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WOORD VOORAF

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Doe wat je moet doen in deze wereld
met een groot en mooi lawaai
doe het met de dingen die je drijven
wees naïef maar wees niet saai

Tijd is kort
ik heb het gezien
tijd is kort
en aan het eind vergetelheid

Thé Lau (The Scene) – Tijd is kort

SUMMARY

Transgenerational signaling allows plants to adapt in a fast and efficient manner to biotic and abiotic stresses. Transgenerational plasticity can be mediated by maternally derived proteins, mRNAs and hormones, via DNA methylation and histone modifications, or via seed provisioning. Seed-transmitted endophytes could also play a role in this process, as was already shown for fungal endophytes of grasses. Vertically transmitted endophytes usually evolve to have a mutualistic relationship with their host in which they are able to improve plant growth and health.

In the **first section**, the objective was to understand how different factors affect the bacterial community present in seeds. To study the impact of Cd on the seed endophytic community, a selection pressure was simulated in *Arabidopsis thaliana* in order to create 2 types of seeds: from plants that were exposed to 2 µM Cd for several generations (Cd seeds) and from plants that were grown in parallel but never exposed to Cd (control seeds). The cultivable as well as the total endophytic communities of both seed types were characterized. Some bacterial genera, such as *Rhizobium* and *Pseudomonas*, appeared to be tightly associated with *A. thaliana* seeds, while others were specialized endophytes that are selectively taken up. Important bacterial characteristics for transfer to the next generations of plants were ACC deaminase activity and Cd tolerance in Cd seeds, and IAA production in control as well as Cd seeds. The presence of these selected endophytic communities in seedlings makes rapid adaptation to environmental conditions possible by stimulating different beneficial functions.
To verify whether the selection pressure induced in the lab-grown *A. thaliana* was comparable with a selection pressure occurring in the field, the cultivable endophytic communities associated with seeds of *Agrostis capillaris* from a long-term Cd/Ni contaminated field site and a non-contaminated field site were identified and characterized. Also in *A. capillaris*, bacterial ACC deaminase activity appeared to be very important for coping with Cd stress. Surprisingly, Cd tolerance was almost absent in seed endophytes from long-term Cd/Ni-exposed *A. capillaris*. This can be attributed to the pseudometallophyte nature of *A. capillaris*: metal exclusion from aerial parts as well as metal

compartmentalization and complexation makes the development of metal tolerance mechanisms in endophytes present in *A. capillaris* redundant.

In order to determine the influences of the substrate type, the seed endophytic communities of *A. thaliana* were also identified and characterized after generations of culturing on sand and on a mixture of sand and potting soil, 2 commonly used substrates for the production of *A. thaliana* seeds. The seeds harvested from plants growing on sand and potting soil were sown again, but this time on the same substrate, to determine if the substrate type still had an influence on the establishment of the plant endophytic community. Despite differences in the soil bacterial communities, the seed and radicle endophytic communities were very similar, which indicates that plants are able to select their endophytes. On the other hand, the leaf endophytic community showed to be mainly derived from the environment and not from the seed. This indicates that bacteria are recruited from the environment during plant growth to complement the endophytic community from which the seed endophytes of the next generation can again be selected. Moreover, it appeared that the genera that were most abundant in the cultivable endophytic community, mainly *Rhizobium* and *Pseudomonas*, coincide with the genera frequently detected in the total community.

In the **second section**, the importance of bacterial seed endophytes for seedling establishment and plant growth were studied. It was verified whether the *in vitro* plant growth promoting characteristics of *A. thaliana* and *A. capillaris* seed endophytes could be a predictor of *in planta* growth promotion. The effect of inoculation with the isolated seed endophytes on the growth and metal uptake of their host during Cd exposure was studied in order to evaluate their possible contribution to bacteria-assisted phytoremediation as a clean-up strategy.

Inoculation of *A. thaliana* with its seed endophytes on vertical agar plates or in a hydroponic culture led to improved plant growth upon Cd exposure, however results were not consistent. Therefore several features possibly involved in bacterial colonization and inoculation success were analysed. Different bacterial growth stages were associated with changes in motility, chemotactic behavior and pectinase activity, which could influence the plant colonizing capacity of the inoculated endophytes.

Inoculation of *A. capillaris* growing in a hydroponic culture with its seed endophytes resulted in a significantly improved plant growth in case of non-exposed plants. After inoculation of Cd-exposed plants, an increased Cd uptake was achieved without affecting plant growth. This indicates that inoculation of *A. capillaris* with its seed endophytes might be beneficial for plant establishment and growth during phytostabilization and phytoextraction of Cd-contaminated soils.

In conclusion, plants appear to be able to select which endophytes are transferred to the next generation, primarily on a phenotypic but probably also on a genotypic level. Understanding how different factors influence the seed endophytic community can improve seed quality and plant growth during different biotechnological applications. Seed endophytes seem to have the potential to contribute to a more efficient phytostabilization and phytoextraction of metal-contaminated areas, although it remains difficult to predict *in planta* behavior based on *in vitro* plant growth promoting characteristics. Moreover, optimization of the inoculation procedure is needed to generate a more stable and repeatable inoculation outcome.

SAMENVATTING

Transgenerationele signalisatie laat planten toe om zich op een snelle en efficiënte wijze aan te passen aan biotische en abiotische stressfactoren. Transgenerationele plasticiteit kan verlopen via maternale eiwitten, mRNA en hormonen, via DNA methylatie en modificatie van histonen, en via zaadvulling. Endofyten die overgedragen worden via de zaden kunnen ook bijdragen aan dit proces zoals reeds werd aangetoond voor endofytische schimmels in grassen. Verticaal overgedragen endofyten hebben meestal een mutualistische relatie met hun gastheer waarbij ze de groei en gezondheid van de plant kunnen bevorderen.

In **deel 1** werden de effecten van verschillende factoren op de bacteriële gemeenschap aanwezig in zaden bestudeerd. Om de impact van Cd op de zaadendofyt-gemeenschap te bestuderen, werd een selectiedruk gesimuleerd bij *Arabidopsis thaliana* waardoor 2 zaadtypes tot stand kwamen: van planten die blootgesteld werden aan 2 µM Cd gedurende verschillende generaties (Cd zaden) en van planten die in parallel groeiden maar nooit blootgesteld werden aan Cd (controlezaden). Zowel de cultiveerbare als de totale endofytische gemeenschappen van beide zaadtypes werden gekarakteriseerd. Enkele bacteriële genera, zoals *Rhizobium* en *Pseudomonas*, waren nauw geassocieerd met de zaden van *A. thaliana*, terwijl andere genera meer gespecialiseerde endofyten bleken te zijn die selectief werden opgenomen. Belangrijke bacteriële kenmerken voor transfer naar de volgende generatie van planten waren ACC deaminase activiteit en Cd tolerantie in de Cd zaden, en IAA productie in beide zaadtypes. De aanwezigheid van geselecteerde endofytische gemeenschappen in de zaailingen maakt snelle aanpassingen aan veranderingen in de omgeving mogelijk doordat verschillende gunstige functies gestimuleerd kunnen worden.
Vervolgens werd nagegaan of de selectiedruk die geïnduceerd werd in de labo-gekweekte *A. thaliana* vergelijkbaar is met een selectiedruk aanwezig in een veldsituatie. Hiervoor werden de cultiveerbare endofytische gemeenschappen geassocieerd met de zaden van *Agrostis capillaris* groeiend op een veldsite die langdurig gecontamineerd is met Cd en Ni en op een niet-gecontamineerde controlesite geïdentificeerd en gekarakteriseerd. Ook in *A. capillaris* bleek

bacteriële ACC deaminase activiteit belangrijk om te kunnen omgaan met Cd stress. Opmerkelijk was dat er nauwelijks Cd tolerante endofyten aanwezig waren in de zaden van Cd/Ni-blootgestelde *A. capillaris*. Dit kan te wijten zijn aan het pseudometallofyt karakter van *A. capillaris*: exclusie van metalen uit de bovengrondse delen alsook compartimentalisatie en complexatie maken de ontwikkeling van metaaltolerantie mechanismen in endofyten aanwezig in *A. capillaris* overbodig.

Om het effect van het substraat te bepalen, werd de zaadendofyt-gemeenschap van *A. thaliana* ook geïdentificeerd en gekarakteriseerd na verschillende generaties gekweekt te zijn op zand en op een mengeling van zand en potgrond, 2 veelgebruikte substraten voor het produceren van *A. thaliana* zaden. De zaden geoogst van op zand en op potgrond gegroeide planten werden vervolgens opnieuw gezaaid, maar dan op hetzelfde substraat, om na te gaan of het substraat nog altijd een effect heeft op de endofytische gemeenschap van de plant. Ondanks verschillen in de aanwezige bodembacteriën waren de endofytische gemeenschappen in de zaden en kiemwortels zeer gelijkaardig. Dit wijst erop dat de plant in staat is om bepaalde bacteriële stammen te selecteren als endofyt. Daarentegen was de endofytische gemeenschap in de bladeren hoofdzakelijk afkomstig van de omgeving en niet van de zaden. Dit wijst erop dat tijdens de groei van de plant bacteriën gerekruteerd worden uit de omgeving om de endofytische gemeenschap te vervolledigen. Vanuit deze gemeenschap kunnen dan opnieuw de zaadendofyten van de volgende generatie geselecteerd worden. Bovendien bleken de genera die het meest voorkwamen in de cultiveerbare gemeenschap, hoofdzakelijk *Rhizobium* en *Pseudomonas*, ook de genera te zijn die het meest gedetecteerd werden in de totale gemeenschap.

In **deel 2** werd het belang van bacteriële zaadendofyten voor de ontwikkeling van de zaailing en de verdere groei van de plant bestudeerd. Er werd nagegaan of de plantengroei bevorderende eigenschappen die *in vitro* gedetecteerd werden in de zaadendofyten van *A. thaliana* en *A. capillaris* een goede indicatie vormen voor hun *in planta* effecten. Het effect van inoculatie met de geïsoleerde zaadendofyten op de groei en metaalopname van hun gastheer tijdens blootstelling aan Cd werd bestudeerd om zo hun mogelijke bijdrage aan bacterie-geassisteerde fytoremediatie als saneringsstrategie in te schatten.

Inoculatie van *A. thaliana* met zijn zaadendofyten op verticale agarplaten of in een hydrocultuur bevorderde de plantengroei bij blootstelling aan Cd, maar deze resultaten waren niet consistent. Daarom werden verschillende factoren die betrokken zijn bij bacteriële kolonisatie van de plant, en die dus het succes van de inoculatie kunnen beïnvloeden, onderzocht. Verschillende bacteriële groeifasen bleken geassocieerd met veranderingen in motiliteit, chemotaxis en pectinase activiteit, hetgeen de capaciteit van de geïnoculeerde endofyten om de plant te koloniseren kan beïnvloeden.
Inoculatie van *A. capillaris* met zijn zaadendofyten in een hydrocultuur resulteerde in een significant verbeterde groei in de niet-blootgestelde planten. Na inoculatie van de Cd-blootgestelde planten werd een verhoogde Cd opname bekomen zonder dat dit een negatief effect had op de plantengroei. Dit wijst erop dat inoculatie van *A. capillaris* met zijn zaadendofyten gunstig kan zijn voor de ontwikkeling en groei van de plant tijdens fytostabilisatie en fytoextractie van bodems verontreinigd met Cd.

Planten lijken dus in staat om te selecteren welke endofyten er naar de volgende generatie worden overgedragen, hoofdzakelijk op een fenotypisch, maar ook op een genotypisch niveau. Kennis van de effecten van verschillende factoren op de zaadendofyt-gemeenschap is belangrijk aangezien het de zaadkwaliteit en plantengroei kan verbeteren tijdens uiteenlopende biotechnologische toepassingen. Zaadendofyten lijken te kunnen bijdragen aan een efficiëntere fytostabilisatie en fytoextractie van metaalverontreinigde gronden, hoewel het moeilijk blijft om *in planta* gedrag te voorspellen op basis van *in vitro* bestudeerde plantengroei promoverende eigenschappen. Bovendien is er een optimalisatie van de inoculatieprocedure nodig zodat een consistenter en beter herhaalbaar resultaat verkregen kan worden.

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CHAPTER 1 Introduction to plant-bacteria interactions

Bacteria can interact with plants and impact their growth in many ways. They can bind to roots (rhizosphere bacteria), leaves (phyllosphere bacteria) or are located within plant tissues (endophytes) (Glick *et al.*, 2007). Some bacteria can establish an active relationship with their host and promote plant growth and health both directly as well as indirectly. In turn, plants provide the bacteria with nutrients and protect them from environmental stresses.

1.1 Direct plant growth promoting mechanisms

There are several ways in which plant-associated bacteria can improve plant growth. They can produce phytohormones that regulate the growth of their host, increase the availability of several important plant nutrients or counteract stress-induced ethylene production. Bacteria can possess one or more of these mechanisms, and can use different mechanisms under different conditions.

1.1.1 Plant growth regulating compounds

Auxins influence almost every aspect of plant growth and development. They modulate responses to light and gravity, regulate root and shoot architecture, organ patterning and vascular development (Davies, 1995). Production of the auxin indole-3-acetic acid (IAA), from the precursor tryptophan, is widespread among plant-associated bacteria (Patten and Glick, 1996).

Several studies demonstrated that bacterial IAA plays a major role in the development of the host plant root system. Dobbelaere *et al.* (1999) showed that an IAA-producing strain of *Azospirillum* increased root hair formation in wheat. Primary root length of canola seedlings inoculated with an IAA-producing *Pseudomonas putida* increased with 35 to 50% compared to inoculation with an IAA-deficient mutant or non-inoculated plants (Patten and Glick, 2002). Also Long *et al.* (2008) found that root growth promotion of *Solanum nigrum* and *Nicotiana attenuata* was associated with IAA-producing bacterial isolates. More

recently, Masciarelli *et al.* (2013) reported that bacterial release of IAA was the main activator of early growth promotion in inoculated maize seedlings.

Gibberellins are a large family of plant growth regulators that are involved in a number of developmental and physiological processes, such as seed germination, root growth and root hair abundance, and flower and fruit growth (Pharis and King, 1985; Tanimoto, 1987). The biosynthetic pathway leading to gibberellin synthesis as well as the function of this hormone in bacteria remains obscure. Bacterial gibberellins seem to be secondary metabolites that play a role as signaling molecules towards the host plant (Bottini *et al.*, 2004; Hershey *et al.*, 2014). Gibberellins produced by plant-associated bacteria can influence the growth and development of their host plant. For example, Gutiérrez-Mañero *et al.* (2001) demonstrated that promotion of stem elongation in alder by 2 rhizobacteria could be attributed to their gibberellin production: the dwarf phenotype in alder seedlings induced by adding an inhibitor of the plant's gibberellin biosynthesis was reversed by adding extracts from media incubated with these bacteria. Recently, Khan *et al.* (2014) demonstrated that inoculation of tomato with an endophytic gibberellin producing *Sphingomonas* strain significantly increased plant growth.

Cytokinins regulate many aspects of plant development and growth in aboveground as well as below ground plant parts. They regulate apical meristem function, cambial activity and vascular differentiation, lateral root formation, leaf senescence and sink-source relationships (Argueso *et al.*, 2009; Werner and Schmülling, 2009). Cytokinins also mediate the responses to environmental cues such as light, availability of nutrients and water, and biotic and abiotic stresses (Werner and Schmülling, 2009). Arkhipova *et al.* (2007) demonstrated that inoculation with cytokinin-producing bacteria could compensate for the loss of natural cytokinins in drought induced plants by interfering with the suppression of shoot growth and the enhancement of root elongation normally seen in these plants. Also Ortíz-Castro *et al.* (2008) showed the involvement of bacterial cytokinin signaling in plant growth stimulation: they identified a cytokinin-producing *Bacillus* strain that inhibited primary root growth of *Arabidopsis thaliana* followed by an increase in the number of lateral roots and the length of root hairs.

In the absence of physiological contact with plants, bacterial **volatiles** can also trigger plant growth promotion. Due to the high complexity of the volatile blend produced by bacteria, not many components have been unequivocally identified as plant growth promotors. Xie *et al.* (2009) found that a *Bacillus* strain, producing a blend of more than 25 volatile components, augmented growth, photosynthetic capacity and seed-set of *Arabidopsis thaliana*. Also Ryu *et al.* (2003) identified some bacterial strains that could enhance growth of *A. thaliana* by releasing volatile components, such as 2,3-butanediol and acetoin.

1.1.2 Availability of nutrients

Iron is an essential nutrient for plants with a main function in the electron-transport chain during photosynthesis and respiration (Connolly and Guerinot, 2002). The availability of Fe to the plant is limited by the low rate at which inorganic iron minerals dissolve in the rhizosphere (Lindsay, 1995). During Fe deficiency, bacteria can produce low molecular weight chelating agents, called siderophores, which can scavenge Fe in solution, directly mobilize Fe from solid phase minerals and remove Fe from organic Fe complexes (Crowley, 2006). Siderophores can be divided in 4 classes: (1) phenol-catecholates which have the highest affinity for Fe but which are extremely labile, (2) hydroxamates which are produced by the common colonizers of the rhizosphere, (3) rhizobactin which is produced by *Rhizobium* and which is a highly effective Fe source for plants and (4) pyoverdines which are primarily produced by *Pseudomonads* and *Azotobacter* but which do not seem to have a major role as potential Fe sources for plants (Crowley, 2006). Sharma and Johri (2003) demonstrated that several siderophore producing strains of *Pseudomonas* were able to increase root and shoot biomass and decrease chlorosis in maize grown under Fe limiting conditions. Rungin *et al.* (2012) demonstrated that inoculation of rice and mungbean plants with a siderophore producing endophytic *Streptomyces* strain enhanced plant growth significantly compared to the untreated controls and siderophore-deficient mutant treatments.

Phosphorus (P) plays a major role in plant energy metabolism, biosynthesis of nucleic acids and membranes, photosynthesis, respiration, and regulation of a number of enzymes (Raghothama, 1999). Large amounts of P applied as

fertilizer become unavailable to plants, accumulating in inorganic P fractions, which precipitate due to high reactivity with calcium, iron or aluminum, and organic P fractions that are immobilized in soil organic matter (Sanyal and De Datta, 1991; Gyaneshwar *et al.*, 2002). Soil bacteria play a major role in soil P dynamics and subsequent availability of phosphate to plants. They can have the ability to solubilize inorganic P by reducing the pH, either by producing organic acids or by releasing protons (Gyaneshwar *et al.*, 2002). Vyas and Gulati (2009) underlined the importance of bacterial phosphate solubilizing activity for plant growth promotion by demonstrating that treatment with an organic acid-producing *Pseudomonas* increased growth and P content in maize. To be available to plants, organic P fractions must be hydrolyzed by phosphatases, which are predominantly produced by soil microorganisms, although plant roots possess limited phosphatase activity (Richardson, 1994). The growth and P nutrition of several pasture plants was improved after inoculation with a cultured population of total soil microorganisms as well as with a specific isolate that was selected for its ability to release P from phytate (Richardson *et al.*, 2001). Also Idriss *et al.* (2001) showed strong evidence that bacterial phytase activity is important in plant growth stimulation under P limitation: a phytase-producing *Bacillus* was able to stimulate growth of maize seedlings under phosphate limitation in the presence of phytate, while a phytase-negative mutant strain did not stimulate plant growth.

The ultimate reservoir of soil **nitrogen** (N) is the atmosphere, where the very stable molecule dinitrogen is the predominant gas (Stevenson and Cole, 1999). Nitrogen is very important as it is a central element in proteins and nucleic acids. Nevertheless, most soils are deficient in N and application of fertilizer is inefficient. Less than 50% of the applied urea is used by plants due to NH_3 volatilization, denitrification and leaching to the groundwater (De Datta and Buresh, 1989; Garabet *et al.*, 1998). Diazotrophic bacteria can convert atmospheric N_2 to NH_3, a form of N that can be used by plants. In most cases, it concerns associative diazotrophs, which are growing in the rhizosphere, on the rhizoplane or in non-specialized intercellular spaces in plants (Vessey, 2003). Inoculation with these N_2-fixing bacteria significantly increased chlorophyll content as well as the uptake of different macro- and micro-nutrients by red pepper (Islam *et al.*, 2013). Other studies on the beneficial association of N_2-

fixing bacteria and their host plant include *Burkholderia* and rice (Trân Van *et al.*, 2000), *Azotobacter* and wheat (Soliman *et al.*, 1995) and *Herbaspirillum* and sugarcane (Muthukumarasamy *et al.*, 1999). In some cases, the N_2-fixing bacteria are living in specialized plant structures, called nodules. In these mutualistic symbioses, the transfer of fixed N from the bacteria to the plant is much higher due to the intimate association between both.

1.1.3 Counteracting stress-induced ethylene

Plant growth can be inhibited by a large variety of biotic and abiotic stresses, such as drought, pathogens or the presence of toxic contaminants. A general response of plants to stress is the production of ethylene, which occurs in 2 phases. In the first phase, a small amount of ethylene is produced which consumes the existing pool of 1-aminocyclopropane-1-carboxylate (ACC) within plant tissues (Robison *et al.*, 2001). This peak initiates a protective response, for example transcription of pathogenesis-related genes (Stearns and Glick, 2003). In the second phase, a much larger amount of ethylene is produced due to increased transcription of ACC synthase genes. This peak activates processes that inhibit plant development and growth, such as senescence, chlorosis and abscission (Ciardi *et al.*, 2000). The negative impact of this second ethylene peak can be counteracted by **ACC deaminase** produced by plant-associated bacteria (Li *et al.*, 2000; Grichko and Glick, 2001; Belimov *et al*., 2005). This enzyme hydrolyzes ACC into ammonia and α-ketobutyrate (Hontzeas *et al.*, 2006). As ACC is the immediate precursor for ethylene, lowering the level of ACC in the plant also lowers the amount of ethylene that can be produced. Bal *et al.* (2013) demonstrated that inoculation of rice with ACC deaminase-producing rhizobacteria increased root and shoot length and biomass, and enhanced shoot chlorophyll content.

1.2 Indirect plant growth promoting mechanisms

The ability of plant growth promoting bacteria to act as biocontrol agents against phytopathogens and thus indirectly improve plant growth may be due to different mechanisms including competition for space and nutrients, antibiosis,

iron-chelating siderophores, production of hydrolytic and detoxifying enzymes, and induction of plant defense mechanisms.

1.2.1 Competition for space and nutrients

The root surface and surrounding rhizosphere are nutrient-rich niches attracting many microorganisms, including phytopathogens that can infect the host and lead to disease. Competition for these niches and nutrients is a fundamental mechanism by which plant-associated bacteria improve plant health and growth (Duffy, 2001). Ryan and Kinkel (1997) suggested that the suppressing effect of certain *Streptomyces* strains on potato scab caused by *Streptomyces scabies* involves density-dependent interactions between these antagonists as the ability of the suppressive strain to increase its population was hindered at high inoculum doses of the pathogen.

1.2.2 Synthesis of allelochemicals

A variety of **antibiotics** have been identified that are produced by plant-associated bacteria. These biocontrol strains not only exhibit a wide range of diversity in the type but also in the number of antibiotics produced, with many of these molecules having broad-spectrum activity. Some examples of antibiotics produced by bacterial biocontrol agents are 2,4-diacetylphloroglucinol, phenazines, oomycin A, zwittermycin A, pyoluteorin, pyrrolnitrin, kanosamine and xanthobaccins (Raaijmakers *et al.*, 2002).

Under Fe-limiting conditions, plant-associated bacteria can produce **siderophores**, which have a very high affinity for Fe^{3+}. They can deprive pathogenic fungi and bacteria from Fe, thereby restricting their growth (Whipps, 2001). The role of siderophores in biocontrol of plant pathogens was first demonstrated with pseudobactin, a siderophore produced by *Pseudomonas* strain B10 that rendered soils inoculated with *Fusarium oxysporum* or *Gaeumannomyces graminis* disease-suppressive (Kloepper *et al.*, 1980a). Yu *et al.* (2011) found that a siderophore-producing *Bacillus subtilis* strain reduced the incidence of *Fusarium* wilt in pepper, while increasing plant growth.

Some microorganisms can also attack pathogens by secreting cell wall **hydrolases**. The production of chitinase appeared crucial for *Serratia*

marcescens to suppress *Sclerotium rolfsii* (Ordentlich *et al.*, 1988) and for *Streptomyces* and *Paenibacillus* to antagonize *Fusarium oxysporum* (Singh *et al.*, 1999). Lee *et al.* (2012) demonstrated the synergistic effect of chitinase, protease, glucanase and lipase produced by a strain of *Streptomyces cavourensis* against *Colletotrichum gloeosporioides* infecting pepper plants.

Another mechanism of biological control is the **detoxification** of pathogen virulence factors. Some bacteria can detoxify albicidin which is produced by *Xanthomonas albilineans* (Walker *et al.*, 1988; Basnayake and Birch, 1995; Zhang and Birch, 1997) and hydrolyse fusaric acid, a toxin produced by *Fusarium* species (Toyoda *et al.*, 1991), or they are able to quench quorum-sensing molecules thereby blocking the expression of pathogen virulence genes (Dong *et al.*, 2000; Molina *et al.*, 2003).

1.2.3 Induction of plant defense mechanisms

Priming plants with certain bacteria can induce **systemic resistance** (ISR) against a broad range of pathogens, including fungi, bacteria, viruses and sometimes even nematodes and insects (Ramamoorthy *et al.*, 2001). Plant growth promoting bacteria can activate the plant defense mechanisms without causing visible symptoms (Van Loon *et al.*, 1995). Changes occurring in plants with induced systemic resistance are (1) strengthening of cell walls (Benhamou *et al.*, 1996), (2) increased levels of enzymes such as chitinase and peroxidase (Chen *et al.*, 2000), (3) enhanced production of antimicrobial phytoalexins (van Peer *et al.*, 1991; Verhagen *et al.*, 2010) and (4) increased expression of stress-related genes (Timmusk and Wagner, 1999). Cartieaux *et al.* (2003) found that various genes involved in plant protection against pathogen attack and oxidative stress linked to infections were activated in *Arabidopsis thaliana* after inoculation with *Pseudomonas thivervalensis*. The hypothesis that prior colonization by this *Pseudomonas* strain could induce ISR was confirmed by challenging the inoculated plants with *Pseudomonas syringae* pv. *tomato*, which normally causes leaf speck disease on *A. thaliana*.

1.3 The bacterial microbiome of *Arabidopsis thaliana*

Arabidopsis thaliana is a widely used model organism in plant biology due to its short generation time, small size and self-pollinating capacity (Koornneef and Meinke, 2010). Moreover, it has a completely sequenced genome, it can be transformed efficiently using *Agrobacterium tumefaciens*, and a lot of mutants are available (Koornneef and Meinke, 2010). Nevertheless, knowledge about the bacterial microbiome of this model plant is scarce. As *A. thaliana* does not interact with mycorrhizal fungi or nodule-forming N_2-fixing rhizobia, it could be an ideal candidate for a simplified study of plant-bacteria interactions (Rott, 2012).

Several authors studied the bacterial communities present in the rhizosphere, roots, phyllosphere and leaves of *A. thaliana*. Comparison of the detected communities remains difficult because different procedures are used, including cultivation-independent techniques, such as terminal restriction fragment length polymorphisms (T-RFLP), ribosomal intergenic spacer analysis (RISA), denaturing gradient gel electrophoresis (DGGE) and pyrosequencing, as well as cultivation-dependent techniques.

Some authors found only a weak rhizosphere effect in *A. thaliana*: the bacterial communities in soil and rhizosphere resembled each other but were different from the community present in the root endophytic compartment (Schlaeppi *et al.*, 2014; Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012). However, Bressan *et al.* (2009) and Micallef *et al.* (2009a, b) found a significant change in the rhizosphere community compared to the bulk soil. Nevertheless, this difference between rhizosphere and bulk soil seems to disappear towards the end of the *A. thaliana* life cycle probably due to a decrease in rhizodeposition (Micallef *et al.*, 2009b). The most detected bacterial taxa in the *A. thaliana* rhizosphere are members of the Proteobacteria, Actinobacteria, Bacteroidetes, Acidobacteria and Firmicutes (Schlaeppi *et al.*, 2014; Bodenhausen *et al.*, 2013; Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012; Micallef *et al.*, 2009a).

In general, the root endophytic community seems to be enriched for Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes (Schlaeppi *et al.*, 2014; Bodenhausen *et al.*, 2013; Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012; Bressan *et al.*, 2009). Among the Proteobacteria, mainly *Rhizobiaceae*, *Burkholderiales*, *Comamonadaceae*, *Rhodocyclales*, *Pseudomonadaceae*,

Xanthomonadaceae and *Moraxellaceae* are detected (Schlaeppi *et al.*, 2014, Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012, Bressan *et al.*, 2009). *Streptomycetaceae* and *Flavobacteriaceae* are the families that are most observed among respectively the Actinobacteria and Bacteroidetes (Schlaeppi *et al.*, 2014; Bodenhausen *et al.*, 2013; Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012).

Bulgarelli *et al.* (2012) found that several plant cell wall features are sufficient as colonization cues, while additional cues from metabolically active cells are necessary for *A. thaliana* specific selection of soil bacteria. It is possible that each separate bacterial lineage responds to these host-derived cues, or that interactions among bacteria enable selective advantage for colonization of certain lineages (Schlaeppi *et al.*, 2014). Some bacterial phyla seem to be depleted in the endophytic root microbiome compared to the rhizosphere: they can be actively excluded from the host plant, outcompeted by other endophytic colonizers or metabolically unable to colonize the endophytic compartment (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012).

Besides the core microbiome, which provides retrievable functions independent of environmental or host parameters, there also seems to be an environment and host-species specific subcommunity (Schlaeppi *et al.*, 2014). Soil type seems to be the most important environmental parameter as it provides the start inoculum as well as the nutrients available for plants (Schlaeppi *et al.*, 2014; Bulgarelli *et al.*, 2012). Micallef *et al.* (2009a) found that different *A. thaliana* accessions have different exudation patterns and by consequence attract different bacterial assemblages. This was also demonstrated by Badri *et al.* (2009) who found that an ATP binding cassette (ABC) transporter mutant of *A. thaliana* had a changed exudation pattern and associated rhizospheric bacterial community in comparison to the wild type.

Glucosinolates and their hydrolysis products seem to be an important factor for microbial selection in the rhizosphere and root environment of *Brassicaceae*: Alphaproteobacteria, and especially *Rhizobium*, in the rhizosphere and roots of *A. thaliana* were influenced by changing levels of glucosinolates (Bressan *et al.*, 2009). Glucosinolates and their hydrolysis products can serve as nutritional substrates but can also have anti-microbial properties (Bressan *et al.*, 2009). In general, the effect of the host's innate immune system on the indigenous plant-

associated bacterial community remains obscure. Doornbos *et al.* (2011) detected a lower number of cultivable bacteria in the rhizosphere of *A. thaliana* genotypes affected in jasmonic acid-, ethylene- and salicylic acid-related defense signaling, however this effect was soil type-dependent. Chemical activation of systemic acquired resistance (SAR) or induced systemic resistance (ISR) had no effect on the number of cultivable bacteria and total community composition, based on DGGE fingerprints, in the rhizosphere (Doornbos *et al.*, 2011). By contrast, Hein *et al.* (2008) found changes in the rhizospheric communities as a result of SAR activation in *A. thaliana* based on T-RFLP profiles. Remarkably, the mutant that lacked the ability to undergo SAR had less rhizosphere diversity than the mutant with constitutive salicylic acid-dependent defense responses. Kniskern *et al.* (2007) also found an effect of the plant defense system on the leaf endophytic and epiphytic communities of *A. thaliana*, where salicylic acid-mediated defenses reduced endophytic diversity, while jasmonic acid-related defenses increased epiphytic diversity.

Comparable to the root endophytic community, also the leaf endophytic community contains mainly Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes (Bodenhausen *et al.*, 2013; Kniskern *et al.*, 2007). *Pseudomonas*, *Sphingomonas*, *Xanthomonas* and *Agrobacterium* were abundantly detected among the Proteobacteria present in leaves, while *Flavobacterium* and *Bacillus* were the most abundant genera among respectively the Bacteroidetes and Firmicutes (Bodenhausen *et al.*, 2013; Kniskern *et al.*, 2007).

Bodenhausen *et al.* (2013) found many of the same genera in roots and leaves of *A. thaliana* pointing towards a similar source. Most probably, seeds are colonized from the soil, and bacteria colonize the roots and leaves as the plant grows (Bodenhausen *et al.*, 2013). Alternatively, rain splashing off the soil can bring soil bacteria into contact with the leaves. However, this route of endophytic colonization is less likely as the richness of epiphytes on the leaves is lower than that of the endophytes, suggesting that bacteria are probably migrating from the roots to the leaves (Bodenhausen *et al.*, 2013).

The *A. thaliana* phyllosphere community is mainly composed of members of the Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes (Bodenhausen *et al.*, 2013; Reisberg *et al.*, 2013; Reisberg *et al.*, 2012; Delmotte *et al.*, 2009; Kniskern *et al.*, 2007). Especially *Pseudomonas* and *Sphingomonas* appear to be

abundant in the leaf epiphytic community (Bodenhausen *et al.*, 2013; Reisberg *et al.*, 2013; Reisberg *et al.*, 2012; Delmotte *et al.*, 2009). There seems to be a core phyllospheric community which is complemented by plant-line specific communities, as was shown by epicuticular leaf wax composition mutants of *A. thaliana* (Reisberg *et al.*, 2013). Moreover, using a metaproteomics study, Delmotte *et al.* (2009) found some common adaptation mechanisms among the phyllosphere bacteria, which were related to carbon and nitrogen metabolism, motility and stress resistance.

It is obvious that information on the endophytic and epiphytic communities of *A. thaliana* roots and leaves remains scarce, and that information on the seed-associated bacterial community seems to be completely absent. Nevertheless, these bacteria could be important during seed preservation, germination and plant growth.

1.4 The potential of seed endophytes

Truyens S, Weyens N, Cuypers A, Vangronsveld J. 2015. Bacterial seed endophytes: genera, vertical transmission and interaction with plants. Environmental Microbiology Reports 7(1): 40-50.

