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The potential role of plant-associated bacteria in metal uptake and metal translocation in *Nicotiana tabacum*

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Summary

It is known that rhizospheric and endophytic bacteria may carry out a variety of functions improving plant growth (Mastretta *et al.*, 2007). In agriculture they are under investigation as an alternative for chemical fertilizers to improve plant yield. These bacteria can also improve the nutrient status of their host plant due to biological nitrogen fixation (diazotrophy) and increasing nutrient availability into the rhizosphere through solubilization of minerals. Plant biomass production can also be increased via the synthesis of plant hormones by the bacteria. Plant-associated bacteria further seem to be able to reduce or prevent the deleterious effects of phytopathogenic organisms through competition, antibiosis and Induced Systemic Resistance (ISR) in plants. During recent years, their ubiquitous existence combined with the large variability of metabolic functions that they may carry out, attracted attention for their potential to improve phytoremediation.

This thesis aims to improve our understanding of the potential contribution of plant-associated bacteria, which naturally occur in *Nicotiana tabacum* grown on soils with increased metal contents, to improve metal uptake and translocation in their host plant. We compared the endophytic consortia associated with tobacco plants growing on two metal contaminated sites. These sites were different in physico-chemical soil characteristics but received similar fertilization treatments (Chapter 3). On both sites a phytoextraction feasibility study was being performed.

Besides a high phenotypic variability, similar taxa of plant-associated rhizosphere and endophytic bacteria were observed on both sites. A strong interaction between plant-associated bacteria and their host was suggested by the observed "correlation" between the percentage of metal tolerant bacteria and the plant's metal content. This indicated that the metals inside the tobacco plant are highly available to the endogenous bacterial community. A number of representative metal tolerant bacteria and a defined consortium composed of three strains were used for a series of re-inoculation experiments, whose aim was to investigate the effects of inoculation on plant growth and metal mobilization, uptake and distribution inside the plant. Cadmium was chosen as the test metal, as it represents one of the metals of major environmental

concern present in both field sites. The cadmium concentration used in the experiments was chosen to cause slightly visible symptoms of metal toxicity on the tobacco plant. The different inocula variably influenced the host plant (Chapter 5). A negative effect on plant growth was common to all the inocula with the exception of the consortium. This result could be due to a synergic effect of the strains composing the consortium.

We analysed the endophytic bacteria present in the seeds harvested from the tobacco plants grown on one of the two field sites (Chapter 6). The consortium composition was studied after three different periods of seed storage. With increasing storage time, an evolution in metal tolerance of the seed endophytes from a high to a lower percentage was observed in parallel with a decrease in the cultivable population. The bacteria inhabiting the seeds were mainly isolated from the embryo. In order to establish a level of correlation between them and the previously isolated bacteria from the different plant parts and the rhizosphere their phylogenetic relationship was investigated by constructing a Neighbour Joining tree based on their 16S rDNA. The results suggest a systematic "migration" of bacteria from the rhizosphere up inside the plant till they reach the seed interior. *Pseudomonas fulva*, isolated from the seeds, was an exception as it formed a separate phylogenetic group from the other plant-associated Pseudomonads, suggesting another origin than the rhizosphere. The strict interaction between the seed endophytes and the embryo led us to hypothesize effects of these bacteria on plant growth. A series of experiments was performed in which sterile tobacco plants were inoculated with either single strains or defined consortia of seed endophytes, and subsequently exposed or not to cadmium (Chapter 7). In general a positive effect of the inocula on plant growth, metal uptake and translocation was observed. These results strongly suggest an active role of the seed endophytic bacteria in plant metal uptake and translocation. This positive result obtained with the seed endophytes opens the way for future tests in the greenhouse and the field, aiming to ameliorate and increase the efficiency of metal phytoextraction.

Sunto

E' noto che i batteri endofitici e rizosferici svolgono diverse funzioni che possono contribuire ad una migliore crescita della pianta (Mastretta *et al.*, 2007). In agricoltura questi batteri vengono studiati come alternativa ai fertilizzanti chimici per un incremento della produzione vegetale. Questi batteri possono inoltre migliorare lo *status* dei nutrienti nella pianta ospite grazie alla fissazione biologica dell'azoto (diazotrofia) e, attraverso la solubilizzazione dei minerali, all'incremento della disponibilità di nutrienti nella rizosfera. La produzione di biomassa vegetale può inoltre essere accresciuta attraverso la sintesi batterica di fitormoni. Tra gli effetti rilevati finora nei batteri associati alle piante è inclusa la riduzione o prevenzione degli effetti deleteri apportati da organismi fitopatogeni, attraverso diversi meccanismi quali competizione tra organismi, produzione di antibiotici ed induzione del sistema di resistenza delle piante (ISR). Recentemente, la loro onnipresenza unita alla grande variabilità di funzioni metaboliche che sono in grado di svolgere, hanno attratto l'attenzione per un potenziale utilizzo dei batteri associati alle piante nel miglioramento della *phytoremediation*.

Questa tesi si propone di ampliare le nostre conoscenze sul potenziale contributo dei batteri associati, naturalmente presenti in *Nicotiana tabacum* L. cresciuta su suoli con maggiore contenuto di metalli, nel miglioramento dell'assorbimento e della traslocazione dei metalli nella pianta ospite. Abbiamo paragonato i consorzi endofitici associati con piante di tabacco cresciute in due diversi siti contaminati da metalli pesanti. Questi siti si differenziavano per caratteristiche fisico-chimiche, ma sono stati sottoposti a fertilizzazione simile (capitolo 3). In entrambi i siti era in corso uno studio di fattibilità sull'utilizzo della fitoestrazione quale tecnica di bonifica.

Accanto ad un'elevata variabilità fenotipica, in entrambi i siti si è riscontrata, nella rizosfera e nei batteri endofitici, una chiara omogeneità a livello di *taxa*. La correlazione osservata tra la percentuale di batteri metallo-tolleranti e il contenuto di metalli all'interno della pianta ha suggerito una forte interazione tra i batteri associati alle piante e le piante stesse. Ciò suggerisce che i metalli all'interno della pianta di tabacco sono altamente disponibili alla comunità batterica endogena. Un certo numero di batteri metallo-tolleranti, ed un

consorzio definito composto da tre famiglie batteriche, sono stati usati per una serie di esperimenti di re-inoculo mirati allo studio degli effetti apportati dagli inoculi stessi sulla crescita della pianta e sulla mobilizzazione, assorbimento e distribuzione dei metalli all'interno di questa. Dal momento che il cadmio rappresenta uno dei più pericolosi metalli inquinanti presente in entrambi i suoli di provenienza delle piante, esso è stato scelto anche come metallo test. La concentrazione di cadmio usata negli esperimenti è stata scelta in modo che causasse lievi sintomi di tossicità nelle piante di tabacco testate. I vari inoculi hanno diversamente influito sulla pianta ospite (capitolo 5). Generalmente tutti gli inoculi, con eccezione del consorzio, hanno negativamente influito sulla sua crescita. Questo risultato potrebbe essere dovuto all'effetto sinergico tra le diverse famiglie formanti il consorzio.

Abbiamo analizzato i batteri endofitici presenti all'interno dei semi raccolti da piante di tabacco cresciute su uno dei due campi in esame (capitolo 6). La composizione del consorzio è stata studiata durante tre diversi periodi di stoccaggio dei semi. All'aumento del tempo di stoccaggio, è stata osservata una diminuzione della tolleranza al metallo negli endofiti del seme, in parallelo ad un'analoga diminuzione della popolazione dei batteri coltivabili. I batteri presenti all'interno dei semi sono stati isolati prevalentemente dall'embrione. Allo scopo di valutare un'eventuale relazione filogenetica tra questi batteri e quelli precedentemente isolati sia dalle diverse porzioni della pianta sia dalla rizosfera, è stato costruito un albero Neighbour Joining basato sul 16S rDNA. Il risultato suggerisce una migrazione sistematica dei batteri dalla rizosfera all'interno della pianta e da questa ai semi. *Pseudomonas fulva*, isolata dai semi, ha formato un gruppo filogenetico separato dalle altre *Pseudomonadales* associate alle piante, risultando un'eccezione che suggerisce un'origine diversa da quella della rizosfera. La stretta interazione esistente tra endofiti del seme ed embrione ci ha permesso di ipotizzare l'esistenza di effetti di questi batteri sulla crescita della pianta. E' stata condotta una serie di esperimenti in cui piante di tabacco sterili sono state inoculate o con singole famiglie batteriche o con consorzi definiti di endofiti dei semi, e successivamente esposte o no al cadmio (capitolo 7). In generale tutti gli inoculi hanno positivamente influito sulla crescita della pianta, sull'assorbimento del metallo e sulla sua traslocazione. Questi risultati supportano fortemente l'idea di un ruolo attivo giocato dai batteri endofitici dei

semi nell'assorbimento del metallo da parte della pianta e nella traslocazione dello stesso. Questo risultato positivo ottenuto con gli endofiti del seme apre la strada a sperimentazioni future in serra e in campo, con lo scopo di migliorare ed incrementare l'efficienza della fitoestrazione dei metalli.

Chapter 1: Introduction

Bacterial and fungal symbionts exist across all areas of life, from the bacteria that colonize the human intestinal tract to the ancient cells that became the plastids in plants cells, and the mitochondria that are present in almost all eukaryotic cells. The associations of plant associated microorganisms with their hosts are varied and complex and we are only approaching to understand these interactions starting analysing from the part surrounding the root up to the leaf epidermide.

In the soil, the population density of microorganisms, especially bacteria, is several orders of magnitude higher in the rhizosphere compared with the bulk soil (Reynolds *et al.*, 1999). Roots, in fact, act as a source of organic carbon, controlling in this way the exogenous metabolic activities of rhizosphere microorganisms consisting of help acquire phosphorous, potassium and to some extent nitrogen from soils mainly via their effects on root morphology and physiology (Cocking, 2003). The positive effect of rhizospheric bacteria on plant growth and yield includes the secretion of plant growth regulators such as auxins, gibberellins and cytokinins. This has led to speculation that such complementary interactions may be part of a plant's response to chemical stress in the soil (Walton *et al.*, 1994).

Motility and chemotaxis are key characteristics of plant-growth-promoting rhizobacteria and could thus play an important role in the interaction process, yet the role of motility and chemotaxis in plant-microbe interaction is poorly understood (Sood, 2003). Chemotactic responses of plant-growth-promoting bacteria and endophytic bacteria towards root exudates have been reported from rice (Bacilio-Jimenez *et al.*, 2003). Recently has been identified an operon in which are organized two components, hlpA and hlpB, that revealed to be connected with the bacterial cell capability to attach to the root surface (Molina *et al.*, 2006).

Rhizobium and other bacteria are known to colonize root nodules and enter into a symbiotic relationship with a plant, whereby the plant protects and supplies nutrients to the bacteria, and the bacteria provide nitrogen to the plant (Schultze and Kondorosi, 2002). The host plant preinfection steps take place with the bacterial cells outside the root tissues, they comprise: rhizobial

chemotactic approach to the root, root colonization, attachment to root surfaces, particularly to emerging root hairs, hair deformation and curling, and induction at a distance of a meristem and cortex proliferation at special locations or foci in the root (Long, 1989; Vincent, 1980). Some of these events require the participation of the legume root exudates among which many are rhizobial chemoattractants (Bergman *et al.*, 1988; Currier and Strobel, 1987; Gaworzewska and Carlile, 1982; Gotz *et al.*, 1982).
However, we now know that plant endophytes go well beyond the well-studied root nodules, and exist within the leaf, root and vascular tissue of the plant (Zinniel *et al.,* 2002; Lodewyckx *et al.,* 2002). The large number of reports on these so called endophytic bacteria clearly indicates that such bacteria exist in a variety of tissue types within numerous plant species, suggesting an ubiquitous existence in most if not all higher plants.

Plant endophytes have been studied since the 1940's (Tervet and Hollis, 1948; Hollis, 1951), with a great deal of research done on fungi. Endophytes undertake a variety of interactions with plants, from active pathogens, opportunist pathogens, bacteria that just exist within the plant and gain some physical protection, to bacteria that actively interact with their host plant for the betterment of both. As a result of these various plant-endophyte interactions, a variety of definitions has been proposed to the term endophyte and consideration of each leads to different interpretations**.** Kloepper *et al.* (1992) called bacteria, found within tissues internal to the epidermis, endophytes. Since quiescent endophytic bacteria can become pathogenic under certain conditions and or within different host genotypes (Misaghi and Donndelinger, 1990), James and Olivares (1997) adjusted the definition and stated that all bacteria that colonise the interior of plants, including active and latent pathogens, can be considered as endophytes. Considering all bacteria that colonise the interior of plants, one should also take into account those bacteria that reside within living plant tissues without doing substantive harm or gaining benefit other than securing residency (Kado, 1992) and bacteria that establish endosymbiosis with the plant whereby the plant receives an ecological benefit from the presence of the symbiont (Quispel, 1992). All these sub-definitions may give an overview of what is considered to be endophytic by the quoted authors and this might

consequently be regarded as the most general definition of what an endophyte stands for.

1.2. Ecology of Endophytic Bacteria

Endophytic bacteria have been isolated from both monocotyledonous and dicotyledonous plants that range from woody tree species such as oak (Brooks *et al.,* 1994), pear (Whitesides and Spotts, 1991) poplar (Germaine *et al.*, 2004, Taghavi *et al.*, 2005, Porteous-Moore *et al.*, 2006), citrus plants (Araújo *et al.,* 2002), *Mimosa pudica* (Pandey *et al.,* 2005) and pine seeds (Cankar *et al.,* 2005), to herbaceous crop plants such as sugar beets (Jacobs *et al.,* 1985), sugarcane (Loiret *et al.,* 2004), wheat (Coombs and Franco, 2003), maize (Fischer *et al.,* 1992; Gutierrez-Zamora and Martinez-Romero, 2001; Lalande *et al.,* 1989; McInroy and Kloepper, 1995), *Thlaspi caerulescens* (Lodewyckx *et al.,* 2002), yellow lupine (Lodewyckx *et al.,* 2001), tall festuca (Malinowski *et al.,* 2004), *Echinacea* (Lata *et al*. 2006), *Brassica campestris* (Poonguzhali *et al*. 2006), *Conzattia multiflora* (Wang *et al*. 2006) and different grass species (Dalton *et al.,* 2004; Zinniel *et al.,* 2002).

1.2.1 Most common species of endophytic bacteria

In general, the highest endophytic densities are observed in the roots and decrease from the stem to the leaves (Quadt-Hallman and Kloepper, 1996; Lamb *et al.,* 1996; Porteous-Moore *et al.,* 2005). This is consistent with the fact that many endophytic bacteria can also exist in the rhizosphere and that their preferred path of entering the plant is via the lateral roots (Sprent and de Faria, 1988; Sturz and Nowak, 2000). It should be noted that attempts to evaluate total populations of cultivable bacteria in plants may produce varied results according to the growth medium used for isolation, the method of surface disinfection used, variations in growth conditions of the host plant, and the state in which the plant tissue was used, as is also the case for plant associated rhizosphere bacteria (Kloepper and Beauchamp, 1992; Porteous Moore *et al.*, 2005). In general, *Pseudomonaceae*, *Burkholderiaceae* and *Enterobacteriaceae* are among the most common genera of cultivable endophytic species found. This was first observed by Gardner *et al.* (1982) who identified bacteria present

in the xylem fluid from the roots of the rough lemon rootstock of the Florida citrus tree. Among the thirteen genera found, the most frequently occurring genera were *Pseudomonas* (40%) and *Enterobacter* (18%). A similar observation has been made when, isolating cultivable endophytic bacteria from poplar, *Pseudomonas*, *Stenotrophomonas* and *Enterobacter* were fond to be the dominant cultivable bacteria (Taghavi *et al.*, 2005). At this moment, the genomes of several of these endophytes, *P. putida* W619, *Enterobacter sp*. 638, *Stenotrophomonas maltophilia* R551-3 and *Serratia proteamaculans* 568 are being sequenced. The genomes of these strains will be compared with those of closely related rhizosphere strains in order to determine which properties are necessary for endophytic behaviour (van der Lelie D., personal communication).

In another study the diversity of endophytic bacteria found in association with poplar was investigated as part of a larger study to assess the possibility and practicality of using endophytic bacteria to enhance *in situ* phytoremediation (Porteous Moore *et al.*, 2006). Endophytic bacteria were isolated from the root, stem and leaf from two cultivars of *Populus trichocarpa* x *deltoides* growing on a site contaminated with BTEX compounds. One hundred and twenty-one stable, morphologically-distinct isolates were obtained, belonging to twenty-one genera. Most of them were Gram-negative, from which the Gamma-proteobacteria dominated the collection of isolates, comprising 59% of the total strains. They included *Pseudomonas* sp. (42%), with smaller percentages of *Xanthomonas*, *Acinetobacter* and *Enterobacter*. The dominance of *Pseudomonas* sp. was not unexpected, as it has been previously observed that in many cases Pseudomonads are abundant in both the soil environment and the plant interior (Gardner *et al.*, 1982; Hallmann *et al.*, 1995; Rademaker *et al.*, 1998; Siciliano *et al.*, 2001). The Beta-proteobacteria made up 18% of the isolate collection, with *Burkholderia* sp. and *Herbaspirillum* sp. representing the majority of this group. The Alpha-proteobacteria group formed 10% of the total number of isolates and was largely represented by *Sphingomonas* (9%). Gram-positive bacteria comprised 13% of the total number of isolates, and were represented largely by *Arthrobacter*, *Bacillus*, *Paenibacillus* and *Agreia* species (Porteous Moore *et al.*, 2005). Six isolates could not be identified with confidence to a genus. The distribution of the isolated endophytic bacteria exhibited marked

spatial compartmentalisation within the plant, suggesting there are likely to be species-specific and non-specific associations between bacteria and plants.

Similar results were also found by Poonguzhali *et al*. (2006) during the identification of endophytic bacteria isolated from *Brassica campestris* ssp *pekinensis.* 83% of the identifications represented 9 and 14 different known bacterial genera from the rhizosphere and the root interior respectively from *Proteobacteria* (*α, β* and *γ*), firmicules (Actinobacteria and the *Bacillus* group) and Bacteroides.

For practical applications, where endophytic bacteria are engineered and subsequently used as a carrier to introduce new properties into their host plant, we are only interested in the subset of cultivable microorganisms. The advantage of the above-mentioned bacterial families is that many of their members can be easily manipulated to express new properties by either natural gene transfer or transformation. The application of these engineered endophytic strains for improved phytoremediation will be discussed below.

1.2.2 Culture dependent and independent methods to enumerate and characterize plant-associated communities

The research on endophytic bacteria has concentrated on the cultivable members of different endophyic communities. These are in general isolated after surface sterilization of the plant material. However, no surface disinfection protocols exist that result in the complete killing of surface bacteria on 100% of samples without penetrating interior tissues and thereby killing internal colonists, thus influencing the recoverable endophytic population. Therefore, comparisons between different studies should be carefully evaluated taking into account the different surface sterilization methods and conditions used. We optimized sterilization protocols for the recovery of endophytic bacteria from different parts of poplar (Porteous-Moore *et al.*, 2005), willow, yellow lupine (Lodewyckx *et al.*, 2001), *Thlaspi caerulescens* (Lodewyckx *et al.*, 2002), Brassica (Lodewyckx, PhD thesis), and tobacco (Mastretta *et al.*, unpublished see chapter 3).

In addition to cultivation-based methods, culture-independent methods should be used to obtain a more complete insight into the composition of plant associated microbial communities. The recent surge of research in molecular microbial ecology and metagenomic sequencing has provided compelling evidence for the existence of many novel types of microorganisms, in numbers and varieties far greater than those amenable to laboratory cultivation, and it has been estimated that cultivable organisms comprise less than 1% of all microorganisms (Amann *et al.,* 1995). In addition we observed that many endophytic bacteria, even after their initial isolation in cultivation, could not be propagated under laboratory conditions, justifying the analysis of their communities with molecular techniques.

A variety of comprehensive DNA-based techniques have been developed to identify, characterize and compare whole genomes of organisms, either independently or as members of communities. These techniques include cloning plus sequencing of ribosomal rRNA genes (Amann *et al.,* 1995), amplified fragment length polymorphism (AFLP) (Vos *et al.,* 1995), terminal restriction fragment length polymorphism (T-RFLP) (Marsh, 1997), denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.,* 1993), amplified rDNA restriction analysis, (ARDRA) (Vaneechoutte *et al.,* 1992), and restriction landmark genome scanning (RLGS) (Hirotsune *et al.,* 1992). Recently, serial analysis of ribosomal sequence tags (SARST) was developed as a novel technique for characterizing microbial community composition. The SARST method captures sequence information from concatenates of short PCR amplicons (tags) derived from the 16S rDNAs from complex bacterial populations. Depending on the protocol, tags are generated from either the V1 (Neufeld *et al.,* 2004) or V6 hyper-variable regions (Kysela *et al.,* 2005) of bacterial 16S rDNA genes. The major advantage of the SARST method is the high throughput generation of sequence data that can be directly used for species identification and comparisons between different experiments. Some of these molecular techniques have been employed to analyse to composition of endophytic communities. Sessitsch *et al.* (2001) amplified and subsequently sequenced 16S rRNA genes to analyse the populations of endophytic bacteria in three potato cultivars. Conn and Franco (2004) used T-RFLP to study the effect of microbial inoculants on the indigenous actinobacterial endophyte population in the roots of wheat. They observed that

the application of a commercial mixed inoculum in the soil reduced the endophytic actinobacterial diversity from 40 genera to 21 genera and reduced the detectable root colonization by approximately half. Their results indicate that the addition of a non-adapted microbial inoculum to the soil disrupted the natural actinobacterial endophyte population, reducing diversity and colonization levels. This finding was in contrast to the results obtained after the addition of a single actinobacterial endophyte to the wheat plant, where an increase in colonization level could be confirmed even though the indigenous endophyte population was not adversely affected. Tan *et al.* (2001) used 16S-23S Ribosomal DNA Intergenic Spacer-Targeted PCR for the differentiation of closely related rhizobial taxa and for the development of PCR protocols allowing the specific detection of strains to follow up the colonization of rice (*Oryza sativa*) roots by *Bradyrhizobium* and *Rhizobium* strains.

Besides their application for phylogenetic identification, molecular tools can be used to specifically address the metabolic potential of the plant-associated bacteria. Primers that allow the specific amplification of genes encoding important bacterial traits, such as the *nif* genes required for nitrogen fixation (Simonet *et al.,* 1991), genes involved in the degradation of organics (Hendrickx *et al.,* 2005) or the synthesis of secondary metabolites (Wawrik *et al.,* 2005), can be used to examine the potential of the endophytic population to participate in important processes within its host plant.

A new approach, made possible by the breakthrough in sequencing output, is metagenome sequencing. Examples so far include the construction and sequencing of metagenome libraries from the Saragossa sea (Venter *et al.*, 2004) and a microbial community found in acid mine drainage. Metagenomic analysis involves DNA isolation from an environmental sample, library construction and functional analyses (Handelsman, 2004). Efficiently extracting bacterial DNA from host plant tissues without the presence of contaminating plant DNA is central to constructing a metagenomic library that harbours sufficient copies of interesting genomes from plant associated bacteria. Jao *et al.* (2006) first attempted to enrich for plant-associated microbes of cultured- and uncultured-origin, and then used seeds of another maytansinoid-producing plant (*Trewia nudiflora*) to confirm the feasibility of their method for microbe

enrichment in plant tissues. However unless technical breakthroughs in DNA isolation of non-cultivable microorganisms from plant tissue without contaminating plant material, metagenome analysis will not be feasible. The recent sequencing of the poplar genome (Tuskan *et al*., 2006) provides however a first insight in the total endophytic community associated with tree. Though the plant material used for plant DNA extraction was surface sterilized, the 0.16% of the total number of end reads was from DNA from archaea, bacteria and fungi, suggesting that these organisms may in fact be *Populus* endophytes. Moreover, Tuskan *et al.* (2006) confirmed the presence of several taxa as *Rhizobium tropic* and *Pseudomonas putida*, already isolated from *Populus* in other studies (Doty *et al*., 2005; Germaine *et al*., 2004).

1.2.3 Inoculation of plants with endophytic bacteria

The fact that endophytic colonisation appears especially abundant in root tissue (Quadt-Hallman and Kloepper, 1996; Lamb *et al.,* 1996; Porteous-Moore *et al.,* 2005) may reflect that the root is the primary site where endophytes gain entry into plants. With the exception of seed transmitted bacteria, which are already present in the plant, potential endophytes must first colonise the root surface prior to entering the plant. This might explain the close relationship between endophytic and rhizosphere colonizing bacteria: many facultative endophytic bacteria can also survive as rhizosphere bacteria.

Under natural conditions, endophytic bacteria find their host by chemotaxis, electrotaxis, or accidental encounter. Root colonization is relatively fast, as Gamalero *et al.* (2004) observed monitoring the colonization of tomato roots by *Pseudomonas fluorescens* A6RI during the first seven days. It starts from single cells distributed in two root zones, (1) the apex and (2) elongation zone and the hairy zone, already within the first three days, followed by a redistribution of the inocula. In 5-7 days old plants in the apex and elongation as well as the young hairy zone, only few bacteria were detectable, while the hairy zone and the old hairy zone with the collar were mostly covered by cells localized between epidermal root cells and organized in pairs and strings respectively. In *Zea mays* Humphris *et al*. (2005) could observe that the root tip is protected by the border cells and their associated mucilage not only from the immediate infection of

pathogens but also from the complete colonization of benefit bacteria as *Pseudomonas fluorescens* SBW25, probably by acting as a disposable surface or sheath around the cap. The main entry for endophytic bacteria appears to be through wounds that naturally occur as a result of plant growth, or through root hairs and at epidermal conjunctions (Sprent and de Faria, 1988). This was confirmed by several microscopic studies (Wiehe *et al.,* 1994; Benhamou *et al.* 1996a, 1996b, Pan *et al.,* 1997). Artificial wounding was shown to contribute to increased endophytic colonization in roots of plants compared to intact roots (Gagné *et al.,* 1987). Besides providing entry avenues, wounds also create favourable conditions for the approaching bacteria by allowing leakage of plant exudates, which serve as a nutrients source for the bacteria.

Wounds and lateral roots are not, however, absolutely required for entrance of endophytic bacteria. Endophytic bacteria penetrated seedlings grown with minimal disturbance in liquid media or on agar long before lateral root emerged (Levanoy *et al.,* 1989; Quadt-Hallmann *et al.,* 1997). Since untreated control plants were endophyte free, the observed bacterial behaviour indicated active penetration. It is known that some PGPR endophytic species possess cellulase and pectinase activities (Kovtunovych *et al.,* 1999; Verma *et al.,* 2001) that could contribute to the infection of their host plant, even if the most lignified and/or suberized layers could still act as a plant barrier to such bacteria (McCulley, 2001). This hypothesis is for instance supported by the presence of cellulytic and pectinolytic enzymes produced by numerous endophytic bacteria such as *Azoarcus* sp. (Hurek *et al.,* 1994), *Azospirillum irakense* (Khammas and Kaiser, 1991), and *Pseudomonas fluorescens* (Duijff *et al.,* 1997; Benhamou *et al.,* 1996a; Quadt-Hallmann *et al.,* 1997). Enzymatic degradation of plant cell walls by these bacteria was only observed during colonization of the root epidermis, but never after colonising the intercellular spaces of the root cortex. These results suggest that the endophyte induced production of cellulase and pectinase only serves for penetration into the host plant. Although these observations demonstrate the possibility of active penetration mechanisms for some endophytic bacteria, very little is known about the origin and regulation of these enzymes. It is assumed that these bacteria must possess some regulatory mechanism to specifically regulate their enzyme production in terms of quantity and time of expression.

Another possibility to colonize their host plant is the use of vector organisms to gain entrance to the apoplastic spaces, as was shown by several authors (Ashbolt and Inkerman, 1990; Franke *et al.,* 2000) who could relate *Gluconacetobacter diazotrophicus* colonization of its host plants to infection via pink sugarcane mealy bug (*Saccharicoccus sacchari*) or via arbuscular mycorrhizae. Also insects can be used as vectors for endophytic infection, as was shown by Kluepfel (1993).

Although the root zone offers the most obvious site of entry for many endophytes, entry may also occur at sites on aerial portions of plants. Sharrock *et al.* (1991) suggested that in some cases endophytic populations within fruit might arise by entry through flowers. Penetration is also supposed to occur through natural openings on the leaves (e.g. stomata) or through stem lenticels (Kluepfel, 1993).

Once inside plant tissue, endophytic bacteria either remain localised in a specific plant tissue like the root cortex, or colonise the plant systematically by transport through the vascular system or the apoplast (Hurek *et al.,* 1994; James *et al.,* 1994; Mahaffee *et al.,* 1997; Quadt-Hallmann *et al.,* 1997, Patriquin and DÖbereinner, 1978). This difference in distribution might be due to interactions with other bacteria or to different requirements of each microorganism that let them inhabit different niches, represented by a tissue and more specifically by intercellular spaces inside each tissue (Di Fiori and Del Gallo, 1995).

Inside the plant endophytic populations have been observed to grow to between 10^{+2} and 10^{+7} cells per gram of fresh tissue (Shishido *et al.,* 1999; Taghavi *et al.,* 2005). The major difference between beneficial endophytic bacteria and pathogenic species is that they don't cause disease symptoms, this despite their high numbers. For *Xylella fastidiosa*, which causes Pierce's disease of grapevine (*Vitis vinifera*) as well as several other major agricultural diseases but is a benign endophyte in most host plants, a direct correlation seems to exist between pathogenic behaviour and blockage of vessels by increased levels of colonization (Newman *et al.,* 2003). Using a *gfp*-labeled *X. fastidiosa* strain it was observed that in symptomatic leaves, the fraction of vessels colonized by *X. fastidiosa* was fivefold higher than in nearby asymptomatic leaves. The fraction

of vessels completely blocked by *X. fastidiosa* colonies increased 40-fold in symptomatic leaves and was the feature of colonization most dramatically linked to symptoms. Therefore, the extent of vessel blockage by bacterial colonization is highly likely to be a crucial variable in symptom expression. Intriguingly, the authors also observed that a high proportion (>80%) of colonized vessels were not blocked in infected leaves and instead had small colonies or solitary cells, suggesting that vessel blockage is not a colonization strategy employed by the pathogen but, rather, a by-product of endophytic colonization.

There are a few studies that report on the intracellular presence of endophytes. This seems to be an occasional finding, except for particular cases such as *Rhizobium* or *Alcaligenes faecalis* (You *et al.,* 1983 and 1991) where both bacteria are enveloped by the plant in specialized structures.

There exists increasing evidence indicating that plants, like animals, use a component of the innate immune system, the basal resistance (BR), to defend themselves against foreign organisms. Tao *et al.* (2003), studying the interaction between *Arabidopsis* and *P. syringae*, differentiated between two interaction levels, low (compatible interaction) and high (incompatible and non-host interaction). This observation was supported by Szatmari *et al.* (2006) for tobacco plants. They also assumed that the general defence response of plants contributed to the inhibition of the proliferation of a wide range of bacteria that were able to enter the intercellular spaces, unless these bacteria used special suppressor efforts. The fact that bacteria seem to be capable of colonising the internal tissues of plants could confer an ecological advantage over bacteria that can only colonise plants epiphytically. The internal tissues of plants are thought to provide a more uniform and protective environment for microorganisms than plant surfaces, where exposure to extreme environmental conditions such as temperature, osmotic potentials, and ultraviolet radiation are major factors limiting long-term bacterial survival (Reinhold-Hurek and Hurek, 1998). However, there are probably other limiting factors that must be overcome when establishing populations in the internal tissues of plants. Thus, establishing and maintaining an introduced bacterial population would still be limited and influenced by the same factors that affect plant health.

In practice, it turns out to be quite easy to inoculate plants with endophytic bacteria. In the case of poplar it is sufficient to place rooting cuttings for 2 to 3 days in a solution of endophytic bacteria, after which the plants are transferred onto a solid substrate. However, evidence of their distribution as endophytes is required to prove their endorhizosphere competence. A commonly used strategy to visualize bacteria-plant interactions, including endophytic colonization, is to construct derivatives of endophytic strains that are expressing the green fluorescent protein (*gfp*) and to use confocal laser-scanning microscopy to visualize colonization of the host plant. Gage and colleagues (1996) used *gfp* expression to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). Expression of *gfp* was also used to study the endophytic colonisation of plants by the biocontrol agent *Rhizobium etli* G12 in relation to *Meloidogyne incognita* infection (Hallmann *et al.,* 2000), the colonization pattern of the biocontrol strain *Pseudomonas chlororaphis* MA 342 on barley seeds (Tombolini *et al.,* 1999), and the colonization of *Vitis vinifera* by *Xylella fastidiosa* (Newman *et al.,* 2003). Coomb and Franco (2003) isolated endophytic filamentous actinobacteria from surface-sterilized roots of wheat plants and subsequently one of these endophytes, *Streptomyces* sp. strain EN27 that was tagged with the *egfp* gene, was used to study the colonization of germinating wheat seed. The *egfp* gene encodes a derivative of the green fluorescent protein (*gfp*) gene whose expression has been optimized for actinobacteria (Sun *et al.,* 1999) and has been coupled to a constitutively expressed promoter, *ermEp* (Schmitt-John and Engels, 1992). Using this *egfp* labelled derivative the authors observed endophytic colonization from a very early stage of plant development, with colonization of the embryo, endosperm, and emerging radicle.

Germaine *et al.* (2004) also used *gfp* to study the re-colonisation patterns of three green fluorescent protein (*gfp*):kanamycin-resistance labelled endophytic *Pseudomonas* sp. when they were re-introduced into poplar trees, their original host plant. Two of these endophytes showed considerable colonisation in the roots and stems of inoculated plants, and *gfp* expressing cells of all three strains were observed to colonise the xylem tissue of the root. All three strains also proved to be efficient rhizosphere colonisers, supporting the theory that the rhizosphere can serve as a source of bacterial endophytes.

1.2.4 Rhizospheric and endophytic bacteria from metal hyperaccumulator plants

Hyperaccumulating plants are able to concentrate metals in their above ground parts to levels much higher than the substrate concentration (Baker, 1981). These plants are often the result of a genetic adaptation to soils naturally containing high metals concentrations but they also are found on soil with increased metal levels due to anthropogenic activities. As a consequence, their associated bacteria could require specific adaptation to the increase of heavy metals in the plants. Mengoni *et al.* (2001) found a direct relation between the metal plant concentration and the proportion of nickel resistant bacteria in *Alyssum bertolonii*, an endemic plant found on the serpentine outcrops of central Italy that hyper-accumulates nickel. Moreover their results showed that the proportion of nickel-resistant colony forming units was higher in the rhizosphere of *A. bertolonii* than in the soil, confirming the results of Schlegel *et al.* (1991). Confirmation of their results was obtained in another experiment (Mengoni *et al.,* 2004) using cultivation independent methods. Based on the results they hypothesised that the presence of the Ni hyperaccumulating *A. bertolonii* plants could affect the microbial community composition along a distance gradient from the roots of at least 5 cm. High proportions of metal resistant bacteria also persist in the rhizosphere of *Alyssum murale* (Abou-Shanab *et al.,* 2003).

Delorme *et al.* (2001) studied another well know metal (primarily zinc and cadmium) hyperaccumulator plant, *Thlaspi caerulescens*, and its effect on total (cultivable) bacteria, total fungi, as well as cadmium- and zinc-resistant microbial populations in non-rhizospheric and rhizospheric soils. Microbial populations in rhizosphere soil increased as compared with non-rhizospheric bulk soil. Also, the ratios of metal-resistant bacteria found in the presence of *T. caerulescens* were significantly higher than those in the rhizosphere of the non-hyperaccumulator plant *Trifolium pratense* L. It was suggested that *T. caerulescens* could increase the selection of metal resistant bacteria around its root system by increasing the soluble and thus bioavailable metal concentration in that zone through an active acidification caused by roots exudates. Whiting *et al.* (2001) demonstrated that the rhizosphere bacteria associated with *T. caerulescens* increased the amount of water-soluble zinc in the soil and as such contributed to the enhanced zinc accumulation in *T. caerulescens* shoots.

Lodewyckx *et al.* (2002) investigated the bacterial populations associated with *Thlaspi caerulescens* subsp. *calaminaria* grown on a soil collected from the abandoned Zn and Pb mining and smelter site of Plombières (Belgium). The non-rhizospheric soil population consisted of many bacteria equipped with multiple heavy metal resistance systems: 7.8% and 4% of the cultivable were able to survive elevated levels of the major pollutants Zn (1mM) and Cd (0.8mM). As observed by Delorme *et al.* (2001), the rhizosphere population was well-adapted to heavy metals and showed a survival rate of 88% and 78% on the respective selective heavy metals, which indicates a difference in metal availability in the vicinity of the root compared to the non-rhizospheric bulk soil (Lodewyckx *et al.,* 2002). Characterization and identification of the endophytes of the roots and shoots of *T. caerulescens* demonstrated that although similar species were isolated in both tissues, clear differences could be observed. In the presence of Zn and Cd, rhizoplane and root endophytic isolates showed to have a much lower survival rate under the same conditions and root endophytic bacteria even seemed to have different growth requirements. Additionally, only some bacteria residing in the plant root were able to produce siderophores under iron limiting conditions. In contrast to the root residing endophytes, the shoot represented a niche rich in metal resistant bacteria and was even shown to contain species that were exclusively abundant in this environment. These differences in the characteristics of the bacterial microflora associated with *T. caerulescens* might possibly reflect, among others, altered metal speciation in the different compartments studied (Lodewyckx *et al.,* 2002).

1.3. Beneficial effects of plant-associated bacteria

Endophytic bacteria that provide a beneficial effect to their host plant may generally be organized into two different systems: either they are forming a symbiotic relationship through the construction of specialized structures such as nodules where nitrogen fixation occurs, or they are free living in the plant's vascular system. The latter is very similar to plant growth-promoting rhizobacteria (PGPR) that are generally found near the roots in the rhizoplane or on plant roots (Frommel *et al.,* 1991). This is not surprising, as most of the endophytes isolated are capable of surviving outside their host plant as rhizospheric bacteria (Di Fiori and Del Gallo, 1995), and many endophytic

bacterial taxa, such as those isolated from sweet corn or cotton, were reported to be common soil bacteria (McInroy and Kloepper, 1994). Recently, Kishore *et al.* (2005) analysing the *Arachis hypogaea* L. associated bacteria, proved that phylloplane isolates, who normally survive on a plant surface under low moisture and other adverse conditions, may effectively colonize the nutrient rich rhizosphere. Therefore it is not unexpected then that the mechanisms used by endophytic bacteria and plant growth promoting rhizobacteria (PGPR) to beneficially affect their host plants seem to be similar (Kloepper *et al.,* 1991; Höflich *et al.,* 1994).

Plant growth-promoting bacteria, under nutrient-imbalanced conditions, have been reported to be key elements for plant establishment. For this reason their use can support eco-friendly crop production favouring to reduce the use of agrochemicals (Herrera *et al.,* 1993; Glick, 1995; Requena *et al.,* 1997). However, at present there are fewer than 20 different commercially available biocontrol PGPR strains (Penrose and Glick, 2003). Recent studies proved that inoculation with N-fixing and P-solubilizing bacteria isolated from the rhizosphere of barley and wheat not only increased barley root weight, but had a beneficial potential on the general barley growth activity (Cambolat *et al.,* 2006).

1.3.1 Direct plant growth promoting activity of plant-associated bacteria

The means by which PGPR can enhance the nutrient status of their host plant may be summarized into three main points: biological nitrogen fixation (diazotrophy), increased nutrient availability into the rhizosphere through the solubilization of unavailable minerals, and increased plant biomass production via the synthesis of phytohormones. Direct evidence for plant growth promoting activity by endophytic bacteria came from Sturz (1995). According to his study, approximately 10% of bacterial isolates recovered from within potato tubers were shown to promote plant growth. Other experiments with clover and potatoes in a crop rotation setup revealed that 21% of the isolated endophytic bacteria were plant growth promoting and this was reflected in increased shoot height (63%), shoot wet weight (66%), and increased root wet weight (55%) (Sturz *et al.,* 1998). Encouraging data have also been achieved by Egamberdiyeva and Höflich (2004) who demonstrated that selected growth

stimulating bacteria isolated from the soil of different crop root zones (cotton, wheat, tomato, melon and alfalfa) were able to increase the growth and nutrient uptake of cotton and pea in nutrient poor Calcisol soil compared with the control plants that performed poorly under the same conditions.

1.3.2 Diazotrophy

Biological nitrogen fixation supplies more than the 60% of the world's annual resource of new ammonia (Schlesinger, 1991). This process is performed by a diverse array of prokaryotes (including many cyanobacteria) possessing the enzyme nitrogenase, an O_2-sensitive enzyme that catalyzes the reduction of atmospheric nitrogen to ammonia. A profound study of the symbiotic diazotrophs, such as rhizobia, served as a first break to better understand the growth promotion mechanisms linked to diazotrophy. The symbiotic relation between rhizobia and plants is based on an exchange of carbon source provided to the bacteria by the plant for nitrogen fixed by the bacteria. It was initially thought that this mechanism formed the general basis of the relation between PGPR and their host plants. Further studies, however, showed that not all PGPR are diazotrophic, and that many of the diazotrophes are able to only fix a limited amount of nitrogen, sometimes not nearly enough to provide for their own needs (Hong *et al.,* 1991). In general, diazotrophic bacteria present in the rhizosphere tend to retain the nitrogen fixation products for their own use, and any benefit to the plant is only realized after the bacteria die (Okon, 1985). The general lack of bacterial nitrogen release is thought to be the main reason why nitrogen fixation in the rhizosphere only poorly contributes to the nitrogen supply for the plant.

The variables that influence the quantity of fixed nitrogen by free-living and associative bacteria are the amount of carbon available and the ability of the heterotrophic N_2-fixing bacteria to capture and use it efficiently (Rao *et al.,* 1998). Free-living N_2-fixing bacteria are distant from the main sources of C substrates, as was underlined by Kennedy and Tchan (1992), and are in competition with other microorganisms for these substrates. Experiments adding C sources directly to the rhizosphere have been performed to study the changes in nitrogen fixed into the plant tissues. Van Nieuwenhove *et al.* (2001) who

worked with rice and *Azorhizobium caulinodans* suggested that in order to fully exploit the possible benefits of this association, the inoculum should be used in a low-input, nutrient-deficient system. Nitrogen fixing bacteria associated with the rhizosphere have been isolated from several tropical grasses such as *Paspalum* and *Digitaria* (e.g. *Azotobacter*, *Azospirillum* and *Herbaspirillum* species) (Döbereiner *et al.,* (1976), Vose (1983)). A novel nitrogen-fixing bacterium, an *Azoarcus* species (Reinhold-Hurek *et al.,* 1993), was isolated from Kallar grass (*Leptochloa fusca*), a non-domesticated plant from Pakistan.

N_2-fixing bacteria were also isolated from plant tissues. In these tissues the fight for C-sources is reduced and the quantity of free oxygen that inhibits the nitrogenase activity is almost zero. Dalton *et al.* (2004) showed that endophytic, nitrogen-fixing bacteria were present in a symbiotic relationship within plant tissues of *Ammophila arenaria*. Another studied association is the one of sugarcane (*Sorghum officinarum*) in which endophytic nitrogen-fixation is carried out by *Gluconoacetobacter diazotrophicus* and *Herbaspirillum seropedicae*, which live in the intercellular spaces and xylem (Cavalcante and DÖbereiner, 1988; Sevilla *et al.,* 2001). *G. diazotrophicus* was also isolated from other sucrose-rich plants that are propagated vegetatively, such as sweet potato and Cameroon grass (Döbereiner *et al.,* 1998; Döbereiner *et al.,* 1988; Paula *et al.,* 1991). There are also numerous accounts of other grass species, some of agricultural importance such as maize, wheat, oats, barley, and rice that may be associated with endophytic nitrogen-fixing bacteria, although it is unclear whether or not significant amounts of nitrogen are fixed in these systems (Vose, 1983; Riggs *et al.,* 2001; Engelhard *et al.,* 2000). On the other hand, many studies have proved a direct contribution of endophytic bacteria to plant nitrogen fixation, such as in sugarcane (Boddey *et al.,* 1995), rice (Ladha and Reddy, 1995; Yanni *et al.,* 1997; Balachandar *et al.*, 2006) and wheat (Webster *et al.,* 1997). According to a review from Boddey *et al.* (1995), certain Brazilian cultivars of sugarcane, after ^{15}N nitrogen balance studies, obtained over half their needs for nitrogen from biological nitrogen fixation (>150kg N ha^{-1} $year^{-1}$). In rice plants, inoculation with *Azospirillum* contributed to 66% of the total ammonium in the plants (Malik *et al.,* 1997). It was also shown that azotobacterisation is beneficial in raising vigorous seedlings of mangroves in coastal wetlands (Ravikumar *et al.,* 2004). The results of the azotobacters,

which were inoculated in *Rhizophora* seedlings, revealed a direct effect on the host plant: inoculation significantly increased the root biomass by an average of 98%, the root length by 48%, the leaf area by 278% and the shoot biomass by 29% as compared to non inoculated controls. Inoculation also resulted in increased levels of total chlorophylls and carotenoids by 151% and 159%, respectively. Diazotrophs are also present in extreme environments like the artic glacial low lands. In these environments, the low temperature and the poor substrate quality limit the decomposition and N mineralization. Deslippe *et al.* (2006) examined the diazotroph community associated with three common, woody, perennial plants, *Dryas integrifolia*, *Salix arctica*, and *Cassiope tetragonal* reporting high *nifH* diversity present in these communities.

1.3.3 Phosphate solubilization

Phosphorous is one of the most import plant nutrients. The vast majority of soil P is found as insoluble forms. Plants can absorb P when it is in its monobasic ($H_2PO_4^-$) or dibasic (HPO_4^{2-}) soluble form (Glass, 1989). Even when P is applied to the soil as a fertilizer, it is rapidly immobilized and becomes unavailable to plants (Nautiyal, 1999; Rodriguez and Fraga, 1999). Solubilization of P in the rhizosphere is the most common way used by PGPR to increase nutrient availability to their host plant (Richardson, 2001). Phosphate solubilizing bacteria are common in the rhizosphere (Nautiyal *et al.,* 2000; Vazquez *et al.,* 2000) and they use secretion of organic acids and phosphatases to convert phosphate to a plant available form (Kim *et al.,* 1998). The solubilization of insoluble phosphate by *Pantoea agglomerans* isolated from the soybean rhizosphere was associated with a drop of pH values in the culture medium (Hong-Joo *et al.,* 2006). Same drop in pH was reported during the study of 36 phosphate solubilizing bacteria belonging to different genus like *Bacillus*, *Serratia*, *Rhodococcus*, *Arthrobacter*, *Chryseobacterium*, *Delftia*, *Gordonia* and *Phyllobacterium* (Chen *et al.*, 2006). Recent studies focusing on the association of phosphate-solubilizing rhizospheric bacteria and their host plant included: *Azotobacter chroococcum* and wheat (Kumar and Narula, 1999); *Bacillus circulans* and *Cladosporium herbarum* with wheat (Singh and Kapoor, 1999), *Bacillus* sp. and five crop species (Pal, 1998); *Enterobacter agglomerans* and tomato (Kim *et al.,* 1998); *Pseudomonas chlororaphis* and *P. putida* with

soybean (Cattelan *et al.,* 1999); *Rhizobium* sp. and *Bradyrhizobium japonicum* with radish (Antoun *et al.,* 1998); *Rhizobium leguminosarum* bv. p*haseoli* and maize (Chabot *et al.,* 1998). Endophytic bacteria have also been reported to solubilize immobilized mineral phosphate. Rodriguez and Fraga (1999) and Verma *et al.* (2001) suggested that during initial colonization, endophytic bacteria could enhance phosphate availability to soybean plants. Results from Kuklinsky-Sobral *et al.* (2004) supported this suggestion, showing that 52% of the endophytic bacteria isolated from soybean could solubilize mineral phosphate.

