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WOORD VOORAF

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Nele Weyens
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SUMMARY

Established methods to remediate contaminated soils and groundwater are frequently expensive, environmentally invasive, labour intensive, and do not make cost-effective use of existing resources. Especially in case of large-scale contaminated areas phytoremediation is considered to be a cost-effective and sustainable alternative for remediation since it works *in situ*, is solar powered and demands minimal site disturbance and maintenance. However, phytoremediation still has to deal with some important shortcomings like phytotoxicity, a limited contaminant uptake, and evapotranspiration of volatile organic contaminants. Plant-associated bacteria can be exploited to overcome these constraints. In case of phytoremediation of organic contaminants, endophytes equipped with the appropriate degradation pathway can diminish phytotoxicity and evapotranspiration. To increase metal bioavailability and to decrease metal phytotoxicity during phytoremediation of toxic metals, plant-associated bacteria that are provided with a metal resistance/sequestration system and that are capable of producing siderophores and/or organic acids can be used. Proof of this concept was already provided under controlled laboratory conditions for single contaminations of toluene or nickel. However, at most contaminated sites, plants and their associated microorganisms have to deal with mixed pollutions of organics and toxic metals. Moreover, this proof of concept on laboratory scale is no guarantee that (a) *in situ* inoculation of bacteria is possible, (b) the inoculated strains will become an integrated part of the endogenous community, and (c) the selection pressure will be sufficient to maintain a stable degradation capacity and/or metal sequestration. Since the main objective of this work was to move endophyte-enhanced phytoremediation towards application in the field, these critical issues were further investigated.

In section 3 it was investigated on laboratory scale if endophytes can also improve phytoremediation of mixed contaminations of organic contaminants and toxic metals. For this purpose, yellow lupine (*Lupinus luteus*) plants were inoculated with a Ni-resistant, toluene- and TCE-degrading endophyte and plants were exposed to combinations of Ni and toluene (chapter 3.3) and of Ni and TCE (chapter 3.4). To examine if the inoculated endophyte could improve efficiency of phytoremediation, Ni, toluene and TCE phytotoxicity, Ni uptake and toluene or

TCE evapotranspiration were investigated. Inoculation of plants exposed to Ni and toluene with the Ni-resistant toluene degrading endophyte resulted in decreases of Ni and toluene phytotoxicity and of toluene evapotranspiration. When plants were exposed to Ni and TCE, inoculation lead to a reduced toxicity of Ni and TCE in the roots of the host plant, a slightly decreased TCE evapotranspiration and a strong increase in Ni uptake. From these results it can be concluded that endophytic bacteria possessing the right properties, can improve phytoremediation of mixed contaminations by decreasing metal phytotoxicity and increasing degradation of organic contaminants.

In a next step towards field application, yellow lupine, our model test plant, was replaced by poplar cuttings [*Populus deltoides x (trichocarpa x deltoides) cv. Grimminge*] since poplar is an excellent tree species for both biomass production and also phytoremediation purposes. The toluene- and TCE-degrading poplar endophyte *Pseudomonas putida* W619 colonized the rhizosphere, the root cortex and the xylem vessels of the root (chapter 4.1). Inoculation with *P. putida W619* resulted in (a) remarkable plant growth promotion, (b) decreased activities of stress enzymes in roots and leaves, and (c) a strongly reduced stomatal resistance, all indicative of improved plant fitness in comparison with the non-inoculated control cuttings. In chapter 4.2 the potential of *P. putida* W619-TCE to reduce TCE phytotoxicity and evapotranspiration was investigated in a short-term experiment in hydroponics and in a mid-term experiment in potting soil. In the short-term experiment inoculation with *P. putida* W619-TCE (a) strongly promoted plant growth, (b) reduced the TCE phytotoxicity and (c) reduced the amount of TCE that was extracted from the leaves. In the mid-term experiment, the plant growth-promoting effect of *P. putida* W619-TCE and the reduction of TCE phytotoxicity were less pronounced. However, the amounts of TCE that were extracted from the leaves and the roots were significantly lower in inoculated plants supplied with 400 mg l^{-1} TCE. Also the amount of TCE that was evapotranspired through the leaves was significantly lower after inoculation.

Section 5 reports the field trials we performed to achieve a successful endophyte-enhanced phytoremediation in the field.
In the first field experiment, poplar and its naturally associated microorganisms were applied for the *in situ* remediation of a BTEX-contaminated groundwater plume (chapter 5.2). Analysis of the poplar-associated microorganisms

demonstrated that, both rhizosphere and endophytic bacteria that were able to degrade toluene were enriched once the plant roots got in contact with the BTEX-contaminated groundwater. Interestingly, once the BTEX plume was remediated, the numbers of toluene-degrading rhizosphere and endophytic bacteria decreased below detection limits, indicating that their population resulted from selective enrichment by the presence of the contaminants and demonstrating the plasticity of the endogenous microbial communities to assist their host plant in the phytoremediation of organic contaminants.

For the other field experiments, a TCE-contaminated site was chosen where a mixed woodland of English oak (*Quercus robur*) and common ash (*Fraxinus excelsior*) was already present on the contaminated groundwater plume. TCE concentrations sharply decreased along transects under this mixed woodland suggesting that phytoremediation was already taking place. Therefore, in chapter 5.3 (a) the bacteria associated with oak and ash were characterized and (b) *in situ* TCE evapotranspiration was investigated. Although the majority of the isolated strains showed increased tolerance to TCE, and TCE degradation capacity was observed in some of them, significant amounts of TCE were evaporating through the leaves to the atmosphere.

To test if *in situ* inoculation of the TCE-degrading *P. putida* W619 could reduce TCE evapotranspiration, poplar trees were planted and provided with a drainage tube around the roots to allow inoculation. In chapter 5.5, the first successful *in situ* inoculation of these poplar trees with *P. putida* W619 is reported. The establishment and enrichment of the inoculated *P. putida* W619, and the horizontal transfer of the genes coding for TCE degradation to other members of the poplar's endogenous endophytic population resulted in a 90% reduced TCE evapotranspiration.

Considering that (a) the major obstacles to the implementation of phytoremediation still are the mixed pollutions that are frequently present at contaminated sites and the evapotranspiration of volatile contaminants and that (b) the results of this work offer a possible solution for these constraints, endophyte-enhanced phytoremediation will offer a safe, break-through approach to the large-scale application of phytoremediation.

SAMENVATTING

Conventionele methoden om gecontamineerde bodems en grondwaters te remediëren zijn vaak duur en zeer arbeidsintensief, leiden vaak tot drastische wijzigingen van de bodemkarakteristieken (o.a. zeer sterke daling van het gehalte aan organische stof), en maken inefficiënt of zelfs geen gebruik van bestaande hulpbronnen. Vooral in geval van zeer uitgestrekte verontreinigde gebieden, wordt fytoremediatie beschouwd als een kosten-efficiënt, duurzaam alternatief aangezien het *in situ* wordt toegepast, het volledig draait op zonne-energie, en bovendien slechts een minimale verstoring van de omgeving veroorzaakt en ook zeer onderhoudsvriendelijk is. Toch heeft fytoremediatie nog steeds te kampen met enkele belangrijke tekortkomingen zoals fytotoxiciteit, een beperkte opname van de contaminanten (omwille van een lage biobeschikbaarheid en een beperkte opname en translocatie door de plant), en evapotranspiratie van vluchtige organische contaminanten. Om aan deze tekortkomingen een oplossing te bieden kunnen plant-geassocieerde bacteriën gebruikt worden. In geval van fytoremediatie van organische contaminanten kunnen endofyten, uitgerust met de geschikte afbraakroute, de fytotoxiciteit en de evapotranspiratie reduceren. Om de biobeschikbaarheid van metalen te verhogen en de metaal fytotoxiciteit te verlagen tijdens fytoremediatie van toxische metalen, kunnen plant-geassocieerde bacteriën aangewend worden die (a) uitgerust zijn met een metaal resistentie/sequestratie systeem en die (b) in staat zijn om sideroforen en/of organische zuren te produceren. De werkzaamheid van dit concept werd reeds op laboschaal aangetoond voor enkelvoudige verontreinigingen van tolueen of nikkel. Op de meeste verontreinigde sites worden planten en hun geassocieerde micro-organismen echter geconfronteerd met gemengde verontreinigingen van organische contaminanten en toxische metalen. Bovendien is het bewijs van de werkzaamheid van het concept op laboschaal nog geen garantie dat (a) *in situ* inoculatie van bacteriën mogelijk is, (b) de geïnoculeerde bacteriën effectief geïntegreerd zullen worden in de endogene bacteriële gemeenschap, en dat (c) de selectiedruk voldoende zal zijn om een stabiele afbraak capaciteit en/of metaal sequestratie te bewerkstelligen. Aangezien het hoofddoel van dit werk was om endofyt-gestimuleerde fytoremediatie dichter bij toepassing in de praktijk te brengen, werden deze knelpunten verder onderzocht.

In deel 3 werd op laboschaal onderzocht of endofyten in staat zijn om fytoremediatie van gemengde verontreinigingen van organische contaminanten en toxische metalen te verbeteren. Om dit na te gaan werden gele lupine (*Lupinus luteus*) planten geïnoculeerd met een Ni-resistente, tolueen- en TCE-afbrekende endofyt en blootgesteld aan combinaties van Ni en tolueen (hoofdstuk 3.3) en van Ni en TCE (hoofdstuk 3.4). Om uit te maken of de geïnoculeerde endofyten de fytoremediatie-efficiëntie konden verbeteren, werden de Ni, tolueen en TCE fytotoxiciteit, de Ni opname en de tolueen en TCE evapotranspiratie onderzocht. Inoculatie van planten die blootgesteld werden aan Ni en tolueen met de Ni-resistente, tolueen-afbrekende endofyt resulteerde in een daling van de Ni en tolueen fytotoxiciteit en van de tolueen evapotranspiratie. Wanneer de planten blootgesteld werden aan Ni en TCE, had inoculatie (a) een gereduceerde Ni en TCE fytotoxiciteit in de wortels van de gastheer, (b) een licht verlaagde TCE evapotranspiratie en (c) een sterk verhoogde Ni opname voor gevolg. Uit deze resultaten kan besloten worden dat endofytische bacteriën, voorzien van de geschikte eigenschappen, fytoremediatie van gemengde verontreinigingen kunnen verbeteren via een verlaging van de fytotoxiciteit van de metalen en een verhoging van de afbraakcapaciteit van organische contaminanten.

In een volgende stap richting toepassing in het veld, werden de gele lupine planten, onze model testplanten, vervangen door stekken van populier [*Populus deltoides x (trichocarpa x deltoides) cv. Grimminge*] aangezien populier zeer geschikt is voor zowel biomassaproductie als voor fytoremediatie doeleinden. De tolueen- en TCE-afbrekende populier endofyt *Pseudomonas putida* W619 koloniseerde de rhizosfeer, de wortelcortex en de xyleemvaten van de wortel (hoofdstuk 4.1). Inoculatie met *P. putida* W619 resulteerde in (a) een opmerkelijke groeipromotie, (b) verlaagde activiteiten van stress-gerelateerde enzymen in de wortels en de bladeren en (c) in een sterk gereduceerde stomatale weerstand. Al deze parameters wijzen op een verbeterde 'fitness' van de geïnoculeerde stekken in vergelijking met de niet-geïnoculeerde populierstekken. In hoofdstuk 4.2 werd onderzocht of de TCE-afbrekende *P. putida* W619-TCE in staat is om de TCE fytotoxiciteit en evapotranspiratie te reduceren. Hiervoor werden een korte termijn experiment in hydrocultuur en

een langere termijn experiment in potgrond uitgevoerd. In het korte termijn experiment zorgde inoculatie met *P. putida* W619-TCE voor (a) een sterke plantengroeipromotie, (b) een gereduceerde TCE fytotoxiciteit, en (c) een verlaging van de hoeveelheid TCE die kon geëxtraheerd worden uit de bladeren. In het langere termijn experiment was het positief effect van *P. putida* W619-TCE op de groei van de planten en de TCE fytotoxiciteit minder uitgesproken. Echter, in de bladeren en de wortels van met *P. putida* W619-TCE geïnoculeerde planten behandeld met 400 mg l^{-1} TCE waren de gehalten aan TCE veel lager dan in niet-geïnoculeerde planten en ook de hoeveelheid aan TCE die geëvapotranspireerd werd via de bladeren was significant lager na inoculatie met *P. putida* W619-TCE.

Deel 5 geeft de veldexperimenten weer die werden uitgevoerd om een succesvolle endofyt-gestimuleerde fytoremediatie in het veld aan te tonen.
In het eerste veldexperiment werden populier en zijn van nature uit geassocieerde micro-organismen gebruikt voor de *in situ* remediatie van een BTEX-gecontamineerde grondwaterpluim (hoofdstuk 5.2). Onderzoek van de populier-geassocieerde micro-organismen toonde aan dat zowel rhizosfeer als endofytische bacteriën die in staat zijn om tolueen af te breken, aangerijkt werden vanaf het moment dat de plantenwortels in contact kwamen met de BTEX-gecontamineerde grondwaterpluim. Een opvallende en hoogstinteressante vaststelling was dat wanneer de BTEX-pluim geremedieerd was, het aantal tolueen afbrekende rhizosfeer en endofytische bacteriën daalde tot beneden de detectielimiet. Dit wijst erop dat het voorkomen van een natuurlijk BTEX-afbraakpotentieel op de betreffende site het resultaat was van een selectieve aanrijking ten gevolge van de selectiedruk van de aanwezige contaminanten, hetgeen de plasticiteit aantoont van de endogene microbiële gemeenschap om hun gastplant te helpen bij de fytoremediatie van organische contaminanten.
Voor de andere veldexperimenten werd een TCE-gecontamineerde site gekozen waar reeds een ongeveer 20 jaar oud gemengd bos van zomereik (*Quercus robur*) en gewone es (*Fraxinus excelsior*) aanwezig was boven de gecontamineerde grondwaterpluim. De TCE concentraties daalden zeer sterk in grondwaterstalen van de transecten doorheen het bos, hetgeen suggereerde dat er reeds fytoremediatie plaatsvond. Om deze reden werden in hoofdstuk 5.4 (a) de bacteriën geassocieerd met de op de site voorkomende eik en es

gekarakteriseerd en (b) de *in situ* TCE evapotranspiratie onderzocht. Hoewel de meerderheid van de geïsoleerde bacteriële stammen een verhoogde TCE tolerantie vertoonden en er ook een TCE afbraak capaciteit werd vastgesteld voor enkele van deze stammen, bleken toch nog significante hoeveelheden TCE via de bladeren naar de atmosfeer geëvapotranspireerd te worden.
Om na te gaan of *in situ* inoculatie met de TCE-afbrekende *P. putida* W619-TCE de TCE evapotranspiratie kan reduceren, werden populieren aangeplant op dezelfde site en werden deze met het oog op inoculatie voorzien van een drainagesysteem in de wortelzone. In hoofdstuk 5.5 wordt de succesvolle *in situ* inoculatie van deze populieren met *P. putida* W619-TCE beschreven. De vestiging en aanrijking van de geïnoculeerde *P. putida* W619-TCE, en de horizontale gentransfer van de genen die coderen voor de TCE afbraak naar andere leden van de endogene endofytische populatie van populier resulteerden in een 90% gereduceerde TCE evapotranspiratie. Voor zover we konden nagaan is dit de eerste succesvolle en stabiele *in situ* inoculatie met contaminant afbrekende endofyten.

Aangezien het voorkomen van gemengde verontreinigingen en de evapotranspiratie van vluchtige organische contaminanten de toepassing van fytoremediatie in het veld tot op heden vaak bemoeilijkten en de resultaten van dit werk hiervoor een oplossing bieden, kan endofyt-gestimuleerde fytoremediatie een belangrijke doorbraak betekenen naar de veilige, grootschalige toepassing van fytoremediatie.

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LIST OF ABBREVIATIONS

2,4-D	2,4 dichlorophenoxyacetic acid
ACC	1-aminocyclopropane-1-carboxylate
ARDRA	amplified rDNA restriction analysis
Aut	autotrophic
BTEX	benzene, toluene, ethylbenzene and xylene
$CaCl_2$	calciumchloride
CAT	catalase
CFU	colonie forming units
C-mix	carbon mix
CO_2	carbon dioxide
DAPG	2,4-diacetyl phloroglucinol
DCAA	dichloroacetic acid
EDTA	ethylenediaminetetraacetic acid
Egfp	enhanced green fluorescent protein
Fe	iron
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
Gfp	green fluorescent protein
GMO	genetic manipulated organisms
GPOD	guaiacol peroxidase
GR	glutathione reductase
IAA	indole-3-acetic acid
ISR	induced systemic resistance
K_{ow}	octanol-water coefficient
$MgSO_4$	magnesiumsulphate
MtBE	methyl tert-butyl-ether
N_2	nitrogen
Ni	nickel
$NiSO_4$	nickel sulphate
OD	optical density
P	phosphorous
PGPR	plant growth promoting rhizobacteria

SE	standard error
SEM	scanning electron microscopy
SOD	superoxide dismutase
TC	transfer coefficient
TCAA	trichloroacetic acid
TCE	trichloroethylene
TF	translocation factor
Tol	toluene
TOM	toluene ortho-monooxygenase
VOA	volatile organic analysis

Section 1

INTRODUCTION

Parts of the introduction section were published in:

- Weyens N, van der Lelie D, Taghavi S, Vangronsveld J (2009) Phytoremediation: plant-endophyte partnerships take the challenge. Current Opinion in Biotechnology 20:248-254

- Weyens N, van der Lelie D, Taghavi S, Newman L, Vangronsveld J (2009) Exploiting plant–microbe partnerships for improving biomass production and remediation. Trends in Biotechnology 27:591-598

- Weyens N, Monchy S, Vangronsveld J, Taghavi S, van der Lelie D (xxxx) Plant-Microbe Partnerschips: In: Handbook of hydrocarbon microbiology: microbial interactions with hydrocarbons, oils, fats and related hydrophobic substrates and products (chapter 402). Kenneth N Timmis (editor) ISBN: 978-3-540-77584-3 (print) 978-3-540-77587-4 (Online) Springer-Verlag Berlin Heidelberg

- Weyens N, Monchy S, Vangronsveld J, Taghavi S, van der Lelie D (xxxx) Role of Plant-Microbe Partnerships to Deal with Environmental Challenges: In: Handbook of hydrocarbon microbiology: microbial interactions with hydrocarbons, oils, fats and related hydrophobic substrates and products (chapter 623). Kenneth N Timmis (editor) ISBN: 978-3-540-77584-3 (print) 978-3-540-77587-4 (Online) Springer-Verlag Berlin Heidelberg

- Weyens N, Monchy S, Vangronsveld J, Taghavi S, van der Lelie D (xxxx) Plant-endophyte partnerships. In: Phytoremediation. Edited by : Marinus L. Otte and Donna L. Jacob, Encyclopedia of Life Support Systems, Eolss Publishers, Oxford UK

- Vangronsveld J, Weyens N, Ruttens A, Vassilev A, Mench M (xxxx) Land revitalisation. In: Vanek T (Ed) Phytotechnologies solutions for sustainable land management, Encyclopedia of Life Support Systems, Eolss Publishers, Oxford UK

Interest in using biofuels as an alternative energy source (Cassman and Liska, 2007) is now high on the agenda of policy makers in many countries. In 2007, the US Congress passed the Energy Independence and Security Act, which requires that biofuel production should increase more than sevenfold by 2022. In response to the global increase in the use of biofuels, there has been a rapid expansion of biofuel production capacity from food crops in the USA, Brazil, Europe, and several Southeast Asian countries. Food crops currently used for biofuel production include grains (maize, sorghum, wheat), sugar crops (sugarcane, sweet sorghum, sugar beet), starch crops (cassava), and oilseed crops (soybean, oil palm, castor bean, rapeseed). To meet the rapidly increasing demand without infringing upon the food supply, additional ways of growing plants as energy sources without seizing fertile land or food crops need to be explored. Indeed, the most popular biofuels, such as corn-based ethanol, are starting to cause their own problems. More and more cropland is being devoted to growing crops for fuel instead of for food, and a sustainable method of producing biofuel crops is urgently needed. The best example of the food–fuel conflict is the North American corn crop. It is believed that the recent increases in corn prices, which peaked in summer 2008, were at least partly caused by the fact that almost a third of the production is being used for ethanol fuel production. The additional demand during the next years is likely to lead to further increases. Another example is the land used for biofuel production in Brazil; Brazilian farmers are clearing huge swaths of land for palm and sugarcane plantations, destroying native ecosystems. Currently 8.7 million hectares are used for sugarcane growth and a 50% increase is predicted by 2018 (http://www.iddri.org/Activites/Ateliers/081009_Conf-Ethanol_Presentation_Andre_Nassar.pdf). Palm oil, a popular biodiesel fuel, is extracted from palm trees, which grow well in places such as Brazil.

One way of avoiding the conflict between food and energy crops is to produce biofuel feedstocks on marginal land that is not suited for agriculture (figure 1.1). This marginal land comprises soils that either, lack nutrients, receive little rain, or have been contaminated due to previous industrial or agricultural activities.

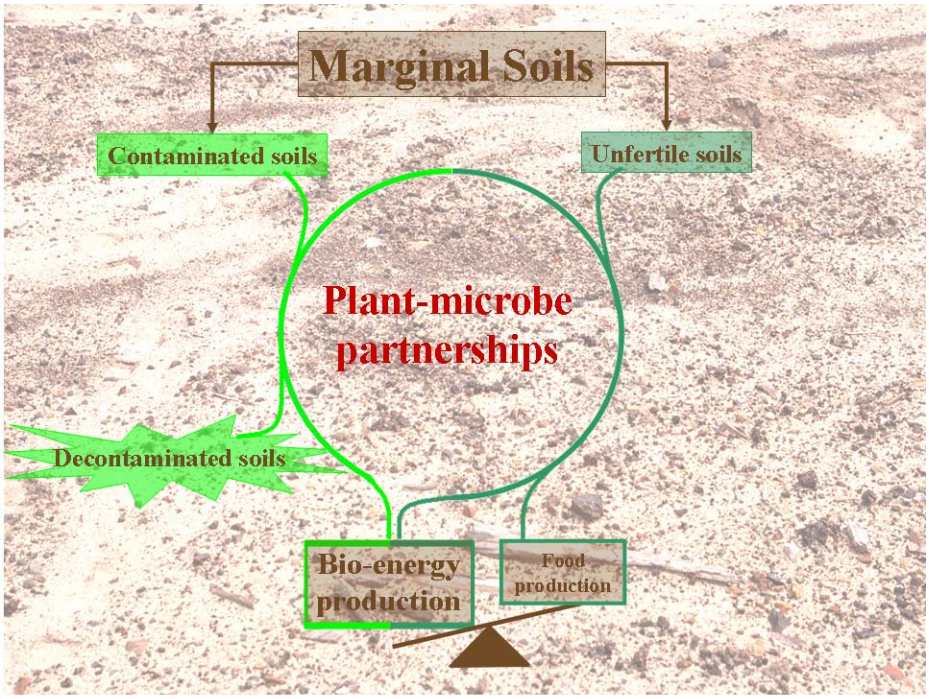


Figure 1.1: For avoiding a conflict between food and energy, energy crops can be produced on contaminated and unfertile soils, exploiting plant-microbe partnerships. In an idealized scenario, on contaminated soils, energy crop production is 'coupled' with decontamination of the soil; on unfertile soils, both, food and energy crop production can be considered.

Plant-associated bacteria can be of great value in enabling plants to establish or to grow better on marginal land, and could aid in the economic production of biofuels. Also on arable land, plant-microbe partnerships can be exploited to increase yields of food and feed production, food quality (increased concentrations of essential elements) and certainly could contribute to improve sustainability of agriculture.

Furthermore, plant-associated bacteria can assist in remediating marginal land polluted with organic contaminants and/or toxic metals. During phytoremediation of organic contaminants, plants can further benefit from endophytes possessing appropriate degradation pathways and metabolic capabilities, leading to more efficient contaminant degradation and reduction of both phytotoxicity and evapotranspiration of volatile contaminants. For phytoremediation of toxic metals, endophytes possessing a metal-

resistance/sequestration system could lower metal phytotoxicity and affect metal translocation to the above-ground plant parts. Furthermore, endophytes that can degrade organic contaminants and deal with, or even better, improve extraction of the metals offer promising ways to improve phytoremediation of mixed pollution.

In order to further reveal the potential of plant-microbe partnerships, the ecology of rhizosphere, phyllosphere and endophytic bacteria will be described. Mechanisms that are used by plant-associated bacteria to promote plant growth directly or indirectly are discussed. Moreover, the possibilities of using plant-associated bacteria to improve phytoremediation will be explored and some future perspectives for research and applications will be discussed.

Chapter 1.1 *Ecology of plant-associated bacteria*

1.1.1 Most common species hitherto

Plant-associated bacteria include endophytic, phyllosphere and rhizosphere bacteria. Endophytic bacteria can be defined as those bacteria that colonize the internal tissue of the plant without causing visible external sign of infection or negative effect of their host (Schulz and Boyle, 2006). Since these endophytic bacteria can proliferate inside the plant tissue, they are likely to interact closely with their host, face less competition for nutrients, and are more protected from adverse changes in the environment than bacteria in the rhizosphere and phyllosphere (Reinhold-Hurek and Hurek, 1998; Beattie, 2007).

Endophytic bacteria have been isolated from a wide variety of healthy plant species ranging from woody tree species such as oak (Brooks *et al*., 1994; chapter 5.3), poplar (Germaine *et al*., 2004; Taghavi *et al*., 2005; Portous-Moore *et al*., 2006; chapter 5.2) and ash (chapter 5.3), to herbaceous crop plants such as sugar beets (Jacobs *et al.*, 1985), sugar cane (Loiret *et al.*, 2004), wheat (Coombs and Franco, 2003a and b), maize (Gutierrez-Zamora and Martinez-Romero, 2001), the metal hyperaccumulating alpine pennycress (*Thlaspi caerulescens*) (Lodewyckx *et al.*, 2002a), yellow lupine (Lodewyckx *et al.*, 2001), tall fescue (Malinowski *et al.*, 2004), tobacco seeds (Mastretta *et al*., 2009) and different grass species (Zinniel *et al.*, 2002; Dalton *et al.*, 2004). However, endophytic bacteria show a tremendous amount of diversity not only in plant hosts but also in bacterial taxa. Listing for host species with their associated endophytic bacterial diversity can be found in earlier reviews (Lodewyckx *et al*., 2002b; Bacon *et al*, 2007; Hallman *et al*., 2006b). All of these isolates belong to the Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes phyla of the domain bacteria (Beattie, 2007). In general Pseudomonaceae, Burkholderiaceae, and Enterobacteriaceae are among the most common taxa of cultivable endophytic species found (Mastretta *et al*., 2006).

The phyllosphere can be referred to as the external regions of the above-ground plant parts, including leaves, stems, blossoms and fruits. Since the major

surface area available for colonization is located on the leaves, this is the dominant tissue of the phyllosphere. Bacteria residing in the phyllosphere are exposed to large and rapid fluctuations in temperature, solar radiation and water availability. In general, phyllosphere communities contain a few taxa that have a relatively large number of individuals and many taxa with a small number of individuals (Hirano and Upper, 2000). For example, Ercolani (1991) cultivated 1,701 isolates from olive leaves including 51% *Pseudomonas syringae* whereas each of the other taxa comprised less than 10%. Among the cultivable phyllosphere bacteria, Gram-negative bacteria are more common then Gram-positive bacteria. However it is complicated to make generalizations regarding these communities since the size and composition of phyllosphere communities varies greatly with plant species, leaf physiology, leaf age, and environmental conditions. This taxonomic diversity was illustrated by the work of Legard *et al*. (1994), where isolates from spring wheat phyllosphere collected over a growing season represented 37 genera and 88 species.

Root exudates are believed to have a major influence on the diversity of microorganisms within the rhizosphere (Lemanceau *et al*., 1995). Brimecombe *et al.* (2007) presented an overview of rhizosphere microorganisms associated with germinating seeds and young roots as well as with mature roots. An interesting group of bacteria within the rhizosphere community that exerts beneficial effects on plant growth was first defined by Kloepper and Schroth (1978) and termed as plant growth-promoting rhizobacteria (PGPR). As PGPR are an important subset of rhizosphere microorganisms, several studies were performed to identify PGPR in various cropping systems and agro-ecological zones (reviewed by Zahir *et al*., 2004; Ping and Boland, 2004; Tilak *et al*., 2005). Most common genera identified hitherto are *Aeromonas*, *Azoarcus*, *Azospirillum*, *Azobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Glucanobacter*, *Klebsiella*, *Pseudomonas and Serratia* (Podile and Hishore, 2007) while the exploration for new species continues.

1.1.2 Determining factors in the isolation of plant-associated bacteria

Until now, the research on plant-associated bacteria has mainly focussed on the cultivable members. Investigating plant-associated bacterial communities, the isolation procedure is a critical and essential step, especially when concentrating

on endophytic bacteria. Commonly used protocols for isolation of endophytes combine surface sterilization of the plant tissue with subsequently either maceration of the plant tissue and plating onto nutrient agar, or plating small sterilized pieces onto nutrient agar (Hallmann *et al*., 2006a). In general, surface sterilization consists of: (1) thorough washing of the plant tissue, (2) surface sterilization, (3) several aseptic rinses and (4) a sterility check. Hallmann *et al.* (2006a) listed the most frequently used sterilizing agents like sodium hypochlorite, ethanol and hydrogen peroxide, and conditions for their application. Considering the large variety in isolation procedures, comparison between different studies should be carefully evaluated. In our laboratory, sterilization procedures were optimized for the isolation of endophytic bacteria from different parts of poplar (Porteous-Moore *et al*., 2006), willow, yellow lupine (Lodewyckx *et al*., 2001), alpine pennycress (*Thlaspi caerulescens*) (Lodewyckx *et al*., 2002a), rapeseed (Lodewyckx, 2001), and tobacco (Mastretta *et al*., 2009). Beside the sterilization procedure, the choice of the growth medium is crucial in the isolation procedure as it directly affects the number and type of strains that can be isolated. The composition of most commonly used microbial growth media are listed on the webpage of the German National Resource Centre for Biological Material: http://www2.dsmz.de/media/media.htm. It should also be noticed that so far the isolation of plant-associated bacteria has mainly focussed on aerobic species, with the exception of anaerobic bacteria associated with rice.

However, even after optimization of these crucial steps in the isolation protocol, cultivation-dependent techniques still strongly underestimate the bacterial numbers, as they do not record viable but non-cultivable bacteria. Given that cultivation-independent methods for exploring microbial communities in natural habitats have suggested that cultivated isolates represent less than 1% of the bacterial taxa present (Torsvik and Øvreås, 2002), cultivation-independent methods based on rRNA gene identification should be used to obtain a more complete insight in the composition of plant-associated microbial communities. Besides, we observed that even after their initial isolation and cultivation, many endophytic bacteria could not be further propagated under laboratory conditions, supporting the importance of the analysis of their communities with molecular cultivation-independent techniques. Furthermore, the design of new cultivation-

dependent approaches provided access to an immense reservoir of unexploited microbial diversity by cultivation (Zengler *et al.*, 2005; 2002).

1.1.3 Colonization

Studies have shown that bacterial densities decrease progressively from the rhizosphere, the roots, and the stem to the leaves (Quadt-Hallman and Kloepper, 1996; Lamb *et al*., 1996; Porteous-Moore *et al*., 2006; chapter 5.3). Root colonization by rhizosphere bacteria (figure 1.2) can be considered to involve 4 stages (Brimecombe *et al*., 2007). The initial step consists of bacteria moving to the plant roots. This movement can either be passive via soil water fluxes, or active, via specific induction of flagellar activity by plant-released compounds (chemotaxis). In the second step, non-specific adsorption of bacteria to the roots takes place which is followed by anchoring (3rd step), meaning the firm attachment of a bacterial cell to the root surface. Finally, specific or complex interactions between the bacterium and the host plant may arise that can result in the induction of the expression of specific bacterial genes. Root exudate components involved in these processes are listed by Brimecombe *et al*. (2007).

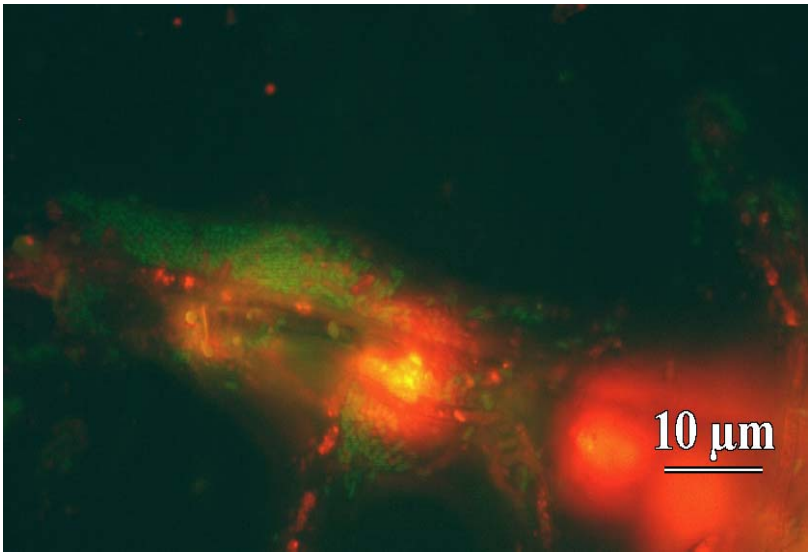


Figure 1.2: Poplar root colonization by green *gfp*-fluorescent *Pseudomonas veronii* VM1449 rhizosphere bacteria (Germaine *et al.*, 2004)

As many facultative endophytic bacteria (endophytes colonizing the plant via the roots) can also survive in the rhizosphere, it is clear that there exists a close relationship between endophytic and rhizosphere colonizing bacteria. With the exception of seed transmitted bacteria, which are already present in the embryo, the root is the primary site where endophytes gain entry into plants (Quadt-Hallman and Kloepper, 1996; Lamb *et al*., 1996; Germaine *et al*., 2004). Bacterial entry into plants mainly occurs at locations of epidermal damage, that naturally arise as a result of plant growth (lateral root formation), or through root hairs and at epidermal conjunctions (Sprent and de Faria, 1988). In addition, plant exudates leaking through these wounds are a nutrients source for the colonizing bacteria and hence create favourable conditions. This colonization strategy was confirmed by several microscopic studies (Wiehe *et al*., 1994; Benhamou *et al*. 1996a, 1996b, Pan *et al*., 1997) and supported by the analysis of the genome of *Enterobacter* sp. 638, a plant growth-promoting endophyte from poplar (Taghavi *et al*., 2009) whose genome was sequenced by the JGI (http://genome.jgi-psf.org/finished_microbes/ent_6/ent_6.home.html). This analysis revealed the presence of several gene clusters important for cell mobility.

In addition, the *Enterobacter* sp. 638 genome contains a number of genes associated with agglutination and cell adhesion, similar to those found in both animal and plant pathogens. Many of these genes are hypothesized to be important for plant colonization (Monchy *et al.*, in prep.). The genes include filamentous haemagglutinin (which is implicated in cell aggregation as a surface-exposed and secreted protein, and acts as a major virulence attachment factor) (Relman *et al.*, 1998), autotransporters with a pertactin or hemagglutinin domain (which are adhesins that are exported via the autotransporter protein mechanism) (Henderson *et al*., 1998), and virulence factors (such as those encoded by the *srfABC* operon (Worley *et al.*, 2000) located on both the chromosome and plasmid pENT638-1). In addition, plasmid pENT638-1 encodes the NdvB protein, involved in the production of beta-(1,2)-glucan and important for invasion (Ielpi *et al.,* 1990).

However, wounds and lateral roots are not absolutely required for the entrance of endophytic bacteria. For instance, the use of vector organisms (e.g.

arbuscular mycorrhizae, *Saccharicoccus sacchari* and other insects) to gain entrance to the apoplastic spaces is a possibility to colonize the host plant (Ashbolt and Inkerman, 1990; Kluepfel, 1993; Franke *et al*., 2000).