Seeds represent a remarkable phase in the life cycle of spermatophytes: they can persist for years in a dormant state and, when the appropriate conditions are met, develop into a new plant (Nelson, 2004). Seeds can benefit from seed-associated microorganisms as these can play a role in seed preservation and preparation of the environment for germination (Chee-Sanford *et al.*, 2006). When seeds start to germinate, they imbibe water and start to secrete exudates that attract bacteria which colonize the spermosphere, rhizosphere and the seedling where they can directly and indirectly promote plant growth and health (Nelson, 2004). Direct plant growth promoting mechanisms involve nitrogen fixation, mobilization of nutrients such as phosphorus and iron by production of organic acids and siderophores, production of phytohormones such as auxins and cytokinins, and suppression of stress ethylene production by 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Weyens *et al.*, 2009a). Indirect growth promotion occurs by preventing the growth or activity of

phytopathogens through competition for space and nutrients, antibiosis, production of hydrolytic enzymes, inhibition of toxins and induction of plant defense mechanisms (Weyens *et al.*, 2009a).

Apart from bacteria present in the seed surroundings, bacteria that are already present inside the seed can be equally important for the evolution of the microbial community of the seedling (Johnston-Monje and Raizada, 2011). Many seeds carry a diversity of endophytes (Table 1.1). Bacterial endophytes are described by Azevedo and Araújo (2007) as all bacteria that may or may not be successfully cultured, that internally colonize the plant and do not cause apparent damage and/or visible external structures.

Seed endophytes are of particular interest as they are transmitted from generation to generation. By being seed-borne, endophytes assure their presence in new plants. This vertical transmission, defined as the direct transfer from parent to progeny, should select against pathogenicity and favour mutualism as these endosymbionts depend entirely on their host for survival and reproduction (Ewald, 1987; Rudgers *et al.*, 2009). At the same time, the host plant benefits by providing their progeny with beneficial endosymbionts (Ewald, 1987).

Many studies have focused on fungal endophytes in seeds, while reports concerning the presence and role of bacterial seed endophytes during germination and seedling development are less numerous. Below, an overview is given of the bacterial seed endophytes that have been isolated from different plant species. The colonization routes of these endophytes, their localization inside the seeds and their mode of transmission are discussed. Further, their role and fate during germination and seedling development are reviewed. Finally, examples of seed endophytes with direct plant growth promoting capacities or biocontrol properties are discussed.

1.4.1 Frequently reported bacterial seed endophytes

The conditions within the seed change during seed maturation and this can affect which bacteria will be able to inhabit the seed. By consequence, some characteristics are typically found in seed endophytes and not in endophytes isolated from other plant tissues such as roots or shoots. The accumulation of

starch and loss of water during the maturation process seem to favor seed endophytes that are tolerant to a high osmotic pressure (Ebeltagy *et al.*, 2000; Mano *et al.*, 2006). Endospore formation can also be an important feature for seed colonizers as this protects them from the changes that are happening inside the seed (Mano *et al.*, 2006; Compant *et al.*, 2011). Seed endophytes often seem to possess amylase activity in order to utilize starch and resume growth after long-term survival inside the seeds (Mano *et al.*, 2006). Some seed endophytes were also reported to be able to use phytate, which is the main storage form of phosphorus in seeds, as a source of phosphate (López-López *et al.*, 2010). Moreover, Okunishi *et al.* (2005) and Ebeltagy *et al.* (2000) found that all the seed endophytes from rice seeds were motile which enables them to migrate into the seeds before they harden. Johnston-Monje and Raizada (2011) reported that most of the bacterial isolates from seeds of different maize genotypes were able to solubilize phosphorus, secrete acetoin and fix nitrogen. Also ACC deaminase activity and antibiosis were moderately conserved among seed endophytes from different maize genotypes (Johnston-Monje and Raizada, 2011). These conserved endophyte properties could reflect common needs of the host seeds and their spermosphere (Nelson, 2004).

The seed maturation process not only selects bacteria based on their phenotypic properties, also the diversity of bacterial genera, at least of the cultivable ones, seems to be influenced. Mano *et al.* (2006) found that there was a dominance of Gram-negative isolates in the early stages of seed development, but more Gram-positive isolates appeared when the seeds were maturing. In the very early stages they found mainly *Methylobacterium spp.* and *Sphingomonas spp.*, while later on *Bacillus spp.* and *Curtobacterium spp.* were more abundant (Mano *et al.*, 2006).

Until now, 131 bacterial genera from 4 different phyla and 25 different plants or plant species have been reported as naturally occurring seed endophytes (Table 1.1 and references herein). The most predominant seed endophytes belong to the Proteobacteria (80 genera) and mainly the γ-Proteobacteria (41 genera). Also Actinobacteria and Firmicutes are well represented with 25 and 20 genera respectively. The phylum Bacteroidetes is represented by only six genera. It seems as if the phylum composition of bacterial seed endophytes is a good reflection of the endophytic phyla found in other plant tissues. In general,

Proteobacteria, and especially γ-Proteobacteria, were found to be the dominant endophytic phylum isolated from a wide variety of plant species, while Actinobacteria, Firmicutes and certainly Bacteroidetes were much less represented (Rosenblueth and Martínez-Romero, 2006).

Some studies considered only the cultivable strains, while others investigated the total bacterial community present in seeds. To identify the cultivable strains, the more recent studies used 16S rDNA sequencing (e.g. Ferreira *et al.*, 2008; Mastretta *et al.*, 2009) while earlier studies used fatty acid methyl ester (FAME) profile analysis (Granér *et al.*, 2003; Vega *et al.*, 2005) or biochemical methods (Mundt and Hinkle, 1976; Kremer, 1987; Dunleavy, 1989). To identify the total bacterial community, 16S rRNA libraries in combination with 16S rRNA gene sequencing (e.g. Johnston-Monje and Raizada, 2011; Liu *et al.*, 2012) or polymerase chain reaction-based differential denaturing gel electrophoresis (PCR-DGGE) whether or not in combination with 16S rDNA sequencing were used (e.g. Hardoim *et al.*, 2012).

Although several of the studies listed in table 1.1 had the description of the seed bacterial community as a primary objective, others discovered seed endophytes while studying other plant-associated bacterial communities or other seed characteristics. This might explain the low numbers of bacterial genera recovered from some types of seeds as well as the differences in community size. The cultivation of seed endophytes is also challenging because of the specific habitat they are derived from. Seed endophyte community sizes range from 55 cfu.g^{-1} in bean (Rosenblueth *et al.*, 2010) to as high as 10^7 cfu.g^{-1} in rapeseed (Granér *et al.*, 2003). In general, common bacterial genera reported in seeds of very different plant species are *Bacillus* and *Pseudomonas*. Also *Paenibacillus*, *Micrococcus*, *Staphylococcus*, *Pantoea* and *Acinetobacter* are often found inhabiting the seed.

Table 1.1: Summary of the known bacterial seed endophytes isolated from different plant species.

Plant	Actinobacteria	Bacteroidetes	Firmicutes	α-proteobacteria	β-proteobacteria	γ-proteobacteria	δ-proteobacteria	References
Norway spruce (*Picea abies*)	0	0	0	0	0	2	0	Cankar *et al.*, 2005
Grape vine (*Vitis vinifera*)	0	0	3	0	0	0	0	Compant *et al.*, 2011
Eucalyptus (*Eucalyptus*)	1	0	3	3	0	0	0	Ferreira *et al.*, 2008
Rice (*Oryza sativa*)	11	1	3	4	0	9	0	Mukhopadhyay *et al.*, 1996; Ebeltagy *et al.*, 2000; Bacilio-Jiménez *et al.*, 2001; Cottyn *et al.*, 2001; Okunishi *et al.*, 2005; Mano *et al.*, 2006; Tripathi *et al.*, 2006; Kaga *et al.*, 2009; Ruiz *et al.*, 2011; Hardoim *et al.*, 2012
Bean (*Phaseolus vulgaris*)	4	0	4	3	0	2	0	Pleban *et al.*, 1995; Pérez-Ramírez *et al.*, 1998; López-López *et al.*, 2010; Rosenblueth *et al.*, 2010
Tobacco (*Nicotiana tabacum*)	1	0	1	0	0	4	0	Mastretta *et al.*, 2009
Cactus (*Pachycereus pringlei*)	0	0	2	0	0	3	0	Puente *et al.*, 2009b
Coffee (*Coffea arabica*)	3	0	1	0	1	5	0	Vega *et al.*, 2005
Rapeseed (*Brassica napus*)	1	1	2	0	2	4	0	Granér *et al.*, 2003
Maize (*Zea mays*)	18	5	14	16	17	33	1	Dunleavy, 1989; Rijavec *et al.*, 2007; Rosenblueth *et al.*, 2010; Johnston-Monje and Raizada, 2011; Liu *et al.*, 2012; Liu *et al.*, 2013
Ash (*Fraxinus*)	3	0	4	0	0	3	0	Donnarumma *et al.*, 2010

Soybean (*Glycine max*)	1	1	1	2	0	2	1	Kremer, 1987; Dunleavy, 1989; Oehrle *et al.*, 2000
Wheat (*Triticum aestivum*)	3	0	2	0	0	4	0	Coombs and Franco, 2003; Ringelberg *et al.*, 2012
Sugar beet (*Beta vulgaris*)	0	0	1	0	0	0	0	Dent *et al.*, 2004
Garden pea (*Pisum sativum*)	0	0	1	0	0	0	0	Smerda *et al.*, 2005
Pumpkin (*Cucurbita pepo*)	0	0	1	0	0	1	0	Fürnkranz *et al.*, 2012
Peanut (*Arachis hypogaea*)	0	0	2	0	0	1	0	Sobolev *et al.*, 2013
Cauliflower (*Brassica oleacea*)	0	0	1	0	0	0	0	Pleban *et al.*, 1995
Wild mustard (*Sinapis arvensis*)	0	0	1	0	0	0	0	Pleban *et al.*, 1995
Tomato (*Lycopersicum esculentum*)	0	0	1	0	0	0	0	Xu *et al.*, 2014
Strawberry (*Fragaria*)	0	0	0	0	0	1	0	Kukkurainen *et al.*, 2005
Thale cress (*Arabidopsis thaliana*)	3	0	3	5	0	1	0	Truyens *et al.*, 2013
Grasses (*Lolium multiflorum*; *Phleum pretense*; *Panicum virgatum; Agrostis capillaris*)	1	0	3	0	0	3	0	Ikeda *et al.*, 2006; Gagne-Bourgue *et al.*, 2013; Truyens *et al.*, 2014
Broadleaf weed species	0	1	1	0	1	7	0	Kremer, 1987
Herbaceous and woody species	5	2	3	0	2	7	0	Mundt and Hinkle, 1976

1.4.2 Seed endophyte dynamics

Sources of seed endophytes

The majority of the bacterial taxa detected inside seeds were similar to common soil strains. Plant roots are exposed to bacteria in soil during development and growth, enabling entry of bacteria into the plants and making this an obvious colonization route towards the seeds. Several bacterial traits are important for plant colonization and endophytic capacity. Chemotaxis-induced motility leading to root colonization is probably one of the most important mechanisms determining the endophytic potential of soil bacteria (Bacilio-Jiménez *et al.*, 2003). For example, cheA or pilA mutants of several *Pseudomonas* strains defective in flagella-driven chemotaxis or twitching motility showed reduced competitive root colonizing abilities (Camacho Carvajal, 2001; de Weert *et al.*, 2002). The next step in the colonization route towards the seeds requires bacteria to enter the root and become endophytes either through passive penetration at the root tip, side root emergence or pathogen entry sites, or through active penetration using cell wall degrading enzymes, such as cellulase and pectinase (Hurek *et al.*, 1994; Ebeltagy *et al.*, 2000; James *et al.*, 2002). Recently, also transport proteins for uptake of plant-synthesized nutrients, secretion and delivery systems involved in switching from a free-living to an endophytic life style, and transcriptional regulators for metabolic adaptation and quorum sensing have been shown to be determinants of competent endophytes (Sessitsch *et al.*, 2012; Ali *et al.*, 2014). Moreover, detoxification mechanisms used in the protection against oxidative stress induced after host infection were found to be associated with endophytic behavior (Sessitsch *et al.*, 2012; Ali *et al.*, 2014). Colonization of the plant interior appears to be very attractive as plant nutrients can be used more efficiently without competition with the very high numbers of other bacteria colonizing the root exterior (Rosenblueth and Martínez-Romero, 2006). Moreover, endophytic bacteria seem to be better protected from abiotic stresses compared with rhizospheric bacteria (Hallmann *et al.*, 1997). Once inside the plant, some endophytes are able to spread systemically and ultimately reach the flowers, fruits and seeds. Some endophytes use the root xylem vessels of their hosts where they are assisted by the movement of their flagella and by the plant transpiration stream (James *et*

al., 2002; Compant *et al.*, 2005), while others use the nutrient-rich intercellular spaces, but this requires the secretion of cell wall degrading enzymes (Dong *et al.*, 1994). The bacteria that become seed endophytes are not exclusively soil derived. Alternative entry points might be the caulosphere, the phyllosphere, the anthosphere as well as the carposphere from where they can colonize plant reproductive organs (James *et al.*, 2002; Berg *et al.*, 2005; Compant *et al.*, 2010; 2011). Some bacteria can already interact with seeds present in soil, thereby increasing their chances of subsequent plant colonization. The amount and composition of seed exudates released during imbibition and germination influences the bacterial community that can be supported in the spermosphere (Schenck and Stotzky, 1975; Roberts *et al.*, 2009). The pfkA gene, encoding a phosphofructokinase, seems to be very important for colonization of seeds, which release only a limited amount of reduced carbon sources (Roberts *et al.*, 1999). Also fimbriae and several adhesion factors, efflux pumps, calcium-binding proteins and transport systems involved in iron acquisition seem to be involved in bacterial seed attachment (Hood *et al.*, 1998; Espinosa-Urgel *et al.*, 2000; Molina *et al.*, 2005; 2006). Several other factors can influence the bacterial communities of seeds. A contaminant-dependent effect on the genotypic and phenotypic characteristics of the cultivable seed endophytic community was reported in *Arabidopsis thaliana* and *Agrostis capillaris* (Truyens *et al.*, 2013; 2014; chapter 4 and 5). Also differences in seed endophytic communities were found between different cotton cultivars (Adams and Kloepper, 2002) and between different maize genotypes (Liu *et al.*, 2012). Certain plant genes, such as the receptor-like kinase shr5 or members of the ethylene signaling pathway, could regulate the plants' interaction with bacteria and determine whether the bacteria are able to endophytically colonize the plant (Vinagre *et al.*, 2006; Cavalcante *et al.*, 2007; Liu *et al.*, 2012).

Vertical transmission, seed location and seed quality

Bacterial endophytes can get in seeds in different ways. They can be transmitted from the vegetative parts of the plant to the seed via vascular connections from the maternal plant through the funiculus and chalaza into the seed endosperm as well as via the micropyle (Ivanoff, 1933; Rand and Cash, 1933; Samish and

Etinger-Tulczynska, 1963; Agarwal and Sinclair, 1996). Endophytes can also be transferred through gametes directly, colonizing the resulting embryo and endosperm (Agarwal and Sinclair, 1996; Madmony *et al.*, 2005; Malfanova *et al.*, 2013). Finally, vertical transfer of bacteria might also be possible if shoot meristems, which later become reproductive meristems, are colonized as these give eventually rise to ovules and thus seeds (Pirttilä *et al.*, 2000) (Fig. 1.1). Transfer of endophytes from mature fruits to seeds was shown by Puente *et al.* (2009b). They found bacteria in the endocarp, mesocarp, exocarp, on the surface and at the embryonic site of cactus seeds. Most bacteria found inside seeds were associated with the seed husk, seed coat or cortex (Mukhopadhyay *et al.*, 1996; Cankar *et al.*, 2005; Puente *et al.*, 2009b). Some endophytes were also found in endosperm and embryonic tissues (Mukhopadhyay *et al.*, 1996; Cankar *et al.*, 2005). Compant *et al.* (2011) used fluorescence *in situ* hybridization (FISH) to demonstrate the presence of *Bacillus spp.* along the cell walls of some cells inside grapevine seeds. FISH was also used by Pirttilä *et al.* (2000) to show the presence of bacteria in (ovuliferous) pine buds. They detected bacterial RNA particularly in the cells of scale primordia and in the epithelial cells of the resin ducts. Bacterial RNA was less abundant in the apical meristem itself, but some could be found in the outermost cells and in the cells of the developing stem, right below the apical meristem (Pirttilä *et al.*, 2000). Vertical transmission of bacterial endophytes has already been suggested in several plant species. Gagne-Bourgue *et al.* (2013) described the presence of the same *Bacillus spp.* and *Microbacterium spp.* in seeds harvested in 1 year and switchgrass plants grown from these seeds the next year. Also, Ringelberg *et al.* (2012) suggested that seeds are a major contributor of mature wheatgrass endophytes as they recovered the same genera from seeds and from mature plant tissues. Moreover, the same *Enterobacter spp.* were reported in seeds of consecutive rice generations (Mukhopadhyay *et al.*, 1996). Johnston-Monje and Raizada (2011) demonstrated in maize that a core microbiota, consisting of the same bacterial species, exists which is conserved even across boundaries of evolution, human selection and following cross-continental migration. Also Liu *et al.* (2012) found continuity of endophytic bacterial species from parental to offspring maize seeds. Moreover, the seed endophytic community of genetically related maize hybrids consisted of similar species and genera (Liu *et al.*, 2012).

Correspondence analysis of the cultivable endophytic communities of roots of *Brassica napus* grown on a Cd, Zn and Pb-contaminated field and a non-contaminated control field revealed a correlation between endophytic communities of roots between fields, suggesting that plants on the two sites contained similar obligate endophytes possibly derived from a common seed endophytic community (Croes *et al.*, 2013). The above mentioned findings are all based on 16S rRNA gene sequencing generating only genus and species level information. Confirmation of the vertical transmission of bacterial endophytes can only be obtained if strain information is available. Nevertheless, the possible existence of vertical transmission is very interesting as it enables a plant with an established endophytic community to pass bacteria with beneficial characteristics to their offspring (Ferreira *et al.*, 2008). Conserved bacteria that are vertically transmitted point towards an evolved form of mutualism or benign parasitism with their host plant (Ewald, 1987; Johnston-Monje and Raizada, 2011). For example, Ferreira *et al.* (2008) suggested that vertical transmission of endophytes might maintain the stability of bacterial communities in eucalyptus plants. On the contrary, it could even be possible that seed endophytic bacteria use seeds for their own dispersion as many of these bacteria are ubiquitous in different environmental niches (Hardoim *et al.*, 2012) (Fig. 1.1). The same taxa indeed were commonly found in the endosphere of rice (Okunishi *et al.*, 2005; Mano *et al.*, 2006), maize (Johnston-Monje and Raizada, 2011) and leguminous plants such as soybean (Oehrle *et al.*, 2000), and in the soil where these plants were growing.

A possible link exists between the endophyte content of seeds and seed quality. A high quality seed lot is disease-free and shows a high germination percentage and high vigor (Perry, 1978; Dent *et al.*, 2004). Hill *et al.* (2005) demonstrated that fungal endophyte infection rates of tall fescue seedlings were lower when seeds were harvested before physiological maturity. Seeds harvested before maturity showed a lower germination percentage and a reduced seedling vigor (Hill *et al.*, 2005). Moreover, certain seed storage conditions such as increased temperature or humidity can reduce fungal endophyte viability in tall fescue seeds (Hill and Roach, 2009). Also the amount of bacterial endophytes that can be isolated decreases with the duration of seed storage (Mundt and Hinkle, 1976; Bacon and Hinton, 1996; Cankar *et al.*, 2005; Mastretta, 2007). The seed

sterilization protocol used before sowing was a major determinant of the amount of bacterial endophytes found in seeds of *Arabidopsis thaliana* (Truyens *et al.*, 2013). Using a mild seed sterilization protocol increased the homogeneity of germination and reduced variability in plant growth (Truyens *et al.*, unpublished results). Also Holland and Polacco (1994) suggested that seed endophytes may be beneficial during germination as the rate of germination declined when bacteria were removed from seeds.

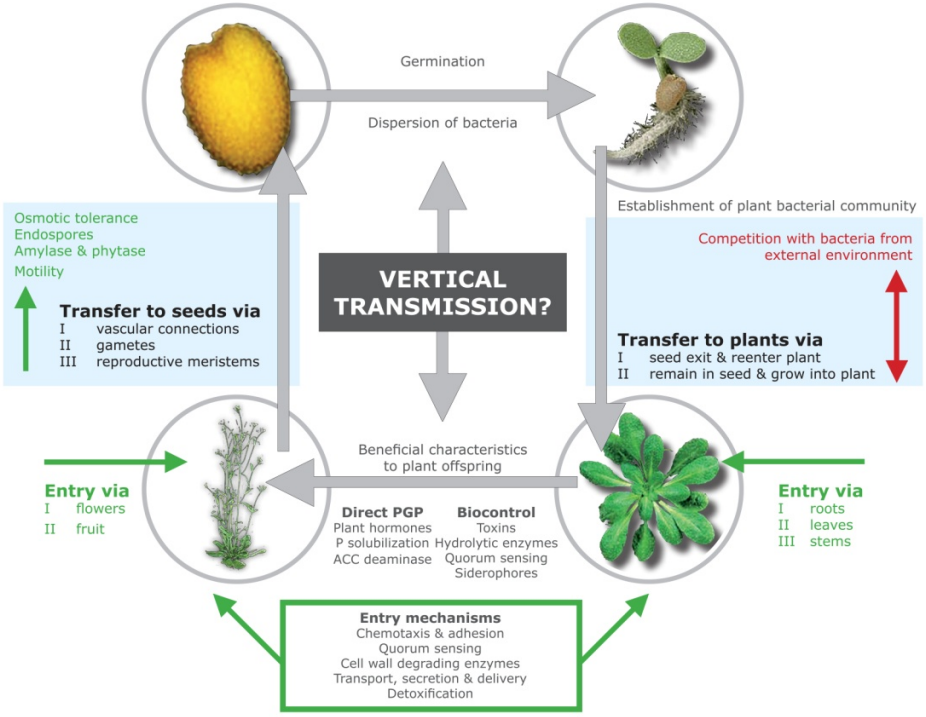

Figure 1.1: Schematic summary of colonization routes and determinants, transmission modes and functions of bacterial seed endophytes. After seed germination, the plant bacterial community is established from seed endophytes and from bacteria present in the external environment. Some of these bacteria could be transferred to the next generation of plants via the seeds. This would not only lead to dispersion of bacteria, but also ensures the transfer of beneficial bacterial characteristics to the next generation of plants.

Role and fate of seed endophytes during germination and growth

Seed endophytes are believed to be capable of infecting the next generation of plants through 2 infection routes or a combination of both: (1) exiting the seed and then entering the plant via other places on the plant surface and (2) remaining inside the seed to be spread through plant growth or move within the

plant tissue (Kaga *et al.*, 2009) (Fig. 1.1). A germinating seed starts to release organic molecules from the developing root system which leads to an increase in the numbers of bacteria compared to the low nutrient environment in the soil around the seed (Baker and Cook, 1974). Endophytic bacteria inhabiting the seeds have the advantage of rapid infection of the new generation of plants because they experience less competition with bacteria originating from the external environment for space and nutrients available in the host plant and because they are already adapted to living inside plant tissue (Kaga *et al.*, 2009; Hardoim *et al.*, 2012) (Fig. 1.1). Nevertheless, bacteria from the external environment will also colonize the host leading to shifts in bacterial community structure during seedling development (Hallmann *et al.*, 1997; Mahaffee and Kloepper, 1997).

In rice seeds, seed endophytes were important founders of the bacterial community present during the early plant growth stages (Hardoim *et al.*, 2012). They mainly colonize rice shoots, where there is less competition than in the roots, which are surrounded by large bacterial communities in soil. Some strains were even able to spread out into the rhizosphere and soil. In mature rice plants, endophytes originating from the seeds seemed to coexist with bacteria that originated from the external environment (Mano *et al.*, 2006). Also in bean it was demonstrated that seed-borne bacteria are able to multiply and persist in the seedlings as almost all bacterial genera isolated from seeds were also recovered from bean roots (López-López *et al.*, 2010).

Bacterial communities hosted by seeds can support the development of their host. Due to their metabolic diversity and depending on the local conditions, different beneficial bacterial functions can be stimulated thereby increasing plant fitness and giving the host a competitive advantage over other plants which can affect whole-ecosystem functioning (Klironomos, 2002). Puente *et al.* (2009b) found that the endophytic bacteria from cactus seeds contributed to rock weathering, soil formation and plant growth thereby supporting the establishment of cactus seedlings in barren desert areas without the need for acquiring bacteria from the environment. When these endophytic bacteria were removed from the seeds, seedling development was stopped.

Several authors have tried to determine the exact location of seed-borne bacteria inside the seedling using histological cuttings or green fluorescent

protein (gfp)-labelled seed endophytes. Puente *et al.* (2009b) found seed-borne endophytic bacteria mainly in the root cortex and the vascular system of cactus. In rice, some bacterial cells were localized in the root cortex, but the highest concentration was found in the stele (Mukhopadhyay *et al.*, 1996). A gfp-labelled *Pantoea sp.* inoculated on the seeds of *Eucalyptus* was carried into the embryo through breaks in the seed husk and continued to colonize the seedlings: the gfp-labelled strain was present on the rhizoplane, in the intercellular spaces of the root and in the interior of the stem xylem vessels (Ferreira *et al.*, 2008). Johnston-Monje and Raizada (2011) injected a gfp-labelled seed-borne *Pantoea* or *Enterobacter* strain into the stem of maize plants. They demonstrated that both strains were able to move systemically through vascular tissues: the *Pantoea* strain was found in the metaxylem vessels and the *Enterobacter* strain in phloem cells at the base of the stem. The latter was even able to exit the plant and colonize the rhizosphere.

1.4.3 Seed endophyte functions

Until now, only limited research is conducted to unravel the exact role and possible applications of seed endophytes. The existing studies mainly focus on the use of seed endophytes as plant growth promoting agents, while some also discuss their biocontrol potential. In general, the beneficial functions found in seed endophytes do not seem to be different from those found in endophytes isolated from other plant tissues.

Direct plant growth promotion

Inoculating plants with bacteria isolated from seeds can increase plant growth, especially in suboptimal or harsh environmental conditions. Gagne-Bourge *et al.* (2013) showed that the inoculation of switchgrass seedlings with seed-borne *Bacillus* or *Microbacterium* strains improved plant growth compared to the non-inoculated controls. Both strains produced indole-3-acetic acid (IAA), cytokinins and volatiles such as butanediol and acetoin, and the *Bacillus* strain was able to solubilize phosphorus. Also, Xu *et al.* (2014) found a seed-borne *Bacillus* strain that improved the root and shoot growth of tomato, probably through the production of ACC deaminase and nitrogen fixation. Johnston-Monje and Raizada

(2011) used a potato bioassay to test the plant growth promoting ability of maize seed endophytes. They found a *Burkholderia* strain, producing ACC deaminase, that was able to improve potato shoot growth and a *Hafnia* strain, producing acetoin, that increased potato root growth. Seed endophytes of cactus allowed the growth of cactus seedlings in extreme conditions for at least a year without fertilization and without showing distress (Puente *et al.*, 2009a). These seed endophytes were able to release inorganic nutrients, such as phosphorus, from pulverized rock through the production of organic acids and possessed the capacity to fix nitrogen (Puente *et al.*, 2009b). Several other studies have assumed that the seed endophytes they have isolated are beneficial for the plant based on plant-growth promoting effects described in literature, but did not verify this experimentally (Cankar *et al.*, 2005; López-López *et al.*, 2010).

The plant growth promoting effect of seed endophytes in suboptimal/harsh environmental conditions can be exploited in practical applications such as phytoremediation. Mastretta *et al.* (2009) demonstrated that inoculation of tobacco with its seed endophytes improved biomass production under Cd exposure and increased plant Cd content compared to the non-inoculated plants due to increased uptake of essential nutrients from the growth medium. Also the inoculation of *Agrostis capillaris* with its seed endophytes, which were capable of producing ACC deaminase, IAA, siderophores and acetoin, able to solubilize phosphorus, and were tolerant to Cd, might be beneficial for its establishment during phytoextraction and phytostabilization of Cd-contaminated areas (Truyens *et al.*, 2014; chapter 5).

Biocontrol

Several of the endophytes isolated from seeds were found to possess anti-fungal properties. *Bacillus* and *Microbacterium* strains isolated from seeds of switchgrass inhibited the mycelial growth of fungal plant pathogens due to the production of several toxins such as surfactins and the lipopeptides iturin and mycobacilli (Gagne-Bourgue *et al.*, 2013). Among rice seed endophytes, the strongest anti-fungal properties against *Rhizoctonia solani*, *Pythium myriotyum*, *Guamannomyces graminis* and *Heterobasidium annosum* were found in 2 *Enterobacter* strains because of the production of a volatile anti-fungal

compound, probably ammonia, and/or the chitinolytic enzyme N-acetyl-β-D-glucosaminidase (Mukhopadhyay *et al.*, 1996). Also Cottyn *et al.* (2001) detected anti-fungal activity among rice seed endophytes: nearly half of the antagonists of *Rhizoctonia solani* and *Pyricularia grisea* were *Bacillus* strains, but also in some of the *Pantoea*, *Enterobacter*, *Stenotrophomonas*, *Xanthomonas*, *Acinetobacter*, *Paenibacillus* and *Cellulomonas* strains antagonistic activity against one or both of these fungi was detected. In addition, Ruiz *et al.* (2011) reported anti-fungal activity against *Curvularia sp.*, *Fusarium oxysporum* and *Phytium ultimum* in several strains of *Pantoea*, *Microbacterium*, *Pseudomonas*, *Paenibacillus* and *Curtobacterium* isolated from rice seeds. Besides the production of anti-fungal compounds and hydrolytic enzymes, also quorum sensing and the release of siderophores, properties often found in seed endophytes, could contribute to their anti-fungal activity (Ruiz *et al.*, 2011; Sessitsch *et al.*, 2012). The production of siderophores leads to competition with pathogens for iron (Loper and Henkels, 1999), while quorum-sensing molecules may participate in the interaction of plants with pathogens and symbionts by induction of plant gene expression (Mathesius *et al.*, 2003).

Conclusions and future research needs

Bacteria inhabiting seeds form an important group among the plant-associated bacteria. They are presumably present in seeds of all plant species and numerous bacterial genera of different phyla were reported as seed endophytes. They can play important roles during germination, seedling development and plant growth. As they possess plant growth promoting and biocontrol properties, the study of their application in diverse processes such as biofertilization, bioenergy production and bioremediation should be encouraged.

Most seed endophytes isolated until now are cultivable bacteria and several of them are accidentally discovered during a sidetrack of the main research issue. A large fraction of the bacterial endophytic community in seeds probably has unknown cultivation conditions or is in a viable but not cultivable state. Metagenomic studies will shed more light on this part of the seed endophytic community and provide a better understanding of the total bacterial communities present in seeds, both concerning the genera that are present as

well as their phenotypic characteristics and possible role(s) in germination and plant development.

Until now, the studies suggesting vertical transmission of bacterial endophytes have used 16S rRNA gene sequencing, leading to identification at genus or species level. To confirm the existence of vertical transmission, techniques allowing to generate strain level identification, such as repetitive element palindromic PCR, single nucleotide polymorphism analysis or pulsed-field gel electrophoresis, should be applied in order to detect the presence of the same bacterial strains in consecutive plant generations (Lopez-Velasco *et al.*, 2013). Moreover, more research is needed focusing on the (genetic) determinants allowing seed colonization, seed endophyte dispersal and vertical transmission. Due to the unique seed environment and the selection process required for possible transfer to the next generation, seed endophytes often possess attractive characteristics that can be exploited in several biotechnological applications.

To use seed endophytes in agricultural, industrial or environmental applications, several questions still need to be addressed. The fate of seed endophytes during germination and plant development has to be elucidated: are they important founders of the newly established endophytic community or will they get (at least partly) outcompeted by soil bacteria? Moreover, are seed endophytes truly selected by the host plant to benefit the next generation or do these bacteria use seeds as a vector for their own dispersion and colonization of new environments? Also the evolutionary significance of these associations is not sufficiently clear yet: how can seed endophytes contribute to plant growth and development in ways that the plant cannot accomplish by itself? More fundamental knowledge is needed concerning their interactions with each other, with other plant-associated microorganisms (e.g. mycorrhiza) as well as with their host plant after inoculation. Especially seed endophytes from economically important crops, such as maize, rice or rapeseed, are worthwhile studying as they can be applied in biocontrol, biofertilization, bioenergy production and bioremediation. Finally, thorough research should be performed to unravel the changes that occur in the seed associated bacterial community due to seed maturation, storage time and storage conditions to guarantee the production of high quality seeds.

CHAPTER 2 Scope and objectives

Transgenerational signaling through maternally derived compounds or epigenetic mechanisms allows plants to adapt in a fast and efficient manner to biotic and abiotic stresses. Recently, the plant microbiome emerged as a novel trait that extends the capacity of plants to adapt to the environment (Bulgarelli *et al.*, 2013). Due to the unique niche they occupy within the group of plant-associated bacteria, vertically transmitted endophytes are ideally suited to prepare the next generation of plants for certain conditions already present in the maternal environment. Seed endophytes are perfect candidates for use in agricultural, industrial or environmental applications. Nevertheless, knowledge on their fate and function during the life cycle of plants is still scarce. The general goal of this thesis was to better understand the factors that shape seed endophytic communities and the importance of seed endophytes for plant growth.

In the **first section**, several factors affecting seed endophytic communities are considered. The toxic metal Cd was chosen because a large area in the northeastern part of Belgium is contaminated with Cd due to the historic presence of zinc smelters (Hogervorst *et al.*, 2007). Moreover, due to the diffuse nature of this contamination, bacteria-assisted phytoremediation could be a possible solution for clean-up of this contaminated area (Thewys *et al.*, 2010). To study the impact of Cd on the seed endophytic community, a selection pressure was simulated in *Arabidopsis thaliana* in order to create 2 types of seeds: from plants that were exposed to 2 µM Cd during 8 subsequent generations (Cd seeds) and from plants that were grown in parallel for also 8 generations but never exposed to Cd (control seeds). The cultivable as well as the total endophytic communities of both seed types were isolated and the species composition was determined (**chapter 4**). The composition and characteristics of the seed endophytic communities of both seed types were again determined after 4 and 6 more generations to check if they remained stable.

To verify whether the selection pressure induced in the laboratory-grown *A. thaliana* was comparable with a selection pressure occurring in the field, the cultivable endophytic communities associated with seeds of *Agrostis capillaris*

from a Cd/Ni contaminated field site and a non-contaminated field site were identified and characterized (**chapter 5**).

