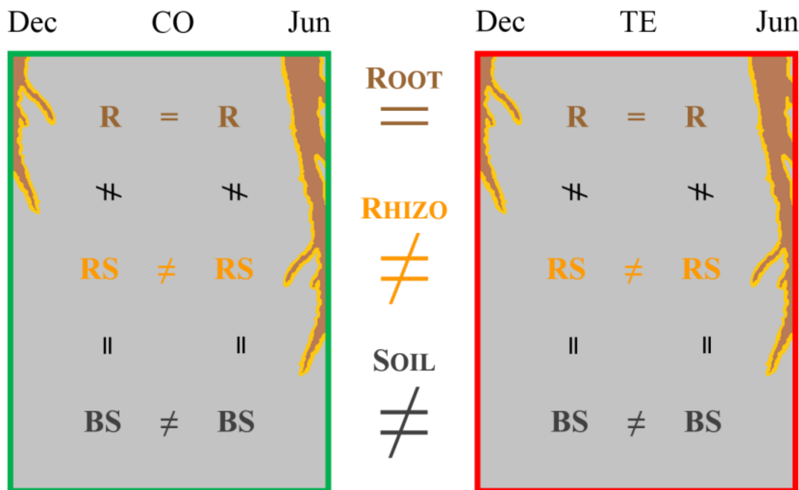

Figure 8.1 Schematic overview of the most important genotypic variations between bacterial bulk soil (BS), rhizosphere soil (RS) and root (R) communities isolated in December (D) and June (J) on a non-contaminated control field (CO) and a trace element-contaminated field (TE). Variations are based on the correlation coefficients generated from a correspondence analysis (appendix 5.2). Similar bacterial communitites share correlation coefficients ≥ 0.45.

From chapters 4 and 5 we can conclude that the bacterial communities isolated from the bulk soil (BS) and rhizosphere soil (RS) at both fields (CO and TE) are genotypically correlated in June (J) and December (D), but differ between fields and seasons (figure 4.3 and 5.3). On the contrary, endophytic root communities were genotypically similar between fields and seasons, but differed from the communities living in the rhizosphere and bulk soil. These data suggest that local conditions play a more important role in influencing the composition of rhizosphere and bulk soil bacterial communities than root exudates and that the bacterial strains composing the endophytic root communities are mainly derived from the seeds as plants selected only a few strains from the rhizosphere (figure 4.1/4.2 and 5.2). Also Lundberg *et al.* (2012) found that bacterial communities in rhizosphere and bulk soil were strongly influenced by soil type although endophytic communities from different soils feature overlapping, low-complexity communities.

Seasonal differences, primarily based on changes in environmental conditions (*e.g.* temperature, light and humidity) and the composition of root exudates, present in the rhizosphere and bulk soil bacterial communities were not

observed in the roots (figure 5.3). There indeed exists a rich literature describing seasonal dynamics of many components of the belowground community in a range of natural and farming ecosystems (Wardle 2002; Bardgett, 2005).

Based on these genotypic results (figure 8.1), we suggest that endophytes in contrast to (rhizosphere) soil bacteria could be protected against various effects from outside (Lundberg *et al.*, 2012). In other words, the plant interior may buffer and maintain some conserved bacterial lineages.