Several studies reported increased cellulase and pectinase activities during colonization of endophytes indicating that active penetration is also an option (Verma *et al*., 2001; McCulley, 2001). Although *Enterobacter* sp. 638 is not able to grow on pectin (poly(1,4-α-D-galacturonate)) as a sole carbon source, its genome carries the genes involved in pectate degradation, a demethylated backbone of pectin (Monchy *et al.*, in prep.). Furthermore, other regions on the *Enterobacter* sp. 638 genome encode for carbohydrate uptake and metabolism. No cellulose hydrolase were found on the genome of *Enterobacter* sp. 638 (Monchy *et al.*, in prep.).
Once inside the plant, endophytic bacteria either remain localized in a specific plant tissue like the root cortex or the xyleme (figure 1.3), or colonize 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).
Bacteria primarily colonize intercellularly (McCulley, 2001; Bacon and Hinton, 2002), except for particular cases such as *Azoarcus* spp. and *Rhizobium* in grasses, or *Pseudomonas stutzeri* (previously classified as: *Alcaligenes faecalis*) in rice (You *et al*., 1983 and 1991; Hurek *et al*. 1994; Vermeiren *et al.*, 1999 Rediers *et al*., 2003). In an attempt to understand the modes and sites of entry of endophytic bacteria, *gfp* (green fluorescent protein) and *egfp* (enhanced gfp) marked strains were inoculated in various plant species and studied using confocal microscopy (Coomb and Franco, 2003; Newman *et al*., 2003; Germaine *et al*., 2004; Bloemberg *et al*., 2006; chapter 4.1).

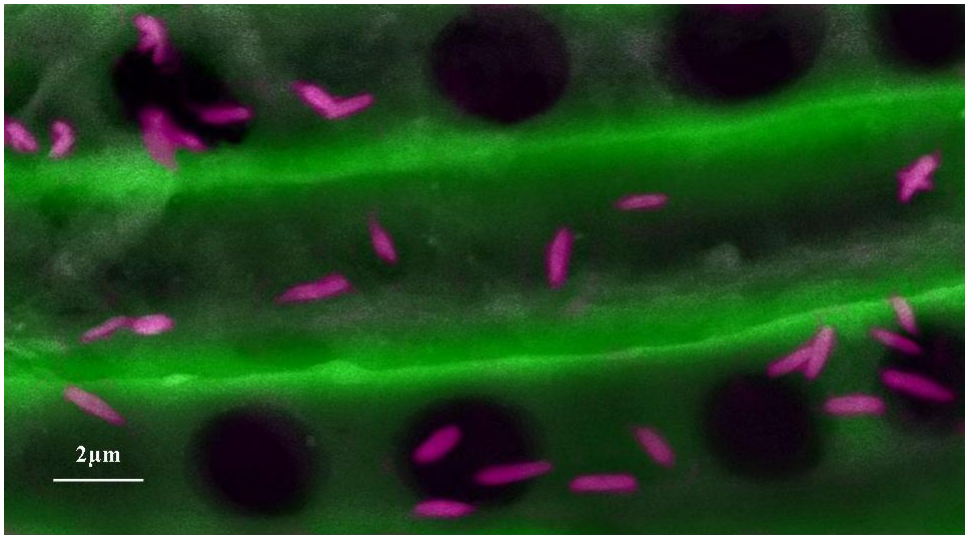


Figure 1.3: *Pseudomonas putida* W619 colonizing the root (5cm from the apex) xylem of poplar.

Although the root zone offers the most obvious site of entry for many endophytes, entry may also occur through the seeds and through natural openings in the phyllosphere such as leaf stomata and lenticels (pores for gas exchange in stems) (Kluepfel, 1993), hydathodes (water pores), and nectarthodes (opening in the nectary of blossoms) (Sharrock *et al*., 1991). Beattie and Lindow (1999) developed a general model of leaf colonization by phyllobacteria consisting of 8 important steps: bacterial immigration (1), habitat modification (2), bacterial division (3), microcolony formation (4), large aggregate formation (5), entry into internal spaces (6), habitat modification and bacterial division (7), and egression onto the leaf surface (8). However, they acknowledged that these steps will vary with different bacterial species.

Chapter 1.2 *Plant-microbe partnerships to increase biomass*

Until now, plant growth promotion has been intensively studied for rhizobacteria, as reviewed by Whipps (2001), Lugtenberg *et al.* (2001), Bloemberg *et al.* (2001) and van Loon (2007). More recently, however, attention has focussed on the plant growth-promoting capacity of endophytes (Taghavi *et al*., 2009; Mano and Morisaki, 2008). As mentioned before, there is a close relationship between rhizosphere, endophytic and phyllosphere bacteria suggesting that, presumably, they all use similar mechanisms to benefit their host plant. Mechanisms of plant growth promotion by plant-associated bacteria vary greatly, and have been broadly categorized into two groups, direct and indirect (Kloepper *et al.*, 1989) (figure 1.4). Many efforts have been made in the past two decades to elucidate both, the direct and indirect mechanisms by which plant-associated bacteria enhance plant growth. Direct plant growth-promoting mechanisms may involve nitrogen fixation by free living endophytic bacteria, especially diazotrophs, the supply of unavailable nutrients such as phosphorus and other mineral nutrients, production of plant growth regulators such as auxins, cytokinins and gibberellines, and suppression of ethylene production by 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. Plant-associated bacteria can indirectly benefit the growth of their host plant by preventing the growth or activity of plant pathogens through competition for space and nutrients, antibiosis, production of hydrolytic enzymes, inhibition of pathogen-produced enzymes or toxins and through induction of plant defence mechanisms. In general, free-living bacteria usually do not rely on one single mechanism of plant growth promotion (Glick *et al.*, 1999), and may involve a combination of the individual mechanisms listed above. Understanding the environmental factors that regulate the biosynthesis of growth-promoting and antimicrobial compounds by plant-associated bacteria is an initial step towards improving the level and reliability of their growth-promoting activity. Details of these mechanisms and their individual contribution to plant growth promotion are discussed below.

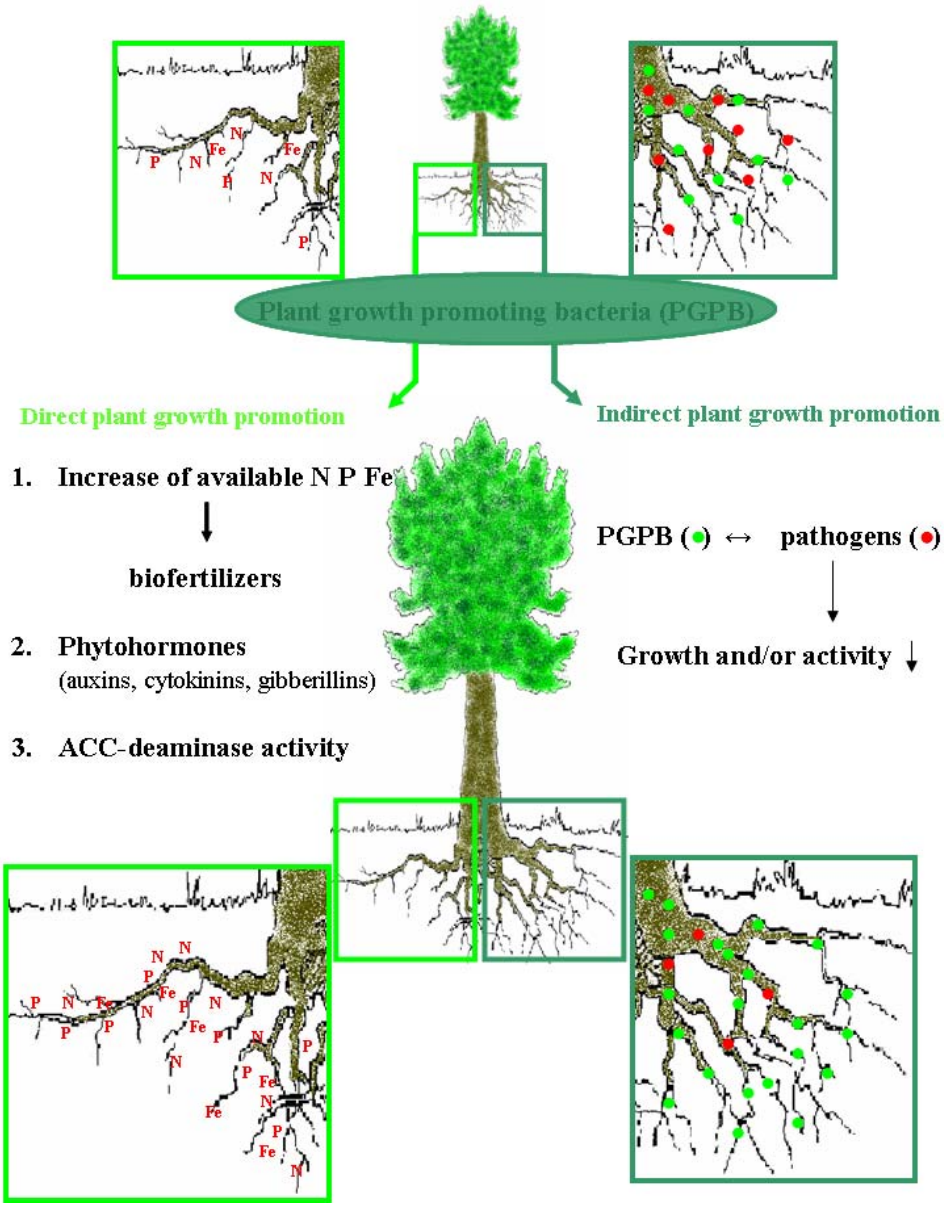


Figure 1.4: Plant-associated bacteria can promote plant growth by (1) increasing the plant-available amounts of growth limiting elements (fixating atmospheric nitrogen and solubilizing mineral nutrients unavailable for plants (P, Fe)), (2) producing phytohormones (auxins, cytokinins, gibberellins) or (3) exerting ACC-deaminase activity (resulting in a decrease stress ethylene production). Bacteria can indirectly benefit plant growth competing with pathogens and reducing their growth and/or activity.

1.2.1 Direct plant growth-promoting activity

1.2.1.1 Plant growth-promoting bacteria as bio fertilizers

A number of mineral nutrients in the soils, including nitrogen, phosphorus and iron, can frequently be limiting, thus restricting the growth of terrestrial plants. Requirements for adding these nutrients accounts for the major portion of fossil fuels used in agricultural systems (World Watch Institute, 2007) and minimal application of fertilizers is therefore desirable in order to make feedstock production economically and energetically viable. Thus, strategies to minimize fertilizer inputs by promoting biological nitrogen fixation, as well as acquisition of phosphorus and iron are important to achieve sustainable production of bioenergy feedstocks. Through to their ability to fix and solubilize mineral nutrients unavailable for plants, plant-associated bacteria can act as bio fertilizers.

Nitrogen is often a limiting factor for plant growth, despite the fact that 78% of the earth's atmosphere is nitrogen. This is because atmospheric nitrogen exists as dinitrogen (N_2), a form of the element that is inaccessible to all except a few specially adapted prokaryotic organisms including some eubacteria, cyanobacteria and actinomycetes. For all other organisms (also plants), nitrogen should occur under the form of ammonia or nitrate before it can be incorporated into organic molecules. Higher plants cannot carry out this process in the absence of associated diazotrophic bacteria. These bacteria possess the enzyme nitrogenase, an O_2-sensitive enzyme catalyzing the reduction of atmospheric nitrogen to ammonia. Diazotrophic bacteria can be grouped into those that involve close associations with plants, often called symbioses and those that involve only loose associations, often called associative interactions.

Symbiotic diazotrophic bacteria exhibit highly specific, intimate interactions leading to the induction of new organs or organ-like structures in the host. The intimacy of such associations maximizes the transfer of fixed nitrogen to the host plant. The plant provides the prokaryotic symbiont with a protected environment and supplies it with energy-rich compounds, which are necessary to support the high energy demands of nitrogen fixation. Symbiotic diazotrophs consist of (a) legume symbionts, which are Gram-negative bacteria that form

nodules on roots of leguminous plants, (b) members of the genus *Frankia*, which are Gram-positive bacteria that form nodules on roots of woody, dicotyledonous trees and shrubs and (c) cyanobacteria that form mutualisms with many Pteridophytes. All legume symbionts were originally classified into the genus *Rhizobium*, although currently these symbionts are classified into several genera (for review: Beattie, 2007) with most species belonging to the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Bradyrhizobium*.

Associative diazotrophic bacteria vary in how closely they interact with their host plant. It is tempting to speculate that increasing closeness in this association is corresponding to an increasing capacity to transfer fixed nitrogen to the plant. Furthermore, it is reasonable to suppose that nitrogen fixed by free-living diazotrophs on plant surfaces is transferred less efficiently to the plant host and is subject to greater losses than nitrogen fixed by endophytic bacteria. The key factors that influence the quantity of fixed nitrogen by associative nitrogen-fixing bacteria are the carbon availability and the capability of the heterotrophic N_2-fixing bacteria to capture and use it efficiently (Rao *et al.*, 1998). Kennedy and Tchan (1992) underlined that free-living N_2-fixing bacteria are distant from the main sources of carbon compounds, and are in competition with other microorganisms for these substrates. However, in case of endophytic diazotrophic bacteria, the competition for C-sources is much lower and the quantity of free oxygen that inhibits the nitrogenase activity is low. Associative diazotrophic endophytes have been primarily isolated from grasses such as maize, rice, sorghum, sugarcane and wheat. The most profoundly investigated diazotrophic endophytes are *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp. in sugarcane, *Azoarcus* spp. in Kallar grass, and *Alcaligenes*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas* and *Rhizobium* in rice and maize (James, 2000).

The efficiency of diazotrophic endophytes to promote plant growth has been demonstrated in several greenhouse and field studies of different plant species; for example, sugarcane (Boddey *et al.*, 1995), wheat (http://www.cropscience.org.au/icsc2004/poster/2/5/6/863_anyiaa.htm), soybean (Mishra *et al.*, 2009) and rice (Mano and Morisaki, 2008; Boddey *et al.*, 1995). These studies have reported considerable promotion of growth and of biomass production. For example, grain yield and total biomass of wheat that had been

inoculated with the diazotroph *Azorhizobium caulinodans* increased by 34 and 39%, respectively (http://www.cropscience.org.au/icsc2004/poster /2/5/6/863_ anyiaa.htm). Dobbelaere *et al.* (2003) demonstrated that, apart from fixing nitrogen, associative diazotrophs exert these positive effects on plant growth directly or indirectly through (a combination of) different mechanisms. Recently, Mishra *et al.* (2009) demonstrated that the beneficial effect of nodule forming diazotrophs could be enhanced when inoculation with diazotrophic rhizobacteria was combined with that of plant growth-promoting bacteria. The greatest nodule number and subsequent biomass yield could be achieved after co-inoculating soybean with the rhizobacteria *Bradyrhizobium japonicum*-SB1 and the plant growth-promoting *Bacillus thuringiensis*-KR1. Zakria *et al.* (2008) investigated the effects of inoculating dicot plants with N_2-fixing endophytic bacteria isolated from monocots and found that these monocot isolates could indeed promote plant growth.

Several studies, such as N-balance, ^{15}N isotope dilution and ^{15}N natural abundance, have supplied evidence that some plants can obtain at least part of their nitrogen from associative nitrogen fixation. This transfer of fixed nitrogen to the host plant can be directly or indirectly due to the death and subsequent mineralization of the bacterial cells. Many reports proved a direct contribution of endophytic bacteria to plant nitrogen fixation, such as in sugarcane (Boddey *et al*., 1995; James, 2000), rice (Yanni *et al*., 1997; Malik *et al*., 1997; Ladha and Reddy, 2005) and wheat (Webster *et al*., 1997; Iniguez *et al.*, 2004). Certain Brazilian cultivars of sugarcane obtain over half their needs for nitrogen from biological nitrogen fixation (Boddey *et al*., 1995). Other studies have shown that up to 70% of the nitrogen in sugarcane grown with *Gluconobacter diazotrophicus* can originate from biological nitrogen fixation (James, 2000). In rice plants, inoculation with *Azospirillum* contributed to 66% of the total ammonium present in the plants (Malik *et al*., 1997). Poplars (*Populus* spp.) and willows (*Salix* spp.) are suitable tree species for both biomass production, but also for phytoremediation purposes and an investigation of their associated bacteria found that some of their endophytes appear to be capable of fixing nitrogen (Doty, 2008). The presence of these diazotrophic microorganisms could help to explain how these tree species are able to grow under nitrogen limitation

on marginal land. However, until now, the biological role of endophytes in supplying nitrogen to their host plant has not been confirmed.

Phosphorous is second to nitrogen among the mineral nutrients that limit terrestrial plant growth. Phosphorous often is abundant in soil, but mostly in an insoluble form. Even in phosphorous rich soils most of the P is insoluble and only a very limited proportion (≈ 0.1%) is available to plants (Stevenson and Cole, 1999). Plants only can take up P when it is in its monobasic ($H_2PO_4^-$) or dibasic (HPO_4^{2-}) soluble form (Glass, 1989). In addition, 75% of the phosphate fertilizers applied to soil is rapidly immobilized and becomes unavailable to plants (Nautiyal, 1999; Rodriguez and Fraga, 1999). Phosphate solubilizing and phosphate mineralizing bacteria that can enhance plant growth are common in the rhizosphere (Nautiyal *et al*., 2000; Vazquez *et al*., 2000) and include *Azotobacter chroococcum*, *Bacillus* spp., *Enterobacter agglomerans*, *Pseudomonas chlororaphis*, *Pseudomonas putida*, and *Rhizobium* and *Bradyrhizobium* spp. (Vessey, 2003). They can solubilize inorganic phosphates by releasing organic acids such as gluconic acid and 2-ketogluconic acid, or can mineralize organic phosphates by secreting extracellular phosphatases (Kim *et al*., 1998). Some recent studies on the beneficial association of phosphate-solubilizing rhizospheric bacteria and their host plant include *Bacillus circulans* and *Cladosporium herbarum* (Singh and Kapoor, 1999), and *Azotobacter chroococcum* (Kumar and Narula, 1999) with wheat; *Pseudomonas chlororaphis* and *P. putida* with soybean (Cattelan *et al*., 1999); *Bacillus* sp. with five crop species (Pal, 1998); *Rhizobium* sp. and *Bradyrhizobium japonicum* with radish (Antoun *et al*., 1998); *Enterobacter agglomerans* with tomato (Kim *et al*., 1998); and *Rhizobium leguminosarum* with maize (Chabot *et al*., 1998). Also endophytic bacteria have 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. This suggestion was further supported by Kuklinsky-Sobrial *et al.* (2004), showing that 52% of the endophytic bacteria isolated from soybean could solubilize mineral phosphate.

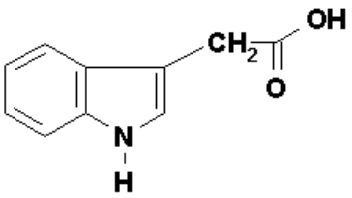
In *Brassica juncea*, inoculation with *Bacillus subtilis* SJ-101, which is also considered a potential phosphate solubilizer, resulted in increases in shoot and root length and in biomass (Zaidi *et al*., 2006). Hameeda *et al.* (2008) selected several bacterial strains that solubilized phosphorus in maize rhizosphere and that were efficient in enhancing plant growth and grain yield. Use of such bacteria, either as seed inoculants, or in compost production, would increase plant productivity and reduce the need for additional phosphorus application.
It is evident that phosphate mobilization will especially be important in the rhizosphere. Therefore it is not surprising that the rhizosphere strain *Pseudomonas putida* KT2440, possesses three ABC-type phosphate transport operons (Dos Santos *et al.,* 2004) compared to only one in the closely related endophytic strain *P. putida* W619 (Monchy *et al.*, in prep.). Consistently, other endophytic bacteria like *Enterobacter* sp. 638 and *Serratia proteamaculans* 568 were found to possess only one ABC phosphate transport operon (Monchy *et al.*, in prep.).

Iron in the environment is, like phosphorous, often present in an insoluble form, more specifically the highly insoluble ferric hydroxide form. Many bacteria produce organic compounds, called siderophores, which bind Fe^{3+} and render it available for conversion to the preferred form, Fe^{2+}. The plant-microbe interactions involved in the regulation of siderophore production and their role in mediating competition for iron in the rhizosphere were reviewed by Crowley *et al.* (2007). Bacterial Fe^{3+}-siderophore complexes may not only facilitate uptake of iron into bacteria. Moreover, evidence exists that several plant species can recognize and take up bacterial Fe^{3+}-siderophore complexes, and that, especially in calcareous soils, this process is crucial for the uptake of iron by plants (Masalha *et al*., 2000; Sharma and Johri, 2003; Sharma *et al*., 2003; Katiyar and Goel, 2004).
Bacteria developed several distinct mechanisms to compete against each other for iron resources, a concept used by plant growth-promoting bacteria to protect their host plant against pathogens. They can produce a high number of specific iron uptake transporters, secrete large numbers of diverse siderophores (which is energy costly), or synthesize siderophore receptors to utilize siderophores excreted by other bacteria.

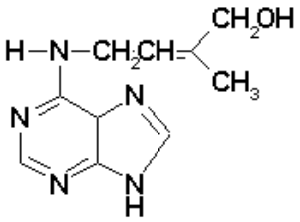
During its adaptation to survive in the soil prior to colonization of a host plant, or as a plant growth-promoting endophyte, *Enterobacter* sp. 638 has developed an intermediate solution to deal with iron uptake. *Enterobacter* sp. 638 contains two ferrous iron uptake systems and nine iron ABC transporters (Monchy *et al.*, in prep.). This number is much higher than the four iron ABC transporters present in *E. coli* K12 or the three found in *P. putida* KT2440 (Dos Santos *et al.*, 2004). *Enterobacter* sp. 638 is also able to synthetize the siderophore enterobactin, to secrete it, recover the iron-enterobactin complex using a ferric siderophore uptake system, and to extract the iron using an enterobactin esterase after internalization of the iron-enterobactin complex. In addition, *Enterobacter* sp. 638 possesses 12 outer membrane ferric and ferric-related siderophore receptors. In contrast, *E. coli* only possesses 6 siderophore receptors, while *P. putida* KT2440 is equipped with 18 (Dos Santos *et al.*, 2004), which is consistent with the concept of a plant growth-promoting rhizosphere bacterium that needs to compete for iron resources present in the environment (see 1.3.2.1).

1.2.1.2 Phytohormones and plant growth-promoting compounds

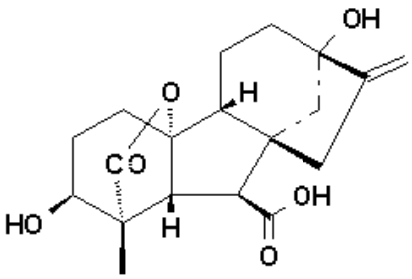
Phytohormones produced by plant-associated bacteria often stimulate plant growth. However, this bacterial production of phytohormones does not have a direct benefit for the bacteria itself and should be motivated in an indirect way. The stimulated plant growth that is induced by bacterial phytohormone production will provide more nutrients to the plant-associated bacteria. Auxins, cytokinins and gibberellins (figure 1.5) may be considered as causal agents for altering plant growth and development (Leifert *et al.*, 1994; Bashan and Holguin, 1997; Tanimoto, 2005; Taghavi *et al.*, 2009). However, because phytohormone production by bacteria does not directly benefit the bacteria, such production needs to be achieved in an indirect way because stimulated plant growth in turn provides more nutrients to these plant-associated bacteria.



Auxins



Cytokinins



Gibberellines

Figure 1.5: Structure of most common auxins, cytokinins and gibberellines.

The most investigated phytohormone produced by plant-associated bacteria is the **auxin** indole-3-acetic acid (IAA). IAA produced by *Azospirillum brasilense*, *Aeromonas veronii*, *Agrobacterium* spp., *Alcaligenes piechaudii*, *Bradyrhizobium* spp., *Comamonas acidovorans*, *Enterobacter* spp, and *Rhizobium leguminosarum*, can contribute to plant growth promotion (Vessey, 2003).

The production of IAA can increase root growth and root length; it also has been associated with proliferation and elongation of root hairs (Taghavi *et al*., 2009). Depending on the type of bacteria, the IAA production seems to follow different pathways. In plant beneficial bacteria, IAA is predominantly synthesized via indolepyruvic acid, although in phytopathogenic bacteria, IAA is generally produced from tryptophan via the intermediate indoleacetamide (Manulis *et al*., 1998; Patten and Glick, 2002).

IAA-producing plant beneficial bacteria have been isolated from lettuce (Barazani and Friedman, 1999), wheat (Kaushik *et al*., 2000), rice (Mehnaz *et al*., 2001), sugarcane (Mirza *et al*., 2001) and poplar (Taghavi *et al*., 2005; Taghavi *et al.*, 2009). In association with poplar, the endophytes *Enterobacter* sp. 638, *Serratia proteamaculans* 568, *Stenotrophomonas maltophilia* R551-3,

and *Pseudomonas putida* W619 were shown to produce IAA (Taghavi *et al.*, 2009).

In studies conducted to determine the specific role of IAA production in plant growth promotion, contrasting observations were made. A mutant strain of *Pseudomonas putida* showing a four-fold increase in IAA production lost its capacity to induce root elongation in canola seedlings, despite the fact that its growth rate and production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase and siderophores remained unaffected (Xie *et al*., 1996). However, in other studies, bacterial IAA production was positively related to changes in plant growth and development. In *Brassica* spp., a positive correlation was observed between auxin production by different PGPR strains and their ability to increase the grain yield and numbers of branches and pods per plant (Asghar *et al*., 2002). This observation was further supported by the positive relation between auxin production by PGPR and the increase in number of branches and oil content in *B. napus* inoculated with these PGPR (Asghar *et al*., 2004). Root elongation in *B. napus* was also shown to be stimulated by IAA-synthesizing PGPR (Sheng and Xia, 2006), as well as by yet unidentified rhizobacteria occurring on *B. juncea* roots (Belimov *et al*., 2005). In addition, roots of *B. juncea* elongated by up to 41% after inoculation with *Variovorax paradoxus* 5C-2 (Belimov *et al*., 2005), and root dry weight was increased by up to 20% (Sheng and Xia, 2006). In lodgepole pine seedlings inoculated with auxin-producing *Paenibacillus polymyxa* L6, growth promotion was correlated with the increased levels of auxin present in the roots (Bent *et al*., 2001). In greenhouse studies, it was shown that the growth of poplar, a potential biofuel plant, was improved by up to 60% after inoculation with different IAA-producing endophytic strains (Taghavi *et al.*, 2009; chapter 4.1).

In order to identify the plant genetic determinants of bacterial colonization Persello-Cartieaux *et al*. (2001) used mutants of *Arabidopsis thaliana* affected in root hair development and possible hormone perception. In this study, the role of bacterial auxin altering the root morphology was supported by the observation that two auxin-resistant mutants were insensitive to *Pseudomonas thivervalensis* colonization.

Concerning the role of hormones other than auxin, such as cytokinins and gibberellins, less detailed information is available. **Cytokinins** are known to

stimulate cell division, cell enlargement and tissue expansion in certain plant parts (Salisbury, 1994). Furthermore, they can promote stomatal opening, stimulate shoot growth and decrease root growth. When soil is drying, natural cytokinin concentrations decrease in association with stomatal closure and growth is redirected away from the shoots to the roots (Hare *et al*., 1997). Although decreased cytokinin levels induced by water shortage contribute to drought tolerance, Arkhipova *et al.* (2007) demonstrated that preventing the loss of cytokinin in soil by inoculation with cytokinin-producing bacteria was beneficial for plant growth under moderate drought conditions. Helping food and feed plants to tolerate drought (as well as other forms of abiotic stress) will become very important in a changing climate (Yang *et al*., 2009). Cytokinin-producing PGPR have been isolated from rape and lettuce (Noel *et al*., 1996), wheat (Timmusk *et al*., 1999), soybean (de Salamone *et al*., 2001), and pine (Bent *et al*., 2001). However, to which extent these compounds play a role in plant growth promotion is still not unravelled. **Gibberellins** (gibberellic acid) are involved in modifying plant morphology by the extension of plant tissue, in particular of the stem (Salisbury, 1994). Compared with auxins, gibberellin functions in roots are less important, but they nevertheless play an indispensable role in normal root development (Tanimoto, 2005). Although gibberellin production by plant-associated bacteria seems to be less widespread, *Bacillus pumilus* and *Bacillus licheniformis* spp. (Gutierrez-Manero *et al*., 2001) and *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* spp. (Bastián *et al.*, 1998) that were able to produce this phytohormone, were isolated and were shown to improve plant growth and yield.

Volatile compounds such as 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol are emitted by rhizobacteria to enhance plant growth. *Enterobacter* sp. 638 and *Serratia proteamaculans* 568 possess the genetic capability to convert pyruvate to acetolactate (acetolactate synthase) (Monchy *et al.*, in prep). Acetolactate is converted either into diacetyl (spontaneously under aerobic conditions), or into acetoin (acetolactate decarboxylase). *Enterobacter* sp. 638 possesses an additional gene to convert diacetyl into acetoin (acetoin dehydrogenase), which can be released and converted into 2,3-butanediol (2,3-butanediol dehydrogenase) by the plant. Interestingly, the poplar genome encodes the genes necessary for the conversion of acetoin into 2,3-butanediol, but not for

the biosynthesis of acetoin itself. Therefore it seems to rely on endophytic bacteria like *Enterobacter* sp. 638 to synthesize the plant growth hormones acetoin and 2,3-butanediol (Monchy *et al.*, in prep.).

1.2.1.3 Counteracting stress-induced ethylene

Ethylene is a phytohormone that is known to be increased in plants exposed to both abiotic and biotic environmental stress conditions. Among its multiple effects on plant development (from seed germination, morphogenesis, flowering induction up to senescence), frequently investigated effects include the inhibition of root elongation, lateral root growth and root hair formation (Mayak *et al.*, 2004). Therefore, reductions in ethylene production should lead to an indirect promotion of root elongation. Bacteria can affect ethylene production via two main mechanisms (Mastretta *et al.*, 2006): (a) some bacteria can balance ethylene hormone levels through auxin production; (b) however, the most commonly observed mechanism to reduce ethylene production levels is bacterial ACC-deaminase activity. ACC-deaminase was observed in bacterial strains of *Alcaligenes* spp., *Bacillus pumilus*, *Enterobacter cloacae*, *Burkholderia cepacia*, *Pseudomonas putida*, *Pseudomonas* spp. and *Variovorax paradoxus* (Vessey, 2003; Babalola *et al*., 2003), but is not produced by the endophytic bacteria *Enterobacter* sp. 638, *Serratia proteamaculans* 568, *Stenotrophomonas maltophilia* R551-3 and *Pseudomonas putida* W619 (Taghavi *et al.*, 2009). The working mechanism is believed to be via cleavage of ACC, which is the immediate precursor to ethylene during ethylene biosynthesis in roots, with a net result of increased root growth (Glick *et al*., 1998). Bacteria originating from different soils and containing ACC-deaminase activity, stimulated plant growth, even in soils containing phytotoxic cadmium concentrations (Belimov *et al*., 2005). Furthermore, Dell'Amico *et al*. (2005) underlined that most of the PGPR isolated from grasses (*Poaceae*) growing on a heavy metal polluted meadow show ACC-deaminase activity resulting in plant growth promotion.

1.2.2 Indirect plant growth-promoting activity

1.2.2.1 Competition

Competition has been claimed as an important mechanism of biocontrol, since both, pathogens and non-pathogenic plant-associated bacteria colonize similar

niches and utilize the same nutrients. However, experimental evidence for this claim still hardly exists.

Many plant growth-promoting bacteria, especially pseudomonads, produce high-affinity Fe^{3+} binding siderophores under iron-limiting conditions (Kloepper *et al.*, 1980; Teintze *et al.*, 1981). By binding available iron, these bacteria deprive pathogenic bacteria and fungi of iron, which could limit their growth. Many authors have demonstrated the importance of siderophores in the inhibition of both fungal and bacterial pathogens (Geels *et al.*, 1983; Vandenbergh *et al.*, 1983; Xu *et al.*, 1986; Buyer and Leony, 1986; O'Sullivan and O'Gara, 1992; Compant *et al.*, 2005; Miethke *et al.*, 2007).

1.2.2.2 Antibiosis

Antibiosis is the production and release of molecules that kill or inhibit the growth of the target pathogen, and is the best-known mechanism by which microbes can control plant diseases (Dowling and O'Gara, 1994). The antibiotics generally produced by diverse antagonistic bacteria consist of ammonia, butyrolactones, 2,4-diacetyl phloroglucinol (DAPG), kanosamine, oligomycin A, oomycin A, phenazine-1-carboxylic acid, pyoluteorin, pyrrolnitrin, viscosinamide, xanthobaccin, and zwittermycin A (Whipps, 2001). Many of these antibiotics possess a broad-spectrum activity, and DAPG was the most effective and most extensively studied antibiotic (Raaijmakers *et al.*, 2002). The role of individual antibiotic compounds in suppression of root pathogens has been clearly established using mutation analysis, molecular genetic tools, and using purified antibiotic compounds. In addition, the structures and mode of action of many antimicrobial compounds have been extensively reviewed (Chin-A-Woeng *et al.*, 2003; Ramoz-Gonzalez *et al.*, 2005).

Recently, biosurfactants have been investigated as antimicrobial compounds (Bais *et al.*, 2004; Nielson *et al.*, 1999 and 2000; Thrane *et al.*, 2000). As pathogens frequently form a biofilm on the root surface, it is interesting to note that some biosurfactants prevent biofilm formation and even degrade existing biofilms (Kuiper *et al.*, 2003; Bais *et al.*, 2004). For instance, *Pseudomonas fluorescens* produces cyclic lipopeptide surfactants such as viscosinamide (Nielson *et al.*, 1999) and tensin (Nielson *et al.*, 2000), which have antifungal activity against *Rhizoctonia solani* and *Pythium ultimum* (Thrane *et al.*, 2000).

1.2.2.3 Production of hydrolytic enzymes

Cell wall lysis is another potential mechanism for plant-associated bacteria to control fungal pathogens. This mechanism is well established in the biocontrol of fungal pathogens in the rhizosphere (Backman and Sikora, 2008). Endophytic bacteria isolated from potato roots express high levels of hydrolytic enzymes such as cellulase, chitinase and glucanase (Krechel *et al.*, 2002). However, the endophytic bacteria *Enterobacter* sp. 638, *Serratia proteamaculans* 568 and *Pseudomonas putida* W619 did not possess members of the cellulase/endoglucanase, lichenase and xylanase families of glycosyl hydrolases (GH). This observation is consistent with the non-phytopathogenic behavior of these bacteria (Monchy *et al.*, in prep.).

Among the hydrolytic enzymes, chitinases are of major importance since chitin is a main cell wall component in the majority of the phytopathogenic fungi. The endophytic bacteria *Serratia proteamaculans* 568, *Pseudomonas putida* W619 and *Stenotrophomonas maltophilia* R551-3 both possess chitinases and are able to grow on chitin as carbon source. (Monchy *et al.*, in prep.). Purified chitinases of *Bacillus subtilis* AF 1 (Manjula *et al.*, 2004), *Serratia plymuthica* (Frankowski *et al.*, 2001), and *Serratia marcescens* (Ordentlich *et al.*, 1988; Kishore *et al.*, 2005) performed high antifungal activity. Furthermore, the role of chitinolysis in root pathogen suppression has been supported by improved disease control in chitin-supplemented treatments of chitinolytic PGPR (Manjula and Podile, 2001; Hallmann *et al.*, 1999). Gluconases are another important group of hydrolytic enzymes since they degrade the β-1,3-glucans of the fungal cell walls. The production of β-1,3-glucanases by *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) induced an inhibition in rhizosphere proliferation of various phytopathogenic fungi including *Rhizoctonia solani* and *Pythium ultimum* (Fridlender *et al.*, 1993). Furthermore, Tanaka and Watanabe (1995) observed that a synergistic action of two hydrolytic enzymes chitinases and β-1,3-glucanases resulted in a more effective inhibition of fungal pathogens than by individual enzymes.

1.2.2.4 Inhibition of pathogen-produced enzymes or toxins

In order to degrade the polymers in plant cell walls and so facilitate the fungal infection, pathogenic fungi produce extracellular hydrolytic enzymes including pectolytic enzymes (exo- and endo- polygalacturonases, pectin lyases),

cellulases and cutinase. A suppression of the activity of these enzymes correlates with a reduction in virulence (Beraha *et al*., 1983). For instance, the activities of extracellular enzymes, such as cellulase, pectin lyase and pectinase produced by *Rhizoctonia solani*, were inhibited by *Bacillus megaterium* B 153-2-2 producing an extracellular endoproteinase (Bertagnolli *et al*., 1996).