1.3.4 Phytohormones

There is plenty of evidence for bacterial production of phytohormones. Ethylene, auxins and cytokinins were found to be produced by strains of *Pseudomonas*, *Enterobacter*, *Staphylococcus*, *Azotobacter* and *Azospirillum.* These substances, together with two other phytohormones, gibberellins and indole-3-acetic acid (IAA), may be considered as causal agents for altering plant growth and development (Arshad and Frankenberger, 1991; Bashan and Holguin, 1997; Leifert *et al.,* 1994). Phytohormones production by bacteria is considered without a direct benefit for the bacteria itself, and should be motivated in an indirect way: phytohormones production influences plant growth, but at the same time stimulated plant growth will provide more nutrients to the plant associated bacteria. An example of this equilibrium is the production of Indole-3-acetic acid (IAA), a phytohormone known to be involved in root initiation, cell division and cell enlargement (Salisbury, 1994). The presence of IAA-producing PGPR will result in an enlargement of the root area, which will allow the plant to reach more nutrients and, at the same time, will provide more root surface area for microbial colonization. For instance, *Azospirillum* inoculated roots showed a stimulation of root cell membrane activity as well as an increase in the levels of free IAA, indole-3-butyric acid and the specific activities of both the tricarboxylic acid cycle and the glycolysis pathway (Fallik *et al.,* 1994). *Azospirillum brasiliense FT326* inoculated tomato plants have increased IAA and ethylen levels (Ribaudo *et al.*, 2006). IAA-producing PGPR have been isolated lately from rice (Mehnaz *et al.,* 2001), sugarcane (Mirza *et al.,* 2001), lettuce (Barazani and Friedman, 1999) and wheat (Kaushik *et al.,* 2000). Recently an *Enterobacter*

cloacaea IAA-producer strain has been found as an obligatory endophyte of pollen grains of Mediterranean pines (Madmony *et al.,* 2005), being probably the responsible for an increased IAA content in the germination medium of pollen. The same strain was found to promote the adventitious root formation in mung bean cuttings as well.

IAA production is common in plant-associated bacteria and depending on the bacteria the IAA production seems to follow different pathways. In case of phytopathogenic bacteria, IAA is in general produced from tryptophan via the intermediate indoleacetamide. In plant beneficial bacteria, IAA is predominantly synthesised via indolepyruvic acid (Manulis *et al.,* 1998; Patten and Glick, 2002).

Cytokinins are another class of phytohormones known to stimulate cell division, cell enlargement and tissue expansion in certain plant parts (Salisbury, 1994). Cytokinin-producing PGPR have been isolated from wheat (Timmusk *et al.,* 1999), soybean (de Salamone *et al.,* 2001), pine (Bent *et al.,* 2001), rape and lettuce (Noel *et al.,* 1996).

Gibberellins (gibberellic acid) are involved in modifying plant morphology by the extension of plant tissue, in particular of the stem (Salisbury, 1994). Their production by PGPR is rare; however, *Bacillus pumilus* and *Bacillus licheniformis* species able to produce this phytohormone have been isolated (Gutierrez-Manero *et al.,* 2001). Also *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* species were identified as indole-3-acetic and gibberellin producing endophytic bacteria present in *Gramineae* species where they increased plant growth and yield (Bastián *et al.*, 1998).

1.3.5 Counteracting stress induced ethylene

Ethylene is a phytohormone involved in different plant processes ranging from seed germination, cell differentiation, stress responses, flowering induction, fruit ripening and senescence. It induces decreased membrane fluidity, influences the turnover of phospholipids in membranes, enhances the leakage of solutions from plant cells, and decreases root elongation (Mayak *et al.,* 2004). Ethylene release

is known to be increased in plants exposed to both abiotic and biotic environmental stress conditions such as heavy metals, flooding, drought, infection by fungal or bacterial pathogens, and is synthesized from L-methionine via S-adenosyl-L-metionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC), which is the immediate precursor of ethylene.

Bacteria can influence ethylene production via two main mechanisms. The most commonly observed is bacterial ACC-deaminase activity, which results in decreased ACC and ethylene levels. ACC-deaminase activity is widely distributed, especially among different microbial genera found in the rhizosphere, where their presence was reported to be strain dependent (Dell'Amico *et al.,* 2005). ACC-deaminase cleaves ACC into NH_3 and α-ketobutyrate and some bacteria can use these two compounds as N and C sources respectively (Belimov *et al.,* 2004; Ma *et al.,* 2004). However, some bacteria can also balance hormone levels through auxin production. Plants can take up bacterial auxins, which will stimulate the ACC synthase activity, which in turn will result in an enhanced ACC synthesis.

Ethylene is also a component of the signalling pathway controlling the rhizobial infection in legumes. A correlation was observed between decreased ethylene production and increased nodulation levels (Ma *et al.,* 2004). Low ethylene levels are required for the entry of infection threads into the outermost layer of cortical cells by allowing proper disposition of cytoskeleton and formation of cytoplasmic bridges. On the other hand, a high ethylene concentration will induce the abortion of infection threads. For instance in alfalfa about 95-99% of infection thread progressions abort in the cortex (Vasse *et al.,* 1993). The infection threads containing ACC-deaminase-producing bacteria can better suppress the defence signals in the plant cells by decreasing ethylene levels, thus increasing the persistence of infection threads. ACC-deaminase activity has been found in several rhizobial strains including *Rhizobium leguminosarum* bv. *viciae* strains, *Rhizobium hedysari* (Ma *et al.,* 2003), and *Mesorhizobium loti* (Kaneko *et al.,* 2000; Sullivan *et al.,* 2002). Ma *et al.* (2004) found a 40% increase in nodulation numbers in alphalpha plants inoculated with an engineered ACC-deaminase overproducing *S. meliloti* strain, this compared to plants inoculated with the wild type strain. An investigation conducted by

Donate-Correa *et al.* (2004) on the rhizosphere microbial population associated with *Chamaecytisus proliferus* (tagasaste) reported that 50% of the isolated *Pseudomonas* strains possesed ACC-deaminase activity; moreover, they observed an increase in nodule numbers in co-inoculation experiments run with the *Bradyrhizobium* strain BTA-1. These experiments resulted in a positive effect on root elongation, plant growth and nodulation levels, and underline the importance of the bacterial influence on plant growth and yield production. An alternative strategy to overcome high ethylene levels was found for *Bradyrhizobium elkanii*, which produces rhizobitoxine, an ethylene biosynthesis inhibitor: rhizobitoxine production by *B. elkanii* decreased ethylene production in plant roots and enhances nodulation on *M. atropurpureum* Urb. cv. Siratro (Yuhashi *et al.,* 2000).

Ethylene production is increased as a stress response when plants are exposed to sub-lethal concentrations of toxic metals. Belimov *et al.* (2004) found that *Variovorax paradoxus* was the dominant cultivable strain in the rhizosphere population of *Brassica juncea* grown on Cd contaminated soil. This strain was shown to produce ACC-deaminase and to be capable of using ACC as energy source, characteristics that probably gave it a competitive advantage to strains when the plants were grown under stress conditions. *In vitro* they observed a positive correlation between ACC-deaminase activity and the bacterial effect on plant root elongation. This result allowed them to conclude that inoculation of *Brassica juncea* by *V. paradoxus* under cadmium stress could promote root growth, the latter being positive for the phytoremediation process. Dell'Amico *et al.* (2005) underlined that most of the PGPR isolated from *Graminaceae* grasses grown on a heavy metal polluted water meadow were ACC-deaminase producers and that these strains were helping the plant growth. Hasnain and Sabri (1996) found that wheat plants, grown under chromium toxicity and inoculated with IAA- producing *Pseudomonas* sp., gained in root length growth and plant auxin production; at the same time the chromium concentration inside the plants decreased.

1.3.6 Indirect plant growth promoting activity

PGPR as well as endophytic bacteria seem to be able to lessen or prevent the deleterious effects of phytopathogenic organisms through nice competition, antibiosis and Induced Systemic Resistance (ISR) in plant. This ability can be considered as an indirect way to promote plant growth. For instance, treatment of maize seeds with a culture of *Burkholderia cepacia*, isolated from the rhizosphere of maize, resulted in disease suppression and growth promotion (Bevivino *et al.,* 1998). Similar observations were made by Hebbar *et al.* (1992 a and b), who described an antagonistic effect of *B. cepacia* against *Fusarium moniliforme*, a soil borne fungal pathogen of maize. From healthy wheat, Coombs and Franco (2003) could isolate and characterize a*ctinobacteria* endophytic organisms responsible for the production of the two-thirds of the microbially derived antibiotics involved in plant pathogen defence. A decrease in anthracnose severity was found in soybean plants whose seeds were treated with four *Pseudomonas* strains, this compared with the non bacterized seeds (Tripathi *et al.,* 2006). Soybean seeds inoculated at the same time with *Pantoea agglomerans*, an endophytic strain, and with *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, a pathogen that causes soybean wilt, resulted in a decreased frequency of infection by the pathogen, improved number of seedlings and height, and reduced disease severity compared to the seeds only inoculated with the pathogen (Hsieh *et al.,* 2005). Another cultivable strain frequently found as plant associated, *Stenotrophomonas maltophilia*, was shown to be the most effective of the antagonists for its efficiency in suppressing dumping-off, a common leaf disease, at the same time increasing wheat plant biomass production, presenting itself as a potential biocontrol agent (Dal Bello *et al.,* 2002). The same species in combination with *Bacillus mycoides* and *Pseudomonas sp*. was found to consistently reduce nematode densities (by 56.7-74.4%) in potatoes tubers with no visual effect on plant growth (Insunza *et al.,* 2002).

Another way that endophytic bacteria control pathogens proliferation is via iron control. They can excrete organic compounds (siderophores) that are with very high affinity able to bind the Fe^{3+} that is available in the rhizosphere, and as a result prevent the proliferation of pathogens (O'Sullivan and O'Gara, 1992). Evidence for this mechanism comes from several studies including a report where a mutant strain of *Pseudomonas aeruginosa*, deficient in siderophore

production, no longer protected tomato plants against *Pythium* (Buysens *et al.,* 1994). Generally, *Pseudomonas* sp. have been considered to play a major role in disease inhibition through the production of siderophores and thanks to this efficient iron uptake mechanism, they can quickly colonise the rhizosphere and thus physically displace deleterious organisms. Strains of *Pseudomonas fluorescens* were recently investigated for biocontrol against tomato spotted wilt virus (TSWV) in tomato. Analyses indicated a reduction in viral antigen concentration in *P. fluorescens*- treated tomato plants corresponding with a reduced disease rating. Moreover, all *P. fluorescens*-treated tomato plants showed enhanced growth and yield compared to control plants (Kandan *et al.,* 2005). A more general effect was observed after application of dialysates from *Bacillus cereus* isolated from the rhizosphere of healthy tomato plants. The plants treated in this way were protected against leaf fungal and bacterial pathogens, emphasizing that macromolecules synthesized by PGPR and released into the environment can act as elicitors of systemic resistance (Romeiro *et al.,* 2004). Interesting results were also achieved with rust affected *Pinus taeda* L. *fusiform*. This infection is caused by *Cronartium quercuum* f. sp. *fusiform* and it is the most damaging stem disease for Pinus spp. in the southern United States. After treating Pinus seeds with three different bacterial strains, the seedlings analysed at the end of one season showed reduced rust gall infections and also a higher biomass production compared to untreated control plants (Enebak and Carey, 2004).

In the case of endophytic bacteria, some studies reported the ability of fungal suppression. Several endophytic bacteria isolated from rice seeds exhibited strong anti-fungal activity against *Rhizoctonia solani*, *Pythium myriotylum*, *Gaeumannomyces graminis*, and *Heterobasidium annosum* (Mukhopadhyay *et al.,* 1996). *Enterobacter cloaca*, an endophyte isolated from corn, seemed to be involved in antibiosis against *Fusarium moniliforme* (Hinton and Bacon, 1995). This result could be added to the experiments performed by Chen *et al.* (1994) who showed that wilt disease symptoms (caused by a *Fusarium* sp.) could be successfully reduced when cotton plants were artificially inoculated with naturally occurring endophytes isolated from the same host plant. Kremer *et al.* (2006) illustrated that the most effective condition for inducing disease and subsequent mortality of leafy spurge, a seriously invasive weed of grasslands of

the northern Great Plains in the U.S.A., and the prairie provinces of Canada, includes a synergism between plant-associated microorganisms and root-damaging insects. The results were valuable to identify sites where collect, in their native range, soilborne microorganisms on weeds for evaluation as biocontrol agents in their invasive range.

1.4. Application of plant-associated bacteria to improve phytoremediation

The potential that plant-associated bacteria have to offer for agricultural gains has been realised beyond their use as natural biocontrol agents. The inherent nature of certain endophytes to potentially colonise plants in a systemic manner provides a novel approach as a delivery system to plants for various beneficial traits. This paragraph specifically addresses the potential of plant-associated bacteria to improve phytoremediation of heavy metals and organic contaminants.

1.4.1 Phytoremediation

Remediation options currently applicable to contaminated soils and groundwater are frequently expensive, environmentally invasive and do not make cost-effective uses of existing resources. These techniques are based upon civil engineering methodologies, involving either the excavation and removal of contaminated soil (dig and dump), pumping and treatment of contaminated groundwater or an *ex situ* treatment of the soil that drastically alters soil structure, biological activity and subsequent function. Certainly in case of very large contaminated areas there is a clear need for cost-effective, durable and validated alternative remediation strategies to those that are in current use (van der Lelie *et al.,* 2001). The focus of much recent experimental work has been directed towards these ends, developing techniques that exploit biological (plant and microorganisms) and chemical (use of metal-binding agents) processes to reduce the inherent risk associated with contaminated soils and groundwater. Strategies of this nature are generally classified under the generic heading of phytoremediation. One clear distinction in the use of this technology relates to

whether inorganic or organic compounds are the primary targets of remediation, although mixed pollution situations of course also exist.

Phytoremediation of the contaminated groundwater is an option that appeals not only to site owners but also community and oversight groups. The chance to use plants to remediate sites while restoration activities are taking place is very much in favour. And while there are groundwater depth limitations to direct application, combination of technologies can easily overcome those limitations. A more pressing issue would be the concentration of the contaminant in the groundwater, as the potential toxicity of the chemical would limit application.

There is documented evidence that plants can degrade a wide range of organic compounds, following the `green liver' model proposed by Sandermann (1994). However, there is the potential for degradation to be incomplete for some of the more recalcitrant compounds, such as benzene, toluene, and trichloroethylene and perchloroethylene. These compounds do not accumulate in the plant tissues, but due to their chemical nature either transpire from the leaves or volatilize directly from the stems and trucks of the trees. For areas that have zero emission policies for remediation technologies, this could prevent phytoremediation from being deployed.

1.4.2 Plant-associated bacteria assisted phytoremediation of organic contaminants

The fate of contaminants in the rhizosphere-root system largely depends on its physicochemical properties. Organic xenobiotics with a log K_{ow} < 1 are considered to be very water-soluble, and plant roots do not generally accumulate them at a rate surpassing passive influx into the transpiration stream (Cunningham and Berti, 1993). Contaminants with a log K_{ow} >3.5 show high sorption to the roots, but slow or no translocation to the stems and leaves (Trapp *et al.,* 2001). However, plants readily take up organic xenobiotics with a log K_{ow} between 0.5 and 3.5, as well as weak electrolytes (weak acids and bases or amphometers as herbicides). These compounds seem to enter the xylem faster than the soil and rhizosphere microflora can degrade them, even if the latter is enriched with degrader bacteria (Trapp *et al.,* 2000). Once taken up,

plants metabolize these contaminants, although some of them, or their metabolites, such as trichloroethylene (TCE), which is transformed into tricarboxylic acid (TCA), can be toxic (Doucete *et al.,* 1998). Alternatively, plants preferentially release volatile pollutants, such as BTEX compounds and TCE and their metabolites, into the environment by evaporation via the leaves, which questions the merits of phytoremediation (van der Lelie *et al.,* 2001; Schwitzguebel *et al.,* 2002; Ma and Burken, 2003; Burken and Schnoor, 1999).

While the degradation of compounds such as TCE by plants has been well documented by many groups, other groundwater contaminants are not. One of these is methyl-t-butyl ether (MtBE), a major additive of gasoline. MtBE is extremely water-soluble and does not adhere well to soil, thus plumes of undetermined origin and several miles in length are not uncommon. Also, MtBE has been found in groundwater in states that do not use MtBE, perhaps the result of volatilization and deposition during rain events. Plants readily take up MtBE, but limited degradation has been observed. Instead, the majority of the compound is transpired unaltered, thus potentially increasing this spread of MtBE contamination.

The use of engineered endophytes has the potential to overcome the problems of phytotoxicity and incomplete or limited degradation. Work that has previously been published by some of us (Barac *et al.* 2004) has proven this concept. Lupine plants were inoculated with endophytic bacteria that had acquired the plasmid pTOM, which encodes for enzymes involved in toluene degradation. The bacteria thrived in these plants, and more importantly for this work, showed that they enabled the plants to overcome both plant toxicity and air release limitations. Inoculated plants grew in soils watered with 500 mg L^{-1} toluene, while control plants showed serious signs of stress at 100 mg L^{-1}. Additionally, there was greater than 50% reduction in toluene released from the above-ground portions of the plants. Recently, this concept was successfully extended to poplar (Taghavi *et al.,* 2005): inoculation of poplar with an endophytic strain able to degrade toluene resulted in a significant decrease of the amount of the contaminant released via evapotranspiration.

1.4.3 Horizontal gene transfer to the endogenous endophytic community

Although the application of engineered endophytic bacteria to improve phytoremediation of volatile organic contaminants has several obvious advantages over the application of engineered rhizosphere bacteria or the genetic engineering of the plant's metabolism, several obstacles have to be overcome before this technology can move towards application (Newman and Reynolds, 2005). One major point of concern is the persistence and the stability of the engineered organisms and their degradation capabilities in field grown plants, as phytoremediation projects can conceivably last decades. As long as a selection pressure is present, there will be an advantage for those endophytic community members possessing the appropriate degradation characteristics. However, this is no guarantee that strains of the inoculum will become an integrated part of the endogenous endophytic community. While marked endophytic strains, isolated from the endogenous endophytic community from poplar, were successfully used to re-inoculate their original host plants (Germaine *et al.,* 2004), attempts to inoculate poplar with a toluene metabolizing *Burkholderia cepacia* strain that has yellow lupine as its natural host seemed unsuccessful: ten weeks after inoculation the inoculum could no longer be detected among the cultivable bacteria (Taghavi *et al.,* 2005). However, despite the absence of the inoculum, improved toluene degradation and a reduction of toluene phytotoxicity and release via evapotranspiration were observed. Horizontal gene transfer of the pTOM-Bu61 encoded toluene-*ortho*-monooxygenase had occurred from *Burkholderia cepacia* to different members of the endogenous endophytic community, both in the presence and absence of toluene, allowing the endogenous endophytic communities to adapt and to deal with the toluene contamination.

Horizontal gene transfer has been shown to play an important role in rapidly adapting a microbial community to a new environmental stress factor (Dong *et al.,* 1998), including rhizosphere communities (Devers *et al.,* 2005; Ronchel *et al.,* 2000; van Elsas *et al.,* 1998), and we had speculated that it could play an important role in adapting the endogenous endophytic community (van der Lelie *et al.,* 2005): rather than integrating a new bacterium in a stable community, the degradation pathway is transferred among the members of the community. It can be hypothesized that horizontal gene transfer also played a role in the selective enrichment of catabolic genotypes observed by Siciliano *et al.* (2001).

This enrichment in the root interior was found to be both plant and contaminant dependent: selective enrichment for endophytic bacteria containing the appropriate catabolic genes was found for plants grown on petroleum hydrocarbon contaminated sites, on field sites contaminated with nitroaromatics, and after the addition of petroleum to sediment. Similarly, the numbers of *alk*B- or *ndo*B-positive endophytes in *Festuca arundinacea* were correlated with the concentration of creosote in the soil but not with the numbers of *alk*B- or *ndo*B-positive bacteria in the bulk soil.

1.4.4. Cadmium contamination of soils and its effects on plants

Pollution of soils and waters with toxic metals, caused mainly by mining and refining of metals ores, the burning of fossil fuels, manufacturing waste, and the indiscriminate use of chemicals has accelerated dramatically due to world industrialization. A serious soil-related pollution problem is the elevated levels of contaminating Cd in products used in agriculture such as phosphate fertilizers and sewage sludge (Ryan *et al.*, 1982; Nicholson & Jones, 1994).

Heavy metals and metalloids such as Hg, As, Cd, and Pb are extremely toxic, and pollution caused by these metals is a major environmental concern.

Over 300 km^2 of soil area in the northeast of Belgium are contaminated with heavy metals due to historical soil pollution caused by intensive zinc refining in several zinc smelters in the 19th and 20th century. Although these smelters have been shut down or have drastically reduced their emissions, the soil in the area continues to be polluted with metals due to the lack of biodegradability of metals. The area is considered to be too large for extensive clean up, which would require enormous financial means and which would result in enormous amounts of polluted soil that have to be stored or used safely elsewhere. Therefore, the soil contamination leads to continued exposure of the inhabitants of the area to heavy metals. To reduce the exposure of the inhabitants, the Ministry of Public Health, the Province of Limburg and Antwerp and several municipalities in the contaminated area have educated people about ways to reduce exposure, for instance by advising them not to use well water for consumption, to lime their garden soils and by distributing a leaflet about which vegetables can be safely grown and which vegetables should better be avoided. Also agricultural plants were shown to regularly exceed threshold levels for Cd and Pb. Therefore, one of the measures actually under investigation is the

production of non-food and non-feed agricultural products, allowing a sustainable use and economic valorisation of the contaminated soils, on the mid- or long-term leading to a reduction of the metal levels in the soils. This plant-based technology commonly is termed as "phytoremediation" (see section 1.4 of this introduction).

Cd is strongly phytotoxic; it causes growth inhibition, and may cause plant death by interfering with important biochemical pathways (Steffens, 1990). Disturbance in the uptake and distribution of macro and micronutrients in plants is also shown to be correlated with Cd toxicity (Sandalio *et al*., 2001). For example, Cd(II) appears to inhibit Zn(II) uptake by a major class of zinc transporters (Grotz *et al*., 1998). Its phytotoxicity is related to its reactivity with O-, N- and S-containing ligands (Van Assche and Clijsters 1990a). This metal as Hg, As and Pb, may cause oxidative damage such as lipid peroxidation, enzyme inactivation, and DNA damage and by binding to protein sulfhydryl groups (Stohs *et al*., 2001; Bal & Kasprzak, 2002; Ramirez & Gimenez, 2002).

Cd inhibits photosynthesis (Clijsters and Van Assche 1985) but stimulates respiration. The activities of the tricarboxylic acid cycle and of other pathways of carbohydrate utilisation are induced by Cd accumulation in leaves. Cd induces the synthesis of cysteine-rich peptides with the general structure (γ -EC)nG, called phytochelatins (Rauser 1995), and of other thiol peptides (γ -EC)n and (γ -EC)nE (Meuwly *et al.* 1995). Phytochelatins form complexes with Cd in the cytosol and are important in subsequent Cd sequestration in the vacuoles (Ortiz *et al.,* 1995). They participate in the maintenance of cellular metal homeostasis (Zenk 1996) and are involved in limiting the transport of heavy metal ions from roots to shoots (Galli *et al.,* 1996). Phytochelatins are synthesised by γ -EC dipeptidyl transpeptidase from the precursor reduced glutathione (GSH; Grill *et al*., 1989). GSH is synthesised by two sequential reactions, catalysed by γ-glutamyl cysteine synthetase (γ -ECS) and glutathione synthetase (GS) in the chloroplasts and cytosol of plant cells (Noctor *et al.,* 1998a,b).

Cd is widespread in the environment and in foods consumed by man (Sherlock, 1984). A recent research by Edrydstephens and Anguscalder (2005) points out smoking together with dietary as the main intake routes for metals and in particular for cadmium. Hogervorst *et al.* (2006) indicate metal-enriched house dust to be also a significant contributor to the total body burden of Cd and Pb.

Foods such as potatoes, grain, and cereal (especially rice) deliver relatively high quantities of Cd, whereas meat delivers less and legumes and fruits deliver very much less (Wagner, 1993). Cadmium is readily accumulated by many organisms, particularly by microorganisms and molluscs where the bioconcentration factors are in the order of thousands. Soil invertebrates also concentrate cadmium markedly. Most organisms show low to moderate concentration factors of less than 100. In animals, cadmium concentrates in the internal organs rather than in muscle or fat. It is typically higher in kidney than in liver, and higher in liver than in muscle. Cadmium levels usually increase with age. Mammals can tolerate low levels of cadmium exposure by binding the metal to a special protein that renders it harmless. In this form, the cadmium accumulates in the kidney and liver. Higher levels of exposure, however, lead to kidney damage, disturbed calcium and vitamin D metabolism, and bone loss. The body takes decades to remove cadmium from its tissues and organs (Nordic Council of Minister, 2003).

Recent studies indicate that a threshold of Cd in urine of 10 µg/g creatinine must be exceeded before this disorder becomes evident in the population (Ikeda *et al*., 2003). In polluted areas the value has been found to be several hundred µg per day while average daily intakes from food in most areas not polluted with cadmium are 10-40 µg (WHO 1992). It is unlikely that smoking high Cd tobacco will cause many to exceed this threshold from this source alone. However low-level exposure such as that from combined dietary and smoking sources can result in unrecognized but potentially significant burdens on health (Borgden *et al.,* 1981, Satarug *et al.*, 2002). Recently Navas-Ancient *et al.* (2005) indicated that levels of Cd and Pb well below exposure limits recommended by World Health Organization (WHO) can still increase the risk of peripheral arterial disease. Another concern is that other heavy metals may also be entering the lungs and bloodstream, and the effects may be additional or even synergistic with cadmium (Carpenter *et al*., 2002). It is evident the necessity to decrease the levels of cadmium in the soil used for agriculture purposes.

1.4.5. Bacterial enhanced phytoremediation of heavy metals

Metal phytoextraction is a promising approach but it is still in its infancy stage and needs further research and development. Recent studies investigating the feasibility of phytoextraction confirmed that both biomass production and metal concentration factor (metal (hyper)accumulation) determine the efficiency of the remediation process (McGrath and Zhao, 2003; Vassilev *et al.,* 2004). Several studies claiming to have demonstrated a high potential of different plant species for remediation of contaminated sites in fact were using plants showing a bioconcentration factor of lower than 0.5. A simple calculation using realistic biomass production levels leads to the conclusion that remediation of a moderately contaminated soil should take at least more than 100 years. In (hyper)accumulator plants, the bioconcentration factor is usually higher than 1 and in some cases even up to 100 (Baker *et al.*, 2000; Zhao *et al.,* 2003). For easy harvesting, the root to shoot transport should be efficient, resulting in a shoot to root of metal concentration higher than 1. Few studies report on the use of natural metal hyperaccumulators under field conditions. The metal concentration factor depends not only on plant but also soil factors (soil type, pH, organic matter content, etc.).

Beside of the metal uptake and translocation in the plants, metal availability in soils is a second key factor in the efficiency of the remediation process. The use of amendments for mobilization (e.g. chelating or acidifying agents) of metals in the soil can sometimes improve the metal accumulation by plants. Chemically assisted phytoextraction indeed is based on the use of non-accumulator plants with metal accumulation levels far below those of hyperaccumulators, but with high biomass potential (Vassilev *et al.*, 2004). Restrictions apply, however, to both the use of complexing agents and artificial soil acidification. It was found that EDTA and EDTA heavy metal complexes are toxic for some plants and that high dose of EDTA inhibited, for example, the development of arbuscular mycorrhiza (Vassilev *et al.*, 2004). Furthermore, EDTA is poorly photo-, chemo- and bio-degradable. *In situ* application of both poorly degradable but also easily degradable chelating agents can cause groundwater pollution by uncontrolled metal dissolution and leaching (Wenzel *et al.*, 2003).

Since in many cases metal uptake by plants is limited by low metal solubility, it is necessary that the efforts for selection of appropriate rhizosphere

manipulation is continued. There is a need to find cheaper, environmentally benign chemical compounds with chelating properties as well as to better understand the role of rhizospheric bacteria in metal solubility, plant uptake and tolerance. The plant-rhizosphere interactions controlling metal uptake by roots are indeed of primary interest. It is necessary to identify which are the main limiting factors and to find appropriate solutions to overcome them. There is some evidence that diffusion of metals is such a limitation that, even in moderately contaminated soils, mass flow contribution is less than 10% of total metal uptake (McGrath *et al.,* 2001). The diffusion rate in soil generally depends on metal availability in soil solution and, on the other hand, on the concentration gradient driven by metal ions' uptake by roots. It was found that roots of *T. caerulescens* responded positively to Zn and Cd supply (Whiting *et al.,* 2000), but not to enhanced metal solubility by changes in rhizosphere pH (Knight *et al.,* 1997). To what extend root exudates can mobilize metals (as was shown for Fe and possibly Zn; Marschner, 1995) or if microbial rhizosphere communities stimulated by these root exudates (Anderson, 1997) can contribute to metal phytoavailability, remains to be further examined. As certain plants can use microbial siderophores to improve their iron uptake, it has been hypothesized that bacterial metal chelators, such as siderophores, can eventually improve the uptake of heavy metals by plants (van der Lelie *et al.,* 1998). Bacterial siderophores can be considered as natural chelators and the bacterial production of which is in tight equilibrium with plant activity, thus improving heavy metal uptake and translocation as part of the phytoextraction process. Another possibility that should be considered is the use of Plant Growth Promoting Bacteria that stimulate root formation by plants. A better-developed root system can lead to increased metal uptake.

Endophytic bacteria can be engineered for increased heavy metal sequestration. The activities of these bacterial strains could enhance heavy metal uptake and translocation by the host plants. In order to improve phytoremediation of heavy metals, Lodewyckx *et al.* (2001) introduced the *ncc-nre* nickel resistance system of *Ralstonia metallidurans* 31A in *Burkholderia cepacia* L.S.2.4 and *Herbaspirillum seropedicae* LMG2284. *Lupinus luteus* L, when grown on a nickel enriched substrate and inoculated with *B. cepacia* L.S.2.4::*ncc-nre*, showed a significant increase (30%) of nickel concentration in the roots, whereas the

nickel concentration in the shoots remained comparable with that of the control plants. The inoculation of *Lolium perenne* (cv Atlas) with the nickel resistance derivative of *H*. *seropedicae* LMG2284::*ncc-nre* resulted in a significant decrease of the nickel concentration in the roots (11%) as well as in the shoots (14%). However, a similar observation was made when *Lolium perenne* plants were inoculated with the wild type strain LMG2284, indicating that the nickel resistance characteristics are not responsible for the altered nickel uptake observed.

Other studies conducted on non-hyperaccumulator plant species and their associated bacteria confirmed the possibility of an enhanced plant metal uptake in case of plant bacteria interaction. Working with Trifolium growing on cadmium contaminated soil, Vivas *et al.* (2003) demonstrated a positive influence of Cd-adapted autochthonous *Brevibacillus sp*. and the arbuscular mycorrhiza (AM) *Glomus mosseae* on biomass production, and N, P and even Cd content in plants. They observed an increment in symbiotic structures (nodule number and AM colonization) as well; these in general decrease at an increased cadmium concentration. Further studies (Vivas *et al.,* 2005) demonstrated that the inoculated Cd-adapted bacteria increased dehydrogenase, phosphatase, and β-gluconase activities in the mycorrhizosphere, indicating an enhancement of microbial activities related to plant development.
Experiments with Indian mustard (*Brassica juncea*), a high-biomass producing metal tolerant plant species, showed, in case of soil contamination by lead, root hyperaccumulation of the metal concomitant with the enrichment in the rhizosphere of a Pb and Cu resistant strain of *Arthrobacter* (Roy *et al.,* 2005). An increment in total nickel content per plant was noticed after inoculation of *Brassica napus* with *Pseudomonas putida* HS-2 grown in a Ni contaminated site (Farwell *et al.,* 2006). Chen *et al.* (2005) isolated Cu tolerant bacteria from the rhizosphere of *Elsholtzia splendens*, a copper accumulator plant generally growing on copper mines. The re-inoculation of these strains in the plant rhizosphere resulted in an increased water copper concentration and facilitated Cu accumulation by the plants. This role of the bacteria was confirmed using ampicillin, an antibiotic that inhibits microbial activity: in the presence of the antibiotic the added bacteria failed to exert their mobilizing effect on Cu. It was concluded that the strains used as inoculum were able to excrete low molecular

weight compounds that increased heavy metal availability to their host plants.

1.5. Conclusions

Plant-associated bacteria are a very challenging field of research, both from a fundamental and applied point of view. The first attempts to use these bacteria for the improvement of pest control or phytoremediation processes of both organic contaminants and metals are promising, but considerable research efforts are required to optimise the practical applications. More knowledge is required on the population dynamics and activity of endophytic bacteria in their host plants. Also the effects of contaminants on these processes should be further evaluated. Further studies must address the consequences of the co-operation between microbes in the rhizosphere under field conditions to assess their ecological impacts and biotechnological potential. A deeper knowledge of the population dynamics in response to different fertilization treatment, for example, could be of great help in the manipulation of the microbial consortia. For the construction of endophytic strains with new catabolic functions, natural gene transfer offers great potential. Many catabolic functions are found in soil bacteria where they are encoded on self-transferable plasmids or transposons. These can be easily transferred using natural gene transfer. Heterologous expression of the catabolic functions might not be a major problem, especially when the donor strain and the recipient endophytic strain are closely related, as is frequently the case. A considerable research effort will also be required to design strategies for the reinoculation of endophytic bacteria under field conditions. In order to guarantee reproducibility, reliable methods of inoculum delivery should be developed. This is especially the case for the inoculation of trees, such as poplar, with endophytic bacteria. Intense testing of different delivery systems indicated that the application method for introducing endophytic bacteria into plant tissue is strain specific (Musson *et al.,* 1995). Some methods that proved to be successful include the infusion of a bacterial suspension into imbibed seeds (Turner *et al.,* 1993) or the bacterial application via alginate beads (Bashan, 1986), which have the advantage of adding bacteria-specific nutrients to the alginate to improve bacterial survival rates. It should be noted that the development of successful application technologies would fully depend on improving our understanding of how bacterial endophytes

enter and colonize plants. This remark could be applied to all aspects of the ecology of bacterial endophytes and only under those circumstances the potential use of bacterial endophytes for plant beneficial purposes can be fully evaluated.

The observation of horizontal gene transfer presents the possibility of adapting the plant's endogenous endophytic and rhizospheric population directly without the need to select the appropriate plant-associated microorganisms from the plant species of interest. However, in order to be successful the genetic information encoding the desired metabolic properties should be present on a broad host range plasmid that can be efficiently transferred within the endophytic community, and it should have a broad expression range. If this is the case, the combination of horizontal gene transfer and heterologous expression has several obvious advantages over our original approach (Barac *et al.,* 2004) where an endophytic strain is optimized in a laboratory setup before being introduced into its host plant: there is no need to isolate plant-specific endophytic bacteria, there is no need for genetic manipulation of isolated plant-specific endophytes, and there is no need to establish the endophytic inoculum in the plant's endogenous endophytic community as the genetic information will be transferred to many members of the endogenous endophytic population (Taghavi *et al.,* 2005). Additional work needs to be done in this area, and is planned. First, endophytes such as these needs to be introduced into plant species that already show promise for remediation purposes due to their phenotypic characteristics, such as rapid growth and high water uptake levels. These plants will then need to be monitored for such things as retention of the endophyte as well as production and potential accumulation of metabolites of the pollutants, and volatilization under field conditions.

We also need to consider the potential role of naturally occurring plant-associated bacteria, and what their role may be in phytoremediation. We know that endophytes have multiple roles in plants, but understanding what that role may be in remediation has not been explored in any depth. Work by Van Aken *et al.* (2004a and b) showed that an endophytic *Methylobacterium populum* sp. isolated from poplar had the ability to degrade 2,4,6-trinitrotoluene (TNT) in the laboratory. However, the role of this bacterium, and the extent of its

contribution to TNT degradation within the intact plant system is still under investigation.

When we consider the diversity of endophytic colonization of plants, it is apparent that many of these organisms may play a major role in the degradation of organic contaminants, but what the role may be or how extensive that contribution is to the overall degradation of the contaminant is completely unknown. Better understanding of these contributions, as well as techniques to utilize and manipulate these processes is vital for the improvement of phytodegradation capabilities as well as enhanced survival. These improvements may come in the form of introduction of novel bacteria that have the needed degradative properties, enrichment of naturally occurring endophytes, or re-introduction of natural endophytes that have been altered for enhanced degradation capabilities. All of these options have potential for improving phytoremediation of organic contaminants.

Despite the difficulty in selecting effective multifunctional microbial inoculates, appropriate combinations can already be recommended. New environmentally friendly, genetically modified, microbial inoculates are being produced commercially and used to protect plants from disease and to promote plant growth. In the same way, plant-associated bacteria, both rhizospheric and endophytic, could play a major role in metal solubility and sequestration by the plants. For these reason further investigations in the role that these communities of bacteria have on metal solubility, plant uptake and tolerance is of main importance to improve phytoremediation of metal contaminants.

Chapter 2: Aim and outline of the thesis

Phytoextraction is a promising technology that assures soil recovery exploiting the natural uptake process that plants use for nutrients. It is a promising approach but it is still in its infancy stage and needs further research and development. Two factors determine the efficiency of the phytoextraction process: the plant (metal uptake, -translocation and -tolerance) and the soil (plant-availability of metals)

The ideal plant for phytoextraction should be able to tolerate and accumulate high levels of heavy metals (in harvestable plant parts) but also have a rapid growth rate and the potential to produce a high biomass, with an economical value. Unfortunately, till now, a plant possessing all these characteristics has not been found. Recent studies investigating the feasibility of phytoextraction confirmed that both biomass production and metal concentration factor (metal (hyper)accumulation) determine the efficiency of the remediation process.

Also metal availability in soils plays a crucial role in the efficiency of the remediation process. In many cases, it is considered as a limiting factor for an efficient phytoextraction. The use of amendments for mobilization (e.g. chelating or acidifying agents) of metals in the soil can sometimes improve the metal accumulation by plants. Chemically assisted phytoextraction is based on the use of non-accumulator plants with metal accumulation levels far below those of hyperaccumulators, but with high biomass potential. Restrictions apply, however, to both the use of complexing agents and artificial soil acidification. Toxicity has been reported. Furthermore, EDTA is poorly photo-, chemo- and bio-degradable. *In situ* application of both poorly degradable but also easily degradable chelating agents can cause groundwater pollution by uncontrolled metal dissolution and leaching.

Since in many cases metal uptake by plants is limited by low metal solubility, it is necessary that the efforts for selection of appropriate rhizosphere manipulation be continued. There is a need to find cheaper, environmentally benign chemical compounds with chelating properties as well as to better understand the role of rhizospheric bacteria in metal solubility, plant uptake and tolerance. The plant-rhizosphere interactions controlling metal uptake by roots are indeed of primary interest. To what extend root exudates can mobilize

metals or if microbial rhizosphere communities stimulated by these root exudates can contribute to metal phytoavailability, remains to be further examined. As certain plants can use microbial siderophores to improve their iron uptake, we hypothesized that bacterial metal chelators, such as siderophores, can eventually improve the uptake of heavy metals by plants. Bacterial siderophores can be considered as natural chelators and the bacterial production of which is in tight equilibrium with plant activity, thus improving heavy metal uptake and translocation as part of the phytoextraction process.

Another possibility that should be considered is the fact that Plant Growth Promoting Bacteria can stimulate root formation and/or aerial biomass production by plants (see Chapter 1). A better-developed root system can lead to increased metal uptake. A higher harvestable aerial biomass would allow an improved export if metal concentrations in the tissue remain at the same level.

Endophytic bacteria can lead to or be engineered for increased heavy metal sequestration. The activities of these bacterial strains could enhance heavy metal uptake and translocation by the host plants. An increased sequestration of toxic metals could also assist the host plant to tolerate higher metal levels in its tissues.

The former illustrates that plant-associated bacteria may have an important potential to 'assist' plants in improving the process of phytoextraction of metals.

In order to get a better understanding of the potential role of the microbial flora associated with plants possessing a potential for phytoextraction, we investigated several aspects of plant-associated bacteria of tobacco (*Nicotiana tabacum*), a plant species known for its already quite high potential for accumulation of several trace elements (Cd, Zn, Pb, ...).

Chapter III describes the isolation and characterization of rhizospheric and endophytic bacteria from different parts of tobacco plants growing on two different field sites (with different soil types) both characterized by a low metal contamination. The cultivable metal tolerant isolates were further identified by comparative sequence analysis of their 16S rDNA.

Chapter IV describes the difficulties we encountered developing a method to grow the plant in complete sterility and the desired concentration of cadmium to apply to the system.

Chapter V explores the results obtained after the inoculation of few rhizospheric and endophytic strains isolated from tobacco plants grown on metal contaminated soil as well as of an artificially built consortium. Influences on metal uptake and distribution and biomass production were examined.

Chapter VI investigates the microbial consortium associated with tobacco seeds isolated at different storage time. The cultivable strains were isolated, characterized and subsequently identified by comparative sequence analyses of their 16S rDNA. The period of preservation showed clearly to influence the colony forming units per seed (CFU's/seed) and the isolates characteristics. After the construction of a Neighbour Joining tree inclusive of the 16S rDNA belonging to the rhizospheric and plant endophytic isolates with the addiction of some 16S rDNA of seed endophytic strains; two main routes of seed colonisation by bacteria were underlined.

Chapter VII describes the influence that some of the seed associated strains, as well as different type of consortia, have on the plant growth, on plant metal toxicity and on plant metal uptake.

Chapter VIII provides a summarizing discussion and the general perspectives of this research.

Chapter 3. Isolation and characterization of cultivable rhizosphere and endophytic bacteria associated with *Nicotiana tabacum*.

Abstract

Rhizosphere and endophytic bacteria were isolated from different varieties of *Nicotiana tabacum* plants grown on two different field sites slightly contaminated with zinc and cadmium. On these sites, Lommel (Belgium) and Rafz (Switzerland), a phytoextraction feasibility study was ongoing. After isolation, the strains were phenotypically characterized testing them for key parameters related to interaction with metals, like minimum inhibiting concentration (MIC) and siderophore production. Differences in percentages of tolerant bacteria were observed in relation with the metal concentration in plants and soils suggesting an increased occurrence of tolerant strains related to higher metals availability. A genotypic characterization was carried out on the strains showing metal tolerance; a dominance of Gammaproteobacteria was observed in the different consortia, and inside this group the presence of *Stenotrophomonas maltophilia*, a strain present in different plant parts and common on the two different sites, was remarkable. This study delivered different endophytic and rhizospheric strains that looked promising for use in re-inoculation experiments in order to clarify their active role in the development of plants in presence of metal stress.

3.1 Introduction

Nicotiana tabacum L. (tobacco), a member of the family *Solanaceae* or the *night shade family,* is a handsome unbranched annual plant with large oval leaves reaching a height of 90cm up to 180cm. This species deserves its name from Joan Nicot, a Portuguese who introduced the tobacco plant into France. The specific name being derived from the pipe in which the herb is smoked. The leaf of the plant is used in the production of cigarettes and cigars in the tobacco processing industries.

In 1987, Reese and Wagner, isolated Cd-binding peptides from tobacco plants and later in 1999, Arazi *et al.* (1999) isolated, always from the same plant

species, NtCBP4 a calmodulin-binding protein. This protein is structurally similar to K^+ and to non-selective cation channels and has the function of modulating plant tolerance to heavy metals. Tobacco was also shown to considerably change its rhizosphere pH. Loosemore *et al.* (2004) demonstrated that, in their experimental conditions, the uptake of Zn by tobacco is only limited by the concentration in exchangeable Zn in soil. Further, the concentration in exchangeable Zn in soil depends on the pH in rhizosphere. That itself depends on the initial soil pH and on the ability of plants to change pH in their rhizosphere. Earlier, Mench *et al.* (1994) proved that tobacco exerted root actions which affect the same soil compartments as EDTA: the plant is able to remove water soluble and exchangeable metal fractions, metals bound to organic matter, and part of the metals bound to or included in metal oxides and clay minerals (Ure, 1995) illustrating its strong capacity of metal mobilization. Thanks to these properties and to the high biomass production, tobacco has been used in investigations of metal remediation (Mench and Martin, 1991; Guadagnini, 2000; Kayser *et al.*, 2000). Moreover, *Nicotiana* species have another important added value, they are repulsive to the herbivores (Glebert *et al.*, 2003).

Studies trying to clarify and eventually to change the interaction between tobacco and metals in the soil focused mainly either on transgenic plants or on arbuscular mycorrhyza.

Dhankher *et al.* (2003), using transgenic tobacco plants overexpressing a bacterial arsenate reductase gene (*arsC*), proved that the plants were significantly more cadmium tolerant than the wild-type. Janoušková *et al.* (2005), on the other hand, studied the influence of arbuscular mycorrhiza (AM) on tobacco plant growth under cadmium stress, they proved that AM may improve the phosphorous nutrition and decreased plants cadmium uptake.

Even if it is known that rhizosphere as well as endophytic population may play a role in the trace elements plant uptake influencing metal availability we couldn’t find any publication concerning *Nicotiana tabacum* associated bacteria.

Since tobacco is often proposed as a promising plant species for phytoextraction purposes (Guadagnini, 2000; Kayser *et al.*, 2000), we decided to investigate the endophytic and rhizospheric population of this plant species growing on two

different sites characterized by soils slightly contaminated with cadmium and zinc.

Tobacco showed to be well adapted to our specific field conditions and metals contamination levels. In this chapter we investigated the cultivable rhizosphere and endophytic bacteria associated with *Nicotiana tabacum*, their metal tolerance and siderophore production capabilities, both being key characteristics interacting with heavy metals tolerance. We intended to compare the cultivable consortia under different fertilization regime and grown in two soils (Rafz, Switzerland and Lommel, Belgium) showing differences in important soil parameters like pH and metals availability. Since cadmium and zinc are the two dominant metals involved in the contamination of both sites, we concentrated on the cadmium and zinc tolerant bacteria associated with tobacco. The cultivable strains were identified through the sequence of their 16S rDNA.

3.2 Materials and Methods

3.2.1 Sites description and soil characteristics

The phytoextraction trials in Rafz (Switzerland) and Lommel (Belgium), were part of the European project PHYTAC (project N° QLRT200100429). The main soil characteristics at both sites are shown in tables 3.1 and 3.2.

3.2.1.1 Switzerland - Rafz site (CH)

The Swiss experimental site at Rafz was located on a sandy loam (classified as haplic luvisol) field at Rafz, near Zürich airport. It has been contaminated by domestic and industrial sewage sludge (originating from an alkaline bath plant) in the 1960s, representing Cd, Zn, Cu, Pb and Cr contamination exceeding the guideline and trigger values, but still below the clean-up values of the Swiss Ordinance of Soil OIS (Karlaganis 2000).

Physical properties, Rafz	**2001-2003**		**2004**
% Sand (1)	54		-
% Silt (1)	28		-
% Clay (1)	18		-
%OM (1)	0.6		1.8 (2)
CEC (meq $100g^{-1}$) (1)	16.9		-
pH-H_2O (1)	6.8	7.0±0.002	6.8 (2)
Total metal content (mg kg DW^{-1}) *[2M HNO_3; CH]*			
Zn_{tot}	813 (1)	1286±157	505 (2)
Cd_{tot}	0.9 (1)	0.93±0.11	0.7 (2)
Cu_{tot}	58 (1)	56±8.0	54 (2)
Pb_{tot}	492 (1)	n.a.	362 (2)
Ni_{tot}			22 (2)
Cr_{tot}			97 (2)

Exchangeable metal (2) content (mg kg DW^{-1}) *[0.1M $NaNO_3$ extractable;CH]*	**Without fertili-sation**	**AN-Fertili-sation** (4)	**AS-Fertili-sation** (4)	**Clone 1 AN**	**Clone 1 AS**	**Clone 2 AN**	**Clone 2 AS**
Zn_{exch}	5.3±0.5			22.663	18.05	17.475	15.23
Cd_{exch}	<0.008			0.030	0.025	0.030	0.030
Cu_{exch}	0.26						
Pb_{exch}	0.04			0.183	0.175	0.215	0.150
pH-H_2O	7.0±0.002	6.7±0.04	6.5±0.7				
pH-KCl	6.0±0.006	5.5±0.02	5.4±0.1				

table 3.1: Soil characteristics at the Rafz field site in Switzerland. Metals extractability referred to two different tobacco clones 1: BAG, the mother clone and 2: NBZn7-51 under two fertilizations treatments, ammonium nitrate (AN) and ammonium sulphate (AS). 1) Parameters measured according to Wenger (2000). 2) Parameters measured XRF according to Keller (personal communication, 2004)

Physical properties	**2003**	**2004**	
% Sand(1)	86		
% Silt(1)	9		
% Clay(1)	5		
%OM(1)	3.9		
pH-H20	6.4±0.1	5.9±0.1	
pH-KCl	5.5±0.1	5.0±0.1	
Total metal content (mg kg DW^{-1}) *[aqua regia; EC]*			
Zn_{tot}	227±5		
Cd_{tot}	5.0±0.2		
Cu_{tot}	33±2		
Pb_{tot}	206±11		
Exchangeable metal content (mg kg DW^{-1}) *[$CaNO_3$ extractable; EC]*			
	NH_4-nitrate	**NH_4-nitrate**	**NH_4-sulphate**
Zn_{exch}	57±2	70.7±0.6	79.2±2.3
Cd_{exch}	0.93±0.09	1.13±0.03	1.23±0.03
Cu_{exch}	<0.25	<0.25	<0.25
Pb_{exch}	<0.5	<0.5	<0.5

table 3.2: Soil characteristics at the Lommel field site in Belgium. (1) Parameters measured according to Meers *et al.* (2005)

Injuries of sensitive crop plants due to heavy metal contamination were recorded for that site. The Rafz soil is very slightly acidic showing a pH (H_2O) of 6.8 and (KCl) 5.8, which is interesting for phytoextraction purposes according to its metal bioavailability. At the Rafz site the tobacco variants were tested simultaneously.