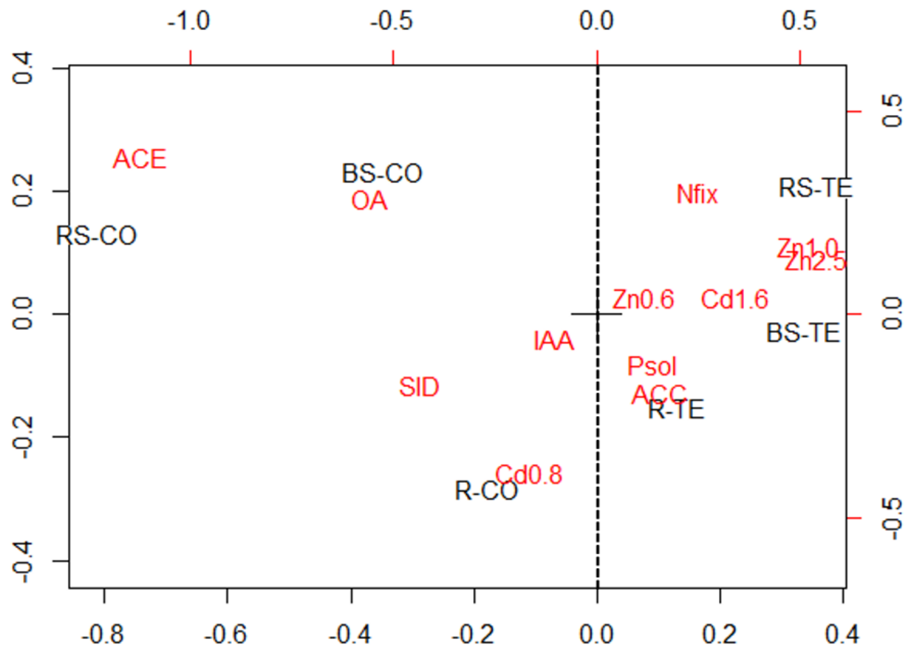

Figure 8.2 Correspondence analysis of bacterial communities isolated from bulk soil (BS), rhizosphere soil (RS) and *B. napus* roots (R) at the non-contaminated control (CO) and the trace element-contaminated (TE) fields in June. Clustered compartments (see left and right side of the dotted line) point out the correlation between bacterial communities concerning phenotypical characteristics plotted in red (tolerance to Cd (0.8 and 1.6 mM) and Zn (1.0 and 2.5 mM), phosphate solubilisation (Psol), nitrogen fixation (Nfix) and production of siderophores (SID), organic acids (OA), indole-3-acetic acid (IAA), acetoin (ACE) and 1-aminocyclopropane-1-carboxylate deaminase (ACC)). Data are based on 3 replicates consisting of 3 independent compartment samples.

Considering the phenotypic characteristics in June, bacterial communities isolated at the trace element-contaminated field (TE) harboured significantly more metal tolerant (Cd and Zn), nitrogen fixating and phosphorus solubilizing

bacteria. Also the majority of bacterial strains producing IAA and ACC deaminase originated from the contaminated field (table 4.2 and figure 8.2). The production of siderophores, organic acids and acetoin was most pronounced in the rhizosphere at the non-contaminated control field (CO). Also the other bacterial characteristics, highly represented at the contaminated field, were predominantly found in the rhizosphere (except for IAA production which was highest in the roots) (table 2.2). Indeed, the root-rhizosphere interface is the nexus of a variety of exchanges between bacteria and their host plant and therefore an ideal model for studying interactions between plants and microorganisms (Gottel *et al*., 2011).

To study the seasonal effects on the phenotypic characteristics of bacterial communities isolated from both fields, only Cd tolerance was considered. Additionally, bacterial counts and bacterial diversity indices were analysed to elucidate seasonal influences on these bacterial communities (figure 8.3). Also the field effects on these characteristics are represented in figure 8.3.

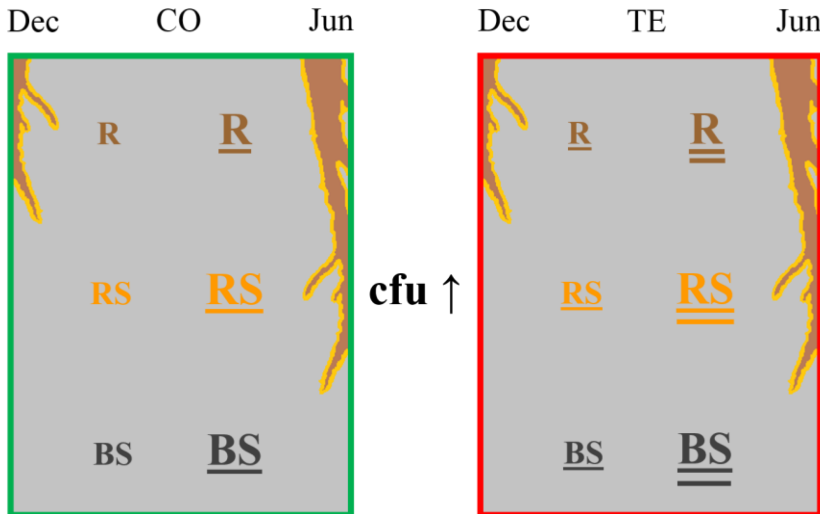

Figure 8.3 Schematic overview of the most important phenotypic (*i.e.* Cd tolerance indicated by underlining) and structural (*i.e.* diversity index indicated by fontsize and bacterial count (*i.e.* amount of colony forming units (cfu))) variations between bacterial bulk soil (BS), rhizosphere soil (RS) and root (R) communities isolated in December (Dec) and June (Jun) on a non-contaminated control field (CO) and a trace-element contaminated field (TE).