The seed endophytic communities of *A. thaliana* were also characterized after generations of bulking on sand and on a mixture of sand and potting soil, 2 commonly used substrates for production of seeds of *A. thaliana*, to determine the influence of the substrate type. The seeds harvested from plants growing on sand and potting soil were sown again, but this time on the same substrate to determine the influence of the seed type on the establishment of the plant endophytic community (**chapter 6**).

In the **second section**, inoculation experiments were performed to investigate the plant growth promoting properties of the isolated seed endophytes on their host plant. Using vertical agar plates and/or a hydroponic system, it was verified whether the *in vitro* plant growth promoting traits of *A. thaliana* and *A. capillaris* seed endophytes could be good indicators for *in planta* growth promotion (**chapter 7**). Several important features for inoculation success were studied in order to optimize plant colonization and plant growth promotion by the inoculated endophytes (**chapter 8**).

SECTION 1 Factors affecting the seed endophytic community

Abstract

Transgenerational adaptation is important to respond rapidly to environmental challenges and increase overall plant fitness. Besides well-known mechanisms such as epigenetic modifications, vertically transmitted endophytic bacteria could play a role in this process. They usually develop a mutualistic relationship with their host plant of which they can improve growth and health. In this part of the thesis, we wanted to find out whether plants are able to select which endophytic bacterial genera are transferred to its next generation.

First, we studied the cultivable and total endophytic communities of several generations of *Arabidopsis thaliana* seeds harvested from plants exposed to Cd or not. Not the genera that were present but rather their phenotypic characteristics were important as selection criteria for which bacteria are transferred to the next plant generation.

Next, we verified whether the changes detected in the lab-grown *A. thaliana* plants are also occuring in a field situation. Therefore the cultivable seed endophytes of *Agrostis capillaris* growing on a long-term Cd/Ni-contaminated experimental plot were compared with those originating from a non-contaminated plot. We observed plant- and contaminant-dependent effects on the community composition as well as on their phenotypic characteristics.

Finally, we determined the effect of 2 plant growth substrates, frequently used during production of *A. thaliana* seeds, on the cultivable and total endophytic communities. Despite differences in the soil bacterial communities, the seed and radicle endophytic communities were very similar, which suggests that plants are able to select their endophytes. The leaf endophytic community showed to be mainly derived from the environment and not from the seed. Moreover, it appeared that, concerning the endophytic communities, the genera that were most abundant in the cultivable bacterial community coincide with the genera frequently detected in the total bacterial community.

Understanding how different factors influence the seed endophytic community can help us to improve seed quality and plant growth throughout different biotechnological applications.

Introduction

As plants are sessile organisms, the ability to respond and adapt rapidly to changing environmental conditions is advantageous (Walbot and Cullis, 1985). When the local maternal environment reliably predicts the offspring environment, transgenerational signaling can prepare the next generation of plants for biotic as well as abiotic stresses (Rasmann *et al.*, 2012). Transgenerational plasticity can be mediated by maternally derived proteins and mRNAs, which mainly have an effect on seed dormancy, seed longevity and germination (Rajjou *et al.*, 2004; Mondoni *et al.*, 2014). Moreover, maternally derived hormone contents can be altered by environmental stresses (Herman and Sultan, 2011). For example, Jha *et al.* (2010) discovered that shading influenced the germination of Palmer amaranth through an increased abscisic acid content in the seeds. Also DNA methylation and histone modifications are a source of heritable phenotypic variation and were shown to be involved in transgenerational adaptation to environmental challenges (Johannes *et al.*, 2009; Rasmann *et al.*, 2012). For example, Whittle *et al.* (2009) reported temperature-induced epigenetic changes in *Arabidopsis thaliana* that persisted over at least 2 generations and improved progeny fitness. Maternal environmental effects can also be transmitted via seed provisioning: all reserves allocated to the seed by the mother plant can influence the seedling from establishment up until reproduction (Metz *et al.*, 2010; Herman and Sultan, 2011). Zas *et al.* (2013) observed an increased pine seedling biomass when seeds originated from a favourable environment that was associated with a higher seed weight.

Also endophytes can play an important role in transgenerational adaptation. Saikkonen *et al.* (2010) demonstrated that vertically transmitted fungal endophytes were able to increase the herbivore resistance of grass. Moreover, the vertically transmitted *Curvularium* and *Fusarium* isolates conferred respectively heat and salt tolerance in a habitat-specific manner (Rodriguez *et al.*, 2008). Vertically transmitted endophytes are likely to evolve towards a mutualistic relationship with their host because there occurs selection for reduced virulence as they are dependent on host fitness for reproduction. In

contrast, horizontal transmission usually promotes virulence as this gives opportunities for contagious spread (Ewald, 1987).

Besides fungal endophytes, bacterial endophytes and their associated beneficial traits could also be transferred over subsequent generations. By consequence, these bacterial seed endophytes can rapidly infect the new generation of plants because they experience less competition with bacteria originating from the external environment and because they are already adapted to living inside plant tissues (Kaga *et al.*, 2009; Hardoim *et al.*, 2012). Moreover, endophytic bacteria have an advantage over other plant-associated bacteria as they are better protected from biotic and abiotic stresses due to their location inside the plant (Hallmann *et al.*, 1997).

Cankar *et al.* (2005) already suggested that seeds could serve as vectors for transfer of beneficial bacteria. Their presence in early growth stages can promote plant growth and health by producing plant growth hormones, increasing the quantities of available nutrients, lowering stress ethylene levels and preventing the growth and activity of phytopathogens (Weyens *et al.*, 2009a). For example, Mastretta *et al.* (2009) demonstrated that inoculation of tobacco with its seed endophytes improved plant growth and reduced metal phytotoxicity in case of Cd exposure.

The composition of the bacterial endophytic community is influenced by several factors such as host plant genotype, seasonal variation, plant growth stage, or geobiochemical conditions (Inceoğlu *et al.*, 2011; Ding *et al.*, 2013; Bokulich *et al.*, 2014). A study by Johnston-Monje and Raizada (2011) demonstrated that certain seed endophytes of *Zea mays* are conserved across boundaries of evolution, ethnography and ecology. They found that the seed bacterial community varied with *Zea* phylogeny, but there was also a core microbiota that was conserved across *Zea* genotypes. Siciliano *et al.* (2001) demonstrated that certain beneficial endophytic bacterial genotypes were enriched dependent on the presence and concentration of contaminants in the soil where the plants were growing.

A better understanding of the factors that specifically shape the seed endophytic community can lead to an improved seed quality and a more efficient application of bacterial seed endophytes in agricultural, industrial or environmental applications. Bokulich *et al.* (2014) pointed out that patterning of bacterial

communities associated with agricultural crops could be associated with downstream quality characteristics. The presence or absence of certain bacterial taxa could also influence the productivity, chemical properties and pathogen defense of the plant (Gilbert *et al.*, 2014).

We investigated the effect of a contaminant on the composition and characteristics of the seed bacterial community. In chapter 4, we characterized the cultivable and total endophytic community associated with the seeds of the model plant *Arabidopsis thaliana*. Two different types of seeds were used: seeds from plants that were exposed for 8, 12 and 14 generations to 2 µM Cd and seeds from plants that were reproduced in parallel but never exposed to Cd.

To make the link between the laboratory and the field situation, we investigated whether changes in the cultivable seed endophytic community of *Agrostis capillaris* after long-term exposure to Cd and Ni in the field are comparable to the laboratory-induced changes found in *A. thaliana*. Therefore the cultivable seed endophytes of *A. capillaris* growing on a Cd/Ni-contaminated site were compared with *A. capillaris*-associated seed endophytes present on a non-contaminated site in chapter 5.

In chapter 6, we investigated the effects of soil type on the cultivable and total bacterial community present in the seeds harvested from plants growing on sand (bacteria-poor) and on a mixture of sand and potting soil (bacteria-rich), 2 common substrates for producing *A. thaliana* seeds. Soil is considered to be the most microbially diverse habitat on Earth (Daniel, 2005). Different soil types harbour different microbial communities due to differences in vegetation type, carbon and nutrient availability, soil moisture and pH (Buckley and Schmidt, 2002; Fierer and Jackson, 2006). Plants can select from this large range of bacteria present in the bulk soil through specific root exudates (Haichar *et al.*, 2008; Berg and Smalla, 2009; Bulgarelli *et al.*, 2013). Together with the plant innate immune system, this results in specific bacterial rhizosphere and endosphere communities (Haichar *et al.*, 2008; Bulgarelli *et al.*, 2013). To determine if the substrate type still has an effect on the establishment of the plant bacterial community, we investigated cultivable and total bacterial endophytic communities of plants grown from these seeds (chapter 6).

CHAPTER 3 Materials and methods used in section 1

3.1 Optimization of the sterilization protocols

To obtain as many (different) cultivable seed endophytes as possible, several seed sterilization protocols were tested.

For *Arabidopsis thaliana* seeds (ecotype Col-0), seeds were immersed in 70% ethanol during 1 min or they were not immersed in ethanol. In a second step, they were further sterilized in a NaClO solution, supplemented with 0.1% Tween 80, with different concentrations and exposure times. In case of the 1% NaClO, seeds were rinsed for 3, 5, 10, 15 or 20 min. For the 0.1% NaClO, they were rinsed during 1, 3, 5 or 10 min. In the last step, the seeds were thoroughly washed in sterile deionized water.

For *Agrostis capillaris*, different seed sterilization protocols were tested on commercially available seeds. First, the seeds were immersed in 70% EtOH during 1 min or they were not immersed in EtOH. Then the seeds were rinsed in 0.1% or 0.01% NaClO for 1 min, or in 0.05% NaClO, for 1 or 0.5 min. Finally, the seeds were thoroughly washed in sterile deionized water.

Sterility of the surface was verified by incubating the last rinsing water and 10 seeds on 869 solid medium (Mergeay *et al.*, 1985) containing per liter deionized water: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 g D-glucose and 0.345 g $CaCl_2.2H_2O$ (pH 7). The remaining seeds were crushed in a sterile mortar with 500 µL sterile 10 mM $MgSO_4$. Dilutions 0 to 10^{-2} were plated on 1/10 869 medium and incubated for 1 week at 30°C. For each condition 2 replicates were used.

3.2 Seed sampling

Arabidopsis thaliana seeds (ecotype Col-0) were sown on quartz sand with a particle size of 0.4-0.8 mm (referred to as sand) or on a 1:1 mixture of this sand with a commercially available universal potting soil (referred to as potting soil). These are 2 often-used substrates for production of *A. thaliana* seeds. Plants were grown in a greenhouse and supplied regularly with a 1/10 diluted Hoagland nutrient solution (Smeets *et al.*, 2008). When flowering was complete,

no more Hoagland solution was applied and seeds were harvested when plants were completely dry. Sowing and harvesting of seeds occurred 2 times a year: once in spring and once in fall. Seeds of spring 2013 (generation 14) were used for the analysis.

Using Cd, an artificial selection pressure was simulated in *A. thaliana* in order to create 2 types of seeds: seeds originating from plants that were grown in a greenhouse on sand with 2 µM Cd (Cd seeds) and seeds originating from plants that reproduced in parallel but that were never exposed to Cd (control seeds).

Production of seeds was done 2 times a year: once in spring and once in fall. Seeds of spring 2010 (generation 8), 2012 (generation 12) and 2013 (generation 14) were used for further analyses.

Agrostis capillaris seeds were collected from a Cd/Ni-contaminated and a non-contaminated control plot at the experimental site of Couhins of the 'Institut National de la Recherche Agronomique' (INRA) in Bordeaux (France). The contaminated plot was treated with sludge from a sewage treatment plant (Louis Fargue, Bordeaux) containing high Cd and Ni concentrations from effluents discharged by a battery manufacturer (Boisson *et al.,* 1998). Treatment consisted of 100 tons DM.ha^{-1} and was applied in 1976, 1978 and 1980 (Boisson *et al.*, 1998). $Ca(NO_3)_2$-extractable amounts of Cd and Ni at sampling time were 0.67 ± 0.018 mg.kg^{-1} DW and 1.02 ± 0.040 mg.kg^{-1} DW respectively on the non-contaminated plot and 3.90 ± 0.17 mg.kg^{-1} DW and 8.05 ± 0.11 mg.kg^{-1} DW respectively on the contaminated plot.

3.3 Metal extraction

Cd and Ni contents were determined in soils and *A. capillaris* shoots and seeds from the non-contaminated and Cd/Ni-contaminated field plots. To determine the total metal contents, 50 to 200 mg soil, crushed shoots or seeds were transferred to glass tubes. Extraction was performed using acid (2x HNO_3 Suprapur, 1x HCl Suprapur) and heat (110°C). Samples were dissolved in 0.5 mL 20% HCl and 4.5 mL Millipore water. To estimate 'bioavailable' metal contents, 25 g of soil was shaken in 25 mL of 0.1 M $Ca(NO_3)_2$ and filtrated through Whatman ashless filter paper. Cd and Ni content were determined using inductively coupled plasma-atomic emission spectroscopy (ICP-AES).

3.4 Isolation of cultivable bacteria

Seeds were surface-sterilized with the optimal protocol as determined above (*A. thaliana*: 1 min 0.1% NaClO; *A. capillaris*: 30 s 0.05% NaClO). Seed endophytes were isolated by crushing 3 times 10 mg (*A. thaliana*) or 30 mg (*A. capillaris*) seeds in 500 µL 10 mM $MgSO_4$. Dilutions 0 to 10^{-2} were incubated on 1/10 869 medium during 1 week at 30°C. Colony forming units (cfu) were counted and calculated per g seed. For each morphologically different colony type, 5 to 10 colonies were chosen for further analyses.

For chapter 6, part of the *A. thaliana* seeds were sown on vertical agar plates (Remans *et al.*, 2006) that were incubated in a growth chamber until the **radicle** emerged. Part of the seeds was sown in a hydroponic culture on sand for 3 weeks with a regular supply of 1/10 diluted Hoagland nutrient solution (Smeets *et al.*, 2008). Three week-old **leaves** harvested in the hydroponic culture were immersed in 0.1% NaClO supplemented with 0.1% Tween 80 for 3 min, and were thoroughly washed in sterile deionized water. Sterility of the surface was checked by incubating the last rinsing water on 869 solid medium (Mergeay *et al.*, 1985). Subsequently, these leaves as well as the radicles from the vertical agar plates were crushed in 1 mL sterile 10 mM $MgSO_4$. Appropriate dilutions were plated on 1/10 869 medium and incubated for 1 week at 30°C. For each condition, 3 replicates of 30 mg (radicles) or 300 mg (leaves) were used. **Soil** bacteria were isolated by shaking 1 g of growth substrate (sand/potting soil) in 10 mL sterile 10 mM $MgSO_4$. Dilutions 10^{-2} to 10^{-7} were plated on 1/10 869 medium and incubated for 1 week at 30°C. Cfu were counted and calculated per gram soil or plant material. For each morphologically different colony type, 5 to 10 colonies were chosen for further analyses.

3.5 Phenotypic characterization of cultivable bacteria

Tolerance to **metals** was estimated visually after incubation of the bacteria for 1 week at 30°C on solid 284 medium (Schlegel *et al.*, 1991) supplemented with 0.4 mM $CdSO_4$, 0.8 mM $CdSO_4$, 0.6 mM $ZnSO_4$ or 0.4 mM $CuSO_4$. This 284 medium contained per liter deionized water: 6.06 g Tris-HCl, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.2 g $MgCl_2.6H_2O$, 0.03 g $CaCl_2.2H_2O$, 40 mg $Na_2HPO_4.2H_2O$, 10 mL 1.8 mM Fe(III)NH_4citrate solution and 1 mL

microelements solution (pH 7). The microelements solution contained per liter deionized water: 1.3 mL 25% HCl, 144 mg $ZnSO_4.7H_2O$, 100 mg $MnCl_2.4H_2O$, 62 mg H_3BO_3, 190 mg $CoCl_2.6H_2O$, 17 mg $CuCl_2.2H_2O$, 24 mg $NiCl_2.6H_2O$ and 36 mg $NaMoO_4.2H_2O$. Five carbon sources (C-mix) were added per liter medium: 0.52 g glucose, 0.66 g gluconate, 0.54 g fructose, 0.81 g succinate and 0.35 g lactate.

Bacterial **siderophore** production was determined using a chrome azurol S (CAS) reagent as described by Schwyn and Neilands (1987) and organic acids were detected with the method of Cunningham and Kuiack (1992). First, bacteria were grown in liquid 869 medium. Next, 20 µL of bacterial suspension was added to 800 µL of the culture medium. For testing siderophore production 284 medium with C-mix containing 0, 0.25 and 3 µM Fe(III)NH_4citrate was used. For **organic acid** production, sucrose tryptone medium was used. This medium contained per liter deionized water: 20 g sucrose, 5 g tryptone and 10 mL microelement solution (containing per liter deionized water: 20 mg $NaMoO_4$, 200 mg H_3BO_3, 20 mg $CuSO_4.5H_2O$, 100 mg $FeCl_3$, 20 mg $MgCl_2.4H_2O$ and 280 mg $ZnCl_2$). After 5 days incubation at 30°C, 100 µl of the CAS reagent (per 10 mL: 0.6 mL 10 mM hexadecyltrimethylammoniumbromide, 1.5 mL 10 mM HCl, 0.1 mL 1 mM $FeCl_3$, 0.75 mL 2 mM CAS, 3 mL 1.67 M piperazine, 1 mL 40 mM sulphosalicylic acid and 3.05 mL deionized water) was added to each sample. After 4 h incubation, orange wells were considered positive for siderophore production. To detect organic acids, 100 µL 0.1% alizarin red S was added to each sample. After 20 min incubation, yellow wells were considered positive for organic acid production.

Production of **IAA** was determined using Salkowski's reagent after incubation of the bacteria for 5 days at 30°C in the dark in liquid 1/10 869 medium supplemented with 0.5 $g.L^{-1}$ tryptophan. After centrifugation, 0.5 mL supernatant was mixed with 1 mL Salkowski's reagent (50 mL 35% $HClO_4$, 1 mL 0.5 M $FeCl_3$) (Gordon and Weber, 1951). After 20 min incubation, pink wells were considered positive for IAA production.

ACC deaminase activity was determined by monitoring the amount of α-ketobutyrate generated by enzymatic hydrolysis of ACC (Belimov *et al.*, 2005). Bacteria were cultured in liquid 869 medium for 3 days at 30°C and harvested by centrifugation. Cell pellets were washed twice with 0.1 M Tris-HCl buffer (pH

7.5) and resuspended in 3 mL liquid SMN medium containing 5 mM ACC as a sole source of nitrogen. After 2 days incubation at 30°C, the bacteria were pelleted and resuspended in 1 mL 0.1 M Tris-HCl buffer (pH 8.5). The cells were disrupted by adding 30 µL toluene and vigorous vortexing. 100 µL of this cell suspension was mixed with 100 µL 0.1 M Tris-HCl buffer (pH 8.5) and 10 µL 0.5 M ACC, and incubated for 30 min at 30°C. Then, 1 mL 0.56 N HCl was added and mixtures were centrifuged. Mixtures without cell suspension were used as controls. Next, 400 µL 0.56 N HCl and 150 µL 0.2% 2,4-dinitrophenylhydrazine in 2 N HCl were added to 500 µL supernatant. After incubation for 30 min at 30°C, 1 mL 2 N NaOH was added and a color change from yellow to brown was considered positive for ACC deaminase activity.

For **acetoin** production, bacteria were cultured in liquid 869 medium for 3 days and washed twice in 10 mM sterile $MgSO_4$. 1 mL of methyl red/Voges-Proskauer (MR/VP) medium was inoculated and incubated for 48 h at 30°C. After centrifugation, 100 µL supernatant was mixed with 10 µL 50 mM L-arginine, 10 µL alpha-naphtol (Barritz reagens, Sigma) and 25 µL KOH (Barritz reagens, Sigma) (Romick and Fleming, 1998). After vortexing for 10 minutes, a red color was indicative for acetoin production.

To test for bacterial **nitrogenase** activity, the bacteria were cultured in liquid 869 medium and washed twice in 10 mM $MgSO_4$. Bacteria were transferred to 1.5 mL semisolid malate-sucrose medium (per L: 10 g sucrose, 5 g L-malic acid, 0.3563 g $MgSO_4.7H_2O$, 0.01 g $FeCl_3$, 0.1 g NaCl, 0.0151 g $CaCl_2.2H_2O$, 0.1 g K_2HPO_4, 0.4 g KH_2PO_4, 0.002161 g $Na_2MoO_4.H_2O$, and 1.75 g Noble agar; pH 7.2) and containing 3 mL bromothymol blue (BTB) (50 mg in 50% ethanol) as an indicator (Xie *et al.*, 2006). The same medium supplemented with 0.12 $g.L^{-1}$ NH_4Cl was used as a positive control. After 1 week incubation at 30°C, a color change to yellow was considered positive for N_2 fixation.

The protocols for testing the **phosphate solubilizing** and **phytate mineralizing** abilities of the bacteria were based on Jorquera *et al.* (2008). In short, bacteria were cultured in liquid 869 medium for 3 days, washed twice in 10 mM $MgSO_4$ and resuspended in 10 mM $MgSO_4$. 50 µL of this suspension was transferred to National Botanical Research Institute's phosphate growth medium (NBRIP) (per L: 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) or to phytate screening

medium (PSM) (per L: 10 g D-glucose, 4 g Na-phytate, 2 g $CaCl_2$, 5 g NH_4NO_3, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.01 g $MnSO_4.H_2O$ and 15 g agar). After 1 week, the appearance of a clear zone around the colonies was considered as an indicator of phosphate solubilization or phytate mineralization.

3.6 Genotypic characterization of cultivable bacteria

Genomic DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen). The quality and quantity of the DNA were checked using the Nanodrop ND-1000 Spectrophotometer (Isogen Life Sciences). The 16S rRNA gene was amplified in a PCR reaction containing 1x High Fidelity PCR buffer, 2 mM $MgSO_4$, 200 µM of each dNTP, 200 nM of the bacteria-specific 26F primer (AGAGTTTGATCCTGGCTCAG, target 16S-23S internal transcribed spacer) (Schauer and Hahn, 2005), 200 nM of the universal 1392R primer (ACGGGCGGTGTGTRC, target 16S bp 1392-1406) (Amann *et al.*, 1995), 1 U High Fidelity Taq DNA polymerase, 1 µL DNA template and RNase free water until a total volume of 50 µL. Cycling conditions were: 5 min at 95°C, 35 cycles of 1 min at 94°C, 30 s at 52°C and 3 min at 72°C, and a final incubation step of 10 min at 72°C. For the amplified ribosomal DNA restriction analysis (ARDRA), 2.9 µL 1x NEB buffer 1 (New England Biolabs), 0.3 µL restriction enzyme $HpyCH_4$ IV (New England Biolabs), 1.1 µL RNase and 4.3 µL RNase free water were added to 20 µL amplified 16S rDNA. This mixture was incubated for 2 h at 37°C. The resulting fragments were separated on a 1.5% agarose gel during 2 h at 90 V. Bacteria with the same ARDRA patterns were grouped and 1 representative isolate of each group was selected for sequencing. Purified PCR products (PCR purification kit, Qiagen) of 16S rDNA were bidirectionally sequenced by Macrogen (Seoul, South-Korea). Consensus sequences were determined using the Staden Package (Staden *et al.*, 1999) and identification was carried out by comparing the obtained sequences with those of reference strains in the Ribosomal Database Project (RDPII). In order to verify the identification, sequences were aligned and a UPGMA tree was constructed using Geneious 5.4 with default settings (Drummond *et al.*, 2011). To assess branch supports, bootstrap values were calculated with 1000 replicates. The obtained 16S rDNA sequences have been submitted into the European Nucleotide Archive

with the accession numbers HG937592-HG937604 and LN830896-LN830948 for *A. thaliana* (chapter 4 and 6) and HG001272-HG001280 for *A. capillaris* (chapter 5).

16S rDNA sequences from the cultivable bacteria were blasted against a local BLAST database created with the OTU sequences obtained with 454 pyrosequencing using Geneious 8.1.3 (Kearse *et al.*, 2012). A match was defined as at least 97% identity over the 394 bp region of the OTU sequence (Rott, 2012).

3.7 454 pyrosequencing workflow

3.7.1 Sampling and DNA extraction

Soil samples were collected by snap freezing and keeping 3x 900 mg at -80°C until further processing. Also 3x 300 mg of *A. thaliana* seeds, 3x 600 mg of radicles and 3x 900 mg of 3 week-old leaves were collected. Surface DNA on seeds, radicles and leaves was removed by rinsing for 1 min in sterile deionized H_2O, 30 s in 70% EtOH, 1 min in 1% NaClO, 30 s in 70% EtOH and 5x in sterile deionized H_2O. Samples were homogenized in 2 mL sterile 10 mM $MgSO_4$ using a mortar and pestle, and frozen at -80°C until further processing. To exclude the presence of DNA from external bacteria as much as possible, a PCR on 1 µL of the last rinsing water was performed. The mastermix contained 1x High Fidelity PCR-buffer, 2 mM $MgSO_4$, 200 µM of each dNTP, 200 nM bacteria-specific 26F primer (AGAGTTTGATCCTGGCTCAG) (Schauer and Hahn, 2005), 200 nM universal 1392R primer (ACGGGCGGTGTGTRC) (Amann *et al.*, 1995), 1 U Taq DNA polymerase and RNase free water until a total volume of 50 µL per reaction. Cycling conditions were: 5 min at 95°C, 35 cycles of 1 min at 94°C, 30 s at 52°C and 3 min at 72°C, and a final incubation step of 10 min at 72°C.

Total genomic DNA was extracted from seeds, radicles and leaves using the Invisorb Spin Plant Mini Kit (Invitek), which was shown to be the best out of 3 commercially available DNA extraction kits for the isolation of bacterial DNA from plant samples (Beckers, 2015). The Powersoil DNA Isolation kit (Mobio) was used for DNA extraction from soil.

3.7.2 Test run

To find a primerset combination that amplifies the bacterial 16S rDNA with a high taxonomic coverage while minimizing interference of chloroplastidal and/or mitochondrial DNA, several primerset combinations were tested on DNA extracted from seeds and radicles (Table 3.1 and 3.2). Different regions of the 16S rDNA were amplified with the 1st primerset (whether or not with a chloroplast mismatch), gel-extracted with the Qiaquick Gel Extraction Kit (Qiagen) (to remove mitochondrial DNA where possible) and amplified with the 2nd primerset to add adaptor sequences and multiplex identifier tags. The PCR reaction contained per sample 1x FastStart High Fidelity Reaction buffer, 1.8 mM $MgCl_2$, 200 µM of each dNTP, 200 nM forward primer, 200 nM reverse primer, 2 U FastStart High Fidelity Taq DNA polymerase, 1 µL DNA template and RNase free water until a total volulme of 25 µL. Cycling conditions were: 2.5 min at 95°C, 35 (1st primerset) or 18 (2nd primerset) cycles of 1 min at 94°C, 40 s at 53°C and 40 s at 72°C, and a final incubation step of 7 min at 72°C.

Table 3.1: Combinations of primersets used to amplify different hypervariable regions of the bacterial 16S rDNA.

No.	Primerset 1	Primerset 2	16S rDNA region	Chloroplast mismatch	Nested PCR
1	68F-783Rabc	68F-518R	V1-V3	Yes	Yes
2	68F-518R	68F-518R	V1-V3	No	No
3	341F-785R	341F-785R	V3-V4	No	No
4	341F-783Rabc	341F-783Rabc	V3-V4	Yes	No
5	799F-1391R	967F-1391R	V6-V7	Yes	Yes
6	967F-1391R	967F-1391R	V6-V7	No	Yes
7	799F-1193R	799F-1193R	V5-V7	Yes	No

Table 3.2: Primer sequences used in this study.

Primer	Sequence 5'-3'	Reference
799F	AACMGGATTAGATACCCKG	Chelius and Triplett, 2001
967F	CAACGCGAAGAACCTTACC	Sogin *et al.*, 2006
68F	TNANACATGCAAGTCGRRCG	McAllister *et al.*, 2011
341F	CCTACGGGNGGCWGCAG	Klindworth *et al.*, 2013
1391R	GACGGGCGGTGWGTRCA	Walker and Pace, 2007
1193R	ACGTCATCCCCACCTTCC	Bodenhausen *et al.*, 2013
518R	WTTACCGCGGCTGCTGG	Lee *et al.*, 2010
785R	GACTACHVGGGTATCTAATCC	Klindworth *et al.*, 2013
783Ra	CTACCAGGGTATCTAATCCTG	Sakai *et al.*, 2004
783Rb	CTACCGGGGTATCTAATCCCG	Sakai *et al.*, 2004
783Rc	CTACCCGGGTATCTAATCCGG	Sakai *et al.*, 2004

Unbound nucleotides, primer dimers and very short amplicons were removed using the PCR Purification Kit (Qiagen). The DNA concentration of each sample was determined with the Picogreen dsDNA Quantitation assay (Life Technologies) and an equimolar mixture of the different samples was made. Generation of a single-stranded template library, emulsion PCR and pyrosequencing were carried out by LGC Genomics (Berlin, Germany) on a 454 Genome Sequencer FLX Titanium (Roche). Each amplicon library (one for each primerset combination), consisting of 26 samples in total from several collaborators, was sequenced on 1/8 of a picotiter plate. The results of the test run can be found in appendix A.

3.7.3 Final run

Sample preparation for the final run was analogous to the test run. During the 1st PCR, primers 799F and 1193R were used to amplify the V5-V7 hypervariable region of the 16S rDNA, while avoiding amplification of chloroplastidal 16 rDNA through a 2 bp mismatch at the 3'-end of the 799F primer (Chelius and Triplett, 2001; Bodenhausen *et al.*, 2013). Samples were run on a 1.5% agarose gel to separate bacterial DNA (394 bp) from mitochondrial DNA if present (approximately 800 bp). The 394 bp band was extracted from the gel. During the 2nd PCR, primers 799F and 1193R were used. After purification, an equimolar mixture of the different samples was made and sent to Macrogen (Seoul, South-Korea) for generation of a single-stranded template library, emulsion PCR and 454 pyrosequencing. Each amplicon library, consisting of 26 samples in total from several collaborators, was sequenced on 1/8 of a picotiter plate.

3.7.4 Data analysis

Sequence reads were processed in Mothur software 1.33.3 following the Standard Operating Procedure available on http://www.mothur.org/wiki/Schloss_SOP (Schloss *et al.*, 2009). Briefly, sequences were trimmed to remove primers, barcodes, homopolymers longer than 8 bases and sequences shorter than 200 bases. Each unique sequence was aligned using the SILVA reference alignment. Chimeric sequences were detected using the Uchime tool and removed. Sequences were classified using the

Ribosomal Database Project reference files. For the test run, sequences matching 'Mitochondria' or 'Chloroplast' were identified and abundance data of these sequences were used to compare the performance of the tested primerset combinations. Next, sequences matching 'Mitochondria', 'Chloroplast', 'Archaea', 'Eukaryota' or 'unknown' were removed. Pairwise distances of the remaining sequences were calculated and species-level operational taxonomic units (OTUs) were defined based on a 0.03% distance level. Singletons (i.e. OTUs represented by only 1 sequence over an entire dataset) were removed and all samples were subsampled to 1285 (test run) or 2000 (final run) sequences per sample. The inverse Simpson diversity index, Chao richness estimator and Good's coverage estimator were calculated based on 1000 iterations. Rarefaction curves were generated based on 1000 iterations. The standard flowgram format (SFF) file was deposited in the NCBI Sequence Read Archive under the accession number SRP056064.

3.8 Statistical analysis

Statistical analyses were performed in R 3.1.1 (R Core Team, 2014) or SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Normal distribution of the data was analysed with the Shapiro-Wilk test and transformations were applied when necessary to approximate normality. Homoscedasticity of variances was checked using a residuals plot. Significant differences were evaluated with an analysis of variances (ANOVA) and post-hoc comparisons were carried out using the Tukey's Honest Significant Differences test for multiple comparisons.

Non-metric multi-dimensional scaling (NMDS) with Bray-Curtis distances was performed using the metaMDS function of the vegan package in R 3.1.1 (Oksanen *et al.*, 2014). Permutational multi-variate analysis of variance (PERMANOVA) with 1000 permutations was performed using the adonis function of the vegan package in R (Oksanen *et al.*, 2014). Indicator species analysis was performed using the multipatt function of the indicspecies package in R 3.1.1 with 1000 permutations (De Cáceres and Legendre, 2009).

CHAPTER 4 Cadmium-induced and transgenerational effects on cultivable and total seed endophytic communities of *Arabidopsis thaliana*

Parts of this chapter were published in:
Truyens S, Weyens N, Cuypers A, Vangronsveld J. 2013. Changes in the population of seed bacteria of transgenerationally Cd-exposed Arabidopsis thaliana. Plant Biology 15: 971-981.
Truyens S, Beckers B, Thijs S, Weyens N, Cuypers A, Vangronsveld J. 201x. Cadmium-induced and transgenerational changes in the cultivable and total seed endophytic community of Arabidopsis thaliana. Submitted to Plant Biology.

In this chapter, the effect of Cd on the seed endophytic community of *Arabidopsis thaliana* was studied. Two different types of seeds were used: seeds from plants that were exposed for 8 generations to 2 µM Cd (Cd seeds) and seeds from plants that were never exposed to Cd (control seeds). The genotypic as well as phenotypic composition of the seed endophytic communities were analyzed over several generations.

4.1 Results and discussion

4.1.1 Optimization of the sterilization protocol

Arabidopsis thaliana is worldwide the most used model plant. In the standard seed sterilization protocol for *A. thaliana*, 1 min rinsing in 70% ethanol followed by 10 min in 1.2% NaClO are described (Balcells, 1993), but our results indicate that this is too harsh, possibly destroying the seed endophytes which can lead to effects on germination and further plant development. An optimal seed sterilization protocol leads to a complete sterilization of the seed exterior while preserving a high number of cfu and morphologically distinct colony types that can be isolated from the seed interior. An important factor appeared to be the strength of the NaClO solution: an increase from 0.1% to 1% led to a strong decrease in the number of cfu.g^{-1} seed (Table 4.1). In case of the 0.1% NaClO

solution, the number of cfu.g^{-1} seed was still low when ethanol was used or when the exposure time in the NaClO exceeded 3 min. Although a higher number of cfu.g^{-1} seed was obtained when the exposure time was 3 min, 1 min was considered optimal because in that case more morphologically distinct colony types were isolated.