1.2.2.5 Induction of plant defence mechanisms

After contact with a necrotizing pathogen or a non-pathogenic biocontrol bacterium, a state of physiological immunity can be induced in plants, protecting the plant against subsequent viral, bacterial or fungal attacks (Wei *et al.*, 1991; Raupach *et al.*, 1996). The induced resistance associated with the colonization of plant roots by certain plant growth-promoting rhizobacteria (PGPR), has been referred as induced systemic resistance (ISR) (reviewed in van Loon *et al*., 1998; Knoester *et al.*, 1999). ISR is characterized by remote action, long-lasting resistance, and protection against a large number of pathogens. The majority of resistance-inducing microbes described hitherto are Gram-negative bacteria, with mainly *Pseudomonas* and *Serratia* strains (Van Loon *et al.*, 1998). However, also a number of Gram-positive bacteria were reported to induce resistance (Kloepper *et al.*, 2004).

ISR can activate multiple potential defence mechanisms, including an increase in activity of chitinases, β-1,3-glucanases, peroxidases, and other pathogensis-related proteins (Lawton and Lamb, 1987), accumulation of antimicrobial low-molecular-weight substances, such as phytoalexins (Kuc and Rush, 1985) and the formation of protective biopolymers, such as lignin, cellulose, and hydroyproline-rich glycoproteins (Hammerschmidt and Kuc, 1982; Hammerschmidt *et al.*, 1982; Hammerschmidt *et al.*, 1984). In addition, a single inducing agent can control a wide variety of pathogens.

Studies on indirect plant growth-promoting activity mainly focus on rhizosphere bacteria and endophytes. However, phyllosphere bacteria present on the leaf surfaces or those introduced as foliar sprays can also suppress plant pathogenic microorganisms of global importance. *Erwinia herbicola*, the phyllosphere bacterium present on the leaf surfaces of rice was known to lower the pH of the rice leaf resulting in a difficult growth for the bacterial pathogen (*Xanthomonas oryzae* pv. *Oryzae*) (Hsieh and Buddenhagen, 1974; Santhi *et al*., 1987).

Gnanamanickam and Immanuel (2007) listed a number of rice phyllosphere associated strains of *Pseudomonas fluorescens* and *Bacillus* spp. that have shown good potential for suppression of four major foliar diseases of rice.

Chapter 1.3 *Plant-Microbe partnerships in phytoremediation*

We often think of plants primarily as a source of food, fuel and fiber. However, the potential of plants and their associated microorganisms to serve as an environmental counterbalance to industrial pollution has recently been recognized. Although the use of marginal land contaminated with organic contaminants and/or toxic metals for biofuel production can reduce the need to use viable cropland, it is also important to remove contaminants from these sites. Established, primarily civil-engineering based physico-chemical methods to remediate contaminated soils and groundwater are frequently expensive, environmentally invasive, labour intensive, and do not make cost-effective use of existing resources. Among these techniques, the excavation and removal of contaminated soil, pump and treat of contaminated groundwater or an *ex situ* treatment of the soil that drastically alters soil structure, biological activity and subsequent function, are commonly applied. Especially in case of large-scale contaminated areas there is an obvious need for cost-effective, sustainable and validated alternative remediation strategies. For this reason, in the last decade much research was focussed on the use of biological remediation methods to minimize the inherent risk associated with contaminated soils and groundwater.
Generally, strategies making use of plant-microbe partnerships are classified under the heading of phytoremediation. Phytoremediation is an *in situ*, solar powered technology that requires minimal site disturbance and maintenance resulting in a low cost and a high public acceptance. This technology can be applied to clean up and/or stabilize both, inorganic as well as organic contaminants (Prasad *et al*., 2009; Schwitzguébel *et al.*, 2009).

1.3.1 Phytoremediation of organic contaminants

1.3.1.1 Plant uptake of organic contaminants

Considering the whole plant metabolism of organics, the entry of the organic chemical in the plant is the first critical step. Organic contaminants can reach the plant through translocation of soil water with the transpiration stream, or through transfer from the soil gas phase, or from atmospheric gases or water to the foliar tissues. Although foliar uptake has been proposed as a major sink of some anthropogenic contaminants in the atmosphere (Jeffers and Liddy, 2003;

Morikawa *et al.*, 2003), root uptake through translocation with the transpiration stream still is the main uptake mechanism considered in phytoremediation of waste streams or contaminated soil and water.

Table 1.1: Log K_{ow} (octanol-water partition coefficient) values of some frequently found organic contaminants

2-Butanone	0.3	3-Chlorobenzoic acid	2.7
4-Acetylpyridine	0.5	Toluene	2.7
Aniline	0.9	1-Naphtol	2.7
Acetanilide	1.0	2,3-Dichloroaniline	2.8
Benzyl alcohol	1.1	Chlorobenzene	2.8
4-Methoxyphenol	1.3	Allyl phenyl ether	2.9
Phenoxyacetic acid	1.4	Bromobenzene	3.0
Phenol	1.5	Ethylbenzene	3.2
2,4-Dinitrophenol	1.5	Benzophenone	3.2
Benzonitrile	1.6	4-Phenyl phenol	3.2
Phenylacetonitrile	1.6	Thymol	3.3
4-Methylbenzyl alcohol	1.6	1,4-Dichlorobenzene	3.4
Acetophenone	1.7	Diphenylamine	3.4
2-Nitrophenol	1.8	Naphtalene	3.6
3-Nitrobenzoic acid	1.8	Phenyl benzoate	3.6
4-Chloraniline	1.8	Isopropylbenzene	3.7
Nitrobenzene	1.9	2,4,6-Trichlorophenol	3.7
Cinnamic alcohol	1.9	Biphenyl	4.0
Benzoic acid	1.9	Benzyl benzoate	4.0
p-Cresol	1.9	2,4-Dinitro-6-sec-butyl phenol	4.1
cis-Cinnamic acid	2.1	1,2,4-Trichlorobenzene	4.2
trans-Cinnamic acid	2.1	Dodecanoic acid	4.2
Anisole	2.1	Diphenyl ether	4.2
Methyl benzoate	2.1	Phenanthrene	4.5
Benzene	2.1	n-Butylbenzene	4.6
3-Methylbenzoic acid	2.4	Fluoranthene	4.7
4-Chlorophenol	2.4	Dibenzyl	4.8
Trichloroethene	2.4	2,6-Diphenylpyridine	4.9
Atrazine	2.6	Triphenylamine	5.7
Ethyl benzoate	2.6	DDT	6.2
2,6-Dichlorobenzonitrile	2.6		

Root uptake of organic compounds from soil is affected by (a) the physicochemical properties of the compound, (b) the environmental conditions (such as organic matter, pH and moisture), and (c) by plant characteristics (such as root surface area) (Ryan *et al*., 1988; Paterson *et al*., 1990). In case of constant plant and environmental characteristics, root uptake has been shown to be directly proportional to the chemical's lipophilicity which is expressed as the chemical's octanol-water partition coefficient (K_{ow}) (table 1.1).

In practice, an optimum range of lipophilicity exists (log K_{ow} between 1 and 3.5) outside of which uptake and translocation of organic molecules in plants is severely restricted. Organic contaminants with a log $K_{ow} < 1$ are considered to be very water-soluble and are missing any specific affinity to be taken up into plant roots and then be translocated through the vascular system to the shoot (Cunningham and Berti, 1993), whereas contaminants with a log $K_{ow} > 3.5$ are so tightly sorbed onto root surfaces that their uptake and translocation to the shoot is limited (Trapp *et al*., 2001).

1.3.1.2 Plant-bacteria synergism during phytoremediation

After being transported into the plant, an organic chemical can be metabolized in plant cells and/or evapotranspired through the stem and the leaves into the atmosphere. Although the plant may often metabolize or sequester environmental toxins, plants are at a significant disadvantage in two ways. At first, being photoautotrophic, plants do not rely on organic compounds as a source of energy or carbon. As such, unlike microbial systems, they were not under selective pressure to develop the capacity to degrade chemically intransigent materials, resulting in a more restrictive set of chemical structures that can be metabolized by plants. Secondly, plant metabolism of organic carbon (other than photosynthates) follows general transformations to more water-soluble forms, and sequestration processes to avoid build-up and potential toxicity to sensitive organelles (green-liver model: Burken, 2003). In contrast, microbial metabolism often ends with the compound being transformed to CO_2, water and cellular biomass.

Therefore, in order to obtain a more efficient degradation of organic contaminants, plants rely on their associated microorganisms (figure 1.6). Plants themselves have a positive effect on the microbial degradation of organic contaminants (Crowley *et al*., 1997). The most commonly proposed explanation

for this positive effect is a higher microbial density and metabolic activity in the rhizosphere as a result of growth on carbon substrates (root exudates and death cells) provided by the plant roots. Furthermore, diverse species of heterotrophic microorganisms are brought together at high population densities in the rhizosphere, the phyllosphere and inside the plant (endosphere), which may enhance stepwise transformation of xenobiotics by consortia, or provide an environment that is conducive to genetic exchange and gene rearrangement. Additionally, the plant's water evapotranspiration may also influence the transport of water soluble compounds by increasing their mass flow to the root surface where they can be acted upon by the rhizosphere microflora. In conclusion, plant-microbe partnerships are an important key for a successful remediation of organic contaminants. The importance of these plant-microbe partnerships in the remediation of organic pollutants was confirmed in studies at the level of the rhizosphere (Ho *et al.*, 2007; Zhuang *et al.*, 2007; Kidd *et al.*, 2008), the phyllosphere (Sandhu *et al.*, 2007) and inside the plant (Siciliano *et al.*, 2001; Porteous-Moore *et al.*, 2006; Barac *et al.*, 2009). An overview of applications of plant-based technologies for the remediation of organic contaminants is provided by McCutcheon and Schnoor (2003).

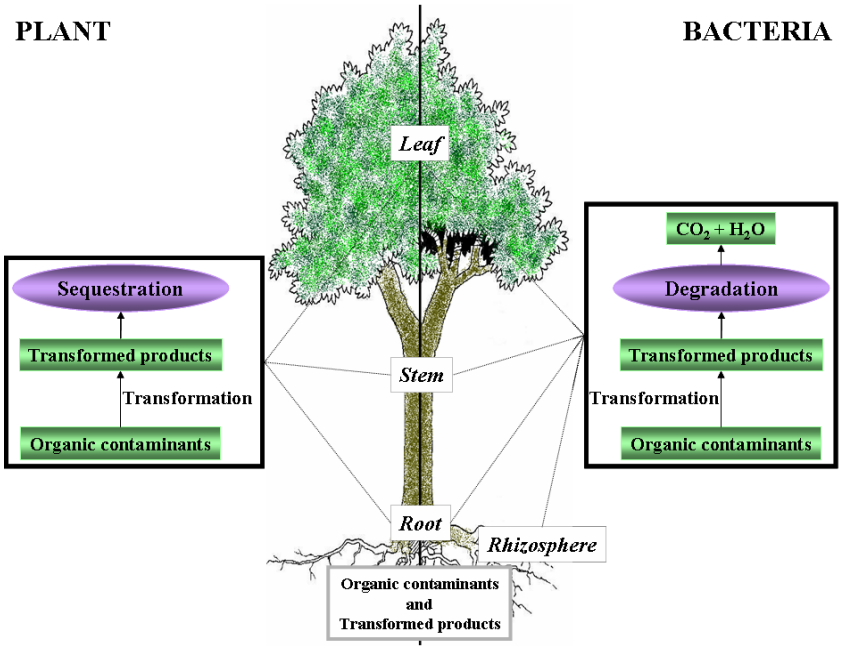


Figure 1.6: Contribution of plants and their associated bacteria to phytoremediation of organic contaminants.

1.3.1.3 Endophytes take the challenges for phytoremediation of organics

Although successfully applied in several demonstration projects, large-scale field application of phytoremediation of organic pollutants is limited by several restrictions including (a) the levels of pollutants tolerated by the plant, (b) the bioavailable fraction of the contaminants and (c), in some cases, the evapotranspiration of volatile organic pollutants to the environment (Gerhardt *et al.*, 2009). A possible option to conquer these constraints is the construction of plants specifically tailored for phytoremediation purposes by genetic manipulations (Macek *et al.*, 2002; Macek *et al.*, 2007; Doty, 2008). However, since bacteria are much easier to manipulate than plants and natural gene transfer is possible (avoiding the limitations of the use of GMO), many studies focussed on the use of engineered plant-associated bacteria. The state of the art of rhizosphere 'engineering' for accelerated rhizodegradation of organic contaminants was recently reviewed by Dzantor (2007). Even when an efficient rhizodegradation seems possible, compounds with a lipophilicity in the optimum range seem to enter the root xylem before the soil and rhizosphere microflora can degrade them (Trapp *et al*., 2000). Since the retention time of contaminants in the xylem ranges from several hours up to 2 days (McCrady *et al*., 1987), (engineered) degrader endophytes colonizing the xylem are ideal candidates to prevent evapotranspiration of pollutants or intermediates through the leaves into the environment and to reduce phytotoxicity. If no naturally occurring endophytes with the desired metabolic properties are available, endophytic bacteria can be isolated, equipped with the appropriate degradation pathways and subsequently re-inoculated in their host plant. The general idea behind the use of engineered endophytes to improve phytoremediation is to complement the metabolic properties of their host plant. Proof of this concept was provided by inoculating lupine plants (Barac *et al.*, 2004) and poplar (Taghavi *et al.*, 2005) with endophytic bacteria able to degrade toluene, which resulted in decreased toluene phytotoxicity and a significant decrease of toluene evapotranspiration. In future work, this concept should be extrapolated to other contaminants. As many catabolic pathways are found in soil bacteria where they are encoded on self-transferable plasmids or transposons, natural gene transfer offers great potential for the construction of endophytic strains with new catabolic functions. Moreover, heterologous expression of these catabolic functions might not be a major problem, especially when the donor strain and

the recipient endophytic strain are closely related, as frequently is the case. In addition, this concept can not only be applied with the purpose of remediation, but also has potential applications in the protection of food chains. For example, Germaine *et al.* (2006) demonstrated the use of bacterial endophytes to reduce levels of toxic herbicide residues in crop plants. Inoculation of pea plants (*Pisum sativum*) with a poplar endophyte able to degrade 2,4 dichlorophenoxyacetic acid (2,4-D) resulted in an increased ability to remove 2,4-D from the soil, while the plants did not accumulate 2,4-D in the tissues nor showed toxic effects.

Although it is obvious that the application of engineered plant-associated bacteria to improve phytoremediation of organic contaminants has high potential, some questions still need to be solved prior to large-scale field use (Newman and Reynolds, 2005). As phytoremediation projects can conceivably last decades, one major point of concern is the persistence and the stability of the engineered organisms and their degradation capabilities in association with field grown plants. As long as a selection pressure is present, there will be an advantage for those community members possessing the appropriate degradation characteristics. Nevertheless, this is no guarantee that inoculated strains will become an integrated part of the natural community. However, instead of integrating a new bacterium in a stable community, the natural community can also be adapted through horizontal gene transfer. Horizontal gene transfer has been shown to play an important role in adapting a microbial community to a new environmental stress factor, including rhizosphere communities (van Elsas *et al*., 1998; Ronchel *et al*., 2000; Devers *et al*., 2005), and endophytic communities (Taghavi *et al*., 2005). This may have the practical advantage that no long-term establishment of inoculants is required.

1.3.2 Phytoremediation of toxic metals

1.3.2.1 Uptake and translocation of toxic metals (phytoextraction)

When plants are growing on a metal-contaminated soil, several processes take place. Physically, plant roots stabilize the soil, thus preventing wind and water erosion, and evapotranspiration reduces percolation of water and metals through the soil profile. Natural sensitivity or tolerance of plants to accumulate metals is considerably affected by plant species and genotypes. In general, plants can be subdivided into three groups: (a) excluders that are insensitive for uptake and

accumulation of potentially toxic metals, (b) indicators whose content of metals is more or less linearly responding to increasing available content of metals in soil, and (c) accumulators that are accumulating higher contents of metals in their tissues according to the concentrations in the soil. Extreme accumulators (hyperaccumulators) exist that can even prosper on highly contaminated soils and accumulate extremely high contents of trace elements. Beside these differences in accumulation and plant biomass yield, metal specific properties and mobility can also significantly affect their uptake and distribution in plant tissue. The transfer coefficient (TC; also called bioconcentration factor, BCF) is defined as the metal content in plant tissues divided by the soil metal content (Kiekens and Camerlyck, 1982; McGrath and Zhao, 2003). As phytoextraction of metal-contaminated soils relies on the use of plants to extract and translocate metals to their above-ground parts, the translocation factor (TF), representing the ratio between the metal concentration in the shoots and the metal concentration in the roots, is an interesting coefficient (McGrath and Zhao, 2003). The plant biomass containing heavy metals should then be harvested, reduced in volume e.g. by composting, low temperature ashing or other methods, stored on special places or ideally used for recuperation of the metals. Metal phytoextraction is a promising remediation technology but for most elements (except for Ni; Chaney *et al.*, 2007) the market value of the metals at this moment is too low to make phytoextraction economically feasible.

Studies investigating the feasibility of phytoextraction stated that both the biomass production and the metal bioconcentration factor together with the plant-availability of the metals determine the efficiency of the remediation process (McGrath and Zhao, 2003; Vassilev *et al*., 2004). The use of energy crops (e.g. willow, poplar and maize) allows for gradual contaminant removal, while the produced biomass can be used for production of renewable energy. This in turn allows for economical valorization of contaminated soils during the remediation process. An increasing number of reports support the opinion that phytoextraction will get only economically feasible if, in addition to metal removal, plants produce biomass with an added economical value (Vassilev *et al*, 2004; Witters *et al.*, 2009).

1.3.2.2 Plant-bacteria synergism during phytoremediation of toxic metals

In the case of phytoremediation of metal-contaminated soils and (ground) water, metal availability, and phytotoxicity are the main limiting factors. Here, bioaugmentation-assisted phytoextraction constitutes a promising method (reviewed in Lebeau *et al*., 2008; Rajkumar *et al.*, 2009).

Metal availability in the soil is a critical factor in the efficiency of the remediation process. Use of chemical amendments for mobilization (e.g. chelating or acidifying agents) of metals can sometimes improve the metal accumulation by plants. However, these chemicals (such as EDTA) are often poorly photo-, chemo- and biodegradable and can cause groundwater pollution by uncontrolled metal dissolution and leaching. Beside these chemical amendments, plant-microbe interactions can significantly affect the metal availability in soils. As certain plants can make use of microbial siderophores to increase their iron uptake, it has been hypothesized that bacterial metal chelators, such as siderophores, can eventually also improve the uptake of other metals by plants (van der Lelie, 1998; Saravanan *et al.*, 2007; Braud *et al*., 2009). The production of these bacterial chelators is in tight equilibrium with plant activity, which means that metal mobilization only takes place when plants are active and by consequence can take up the metals; this may prevent uncontrolled metal dissolution and leaching, as is the case for chemical chelators. Since a better developed root system can also lead to increased metal uptake, plant growth-promoting bacteria that stimulate formation of plant roots can also contribute to an increased metal uptake (Sheng *et al.*, 2008). Also application of plant hormones, that are produced by several plant-associated bacteria can affect metal uptake by plants (Lòpez *et al*., 2007).

Beside the metal availability, **metal phytotoxicity** is often a limiting factor for metal phytoextraction. Some plant-associated bacteria can be adapted to the metal contamination they have to deal with (Lodewyckx *et al*., 2002a; Pal *et al.*, 2007; Mastretta *et al.*, 2009). They can be equipped with a metal-sequestration system in order to decrease the bioavailibity of the heavy metals resulting in decreased toxicity, for themselves as well as for their host plant. Moreover, endophytes possessing such a metal-sequestration system can contribute to an increased translocation of metals to the above-ground plant parts. Recently, Mastretta *et al.* (2009) demonstrated the beneficial effects of seed endophytes

on metal toxicity and translocation. The importance of seed endophytes as a vector for beneficial bacteria was already demonstrated by Cankar *et al.* (2005).

1.3.2.3 Endophytes take the challenges for phytoremediation of toxic metals

The general drawbacks of phytoextraction are well known and its optimization still requires a lot of research (Vangronsveld and Cunningham, 1998; Lebeau *et al.*, 2008). Beside the metal availability and phytotoxicity, there are some other factors that limit the application of phytoextraction. Phytoextraction is a slow process, it may not be able to remove 100% of the contaminants and its efficiency has only been proved for some contaminants (Chaney *et al*., 2007). In order to optimize the phytoextraction process, genetic manipulation of plants (Macek *et al.*, 2002; Macek *et al.*, 2007; Doty, 2008) as well as manipulations of the plant-associated microbial communities have been investigated. The manipulations of the plant-associated community that have a positive effect on the efficiency of phytoextraction include (a) isolation of associated bacteria, followed by genetic modification to equip them with siderophore production and/or metal sequestration systems, and re-inoculation of these modified bacteria (Lodewyckx *et al*., 2001; Valls and de Lorenzo, 2002; Sessitsch and Puschenreiter, 2008); as well as (b) enrichment of *in planta* present bacteria (Roy *et al*., 2005; Chen *et al*., 2005; Li *et al.*, 2007; Abou-Shanab *et al.*, 2007). For instance, Sheng *et al.* (2008) observed increases in biomass production and total lead uptake in *Brassica napus* after inoculation with *Microbacterium* sp. G16, a bacterial strain that is capable of producing IAA, ACC-deaminase and siderophores. *Lupinus luteus* L, when grown on a nickel enriched substrate and inoculated with the engineered nickel-resistant bacterium *Burkholderia 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 (Lodewyckx *et al*., 2001). On the other hand, the inoculation of *Lolium perenne* (cv Atlas) with the nickel-resistant derivative of *Herbaspirillum seropedicae* LMG2284::*ncc-nre* resulted in a significant decrease of the nickel concentration in the roots (11%) as well as in the shoots (14%). As this phenomenon was also observed in the *Lolium perenne* plants inoculated with the wild-type strain LMG2284, the nickel resistance characteristics probably are not responsible for the altered nickel uptake observed. These examples also

show that an in depth study is required before these concepts can be generally applied.

Chapter 1.4 *Conclusions and research needs*

The interactions between plants and their associated microorganisms are intricate and are subject of an increasing number of investigations. The exploitation of plant-endophyte partnerships for increasing biomass production and the remediation of contaminated soils and (ground) water are promising areas. In the case of phytoremediation of organic contaminants, endophytic bacteria possessing the appropriate degradation pathway(s) can assist their host plant by degrading contaminants that are readily taken up by plants, which fail to degrade them to completion, resulting in (a) toxicity due to the accumulation of the original compound and/or degradation intermediates or (b) evapotranspiration of volatile contaminants (figure 1.7). In the case of phytoremediation of toxic metals, endophytes equipped with a metal-sequestration system and/or able to produce natural metal chelators, can increase metal availability in the soil (leading to an increase uptake by the host plant), reduce metal toxicity for their host plant and/or increase metal translocation to the aerial parts (figure 1.7).

Although there is a clear difference in phytoremediation potential whether metals or organic compounds are the primary targets of the remediation, at many contaminated sites, plants and their associated microorganisms have to deal with mixed pollution. Remediation of co-contaminated sites is generally complex. The presence of toxic metals can, for example, affect the biodegradation of a variety of organic pollutants through impacting both the physiology and ecology of degrading microorganisms (Sandrin and Maier, 2003; Lin *et al*., 2006). Studies on remediation of co-contaminated sites have focussed on increasing organic biodegradation by concomitantly reducing metal toxicity through metal sequestration and precipitation. However, this strategy mainly addresses the degradation of the organic contaminants, whereas metal extraction is not considered and, as a consequence, metal remediation is restricted to metal stabilization only. For the remediation of substrates that are co-contaminated with metals and organics, use of metal-tolerant endophytes that are also equipped with degradation pathway(s) for organic contaminants, is a particular promising strategy that needs to be explored (figure 1.7).

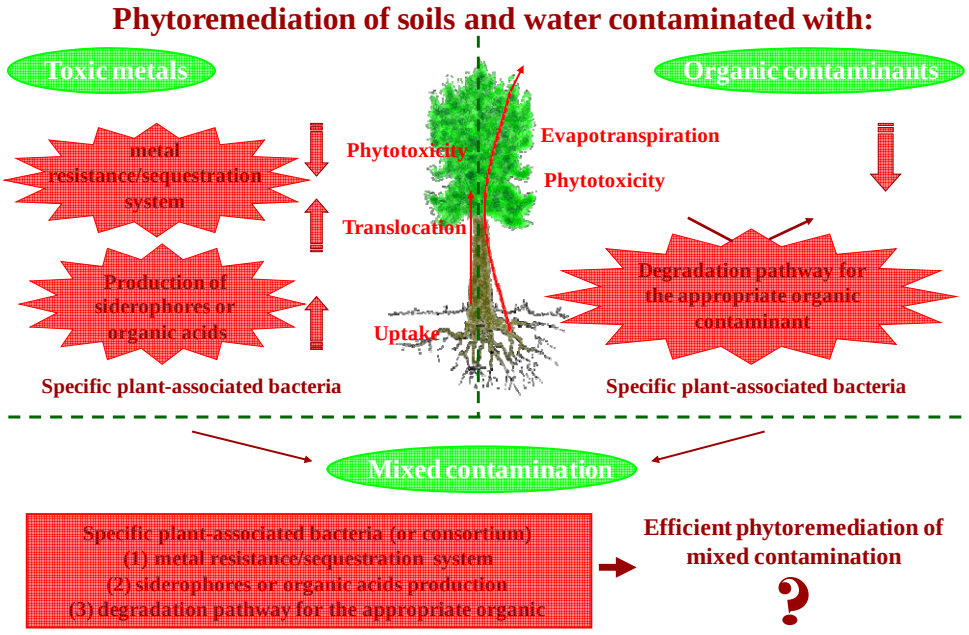


Figure 1.7: To improve phytoremediation of toxic metals, plant-associated bacteria can be equipped with a metal-sequestration system making the heavy metals less toxic to the plant. Bacteria producing siderophores and/or organic acids can increase the bioavailability of heavy metals in the rhizosphere and contribute to an increased root to shoot translocation. Phytoremediation of highly water soluble and volatile organic contaminants is often inefficient because plants do not completely degrade these compounds and the contact time between the microorganisms in the rhizosphere and the pollutants is not long enough. Since the pollutant is transported through the plant vascular system, endophytic bacteria, possessing the genetic information for efficient degradation of the contaminant(s), can promote degradation resulting in a decreased phytotoxicity and evapotranspiration. Combining these traits in one strain or in a bacterial consortium may allow an efficient phytoremediation of mixed contaminations.

Section 2

OBJECTIVES

The general idea behind the use of plant-associated bacteria to improve phytoremediation is to complement the contaminant handling capacities of the host plant. As described in the introduction section, proof of this concept was already provided under controlled laboratory conditions and for single contaminations of toluene or nickel (for Ni: Lodewyckx *et al*., 2001; for toluene: Barac *et al*., 2004 and Taghavi *et al*., 2005). However, at many contaminated sites, plants and their associated microorganisms have to deal with mixed pollutions of organics and toxic metals. Further, this proof of concept on labscale is no guarantee that (a) inoculating bacteria in the field will be successful, (b) the inoculated strains will become an integrated part of the endogenous community, and (c) the selection pressure will be sufficient to maintain a stable degradation capacity and/or metal sequestration.
In order to move endophyte-enhanced phytoremediation further towards application in the field, in this thesis we focused on, these critical issues.

In a first section it was investigated if endophytes can also deal with mixed contaminations. For that purpose, yellow lupine plants were inoculated with a Ni-resistant, toluene- and TCE-degrading endophyte and plants were exposed to Ni and toluene (chapter 3.3) and to Ni and TCE (chapter 3.4). The phytoremediation efficiency of non-inoculated (control) plants was compared with inoculated plants by analyzing Ni, toluene and TCE phytotoxicity, Ni uptake and toluene or TCE evapotranspiration.
Since poplar is a suitable species for both biomass production, but also for phytoremediation, in a next step towards field application, yellow lupine, which is a model test plant, was replaced by poplar cuttings. Chapter 4.1 was dedicated to the exploration of the colonization and plant growth-promoting capacity of the toluene- and TCE-degrading poplar endophyte *Pseudomonas putida* W619-TCE in poplar cuttings. In chapter 4.2 the potential of *Pseudomonas putida* W619-TCE to reduce TCE phytotoxicity and evapotranspiration in poplar was investigated at laboratory scale.
In the last section, the different field experiments that were performed to achieve a successful proof of our concept in the field, are described. In a first field experiment poplar and its natural associated microorganisms were applied for the *in situ* remediation of a BTEX-contaminated groundwater plume (chapter

5.2). For the other field experiments, a site was chosen where TCE was present in the groundwater in concentrations up to 100 mg l^{-1}. At this site, a mixed woodland of English oak and common ash was already present on the contaminated groundwater plume. The sharp decrease in TCE concentrations along transects under this mixed woodland suggested that phytoremediation was already taking place. Therefore, at first the bacteria associated with oak and ash that showed potential to avoid evapotranspiration of TCE were characterized (chapter 5.3). To further contain the groundwater TCE plume and to be capable of inoculating the TCE-degrading poplar endophyte *Pseudomonas putida* W619-TCE, hybrid poplar trees [*Populus deltoides x (trichocarpa x deltoides) cv. Grimminge*] were planted in the spring of 2006 perpendicularly on the plume and were provided with a drainage tube around the roots. In the final chapter (chapter 5.4) the first *in situ* inoculation of these poplar trees, growing on the TCE-contaminated site, with the TCE-degrading strain *Pseudomonas putida* W619-TCE is reported.

Section 3

Endophytes to deal with mixed contaminations

Abstract

The aim of this section was to investigate if engineered endophytes that are capable of degrading organic contaminants, and deal with toxic metals, can improve phytoremediation of organic contaminants and toxic metals. As a model system, yellow lupine was inoculated with the endophyte *Burkholderia cepacia* VM1468 possessing (a) the pTOM-Bu61 plasmid, coding for constitutive toluene/TCE degradation, and (b) the chromosomally inserted *ncc-nre* Ni resistance/sequestration system. As controls, plants were inoculated with *B. vietnamiensis* BU61 (pTOM-Bu61) and *B. cepacia* BU72 (containing the *ncc-nre* Ni resistance/sequestration system). Plants were exposed to mixtures of Ni and toluene, and Ni and TCE.

At first, in chapter 3.2, the effect of the inoculation itself on plant fitness was investigated. None of the inoculated bacterial strains could affect plant growth and at the activity of stress-related enzymes; only inoculation with the endophyte *B. cepacia* VM1468 resulted in a significant increase in superoxide dismutase activity in the roots.

In chapter 3.3, the concept of using endophytes to improve phytoremediation of mixed contaminations was tested: plants were exposed to Ni and toluene and (a) Ni and toluene phytotoxicity, (b) toluene degradation and evapotranspiration, and (c) Ni concentrations in the roots and shoots were determined. Only inoculation with *B. cepacia* VM1468 resulted in decreased Ni and toluene phytotoxicity, as measured by a protective effect on plant growth and decreased activities of enzymes involved in antioxidative defence (catalase, guaiacol peroxidase, superoxide dismutase) in the roots. Besides, plants inoculated with *B. cepacia* VM1468 and *B. vietnamiensis* BU61 released less toluene through the leaves than non-inoculated plants and those inoculated with *B. cepacia* BU72. Ni-uptake in the roots was slightly increased for *B. cepacia* BU72 inoculated plants.

In the last section (chapter 3.4), the concept was extended to mixed contaminations of Ni and TCE. For this purpose, yellow lupine plants were inoculated with the endophytic strain *B. cepacia* VM1468 (Ni^R, TCE^+) and exposed to Ni and TCE. This inoculation resulted in (a) a reduced Ni and TCE phytotoxicity in the roots of the host plant, (b) a slightly decreased TCE evapotranspiration and (c) a strongly increase in Ni uptake.

Introduction

Since phytoremediation is an *in situ*, solar powered remediation technology requiring minimal site disturbance and maintenance, it often is proposed as a valuable alternative for conventional remediation options that are frequently expensive and environmentally invasive. However, as illustrated in the first section of this work (Introduction), phytoremediation still faces a number of obstacles that need to be overcome before it can move towards large-scale application.

Phytoremediation of highly water soluble and volatile **organic contaminants** is often inefficient because plants do not completely degrade these compounds resulting in phytotoxicity and/or evapotranspiration of the organic contaminants to the ambient air. In previous work, Barac *et al.* (2004), already successfully demonstrated that endophytes, equipped with the appropriate degradation pathway can be used to conquer these constraints.

In case of phytoremediation of **metal-contaminated** soils and (ground) water, metal availability, metal uptake and translocation, and phytotoxicity are the main limiting factors. To improve phytoremediation efficiency of toxic metal-contaminated soils, rhizospheric and/or endophytic bacteria can be equipped with (a) metabolic pathways for the synthesis of natural chelators, such as citric acid, to improve metal availability for plant uptake and translocation and with (b) metal sequestration systems to reduce phytotoxicity (Lodewyckx *et al*., 2001).

In addition, the application of phytoremediation is often limited due to the fact that in many field cases plants have to deal with **mixed contaminations** of both, toxic metals and organic contaminants. Remediation of co-contaminated sites is a very complex problem that needs to be investigated. The presence of toxic metals can for example affect the biodegradation of a variety of organic pollutants through impacting both the physiology and ecology of degrading microorganisms (Sandrin and Maier, 2003; Lin *et al*., 2006; Said *et al*., 1991; Burkhardt *et al*., 1993). Consequently, until now, studies on remediation of co-

contaminated sites mainly focus on improving biodegradation of organic contaminants by reducing metal toxicity through metal sequestration and precipitation. However, this strategy mainly addresses the degradation of the organic contaminants while metal extraction is not considered and by consequence the metal remediation is restricted to metal inactivation.

In this section of the thesis, we wanted to explore if engineered endophytes that are capable of (a) degrading organic contaminants, and (b) dealing with, or in the ideal scenario of enhancing uptake and translocation of toxic metals, can be used to improve the remediation of both, organic contaminants and toxic metals in a mixed waste situation. For this purpose, yellow lupine plants (*Lupinus luteus*) were inoculated with (a) the endophytic strain *Burkholderia cepacia* VM1468 possessing the pTOM-Bu61 plasmid coding for toluene and trichloroethylene (TCE) degradation and the *ncc-nre* Ni resistance/sequestration system (Ni^R, Tol^+, TCE), (b) a soil bacterium *B. vietnamiensis* BU61 only containing the pTOM-Bu61 plasmid (Tol^+, TCE) and (c) the endophytic strain *B. cepacia* BU72 equipped with the *ncc-nre* Ni resistance/sequestration system (Ni^R). At first, the effect of the inoculation on plant growth was investigated. To test the concept of using endophytes to improve phytoremediation of mixed contaminations, plants were exposed to toluene and Ni and (a) toluene and Ni phytotoxicity, (b) toluene degradation and evapotranspiration, and (c) Ni concentration in the roots and shoots were determined. In the last section the concept was extended to mixed contaminations of TCE and Ni.

Chapter 3.1 *Materials and methods*

3.1.1 Inoculation of yellow lupine plants with Burkholderia strains

Yellow lupine plants were inoculated with *B. cepacia* BU72, *B. vietnamiensis* BU61 and *B. cepacia* VM1468. These strains were constructed as described by Barac *et al*. (2004): (a) a natural endophyte of yellow lupine (*B. cepacia* L.S.2.4.) was equipped with Ni resistance by conjugation between *E. coli* CM2520 carrying the *ncc-nre* Ni resistance and the natural endophyte resulting in *B. cepacia* BU72; (b) conjugation between this Ni-resistant *B. cepacia* BU72 as a receptor and *B vietnamiensis* BU61, a soil isolate possessing the pTOM plasmid as a donor, resulted in a Ni-resistant, toluene-degrading endophyte *B. cepacia* VM1468. In previous work, the potential of *B. vietnamiensis* BU61, *B. cepacia* BU72, and *B. cepacia* VM1468 to reduce toluene phytotoxcicity and evapotranspiration (Taghavi *et al*., 2005) and of *B. cepacia* BU72 to increase Ni uptake and translocation have been demonstrated (Lodewyckx *et al*., 2001). Fresh cultures of all three *Burkholderia* strains were grown in 869 medium (Mergeay *et al*., 1985) at 30°C until an approximate absorbance (A_{660}) value of 1 was reached. The cells were collected by centrifugation (15 min at 3900 g), washed in 10 mM $MgSO_4$ and resuspended in the original volume of 10 mM $MgSO_4$.