3.2.1.2 Belgium - Lommel site (B)

The Belgian experimental field is situated in Lommel. The soil contains increased levels of Zn, Cd and Pb due to atmospheric depositions caused by the activities of a metal smelter situated at about 500m SW of the experimental field. The site is a former maize field, which has been excluded from production by the government because of the contaminated character of the soil and exceeding of consumption limits of food and feed when cultivated on this soil. Soil characteristics are presented in table 3.2.

3.2.2.1 Plant material - Switzerland

For isolation and characterization of the plant associated bacteria, the following tobacco cultivars were sampled: cv. Badischer Geudertheimer (BaG) originating from the Landesanstalt für Pflanzenbau Rheinstetten, Germany and two pre-selected tobacco clones developed by Guadagnini (2000) derived from the mother clone BaG (1), NBZn7-51 (2) and NBZn5-57 (4). We were not able to harvest the stems of the plants as it would have meant lose of the samples for metals analyses foreseen for a later period.

3.2.2.2 Plant material – Belgium

On the Belgian site of Lommel the selected tobacco variants and their mother clone were tested simultaneously. The 5-6-weeks old *in vitro*-bred tobacco plants already acclimatized to freeland were planted in the field. The plants were planted in 3 replicates (2003) or 4 replicates (2004) in a completely randomized design (1 plant/replicate). During 2003 only the mother clone, 1, and the clone 2, were tested on Lommel soil, once tested the healthy plant growth on that kind of soil and in a different environment; during 2004 another clone, 4, was added to the experiment.

3.2.3 Fertilization treatment

In both years, in Lommel, growth and metal accumulation of the different tobacco variants were compared under different fertilisation scenarios. In 2003, a single (NF) and a double dose (F) of fertilisation were compared. In the first case the fertiliser (NPK 17-9-18 + 3%Mg) was applied only at the time of planting; in the second scenario fertilisation was repeated one month later, always at a rate of 500 kg/ha. In 2004 a double dose (2xF) of NH_4-nitrate fertilisation was compared with the same dose of a NH_4-sulphate fertiliser.

In Rafz in 2004, many fertilizations scenarios were tested, in order to optimize the plant growth and the metals uptake, (2x) of NH_4-nitrate and (2x) of NH_4-sulphate were part of the scenarios. Plant and soil analyses were provided by Dr. Ann Ruttens from Hasselt University (Belgium), while data from Rafz has being provided from Dr. Erika Nehnevajova from Phytotech-Foundation (PTF, Switzerland).

3.2.4 Isolation of plant-associated bacteria

In order to minimize field site effects/heterogeneity, rather than sampling five plants and individually analyzing them, combined samples consisting of equal amounts of the same tissues type from each individual plant were prepared. The different plant parts (roots, stems and leaves), grouped as described above, were sterilely sampled from the field and kept at 4 °C until analysis. For the isolation of rhizosphere, the roots were shaken for 1 h in 10 ml of sterile 10 mM $MgSO_4$ to remove the bacteria associated with the rhizosphere. Dilutions were made in sterile 10 mM $MgSO_4$ and plated on ten times diluted 869 medium (Mergeay *et al.* 1985). The plates were incubated at 30 °C for 7 d. Roots, stem and leaves were separately surface sterilized using a solution of active chloride supplemented with one droplet of Tween 80 per 100ml (table 3.3), rinsed 3 times with sterile deionised water and subsequently dried with sterile filter paper. A 100 µl sample of the third rinsing water was plated on 869 medium diluted ten times to verify the efficiency of sterilization (Germaine *et al.* 2004). After sterilization, roots, leaves and stems were macerated in 10 mM $MgSO_4$ using Polytron PT1200 mixer (Kinematica A6). Samples (100 µl) and their dilutions were plated on ten times diluted 869 medium. After 7 d incubation at 30 °C, colony forming units were determined and purification of the different morphological types was performed.

Plant species	Roots	Stems	Leaves
Nicotiana tabacum	3 min with 0.2% active chloride	5 min with 1% active chloride	6 min with 0.5% active chloride

table 3.3: Surface sterilization protocol of different tobacco plant parts applied to plant harvested from the fields in Lommel and Rafz.

3.2.5 Bacteria heavy metals resistance

The isolated strains, after at least three times sub-cultures to ensure purity, were tested for their heavy metals resistance using the minimal Tris buffered medium 284 (Schlegel *et al.*, 1961). This medium contains per liter deionised water, 6.06g Tris-HCl, 4.68g NaCl, 1.49g KCl, 1.07g NH_4Cl, 0.43 Na_2SO_4, 0.2g $MgCl_2 \cdot 6H_2O$, 0.03g $CaCl_2 \cdot 2H_2O$, 40mg $Na_2HPO_4 \cdot 2H_2O$ 1%, 10ml Fe(III)NH_4 citrate solution (containing 48mg:100ml), 1ml microelements solution, final pH 7. Four different carbon sources (1.3ml glucose 40%, 2.2ml gluconate 30%, 2.7ml fructose 20% and 3ml succinate 1M) were added. The microelements solution contains per liter distilled water: 1.3ml 25% HCl, 144mg $ZnSO_4 \cdot 7H_2O$, 100mg $MnCl_2 \cdot 4H_2O$, 62mg H_3BO_3, 190mg $CoCl_2 \cdot 6H_2O$, 17mg $CuCl_2 \cdot 2H_2O$, 24mg $NiCl_2 \cdot 6H_2O$ and 36mg $NaMoO_4 \cdot 2H_2O$. This medium was supplemented with different concentrations of $CdCl_2$ and separately of $ZnSO_4$. The minimum Inhibiting Concentration (MIC) was determined and defined as the minimal concentration of the heavy metal inhibiting growth of the bacterial strains. The range tested for Zn was from 0mM up to 4mM, and the one for Cd was from 0mM up to 1.5mM.

3.2.6 Bacteria carbon sources

The pure isolated strains were tested for their favoured carbon source, choosing between glucose, gluconate, fructose and succinate, using a minimal Tris buffered medium 284 (Schlegel *et al.*, 1961). The carbon sources were supplemented on separate Petri dish using either 1.3ml glucose 40% or 2.2ml gluconate 30% or 2.7ml fructose 20% or 3ml succinate 1M per liter of medium.

Bacteria were tested without carbon sources in order to check their eventual heterotrophy, with all the carbon sources and on 869 diluted ten times to have a positive control.

3.2.7 Siderophore assay

A chrome azurol S (CAS) shuttle solution (Schwyn and Neilands, 1987) was used for routine testing of siderophore production in liquid media. This test was carried out using liquid medium 284 (Schlegel *et al.*, 1961) in glass tubes. The test was carried out using both minimal medium without iron and with minimal medium with 0.25µM Fe(III) citrate.

3.2.8 Bacterial DNA extraction

The pure strains were grown in their appropriate medium (1/10 869) at 30°C on a shaker. The bacterial growth was stopped when an OD_{660} included between 0.5 and 0.8. The culture was centrifuged in eppendorf tubes at maximum speed during 10min. The pellet was frozen after medium removal. The defrosted pellet was re-suspended in 300µl 1xSSC +2mg/ml Lysozyme + 1:100 of 10mg/ml RNase. After incubation at 37°C during 15min, 17 µl of SDS (10%) were added and the solution was again incubated during 10min. 3.3µl of proteinase K (20mg/ml) were added to finish the lysis step and incubated during one hour at 65°C.

A phenol chloroform extraction was carried out. The DNA precipitation was obtained adding 1/10 volume of 3M Na Acetate pH 6 and 2.5x 100% ethanol (-20°C). The DNA was subsequently fished out and solubilized in sterile ddH_2O.

DNA quality was checked on 0.8% Agarose gel.

3.2.9 Box-PCR genomic DNA profile

The DNA extracted was amplified by PCR using the BOX1 primer. The PCR reaction contains 5µl 10x Taq-Buffer, 4µl 10mM dNTP's, 2µl Box-primer (forward + reverse, sequence: 5'-CTACGGCAAGGCGACGCTGACG-3'), 0.25µl Taq polymerase and 5µl template, in a total final volume of 50µl. The thermocycling conditions were: 1 min. at 95°C, 35 cycles of 1min. at 95°C, 1.5min. at 50°C,

8min. at 65°C, and finally one cycle at 65°C during 8 min. The obtained PCR products were separated by means of a 2% agarose gel electrophoresis with 4µl/100ml of ethidium bromide run during 2 hours at 75V.

3.2.10 PCR amplification

PCR amplification was carried out, targeting the 16S gene using primers P0 (27f sequence: 5' GAGAGTTTGATCCTGGCTCAG) and P6 (1495r sequence: 5' CTACGGCTACCTTGTTACGA).

The extracted DNA (1µl) was used in a final volume of 50µl PCR's, each reaction, consisting of: 5µl buffer (10x), 2µl $MgCl_2$ (50mM), 1µl of each primer (10µM each provided by Invitrogen), 1µl 10mM dNTP's (Invitrogen), 0.4µl *Taq* (Invitrogen), prepared as a master mix, with addition of sterile ddH_2O till the final volume, prior to DNA addition.

Cycling conditions were: 1.5 min at 95°C, 5 cycles of 30 sec at 94°C, 30 sec at 60°C, 2 min. at 72°C, 5 cycles of 30 sec. at 94°C, 30 sec. at 55°C, 2 min. at 72°C, 25 cycles of 30 sec. at 94°C, 30 sec. at 50°C, 2 min. at 72°C and finally one cycle of 10 min. at 72°C.

5µl of the resulting PCR-DNA product was then checked by gel electrophoresis at 80V during 30min on a 0.8% Agarose gel with 4µl/100ml of ethidium bromide. The remaining PCR products were cleaned by GFX PCR DNA and gel band purification kit (Amersham Biosciences) before sequence.

3.3 Results and discussion

3.3.1 Lommel and Rafz soils

A phytoextraction field trial was started in Lommel (Belgium) and in parallel in Rafz (Switzerland) in order to compare the heavy metals accumulation by the same plants grown on two soils with quite different characteristics. Rafz soil, compared with the one in Lommel, had different traits, like a higher pH, a higher total contamination, but a lower bioavailable fraction of metals (table 3.1 and

3.2). A first experiment was performed in Lommel during summer 2003, in order to set up the experimental procedures, like feasibility of plant growth and bacterial isolations from the different plant parts.

From the sample harvested from these fields' experiments we isolated the endophytic and rhizospheric bacteria were isolated.

picture 1: Different flowering variants of *Nicotiana tabacum* growing on the field site in Lommel during summer 2003. Spade handle as reference in first stage.

3.3.2 Isolation of cultivable bacteria from plant and rhizosphere material

We separately analysed leaf, stem, root and rhizosphere of three months old plants, grown on Lommel or Rafz soil. From Rafz site, the stems were not analysed as explained in paragraph 3.2.2. We carried out the bacteria counting (CFU/gFW) on surface sterilized plant material for Lommel 2003 (table 3.4) and for both Lommel and Rafz during summer 2004 (table 3.5). In Lommel 2003

only the mother clone 1 (BaG) and one of its mutants, 2, were sampled for associated bacteria, while in 2004 another mutant, 4, has been added to the study. Analysing the results of 2003, the cultivable bacteria population of tobacco plants tents to be slightly higher in the plants fertilized only once than in the plants fertilized twice, except for stem of 1 and for leaf of 2 that have higher values in the double fertilized plants. Analyzing data from 2004, the bacterial concentration in Rafz samples is in general higher. In clone 2, as for the mother clone 1, the cultivable bacteria seem to be more abundant under ammonium fertilization both in Lommel and Rafz, while the opposite seems to happen for 4 who show lower cultivable concentrations under this fertilization treatment.

Clone		Plant part	CFU/g		Clone	Plant part	CFU/g
2NF		L	0		2F	L	$2,2x10^2$
		S	$2,6x10^3$			S	$4,1x10^1$
		R	$8x10^2$			R	$6,6x10^2$
		RH	$5,2x10^9$			RH	$3,8x10^9$
1NF		L	0		1F	L	0
		S	$1,1x10^2$			S	UC
		R	$1,1x10^4$			R	$4,2x10^3$
		RH	$5,1x10^9$			RH	$4x10^9$

table 3.4: Colony Forming Units (CFU), per gram of plant material, found in the plant materi sampled during summer 2003 in Lommel, (Belgium). In the column sample, 2 stands f NBZn7-51 and it is a mutant of 1 (BaG), while F stands for fertilized twice and NF fertilize once. In the column plant parts, L stands for leaf, S for stem, R for root and RH f rhizosphere. UC stands for uncountable.

	Fertilization: ammonium sulphate			Fertilization: ammonium nitrate		
Field Site	Clone	Plant part	CFU/g	Clone	Plant part	CFU/g
	1	L	0	1	L	0
		S	n.h.		S	n.h.
		R	$2x10^2$		R	$9x10^3$
B		RH	$1.8x10^8$		RH	$1.1x10^8$
A	2	L	$1.8x10^2$	2	L	$1.69x10^4$
L		S	$2.3x10^3$		S	$1.74x10^4$
E		R	$9.1x10^3$		R	$1.47x10^4$
N		RH	$2.2x10^8$		RH	$5.6x10^9$
	4	L	$9x10^2$	4	L	0
		S	$4.85x10^5$		S	$3.7x10^3$
		R	$4.38x10^5$		R	$2.1x10^4$
		RH	$1.46x10^8$		RH	$3x10^8$
	1	L	$3.5x10^3$	1	L	0
		R	u.c.		R	$1.2x10^6$
		RH	$3.9x10^{10}$		RH	$1.4x10^{10}$
R	2	L	0	2	L	0
A		R	$1.8x10^5$		R	$3.3x10^5$
F		RH	$2.6x10^{10}$		RH	$1.4x10^{12}$
Z				$2^{\diamond}$	L	$6.7x10^2$
					R	$7.4x10^5$
					RH	$2.2x10^{11}$
	4	L	0	4	L	0
		R	$2.2x10^7$		R	$6.1x10^5$
		RH	$2.5x10^{10}$		RH	$6.2x10^{10}$

table 3.5: Colony Forming Units (CFU), per gram of plant material, isolated in plant material sampled during summer 2004 in the two different field experiments, one running in Lommel (Belgium) and the second in Rafz (Switzerland). In the column sample, 1 stands for BAG and it is the mother clone, 2 stands for NBZn7-51 and 4 stands for NBZn5-57; these last two are BAG mutants. In the column plant parts, L stands for leaf, S for stem, R for root and RH for rhizosphere. $^{\diamond}$ were plants fertilized only once with ammonium nitrate and not twice like all the others samples. n.h. is not harvested, while u.c. is uncountable.

3.3.3 Plant growth

Comparing the plants harvested on the two fields, Rafz and Lommel during summer 2004, the biomass production was in general from 2 to 4 times higher in Rafz (table 3.8). The tobacco mother clone (1) treated with the lowest fertilization treatment in Rafz has an estimated production of 7.4 ton ha^{-1} that reaches 9.5 ton ha^{-1} using the triple amount of fertilization (Herzig and Nehnevajova, not published). On the contrary the metal concentration inside the plants is much higher in Lommel especially for cadmium (table 3.8). This was most probably due to the difference in pH values and soil texture present in the two fields; in Lommel the pH of the sandy was 1 unit lower compared to Rafz (tables 3.1 and 3.2), this resulted in an increased plant-availability of the metals.

In Lommel the best biomass production was achieved by clone 2 under both fertilization treatments, while in Rafz the mother clone (1) was showing the best growth.

In Lommel ammonium sulphate seemed to enhance the plant metals accumulation for both mother clone 1 and clone 2, while clone 4 seemed less sensible to the fertilization showing comparable metals concentration under both treatments. Ammonium sulphate increased, even if only slightly, the cadmium concentration in plant growing in Rafz as well, while clone 4 showed a higher zinc concentration too. The opposite happened for zinc in mother clone 1 and clone 2 grown in Rafz: ammonium nitrate fertilization was leading to a more efficient zinc extraction.

Field	Fertilization	Tobacco clone	Biomass (g)	Zn (mg kg^{-1})	Cd (mg kg^{-1})
Lommel 2004	Ammonium sulphate	1	31.38 (6.34)	822.98 (149.26)	23.77 (2.59)
Lommel 2004	Ammonium sulphate	2	56.28 (14.48)	674.67 (154.34)	16.43 (3.93)
Lommel 2004	Ammonium sulphate	4	37.9 (27.84)	599.42 (71.05)	13.39 (1.12)
Lommel 2004	Ammonium nitrate	1	15.15 (14.88)	609 (114.53)	16.58 (3.4)
Lommel 2004	Ammonium nitrate	2	55.57 (12.41)	681.4 (231.79)	14.99 (4.07)
Lommel 2004	Ammonium nitrate	4	35.96 (27.18)	633.26 (114.14)	13.46 (0.75)
Rafz 2004	Ammonium sulphate	1	186 (29.1)	540.43 (10.16)	3.79 (0.18)
Rafz 2004	Ammonium sulphate	2	115 (21)	446.67 (9.77)	2.91 (0.09)
Rafz 2004	Ammonium sulphate	4	143 (8)	554.88 (18.17)	3.79 (0.28)
Rafz 2004	Ammonium nitrate	1	196 (12.8)	560.4 (28.11)	3.12 (0.08)
Rafz 2004	Ammonium nitrate	2	146 (27.3)	619.09 (10.76)	2.88 (0.24)
Rafz 2004	Ammonium nitrate	4	156 (25.6)	392.37 (8.68)	2.88 (0.08)

table 3.8: Plant biomass production, dry weight of shoots (g) and metal concentration of zinc and cadmium in mg kg^{-1}. Tobacco clone analysed are: 1 the mother clone (BaG), 2 NBZn7-51, and 4 NBZn5-57. Standard deviation is expressed in brackets. Data from Lommel provided from Dr. Ann Ruttens from University of Hasselt (Belgium), while data from Rafz has being provided from Dr. Erika Nehnevajova from Phytotech-Foundation (PTF, Switzerland).

3.3.4 Characterization of the plant associated bacteria

From the different plant parts, we isolated the strains belonging to the different colony types observed. In a next step, they were phenotypically characterized (MIC, siderophore production and preference in carbon sources used). Strains isolated from the two different sites in 2004 that showed metal tolerance were identified by the mean of the sequence of their 16S rDNA gene.

3.3.4.1 Phenotypic characterization

Since the plants were growing on a metal contaminated soil, we hypothesized that the endophytic and/or the rhizospheric population could have developed characteristics related with the presence of metals. For this reason, the isolated bacteria were analysed for important characteristics interacting with heavy metals stress and bioavailability such as siderophore production and minimum inhibiting concentration (MIC, data resumed in tables 3.11 to 3.23). MIC values were determined for zinc and cadmium, the elements of major concern present into the two different soils. Comparing the data concerning the soluble metal concentration in Rafz soil during 2004 for the different combinations of fertilization treatment and tobacco clones (table 3.1) with the MIC values obtained for the cultivable rhizosphere strains (table 3.10) there seems to be a relation. Tobacco clone 1 fertilized with ammonium nitrate showed both the highest metal availability for cadmium and zinc and the highest percentage of cadmium and zinc tolerant strains as well as the highest percentage of total metal tolerant strains (S1N in table 3.10). The second highest in total percentage of metal tolerant strains was tobacco clone 2 fertilized again with ammonium nitrate (S2N in table 3.10); it showed the second highest concentration of soluble metals in soil (table 3.1). The relation was confirmed by the data obtained for tobacco clone 1 and 2 fertilized with ammonium sulphate. In this case the total percentage of tolerant strains is higher in clone 2, where the zinc concentration was lower, but the cadmium concentration was higher; the percentage of both zinc and cadmium tolerant strains was also higher when comparing with tobacco clone 1. These results were confirmed by the results of the metal concentrations in the plants (table 3.8). The plant metal concentrations in Lommel 2004 and the metal tolerance data of the bacteria isolated from the same plants showed similar trends. As already underlined, in

Lommel under ammonium sulphate fertilization the metal concentration inside the plants is higher (table 3.8) compared to the same clones treated with ammonium nitrate; in the same way, tobacco clone 2 hosted a higher percentage in tolerant strains when fertilized with ammonium sulphate (B2S and B2N in table 3.10). Tobacco clone 4 that didn't show any difference in metals concentration in relation with fertilization (table 3.8), didn't present any difference in percentage of tolerant strains as well (B4N and B4S in table 3.10). This observed trend seemed not to be applicable to clone 2 that showed the opposite trend; this result, anyway, could be due to the high difference in number of strains analysed.

Comparing the percentages of metal tolerant strains in Lommel during the two experiments, the first in 2003 and the second in 2004, a much higher percentage of cadmium tolerant strains was noticed in 2003 compared with 2004 when the strains exclusively cadmium tolerant were rare (table 3.9 and 3.10).

Variety	N.G.(%)	Total colonies in different plant parts	Only Zn tolerant (%)	Only Cd tolerant (%)	Both Zn and Cd tolerant (%)	Total of tolerants (%)
1F	50	Leaf: 4	0	0	0	0
	52.17	Stem:23	0	0	17.4	17.4
	15.4	Root: 39	10.25	7.7	2.6	20.55
		Rhizosphere:29	10.34	20.7	17.24	48.28
1NF	0	Stem:4	0	0	0	0
	16.7	Root:12	8.3	0	8.3	16.6
	10.5	Rhizosphere:19	0	5.26	26.3	31.56
2F	0	Leaf: 3	33.3	0	0	33.3
	16	Stem: 25	0	8	0	8
	0	Root: 25	0	40	4	44
	5	Rhizosphere: 20	0	25	5	30
2NF	100	Leaf: 1	0	0	0	0
	4.5	Stem: 22	0	0	0	0
	3.6	Root: 28	3.6	57.1	0	60.7
	5.6	Rhizosphere: 18	0	11.1	5.6	16.7

table 3.9: Percentage of cultivable strains zinc tolerant (≥0.5mM), cadmium tolerant (≥0.6mM) and tolerant to both cadmium and zinc found in the plant material sampled during summer 2003 in Lommel (Belgium). 1F: BaG mother clone, fertilized twice; 1NF: BaG mother clone, fertilized once; 2F: clone 2 (NBZn7-51), fertilized twice; 2NF: clone 2 (NBZn7-51), fertilized once. N.G.% are the percentages of colonies not grown on the media in our use for the test.

Variety	N.G.%	Total colonies isolated in the different plant parts	Only Zn tolerant (%)	Only Cd tolerant (%)	Both Zn and Cd tolerant (%)	Total of tolerates (%)
S2N	50	Root:8	0	0	0	0
	52.6	Rhizosphere:19	26.3	5.26	0	31.56
B2N	75	Leaf:4	0	0	0	0
	6.5	Stem:16	18.75	0	0	18.75
	36.4	Root:11	3.3	0	3.3	6.6
	20	Rhizosphere:5	20	0	20	40
S1S	50	Leaf:2	0	0	50	50
	66.7	Root:3	0	0	33.3	33.3
	22.2	Rhizosphere:17	11.8	0	5.9	17.7
B1S	0	Root:1	0	0	0	0
	6.67	Rhizosphere:12	8.3	0	8.3	16.6
S4N	40	Root:10	10	0	20	30
	45	Rhizosphere:20	10	0	5	15
B4N	50	Stem:6	0	33.3	0	33.3
	40	Root:10	10	0	20	30
	38.9	Rhizosphere:18	22.2	0	11.1	33.3
B2S	10	Stem:10	30	10	0	40
	37.5	Root:8	12.5	0	12.5	25
	28.6	Rhizosphere:7	28.6	14.3	0	42.9
S2S	25	Root:4	0	0	0	0
	36.4	Rhizosphere:22	9.1	0	18.2	27.3
S1N	57.1	Root:7	0	0	0	0
	25	Rhizosphere:12	8.3	8.3	41.6	58.2
B1N	40	Root:5	0	0	0	0
	60	Rhizosphere:5	20	0	0	20
B4S	0	Leaf:2	0	0	0	0
	30.8	Stem:13	30.8	7.7	0	38.5
	80	Root:10	0	0	0	0
	25	Rhizosphere:12	33.3	0	0	33.3
S4S	26.3	Rhizosphere:19	21	0	21	42
S21N	50	Root:8	25	0	12.5	37.5
	55.6	Rhizosphere:9	11	0	11	22

table 3.10: Percentage of cultivable strains zinc tolerant (≥0.5mM), cadmium tolerant (≥0.6mM) and tolerant to both cadmium and zinc found in the plant material sampled during summer 2004 in the two different field experiments. S2N: Rafz, clone 2 (NBZn7-51), ammonium nitrate; B2N: Lommel, clone 2 (NBZn7-51), ammonium nitrate; S1S: Rafz, clone 1 (BaG), ammonium sulphate; B1S: Lommel, clone 1 (BaG), ammonium sulphate; S4N: Rafz, clone 4 (NBZn5-57), ammonium nitrate; B4N: Lommel, clone 4 (NBZn5-57), ammonium nitrate; B2S: Lommel, clone 2 (NBZn7-51), ammonium sulphate; S2S: Rafz, clone 2 (NBZn7-51), ammonium sulphate; S1N: Rafz, clone 1 (BaG), ammonium nitrate; B1N: Lommel, clone 1 (BaG), ammonium nitrate; B4S: clone 4 (NBZn5-57), ammonium sulphate; S4S: Rafz, clone 4 (NBZn5-57), ammonium sulphate; S21N: Rafz, clone 2 (NBZn7-51), ammonium nitrate only one treatment instead of two like all the others. N.G.% are the percentages of colonies that were impossible to test with the media in our use.

3.3.4.2 Carbon sources

To be able to start grouping them based on something more than the colony characteristics the cultivable strains isolated from rhizosphere and plant material were screened using simple carbon sources like sucrose, gluconate, succinate and glucose. Testing on a medium without carbon sources allowed us to identify the autotrophic strains, their growth on medium with metals should be considered as not trustable. The results are presented in tables 3.11 to 3.23.

3.3.4.3 Siderophores production

Siderophores are organic compounds that can bind Fe^{3+} with very high affinity (O'Sullivan and O'Gara, 1992). They can be excreted by both rhizosphere and endophytic bacteria (Sessitsch *et al.*, 2004; Buysens *et al.,* 1994). The effect of this characteristic is double. On one hand, the bacteria producing these molecules may immobilize the iron rendering it unavailable and preventing, in this way, the proliferation of pathogens (Buysens *et al.*, 1994). On the other hand, bacteria producing siderophores may help plant growth capturing the iron present in the soil in bio-unavailable forms and rendering it available to the plants as Fe-siderophore complex and reducing the toxicity of the metals present in the environment (Rajkumar *et al.*, 2005). This characteristic added to

the MIC values and the carbon sources used allowed us to make different groups of bacteria giving us an idea of the heterogeneity of the microbial population associated to tobacco plants, presented from table 3.11 to 3.23.

3.3.4.4 BOX-PCR genomic DNA fingerprinting

Strains showing metals tolerance were screened for their belonging to similar species by means of BOX PCR before their 16S rDNA was sequenced. The use of this method allowed us to group the bacteria and to limit the number of strains that had to be sequenced.

An example of BOX PCR result is presented in figure 3.2. It has to be mentioned that in some cases even strains showing different BOX-PCR fingerprint were determined as the same species based on their 16S rDNA sequence (see section 3.3.4.5); this was for instance the case of *Stenotrophomonas maltophilia* (fig. 3.2) proving a high diversity inside the same species. In figure 3.2 are presented 6 strains isolated from different plant parts, apart 150 and 427 that have same BOX PCR fingerprint, in fact, the other four show differences in the BOX profile, suggesting a possible difference in strain, this is confirmed by the analyses of the sequences.

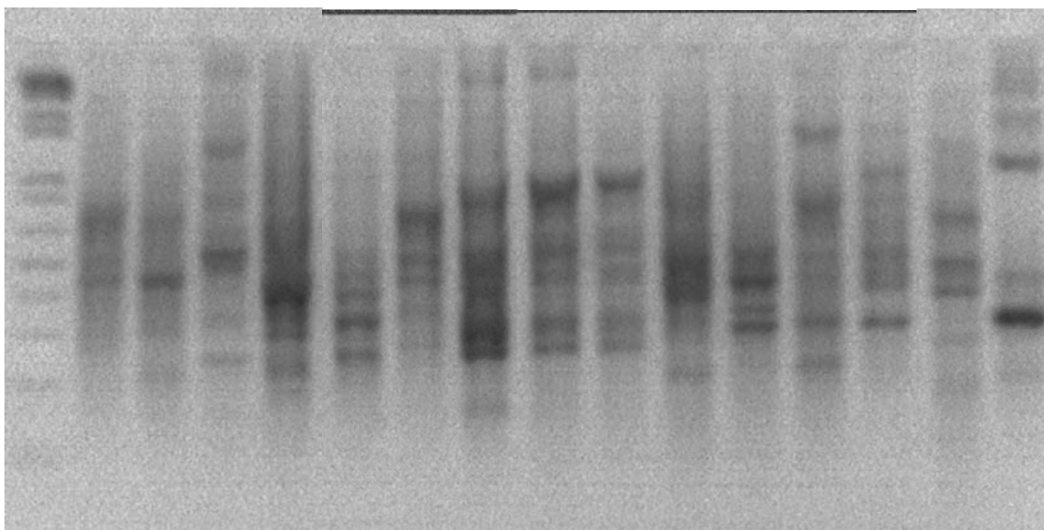


figure 3.2: Box-PCR fingerprinting of endophytic bacteria isolated from *Nicotiana tabacum* var. BaG. In lane 1: 1kb+ DNA ladder, lane2: *Stenotrophomonas maltophilia* (259), lane3: *Microcella putealis* (183), lane4: *Stenotrophomonas maltophilia* (365), lane5: *Pantoea agglomerans* (409), lane6: *Wautersia respiraculi* (BS3), lane7: *Stenotrophomonas maltophilia* (164), lane8: *Stenotrophomonas maltophilia* (339), lane9: *Stenotrophomonas maltophilia* (150), lane10: *Stenotrophomonas maltophilia* (427), lane11: *Arthrobacter nicotinivorans* (419), lane12: *Pseudomonas sp*. (188), lane13: *Arthrobacter sp*. (285), lane14: *Variovorax sp*. (275), lane15: *Pseudomonas sp*. (293), lane16: *Cryseobacterium sp*. (311)

Strain	Isolated from	284+ FR	284+ SC	284+ GS	284+ GN	284-C	284+ 4C	Zn mM	Cd mM	Sid.	Similar to
S. maltophilia (358)	S2NRH	-	-	-	-	-	-	0.5	0.15	N.T.	
S. maltophilia (363.b)	S2NRH	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	0.75	0.45	N.G.	
S. maltophilia (295)	S1SL	-	+	-	+	-	+	0.5	0.6	N.G.	
S. maltophilia (300)	S1SR	+	+	+	-	-	+	1	1.5	N.T.	
S. maltophilia (359)	S2NRH	-	-	-	+	-	+	0.5	<0.15	N.T.	
S. maltophilia (365)	S2NRH	-	-	-	-	-	-	0.5	0.15	N.T.	
S. maltophilia (50)	B1SRH	-	-	-	-	-	+	0.25	<0.15	-	b
S. maltophilia (405)	S4NR	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	0.5	0.45	N.G.	
S. maltophilia (259)	B4NS	-	-	-	-	-	-	<0.25	<0.15	N.G.	
S. maltophilia (427)	B4NS	+	-	+	+	-	+	<0.25	0.75	N.G.	c
S. maltophilia (273)	B4NR	+	+	+	+	-	+	1	0.75	-	
S. maltophilia (49)	B4NRH	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	1.5	0.15	N.T.	
S. maltophilia (150)	B2SR	-	+	-	-	-	+	0.75	0.75	N.G.	c
S. maltophilia (160)	B2SRH	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	0.5	<0.15	+	
S. maltophilia (164)	B2SRH	-	-	-	-	-	+	0.75	0.45	-	b
S. maltophilia (328)	S2SRH	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	0.5	0.6	N.T.	
S. maltophilia (338)	S2SRH	-	-	-	-	-	+	0.75	0.75	N.G.	
S. maltophilia (339)	S2SRH	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	0.5	<0.15	N.G.	
S. maltophilia (342)	S2SRH	+	+	+	+	-	+	1	1.5	N.T.	
S. maltophilia (343)	S2SRH	+	+	+	+	-	+	0.75	0.75	N.G.	
S. maltophilia (320)	S1NRH	+	+	+	+	-	+	1	0.75	N.G.	

Strain	Isolated from	284+ FR	284+ SC	284+ GS	284+ GN	284-C	284+ 4C	Zn mM	Cd mM	Sid.	Similar to
S. maltophilia (322)	S1NRH	+	+	+	+	-	+	0.75	0.9	-	b
S. maltophilia (96)	B4SS	-	-	-	-	-	+	0.5	0.3	-	a
S. maltophilia (100)	B4SS	-	+	-	+	-	+	0.5	0.45	-	a
S. maltophilia (398)	S4SRH	+	+	+	+	-	+	0.5	<0.15	N.G.	
S. maltophilia (75)	S21NRH	-	-	-	-	-	+	<0.25	<0.15	N.G.	
S. maltophilia (62)	S21NRH	-	-	-	-	-	+	0.5	0.3	-	a
S. maltophilia (384)	S4SRH	-	-	-	-	-	+	0.35	0.45	N.G.	
Pseudomonas sp. (188)	B2NS	+	+	-	-	-	+	1	0.6	-	
Pseudomonas sp. (354)	S2NRH	+	+	+	+	-	+	0.5	<0.15	N.T.	
Pseudomonas sp. (303)	S1SRH	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	0.75	0.15	-	
Pseudomonas sp. (293)	B4NRH	+	+	-	-	-	+	0.75	0.9	+/-	
Pseudomonas sp. (137)	B2SS	-	-	-	-	-	+	0.75	0.45	+/-	
Pseudomonas sp. (141)	B2SS	+	+	-	+	-	+	0.5	0.3	N.T.	
Pseudomonas sp. (143)	B2SS	+	+	-	+	-	+	0.5	0.15	N.T.	
Pseudomonas sp. (54)	S1NRH	+	+	+	+	-	+	0.35	0.75	+/-	
Pseudomonas sp. (55)	B4SS	+	+	-	+	-	+	<0.25	<0.15	N.G.	
Pseudomonas sp. (246)	B4SRH	+	+	+	+	-	+	0.5	0.15	N.T.	
Arthrobacter sp. (107)	S1SRH	+	+	+	+	-	+	0.75	<0.15	+	
Arthrobacter sp. (418)	S4NRH	-	-	-	-	-	+	1	0.9	N.T.	
Arthrobacter sp. (419)	S4NRH	+	+	+	+	-	+	1.5	0.15	+/-	
Arthrobacter sp. (285)	B4NRH	+	-	+	+	-	+	1.5	<0.15	-	
Arthrobacter sp. (286)	B4NRH	+	+	-	+	-	+	0.5	<0.15	-	

Strain	Isolated from	284+ FR	284+ SC	284+ GS	284+ GN	284-C	284+ 4C	Zn mM	Cd mM	Sid.	Similar to
Arthrobacter sp. (333)	S2SRH	+	+	+	+	-	+	0.5	<0.15	-	
Pantoea agglomerans (409)	S4NR	+	+	-	+	-	+	0.35	0.6	+	
Pantoea agglomerans (138)	B2SS	+	+	+	+	-	+	0.25	0.6	N.T.	
Pantoea agglomerans (85)	S2SR	+	+	-	+	-	+	0.25	0.6	+/-	
Pantoea agglomerans (387)	S4SRH	+	-	+	+	-	+	1.5	0.6	N.T.	
Enterobacter sp. (319)	S1NRH	+	+	-	+	-	+	1.5	1.05	+	
Enterobacter sp. (321)	S1NRH	+	+	+	+	-	+	1	0.75	+	
Enterobacter sp. (392)	S4SRH	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	1	0.75	-	
Enterobacter sp. (401)	S4SRH	+	-	+	+	-	+	0.75	0.45	N.T.	
Enterobacter sp. (395)	S4SRH	+	+	+	+	-	+	0.75	0.45	N.G.	
Enterobacter sp. (389)	S4SRH	+	+	+	+	-	+	0.5	0.6	N.T.	
Enterobacter sp. (399)	S4SRH	+	+	+	+	-	+	0.5	0.9	N.T.	
Enterobacter sp. (400)	S4SRH	+	+	+	+	-	+	0.5	0.3	+	
Variovorax sp. (275)	B4NR	+	+	+	+	-	+	1	0.45	+	
Variovorax sp. (280)	B4NR	-	+	-	+	-	+	1	0.75	N.T.	
Leifsonia aquatica (316)	S1NRH	-	-	-	-	-	+	0.5	<0.15	N.G.	
Crustibacterium reblochoni (48)	B4SS	+	+	+	-	-	+	0.75	0.15	N.T.	
Microcella putealis (183)	B2NR	-	-	-	-	-	-	0.5	0.15	N.G.	
Cryseobacterium sp. (311)	S1SRH	-	-	+	-	-	+	0.5	0.15	-	
Sphingobium herbicidovorans (71)	B4NRH	-	-	-	-	-	+	0.5	0.15	-	

table 3.24: Resume of phenotypic and genotypic characteristics of metals tolerant cultivable strains isolated from different plant parts of different tobacco clones grown in Rafz or Lommel in 2004. 284+FR is the medium 284 supplemented with fructose as carbon source, in the same way 284+SC, is supplemented with sucrose, 284+GS with glucose, 284+GN with gluconate, 284-C, no carbon sources were added, 284+4C all the carbon sources were added at the same time. + if the strain could use the carbon sources supplied, - if the strain couldn't use it. MIC values are reported for zinc and cadmium in mM. Sid is siderophores production, + if producer, - if no producer, N.G. if not grown in the medium for the test, N.T. is Not tested. In the column "similar to" same letter corresponds to identical BOX PCR profile. S2N: Rafz, clone 2 (NBZn7-51), ammonium nitrate L,S,R,RH stands respectively for Leaf, Stem, Root, RHizosphere; B2N: Lommel, clone 2 (NBZn7-51), ammonium nitrate L,S,R,RH stands respectively for Leaf, Stem, Root, RHizosphere; S1S: Rafz, clone 1 (BaG), ammonium sulphate, L,S,R,RH stands respectively for Leaf, Stem, Root, RHizosphere; B1S: Lommel, clone 1 (BaG), ammonium sulphate L,S,R,RH stands respectively for Leaf, Stem, Root, RHizosphere; S4N: Rafz, clone 4 (NBZn5-57), ammonium nitrate L,S,R,RH stands respectively for Leaf, Stem, Root, RHizosphere; B4N: Lommel, clone 4 (NBZn5-57), ammonium nitrate L,S,R,RH stands respectively for Leaf, Stem, Root, RHizosphere; B2S: Lommel, clone 2 (NBZn7-51), ammonium sulphate L,S,R,RH stands respectively for Leaf, Stem, Root, RHizosphere; S2S: Rafz, clone 2 (NBZn7-51), ammonium sulphate L,S,R,RH stands respectively for Leaf, Stem, Root, RHizosphere; S1N: Rafz, clone 1 (BaG), ammonium nitrate L,S,R,RH stands respectively for Leaf, Stem, Root, RHizosphere; B1N: Lommel, clone 1 (BaG), ammonium nitrate L,S,R,RH stands respectively for Leaf, Stem, Root, RHizosphere; B4S: clone 4 (NBZn5-57), ammonium sulphate L,S,R,RH stands respectively for Leaf, Stem, Root, RHizosphere; S4S: Rafz, clone 4 (NBZn5-57), ammonium sulphate L,S,R,RH stands respectively for Leaf, Stem, Root, RHizosphere; S21N: Rafz, clone 2 (NBZn7-51), ammonium nitrate only one treatment instead of two like all the others L,S,R,RH stands respectively for Leaf, Stem, Root, RHizosphere.

3.3.4.5 16S rDNA sequence analysis

The cultivable isolates were characterized by comparative sequence analysis of 16S rDNA generated by PCR, obtaining a determinations of approximately 1468 nucleotide positions upstream from 5'- terminus.

The characterization has been performed only on the strains showing a metal tolerance. The results of the analyses evidenced that bacteria of the Gammaproteobacteria (Ludwig and Klenk, 2001) dominated the collection of metal tolerant isolates with a total of 81.96%. It included 45.9% *Stenotrophomonas maltophilia*, 16.4% *Pseudomonas sp*., 13.1% *Enterobacter sp.*, 6.56% *Pantoea agglomerans.* Betaproteobacteria were present as well represented by *Variovorax sp*. with a 3.3%. Gram positive bacteria were represented by *Arthrobacter sp.* with a 9.8% and a rare presence of *Crustibacterium sp.* (1.6%), *Leifsonia aquatica* (1.6%) and *Microcella putealis* (1.6%).

3.4 Conclusions

After a first preliminary field experiment in Lommel during summer 2003, another experiment growing tobacco was carried out in parallel in Lommel (Belgium) and Rafz (Switzerland) in summer 2004.

A comparison between the two years is difficult; the only observation that we may underline is a higher percentage of cadmium tolerant strains isolated in Lommel 2003 comparing to Lommel 2004 (table 3.9 and 3.10).

The phenotypic analyses of the cultivable strains isolated in 2004 revealed a high diversity of the bacteria associated to the tobacco plants both in Lommel and Rafz as well as an influence of the fertilizers on the different consortia (from table 3.11 to 3.23).

It is interesting to notice that there existed a "correlation" between heavy metal concentrations inside the plants and percentages of metals tolerant strains (table 3.8 and table 3.10). This suggests that at least a part of the metals inside tobacco plant should be physiologically available and thus toxic. High

percentages of metals tolerant strains were also found in association with the hyperaccumulator *Thlaspi caerulescens* (Lodewyckx *et al.*, 2001).
Looking at the genotypic results, and in particular to the BOX PCR fingerprintings, the presence of the same bacterial species in different conditions is remarkable. *Stenotrophomonas maltophilia* was found in different tobacco clones and different parts of these plants, like f.i. the strains (150) and (427), and even at the two different field locations as in case of (62), (96) and (100) for one type and (50), (164) and (322) for another (table 3.24).

Combining phenotypic and genotypic results, it becomes possible to draw hypothesis concerning to the isolated strains, like in case of *Stenotrophomonas maltophilia* (150) and *S. maltophilia* (427). Their phenotypic characterization shows differences in carbon sources preference, and metals tolerance, while BOX PCR results showed identity (table 3.24). This could mean that the strain originally was the same, but, inhabiting different plant parts, probably exposed to different environmental conditions, evolved different characteristics not visible on the genome because the genetic information codifying for them is probably located on plasmids. It is possible, in fact, that *S. maltophilia* (150) which was isolated from the roots, a plant part where in general the metal concentration is higher, shows tolerance to both cadmium and zinc, while the same *S. maltophilia*, but isolated from the stem, (427) is only tolerant to cadmium, being more toxic than zinc.

Another example of differences in phenotypic results of strains showing similar BOX PCR profiles was the case of *S. maltophilia* (50), *S. maltophilia* (164) and *S. maltophilia* (322). All of them were not siderophore producing (table 3.24), but the first two showed similarities in carbon sources preference and differences in MIC results; the first was not tolerant to metals, while the second was zinc tolerant. The third strain preferred different carbon sources compared to the other two, and was both cadmium and zinc tolerant. All three were isolated from the rhizosphere, the first two from Lommel and the third from Rafz. The difference in metal tolerance might have been due to the different fertilization treatment and different tobacco clone, *S. maltophilia* (322) was isolated from rhizosphere of clone 1 fertilized with ammonium nitrate and looking at the data

referred to the concentration of metal availability into the soil (table 3.1), this concentration was the highest present. It was interesting to observe that all three strains were not siderophore producing; this genetic information could be more conservative into the genome.

Similarities in phenotypic characteristics and BOX PCR fingerprinting were observed in case of *S. maltophilia* (62), (96), and (100). Strains 96 and 62 showed identical results although they were originating from different environments; the first was isolated from the rhizosphere and the second from a stem of tobacco. Slight differences in carbon sources and cadmium tolerance values were found for the third strain which was, like strain 96, isolated from the stem of tobacco.

The information provided from this work could be a first basis for the selection of single strains or consortia that could be tested in order to improve the efficiency of phytoextraction already in process on these field sites. The associated bacteria isolated in this work, constitute a bank of non pathogenic strains with different phenotypic characteristics eventually able to influence growth and metal uptake characteristics of the host plants under conditions of trace elements toxicity. Further work is needed to better characterize all (not only the metal tolerant) isolated strains. Sequencing of the bacteria that didn’t show a metal tolerance as well as the strains that were difficult to be maintained on growth media should be performed.

An effect of the fertilizers has been found, mainly on metal mobility and consequently on plant metal uptake. A more profound investigation is needed on how the population of plant associated bacteria is affected by the different fertilizers; knowledge in this field could allow to ’manipulate’ the composition of the plant associated bacteria simply modulating the fertilization treatment.

Isolate	284+ FR	284+ SC	284+ GS	284+ GN	284- C	284+ 4C	Zn mM	Cd mM	Sid.
S2N									
Root									
345	+	+	-	-	-	+	<0.25	0.45	N.T.
352	-	+	+	-	-	+	0.25	<0.15	N.G.
346	+	+	+	-	-	+	0.25	<0.15	N.G.
347	+	+	+	-	-	+	<0.25	<0.15	N.G.
348	-	-	-	-	-	+	<0.25	<0.15	N.G.
349	+	+	-	-	-	+	0.25	<0.15	N.G.
350	-	-	-	-	-	+	<0.25	<0.15	N.G.
351	-	+	-	+	-	+	<0.25	<0.15	N.G.
Rhizosphere									
355	-	-	-	-	-	+	<0.25	<0.15	N.G.
366	+	+	+	+	-	+	0.25	0.45	N.T.
358	-	-	-	-	-	-	0.5	0.15	N.T.
362	+	+	+	+	-	+	0.25	<0.15	-
360	-	-	-	-	-	+	<0.25	<0.15	N.G.
364	-	-	-	-	-	+	<0.25	<0.15	N.G.
357	+	+	+	+	-	+	<0.25	<0.15	-
363	-	-	-	-	-	-	0.35	<0.15	N.G.
369	-	-	-	-	-	-	<0.25	<0.15	N.G.
57	-	-	-	-	-	-	<0.25	<0.15	+
370	-	-	-	-	-	+	<0.25	<0.15	N.G.
353	-	-	-	-	-	+	<0.25	<0.15	N.G.
354	+	+	+	+	-	+	0.5	<0.15	N.T.
359	-	-	-	+	-	+	0.5	<0.15	N.T.
367	-	-	-	-	-	+	<0.25	<0.15	N.G.
356	+	-	-	-	-	+	<0.25	0.75	-
361	-	+	-	+	-	+	<0.25	<0.15	-
365	-	-	-	-	-	-	0.5	0.15	N.T.
363.b	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	0.75	0.45	N.G.

table 3.11: Resume of phenotypic characteristics of cultivable strains isolated from different plant parts of clone 2 (NBZn7-51) grown in Rafz in 2004, fertilized with ammonium nitrate. 284+FR is the medium 284 supplemented with fructose as carbon source, in the same way 284+SC, is supplemented with sucrose, 284+GS with glucose, 284+GN with gluconate, 284-C, no carbon sources were added, 284+4C all the carbon sources were added at the same time. + if the strain could use the carbon sources supplied, - if the strain couldn't use it. MIC values are reported for zinc and cadmium in mM. Sid is siderophores production, + if producer, - if no producer, N.G. if not grown in the medium for the test. N.T. is Not Tested.