Table 4.1: Overview of the different sterilization protocols tested.

EtOH	Strength NaClO solution (%)	Time in NaClO solution (min)	Seed surface	Cfu.g^{-1} seed	Morphologically distinct colony types
Yes	1	3	Sterile	83	1
		5	Not Sterile	5833	2
		10	Sterile	11500	3
		15	Not Sterile	0	-
		20	Sterile	2167	1
	0.1	3	Sterile	11833	3
		5	Not Sterile	31583	2
		10	Not Sterile	36833	2
No	1	3	Sterile	0	-
		5	Not Sterile	16917	2
		10	Not Sterile	0	-
		15	Not Sterile	83	1
		20	Sterile	0	-
	0.1	1	Sterile	135000	4
		3	Sterile	420000	3
		5	Sterile	17000	2
		10	Sterile	3833	2

4.1.2 Seed endophytic community composition

Control and Cd seeds of *A. thaliana* contained similar amounts of cfu.g^{-1} seed (respectively 4.3 x 10^7 cfu.g^{-1} seed and 3.7 x 10^7 cfu.g^{-1} seed). With increasing number of generations, there was a decreasing number of cfu that can be isolated: 5.7 x 10^6 and 6.9 x 10^7 cfu.g^{-1} seed after 12 generations and 5.6 x 10^5 and 8.7 x 10^3 cfu.g^{-1} seed after 14 generations in control and Cd seeds respectively. This is in accordance with Hardoim *et al.* (2012) who found a 100-fold decrease in seed endophytes when comparing 2 consecutive generations of rice seeds grown under greenhouse conditions. The number of cultivable endophytes isolated from both *A. thaliana* seed types was much higher than expected. It is generally assumed that the number of cultivable bacteria decreases from rhizosphere (10^7-10^9 cfu.g^{-1}), to root (10^5-10^7 cfu.g^{-1}), to stem and leaves (10^3-10^4 cfu.g^{-1}) and that it reaches approximately 10^2-10^3 cfu.g^{-1} in seeds (Benizri *et al.*, 2001; Hallmann, 2001).

The number of genera that was isolated also diminished when the number of generations increased: 9 genera in generation 8, 3 in generation 12, and only 2 in generation 14 (Table 4.2). These findings demonstrate that production of seeds on a bacteria-poor substrate, such as the quartz sand used in this study, reduces the number of seed endophytes compared to previous generations. This indicates that only a minor part of the community is transferred to the next generation.

Table 4.2: Percentages of the cultivable bacterial genera isolated from control and Cd seeds after 8, 12 and 14 generations of plant growth. Percentages of the top 10 most abundant genus-level OTUs from each sample are shown for the total bacterial community from control seeds after 12 and 14 generations and from Cd seeds after 14 generations. Co = control seeds, Cd = Cd seeds.

	Cultivable						Total		
Generation	**8**		**12**		**14**		**12**	**14**	
Seed type	**Co**	**Cd**	**Co**	**Cd**	**Co**	**Cd**	**Co**	**Co**	**Cd**
Actinomyces	-	-	-	-	-	-	-	1.5	-
Cellulomonas	-	-	-	-	-	-	-	-	0.42
Corynebacterium	-	-	-	-	-	-	0.52	2.3	0.76
Microbacterium	-	-	-	-	-	-	0.48	-	1.5
Mycobacterium	-	-	-	-	-	-	-	-	0.34
Aeromicrobium	-	-	-	-	-	37.5	-	-	1.1
Nocardioides	-	-	-	-	-	-	-	-	1.2
Propionibacterium	-	-	-	-	-	-	1.7	2.3	1.8
Pseudonocardia	-	-	-	-	-	-	-	-	1.4
Patulibacter	-	-	-	-	-	-	-	1.0	0.34
Micrococcus	0.01	-	28.9	-	-	-	-	-	-
Chryseobacterium	-	-	-	-	-	-	0.65	-	0.75
Flavobacterium	-	-	-	-	-	-	-	-	0.69
Sediminibacterium	-	-	-	-	-	-	1.7	1.7	0.68
Paenibacillus	-	0.001	-	-	-	-	-	-	-
Streptococcus	-	-	-	-	-	-	1.1	1.7	-
Staphylococcus	0.52	1.89	-	-	-	-	1.2	0.76	1.45
Bacillus	2.07	0.07	-	0.81	-	12.5	-	-	-
Devosia	-	-	-	-	-	-	-	-	0.76
Brevundimonas	-	-	-	-	-	-	0.65	0.76	1.4
Afipia	-	-	-	-	-	-	6.0	2.7	1.4
Sinorhizobium	8.05	-	57.8	-	-	-	-	-	-
Mesorhizobium	-	-	-	-	-	-	1.5	-	0.76
Rhizobium	40.2	12.1	13.3	99.2	58.9	50	8.5	11.9	2.9
Azospirillum	-	-	-	-	-	-	2.0	-	-
Pseudolabrys	-	-	-	-	-	-	-	-	0.34
Sphingomonas	33.1	64.7	-	-	-	-	-	0.95	2.2
Bosea	-	0.13	-	-	-	-	1.6	-	-
Methylobacterium	14.3	0.06	-	-	-	-	2.6	0.76	5.9
Acidovorax	0.76	9.16	-	-	-	-	-	2.0	0.34
Variovorax	1.02	12.0	-	-	-	-	-	-	-
Delftia	-	-	-	-	-	-	0.7	-	-
Escherichia/Shigella	-	-	-	-	-	-	1.7	0.76	0.76
Pseudomonas	-	0.003	-	-	41.1	-	12.3	5.4	2.1
Stenotrophomonas	-	-	-	-	-	-	0.65	-	0.34
% of community	**100**	**100**	**100**	**100**	**100**	**100**	**45.6**	**36.5**	**31.6**

This was confirmed by the results of the 454 pyrosequencing. Sequencing of the 2 amplicon libraries generated a total of 283146 reads with an average read length of 403 bp. After quality trimming and assigning the reads to the different samples, a total of 145526 high quality reads remained. For the 9 seed samples together, 43214 high quality bacterial reads were finally recovered. When comparing the total bacterial community of seeds of generation 12 with that of generation 14, the number of OTUs detected decreased from 34.8 ± 2.17 to 20.8 ± 1.92, the inverse Simpson diversity index decreased from 1.8 ± 0.026 to 1.7 ± 0.027 and the Chao richness estimator decreased from 44.1 ± 8.73 to 29.4 ± 9.00 (Table 4.3). These values were obtained by subsampling all samples at 2000 sequences, which was sufficient according to the rarefaction curves (Fig. 4.1).

Table 4.3: Good's coverage estimator, number of OTUs (Sobs), inverse Simpson diversity index and Chao richness estimator at similarity level 97%. Values are based on 1000 subsamplings of 2000 sequences per sample. Co = control seeds, Cd = Cd seeds. * = p < 0.1 compared to generation 12.

Seed type	Generation	Coverage ± stdev	Sobs ± stdev	InvSimpson ± stdev	Chao ± stdev
Co	12	99.5 ± 0.13	34.8 ± 2.17	1.8 ± 0.026	44.1 ± 8.73
Co	14	99.6 ± 0.11	20.8 ± 1.92*	1.7 ± 0.027	29.4 ± 9.00*
Cd	14	99.1 ± 0.16	48.4 ± 2.69	2.5 ± 0.051	66.5 ± 11.8

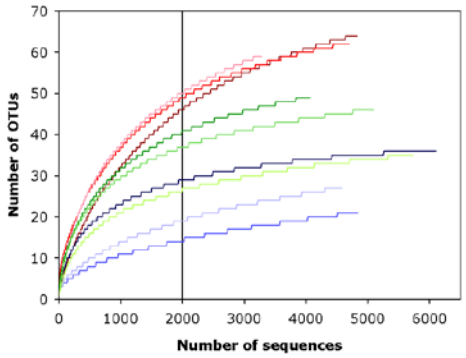

Figure 4.1: Rarefaction curves for endophytic samples from control seeds generation 12 (green), control seeds generation 14 (blue) and Cd seeds generation 14 (red). For each sample type 3 biological replicates were used. The vertical line indicates the number of sequences (2000) subsampled from each sample for further analysis.

The cultivable bacterial community associated with **control seeds** of generation 8 was dominated by *Rhizobium* (40.2%) (Table 4.2). Also *Sphingomonas* was isolated in high numbers (33.1%). *Variovorax* made up 1.02% of the community and *Acidovorax* represented 0.76%. *Sinorhizobium* and *Methylobacterium* accounted for 8.05% and 14.3%, respectively, of the bacterial community associated with control seeds. The remaining part of the community consisted of *Micrococcus* (0.01%), *Bacillus* (2.07%) and *Staphylococcus* (0.52%). Concerning the cultivable community, *Rhizobium* is the only genus that was found in control seeds from all generations. The other genera did not seem to be transferred from generation to generation via the seed. Also in the total community, *Rhizobium* was the most abundant genus that was present in both generations studied (Table 4.2). Likewise, *Pseudomonas* was abundant in both generations of the total community (Table 4.2). *Rhizobium* as well as *Pseudomonas* are often found to colonize plants and thereby increase plant growth and fitness (Biswas *et al.*, 2000; Chi *et al.*, 2005; Mercado-Blanco and Bakker, 2007; Gómez-Lama Cabanás *et al.*, 2014).

In **Cd seeds** of generation 8, *Sphingomonas* was the predominant genus with a relative abundance of 64.7% (Table 4.2). *Sphingomonas* is abundant in nature and is often found in association with plants where it exerts plant growth promoting and biocontrol functions (Innerebner *et al.*, 2011; Khan *et al.*, 2014). *Rhizobium* (12.1%) was much less represented in Cd seeds compared to control seeds. In contrast to control seeds, a fairly large portion of the cultivable endophytic community associated with Cd seeds consisted of *Variovorax* (12.0%) and *Acidovorax* (9.16%). *Methylobacterium* made up 0.06% of the community and *Bacillus* represented 0.07%. The remainder of the community consisted of *Staphylococcus* (1.89%), *Paenibacillus* (0.001%), *Bosea* (0.13%) and *Pseudomonas* (0.003%). *Sinorhizobium* and *Micrococcus* were only found in control seeds, while *Paenibacillus*, *Pseudomonas* and *Bosea* were only found in Cd seeds. *Methylobacterium* was also predominantly isolated from control seeds. *Sphingomonas*, *Variovorax*, *Acidovorax*, *Bacillus*, *Rhizobium* and a low number of *Staphylococcus* were found in both control and Cd seeds.

Several of the bacterial genera isolated from the seeds of *A. thaliana* were also recovered from seeds of other plant species. *Methylobacterium sp.* and *Sphingomonas sp.* were found in seeds of rice (Okunishi *et al.*, 2005; Kaga *et*

al., 2009) and eucalyptus (Ferreira *et al.*, 2008). *Paenibacillus sp.* was isolated from seeds of eucalyptus (Ferreira *et al.*, 2008) and bean (López-López *et al.*, 2010). *Bacillus sp.* and *Pseudomonas sp.* were, for example, found in seeds from cactus (Puente *et al.*, 2009b), coffee (Vega *et al.*, 2006) and rapeseed (Granér *et al.*, 2003). *Micrococcus sp.* were isolated from seeds of rice (Okunishi *et al.*, 2005; Kaga *et al.*, 2009), coffee (Vega *et al.*, 2006), bean (López- López *et al.*, 2010) and rapeseed (Granér *et al.*, 2003). To our knowledge, *Sinorhizobium sp.*, *Rhizobium sp.*, *Bosea sp., Variovorax sp.* and *Acidovorax sp.* (except for *avenae ssp.* as a pathogen in rice (Song *et al.*, 2004)) have never been isolated from seeds. This suggest that, besides those endophytic taxa commonly found in seeds, there also exist seed endophytes that are specific for certain plant species, genera or even families.

The presence of *Staphylococcus sp.* is often thought to be the result of contamination but Prithiviraj *et al.* (2005) demonstrated that *Staphylococcus sp.* is also able to infect *A. thaliana*. Moreover, previous studies reported the isolation of *Staphylococcus sp.* from plant tissues such as poplar (*Populus trichocarpa*) (Porteous-Moore *et al.*, 2006) and sugarcane (*Saccharum officinarum*) (Velazquez *et al.*, 2008) and from seeds of cactus (Puente *et al.*, 2009b) and bean (López- López *et al.*, 2010). Due to the fact that 16S rDNA based identification usually does not provide information on the strain level, the presence of other potentially pathogenic strains among the genera present in the seeds can not be excluded.

When plants are exposed to 2 µM Cd for several generations, some of the genera present appear to be contaminant-related: *Aeromicrobium*, *Paenibacillus* and *Bosea* were only isolated from Cd seeds but were at no stage recovered from control seeds. These findings were confirmed when comparing the results of the total bacterial community from generation 12 and 14 for control and Cd seeds: *Cellulomonas*, *Mycobacterium*, *Aeromicrobium*, *Nocardioides*, *Pseudonocardia*, *Flavobacterium*, *Devosia* and *Pseudolabrys* were only found in Cd seeds and not in control seeds (Table 4.2). The indicator species analysis, which was performed on the full community matrix and not only on the top 10 genus-level OTUs, also revealed *Aeromicrobium* and *Pseudonocardia* as indicator species in Cd seeds, in addition to unclassified Bacteroidetes and *Acetobacteraceae* (Table 4.4). No indicator species were found for control seeds

from generation 12 and 14. This suggests a correlation between the presence of Cd and the composition of the endophytic community present in seeds. This could be a consequence of different abilities of the individual strains to adapt to the presence of increased Cd concentrations in the plant. Similar results were reported by Frostegård *et al.* (1993): they indicated that a correlation exists between changes in the community tolerance pattern and changes in the community species composition after metal contamination of the soil. However, in consecutive generations it is not always the same cultivable genera that are present in Cd seeds but absent in control seeds. Together with the fact that *Rhizobium* appeared to be the only genus transferred from one generation to the next, this suggests that transfer of seed endophytes to the next generation of seeds is not so much based on the genera that are present but more on their phenotypic characteristics.

Table 4.4: Indicator species of Cd seeds. Uc = unclassified.

OTU	Indicator value	p-value	Relative abundance (%)
Aeromicrobium	1	0.04	1.1
Pseudonocardia	1	0.04	1.4
Uc Bacteroidetes	1	0.04	2.2
Uc *Acetobacteraceae*	1	0.04	1.1

Also concerning the **phenotypic** characteristics, differences were observed between the bacteria isolated from control seeds and those isolated from Cd seeds from generation 8 (Table 4.5).

To survive under conditions with excess metal exposure, bacteria have evolved several mechanisms to cope with toxic metals. These include the efflux of metal ions, accumulation and complexation of the metal ions inside the cell and reduction of metal ions to a less toxic state (Nies, 1999). In control seeds 64.56% of all bacteria were tolerant to 0.4 mM Cd. This number decreased to 53.22% when they were exposed to 0.8 mM Cd. In Cd seeds, 76.58% of the cultivable bacteria were tolerant to 0.4 mM Cd and approximately the same number was tolerant to 0.8 mM Cd. These mechanisms not only protect the bacteria but also their host plant against high concentrations of toxic metals, since metal sequestration by the endophytes leads to a decreased internal availability of the metals and by consequence a lowered phytotoxicity (Lodewyckx *et al.*, 2001a; Weyens *et al.*, 2009b). The latter allows the plant to

accumulate higher amounts of metals without experiencing toxicity. For instance, after inoculation of *Lupinus luteus* grown on a nickel-enriched substrate with the engineered nickel resistant bacterium *Burkholderia cepacia* L.S.2.4::nccnre, nickel concentrations in the roots increased significantly (Lodewyckx *et al.*, 2001a). Díaz-Raviña and Bååth (1996) demonstrated that the bacterial community in experimentally polluted soil was tolerant to higher levels of metals than those from unpolluted environments. This was due to the elimination of sensitive species, selection of bacteria with metal tolerance abilities and adaptation of bacteria due to physiological or genetic changes. This could explain the higher metal tolerance found in endophytes from Cd seeds compared to control seeds (Table 4.5). The presence of one metal not only increases tolerance to that specific metal but can also influence tolerance to other metals (Díaz-Raviña *et al.*, 1994). The development of such multiple tolerances was also observed in the bacteria isolated from the Cd seeds: Zn and Cu tolerance were higher in endophytes from Cd seeds than in those from control seeds (68.05% vs. 53.73% and 66.7% vs. 54.44% respectively), even though these metals were not present at increased concentrations (Table 4.5).

There exist also other bacterial traits that can alleviate metal stress for the bacteria themselves as well as for their host plant. Bacterial siderophore production, for example, can be important to obtain sufficient Fe (Dimpka *et al.*, 2008). Metal contamination of the soil is often associated with Fe deficiency in a wide range of plant species due to the competition between metal ions and Fe for uptake by siderophores (Wallace *et al.*, 1992). Production of siderophores was more frequently found in endophytes from control seeds (24.8%) compared to Cd seeds (4.5%). Mechanisms providing Fe to the plant do not seem to be essential during that stage as seeds probably contain sufficient Fe for the development of 4- to 10-day old seedlings (Burd *et al.*, 1998).

Also production of IAA was more frequently found in endophytes from control seeds (40.1%) than in endophytes from Cd seeds (12.5%). This is in contrast with the findings of Dell'Amico *et al.* (2008) concerning rhizobacteria of *Brassica napus*: they noticed that bacterial IAA production is enhanced in the presence of Cd. IAA can stimulate plant cell proliferation and elongation (Glick *et al.*, 1998). It can promote root growth resulting in a larger root surface which has positive effects on water and nutrient uptake (Patten and Glick, 2002).

Also, the release of organic acids by bacteria can lead to an improved plant growth due to the solubilization of inorganic phosphates (Dimpka *et al.*, 2009b). Nevertheless, production of organic acids was more frequently found in endophytes from control seeds compared to Cd seeds (10.28% vs. 3.64%), suggesting that it is probably of less importance for seed development and seedling establishment in the presence of Cd.

On the other hand, bacterial ACC deaminase activity appeared to be of major importance in the early stages of development as it was almost 2.5 times more present in endophytes from Cd seeds compared to control seeds (86.11% vs. 36.97%). Also Timmusk *et al.* (2011) observed that areas with stressful plant growth conditions contained significantly higher numbers of ACC deaminase producing bacterial strains compared to areas with better growth conditions. Glick *et al.* (1998) demonstrated that bacterial ACC deaminase activity may lower the levels of stress ethylene in the plant thereby allowing the plant to develop longer roots and thus establish itself better during the early stages of growth. They also mentioned that ACC deaminase-producing bacteria become ineffective for promoting root growth if they are added to the seeds more than 1 day after imbibition. If ACC deaminase is not present prior to the induction of ACC oxidase during imbibition, the ratio of ACC deaminase to ACC oxidase becomes less favorable to the lowering of ACC levels and hence ethylene production (Glick *et al.*, 1998). In addition, plants or seeds that are provided with ACC deaminase-producing bacteria were reported to be more resistant to the deleterious effects of stress ethylene that is synthesized in harmful conditions such as the presence of toxic metals (Burd *et al.*, 1998; Arteca and Arteca, 2007; Dell'Amico *et al.*, 2008).

Some genera displayed certain characteristics in very high numbers (95-100%) regardless of whether they were present in control seeds or Cd seeds. For example, *Methylobacterium sp.* and *Sphingomonas sp.* showed high metal tolerance in both types of seeds (underlined in table 4.5). Other genera displayed a certain characteristic only in high numbers (95-100%) if they were isolated from Cd seeds. For example, *Bacillus sp.* showed clearly increased Cd-tolerance, production of siderophores, IAA and organic acids, and ACC deaminase activity in Cd seeds compared to control seeds (dotted line in table 4.5).

It appears that some bacterial genera display specific phenotypes only under Cd-stress. During the colonization of the plant, this could result in a selective advantage over other bacteria present in the environment that do not possess these characteristics. The selective advantage is even greater when these bacterial phenotypes are already present in the seeds because the earlier in the plant's development a bacterium can be established, the better the chances that it might out-compete others (Dimpka *et al.*, 2009b).

Table 4.5: Percentages of genera isolated from control and Cd seeds that display a certain characteristic. Sid = siderophores, OA = organic acids, ACCD = 1-aminocyclopropane-1-carboxylic acid deaminase. Percentages reflect the number of bacteria belonging to a certain genus/total community that show a certain phenotypic characteristic relative to the total number of bacteria belonging to this genus/total community. Underlined values are referred to in the discussion.

Genus	Accession no.	0.4 mM Cd	0.8 mM Cd	0.6 mM Zn	0.4 mM Cu	Sid	IAA	OA	ACCD
				Control seeds					
Methylobacterium	GU294332 EF126750	97.1	97.1	97.1	97.1	42.4	22.2	22.7	28.9
Sinorhizobium	HM011059	5.6	5.6	5.6	5.6	5.7	9.9	13.1	64.9
Micrococcus	AJ717369	0	0	0	0	0.37	0.37	0	1.5
Bacillus	AB245378 EU221335	86.7	12.8	14.2	12.8	87.0	12.8	73.9	0.31
Staphylococcus	AM945546 HM355720	51.1	51.1	51.1	51.1	0	0	100	0
Variovorax	EU341214 AY512635	75.1	75.1	75.1	75.1	75.1	75.1	75.1	0
Rhizobium	EU256433 AB453869 FJ719352 EF437256 HM032840	36.7	12.3	12.5	12.5	32.8	68.4	5.3	3.1
Sphingomonas	HM629444 AY563441	96.4	96.4	97.6	99.8	7.7	22.4	3.1	79.6
Acidovorax	AM989684 HM027578	99.2	99.2	100	99.2	0	33.1	0	0
Total		**64.6**	**53.2**	**53.7**	**54.4**	**24.8**	**40.1**	**10.3**	**37.0**

Table 4.5: continued

Genus	Accession no.	0.4 mM Cd	0.8 mM Cd	0.6 mM Zn	0.4 mM Cu	Sid	IAA	OA	ACCD
				Cd seeds					
Methylobacterium	GU294332	100	100	100	100	20.0	40.0	0	100
Bacillus	EU888579	100	100	0	0	100	100	100	100
Staphylococcus	AJ717375 HM355667 FJ613577	14.9	13.2	13.2	13.2	0	10.3	13.2	1.8
Variovorax	EU341214	0	0	0	0	0	0	0	93.0
Rhizobium	EU256433 AB453869 FJ719352	23.9	21.7	11.1	0.05	36.0	98.7	22.7	26.1
Sphingomonas	AY563441	99.9	99.9	100	99.9	0.05	0.40	0.02	97.6
Acidovorax	AM989684 HM027578	93.4	93.1	19.1	19.3	0	0.34	6.3	93.4
Paenibacillus	AJ297715	100	100	100	100	0	0	0	0
Bosea	FJ774000	98.8	0	0	49.4	0.59	0	0	9.9
Pseudomonas	AY236959	100	0	0	100	100	0	0	100
Total		**76.6**	**76.1**	**68.1**	**66.7**	**4.5**	**12.5**	**3.6**	**86.1**

Regardless from which generation or seed type the cultivable endophytes were derived, a large fraction of the community was able to produce IAA (Table 4.6). This indicates that certain plant growth promoting characteristics are important to the plant and thus are transferred to the next generation. ACC deaminase activity was abundant in Cd seeds of all generations, while it occured in varying amounts in control seeds (Table 4.6). This indicates that this is an important trait to cope with Cd stress. The percentage of the bacterial community tolerant to 0.4 mM Cd remained high, between 59.3% and 96.2%, over generations in Cd seeds, but decreased from 64.6% to 17.7% in control seeds (Table 4.6), again indicating that certain characteristics are only transferred to the next generation if they are useful to the plant. The presence of selected endophytic communities in seedlings makes rapid adaptation to environmental conditions possible by stimulating different beneficial functions (Hardoim *et al.*, 2012). Johnston-Monje *et al.* (2014) also observed that the bacterial traits beneficial to young maize plants are not derived from uptake of soil bacteria but rather from bacteria supplied by vertical transmission.

Some additional phenotypic tests were performed on the seed endophytes selected for the plant growth promotion assay (chapter 7). In order to survive and proliferate during germination, seed endophytes must be able to use the nutrients present in the seed. As phytate is the main storage form of P in seeds (López-López *et al.*, 2010), it is not surprising that all 10 selected seed endophytes were able to mineralize phytate. During plant development, extracellular phytase activity can also contribute to plant growth promotion in case of P limitation (Idriss *et al.*, 2002). Moreover, the presence of bacterial phytase in the rhizosphere can lower chelate-forming phytate, which binds nutritionally important minerals (Reddy *et al.*, 1989). Another plant nutrient that generally is limiting is nitrogen. Remarkably, 2 of the selected *Rhizobium sp.* and 3 of the selected *Sphingomonas sp.* were able to fix N_2. Although N_2 fixation is a typical feature associated with leguminous plants, a number of non-leguminous plants are also recognized to possess the ability to fix N_2 through exogenous or endogenous symbiosis with N_2-fixing bacteria (Trinick, 1973; Boddey *et al.*, 1995; Gopalaswamy and Kannaiyan, 2000). The transmission of this ability to the next generation via seeds further underscores the importance

of N_2 fixation in non-leguminous plants. None of the selected endophytes showed acetoin production or phosphate solubilization capacities.

Table 4.6: Percentages of the cultivable bacterial communities isolated from control and Cd seeds after 8, 12 and 14 generations of plant growth that display a certain phenotypic characteristic. IAA = indole-3-acetic acid, OA = organic acids, SID = siderophores, ACCD = 1-aminocyclopropane-1-carboxylate deaminase. Co = control seeds, Cd = Cd seeds.

Generation	**8**		**12**		**14**	
Seed type	**Co**	**Cd**	**Co**	**Cd**	**Co**	**Cd**
0.4 mM Cd	64.6	76.6	8.0	59.3	17.7	96.2
IAA	40.1	12.5	100	98.9	86.1	97.1
ACCD	37.0	86.1	12.0	98.8	100	97.1

4.2 Conclusion

We established that the standard sterilization protocol for *A. thaliana* seeds is too harsh. It ensures a profound sterilization of the surface, but at the same time destroys the bacterial seed endophytes. Since most of the strains described here display growth-promoting characteristics, it is reasonable to hypothesize that their colonization of seeds is beneficial for germination and/or seedling development, because it ensures the presence of potentially important bacteria at the time of germination and further growth. Moreover, it appeared that the selection of endophytes for transfer to the next generation is not absolutely random. It seems, at least in part, based on the bacterial traits and the environmental stressors experienced by the parental plant. Plants may enrich specific endophytic bacterial genotypes in response to soil contamination via an as-yet-unknown plant process or the numbers of certain genotypes may increase due to contaminant flux through the plant (Siciliano *et al.*, 2001).

The production of seeds on a bacteria-poor substrate reduced the size, diversity and richness of the seed endophytic community. Based on the composition of the cultivable and total communities, *Rhizobium* and *Pseudomonas* belong to the core microbiota present in *A. thaliana* seeds as they were both very predominant in control and Cd seeds of all generations. The conservation of certain species or their associated phenotypic abilities could reflect common needs of *A. thaliana* during germination, growth and development, and pathogen defense (Nelson, 2004).

Our data indicate that the seed endophytic community of *A. thaliana* seeds is composed of some bacterial genera that are tightly associated with these seeds, while others are specialized endophytes that are selectively taken up from the environment (Johnston-Monje *et al.*, 2014). For example, IAA-producing endophytes were abundant in both seed types studied, while Cd-tolerant endophytes were mainly recruited by seeds from Cd-exposed plants. This indicates that certain endophyte associations might be essential to meet environment-specific host needs (Lundberg *et al.*, 2012).

CHAPTER 5 The effect of Cd and Ni on the cultivable seed endophytic community of *Agrostis capillaris*

Truyens S, Jambon I, Croes S, Janssen J, Weyens N, Mench M, Carleer R, Cuypers A, Vangronsveld J. 2014. The effect of long-term Cd and Ni exposure on seed endophytes of Agrostis capillaris and their potential application in phytoremediation of metal-contaminated soils. International Journal of Phytoremediation 16(7-8): 643-659.

To compare the effects of the lab-induced Cd selection pressure found in *Arabidopsis thaliana* (chapter 4) with a field situation, the cultivable seed endophytic communities of *Agrostis capillaris* growing on a long-term Cd/Ni contaminated plot and a control plot were isolated. The composition of these communities and their associated phenotypic characteristics were determined.

5.1 Results

5.1.1 Optimization of the sterilization protocol

Different seed sterilization protocols were tested in order to determine a protocol that delivers the highest number of cfu.g^{-1} seed and as many morphologically distinct colony types, while still ensuring a sterile seed surface (Table 5.1). The optimal protocol for *A. capillaris* seed sterilization appeared to be: no exposure to ethanol and 0.5 min rinsing in 0.05% NaClO solution.

Table 5.1: Overview of the different sterilization protocols and their effect on the sterility of the seed surface, cfu.g^{-1} seed and number of morphologically distinct colony types that could be isolated.

70% EtOH	Time in NaClO (min)	Strength NaClO (%)	Seed surface	Cfu.g^{-1} seed	Morphologically distinct colony types
Yes	1	0.1	Not sterile	0	-
		0.05	Sterile	144	1
No	1	0.1	Sterile	64269	1
		0.01	Not sterile	6394139	3
		0.05	Not sterile	21451	2
	0.5	0.05	Sterile	6251722	3

5.1.2 Isolation of seed endophytes

Isolation of the cultivable seed endophytes resulted in 7.5 x 10^6 cfu.g^{-1} for seeds collected on the control plot (control seeds) and 1 x 10^6 cfu.g^{-1} for seeds from the Cd/Ni-contaminated plot (Cd/Ni seeds). The diversity of the cultivable bacterial isolates was similar for both seed types. *Pantoea sp.* was the most abundant genus among the isolates obtained from both seed types: 40% and 51% in control and Cd/Ni seeds respectively (Table 5.2). Also *Lysinibacillus sp.* was present in high amounts in both control (27%) and Cd/Ni seeds (33%). The remainder of the cultivable endophytes isolated from control seeds consisted of *Pseudomonas sp.* (23%), *Bacillus sp.* (9%) and *Brevibacillus sp.* (1%) (Table 5.2). *Bacillus sp.* and *Brevibacillus sp.* were also present among the isolates from Cd/Ni seeds, although in slightly different amounts (respectively 6% and 10%), while *Pseudomonas sp.* was not isolated from Cd/Ni seeds (Table 5.2). The 16S rDNA-based identification procedure was confirmed with a UPGMA tree. Clustering of the same genera confirmed the reliability of the identification procedure (results not shown).

Table 5.2: Number of isolates used for analysis, relative abundance (RA) compared to the total cultivable community, percentage sequence identity with the 16S rDNA of the reference strain and accession number in the EMBL database of the genera isolated from control and Cd/Ni seeds.

Genus	Number of isolates (RA)		% identity (accession number)	
	Control	Cd/Ni	Control	Cd/Ni
Pantoea	17 (40%)	4 (51%)	0.994 (FJ357836)	0.949 (DQ365569)
Lysinibacillus	2 (27%)	1 (33%)	1.000 (EF472269)	1.000 (EF472269)
Bacillus	1 (9%)	6 (10%)	1.000 (AY124766)	0.994 (AB508884)
Brevibacillus	1 (1%)	14 (6%)	0.997 (AB215101)	0986 (AB215101)
Pseudomonas	4 (23%)	-	0.831 (DQ813308)	-

Isolated seed endophytes were tested for ACC deaminase activity, production of organic acids, siderophores, IAA and acetoin, ability to fix N_2 and to solubilize phosphates, and their tolerance to Cd, Ni, Zn and Cu (Table 5.3). The most distinctive characteristics among isolates obtained from Cd/Ni seeds were ACC deaminase activity (78.4%), production of siderophores (42.4%), IAA (82.6%) and acetoin (43.4%), and solubilization of phosphates (95.2%). Acetoin production and phosphate solubilization were also abundant in endophytes isolated from control seeds (57.3% and 88.8%), while ACC deaminase activity and siderophore and IAA production were strikingly lower (27.6%, 0.6% and

32.2%). Organic acid production and N_2 fixation were more frequently found in endophytes obtained from control seeds than from Cd/Ni seeds (16.4% vs. 0% and 32.8% vs. 3.7% respectively).

Although mainly the same genera were identified in control and Cd/Ni seeds, their phenotypic characteristics differed widely. Several genera showed a certain characteristic for 80% or more of the strains in one seed type, while they showed that same characteristic for 5% or less in the other seed type. For example, all *Lysinibacillus* strains isolated from Cd/Ni seeds exhibited IAA production, while none of the *Lysinibacillus* strains from control seeds possessed this capacity (examples are indicated with a full line in table 5.3). Other characteristics were present in comparable amounts whether the genus was isolated from control or from Cd/Ni seeds. For example, *Lysinibacillus sp.* showed 96.6% to 100% ACC deaminase activity irrespective of which seed type it was isolated from (examples are indicated with a dotted line in table 5.3).

Surprising was the high percentage of endophytes obtained from control seeds (99.7%) that were tolerant to 0.2 mM Cd. In Cd/Ni seeds only 2.7% of the isolated endophytes showed tolerance to this Cd exposure. In control seeds, only *Lysinibacillus sp.* showed almost no Cd tolerance, while 4 other genera isolated from these seeds showed 100% tolerance to that Cd concentration. However, the number of isolated strains tolerant to 0.4 mM Cd was much lower: only 5.8% in control seeds and even 0% in Cd/Ni seeds. Also Zn tolerance was remarkably higher in control seeds compared to Cd/Ni seeds (40.8% vs. 8.9%). Cu tolerance was only detected in *Bacillus sp.* from Cd/Ni seeds: 73.1% of the *Bacillus* isolates were Cu-tolerant. None of the endophytes from both seed types showed tolerance to the Ni concentrations tested. Although metal tolerance was generally low in the bacterial isolates from Cd/Ni seeds, *Bacillus sp.* showed 61.4% Cd tolerance, 96.7% Zn tolerance and 73.1% Cu tolerance.

Table 5.3: Percentages of genera isolated from control and Cd/Ni seeds that display a certain phenotypic characteristic. ACC = 1-aminocyclopropane-1-carboxylic acid, IAA = indole-3-acetic acid. Percentages reflect the number of bacteria belonging to a certain genus/total community that show a certain phenotypic characteristic relative to the total number of bacteria belonging to this genus/total community. Underlined values are referred to in the text.