Seeds of yellow lupine plants were surface sterilized for 20 min at room temperature in a 1% active chloride solution supplemented with 1 drop Tween 80 (Merck) per 100 ml solution. After surface sterilization, seeds were rinsed 3 times in sterile tap water and were soaked overnight in the last rinsing water.

In case the concept was tested for **nickel and toluene**, sterile seeds were planted in 400 ml plastic pots filled with perlite and saturated with half strength Hoagland's nutrient solution (Barac *et al*., 2004) supplemented with 0, 0.05, and 0.25 mM (or 0, 7.7 and 38.5 mg l^{-1}) $NiSO_4$. The bacterial inocula were added to each pot at a final concentration of 10^8 CFU ml^{-1}. Non-inoculated plants were used as controls.

To test the concept for **nickel and TCE** mixes, a slightly different pre-culture was used: seeds were planted in 400 ml plastic jars filled with perlite and saturated with half strength Hoagland's nutrient solution to which the bacterial

inocula were added at a final concentration of 10^8 CFU ml^{-1}. Non-inoculated plants were used as controls. Only after 2 weeks, plants were transferred to 400 ml pots filled with sand and were exposed to TCE and $NiSO_4$ as described below.

3.1.2 Recovery of inoculated bacteria

To ensure the inoculation was successful, bacterial endophytes were re-isolated from shoots and roots of lupine plants after 4 weeks of growth. The endophytes were isolated as described earlier (Barac *et al*., 2004). Samples were plated on (a) 284 medium with addition of a carbon mix (per liter medium: 0.52 g glucose, 0.35 g lactate, 0.66 g gluconate, 0.54 g fructose and 0.81 g succinate) and 1 mM Ni (284+Ni) that is selective for *B. cepacia* VM1468 and BU72; (b) 284 medium that was incubated in sealed 10 liter vessels with addition of 600 µl toluene (to create a toluene saturated atmosphere) (284+Tol) and by consequence is selective for *B. vietnamiensis* BU61 and *B. cepacia* VM1468 and on (c) 284 medium with addition of 1 mM Ni, and incubated in sealed 10 liter vessels with addition of 600 µl toluene (284+Ni+Tol) that is selective for *B. cepacia* VM1468. The 284 medium contains per liter distilled water 6.06 g Tris-HCl, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g $NaSO_4$, 0.20 g $MgCl_2$ x $6H_2O$, 0.03 g $CaCl_2$ x $2H_2O$, 40 mg Na_2HPO_4 x $2H_2O$, 0.48 mg Fe(III)NH_4 citrate, 1 ml microelements solution, final pH 7. The microelement solution contains per liter distilled water: 1.3 ml 25% HCl, 144 mg $ZnSO_4$ x $7H_2O$, 100 mg MnCl x 4 $2H_2O$, 62 mg H_3BO_3, 190 mg $CoCl_2$ x $6H_2O$, 17 mg $CuCl_2$ x $2H_2O$, 24 mg $NiCl_2$ x $6H_2O$ and 36 mg $NaMoO_4$ x $2H_2O$.

3.1.3 Phytotoxic effects of exposure to nickel, toluene and TCE

After 14 days of growth under the above conditions, non-inoculated and inoculated plants exposed to 0, 0.05, and 0.25 mM (or 0, 7.7 and 38.5 mg l^{-1}) $NiSO_4$ respectively, were irrigated every other day with half strength Hoagland's solution to which **toluene** was added to obtain a final toluene concentration of respectively 0, 250, and 500 mg l^{-1}.

To extend the concept to mixed contaminations of **TCE** and Ni, non-inoculated and inoculated plants were exposed to 0 and 0.25 mM (or 0 and 38.5 mg l^{-1}) $NiSO_4$ respectively, and were irrigated every other day with half strength Hoagland's solution to which TCE was added to obtain a final TCE concentration of respectively 0 and 10 mg l^{-1}. After two weeks exposure plants were

harvested. To investigate toluene, TCE and Ni phytotoxicity, changes in growth and activity of some 'stress-enzymes' (involved in antioxidative defence) were analyzed. Plant growth and enzyme activities can be affected by both the microbial inoculation and by exposure to contaminants (Taghavi *et al.*, 2009 and 2005). To eliminate the effects caused by inoculation, the phytotoxic effects induced by toluene, TCE and/or Ni were determined by calculating biomass and enzyme activities relative to corresponding non-exposed control plants that were inoculated with the same bacterial strain.

3.1.3.1 Growth reduction

During harvest, roots and shoots were separated and their biomass was determined. The biomass relative to corresponding non-exposed plants was calculated as follows:

$Biomass_{relative\ to\ non\text{-}exposed}$ of plant inoculated with x and exposed to y (%) =
[(biomass of plant inoculated with x and exposed to y) *
(biomass of non-exposed plant inoculated with x)$^{-1}$] * 100

x, being no bacterial strain, *B. cepacia* BU72, *B. vietnamiensis* BU61 or *B. cepacia* VM1468
y, being 0.05 mM $NiSO_4$ and 250 mg l^{-1} toluene or 0.25 mM $NiSO_4$ and 500 mg l^{-1} toluene or 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE

3.1.3.2 Activity of enzymes involved in antioxidative defence

To determine the activities of enzymes involved in antioxidative defence, root samples were harvested and immediately snap-frozen into liquid nitrogen before storing them at -80°C. These frozen root tissues were homogenized in ice-cold 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiotreitol and 4% insoluble polyvinylpyrrolidone (1 ml buffer per 100 mg fresh weight), after which this homogenate was squeezed through a nylon mesh and centrifuged for 10 minutes at 20000 g and 4°C. Catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (GPOD, EC 1.11.1.7) and superoxide dismutase (SOD, EC 1.15.1.1) activities as markers for oxidative stress (Vangronsveld and Clijsters, 1994) were measured spectrophotometrically in the supernatant at 25°C. CAT and GPOD activities were determined at 240 nm and 436 nm respectively according to Bergmeyer *et*

al. (1974). Analysis of SOD activity was based on the inhibition of the reduction of cytochrome c measured at 550 nm (McCord and Fridovich, 1969).

The enzyme activities relative to corresponding non-exposed plants were calculated as follows:

Enzyme activity$_{\text{relative to non-exposed}}$ of plant inoculated with x and exposed to y (%) =
[(enzyme activity of plant inoculated with x and exposed to y) *
(enzyme activity of non-exposed plant inoculated with x)$^{-1}$] * 100

x, being no bacterial strain, *B. cepacia* BU72, *B. vietnamiensis* BU61 or *B. cepacia* VM1468
y, being 0.05 mM $NiSO_4$ and 250 mg l^{-1} toluene or 0.25 mM $NiSO_4$ and 500 mg l^{-1} toluene or 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE

3.1.4 Ni concentrations in roots and shoots

During harvest, fresh root and shoot material was vigorously washed with distilled water to remove all traces of Ni that could be present on the surface. Root and shoot samples were oven-dried (48 h at 65°C) and subsequently crushed to a fine powder with a mortar and pestle, and wet digested in Pyrex tubes in a heating block. The digestion included 3 cycles in 1 ml HNO_3 (65%) and 1 cycle in 1 ml HCl (37%) at 120°C for 4 h. Samples were then dissolved in HCl (37%) and diluted to a final volume of 5 ml (2% HCl). Ni concentrations were determined by means of flame atomic absorption spectrometry (AAS).

3.1.5 Toluene or TCE evapotranspiration

After plants were grown for 3 weeks under conditions described in 3.1.1, non-inoculated and inoculated (*B. cepacia* BU72, *B. vietnamiensis* BU61 or *B. cepacia* VM1468) plants exposed to 0.05 mM $NiSO_4$ or 0.25 mM respectively, were used to evaluate respectively toluene or TCE degradation and evapotranspiration. The lupine plants were taken out of the jars and their roots were vigorously rinsed in sterile water to remove bacteria from the surface. Subsequently, plants were transferred into a two-compartment glass cuvette system (figure 3.1) (Barac *et al.*, 2004) and grown hydroponically in half strength Hoagland's nutrient solution

supplemented with 0.05 mM $NiSO_4$ and 100 mg l^{-1} toluene or with 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE to avoid gas exchange between the two compartments, the upper and lower compartments were separated by a glass plate, which has an insertion to bring in the stem of the plant; the space around the stem was made gas tight with a Polyfilla exterior mixture. Each compartment was connected with an air source (Air Liquide, Liège, Belgium) with an inflow of 1 liter per hour. In order to capture any transpired or volatilized toluene or TCE, Chromosorb 106 traps were inserted in the out flow of each compartment. The traps were changed at regular intervals. Between the cuvettes and the Chromosorb traps, a column filled with $CaCl_2$ was installed in order to prevent condensation of water in the traps.

The toluene or TCE evapotranspiration through the shoots located in the upper compartment of the cuvette system was analyzed by gas chromatography/mass spectrometry (GC-MS) as described by Barac *et al.* (2004).

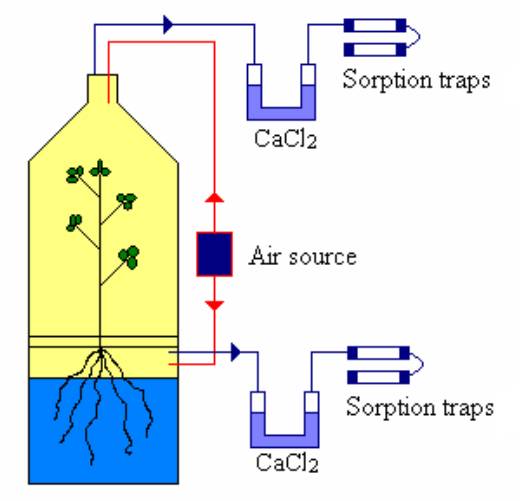


Figure 3.1: Schematic representation of the experimental set up for measuring toluene or TCE evapotranspiration

3.1.6 Statistical analysis

All datasets were statistically compared using one way or two way ANOVA and *post hoc* multiple comparison testing (Tukey Kramer). Transformations were applied when necessary to approximate normality and/or homoscedasticity. The statistical analyses were performed in SAS 9.1.3.

Chapter 3.2 *Effect of inoculation on plant fitness*

To study the plant fitness after inoculation, (a) plant growth and (b) the activity of enzymes strongly related to the general plant fitness were analyzed.
Before the effect of inoculation on plant fitness was investigated, for all inoculated bacteria, a successful inoculation was confirmed by re-isolation (see 3.3.1 and 3.4.1).

3.2.1 Plant growth

After 4 weeks of growth, non-inoculated (control) plants and plants inoculated with *B. cepacia* VM1468, *B. vietnamiensis* BU61 and *B. cepacia* BU72 were harvested and shoot and root biomass were determined (figure 3.2).

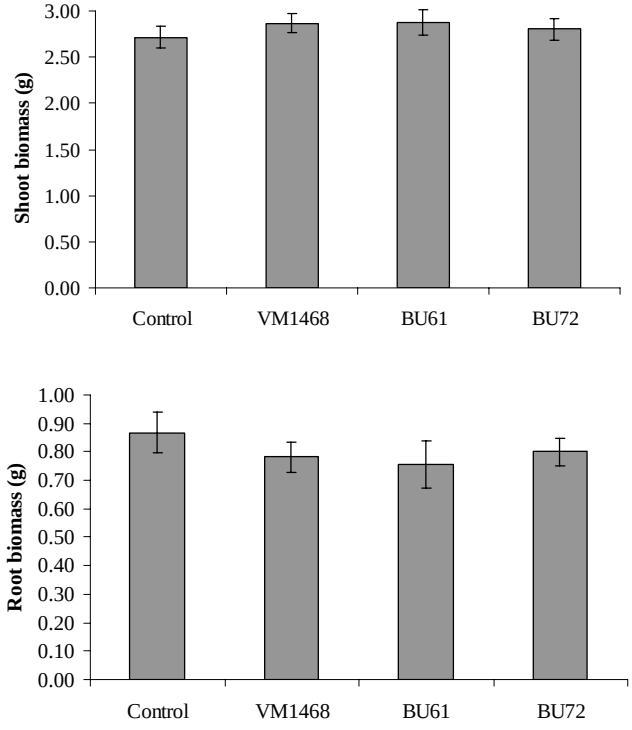


Figure 3.2: Shoot and root biomass (g) of control plants and plants inoculated with *B. cepacia* VM1468, *B. vietnamiensis* BU61 and *B. cepacia* BU72. Values are mean ± standard error of at least 15 biological independent replicas.

None of the inoculated bacterial strains had a significant effect on plant growth: root biomass as well as shoot biomass were similar for control plants and inoculated plants.

3.2.2 Activity of enzymes strongly related to the general plant fitness

Since enzyme activity is a more sensitive parameter than plant biomass to study plant fitness, the activities of catalase, guaiacol peroxidize (GPOD) and superoxide dismutase (SOD) in the roots and leaves of non-inoculated control plants and plants inoculated with *B. cepacia* VM1468, *B. vietnamiensis* BU61 and *B. cepacia* BU72, were determined (figure 3.3) to further investigate the effect of inoculation on plant fitness.

However, inoculation of lupine plants with *B. vietnamiensis* BU61 and *B. cepacia* BU72 had no effect on the activity of enzymes strongly related to the plant fitness. Inoculation with the endophyte *B. cepacia* VM1468 resulted in a significant increase in superoxide dismutase activity in the roots. The same trend was observed for superoxide dismutase activity in the leaves though this increase was not significant. Further, the catalase activity in the roots of plants inoculated with *B. cepacia* VM1468 was lower than for the control plants, however this decreasing trend was not significant.

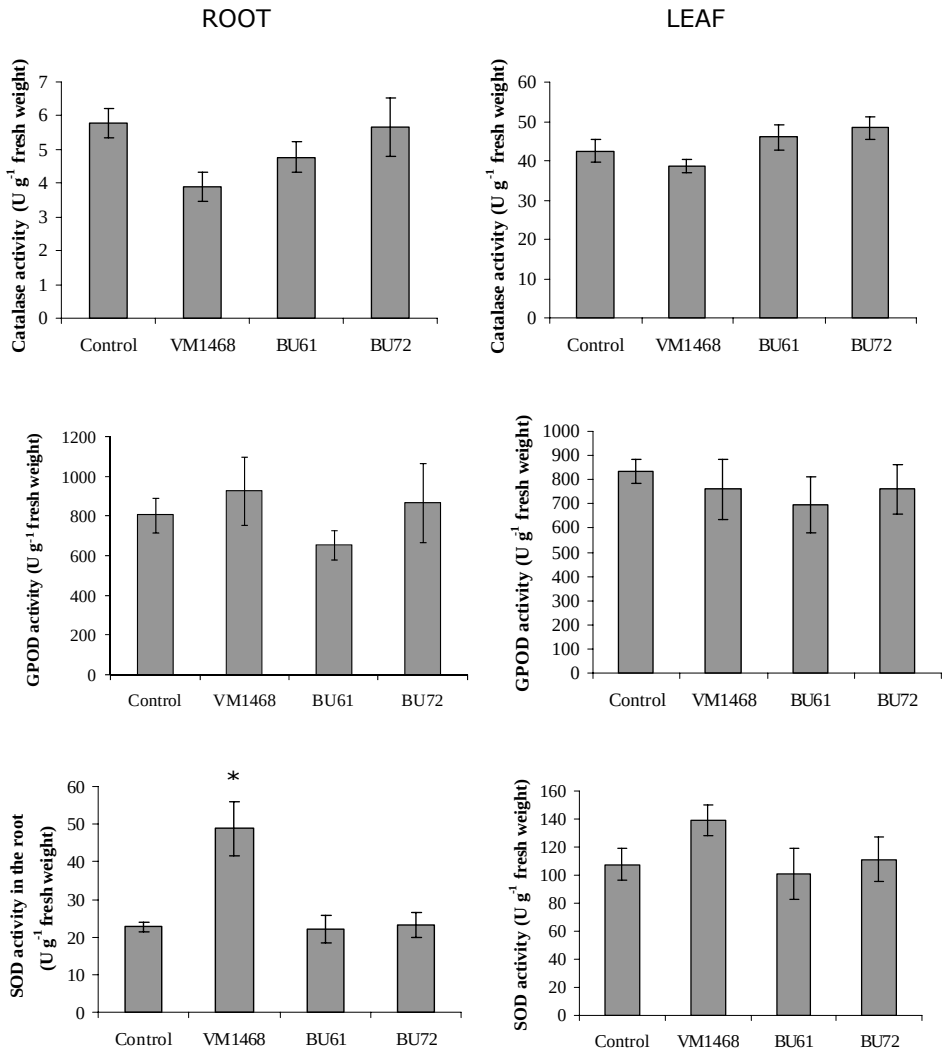


Figure 3.3: Catalase, guaiacol peroxidase and superoxide dismutase activity in the roots (left) and the leaves (right) of control (non-inoculated) lupine plants and plants inoculated with *B. cepacia* VM1468, *B. vietnamiensis* BU61 and *B. cepacia* BU72. Values are mean ± standard error of at least 6 biological independent replicas (significance level: *: $p<0.05$).

Chapter 3.3 *Co-contamination of nickel and toluene*

***Accepted for publication in**: Weyens N, Truyens S, Saenen E, Boulet J, Dupae J, van der Lelie D, Carleer R, Vangronsveld J. (xxxx) Endophytes and their potential to deal with co-contamination of organic contaminants (toluene) and toxic metals (nickel) during phytoremediation. International Journal of Phytoremediation.*

In this work, yellow lupine plants were inoculated with the endophytic strain *Burkholderia cepacia* BU72 (Ni^R), its derivative *B. cepacia* VM1468 (Ni^R, Tol^+), or the soil bacterium *B. vietnamiensis* BU61 (Tol^+). Plants were exposed to mixed contaminations of **nickel and toluene**. At first all cultivable endophytes were isolated to verify if the inoculation was successful. To evaluate the phytoremediation efficiency, phytotoxic effects of Ni and toluene, toluene evapotranspiration and Ni uptake and translocation were determined.

3.3.1 Recovery of endophytic bacteria

To verify if inoculation of *B. cepacia* BU72 (Ni^R), *B. vietnamiensis* BU61 (Tol^+) and *B. cepacia* VM1468 (Ni^R, Tol^+) was successful, after 4 weeks of growth, the cultivable endophytic bacteria were isolated from root and shoot samples and plated on media selective for the inoculated strains (table 3.1). All three *Burkholderia* strains could be re-isolated on the selective media. As expected for each isolation only one morphotype, corresponding to the inoculated strain, could be observed on the selective media. Bacteria isolated from the shoots and the roots of lupine plants inoculated with *B. cepacia* VM1468 (Ni^R, Tol^+) were able to grow on all 3 selective media; plants inoculated with *B. cepacia* BU72 (Ni^R) contained no toluene-degrading strains and from plants inoculated with *B. vietnamiensis* BU61 (Tol^+), isolated bacteria were growing exclusively on 284+Tol.

Furthermore, when plants were exposed to mixes of Ni and toluene, the colony forming units (CFU) of *B. vietnamiensis* BU61 and *B. cepacia* BU72 that could be re-isolated slightly decreased in comparison with non-exposed plants. In contrast, the re-isolated CFU of *B. cepacia* VM1468 slightly increased after exposure to mixes of Ni and toluene.

Table 3.1: Number of colony forming units isolated from roots and shoots of lupine plants

	Inoculum[a]	Plant part	284+Ni[b]	284+Tol[b]	284+Ni+Tol[b]
Non-exposed plants	No	Shoot	0	0	0
	No	Root	0	0	0
	VM1468	Shoot	3.5×10^2 (1)[c]	4.3×10^2 (1)	3.9×10^2 (1)
	VM1468	Root	8.6×10^3 (1)	9.2×10^3 (1)	1.1×10^4 (1)
	BU61	Shoot	0	2.6×10^2 (1)	0
	BU61	Root	0	3.4×10^3 (1)	0
	BU72	Shoot	8.4×10^2 (1)	0	0
	BU72	Root	9.8×10^3 (1)	0	0
Plants exposed to 0.05 mM $NiSO_4$ + 250 mg l^{-1} toluene	No	Shoot	0	0	0
	No	Root	0	0	0
	VM1468	Shoot	4.1×10^2 (1)	3.9×10^2 (1)	4.2×10^2 (1)
	VM1468	Root	9.8×10^3 (1)	9.3×10^3 (1)	2.3×10^4 (1)
	BU61	Shoot	0	8.0×10^1 (1)	0
	BU61	Root	0	1.1×10^3 (1)	0
	BU72	Shoot	1.2×10^2 (1)	0	0
	BU72	Root	2.1×10^3 (1)	0	0
Plants exposed to 0.25 mM $NiSO_4$ + 500 mg l^{-1} toluene	No	Shoot	0	0	0
	No	Root	0	0	0
	VM1468	Shoot	4.0×10^2 (1)	4.6×10^2 (1)	5.3×10^2 (1)
	VM1468	Root	1.5×10^4 (1)	9.9×10^3 (1)	3.5×10^4 (1)
	BU61	Shoot	0	6.0×10^1 (1)	0
	BU61	Root	0	8.9×10^2 (1)	0
	BU72	Shoot	9.0×10^1 (1)	0	0
	BU72	Root	1.3×10^3 (1)	0	0

[a] Lupine plants were inoculated with *Burkholderia* strains VM1468, BU61, and BU72. As controls, plants without inoculum were analyzed. [b] Ni: 1mM nickel; Tol: toluene vapour as only carbon source. [c] After 4 weeks of growth, endophytic bacteria were isolated and the number of bacteria was determined and expressed per g fresh weight. Numbers in parentheses are the numbers of different morphological types of bacteria as observed visually.

3.3.2 Phytotoxic effects of nickel and toluene

To examine Ni and toluene phytotoxicity, changes in growth (figure 3.4) and enzyme activity (figure 3.5) caused by the exposure were analyzed. As growth and enzyme activity can also be influenced by the inoculation itself (Taghavi *et al.*, 2009), we eliminated the effect of the inoculation by calculating biomass and enzyme activities relative to corresponding non-exposed plants as described in materials and methods.

3.3.2.1 Growth reduction

All plants, except plants inoculated with *B. cepacia* VM1468 (capable of degrading toluene and equipped with a Ni resistance/sequestration system) did suffer when exposed to a mixed contamination of 0.05 mM $NiSO_4$ and 250 mg l^{-1} toluene (figure 3.4): root and shoot biomass were both reduced to approximately 70% of the biomass of the corresponding non-exposed plants. Since no growth reduction was observed for plants inoculated with *B. cepacia* VM1468, this endophyte clearly had a protective effect on its host plant. Exposure of plants to 0.25 mM $NiSO_4$ and 500 mg l^{-1} toluene was strongly phytotoxic. However, inoculation of lupine plants with *B. cepacia* VM1468 resulted in a reduction of the phytotoxicity response. In the roots, inoculation with *B. cepacia* VM1468 led to an increase in relative biomass from approximately 15% to 40% of the biomass of the corresponding non-exposed roots, while in the shoots this effect was less significant (figure 3.4).

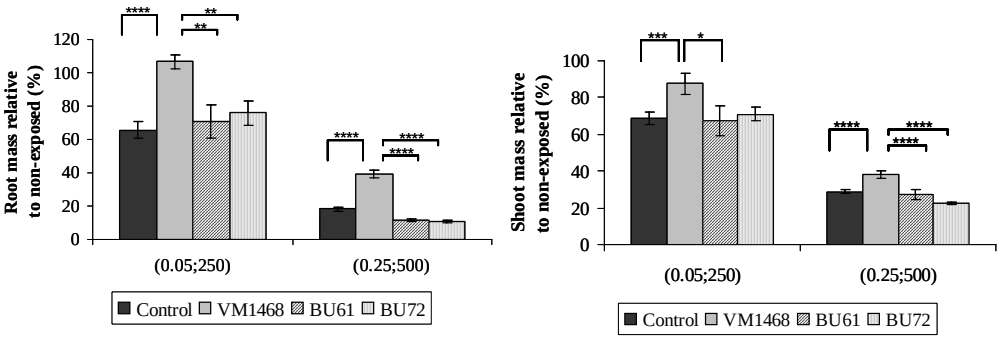


Figure 3.4: Root (left) and shoot (right) mass relative to corresponding non-exposed plants of control (non-inoculated) lupine plants and plants inoculated with *B. cepacia* BU72, *B. vietnamiensis* BU61 or *B. cepacia* VM1468. The (root or shoot) mass relative to corresponding non-exposed plants was calculated as follows: $Mass_{relative\ to\ non\text{-}exposed}$ of plant inoculated with x and exposed to y (%) = [(biomass of plant inoculated with x and exposed to y)*(biomass of non-exposed plant inoculated with x)$^{-1}$]*100; with x, being no bacterial strain, *B. cepacia* BU72, *B. vietnamiensis* BU61 or *B. cepacia* VM1468; y, being 0.05 mM $NiSO_4$ and 250 mg l^{-1} toluene (0.05;250) or 0.25 mM $NiSO_4$ and 500 mg l^{-1} toluene (0.25;500). Values are mean ± standard error of at least 15 biological independent replicas. (significance levels: *: $p < 0.1$; **: $p < 0.05$; ***: $p < 0.01$; ****: $p < 0.001$).

3.3.2.2 Activity of enzymes involved in antioxidative defence

The phytotoxic effects that were obvious from the growth reduction were further supported by changes in activities of enzymes involved in antioxidative defence (figure 3.5). After exposure to 0.05 mM $NiSO_4$ and 250 mg l^{-1} toluene, lupine plants inoculated with *B. cepacia* VM1468 showed slightly decreased GPOD and SOD activities in the roots while in the roots of non-inoculated plants and plants inoculated with *B. vietnamiensis* BU61 and *B. cepacia* BU72 CAT, GPOD and SOD activities remained similar in comparison with corresponding non-exposed plants. Exposure to 0.25 mM $NiSO_4$ and 500 mg l^{-1} toluene resulted in a significant increase in CAT, GPOD, and SOD activity in the roots of non-inoculated plants and plants inoculated with *B. vietnamiensis* BU61 and *B. cepacia* BU72. In case of inoculation with the soil isolate *B. vietnamiensis* BU61, the induction of CAT and GPOD caused by this exposure was even significantly higher than in the roots of non-inoculated plants, which might be explained by the previously observed opportunistic pathogenic behaviour of this strain (derived from *B. vietnamiensis* G4) on yellow lupine (Barac *et al*., 2004). Only

after inoculation with *B. cepacia* VM1468, CAT, GPOD and SOD activities in the roots remained at the same level as in the roots of corresponding non-exposed plants.

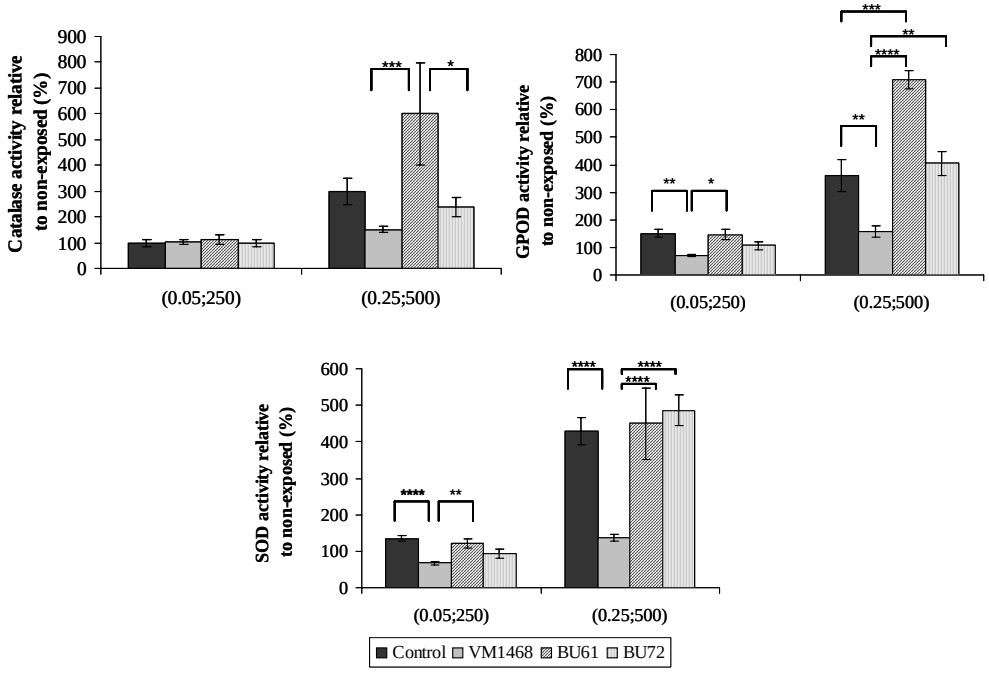


Figure 3.5: Catalase, guaiacol peroxidase and superoxide dismutase activity relative to corresponding non-exposed plants in the roots of control (non-inoculated) lupine plants and plants inoculated with *B. cepacia* BU72, *B. vietnamiensis* BU61 or *B. cepacia* VM1468. The enzyme activities in the roots relative to corresponding non-exposed plants were calculated as follows: $Activity_{relative\ to\ non\text{-}exposed}$ of plant inoculated with x and exposed to y (%) = [(activity of plant inoculated with x and exposed to y)*(activity of non-exposed plant inoculated with x)$^{-1}$]*100; with x being no bacterial strain, *B. cepacia* BU72, *B. vietnamiensis* BU61 or *B. cepacia* VM1468; y, being 0.05mM $NiSO_4$ and 250 mg l^{-1} toluene (0.05;250) or 0.25 mM $NiSO_4$ and 500 mg l^{-1} toluene (0.25;500). Values are mean ± standard error of at least 6 biological independent replicates (significance levels: *: $p < 0.1$; **: $p < 0.05$; ***: $p < 0.01$; ****: $p < 0.001$).

3.3.3 Toluene evapotranspiration

After plants were grown for 3 weeks, they were transferred into a two-compartment glass cuvette system (Barac *et al.*, 2004) and grown hydroponically in half strength Hoagland's nutrient solution supplemented with a sub-phytotoxic toluene concentration of 100 mg l^{-1} and with 0.05 mM $NiSO_4$. The amount of toluene evapotranspired through the aerial plant parts located in the

upper compartment of the cuvette system was determined using GC-MS. As shown in figure 3.6, compared to control plants and plants inoculated with *B. cepacia* BU72, those inoculated with *B. vietnamiensis* BU61 and *B. cepacia* VM1468 seemed to evapotranspire less toluene through their shoot. Because of the high degree of variability in the controls, the differences were not significant. However, it suggests that both toluene-degrading strains were capable of decreasing toluene release, even when the plants were exposed to a mixed contamination of 0.05 mM $NiSO_4$ and 100 mg l^{-1} toluene.

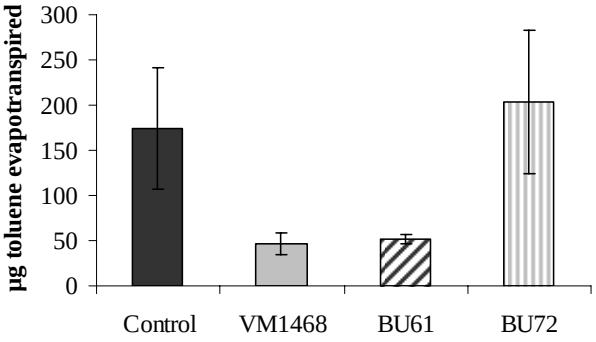


Figure 3.6: Total amount of toluene (µg) evapotranspired through the shoot, located in the upper compartment of the cuvette system, of control (non-inoculated) lupine plants and plants inoculated with *B. cepacia* BU72, *B. vietnamiensis* BU61 or *B. cepacia* VM1468, all exposed to 0.05 mM $NiSO_4$ and 100 mg l^{-1} toluene. Values are mean ± standard error of at least 3 biological independent replicates.

3.3.4 Ni concentrations in roots and shoots

The Ni concentrations in both roots and shoots of plants exposed to 0.05 mM $NiSO_4$ and 250 mg l^{-1} toluene or 0.25 mM $NiSO_4$ and 500 mg l^{-1} toluene, were determined (figure 3.7). As expected, Ni concentrations in the roots after exposure to 0.25 mM $NiSO_4$ and 500 mg l^{-1} toluene were significantly ($p<0.05$) higher than after exposure to 0.05 mM $NiSO_4$ and 250 mg l^{-1} toluene for all plants (non-inoculated and inoculated). However, Ni concentrations in the shoots of all plants were very similar for both exposures. Only after inoculation with *B. cepacia* BU72, an increased Ni uptake, as previously reported by Lodewyckx *et al.* (2001), was achieved. Inoculation with *B. vietnamiensis* BU61 and *B. cepacia* VM1468 did not affect Ni uptake in the roots, neither in the shoots.

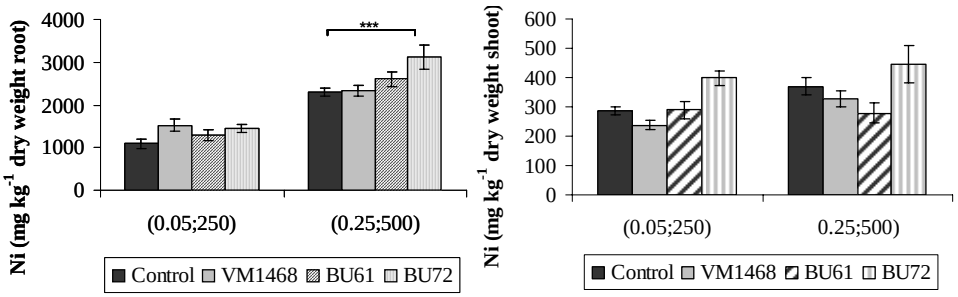


Figure 3.7: Ni concentration (mg kg^{-1} dry weight) in roots (left) and shoots (right) of control (non-inoculated) lupine plants and plants inoculated with *B. cepacia* BU72, *B. vietnamiensis* BU61 or *B. cepacia* VM1468 that were exposed to 0.05 mM $NiSO_4$ and 250 mg l^{-1} toluene (0.05;250) or 0.25 mM $NiSO_4$ and 500 mg l^{-1} toluene (0.25;500). Values are mean ± standard error of at least 3 biological independent replicates. (significance levels: ***: $p < 0.01$)

Chapter 3.4 *Co-contamination of nickel and TCE*

***Submitted for publication in**: Weyens N, Croes S, Dupae J, Newman L, van der Lelie D, Carleer R, Vangronsveld J. (xxxx) Endophytic bacteria improve phytoremediation of Ni and TCE co-contamination. Environmental pollution*

In this section, the concept of using endophytes to improve phytoremediation of co-contaminated soils was extended to mixed contaminations of Ni and TCE. In contrast to toluene, TCE cannot be used as a carbon source, and is degraded via co-metabolization of BTEX compounds. For the pTOM plasmid, the presence of toluene or phenol induces the activity of tomA, the toluene-ortho-monooxigenase gene that also catalyzes the first step in TCE degradation. The co-metabolization requirement has several disadvantages: in the absence of the inducer, regulations will forbid sparking of groundwater to induce TCE breakdown. Furthermore, the *in planta* concentrations of inducing compounds, such as toluene, might be too low to get efficient TCE co-metabolization. To overcome these limitations, we introduced a derivative of the pTOM plasmid, pTOM-Bu61 which constitutively expresses the tomA gene, into *B. cepacia* BU72. This resulted in strain VM1468 (Taghavi *et al.*, 2005). By consequence, co-metabolism is not longer a limiting factor for using endophytes to improve TCE phytoremediation.