Isolate	284+ FR	284+ SC	284+ GS	284+ GN	284- C	284+ 4C	Zn mM	Cd mM	Sid.
B2N									
165	-	-	-	-	-	+	<0.25	<0.15	N.G.
168	-	-	-	-	-	+	<0.25	<0.15	N.G.
166	+	+	+	-	-	+	<0.25	0.15	-
167	+	-	+	-	-	+	<0.25	<0.15	N.G.
Stem									
183	-	-	-	-	-	-	<0.25	<0.15	N.G.
175	-	-	-	-	-	-	0.35	<0.15	+/-
179	-	-	-	-	-	+	0.25	<0.15	N.G.
180	-	-	-	-	-	+	0.25	<0.15	N.T.
177	-	-	-	-	-	+	0.35	<0.15	N.T.
178	-	-	-	-	-	-	<0.25	<0.15	N.G.
181	-	-	-	-	-	+	0.5	0.3	-
173	-	-	-	-	-	+	0.35	<0.15	N.G.
73	+	-	+	+	-	+	<0.25	0.15	N.G.
183	-	-	-	-	-	-	0.5	0.15	N.G.
174	-	-	-	-	-	-	<0.25	<0.15	N.G.
170	+	+	+	+	-	+	0.25	0.3	+/-
171	+	+	+	+	-	+	<0.25	0.15	+
169	-	+	+	+	-	+	1	0.45	N.G.
176	-	-	-	-	-	-	0.25	<0.15	N.G.
Root									
79	+	+	+	+	-	+	0.25	0.3	N.G.
188	+	+	-	-	-	+	1	0.6	-
190	+	-	-	-	-	+	0.35	<0.15	N.T.
186	-	-	-	-	-	+	0.35	<0.15	-
187	-	-	-	-	-	-	<0.25	<0.15	N.G.
199	-	-	-	-	-	+	0.25	<0.15	N.G.
192	-	-	-	-	-	+	<0.25	<0.15	N.G.
198	+	+	-	+	-	+	<0.25	<0.15	N.G.
194	-	-	-	-	-	-	<0.25	<0.15	N.G.
189	+	-	-	+	-	+	0.35	<0.15	N.T.
196	-	-	+	-	-	+	0.75	0.3	-
Rhizosphere									
200							0.25	<0.15	N.G.
201	-	-	-	-	-	+	0.25	<0.15	N.G.

206	-	+	-	+	-	+	<0.25	<0.15	N.G.
204	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	0.5	<0.15	-
207	+	+	+	+	-	+	0.5	1.05	N.G.

table 3.12: Resume of phenotypic characteristics of cultivable strains isolated from different plant parts of clone 2 (NBZn7-51) grown in Lommel in 2004, fertilized with ammonium nitrate. 284+FR is the medium 284 supplemented with fructose as carbon source, in the same way 284+SC, is supplemented with sucrose, 284+GS with glucose, 284+GN with gluconate, 284-C, no carbon sources were added, 284+4C all the carbon sources were added at the same time. + if the strain could use the carbon sources supplied, - if the strain couldn't use it. MIC values are reported for zinc and cadmium in mM. Sid is siderophores production, + if producer, - if no producer, N.G. if not grown in the medium for the test. N.T. is Not Tested.

Isolate	284+ FR	284+ SC	284+ GS	284+ GN	284- C	284+ 4C	Zn mM	Cd mM	Sid.
S1S									
Leaf									
295	-	+	-	+	-	+	0.5	0.6	N.G.
298	-	+	-	-	-	+	<0.25	<0.15	N.G.
Root									
299	-	-	-	-	-	-	<0.25	<0.15	N.G.
300	+	+	+	-	-	+	1	1.5	N.T.
87	-	-	-	-	-	-	<0.25	<0.15	N.G.
Rhizosphere									
310	-	-	-	-	-	-	<0.25	<0.15	N.G.
304	-	+	-	-	-	+	<0.25	<0.15	N.G.
309	+	+	+	-	-	+	0.35	0.15	+
302	-	-	-	-	-	+	0.35	<0.15	N.G.
305	-	-	-	+	-	+	0.35	<0.15	N.G.
301	-	+	-	+	-	+	0.25	<0.15	N.T.
307	-	-	-	-	-	+	0.35	<0.15	N.G.
306	-	-	-	-	-	-	<0.25	<0.15	N.G.
107	+	+	+	+	-	+	0.75	<0.15	+
311	-	-	+	-	-	+	0.5	0.15	-
308	-	-	-	-	-	-	<0.25	<0.15	N.G.
312	+	+	+	+	-	+	0.5	0.75	-
313	+	+	+	+	+	+	0.35	<0.15	+
58	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	0.25	<0.15	N.G.
303	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	0.75	0.15	-

table 3.13: Resume of phenotypic characteristics of cultivable strains isolated from different plant parts of clone 1 (BaG) grown in Rafz in 2004, fertilized with ammonium sulphate. 284+FR is the medium 284 supplemented with fructose as carbon source, in the same way 284+SC, is supplemented with sucrose, 284+GS with glucose, 284+GN with gluconate, 284-C, no carbon sources were added, 284+4C all the carbon sources were added at the same time. + if the strain could use the carbon sources supplied, - if the strain couldn't use it. MIC values are reported for zinc and cadmium in mM. Sid is siderophores production, + if producer, - if no producer, N.G. if not grown in the medium for the test. N.T. is Not Tested.

Isolate	284+ FR	284+ SC	284+ GS	284+ GN	284- C	284+ 4C	Zn mM	Cd mM	Sid.
B1S									
Root									
59	+	-	+	-	-	+	0.25	<0.15	N.T.
Rhizosphere									
45	-	-	-	-	-	+	0.25	<0.15	N.T.
50	-	-	-	-	-	+	0.25	<0.15	-
52	-	-	-	-	-	+	0.25	<0.15	N.G.
51	-	-	-	-	-	+	0.25	<0.15	N.G.
110	-	+	-	-	-	-	0.35	<0.15	-
114	-	+	-	+	-	+	0.5	0.6	+
111	-	+	-	-	-	+	0.5	<0.15	N.G.
108	+	+	+	+	-	+	<0.25	<0.15	-
109	+	+	+	+	-	+	0.35	<0.15	+
113	-	-	-	-	-	+	0.35	0.3	+
112	+	+	+	+	-	+	0.35	0.3	+

table 3.14: Resume of phenotypic characteristics of cultivable strains isolated from different plant parts of clone 1 (BaG) grown in Lommel in 2004, fertilized with ammonium sulphate. 284+FR is the medium 284 supplemented with fructose as carbon source, in the same way 284+SC, is supplemented with sucrose, 284+GS with glucose, 284+GN with gluconate, 284-C, no carbon sources were added, 284+4C all the carbon sources were added at the same time. + if the strain could use the carbon sources supplied, - if the strain couldn't use it. MIC values are reported for zinc and cadmium in mM. Sid is siderophores production, + if producer, - if no producer, N.G. if not grown in the medium for the test. N.T. is Not Tested.

Isolate	284+ FR	284+ SC	284+ GS	284+ GN	284- C	284+ 4C	Zn mM	Cd mM	Sid.
S4N									
Root									
402	+	+	+	+	-	+	0.25	<0.15	+
403	-	-	-	-	-	+	<0.25	<0.15	N.G.
404	-	+	-	-	-	+	<0.25	<0.15	+/-
406.a	-	+	-	+	-	+	<0.25	<0.15	-

406.b	-	-	-	-	-	-	<0.25	<0.15	N.G.
407	+	+	+	+	-	+	0.25	0.3	-
408	-	-	-	-	-	+	0.25	0.45	N.G.
409	+	+	-	+	-	+	0.35	0.6	+
410	+	+	+	+	-	+	<0.25	0.3	-
405	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	0.5	0.45	N.G.
Rhizosphere									
411	+	+	+	+	-	+	<0.25	<0.15	N.G.
413	-	-	-	-	-	+	<0.25	<0.15	-
418	-	-	-	-	-	+	1	0.9	N.T.
422	-	+	-	+	-	+	<0.25	<0.15	N.G.
419	+	+	+	+	-	+	1.5	0.15	+/-
420	-	-	-	-	-	+	<0.25	<0.15	+
92	+	+	+	+	-	+	<0.25	0.3	+
93	+	+	+	+	-	+	0.25	<0.15	N.T.
105	+	+	+	+	-	+	<0.25	0.15	N.T.
106	+	+	+	+	-	+	<0..25	0.15	N.T.
99	-	+	+	-	-	+	0.25	<0.15	N.T.
414	-	-	-	-	-	-	<0.25	<0.15	N.G.
415	-	-	-	-	-	-	<0.25	<0.15	N.G.
416	-	+	-	+	-	+	0.25	0.3	N.T.
417	-	+	-	+	-	+	<0.25	<0.15	N.G.
420	-	-	-	+	+	+	<0.25	<0.15	N.G.
92	+	+	+	+	+	+	<0.25	0.3	

table 3.15: Resume of phenotypic characteristics of cultivable strains isolated from different plant parts of clone 4 (NBZn5-57) grown in Rafz in 2004, fertilized with ammonium nitrate. 284+FR is the medium 284 supplemented with fructose as carbon source, in the same way 284+SC, is supplemented with sucrose, 284+GS with glucose, 284+GN with gluconate, 284-C, no carbon sources were added, 284+4C all the carbon sources were added at the same time. + if the strain could use the carbon sources supplied, - if the strain couldn’t use it. MIC values are reported for zinc and cadmium in mM. Sid is siderophores production, + if producer, - if no producer, N.G. if not grown in the medium for the test. N.T. is Not Tested.

Isolate	284+ FR	284+ SC	284+ GS	284+ GN	284- C	284+ 4C	Zn mM	Cd mM	Sid.
B4N									
Stem									
259	-	-	-	-	-	-	<0.25	<0.15	N.G.
260	+	+	-	+	+	+	<0.25	<0.15	N.G.
265	-	-	-	-	-	-	<0.25	<0.15	N.G.
426	+	+	+	+	-	+	<0.25	0.6	+
427	+	-	+	+	-	+	<0.25	0.75	N.G.
70	+	+	+	-	-	+	<0.25	0.3	+
Root									
271	-	+	-	-	-	+	<0.25	<0.15	N.G.
273	+	+	+	+	-	+	1	0.75	-
274	-	-	-	-	-	+	0.25	<0.15	N.G.
275	+	+	+	+	-	+	1	0.45	+
276	-	-	-	-	-	-	<0.25	<0.15	N.G.
277	-	+	-	-	-	+	0.25	<0.15	N.G.
278	-	-	-	-	-	-	<0.25	<0.15	N.G.
279	+	-	-	-	-	-	<0.25	<0.15	N.G.
281	+	+	-	+	-	+	0.25	<0.15	N.T.
280	-	+	-	+	-	+	1	0.75	N.T.
Rhizosphere									
289	+	+	+	+	-	+	1	0.9	N.T.
293	+	+	-	-	-	+	0.75	0.9	+/-
576	-	-	-	-	-	-	<0.25	<0.15	N.G.
71	-	-	-	-	-	+	0.5	0.15	-
285	+	-	+	+	-	+	1.5	<0.15	-
288	+	+	+	+	-	+	<0.25	0.15	-
290	+	+	+	+	-	+	<0.25	0.15	N.T.
286	+	+	-	+	-	+	0.5	<0.15	-
88	+	+	+	+	-	+	<0.25	<0.15	-
89	+	+	+	+	-	+	<0.25	<0.15	N.G.
90	+	+	-	+	-	+	<0.25	<0.15	+
91	-	-	-	-	-	-	<0.25	<0.15	N.G.
283	-	-	-	-	-	-	<0.25	<0.15	N.G.
282	+	+	+	+	-	+	<0.25	0.3	+/-
292	+	+	+	+	-	+	0.35	<0.15	-

49	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	1.5	0.15	N.T.

table 3.16: Resume of phenotypic characteristics of cultivable strains isolated from different plant parts of clone 4 (NBZn5-57) grown in Lommel in 2004, fertilized with ammonium nitrate. 284+FR is the medium 284 supplemented with fructose as carbon source, in the same way 284+SC, is supplemented with sucrose, 284+GS with glucose, 284+GN with gluconate, 284-C, no carbon sources were added, 284+4C all the carbon sources were added at the same time. + if the strain could use the carbon sources supplied, - if the strain couldn't use it. MIC values are reported for zinc and cadmium in mM. Sid is siderophores production, + if producer, - if no producer, N.G. if not grown in the medium for the test. N.T. is Not Tested.

Isolate	284+ FR	284+ SC	284+ GS	284+ GN	284- C	284+ 4C	Zn mM	Cd mM	Sid.
B2S									
Stem									
104	+	+	+	+	-	+	<0.25	0.45	N.T.
137	-	-	-	-	-	+	0.75	0.45	+/-
138	+	+	+	+	-	+	0.25	0.6	N.T.
141	+	+	-	+	-	+	0.5	0.3	N.T.
143	+	+	-	+	-	+	0.5	0.15	N.T.
145	+	+	-	+	-	+	0.25	<0.15	N.T.
140	+	+	+	+	-	+	0.25	0.3	+
144	+	+	+	+	-	+	<0.25	0.15	N.T.
142	+	+	+	+	-	+	0.35	0.15	N.T.
146	-	-	-	-	-	-	<0.25	<0.15	N.G.
Root									
148	+	+	+	-	-	+	<0.25	0.15	N.G.
149	+	-	+	+	-	+	<0.25	0.15	N.T.
150	-	+	-	-	-	+	0.75	0.75	N.G.
151	-	+	-	-	-	+	<0.25	<0.15	N.G.
155	+	+	+	-	-	+	0.5	<0.15	N.G.
153	-	-	-	-	-	-	<0.25	<0.15	N.G.
154	-	-	-	+	-	+	0.25	0.15	N.G.
156	-	-	-	-	-	-	<0.25	<0.15	N.G.
Rhizosphere									
157	-	-	-	-	-	-	<0.25	<0.15	N.G.

158	-	-	-	-	-	+	0.25	<0.15	-
159	+	+	+	+	-	+	<0.25	0.3	N.G.
160							0.5	<0.15	+
162	+	+	+	+	-	+	<0.25	0.6	N.G.
163	+	+	+	+	-	+	<0.25	<0.15	N.G.
164	-	-	-	-	-	+	0.75	0.45	-

table 3.17: Resume of phenotypic characteristics of cultivable strains isolated from different plant parts of clone 2 (NBZn7-51) grown in Lommel in 2004, fertilized with ammonium sulphate. 284+FR is the medium 284 supplemented with fructose as carbon source, in the same way 284+SC, is supplemented with sucrose, 284+GS with glucose, 284+GN with gluconate, 284-C, no carbon sources were added, 284+4C all the carbon sources were added at the same time. + if the strain could use the carbon sources supplied, - if the strain couldn't use it. MIC values are reported for zinc and cadmium in mM. Sid is siderophores production, + if producer, - if no producer, N.G. if not grown in the medium for the test. N.T. is Not Tested.

Isolate	284+ FR	284+ SC	284+ GS	284+ GN	284- C	284+ 4C	Zn mM	Cd mM	Sid.
S2S									
Root									
83	-	-	+	-	-	+	0.25	0.3	N.T.
84	-	-	+	-	-	+	0.25	0.3	N.T.
85	+	+	-	+	-	+	0.25	0.6	+/-
86	-	-	-	-	-	-	<0.25	<0.15	N.G.
Rhizosphere									
326	-	-	-	-	-	-	<0.25	<0.15	N.G.
330	-	-	-	-	-	+	0.25	0.3	N.G.
327	-	-	-	-	-	-	<0.25	<0.15	N.G.
329	+	+	-	-	-	+	<0.25	<0.15	N.G.
331	+	+	+	+	-	+	0.35	0.45	N.G.
335	-	-	-	-	-	-	<0.25	<0.15	N.G.
332	+	-	-	-	-	+	<0.25	<0.15	N.G.
333	+	+	+	+	-	+	0.5	<0.15	-
344	+	+	+	+	-	+	0.25	0.15	N.G.

337	-	-	-	-	-	-	<0.25	<0.15	N.G.
336	-	-	-	-	-	-	<0.25	<0.15	N.G.
341	+	+	+	+	-	+	<0.25	0.3	N.G.
338	-	-	-	-	-	+	0.75	0.75	N.G.
340	-	-	+	-	-	+	0.25	<0.15	N.G.
342	+	+	+	+	-	+	1	1.5	N.T.
343	+	+	+	+	-	+	0.75	0.75	N.G.
328	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	0.5	0.6	N.T.
339	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	0.5	<0.15	N.G.

table 3.18: Resume of phenotypic characteristics of cultivable strains isolated from different plant parts of clone 2 (NBZn7-51) grown in Rafz in 2004, fertilized with ammonium sulphate. 284+FR is the medium 284 supplemented with fructose as carbon source, in the same way 284+SC, is supplemented with sucrose, 284+GS with glucose, 284+GN with gluconate, 284-C, no carbon sources were added, 284+4C all the carbon sources were added at the same time. + if the strain could use the carbon sources supplied, - if the strain couldn't use it. MIC values are reported for zinc and cadmium in mM. Sid is siderophores production, + if producer, - if no producer, N.G. if not grown in the medium for the test. N.T. is Not Tested.

Isolate	284+ FR	284+ SC	284+ GS	284+ GN	284- C	284+ 4C	Zn mM	Cd mM	Sid.
S1N									
Root									
81	-	-	+	-	-	+	<0.25	0.45	N.G.
82	-	-	+	+	-	+	<0.25	<0.15	N.G.
314	-	+	-	-	-	+	<0.25	0.15	N.G.
424	-	+	-	-	-	+	<0.25	<0.15	N.G.
423	-	+	+	-	-	+	0.25	<0.15	N.G.
425	-	-	-	-	-	-	<0.25	<0.15	N.G.
Rhizosphere									
54	+	+	+	+	-	+	0.35	0.75	+/-
315	-	+	+	+	-	+	0.35	<0.15	N.T.
316	-	-	-	-	-	+	0.5	<0.15	N.G.
317	-	-	-	-	-	-	<0.25	<0.15	N.G.
318.a	-	+	-	+	-	+	<0.25	<0.15	N.G.
318.b	-	-	-	+	-	+	<0.25	<0.15	N.G.

319	+	+	-	+	-	+	1.5	1.05	+
320	+	+	+	+	-	+	1	0.75	N.G.
321	+	+	+	+	-	+	1	0.75	+
322	+	+	+	+	-	+	0.75	0.9	-
323	+	+	+	+	-	+	0.25	0.3	+/-
324	+	-	+	+	-	+	0.75	0.75	+

table 3.19: Resume of phenotypic characteristics of cultivable strains isolated from different plant parts of clone 1 (BaG) grown in Rafz in 2004, fertilized with ammonium nitrate. 284+FR is the medium 284 supplemented with fructose as carbon source, in the same way 284+SC, is supplemented with sucrose, 284+GS with glucose, 284+GN with gluconate, 284-C, no carbon sources were added, 284+4C all the carbon sources were added at the same time. + if the strain could use the carbon sources supplied, - if the strain couldn't use it. MIC values are reported for zinc and cadmium in mM. Sid is siderophores production, + if producer, - if no producer, N.G. if not grown in the medium for the test. N.T. is Not Tested.

Isolate	284+ FR	284+ SC	284+ GS	284+ GN	284- C	284+ 4C	Zn mM	Cd mM	Sid.
B1N									
Root									
44	-	-	-	+	-	+	0.35	<0.15	N.T.
119	-	-	-	-	-	+	<0.25	<0.15	-
120	+	+	+	+	-	+	<0.25	<0.15	-
118	+	+	+	+	-	+	0.35	0.3	-
Rhizosphere									
124	+	+	+	+	-	+	<0.25	<0.15	-
125	-	-	-	-	-	+	<0.25	<0.15	N.G.
126	+	-	-	-	-	+	0.5	<0.15	-
129	-	+	-	-	-	+	<0.25	<0.15	N.G.

table 3.20: Resume of phenotypic characteristics of cultivable strains isolated from different plant parts of clone 1 (BaG) grown in Lommel in 2004, fertilized with ammonium nitrate. 284+FR is the medium 284 supplemented with fructose as carbon source, in the same way 284+SC, is supplemented with sucrose, 284+GS with glucose, 284+GN with gluconate, 284-C, no carbon sources were added, 284+4C all the carbon sources were added at the same time. + if the strain could use the carbon sources supplied, - if the

strain couldn't use it. MIC values are reported for zinc and cadmium in mM. Sid is siderophores production, + if producer, - if no producer, N.G. if not grown in the medium for the test. N.T. is Not Tested.

Isolate	284+ FR	284+ SC	284+ GS	284+ GN	284- C	284+ 4C	Zn mM	Cd mM	Sid.
B4S									
Leaf									
216	-	-	-	-	-	+	0.35	<0.15	N.G.
217	-	-	-	-	-	+	0.35	<0.15	N.G.
Stem									
48	+	+	+	-	-	+	0.75	0.15	N.T.
55	+	+	-	+	-	+	<0.25	<0.15	N.G.
56	-	-	-	-	-	-	<0.25	<0.15	N.G.
95	-	+	+	+	-	+	0.35	0.15	N.T.
94	-	-	-	-	-	+	<0.25	0.3	N.G.
97	-	-	-	+	-	+	0.5	0.3	N.G.
96	-	-	-	-	-	+	0.5	0.3	-
98	-	+	-	-	-	+	<0.25	<0.15	N.G.
100	-	+	-	+	-	+	0.5	0.45	N.G.
220	+	+	+	-	-	+	0.25	0.15	N.G.
223	+	-	+	+	-	+	0.35	0.15	N.T.
103	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	0.3	0.9	N.G.
Root									
76	-	-	-	-	-	-	<0.25	<0.15	N.G.
77	-	-	-	-	-	-	<0.25	<0.15	N.G.
78	-	-	-	-	-	-	<0.25	<0.15	N.G.
230	-	-	-	-	-	+	0.35	<0.15	N.T.
231	-	-	-	-	-	+	<0.25	<0.15	N.G.
232	-	-	+	-	-	+	0.25	<0.15	N.T.
237	-	-	-	-	-	+	<0.25	<0.15	N.G.
238	-	-	-	-	-	+	<0.25	<0.15	N.G.
239	-	-	-	-	-	+	<0.25	<0.15	N.G.
240	+	-	+	-	-	+	0.35	<0.15	N.G.
243	-	-	+	-	-	+	0.5	<0.15	N.G.
244	-	-	+	-	-	+	0.35	<0.15	N.G.
247	+	-	+	+	-	+	0.5	0.45	+

246	+	+	+	+	-	+	0.5	0.15	N.T.
250	+	-	+	+	-	+	<0.25	<0.15	-
251	+	+	+	+	-	+	1	0.3	-
252	+	+	+	+	-	+	<0.25	0.15	-
253	+	-	-	+	-	+	<0.25	<0.15	N.G.
254	+	+	+	+	-	+	<0.25	<0.15	-
255	+	+	+	+	-	+	0.35	0.15	-
256	+	+	+	+	-	+	<0.25	0.15	+/-

table 3.21: Resume of phenotypic characteristics of cultivable strains isolated from different plant parts of clone 4 (NBZn5-57) grown in Lommel in 2004, fertilized with ammonium sulphate. 284+FR is the medium 284 supplemented with fructose as carbon source, in the same way 284+SC, is supplemented with sucrose, 284+GS with glucose, 284+GN with gluconate, 284-C, no carbon sources were added, 284+4C all the carbon sources were added at the same time. + if the strain could use the carbon sources supplied, - if the strain couldn't use it. MIC values are reported for zinc and cadmium in mM. Sid is siderophores production, + if producer, - if no producer, N.G. if not grown in the medium for the test. N.T. is Not Tested.

Isolate	284+ FR	284+ SC	284+ GS	284+ GN	284- C	284+ 4C	Zn mM	Cd mM	Sid.
S4S									
Rhizosphere									
385	+	+	+	+	-	+	0.35	0.45	+
384	-	-	-	-	-	+	0.35	0.45	N.G.
386	-	-	-	-	-	-	<0.25	<0.15	N.G.
387	+	-	+	+	-	+	1.5	0.6	N.T.
388	-	-	-	-	-	-	<0.25	<0.15	N.G.
389	+	+	+	+	-	+	0.5	0.6	N.T.
390	-	-	-	-	-	-	<0.25	<0.15	N.G.
391	-	-	-	-	-	+	0.25	<0.15	N.G.
392	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	1	0.75	-
393	+	+	+	+	-	+	0.25	<0.15	-
398	+	+	+	+	-	+	0.5	<0.15	N.G.
397	-	-	-	-	-	-	<0.25	<0.15	N.G.
395	+	+	+	+	-	+	0.75	0.45	N.G.
394	-	-	-	-	-	+	0.35	<0.15	N.G.

396	-	-	-	-	-	+	0.35	<0.15	N.G.
399	+	+	+	+	-	+	0.5	0.9	N.T.
400	+	+	+	+	-	+	0.5	0.3	+
401	+	-	+	+	-	+	0.75	0.45	N.T.

table 3.22: Resume of phenotypic characteristics of cultivable strains isolated from different plant parts of clone 4 (NBZn5-57) grown in Rafz in 2004, fertilized with ammonium sulphate. 284+FR is the medium 284 supplemented with fructose as carbon source, in the same way 284+SC, is supplemented with sucrose, 284+GS with glucose, 284+GN with gluconate, 284-C, no carbon sources were added, 284+4C all the carbon sources were added at the same time. + if the strain could use the carbon sources supplied, - if the strain couldn't use it. MIC values are reported for zinc and cadmium in mM. Sid is siderophores production, + if producer, - if no producer, N.G. if not grown in the medium for the test. N.T. is Not Tested.

Isolate	284+ FR	284+ SC	284+ GS	284+ GN	284- C	284+ 4C	Zn mM	Cd mM	Sid.
S21N									
Leaf									
372	-	+	-	-	-	+	0.35	<0.15	N.G.
373	-	-	-	-	-	-	<0.25	<0.15	N.G.
Root									
374	+	+	-	-	-	+	<0.25	<0.15	-
375	-	+	-	-	-	+	0.5	0.75	N.T.
376	-	-	-	-	-	-	<0.25	<0.15	N.G.
377	-	-	+	-	-	+	0.25	<0.15	N.G.
379	-	+	+	-	-	+	0.5	<0.15	N.G.
380	+	+	+	+	-	+	<0.25	<0.15	N.G.
381	-	-	-	-	-	+	<0.25	<0.15	-
Rhizosphere									
60	-	-	-	-	-	-	<0.25	<0.15	N.G.
61	-	+	+	+	-	+	<0.25	<0.15	N.G.
62	-	-	-	-	-	+	0.5	0.3	-
63	-	-	-	-	-	-	<0.25	<0.15	N.G.
66	+	+	+	+	-	+	1	0.6	+
67	-	-	-	-	-	+	0.35	<0.15	N.G.
75	-	-	-	-	-	+	<0.25	<0.15	N.G.

382	+	+	-	+	-	+	0.25	0.15	

table 3.23: Resume of phenotypic characteristics of cultivable strains isolated from different plant parts of clone 2 (NBZn7-51) grown in Rafz in 2004, fertilized with ammonium sulphate just once on the contrary of all the others that were fertilized with the double amount. 284+FR is the medium 284 supplemented with fructose as carbon source, in the same way 284+SC, is supplemented with sucrose, 284+GS with glucose, 284+GN with gluconate, 284-C, no carbon sources were added, 284+4C all the carbon sources were added at the same time. + if the strain could use the carbon sources supplied, - if the strain couldn't use it. MIC values are reported for zinc and cadmium in mM. Sid is siderophores production, + if producer, - if no producer, N.G. if not grown in the medium for the test. N.T. is Not Tested.

Chapter 4. Development of a method for "*in vitro*" plant culture, exposure to cadmium and growth variations in control plants

Abstract

A method for cultivation of *Nicotiana tabacum* in complete sterility was developed. An appropriate cadmium concentration to apply to the culture medium was determined for just causing a slight growth inhibition. The aim was to perform tests at a low level of cadmium exposure, in the same order as the one present on the field sites used for pilot experiments on phytoextraction. Differences in biomass production were observed when comparing the non-inoculated and not cadmium treated control plants from the different re-inoculation experiments we performed during our study. The differences were hypothesized to occur due to differences in age of the seeds and on the slight differences in light intensity and quality. These variations underline the necessity of a control set run with each experiment.

4.1 Introduction

Before starting up the re-inoculations experiments, it was necessary to develop a method for a cultivation of *Nicotiana tabacum* plants in complete sterility. Also growth conditions and the level of cadmium exposure had to be determined. In order to do so, different method for plant culture were tested. Furthermore, a level of cadmium exposure had to be determined for use in re-inoculation experiments. The aim was to keep the exposure at a low level in order to obtain only slight effects caused by cadmium toxicity, similar to the conditions on the field sites in Belgium and Switzerland used for pilot experiments on phytoextraction (see table 3.2). A last aim of this chapter is to compare biomass production of non-inoculated tobacco plants throughout the different growth and re-inoculation experiments performed during 2 subsequent years.

4.2 Materials and Method

4.2.1 Cultivation of plants

Seeds of BAG, the *Nicotiana tabacum* mother clone, were completely sterilized. This was obtained by submerging the seeds for 30s in ethanol 70%, after which they were rinsed once in sterile Millipore water during 30 seconds. Subsequently, the seeds were placed during 35 minutes in $HOCl^-$ 16Volumes, after which the seeds were rinsed three times for 10 min in sterile Millipore water and dried using sterile filter paper. The seeds' sterility was checked by incubating some of them for 3 days at 30°C on ten times diluted 869 medium. Seeds were considered sterile when no bacterial growth was observed after seed germination.

The seeds were incubated on a Petri dish containing MSM0 medium (Sigma) in the plant growth chamber during 3 days in the dark followed by 2 days at normal growth chamber conditions (temperature of 25°C (day) - 17°C (night), relative humidity of 60-65% and a 12 h (day) photoperiod provided by Philips TDL 58WT33 fluorescent tubes (photosynthetic active radiation at plant level 160 µmol m^{-2} s^{-1})). Germinated seeds were moved into sterile Magenta (Sigma) containing 100ml of MSM0 medium (pH 5.6) supplemented with 5.5g agar per litre. The plants were left to grow during 2 more weeks before being harvested. Three plants were used to analyse plant heavy metals uptake while all plants were used to analyse biomass production.

4.2.2 Cadmium concentration in plant growth medium

The cadmium concentration to apply to the plant medium was established after a range finding test. The plants were grown as described above, but when the MSM0 medium was still warm and liquid different concentrations of $CdCl_2$ were added. The concentration range tested started from 0µM up to 35µM.

4.2.3 Biomass production

The plant material was harvested, keeping the shoots and the roots of the same plant separated, and vigorously washed with deionised water. The roots were washed with 10mM $Pb(NO_3)_2$ at 4°C for 10 min in order to remove the adhering metals and rinsed three times with distilled water. Plants fresh weight was determined on an analytical balance (Mettler H54). The dry weight was obtained after drying the shoots and roots during 48 hours at 60°C. Length and width of the biggest leaf were measured by means of a normal scale at harvesting time.

4.2.4 Plant metal analyses

Dry plant material, shoot and root, were separately homogenized and mineralised by wet ashing with a mixture of concentrated HNO_3 and $HClO_4$ (5:2 v/v). Cd content was determined by means of Inductively Coupled Plasma Optical Emission Spectroscopy (I.C.P.-O.E.S).

4.2.5 Statistical analysis

All treatments were performed in a minimum of three replicas, except the ones related to the cadmium range finding test. The significance of differences was analysed by one-way and factorial ANOVA followed by or Tukey test or HSD-Tukey test for post-hoc comparisons between unequal samples performed with Statistica 6 (StatSoft, 2003). Using the same statistical program, in case of not normal distribution a Mann-Whitney U test was used. The data referred to concentration, ratios and metals content were previously transformed using the arctangent function in order to delete the effect of no pure measure units.

For the analysis of data SigmaPlot 8.0 (SPSS, Chicago, IL) was used.

4.3 Results and discussion

4.3.1 Cadmium range finding test

After a few tests using a mixture of perlite and sand that showed to not allow to work under complete sterile conditions, a medium (MSM0 by SIGMA) with agar (0,55%) was chosen to grow the plants. Using a medium supplemented with agar (and thus having a certain level of metal binding capacity), it was difficult to predict the cadmium concentration effectively available to the plants. The aim was to keep the exposure at a low level in order to obtain only slight effects caused by cadmium toxicity, similar to the conditions on the field sites in Belgium and Switzerland used for pilot experiments on phytoextraction (table 3.2). For this reason we performed a range-finding cadmium test; addition of 10µM of $CdCl_2$ to the culture medium was selected as the concentration for the future inoculation tests since from this level of exposure a decrease in biomass production in the exposed plants was observed (red spot in the figures 4.1 and

4.2). This parameter is considered to be one of the first macroscopic observable effects of cadmium toxicity in plants (Steffens, 1990).

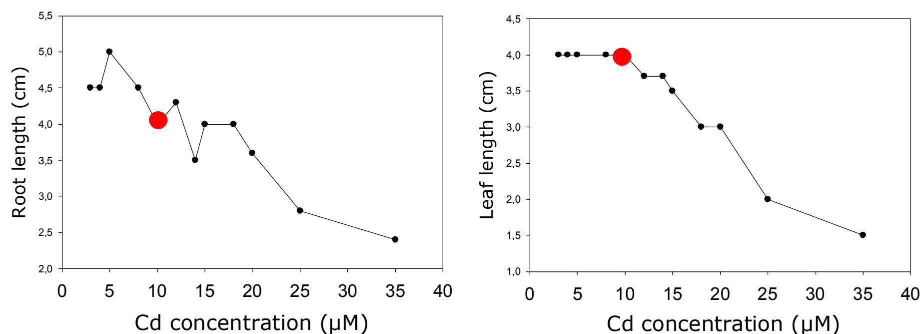


figure 4.1: Influence of different Cd (µM) concentrations on tobacco plant growth (length of leaves and roots (cm)) in MSM0 medium with 0,55% agar.

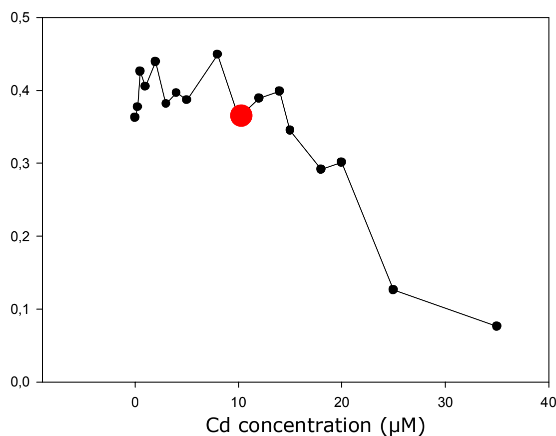


figure 4.2: Influence of different Cd (µM) concentrations on tobacco plant growth (Shoots fresh weight (g)) in MSM0 medium with 0,55% agar.

4.3.2 Comparison of the controls used in the re-inoculations experiments

Plants are living organisms sensible to many variables, for this reason tobacco plants used for re-inoculations experiments during this thesis work, were kept in growth chamber with standardised conditions of light, temperature and humidity that allowed us, during two years, to work independently from the outside

conditions. Nevertheless, analysing and comparing all the data, we observed a variation within the non inoculated control plants, grown in parallel with each experiment. In this paragraph we want to compare the data referred to the control plants used in the different experiments (table 4.1) that will be described in more details in the next chapters of the thesis.

Code	Seeding day	Harvesting day
C.01	2-03-05	30-03-05
C.02	25-05-05	22-06-05
C.03	15-02-06	15-03-06
C.04	1-06-06	29-06-06
C.05	6-07-06	2-08-06

table 4.1: Code used in the following graphs is specified as well as the seedling and harvesting dates.

4.3.2.1 Biomass production

Biomass production was the parameter showing the highest variability. After the first experiment, lasted four weeks, the plant biomass production was lower than expected; for this reason, light intensity was increased during the next experiments. This could explain the different result in biomass production between C.01 and the next two experiments C.02 and C.03 (figure 4.3). After C.03, a significantly lower biomass production was observed. This could probably be attributed to the fact that the seeds were getting older. The seeds we worked with during all these experiments belonged to the same lot collected from the BAG mother clone during summer 2004 in Rafz. The last two experiments were run approximately two years after collection of the seeds.

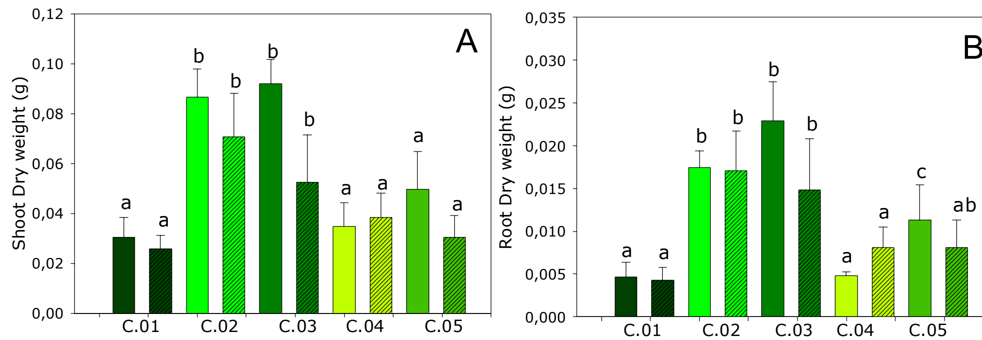


figure 4.3: Biomass production dry weight of shoot (A) and root (B) in control plants without inoculation (control is specified under each histograms). Histograms with shading refer to the plants grown in presence of 10µM cadmium. Means and standard deviation are given of minimum of four different biological replicas. The statistical significances obtained from the comparison between one each other are evidenced by a letter. No statistical difference was find between histograms marked with the same letter. Statistical significance was confirmed at 5% level using a factorial and one-way ANOVA model in data normal distribution case, U Mann-Whitney test was used in alternative case.

4.3.2.2 Cadmium concentration in the shoot part

Cadmium concentration in the shoots of cadmium exposed plants showed to be more stable than was the case for biomass production. This result seems logic as this parameter is more related to the cadmium concentration in the growth media, that was kept the same during all the experiments, and to the plant species used.

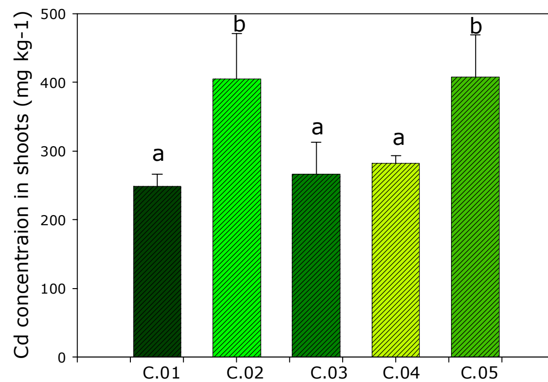


figure 4.4: Cadmium concentration in mg kg^{-1} dry matter in shoot part. The different experiments are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison between one each other are evidenced by a letter. No statistical difference was find between histograms marked with the same letter. Statistical significance was confirmed at 5% level using a one-way ANOVA model in data normal distribution case, U Mann-Whitney test was used in alternative case.

4.4 Conclusions

Incubation conditions, such as temperature and light intensity, are the major factors influencing the embryogenic response and plant generation (Thorpe, 1994).

Light requirements for plant growth involves a combination of several components, including intensity, spectral composition and species specific photoperiodism (Támas *et al.*, 2004). In our experiments we kept the photoperiodism constant, but spectral composition and light intensity may have slightly changed with lamp age. A second important factor that might explain the changes in biomass production we observed throughout our experiments is the age of the seeds used. Seeds age can influence both germination rate and biomass yield. Incubation conditions and seed age are just a few of the possible explanations for the variability we observed. Plant growth is sensible to many variables, for this reason it is very important and very necessary to run always controls in parallel to each experiment, allowing to compare results of experiments run in different periods.

Chapter 5: Inoculation of *Nicotiana tabacum* with rhizosphere and endophytic bacteria

Abstract

The weakest point of phytoextraction is the extremely long time needed to reach soil remediation goals. In this work, the potential role of plant–associated bacteria in reducing the time period for remediation was investigated. Endophytic bacteria are known to interact with their plant hosts in key processes that improve the plant growth, sometimes overcoming the stresses present and decreasing the toxic effects of the contaminant. From this point of view, different strains isolated from *Nicotiana tabacum* plants growing on phytoextraction field trial were inoculated on tobacco seedlings grown from completely sterile seeds cultivated with and without cadmium in sterile conditions during four weeks. The results obtained underlined the different bacterial effects on plants growth and on trace elements uptake by plants. The most promising results were obtained inoculating a consortium of three different bacterial strains, previously inoculated in purity, which showed to increase cadmium translocation and plant cadmium content without affecting the plant growth.

5.1 Introduction

During the last years, phytoremediation, i.e. the use of plants to remove, contain or render harmless environmental pollutants, gained an increased interest as the other remediation options currently used and generally based upon civil engineering methodologies, are considered to be more expensive and environmentally invasive. The use of phytoremediation to decontaminate metals polluted soils is referred to as phytoextraction. The main advantage of this technique is the possibility to remove the hazardous element(s) without destroying the soil structure and with a limited impact on soil fertility (Dushenkov, 2003). This technique, in fact, exploits the plant natural capability to take up toxic metals along with nutrients (Meagher & Rugh, 1996). However, in order to avoid toxicity, plants must be able to resist toxic metals and maintain homeostasis. For most phytoremediation strategies to be successful plants

should tolerate and if possible hyperaccumulate toxic metals in above-ground tissue/organs (Goldsbrough, 1998; Salt *et al*., 1998).

Hyperaccumulators, such as *Thlaspi caerulescens* (Màdico *et al.*, 1992) or *Alyssum bertolonii* (Gabbrielli *et al.*, 1991, Brooks and Radford, 1978), are defined as plants able to actively accumulate and concentrate in their shoots a minimum of 0.1% metal /dry biomass. In general these plants colonize soils geologically rich in heavy metals, such as mining sites or serpentine soils, but are also able to grow on anthropologically contaminated soils. These plants could be used to lower certain levels of contamination to levels that are environmentally acceptable and meet regulatory requirements, but their slow growth and their low biomass production render the metals extractive process excessively time consuming. During an experiment on agricultural soil amended with heavy metals urban sludge for a term of several years, Schwartz *et al.* (2003) found that two crops of *Thlaspi caerulescens* extracted about 9% of the total cadmium and 7% of the total zinc. The yield of this plant is really low, estimated in some cases in field trials of 1t ha^{-1} due to the poor growth and weak resistance to hot environments (Kayser *et al.*, 2000).

Phytoextraction efficiency is the result of both metal accumulation and biomass production, therefore, the ideal plant for metal phytoextraction has to be highly productive in biomass and to uptake and translocate to its shoots a significant part of the metals of concern. Additional favourable traits are fast growth, easy propagation and a deep root system. *Salix* (Greger and Landberg, 1999), *Populus* (Punshon and Adriano, 2003), *Brassica juncea* (Bañuelos *et al.*, 1997), *Helianthus annuus* (Elkatib *et al.*, 2001), *Phragmites australis* (Massacci *et al.*, 2001) and *Nicotiana tabacum* (Mench and Martin, 1991; Guadagnini, 2000; Kayser *et al.*, 2000) are just few of the numerous plants species investigated for their potential use in phytoextraction.

A possible amelioration aiming the increase of metal accumulation in high biomass producing plants could be genetic manipulation of these plants with genes responsible for hyperaccumulations (Kärenlampi *et al.*, 2000). This strategy was explored by many researchers (Boominathan and Doran, 2003,

Douchkov *et al.*, 2005), but it still meets the scepticism and the resistance of the governments, especially in Europe, and of the majority of the public opinion.

Besides of metal accumulation capacity of the plants, plant-availability of metals in the soil is the second important limiting factor for an efficient phytoextraction. In general, only a part of the total metal content of a soil is plant-available, mainly that one that is present as free ions, soluble forms and absorbed to inorganic constituents at ion exchange sites. Some metals such as Zn and Cd occur in exchangeable forms, while others as Pb are less bioavailable and are mainly being precipitated (Puschenreiter *et al.*, 2001). In any case, to achieve the requested metal uptake value, the concentration of soluble metals in soil must be enhanced. It has been identified that it is possible by rhizosphere manipulation based on the application of chemical agents.

In order to increase the amount of metals in the soil solution, the use of synthetic chelators, like EDTA, EGTA, DTPA. etc. (Blaylock *et al.* 1997, Epstein *et al.*, 1999, Creman *et al.*, 2001) and artificial soil acidification have been proposed and tested. Restrictions apply, however, to both the use of complexing agents and artificial soil acidification. It was found that EDTA and EDTA-heavy metal complexes are toxic for some plants and that high dose of EDTA inhibited the development of arbuscular mycorrhiza (Geebelen *et al.*, 2002, Dirilgen, 1998, Creman *et al.*, 2001). Furthermore, EDTA is poorly photo-, chemo- and biodegradable (Nortemann, 1999). *In situ* application of chelating agents can cause groundwater pollution by uncontrolled metal dissolution and leaching. Some evidence supporting this apprehension has been found (Sun *et al.*, 2001, Creman *et al.*, 2001), thus mass balances to confirm that metals are not leached to groundwater have been recommended (Schwitzgubel *et al.*, 2002). Wenzel *et al.* (2003) (using outdoor pot and lysimeter experiments) confirmed that EDTA considerably increased metal liability in soil, but also observed enormously increased metal concentrations in the leachates collected below the root zone. Furthermore, they found that the enhanced metal liabilities and leachate concentrations persisted for more than 1 year after harvest.

An alternative strategy to the previous ones is the role of plant-associated bacteria in order to both, ameliorate the growth of the plant and/or improve its

metal uptake. Plant Growth Promoting Bacteria (PGPB) that stimulate root formation by plants and also produce siderophores have been described (Lodewyckx *et al.*, 2002, Mastretta *et al.* in press, Rajkumar *et al.*, 2005). These siderophores can interact with heavy metals, in certain cases reducing their toxicity and increasing their bioavailability and uptake by plants. Endophytic bacteria can be engineered for increased heavy metal sequestration (Lodewyckx *et al.*, 2001. 2002). The (combined) activities of these new bacterial strains can be used to enhance heavy metal uptake and translocation by the host plants. These bacterial siderophores can be considered as natural chelators and the bacterial production of which is in tight equilibrium with plant activity, thus improving heavy metal uptake and translocation as part of the phytoextraction process.

Many examples of the influence of endophytic bacteria on plants both under metal or no metal stress can be found in literature. Working with Trifolium, Vivas *et al.* (2005) demonstrated that plants inoculated with Cd-adapted *Brevibacillus sp*., increased dehydrogenase, phosphatase, and β-gluconase activities in the mycorrhizosphere, indicating an enhancement of microbial activities related to plant development. Belimov *et al.* (2005) observed a stimulation in growth elongation of *Brassica juncea* seedlings in presence or absence of toxic cadmium concentrations in plants inoculated with cadmium-tolerant strains isolated from the root zone of this plant species. The results of bacterial inoculations of plants delivered, anyway, contrasting data depending on the strain concentration used for the inocula (Ruppel *et al.*, 2005) and on the application method (Ciccillo *et al.*, 2002).

Cadmium is one of the main contaminants in Rafz (Switzerland) and Lommel (Belgium), two field trials for testing possibilities of phytoextraction. This non essential element is extremely toxic for human and plant health, and, for this reason, one serious soil-related pollution problem is the elevated level of contaminating Cd in agricultural products such as phosphate fertilizers and sewage sludge (Ryan *et al*., 1982; Nicholson & Jones, 1994). Cadmium is a pollutant that accumulates in soil as a result of industrial processes or intensive use of fertilisers in agriculture.

In this chapter we investigated the influence of tobacco associated bacteria isolated from plants used for phytoextraction purposes and inoculated "in vitro" in tobacco plants with and without cadmium stress. The main aims were to (1) identify suitable candidates bacteria for improving the phytoextraction process run in those fields and (2) to clarify the role played by some strain in plant-metal interaction.

5.2 Material and Methods

5.2.1 Cultivation of plants

Nicotiana tabacum was chosen as a model plant. Seeds of BAG, the *Nicotiana tabacum* mother clone, were completely surface sterilized. This was done by submerging the seeds for 30s in ethanol 70%, after which they were rinsed once in sterile Millipore water during 30 seconds. Subsequently, the seeds were placed during 35 minutes in $HOCl^-$ 16Volumes, after which the seeds were rinsed three times for 10 min in sterile Millipore water and dried using sterile filter paper. The seeds' sterility was checked by incubating some of them for 3 days at 30°C on ten times diluted 869 medium. Seeds were considered sterile when no bacterial growth was observed.