Genus	Accession no.	0.2 mM Cd	0.4 mM Cd	1 mM Ni	0.3 mM Zn	0.2 mM Cu	ACC deaminase	Organic acids	Siderophores	IAA	Acetoin	N_2 fixation	P solubilization
Control seeds													
Pantoea	AF130946 EU598802 AY924374 FJ357836	100	2.9	0	87.4	0	13.3	19.2	0.54	42.5	86.5	16.7	100
Pseudomonas	DQ813308 FJ662891 GQ900589	100	2.9	0	20.3	0	100	0	0	1.45	0	58.0	40.6
Brevibacillus	AB215101	100	0	0	100	0	0	0	100	100	100	0	100
Lysinibacillus	EF472269	3.4	0	0	0	0	96.6	0	0	0	3.4	96.6	100
Bacillus	AY124766	100	0	0	100	0	0	100	0	100	0	0	100
Total		**99.7**	**5.8**	**0**	**40.8**	**0**	**27.6**	**16.4**	**0.6**	**32.2**	**57.3**	**32.8**	**88.8**
Cd / Ni seeds													
Brevibacillus	AB215101	0	0	0	11.1	0	82.9	0	2.73	100	2.51	0.11	83.6
Pantoea	DQ365569 AF130946	0	0	0	0	0	66.3	0	66.9	66.9	66.3	0.58	99.4
Lysinibacillus	EF472269	0	0	0	0	0	100	0	0	100	0	0	100
Bacillus	DQ523500 AB508884	61.4	0	0	96.7	73.1	96.7	0	73.1	100	88.2	11.8	88.2
Total		**2.7**	**0**	**0**	**6.9**	**8.9**	**78.4**	**0**	**42.4**	**82.6**	**43.4**	**3.7**	**95.2**

The above mentioned results are not in line with the results obtained with *Arabidopsis thaliana* where more metal-tolerant endophytes were isolated from seeds of plants grown on the Cd-contaminated soil (Truyens *et al.*, 2013). Furthermore, these results do not meet the expectations based on 'bioavailable' amounts of Cd and Ni at the experimental plots (Table 5.4). For this purpose, we determined Cd and Ni concentrations in *A. capillaris* shoots and seeds from both the control and Cd/Ni-contaminated plot as these directly impact the endophyte metal tolerance. While total Cd and Ni concentrations were much higher in soils from the contaminated plot compared with the control plot, these differences appeared to be much lower when considering $Ca(NO_3)_2$-extractable amounts in soils and concentrations in plant shoots and seeds (Table 5.4).

Table 5.4: Cd and Ni content of soil and *A. capillaris* shoot and seed samples from both the control and Cd/Ni-contaminated site. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ compared to the same metal on the control plot.

Metal	Plot	Soil (total)	Soil ($Ca(NO_3)_2$-extractable)	Shoot (total)	Seed (total)
Cd ($mg.kg^{-1}$)	Control	2.94 ± 1.1	0.67 ± 0.018	0.77 ± 0.065	< 0.25
	Cd/Ni	82.4 ± 32.2***	3.90 ± 0.17***	4.48 ± 1.44**	3.90 ± 0.29***
Ni ($mg.kg^{-1}$)	Control	6.75 ± 1.53	1.02 ± 0.040	6.89 ± 0.23	10.20 ± 3.81
	Cd/Ni	160 ± 49.1***	8.05 ± 0.11***	33.5 ± 1.1*	24.12 ± 3.92

5.2 Discussion

The number of cultivable endophytes isolated from *A. capillaris* seeds was on average 10^6 $cfu.g^{-1}$ seed. This is higher than expected as it is generally reported that the number of cultivable endophytes decreases from the rhizosphere to the roots, to stems and leaves, and reaches 10^2-10^3 $cfu.g^{-1}$ in seeds (Benizri *et al.*, 2001; Hallmann, 2001). This might be due to the fact that the isolation procedure was carefully optimized and adapted for *A. capillaris* seeds. Especially the minimal strength of the NaClO solution (0.05%) could preserve the bacteria in the seed interior, while at the same time ensuring a sterile seed surface. The number of cfu isolated from Cd/Ni seeds was 7.5 times lower compared to the control seeds, which suggests a reduction in bacterial abundance after long-term metal exposure (Chen *et al.*, 2014).

To our knowledge, this was the first time that *Brevibacillus sp.* was isolated from seeds. *Lysinibacillus sp.* was only isolated from seeds of rapeseed (Croes,

unpublished results). *Pseudomonas sp.* were already isolated from seeds of rapeseed (Granér *et al.*, 2003), spruce (Cankar *et al.*, 2005), coffee (Vega *et al.*, 2006), cactus (Puente *et al.*, 2009b), ash (Donnarumma *et al.*, 2010) and tobacco (Mastretta *et al.*, 2009). Also *Bacillus sp.* were found in seeds of rapeseed (Granér *et al.*, 2003), coffee (Vega *et al.*, 2006) and ash (Donnarumma *et al.*, 2010). Moreover, *Bacillus sp.* have been isolated from seeds of rice (Okunishi *et al.*, 2005; Mano *et al.*, 2006; Kaga *et al.*, 2009), eucalyptus (Ferreira *et al.*, 2008), bean (López-López *et al.*, 2010), grapevine (Compant *et al.*, 2011) and rapeseed (Croes, unpublished results). *Pantoea sp.* has been isolated from seeds of rapeseed (Granér *et al.*, 2003), rice (Okunishi *et al.*, 2005; Kaga *et al.*, 2009), coffee (Vega *et al.*, 2006) and ash (Donnarumma *et al.*, 2010).

Most of the genera isolated from *A. capillaris* seeds occurred in both control and Cd/Ni seeds. Long *et al.* (2008) suggested 2 possible scenarios for the recruitment of endophytes: (1) bacteria with general growth promoting characteristics that are readily recruited by all plant species and (2) bacteria, once recruited by a particular host, that undergo specific adaptations which result in a finely tuned mutualism. In our case, obviously fine-tuning of phenotypic characteristics of the bacteria occurred and was induced by the presence or absence of Cd and Ni in the soil. Further, *Pseudomonas sp.* occurred in control seeds but not in Cd/Ni seeds which could be related to a contaminant-dependent effect. Frostegård *et al.* (1993) indicated that a relationship exists between changes in the community tolerance pattern and changes in the community species composition due to metal contamination of the soil. However, we did not observe many changes in community composition (Table 5.2), but great differences in metal tolerance between seed endophytes from the control and Cd/Ni-contaminated plot (Table 5.3).

Surprisingly, endophytes from control seeds displayed much more tolerance to 0.2 mM Cd than endophytes from Cd/Ni seeds (99.7% vs. 2.7%), although this difference was lower at higher Cd exposure (5.8% vs. 0%). The excluder mechanisms reported to be developed by *A. capillaris* in response to toxic metal exposure could explain this observation (Symeonidis *et al.*, 1985; Dahmani-Muller *et al.*, 2000). *A. capillaris* is known as a pseudometallophyte, growing on both contaminated and non-contaminated soils, that develops metal exclusion

from aerial parts as a metal tolerance strategy (Turner and Marshall, 1972; Baker, 1987; Dahmani-Muller *et al.*, 2000). On the Cd/Ni contaminated soil, plants could have developed an excluder mechanism preventing the endophytes from being exposed to Cd. Consequently, these endophytes do not need to acquire Cd tolerance. By contrast, development of an excluder mechanism was not expected on the control plot since plants were exposed to low, for plants harmless amounts of Cd which gave the endophytes the opportunity to develop Cd tolerance. However, no evidence for an exclusion mechanism was found. On the contrary, the metal content of shoots from the Cd/Ni-contaminated plot is much higher than that of shoots from the control plot (Table 5.4). Moreover, a very good correlation was found between the $Ca(NO_3)_2$-extractable metals in the soils and the metal contents in the shoots (Table 5.4). Also the Cd content in the seeds was still higher for seeds from the contaminated plot compared to seeds from the control plot (Table 5.4). Mechanisms reducing internal 'bioavailability' of the root-to-shoot transported metals can be another explanation for this lower metal tolerance in endophytes associated with plants growing on the metal contaminated plot. It is possible that the plants growing on the contaminated plot developed mechanisms such as vacuolar compartmentalization and phytochelatine complexation (Vögeli-Lange and Wagner, 1996; Sanita di Toppi and Gabbrielli, 1999). Lodewyckx (2001b) also reported a lower metal tolerance for endophytes from roots of *Thlaspi caerulescens* growing on a heavily contaminated soil compared to those of *Brassica napus* growing on an uncontaminated garden soil.

Concerning the plant growth promoting characteristics, ACC deaminase activity was found in a major part of the isolated cultivable endophytes from Cd/Ni seeds. This might be due to the elevated Cd and Ni concentrations in the roots of plants growing on the contaminated soil. Bacterial ACC deaminase is normally present in the cell in low levels until it is induced. As its induction is a slow and complex process, bacterial ACC deaminase can lower harmful stress ethylene levels in the plant without affecting the small early peak of ethylene production that is important to activate plant defense responses (Glick and Stearns, 2011). By consequence, seed endophytes with ACC deaminase activity can allow the plant to establish itself better during the early stages of growth (Glick *et al.*, 1998). In addition, plants or seeds that are colonised by ACC deaminase-

producing bacteria were reported to be more resistant to the deleterious effects of stress ethylene that is synthesized in harmful conditions such as the presence of toxic metals (Burd *et al.*, 1998; Arteca and Arteca, 2007; Dell'Amico *et al.*, 2008). Beside ACC-deaminase activity, the production of siderophores and IAA was also increased in the endophytic strains from Cd/Ni seeds compared to control seeds. On metal-contaminated soils, siderophores are important for acquiring sufficient Fe, as metal contamination is often associated with Fe deficiency due to the competition between toxic metal ions and Fe for uptake (Wallace *et al.*, 1992; Dimpka *et al.*, 2008). Siderophores can also protect bacteria, and their host plant, against metal toxicity as they bind the metal ions in the extracellular medium and prevent their diffusion into the cell (Schalk *et al.*, 2011). IAA can promote root growth and induces proliferation and elongation of root hairs, which results in a larger root surface that positively influences water and nutrient uptake (Patten and Glick, 2002). Moreover, a high percentage of the endophytes isolated from Cd/Ni seeds were able to solubilize phosphate despite the absence of organic acid production. This implies that phosphate is solubilized through the secretion of extracellular phosphatases rather than the production of organic acids (Jorquera *et al.*, 2008). These endophytes can thus increase the phosphate uptake by the plant and hence stimulate its growth (Kim *et al.*, 1998; Gyaneshwar *et al.*, 2002). The above-mentioned findings are in line with results of a study on field grown *Brassica napus* where plants were grown from the same seed stock on both uncontaminated control and Cd/Zn contaminated field sites (Croes *et al.,* 2013). In this study, they found that the majority of Cd/Zn tolerant strains showing phosphate solubilization, N_2 fixation, IAA production and ACC deaminase activity were found in the rhizosphere and roots of plants growing on the Cd/Zn contaminated field. Also the seed endophytes from *Arabidopsis thaliana* showed high Cd tolerance, IAA production and ACC deaminase activity when plants were grown on a Cd contaminated soil (Truyens *et al.*, 2013; chapter 4).

5.3 Conclusion

Long-term growth on a Cd/Ni-contaminated field site induced changes in the seed endophytic community of *Agrostis capillaris*. These changes were mainly on

the level of community characteristics rather than community composition. The results obtained here are not completely in agreement with the results obtained with the lab-induced Cd selection pressure in *Arabidopsis thaliana* described in the previous chapter. While bacterial ACC deaminase activity appeared to be very important in coping with Cd stress in both *A. thaliana* and *A. capillaris*, Cd tolerance was found in most endophytes from Cd-exposed *A. thaliana* seeds but was almost absent in endophytes from Cd/Ni-exposed *A. capillaris* seeds. This can be attributed to the pseudometallophyte nature of *A. capillaris*: metal exclusion from aerial parts as well as metal compartmentalization and complexation prevent the development of metal tolerance mechanisms in endophytes present in *A. capillaris*.

CHAPTER 6 The effects of the growth substrate on cultivable and total endophytic communities of *Arabidopsis thaliana*

Truyens S, Beckers B, Thijs S, Weyens N, Cuypers A, Vangronsveld J. 201x. The effects of the growth substrate on cultivable and total endophytic communities of Arabidopsis thaliana. Submitted to Plant and Soil.

In this chapter, we studied the effect of 2 frequently used substrates for production of *Arabidopsis thaliana* seeds on the cultivable and total seed endophytic communities of this model plant. Bacteria present in sand (bacteria-poor) and a mixture of sand and potting soil (bacteria-rich) were identified and compared with the endophytes present in the seeds that were harvested from plants grown on these substrate types. Furthermore, endophytic communities of plants grown from these seeds were compared to determine if the substrate type still has an effect on the establishment of the plant bacterial community.

6.1 Results and discussion

The total bacterial communities of soil, seed, radicle and leaf samples were determined using 454 pyrosequencing. Sequencing of the 2 amplicon libraries generated a total of 283146 reads with an average read length of 403 bp. After quality trimming and assigning the reads to the different samples, a total of 145526 high quality reads remained. For the 22 samples (soil, seeds, radicles and leaves) together 93202 high quality bacterial reads were finally recovered. The rarefaction curves indicated that subsampling at 2000 sequences per sample was sufficient (Fig. 6.1). This was also confirmed by the Good's coverage estimator, which was between 95.8% and 99.9% for all samples (Table 6.1). The observed number of OTUs, the inverse Simpson diversity index and the Chao richness estimator were generally higher in potting soil and plant samples originating from potting soil (Table 6.1). These parameters were also higher for soil and leaf samples compared to seed and radicle samples (Table 6.1).

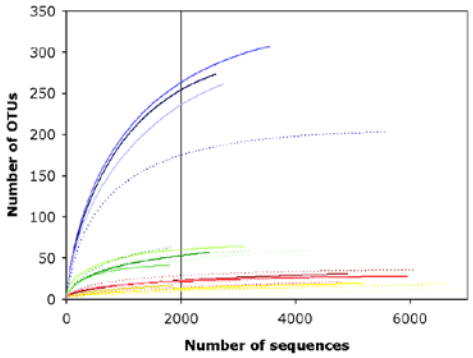

Figure 6.1: Rarefaction curves for soil (blue), seed (red), radicle (yellow), and leaf (green) samples. Dotted lines are samples from sand; full lines are samples from potting soil. The vertical line indicates the number of sequences (2000) subsampled from each sample for further analysis.

Table 6.1: Good's coverage estimator, number of OTUs (Sobs), inverse Simpson diversity index and Chao richness estimator at similarity level 97%. Values are based on 1000 subsamplings of 2000 sequences per sample. S = sand, PS = potting soil. ** = $p < 0.01$, *** = $p < 0.001$ compared to the same matrix but other substrate. a = $p < 0.05$, b = $p < 0.01$ and c = $p < 0.001$ compared to the same substrate but different matrix (indicated in capital letter: S = soil, L = leaf).

Matrix	Substrate	Coverage ± stdev	Sobs ± stdev	InvSimpson ± stdev	Chao ± stdev
Soil	S	97.9 ± 0.26	175.3 ± 4.15	12.3 ± 0.44	202.8 ± 11.4
	PS	95.8 ± 0.31	251.1 ± 4.49**	31.6 ± 0.78	312.4 ± 14.6**
Seed	S	99.6 ± 0.11	20.8 ± 1.92^{cSL}	1.7 ± 0.03^{cS}	29.4 ± 9.00^{cS,bL}
	PS	99.6 ± 0.10	22.6 ± 1.77 ^{cSL}	3.3 ± 0.05^{**,cL}	29.6 ± 7.48^{cSL}
Radicle	S	99.9 ± 0.08	9.4 ± 1.51 ^{cSL}	1.1 ± 0.01^{cSL}	13.8 ± 5.96^{cSL}
	PS	99.8 ± 0.07	12.4 ± 1.27 ^{cSL}	2.3 ± 0.04$^{***,\ cS,aL}$	16.5 ± 4.97^{cSL}
Leaf	S	99.2 ± 0.15	49.9 ± 2.37	2.2 ± 0.03	64.1 ± 9.48
	PS	99.3 ± 0.12	56.7 ± 1.75	9.4 ± 0.14**	69.0 ± 7.30

The higher microbial presence in potting soil compared to sand is expected since microbial growth in sand is usually resource-limited and addition of organic matter rapidly induces growth (Wardle, 1992). Tiquia *et al.* (2002) found that soil amended with composted yard waste had a higher total microbial biomass than unamended soil. This was confirmed in our counts of the cultivable bacterial community: the total number of cultivable cfu.g^{-1} isolated from sand and potting soil were respectively 6.7 x 10^4 and 1.6 x 10^5 (Table 6.2). The higher number of cultivable bacteria present in potting soil also resulted in higher numbers of cfu isolated from seeds harvested from plants grown on potting soil compared to seeds harvested from sand-grown plants. After

emergence of the radicle this difference disappeared. Also community sizes of cultivable bacteria in leaves from both seed types were very similar (Table 6.2).

Table 6.2: Amounts of cultivable bacteria (cfu.g^{-1}) for the different matrices. ** = p < 0.01 compared to the same matrix but other substrate.

	Cfu.g^{-1}	
	Sand	Potting soil
Soil	6.7×10^4	1.6×10^5
Seeds	5.6×10^5	$3.4 \times 10^{7**}$
Radicles	6.1×10^7	2.3×10^7
Leaves	4.0×10^4	2.9×10^4

The composition of the cultivable bacterial community isolated from sand was less diverse compared to the community isolated from potting soil. It consisted mainly of *Bacillus* (66%) and *Micrococcus* (33.3%) with a minor share of *Staphylococcus* (0.7%) (Fig. 6.2A). The bacterial community present in potting soil was also dominated by *Bacillus* (23.1%), but in addition also *Streptomyces* (22.1%) and *Cellulomonas* (17.2%) were abundant (Fig. 6.2A). Looking at the total bacterial community present in soil, many more genera could be detected as expected based on the results of the cultivable community, especially in sand samples (Fig. 6.2B).

Although the cultivable bacterial community in sand is very different from that in potting soil, the communities isolated from seeds harvested on these 2 substrate types were highly similar (Fig. 6.2A). *Pseudomonas* and *Rhizobium* comprised a large part of the cultivable community in both seed types: respectively 58.9% and 41.1% in seeds harvested from plants grown on sand and 43.8% and 16.1% in seeds harvested from potting soil-grown plants. In seeds harvested on potting soil also *Stenotrophomonas* (36.9%) and *Variovorax* (3.2%) were found. Moreover, the total bacterial communities of seeds were very different from the soil ones and were dominated by the same genera as the cultivable bacterial communities (Fig. 6.2A+B). From seeds harvested from sand- and potting soil-grown plants respectively 11.9% and 7.5% of the total bacterial community was composed of *Rhizobium* and respectively 5.4% and 6.0% of *Pseudomonas*. Several other authors found that, although there is similarity between soil and rhizospheric bacterial communities, there is a disparity between the rhizospheric and endophytic communities (Gottel *et al.*, 2011; Lundberg *et al.*, 2012; Croes *et al.*, 2013). Also Green *et al.* (2006) found that root bacterial community

profiles differed significantly from those in the initial potting substrate. They suggested that persistence was a result of rhizosphere competence rather than abundance in the potting substrate. Rhizosphere competence of soil bacteria together with other plant characteristics could determine which bacteria will be able to colonize the plant and finally the seeds. Bulgarelli *et al.* (2013) suggested a 2-step selection model where plant root exudates drive a community shift in the rhizosphere, and where fine-tuning of the endosphere is accomplished by host-controlled mechanisms such as the innate immune system.

After germination, the bacterial community present in the radicle emerging from seeds harvested from sand-grown plants was similar to that of seeds harvested from plants growing on potting soil (Fig. 6.2A+B). Moreover, these communities were very similar to those isolated from the ungerminated seeds (Fig. 6.2A+B). In the cultivable community of the radicle from seeds harvested from plants grown on sand only *Pseudomonas* was found, while in the total community also *Rhizobium* (11.4%) was shown to be an important component. The cultivable community in the radicle emerging from germinating seeds harvested on potting soil consisted of *Pseudomonas* (37.2%), *Rhizobium* (42.8%) and *Stenotrophomonas* (20%). *Pseudomonas* (4.6%), *Rhizobium* (10.8%) and *Stenotrophomonas* (8.6%) were also found as the main constituents of the total bacterial community in this sample.

In contrast, the bacterial community present in the leaves of 3-week old plants was very different from the seed and radicle endophytic communities (Fig. 6.2A+B). Nevertheless, the bacterial communities isolated from leaves of plants grown from seeds harvested on sand and those harvested on potting soil were very similar. They had several cultivable bacterial genera in common: *Hydrocarboniphaga* (42% and 30%), *Pimelobacter* (20.4% and 20.6%), *Sphingobacteriaceae* (8.3% and 11.2%), *Variovorax* (5.6% and 4.9%), *Sphingopyxis* (4.6% and 4.4%), *Runella* (2.9% and 4.4%), *Pedobacter* (1.7% and 2.3%) and *Pseudomonas* (0.9% and 7.5%). *Hydrocarboniphaga* (1.9% and 2.4%), *Runella* (2.3% and 1.2%) and *Pseudomonas* (2.3% and 3.1%) were also important components of the total bacterial community. In addition, 8.8% of the total bacterial community in leaves from seeds harvested on sand was composed of *Varibaculum*. The above mentioned findings suggest that the major

part of the leaf endophytic community was derived from the environment while only a minor part was derived from the seeds. This is in accordance with the findings of Normander and Prosser (2000) where barley was mainly colonized by soil-borne and not seed-borne bacteria. Also Ringelberg *et al.* (2012) found a contribution of the growing medium to the mature plant endophytic community, although in addition to the contribution of seeds. Remarkably, *Rhizobium*, a diazotroph, was one of the most abundant genera in this study. Several other studies have indicated that *Rhizobium* is able to endophytically colonize plants thereby increasing seedling vigour and plant growth (Biswas *et al.*, 2000; Chi *et al.*, 2005; García-Fraile *et al.*, 2012). Also *Pseudomonas* was found to be a prominent member of the bacterial community, especially in seeds and radicles. *Pseudomonas* is abundant in nature and known to be able to increase plant host fitness (Mercado-Blanco and Bakker, 2007; Gómez-Lama Cabanás *et al.*, 2014; Kloepper *et al.*, 1980b).

Rhizobium, *Methylobacterium* and *Pseudomonas*, which are 3 of the top 13 most abundant genus-level OTUs from leaf samples, were also found to be part of the *A. thaliana* leaf community by Bodenhausen *et al.* (2013). Also the core bacterial community on the cuticle of *A. thaliana* ecotype Landsberg erecta bears resemblance to our leaf endophytic community: *Methylobacterium*, *Rhizobiales* and *Sphingomonadales* are part of both communities (Reisberg *et al.*, 2013). A large part of the core community of *A. thaliana* roots consists of members of the *Rhizobiaceae*, *Caulobacteriaceae*, *Pseudomonadaceae* and *Xanthomonadaceae* (Lundberg *et al.*, 2012; Bulgarelli *et al.*, 2012). Members of these families were also isolated from the radicles of our seedlings: *Rhizobium*, *Brevundimonas*, *Pseudomonas* and *Stenotrophomonas* (Fig. 6.2B).

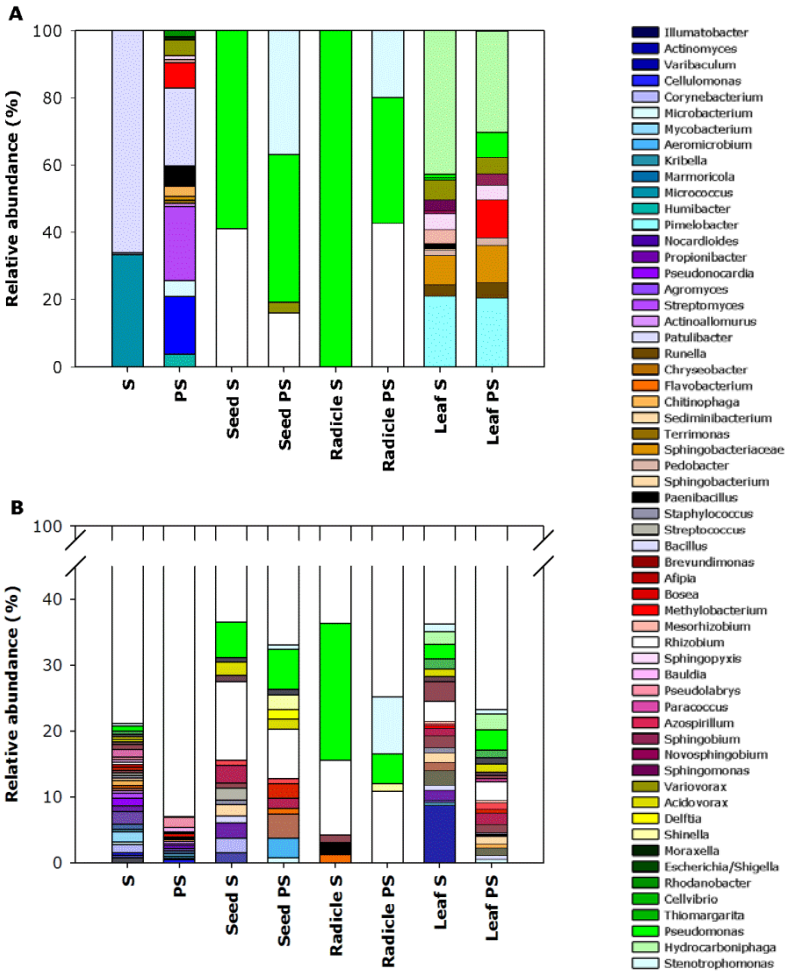

Figure 6.2: Relative abundances (%) of the isolated genera in each compartment (S = sand, PS = potting soil). A. Cultivable bacterial community. B. Total bacterial community: top 10 of most abundant genus-level OTUs of each sample are shown. The upper white part of the bars represents the remaining part of the community.

It appeared that for plants grown on the 2 different substrate types the seed and radicle endophytic communities are very similar, while the leaf endophytic community is again different. These observations were confirmed by the NMDS plot of the cultivable communities: the samples formed 4 groups based on their genera composition: (1) sand, (2) potting soil, (3) seeds and radicles from sand as well as potting soil, and (4) leaves from both sand and potting soil (Fig. 6.3A). The PERMANOVA also indicated that the highest amount of variation was

explained by the compartment from which the bacteria were isolated (R^2 = 0.56; p = 0.001) and not by the substrate type (R^2 = 0.06; p = 0.04). For the total bacterial community, 3 main groups were formed: (1) sand and potting soil, (2) seeds and radicles from both sand and potting soil, and (3) leaves from sand as well as potting soil (Fig. 6.3B). The PERMANOVA again indicated that most of the variation was explained by the compartment where the bacteria were isolated from (R^2 = 0.57; p = 0.001) and not by the substrate type (R^2 = 0.05; p= 0.03).

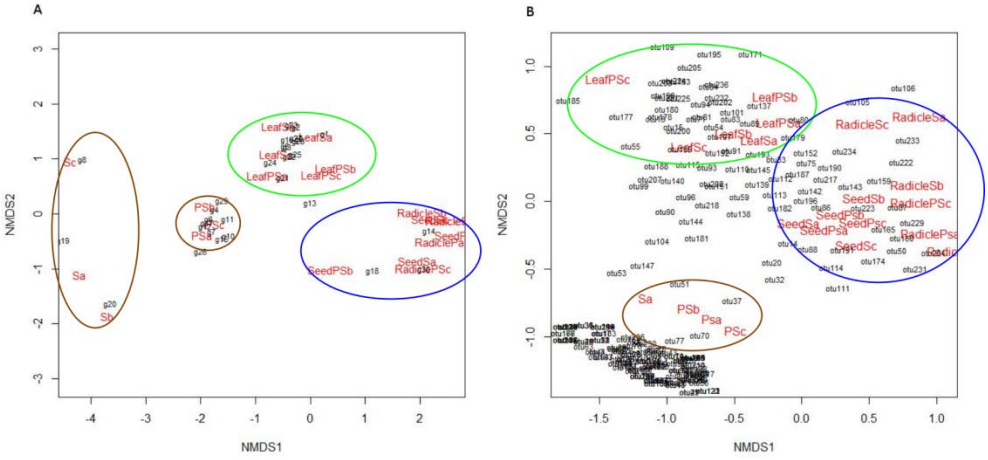

Figure 6.3: NMDS plot of the cultivable (A) and total (B) bacterial communities isolated from the different compartments. Brown = soil samples, blue = seed and radicle samples, green = leaf samples. S = sand, PS = potting soil. Each number on the NMDS plot corresponds to an isolated bacterial genus or OTU as indicated in table S6.1 and S6.2.

6.2 Conclusion

We demonstrated that 2 different substrate types, frequently used for the production of *A. thaliana* seeds and containing distinct bacterial communities, delivered *A. thaliana* plants with seeds containing very similar endophytic communities. This indicates that the plant possesses selection mechanisms determining which bacteria can become seed endophytes. Johnston-Monje *et al.* (2014) pointed out that the establishment of the bacterial endophytic community is not only based on passive entry from soil, but also on selective soil uptake and vertical transmission from one generation to the next. They demonstrated that maize plants are able to select similar bacteria from diverse

soils. Also Lundberg *et al.* (2012) showed that a limited number of bacterial taxa are responsible for the establishment of the endophytic microbiome of *A. thaliana* roots across diverse soils.

The bacterial endophytic communities present after radicle emergence were also very similar to one another as well as to the seed endophytic communities. In contrast, the communities isolated from leaves of plants grown from these seeds (on the same substrate) did not resemble the communities present in the seeds, but were nevertheless very similar to each other suggesting that they are mainly composed of bacteria originating from the environment.

Finally, a BLAST of the 16S rDNA sequences of the cultivable bacteria with the OTU sequences of the corresponding pyrosequencing sample showed that matches were found for all cultivable bacteria from seed and radicle samples. In leaf samples, matches (> 97% identity) were found for 14 out of 21 sequences. Five 16S rDNA sequences showed 88 to 94% identity with the corresponding OTU sequences. The 16S rDNA sequences of *Stenotrophomonas* and *Thiomargarita* isolated from leaves were not retrieved from the OTU sequence database of leaf samples. Our data indicate that isolation and identification of the cultivable endophytic bacteria gives a good estimate of the most important members of the total community: key members of the total endophytic community were also detected in the cultivable endophytic community. This is in accordance with the findings of Hodgson (2010) concerning fungal endophytes of forbs. She reported that in herbaceous plants, such as *Plantago lanceolate*, PCR methods followed by T-RFLP produced an identical number of OTUs as identified by culturing.

Supplementary data

Table S6.1: Genera of the cultivable community corresponding to the numbers used on the NMDS plot of figure 6.3A.

1	*Nocardioides*
2	*Thiomargarita*
3	*Pedobacter*
4	*Chitinophaga*
5	*Sphingobacteriaceae*
6	*Cellulomonas*
7	*Streptomyces*
8	*Micrococcus*
9	*Pimelobacter*
10	*Agromyces*
11	*Humibacter*
12	*Microbacterium*
13	*Variovorax*
14	*Pseudomonas*
15	*Moraxella*
16	*Hydrocarboniphaga*
17	*Rhodanobacter*
18	*Stenotrophomonas*
19	*Staphylococcus*
20	*Bacillus*
21	*Devosia*
22	*Mesorhizobium*
23	*Sphingomonas*
24	*Sphingopyxis*
25	*Sphingobium*
26	*Terrimonas*
27	*Novosphingobium*
28	*Runella*
29	*Paenibacillus*
30	*Rhizobium*
31	*Sphingobacterium*

Table S6.2: OTUs of the total community corresponding to the numbers used on the NMDS plot of figure 6.3B. Uc = unclassified.