Since (a) the results of previous chapter demonstrated that the concept of using endophytes to improve phytoremediation of mixed contaminations was successful, though only in case *B. cepacia* VM1468 was inoculated, and (b) this experiment concerns no more than an extension to mixes of Ni and TCE, lupine plants were only inoculated with *B. cepacia* VM1468 (Ni^R, TCE^+) in this experiment. Yellow lupine plants were exposed to 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE. The inoculated strain *B. cepacia* VM1468 was re-isolated to verify if the inoculation was successful. Non-inoculated plants were used as controls. To evaluate the phytoremediation efficiency, phytotoxic responses due to Ni and TCE exposure, TCE evapotranspiration and Ni uptake and translocation were determined.

3.4.1 Recovery of endophytic bacteria

After 4 weeks of growth, yellow lupine plants were harvested and the cultivable endophytic bacteria were isolated from root and shoot samples and plated on 284 medium with the addition of 1 mM Ni, which allows selective growth of the inoculated strain *B. cepacia* VM1468 (table 3.2).

Burkholderia cepacia VM1468 could only be re-isolated from the roots of inoculated plants and here the number of CFU that could be re-isolated slightly increased when plants were exposed to 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE. This increasing trend for *B. cepacia* VM1468 was also observed when plants were exposed to Ni and toluene (see 3.3.1).

Table 3.2: Number of colony forming units isolated from roots and shoots of lupine plants

	Inoculum[a]	Plant part	284+Ni[b]
Non-exposed plants	No	Shoot	0
	No	Root	0
	VM1468	Shoot	0
	VM1468	Root	1.8×10^2 (1)[c]
Plants exposed to 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE	No	Shoot	0
	No	Root	0
	VM1468	Shoot	0
	VM1468	Root	2.3×10^2 (1)

[a] Lupine plants were inoculated with *Burkholderia cepacia* VM1468. As controls, plants without inoculum were analyzed. [b] 284+Ni: selective 284 medium with the addition of 1mM nickel. [c] After 4 weeks growth, endophytic bacteria were isolated and the number of bacteria was determined and expressed per g fresh weight. Numbers in parentheses are the numbers of different morphological types of bacteria as observed visually.

3.4.2 Phytotoxic effects of nickel and TCE

The phytotoxic effects of nickel and TCE were investigated by analyzing changes in plant growth (figure 3.8) and activities of antioxidative enzymes (figure 3.9) after exposure to nickel and TCE. To eliminate the effect of the inoculation, biomass and enzyme activities were calculated relative to corresponding non-exposed plants as described in the materials and methods.

3.4.2.1 Growth reduction

Exposure of non-inoculated plants to 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE was clearly toxic: root and shoot biomass were both reduced to approximately 70% and 80% respectively of the biomass of the corresponding non-exposed plants (figure 3.8). Inoculation with *B. cepacia* VM1468 resulted in a reduced toxicity of nickel and TCE for the host plant. For inoculated plants the root biomass of plants exposed to 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE was significantly increased to levels similar to non-exposed control plants.

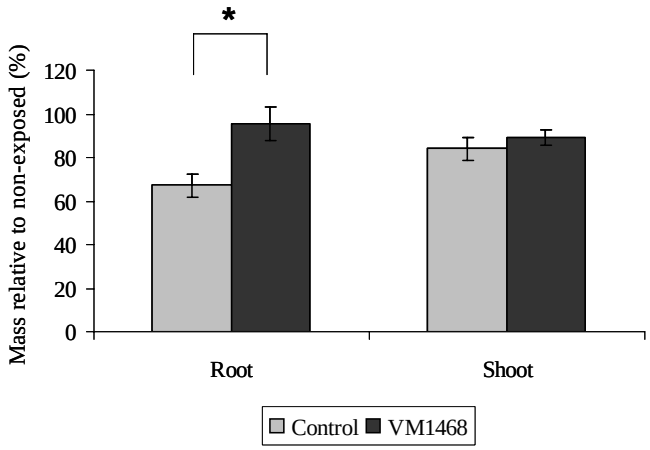


Figure 3.8: Root and shoot mass of plants exposed to 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE relative to corresponding non-exposed plants of control (non-inoculated) lupine plants and plants inoculated with *B. cepacia* VM1468. The (root or shoot) mass relative to corresponding non-exposed plants was calculated as follows: $Mass_{relative\ to\ non\text{-}exposed}$ of plant inoculated with x and exposed to y (%) = [(biomass of plant inoculated with x and exposed to y)*(biomass of non-exposed plant inoculated with x)$^{-1}$]*100; with x, being no bacterial strain or *B. cepacia* VM1468; y, 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE. Values are mean ± standard error of at least 15 biological independent replicas. (significance levels: *: $p < 0.05$).

3.4.2.2 Activity of enzymes involved in antioxidative defence

To further investigate the effect of inoculation with *B. cepacia* VM1468 on the phytotoxicity of nickel and TCE on lupine plants, CAT, GPOD and SOD activities were determined for roots and leaves (figure 3.9). After exposure to 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE, the CAT and GPOD activities in the roots of non-inoculated plants significantly increased while these activities remained at the control level or even slightly decreased in the roots of plants inoculated with *B. cepacia* VM1468. In the leaves of both non-inoculated and inoculated plants, CAT and GPOD activities remained at the control level or slightly decreased after exposure. Furthermore, exposure to 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE resulted in a decreased SOD activity in the roots and the leaves of non-inoculated plants and an even (significantly) stronger decreased SOD activity in the roots and the leaves of inoculated plants.

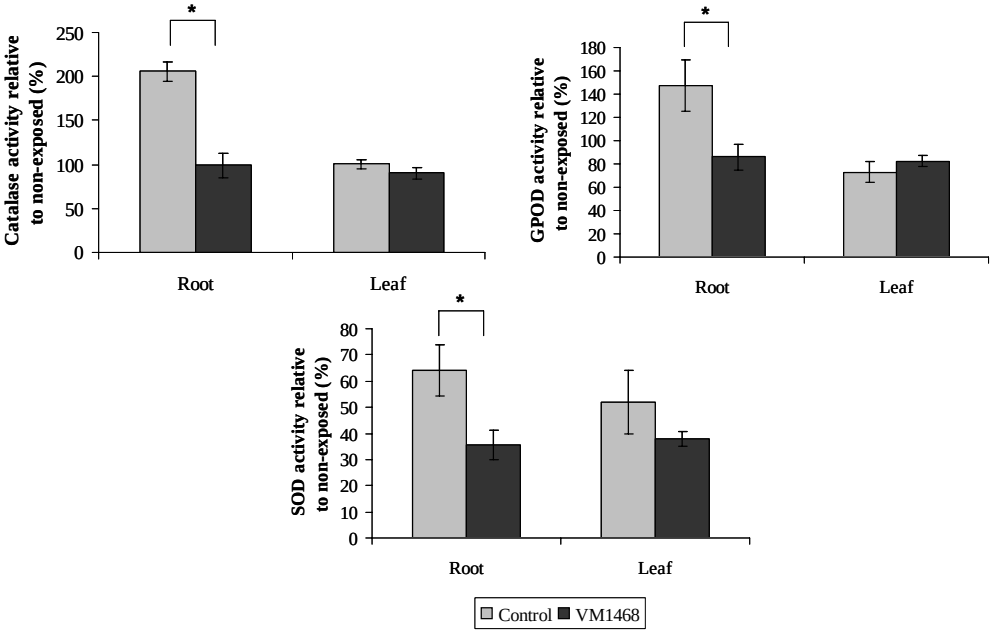


Figure 3.9: Catalase, guaiacol peroxidase (GPOD) and superoxide dismutase (SOD) activity in the roots and the leaves of plants exposed to 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE relative to corresponding non-exposed control (non-inoculated) lupine plants and plants inoculated with *B. cepacia* VM1468. The enzyme activities in the roots and leaves relative to corresponding non-exposed plants were calculated as follows: $Activity_{relative\ to\ non\text{-}exposed}$ of plant inoculated with x and exposed to y (%) = [(activity of plant inoculated with x and exposed to y)*(activity of non-exposed plant inoculated with x)$^{-1}$]*100; with x being no bacterial strain or *B. cepacia* VM1468; y, being 0.25 mM $NiSO_4$ and 10 mg l^{-1}

TCE. Values are mean ± standard error of at least 6 biological independent replicates (significance levels: *: $p < 0.05$).

3.4.3 TCE evapotranspiration

To determine TCE evapotranspiration, plants were transferred into a two-compartment glass cuvette system (Barac *et al.*, 2004) after they were grown for 4 weeks as described in materials and methods. In this cuvette system, plants were grown hydroponically in half strength Hoagland's nutrient solution supplemented with sub-phytotoxic concentrations of $NiSO_4$ (0.25 mM) and TCE (10 mg l^{-1}). The amount of TCE evapotranspired through the shoot, located in the upper compartment of the cuvette system, was determined using GC-MS.
Lupine plants inoculated with *B. cepacia* VM1468 seemed to evapotranspire less TCE through their shoots in comparison to non-inoculated plants (figure 3.10). Although the decrease in TCE evapotranspiration was not significant, due to the high degree of variability observed for the controls, the results suggest that inoculation with the TCE-degrading Ni-resistant *B. cepacia* VM1468 decreased the TCE release, even when the plants were exposed to mixed contamination of 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE.

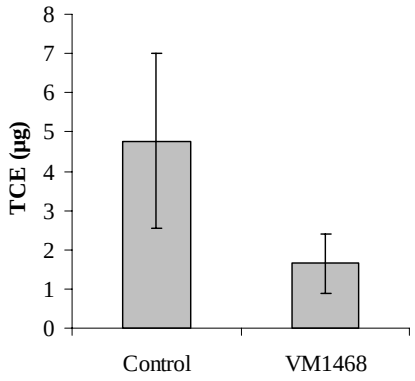


Figure 3.10: Total amount of TCE (µg) evapotranspired through the shoot, located in the upper compartment of the cuvette system, of control (non-inoculated) lupine plants and plants inoculated with *B. cepacia* VM1468, all exposed to 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE. Values are mean ± standard error of at least 3 biological independent replicates.

3.4.4 Ni concentrations in roots and shoots

The Ni concentrations in roots and shoots of control plants and plants inoculated with *B. cepacia* VM1468, exposed to 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE, were determined (figure 3.11). Despite the fact that inoculation with the toluene- and

TCE-degrading Ni-resistant lupine endophyte *B. cepacia* VM1468 did not affect Ni uptake when plants were exposed to mixes of Ni and toluene (figure 3.7), a significantly increased Ni uptake by the roots of plants exposed to 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE was achieved after inoculation with *B. cepacia* VM1468. A similar increased Ni uptake by roots was previously reported by Lodewyckx *et al.* (2001) for plants only exposed to 0.25 mM $NiSO_4$ and inoculated with the Ni-resistant lupine endophyte *B. cepacia* BU72, of which VM1468 is a derivative.

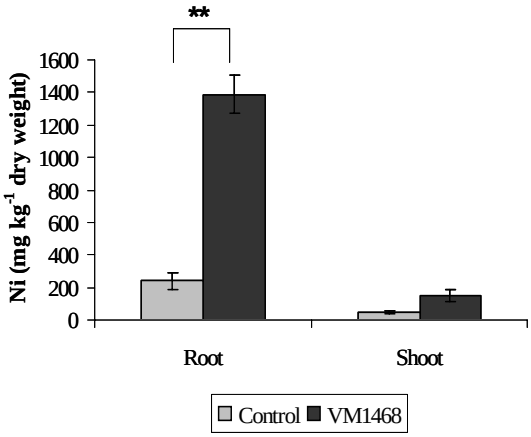


Figure 3.11: Ni concentration [mg (kg dry weight)$^{-1}$] in roots and shoots of control (non-inoculated) lupine plants and plants inoculated with *B. cepacia* VM1468 that were exposed to 0.25 mM $NiSO_4$ and 10 mg l^{-1} TCE. Values are mean ± standard error of at least 3 biological independent replicates. (significance levels: **: $p < 0.01$)

Chapter 3.5 *Conclusions*

So far, most studies on (phyto)remediation of sites with mixed contaminations of metals and organics have focussed on the degradation of the organic contaminants (Majumdar *et al*., 1999; Roane *et al*., 2001; Lin *et al*., 2006). Metal extraction has not been considered and attention has been restricted to inactivation of these elements. Here we described how endophytic bacteria possessing the right properties, can improve phytoremediation of mixed contaminations by decreasing metal phytotoxicity and increasing degradation of organic contaminants.

Inoculation of yellow lupine with *B. cepacia* VM1468, an endophytic bacteria possessing the pTOM-Bu61 plasmid coding for toluene and TCE degradation and the *ncc-nre* Ni resistance/sequestration system, resulted in a strongly reduced phytotoxicity in plants exposed to Ni and toluene and to Ni and TCE. However, after inoculation with *B. vietnamiensis* BU61 (pTOM-Bu61) or *B. cepacia* BU72 (*ncc-nre*), both of which lack one of the properties to deal with the mixed contamination, no reduction in toluene phytotoxicity was achieved.

The combined Ni resistance and toluene degradation capabilities allow *B. cepacia* VM1468 to survive at higher numbers under combined Ni and toluene exposure as compared to *B. vietnamiensis* BU61 and *B. cepacia* BU72. Since *B. cepacia* VM1468 shows ACC deaminase activity (Taghavi *et al*., 2009), its higher colonization levels might also contribute to a more efficient breakdown of 1-aminocyclopropane-1-carboxylate (ACC), the precursor for ethylene production, thus reducing the plants' stress response. The perspectives of bacterial ACC deaminase production to improve phytoremediation were recently reviewed (Arshad *et al*., 2007).

Evapotranspiration of toluene and TCE through the leaves after exposure to a mixture of Ni and toluene, and Ni and TCE respectively decreased for plants inoculated with VM1468. Moreover, the toluene degradation capacity clearly was not affected by the presence of Ni: the reduction of toluene evapotranspiration was similar as previously measured for plants exposed to the same toluene concentration (Barac *et al*., 2004).

In addition, the Ni uptake in roots and shoots of plants exposed to the mixed contaminations of Ni and toluene is comparable to the Ni uptake after exposure

to the same concentration of Ni only (Lodewyckx *et al*., 2001). When plants were exposed to Ni and TCE, Ni uptake was significantly higher for plants inoculated with *B. cepacia* VM1468 in comparison with non-inoculated plants.

Although for practical reasons genetically modified endophytes were used in this study, the same concept can easily be realized using naturally modified endophytes (obtained via horizontal gene transfer). This may facilitate public acceptance of endophyte-assisted phytoremediation and by consequence its use in practice. It will be relatively straightforward to equip endophytes by horizontal gene transfer with the appropriate degradation pathways as many endophytic bacteria are closely related to environmental strains that carry pathways against a broad spectrum of organics on mobile DNA elements. Secondly, horizontal gene transfer can also be used to improve heavy metal resistance of endophytic bacteria. For instance, retrotransfer by broad host range plasmids, such as RP4, can be used as a straightforward approach to acquire the desired heavy metal resistances (Top *et al*., 1992). In this way, endophytes can be specifically tailored for many mixed contaminations. In case the energy requirements to maintain concurrent metal resistance and organic degradation are so high that the introduced endophyte becomes unable to perform both activities under field conditions, a consortium of metal-detoxifying endophytes and endophytes capable of degrading the organic contaminants can be applied. Roane *et al.* (2001) have successfully used a consortium of metal-detoxifying and organic-degrading bacteria to facilitate organic degradation during bioaugmentation of co-contaminated soil. The results obtained in this work did indicate that engineered metal-detoxifying and organic-degrading endophytes represent a break-through for the improved phytoremediation of organics in co-contaminated sites.

Section 4

Exploration of poplar cutting (Grimminge) – *Pseudomonas putida* W619 partnerships

Abstract

In the work presented in chapter 4.1, poplar cuttings were inoculated with the poplar endophyte *Pseudomonas putida* W619 (wild-type or *gfp*-labelled). Analysis of various morphological, physiological and biochemical parameters showed that inoculation with *P. putida* W619 (wildtype) resulted in (a) remarkable plant growth promotion, (b) decreased activities of antioxidative defence related enzymes in roots and leaves, and (c) strongly reduced stomatal resistance, all indicative for improved plant fitness in comparison with the non-inoculated control cuttings. In contrast, inoculation with *gfp*-labelled *P. putida* W619::*gfp*1, obtained after insertion of miniTn5-Km2-*gfp*, did not at all promote plant growth. It even had a negative effect on plant fitness, as illustrated by (a) inhibiting effects on plant growth, (b) an increased activity of the antioxidative enzyme superoxide dismutase in the roots, and (c) a significantly increased stomatal resistance as compared to the non-inoculated control. Further, compared to the wild-type strain, colonization by the *gfp*-labelled *P. putida* W619::*gfp*1 was much lower; moreover, it only colonized rhizosphere and root cortex while the wild-type strain also colonized the root xylem vessels. The negative responses of cuttings to inoculation were confirmed using another *gfp*-labelled derivative *P. putida* W619::*gfp*2.

Since the *gfp*-transposon did not insert in an area of the *P. putida* W619 genome coding for genes putatively involved in colonization or production of phytohormones, the observed differences after inoculation with the wild-type and *gfp*-labelled strains might be related to *gfp* expression, probably causing a 'stress effect' on the plant, more specifically on root cells, leading to an inhibition of root development and decreased plant fitness. It cannot be excluded that insertion of *gfp* has induced secondary mutations leading to changes in background DNA, which also could be responsible for negative effects on plant fitness.

In the next chapter (chapter 4.2), the same poplar endophyte *P. putida* W619 equipped with the pTOM plasmid coding for TCE degradation (*P. putida* W619-TCE) was inoculated in poplar cuttings exposed to 0, 200 and 400 mg l^{-1} TCE that were grown in two different experimental setups. During a short-term experiment, plants were grown hydroponically in half strength Hoagland nutrient solution and were exposed to TCE for 3 days. Inoculation with *P. putida* W619-

TCE promoted plant growth, reduced the TCE phytotoxicity and reduced the amount of TCE present in the leaves.

During a mid-term experiment, plants were grown in potting soil and were exposed to TCE for 3 weeks. In comparison with half strength Hoagland's nutrient solution, potting soil contains much more essential plant available nutrients. This probably resulted in a less pronounced positive effect of *P. putida* W619-TCE on plant growth and TCE phytotoxicity. In this mid-term experiment, the amount of TCE present in the leaves and the roots of plants exposed to 400 mg l^{-1} and inoculated with *P. putida* W619-TCE was strongly reduced and the amount of TCE that was evapotranspired was significantly lower after inoculation with the TCE-degrading *P. putida* W619-TCE. These results confirm that *P. putida* W619-TCE is degrading TCE during its transport through the poplar xylem leading to a reduced amount of TCE present in the plant and a decreased evapotranspiration.

In conclusion, given that *P. putida* W619-TCE is capable of (a) colonizing poplar, (b) improving plant fitness, (c) reducing TCE phytotoxicity and evapotranspiration, poplar–*P. putida* W619 partnerships are ideal candidates for endophyte-enhanced phytoremediation in the field.

Introduction

Endophytic bacteria are defined as bacteria that reside within living plant tissue without causing substantive harm to the host plant. Bacterial entry into plants predominantly takes place in the roots, more precisely at sites of epidermal damage, that naturally arise as a result of plant growth (lateral root formation), or through root hairs and at epidermal conjunctions (Sprent and de Faria, 1988). Once inside the plant, endophytic bacteria either colonize the plant systematically by migration through the vascular system or the apoplast, or remain localized in specific plant tissues like the root cortex or the xylem, (Hurek *et al*., 1994; James *et al*., 1994; Mahaffee *et al*., 1997; Quadt-Hallmann *et al*., 1997).

Plant-endophyte associations rely on very close beneficial interactions where plants provide nutrients and residency for bacteria, which in exchange can directly or indirectly improve plant growth and health (see section 1: introduction). These mechanisms can improve biomass production of plants suitable for biofuels and carbon sequestration.

In addition to their beneficial effects on plant growth, endophytic bacteria can contribute to an improved phytoremediation of organic contaminants. Since the organic pollutants that have been taken up through the roots move through the plant vascular system, endophytic bacteria, possessing the genetic information required for the efficient degradation of a contaminant, can promote its degradation and decrease phytotoxicity and evapotranspiration (Barac *et al*., 2004; Taghavi *et al*., 2005).

Since poplar is an excellent candidate for both phytoremediation and as a biofuels feedstock, it was chosen for a more in depth study of (a) the colonization process and the plant growth promotion capacity (chapter 4.1) and (b) the potential to reduce TCE phytotoxicity and evapotranspiration (chapter 4.2), of the endophyte *Pseudomonas putida* W619-TCE that is also equipped with a pTOM plasmid coding for TCE degradation via *in planta* horizontal gene transfer.

Chapter 4.1 *Colonization and plant growth-promoting capacity of the endophyte Pseudomonas putida W619 in hybrid poplar*

***Submitted in**: Weyens N, Boulet J, Adriaensen D, Timmermans J-P, Prinsen E, Van Oevelen S, D'Haen J, Smeets K, van der Lelie D, Taghavi S, Vangronsveld J (xxxx) Colonization and plant growth-promoting capacity of the endophyte Pseudomonas putida W619 in hybrid poplar. Plant Biology*

4.1.1 Materials and methods

4.1.1.1 Construction of gfp-labelled Pseudomonas putida W619

The *gfp:kanamycin*-labelled strain was constructed by conjugation between *E. coli* CM2780 carrying the pFAJ1819 plasmid (miniTn5-Km2-*gfp*) as a donor and *P. putida* W619 as a receptor as described previously (Taghavi *et al.*, 2009). Kanamycin (100 μg ml^{-1}) resistant transconjugants were selected on 284 minimal medium (see 3.1.2) complemented with a carbon mix (a mixture of glucose, gluconate, lactate, succinate and acetate was added at 0.05 % (wt/vol) each) and subsequently tested for *gfp* expression under UV light. The stability of the *gfp* insertions was verified by growing 100 individual transconjugants for 100 generations on non-selective 869 medium (Mergeay *et al.*, 1985) and subsequently replica-plating them on 869 medium supplemented with kanamycin, after which they were tested for *gfp* expression. All *gfp*-labelled strains gave transconjugants that stably maintained the insertion (less than 1% loss). A transconjugant, referred to as *P. putida* W619::*gfp*1 that showed 100% stable expression of *gfp* under non-selective conditions, was used for in depth studies of interaction with the host plant. A second derivative, *P. putida* W619::*gfp*2 was used to confirm effects of *gfp*-labelled strains on plant growth.

The insertion of miniTn5-Km2-*gfp* in *P. putida* W619::*gfp*1 was determined as recently described (Taghavi *et al.*, 2009). Genomic DNA of W619::*gfp*1 was isolated (Bron and Venema, 1972) and digested with *Hpy*CH4IV (New England Biolabs), which has no recognition site between the 5'-end of *gfp* and the upstream end of the miniTn5 transposon. After digestion, *Hpy*CH4IV was heat inactivated by incubating the digestion mixture for 20 min at 65°C.

Subsequently, the restriction fragments were ligated to a Y-shaped linker cassette, which was obtained by annealing two oligonucleotides with sequences 5' TTTGGATTTGCTGGTCGAATTCAACTAGGCTTAATCCGACA-3' and 5'-CGTGTCG GATTAAGCCTAGTTGAATTTATTCCTATCCCTAT-3' as described previously (van der Lelie *et al.*, 2006). The ligation mixture was GFX purified and used as a template for a linear amplification, using a single primer complementary and pointing outwards of the 5' end of *gfp* (GFP primer: 5'-GAAAAGTTCTTCTCCTTTAC-3'). The linear amplification results in repair of the Y-shaped linker cassette only for those fragments that contain the *gfp* insertion region. Subsequently, PCR was performed using the GFP primer plus the LINKER primer (5'-GGATTTGCTGGTCGAATTCAAC-3'), which will only hybridize to the repaired linker. This PCR reaction resulted in the amplification of a single DNA fragment, indicative for a single insertion of miniTn5-Km2-*gfp*, of approximately 1200 bp. Sequence analysis of this fragment revealed that one end corresponded to the *gfp*-end of miniTn5-Km2-*gfp*. The site of the miniTn5-Km2-*gfp* insertion was found to be located at position 341748 of the *P. putida* W619 genome (http://genome.jgi-psf.org/finished_microbes/psepw/psepw.home. html), within ORF326, which encodes for a putative heavy metal translocating P-type ATPase (CadA-like). This gene is located in a conserved genome region of several *P. putida* genomes, including F1, BG-1 and KT2440, where it involved in resistance to Cd (Cànovas *et al.*, 2003)

We also determined the site of miniTn5-Km2-*gfp* insertion in the second *gfp*-labelled derivative *P. putida* W619::*gfp*2. The site of the miniTn5-Km2-*gfp* insertion of this strain was located at position 524756 of the W619 genome in ORF478 which encodes a putative hemolysin III family channel protein. Again, this gene is located in a region conserved between the genomes of *P. putida* F1, BG-1 and KT2440, and is not considered to be a specific property of W619. However, it can not be excluded that hemolysin plays a role in the interaction of *P. putida* W619 with poplar.

4.1.1.2 IAA and cytokinin production of the bacteria

Pre-cultures of the *P. putida* W619 and W619-*gfp* strains were started by inoculating 20 ml of liquid LB medium from glycerol stocks and growing them at

30°C / 200 rpm. After 24 h the OD (at 600 nm; cm^{-1}) of both cultures was adjusted and 100 µl of an OD = 0.2 suspension was used for inoculation of 25 ml fresh medium. All subsequent cultures were grown at 30°C and 200 rpm in liquid MMAB medium (per liter distilled water: 3 g K_2HPO_4, 1 g Na H_2PO_4, 1 g NH_4Cl, 0.3 g $MgSO_47H_2O$, 0.15 g KCl, 0.01 g $CaCl_22H_2O$, 0.0025 g $FeSO_47H_2O$, 5 g sodium malate) with and without addition of 10 mg l^{-1} tryptophan. Tryptophan was added in this concentration to mimic the tryptophan concentration as was found in the rhizosphere of maize (*Zea mays*) and tobacco (*Nicotiana tabacum*) (Prinsen *et al*., unpublished results). Analogous to the Salkowsky protocol (Mayer, 1958) hormones were analyzed after 72 h of growth. Cultures were centrifuged (10 min, 1700 g, 4°C) and supernatant was isolated (per sample 8 ml was taken for indole-3-acetic acid (IAA) extraction, and 10 ml for cytokinin extraction).

IAA was purified from bacterial samples as described by Prinsen *et al*. (1997). For recovery and quantification purposes 500 pmol 13C6-IAA (phenyl-13C6-indole-3-acetic acid; Cambridge Isotope Laboratory, Inc., Andover, MA, USA) was added as an internal standard. Cytokinin extraction was based on the protocol previously described by Redig *et al.* (1996). Following deuterated cytokinins were added as internal standards: $[^2H_3]$DHZ, $[^2H_3]$DHZR, $[^2H_5]$Z-N-G, $[^2H_6]$2iP, $[^2H_6]$2iPA, $[^2H_6]$2iP-G (Apex Organics, Honiton, UK).

An ACQUITY UPLC® System was combined with an ACQUITY® TQD (Waters, Milford, MA, USA) for analysis of IAA. Samples were injected on a VanGuard™ pre-column (BEH C18, 1.7 µm, 2.1 x 5 mm; Waters, Milford, MA, USA) coupled to a reversed-phase column (BEH C18, 1.7 µm, 2.1 x 50 mm; Waters, Milford, MA, USA). For IAA analysis, after a 2 min equilibration at 10:90 A:B samples were eluted from the column by changing solvent composition to 90:10 A:B in a 2 min linear gradient (where A is methanol and B is 1mM ammonium acetate), using a constant flow-rate of 300 µl min^{-1} and a column temperature of 30°C. For cytokinin analysis, solvent composition was changed from 10:90 A:B to 48/52 A:B over a 7.5 min linear gradient, followed by a linear increase to 70/30 A:B over 1.5 min. In both cases the column was rinsed at 100:0 A:B for 2 min and equilibrated at 10:90 A:B before the next run.

Quantification was done in ESI(+)-MRM mode. Chromatograms obtained were processed using QuanLynx v4.1 (Waters, Milford, MA, USA). Concentrations

were calculated using the principle of isotope dilution. Data are expressed in pmol OD^{-1} ml^{-1}.

4.1.1.3 Inoculation

Poplar cuttings [*Populus deltoides x (trichocarpa x deltoides) cv. Grimminge*] of 30 cm long were placed in aerated tap water for 4 weeks in order to allow establishment of roots, after which they were weighed and root inoculation was performed under greenhouse conditions. The cuttings were inoculated with *P. putida* W619 (wild-type), *P. putida* W619::*gfp*1 or *P. putida* W619::*gfp*2. Fresh cultures of the strains were grown in 869 medium (Mergeay *et al.*, 1985) at 30°C until an approximate absorbance (A_{660}) value of 1 was reached. Bacterial cells were washed in 10 mM $MgSO_4$ and resuspended in the original volume of 10 mM $MgSO_4$. Cuttings were inoculated for 1 week in 1.8 liter half strength Hoagland's nutrient solution to which 200 ml of the inocula was added. Control plants were placed in the same solution without bacteria. After inoculation, cuttings were grown hydroponically in 2 liter jars filled with half strength Hoagland's solution for an additionally three weeks before harvesting.

4.1.1.4 Colonization

Four weeks after the start of the inoculation, colonization of the root by *P. putida* W619::*gfp* was visualized *in planta* using confocal microscopy. Hand-cut longitudinal and transverse sections as well as intact fresh root material (5 cm from the apex; at least 45 replicas), were examined using an inverted microscope (Zeiss Axiovert 200; Carl Zeiss, Jena, Germany), attached to a microlens-enhanced dual spinning disc confocal microscope system (Ultra*VIEW* ERS, PerkinElmer, Seer Green, UK) equipped with a three-line (488, 568 and 647 nm) argon-krypton laser. Excitation with blue light (488 nm) was used for visualization of the green fluorescence (emission filter 527 ± 30 nm) of *gfp*-labelled *Pseudomonas* but also resulted in an unavoidable autofluorescence of poplar tissues. Double exposure with green light (568 nm; emission filter 640 ± 60 nm) however appeared to provide the possibility for differentiating *gfp*

fluorescence from autofluorescence by creating an additional unspecific red autofluorescence of the tissue without interfering with the *gfp* fluorescence.
In addition to confocal analysis, scanning electron microscopy (SEM) was used to further confirm the presence/absence of both inoculated bacteria. For this, pure bacteria and hand-cut longitudinal sections (at least 45 of each) of 3 times surface washed fresh root material (4 weeks after the start of the inoculation) of both inoculated as well as non-inoculated cuttings were analyzed. SEM studies were performed using a FEI Quanta 200 FEG-SEM. The images, mostly back-scattered electron images revealing better atomic number contrast, were recorded under low vacuum conditions.
Finally, colonization by the inocula was examined by means of re-isolation. Root, stem, twig and leaf samples were surface sterilized for 10 (roots and leaves) or 5 (stems and twigs) minutes in a 2% (roots and leaves) or a 1% (stems and twigs) active chloride solution supplemented with 1 drop Tween 80 (Merck) per 100 ml solution. After surface sterilization, samples were rinsed 3 times for 1 min in sterile distilled water. The third rinsing solution was plated on 869 medium (Mergeay *et al*., 1985) to check surface sterility (if no growth was observed after 7 days, surface sterilization was considered to be successful). Surface sterile poplar samples were macerated for 60 (roots and leaves) or 120 (stems and twigs) sec in 10 ml 10 mM $MgSO_4$ using a Polytron PR1200 mixer (Kinematica A6) immediately after the third rinse. Serial dilutions were plated on selective 284 medium (see 3.1.2) supplemented with C-mix (per liter medium: 0.52 g glucose, 0.35 g lactate, 0.66 g gluconate, 0.54 g fructose and 0.81 g succinate) with addition of 1mM $NiCl_2$ as a selective agent for W619 (wild-type) and 1mM $NiCl_2$ + 100 mg l^{-1} kanamycin as a selective agent for W619::*gfp*; and incubated for 7 days at 30°C before the CFU were counted and calculated per gram fresh plant weight.

4.1.1.5 Plant responses

Morphological parameters

Four weeks after the start of the inoculation (*i.e*. after eight weeks of growth), plants were harvested and their growth index [(final $biomass_{fresh\_weight}$ – initial

biomass$_{fresh_weight}$) / initial biomass$_{fresh_weight}$], biomass (fresh weight) of different plant parts, root length, leaf surface and the number of leaves were determined.

Physiological and biochemical parameters

During the experiment (one week after inoculation) the stomatal resistance as a general indicator for plant fitness (Gao *et al*., 2003) was determined using a Delta-T AP4 porometer.

During harvest, leaf and root samples for determining of the activities of enzymes involved in antioxidative defence were taken and snap-frozen immediately into liquid nitrogen before storing them at -80°C. These frozen leaf and root tissues were homogenized in ice-cold 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiotreitol and 4% insoluble polyvinylpyrrolidone (1 ml buffer per 100 mg fresh weight). Subsequently, this homogenate was squeezed through a nylon mesh and centrifuged for 10 minutes at 20000 g and 4°C. The enzyme activities of glutathione reductase (GR, EC 1.6.4.2) and superoxide dismutase (SOD, EC 1.15.1.1) as marker enzymes for oxidative stress (Vangronsveld and Clijsters, 1994) were measured spectrophotometrically in the supernatant at 25°C. Analysis of SOD activity was based on the inhibition of the reduction of cytochrome C measured at 550 nm (McCord and Fridovich, 1969). Analysis of GR activity was based on the reduction of GSSG measured at 340 nm, and using NADPH as described by Bergmeyer *et al*. (1974).

4.1.1.6 Statistical analysis

Different inoculations were statistically compared using one-way ANOVA and post-hoc multiple comparison testing (Tukey-Kramer). Transformations were applied when necessary to approximate normality and/or homoscedasticity. The stomatal resistance datasets were analyzed using linear mixed-effects models (Verbeke and Molenberghs, 2000) to correct for the correlation between different leaves of the same plant. The statistical analyses were performed using the ANOVA and MIXED procedure in SAS 9.1.3. Measurements of plant responses were performed on 10 control cuttings, 10 W619-inoculated cuttings

and 5 W619 + *gfp*-inoculated cuttings; and stomatal resistance was measured for at least 2 leaves per cutting. IAA- and cytokinin production was determined on 5 biological independent replicates. Furthermore, all results shown in this work were confirmed in an additional independent experiment.

4.1.2 Results

4.1.2.1 IAA and cytokinin production of the bacteria

Unless otherwise stated, *P. putida* W619::*gfp*1 was used for plant interaction studies. In this strain the site of the chromosomal miniTn5-Km2-*gfp* insertion was found to be located in a zone encoding for a putative cadmium translocating P-typeATPase (CadA-like), and therefore no effects on colonization and/or production of plant growth regulating phytohormones should be expected. Nevertheless, to be sure that no indirect effects of *gfp* insertion on hormone production were occurring, production of both, IAA and cytokinin by the inoculated bacteria were determined in MMAB medium with and without addition of 10 mg l^{-1} tryptophan. Auxins indeed play a central role in root development (Tanimoto *et al*., 2005). Bacterial cytokinin production also may be important for root and shoot growth (Arkhipova *et al*., 2007). Tryptophan was added in a concentration of 10 mg l^{-1} to mimic the tryptophan concentration as was found in the rhizosphere of maize (*Zea mays*) and tobacco (*Nicotiana tabacum*) (Prinsen *et al*., unpublished results).

In absence of tryptophan, there was no difference in the IAA production between *P. putida* W619 wild-type and *gfp*-labelled (figure 4.1). After addition of 10 mg l^{-1} tryptophan, the IAA production of both bacteria slightly increased, but also for this condition the IAA production of both strains (wild-type and *gfp*-labelled) was similar (figure 4.1). Furthermore, no significant difference in cytokinin production was observed for the wild-type and *gfp*-labelled *P. putida* W619::*gfp*1 (results not shown).