At both fields, bacterial communities were more diverse and Cd tolerant in June (respectively indicated by increasing font size and underlining in figure 8.3). The highest percentages of Cd tolerant strains were consistently found at the contaminated field (figure 5.2). In specific fields and seasons similar bacterial diversities and tolerance parameters were determined between compartments (*i.e.* BS, RS and R) (figure 5.2). In general, rhizosphere communities contained the highest numbers of colony forming units (cfu ↑, see figure 8.3), while neither field nor seasonal effects were observed concerning bacterial counts (figure 5.1). Based on the phenotypic results from chapters 4 and 5, bacteria with the potential to increase phytoextraction efficiency are likely to be found at the contaminated field in June (figure 3.2). That the presence of specific bacterial phenotypes is correlated with the presence of contaminants as a result of selective pressure was already reported before (Siciliano *et al*., 2001). It further makes sense that bacteria are more active in summer when soil labile C from root exudates is higher than in winter (Guicharnaud *et al*., 2010). Other factors influencing bacterial activity are soil temperature, soil humidity, soil fertilization, etc.

Particular attention could be paid to the strains that are present during the entire growing season on both sites because they might be promising to improve plant growth and Cd uptake. In this respect it is important to notice that *B. napus* plants grown at both fields were persistently colonised by *Bacillus, Pseudomonas* and *Variovorax* species in the rhizosphere and by *Pseudomonas* strains in the roots (figure 5.2). At the end of the growing season, high numbers of *Bacillus* and *Pseudomonas* strains were recovered from the shoots harvested at both fields, possibly implying transfer of essential endophytes to the seeds. Indeed, high amounts of *Bacillus* strains were found in the harvested seeds (figure 5.4). Moreover, bacterial communities isolated from the shoots in June tend to be more similar to the seed endophytic communities (figure 5.5). Besides, the most promosing seed endophyte isolated at the contaminated field was a *Pseudomonas* strain (appendix 7.1 and table 7.2).
Because the main goal of this study was to identify appropriate bacterial strains for inoculation purposes, we focussed on the cultivable bacterial communities. However, it would be interesting to repeat the work presented in chapters 4 and

5 including non-cultivable bacterial strains, for determining if similar conclusions can be made. Indeed, the emerging use of molecular biology in microbial ecology has revealed that 0.1-10 % of the microorganisms present in numerous environments are readily cultivable (Amann *et al*., 1995; Hugenholtz, 2002; Schloss and Handelsman, 2004; Hall, 2007). Lately, genomic analysis of environmental samples is becoming an important tool for understanding evolutionary history and functional and ecological biodiversity (Shokralla *et al*., 2012). Since 2005 (Roche 454 genome sequencers), advances in next-generation sequencing technologies have revolutionized biological science. The analysis of environmental DNA through the use of specific gene markers such as species-specific DNA barcodes has been a key application of next-generation sequencing technologies in ecological and environmental research. For bacterial identification, 16S ribosomal RNA (16S) is commonly used (Sogin *et al*., 2006; Flanagan *et al*., 2007; Rousk *et al*., 2010; Nacke *et al*., 2011). Recovering DNA sequences from the thousands of specimens present in an environmental bulk sample requires the ability to read DNA from multiple templates in parallel; something that next-generation sequencing technologies do effectively, and with ever-lowering costs. By comparing obtained sequences to a growing standard reference library of known organisms, taxa present in an environmental sample can be identified with high confidence.