1	Uc *Acidipilia*	120	*Anaerovorax*
2	Uc *Granulicella*	121	Uc *Gracilibacteriaceae*
3	Uc *Talmatobacter*	122	*Clostridium_XIVa*
4	Uc Acidobacteria_Gp1	123	*Roseburia*
5	Uc Acidobacteria_Gp2	124	Uc *Lachnospiraceae*
6	Uc *Bryobacter*	125	*Desulfosporosinus*
7	Uc Candidatus Solibacter	126	Uc *Peptococcaceae_1*
8	Uc Acidobacteria_Gp3	127	*Clostridium_XI*
9	*Holophaga*	128	*Clostridium_III*
10	Uc *Holophagaceae*	129	*Ethanoligenes*
11	Uc Acidobacteria	130	Uc *Ruminococcaceae*
12	*Illumatobacter*	131	Uc *Clostridiales*
13	Uc *Acidimicrobiaceae*	132	Uc Firmicutes
14	*Actinomyces*	133	*Ignavibacterium*
15	*Varibaculum*	134	*Nitrospira*
16	Uc *Actinomycetaceae*	135	Uc *Planctomycetaceae*
17	*Brevibacterium*	136	*Asticcacaulus*
18	*Cellulomonas*	137	*Brevundimonas*
19	Uc *Cellulomonadaceae*	138	*Phenylobacterium*
20	*Corynebacterium*	139	Uc *Caulobacteraceae*
21	*Turicella*	140	Uc *Hyphomonadaceae*
22	*Demequina*	141	*Aurantimonas*
23	*Brachybacterium*	142	*Afipia*
24	*Blastococcus*	143	*Bosea*
25	*Phycicoccus*	144	*Bradyrhizobium*
26	Uc *Intrasporangiaceae*	145	Uc *Bradyrhizobiaceae*
27	*Angustibacter*	146	*Cucumibacter*
28	*Quadrispaera*	147	*Devosia*
29	*Agrococcus*	148	*Hyphomicrobium*
30	*Agromyces*	149	*Pedomicrobium*
31	*Curtobacterium*	150	*Rhodomicrobium*
32	*Leifsonia*	151	Uc *Hyphomicrobiaceae*
33	*Microbacterium*	152	*Methylobacterium*
34	*Microcella*	153	*Microvirga*
35	*Rathayibacter*	154	*Aminobacter*
36	*Zimmermannella*	155	*Mesorhizobium*
37	Uc *Microbacteriaceae*	156	*Phyllobacterium*
38	*Arthrobacter*	157	Uc *Phyllobacteriaceae*
39	*Micrococcus*	158	*Kaistia*
40	*Rothia*	159	*Rhizobium*
41	Uc *Micrococcaceae*	160	Uc *Rhizobiaceae*
42	*Dactylosporangium*	161	*Bauldia*
43	Uc *Micromonosporaceae*	162	*Pseudolabrys*
44	*Mycobacterium*	163	*Xanthobacter*
45	Uc *Mycrobacteriaceae*	164	Uc *Xanthobacteraceae*
46	*Nakamurella*	165	Uc *Rhizobiales*
47	*Gordonia*	166	*Paracoccus*
48	*Nocardia*	167	Uc *Rhodobacteraceae*
49	*Pimelobacter*	168	*Acidocella*
50	Uc *Nocardioidaceae*	169	*Belnapia*
51	*Kribella*	170	Uc *Acetobacteraceae*
52	*Marmoricola*	171	*Azospirillum*
53	*Nocardioides*	172	*Dongia*
54	*Pimelobacter*	173	Uc *Rhodospirillaceae*
55	Uc *Nocadioidaceae*	174	Uc *Rhodospirillales*
56	Uc *Promicromonosporaceae*	175	*Porphyrobacter*
57	*Friedmanniella*	176	*Blastomonas*
58	*Microlunatus*	177	*Novosphingobium*
59	*Propionibacterium*	178	*Sphingobium*

60	Uc *Propionibacteriaceae*
61	*Actinomycetospora*
62	*Pseudonocardia*
63	Uc *Pseudonocardiaceae*
64	*Sporichthya*
65	*Streptomyces*
66	Uc *Streptomycetaceae*
67	*Microbispora*
68	*Actinoallomurus*
69	Uc *Thermomonosporaceae*
70	Uc *Actinomycetales*
71	Uc *Bifidobacteriaceae*
72	*Gaiella*
73	*Rubrobacter*
74	*Conexibaacter*
75	*Patulibacter*
76	Uc *Solirubrobacterales*
77	Uc Actinobacteria
78	Uc Actinobacteria
79	Uc *Armatimonadetes_Gp5*
80	*Dysgomonas*
81	*Prevotella*
82	*Adhaeribacter*
83	*Larkinella*
84	*Runella*
85	*Capnocytophaga*
86	*Chryseobacterium*
87	*Flavobacterium*
88	*Moheibacter*
89	Uc *Flavobacteriaceae*
90	*Chitinophaga*
91	*Sediminibacterium*
92	*Terrimonas*
93	Uc *Chitinophagaceae*
94	Uc *Sphingobacteriacae*
95	Uc Bacteroidetes
96	Uc *Saccharibacteria*
97	Uc *Parachlamydiaceae*
98	*Simkania*
99	Uc *Simkaniaceae*
100	Uc *Ktedonobacteriales*
101	*Deinococcus*
102	*Alicyclobacillus*
103	*Tumebacillus*
104	Uc *Bacillaceae_1*
105	*Paenibacullus*
106	Uc *Paenibacillaceae_1*
107	*Ammoniphilus*
108	*Chryseomicrobium*
109	*Bacillus*
110	*Staphylococcus*
111	Uc *Staphylococcacea*
112	Uc *Bacillales*
113	*Aerococcus*
114	*Streptococcus*
115	Uc *Bacilli*
116	*Clostridium_sensu_stricto*
117	*Oxobacter*
118	*Peptonipholus*
119	*Soehngenia*
179	*Sphingomonas*
180	Uc *Sphingomonadaceae*
181	Uc *Sphingomonadales*
182	Uc Alphaproteobacteria
183	*Bordetella*
184	Uc *Alicaligenaceae*
185	*Burkholderia*
186	*Limnobacter*
187	Uc *Burkholderiaceae*
188	*Aquabacterium*
189	Uc *Burkholderiaceae_incertae_sedis*
190	*Acidovorax*
191	*Delftia*
192	*Polaromonas*
193	Uc *Comamonadaceae*
194	*Massilia*
195	*Duganella*
196	Uc *Oxalobacteraceae*
197	Uc *Burkholderiales*
198	*Thiobacillus*
199	*Methylophilus*
200	Uc *Methylophilaceae*
201	*Leeia*
202	Uc *Neisseriaceae*
203	*Methyloversatilis*
204	*Shinella*
205	*Zoogloea*
206	Uc *Rhodocyclaceae*
207	Uc *Betaproteobacteria*
208	*Bdellovibrio*
209	*Geobacter*
210	*Cystobacter*
211	Uc *Nannocystaceae*
212	Uc *Polyangiaceae*
213	Uc *Myxococcales*
214	Uc Deltaproteobacteria
215	*Marinobacter*
216	*Rheinheimera*
217	*Escherichia/Shigella*
218	Uc *Enterobacteriaceae*
219	*Acinetobacter*
220	*Enhydrobacter*
221	*Cellvibrio*
222	*Pseudomonas*
223	Uc *Pseudomonadaceae*
224	*Hydrocarbinophaga*
225	*Nevskia*
226	Uc *Sinobacteraceae*
227	*Dokdonella*
228	*Lysobacter*
229	*Stenotrophomonas*
230	*Xanthomonas*
231	Uc *Xanthomonadaceae*
232	Uc *Xanthomonadales*
233	Uc Gammaproteobacteria
234	Uc Proteobacteria
235	Uc Subdivision 3
236	*Prosthecobacter*
237	Uc Verrucomicrobia

SECTION 2 Inoculation experiments

Abstract

Bacterial endophytes can possess plant growth promoting characteristics that can be exploited during phytoremediation of metal-contaminated soils. Inoculation with these endophytes can increase plant biomass and metal uptake from soil in order to increase phytoremediation efficiency.

We studied the effects of inoculation with seed endophytes isolated from *Arabidopsis thaliana* and *Agrostis capillaris* on plant growth and Cd uptake. Several promising seed endophytes were selected for inoculation based on the results of the phenotypic screening (chapters 4 and 5).

Inoculation of *A. thaliana* with its seed endophytes on vertical agar plates or in a hydroponic culture could lead to improved plant growth during Cd exposure, although results were not consistent (chapter 7). Therefore, we investigated several factors possibly involved in bacterial colonization and inoculation success (chapter 8). Different bacterial growth stages are associated with changes in motility, chemotactic behavior and pectinase activity which could influence the plant colonizing capacity of the inoculated endophytes.

Inoculation of hydroponically grown *A. capillaris* with its seed endophytes improved plant growth and/or increased Cd uptake (chapter 7). This can be advantageous for plant establishment and growth during phytostabilization and phytoextraction of metal-contaminated soils.

Seed endophytes seem to possess the potential to contribute to a more efficient phytostabilization and phytoextraction of metal-contaminated areas, but further optimization of the inoculation process is needed to obtain a more consistent and reproducible inoculation outcome.

CHAPTER 7 Plant growth promoting capacity of selected seed endophytes of *Arabidopsis thaliana* and *Agrostis capillaris*

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Truyens S, Jambon I, Croes S, Janssen J, Weyens N, Mench M, Carleer R, Cuypers A, Vangronsveld J. 2014. The effect of long-term Cd and Ni exposure on seed endophytes of Agrostis capillaris and their potential application in phytoremediation of metal-contaminated soils. International Journal of Phytoremediation 16(7-8): 643-659.

7.1 Introduction

As a consequence of both the growing population and industrial technology, large areas of our planet are contaminated with various toxic compounds (Glick, 2010). Many of the current remediation technologies include the removal of soil to landfill sites or contaminant extraction through chemical or physical methods. These technologies are rapid but costly and disturb the physical, chemical and biological properties of the soil. An alternative and sustainable approach could be phytoremediation: the use of plants and their associated microorganisms to remove, degrade or sequester hazardous substances from the environment (Cunningham and Berti, 1993).

The most critical metals contaminating soil and water systems are lead, zinc, cadmium, selenium, chromium, cobalt, copper, nickel and mercury (Glick, 2003). The sources of this contamination are the metal smelting industry, residues from metalliferous mining, combustion of fossil fuel, and waste incineration, as well as some pesticides and fertilizers used in agriculture (Vassilev *et al.*, 2004). For the removal of toxic metals either decontamination as well as stabilization strategies can be applied.

Phytoextraction involves the uptake of contaminants via the roots and translocation to the harvestable plant parts (Chaney, 1983). In addition to metal removal, these plants can produce biomass with an added economic value. The biomass itself can be used for the production of bioenergy or, in case the market value is high, metals recovered from the harvested biomass can be sold (Vassilev *et al.*, 2004). The concept of phytoextraction was initially based on the use of hyperaccumulating plants, which can take up and tolerate extraordinary levels of metals. The criterion of hyperaccumulation of Cd is 100 $mg.kg^{-1}$ in the aboveground dry mass (Reeves and Baker, 2000). Another approach is based on the use of high biomass-producing plants. According to Blaylock and Huang (2000) plants that yield 20 $tons.ha^{-1}.year^{-1}$ with a concentration of the targeted metal of about 1% of the harvestable dry mass are needed for successful phytoextraction. Metal availability, metal uptake and translocation, and phytotoxicity are the main limiting factors for the application of phytoextraction (Weyens *et al.*, 2009a). Plant-associated bacteria can be used to improve the efficiency of phytoextraction: they can produce siderophores which on the one hand deliver sufficient Fe to counteract the Fe depletion in the plant due to high levels of metals, and on the other hand can bind the toxic metals. (Glick, 2003; Dimpka *et al.*, 2009a). The latter not only diminishes phytotoxicity but also increases translocation of the metals to aboveground plant parts. Plant-associated bacteria can also promote plant growth, and by consequence metal uptake, by producing phytohormones or by alleviating metal-induced stress by degrading the ethylene precursor ACC (Glick, 2010). In addition, they can also possess or be equipped with metal tolerance systems that work according to one of these basic mechanisms: (1) active extrusion of the metal ion from the cell, (2) complexation by thiol-containing molecules or (3) reduction to a less toxic oxidation state (Nies, 1999). Burd *et al.* (1998) demonstrated that a Ni-resistant ACC deaminase-producing bacterium could decrease the toxicity of Ni to canola plants. Kuffner *et al.* (2010) observed increases in Cd and Zn accumulation in leaves of *Salix caprea* after inoculation with an endophytic *Microbacterium* strain. More recently, Ma *et al.* (2015) demonstrated that the hyperaccumulator *Sedum plumbizincicola* harbors metal-resistant endophytes that increase biomass production and metal uptake after inoculation.

Phytostabilization reduces the environmental risks associated with contaminated soils by decreasing metal bioavailability using a combination of plants and soil amendments (Vangronsveld *et al.*, 1995). The vegetative cover protects the soil from wind and water erosion and reduces water percolation through the soil in order to diminish leaching of the contaminant to the groundwater (Vangronsveld *et al.*, 1995). In addition, phytostabilization renders the site aesthetically pleasing and enhances biodiversity by restoring the local ecosystem (Vassilev *et al.*, 2004). Vásquez *et al.* (2006) used lupine plants for Cd phytostabilization as these crops decreased the soluble Cd fractions by binding the metal to root nodule cell walls and by increasing the acid soil pH, probably due to citrate excretion. Moreover, inoculation with beneficial plant-associated bacteria can help plants to cope with contaminants and increase the efficiency of phytostabilization. Grandlic *et al.* (2008) reported that plant growth-promoting bacteria enhanced the revegetation of metal-rich mine tailings and minimized the need for compost amendment.

Seed endophytes occupy a special niche among the plant-associated bacteria as they are transmitted from one plant generation to the next. Mastretta *et al.* (2009) already demonstrated that inoculation of tobacco with its seed endophytes improved plant growth and reduced metal phytotoxicity in case of Cd pollution. Kolbas *et al.* (2015) found that inoculation of sunflower with a seed extract of Cu-tolerant *Agrostis capillaris* increased shoot and root dry weight upon Cu exposure.

In this chapter, isolated seed endophytes with promising characteristics for plant growth promotion under metal-stressed conditions were tested in several inoculation experiments. *Arabidopsis thaliana* grown on vertical agar plates was inoculated with different seed endophytes in order to monitor root growth responses upon exposure to Cd. *A. thaliana* and *A. capillaris* were also grown in a hydroponic culture and inoculated with different seed endophytes to determine the effects on plant growth and metal uptake during Cd exposure.

7.2 Materials and methods

7.2.1 Range finding experiment

Vertical agar plates

A. thaliana Col-0 seeds were surface-sterilized for 1 min in 0.1% NaClO with 0.1% Tween 80, washed in sterile deionized water and dried in a laminar air flow. Seeds were stored at 4°C to ensure a homogenous germination. Seeds were planted with a sterile toothpick in 12 x 12 cm transparent plates on 40 mL of solid medium (1% agar) (Remans *et al.*, 2006). The growth medium of the vertical agar plates was based on a 50-fold dilution of Gamborg's B5-medium (Zhang and Forde, 1998). It contained 0.5 mM KNO_3, 0.02 mM $MgSO_4.7H_2O$, 0.02 mM $CaCl_2.6H_2O$, 0.022 mM NaH_2PO_4, 0.94 µM $MnSO_4.H_2O$, 0.02 mM $(NH_4)_2SO_4$, 90 nM KI, 0.97 nM H_3BO_3, 0.14 nM $ZnSO_4.7H_2O$, 2 nM $CuSO_4.5H_2O$, 20.6 nM $Na_2SO_4.H_2O$, 2.6 nM $CoSO_4.H_2O$, 3.6 µM $FeCl_3$, 2.56 mM MES and 15 mM sucrose. After 6 days, plants were transferred to plates with the same growth medium but without sucrose and with the appropriate amount of $CdSO_4$ to obtain plates with 0, 3, 5, 7 or 10 µM Cd. Plates were incubated in a growth chamber at day/night temperatures of 22/18°C with a 12 h light/12 h dark regime, a relative humidity of 65% and a photosynthetic active radiation (PAR) of 170 $\mu mol.m^{-2}.s^{-1}$ at the shoot level. Light was provided by Philips GreenPower LED modules. A combination of blue, red and far-red modules was used to obtain a spectrum simulating the PAR in sunlight. Primary root length of 18 day-old plants was determined using the computer program Optimas6 (Media Cybernetics, Rockville, MD, USA).

Hydroponic culture

A. thaliana and *A. capillaris* were grown in a hydroponic system as described by Smeets *et al.* (2008), except that sand was used instead of rock wool. *A. thaliana* Col-0 seeds were surface-sterilized for 1 min in 0.1% NaClO (supplemented with 0.1% Tween 80) and incubated at 4°C to ensure a homogenous germination. For *A. capillaris*, commercially available seeds were used that were surface-sterilized for 30 s in 0.05% NaClO (supplemented with

0.1% Tween 80). Plants were exposed to 0, 3, 5, 7 and 10 µM $CdSO_4$ in case of *A. thaliana* and to 0, 5, 10, 15, 30 and 50 µM $CdSO_4$ in case of *A. capillaris*, and this throughout the entire experiment. New modified 1/10 (*A. thaliana*) or 1/5 (*A. capillaris*) Hoagland solution (Smeets *et al.*, 2008) with or without Cd was provided twice every week to maintain nutrient availability. Plants were grown under the same conditions as in the previous paragraph. After 3 (*A. thaliana*) or 4 (*A. capillaris*) weeks, root and shoot weight were determined. For each condition 9 (*A. thaliana*) or 30 (*A. capillaris*) biological replicates were used.

7.2.2 Growth promotion screening tests and inoculation experiments

Vertical agar plates

Seed endophytes were selected based on their phenotypical characteristics and tested for plant growth promotion on vertical agar plates. *A. thaliana* Col-0 seeds were sterilized, planted and cultivated as described above. After 6 days, plants were transferred to agar plates that were smeared with 400 µL of a bacterial suspension containing 10^8 cfu.mL^{-1} (Table 7.1). For each endophyte tested, 15 biological and 3 technical replicates were used. Non-inoculated plants were used as a control (50 biological and 10 technical replicates). One week after inoculation, all plants were transferred to new non-inoculated agar plates. All plates used during this experiment contained 2 µM Cd applied as $CdSO_4$. From day 6 until day 19 after sowing, root length was recorded each day. Primary root length was determined using the computer program Optimas6 (Media Cybernetics, Rockville, MD, USA).

Endophytes that improved root growth in the screening test were used in a more elaborate inoculation experiment. This experiment proceeded in the same way as the screening test, except that plants were transferred after 6 days to plates containing 0, 2 or 10 µM Cd smeared with a bacterial suspension of 10^6 cfu.mL^{-1}. Moreover, plants from both control and Cd seeds were used. Cd seeds were harvested from plants that had been exposed to 2 µM Cd during several generations, while control seeds were harvested from plants that were grown in parallel but that were never exposed to Cd. One week after inoculation, all plants were transferred to new non-inoculated agar plates containing 0, 2 or 10 µM Cd. For each condition 15 biological and 3 technical replicates were used.

Table 7.1: For each bacterium, the corresponding number that is used on the graphs, the genus, the accession number of the closest reference strain, the seed type it was isolated from and the phenotypic traits are given. + = trait is present. IAA = indole-3-acetic acid, OA = organic acids, ACCD = 1-aminocyclopropane-1-carboxylic acid deaminase.

No.	Genus Accession no.	Seed type	0.4 mM Cd	0.8 mM Cd	0.6 mM Zn	0.4 mM Cu	Siderophores	IAA	OA	ACCD	Acetoin	N_2 fixation	Phosphate	Phytate
1	*Rhizobium* FJ719352	Control	+	+	+	+	+	+	+	+				+
2	*Sphingomonas* AY563441	Control	+	+			+	+	+	+		+		+
3	*Rhizobium* FJ719352	Control	+	+			+	+	+	+		+		+
4	*Sphingomonas* AY563441	Control	+	+	+	+	+	+	+	+		+		+
5	*Sphingomonas* HM629444	Control	+	+	+	+		+	+	+		+		+
6	*Rhizobium* FJ719352	Cd	+	+			+	+	+	+		+		+
7	*Bacillus* EU888579	Cd	+	+			+	+	+	+				+
8	*Rhizobium* FJ719352	Cd	+	+	+	+	+	+	+	+				+
9	*Rhizobium* FJ719352	Cd	+	+	+		+	+	+	+				+
10	*Rhizobium* FJ719352	Cd	+	+			+	+	+	+				+
11	*Bacillus* EU888579	Cd	+	+	+	+	+			+		+	+	

Hydoponic culture

Seed endophytes of *A. thaliana* with promising characteristics for plant growth promotion under Cd stress were tested in a hydroponic system as described above. During the first week of plant growth, bacteria were present in the modified 1/10 Hoagland nutrient solution (Smeets *et al.*, 2008) at a final concentration of 10^6 cfu.mL^{-1} (Table 7.1). All plants were exposed to 2 µM $CdSO_4$ throughout the entire experiment. After 3 weeks, root and shoot weight, root length and rosette diameter were determined. For each condition, 2 technical and 20 (inoculated) or 40 (non-inoculated) biological replicates were used.

The best plant growth promoter from the screening test was used in a more extensive inoculation experiment: *A. thaliana* plants from both control and Cd

seeds were grown hydroponically as described previously and exposed to 0, 2 or 5 µM $CdSO_4$ throughout the entire experiment. Bacteria were added to the modified 1/10 Hoagland solution (Smeets *et al.*, 2008) during the first week of plant growth. After 3 weeks, root and shoot weight, root length and rosette diameter were determined.

Seed endophytes of *A. capillaris* with the most promising characteristics for plant growth promotion under Cd stress were also tested in a hydroponic system. Two *Pantoea* strains isolated from control seeds and 1 *Bacillus* strain isolated from Cd/Ni seeds were selected for inoculation based on their phenotypic traits (Table 7.2). Bacteria were added to the 1/5 Hoagland nutrient solution (Smeets *et al.*, 2008) at a final concentration of 10^5 cfu.mL^{-1}. Plants were exposed to 0 or 10 µM $CdSO_4$ throughout the entire experiment. Fresh 1/5 Hoagland solution with or without Cd was provided twice per week. After 4 weeks of growth, root and shoot length and weight were determined. For each condition 2 technical and 25 biological replicates were used.

Table 7.2: For each bacterium, the corresponding number that is used on the graphs, the genus, the accession number of the closest reference strain, the seed type it was isolated from and the phenotypic traits are given. + = trait is present. Sid = siderophores, IAA = indole-3-acetic acid, OA = organic acids, ACCD = 1-aminocyclopropane-1-carboxylic acid deaminase, P = phosphate solubilization.

No.	Genus Accession no.	Seed type	0.2 mM Cd	Sid	IAA	OA	ACCD	Acetoin	P
1	*Bacillus* DQ523500	Cd/Ni	+	+	+		+	+	+
2	*Pantoea* EU598802	Control	+	+	+		+		+
3	*Pantoea* FJ357836	Control	+		+	+	+	+	+

Re-isolation

Colonization was confirmed by re-isolation of the endophytes from *A. thaliana* and *A. capillaris* roots as these are the main entry route for colonization. Roots were surface-sterilized by immersing them in 0.1% NaClO supplemented with 0.1% Tween 80 during 5 min. Next, roots were thoroughly washed in sterile

deionized water and the last rinsing water was incubated on solid 869 medium (Mergeay *et al.*, 1985) to check sterility of the surface. Samples were crushed in a sterile mortar containing 500 µL sterile 10 mM $MgSO_4$. Dilutions 0 and 10^{-1} were plated on 1/10 869 medium (Mergeay *et al.*, 1985) and incubated at 30°C during 1 week. The number of cfu.g^{-1} fresh weight (FW) isolated from inoculated plants was compared with that of non-inoculated controls. Only inoculated conditions showing plant growth promotion were used for re-isolation. For each condition, 2 replicates were used.

7.2.3 Metal extraction

Cd content was determined in *A. thaliana* and *A. capillaris* roots and shoots grown in the hydroponic culture. To determine the total metal contents, 50 to 200 mg crushed shoots or roots were transferred to glass tubes. Extraction was performed using acid (2x HNO_3 Suprapur, 1x HCl Suprapur) and heat (110°C). Samples were dissolved in 0.5 mL 20% HCl and 4.5 mL Millipore water. Cd content was determined using inductively coupled plasma-atomic emission spectroscopy (ICP-AES).

7.2.4 Statistical analysis

Statistical analyses were performed in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Normal distribution of the data was analysed with the Shapiro-Wilk test and transformations were applied when necessary to approximate normality. Homoscedasticity of variances was checked using a residuals plot. Significant differences were evaluated with an analysis of variances (ANOVA) and post-hoc comparisons were carried out using the Dunnett's test or Tukey's Honest Significant Difference test. If normality could not be reached, a non-parametric Kruskal-Wallis Rank Sum test and Pairwise Wilcoxon Rank Sum test for post-hoc comparisons were used.

7.3 Results

7.3.1 Range finding experiment

Arabidopsis thaliana

Arabidopsis thaliana was grown on vertical agar plates and in a hydroponic culture, and exposed to different Cd concentrations in order to determine its Cd sensitivity. *A. thaliana* plants showed a gradual reduction in root length on vertical agar plates (Fig. 7.1A), and in root (Fig. 7.1B) and shoot weight in a hydroponic culture (Fig. 7.1C) with increasing Cd concentrations. Cd content in roots and shoots grown in the hydroponic culture was comparable after exposure to 3 and 5 µM Cd. Exposure to 7 and 10 µM Cd increased root Cd content approximately 3-fold compared to 3 and 5 µM Cd, while shoot Cd content was significantly reduced (Fig. 7.1D).

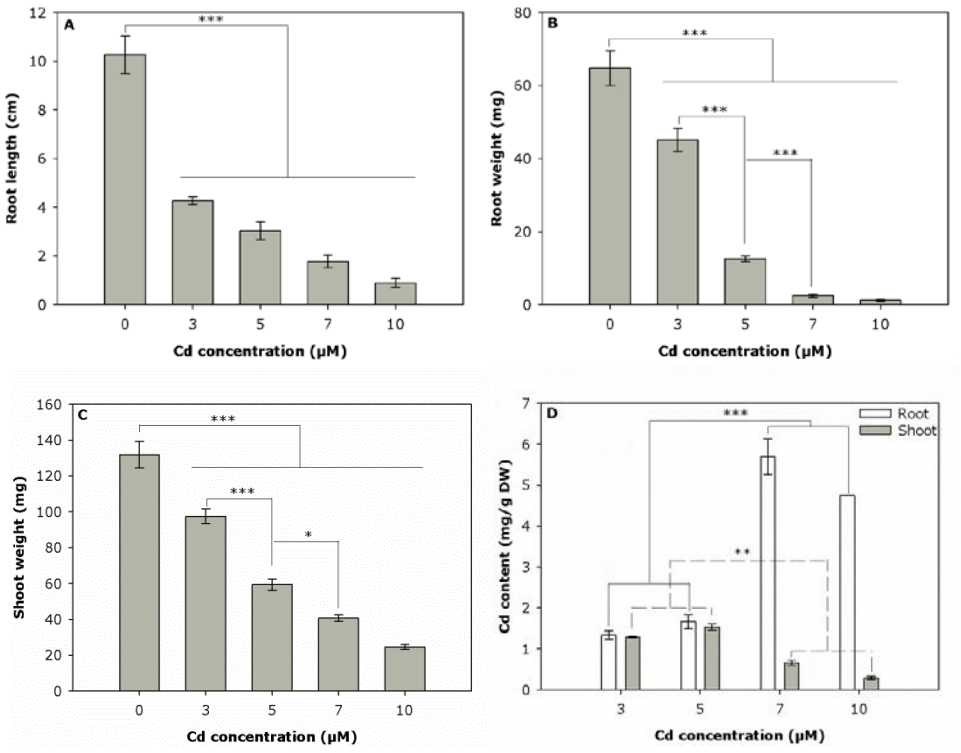

Figure 7.1: Root length (cm) on vertical agar plates (A), and root (B) and shoot (C) weight (mg) and Cd content (D) (mg.g^{-1} dry weight) in a hydroponic culture of *A. thaliana* upon exposure to different Cd concentrations. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

Agrostis capillaris

Agrostis capillaris growing in a hydroponic system was exposed to 0, 5, 10, 15, 30 and 50 µM Cd to determine a sublethal Cd concentration. 10 µM Cd was chosen as a suitable concentration to work with in further experiments as it reduced root weight, shoot weight, root length and shoot length with 30 to 50% (Fig. 7.2).

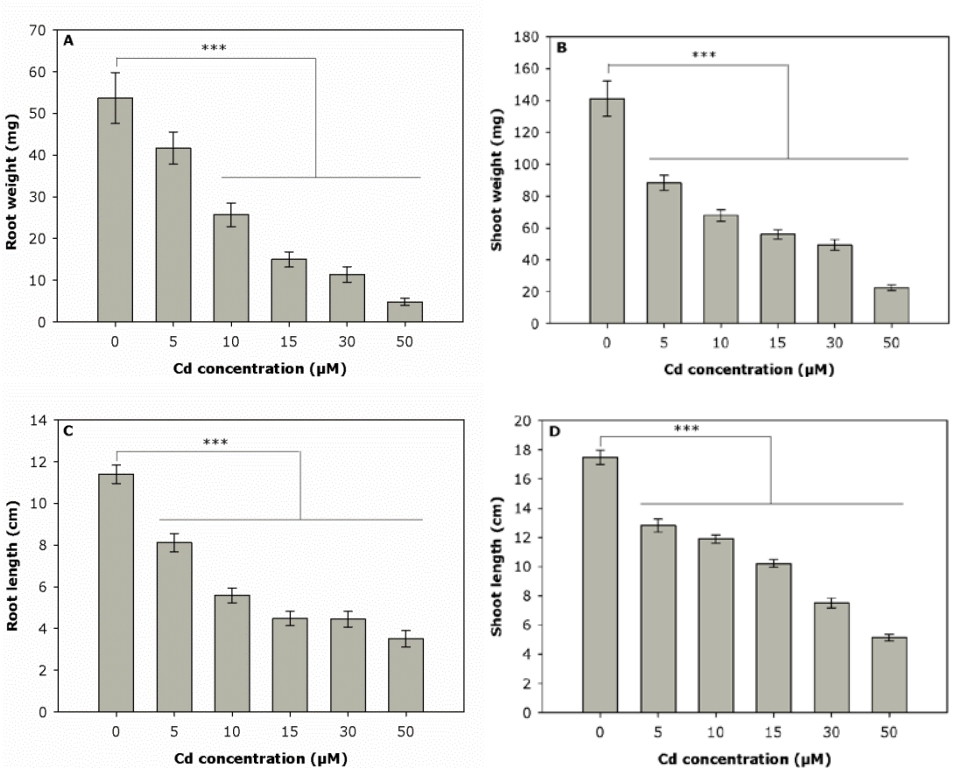

Figure 7.2: Root (A) and shoot (B) weight (mg), and root (C) and shoot (D) length (cm) of *A. capillaris* exposed to different Cd concentrations. *** = $p < 0.001$.

7.3.2 Growth promotion screening tests and inoculation experiments

Arabidopsis thaliana

Eleven different seed endophytes (Table 7.1) were tested for their ability to promote root growth of *A. thaliana* on vertical agar plates with 2 µM Cd. Two *Bacillus* strains (endophyte 7 and 11) and 1 *Rhizobium* strain (endophyte 6)

increased primary root length by approximately 20% compared to the non-inoculated condition (Fig. 7.3). Re-isolation of the inoculated bacteria from the roots confirmed that colonization was successful: respectively 0, 4.2 x 10^5, 1.4 x 10^6 and 6.9 x 10^6 cfu.g^{-1} FW were re-isolated from non-inoculated plants and plants inoculated with seed endophytes 6, 7 and 11.

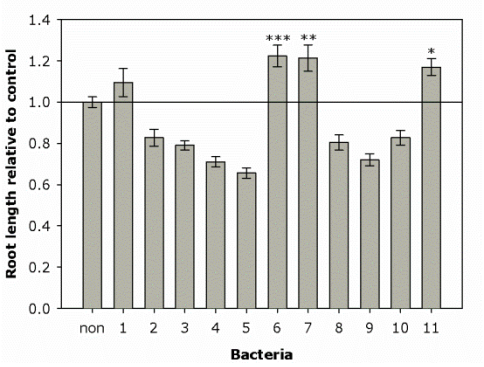

Figure 7.3: Primary root length of *A. thaliana* (relative to non-inoculated control) 2 weeks after inoculation with different seed endophytes on vertical agar plates with 2 µM Cd. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to non-inoculated control. The numbers of the bacteria correspond to the bacteria in table 7.1.

Seed endophyte 11 (*Bacillus sp.*) was chosen for further inoculation experiments based on the results in the growth promotion screening test (Fig. 7.3). Growth promoting abilities were tested in plants from control seeds and from Cd seeds. Moreover, plants were exposed to 0, 2 or 10 µM Cd. Surprisingly, root length was only increased significantly in the case of control seeds exposed to 2 µM Cd (Fig. 7.4).

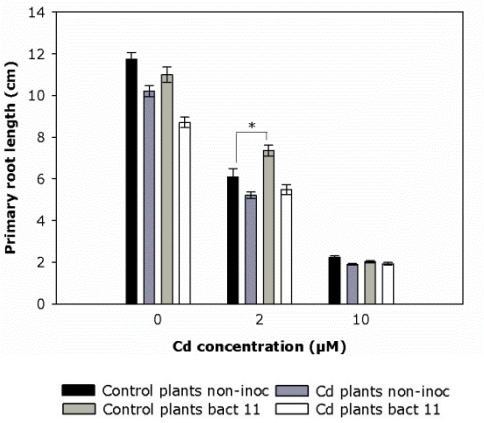

Figure 7.4: Primary root length (cm) of *A. thaliana* grown on vertical agar plates and exposed to 0, 2 or 10 µM Cd. * = $p < 0.05$.

In the hydroponic culture, *A. thaliana* was inoculated with 5 different seed endophytes and exposed to 2 µM Cd. Inoculation with endophyte 2 (*Sphingomonas sp.*) doubled the root weight, increased the shoot weight and root length by approximately 50%, and increased rosette diameter by approximately 20% (Fig. 7.5). Inoculation with endophyte 7 (*Bacillus sp.*) led to an increase in root weight by approximately 50% and an increase in shoot weight, root length and rosette diameter of between 10% and 30% (Fig. 7.5). Endophyte 10 (*Rhizobium sp.*) increased shoot weight by approximately 30% (Fig. 7.5B). Re-isolation of the inoculated bacteria from the roots confirmed that colonization was successful: respectively 6.6 x 10^4 and 7.4 x 10^3 cfu.g^{-1} FW were re-isolated from roots of plants inoculated with endophyte 2 and 7.

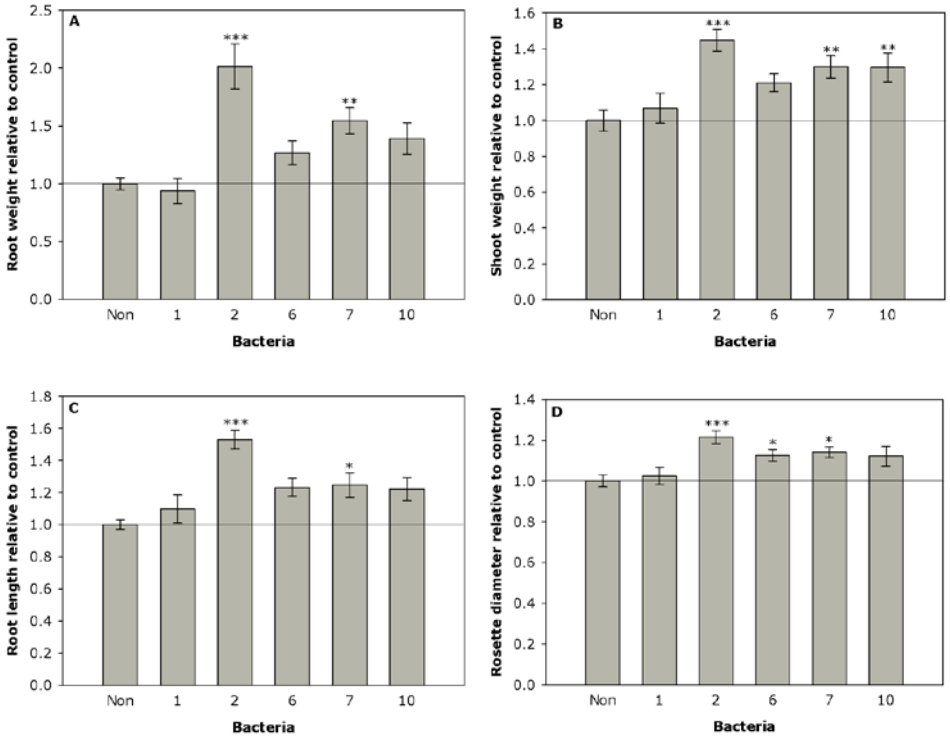

Figure 7.5: Effect of inoculation with selected seed endophytes in an *A. thaliana* hydroponic culture on root (A) and shoot (B) weight, root length (C) and rosette diameter (D) upon exposure to 2 µM Cd relative to non-inoculated controls. Numbers on the graph correspond to the bacteria in table 7.1. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to non-inoculated controls.