The seeds were incubated on a Petri dish containing MSM0 medium (Sigma) in the plant growth chamber during 3 days in the dark followed by 2 days at normal growth chamber conditions (constant temperature of 25°C (day) - 17°C (night), relative humidity of 60-65% and a 12 h (day) photoperiod provided by Philips TDL 58WT33 fluorescent tubes (photosynthetic active radiation 160 µmol m^{-2} s^{-1})). Germinated seeds were moved into sterile Magenta (Sigma) containing 100ml of MSM0 medium (pH 5.6) supplemented with 5.5g agar per litre. Depending on the test conditions, 10µM $CdCl_2$, and/or 56.6µl of inoculums were sterilely plated on the surface of the medium. All the plants were kept during 2 weeks under complete sterility in the growth chamber; after this period the covers were removed and replaced with a sterile tissue that allows gas exchange. The plants were left to grow during more 2 weeks before being

harvested. One plant for each combination of inoculum and/or Cd-challenge was used to check for the survival of the inoculum in the plant, three plants were used to analyse plant heavy metals uptake while all plants were used to analyse biomass production.

5.2.2 Inoculation of Nicotiana tabacum

The bacteria used for the inoculation experiment were originally isolated from *Nicotiana tabacum* grown in the phytoextraction field experiments carried out in Belgium (Lommel) and in Switzerland (Rafz) (see Chapter 3). The strains for inoculation were chosen based on their tolerance to Cd and Zn; also a consortium of bacteria (A+P+S) was selected for same purposes. It was composed of three bacteria inoculated previously as pure cultures (*Arthrobacter sp*. 907, *Pseudomonas sp*. 54 and *Stenotrophomonas sp*. 342), that were selected based on the following criteria: (1) the plant parts colonised by each of them during the previous experiment, and (2) the results obtained during that experiment (e.g. effects plant growth, metal uptake, ...).

The different strains were grown at 30°C on a rotary shaker in ten times diluted 869 medium (Mergeay *et al.*, 1985), containing per litre distilled water: 10g tryptone, 5g yeast extract, 5g NaCl, 1g D-glucose, 0,345g $CaCl_2$*$2H_2O$ (pH 7). The bacterial growth was stopped after approximately–12 hours at a density of 10^{+8} CFU/ml (OD_{660} of 0,5). The cells were collected by centrifugation, washed twice in 10mM $MgSO_4$ and suspended in 1/10 of the original volume. The most important characteristics of the bacteria used for inoculation and their inoculum concentrations are summarized in table 5.1.

Strain n°	Isolated from	Sequence result	mM Cd	mM Zn	Sid. Prod.	Inoculum concentration	
295	BAG leaf	*Stenotrophomonas sp.*	0,60	0,50	N.G.	1,5 x10^8	
328	NBZn7-51 RH	*Stenotrophomonas sp.*	0,60	0,50	N	1,5 x10^8	
342	NBZn7-51 RH	*Stenotrophomonas sp.*	1,50	1,00	N	1 x10^8	
907	BAG RH	*Arthrobacter sp.*	0,75	0,50	N	7,5 x10^7	
54	BAG RH	*Pseudomonas sp.*	0,75	0,35	Y	7,9 x10^7	
338	NBZn7-51 RH	*Stenotrophomonas sp.*	0.75	0.75	N.G.	1x10^9	
A+P+S	Consisting of:					3x10^8	
	907	*Arthrobacter sp.*				A	8x10^7
	54	*Pseudomonas sp.*				P	5x10^7
	342	*Stenotrophomonas sp.*				S	1,6x10^8

table 5.1: Characteristics of bacteria used as inocula. RH stands for rhizosphere; In siderophores production Y, N and N.D. stands respectively for Yes, No and Not Grown (the strain was intolerant to the medium necessary for the test). mM Cd and mM Zn are respectively the Minimum Inhibiting Concentration for cadmium and zinc in mM.

5.2.3 Biomass production

The plant material was collected, keeping the shoots and the roots of the same plant separated, and vigorously washed with deionised water. The roots were washed with 10mM $Pb(NO_3)_2$ at 4°C for 10 min in order to remove the adhering metals and rinsed three times with distilled water. Plants fresh weight was determined on an analytical balance (Mettler H54). The dry weight was obtained after drying the shoots and roots during 48 hours at 60°C.

5.2.4 Recovery of the inocula

Plants were harvested after 4 weeks of growth; leaf, stem, root and rhizosphere samples were kept separately. The plant was removed under sterile conditions out of the Magenta and put into a sterile Petri dish after removing the excess of growth medium. Five ml of 10mM $MgSO_4$ were added to root material, and 100µl of solution and its subsequent dilutions were plated to check the inoculum's survival in strict contact with the root system, considering it as rhizosphere. The biggest leaf was sterilely cut from the plant and surface sterilized using 0.1% active chloride supplemented with 1 droplet of Tween 80 per 100ml solution during 2 minutes, and rinsed 3 times with sterile Millipore water. The leaf was dried using sterile filter paper. In order to verify the efficiency of the sterilization, 100Âµl sample of the third rinsing water was plated on 10 times diluted 869 medium. The same procedure was used for stem and root with a 0.5 % concentration of active chloride and a sterilization time of 1 minute for the stem and 2 minutes for the roots. After sterilization, the different plant parts were macerated into 10mM $MgSO_4$ using a sterile mortar. Samples (100µl) and their dilutions were plated on 10-times diluted 869 medium and checked after 7 days growth at 30°C.

5.2.5 Plant metal analyses

Dry plant material, shoot and root, were separately homogenized and mineralised by wet ashing using a mixture of concentrated HNO_3 and $HClO_4$ (5:2

v/v). Zn, Cd, Ca, Fe content were determined by means of Inductively Coupled Plasma Optical Emission Spectroscopy (I.C.P.-O.E.S).

5.2.6 Statistical analysis

All treatments were performed in a minimum of three replicas. The significance of differences was analysed by one-way and factorial ANOVA followed by or Tukey test or HSD-Tukey test for post-hoc comparisons between unequal samples performed with Statistica 6 (StatSoft, 2003). Using the same statistical program, in case of not normal distribution a Mann-Whitney U test was used. The data referred to concentration, ratios and metals content were previously transformed using the arctangent function in order to delete the effect of no pure measure units.

For the analysis of data SigmaPlot 8.0 (SPSS, Chicago, IL) was used.

5.3 Results

In five days germinated sterile seeds we inoculated singular strains as well as a consortium. The strains forming the consortium were chosen for the effects that showed to have on the plants separately in previous experiments, for their showed preference for inhabiting different plant parts and to be phenotypically different, in this way it has been possible to follow their plant colonization. At the end of the foreseen growth period, roots, stems and leaves of the plants were harvested separately.

5.3.1 Inoculums recovery

After surface sterilization, the plant material was rinsed and macerated. The number of CFUs (colony forming units) was calculated per gram of fresh weight of the respective plant parts. Since it was not possible to quantify the rhizosphere, the weight of the total root system of the analyzed plant was used as a reference. In the control plants without inoculation no cultivable bacteria were found. After four weeks of growth of the inoculated plants, all the inoculated strains could be isolated back from the plants: all of them were found

to be able to colonize the rhizosphere, the root system and the stem; but only when applied as a consortium (A+P+S), also colonization of leaves was detected indicating a synergic effect on plant colonization. *Stenotrophomonas sp*. 338 is the only other case where, in no cadmium treated plants, the strain could colonize the leaves as well. The inocula concentration decreases starting from the rhizosphere, where it is more abundant, to the root and the stem, the lowest concentration, where any, is detected in the leaves. This trend confirms what already found from others researchers working with endophytic populations (Lodewyckx *et al.*, 2001, Taghavi *et al.*, 2005).

Inoculums	Leaf	Stem	Root	Rhizosphere
295	0	$1.6x10^5$	$1x10^3$	$1.4x10^5$
295+	0	$2.5x10^3$	$1.6x10^2$	$3.4x10^4$
54	0	2.9x10	8.3x10	$1.6x10^6$
54+	0	$5x10^3$	0	$1.2x10^7$
907	0	$1.7x10^5$	$3.6x10^3$	$1.3x10^6$
907+	0	$2.1x10^5$	$1.4x10^5$	$1.3x10^7$
342	0	$7.3x10^2$	$5.8x10^2$	$6.9x10^3$
342+	0	$2x10^4$	$1.4x10^3$	$5.8x10^5$
328	0	$9.4x10^2$	0	$7.2x10^4$
328+	0	$2.4x10^4$	$3.2x10^3$	$1.2x10^6$
338	$7.1x10^2$	$1.1x10^5$	$1.8x10^5$	$8.5x10^7$
338+	0	$5.7x10^5$	$4.2x10^2$	$4.9x10^7$
A+P+S	$2.65x10^4$	$5x10^3$	$2.1x10^3$	$3.9x10^3$
A (907)	$2.25x10^4$	$4.7x10^3$	$1.8x10^3$	$2.9x10^3$
P (54)	$1.1x10^3$	0	0	$2.4x10^2$
S (342)	$3x10^3$	$3x10^2$	$3.6x10^3$	$7.3x10^2$
A+P+S+	$1.95x10^4$	$7.4x10^3$	$1.6x10^4$	$2.1x10^4$
A (907)	$1.25x10^4$	$4.5x10^3$	$8.5x10^3$	$1.3x10^4$
P (54)	$2.35x10^3$	$4.8x10^2$	$3.8x10^3$	$2.8x10^3$
S (342)	$4.5x10^3$	$2.4x10^3$	$4.1x10^3$	$5.6x10^3$
Control	0	0	0	0
Control+	0	0	0	0

table 5.2: Inocula recovery. Inocula with + are the ones isolated back from plants grown with 10µM $CdCl_2$. In columns is the plant parts analysed for inocula recovery.

We found that there are strains showing clear preferences for living in certain plant parts. This difference in distribution might be due to different requirements of each microorganism that let them inhabit different niches, represented by a tissue and more specifically by intercellular spaces inside each tissue (Di Fiori and Del Gallo, 1995). *Pseudomonas sp. 54* was isolated from tobacco rhizosphere, and confirmed, both in consortium and in purity, to almost exclude root colonization when there is no cadmium stress, while it is abundant inside the consortium (A+P+S) in the leaf part and in general, in the whole plant, under cadmium stress. This is in contrast with the general finding that *Pseudomonas sp.* is an aggressive root colonizer, as reported by Mehnaz and Lazarovits, 2006. Pseudomonaceae are common inhabitants of the rhizosphere and frequently been reported as biological control agent (Costa *et al.*, 2006). In our experiment as well, we found this strain to fit well in the rhizosphere as confirmed by its higher rhizosphere concentration compared with other inocula (*Stenotrophomonas sp*. 295 and 342) showing lower rhizosphere concentration though a higher inocula concentration (Table 5.1 and 5.2). We compared four different *Stenotrophomonas sp*. strains, one isolated from a tobacco leaf (295) and three isolated from the rhizosphere of another tobacco plant (342, 328 and 338). All of them showed different MIC values for cadmium and zinc as resumed in table 5.1. *Stenotrophomonas* species are known to be plant associated growth promoting bacteria, and have been isolated from different plant parts (Heuer and Smalla, 1997, Wilson *et al.*, 1999, Insunza *et al.*, 2002); it was hardly found outside the rhizosphere (Baker *et al.* 2005) suggesting it to be a common endophyte involved in root pathogen protection (Wolf *et al.* 2003). During our experiments we observed a general distribution of these strains inside the plants from the rhizosphere up to the stem and in case of 338 up to the leaf as well. 295 preferably colonized the plants without cadmium treatment while the other three colonized in higher number the cadmium treated plants (table 5.2). Although it was isolated from a leaf, *Stenotrophomonas sp*. 295 seemed a good rhizosphere colonizer as well; data from literature— show that phylloplane isolates normally survive in low moisture and other adverse conditions and, thus, may effectively colonize the nutrient-rich rhizosphere (Kishore *et al.*, 2005); this could explain our result. *Arthrobacter sp.* 907 is in general more abundant in cadmium treated plants showing its maximum presence in the

rhizosphere, place where this bacteria was isolated from, secondly in stems and finally in roots (table 5.2).

5.3.2 Effects on plant growth

The effects of cadmium (10μM) and inoculation with the bacterial strains and consortium mentioned in table 5.1 were investigated on the four weeks old plants grown in Magenta pots. The growth parameters that were determined were the fresh and dry weight; the results show similar trends (figure from 5.1 to 5.6). The influence of inoculation on the root system of plants without cadmium treatments seems lower than on the shoot: none of the cadmium untreated roots is significantly different from the non-inoculated plants. A negative effect on the shoot growth was observed of all the inocula, with the lowest effect for *Stenotrophomonas sp.* 328 and with an additional negative effect by cadmium treatment (figure 5.1A, 5.2A, 5.3A and 5.4A). The inoculation of the combination of *Arthrobacter sp*. 907, *Pseudomonas sp.* 54 and *Stenotrophomonas sp*. 342 seemed to overcome the negative effects on plants growth even if not statistically significant (figure 5.5 and 5.6). It is interesting to notice that the bacterial concentration generally was higher in plants treated with cadmium than into the untreated; these plants also showed a stronger growth inhibition (table 5.2).

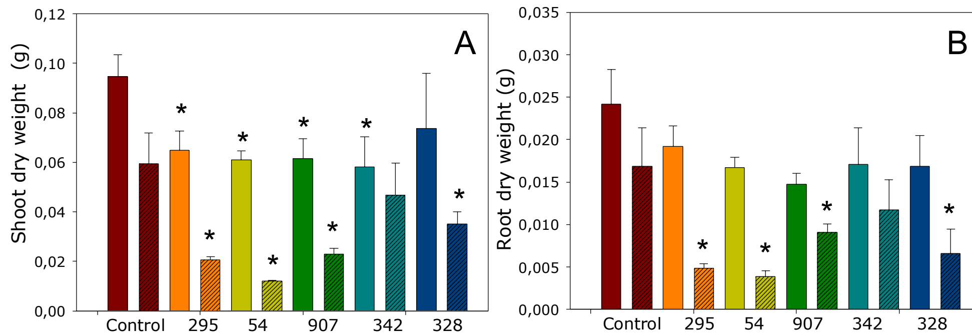


figure 5.1: Biomass production dry weight of shoot (A) and root (B) in control plants without inoculation and plants with different inocula (inocula are specified under each bar). Histograms with shading refer to the plants grown in presence of 10Î¼M cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results, evidenced by a star on the histogram, was confirmed at the 5% level using a two-way and one-way ANOVA model separately exploring inoculums and presence or absence of cadmium with non-inoculated plants U Mann-Whitney test was performed when ANOVA analyses was not allowed. The significance is marked with a star upon the histogram.

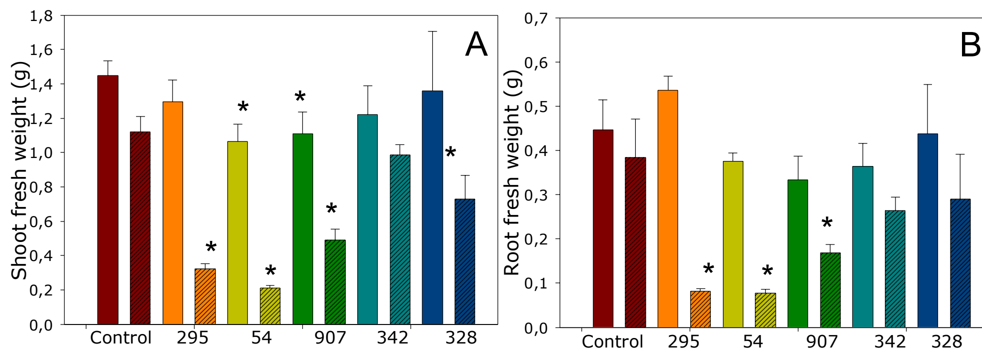


figure 5.2: Biomass production fresh weight of shoot (A) and root (B) in control plants without inoculation and plants with different inocula (inocula are specified under each bar). Histograms with shading refer to the plants grown in presence of $10 \mu M$ cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results, evidenced by a star on the histogram, was confirmed at the 5% level using a two-way and one-way ANOVA model separately exploring inoculums and presence or absence of cadmium with non-inoculated plants U Mann-Whitney test was performed when ANOVA analyses was not allowed. The significance is marked with a star upon the histogram.

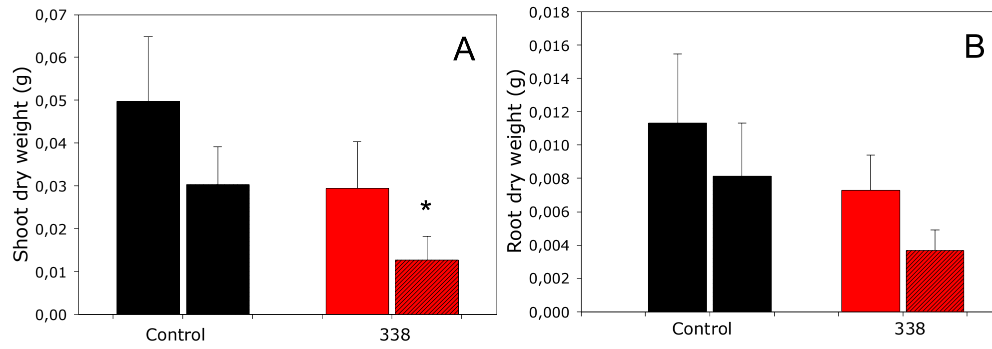


figure 5.3: Biomass production dry weight of shoot (A) and root (B) in control plants without inoculation and plants with different inocula (inocula are specified under each bar). Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results, evidenced by a star on the histogram, was confirmed at the 5% level using a two-way and one-way ANOVA model separately exploring inoculums and presence or absence of cadmium with non-inoculated plants U Mann-Whitney test was performed when ANOVA analyses was not allowed. The significance is marked with a star upon the histogram.

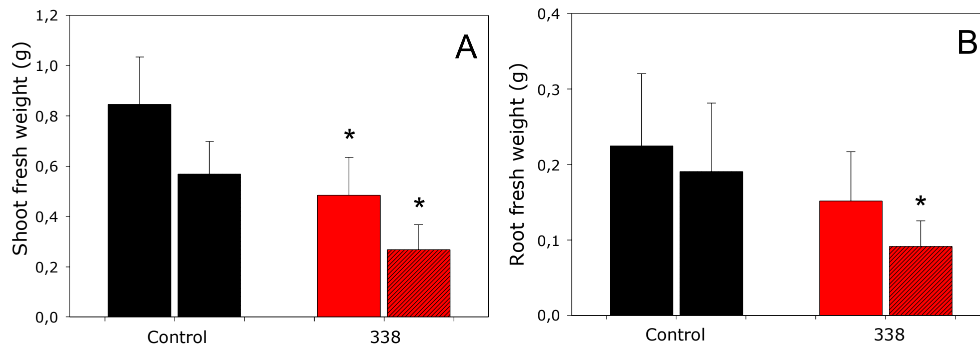


figure 5.4: Biomass production fresh weight of shoot (A) and root (B) in control plants without inoculation and plants with different inocula (inocula are specified under each bar). Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results, evidenced by a star on the histogram, was confirmed at the 5% level using a two-way and one-way ANOVA model separately exploring inoculums and presence or absence of cadmium with non-inoculated plants U Mann-Whitney test was performed when ANOVA analyses was not allowed. The significance is marked with a star upon the histogram.

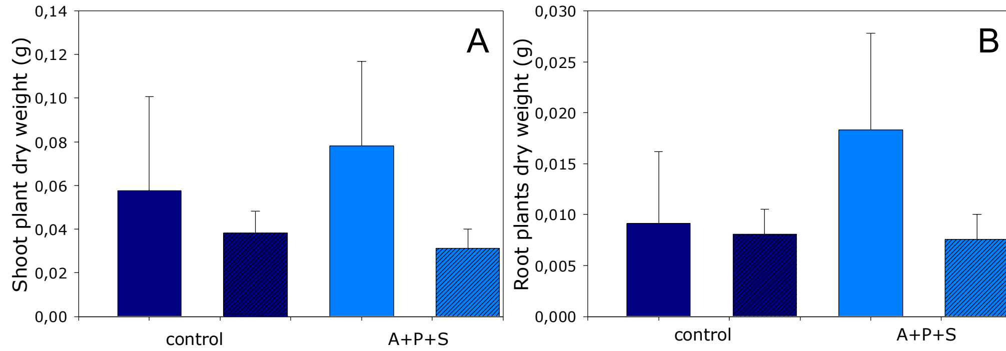


figure 5.5: Biomass production dry weight of shoot (A) and root (B) in control plants without inoculation and plants with different inocula (inocula are specified under the histograms). Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results, evidenced by a star on the histogram, was confirmed at the 5% level using a two-way and one-way ANOVA model separately exploring inoculums and presence or absence of cadmium with non-inoculated plants U Mann-Whitney test was performed when ANOVA analyses was not allowed. The significance is marked with a star upon the histogram.

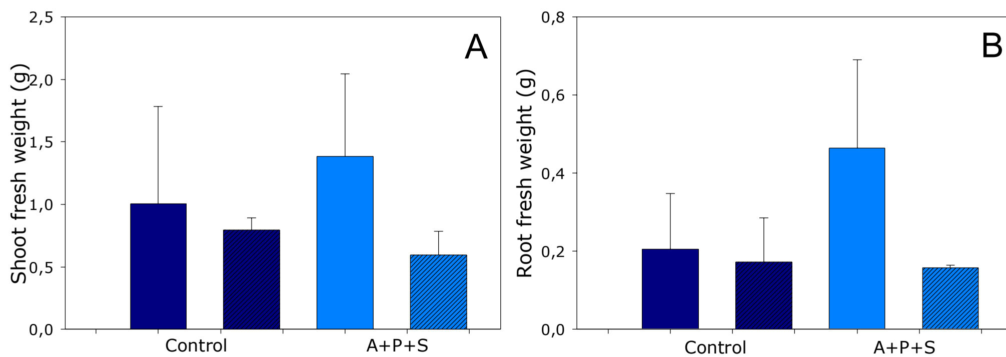


figure 5.6: Biomass production fresh weight of shoot (A) and root (B) in control plants without inoculation and plants with different inocula (inocula are specified under each bar). Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results, evidenced by a star on the histogram, was confirmed at the 5% level using a two-way and one-way ANOVA model separately exploring inoculums and presence or absence of cadmium with non-inoculated plants U Mann-Whitney test was performed when ANOVA analyses was not allowed. The significance is marked with a star upon the histogram.

We could conclude that in order to have a better plant growth, no inoculation with single strains should be performed.

Despite the negative effects on plant growth, we investigated metals accumulation, since phytoextraction efficiency is the product of both parameters.

5.3.3 Essential and non essential trace elements into the plants

Previous research has established that cadmium absorption by plants and its distribution in organs and tissues is influenced by Zn, Fe and lesser extent Ca (Simmons *et al.*, 2003). For this reason, besides of cadmium itself, zinc and iron contents were determined in both roots and shoots. Plants possess extensive families of transporters that are involved in the uptake and efflux of metals. These transporters can be metal specific or specific for `families' of metals, but similarities in ions and higher concentrations of competing ions can lead to uptake of "unwanted" elements (Colangelo and Guerinot, 2006).

5.3.3.1 Cadmium

Only plants grown on cadmium were tested for cadmium concentration analyzing this variable we wanted to find out if the observed inhibition of plant growth could be attributed to an increased uptake of this toxic metal by the plant.

As matter of fact, differences between inoculated and non inoculated plants were observed. *Stenotrophomonas sp*. (295) increased the Cd concentration in the shoot part (figure 5.7A); a similar result was obtained after inoculation of the consortium (figure 5.9A). The *Pseudomonas sp.* (54) and *Stenotrophomonas sp*. (328), on the other hand, increased cadmium concentration in root part, while 295 decreases it (figure 5.7B) suggesting an influence on cadmium translocation.

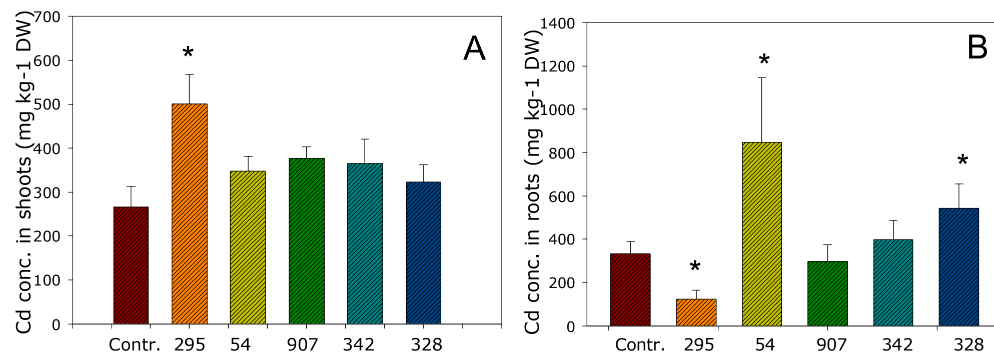


figure 5.7: Cadmium concentration in mg kg^{-1} dry matter in shoot part (A) and in root part (B). The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are evidenced with a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula, in case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

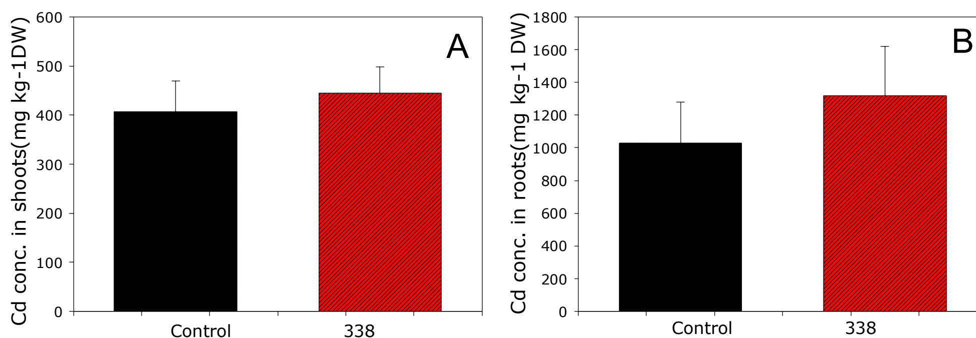


figure 5.8: Cadmium concentration in mg kg^{-1} dry matter in shoot part (A) and in root part (B). The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are evidenced with a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula, in case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

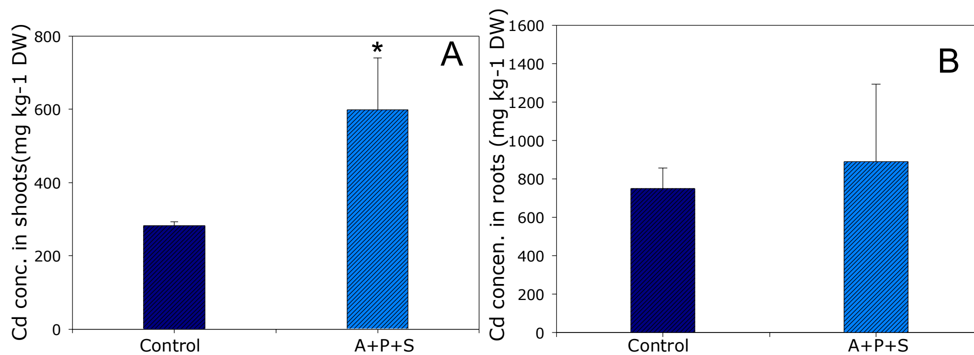


figure 5.9: Cadmium concentration in mg kg^{-1} dry matter in shoot part (A) and in root part (B). The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are evidenced with a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula, in case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

In this experiment *Pseudomonas sp.* 54 influences cadmium concentration in the host plants increasing it into the roots compared with non inoculated plants, while there is no difference in shoots cadmium concentration between inoculated and no inoculated plants (figure 5.7A and B). The consortium (A+P+S) increases the shoot cadmium concentration.

5.3.3.2 Phytoextraction efficiency

Multiplying plant cadmium concentration with the dry weight of roots and shoots the total cadmium uptake in micrograms per plant was calculated. This result allows us to conclude if the lower biomass production is compensated by a much higher cadmium concentration. This parameter is useful in function of phytoextraction purposes.

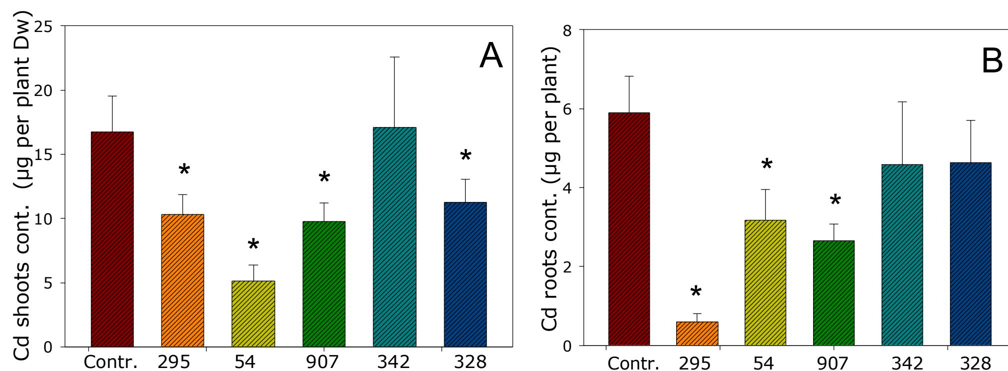


figure 5.10: Plant cadmium content in µg per plant in shoot part (A) and in root part (B). The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are evidenced with a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula, in case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

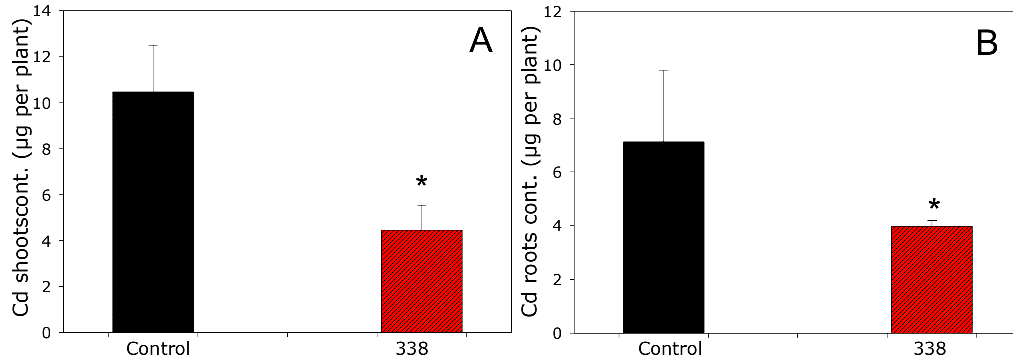


figure 5.11: Plant cadmium content in µg per plant in shoot part (A) and in root part (B). The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are evidenced with a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula, in case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

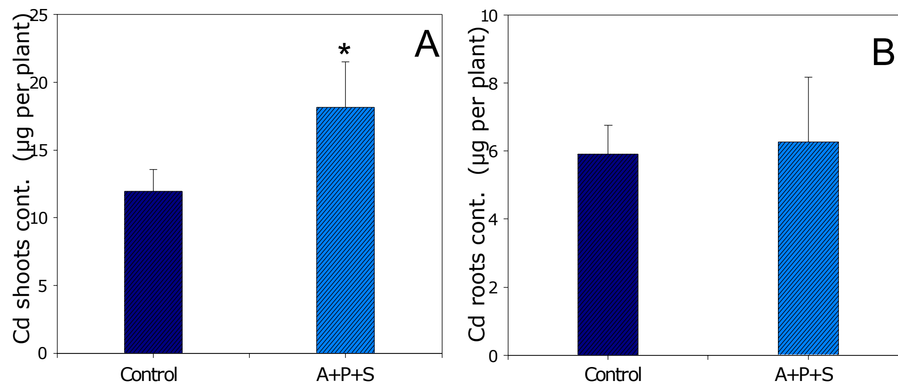


figure 5.12: Plant cadmium content in µg per plant in shoot part (A) and in root part (B). The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are evidenced with a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula, in case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

In both, roots and shoots of plants inoculated with single strains, the cadmium content was lower or at least at the same level compared to the non-inoculated plants (figure 5.10, 5.11 and 5.12B). We may conclude that the higher cadmium concentration, in case of inoculation with single strains (like for instance *Stenotrophomonas sp.* 295), caused a decreased biomass production, which could be an advantage as less metal contaminated biomass has to be processed. While the consortium delivered a higher phytoextraction efficiency index for cadmium in its shoots (figure 5.12A).

5.3.3.3 Zinc

Although zinc was always supplied at the same optimal concentration (not at increased levels) to the growth media significant differences in zinc concentration were observed in both roots and shoots in function of cadmium treatment and/or inoculation.

In absence of cadmium treatment, the roots of plants inoculated with either *Arthrobacter sp*. (907) or *Pseudomonas sp*. (54) or *Stenotrophomonas sp*. 342 showed a significantly lower zinc concentration compared with the non-inoculated plants (figure 5.13B) the zinc concentration in shoots decreased as well (figure 5.13A). On the contrary *Stenotrophomonas sp*. (295) increased the shoots zinc concentration in untreated plants (figure 5.13A).

In presence of cadmium in the growth medium the situation was different. First of all, the zinc concentration in the roots of cadmium-exposed non-inoculated plants was more than 50 % lower than in plants without cadmium treatment (figure 5.13A). Plants inoculated with *Stenotrophomonas sp*. 295 showed higher metal concentration in their roots (figure 5.13A), while *Stenotrophomonas sp*. 342 and 338 increased it into the shoots (figure 5.13A and 5.14A).

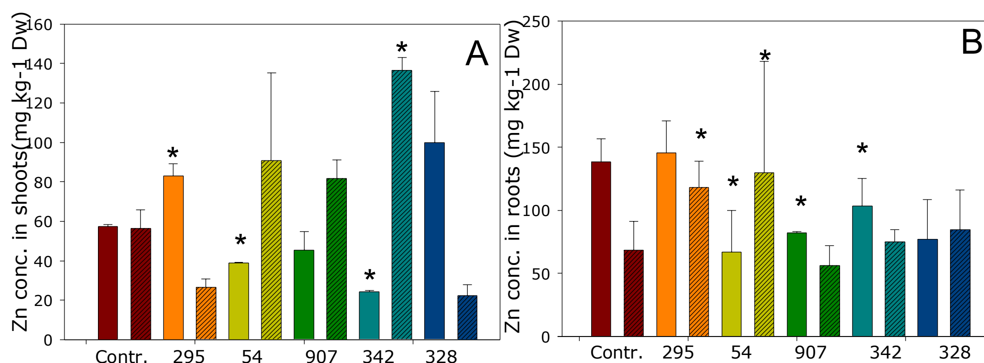


figure 5.13: Zinc concentration in mg kg^{-1} dry matter in shoot part (A) and root part (B). Controls are the non inoculated plants, different inocula are specified under each bar. Histograms with shading refer to the plants grown in presence of 10Î¼M cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results was confirmed at the 5% level using one and two-way ANOVA model separately exploring inocula and presence or absence of cadmium. The difference is marked with a star on the histogram. In case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

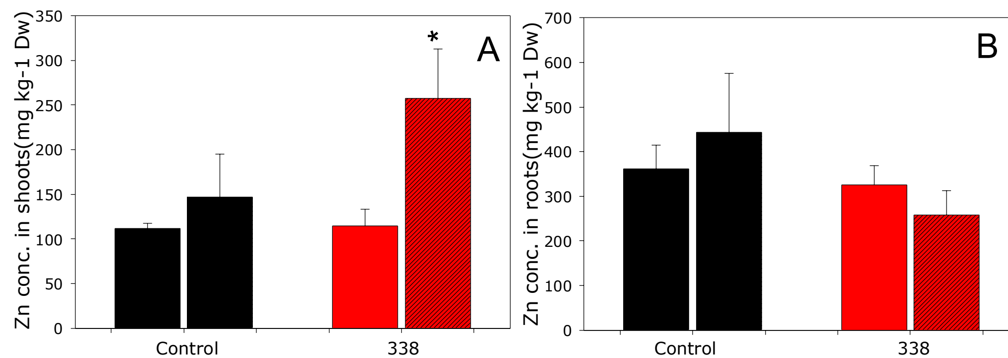


figure 5.14: Zinc concentration in mg kg^{-1} dry matter in shoot part (A) and root part (B). Controls are the non inoculated plants, different inocula are specified under each. Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results was confirmed at the 5% level using one and two-way ANOVA model separately exploring inocula and presence or absence of cadmium. The difference is marked with a star on the histogram. In case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

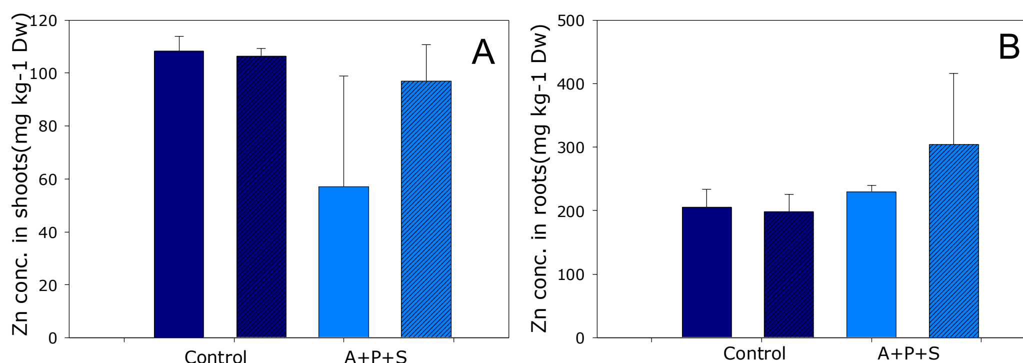


figure 5.15: Zinc concentration in mg kg^{-1} dry matter in shoot part (A) and root part (B). Controls are the non inoculated plants, different inocula are specified under each bar. Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results was confirmed at the 5% level using one and two-way ANOVA model separately exploring inocula and presence or absence of cadmium. The difference is marked with a star on the histogram. In case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

5.3.3.4 Ratio metal-cadmium

As explained before cadmium may interfere with zinc and iron uptake and vice-versa. The ratio between these elements, when the result is > 1, then, becomes high when the plants accumulate more Zn or Fe in comparison to Cd, while when the result is < 1, the ratio becomes high when plants accumulate less Zn or Fe than Cd.

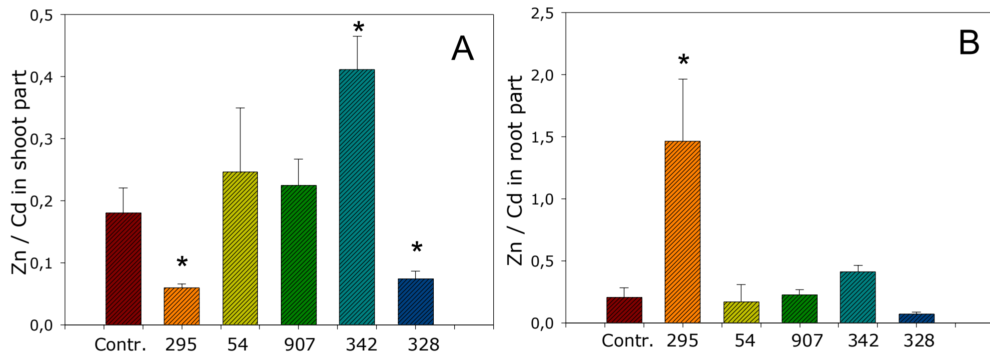


figure 5.16: Zinc-cadmium ratio in shoots (A) and in roots (B). The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are evidenced with a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula. In case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

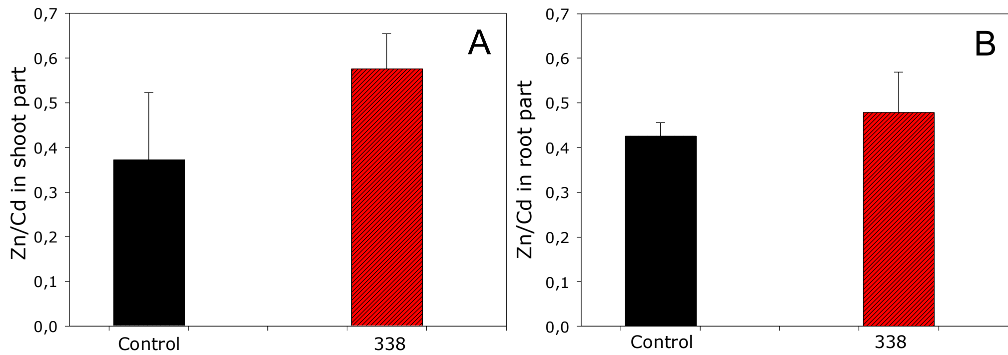


figure 5.17: Zinc-cadmium ratio in shoots (A) and in roots (B). The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are evidenced with a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula. In case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

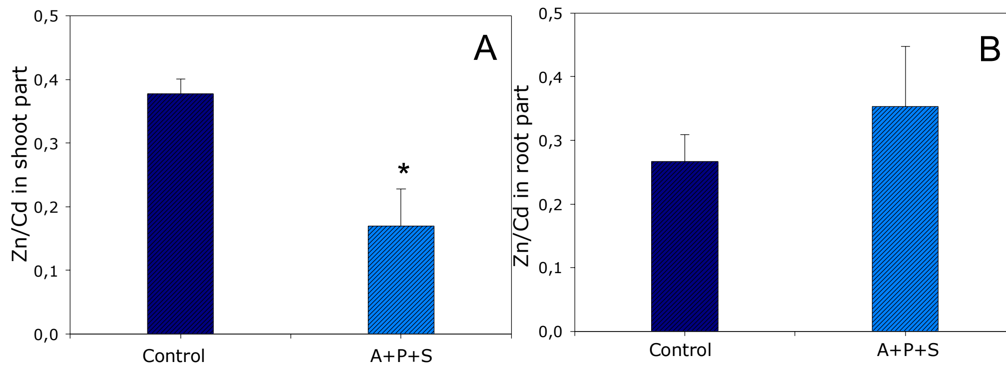


figure 5.18: Zinc-cadmium ratio in shoots (A) and in roots (B). The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are evidenced with a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula. In case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

Plants exposed to cadmium and inoculated with *Stenotrophomonas sp*. 295 and with the consortium A+P+S showed an opposite behaviour compared with plants inoculated with 342. In shoots 295 as well as the consortium decreased the Zn/Cd ratio (figure 5.16A and 5.18A) while 342 increased it significantly compared with non-inoculated plants (figure 5.16A). In the roots plants inoculated with 295 still preferred cadmium to zinc, but the concentration between the both is much less different than in shoots where the difference was ten times more (figure 5.16A). In case of iron (figure 5.19B), inoculation with strain 295 lead to a significantly higher Fe content in the roots when plant were treated with cadmium while in the shoots there was no difference with the non-inoculated plants (figure 5.19A). *Pseudomonas sp*. 54 maintained the opposite behaviour in comparison to 295 leading to a higher uptake of cadmium than iron in the roots compartmentalizing cadmium in that plant part; a similar behaviour was adopted by plants inoculated with *Stenotrophomonas sp*. 328 and 338 and by the ones inoculated with the consortium that decreased the same ratio into the shoots as well (figure 5.19, 5.20 and 5.21).

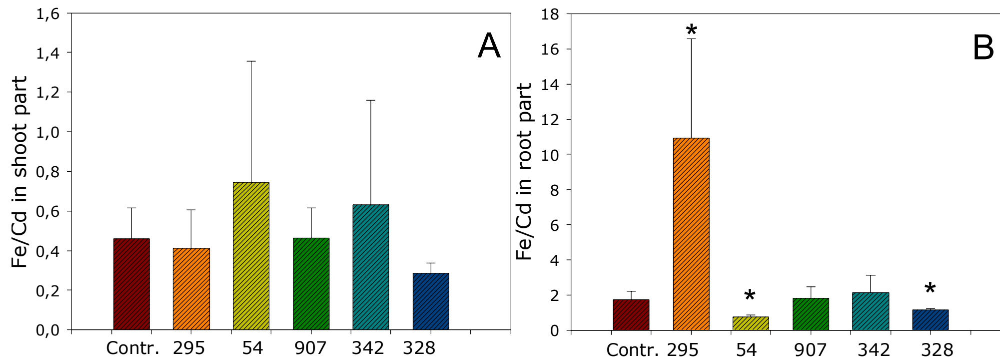


figure 5.19: Iron-cadmium ratio in shoots (A) and in roots (B). The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are evidenced with a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula. In case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

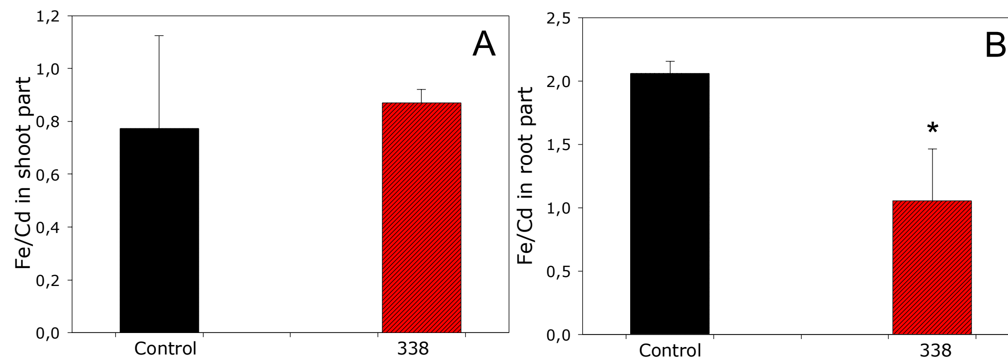


figure 5.20: Iron-cadmium ratio in shoots (A) and in roots (B). The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are evidenced with a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula. In case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

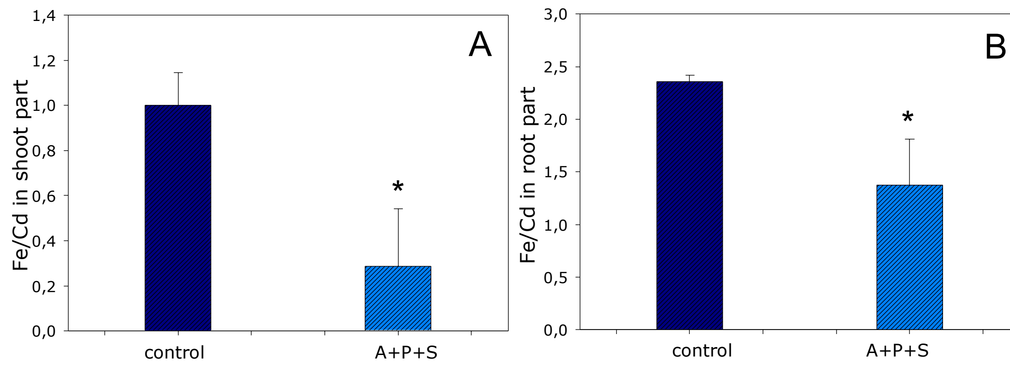


figure 5.21: Iron-cadmium ratio in shoots (A) and in roots (B). The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are evidenced with a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula. In case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

5.3.3.5 Translocation Factor

The translocation Factor (T.F.) is the ratio between the metal concentration present in the shoots and the concentration of the same metal in the roots.

In our experiment, plants inoculated with *Stenotrophomonas sp*. 295 as well as the ones inoculated with the consortium (A+P+S) showed a significantly higher cadmium T.F. Besides 295, significantly lower zinc T.F. were observed in plants treated with cadmium compared with non-inoculated plants (figure 5.22 and 5.24).

The zinc T.F. it was higher in plants without cadmium treatment inoculated with 295 and with 907 (figure 5.22B) while it was lower in plants inoculated with the consortium A+P+S (figure 5.24B) and with 342 (figure 5.22B). Other inoculations show differences with control but standard deviation is too high.