This study mainly aimed at selecting a strain or consortium (from the cultivable communities isolated in chapters 4 and 5) that can be inoculated/enriched to enhance Cd uptake and/or biomass production. Considering the presence of *Bacillus* and *Pseudomonas* species throughout the whole growing season on both sites, it might be relevant to concentrate on these genera. Moreover, data from chapter 3 show their ability to colonize all organs (figure 3.3) of most individual *B. napus* plants (figure 3.1). Predominant strains that are (a) part of some conserved bacterial lineages, (b) hardly affected by factors from outside and (c) transferred via the seeds, might be of great importance in plant development and survival and therefore interesting for enrichment during phytoremediation projects. Nevertheless, all isolated strains were screened *in vitro* for their characteristics to increase plant growth and Cd uptake (chapters 4 and 5). Based on this screening, a first selection of promising bacteria for inoculation

purposes was made (table 6.1). Most selected bacterial strains originated from the contaminated field (65%). Out of these 41 strains, 27 strains originated from the June sampling. From these 27 strains, 20 were isolated from the rhizosphere or roots of *B. napus*. Seven out of these 20 strains were *Pseudomonas* species. The genus *Pseudomonas* was present in the rhizosphere and roots during the entire growing season of *B. napus* on both fields (figure 5.2), and able to colonize all plant tissues (figure 3.3).

All bacterial strains that were promising in *in vitro* tests were enriched in *B. napus* seedlings growing on vertical agar plates (VAPs) containing for the plants a sublethal Cd concentration of 250 μM (figure 6.1 and 6.2). The best-performing strains on VAPs (marked with a '√' in table 6.1) were subsequently tested on sand spiked with 5 mg Cd kg^{-1} (figure 6.3 and 6.4). In table 8.1, the most important *in vitro* characteristics (> 60%) of the strains having a significant effect on the phytoextraction potential of *B. napus* (*i.e.* by increasing plant weights and/or Cd uptake) on VAPs, sand, field soil and in the field are summarized.
A majority of the strains (> 60 %) having a clear effect on Cd availability (determined as $Ca(NO_3)_2$-exchangeable Cd) and uptake in the growth chamber were Cd tolerant and phosphorus solubilizing and also showed able to produce siderophores and IAA (table 8.1). For bacteria, in order to be plant growth promoting under controlled conditions, Cd tolerance and the capacity to produce siderophores are important prerequisites.
The most important *in vitro* characteristics (> 60%) of the strains having a significant effect on the phytoextraction potential of *B. napus* (*i.e.* by increasing plant weights and/or Cd uptake) are represented. Strains were screened for the capacity to solubilise phosphorus (P sol), fixate nitrogen (N_2 fix) and produce siderophores (SID), organic acids (OA), ACC deaminase (ACC), indole-3-acetic acid (IAA) and acetoin (ACE). Tolerance to Cd (0.4, 0.8 and 1.6 mM) was also evaluated. When data were not available, this is indicated by "n.a.".

Table 8.1 Importance of bacterial *in vitro* characteristics to significantly increase $Ca(NO_3)_2$-exchangeable Cd concentrations in the substrate (Cd substrate), total Cd concentrations in the shoot (Cd shoot), root length, shoot weight and root weight of *B. napus* plants grown on Cd polluted media (*i.e.* agar on vertical agar plates (VAPs), sand and field soil) in the growth chamber and in the field.

	VAPs	Sand	Field soil	Field
Cd substrate	n.a.	Cd SID IAA P sol OA ACE	n.a.	Cd SID OA IAA
Cd shoot	Cd SID IAA P sol	Cd SID IAA P sol N_2 fix	n.a.	n.a.
Root length	Cd SID P sol ACC	n.a.	n.a.	n.a.
Root weight	Cd SID P sol IAA ACC	SID P sol ACC	Cd SID IAA ACC	Cd SID IAA ACC
Shoot weight	Cd SID IAA ACC	Cd SID P sol ACC	n.a.	n.a.

Based on VAPs data, the ability to solubilize phosphorus would be a relevant criteria for increasing root growth. A more extended root system exploring a larger volume of topsoil in its turn can contribute to an increased Cd uptake. An increased plant weight on VAPs is likely associated with the bacterial production of IAA and ACC deaminase. Whereas the presence of IAA also positively influences Cd uptake (Du *et al*., 2011), ACC deaminase has a greater role in improving plant growth. For increasing plant weight on spiked sand, strains able to solubilize phosphorus and produce siderophores and ACC deaminase would be of great importance. On spiked sand, bacterial Cd tolerance and IAA production are more important in achieving an increased Cd uptake than a higher plant weight, while on VAPs both characteristics were highly present in strains enhancing plant weight as well as Cd uptake.