Also in the hydroponic set-up, *A. thaliana* control and Cd plants were inoculated with a promising seed endophyte from the screening test and exposed to 0, 2 or

5 µM Cd. Compared to the non-inoculated condition, endophyte 2 (*Sphingomonas sp.*) had a significant positive effect on root weight of Cd plants exposed to 2 µM Cd (Fig. 7.6A), on shoot weight of control and Cd plants exposed to 2 and 5 µM Cd (Fig. 7.6B), on root length of Cd plants exposed to 0 and 2 µM Cd (Fig. 7.6C), and on rosette diameter of Cd plants exposed to 5 µM Cd (Fig. 7.6D). The increases in these parameters were smaller than could be expected based on the growth promotion screening test (Fig. 7.5). Inoculation with endophyte 2 did not affect Cd content of roots and shoots (data not shown).

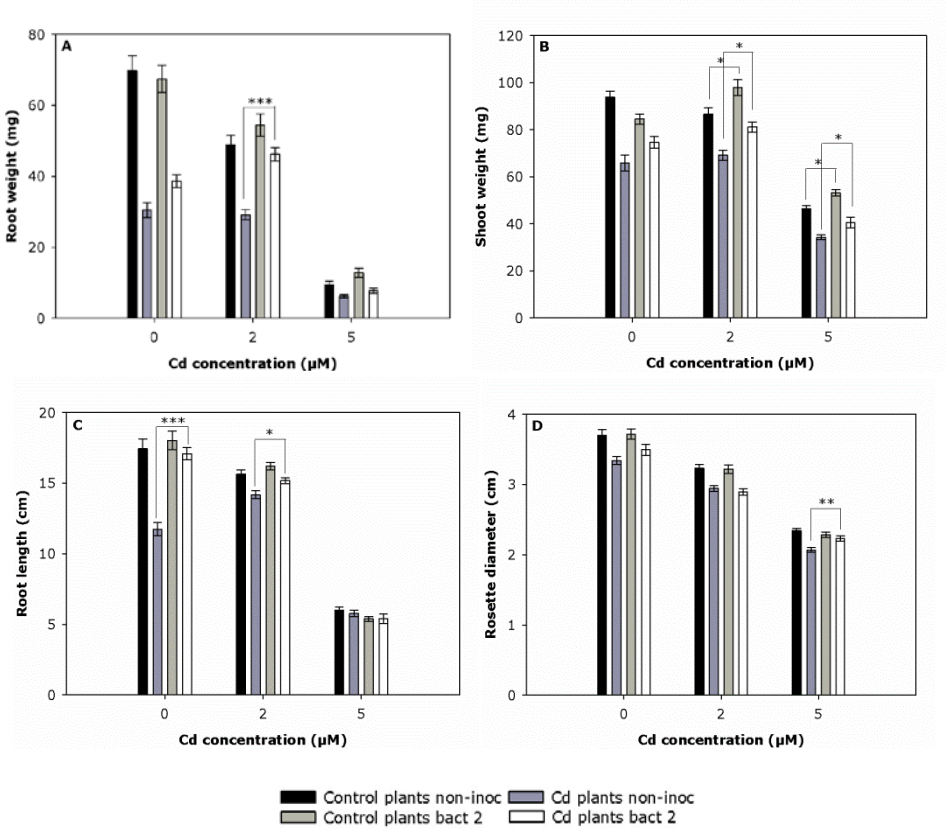

Figure 7.6: The effect of inoculation on root (A) and shoot (B) weight (mg), root length (cm) (C) and rosette diameter (cm) (D) of *A. thaliana* in a hydroponic culture exposed to 0, 2 or 5 µM Cd. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Agrostis capillaris

Also for *A. capillaris*, 3 endophytes with promising traits for improving phytoextraction efficiency were selected to inoculate plants growing in a hydroponic culture (Table 7.2). For the 3 inoculated endophytes, their successful colonization was illustrated by the higher number of bacteria (on average 50 times higher) that were re-isolated from inoculated plants compared to non-inoculated control plants. Inoculation of *A. capillaris* plants with endophytes 1 (*Bacillus sp.*) and 2 (*Pantoea sp.*) improved plant growth when they were not exposed to Cd. Inoculation with endophyte 1 led to a significantly increased root weight, from 23.0 ± 2.7 mg to 37.4 ± 3.3 mg, and a significantly increased shoot length, from 14.5 ± 0.41 cm to 16.7 ± 0.40 cm (Fig. 7.7A+D).

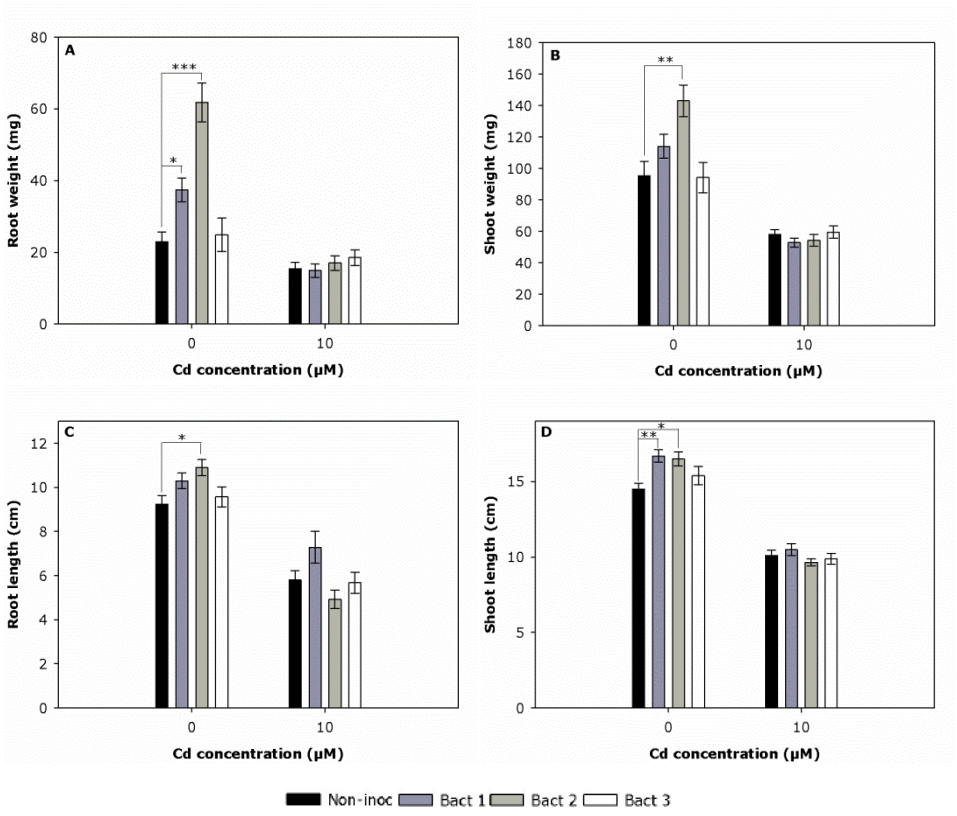

Figure 7.7: Root (A) and shoot (B) weight (mg), and root (C) and shoot (D) length (cm) from *A. capillaris* plants exposed to 0 or 10 µM Cd. The numbers in the legend correspond to the bacteria in table 7.2. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Inoculation with endophyte 2 significantly increased root weight from 23.0 ± 2.7 mg to 61.9 ± 5.4 mg, shoot weight from 95.3 ± 9.3 mg to 143.0 ± 10.1 mg, root length from 9.3 ± 0.37 cm to 10.9 ± 0.36 cm and shoot length from 14.5 ± 0.41 cm to 16.5 ± 0.46 cm (Fig. 7.7). When plants were exposed to 10 µM Cd, none of the inoculated endophytes was able to significantly improve shoot or root length and weight.

Nevertheless, the Cd concentration was significantly higher in roots of plants inoculated with endophyte 3 (*Pantoea sp.*) than in those of non-inoculated plants (Fig. 7.8A). Also the Cd content in shoots was higher in plants inoculated with endophyte 1 and 2 compared to non-inoculated shoots (Fig. 7.8B).

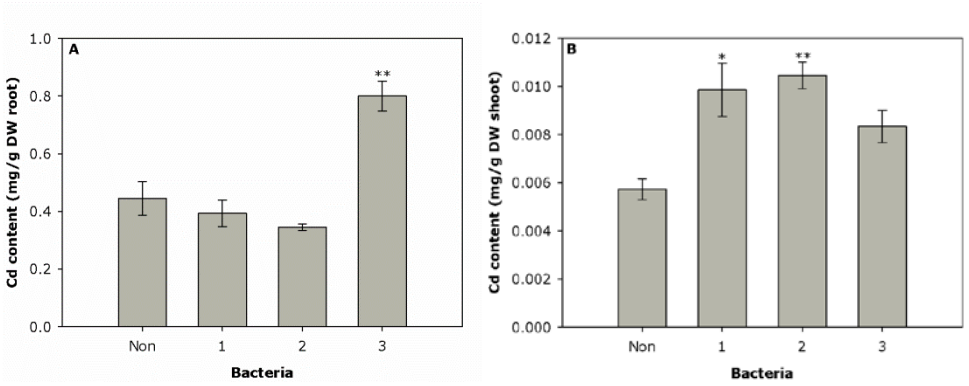

Figure 7.8: Cd content (mg.g^{-1} dry weight) of inoculated *A. capillaris* roots (A) and shoots (B) exposed to 10 µM Cd. * = $p < 0.05$, ** = $p < 0.001$ compared to non-inoculated plants.

7.4 Discussion and conclusion

Several of the selected seed endophytes were shown to be able to improve growth of *A. thaliana*. This indicates that seeds of *A. thaliana* contain bacteria that are beneficial for seedling establishment and growth. However, the *in vitro* phenotypic characteristics were not always a suitable indicator for *in vivo* plant growth promotion. Becerra-Castro *et al.* (2012) already mentioned that the *in vitro* plant growth promoting traits of rhizobacteria isolated from pseudometallophytes growing on Pb/Zn mine tailings not always correlated with their *in vivo* effects after inoculation in willow. Also Weyens *et al.* (2013) mentioned that *in vitro* tests for plant growth promoting characteristics of yellow lupine-associated bacteria were not sufficient to predict *in vivo* plant growth

promotion upon Cd exposure. More recently, Janssen *et al.* (2015) also found that, although the inoculated bacteria showed many beneficial traits in *in vitro* tests, they did not enhance biomass production of willow growing on a Cd/Zn/Pb-contaminated soil.

The results of the inoculation experiments with *A. thaliana* were always below expectations based on the outcomes from the growth promotion screening tests. Only 1 example of an inoculation experiment on vertical agar plates and 1 example of an inoculation experiment in hydroponics are shown above, but these experiments were repeated many times and with several variations in Cd concentration, inoculum size and inoculum composition. Unfortunately, the obtained results were mostly not repeatable. This could indicate that several uncontrolled factors, such as the growth stage of the bacteria or the developmental stage of the plant at the moment of inoculation, could have an important influence on the obtained result. This will be investigated in more depth in chapter 8.

The seed endophytes selected for inoculation of *A. capillaris* improved plant growth and/or increased Cd uptake. The endophytes present in the inoculated plants possibly sequestered Cd, making it less available for its host (Dimpka *et al.*, 2009b). This could explain the higher Cd concentrations in inoculated plants without apparent signs of phytotoxicity. These findings indicate that inoculation of *A. capillaris* with its seed endophytes could be beneficial for its establishment during both phytoextraction and phytostabilization of metal-contaminated soils. Also Mastretta *et al.* (2009) reported an increased Cd uptake after inoculation with bacterial seed endophytes. Inoculation with seed endophytes could be of particular interest as they can be transmitted via the seeds from one generation to the next. Consequently, high numbers of beneficial bacteria could already be present in the very early developmental stages of the plant. Nevertheless, optimization of the inoculation protocol could also lead here to a more consistent and reproducible inoculation outcome.

CHAPTER 8 Features affecting colonization and inoculation success

8.1 Introduction

The use of plant-associated bacteria as inoculants in agricultural or environmental biotechnology applications, including phytoremediation, requires efficient plant colonization. Several features, such as the degree of motility, chemotactic behavior, production of biocontrol agents and secretion of cell-wall degrading enzymes, influence the colonizing ability of bacteria in the rhizosphere.

Bacteria have developed various mechanisms to mediate their movement. Swimming and swarming are both effectuated by rotating flagella. Swimming is performed by individual bacterial cells moving in a liquid environment, while swarming is defined as a multicellular movement over a surface (Jarrel and McBride, 2006; Kearns, 2010). Many bacteria also move without the aid of flagella. Twitching motility is powered by the extension and retraction of type IV pili (Kearns, 2010). In some bacteria, pilus retraction results in smooth movement, called gliding (Jarrel and McBride, 2006). Other bacteria glide using focal adhesion complexes instead of pili (Kearns, 2010). Sliding is a passive form of movement that occurs when a colony is spreading, driven by the outward pressure of cell growth. This does not rely on an extracellular motor but uses surfactants that reduce surface tension (Kearns, 2010).

Gillis *et al.* (2012) demonstrated that the amount of flagella could be correlated with swarming motility in *Bacillus thuringiensis*. Moreover, Bergara *et al.* (2003) found a link between flagellar biosynthesis and bacterial growth stage: in rich complex media, some genes involved in flagellar biosynthesis are repressed during early exponential growth by a DNA-binding protein CodY. When nutrients become limiting, such as in the stationary growth phase or during growth in minimal media, this repression is relieved (Bergara *et al.*, 2003). Several studies have already shown that bacterial motility can be important for efficient root colonization after inoculation. For example, Capdevila *et al.* (2004) demonstrated that a biocontrol *Pseudomonas* strain needed its wild-type motility properties to competitively colonize the alfalfa rhizosphere. Also Shelud'ko *et al.*

(2009) found that a non-swarming mutant of *Azospirillum* possessed a lower capacity of wheat root adsorption. Moreover, de Weert *et al.* (2002) reported that the role of bacterial motility in tomato root colonization by a *Pseudomonas fluorescence* strain was based on chemotaxis towards root exudates. Chemotaxis allows bacteria to rapidly approach chemically favourable environments, such as the rhizosphere, by integrating environmental signals and adapting flagellar rotation accordingly (Bren and Eisenbach, 2000).

Bacteria can also gain a selective advantage over other microorganisms during root colonization by producing metabolites involved in biocontrol. The production of siderophores makes it possible to competitively acquire ferric iron, an essential but low bioavailable element for growth of all living organisms (Whipps, 2001). Bacterial siderophores can scavenge iron from fungal siderophores as these have a lower affinity for iron (Compant *et al.*, 2005; Loper and Buyer, 1991) or they can draw iron from heterologous siderophores produced by other bacteria (Raaijmakers *et al.*, 1995). Bacteria can also attack other microorganisms by producing lytic enzymes such as chitinases or proteases (Kamensky *et al.*, 2003). Besides entry via natural openings and wounds, some endophytic bacteria can actively colonize plant roots by producing cell-wall degrading enzymes, such as pectinase and glucanase (Plazinksi and Rolfe, 1985; Verma *et al.*, 2001; Sessitsch *et al.*, 2004). The production of siderophores, proteases, pectinases as well as glucanases have been shown to be correlated with the bacterial growth stage, with maximum levels usually detected during the late exponential and/or early stationary phases (Oberkotter and Rosenberg, 1978; Membré and Burlot, 1994; Liles *et al.*, 2000; Oleksy *et al.*, 2004; Gaonkar *et al.*, 2012). In this chapter, the effect of the bacterial growth stage on the success of inoculation was studied by looking for a correlation between growth stage and bacterial motility, chemotactic behavior, the cell-wall degrading enzyme pectinase, plant growth promoting capacity and colonization efficiency.

8.2 Materials and methods

Endophyte 7, a Cd-tolerant *Bacillus sp.* isolated from *Arabidopsis thaliana* seeds (Table 7.1), was used in the next experiments as this endophyte demonstrated plant growth promoting properties during previous inoculations of *A. thaliana*.

8.2.1 Growth curve

Endophyte 7 (*Bacillus sp.*) was grown overnight in 869 medium (Mergeay *et al.*, 1985). This culture was diluted 1/50 in fresh 869 medium and incubated at 30°C and 100 rpm. At regular time intervals, culture aliquots were used for measuring the absorption at 660 nm. At OD_{660nm} of 0.3, 0.7, 1.1, 1.6 and 2.4 samples were taken for further analyses.

8.2.2 Bacterial motility and chemotaxis

The capillary assay to determine bacterial motility and chemotaxis was based on Adler (1973) and Mazumder *et al.* (1999) (Fig. 8.1). Briefly, a 2 cm 25 gauge needle with a 0.254 mm internal diameter was attached to a 1 mL syringe containing 100 µL of the compound to be tested for chemotaxis. Important root exudates of *A. thaliana* were used as test compounds (Chaparro *et al.*, 2013): 100 mM glucose, 100 mM glycerol, 30 mM alanine and 50 mM lactate. All compounds were dissolved in chemotaxis buffer containing 10 mM KH_2PO_4 and 0.1 mM disodiumEDTA at pH 7 (Liu and Parales, 2008). To test for motility, syringes were filled with chemotaxis buffer only. Next, the needle was inserted into a 100 µL pipette tip containing 60 µL of the bacterial suspension. Bacterial suspensions were prepared by centrifuging (4000 rpm, 10 min) 10 mL of culture of the appropriate OD_{660nm} (0.3, 0.7, 1.1, 1.6, 2.4) and resuspending the pellet in chemotaxis buffer until an OD_{660nm} of 0.5 was reached. After 45 min incubation at room temperature, the content of the syringe was diluted 10^{-2} in 10 mM sterile $MgSO_4$ and plated on 1/10 869 medium (Mergeay *et al.*, 1985). Motility of the bacteria was expressed as the number of cfu counted on the plates. To determine if a test compound was a chemoattractant, the relative chemotactic response (RCR) was calculated as the ratio of bacteria that entered

the test capillary with the attractant to that in the capillary without the attractant. Results were expressed as the mean of 2 separate assays.

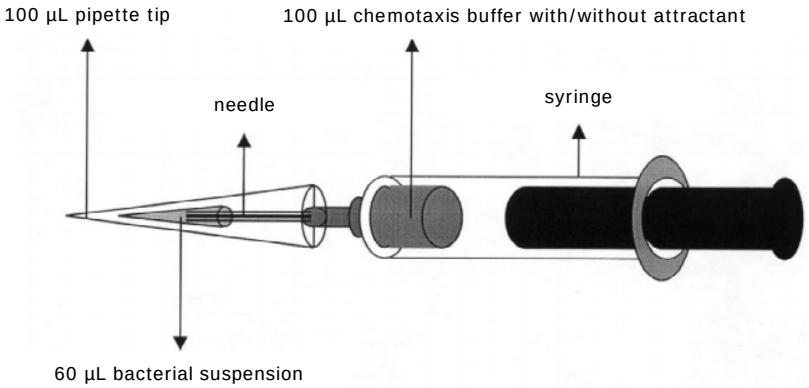

Figure 8.1: Experimental set-up used for determining bacterial motility and chemotaxis. Adapted from Mazumder *et al.* (1999).

8.2.3 Pectinase assay

Pectinase activity was determined spectrophotometrically using an assay adapted from Worthington (2011). Bacterial cultures at the appropriate OD_{660nm} were centrifuged (4000 rpm, 10 min) and 2 mL supernatant was added to 3 mL 0.5% pectin substrate. 2 mL culture medium added to 3 mL 0.5% pectin substrate was used as a sample blank. 0.5 mL of a pectinase solution (dilution series from 3.5 $U.mL^{-1}$ to 0.1 $U.mL^{-1}$ in 0.1 M citric acid/phosphate buffer, pH 5) added to 3 mL 0.5% pectin substrate was used to make a standard curve. 1 U corresponds to the amount of enzyme which liberates 1 µmol galacturonic acid from polygalacturonic acid per minute at pH 4 and 50°C. 0.5 mL buffer added to 3 mL 0.5% pectin substrate was used as a standard curve blank. All assay tubes were incubated for 60 min at 37°C and put on ice to stop the reaction. From each tube 50 µL was added to 1 mL color reagent A (40 g Na_2CO_3, 16 g glycine and 0.45 g $CuSO_4.5H_2O$ in 1 l H_2O) and 1 mL color reagent B (1.2 g neocuprine-HCl in 1 L H_2O). Tubes were incubated in a water bath at 95°C for 13 min and cooled on ice. After adding 1 mL H_2O, absorbance was read at 450 nm against a water blank. For each sample or positive control, 3 technical replicates were measured.

8.2.4 Plant growth promotion and colonization efficiency

To correlate bacterial growth stage at the time of inoculation with plant growth promoting capacity, *A. thaliana* was grown on vertical agar plates prepared as described in 7.2.1. One week-old plants were transferred to plates containing 0 or 3 µM $CdSO_4$. These plates were smeared with 400 µL bacterial suspension of the desired OD_{660nm} (0.3, 0.7, 1.1, 1.6, 2.4) at a concentration of 10^4 cfu.mL^{-1}. At each OD, culture aliquots were plated on 1/10 869 medium (Mergeay *et al.*, 1985) to count the numbers of cfu. Primary root length was determined with Optimas6 (Media Cybernetics, Rockville, MD, USA) 2 weeks after inoculation and compared to the non-inoculated control. For each condition, 3 technical and 15 biological replicates were used. To correlate bacterial growth stage to colonization efficiency, the numbers of bacteria that could be re-isolated from *A. thaliana* roots were determined as described in 7.2.2. The numbers of cfu.g^{-1} fresh weight (FW) are expressed as an average of 2 replicates.

A. thaliana was also grown in a hydroponic culture as described in 7.2.1. Plants were inoculated at the time of sowing by adding bacteria at the appropriate OD_{660nm} (0.3, 0.7, 1.1, 1.6, 2.4) to the Hoagland nutrient solution. The final concentration of bacteria in the Hoagland solution was 10^4 cfu.mL^{-1}. Sterile water was used to prepare the Hoagland solution to eliminate external addition of bacteria in order to facilitate re-isolation of the inoculated bacteria. Root and shoot weight, root length and rosette diameter were recorded 3 weeks after inoculation. For each condition, 10 biological replicates were used. To determine colonization efficiency, bacteria were re-isolated from roots as described in 7.2.2. Cfu.g^{-1} FW is expressed as an average of 2 replicates.

8.2.5 BOX-PCR fingerprinting

To determine which of the re-isolated bacteria are the same as the inoculated bacterial strain, BOX-PCR was performed on the re-isolated colonies that morphologically resembled the inoculated bacteria. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen). Each PCR reaction contained 1x High Fidelity PCR buffer, 2 mM $MgSO_4$, 200 µM of each dNTP, 400 nM BOX-A1R primer (CTACGGCAAGGCGACGCTGACG) (Versalovic *et al.*, 1994), 1 U Taq DNA Polymerase, 1 µL DNA template and RNase free water until a total volume of 50

µL. Cycling conditions were 5 min 94°C, 35 cycles of 1 min 94°C, 1.5 min 50°C and 8 min 68°C, and a final incubation step of 8 min 68°C. The resulting fragments were separated on a 1.5% agarose gel.

8.2.6 Statistical analysis

Data were analysed in R 3.1.1 (R Core Team, 2014). Normal distribution of the data was analysed using the Shapiro-Wilk test and homoscedasticity of variances was checked using a residuals plot. Significant differences were evaluated with a one-way analysis of variances (ANOVA) and post-hoc comparisons were carried out using the Tukey's Honest Significant Difference test or Dunnet's test.

8.3 Results and discussion

A culture of endophyte 7 (*Bacillus sp.*) was sampled at different time points between the late lag and late exponential phase for determining motility and chemotactic behavior, pectinase activity, and plant growth promotion and colonization capacity (Fig. 8.2).

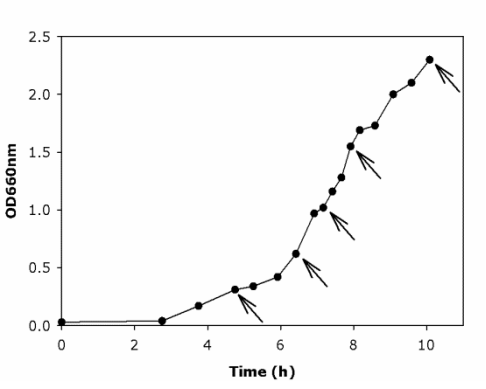

Figure 8.2: Growth curve of endophyte 7 (*Bacillus sp.*). Arrows indicate sampling points.

Motility and chemotaxis towards important root exudates of *A. thaliana* were tested in different growth stages of the bacterial culture. Motility of the bacteria was slightly higher during the early growth stages: on average 1.7 to 2.2 x 10^6 cfu.mL^{-1} were recovered from the syringes at OD_{660nm} of 0.3 and 0.7 compared to 1.2 to 1.4 x 10^6 cfu.mL^{-1} at OD_{660nm} of 1.1, 1.6 and 2.4 (Table 8.1). The

relative chemotactic response (RCR) appeared to be higher in later growth stages, especially towards glucose at OD_{660nm} of 1.1, 1.6 and 2.4, towards glycerol at OD_{660nm} of 1.6, and towards alanine at OD_{660nm} of 2.4 (Table 8.1). Lactate induced no response in the later growth stages (OD_{660nm} of 1.6 and 2.4) or even a negative chemotactic response during the early growth stages (OD_{660nm} of 0.3, 0.7 and 1.1) (Table 8.1).

Table 8.1: Motility ($cfu.mL^{-1}$) and relative chemotactic response (RCR) towards glucose, glycerol, alanine and lactate in the different bacterial growth stages.

OD_{660nm}	**Motility ($cfu.mL^{-1}$)**	**RCR**			
		Glucose	Glycerol	Alanine	Lactate
0.3	1.7×10^6	1	1.3	0.8	0.4
0.7	2.2×10^6	1	0.9	0.7	0.2
1.1	1.2×10^6	2	0.8	0.8	0.6
1.6	1.4×10^6	1.4	1.8	0.9	1.1
2.4	1.2×10^6	1.5	0.8	1.2	1

Pectinase activity was very high in the growth stage with OD_{660nm} of 0.3 compared to all other growth stages that were tested, reaching 0.012 ± 0.00067 U per 10^6 cfu (Fig. 8.3). No significant differences between OD_{660nm} of 0.7, 1.1 and 1.6 were detected. Bacteria at an OD_{660nm} of 2.4 showed a significantly lower pectinase activity compared to all previous growth stages, reaching only 0.00044 ± 0.000079 U per 10^6 cfu (Fig. 8.3). Pectinolytic enzymes can facilitate entry of the bacteria in the plant and thus increase colonization success (Sessitsch *et al.*, 2012). Nevertheless, pectinase activity is not always directly proportional to plant growth, as pectinolytic enzymes are often associated with pathogenicity. Bacteria with pectinolytic activity might be involved in diseases such as soft rots, dry rots, wilts, blights and leafspots (Bateman and Millar, 1966; Potrykus *et al.*, 2014).

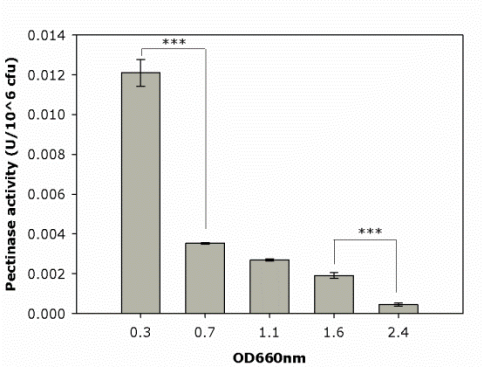

Figure 8.3: Pectinase activity (U/10^6 cfu) in the different bacterial growth stages. *** = $p < 0.001$.

To determine whether the observed differences affect colonization capacity and plant growth, *A. thaliana* was inoculated with the strain at different growth stages. On vertical agar plates without Cd, inoculated *A. thaliana* plants grew faster during the 1st week compared to the non-inoculated controls, although no differences in primary root length could be detected at the end of the experiment (data not shown). Moreover, only minor differences in the number of cfu re-isolated from roots could be detected. As expected, no bacteria were re-isolated from the non-inoculated control plants. The cfu re-isolated from roots increased from inoculation with OD_{660nm} of 0.3 to 1.6, but was the lowest at OD_{660nm} of 2.4 (data not shown).

On vertical agar plates with 3 µM Cd, 2 to 5 times more bacteria could be re-isolated from the roots than on plates without Cd. This is in accordance with the work of Weyens *et al.* (2013) on lupine plants where the amount of re-isolated bacteria was significantly higher after Cd exposure. The amount of re-isolated bacteria was between 1.44 x 10^7 and 5.62 x 10^7 cfu.g^{-1} FW (Table 8.2). The samples of the re-isolation of OD_{660nm} of 0.7 were contaminated with a fungus making an accurate count of the numbers of cfu unfeasible. The primary root lengths were not statistically different but showed a trend where the roots of plants inoculated with OD_{660nm} of 0.3, 0.7 and 1.1 were longer: between 8.09 ± 0.35 and 8.56 ± 0.49 cm compared to 7.88 ± 0.58 cm in the non-inoculated controls (Table 8.2). Roots of plants inoculated with OD_{660nm} of 1.6 and 2.4 were respectively 7.64 ± 0.40 cm and 6.93 ± 0.26 cm, and thus tended to be shorter than the non-inoculated controls. There does not seem to exist a straightforward

correlation between the primary root length and the number of cfu re-isolated from roots, although the least bacteria were re-isolated from the shortest roots (at OD_{660nm} of 2.4) (Table 8.2).

Table 8.2: Primary root length (cm) and number of cfu.g^{-1} fresh weight (FW) re-isolated from roots of *A. thaliana* on vertical agar plates with 3 µM Cd and after inoculation with different bacterial growth stages. NI = not inoculated, SE = standard error, nd = not determined.

OD_{660nm}	Root length ± SE (cm)	Cfu.g^{-1} FW
NI	7.88 ± 0.58	0
0.3	8.17 ± 0.73	5.62×10^7
0.7	8.09 ± 0.35	nd
1.1	8.56 ± 0.49	4.01×10^7
1.6	7.64 ± 0.40	5.33×10^7
2.4	6.93 ± 0.26	1.44×10^7

In the hydroponic culture, clear differences were observed between plants inoculated with the strain at different growth stages. Root weight, shoot weight and rosette diameter were significantly increased in plants inoculated with OD_{660nm} of 0.3, 1.6 and 2.4 compared to the non-inoculated controls (Table 8.3). Also root length was significantly increased in plants inoculated with OD_{660nm} of 1.6 and 2.4 compared to the non-inoculated controls (Table 8.3).

For the non-inoculated plants, none of the re-isolated colonies matched the inoculated bacterial strain as was shown by BOX-PCR fingerprinting. For the plants inoculated with OD_{660nm} of 0.3, up to 12.60% of the re-isolated colonies appeared to be matching the inoculated bacterial strain (Table 8.3). Only very low numbers of the inoculated bacterial strain could be recovered from plants inoculated with OD_{660nm} of 0.7, while intermediate values were obtained for plants inoculated with OD_{660nm} of 1.1, 1.6 and 2.4 (Table 8.3).

The lowest percentage of colonization was linked with the smallest plants (OD_{660nm} of 0.7 in Table 8.3). However, an increase in colonization by the inoculated strain was not linearly correlated to an increase in plant growth. The highest percentage of colonization was observed after inoculation with OD_{660nm} of 0.3, the bacterial growth stage that was also associated with the highest pectinase activity (Fig. 8.3), indicating that this could be used as a mechanism to increase plant colonization.

Table 8.3: Root weight (mg), shoot weight (mg), root length (cm), rosette diameter (cm) and percentage of re-isolated colonies matching the inoculated bacterial strain of plants grown in a hydroponic culture and inoculated with different bacterial growth stages. a = p < 0.05, b = p < 0.01, c = p < 0.001 compared to non-inoculated controls (NI). SE = standard error.

OD_{660nm}	Root weight ± SE (mg)	Shoot weight ± SE (mg)	Root length ± SE (cm)	Rosette diameter ± SE (cm)	Re-isolated matching inoculated (%)
NI	7.60 ± 1.13	31.5 ± 2.55	6.83 ± 0.53	2.43 ± 0.08	0
0.3	17.9 ± 2.32^{c}	51.8 ± 4.73^{c}	8.01 ± 0.25	2.88 ± 0.10^{a}	12.60 ± 4.01
0.7	7.10 ± 0.89	30.9 ± 2.28	7.71 ± 0.57	2.46 ± 0.088	0.74 ± 0.19
1.1	9.50 ± 0.86	47.4 ± 3.20^{a}	8.29 ± 0.45	2.87 ± 0.11^{a}	3.11
1.6	25.0 ± 2.79^{c}	54.8 ± 4.87^{c}	11.81 ± 0.59^{c}	2.96 ± 0.12^{b}	2.89 ± 2.37
2.4	22.7 ± 1.64^{c}	60.0 ± 3.08^{c}	11.81 ± 0.20^{c}	3.06 ± 0.10^{c}	4.34 ± 0.96

8.4 Conclusion

Inoculation at different growth stages of the bacterial culture has different effects on plant growth. However, due to the limited number of bacterial traits that were investigated, it is difficult to correlate differences in bacterial growth stage with changes in plant responses. Each bacterial growth stage clearly has its own characteristics, which could lead to a change in colonizing ability and thus in plant response after inoculation. There are many other features besides motility, chemotactic behavior and pectinase activity, which might affect inoculation success. Further experiments, studying for example the production of siderophores or lytic enzymes, are needed to unravel the effect of the growth stage of the bacterial inoculum on colonization success.