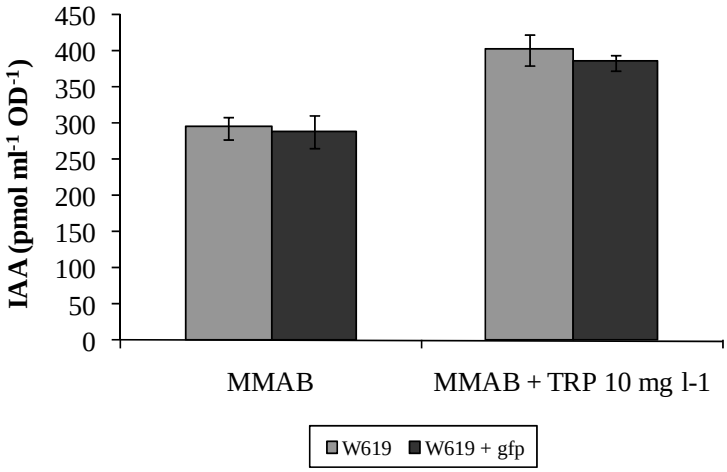


Figure 4.1: IAA production (pmol $ml^{-1}OD^{-1}$) of *P. putida* W619 and *P. putida* W619 + *gfp* in MMAB medium without and with addition of 10 mg l^{-1} tryptophan (TRP). Values are mean ± S.E. of 5 biological independent replicates.

4.1.2.2 Colonization

Poplar cuttings were allowed to grow for 3 weeks after 1 week of inoculation, after which confocal microscopy was used to visualize colonization of the *gfp*-labelled *P. putida* W619::*gfp*1 in living poplar roots. Because the cultured *gfp*-labelled strains were characterized by a strong green fluorescence (blue 488 nm excitation) and the lack of a red fluorescence (green 568 nm excitation) (figure 4.2A, 4.2B and 4.2C), they could easily be distinguished from the broad-spectrum autofluorescence (including green and red fluorescence) of the root tissue using double exposure imaging (figure 4.2D, 4.2E and 4.2F). In hand-cut longitudinal and transverse sections, no *gfp*-labelled bacteria could be detected in the root cortex or in the xylem. In intact fresh roots, *gfp*-labelled *P. putida* W619 could be visualized at the root surface as well as in the root cortex (figure 4.2F), but it was not detected in the root xylem.

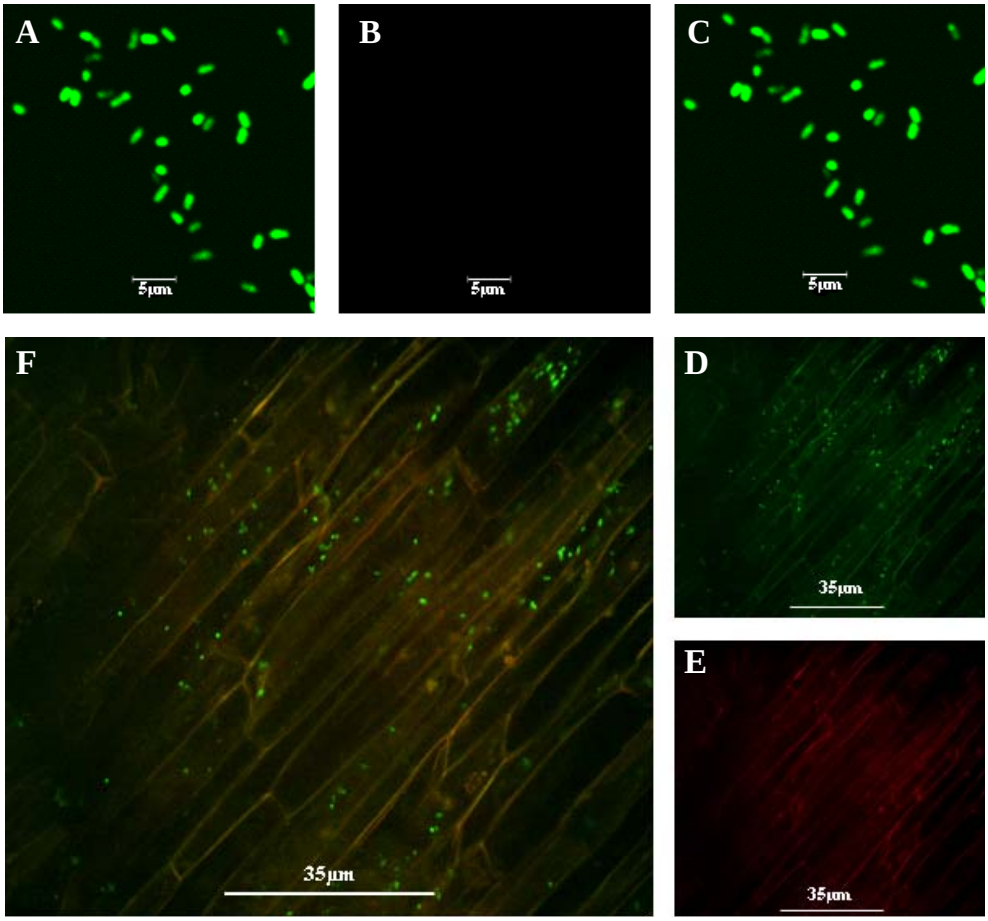


Figure 4.2: A-B-C: Suspension with live *gfp*-labelled *P. putida* W619. A. Green *gfp*-fluorescent bacteria seen with blue light (488 nm) excitation. B. Red fluorescence channel showing that excitation with green light (568 nm) does not interfere with *gfp* fluorescence. C. Combination of the green and red channels. D-E-F: Confocal images of live *gfp*-labelled *P. putida* W619 bacteria in the cortex of a freshly prepared intact poplar root. D. Green *gfp*-fluorescent bacteria seen with blue light (488 nm) excitation. Note the autofluorescence of poplar tissues. E. Red channel showing that excitation with green light (568 nm) also results in tissue autofluorescence. F. Combination of the green and red channels also created an unavoidable autofluorescence of poplar tissues. Double exposure with green light (568 nm; emission filter 640 ± 60 nm) however appeared to provide the possibility for differentiating *gfp* fluorescence from autofluorescence by creating an additional unspecific red autofluorescence of the tissue without interfering with the *gfp* fluorescence.

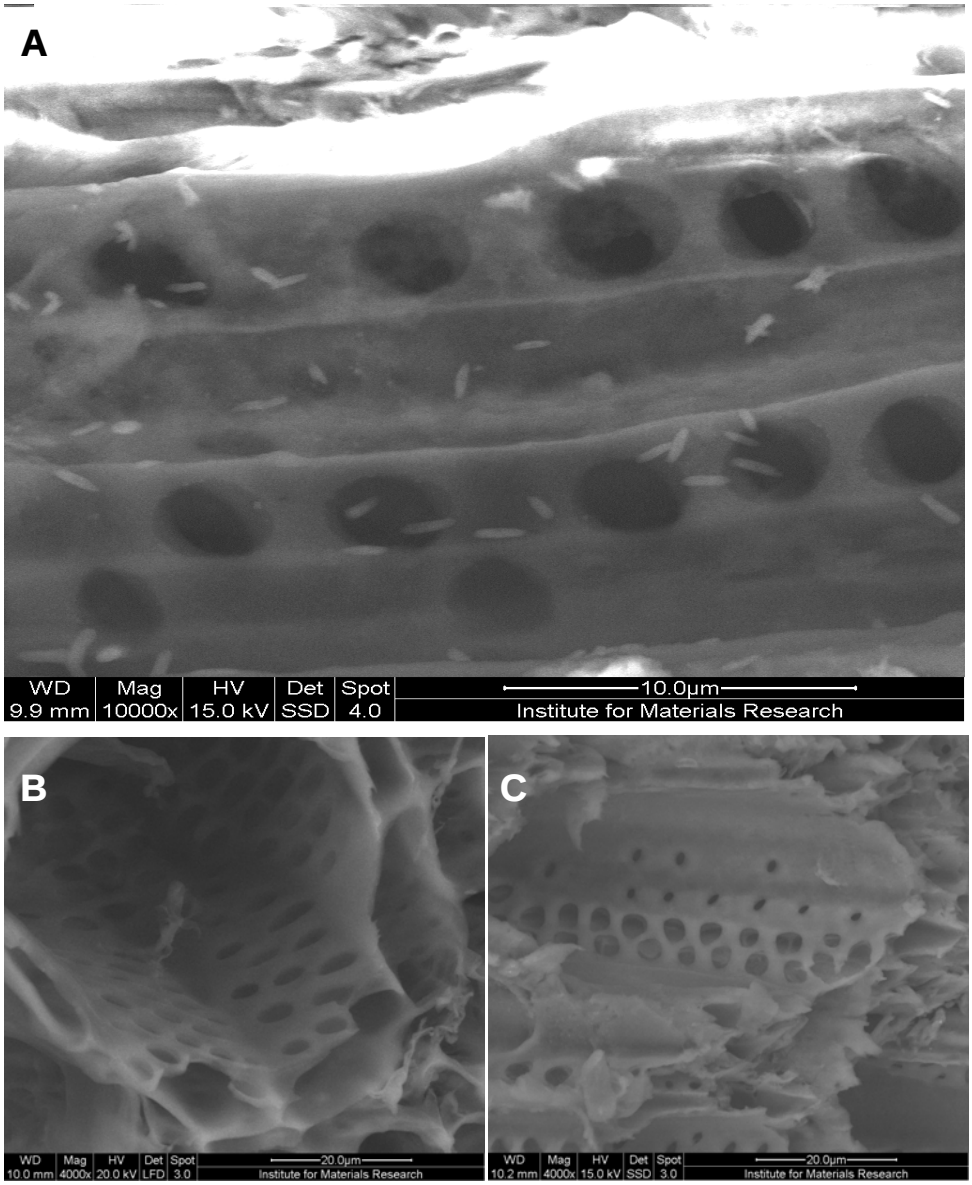


Figure 4.3: SEM of hand-cut longitudinal sections of fresh root material (3 weeks after inoculation) of (A) a *P. putida* W619-inoculated cutting, (B) a *gfp*-labelled *P. putida* W619-inoculated cutting and (C) a non-inoculated cutting.

Subsequently, hand-cut longitudinal sections of fresh root material were studied using SEM. In this way, it was possible to clearly observe the root xylem and, in case of *P. putida* W619-inoculated cuttings, the xylem colonizing bacteria (figure 4.3). The facts that (a) the shape and the size of these bacteria perfectly matched with the inoculated bacterial strain and (b) these bacteria could not be detected in non-inoculated control cuttings, are strong indications that the observed bacteria were *P. putida* W619. In the cuttings of roots inoculated with the *gfp*-labelled strain, no bacteria could be detected in the xylem by SEM, thus confirming the results acquired with confocal microscopy.

Furthermore, the conclusions from confocal microscopy and SEM were confirmed by the results of the re-isolation of the inoculated bacteria. Cuttings inoculated with *P. putida* W619 showed considerable colonization in all tissues except in the leaves. The amount of re-isolated bacteria (CFU per g fresh weight) was decreasing from $3.91x10^7$ in the roots, $6.02x10^5$ in the stem, and $3.82x10^3$ in the twigs to 0 in the leaves. However, compared to the wild-type strain, colonization by the *gfp*-labelled *P. putida* W619 was much lower in the roots ($7.62x10^4$) and the stem ($1.45x10^1$) and no colonization of the twigs and the leaves was observed. Obviously, as the medium used for re-isolation was selective for (*gfp*-labelled) *P. putida* W619, no bacterial strains could be re-isolated out of the non-inoculated control cuttings.

4.1.2.3 Plant responses

At the moment the plants were harvested, their growth index [(final biomass – initial biomass) / initial biomass], biomass of different plant parts (figure 4.4), as well as root length, leaf area, and numbers of leaves (results not shown) were determined. In general, when compared to the non-inoculated controls, cuttings inoculated with *P. putida* W619 showed improved plant fitness, while inoculation with the *P. putida* W619::*gfp*1 seemed to have a negative effect on the host plant. Inoculation with *P. putida* W619 resulted in significant increases of leaf and root biomass and growth-index (figure 4.4), as well as in longer roots, increased leaf area and number of leaves (results not shown). In addition, this improved plant fitness (in comparison to the non-inoculated control) was

further supported by (a) a significantly lower stomatal resistance (figure 4.5) and (b) significantly lower glutathione reductase activity in the root and superoxide dismutase activity in the root and the leaves (figure 4.6).

As stated above, cuttings inoculated with the *gfp*-labelled *P. putida* W619::*gfp*1 did not show any plant growth promotion. On the contrary, there even was a slight decrease in root biomass as well as in growth index (figure 4.4). This negative effect was confirmed by a borderline significant (p=0.05) increase in stomatal resistance (figure 4.5). Furthermore, the activity of glutathione reductase was more or less similar as in the non-inoculated controls and superoxide dismutase activity showed a slight increasing trend in comparison with the control (figure 4.6).

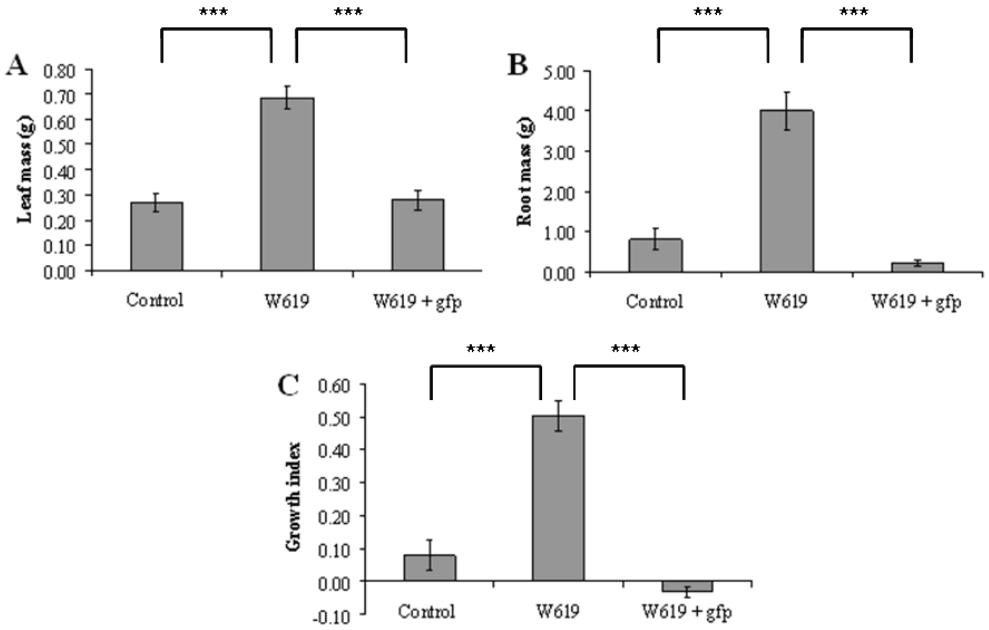


Figure 4.4: Leaf mass (g) (A), root mass (g) (B) and growth index (C) 3 weeks after inoculation for non-inoculated control cuttings and cuttings inoculated with W619 and W619 + *gfp*. Growth index = (final biomass – initial biomass) / initial biomass. Values are mean ± S.E. of 10 (in case of Control and W619) and 5 (in case of W619 + *gfp*) biological independent replicates (significance level: ***: $p < 0.01$).

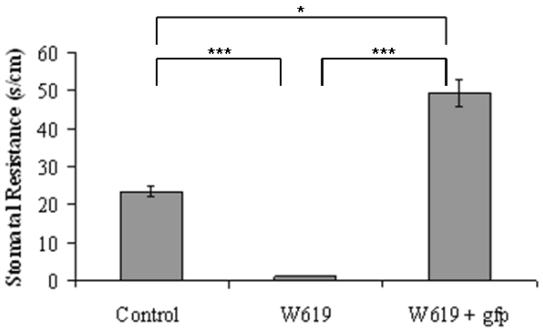


Figure 4.5: Stomatal resistance (s cm^{-1}) 1 week after inoculation for non-inoculated control cuttings and cuttings inoculated with W619 and W619 + *gfp*. Values are mean ± S.E. of 10 (in case of Control and W619) and 5 (in case of W619 + *gfp*) biological independent replicates (significance levels: *: $p < 0.1$; ***: $p < 0.01$). Furthermore, stomatal resistance was measured for at least 2 leaves per cutting.

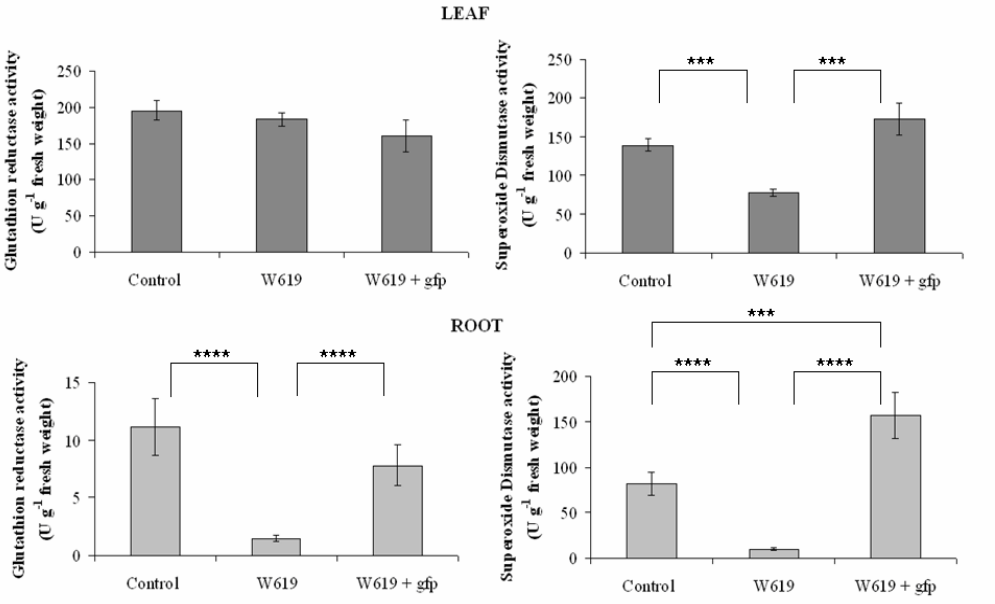


Figure 4.6: Glutathione reductase and superoxide dismutase activity (units g^{-1} fresh weight) in leaves and roots of non-inoculated cuttings and cuttings inoculated with W619 and W619 + *gfp* 3 weeks after inoculation. Values are mean ± S.E. of 10 (in case of Control and W619) and 5 (in case of W619 + *gfp*) biological independent replicates (significance levels: ***: $p < 0.01$; ****: $p < 0.001$).

4.1.3 Conclusions

Inoculation of *Populus deltoides* x (*trichocarpa* x *deltoides*) cv. Grimminge with the wild-type *P. putida* W619 resulted in good colonization (figure 4.3), and had a highly positive effect on plant fitness and growth (figures 4.4, 4.5 and 4.6). On the contrary, colonization of the poplar cuttings with *P. putida* W619::*gfp*1 was limited (figures 4.2 and 4.3) and even resulted in a decreased plant fitness and growth (figures 4.4, 4.5 and 4.6).

Further, the effects of the inoculation on plant growth were confirmed using biochemical and physiological parameters (figures 4.5 and 4.6). Inoculation with wild type *P. putida* W619 resulted in significant decreases of the activities of glutathione reductase in the roots and of superoxide dismutase in both roots and leaves. Since glutathione reductase and superoxide dismutase are both marker enzymes for antioxidative defence (Vangronsveld and Clijsters, 1994), a decrease in their activity suggests a decreased oxidative stress level and consequently improved plant fitness. The latter was also confirmed by a significantly lower stomatal resistance. Reductions of glutathione reductase and superoxide dismutase activities were not observed after inoculation with *P. putida* W619::*gfp*1. On the contrary, superoxide dismutase activity in the roots significantly increased (figure 4.6), indicating that inoculation with the *gfp*-labelled strain induced an oxidative stress response, like in case of infection by a pathogen. Furthermore, cuttings inoculated with *P. putida* W619::*gfp*1 showed a significantly increased stomatal resistance, indicating lowered plant fitness.

The colonization by the *gfp*-labelled strain is quite poor and limited to rhizosphere and root cortex (see data on colonization and figure 4.2). In contrast, the wild-type *P. putida* W619 even colonized the xylem of the roots, which could be visualized using scanning electron microscopy (figure 4.3). These observations indicate that use of *gfp*-labelled bacteria can result in a different colonization pattern in comparison to the wild-type strains. The in comparison to the non-inoculated control plants even negative effects on plant fitness (slight inhibition of root growth, induction of antioxidative defence in roots and increased stomatal resistance) suggest an adverse effect due to the *gfp* expression. Therefore, the observed differences in colonization by the wild-type and *gfp*-labelled strains and their effects on the plant fitness might be related to

the bacterial *gfp* expression. It indeed has been reported that *gfp* can have a toxic effect on plant cells (Haseloff *et al*., 1997; Petri *et al*., 2008), which could explain the negative effects on overall plant fitness after inoculation of poplar cuttings with the *gfp*-labelled *P. putida* W619. Furthermore, similar IAA-production (figure 4.1) and cytokinin production levels measured for wild type and *P. putida* W619::*gfp*1 strains ensure that the production of plant growth promoting compounds was not affected indirectly by the *gfp*-insertion. To further confirm that the observed differences in colonization by the wild-type and *gfp*-labelled strains were not related to the site of the *gfp*-insertion, poplar cuttings were inoculated with *P. putida* W619::*gfp*2. Inoculation with *P. putida* W619::*gfp*2 resulted in a very similar negative effect on plant responses (results not shown), confirming that the site of *gfp*-insertion did not affect the plant responses. It however cannot be excluded that the hemolysin gene, which was inactivated in *P. putida* W619::*gfp*2, plays a role in the interaction of *P. putida* W619 with poplar. Further, for both *gfp*-labelled strains, it cannot be excluded that insertion of *gfp* has induced secondary mutations leading to changes in background DNA, which also could be responsible for negative effects on plant fitness.

The, compared to the wild type, contrasting effects after inoculation with *gfp*-labelled strains illustrate that, despite the interesting results presented in earlier studies (Coombs and Franco 2003; Newman *et al*., 2003; Germaine *et al*., 2004; Bloemberg *et al*., 2006), *gfp*-labelling as a tool to visualize bacterial colonization of plants should be applied with sufficient caution.

The results obtained in this study show that inoculation of *Populus deltoides* x (*trichocarpa* x *deltoides*) cv. Grimminge cuttings with *P. putida* W619 resulted in an increased root mass (leading to an increased nutrient and water uptake) and a remarkably strong plant growth-promoting effect on the short-term. On the long-term, this might be of direct importance to the use of poplar as a feedstock for biofuel production, and for carbon sequestration through biomass production. However, long-term follow up of field grown poplar inoculated with plant growth-promoting strains is needed to support this concept.

Chapter 4.2 *Potential of Pseudomonas putida W619-TCE to reduce TCE phytotoxicity and evapotranspiration in poplar cuttings*

***Submitted in**: Weyens N, Truyens S, Dupae J, Newman L, van der Lelie D, Carleer R, Vangronsveld J (xxxx) Potential of the TCE-degrading endophyte Pseudomonas putida W619-TCE to improve plant growth and reduce TCE phytotoxicity and evapotranspiration in poplar cuttings. Environmental Pollution*

4.2.1 Materials and methods

4.2.1.1 Inoculation of poplar cuttings

Poplar cuttings [*Populus deltoides x (trichocarpa x deltoides) cv. Grimminge*] were placed in aerated tap water for 4 weeks to allow development of roots, after which they were weighed and root inoculation was performed under greenhouse conditions. The cuttings were inoculated with the TCE-degrading *Pseudomonas putida* W619-TCE, the same endophyte as used in chapter 4.1, but equipped via *in planta* horizontal gene transfer with the pTOM plasmid containing the constitutively expressed genes for TCE degradation (Taghavi *et al.*, 2005). Fresh cultures of this strain were grown in 869 medium (Mergeay *et al*., 1985) at 30°C until an approximate absorbance (A_{660}) value of 0.5 was reached. The cells were collected by centrifugation (15 min at 3900 g), washed in 10 mM $MgSO_4$ and resuspended in the original volume of 10 mM $MgSO_4$. Cuttings were inoculated for 4 days in half strength Hoagland nutrient solution to which the inoculum was added in a 1/10 dilution. Control plants were placed in the same solution without bacteria.

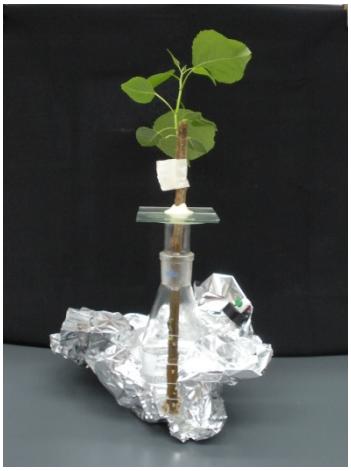
4.2.1.2 Growth, TCE exposure and harvesting of plants

After inoculation, cuttings were grown in two different systems: a gas-tight hydroponic system designed to evaluate TCE degradation during short-term exposure experiments, and a non-gas-tight potting soil system designed to evaluate TCE degradation during mid-term exposure experiments. Plants were

treated with 0, 200 and 400 mg l^{-1} TCE. For each exposure condition 20 cuttings were used, with 10 inoculated and 10 non-inoculated cuttings.

Short-term TCE exposure experiment

After inoculation, cuttings were placed in 500 ml flasks with a TCE injection tube that is equipped with a Mininert™ valve to add TCE (figure 4.7). The flasks were filled with 400 ml half strength Hoagland's nutrient solution and wrapped in aluminium foil to protect the roots from light and to prevent any TCE photodegradation. Cuttings were allowed to stabilize for 1 week under these conditions before the system was made gas-tight with glass plates putted on a ground glass stopper, Apiezon M grease and Polyfilla Interior (figure 4.7). TCE was added to 400 ml fresh half strength Hoagland nutrient solution. After 3 days of TCE exposure, plants were harvested.



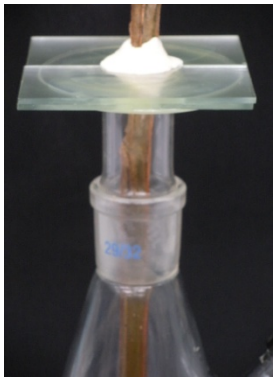


Figure 4.7: Gas-tight set-up to determine TCE degradation in short-term exposure experiments.

Mid-term TCE exposure experiment

For the mid-term exposure experiment, poplar cuttings were transferred from the inoculating solution into 2-liter beakers (1 cutting per beaker) containing a 2 cm thick layer of gravel (1-2 mm size) for preventing anaerobic conditions at the bottom. Above this layer, the beakers were filled up with potting soil and equipped with a 30 cm long glass tubing reaching the bottom for watering and application of TCE (figure 4.8). To protect the roots from light and to avoid any photodegradation of TCE, aluminium foil was wrapped around the beakers

(figure 4.8). After potting, plants were first allowed to grow for 3 weeks without adding TCE (until their roots penetrated into the gravel layer). From then, TCE was added through the glass tubes 3 times a week at concentrations of 200 and 400 mg l^{-1}. The amount of half strength Hoagland nutrient solution added to the potting soil in each beaker depended on the water consumption of the individual plant. Plants were exposed to TCE during 3 weeks before harvesting.

Figure 4.8: Set-up to determine TCE degradation in mid-term exposure experiments.

At harvest root and shoot (= twig and leaves) biomass were determined for all cuttings; subsequently, from 6 cuttings of each condition, samples of roots and leaves were snap frozen in liquid nitrogen to prevent volatilization of TCE. The samples were stored at -80°C until the extraction process.

4.2.1.3 TCE degradation and evapotranspiration

TCE extraction from leaves and roots

All root and leaf samples were extracted and analyzed for TCE. The samples were placed into VOA (volatile organic analysis) vials containing 2 ml of 1M H_2SO_4/ 1.7M NaCl solution. Methyl tert-butyl-ether (MtBE) (10 ml) was added and the mixtures were shaken for 10 minutes at 200 rpm. After shaking, the samples were allowed to settle for 10 minutes, after which the MtBE layer was transferred onto 2 g of Na_2SO_4 and dried for 45 minutes. The dried MtBE was transferred into 2 ml capillary gas chromatography (GC) vials with an internal

standard of 2 µl of ethylene dibromide (50 µl / 100 ml MtBE). Extracts were analyzed using a gas chromatograph equipped with a Supelco VOLCOL column (30m X 0.25m X 1.25 µm film thickness) and helium as carrier gas. TCE samples were run for 58 minutes at 140°C. 1.0 µl samples were injected into the column at a flow-rate of 20 ml min^{-1}. A mass spectrometer detector was used at a temperature of 340°C.

Metabolite extraction from leaves and roots

Since dichloroacetic acid (DCAA) and trichloroacetic acid (TCAA) are known intermediates of TCE degradation (Burken and Ma, 2006), an attempt was made to quantify these metabolites in the harvested tissues. Extraction was performed on 5 test samples (leaves and roots) for each condition. The samples were placed into VOA vials and 20 ml of nanopure water was added. After shaking for 10 minutes at 200 rpm, 3 ml of H_2SO_4 (pure: 95-97%) was added immediately followed by 9 g of Na_2SO_4 and 1 g of $CuSO_4$. Seven ml MtBE was added to the mixture after which it was shaken for 10 min at 200 rpm and allowed to settle for 5 min. The MtBE layer was transferred to a new VOA vial and 6 ml of 1M H_2SO_4 in methanol was added. Subsequently, the vial was placed in a 50°C water bath for 1 hour. After this warming period, the samples were cooled for 10 min at 4°C and 15 ml of a $CuSO_4/Na_2SO_4$ (50 g l^{-1} and 100 g l^{-1} respectively) solution was added. The mixture was shaken again for 10 min at 200 rpm and allowed to rest for 5 minutes. As a final step, the MtBE layer was transferred to a 2 ml GC vial with an internal standard of 2 µl of ethylene dibromide (50 µl / 100 ml MtBE). The extracts were analyzed using the same GC-setup as for the TCE extracts; the runtime was extended to 2 hours at 100°C and the flow rate was diminished to 10 ml min^{-1}.

TCE evapotranspiration

TCE evapotranspiration measurements were only performed on the cuttings with a mid-term TCE exposure. For each condition, TCE evapotranspiration was measured on 3 cuttings. The setup to determine TCE evapotranspiration is described in more detail in 5.1.5.

4.2.1.4 Statistical analysis

All datasets were statistically compared using two way ANOVA and *post hoc* multiple comparison testing (Tukey Kramer). Transformations were applied when necessary to approximate normality and/or homoscedasticity. The statistical analyses were performed in SAS 9.1.3.

4.2.2 Results

4.2.2.1 Effects of TCE on growth

Short-term TCE exposure / hydroponic growth

Short-term exposure of non-inoculated plants to 200 and 400 mg l^{-1} TCE resulted in significant inhibiting effects on root growth (figure 4.9). In contrast, exposure of inoculated plants to TCE did not cause any significant effect on plant growth: inoculation with the TCE-degrading endophyte *P. putida* W619-TCE significantly increased root and shoot weights, even when the plants were exposed to 200 and 400 mg l^{-1} TCE.

Mid-term TCE exposure / growth in potting soil

In case potting soil grown plants were exposed to TCE for 3 weeks, only supplying 400 mg l^{-1} TCE resulted in significant inhibitory effects on growth of roots and shoots (figure 4.10). For cuttings inoculated with *P. putida* W619-TCE, exposure to both TCE concentrations only had significant effects at the shoot level. Inoculation with *P. putida* W619-TCE only significantly promoted shoot growth of non-exposed cuttings, although inoculation resulted for all conditions in a slight, though not significant, increase in biomass.

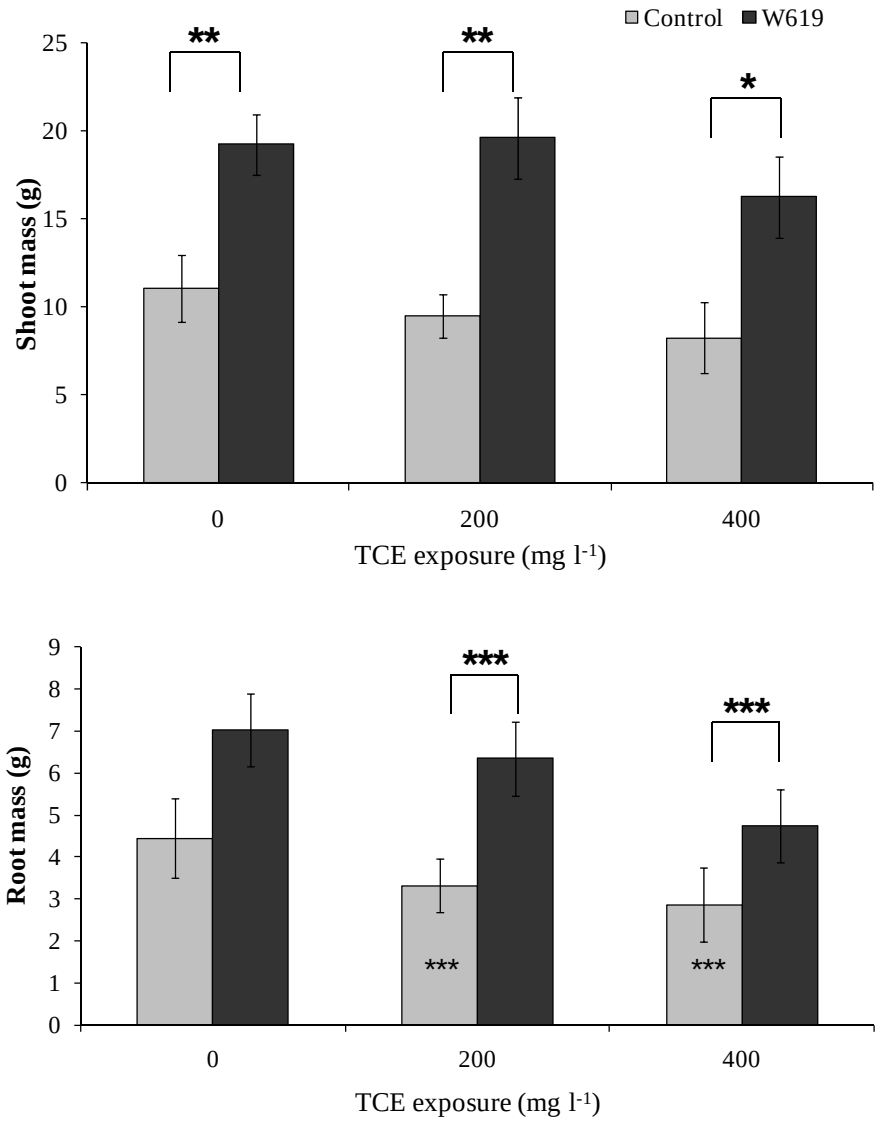


Figure 4.9: Root and shoot biomass (g) of poplar cuttings grown hydroponically and exposed to 0, 200 or 400 mg l^{-1} TCE for 3 days. Control: non-inoculated cuttings; W619: cuttings inoculated with *P. putida* W619-TCE. Values are mean ± standard error of 10 biological independent replicates (significance levels: *: $p < 0.1$; **: $p<0.05$; ***: $p<0.01$). Significance levels shown inside the bars are referring to comparison (a) between non-inoculated cuttings exposed to 200 and 400 mg l^{-1} TCE and non-inoculated cuttings exposed to 0 mg l^{-1} TCE and (b) between inoculated cuttings exposed to 200 and 400 mg l^{-1} TCE and inoculated cuttings exposed to 0 mg l^{-1} TCE.