The most interesting strains on spiked sand were further tested on potted field soil in an attempt to better mimic real field conditions (figure 6.6). However, the expectations of the selected strains (*i.e.* increased plant growth and/or Cd uptake after inoculation) were not fulfilled on field soil, at least not during the time span of the experiment (figure 6.5). The most obvious explanation is that the competition with the indigenous bacterial community in the soil alleviates the beneficial effects of the inoculated bacteria. Another possibility is that the cultivation period of 4 weeks was too short for the inoculated bacteria to compete with the indigenous bacterial community of the field soil. Therefore, all the strains tested on field soil were inoculated in the field.

None of the bacteria selected for the field trial significantly enhanced Cd availability or Cd uptake in the field (figures 7.2 and 7.3). However, many of the selected strains had a highly significant positive effect ($p < 0.01$) on root dry weight in the field (figure 7.4). In the *in vitro* tests, most of these strains showed to be Cd tolerant and capable of producing siderophores, IAA and ACC deaminase. The same *in vitro* characteristics were important to slightly promote root growth on field soil in the growth chamber (figure 6.5). Strains primarily selected for improving Cd uptake in the field had a smaller positive effect on root growth; their most common *in vitro* characteristics were Cd tolerance, phosphorus solubilisation and the production of siderophores and IAA. So, the ability to produce ACC deaminase would be one important prerequisite to improve plant growth on a Cd-contaminated field. Based on these extra results

from the field (chapter 7), we may conclude that the most relevant bacterial traits to improve *in planta* Cd uptake (on VAPs, sand and field soil) are Cd tolerance, phosphorus solubilisation and the production of siderophores and IAA. Indeed, bacterial siderophores, primarily excreted to bind Fe for bacterial uptake, can also be taken up by plants when bound with Fe or other bivalent trace elements like Cd (Burd *et al*., 2000; Rajkumar *et al*., 2010). Therefore, bacterial siderophores can increase Cd availability in the soil thereby facilitating its uptake by plants. Also bacterial strains able to solubilize phosphorus can increase Cd availability in the soil; it is known that those strains often produce (in)organic acids which simultaneously enhance metal solubility (Ivanova *et al*., 2006). Subsequently, an increased root surface can lead to a higher trace element uptake. Indeed, the bacterial production of IAA, a plant growth hormone which especially promotes root growth (Sheng and Xia, 2006; Sheng *et al*., 2008), can be important in plants to increase Cd uptake. After uptake, the deleterious Cd effects may be diminished by the presence of Cd tolerant endophytic bacteria equipped with metal-efflux mechanisms (Lodewyckx *et al*., 2001). Endophytic strains possessing such mechanisms excrete metals after uptake, whereupon precipitation on the bacterial cell wall takes place (Nies *et al.*, 1995). In this way, the fraction of free potentially toxic ions in the plant diminishes resulting in the alleviation of toxicity, enabling the plant to take up more (Cd) ions without experiencing their toxic effects.

From our *in planta* results, with cultivable bacterial strains, we conclude that the most important bacterial *in vitro* characteristics to improve plant weight are Cd tolerance and the production of siderophores, IAA and ACC deaminase (table 8.1). The influence of bacterial ACC deaminase production is more relevant for plant biomass production than for increasing Cd uptake by plants. The reduction of stress induced by the cleavage of ACC, the precursor of the plant stress hormone ethylene, may support the plant ability to resume normal growth. Other studies reported that ACC deaminase-producing strains can increase plant biomass by reducing metal toxicity (Burd *et al*., 1998; Borgmann, 2000; Glick, 2003). Concerning stress reduction, the inoculation of Cd tolerant strains may also reduce levels of free and thus toxic Cd ions inside the plant. Bacteria that can stimulate root development via IAA production are not only important to

increase Cd uptake, but also uptake of macro- and micronutrients resulting in improved plant growth. Iron, as well as other nutrients, is often limited in plants grown in contaminated soils. Indeed, Cd can (in)directly perturb metabolic processes concerning Fe in the plant. Siderophores are important metabolites released by plant growth-promoting bacteria (PGPB) that indirectly alleviate metal toxicity by increasing the supply of iron to the plant (Burd *et al*., 2000).

Taken all results together, the *in vitro* characterization tests that we used did not deliver sufficiently predictive information for what is happening in the field and by consequence are not really satisfying. Hence, for future *in vitro* characterizations, we suggest to incorporate at least some additional tests concerning bacterial colonization efficiency and ability to significantly decrease soil pH as those 2 characteristics turned out to be quite crucial to increase phytoextraction efficiency on field soil (figure 7.2). From the inoculation experiments, we may conclude that the closer we get to the real field conditions, the more complex and less controllable the interactions in the soil become (figure 8.4).