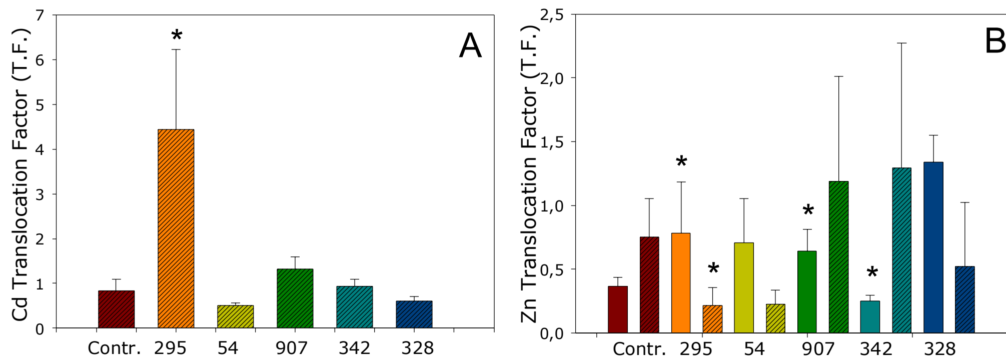


figure 5.22: Translocation Factor (T.F.) of cadmium (A) and of Zn (B) control are the non inoculated plants, different inocula specified under each histogram. Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results was confirmed at the 5% level using one and two-way ANOVA model separately exploring inocula and presence or absence of cadmium. The difference is marked with a star on the histogram. In case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

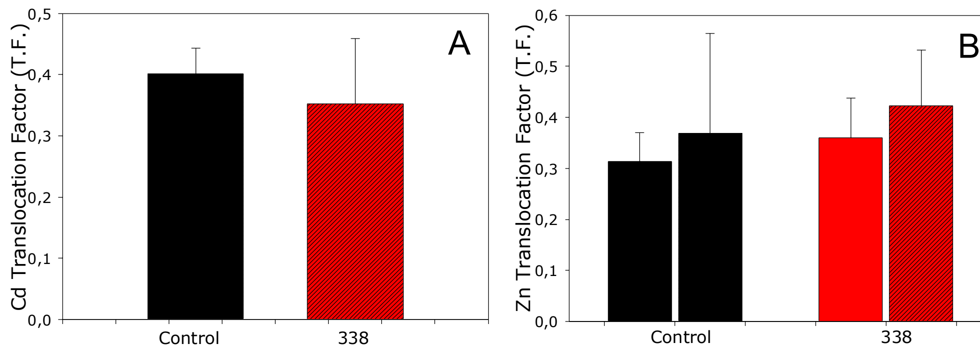


figure 5.23: Translocation Factor (T.F.) of cadmium (A) and of Zn (B) control are the non inoculated plants, different inocula specified under each histogram. Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results was confirmed at the 5% level using one and two-way ANOVA model separately exploring inocula and presence or absence of cadmium. The difference is marked with a star on the histogram. In case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

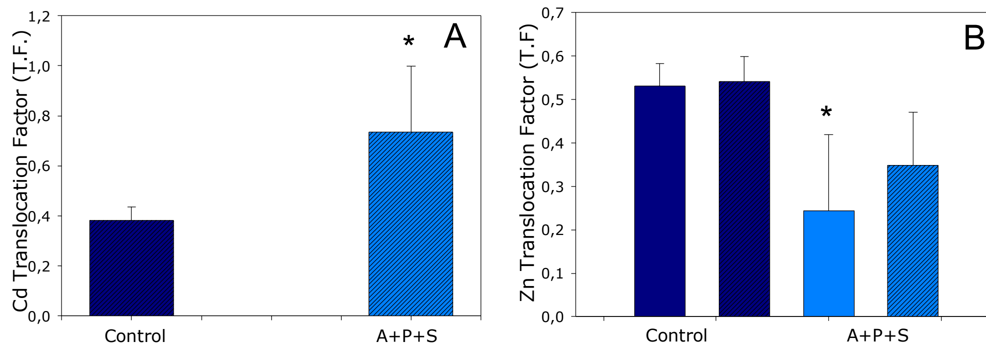


figure 5.24: Translocation Factor (T.F.) of cadmium (A) and of Zn (B) control are the non inoculated plants, different inocula specified under each histogram. Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results was confirmed at the 5% level using one and two-way ANOVA model separately exploring inocula and presence or absence of cadmium. The difference is marked with a star on the histogram. In case of not normal data distribution Mann-Whitney U test was used after arctangent function transformation.

5.4 Discussion and conclusions

Results were shown of three experiments aiming to contribute to a clarification of how inoculation with plant associated bacteria effects plant growth, metals uptake and plant toxicity in normal conditions and under cadmium stress. For this purpose, the following parameters were investigated: 1) root and shoot fresh and dry weight for biomass production; 2) metal concentrations (Cd, Zn and Fe) in roots and shoots. A `derived' parameter considered was the Translocation Factor (T.F. = ratio of concentration of an element in the shoot on the concentration of the same element in the root) that is important in phytoextraction as, one of the desired characteristics of a plant to be efficient in this decontaminating technique, is the ability of metal translocation from the root to the shoot. In general, roots are difficult to be completely harvested and, left in the soil, return the metals to the soil ecosystem.

The ratios of Zn/Cd concentrations as well as Fe/Cd concentrations were also calculated for both inoculated and non inoculated plants. As explained before, cadmium uptake can interfere with the uptake of others essential metals, like zinc and iron resulting in negative effects on plant growth.

Plants inoculated with *Pseudomonas sp. 54* acted as cadmium excluder since in the host plants cadmium concentration inside the roots was higher than non inoculated plants, while there was no difference in shoots cadmium concentration between inoculated and no inoculated plants (figure 5.7A and B). Inoculation of this strain decreased the zinc concentration in the whole plant (figure 5.13) which can (partly) explain the inhibition of plant growth (figure 5.1 and 5.2). In the roots of cadmium treated plants the Fe/Cd ratio is lower than in non inoculated plants; this strain allowed an higher uptake of cadmium compared to iron (figure 5.19B).

Four different *Stenotrophomonas sp*. strains were compared; one originated from a tobacco leaf (295) and three others were isolated from the rhizosphere of another tobacco plant (342, 328 and 338); all of them showed different effects on the inoculated host plants.

Stenotrophomonas sp. 295, highly increased cadmium translocation (figure 5.22A) enhancing its concentration into the shoot and decreasing it into the roots. In cadmium treated plants, this strain modulated the trace elements uptake increasing the essential ones in the roots (figures 5.16B and 5.19B), especially the iron that is ten times higher than in the shoots and than in non inoculated plants. The same elements were decreased in the shoots allowing a higher cadmium concentration compared to the non inoculated plants (figures 5.16A and 5.19A). The resulting unbalance of essential and non essential elements could be one of the possible reasons for the lower biomass production (figure 5.1 and 5.2).

Stenotrophomonas sp. 342 inoculated in non treated plants lowered zinc concentration (figure 5.13) and zinc T.F. (figure 5.22B) but under cadmium stress the plants took up more zinc into the shoots than the non inoculated plants (figure 5.13A). A better plant growth, in addition to a cadmium

concentration comparable to the control, lead, as expectable, to an higher cadmium plant content compared with the others inocula (figure 5.10).

Stenotrophomonas sp. 328 led to an increased Zn/Cd ratio in the shoot (figure 5.16A), while in the roots the higher cadmium concentration (figure 5.7B) seemed to be at the cost of iron (figure 5.19B); this might have been one of the causes for the observed plant growth inhibition (figure 5.1 and 5.2); this growth inhibition together with a similar plant cadmium concentration to that of the non inoculated plants (figure 5.7), resulted in a lower plant cadmium content (figure 5.10).

After inoculation with *Stenotrophomonas* 338, like in case of *Stenotrophomonas* 342, the zinc concentration in the shoots of cadmium treated plants increased (figure 5.14A); just as 328 it decreased the ratio Fe/Cd (figure 5.20A). The plant growth inhibition caused by the inoculation of this strain (figure 5.5 and 5.6) resulted in a lower cadmium content in both roots and shoots (figure 5.11).

The lower biomass production after inoculation of our *Stenotrophomonas* strains, also in case of no cadmium treatment, could be partly explained by work of Suckstorff and Berg (2003), reporting a clear dose-dependent effect of the *Stenotrophomonas* inoculation. They showed, in fact, that only a specific concentration for each strain could enhance plant growth, while a lower or higher concentration lead to a lower biomass production.

Due to the quite high standard deviations on the zinc T.F. (figure 5.22B), it is difficult to draw conclusions, but considering the ratio between zinc and cadmium (figure 5.13) it is interesting to notice that pure inocula, in general, tend to increase this ratio in the shoot part, while inoculation with the consortium led to the opposite, trying to uptake more cadmium than zinc or iron (figure 5.18 and 5.21).

Surprisingly the lower concentrations of iron and zinc didn't affect the biomass production (figure 5.3 and 5.4). Inoculation with the consortium had no negative effect on biomass production allowing a growth similar to the non inoculated plants (figures 5.1, 5.2, 5.5 and 5.6). Mixed bacterial cultures are generally

considered more advantageous than pure cultures for environmental biotechnology (White and Gadd, 1996). The consortium we tested better colonized the plant (table 5.2) even if the inoculum concentration at the start was the same as the strains inoculated in purity (table 5.1). This suggests a beneficial synergism between the different strains inoculated; another factor that could have been positive is that the concentration of bacteria of each individual strain belonging to the consortium is lower than in purity. Different results were obtained by Ruppel *et al.*, 2006; they compared higher inocula concentrations and found the best biomass production with the highest inocula concentration tested (10^9). This difference might be explained by the different inoculation procedure; Ruppel *et al.* (2006) used the inocula on growing plants administering it directly on the leaf. It has been demonstrated that the inoculation of plants with bacteria causes an initial stress for the plants even when the bacteria are not pathogenic (Szatmari *et al.*, 2006); non pathogenic bacteria affected plants more during their initial development. Variable results were also obtained by different inoculating methods. Ciccillo *et al.* (2002), in fact, demonstrated that applying the inoculum they were working with on the seed, it could significantly improve plant growth; when, on the contrary, they applied the same inoculum to the soil, the same strain markedly reduced growth of the plants.

In conclusion, a too high inoculum concentration could be the reason why the bacteria inoculated in purity affected more the growth of our plants, even when these plants were grown in absence of cadmium. In fact, it is true that in nature the microbial concentration of a single strain is much lower due to high competition inside the consortium than the one applied in our "in vitro" experiments. Comparing the results obtained by the use of single strains and the use of the consortium, we obtained better results with the last one from the point of view of phytoextraction. A+P+S, in fact, allowed, beside an healthier plant growth illustrated by a higher biomass production, a higher cadmium translocation into the shoots and an higher plant cadmium content in comparison to non inoculated plants.

Since it was strongly increasing the cadmium T.F., Stenotrophomonas sp. 295 seems an interesting strain to work with, eventually in association with other strains; a suggestion is to use it instead of Stenotrophomonas sp. 342 in the consortium A+P+S.

At this point a soil and/or field experiment could be a next step, using tobacco plants inoculated with the consortium (eventually substituting *Stenotrophomonas sp*. 342 with 295). Another suggestion is to test *Stenotrophomonas sp*. 342 under zinc stress as well.

Chapter 6. Isolation and identification of endophytic bacteria from seeds of *Nicotiana tabacum* (var. BAG) grown on the field site of Rafz (Switzerland)

Abstract

We isolated microbial consortia from surface sterilized seeds of *Nicotiana tabacum* var. BaG grown in Rafz (Switzerland), a field site characterized by metal contamination. The isolation was carried out at three different times, four, five and eight months after the seeds were harvested. The cultivable strains were characterized and identified. In general, the genera identified were similar to the ones isolated from the rhizosphere and from the different parts of tobacco plants, illustrating a systematic 'migration' of part of the bacteria inside the plant. A striking exception was *Pseudomonas fulva* isolated from the seeds that forms a separate group suggesting another origin different from the rhizosphere. A decrease in CFU's/seed could be observed from the first experiment to the third one; at the same time, an evolution in the characteristics of the cultivable strains isolated, like a lowering in metal tolerance, was occurring. A modification in the seed consortia was observed as well underlined by a decrement in *Pseudomonaceae* and in an increment of the percentage of gram positive strains.

6.1 Introduction

Two hypotheses about the seed colonization strategy used by bacteria are existing: 1) bacteria grow epiphytically on the surface of plant reproductive organs and externally contaminate seed; or 2) the bacteria move systemically within the plant and gain access to seed via vascular connections (Barak *et al.* 2002).

Determinants of seed colonization have been studied in *Pseudomonas* spp. and in *Enterobacter cloacae*. In the last case, the importance of amino acids and peptides as carbon sources during spermosphere colonization has been established (Roberts *et al*., 1996 and Roberts and Hartung, 1996). Carbohydrate utilization is also a fundamental trait in the interaction of *E. cloacae* with seeds;

a *pfkA* mutant, defective in phosphofructokinase, a key glycolytic enzyme, is impaired in seed colonization (Roberts *et al*., 1999). The importance of nutrients released by germinating seeds for bacterial colonization is also reflected by the fact that seedling-associated *Pseudomonas chlororaphis* cells are mainly found in the area around the cotyledon and emerging embryo, where nutrient release is presumably maximal (Tombolini *et al.*, 1999). Mutants defective in adhesion to seeds have been isolated in *P. fluorescens* (DeFlaun *et al*., 1990, DeFlaun *et al.*, 1994) and in *Pseudomonas putida* KT2440 (Espinoza-Urgel *et al.*, 2000), a derivative of *P. putida* mt-2 (Franklin *et al.*, 1981) which efficiently colonizes the spermosphere and the rhizosphere of a number of plants (Espinoza-Urgel *et al.*, 2000, Espinoza-Urgel *et al.*, 2004 and Molina *et al.*, 2000). In the first case, two of the three mutants isolated were deficient in the synthesis of flagellin, the structural component of the flagellum (DeFlaun *et al.*, 1990, Deflaun *et al.*, 1994). In *P. putida* KT2440, a number of genes involved in attachment to corn seeds have been identified (Espinoza-Urgel *et al.*, 2000). Among these *lapA*, a gene coding for one of the largest bacterial proteins described to date, also present in *P. fluorescens* WCS365 and essential for adhesion to seeds and for biofilm formation, has been recently characterized (Hinsa *et al*., 2003). Espinoza-Urgel and Ramos (2004) have studied a gene involved in seed colonization by *P. putida* KT2440, whose expression responds to cell density and to seed exudates. This constitutes the first evidence that *P. putida* KT2440 may possess an as-yet-uncharacterized quorum- sensing system and suggests that cell-cell and cell-host communications are important for spermosphere colonization.

Different bacterial genera have been isolated from surface-sterilised seeds, such as *Bacillus*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Cytophaga*, *Leuconostoc*, *Micrococcus* and *Xanthomonas* (Bacon and Hinton, 1996, Mundt and Hinkle, 1976 and Granér *et al*., 2003). *Xanthomonas campestris* pv. *vitians* was isolated from lettuce seeds (Barak *et al.*, 2002), this seems to be common with many other leaf spot bacteria that can be a contaminant of seeds (Umesh *et al.*, 1996; Sahin & Miller, 1997). Vega *et al.* (2005) noticed even the highest bacteria concentration inside the coffee seeds compared with the bacterial concentration inside the rest of the plant parts. From the seeds their group could isolate

Bacillus, *Burkholderia sp.*, *Clavibacter sp.*, *Curtobacterium sp.*, *Escherichia sp.*, *Micrococcus sp.*, *Pantoea sp.*, *Pseudomonas sp.*, *Serratia sp.*, and *Stenotrophomonas sp*. These genera are mostly present in the rhizosphere as well.

Another factor to be considered when studying the microbial consortia of seeds is that these consortia may change in relation with storage time of the seeds. Cankar *et al.*, 2005 were unable to isolate cultivable strain from seeds stored for a few years, while they succeeded with young seeds. Seeds persistence in the soil contrasting with their occurring decay in laboratory, indicates the presence of yet-unknown factors in natural systems that regulate biological mechanisms of seed antagonism by soil microorganisms. Microscopic examinations revealed dense microbial assemblages formed whenever seeds were exposed to soil microorganisms, regardless of whether the outcome was decay. Data from velvetleaf seeds, studied by Chee-Sanford *et al.* (2006) suggested that diverse assemblages of bacteria can mediate decay, whereas fungal associations may be more limited and specific to weed species.

In this chapter we try to identify and characterize the cultivable endophytic consortia isolated from *Nicotiana tabacum* seeds at four, five and eight months after their harvest. To our knowledge this is the first investigation concerning the cultivable endophytic consortium living inside tobacco seeds.

6.2 Material and Methods

6.2.1 Seeds surface sterilization

Nicotiana tabacum capsules seeds were allowed to ripen on the plants; ten of them were collected from different plants belonging to the same tobacco variety and opened, their content was mixed and stored into a sterile Falcon tube and kept in a refrigerator at 4°C. This constituted the seed bank used for bacteria extraction of endophytic bacteria as well as for plant re-inoculation experiments.
The seeds were surface sterilized using a solution of 1% active chloride supplemented with 1 droplet Tween 80 per 100ml of solution during 30 minutes, and rinsing 3 times with sterile Millipore water during 10 minutes each time. To

check the surface sterility, the seeds were rolled on ten times diluted rich medium 869 and subsequently removed. The seeds were considered sterile if no bacterial growth was detected. The same seeds were put into a sterile mortar and squeezed into a sterile solution of 10mM $MgSO_4$. Dilutions of these samples were plated on ten times diluted 869 medium and were checked after 7 days incubation at 30°C.

6.2.2 Bacterial heavy metal resistance

The isolated strains, after at least three times sub-cultures to ensure purity, were tested for their heavy metal resistance using the minimal Tris buffered medium 284 (Schlegel *et al.*, 1961). This medium contained per litre deionised water, 6.06g Tris-HCl, 4.68g NaCl, 1.49g KCl, 1.07g NH_4Cl, 0.43 Na_2SO_4, 0.2g $MgCl_2 \cdot 6H_2O$, 0.03g $CaCl_2 \cdot 2H_2O$, 40mg $Na_2HPO_4 \cdot 2H_2O$ 1%, 10ml Fe(III)NH_4 citrate solution (containing 48mg:100ml), 1ml microelements solution, final pH 7. Four different carbon sources (1.3ml glucose 40%, 2.2ml gluconate 30%, 2.7ml fructose 20% and 3ml succinate 1M) were added. The microelements solution contained per liter distilled water: 1.3ml 25% HCl, 144mg $ZnSO_4 \cdot 7H_2O$, 100mg $MnCl_2 \cdot 4H_2O$, 62mg H_3BO_3, 190mg $CoCl_2 \cdot 6H_2O$, 17mg $CuCl_2 \cdot 2H_2O$, 24mg $NiCl_2 \cdot 6H_2O$ and 36mg $NaMoO_4 \cdot 2H_2O$. This medium was supplemented with different concentrations of $CdCl_2$ and separately of Zn $(SO_4)_2$. The minimum Inhibiting Concentration (MIC) was determined and defined as the minimal concentration of the heavy metal inhibiting growth of the bacterial strains. The range tested for Zn was from 0mM up to 4mM, and the one for Cd was from 0mM up to 1.5mM.

6.2.3 Bacterial carbon sources

The pure isolated strains were tested for their favoured carbon source using a minimal Tris buffered medium 284 (Schlegel *et al*., 1961). The carbon sources were supplemented on separate Petri dish using either 1.3ml glucose 40% or 2.2ml gluconate 30% or 2.7ml fructose 20% or 3ml succinate 1M per liter of medium. Bacteria were also tested without carbon sources in order to check

their eventual heterotrophy, with all the carbon sources and on 869 diluted ten times to have a positive control.

6.2.4 Siderophore assay

A chrome azurol S (CAS) shuttle solution (Schwyn and Neilands, 1987) was used for routine testing of siderophore production in liquid media. This test was carried out using liquid 284 medium (Schlegel *et al.*, 1961) in glass tubes with the addition of four different carbon sources (1.3ml glucose 40%, 2.2ml gluconate 30%, 2.7ml fructose 20% and 3ml succinate 1M). The test was carried out using both 284 medium without iron and with 0.25µM Fe (III) citrate.

6.2.5 Bacterial DNA extraction

The pure strains were grown in their appropriate medium (1/10 869) at 30°C on a shaker. The bacterial growth was stopped when at an OD_{660} included between 0.5 and 0.8. The culture was centrifuged in an eppendorf tube at maximum speed during 10min. The pellet was frozen after medium removal. The defrosted pellet was re-suspended in 300µl 1xSSC +2mg/ml lysozyme + 1:100 of 10mg/ml RNase. After incubation at 37°C during 15min, 17 µl of SDS (10%) were added and the solution was again incubated during 10min. 3.3µl of proteinase K (20mg/ml) were added to finish the lyses step and incubated during one hour at 65°C.
A phenol chloroform extraction was carried out. The DNA precipitation was obtained adding 1/10 volume of 3M Na Acetate pH 6 and 2.5x 100% ethanol (-20°C). The DNA was subsequently fished out and solubilized in sterile ddH_2O.
DNA quality was checked on 0.8% Agarose gel.

6.2.6 Box-PCR genomic DNA profile

The DNA extracted was amplified by PCR using the BOX1 primer. The PCR reaction contained 5µl 10x Taq-Buffer, 4µl 10mM dNTP's, 2µl Box-primer (forward + reverse, sequence: 5'-CTACGGCAAGGCGACGCTGACG-3'), 0.25µl Taq polymerase and 5µl template, in a total final volume of 50µl. The thermocycling

conditions were: 1 min. at 95°C, 35 cycles of 1min. at 95°C, 1.5min. at 50°C, 8min. at 65°C, and finally one cycle at 65°C during 8 min. The obtained PCR products were separated by means of a 2% agarose electrophoresis gel with 4µl/100ml of ethidium bromide run during 2 hours at 75V.

6.2.7 PCR amplification

PCR amplification was carried out, targeting the 16S gene using primers P0 (27f sequence: 5' GAGAGTTTGATCCTGGCTCAG) and P6 (1495r sequence: 5' CTACGGCTACCTTGTTACGA).
The extracted DNA (1µl) was used in a final volume of 50µl PCR's, each reaction, consisting of: 5µl buffer (10x), 2µl $MgCl_2$ (50mM), 1µl of each primer (10µM each provided by Invitrogen), 1µl 10mM dNTP's (Invitrogen), 0.4µl Taq (Invitrogen), prepared as a master mix, with addition of sterile ddH_2O till the final volume, prior to DNA addition.
Cycling conditions were: 1.5 min at 95°C, 5 cycles of 30 sec at 94°C, 30 sec at 60°C, 2 min. at 72°C, 5 cycles of 30 sec. at 94°C, 30 sec. at 55°C, 2 min. at 72°C, 25 cycles of 30 sec. at 94°C, 30 sec. at 50°C, 2 min. at 72°C and finally one cycle of 10 min. at 72°C.
The resulting PCR-DNA product was then checked by gel electrophoresis at 80V during 30min on a 0.8% Agarose gel with 4µl/100ml of ethidium bromide. PCR products were purified with GFX PCR DNA and gel band purification kit (Amersham Biosciences) before sequence.

6.2.8 Sequence alignment and phylogenetic analysis.

The 16S rDNA sequence obtained were compared with 16S rDNA sequence of AY741159, AY837730 and DQ466570 recovered in PubMed database and belonging to the *Pseudomonas fulva* and to two different strains of *Stenotrophomonas maltophilia* respectively. These last ones were used as outgroup. *Pseudomona fulva* AY741159 was a diazotrophic bacterial species isolated from Korean wetland rice, *Stenotrophomonas maltophilia* AY837730 and DQ466570 were isolated the first one from the midgut of mosquitos collected from the field and the second from sugarcane. Multiple alignment was performed

over all the sequences used by means of Multalin program (Corpet, 1988), all characters were weighted equally. Neighbor-Joining (NJ) method was used to analyse the aligned sequences. NJ tree (Saitou and Nei, 1987) was obtained on the basis of a Kimura-2 parameter distance matrix using MEGA2 "*Molecular Evolutionary Genetic Analyses*". Internal support to the branches was estimated by means of 50% Majority-Rule bootstrap analysis with 1000 replicates (Felsenstein, 1985).

6.3 Results and discussion

The analyses of squeezed surface sterilized seed material resulted in CFU's/seed of 1.1×10^2 in the first isolation experiment (SE_I), of 1.2×10^2 one month later (SE_II), and, finally, of 1.1×10^1 more than four months after the first isolation (SE_III). This means that we observed a ten times decrement in CFU's/seed within 130 days of storage at 4°C. The colonies were picked up based on their different morphology. A phenotypic characterisation was started analysing their characteristics interacting with metals resistance and their carbon sources preference. The major part of the isolated seed bacteria were later on used for inoculation experiments (see chapter 7) in order to investigate their potential role in plants growth. Some isolates were difficult to be maintained on media and therefore were removed from further studies.

6.3.1 Phenotypic characterization

The isolated bacteria were analysed for siderophore production and minimum inhibiting concentration (MIC, table 6.1) for zinc and cadmium, the main contaminating trace elements present in the soil of the Rafz site (Switzerland) from where the seeds were originating. The metal content in the seeds is, in general, much lower than in the rest of the plant parts (Rayment, 2005), but we hypothesised that the seed endophytes could originate from more selective environments; for this reason they might possess the capabilities to tolerate toxic metals concentrations.

We also tested capability of the bacteria to produce siderophores (table 6.1); this characteristic may interfere either helping plant growth or protecting the plants against pathogens (Hebbar *et al*., 1992 a and b). The majority of the isolated strains showed to be no siderophores producers; it has to be mentioned that many strains were intolerant to the medium used for this test and for that reason couldn't grow.

In contrast with the bacterial strains isolated from the different plant parts and from the rhizosphere (table 3.10), the bacteria from the seeds showed a tolerance exclusively to cadmium (≥0.6mM), while none of them was found to be zinc tolerant (≥0.5mM), as presented in table 6.1, only about 10% showed a low tolerance to zinc (between 0.25 and <0.5mM). This latter metal tolerance characteristics were found for the cultivable bacteria isolated during the first two experiments; on the contrary, during the third isolation experiment performed 130 days after the first one, the situation was different (table 6.2): the cadmium tolerance disappeared and was replaced by a lower tolerance in a few strains that represent about 25% of the total number of strains isolated; another 25% was showing a slight zinc tolerance (table 6.1). It is clear that at this third moment of sampling we observed not only a decrement in bacterial charge (colony forming units) per seed, but also a change in bacterial characteristics.

Experiment	Only Zn tolerant (%)	Only Cd tolerant (%)	Both Zn and Cd tolerant (%)
SE_I	0	37.5	0
SE_II	0	40.9	0
SE_III	0	0	0

table 6.2: Percentage of cultivable metals tolerant strains isolated from surface sterilised seed material. As zinc tolerant is meant a MIC value ≥0.5mM while for cadmium tolerant is meant a MIC value ≥0.6mM.

The different carbon sources were tested only with the purpose of grouping the different strains in phenotypes through the addition of a variable more. The different characteristics are summarized in table 6.1.

Seq. ID	Isolate	869	284+ C	284-C	284+ SC	284+ GS	284+ GN	284+ FR	Zn (mM)	Cd (mM)	Sid.	Similar to
Pseudomonas fulva	SE_I001	+	+	-	+	+	-	-	<0.25	1.05	N.G.	A
Pseudomonas fulva	SE_I002	+	+	-	+	+	-	-	<0.25	1.05	-	A
Pseudomonas fulva	SE_I003	+	+	-	+	+	-	-	<0.25	1.05	-	A
	SE_I004	+	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	
Pseudomonas sp.	SE_I005	+	+/-	-	-	+	-	-	<0.25	0.45	N.G.	E
Pseudomonas sp.	SE_I006	+	+	-	+	+	+/-	-	<0.25	0.9	-	F
Xanthomonadaceae	SE_I007	+	+	-	+	+	+	-	<0.25	<0.15	N.G.	B
Pseudomonas sp.	SE_I008	+	+	-	+	+	+	-	<0.25	1.05	-	G
Pseudomonas sp.	SE_I009	+	+	-	+	+	+	-	<0.25	0.9	-	G
Stenotrophomonas sp.	SE_I010	+	+	-	-	+	-	-	<0.25	1.05	N.G.	
	SE_I011	+	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	
	SE_I012	+	+	-	+	+/-	+/-	-	<0.25	<0.15	N.G.	H
Pseudomonas sp.	SE_I013	+	+	-	+	+/-	+/-	-	<0.25	0.9	N.G.	C
Pseudomonas sp.	SE_I014	+	+	-	+/-	+/-	-	-	<0.25	0.9	N.G.	F
Stenotrophomonas sp.	SE_I015	+	+/-	-	+/-	+/-	-	-	<0.25	<0.15	N.G.	D
Enterobacter sp.	SE_I016	+	+	-	-	+/-	+/-	-	<0.25	<0.15	N.G.	I
Xanthomonadaceae	SE_I017	+	+	-	-	-	-	-	<0.25	<0.15	N.G.	B
Xanthomonadaceae	SE_I018	+	+/-	-	+/-	-	+/-	-	<0.25	<0.15	N.G.	B
Enterobacter sp.	SE_I019	+	+	-	-	+/-	+/-	-	<0.25	<0.15	N.G.	I
	SE_I020	+	+	-	+	+	-	-	0.35	<0.15	N.G.	
Xanthomonadaceae	SE_I021	+	+/-	-	+/-	+/-	-	+/-	0.35	<0.15	+/-	B

Seq. ID	Isolate	869	284+ C	284-C	284+ SC	284+ GS	284+ GN	284+ FR	Zn (mM)	Cd (mM)	Sid.	Similar to
Pseudomonas sp.	SE_I022	+	+	-	+	+/-	+/-	-	N.T.	N.T.	N.G.	C
	SE_I023	+	-	-	-	-	-	-	N.G.	N.G.	N.G.	
	SE_I024	+	+	-	+	+/-	+/-	-	<0.25	<0.15	N.G.	H
Pseudomonas sp.	SE_II01	+	+	-	+	+	+	-	<0.25	0.6	N.G.	S
Sanguibacter sp.	SE_II02	+	+	-	+	+/-	+/-	-	<0.25	<0.15	N.G.	
Pseudomonas sp.	SE_II03	+	+	-	+	-	-	-	<0.25	0.75	-	
Pseudomonas sp.	SE_II04	+	+	-	+	+	+	-	<0.25	<0.15	-	S
Stenotrophomonas sp.	SE_II05	+	+	-	+/-	+/-	+	+/-	<0.25	0.3	N.G.	
Pseudomonas sp.	SE_II06	+	+	-	+	+	+	+	<0.25	0.45	N.G.	
Pseudomonas sp.	SE_II07	+	+	-	+	+	+/-	-	<0.25	0.3	N.G.	L
Pseudomonas sp.	SE_II08	+	+	-	+	-	-	-	<0.25	0.6	N.G.	L
Pseudomonas sp.	SE_II09	+	+	-	+	-	-	-	<0.25	0.6	N.G.	
Clostridium aminovalericum	SE_II10	+	+	-	+/-	+/-	-	-	<0.25	0.9	N.G.	
Enterobacter sp.	SE_II11	+	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	I
Pseudomonas sp.	SE_II12	+	+	-	+	+	-	-	<0.25	1.5	N.G.	
Pseudomonas sp.	SE_II13	+	+	-	+	+	+	-	<0.25	0.45	-	G
Pseudomonas sp.	SE_II14	+	+	-	+	+	+	-	<0.25	0.75	-	G
Pseudomonas sp.	SE_II15	+	+	-	+	+	+	-	<0.25	0.3	-	G
Pseudomonas sp.	SE_II16	+	+	-	+/-	+/-	+	-	0.25	0.9	+/-	
Enterobacter sp.	SE_II17	+	+	-	+	+	+	-	0.25	0.45	-	
	SE_II18	+	+/-	-	+/-	+/-	+/-	+/-	0.25	<0.15	N.G.	Q

Seq. ID	Isolate	869	284+ C	284-C	284+ SC	284+ GS	284+ GN	284+ FR	Zn (mM)	Cd (mM)	Sid.	Similar to
Pseudomonas sp.	SE_II19	+	+	-	+/-	+	-	-	<0.25	0.3	N.G.	E
	SE_II20	+	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	
Pseudomonas sp.	SE_II21	+	+	-	+	+	+/-	-	<0.25	1.05	-	F
Stenotrophomonas sp.	SE_II22	+	+/-	-	+/-	+/-	-	-	<0.25	<0.15	N.G.	D
	SE_III01	+	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	
	SE_III02	+	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	
	SE_III03	+	+/-	-	+/-	+/-	-	+/-	0.35	<0.15	N.G.	
Pseudomonas sp.	SE_III04	+	+	-	+	+	+/-	-	<0.25	0.45	N.G.	L
	SE_III05	+	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	
	SE_III06	+	+	-	+	+	+	+	<0.25	0.45	N.G.	
	SE_III07	+	+	-	+	+/-	+/-	-	<0.25	<0.15	N.G.	
Sanguibacter sp.	SE_III08	+	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	
Sanguibacter sp.	SE_III09	+	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	
	SE_III10	+	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	
Enterobacter sp.	SE_III11	+	+	-	+/-	+/-	-	+/-	0.5	0.15	N.G.	M
Enterobacter sp.	SE_III12	+	+	-	+	+/-	+/-	-	<0.25	<0.15	N.G.	M
Xanthomonadaceae	SE_III13	+	+/-	-	+/-	-	+/-	-	<0.25	<0.15	N.G.	

table 6.1: Growth media, carbon sources, MIC values and siderophores production characteristics of cultivable bacteria isolated from surface sterilized seeds after four (from SE_I001 to SE_I024), five (from SE_II01 to SE_II22) and eight (from SE_III01 to SE_III13) months after collection from the plants. N.G. is not growth as the strain couldn't grow on the medium tested. In the carbon sources: – where no growth was seen. +/- where only a slight growth was detected, the strain can hardly leave. + the strain well grow on that media. Letters in the column "Similar to" refers to the similar BOX PCR fingerprinting, same letter corresponds to similar profile. N.T. is Not Tested.

6.3.2 Genotypic characterization

A DNA extraction was carried out on the cultivable strains isolated from surface sterilised seeds. On the DNA samples the BOX PCR technique was used to group the strains in species, subsequently a member per group was used for 16S rDNA amplification and identification.

6.3.2.1 BOX-PCR genomic DNA fingerprinting

Running a BOX-PCR on the DNA samples we are able to distinguish the different species present inside the same genus. It became clear that under the same genus a high genotypic diversity and variability was hidden; the results are summarized in the last column of table 6.1 In figure 6.1 an example of BOX PCR fingerprinting identifying different strain species is presented. It is possible to group the *Enterobacter sp*. represented by SE_I016, SE_I037 and SE_I038, the *Xanthomonadacea* family by SE_I017, SE_I018 and SE_I007 and a Pseudomonas sp. in fourth and fifth line in the figure 6.1. Due to technical problems we were not able to identify the SE_I012 and SE_I024.

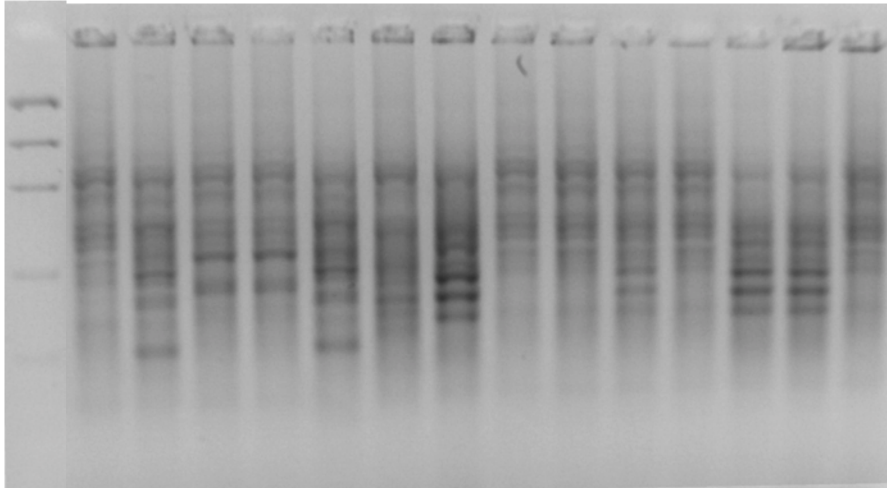


figure 6.1: Box-PCR fingerprinting of bacteria isolated from the seeds of *Nicotiana tabacum* var. BAG. In line 1: 1kb+ DNA ladder; line2: *Xanthomonadacea* SE_I021, line3: *Pseudomonas sp.* SE_I022, line 4: SE_I024, line5: SE_I012, line6: *Pseudomonas sp.* SE_I014, line7: *Stenotrophomonas sp*. SE_I015, line8: *Enterobacter sp.* SE_I016, line9: *Xanthomonadacea* SE_I017, line10: *Xanthomonadacea* SE_I018, line11: *Enterobacter sp.* SE_I019, line12: *Xanthomonadacea* SE_I007, line13: *Enterobacter sp.* SE_I037, line14: *Enterobacter sp.* SE_I038, line15: *Xanthomonadacea* SE_I039

6.3.2.2 16S rDNA sequence analysis

The cultivable isolates were characterized by comparative sequence analysis of 16S rDNA generated by PCR, obtaining a determination of approximately 1468 nucleotide positions upstream from 5'- terminus.

Bacteria of the *Gammaproteobacteria* group (Ludwig and Klenk, 2001) dominated the collection of isolates in the first two experiments. During the first experiment (SE_I), in fact, the *Pseudomonas sp.* represented 41.7%, *Enterobacter sp.* 8.3%, family *Xanthomonadacea* 16.7% and *Stenotrophomonas sp.*, that belongs to the family of *Xanthomonadaceae*, 8%. In the second experiment (SE_II) there were 63.6% of *Pseudomonas sp.*, 9.1% of *Enterobacter sp.*, 9.1% of *Stenotrophomonas sp.* and a low percentage of gram positives represented by 4.5% of *Sanguibacter sp*. Finally in the third experiment (SE_III) there 7.6% of *Pseudomonas sp. Were found*, together with 15% of *Enterobacter sp.*, and 7.6% belonging to the family *Xanthomonadacea,*

the *Sanguibacter sp.,* gram-positive, increased to 15.4%. It seems there occurred an increment in the *Pseudomonas sp.* population between the first and the second experiment followed by a decrease at the third sampling time. It has to be mentioned, anyway, that there remains a percentage of indeterminated strains, 25% in SE_I, 9.1% in SE_II and 53% in SE_III, due to difficulties in their maintenance on media and in their DNA extraction. It is interesting to notice an opposite trend for *Sanguibacter sp.* that did not show up in SE_I, while it increased from 4.5% in SE_II to 15.4% in SE_III. This confirms an evolution in the composition of the endophytic consortium in tobacco seeds as already suggested by the changes in MIC values (table 6.2) and by the decrement of the CFU's/seed.

We decided to build a Neighbour Joining tree using the 16S rDNA sequences belonging to the strains isolated both from the different plant parts (table 3.24) and from the seeds (table 6.1). The aim was to see how close the species colonizing the different plant parts were. The *Gammaproteobacteria* group, including *Pseudomonaceae*, *Enterobacteriaceae* and *Xanthomonadaceae* (represented by *Stenotrophomonas maltophilia*) turned out, as could be expected, to be well separated from the *Betaproteobacteriaceae* that includes *Variovorax sp*., and from the gram positives (figure 6.2). It was interesting to notice that the *Pseudomonas sp*. isolated from the seeds are well separated from the other *Pseudomonas sp*. isolated from the other plant parts. The species isolated from the seeds is presumably *Pseudomonas fulva* according to the close relation to AY741159, a *Pseudomonas fulva* 16S rDNA sequence found in PubMed. This could suggest that the origin of this strain is different from the ones isolated from the rhizosphere and the other plant parts, and that it probably originates from outside the plant, for example from the reproductive organs where the bacteria could have lived before the genesis of the seed. An isolation of the cultivable bacteria growing upon the reproductive organs should be planned in order to prove this hypothesis. On the contrary, the *Stenotrophomonas maltophilia* isolated from the seeds falls perfectly in a big group of other *Stenotrophomonas maltophilia* strains isolated mainly from the rhizosphere of tobacco plants grown on the Rafz site. This could indicate that this strain originated from the rhizosphere of *Nicotiana tabacum*, and colonized the seeds via vascular connections. This should confirm the second hypothesized

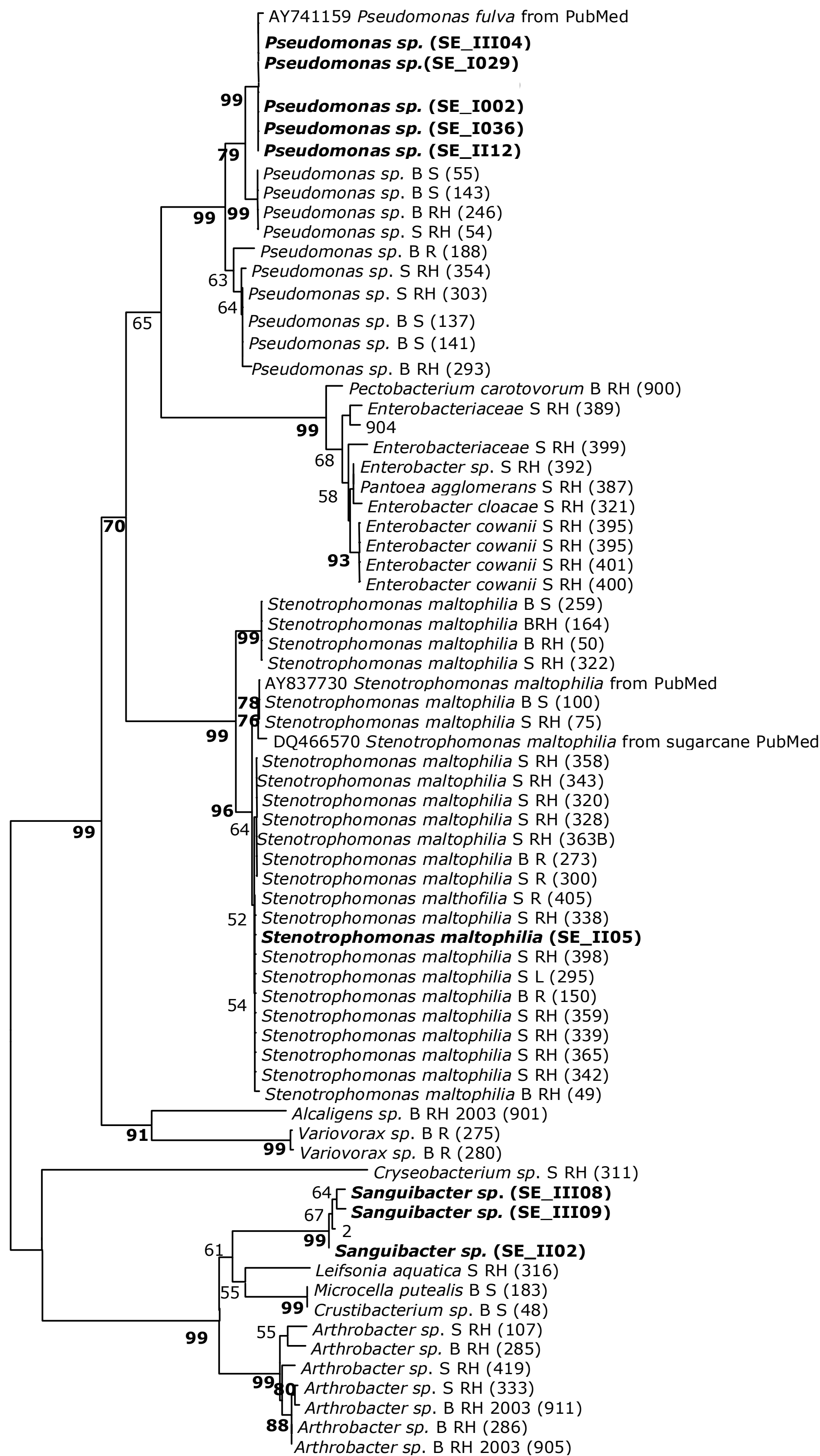
origin of endophytic bacteria inside the seeds (see above). *Stenotrophomonas maltophilia* forms, at least, four different clasta including, in one of them, the strain AY837730 (recovered in PubMed) belonging to a mosquito midgut and a second, the strain DQ466570 (recovered in PubMed), isolated from sugarcane that shows relation with other two strains isolated by us from tobacco plant.

6.4 Conclusions

Taking together the results of phenotypic and genotypic characterizations a general differentiation was noticed mainly in MIC values while the carbon sources used by the identified different genotypic groups were mostly the same (table 6.1). Different results for cadmium tolerance were observed in strains presenting similar BOX PCR profile as observed in *Pseudomonas sp*. SE_I008, SE_I009, SE_II13, SE_II14 and SE_II15; the MIC values ranged from 0.3mM to 1.05mM with the highest tolerances associated to the strains isolated during the first experiment (SE_I008 and SE_I009, table 6.1). No difference in cadmium tolerance range was observed in case of *Pseudomonas sp*., SE_II07, SE_II08 and SE_III04, where it remained between 0.3 and 0.6 mM without any apparent relation with the storage time; in fact, the two extreme values were belonging to consortia isolated during the same experiment.
In literature we couldn't find any reference to metal tolerance values concerning endophytic bacteria of seeds. Our tobacco seeds possess a high percentage of cadmium tolerant strains into the consortia, especially during the first period of storage. This result could be related to the seeds origin, collected from plants growing on a soil containing increased metal concentrations. The fact that only cadmium tolerance was occurring could be due to a higher toxicity of this non essential trace element compared with zinc. Differences in metal tolerance could be expected after comparison with seeds from the same plant grown under less toxic conditions, but further studies should be done.
Analysing the number of cultivable bacteria inside the seeds, our results confirm what was already found by others researchers: the numbers of cultivable strains decreased with increasing storage time (Cankar et a., 2005, Bacon and Hinton, 1996). Observing the families constituting the consortium of endophytic bacteria we isolated from the tobacco seeds, there are similarities with the ones already

found in other plant species seeds, for example in coffee by Vega *et al.*, (2005) where also *Pseudomonas sp*. and *Stenotrophomonas sp*. were found as part of the consortium. Similar bacterial taxa were also found in the rhizosphere and in other tobacco plant parts grown in Rafz and Lommel (Chapter 3, table 3.24). These results, added to the Neighbour Joining tree result, confirm the two main bacterial colonization routes hypothesised so far, one from the rhizosphere and the second from the reproductive organs (Barak *et al.*, 2002). The colonization via used depends, most probably, on the strain species. It has been noticed that the plant may stop *X. campestris* pv. v*itians* colonization, a pathogen, at the seed surface (Barak *et al*., 2002); there seems to exist a kind of code or a group of characteristics that a strain has to possess in order to be able to penetrate the seed coat. Re-inoculation of plants using the strains isolated from the seeds should be performed in order to study their interaction with the plant and the eventual effects on plants exposed to metal stress. More studies need to be addressed to the interaction between endophytic bacteria from seeds and the soil bacteria as well, in order to study their competition capabilities. This could be done, for example, constructing derivatives of these seed endophytic strains expressing the green fluorescent protein (*gfp*) (Germaine *et al.*, 2003). Sawing the seeds inoculated with these new strains directly in soils, it could be possible to visualize the colonization of the host plant using confocal laser-scanning microscopy. It could also be interesting to investigate if any horizontal gene transfer occurs from the seed endophytic bacteria and the bacteria living in the soil so to increase the plant colonization success by the beneficial strains or to increase the bacteria metal tolerance.

figure 6.2 (Below): Neighbour Joining tree built using the 16SrDNA sequence belonging to the strains isolated from different plant parts (table 3.24) and a part of strains isolated from the seeds (table 6.1, in bold in this figure). AY741159, AY837730 and DQ466570 are 16S rDNA sequences respectively of *Pseudomonas fulva* and of two different strains of *Stenotrophomonas maltophilia* recovered in PubMed and used as outgroup. The numbers in brackets are the same codes recoverable in table 3.24 where more data referred to these strains are reported. Bootstrap values are shown above branches when > 50%.