The experiments conducted on vertical agar plates allowed an accurate count of the re-isolated bacteria as this is a sterile system where no external bacteria are introduced. In the hydroponic culture, sterile water was used to minimize external addition of bacteria in order to facilitate the re-isolation of the inoculated bacteria. Our use of (nearly) sterile plant growth systems is an over simplification ruling out any form of biocontrol mechanisms that could contribute to rhizosphere and endosphere competence. In a pot experiment or even field situation, re-isolation of the inoculated bacteria would be much more difficult, but differences between plants inoculated with bacteria in a different growth stage could be more distinguishable because of competition with other microorganisms.

Optimization of the entire inoculation procedure is important to obtain a more consistent and reproducible inoculation outcome. This could accelerate the use of bacteria as inoculants in many agricultural or environmental biotechnological applications.

CHAPTER 9 General discussion, conclusion and future perspectives

Transgenerational signaling through maternally derived compounds or epigenetic mechanisms allows plants to adapt in a fast and efficient manner to biotic and abiotic stresses. Recently, the plant microbiome emerged as a novel trait that extends the capacity of plants to adapt to the environment (Bulgarelli *et al.*, 2013). Vertically transmitted bacteria are ideally placed to prepare the next generation of plants for specific conditions already present in the maternal environment. This makes them ideal candidates for use in different agricultural, industrial or environmental applications. Nevertheless, knowledge on their fate and function during the life cycle of plants is still limited. The overall goal of this thesis was to get a better understanding of the factors that shape seed endophytic communities and the importance of seed endophytes for plant growth and development.

First of all, we searched for a seed surface sterilization protocol that ensures a profound surface sterilization but also preserves a high number of cfu and morphologically distinct colony types that are located in the seed interior. The standard seed sterilization protocol for *A. thaliana*, 1 min rinsing in 70% ethanol followed by 10 min in 1.2% NaClO solution (Balcells, 1993), appeared to be too harsh, possibly destroying most of the seed endophytes. A reduction of the incubation time in and the strength of the NaClO solution was required to reliably isolate the cultivable seed endophytes (Table 4.1). Also for *A. capillaris* seeds, a very mild sterilization protocol was adequate. With the optimized protocol, we were able to isolate between 10^6 and 10^7 cfu.g^{-1} seed (Table 5.1). This is much higher than expected as the number of cultivable endophytes isolated from seeds is usually between 10^2 and 10^3 cfu.g^{-1} (Benizri *et al.*, 2001; Hallmann, 2001).

In the first part of this thesis, cultivation-dependent techniques and next-generation sequencing were used to understand how different factors affect the bacterial community present in seeds. We investigated the effect of long-term Cd exposure on the seed endophytic community of *A. thaliana*. *Rhizobium* and

Pseudomonas appeared to be tightly associated with *A. thaliana* seeds, while other isolates were specialized endophytes that were selectively taken up (Table 4.2, 4.4). *Rhizobium* and *Pseudomonas* are often found colonizing a wide range of plants and are known to contribute to plant growth and health (Biswas *et al.*, 2000; Chi *et al.*, 2005; Mercado-Blanco and Bakker, 2007; Gómez-Lama Cabanás *et al.*, 2014). Nevertheless, *Rhizobium* appeared to be the only genus that was consistently transferred from generation to generation via the seed (Table 4.2, Fig. 9.1). Based on the data of the total community, *Aeromicrobium* and *Pseudonocardia* were identified as indicator species in Cd seeds (Table 4.5). In general, selection for transfer to the next generation of plants appeared to be based more on phenotypic characteristics than on the genera that were present. Selection in Cd seeds was mainly based on the presence of ACC deaminase activity and Cd tolerance, while IAA production was an important trait in both control as well as Cd seeds (Table 4.6, Fig. 9.1). ACC deaminase hydrolyzes ACC into ammonia and α-ketobutyrate thereby lowering the amount of stress-induced ethylene (Glick *et al.*, 1998; Hontzeas *et al.*, 2006). Metal-tolerant bacteria often secrete the metal ions after uptake and precipitate them on the bacterial cell wall. This decreases the internal availability of the metals and by consequence protects the bacteria as well as their host plant against toxicity (Nies, 1999; Lodewyckx *et al.*, 2001a; Weyens *et al.*, 2009b). Bacterial IAA plays a major role in the development of the host plant root system leading to an improved nutrient uptake (Dobbelaere *et al.*, 1999; Patten and Glick, 2002; Long *et al.*, 2008; Masciarelli *et al.*, 2013). The presence of bacteria with these properties in the seeds allows the plant to benefit from their traits during the early developmental stages without being dependent on the external environment as a donor.

Also in *A. capillaris* seeds, plant- and contaminant-dependent effects on the cultivable bacterial community were found. We did not observe many differences in community composition between seeds from the Cd/Ni contaminated and the control site (Table 5.2), but fine-tuning of the phenotypic characteristics of the bacteria, induced by the presence or absence of Cd and Ni in the soil, seemed to have occurred (Table 5.3). Again, bacterial ACC deaminase activity appeared to be very important in coping with metal stress (Fig. 9.1). Surprisingly, Cd tolerance was almost absent in endophytes from Cd/Ni-exposed *A. capillaris*

seeds. The development of metal tolerance mechanisms in these endophytes was possibly not required due to metal exclusion from aerial parts as well as metal compartmentalization and complexation as a consequence of the pseudometallophyte nature of *A. capillaris* (Turner and Marshall, 1972; Baker, 1987; Vögeli-Lange and Wagner, 1996; Sanita di Toppi and Gabbrielli, 1999; Dahmani-Muller *et al.*, 2000).

Further evidence for the selection of endophytes was obtained by growing plants on 2 different substrate types. Despite the different bacterial composition of the substrates, the harvested seeds contained very similar endophytic communities (Fig. 6.2, 6.3). Their presence in the seeds is probably more a consequence of rhizosphere competence and not so much of abundance in the substrate (Green *et al.*, 2006). Again, a strong association of *Rhizobium* and *Pseudomonas* with *A. thaliana* seeds was observed (Fig. 6.2, 9.1). After sowing the seeds from the 2 different origins on the same substrate type, it appeared that the bacterial community present in the leaves was mainly derived from the environment and not from the seed (Fig. 6.2, 6.3). Elucidation of the respective contribution of the growing medium and the seeds to the mature plant endophytic community is needed. Probably, bacteria are recruited from the environment during plant growth to complement the endophytic community already derived from the seeds. Consequently, the seed endophytes of the next generation can again be selected from this community.

The identification of plant-associated bacteria via culture-independent techniques remains very challenging and several aspects of the workflow can generate differences in the obtained community composition. Our choice of DNA extraction kit was based on a comparison of different commercially available kits (Beckers, 2015), as the DNA preparation method can already influence the composition of the analysed bacterial communities (Morgan *et al.*, 2010). Subsequently, we performed a pyrosequencing test run to select the most appropriate primerset combination for our analysis in order to obtain a high taxonomic coverage while minimizing coamplification of host DNA. In the present study, we used primers 799F and 1193R to sequence the V5-V7 hypervariable region of the 16S rDNA. However, the amplification of different hypervariable regions of the 16S rDNA can lead to different results concerning bacterial OTU richness and evenness (Wang and Qian, 2005; Engelbrektson,

2010). Moreover, the OTU with the highest number of sequences is not always the most abundant, as 16S rRNA operon copy numbers vary depending on the species (Bodenhausen *et al.*, 2013). Unfortunately, it was not possible to take this into account as identification on species level would be required. Finally, during the data analysis, we applied strict quality trimming on the amplicons and used a clustering threshold of 97% to reduce sequencing errors which otherwise can inflate diversity estimates (Kunin *et al.*, 2010). A definition of minimal experimental standards would be convenient to minimize the introduction of bias in the final result and to maximize the comparison and integration of data obtained by different studies.

Remarkably, it appeared that the genera that appeared most abundant in the cultivable endophytic community, mainly *Rhizobium* and *Pseudomonas*, coincide with the genera frequently detected during the culture-independent analysis. This indicates that our endophyte isolation procedure provided a representative image concerning the genera most abundantly present in the total bacterial community. Nevertheless, to ensure that also low-abundant bacteria are covered, a lot of colonies need to be picked. Moreover, during cultivation, fast-growing bacteria can mask the presence of slow-growing ones. To culture every cfu present in a sample, the limited dilution approach can be used: the sample is diluted in 96 well plates with liquid growth medium until every well receives a single bacterial cell, which will form a clonal population (Goodman *et al.*, 2011). These cultures can then be identified by sequencing of the 16S rDNA.

Future experiments should shed more light on how endophytes are selected by the plant to be incorporated into the seeds. This process probably already starts at the root-soil interface where plant root exudates drive a shift in the rhizospheric community (Bulgarelli *et al.*, 2013). The endophytic community can be fine-tuned by host-controlled mechanisms, such as interactions with the plant innate immune system, and by external factors, such as the presence of certain contaminants in the plant (Siciliano *et al.*, 2001; Bulgarelli *et al.*, 2013). However, the exact processes underlying these mechanisms are not yet known. Moreover, information concerning the specific functions of seed endophytes during plant development and growth remains limited. Sequencing of the full metagenome could identify enriched functional genes instead of enriched bacterial OTUs. Functions that are useful to the plant could be shared by several

bacterial species or each species could make its own specific contribution (Rott, 2012).

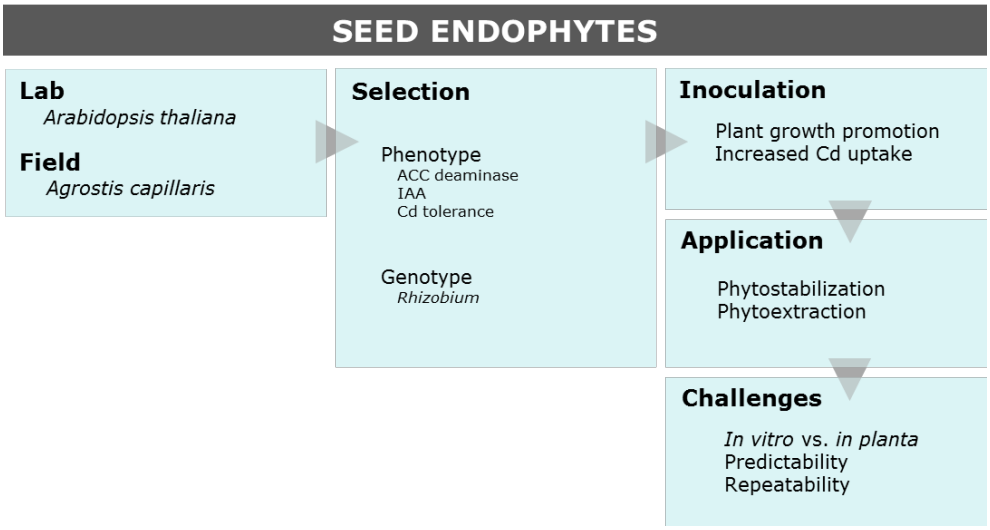

Figure 9.1: Seed endophytic communities of laboratory-grown *A. thaliana* and field-grown *A. capillaris* were studied. Selection of seed endophytes was primarily based on phenotypic characteristics such as ACC deaminase activity, IAA production and Cd tolerance. The only genus that was consistently transferred over generations was *Rhizobium* in *A. thaliana* seeds. Inoculation with selected seed endophytes improved plant growth and increased Cd uptake which could be beneficial during metal phytostabilization and phytoextraction. Increasing the inoculation success remains a future challenge.

In the second part of this thesis, the importance of bacterial seed endophytes for seedling establishment and plant growth was investigated. The effect of inoculation with the isolated seed endophytes on the growth and/or Cd uptake of their host was studied in order to evaluate their possible contribution to bacteria-assisted phytoremediation. Seed endophytes were selected based on their phenotypic traits and tested in a small-scale screening experiment before being applied in a more elaborate inoculation experiment.

We found that inoculation of *A. thaliana* with several of its seed endophytes on vertical agar plates or in a hydroponic culture led to improved plant growth upon Cd exposure (Fig. 7.3–7.6, 9.1). However, results of consecutive experiments were often below our expectations based on the initial screening experiment (Fig. 9.1). Variations in Cd concentration, inoculum size or inoculum composition did not resolve this problem. Recently, Bulgarelli *et al.* (2013) stated that the survival and colonization potential in the rhizosphere is often overlooked when

identifying plant growth promoting bacteria. Features such as motility, chemotaxis, attachment, growth and stress resistance contribute to the rhizosphere competence, which is a key factor for the successful application of plant growth promoting bacteria (Bulgarelli *et al.*, 2013). We demonstrated that different bacterial growth stages were associated with changes in motility, chemotactic behavior, pectinase activity and colonizing capacity (Table 8.1–8.3, Fig. 8.3). Nevertheless, we were not able to robustly correlate the differences in bacterial growth stage with differences in plant responses. Future experiments should also take into account the production of biocontrol agents such as siderophores, lytic enzymes and antibiotics. Our use of (nearly) sterile plant growth systems is an oversimplification, on the one hand allowing accurate re-isolation of the inoculated bacteria, but on the other hand ruling out any form of biocontrol mechanisms and competition that could contribute to rhizosphere and endosphere competence.

Inoculation of hydroponically grown *A. capillaris* with its seed endophytes resulted in a significantly improved plant growth if plants were not exposed to Cd (Fig. 7.7, 9.1). After inoculation of Cd-exposed plants, no effect on plant growth was observed but Cd uptake was significantly increased (Fig. 7.7, 7.8, 9.1). The endophytes present in the inoculated plants probably sequestered Cd, making it less available for its host (Dimpka *et al.*, 2009b), thereby allowing higher Cd concentrations in inoculated plants without apparent signs of phytotoxicity. This indicates that the establishment and growth of *A. capillaris* during phytostabilization and phytoextraction of Cd-contaminated soils might be enhanced by inoculation of the plants with their seed endophytes (Fig. 9.1). Grasses are good candidates for phytoremediation as they have the advantage of a rapid biomass production, extensive root growth and adaptability, and low management costs (Sabreen and Sugiyama, 2008). Grasses belonging to the *Agrostis* genus have been repeatedly used during phytostabilization trials (reviewed in Vangronsveld *et al.*, 2009).

The inoculated seed endophytes of *A. thaliana* and *A. capillaris* were selected based on their phenotypic characteristics. It appeared that these characteristics did not always allow to reliably predict the behavior of the respective strains *in planta* during Cd exposure (Fig. 9.1). Kuffner *et al.* (2010) found that metal mobilization experiments predicted the effects of bacteria inoculated in *Salix*

caprea more reliably than the standard tests for plant growth promoting traits. It would be useful to include this test in order to estimate the potential effect of a bacterial strain on phytoextraction efficiency. However, it remains necessary to test the bacteria in small-scale inoculation experiments, preferably first on a sterile substrate and afterwards in non-sterile soil, before classifying them as phytoextraction improvers (Weyens *et al.*, 2013). Moreover, inoculation of *A. thaliana* mutants impaired in perception of plant growth promoting factors could deliver interesting information concerning the exact mechanisms behind specific plant-bacteria interactions (Rott, 2012).

The selection of inoculants in our experiments was limited to the cultivable fraction of the bacterial endophytes. Recently, Kolbas *et al.* (2015) used crude seed extracts of a Cu-tolerant *A. capillaris* to inoculate sunflower in order to increase Cu tolerance and phytoextraction on a Cu-contaminated soil. This allows inclusion of the effects of the uncultivable fraction of the bacterial community. In addition, a compositional analysis of the filtered seed extract would enable identification of the components that contribute to the observed plant responses (Kolbas *et al.*, 2015).

We have been making attempts to study the colonization and migration pattern of the seed endophytes after inoculation. Seed endophyte 6, a *Rhizobium* strain isolated from *A. thaliana* seeds (Table 7.1) has been labelled with green fluorescent protein (gfp). After inoculation in its host, the gfp-labelled bacterium has only been detected in the root cortex (Fig. 9.2) and not in the xylem vessels. Further experiments using different strains, culturing conditions or labels, are needed to investigate how the inoculated bacteria become part of the seed endophytic community and ultimately if they can be retraced in the next generation of plants.

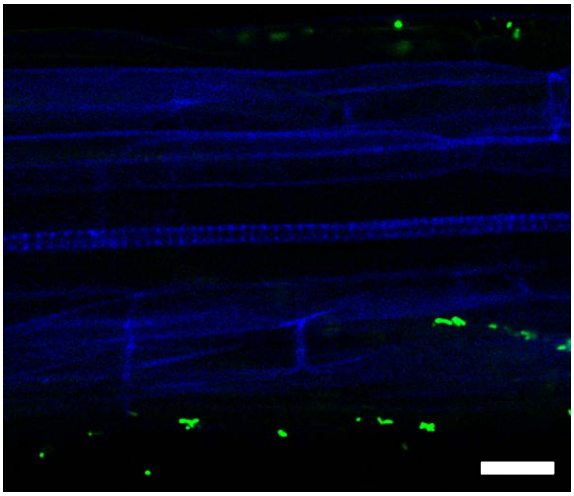

Figure 9.2: Localization of a gfp-labelled *Rhizobium* strain (green) in the roots of *A. thaliana* (blue). Scale bar: 20 µm.

In conclusion, plants appear to be able to select which bacteria will become seed endophytes. These bacteria are vertically transmitted to benefit the next generation of plants by providing plant growth and health promoting properties. The importance of seed endophytes is still often overlooked during the production of high quality seeds and seed storage, as preservation of the seed endophytes could contribute to an improved germination capacity, seedling establishment and plant growth. Moreover, seeds could be an excellent niche for the discovery of bacteria with useful characteristics. Seeds of economically important crops, such as maize, rice or rapeseed, could be an unprecedented source of endophytes applicable in biocontrol, biofertilization, bioenergy production and bioremediation.

APPENDIX

Appendix A

Sequencing of all 7 amplicon libraries generated a total of 799429 raw reads with an average of 114204 ± 12031 per library before quality trimming and assigning the reads to the different samples. The average length of the reads prior to trimming was 348 ± 139 bp, 349 ± 105 bp, 392 ± 105 bp, 361 ± 129 bp, 405 ± 96 bp, 401 ± 101 bp and 364 ± 105 bp for respectively primerset combination 1 until 7.

The homology between bacterial 16S rDNA, chloroplastidal 16S rDNA and mitochondrial 18S rDNA sequences makes the identification of plant-associated bacteria via culture-independent techniques very challenging. Therefore, all primerset combinations (Table 3.1) were compared for the average number of chloroplastidal 16S rDNA, mitochondrial 18S rDNA and bacterial 16S rDNA sequences they amplified from seeds and radicles. Primerset combination 5 and 7 did not amplify chloroplastidal 16S rDNA sequences (Table A1). All other primerset combinations amplified high amounts of chloroplastidal 16S rDNA sequences (between 60.9% and 99.9% of the total sequences obtained), except for primerset combination 4 and 6 used on the radicles where respectively 19.5% and 38.9% of the total sequences obtained were assigned to chloroplastidal sequences (Table A1). The number of mitochondrial 18S rDNA sequences that was coamplified was low for all primerset combinations (Table A1). Only primerset combinations 3 and 4 used on seed samples returned slightly higher amounts of mitochondrial 18S rDNA sequences, accounting for respectively 5.8% and 20.6% of the total sequences obtained (Table A1). The number of bacterial 16S rDNA sequences was high in radicle samples amplified with primerset combinations 4 until 7, accounting for 61.1% to 100% of the total sequences obtained (Table A1). However, the use of primerset combination 5 did not lead to amplification in the seeds. Primerset combination 7 also performed well on seeds where 98% of the obtained sequences were assigned to bacterial 16S rDNA sequences (Table A1).

Table A1: Number of chloroplastidal 16S rDNA, mitochondrial 18S rDNA and bacterial 16S rDNA sequences obtained with the different primerset combinations (1-7) in seeds and radicles. Values are averages of 1 or 3 biological replicates ± standard error. Values between brackets represent the average percentage of chloroplastidal/mitochondrial/bacterial sequences compared to the total number of sequences obtained. Different letters indicate significant differences ($p < 0.05$) between primerset combinations of the same row.

Chloroplastidal	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Seed	2895 ± 328^{a} (85.6)	3042 ± 456^{a} (99.9)	2697 ± 73^{a} (94.1)	1692 ± 214^{a} (78.6)	-	3079^{a} (96.7)	0^{b} (0)
Radicle	3929 ± 333^{a} (71.3)	3635 ± 21^{a} (89.0)	2074 ± 340^{b} (60.9)	781 ± 63^{cd} (19.5)	0^{c} (0)	1485 ± 131^{bd} (38.9)	0^{ce} (0)
Mitochondrial	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Seed	16 ± 7.8^{a} (0.48)	2.7 ± 1.5^{a} (0.09)	167 ± 29^{b} (5.8)	444 ± 47^{b} (20.6)	-	0^{a} (0)	36^{a} (2.0)
Radicle	9.3 ± 2.8^{a} (0.17)	3.7 ± 0.9^{a} (0.09)	31 ± 8^{b} (0.90)	92 ± 8^{c} (2.3)	0^{a} (0)	0.33 ± 0.3^{a} (0.009)	5.7 ± 0.9^{a} (0.24)
Bacterial	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Seed	470^{a} (13.9)	0.67 ± 0.33^{a} (0.02)	0.67 ± 0.33^{a} (0.02)	17 ± 5.7^{a} (0.79)	-	103^{a} (3.2)	1784^{b} (98.0)
Radicle	1569 ± 170^{a} (28.4)	446 ± 90^{b} (10.9)	1302 ± 15^{ab} (38.2)	3126 ± 183^{c} (78.2)	2981 ± 51^{c} (100)	2337 ± 140^{ac} (61.1)	2346 ± 534^{ac} (99.8)

Based on low coamplification of chloroplastidal and mitochondrial sequences and high retrieval of bacterial sequences, primerset combinations 4, 5 and 7 were selected for further analysis. Bacterial 16S rDNA sequences generated by these primerset combinations were subsampled at 1285 sequences. For seed samples, this analysis was only performed for primerset combination 7 as the number of bacterial sequences amplified was very low for primerset combination 4 and primerset combination 5 did not lead to any amplification at all. The Good's coverage estimate was very high for all primerset combinations used on seed and radicle samples (Table A2). In the radicles, the observed number of OTUs was comparable for all primerset combinations, while this appeared to be higher in seeds (Table A2). The inverse Simpson diversity index of primerset combination 4 was significantly lower compared to the other primerset combinations. However, in general, all inverse Simpson diversity indices were low but this could be inherent to the sample matrix (Table A2).

Table A2: Good's coverage estimates, observed number of OTUs (Sobs) and inverse Simpson diversity indices of radicle samples amplified with primerset combinations 4, 5 and 7 and seed samples amplified with primerset combination 7. Different letters indicate significant differences ($p < 0.05$) for the same matrix.

Matrix	**No.**	**Coverage (%) ± Stdev**	**Sobs ± Stdev**	**Inv. Simpson ± Stdev**
Seed	7	0.997 ± 0.00330	45.1 ± 2.00	2.4 ± 1.76
Radicle	4	0.995 ± 0.00022	12.0 ± 1.45	1.9 ± 0.18^{a}
	5	0.997 ± 0.00080	11.6 ± 1.86	2.4 ± 0.12^{b}
	7	0.997 ± 0.00042	13.7 ± 1.60	2.6 ± 0.10^{b}

At the phylum level, the majority of the OTUs in seeds and radicles were assigned consistently by all 3 primerset combinations to Alphaproteobacteria (62.94% to 75.47%) and Deltaproteobacteria (3.65% to 36.48%) (Table A3). The number of phyla detected was higher in seed samples compared to radicle samples, especially when using primerset combination 7 (Table A3). The number of unclassified sequences is low for all 3 primerset combinations in both sample types, ranging from 0.014% to 1.03% (Table A3).

Table A3: Phylum distribution (%) of sequences obtained from seeds and radicles amplified with primerset combinations 4, 5 and 7.

Phylum	**Seed**		**Radicle**		
	4	**7**	**4**	**5**	**7**
Acidobacteria	0	0.027	0	0	0
Actinobacteria	1.89	7.68	0	0	0
Bacteroidetes	3.77	3.10	0	0	0
Alphaproteobacteria	75.47	54.86	67.81	65.38	62.94
Betaproteobacteria	3.77	0.19	0.14	0.18	0.41
Gammaproteobacteria	0	0.082	0	0	0
Deltaproteobacteria	13.21	3.65	31.92	34.36	36.48
Firmicutes	0	1.80	0	0.011	0
TM7	0	27.09	0	0	0
Unclassified	1.89	1.03	0.064	0.067	0.014

OTU abundances generated with primerset combinations 4, 5 and 7 used on seed and radicle samples were compared using non-metric multi-dimensional scaling (NMDS). Replicates from each primerset combination grouped together, except for replicate a of primerset combination 4 used on seeds which separated from the other 2 replicates (Figure A1). This indicates that the primerset combinations produced repeatable results, but also that the choice of the primerset combination influenced the obtained result.

Based on the above mentioned findings, primerset combination 7 appeared to be the most suitable for our analysis and was used during the final run.

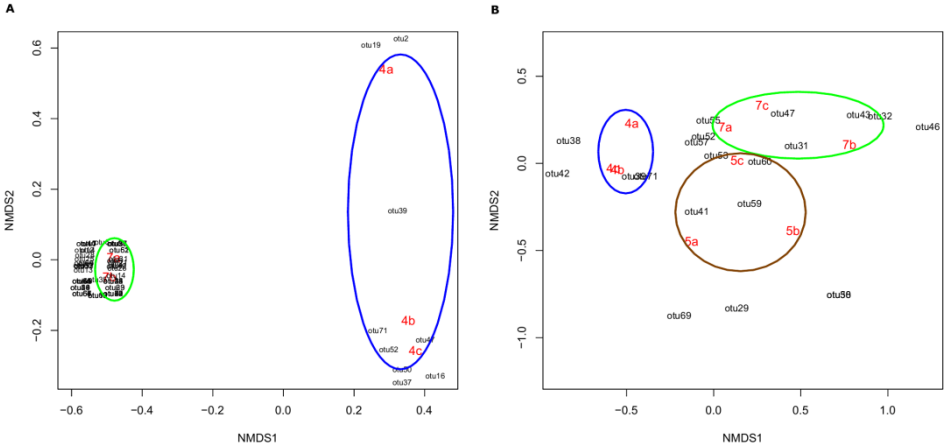

Figure A1: NMDS plot of the OTUs obtained with the 3 replicates (a-c) of the different primerset combinations used on seeds (A) and radicles (B). Blue = primerset combination 4, brown = primerset combination 5, green = primerset combination 7. Each number on the plot corresponds to an OTU as indicated in table A4.

Table A4: OTUs corresponding to the numbers used on the NMDS plot of figure A1. Uc = unclassified.

1	*Bryobacter*	37	Uc *Bradyrhizobiaceae*
2	*Rubrobacter*	38	Uc *Hyphomicrobiaceae*
3	*Corynebacterium*	39	Uc *Rhizobiales*
4	*Blastococcus*	40	Uc *Sphingomonadaceae*
5	*Microbacterium*	41	Uc Alphaproteobacteria
6	*Nocardioides*	42	*Variovorax*
7	*Propionibacterium*	43	*Shinella*
8	*Actinomycetospora*	44	Uc *Burkholderiaceae*
9	*Pseudonocardia*	45	Uc *Rhodocyclaceae*
10	*Kitasatospora*	46	Uc *Burkholderiales*
11	Uc *Geodermatophilaceae*	47	Uc *Comamonadaceae*
12	Uc *Kineosporiaceae*	48	Uc Betaproteobacteria
13	Uc *Microbacteriaceae*	49	Uc *Myxococcales*
14	Uc *Actinomycetales*	50	*Serratia*
15	Uc Actinobacteria	51	*Marinobacter*
16	*Sediminibacterium*	52	*Pseudomonas*
17	*Chryseobacterium*	53	*Stenotrophomonas*
18	*Cloacibacterium*	54	Uc *Enterobacteriaceae*
19	Uc *Cytophagaceae*	55	Uc *Pseudomonadaceae*
20	Uc *Porphyromonodaceae*	56	Uc *Sinobacteraceae*
21	Uc *Chitinophagaceae*	57	Uc *Xanthomonadaceae*
22	Uc *Sphingobacteriales*	58	Uc *Xanthomonadales*
23	Uc *Flavobacteriales*	59	Uc Gammaproteobacteria
24	Uc Bacteroidetes	60	Uc Proteobacteria
25	*Brevundimonas*	61	*Bacillus*
26	*Afipia*	62	*Staphylococcus*
27	*Bosea*	63	*Enterococcus*
28	*Methylobacterium*	64	*Weissella*
29	*Beijerinckia*	65	*Streptococcus*
30	*Ensifer*	66	Uc *Lactobacillales*
31	*Rhizobium*	67	Uc *Bacilli*
32	Uc *Rhizobiaceae*	68	Uc *Ruminococcaceae*
33	*Roseomonas*	69	Uc Firmicutes
34	*Sphingomonas*	70	TM7 genus incertae sedis
35	Uc *Rhodobacteraceae*	71	Uc Bacteria
36	Uc *Caulobacteraceae*		

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SCIENTIFIC OUTPUT

International journals

- Truyens S, Beckers B, Thijs S, Weyens N, Cuypers A, Vangronsveld J. 201x. Cadmium-induced and transgenerational changes in the cultivable and total seed endophytic community of *Arabidopsis thaliana*. Submitted to Plant Biology.
- Truyens S, Beckers B, Thijs S, Weyens N, Cuypers A, Vangronsveld J. 201x. The effects of the growth substrate on cultivable and total endophytic communities of *Arabidopsis thaliana*. Submitted to Plant and Soil.
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Oral presentations

- Vangronsveld J, Croes S, Janssen J, Truyens S, Thijs S, Weyens N. 2014. A role for plant-associated bacteria in metal mobilization and uptake by plants and phytoextraction? Plants, Heavy Metals, Environment Conference. 26-28 June. Katowice, Poland.
- Thijs S, Weyens N, Truyens S, Gkorezis P, Carleer R, Vangronsveld J. 2014. Grasses and bacteria joining forces: bioaugmented rhizoremediation with common bent for clean-up of 2,4,6-trinitrotoluene. The Warsaw Plant Health Initiative, 7FP-REGPOT-2011-1-286093, WP4 "Upgrade MICRO-ECOLOGY research teams mini-symposium and workshop. 28-30 May. Warsaw, Poland.
- Truyens S, Thijs S, Weyens N, Vangronsveld J. 2013. An *Arabidopsis* model for seed endophyte-assisted plant growth upon Cd exposure. 10th International Phytotechnologies Conference. 1-4 October. Syracuse, United States of America.
- Thijs S, Weyens N, Truyens S, Gkorezis P, D'Haen J, Carleer R, Vangronsveld J. 2013. Grasses and bacteria joining forces: bioaugmented rhizoremediation with common bent for clean-up of 2,4,6-trinitrotoluene. 10th International Phytotechnologies Conference. 1-4 October. Syracuse, United States of America.
- Truyens S, Weyens N, Cuypers A, Vangronsveld J. 2012. Long-term Cd exposure induces beneficial shifts in the seed endophytic population of *Arabidopsis thaliana*. 9th International Phytotechnology Society Conference. 11-14 September. Hasselt, Belgium.
- Thijs S, Gkorezis P, Truyens S, Weyens N, Vangronsveld J. 2012. The role of plant-associated bacteria for the improvement of phytoremediation of TNT-contaminated sites. 9th International Phytotechnology Society Conference. 11-14 September. Hasselt, Belgium.
- Vangronsveld J, Thijs S, Truyens S, van der Lelie D, Taghavi S, Weyens N. 2012. A role for plant-associated bacteria in remediation of contaminated soils and groundwater. 5th Symposium on Biosorption and Bioremediation. June 24 - 28. Prague, Czech Republic.
- Remans T, Truyens S, Thijs S, Schellingen K, Gielen H, Weyens N, Cuypers A, Vangronsveld J. 2011. Stress-specific morphological responses in *Arabidopsis thaliana* roots and the effect of plant-associated bacteria. 7th International

Symposium on the structure and function of roots. 5-9 September. Novy Skomovec, Slovakia.

- 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 Bioremediation Conference. 4-7 July. Chania, Greece.
- 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. 16-19 June. Pisa, Italy.
- Vangronsveld J, Truyens S, Dupae J, Taghavi S, van der Lelie D, Weyens N. 2010 Exploiting plant-bacteria partnerships to improve biomass production and remediation of contaminated soils and groundwater. 12th international symposium on biological nitrogen fixation with non-legumes. 3-8 October. Búzios, Brazil.
- Weyens N, Truyens S, Saenen E, Croes S, Beckers B, van der Lelie D, Vangronsveld J. 2010. Modified endophytes for improving phytoremediation of mixed contaminations of toxic metals (Ni) and organic contaminants (toluene or TCE). Phytotechnologies in the 21st century: challenges after Copenhagen 2009. Remediation-Energy-Health-Sustainability. 26-29 September. Parma, Italy.

Poster presentations

- Truyens S, Beckers B, Thijs S, Weyens N, Cuypers A, Vangronsveld J. 2015. Endophytic communities of *Arabidopsis thaliana*: does cultivation substrate matter? 6th Congress of European Microbiologists (FEMS). 7-11 June. Maastricht, The Netherlands.
- 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. 11-14 September. Hasselt, Belgium.
- Remans T, Truyens S, Thijs S, Weyens N, Schellingen K, Gielen H, Cuypers A, Vangronsveld J. 2011. Stress-specific root morphological responses in *Arabidopsis thaliana.* Phenodays. 12-14 October, Wageningen, the Netherlands.
- Truyens S, Weyens N, Saenen E, Opdenakker K, Dupae J, Cuypers A, Vangronsveld J. 2011. Inoculation of *Arabidopsis thaliana* with seed endophytes from Cd-selected plants enhances root growth upon exposure to Cd. 11th ICOBTE International Conference on the Biogeochemistry of Trace Elements. 3-7 July. Florence, Italy.

- Croes S, Weyens N, Truyens S, Vangronsveld J. 2011. Isolation of bacteria associated with *Brassica napus* L.: The characterization of cadmium-tolerant plant growth promoting bacteria and their potential to enhance phytoextraction. 11th ICOBTE International Conference on the Biogeochemistry of Trace Elements. 3-7 July. Florence, Italy.
- Dupae J, Weyens N, Cuypers A, Truyens S, Croes S, Vangronsveld J. 2010. Can DIGE analysis reveal the underlying mechanisms of enhanced plant growth, metal uptake and translocation caused by PGPBs equipped with a metal resistance system? Proteomlux 2010. 18-20 October. Luxembourg, Luxembourg.