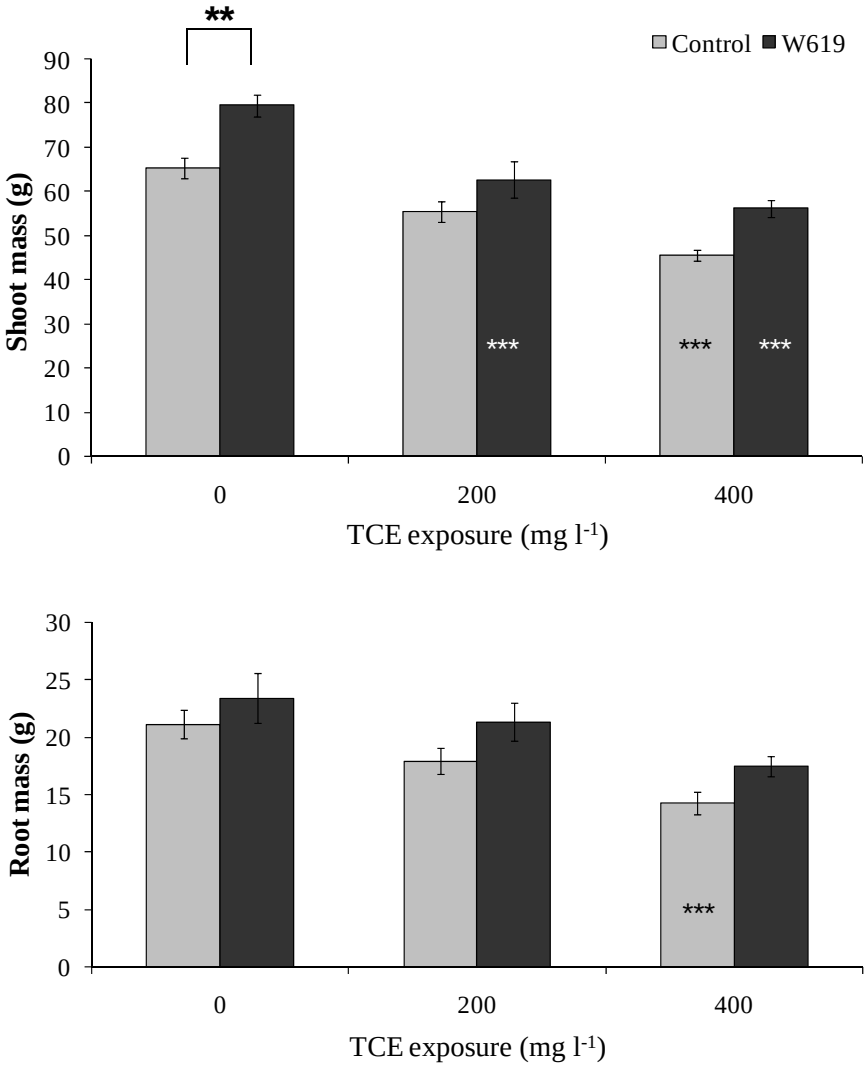


Figure 4.10: Root and shoot biomass (g) of poplar cuttings grown in potting soil. 0, 200 or 400 mg l^{-1} TCE was supplied for 3 weeks. Control: non-inoculated cuttings; W619: cuttings inoculated with *P. putida* W619-TCE. Values are mean ± standard error of 10 biological independent replicates (significance levels: **: $p<0.05$; ***: $p<0.01$). Significance levels shown inside the bars are referring to comparison (a) between non-inoculated cuttings supplied with 200 and 400 mg l^{-1} TCE and non-inoculated cuttings supplied with 0 mg l^{-1} TCE and (b) between inoculated cuttings supplied with 200 and 400 mg l^{-1} TCE and inoculated cuttings supplied with 0 mg l^{-1} TCE.

4.2.2.2 TCE degradation and evapotranspiration

TCE and metabolite extraction from leaves and roots

In case poplar cuttings were grown hydroponically and exposed for 3 days to TCE, the amount of TCE extracted from the leaves of *P. Putida* W619-TCE inoculated plants was reduced (significantly when exposed to 200 mg l^{-1} TCE). The amount of TCE extracted from the roots was remarkably higher than the amount extracted from the leaves (figure 4.11). However, no significant difference was observed between the amount of TCE extracted from the roots of inoculated cuttings and of non-inoculated cuttings (figure 4.11).

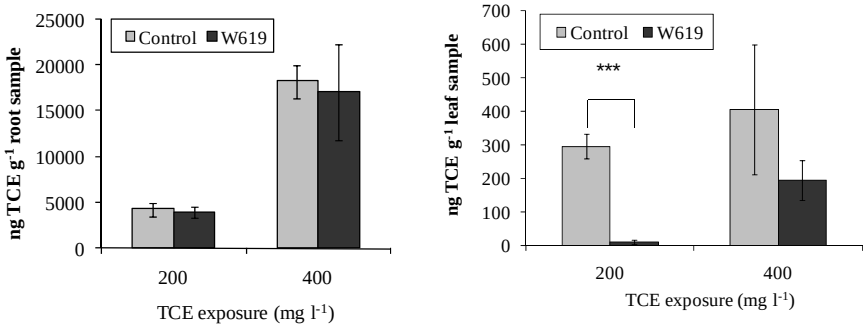


Figure 4.11: Amount of TCE (ng) in the roots (left) and leaves (right) of poplar cuttings grown hydroponically and exposed to 200 or 400 mg l^{-1} TCE for 3 days. Control: non-inoculated cuttings; W619: cuttings inoculated with *P. putida* W619-TCE. Values are mean ± standard error of 6 biological independent replicates (significance level: ***: $p<0.01$).

When poplar cuttings were grown in potting soil and exposed to TCE for 3 weeks, inoculation with *P. putida* W619-TCE resulted in a decreased amount of TCE extracted from the leaves and the roots for cuttings supplied with 400 mg l^{-1} TCE (figure 4.12). Interestingly, in this experimental set up, the amount of TCE extracted from the roots was only slightly higher than the amount extracted from the leaves.

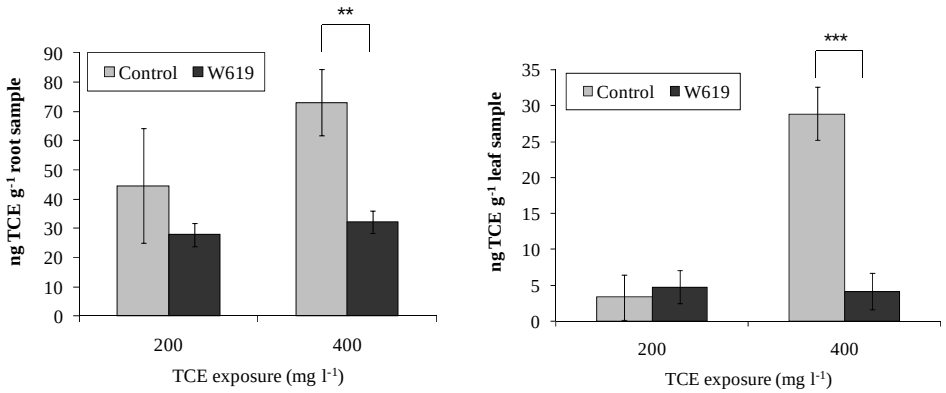


Figure 4.12: Amount of TCE (ng) in the roots (left) and leaves (right) of poplar cuttings grown in potting soil. 200 or 400 mg l^{-1} TCE was supplied for 3 weeks. Control: non-inoculated cuttings; W619: cuttings inoculated with *P. putida* W619-TCE. Values are mean ± standard error of 6 biological independent replicates (significance levels: **: $p<0.05$; ***: $p<0.01$).

DCAA and TCAA, which are known intermediates of TCE degradation, were not detected in any of the samples investigated. Most likely, their concentrations were too low to be detected by the mass spectrometer detector. In a next experiment an electron capture detector could be used and volatilization during extraction should be further minimized.

TCE evapotranspiration

TCE evapotranspiration was only determined on cuttings exposed during 3 weeks to TCE and grown in potting soil. After inoculation with *P. putida* W619-TCE, cuttings treated with 400 mg l^{-1} TCE evapotranspired significantly less TCE through their leaves (figure 4.13). In case 200 mg l^{-1} TCE was supplied to the plants, evapotranspiration of TCE was also lower after inoculation with *P. putida* W619-TCE, but this decrease was not statistically significant. These results demonstrate that inoculation with *P. putida* W619-TCE decreased TCE release by its host plant.

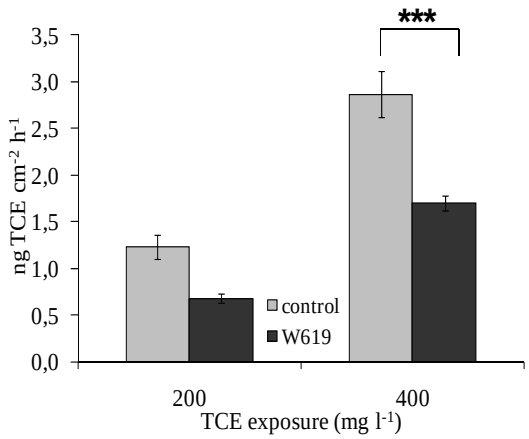


Figure 4.13: Total amount of TCE (ng cm^{-2} h^{-1}) evapotranspired through the leaves of non-inoculated poplar cuttings and cuttings inoculated with *P. putida* W619-TCE, supplied with 200 and 400 mg l^{-1} TCE. Values are mean ± standard error of 3 biological independent replicates (significance level: ***: $p<0.01$).

4.2.3 Discussion and conclusions

In this work, the potential of the TCE-degrading endophyte *P. putida* W619-TCE to reduce TCE phytotoxicity and evapotranspiration was investigated after its inoculation in poplar cuttings. The latter were supplied with 200 and 400 mg l^{-1} TCE in 2 different experimental systems. In the first system, cuttings were grown hydroponically and exposed for 3 days (short-term TCE exposure); in the second system, cuttings were grown in potting soil and exposed for 3 weeks (mid-term TCE exposure). To evaluate TCE phytotoxicity, shoot and root biomasses were determined at harvest time. The fate of TCE was investigated by (a) determining concentrations of TCE in roots and leaves of the cuttings in the two experimental systems, and by (b) measuring evapotranspiration through the leaves of the cuttings grown in potting soil and exposed to TCE for 3 weeks.

When poplar cuttings were grown hydroponically, a 3 days exposure to TCE resulted in a significant reduction in root biomass (figure 4.9). Inoculation with *P. putida* W619-TCE significantly promoted plant growth and also resulted in a strongly decreased TCE phytotoxicity (figure 4.9).

In case poplar cuttings were grown in potting soil and exposed to TCE for 3 weeks, *P. putida* W619-TCE only significantly promoted plant growth (increased shoot biomass) in unexposed cuttings (figure 4.10). However, for TCE-exposed cuttings, inoculation with *P. putida* W619-TCE also resulted in a visual but not statistically significant increase in root and shoot biomass.

It is clear that the plant growth promotion and the protective effect of inoculation with *P. putida* W619-TCE against TCE phytotoxicity was much stronger when poplar cuttings were grown hydroponically and exposed to TCE for 3 days. Since the plant growth-promoting effect was even stronger in the mid-term hydroponic experiment described in chapter 4.1, this difference can not be attributed to the time duration of the experiment. A possible explanation might be that potting soil contains a much better balanced and more easily accessible pool of essential nutrients in comparison to half strength Hoagland nutrient solution. As a result, non-inoculated plants show almost optimal growth, and by consequence, the growth-promoting effect of *P. putida* W619-TCE was "attenuated". This implies that, on marginal land with low nutrient levels, endophytic bacteria will have a much more important role in plant growth promotion and in improving phytoremediation. Another possible explanation for the observed difference in plant growth-promoting effect of *P. putida* W619-TCE in the two experimental systems might be that potting soil, in contrast to half strength Hoagland solution, provides a good substrate favouring growth and colonization by Plant Growth Promoting Bacteria (PGPB). Endogenous PGPBs are probably well represented in potting soil and by consequence *P. putida* W619-TCE will only have a rather limited additional plant growth-promoting effect.

Beside showing a plant growth-promoting effect, *P. putida* W619-TCE could also reduce the TCE concentration in the roots and the leaves (figures 4.11 and 4.12) and the amount of TCE that was evapotranspired through the leaves (figure 4.13) by degrading TCE while it is transported through the xylem vessels.

The amounts of TCE that were extracted from leaves and roots of cuttings that were grown hydroponically were much higher than those of cuttings that were grown in potting soil (figures 4.11 and 4.12). The rather high amounts of TCE extracted from the roots of hydroponically grown cuttings can be explained by a strong adsorption of TCE on the root apoplast. Since the roots of cuttings grown in the hydroponic system are in full contact with the TCE-containing nutrient solution (in contrast with the cuttings grown on potting soil), the TCE uptake in hydroponically grown cuttings might be higher than for cuttings grown in potting soil, which explains the higher amount of TCE found in the leaves of cuttings that were grown hydroponically. In potting soil, a major part of the TCE will adsorb on the high amount of organic matter. For both experimental set ups, the

reduction in TCE concentration after inoculation with *P. putida* W619-TCE was most prominent in the leaves (figures 4.11 and 4.12). Beside the fact that the amounts of TCE that are extracted from the roots also include the TCE that is adsorbed on the root apoplast, in this way masking the effect of inoculation, the effects of inoculation with *P. putida* W619-TCE are much more significant for the TCE concentrations in the leaves, as the strain will degrade TCE during its transport to the leaves.

From the results of (a) the TCE extraction from the roots and the leaves, and (b) the TCE evapotranspiration through the leaves, we can conclude that *P. putida* W619-TCE not only protects its host plant against TCE phytotoxicity, but also improves TCE degradation leading to reduced amounts of TCE accumulation in plant tissue (figures 4.11 and 4.12) and decreased TCE evapotranspiration to the ambient air (figure 4.13). Since phytotoxicity still is a major concern during phytoremediation and evapotranspiration of volatile contaminants often questions the merits of phytoremediation, the results of this work are of major significance in the developments towards large-scale field application of phytoremediation.

Section 5

Field experiments

Abstract

For the first field experiment (chapter 5.2), in 1999, 275 poplar trees were planted on a field site near a car factory in order to install a bioscreen aimed to combine the biodegradation activities of poplar and its associated rhizosphere and endophytic microorganisms for containing a BTEX-contaminated groundwater plume. This BTEX plume occurred as the result of leaking solvents and fuel storage tanks. Monitoring, conducted over a 6 years period (1999-2005) after the planting of the trees, suggested that the poplar trees and their associated microorganisms had, once the tree roots reached the contaminated groundwater zone, an active role in the remediation of the BTEX plume, resulting in full containment of the contamination. Analysis of the microbial communities associated with poplar demonstrated that, once the poplar roots got in contact with the BTEX-contaminated groundwater, enrichment occurred of both rhizosphere and endophytic bacteria that were able to degrade toluene. Interestingly, once the BTEX plume was remediated, the numbers of toluene degrading rhizosphere and endophytic bacteria decreased below detection limits, indicating that their population resulted from selective enrichment due to the presence of the contaminants.

The work described in chapters 5.3 and 5.4 was performed on a site where TCE was present in the groundwater in concentrations up to 100 mg l^{-1}. Along transects under a mixed woodland of English oak (*Quercus robur*) and common ash (*Fraxinus excelsior*) growing on this TCE-contaminated groundwater plume, sharp decreases in TCE concentrations were observed while transects outside the planted area did not show this remarkable decrease. This suggested a possible active role of the trees and their associated bacteria in the remediation process. Therefore, in chapter 5.3, the cultivable bacterial communities associated with both tree species growing on this TCE-contaminated groundwater plume were investigated in order to assess the possibilities and practical aspects of using these common native tree species and their associated bacteria for phytoremediation. Between both tree species, the associated populations of cultivable bacteria clearly differed in composition. In English oak more species-specific, most likely obligate endophytes were found. The majority of the isolated bacteria showed increased tolerance to TCE, and TCE degradation capacity was observed in some of the strains. However, *in situ*

evapotranspiration measurements revealed that a significant amount of TCE and its metabolites was evaporating through the leaves to the atmosphere.

A possible strategy to overcome this evapotranspiration to the atmosphere is to enrich the endogenous plant-associated bacteria by *in situ* inoculation with endophytic strains capable of degrading TCE. For that purpose, hybrid poplar trees [*Populus deltoides x (trichocarpa x deltoides) cv. Grimminge*] were planted in the spring of 2006 perpendicularly to the plume and were provided with a drainage tube in the rooting zone to allow the inoculation.

In chapter 5.5, we report the first *in situ* inoculation of these poplar trees, growing on the TCE-contaminated site, with the TCE-degrading strain *Pseudomonas putida* W619-TCE. *In situ* bioaugmentation with this strain reduced TCE evapotranspiration by 90% under field conditions. This encouraging result was achieved after the establishment and enrichment of *P. putida* W619-TCE as a poplar root endophyte, and by further horizontal gene transfer of TCE metabolic activity to members of the poplar's endogenous endophytic population. Since *P. putida* W619-TCE was engineered via horizontal gene transfer, its deliberate release is not restricted under European GMO regulations. One year after inoculation, the inoculated strain still dominated the cultivable root endophyte community and the amount of evapotranspired TCE was similar as the year before.

Introduction

As described in the introduction section, endophytic bacteria can be exploited for improving phytoremediation of organic contaminants. The fate of organic contaminants in the rhizosphere-root system largely depends on their physical-chemical properties. Plants readily take up organics with a $\log K_{ow}$ between 0.5 and 3.5. These compounds seem to enter the xylem faster than the soil and rhizosphere microflora can degrade them, even if the latter is enriched with degradative bacteria (Trapp *et al.,* 2000). Once these contaminants are taken up, plants may metabolize them, although some of them or their metabolites can be toxic (Doucette *et al.,* 1998). For example, TCE can be transformed into TCA. Alternatively, some plants preferentially release volatile pollutants (such as TCE and BTEX) and/or their metabolites into the environment by evapotranspiration via the leaves. This raises questions about the merits of phytoremediation (Burken and Schnoor, 1999; van der Lelie *et al.,* 2001; Schwitzguébel *et al.,* 2002; Ma and Burken, 2003). The use of engineered endophytic bacteria, which complement the metabolic properties of their host, has the potential to overcome these limitations: while contaminants move through the plant's vascular system, endophytic bacteria, colonizing the xylem (Germaine *et al.,* 2004), can promote their degradation. This may result in both decreased phytotoxicity and evapotranspiration, provided the bacteria possess the genetic information required for efficient degradation of the contaminants. Dominant cultivable endophytic growth-promoting bacteria can be isolated, subsequently equipped with desirable characteristics and re-inoculated in the host plant to enhance their beneficial effects. Proof of concept was provided by inoculating yellow lupine plants (Barac *et al.,* 2004) and poplar cuttings (Taghavi *et al.,* 2005) with endophytic bacteria able to degrade toluene, which resulted in decreased toluene phytotoxicity and a significant decrease in toluene evapotranspiration. Before endophyte-assisted phytoremediation of volatile organic contaminants can be successfully applied under field conditions, several obstacles need to be overcome (Newman and Reynolds, 2005). One major point of concern is the persistence and stability of the engineered organisms and their degradation capabilities in field-grown plants, as phytoremediation projects often last for decades.

In the following section of this thesis, we describe the field trials we performed to achieve a successful application of our concept in the field.

Chapter 5.1 *Materials and methods*

5.1.1 Sampling

Bulk soil, rhizosphere and roots were sampled at a depth of 1.5 m and stored in sterile Falcon tubes (50 ml) filled with 20 ml sterile 10 mM $MgSO_4$; leaf and stem samples were transferred in separate plastic bags. For every compartment, samples were taken from 3 different trees and they were pooled for further analysis.

5.1.2 Isolation of plant-associated bacteria

Soil samples were diluted up to 10^{-7} in 10 mM $MgSO_4$ solution and plated on 1/10 strength 869 solid medium (Mergeay *et al.*, 1985) in order to isolate soil bacteria. Rhizosphere samples were vortexed, roots were removed and serial dilutions up to 10^{-7} were prepared in 10 mM $MgSO_4$ solution and plated on 1/10 strength 869 solid medium. After 7 days incubation at 30°C, colony forming units (CFU) were counted and calculated per gram of bulk or rhizosphere soil.

To isolate the endophytic bacteria, plant samples were surface sterilized for 10 (roots and leaves) or 5 (stems) minutes in a 2% (roots and leaves) or a 1% (stems) active chloride solution supplemented with 1 droplet Tween 80 (Merck) per 100 ml solution, and were subsequently rinsed 3 times for 1 min in sterile distilled water. The third rinsing solution was plated on 869 medium to check surface sterility (if no growth was observed after 7 days, surface sterilization was considered to be successful). Surface sterile plant samples were macerated during 60 (roots and leaves) or 90 (stems) seconds in 10 ml 10 mM $MgSO_4$ using a Polytron PR1200 mixer (Kinematica A6). Serial dilutions were plated on 1/10 strength 869 solid media and incubated for 7 days at 30°C before the CFU were counted and calculated per gram fresh plant weight.

All morphologically different bacteria were purified 3 times and plated on selective 284 medium (see 3.1.2) supplemented with C-mix (per liter medium: 0.52 g glucose, 0.35 g lactate, 0.66 g gluconate, 0.54 g fructose and 0.81 g succinate).

5.1.3 Genotypic characterization of bacteria

After purification, total genomic DNA of all morphologically different bacteria was extracted using the DNeasy® Blood and Tissue kit (Qiagen). Aliquots (1 µl) of the extracted DNA were directly used for PCR without further purification.

The BOX1 primer (5'-CTACGGCAAGGCGACGCTGACG-3') was used for BOX-PCR DNA fingerprinting which was carried out as described earlier (Barac *et al.*, 2004). The obtained PCR products were separated by gel electrophoresis in a 1.5% agarose gel, and visualized by gelred nucleic acid gel staining and UV illumination.

The universal 1392R (5'-ACGGGCGGTGTGTRC-3') and the Bacteria-specific 26F (5'-AGAGTTTGATCCTGGCTCAG-3') primers were used for prokaryotic 16S rRNA gene amplification which was carried out as described by Taghavi *et al.* (2009). PCR products of the 16SrDNA amplification were directly used for amplified 16S rDNA restriction analysis (ARDRA) and sequencing.

For ARDRA, aliquots of these PCR products were digested for 2 h at 37°C with 1 unit of the 4-base-specific restriction endonuclease HpyCH4 IV in 1 x NEB buffer 1 (New England Biolabs). The digestion products obtained were examined electrophoretically in a 1.5% agarose gel, and visualized by gelred nucleic acid gel staining and UV illumination. ARDRA patterns were grouped, and strains with representative patterns were selected for sequencing, which was performed as described earlier (Barac *et al.*, 2004; Taghavi *et al.*, 2009).

Sequence Match at the Ribosome Database Project II was used for nearest neighbour and species identification. In order to verify the identification, a neighbour-joining analysis was performed. Prior to this analysis, the sequences were aligned using Clustal X (Thompson *et al.*, 1997). A neighbour-joining tree was constructed with PAUP*4.0b10 (Swofford, 2003), using default settings. In order to assess branch supports, bootstrap values were calculated with 2000 pseudo-replicates.

5.1.4 Phenotypic characterization of bacteria

Bacterial strains that displayed distinct ARDRA patterns were screened for heavy metal resistance and for TCE and toluene (as a model BTEX compound) tolerance and degradation. To test metal resistance, all different bacteria were plated on selective 284 medium with the addition of 1 mM nickel, 2 mM zinc or

0.8 mM cadmium. A carbon mix (per liter medium: 0.52 g glucose, 0.35 g lactate, 0.66 g gluconate, 0.54 g fructose and 0.81 g succinate) was added and cultures were incubated at 30°C for 7 days. In order to screen the bacteria for toluene and TCE tolerance and degradation, strains were plated on selective medium and incubated for 7 days at 30 °C in sealed 10 liter vessels with addition of 600 µl toluene or TCE to obtain a toluene- or TCE-saturated atmosphere. To detect autotrophic strains, bacteria were also plated on selective medium without any carbon source.

After this screening, head space gas chromatography was used in order to confirm toluene and TCE degradation. For this experiment bacteria were grown in 40 ml Schatz medium (Schatz and Bovell, 1952) with the addition of 100 mg l^{-1} toluene, 100 mg l^{-1} TCE or 100 mg l^{-1} toluene and 100 mg l^{-1} TCE, and in Schatz medium supplemented with C-mix (per liter medium: 0.52 g glucose, 0.35 g lactate, 0.66 g gluconate, 0.54 g fructose and 0.81 g succinate) and 100 mg l^{-1} TCE. Samples of 10 ml were taken at the beginning of the experiment and after 3 days, and placed in 20 ml head space vials to which 4 g Na_2Cl was added to stop all bacterial activity. Samples were analyzed by head space (Teledyne Tekmar HT3^{TM}) gas chromatography (Trace GC Ultra, Interscience). The volatilization of toluene and TCE was taken into account by measuring control samples (without addition of bacteria) and degradation was calculated as a percentage of the non-volatilized fraction.

To verify that the pTOM plasmid was responsible for the toluene or TCE degradation capacity, the presence of the pTOM degradation plasmid was tested with PCR using *tomA4* specific primers 3323F (5'-GTT GCC CTC AAA CCC TAC AA-3') and 3780R (5'-AGG GGC TGA ATG TTG AGT TG-3'). Cycling conditions consisted of: 1 denaturation cycle of 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 60°C for 30 sec, and 72°C for 1.5 min, and completed with an extension cycle of 8 min at 72°C. The PCR products obtained were examined by electrophoresis in a 1.5% agarose gel, and visualized by gelred nucleic acid gel staining and UV illumination.

5.1.5 In situ evapotranspiration

The system designed for these measurements is shown in figure 5.1.

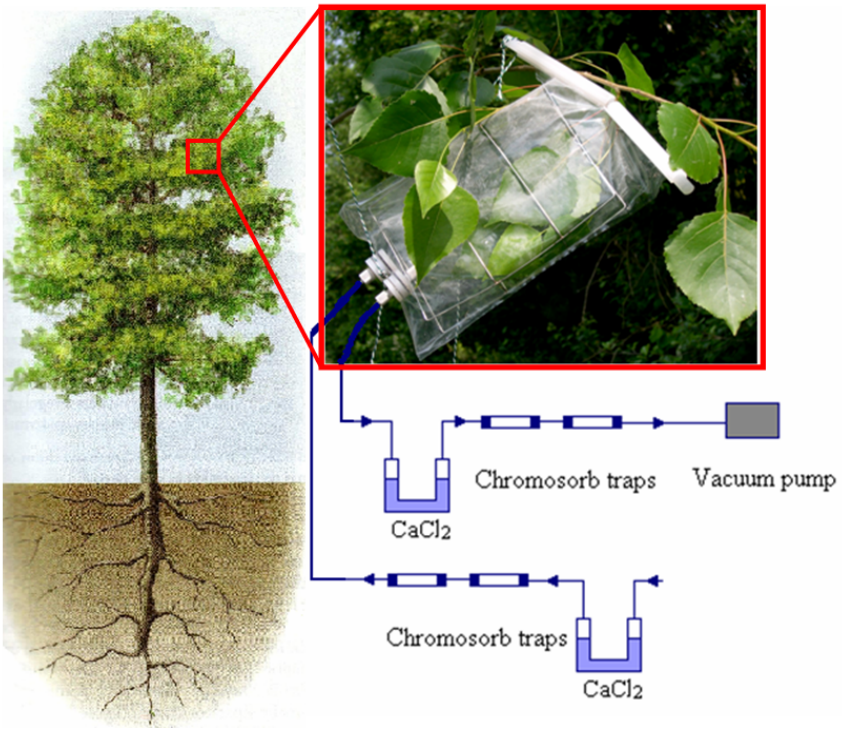


Figure 5.1: Schematic setup to measure *in situ* TCE evapotranspiration

Gas sampling pumps (ADC BioScientific) were connected to Teflon sampling bags (Chemware Laboratory products) via Teflon tubes and Chromosorb 106 traps. A column with $CaCl_2$ was placed between the sampling bags and the Chromosorb traps to prevent water condensation in the traps. In order to have an inflow free of TCE, a column with $CaCl_2$ and 2 Chromosorb 106 traps were also placed before the inflow of the sampling bags. For each tree species, measurements were performed on three independent trees and for each specific tree, TCE evapotranspiration was analyzed on three different twigs. Twigs with 5–6 leaves were placed into the sampling bag which was made gas-tight around the twig and an airflow of 5 l h^{-1} was created for 3 hours. After sampling, the leaves were collected in plastic bags and stored at 4°C until leaf surface area

analysis. The Chromosorb traps were analyzed by gas chromatography-mass spectrometry (GC-MS) with an ATD400 automatic thermal desorption system, an Auto System XLL gas chromatograph and a Turbo mass spectrometer (Perkin-Elmer). The amount of evapotranspired TCE was calculated per hour and unit of leaf area (cm^{-2}).

5.1.6 In situ inoculation of bacteria

Bacteria were cultured in 869 medium (Mergeay *et al*., 1985) and 40 liter of this bacterial suspension (10^8 cfu ml^{-1}) was added to the roots via drainage tubes (figure 5.2), installed when the trees were planted. At the same time, the non-inoculated control trees received 40 liter of 869 medium without bacteria.

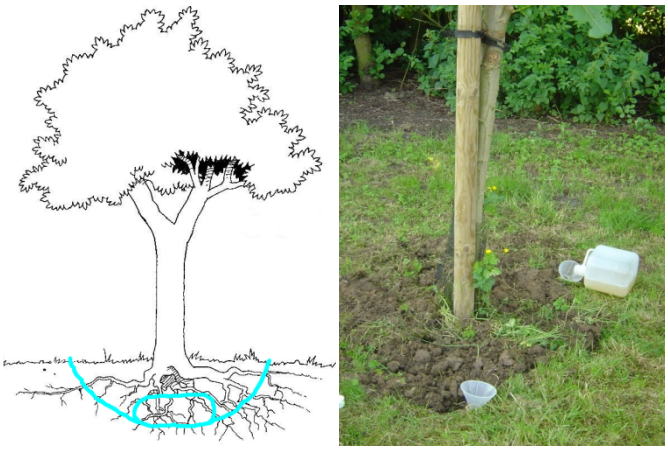


Figure 5.2: *In situ* inoculation via a drainage tube

5.1.7 Statistical analysis

The TCE evapotranspiration datasets employed were analyzed using linear mixed-effects models (Verbeke *et al.*, 2000) to correct for the correlation between different twigs of the same plant (measurements were made on three twigs per plant). TCE degradation was compared statistically using one-way ANOVA (per medium) and post-hoc multiple comparison testing (Tukey-Kramer). When necessary, transformations were applied to approximate normality and/or homoscedasticity. The statistical analyses were performed using the ANOVA and MIXED procedure in SAS 9.1.3.

Chapter 5.2 *Application of poplar and its associated microorganisms for the in situ remediation of a BTEX-contaminated groundwater plume*

***Published in**: Barac T, Weyens N, Oeyen L, Taghavi S, van der Lelie D, Dubin D, Spliet M, Vangronsveld J (2009) Application of poplar and its associated microorganisms for the in situ remediation of a BTEX-contaminated groundwater plume. International Journal of Phytoremediation 11:416-424*

Former leakage of solvents and fuel from underground storage tanks at the site of a car factory in Genk (Belgium) resulted in a contamination of the groundwater with organic solvents (BTEX), fuel and heavy metals. BTEX is the acronym for benzene, toluene, ethyl benzene and xylenes. All BTEX compounds are toxic and cause noticeable health effects at increased concentrations (Mehlman, 1993). Exposure to these compounds from groundwater systems is usually minimal but can be persistent over a long period of time (long-term effect) (Christensen and Elton, 1996). In addition to its toxicity, benzene is a known carcinogen (Bescol-Liversac *et al*., 1982). Upon exposure, benzene will move into the blood stream from where it can get into fatty tissues and undergo reactions that produce phenol, an even more serious carcinogen. The inhalation of toluene and xylenes in concentrations of 0.4 mg l^{-1} causes headache, dizziness and irritation of the mucous membranes (Christensen and Elton, 1996). At higher concentrations toluene and xylenes can lead to a reduced ability of co-ordination.

Pollutant migration is of high concern for BTEX, since these water soluble compounds will follow the flow of the groundwater and migrate off-site. Therefore, several initiatives were undertaken to prevent additional pollution of the groundwater and to stop the contamination leaving the plant property. The sources of contamination were removed: the storage tanks were excavated and replaced by above ground storage tanks in order to stop the input of contaminants in the groundwater. At the core of the contamination, where BTEX concentrations were higher than 500,000 µg l^{-1}, conventional remediation techniques, more specifically "air stripping" (since 2002) and "pump and treat"

(since 2003), were applied. Additionally, natural attenuation under aerobic and anaerobic conditions was investigated and, from the beginning (1999), also phytoremediation was included in the remediation plan in order to contain the contaminant plume.

Hybrid poplar trees (*Populus trichocarpa* x *deltoides* cv. "Hoogvorst" and "Hazendans") were chosen to remediate the BTEX plume. Poplar trees are fast growing and deep rooting with a high water "pumping" capacity (average 260 liter per tree per day or 4200 m^3 ha^{-1} in 6 months during vegetation period: unpublished results) and are phreatophytic. The absorption surface of poplar roots is enormous and can approach 300,000 km ha^{-1} (Newman *et al*., 2004). In order to create a bioreactive root zone, in April 1999, 275 poplar trees were planted in a 2 ha (75 m by 270 m) zone perpendicularly on the migration direction of the groundwater contaminated with BTEX (0-1,000 µg l^{-1}) as well as with nickel (0-100 µg l^{-1}) and zinc (0-1,000 µg l^{-1}). The groundwater depth was 4-5 m, which is within reach of the poplar roots. Two poplar cultivars, *Populus trichocarpa* x *deltoides* "Hoogvorst" and "Hazendans" were chosen in order to reduce the potential effects of fungal disease spreading.

5.2.1 Monitoring of BTEX plume

Since the poplar roots did not yet reach the groundwater table in May 2000 (13 months after planting), the trees were not expected to significantly contribute to the containment and remediation of the BTEX plume. This was confirmed by comparing the contamination plume of October 1998, before planting the trees (not shown) with that of May 2000 (figure 5.3A): no differences in the concentration profile and dispersion of BTEX plume were observed, with the front of the plume leaving the factory site. In the core of the plume, BTEX concentrations up to more than 500,000 µg l^{-1} were found. The BTEX plume followed the groundwater flow in south-west direction: at the location where the trees were planted the concentration varied between 0 and 1,000 µg l^{-1}.

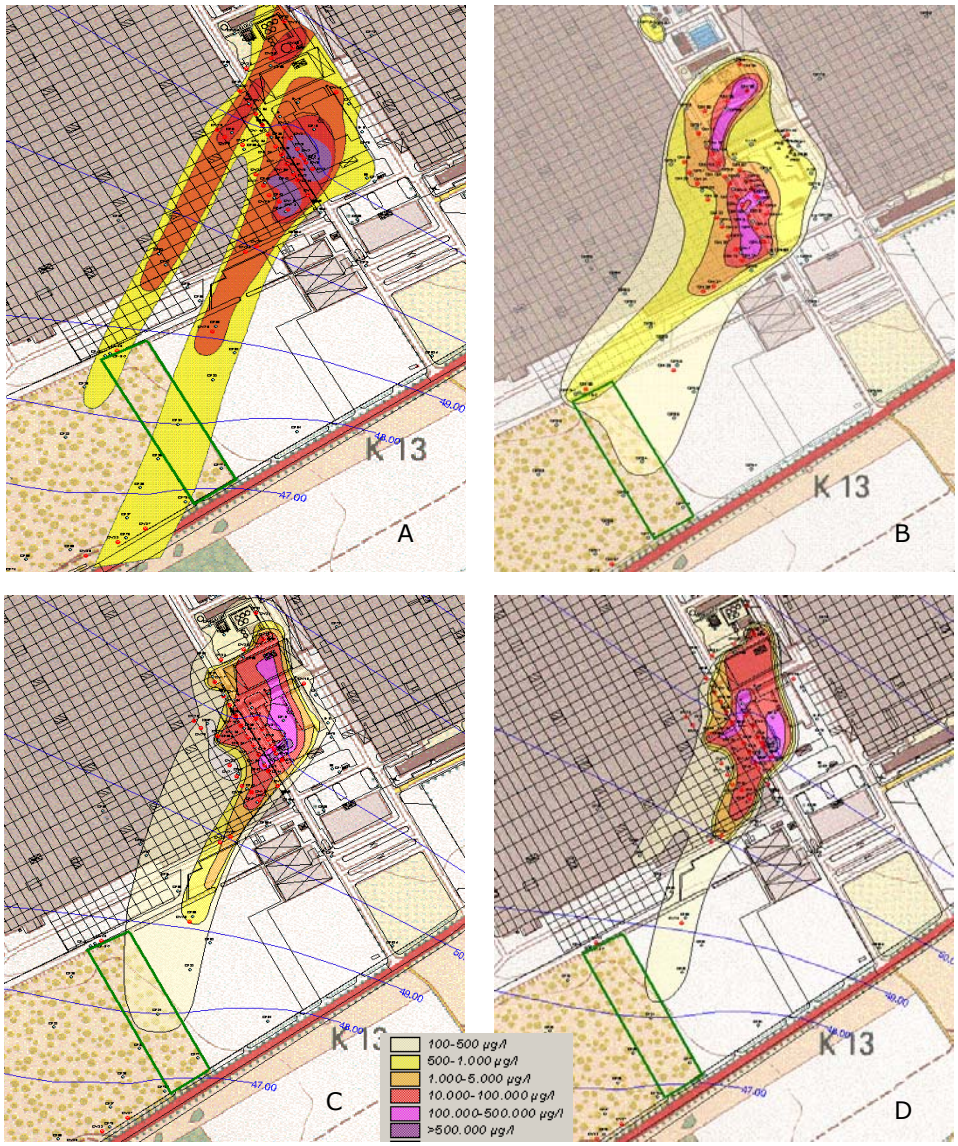


Figure 5.3: BTEX-polluted site: (A) samples taken in May 2000, 13 months after planting of the poplar trees; (B) samples taken in October 2002, 42 months after planting of the poplar trees; (C) samples taken in June 2003, 50 months after planting of the poplar trees; (D) samples taken in November 2003, 55 months after planting of the poplar trees. The green rectangle indicates the phytoremediation area.