AY741159 Pseudomonas fulva from PubMed
Pseudomonas sp. (SE_III04)
Pseudomonas sp.(SE_I029)
Pseudomonas sp. (SE_I002)
Pseudomonas sp. (SE_I036)
Pseudomonas sp. (SE_II12)
Pseudomonas sp. B S (55)
Pseudomonas sp. B S (143)
Pseudomonas sp. B RH (246)
Pseudomonas sp. S RH (54)
Pseudomonas sp. B R (188)
Pseudomonas sp. S RH (354)
Pseudomonas sp. S RH (303)
Pseudomonas sp. B S (137)
Pseudomonas sp. B S (141)
Pseudomonas sp. B RH (293)
Pectobacterium carotovorum B RH (900)
Enterobacteriaceae S RH (389)
904
Enterobacteriaceae S RH (399)
Enterobacter sp. S RH (392)
Pantoea agglomerans S RH (387)
Enterobacter cloacae S RH (321)
Enterobacter cowanii S RH (395)
Enterobacter cowanii S RH (395)
Enterobacter cowanii S RH (401)
Enterobacter cowanii S RH (400)
Stenotrophomonas maltophilia B S (259)
Stenotrophomonas maltophilia BRH (164)
Stenotrophomonas maltophilia B RH (50)
Stenotrophomonas maltophilia S RH (322)
AY837730 Stenotrophomonas maltophilia from PubMed
Stenotrophomonas maltophilia B S (100)
Stenotrophomonas maltophilia S RH (75)
DQ466570 Stenotrophomonas maltophilia from sugarcane PubMed
Stenotrophomonas maltophilia S RH (358)
Stenotrophomonas maltophilia S RH (343)
Stenotrophomonas maltophilia S RH (320)
Stenotrophomonas maltophilia S RH (328)
Stenotrophomonas maltophilia S RH (363B)
Stenotrophomonas maltophilia B R (273)
Stenotrophomonas maltophilia S R (300)
Stenotrophomonas malthofilia S R (405)
Stenotrophomonas maltophilia S RH (338)
Stenotrophomonas maltophilia (SE_II05)
Stenotrophomonas maltophilia S RH (398)
Stenotrophomonas maltophilia S L (295)
Stenotrophomonas maltophilia B R (150)
Stenotrophomonas maltophilia S RH (359)
Stenotrophomonas maltophilia S RH (339)
Stenotrophomonas maltophilia S RH (365)
Stenotrophomonas maltophilia S RH (342)
Stenotrophomonas maltophilia B RH (49)
Alcaligens sp. B RH 2003 (901)
Variovorax sp. B R (275)
Variovorax sp. B R (280)
Cryseobacterium sp. S RH (311)
Sanguibacter sp. (SE_III08)
Sanguibacter sp. (SE_III09)
2
Sanguibacter sp. (SE_II02)
Leifsonia aquatica S RH (316)
Microcella putealis B S (183)
Crustibacterium sp. B S (48)
Arthrobacter sp. S RH (107)
Arthrobacter sp. B RH (285)
Arthrobacter sp. S RH (419)
Arthrobacter sp. S RH (333)
Arthrobacter sp. B RH 2003 (911)
Arthrobacter sp. B RH (286)
Arthrobacter sp. B RH 2003 (905)
0,05

Chapter 7. Beneficial effects of endophytic bacteria present in *Nicotiana tabacum* seeds on cadmium phytotoxicity

Abstract

Little is known about the influence of seed endophytic bacteria on plant development and even less on their interaction with plants under conditions of metal toxicity, though these bacteria seem to have a close association with the host pant. To investigate this close relationship, we isolated endophytic bacteria from surface sterilized *Nicotiana tabacum* seeds, collected from plants grown under heavy metal stress caused by cadmium and zinc contamination. Sterile tobacco plants were inoculated with either the whole microbial consortium, composed of cultivable and non-cultivable strains, or single strains or defined consortia of the most representative cultivable strains. We subsequently explored the role of these endophytes in plant nutrient uptake both in presence and absence of cadmium. Cadmium was preferred to zinc as many of the isolated strains showed a high level of cadmium tolerance. Our results showed that seed endophytes had a general positive effect on plant growth, as was illustrated by an increase in biomass production under normal conditions. In many cases, the endophytes lowered cadmium phytotoxic, which resulted in an improved biomass production of the plants under metal stress. In addition, inoculated plants showed a higher cadmium concentration and cadmium total content compared with non inoculated plants. This demonstrates the beneficial effects of seed endophytes and opens practical applications to use inoculated seeds as a vector for plant beneficial bacteria.

7.1 Introduction

Rhizobacteria are the most studied plant-associated bacteria and are often found to have beneficial effects on plant growth e.g. via inhibited colonisation by pathogenic microorganisms or by helping the plant to overcome stress responses to environmental insults (Hallmann *et al.*, 1997). For their practical application, plant growth-promoting bacteria are frequently applied on seeds. Interestingly, not much research has been documented about plant beneficial

bacteria isolated from plant seeds. Cultivable bacteria have been isolated from the seeds of coffee (Vega *et al.*, 2005) Norwey spruce (Cankar *et al.*, 2005), rice (*Oryza sativa*) (Tripathi *et al.*, 2006) and previously from *Brassica napus* (Granér *et al.*, 2003). However, not much is known about their ecological function. Some of these bacteria were demonstrated to have anti-fungal activity (Mukhopadhyay *et al.*, 1996), but on the other hand pathogenic bacteria may also inhabit the seeds (Schaad *et al.*, 1995 and Grum *et al.*, 1998). For example, infection of carrot seed by *Xanthomonas campestris* pv. *carotae* (Kuan *et al*., 1985) involves the bacterium gaining access to an internal part of the seed, for example the embryo, as was also reported for *X. campestris* pv. *Manihotis* (Elango & Lozano, 1980); *Erwinia stewartii* targets the endosperm (Rand & Cash, 1921), while *X. campestris* pv. *malvacearum* entered the seed coat (Brinkerhoff & Hunter, 1963). Barak *et al.* (2002) infected lettuce plants with *X. campestris* pv. *Vitians* which causes bacterial leaf spot. They concluded that the pathogen had the capacity to enter and translocate within the vascular system of lettuce plants without inducing visible disease symptoms. Seed produced from diseased lettuce plants were contaminated at a level of *c*. 2%, but internally the seeds were not infected. In this case it seems that the pathogen was stopped at the seed surface. This could suggest a kind of communication between bacteria and plant host. Bacteria cell to cell or bacteria host communication was hypothesised by Espinoza-Urgel *et al.* (2004) when they restored the seed adhesion capacity of *Pseudomonas putida* KT2440 by mutating the *ddcA* of this strain, which codes for a putative membrane polypeptide. Expression of *ddcA* revealed to be dependent on cell density, on the addition to a conditioned medium and on seeds exudates, suggesting the existence of a quorum-sensing system in this strain.

Cankar *et al.* (2005) isolated from surface sterilized seeds of Norway spruce strains belonging to the genera *Pseudomonas* and *Rahnella*. Both of them represent well known genera of plant associated bacteria with growth-promoting properties and biological control potential. They hypothesised that the strains present inside the seeds could serve as vector for transmission of beneficial bacteria. Following this hypothesis, we tested if endophytic bacteria found in seeds of tobacco could have a beneficial effect on their host plants, especially

when grown in stressed environments with heavy metals present. More specifically, we examined the effects of the endophytes on 1) in plant growth and nutrients uptake, and 2) on plant-metal interactions.

7.2 Materials and methods

7.2.1 Cultivation of plants

Seeds of the *Nicotiana tabacum* mother clone BAG were completely sterilized. This result was obtained after several sterilization tests using different sterilizing solutions and different procedures (data not showed). This step was crucial as we wanted to have no cultivable bacteria inside the plant used in order to follow the inoculum used and to be able to reduce the interaction between the inocula and the endogenous seed bacteria . At the same time, the sterilization should not affect the seed embryo. The seed endophytic bacteria seem to inhabit mainly the endosperm and this allowed to complete sterilization of the seeds without damaging the plant embryo. In the sterilization protocol used the seeds were submerged for 30s in ethanol 70%, after which they were rinsed once in sterile Millipore water during 30 seconds. Subsequently, the seeds were placed during 35 minutes in $HOCl^-$ 16Volumes, after which the seeds were rinsed three times for 10 min in sterile Millipore water and dried using sterile filter paper. The seeds' sterility was checked by incubating some of them for 3 days at 30°C on ten times diluted 869 medium (Mergeay *et al.*, 1985). Seeds were considered sterile when no bacterial growth was observed after seed germination.

The seeds were incubated on a Petri dish containing MSM0 medium (Sigma) in the plant growth chamber during 3 days in the dark followed by 2 days at normal growth chamber conditions (constant temperature of 25°C (day) - 17°C (night), relative humidity of 60-65% and a 12 h (day) photoperiod provided by Philips TDL 58WT33 fluorescent tubes (photosynthetic active radiation 160 µmol m^{-2} s^{-1})). Germinated seeds were moved into sterile Magenta (Sigma) containing 100ml of MSM0 medium (pH 5.6) supplemented with 5.5g agar per litre. Depending on the test conditions, 10µM $CdCl_2$ was added to the medium, and/or 56.6µl of inoculums was sterilely plated on the surface of the solidified medium. All the plants were kept during 2 weeks under complete sterility in the growth

chamber; after this period the covers were removed and replaced with a sterile tissue that allows gas exchange. The plants were left to grow during more 2 weeks before being harvested. One plant for each combination of inoculum and/or Cd-challenge was used to check for the survival of the inoculum in the plant, three plants were used to analyse plant heavy metals uptake while all plants were used to analyse biomass production.

7.2.2 Surface seeds sterilization

The seeds were surface sterilized using a solution of 1% active chloride supplemented with 1 droplet Tween 80 per 100ml of solution during 30 minutes, and rinsing 3 times with sterile Millipore water during 10 minutes each time. To check the surface sterility, the seeds were rolled on rich medium 869 diluted ten times and subsequently removed. To test for the present of the seed endophytes, the same seeds were put into a sterile mortar and squeezed into a sterile solution of 10mM $MgSO_4$. Samples dilutions were plated on ten times diluted 869 medium and have been checked after 7 days incubation at 30°C.

7.2.3 Preparation of bacterial inoculums

We are aware of the fact that the cultivable bacteria present in the environment are only around the 1% of the total bacteria existing (Amann *et al.*, 1995), for this reason, we also inoculated the entire consortium including the uncultivable bacteria. This approach should give us ideas about the general effect of the bacteria, that inhabit the seeds and presumably the first stage of the next plant generation, on plant growth in the presence and absence of cadmium stress.

The bacteria used for the re-inoculations were isolated from seeds of *Nicotiana tabacum* grown in Rafz during a phytoextractive field trial in the summer of 2004. The seeds belong to the ones described in chapter 6. The most important characteristics of these bacteria and their inoculum concentration are given in table 7.2. For the inoculations, pure strains, combinations of the most represented cultivable strains (SE_Ia, SE_Ib, SE_Ic, SE_IIa and SE_IIb) and the entire consortium (SE_II), containing the non-cultivable bacteria as well, were used. In table 7.1 are the main characteristics of the bacteria constituting the combinations of bacteria and of the cultivable bacteria belonging to SE_II. In

case of SE_II, the cultivable bacteria were identified, but we didn't identify the non cultivable ones (table 6.1).

The different strains were grown at 30°C on a rotary shaker in ten times diluted 869 medium (Mergeay *et al.*, 1985), containing per litre distilled water: 1g tryptone, 0.5g yeast extract, 0.5g NaCl, 0.1g D-glucose, 0,0345g $CaCl_2*2H_2O$ (pH 7). Cells were harvested after approximately 12 hours at a density of 10^{+8} CFU/ml (OD_{660} of 0,5). The cells were collected by centrifugation, washed twice in 10mM $MgSO_4$ and suspended in 1/10 of the original volume.

Defined consortia were artificially created by mixing equal volumes of single strains cultures.

The solution taken directly from the seeds, SE_II, was obtained by squeezing 35 surface sterilized BAG seeds in 2 ml of $MgSO_4$. The suspension was used immediately without knowing in advance its bacterial composition, as we wanted to minimize changes in the community comprised of both cultivable and non-cultivable species. Later on we determined the cultivable bacteria present in the seeds after inoculation. Dilutions of all the inocula were plated on ten times diluted 869 medium and allowed to grow for 7 days incubation at 30°C in order to determined the CFU/ml inoculum.

Strain n°	Isolated from	Sequence result	mM Cd	mM Zn	Sid. prod.	Inoculum concentration
SE_Ia	From SE_I037 to SE_I041	table 7.1	table 7.1	table 7.1	table 7.1	$2.2x10^7$
SE_Ib	From SE_I025 to SE_I036	table 7.1	table 7.1	table 7.1	table 7.1	$3.2x10^7$
SE_Ic	From SE_I042 to SE_I045	table 7.1	table 7.1	table 7.1	table 7.1	$6.7x10^7$
SE_II entire consortium (cultivable and uncultivable strains)	SE_II	table 6.1	table 6.1	table 6.1	table 6.1	$2x10^2$ (only the cultivable)
SE_II17	SE_II	*Enterobacter sp.*	0.45	0.25	-	$2.3x10^8$
SE_II02	SE_II	*Sanguibacter sp.*	0	0	N.G.	$3x10^8$
SE_II13	SE_II	*Pseudomonas sp.*	0.45	0	-	$5x10^7$
SE_II12	SE_II	*Pseudomonas sp.*	1.5	0	N.G.	$7x10^8$
SE_IIa						
	SE_II02	*Sanguibacter sp.*				$1.4x10^8$
	SE_II17	*Enterobacter sp.*				$3x10^7$
	SE_II13	*Pseudomonas sp.*				$5x10^7$
SE_IIb						
	SE_II02	*Sanguibacter sp.*				$1.4x10^8$
	SE_II17	*Enterobacter sp.*				$3x10^7$
	SE_II12	*Pseudomonas sp.*				$7x10^8$
	SE_II13	*Pseudomonas sp.*				$5x10^7$

table 7.2: Characteristics of bacteria used as inoculums. SE_II refers to the whole consortium comprising of both cultivable and uncultivable bacteria (table 6.1). The Minimum Inhibiting Concentrations (M.I.C) values for Cd and Zn (mM) are reported. The last column presents the different inocula concentrations as were used in the inoculation experiments.

7.2.4 Biomass production

The plant material was collected, keeping the shoots and the roots of the same plant separated, and vigorously washed with deionised water. The roots were washed with 10mM $Pb(NO_3)_2$ at 4°C for 10 min in order to remove the adhering metals and rinsed three times with distilled water. Plants fresh weight was determined on an analytical balance (Mettler H54). The dry weight was obtained after drying the shoots and roots during 48 hours at 60°C.

7.2.5 Inoculum recovery

Plants were harvested after 4 weeks of growth; leaf, stem, root and rhizosphere samples were kept separately. The plant was removed under sterile conditions out of the Magenta and put into a sterile Petri dish after removing the excess of growth medium. Five ml of 10mM $MgSO_4$ was added to root material, and 100µl of this solution and its subsequent dilutions were plated to check the inoculum's survival in strict contact with the root system, considering it as rhizosphere. The biggest leaf was sterilely cut from the plant and surface sterilized using 0.1% active chloride supplemented with 1 droplet of Tween 80 per 100ml solution during 2 minutes, and rinsed 3 times with sterile Millipore water. The leaf was dried using sterile filter paper. In order to verify the efficiency of the sterilization, a 100µl sample of the third rinsing water was plated on 10 times diluted 869 medium. The same procedure was used for stem and root with a 0.5 % concentration of active chloride and a sterilization time of 1 minute for the stem and 2 minutes for the roots. After sterilization, the different plant parts were macerated into 10mM $MgSO_4$ using a sterile mortar. Samples (100µl) and their dilutions were plated on 10-times diluted 869 medium and checked after 7 days growth at 30°C.

7.2.6 Plant metal analyses

Dry plant material, shoot and root, were separately homogenized and mineralised by wet ashing with a mixture of concentrated HNO_3 and $HClO_4$ (5:2

v/v). Zn, Cd, Fe content were determined by means of Inductively Coupled Plasma Optical Emission Spectroscopy (I.C.P.-O.E.S).

7.2.7 Statistical analysis

All treatments were performed in a minimum of three replicas. The significance of differences was analysed by one-way and factorial ANOVA followed by or Tukey test or HSD-Tukey test for post-hoc comparisons between unequal samples performed with Statistica 6 (StatSoft, 2003). Using the same statistical program, in case of not normal distribution a Mann-Whitney U test was used. The data referred to concentration, ratios and metals content were previously transformed using the arctangent function in order to delete the effect of no pure measure units.

Other program used for the analysis of data is the SigmaPlot 8.0 (SPSS, Chicago, IL).

7.3 Results

7.3.1 Recovery of inoculated bacteria

After four weeks of growth, the plants treated and untreated with $CdCl_2$ (10µM final concentration in the growth medium) and/or inoculated as described in table 7.1, were harvested. Colonization by the inoculated bacteria was occurring (table 7.3). SE_Ia and SE_Ib showed the best colonization of tobacco, including the leaves, SE_II showed the highest increment, being inoculated with a concentration of 10^2 while found to be present in the rhizosphere, at the end of the plants growth period, at a concentration of 10^8 – 10^9 cfu/µl. It is interesting to notice that SE_Ic, made of two different species of *Pseudomonas sp.* isolated from SE_I (table 7.1), is found back in non Cd-treated plants in stem and in lower concentration in roots while it is absent in rhizosphere. On the contrary in cadmium treated plants, the bacteria fall down to non detectable concentrations inside the plants while seems to be at ease in the rhizosphere (table 7.3). *Pseudomonas sp*. is known to be, in general, a rhizosphere colonizer as already explained in chapter 5. It is known that plant under metals stress secrete more

organics acids into the rhizosphere (Jones, 1998) and this could be the reason of such a preference in our case.

Interestingly *Pseudomonas sp*., SE_II13, showed a different compartmentalization; this strain is abundant in the rhizosphere of cadmium treated and untreated plants and even present inside the leaves. In case of SE_Ic, it is still evident the preference for the interior of untreated plants, where the CFU's/g are always higher with a preference for the stem.

As the others *Pseudomonas sp*., strain SE_II12 inhabits mostly the stems and the rhizosphere.

So, although the strains tested were initially isolated as seed endophytes, they colonize different plant parts, suggesting that they are transferred to the future plants through seeds and subsequently colonize specific plant parts and the rhizosphere.

Seq. ID	Code	869	284+ C	284-C	284+ SC	284+ GS	284+ GN	284+ FR	Zn (mM)	Cd (mM)	Sid.	Similar to
Pseudomonas sp.	SE_I025	+	+	-	+	-	-	+	<0.25	0.6	N.G.	N
Pseudomonas sp.	SE_I026	+	+	-	+	+	+	+	<0.25	0.6	N.G.	N
Pseudomonas sp.	SE_I027	+	+	-	+	+	+	+	<0.25	0.6	N.G.	N
Pseudomonas sp.	SE_I028	+	+	-	+	+	+	+	<0.25	0.6	-	O
Pseudomonas sp.	SE_I029	+	+	-	+	+	+	+	<0.25	0.6	-	O
Pseudomonas sp.	SE_I030	+	+	-	+	+	+	+	<0.25	0.75	-	O
Enterobacter sp.	SE_I031	+	+	-	-	-	-	-	<0.25	0.15	N.G.	R
Enterobacter sp.	SE_I032	+	+	-	+/-	-	-	-	<0.25	0.6	N.G.	R
Pseudomonas sp	SE_I033	+	+	-	+/-	+	+/-	+	<0.25	0.3	-	O
Pseudomonas sp	SE_I034	+	+	-	+/-	+	+/-	+	<0.25	0.45	-	O
Pseudomonas sp	SE_I035	+	+	-	+	-	-	+	<0.25	0.6	N.G.	F (table 6.1)
Pseudomonas fulva	SE_I036	+	+	-	+	+	+	+/-	<0.25	0.9	N.G.	
Enterobacter sp.	SE_I037	+	+	-	-	+/-	+/-	-	<0.25	<0.15	N.G.	I (see table 6.1)
Enterobacter sp.	SE_I038	+	+	-	-	-	-	-	<0.25	0.15	N.G.	I (see table 6.1)
Xanthomonadaceae	SE_I039	+	+	-	+/-	+	+	+	<0.25	<0.15	N.G.	B (see table 6.1)
	SE_I040	+	+/-	-	-	+/-	+/-	-	<0.25	<0.15	N.G.	
	SE_I041	+	+	-	-	+	+/-	+/-	2	0.6	N.G.	Q (see table 6.1)

Seq. ID	Code	869	284+ C	284- C	284+ SC	284+ GS	284+ GN	284+ FR	Zn (mM)	Cd (mM)	Sid.	Similar to
Pseudomonas sp	SE_I042	+	0	-	+	+	+	+	<0.25	0.75	N.G.	N
Pseudomonas sp	SE_I043	+	0	-	+/-	+	+/-	+	<0.25	0.6	-	O
Pseudomonas sp	SE_I044	+	0	-	+/-	+	+/-	+	<0.25	0.75	+	O
Pseudomonas sp	SE_I045	+	0	-	+/-	+	+/-	+	<0.25	0.75	N.G.	O

table 7.1: Growth media, carbon sources, MIC values and siderophores production characteristics of cultivable bacteria isolated from mixtures of colonies extracted from surface sterilized seeds four (from SE_I001 to SE_I024) and five (from SE_II01 to SE_II22) months after seeds collection from the plants. N.G. is not growth as the strain couldn't grow on the medium tested. In the carbon sources: – where no growth was seen. +/- where only a slight growth was detected, the strain can hardly leave. + the strain well grow on that medium. Letters in the column Similar to refers to the similar BOX PCR fingerprinting, same letter corresponds to similar profile

Inoculums	Leaf	Stem	Root	Rhizosphere
SE_Ia	$2.8x10^3$	$1.1x10^3$	n.d.	$5.8x10^6$
SE_Ia+	$1.9x10^4$	$6x10^3$	$4x10^3$	$2.3x10^7$
SE_Ib	$1.8x10^3$	$1x10^6$	$1.7x10^3$	$3.2x10^7$
SE_Ib+	$6.8x10^3$	n.d.	$3.3x10^2$	$2.1x10^7$
SE_Ic	n.d.	$1.1x10^4$	$8.2x10^2$	n.d.
SE_Ic+	n.d.	n.d.	n.d.	$6x10^4$
SE_II	n.d.	$1.6x10^6$	$4x10^4$	$3.4x10^8$
SE_II+	n.d.	$1.8x10^5$	$1.8x10^5$	$2.4x10^9$
SE_II17	$8.8x10^1$	n.d.	$1.9x10^3$	$2.4x10^4$
SE_II17+	n.d.	$2.2x10^2$	$1.5x10^2$	$1.7x10^3$
SE_II02	n.d.	$3.1x10^4$	n.d.	$4.3x10^1$
SE_II02+	n.d.	$1.1x10^4$	$3.7x10^3$	$2.5x10^5$
SE_II13	$1.6x10^2$	$2.5x10^5$	$8.4x10^3$	$3.7x10^7$
SE_II13+	$8.1x10^1$	n.d.	$9.5x10^1$	$7.2x10^4$
SE_II12	n.d.	$5x10^4$	$4.4x10^4$	$3.8x10^5$
SE_II12+	n.d.	$1x10^4$	$3.6x10^3$	$2.4x10^5$
SEP SE_II02 SE_II17 SE_II13	n.d. n.d. n.d. n.d.	$9.6x10^3$ $6.1x10^3$ $6.6x10^3$ $2.4x10^3$	$5.2x10^3$ n.d. $4.9x10^3$ $2.4x10^2$	$7.6x10^4$ n.d. $7x10^2$ $7.5x10^4$
SEP+ SE_II02+ SE_II17+	n.d. n.d. n.d.	$9.1x10^3$ $3.4x10^2$ $3.1x10^3$	$5.8x10^3$ $7.4x10^2$ $2.4x10^3$	$7.7x10^4$ n.d. $7.1x10^2$
SE_II13+	n.d.	$5.7x10^3$	$2.6x10^3$	$7.6x10^4$
SEPP SE_II02 SE_II17 SE_II12 SE_II13	n.d. n.d. n.d. n.d. n.d.	$1.3x10^4$ $1.2x10^3$ n.d. $1.2x10^4$ *	$6.6x10^3$ $3.5x10^2$ n.d. $6.3x10^3$ *	$5x10^5$ n.d. n.d. $5x10^5$ *
SEPP+ SE_II02+ SE_II17+ SE_II12+ SE_II13+	n.d. n.d. n.d. n.d. n.d.	$6.8x10^2$ n.d. n.d. $6.8x10^2$ *	$2.7x10^2$ n.d. n.d. $2.7x10^2$ *	$1.7x10^5$ n.d. $9.9x10^1$ $1.6x10^5$ *

table 7.3: Inocula recovery. Inocula with + are the ones isolated back from plants grown with 10μM $CdCl_2$. In columns are the plant parts analysed for inocula recovery. n.d. is not detected. * phenotipically too similar to SE_II12 to be distinguished.

7.3.2 Effects of cadmium and/or inocula on plant growth

Growth parameters (roots and shoots dry and fresh weight) were determined in order to evaluate the effects of both cadmium and/or inocula on plant development. The analyses were made on four weeks old plants grown in Magenta pots. Comparing with table 5.2, this time a higher bacterial concentration in general correlates to a higher biomass production (table 7.3), as e.g. is the case for SE_II02 (figure 7.3 and 7.4).

A positive effect on shoot and/or root biomass production was observed mainly after inoculation by consortia; SE_Ia and SE_Ic, for example, in cadmium untreated shoots (figure 7.1A and 7.2A) and cadmium treated and untreated roots in both dry and fresh plants (figure 7.1B and 7.2B); SE_Ic also influences positively the fresh weight of shoots treated plants. Inoculation with the SE_IIa consortium had a positive effect on biomass production in Cd-treated shoots and roots, and SE_IIb on shoots and roots of Cd-treated and untreated plants (figure 7.5A, 7.5B, 7.6A and 7.6B). The consortium SE_II had a positive effect mainly on the root system. It increases the roots weight in the absence of cadmium (figure 7.1B and 7.2B), while in presence of cadmium it slightly decreases the shoots fresh weight without affecting the dry weight (figure 7.1A and 7.2A). For the pure strains inoculated, *Sanguibacter sp*. SE_II02, improved the development of shoots and roots in cadmium treated plants (figure 7.3 and 7.4). SE_II13 and SE_II12 slightly increase shoots fresh weight under cadmium stress. On the other hand, a negative effect was observed after inoculation by SE_Ib that decreased biomass of the shoots of the cadmium treated plants (figure 7.1A and 7.2A). For the other strain no significant difference with non-inoculated plants was observed.

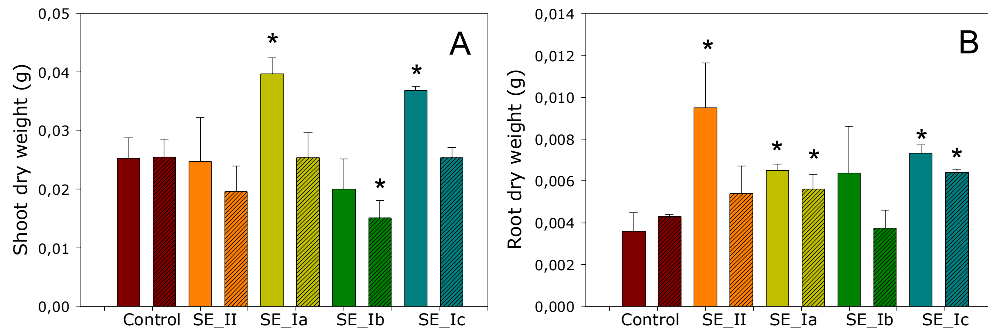


figure 7.1: Biomass production dry weight of shoot (A) and root (B) in control plants without inoculation and plants with different inocula (inocula are specified under each bar). Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are the given of three different biological replicas. The statistical significance of the results, marked by a star on the histogram, was confirmed at the 5% level using a two-way and one-way ANOVA model separately exploring inoculums and presence or absence of cadmium with non-inoculated plants U Mann-Whitney test was performed when ANOVA analyses was not allowed.

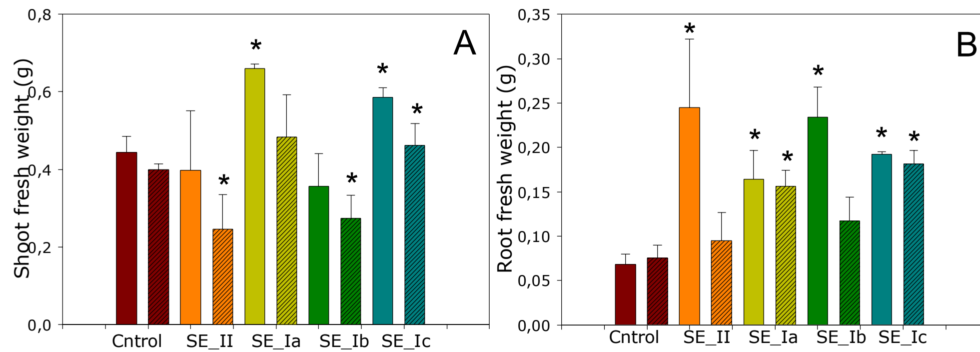


figure 7.2: Biomass production fresh weight of shoot (A) and root (B) in control plants without inoculation and plants with different inocula (inocula are specified under each bar). Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results, marked by a star on the histogram, was confirmed at the 5% level using a two-way and one-way ANOVA model separately exploring inoculums and presence or absence of cadmium with non-inoculated plants U Mann-Whitney test was performed when ANOVA analyses was not allowed.

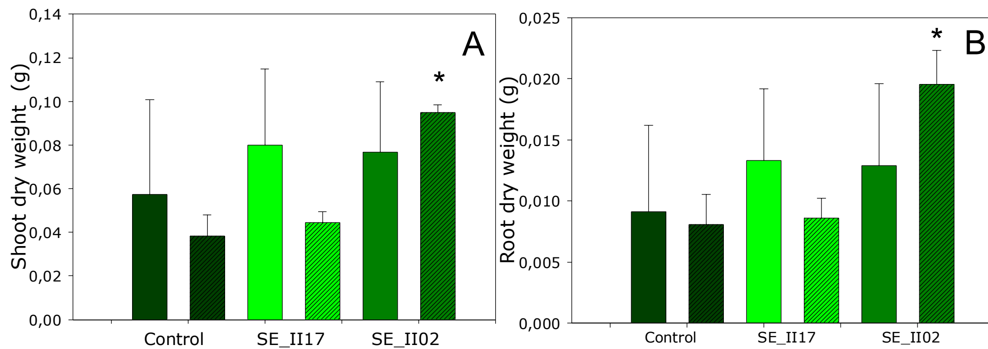


figure 7.3: Biomass production dry weight of shoot (A) and root (B) in control plants without inoculation and plants with different inocula (inocula are specified under each bar). Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results, marked by a star on the histogram, was confirmed at the 5% level using a two-way and one-way ANOVA model separately exploring inoculums and presence or absence of cadmium with non-inoculated plants U Mann-Whitney test was performed when ANOVA analyses was not allowed.

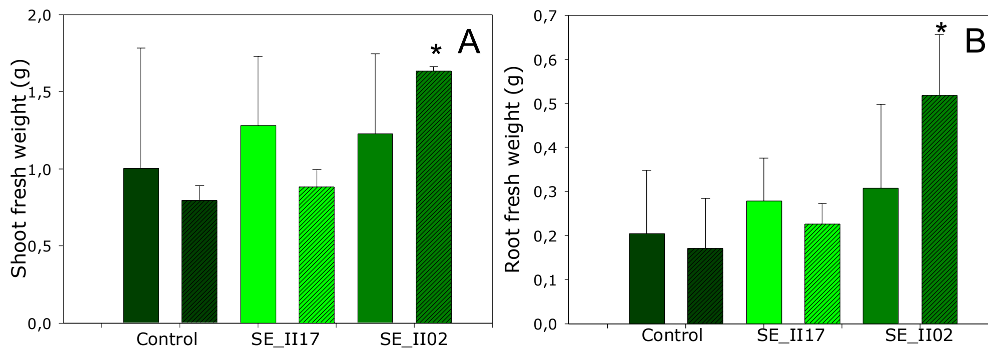


figure 7.4: Biomass production fresh weight of shoot (A) and root (B) in control plants without inoculation and plants with different inocula (inocula are specified under each bar). Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results, marked by a star on the histogram, was confirmed at the 5% level using a two-way and one-way ANOVA model separately exploring inoculums and presence or absence of cadmium with non-inoculated plants U Mann-Whitney test was performed when ANOVA analyses was not allowed.

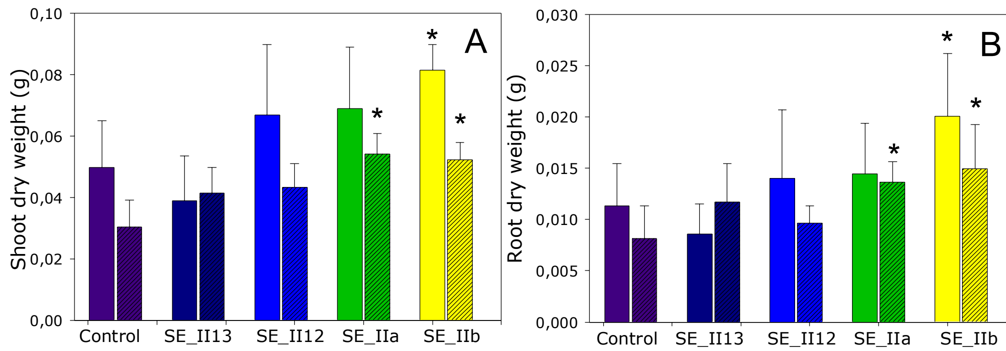


figure 7.5: Biomass production dry weight of shoot (A) and root (B) in control plants without inoculation and plants with different inocula (inocula are specified under each bar). Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results, marked by a star on the histogram, was confirmed at the 5% level using a two-way and one-way ANOVA model separately exploring inoculums and presence or absence of cadmium with non-inoculated plants U Mann-Whitney test was performed when ANOVA analyses was not allowed.

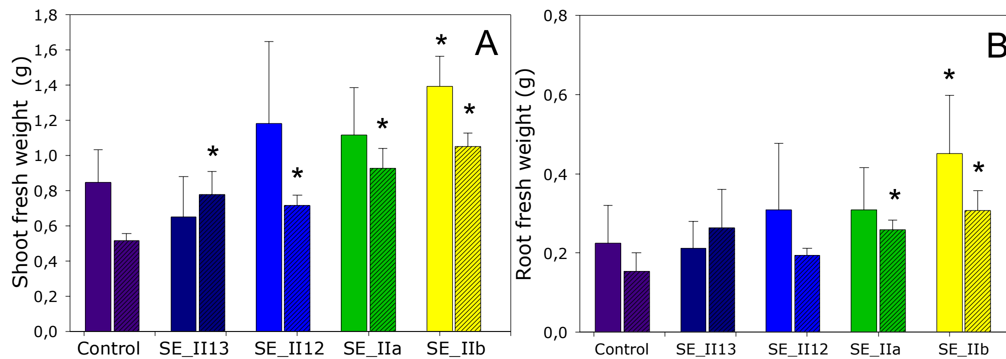


figure 7.6: Biomass production fresh weight of shoot (A) and root (B) in control plants without inoculation and plants with different inocula (inocula are specified under each bar). Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results, marked by a star on the histogram, was confirmed at the 5% level using a two-way and one-way ANOVA model separately exploring inoculums and presence or absence of cadmium with non-inoculated plants U Mann-Whitney test was performed when ANOVA analyses was not allowed.

7.3.3 Plant trace elements uptake

In order to study the influence of the inocula on plant trace elements uptake, cadmium, zinc and iron concentration and content were determined on inoculated plants exposed and non exposed to cadmium. The results were compared with the ones obtained from non-inoculated plants in order to evaluate differences in metals absorption and distribution in organs and tissues.

7.3.3.1 Cadmium

Knowing that cadmium is toxic (paragraph 1.4.4) and analysing the concentration of this metal in the plants, we intended to find a hypothesised relation between biomass production and Cd concentration. Multiplying plant cadmium concentration with the dry weight of roots and shoots the total cadmium content of these plant parts (in micrograms) was calculated. Some clear differences between inoculated and non-inoculated plants were observed (figure 7.7). Inoculation with both the consortia SE_II and SE_Ia (figure 7.7A) lead to increased cadmium concentrations and also total content in the shoots (figure 7.7B). Interestingly, after inoculating with strain *Sanguibacter sp*. SE_II02, that didn't show any cadmium tolerance (table 7.2), a slightly lower cadmium concentration in the shoots, but higher total cadmium content, were found (figure 7.8A and B). On the contrary inoculation with *Pseudomonas sp*. SE_II13, more tolerant to cadmium than SE_II02 (table 7.2), caused a decreased in plant cadmium concentration (figure 7.9A).

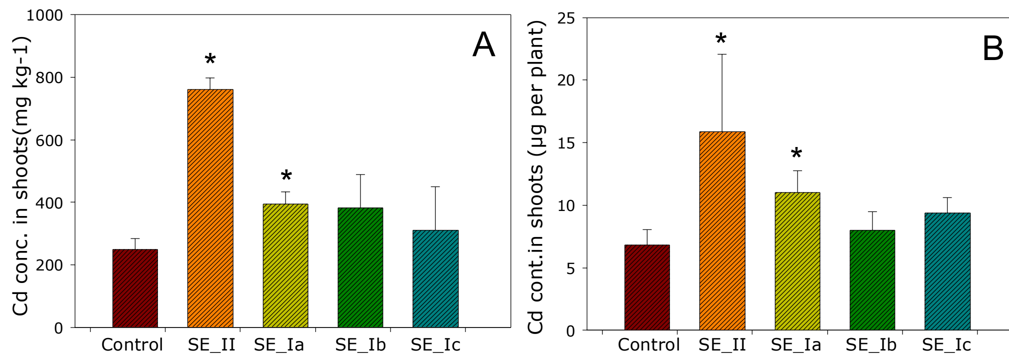


figure 7.7: Cadmium concentration (mg kg^{-1} dry weight) (A) and total cadmium content (µg per plant) (B) in shoots of plants grown in the presence of 10µM cadmium. The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are indicated by a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

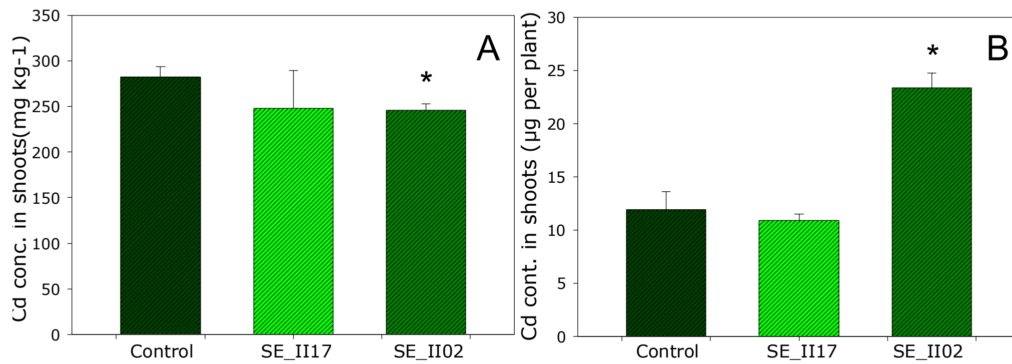


figure 7.8: Cadmium concentration (mg kg^{-1} dry weight) (A) and total cadmium content (µg per plant) (B) in shoots of plants grown in the presence of 10µM cadmium. The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are indicated by a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

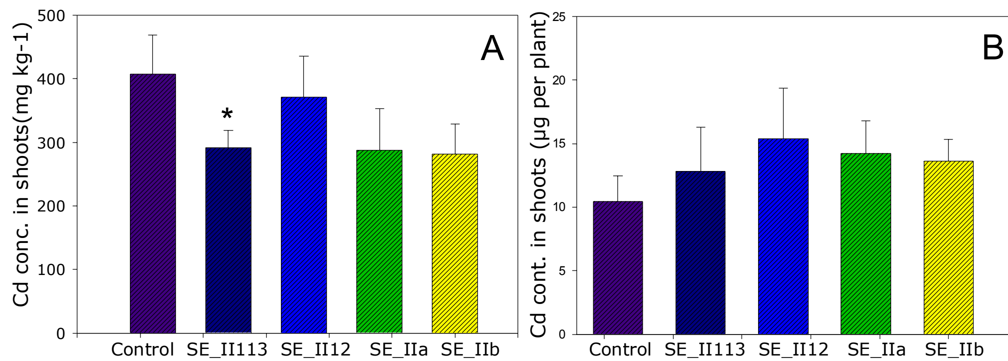


figure 7.9: Cadmium concentration (mg kg^{-1} dry weight) (A) and total cadmium content (µg per plant) (B) in shoots of plants grown in the presence of 10µM cadmium. The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are indicated by a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

Since we initially only focused on the potential effect of inoculation on the metal content in the easily harvestable aerial parts, analysis of the roots was only performed for the last experiments. In the final phase of the work, we widened the objective to all possible effects that the inocula could have on plants growth and elements uptake, thus roots metal content was analyzed as well. Roots of plants inoculated with *Sanguibacter sp*. SE_II02 showed a lower cadmium concentration, while roots of plants inoculated with *Enterobacter sp*. SE_II17 had a lower cadmium concentration and total cadmium content (figure 7.10A and B). *Pseudomonas sp*. SE_II13, and the consortium SE_IIa, lowered the plant root cadmium concentration (figure 7.11A).

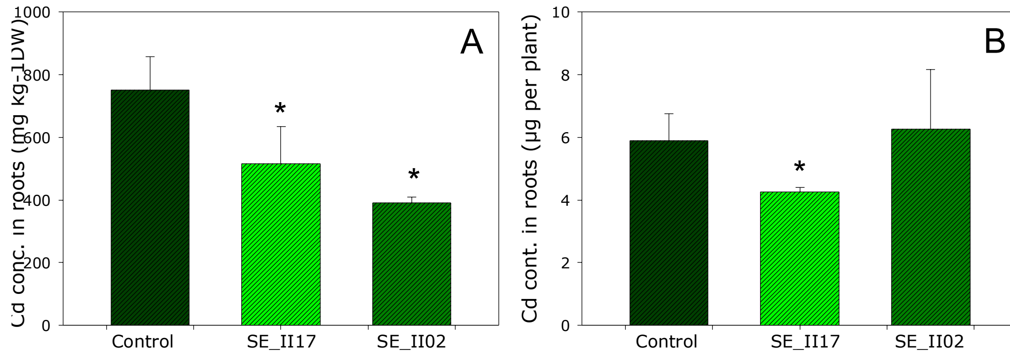


figure 7.10: Cadmium concentration (mg kg^{-1} dry weight) (A) and total cadmium content (µg per plant) in roots (B) of plants grown in the presence of 10µM cadmium. The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are indicated by a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

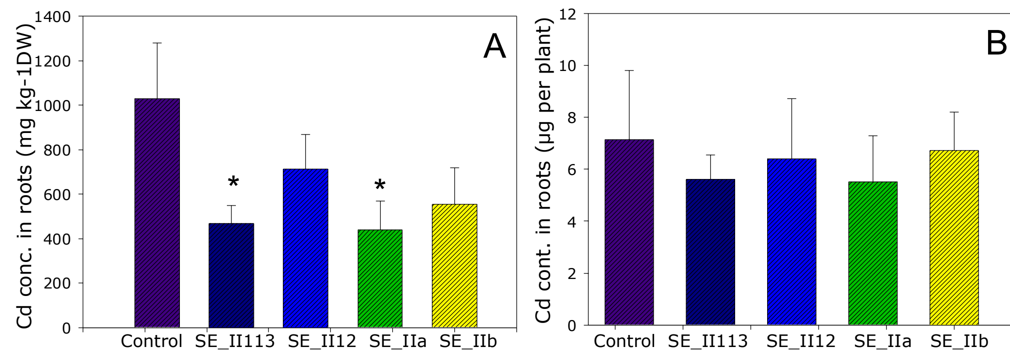


figure 7.11: Cadmium concentration (mg kg^{-1} dry weight) (A) and total cadmium content (µg per plant) in roots (B) of plants grown in the presence of 10µM cadmium. The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are marked by a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

7.3.3.2 Zinc

Zinc in the plant growth medium was always maintained within the optimal concentration range. Nevertheless significant differences in plant zinc concentration were found when comparing inoculated and no-inoculated plants. Shoot zinc concentration is increased in cadmium treated plants after inoculation with the consortia SE_II, SE_Ib and SE_Ic (figure 7.12A) and in non treated plants inoculated, with *Pseudomonas sp*. SE_II13 (figure 7.14A). The zinc concentration decreased in both cadmium treated and untreated plants inoculated with *Sanguibacter sp*. SE_II02 (figure 7.13A) or with consortium SE_IIa (figure 7.14A), while the concentration decreases, but only in non treated plants, when plants were inoculated with *Pseudomonas sp*. SE_II12 and with the consortium SE_IIb (figure 7.14A). Plant zinc content in μg per plant increased in cadmium treated plants inoculated with consortium SE_Ic (figure 7.12B).

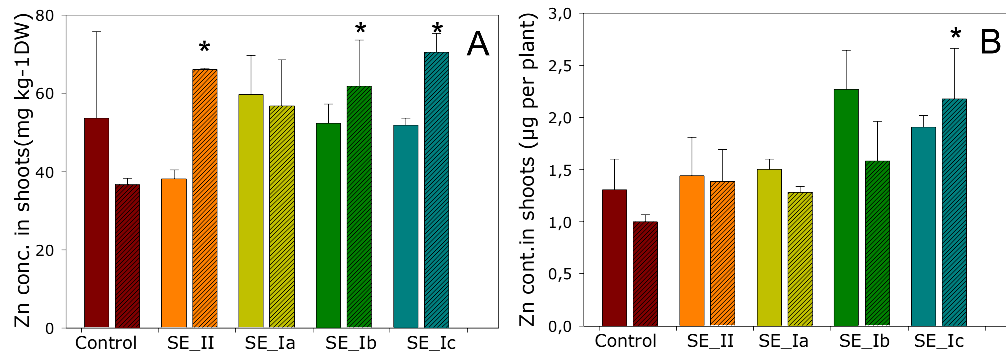


figure 7.12: Zinc concentration (mg kg^{-1} dry weight) (A) and total zinc content (in μg per plant) (B) in shoots. Control are the non inoculated plants, different inocula are specified under each histogram. Histograms with shading refer to the plants grown in presence of 10μM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results was confirmed at the 5% level using one and two-way ANOVA model separately exploring inocula and presence or absence of cadmium, and is indicated by a star on the histogram.

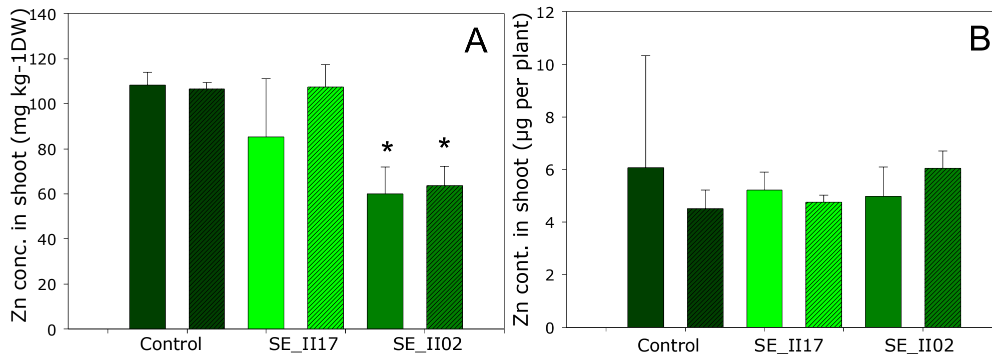


figure 7.13: Zinc concentration (mg kg^{-1} dry weight) (A) and total zinc content (µg per plant) (B) in shoots. Control are the non inoculated plants, different inocula are specified under each histogram. Histograms with shading refer to the plants grown in presence of 10µM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results was confirmed at the 5% level using one and two-way ANOVA model separately exploring inocula and presence or absence of cadmium and is indicated by a star on the histogram.

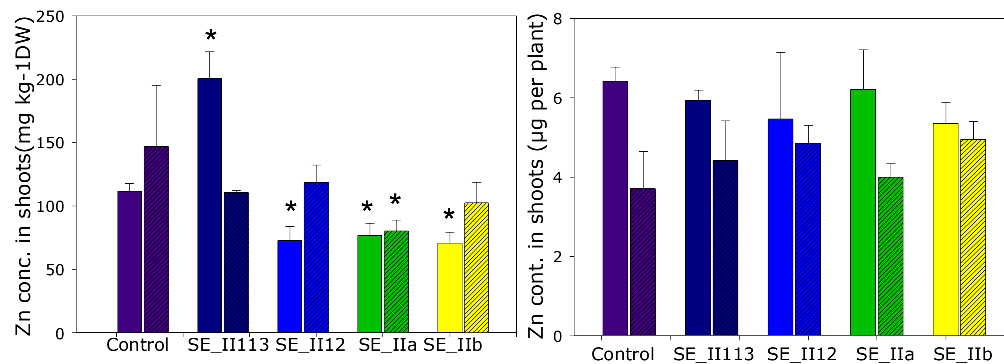


figure 7.14: Zinc concentration (mg kg^{-1} dry weight) (A) and total zinc content (µg per plant) (B) in shoots. Control are the non inoculated plants, different inocula are specified under each histogram. Histograms with shading refer to the plants grown in presence of 10µM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results was confirmed at the 5% level using one and two-way ANOVA model separately exploring inocula and presence or absence of cadmium, and is indicated by a star on the histogram.