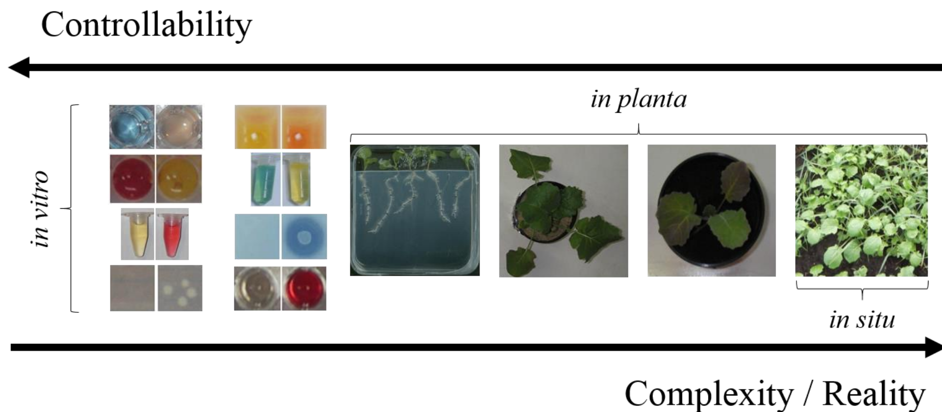

Figure 8.4 The relationship between the complexity and the controllability of *in vitro* and *in planta* experiments with bacteria in order to test their potential to increase phytoextraction efficiency in real field (*in situ*) conditions.

It is difficult for an inoculated strain to compete with the natural abundant bacterial communities in the field soil (Newman and Reynolds, 2005), although we chose for strains previously isolated from the field. Therefore it is of crucial

importance that strains that are selected for inoculation also possess the ability to efficiently colonize the phytoextractor plant. Colonisation efficiency can be estimated *in vitro* by testing the bacteria on their ability to produce enzymes involved in degrading plant cell walls (*e.g.* protease, cellulose, pectinase); also mobility (flagella) and positive chemotaxis can be indicative for a good colonisation potential (Truyens *et al*., 2014). To be able to select strains that can effectively solubilize metals in the field, we suggest to determine the *in vitro* activation of poorly soluble Cd by bacteria in fluid medium as described by Sheng and Xia (2006). Bacterial strains able to decrease pH and increase the water-soluble Cd concentration in the medium should be included in future inoculation experiments. Nevertheless, even a thorough *in vitro* and *in planta* screening of bacterial strains is no guarantee for a successful application in a highly complex field situation.

Moreover, as we mentioned in chapters 6 and 7, we have to be aware of the possible effects of the use of fertilizers and especially compost during the inoculation experiments since they can influence the bioavailability of some metals/metalloids in the substrate (Wang *et al*., 2008; Moreira *et al*., 2013) as well as bacterial performance. Indeed, the addition of nutrients stimulates the growth of all bacterial strains present in the rhizosphere making it even more difficult for the inoculated strain to compete with the indigenous bacterial community. Therefore, for obtaining more realistic results on sand under controlled conditions, similar nutrient levels as in the field need to be applied. In this scenario, not only Cd toxicity but also the shortage of nutrients will underlie the reduced growth. By consequence, the effect of inoculation cannot anymore be studied only in function of Cd toxicity.
In conclusion, a more extended *in vitro* screening (*e.g.* colonization efficiency and pH influence as additional tests) might be useful to identify interesting strains/consortia for inoculation in the field. After a more extended *in vitro* screening, tests on VAPs might be deleted. Strains can directly be tested on potted sand with nutrient supply. Based on the inoculation results on less fertilized sand, better bacterial selections can be made to test on potted field soil. Performing them in open air can increased the value of experiments on potted field soil. Indeed, in this way plants can grow longer so that the

inoculated bacterial strain has more time to compete with the native plant-associated bacterial communities. Also the more realistic soil temperatures present in open air might diminish the discrepancies with the field experiments. Indeed, growth chamber conditions differ from those in the field rendering different inoculation results. Especially, the lower soil temperatures in the field can lead to significant differences in metabolic activity of roots and the inoculated bacteria and of course the activity and even composition of the native plant-associated bacterial communities.

All selected strains for the field experiment originated from the rhizosphere and roots of *B. napus* grown on the contaminated field. Additionally, except one strain, all were *Pseudomonas* species. We found *Pseudomonas* species in all tissues of each studied individual plant from the field (chapter 3). This genus was also associated with *B. napus* throughout the growing seasons and on both the non-contaminated and contaminated field (chapter 5). The seed endophyte UH1, incorporated in the field inoculation experiment, was isolated from the seeds of *B. napus* grown on the contaminated field (appendix 7.1). This seed endophyte was identified as a *Pseudomonas* strain and tested positive to all but one *in vitro* test (*i.e.* acetoin production) (table 7.2). From the former we suggest that in future selection and inoculation experiments strains should be isolated in June from plants growing on contaminated sites and to concentrate on strains that are permanently associated with their host plant.

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CHAPTER 9

Scientific output

Papers (A1)

Croes S, Weyens N, Colpaert JV, Vangronsveld J. (2014) Characterization of the cultivable bacterial populations associated with field-grown *Brassica napus* L.: an evaluation of sampling and isolation protocols. *Environmental Microbiology Reports* (accepted for publication, Manuscript ID: EMI-2014-0755.R2)

Truyens S, Jambon I, **Croes S**, Janssen J, Weyens N, Mench M, Carleer R, Cuypers A, Vangronsveld J (2013) 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

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: 371-384

Weyens N, Beckers B, Schellingen K, Ceulemans R, **Croes S**, Janssen J, Haenen S, Witters N, Vangronsveld J (2013) 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, **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. Environmental Pollution, 158: 2422-2427

Other publications (C2)

Montalbán B, Lobo C, **Croes S**, Weyens N, Vangronsveld J, Pérez-Sanz A (2014) Improvement of growth of *Helianthus tuberosus* L. by rhizosphere and root endophytic bacteria on a Cd-Zn contaminated soil. 11th International Phytotechnologies Society Conference, September 30 - October 3, Heraklion, Crete, Greece (poster presentation)

Croes S, Janssen J, Vercampt H, Carleer R, Weyens N, Vangronsveld J (2013) Inoculation of phenotypically promising *Brassica napus*-associated bacteria to enhance biomass production and Cd phytoextraction. 10th International Phytotechnology Society Conference, October 1-4 2013, Syracuse, New York, USA (poster presentation)

Janssen J, **Croes S**, Weyens N, Vangronsveld J, Carleer R (2013) Valorization of metal-contaminated soil with willow: using plant-associated bacteria to improve biomass production and metal uptake. 10th International Phytotechnology Society Conference, October 1-4 2013, Syracuse, New York, USA (poster presentation)

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Gielen M, Weyens N, Beckers B, **Croes S**, Truyens S, Boerjan W, Vangronsveld J (2012) Comparison of *Arabidopsis thaliana* with normal and reduced lignin: differences in growth, bacterial population and cadmium responses. 9th International Phytotechnology Society Conference, September 11-14 2012, Diepenbeek, Belgium (poster presentation)

Janssen J, Weyens N, **Croes S**, Witters N, Carleer R, Ceulemans R, Van Peteghem P, Vangronsveld J (2012) Improving biomass production of willow for phytoremediation of metal-contaminated soils using plant growth promoting bacteria and fertilization strategies. 9th International Phytotechnology Society Conference, September 11-14 2012, Diepenbeek, Belgium (poster presentation)

Weyens N, Truyens S, Saenen E, **Croes S**, Beckers B, van der Lelie D, Taghavi S, Vangronsveld J (2011) Modified endophytes for improving phytoremediation of mixed contaminations of toxic metals (Ni) and organic contaminants (toluene or TCE). 5th European Fout! Bladwijzer niet gedefinieerd.Bioremediation Conference, July 4-7 2011, Chania, Crete, Greece (oral presentation)

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Dupae J, Weyens N, Cuypers A, Truyens S, **Croes S**, Vangronsveld J. (2010) Equipping plant growth promoting bacteria (PGPBs) with a metal resistance system: effects on metal uptake and translocation in poplar. International Conference on Environmental Pollution and Clean Bio/Phytoremediation, June 16-19 2010, Pisa, Italy (oral presentation)

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