In the roots a lower variability was observed: zinc concentration was lower in treated plants inoculated with *Pseudomonas sp*. SE_II13, while in non-treated plants the concentration was lowered after inoculation with the consortia SE_IIa and SE_IIb (figure 7.16A). Zinc content was higher only in treated plants inoculated with *Sanguibacter sp*. SE_II02 (figure 7.15B).

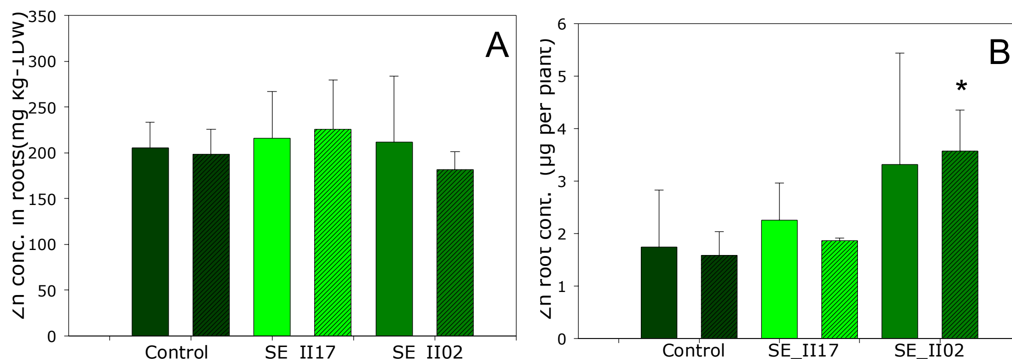


figure 7.15: Zinc concentration (mg kg^{-1} dry weight) (A) and total zinc content (µg per plant) (B) in roots. Control are the non inoculated plants, different inocula are specified under each histogram. Histograms with shading refer to the plants grown in presence of 10µM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results was confirmed at the 5% level using one and two-way ANOVA model separately exploring inocula and presence or absence of cadmium, and is indicated by a star on the histogram.

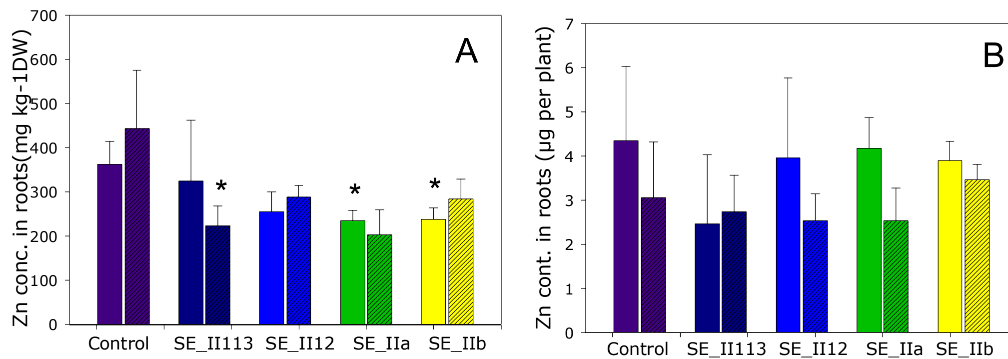


figure 7.16: Zinc concentration (mg kg^{-1} dry weight) (A) and total zinc content (µg per plant) (B) in roots. Controls are the non inoculated plants, different inocula specified under each histogram. Histograms with shading refer to the plants grown in presence of 10µM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results was confirmed at the 5% level using one and two-way ANOVA model separately exploring inocula and presence or absence of cadmium, and is indicated by a star on the histogram.

7.3.3.3 Ratios metal-cadmium

Disturbances in the uptake and distribution of macro and micronutrients in plants are also shown to be correlated with Cd toxicity (Sandalio *et al*., 2001). For example, Cd appears to inhibit Zn uptake by a major class of zinc transporters (Grotz *et al*., 1998). By calculating the ratios between metals, in our case Zn or Fe with Cd, we intend to determine a preferential accumulation of a specific metal in comparison with non inoculated plants. This would point to an effect of the inocula on metal distribution. It is known that the uptake of different nutrients improves the growth of plants on contaminated soils (Belimov *et al.*, 2004, Burd *et al.*, 2000; Verkleij and Schat, 1990). It is therefore relevant not only to determine the plants' metal concentrations, but also if the ratios of essential elements and cadmium change in inoculated plants compared to non-inoculated control plants.

Plants inoculated with the consortium SE_II (figure 7.17A) as well as plants inoculated with *Sanguibacter sp*. SE_II02 (figure 7.18A) showed a lower Zn/Cd ratio compared to non inoculated plants. This means that when the Cd concentration in shoots increased, at least partly, this occurred to detriment of

the Zn concentration. Inoculation with *Sanguibacter sp*. SE_II02 had a similar effect on the Fe/Cd ratio (figure 7.18B).

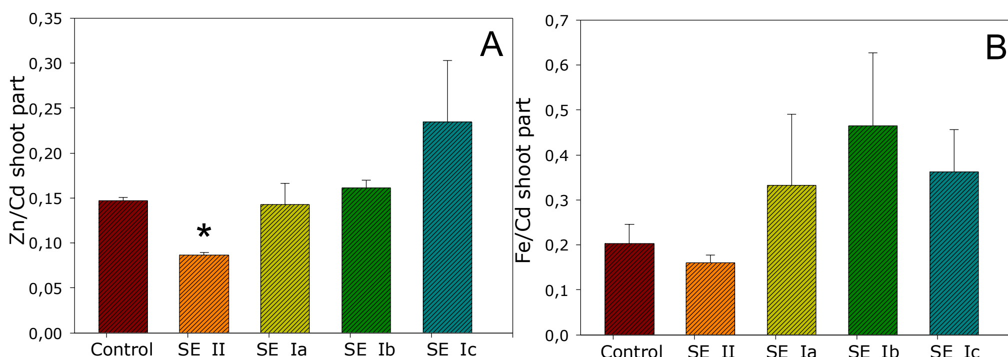


figure 7.17: Zinc-cadmium (A) and iron-cadmium (B) ratio in shoots. The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are indicated by a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

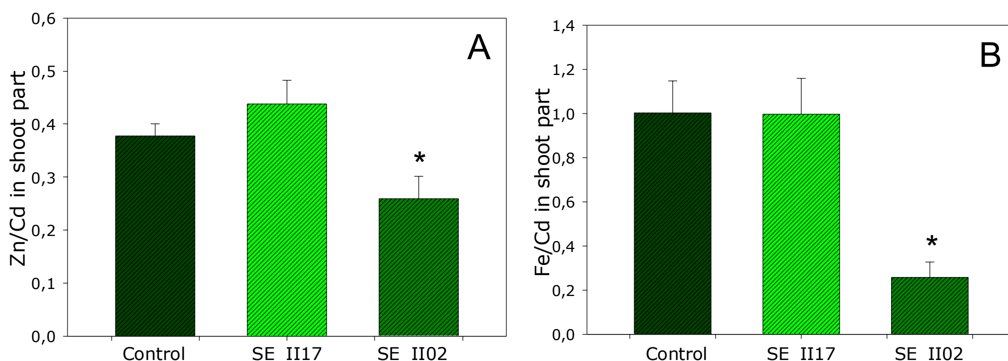


figure 7.18: Zinc-cadmium (A) and iron-cadmium (B) ratio in shoots. The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are indicated by a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

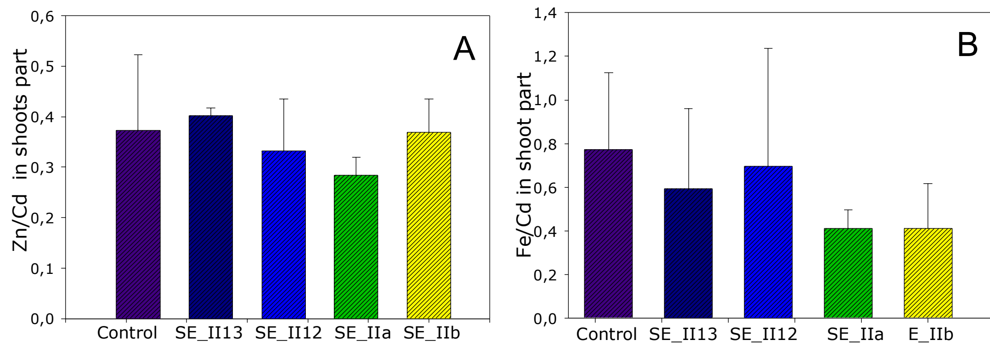


figure 7.19: Zinc-cadmium (A) and iron-cadmium (B) ratio in shoots. The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are indicated by a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

Inoculation with *Enterobacter sp*. SE_II17 seems to cause a higher zinc and iron uptake compared to cadmium. A similar result was obtained after inoculation with *Sanguibacter sp*. SE_II02 (figure 7.20A and B). The two *Pseudomonas sp*. SE_II12 and SE_II13 and the two consortia SE_IIa and SE_IIb cause increased iron to cadmium ratios in the roots (figure 7.21A and B). In the latter case, the Zn/Cd ratio did not significantly change.

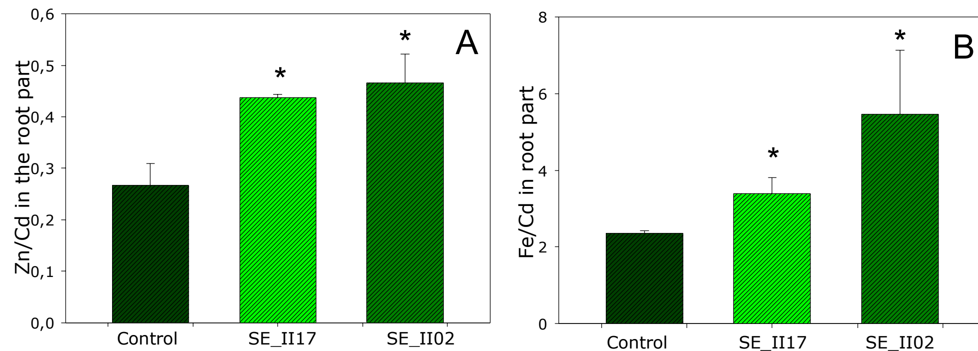


figure 7.20: Zinc-cadmium (A) and iron-cadmium (B) ratio in root part. The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are indicated by a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

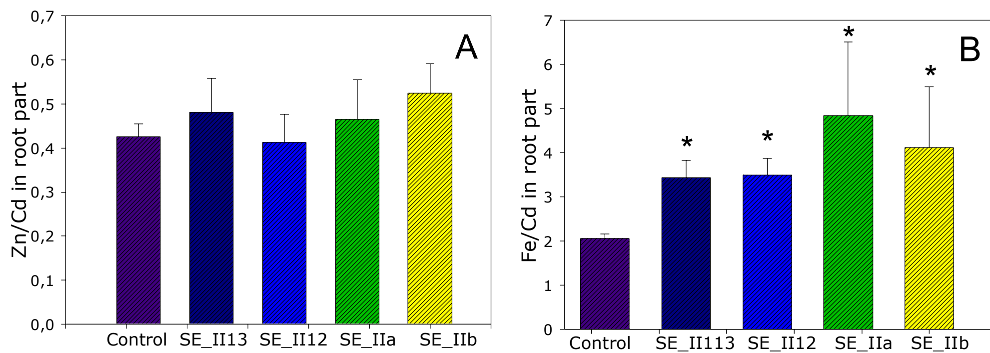


figure 7.21: Zinc-cadmium (A) and iron-cadmium (B) ratio in root part. The different inocula are specified under each bar. Means and standard deviations are shown. The statistical significances obtained from the comparison with non-inoculated plants (control) are indicated by a star on the histogram and are confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

7.3.3.4 Translocation Factor (T.F.)

The Translocation Factor (T.F.) is the ratio between the metal concentrations in the shoot and the metal concentration in the roots. Especially endophytic bacteria, but eventually also rhizosphere bacteria may have effects on the translocation of metals from roots to shoots. In cadmium challenged plants inoculation with *Sanguibacter sp*. SE_II02 (figure 7.22A) as well as *Enterobacter sp*. SE_II17, *Pseudomonas sp*. SE_II12 and the consortium SE_IIa (figure 7.23A) increased the cadmium translocation compartmentalizing more of this metal in the shoots than in the roots. At the same time, *Sanguibacter sp*. SE_II02 decreased zinc translocation resulting in an increased amount of this metal in the roots (figure 7.22B).

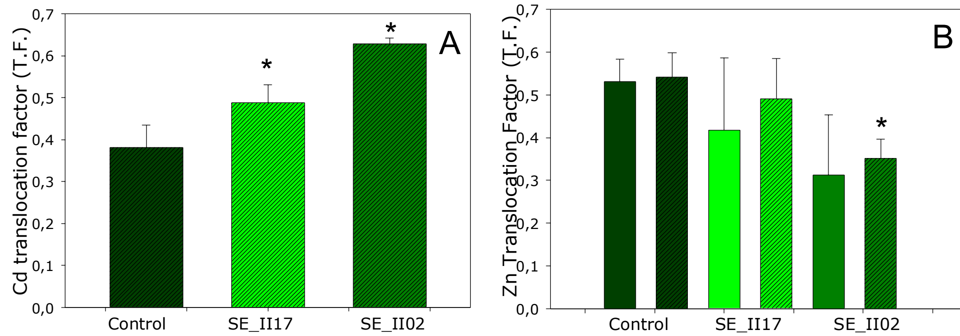


figure 7.22: Translocation Factor (T.F.) of cadmium (A) and of Zn (B). Non inoculated plants are used as controls. The different inocula are specified under each histogram. Histograms with shading refer to the plants grown in presence of 10µM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results was confirmed at the 5% level using one and two-way ANOVA model separately exploring inocula and presence or absence of cadmium, and is indicated by a star on the histogram.

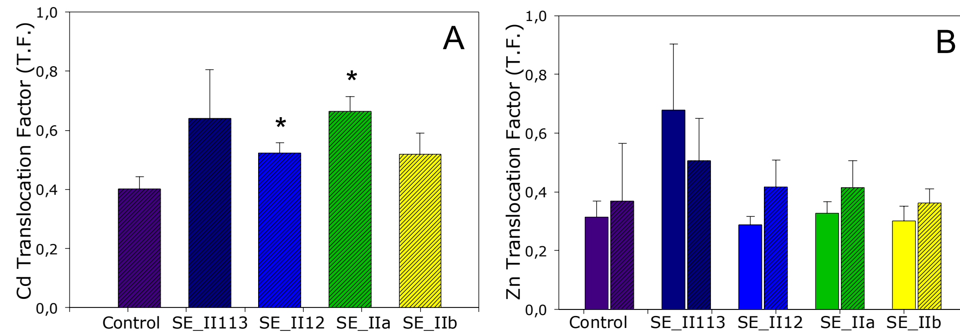


figure 7.23: Translocation Factor (T.F.) of cadmium (A) and of Zn (B). Non-inoculated plants are used as controls. The different inocula are specified under each histogram. Histograms with shading refer to the plants grown in presence of 10µM cadmium. Means and standard deviation are given of three different biological replicas. The statistical significance of the results was confirmed at the 5% level using one and two-way ANOVA model separately exploring inocula and presence or absence of cadmium, and is indicated by a star on the histogram

7.4 Discussion and conclusions

In this chapter we analyzed different parameters that should help us to better delineate the role played by endophytic bacteria, which are naturally present inside tobacco seeds, in the complex interaction with the plant and between plant and cadmium.

SE_II is the consortium found in the seed inclusive of both cultivable and non-cultivable strains (table 6.1). The plants that are inoculated with this consortium show an increased cadmium concentration in shoots (figure 7.7A). The fact that the zinc concentration is also higher than the one present in non-inoculated plants (figure 7.12A) could be read as an attempt to buffer the toxic effect of cadmium. However, the ratio between these two metals shows that cadmium is taken up to a higher concentration than zinc compared with non-inoculated plants (figure 7.17A). Inoculation by this consortium allows for a normal plant growth on 10µM cadmium, and permits at the same time a higher cadmium plant concentration. This results in a significant difference in plant cadmium content extracted from the plant growth media (figure 7.7B).

A general positive effect is observed for plants inoculated by consortium SE_Ia. This consortium is composed of dominant cultivable bacteria isolated from SE_I (chapter 6). Plants inoculated with this inoculum have a higher biomass production (figure 7.1 and 7.2), a general increased cadmium concentration (figure 7.4A) that, added to a non significant difference in biomass of treated plants, lead to a higher cadmium content compared to non-inoculated plants (figure 7.7B).

Inoculation by consortium SE_Ib, on the other hand, shows to higher the plants sensibility to cadmium. This consortium increases zinc concentration in shoots when plants are grown in the presence of cadmium (figure 7.9B). The presence of the consortium also results in a decreased shoot biomass production and an increased the fresh weight of roots when plants are grown in the presence of cadmium (figure 7.1 and 7.2).

As mentioned in chapter 6, the majority of the cultivable bacteria isolated from the seeds were belonged to the Pseudomonads. The study of their Box-PCR

products (tables 7.1 and 6.1) revealed that in many cases they represented different species. Therefore it is reasonable to expect different results from their inoculations. SE_Ic, is a consortium of strains isolated from SE_I, (chapter 6) forming similar colonies, but that after more detailed analyses (BOX PCR, table 7.1) revealed to belong to two different species of *Pseudomonas*. SE_II12 is a strain highly cadmium tolerant isolated from SE_II, while SE_II13 also isolated from SE_II, displayed low level of cadmium tolerance (table 7.1).
SE_Ic, despite its peculiar colonizing behaviour (table 7.3), increases zinc concentration and content in cadmium treated shoots (figure 7.12), and generally has a positive effect on plant growth (figure 7.1 and 7.2). We may conclude that this consortium decreases cadmium toxicity by increasing the zinc uptake. The situation is the opposite in the other two experiments where two different strains of *Pseudomonas sp*. were inoculated separately (SE_II13 and SE_II12). This indicates that in case of the consortium there could be a positive synergic effect of the different species working together.

Pseudomonas sp. SE_II13 seems to increase, in case of untreated plants, the shoots zinc concentration (figure 7.14A) coherently with what was found for consortium SE_Ic. Differently from the consortium, this single strain decreases both cadmium and zinc concentration in roots under cadmium stress (figure 7.11A and 7.16A), while only cadmium concentration is decreased in the shoots (figure 7.9A). *Pseudomonas sp.* SE_II13 seems to enhance iron to cadmium uptake in the roots, as shown by an increment of the ratio Fe/Cd (figure 7.21B).
From the plant colonization result (table 7.3) with *Pseudomonas sp.* SE_II12 we can underline a lower sensibility to cadmium compared to plants inoculated with the others *Pseudomonas sp.* This strain showed a similar inoculum concentration in plants treated and untreated with cadmium.
Sanguibacter sp. SE_II02, despite its low levels of zinc and cadmium tolerance, revealed to be an interesting strain. It slightly decreases cadmium concentration in shoots and roots (figure 7.8A and 7.10A), but this fact, added to a bigger shoots development (figure 7.3A and 7.4A), ends in a higher total plant cadmium content (figure 7.8B). It has also a positive effect on the cadmium T.F. (figure 7.22). Plants inoculated with this strain take up more metal in the shoots than in the roots. This makes this strain as promising candidate for improved Cd

phytoextraction as part of a field trial. Analyzing the ratios of Zn/Cd and Fe/Cd indicates that the host plants preferentially accumulate cadmium over zinc and iron in the shoot, as compared with non inoculated control plants (figure 7.18). On the contrary analyses of these ratios for roots (figure 7.20) show a preferential storage of iron and zinc. This behaviour doesn't result in a plant growth inhibition caused by higher levels of trace elements, but, on the contrary, in an increase, under cadmium stress, of both shoot and root dry weights (figure 7.3 and 7.4).

Plants inoculated with *Enterobacter sp*. SE_II17 don't differ significantly from non-inoculated plants. This strain seems to inhabit mostly the roots and the rhizosphere. Cfu numbers were higher in non treated plants, but the strain could also be found in the in stems of cadmium treated plants (table 7.2). It is in the roots where it mainly showed its influence, decreasing plant roots cadmium concentration and content (figure 7.10) while stimulating zinc and iron levels (figure 7.20). To decrease the cadmium concentration in the roots, this strain stimulates the cadmium translocation (figure 7.22) as a strategy to protect the root part. All these results result in a biomass production not significantly different from the non inoculated plants (figure 7.3 and 7.4).

Inoculation with the others consortia, SE_IIa and SE_IIb, resulted in a decreased zinc concentration in the shoots and roots (figure 7.14A and figure 7.16A) and an increase in the root's Fe/Cd ratio (figure 7.21B). This is translated in a preferential uptake of iron over cadmium compared to non inoculated plants, and in a higher biomass production (figure 7.5 and 7.6). We can therefore presume a decrease in cadmium phytotoxicity. Interestingly inoculation by the consortium SE_IIa also increases the cadmium T.F. (figure 7.23A).

In conclusion, it is evident that seed endophytic bacteria can have a beneficial effect on both biomass production, even under conditions of Cd stress, and trace elements uptake and distribution by their host plant. We also showed that it is important to study these bacteria as consortia as well and not only as individual strains. The bacteria composing the consortium, in fact, prove to have a general

positive influence on plant development and to be more connected with the host plants. Of the ten inocula tested, only SE_Ib decreases the biomass production and just in case of cadmium treated plants. In general the inocula either as single defined consortia or whole consortia, try to lower cadmium uptake. In case inoculation results in an increased Cd uptake, the inocula was found to reduce its toxicity by finding an equilibrium with the uptake of essential nutrients present in the growth medium. Our findings for endophytic bacteria isolated from tobacco seeds support the suggestion by Cankar *et al.* (2005) that the seeds can serve as vector for beneficial bacteria. In our case, many of the endophytic strains isolated, like *Sanguibacter sp*. SE_II02 and *Pseudomonas sp*. SE_II12, as well as the consortia like SE_Ia, SE_Ic, SE_II, SE_IIa and SE_IIb, could be promising in a phytoextraction field trials. All of them increased biomass production and plants cadmium content. This could help in lowering the time needed for the soil remediation. Their test in field experiments seems a logic consequence of our results.

Chapter 8: General conclusions and perspectives

The close relationship existing between plant-associated bacteria and their hosts is of main interest in different fields of research. In agriculture they are investigated as biofertilizers for a more sustainable production, as their use can help to decrease the quantity of fertilizer to be applied and to optimize the soil conditions increasing plant yield (Barea *et al*., 2005). There are several means by which these bacteria can improve the nutrient status of their host plant. These include biological nitrogen fixation (diazotrophy) and increase of nutrient availability into the rhizosphere through the solubilization of minerals (reviewed in Mastretta *et al.* 2007). Synthesis of plant hormones by plant associated bacteria can lead to increased plant biomass production. Plant-associated bacteria further seem to be able to reduce or prevent the deleterious effects of phytopathogenic organisms through competition, antibiosis and Induced Systemic Resistance (ISR) in plants (Bevivino *et al.,* 1998; Hebbar *et al*., 1992a and b). Lately, their ubiquitous existence, combined with the large variability of metabolic functions that they may carry out, attracted the attention for their potential application to improve phytoremediation.

In this context we isolated and characterised naturally occurring rhizospheric and endophytic bacteria that were found in association with *Nicotiana tabacum* plants. These plants were tested *in situ* for their phytoextraction capacities on two soils containing increased metal concentrations and showing different physical-chemical characteristics (Chapter 3). The majority of the cultivable microorganisms behaved as facultative endophytes, being present in all the niches (rhizosphere and different plant organs) studied. However, they presented different metal resistance phenotypes depending on the niche from which they were isolated. An example is *Stenotrophomonas maltophilia*: based on BOX PCR results, the same strain was found to exhibit different levels of metal tolerance and to be present as a rhizosphere isolate as well as an endophyte, occupying different niches from the roots up to the shoots (table 3.24). We found the existence of a general "correlation" between the plant's heavy metal concentration and the percentage of metal tolerant bacteria associated with the same plant (from the comparison of table 3.1 and 3.10, see

paragraph 3.3.4.1). In future experiments, new isolations from plants growing in the field on soils with different characteristics and with different fertilization treatment should be performed. A parallel analysis on the whole endophytic community should be performed, using the same samples for both molecular and phenotypical studies.

The outcomes of Chapter 3 prompted us to investigate the effect that the associated bacteria had on their host plant when it was being exposed to metal stress. To achieve this aim we performed experiments using sterile tobacco plants inoculated either with single strains of plant-associated bacteria isolated from the two field sites where tobacco plants were grown on Zn and Cd polluted soils, or with a defined consortium consisting of three of the bacteria previously inoculated as single isolates (Chapter 5). Each inoculum was found to differently affect plant growth (figures 5.1, 5.2, 5.3, 5.4, 5.5 and 5.6) and metal uptake (figures 5.7, 5.8, 5.9) and distribution inside the plant, including the translocation of the metals from the root to the shoot (figures 5.22, 5.23, 5.24). The best results were obtained after inoculation with the consortium, probably in force of the interaction between its members. Although it didn't affect the plant growth, the consortium conferred a higher metal tolerance to the plants and resulted in an increased cadmium translocation factor compared to non inoculated plants (figure 5.24). On the contrary, when single strains were used, plant growth was inhibited probably due to a too high concentration of bacteria applied. In future experiments, reinoculation of consortia originating from the natural plant associated bacteria should be tested on soils and/or in the field. Eventual horizontal gene transfer (Taghavi et al. 2005) to the original microbial population should be investigated.

Since our work represented a first, preliminary experiment, a more detailed study of the microbial community inhabiting the plants should be performed, not only taking into account the cultivable bacteria but widening the research to the uncultivable members of the community. This could be done using molecular tools, extracting the total genomic DNA of the microbial community directly from the different plant parts and, through DGGE or SARST, investigate the fluctuations present in the different populations. Another way to reach a similar aim could be the construction and analysis of a 16S rDNA genomic library for the different plant parts.

We observed that the fertilization regime affected the composition of the plant associated microbial community (table 3.10). One of the possible explanations of this result is that fertilization influenced metal availability and, in this way, affected the composition of the plant-associated bacterial population. It is known that mineral fertilizers can enhance soil biological activity via increases in system productivity, crop residue return and soil organic matter (Bünemann *et al.*, 2006). Moreover, Bünemann *et al*. (2006) observed that an important indirect negative effect on soil organisms can occur after N fertilization, this as a consequence of soil acidification. In order to better study these changes, in a future experiment our focus should again include the non-cultivable community members, which often represent up to 95% of the total microbial community. This can be done using a suite of molecular tools, including DGGE, SARST, and ribosomal rRNA gene sequencing (Yu *et al*., 2006; Smalla *et al.* 2001; paragraph 1.2.2). Understanding the effects of fertilization and other agronomical techniques is of great importance for the manipulation of the communities plant associated bacteria and can be linked to their application for improved remediation of metal contaminated soils.

A study of endophytic bacteria present inside seeds collected from tobacco plants grown on metal contaminated site was performed (Chapter 6). The aim of this study was to investigate the main characteristics of the cultivable bacterial consortium associated to the plant seed. Important points to be clarified were (1) a possible evolution of this consortium with increasing storage time of the seeds and (2) the discovery of their origin. For these purposes the strains were isolated and further characterized in function of different seed storage periods. The results showed an evolution evidenced by two main changes: 1) metal tolerance of the isolated strains (table 6.2) and 2) a decreasing number in cultivable strains (paragraph 6.3). We used the 16S rDNA sequences results obtained from the plant-associated bacteria in the different fields and from the seed endophytic strains to study their phylogenetic relationship, as illustrated via a Neighbour Joining tree. We observed that *Stenotrophomonas maltophilia* strains isolated from the seed community are included in a bigger group of *Stenotrophomonas maltophilia* strains isolated from the rhizosphere of tobacco plants. Interestingly, some of the *Pseudomonas sp*. isolated from the seed community formed a separate group from the rest of the *Pseudomonas sp*.

isolated from other plant parts and the rhizosphere (figure 6.2). This result made us hypothesize that endophytic bacteria use at least two main strategies to colonize tobacco seeds. One strategy is from the rhizosphere via the roots and the vascular connections, obviously used in our case by *Stenotrophomonas maltophilia*. The second strategy, which we hypothesise for *Pseudomonas sp*., seems to imply epiphytic colonization, probably of the reproductive organs and eventual colonization of the seeds. Both seed colonization pathways correspond to those proposed for *Xanthomonas campestris* pv. v*itians* (Barak *et al.* 2002). To confirm the identity of xanthomonad-like colonies recovered from lettuce plants, they adopted a PCR with an *X. campestris* pv. *vitians*-specific primer pair. In our case, the seed endophytic bacteria isolated were most probably colonizing the embryo.

The high percentage of cadmium tolerant strains present inside the seed consortia SE_I and SE_II (table 6.2) encouraged us to investigate which effects these plant-associated bacteria had on plant development in the presence and absence of Cd stress. To do so, a series of re-inoculations experiments using single strains and consortia were performed on sterile plants with and without exposure to Cd (Chapter 7). In general, inoculation of sterile tobacco plants with endophytes from the seeds had a positive effect, both on biomass production (figures 7.1, 7.2, 7.3, 7.4, 7.5 and 7.6) and on plant metal uptake and distribution (figures from 7.7 to 7.11). This made us conclude that the future work on plant-associated bacteria that positively influence *Nicotiana tabacum* development should include the seed endophytes. A positive effect of seed endophytes was also hypothesised Cankar *et al*. (2005). They identified genera like *Pseudomonas sp*. and *Rahnella aquatilis*, known to possess plant-growth promoting properties, among the bacteria associated with Norway spruce seeds. We may therefore hypothesise the possibility that tobacco, as probably happens for Norway spruce, is transferring these beneficial endophytes via the seeds, making sure that its offspring has the best possible endophytic association with plant growth promoting properties that assist the developing seedling to deal with environmental stresses like, in our case, the ones caused by heavy metals.

Our work presents a preliminary but promising study, which merits that further research to be performed on the endophytic community associated with tobacco seeds, including an analysis of the uncultivable members. A relevant increment

in cadmium concentration and total plant cadmium content, in fact, have been obtained via seed inoculation with the original seed associated endophytic consortium, composed by both cultivable and non cultivable strains. A more detailed study on the changes that occur in the seed endophytic community in function of storage time should be performed, this to understand if and how much changes in community composition occur, and if and how these changes may influence germination and plant development. Different storage procedures should be suggested in case of a deterioration of the positive effects of this community on plant development. A comparison with the endophytic community belonging to seeds coming from non-contaminated sites should be performed to study possible differences in competences of the strains forming the consortia.

Another open question is the fate of the seed endophytes once germination starts. For instance, will these endophytes become a major fraction of the newly established endophytic community, or will they be out-competed by the microbial population present in the soil once the seeds start to germinate. One should also investigate the composition of the next generation of seed endophytes in function of the plant growth regime and the composition of the total endophytic community as part of a population dynamics approach. Finally, it will be interesting to understand if and how much the seed bacteria influence the bacteria present in the soil, e.g. increasing their metal tolerance via horizontal gene transfer. The plant’s improved cadmium tolerance and metal translocation suggest greenhouse and field experiments as logic follow up, using these strains as inocula. Their use could lead to an improvement of the phytoextraction results, reducing the time period normally needed for the process.

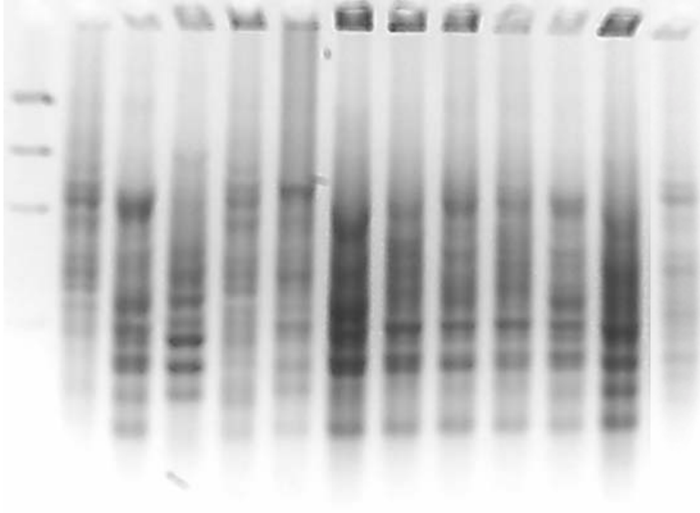


figure 2: Box-PCR fingerprinting of bacteria isolated from seeds of *Nicotiana tabacum* var. BAG. Lane1: 1kb+ DNA ladder, lane2: SE_I041, lane3: *Clostridium aminovalericum* (SE_II10), lane4: *Enterobacter sp.* (SE_II11), lane5: (SE_II18), lane6: *Pseudomonas sp.*(SE_II01), lane7: *Pseudomonas sp.* (SE_II07), lane8: *Pseudomonas sp.* (SE_II08), lane9: *Pseudomonas sp.* (SE_II24), lane10: *Pseudomonas sp.* (SE_II28), lane 11: *Pseudomonas sp.* (SE_II30), lane12: *Clostridium aminovalericum* (SE_II32), lane13: *Stenotrophomonas sp.* (SE_I015).

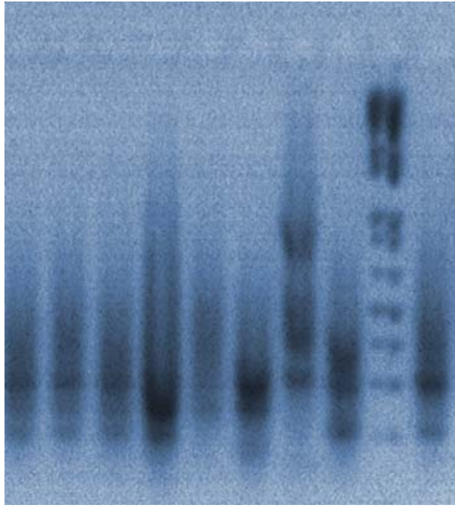


figure 3: Box-PCR fingerprinting of bacteria isolated from seeds of *Nicotiana tabacum* var. BAG. Lane1: *Pseudomonas sp.* (SE_I028), lane2: *Pseudomonas sp.* (SE_I029), lane3: *Pseudomonas sp.* (SE_I030), lane4: *Pseudomonas fulva* (SE_I36), lane5: (SE_I041), lane6: positive control, lane7: *Stenotrophomonas sp.* (SE_II05), lane8: *Pseudomonas sp.* (SE_II16), lane9: 1kb+ DNA ladder, lane10: *Pseudomonas sp.* (SE_III04).

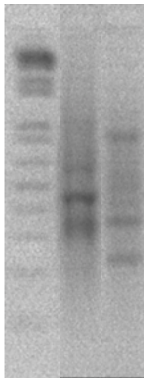


figure 4: Box-PCR fingerprinting of bacteria isolated from seeds of *Nicotiana tabacum* var. BAG.
Lane1: 1kb+DNA ladder,
lane2: *Pseudomonas sp.* (SE_I045),
lane3: *Pseudomonas sp.* (SE_II06).

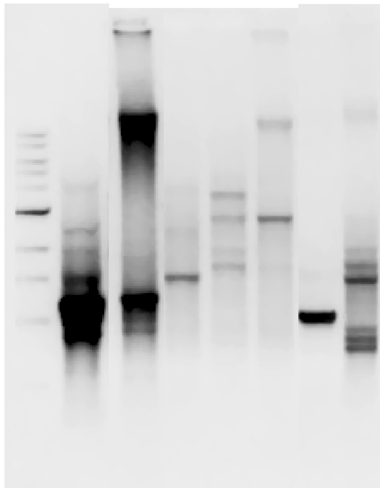


figure 5: Box-PCR fingerprinting of bacteria isolated from seeds of *Nicotiana tabacum* var. BAG. Lane1: 1kb DNA ladder, lane2: *Pseudomonas sp.* (SE_II005), lane3: *Pseudomonas sp.* (SE_III04), lane4: *Pseudomonas sp.* (54), lane5: *Pseudomonas sp*. (SE_II12), lane6: *Pseudomonas sp*. (SE_II27), lane7: *Stenotrophomonas sp.* (SE_I010), lane8: *S. maltophilia* (49).

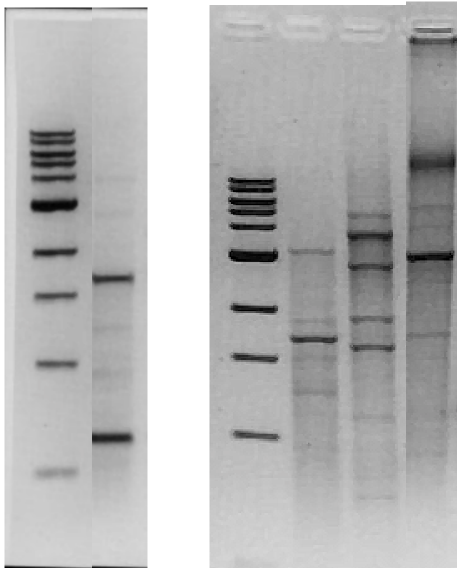


figure 6: Box-PCR fingerprinting of bacteria isolated from seeds of *Nicotiana tabacum* var. BAG. Left part: lane1: 1kb DNA ladder, lane2: *Sanguibacter sp.* (SE_II02). Right part: lane1: 1kb DNA ladder, lane2: *Enterobacter sp.* (SE_II11), lane3: *Pseudomonas sp*. (SE_II12), lane4: *Pseudomonas sp*. (SE_II27).

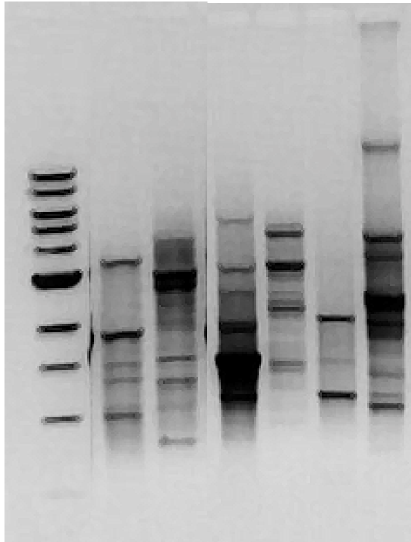


figure 7: Box-PCR fingerprinting of bacteria isolated from seeds of *Nicotiana tabacum* var. BAG. Lane1: 1kb DNA ladder, lane2: *Enterobacter sp.* (SE_III12), lane3: *Sanguibacter sp.* (SE_III08), lane4: *Pseudomonas sp.* (SE_I005), lane5: *Sanguibacter sp.* (SE_III09), lane6: *Enterobacter sp.* (SE_III11), lane7: positive control.

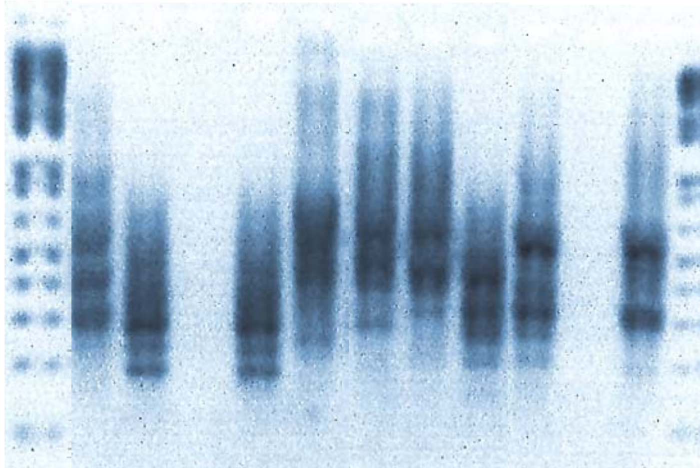


figure 8: Box-PCR fingerprinting of endophytic bacteria isolated from *Nicotiana tabacum* var. BAG. In lane 1: 1kb+ DNA ladder, lane2: *Pseudomonas sp.* (SE_I008), lane3: *Pseudomonas fulva* (SE_I002), lane4: empty, lane5: *Pseudomonas fulva* (SE_I003), lane6: *Stenotrophomonas maltophilia* (50), lane7: *Pseudomonas sp.* (137), lane8: *Pantoea agglomerans* (138), lane9: *Pseudomonas sp.* (143), lane10: *Stenotrophomonas maltophilia* (273), lane11: empty, lane12: *Variovorax sp.* (280), lane13: 1kb+DNA ladder

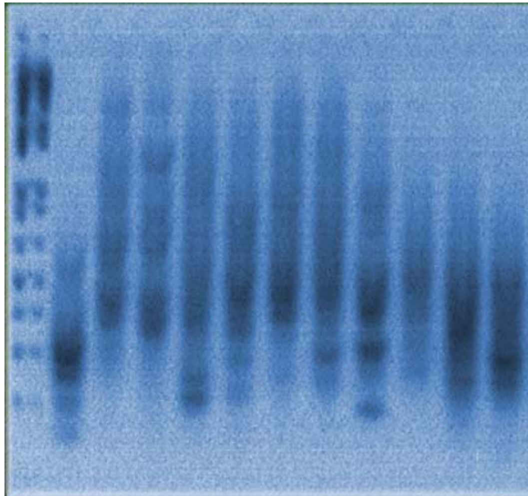


figure 9: Box-PCR fingerprinting of endophytic bacteria isolated from *Nicotiana tabacum* var. BAG. In lane 1: 1kb+ DNA ladder,lane2: *S. maltophilia* (300), lane3: *Pseudomonas sp.* (303), lane4: *Leifsonia aquatica* (316), lane5: *Enterobacter sp.* (319), lane6: *Pseudomonas sp*. (354), lane7: *Pantoea agglomerans* (387), lane8: *Enterobacter sp.* (395), lane9: *S. maltophilia* (398), lane10: blanc, lane11: *Enterobacter sp.* (399), lane12: *Enterobacter sp.* (400)

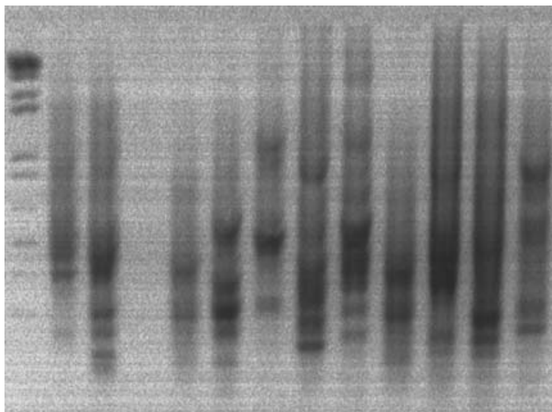


figure 10: Box-PCR fingerprinting of endophytic bacteria isolated from *Nicotiana tabacum* var. BAG. In lane 1: 1kb+ DNA ladder, lane2: *Enterobacter sp.* (321), lane3: *Arthrobacter sp.* (333), lane4: empty, lane5: *S. maltophilia* (343), lane6: *S. maltophilia* (358), lane7: *S. maltophilia* (359), lane8: positive control, lane9: *S. maltophilia* (363b) lane10: *S. maltophilia* (384), lane11: *Enterobacter sp.* (392), lane12: *Enterobacter sp.* (401), lane13: *S. maltophilia* (405)

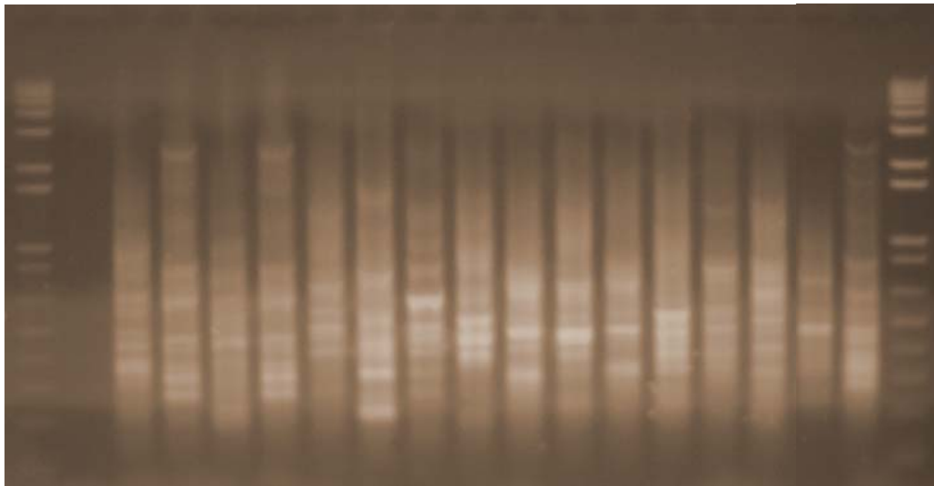


figure 11: Box-PCR fingerprinting of endophytic bacteria isolated from *Nicotiana tabacum* var. BAG. In lane 1: 1kb+ DNA ladder, lane2: *Pseudomonas sp.* (137), lane3: *Sphingobium herbicidovorans* (71), lane4: *S. maltophilia* (100), lane5: *Crustibacterium reblochoni* (48), lane6: *S. maltophilia* (96), lane7: *Pseudomonas sp.* (141), lane8: *Pseudomonas fulva* (SE_I001), lane9: *S. maltophilia* (320), lane10: *Arthrobacter sp.* (286), lane11: *Pseudomonas sp.* (55), lane12: *Pantoea agglomerans* (85), lane13: *Pseudomonas sp.* (246), lane14: *Enterobacter sp.* (389), lane15: *S. maltophilia* (322), lane16: *Arthrobacter sp.* (107), lane17: blanc, lane18: *S. maltophilia* (62), lane19: 1kb+DNA ladder

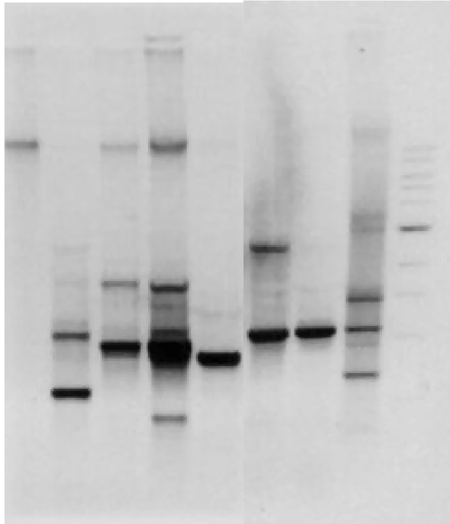


figure 12: Box-PCR fingerprinting of endophytic bacteria isolated from *Nicotiana tabacum* var. BAG. In lane 1: *Stenotrophomonas maltophilia* (912), lane2: *S. maltophilia* (160), lane3: *S. maltophilia* (338), lane4: *S. maltophilia* (295), lane5: *S. maltophilia* (328), lane6: *Alcaligens* (901), lane7: *Alcaligens* (909), lane8: *Ralstonia sp.* (910), lane9: 1kb DNA ladder.

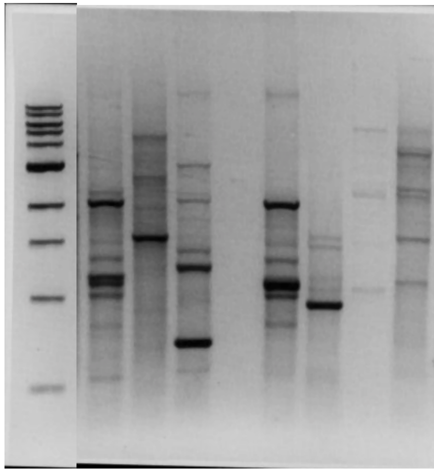


figure 13: Box-PCR fingerprinting of endophytic bacteria isolated from *Nicotiana tabacum* var. BAG. In lane 1: 1kbDNA ladder, lane2: *S. maltophilia* (49), lane3: *Pseudomonas sp.* (54), lane4: *S. maltophilia* (160), lane5: empty, lane6: *S. maltophilia* (342), lane7: positive control, lane8: negative control, lane9: *Arthrobacter sp.* (418).

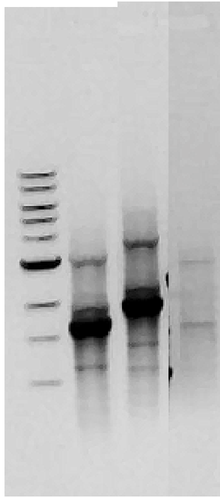


figure 14: Box-PCR fingerprinting of endophytic bacteria isolated from *Nicotiana tabacum* var. BAG. In lane 1: 1kbDNA ladder, lane2: *Arthrobacter sp*. (911), lane3: *Arthrobacter sp*. (907), lane4: *Enterobacter sp.* (399).

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