Monitoring performed in October 2002, 42 months (3 growing seasons) after planting of the trees (figure 5.3B), showed that the concentrations of BTEX in

the core of plume still were between 100,000 and 500,000 µg l^{-1}. Although these concentrations were similar to those measured in 1999 and 2000, the very important observation was made that the BTEX plume was "cut off" at the location where the poplar trees were planted. Before, the plume reached much further and even passed under the motorway, leaving the plant property.
In June 2003 (figure 5.3C), the BTEX plume under the poplars became much smaller with total BTEX concentrations varying between 100 and 500 µg l^{-1}. Interestingly, the concentrations in the core of the plume remained high, without significant changes, pointing to several attenuation processes that actively remediated the BTEX contamination. After the summer period, when the poplar trees exhibited their highest activity, pumping huge amounts of contaminated water, the plume completely disappeared from the zone were the trees were planted (figure 5.3D). These results indicate that the combination of natural attenuation and phytoremediation was successful in containing and remediating the BTEX plume.

5.2.2 The microbial community associated with poplar cv. "Hoogvorst" growing inside and outside the BTEX plume

Using cell cultures of the poplar cultivar "Hoogvorst" it was shown that the plant cells were unable to degrade BTEX (Barac, 2004). An important role in BTEX degradation can be attributed to plant-associated microorganisms equipped with the appropriate degradation pathways (Barac *et al*., 2004). *In planta* horizontal gene transfer was also observed among endophytic bacteria from poplar and demonstrated its role in the adaptation of natural endophytic microbial communities to improve the remediation of toluene (Taghavi *et al*., 2005). This prompted us to compare the potential of the endophytic bacteria isolated from the rhizosphere, roots, stems and leaves of *Populus trichocarpa* x *deltoides* cv. "Hoogvorst" (planted in April 1999) growing either inside or outside the BTEX plume for their growth potential on toluene. This was done in June 2003 by plating them on selective medium in the presence or absence of toluene as sole carbon source (table 5.1a). From the trees growing inside the BTEX plume, the number of endophytic bacteria growing on non-selective medium (representing

the total number of cultivable bacteria, indicated as total CFU) was more than twice as high compared with trees growing outside the plume. In the rhizosphere, the total numbers of cultivable bacteria from trees growing inside and outside the plume were quite similar.

The number of endophytic bacteria showing autotrophy and toluene tolerance (growing on a selective medium without any carbon source and on the same medium in the presence of toluene; indicated as $Aut^{+}Tol^{+}$) isolated from the trees growing inside the plume was higher than for the trees growing outside the plume. Furthermore, the total number of CFU of non-autotrophic bacteria able to grow on a selective medium in the presence of toluene (using toluene as a carbon source and by consequence degrading it) was approximately three times higher in the trees growing inside the plume (54×10^5) compared with trees growing outside the plume (18×10^5). This suggests that growing trees on the BTEX plume was leading to an enrichment of endophytes able to degrade these compounds.

In the rhizosphere of trees growing inside the BTEX-contaminated area, the number of CFU growing on a selective medium with only toluene as a potential carbon source was 10×10^6 CFU per gram, compared to 18×10^5 CFU for trees growing outside the plume. This indicates that, over time, growth in the BTEX-contaminated zone results in an enrichment of rhizosphere isolates able to use BTEX compounds as their carbon source.

In June 2006, after the BTEX plume had retreated from the phytoremediation zone, the same method to screen for toluene-degrading bacteria was repeated.

Neither endophytic nor rhizosphere bacteria with toluene degradation capacity could be isolated from trees growing either inside or outside the former BTEX plume (table 5.1b). This suggests that after disappearance of the BTEX as potential carbon source, the capacity to metabolize these components is rapidly lost.

Table 5.1: The numbers of CFU calculated per gram fresh weight of rhizosphere soil or plant material isolated from *Populus trichocarpa* x *deltoides* cv. "Hoogvorst" growing outside (upper part of table) and inside (lower part of table) the BTEX contamination plume in June 2003 (table 5.1a) and June 2006 (table 5.1b). All bacterial strains were separately tested on selective medium without any carbon source and on selective medium with C-mix or toluene as a carbon source. **Total CFU**: cultivable bacterial strains grown on selective medium with addition of C-mix. Aut^+: stands for bacterial strains able to grow on selective medium without any carbon source (autotrophic growth). Aut^-: are bacterial strains not able to grow without any carbon source. Tol^+: means that the bacterial strains are growing on toluene as only carbon source. **Aut^+ Tol^+ CFU**: bacterial strains grown on selective medium without addition of any carbon source and on the same medium with addition of toluene, autotrophic growth present, no evidence of toluene degradation; **Aut^- Tol^+ CFU**: bacterial strains were not able to grow on selective medium without any carbon source, but could grow when toluene was supplied: this means toluene degradation was present.

		Total CFU	Aut^+ Tol^+ CFU	Aut^- Tol^+ CFU
Outside BTEX plume	**Endophytic bacteria**	30×10^5	18×10^5	18×10^5
	Rhizosphere bacteria	21×10^6	19×10^6	18×10^5
Inside BTEX plume	**Endophytic bacteria**	73×10^5	49×10^5	54×10^5
	Rhizosphere bacteria	**31×10^6**	**24×10^6**	**10×10^6**

Table 5.1a

		Total CFU	Aut^+ Tol^+ CFU	Aut^- Tol^+ CFU
Outside BTEX plume	**Endophytic bacteria**	96×10^4	17×10^4	0
	Rhizosphere bacteria	10×10^6	10×10^6	0
Inside BTEX plume	**Endophytic bacteria**	80×10^5	79×10^5	0
	Rhizosphere bacteria	52×10^5	27×10^5	0

Table 5.1b

5.2.3 Conclusions

This six-year study was conducted to assess the effectiveness of hybrid poplar trees (*Populus trichocarpa* x *deltoide*s cv. "Hoogvorst" and "Hazendans") and their rhizosphere and endophytic bacterial population to remediate a BTEX-contaminated groundwater plume. These poplar trees were growing without any symptoms of phytotoxicity in the presence of BTEX in concentrations varying between 0 and 1,000 µg l^{-1}.

Monitoring data of the BTEX plume during the period 1999-2005 indicated a clear role for the plant-associated bacteria in containing the plume and degrading the BTEX compounds. After planting (April 1999) but before the roots reached the groundwater no changes in the characteristics of the BTEX plume were observed and no indication of natural attenuation was observed. Once the poplar roots reached the groundwater (after three growing seasons, in 2002) the BTEX plume was "cut off" in the treatment zone by the combined activity of poplar and its associated microorganisms. In time, the plume became smaller and completely retreated from the treatment zone after 55 months (November 2003). The presence of BTEX had a positive effect on the percentage of cultivable toluene-degrading endophytic and rhizosphere bacteria associated with poplar. An increase in the number of toluene-degrading phenotypes was observed once the tree roots reached the BTEX plume approximately 30 months after planting. Such an enrichment of toluene-degrading strains was not observed for the bacterial communities associated with trees that were growing outside the contaminated zone. This observation is in accordance with previous studies by Siciliano *et al.*, (2001), who noticed an increase of endophytic bacteria able to degrade the organic contaminants on a contaminated site. In addition, horizontal gene transfer to adapt the endogenous microbial communities, as previously reported by Taghavi *et al.*, (2005), could have occurred. These observations may also explain the increase in toluene-degrading bacteria in 2003 compared to earlier studies conducted in 2001 before the roots of the trees had reached the BTEX-contaminated groundwater (Porteous-Moore *et al.*, 2006). Furthermore, in tree and rhizosphere samples collected in 2006, when the concentrations of BTEX under the selected trees had decreased below detection limit, no bacteria able to grow on toluene as a sole carbon source could be found any more. This further illustrates the plasticity of the endogenous

microbial communities to assist their host plant in the phytoremediation of organic contaminants.

Chapter 5.3 *Bacteria associated with oak and ash on a TCE-contaminated site: characterization of isolates with potential to avoid evapotranspiration of TCE*

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For this work, a site was chosen where TCE was present in the groundwater at concentrations up to 100 mg l^{-1} due to former large-scale use of TCE as a degreaser during the production of metal barrels. TCE concentrations were determined in two transects through a small woodland of English oak *(Quercus robur)* and common ash *(Fraxinus excelsior)* planted about 25 years ago (figure 5.4).

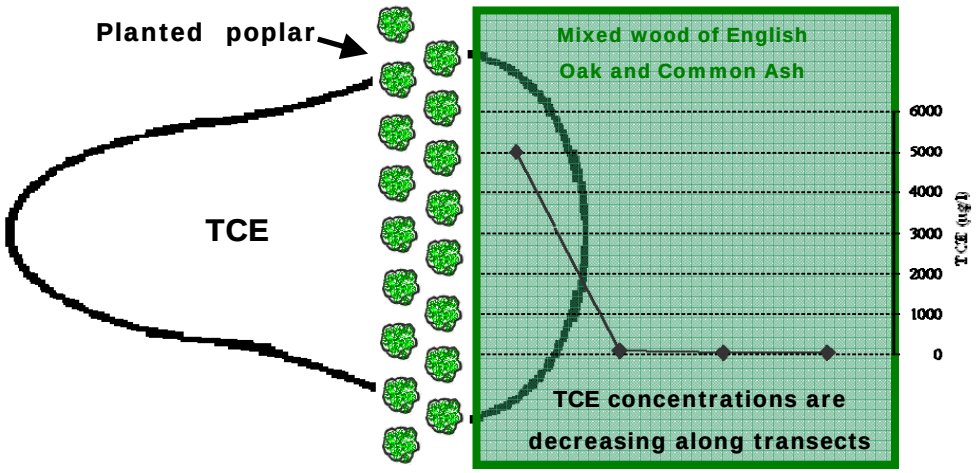


Figure 5.4: Schematic presentation of the TCE-contaminated site

The presence of this woodland seems to be responsible for a sharp decrease in TCE concentrations (figure 5.4) along these transects since transects outside the planted area did not show this remarkable decrease; concentrations there were stable around 9.5 mg l^{-1}. The main objectives of this work were to investigate (a) the role that bacteria associated with both tree species can play in the TCE-degradation and (b) if the natural bacterial community is sufficient to prevent evapotranspiration from the leaves to the atmosphere. Given that TCE is one of the most widespread groundwater contaminants, and that English oak and common ash are both native tree species, it is important to explore the cultivable associated bacterial diversity and its TCE degradation capacity as part of a larger study on the potential of *in situ* inoculation with (cultivable) plant-associated bacteria to enhance phytoremediation (see next chapter). Additionally, in 2006, rows of hybrid poplar trees were planted perpendicularly to the contamination plume (figure 5.4) in order to augment the already existing bioscreen of English oak and common ash.

5.3.1 Isolation of bacteria associated with oak and ash

Bacteria were isolated from bulk soil, rhizosphere, root, stem and leaf from English oak and common ash. For both tree species, the number of cultivable bacteria recovered was an order of magnitude higher for rhizosphere than for soil samples (table 5.2).

The number of endophytic bacteria recovered was the highest in roots and stems, and was lower in the leaves. For both tree species the number of different bacterial morphotypes was the highest in the rhizosphere, rather similar in roots and stems, and the lowest in the leaves. For soil, the number of morphologically different species was clearly higher in association with common ash than in association with English oak.

Table 5.2: The total numbers of CFU calculated per gram fresh weight of soil, rhizosphere or plant material isolated from English oak and common ash, growing on the TCE-contaminated groundwater plume. The number of phenotypically distinct colony morphologies observed is marked in parentheses and total numbers for soil-rhizosphere and endophytes are highlighted in gray.

	Compartment	cfu g^{-1} fresh weight
Oak	Soil	$14.7 * 10^4$ (12)
	Rhizosphere	$37.1 * 10^5$ (17)
	Total Soil-Rhizosphere	$38.6 * 10^5$ (21)
	Root	$94.7 * 10^3$ (16)
	Shoot	$28.2 * 10^4$ (16)
	Leaf	$13.9 * 10^3$ (10)
	Total Endophytes	$39.1 * 10^4$ (27)
Ash	Soil	$19.1 * 10^4$ (17)
	Rhizosphere	$14.8 * 10^5$ (18)
	Total Soil-Rhizosphere	$16.7 * 10^5$ (23)
	Root	$18.0 * 10^4$ (14)
	Shoot	$28.2 * 10^4$ (16)
	Leaf	$13.9 * 10^3$ (10)
	Total Endophytes	$47.6 * 10^4$ (17)

5.3.2 Genotypic characterization

After purification, all morphologically different bacteria were characterized by ARDRA using *Hpy*CH4 IV. Closely related strains were determined and out of these strains 16S rRNA genes of representative members were sequenced for species identification by means of Sequence Match at the Ribosome Database Project II (figure 5.5). The sequence match numbers marked in figure 5.5 were all (except bacterial strain 2) higher than 0.900, which indicated that the identification to the genus level was confident. Moreover, in the neighbour-joining tree, strains belonging to the same genus cluster together in distinct clades (bootstrap values of 100%), which confirms the results of the 16S rRNA

identification procedure. The 16S rRNA-based identification resulted in 41 and 30 genotypically different bacterial strains associated with English oak and common ash, respectively. We have numbered the different bacterial strains (numbers 1–56 in figure 5.5). These numbers are further used in figures 5.6 and 5.7 and in table 5.3.

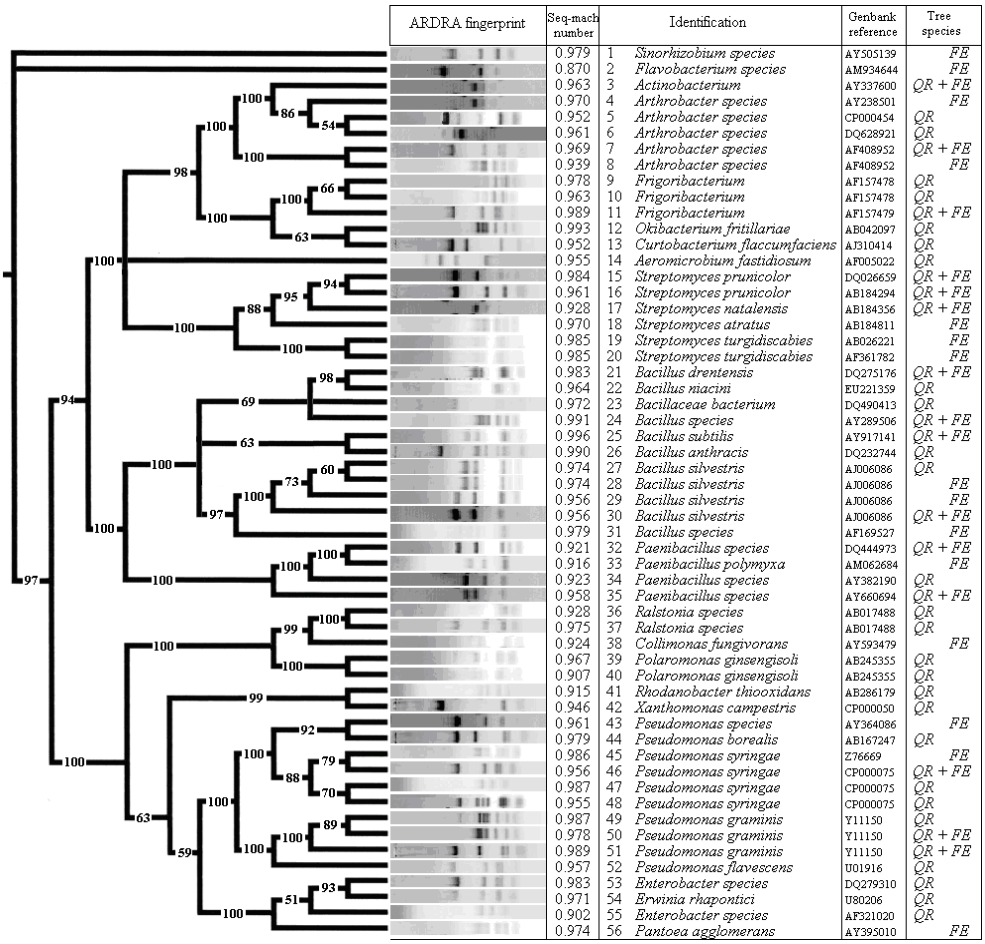
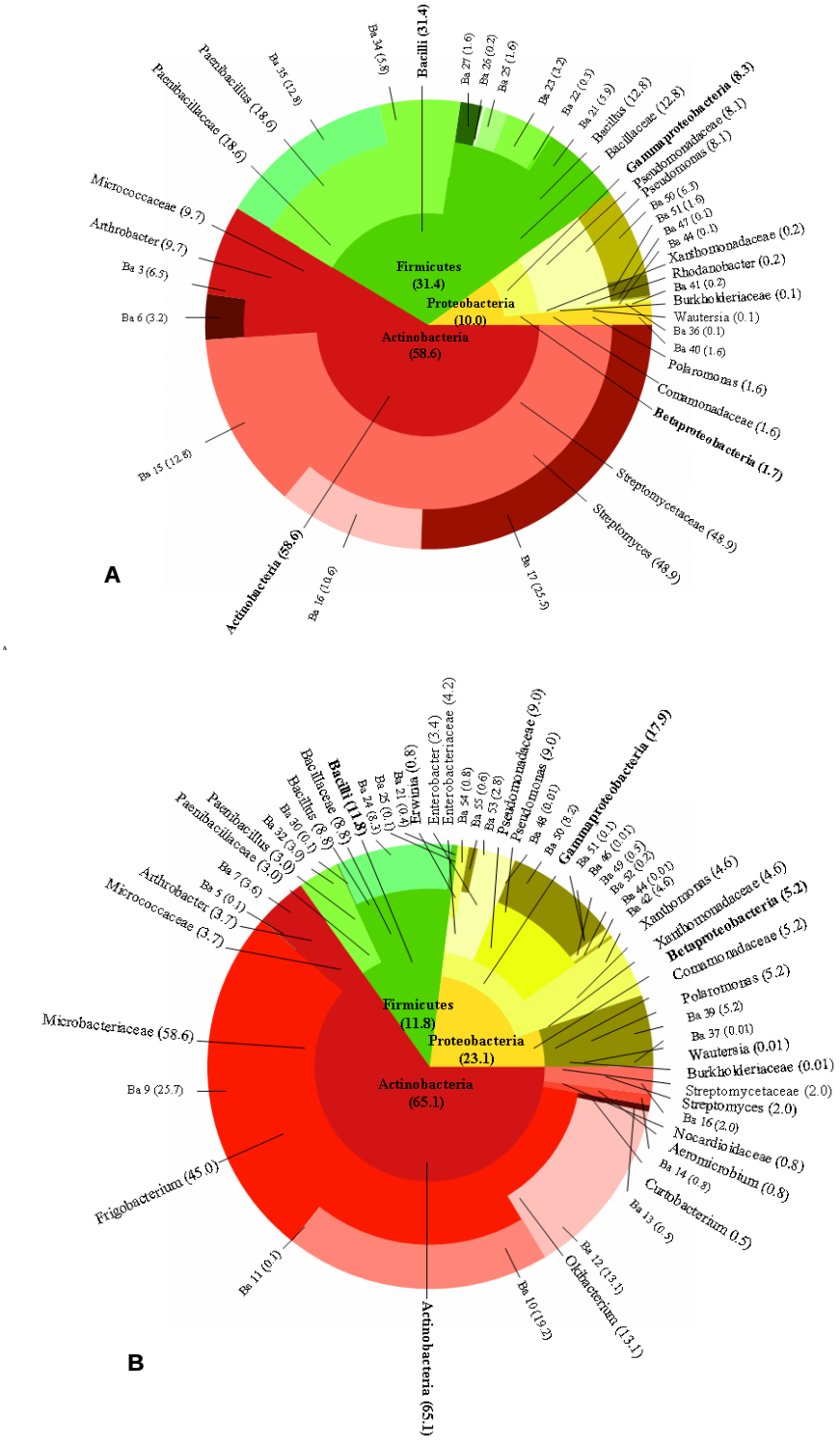
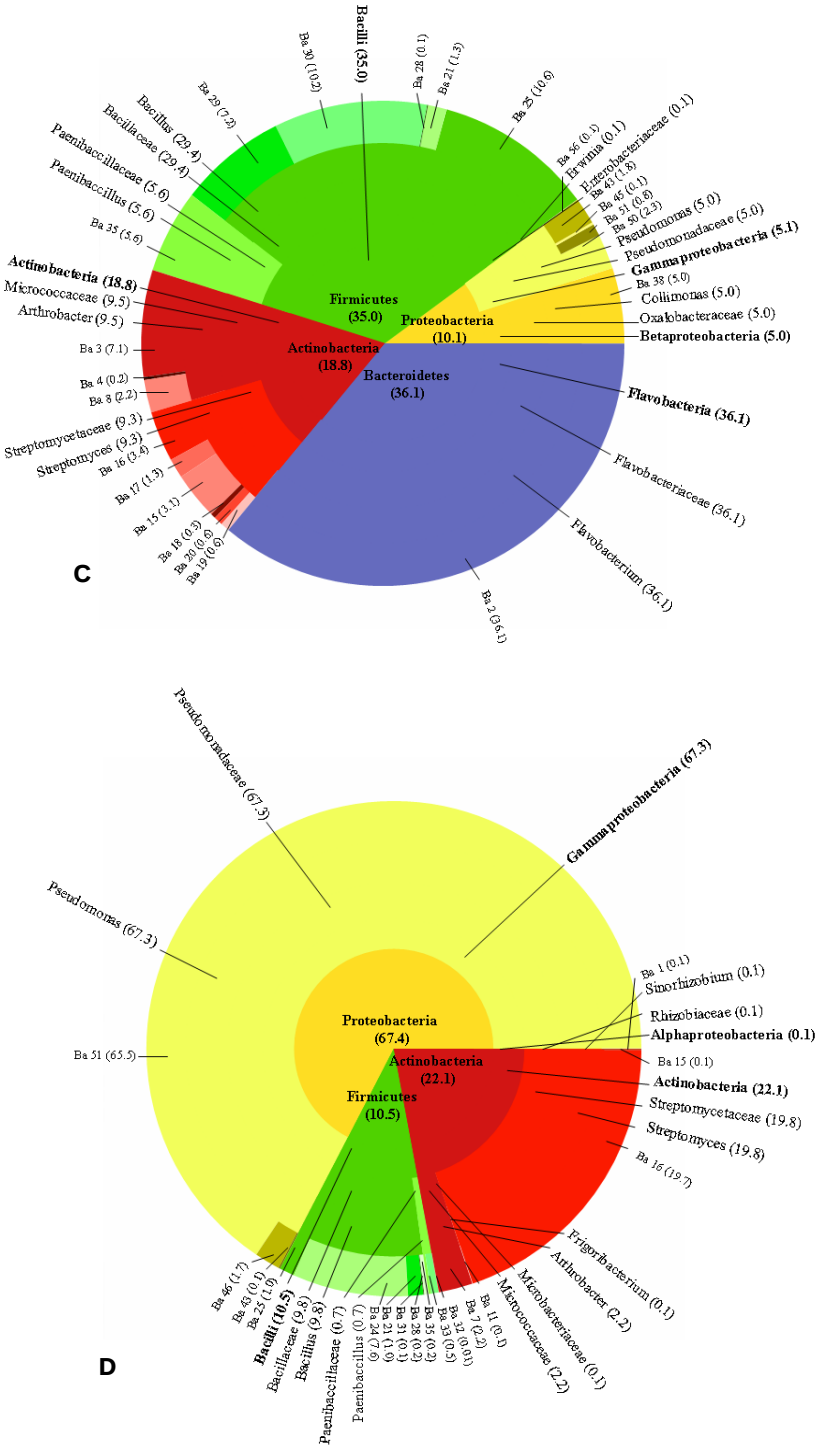


Figure 5.5: Neighbour-joining tree of 16S rDNA of cultivable bacteria associated with English oak and common ash growing on the TCE-contaminated groundwater plume. On the right of the ARDRA fingerprint, the sequence match number, the 16S rDNA identification (with unique number), the accession number of the closest reference strain, and the associated tree are shown. QR: *Quercus robur* (oak); FE: *Fraxinus excelsior* (ash).

To visualize the diversity of cultivable bacteria associated with oak and ash (figure 5.6), soil and rhizosphere bacteria were distinguished from endophytic bacteria. The relative abundance of each genotypically different strain was expressed as a percentage of the total number of cultivable isolates per gram fresh weight in soil and rhizosphere (oak: 100% = $14.7*10^4$ + $37.1*10^5$ = $38.6*10^5$; ash: 100% = $19.1*10^4$ + $14.8*10^5$ = $16.7*10^5$; see table 5.2) or inside the plant (oak: 100% = $94.7*10^3$ + $28.2*10^4$ + $13.9*10^3$ = $39.1*10^4$; ash: 100% = $18.0*10^4$ + $28.2*10^4$ + $13.9*10^3$ = $47.6*10^4$; see table 5.2).

Figure 5.6: (A) Diversity of cultivable bacteria in the soil and rhizosphere associated with English oak; (B) Diversity of cultivable endophytic strains associated with English oak. (C) Diversity of cultivable bacteria in the soil and rhizosphere associated with common ash; (D) Diversity of cultivable endophytic strains associated with common ash. Central pie shows percentages by phyla; each outer ring progressively breaks these down by finer taxonomic levels with the bacterial number in the outermost ring. Numbers in parentheses indicate the relative abundance, expressed as a percentage, of the total number of cultivable isolates per gram fresh weight that are present in the soil and rhizosphere (A) and inside (B) of English oak, and in the soil and rhizosphere (C) and inside (D) of common ash. Pie diagrams were generated using sigmaplot. **p166-167**


Bacilli (31.4)
Ba 34 (5.8)
Ba 27 (1.6)
Ba 26 (0.2)
Ba 25 (1.6)
Ba 23 (3.2)
Ba 22 (0.5)
Ba 21 (5.9)
Bacillus (12.8)
Bacillaceae (12.8)
Gammaproteobacteria (8.3)
Pseudomonadaceae (8.1)
Pseudomonas (8.1)
Ba 35 (12.8)
Paenibacillus (18.6)
Paenibacillaceae (18.6)
Micrococcaceae (9.7)
Arthrobacter (9.7)
Ba 3 (6.5)
Ba 6 (3.2)
Ba 50 (6.5)
Ba 51 (1.6)
Ba 47 (0.1)
Ba 44 (0.1)
Xanthomonadaceae (0.2)
Rhodanobacter (0.2)
Ba 41 (0.2)
Burkholderiaceae (0.1)
Wautersia (0.1)
Ba 36 (0.1)
Ba 40 (1.6)
Polaromonas (1.6)
Commamonadaceae (1.6)
Betaproteobacteria (1.7)
Firmicutes (31.4)
Proteobacteria (10.0)
Actinobacteria (58.6)
Ba 15 (12.8)
Actinobacteria (58.6)
Ba 16 (10.6)
Streptomycetaceae (48.9)
Streptomyces (48.9)
Ba 17 (25.5)
A
Enterobacter (3.4)
Enterobacteriaceae (4.2)
Erwinia (0.8)
Ba 21 (0.4)
Ba 25 (0.1)
Ba 24 (8.3)
Bacilli (11.8)
Bacillaceae (8.8)
Bacillus (8.8)
Ba 30 (0.1)
Ba 32 (3.0)
Paenibacillaceae (3.0)
Paenibacillus (3.0)
Ba 7 (3.6)
Ba 5 (0.1)
Arthrobacter (3.7)
Micrococcaceae (3.7)
Ba 54 (0.8)
Ba 55 (0.6)
Ba 53 (2.8)
Pseudomonadaceae (9.0)
Pseudomonas (9.0)
Ba 48 (0.01)
Ba 50 (8.2)
Gammaproteobacteria (17.9)
Ba 51 (0.1)
Ba 46 (0.01)
Ba 49 (0.3)
Ba 52 (0.2)
Ba 44 (0.01)
Ba 42 (4.6)
Xanthomonas (4.6)
Xanthomonadaceae (4.6)
Betaproteobacteria (5.2)
Commamonadaceae (5.2)
Polaromonas (5.2)
Ba 39 (5.2)
Ba 37 (0.01)
Wautersia (0.01)
Burkholderiaceae (0.01)
Streptomycetaceae (2.0)
Streptomyces (2.0)
Ba 16 (2.0)
Nocardioidaceae (0.8)
Aeromicrobium (0.8)
Ba 14 (0.8)
Curtobacterium (0.5)
Ba 13 (0.5)
Ba 12 (13.1)
Oldibacterium (13.1)
Ba 10 (19.2)
Actinobacteria (65.1)
Ba 11 (0.1)
Frigobacterium (45.0)
Ba 9 (25.7)
Microbacteriaceae (58.6)
Firmicutes (11.8)
Proteobacteria (23.1)
Actinobacteria (65.1)
B


Bacilli (35.0)
Ba 30 (1.0.2)
Ba 28 (0.1)
Ba 21 (1.5)
Ba 29 (7.2)
Ba 25 (10.6)
Bacillus (29.4)
Bacillaceae (29.4)
Paenibacillaceae (5.6)
Paenibacillus (5.6)
Ba 35 (5.6)
Ba 56 (0.1)
Erwinia (0.1)
Enterobacteriaceae (0.1)
Ba 43 (1.8)
Ba 46 (0.1)
Ba 51 (0.8)
Ba 50 (2.3)
Pseudomonas (5.0)
Pseudomonadaceae (5.0)
Gammaproteobacteria (5.1)
Actinobacteria (18.8)
Micrococcaceae (9.5)
Arthrobacter (9.5)
Ba 38 (5.0)
Collimonas (5.0)
Oxalobacteraceae (5.0)
Betaproteobacteria (5.0)
Firmicutes (35.0)
Proteobacteria (10.1)
Ba 3 (7.1)
Actinobacteria (18.8)
Ba 4 (0.2)
Ba 8 (2.2)
Bacteroidetes (36.1)
Flavobacteria (36.1)
Streptomycetaceae (9.3)
Streptomyces (9.3)
Ba 16 (3.4)
Ba 17 (1.3)
Ba 15 (3.1)
Ba 18 (0.3)
Ba 20 (0.6)
Ba 19 (0.6)
Flavobacteriaceae (36.1)
Flavobacterium (36.1)
Ba 2 (36.1)
C
Pseudomonadaceae (67.3)
Gammaproteobacteria (67.3)
Pseudomonas (67.3)
Ba 1 (0.1)
Sinorhizobium (0.1)
Rhizobiaceae (0.1)
Alphaproteobacteria (0.1)
Proteobacteria (67.4)
Ba 15 (0.1)
Actinobacteria (22.1)
Ba 51 (65.5)
Actinobacteria (22.1)
Firmicutes (10.5)
Streptomycetaceae (19.8)
Streptomyces (19.8)
Ba 16 (19.7)
Frigoribacterium (0.1)
Arthrobacter (2.2)
Ba 46 (1.7)
Ba 43 (0.1)
Ba 25 (1.9)
Bacilli (10.5)
Bacillaceae (9.8)
Bacillus (9.8)
Paenibacillaceae (0.7)
Paenibacillus (0.7)
Ba 24 (7.9)
Ba 21 (1.9)
Ba 31 (0.1)
Ba 28 (0.2)
Ba 35 (0.2)
Ba 33 (0.5)
Ba 32 (0.03)
Ba 7 (2.2)
Ba 11 (0.1)
Micrococcaceae (2.2)
Microbacteriaceae (0.1)
D

A

B

Figure 5.7: Schematic representation of endophytic strains as appearing within the different compartments of English oak (A) and common ash (B). The numbers in parentheses refer to the bacterial strain numbers used in figure 5.5.

In the soil and rhizosphere associated with English oak (figure 5.6A), 58.6% of the total number of isolates were Actinobacteria mostly of the genera *Streptomyces* (48.9%) and *Arthrobacter* (9.7%). Firmicutes made up 31.4% of the total number of isolates represented by Paenibacillaceae (18.6%) and Bacillaceae (12.8%). The remaining 10.0% of the collection was represented by Proteobacteria with a majority of gamma-Proteobacteria (8.3%) with *Pseudomonas* as dominant genus, and 1.7% of beta-Proteobacteria. The cultivable endophytic bacterial community associated with English oak (figure 5.6B) was also dominated by Actinobacteria (65.1%), with *Frigobacterium* spp. (45.0%) and *Okibacterium* spp. (13.0%) forming the majority of the group. *Arthrobacter* (3.7%) and *Streptomyces* (2.0%) were much less represented. Proteobacteria represented 23.1% of the endophytic collection associated with English oak and were dominated by gamma-Proteobacteria (17.9%) including *Pseudomonas* spp. (9%), *Xanthomonas* spp. (4.6%), *Enterobacter* spp. (3.4%) and *Erwinia* (0.8%). The remaining part of the endophytic community associated with English oak were Firmicutes (11.8%) with 8.8% Bacillaceae and 3.0% Paenibacillaceae. The compartmentalization of the dominant endophytic taxa in the different plant parts associated with English oak is shown in figure 5.7A.

The community of cultivable soil and rhizosphere bacteria associated with common ash (figure 5.6C) was dominated by Bacteroidetes, more specifically species of *Flavobacterium* (36.1%), and by Firmicutes (35.0%), including 29.4% Bacillaceae and 5.6% Paenibacillaceae. Actinobacteria made up 18.9% of the community (9.5% *Arthrobacter* spp. and 9.4% *Streptomyces* spp.) and Proteobacteria represented 10.1% (5.1% *Pseudomonas* spp. and 5.0% *Collimonas*). Proteobacteria accounted for 67.4% of the common ash endophyte isolates (figure 5.6D). Nearly all of these (67.3%) were gamma-Proteobacteria of the genus *Pseudomonas*; the remaining 0.1% were identified as alpha-Proteobacterial *Sinorhizobium*. Further, Actinobacteria made up 22.1% of the endophytic community, including a majority of 19.9% *Streptomyces* and a minority of 2.2% *Arthrobacter*. The remaining part of the common ash associated endophytic community were Firmicutes (10.5%), comprising mainly Bacillaceae (9.9%), and a small fraction of Paenibacillaceae (0.6%). The

compartmentalization of the bacterial endophytic community associated with common ash is presented in figure 5.7B. No typical leaf strains could be isolated from common ash.

5.3.3 Phenotypic characterization

In a first test, all genotypically different bacteria associated with English oak and common ash were phenotypically characterized for their heavy metal resistance and for their tolerance to the target pollutants TCE and toluene (table 5.3). Metal resistance was also tested because in future experiments metal-resistant strains may allow an easier tracebility. From all the isolated bacteria, six strains (6, 20, 36, 40, 41 and 49) could grow in the presence of Ni and one strain (23) was resistant to Cd and Zn.

The screening for TCE and toluene tolerance resulted in 82% of the bacteria that could grow in the presence of TCE, and 77% in the presence of toluene. In order to test TCE and toluene degradation capacity, a selection was made of bacteria that probably degrade TCE and/or toluene, based on the fact that these bacteria were growing better in a TCE and/or toluene saturated atmosphere as compared to autotrophic conditions, which suggests that they were able to use these components as a carbon source.

These bacteria (strains 4, 19, 27, 34 and 37) were screened for TCE and/or toluene degradation. Strains 4 (*Arthrobacter* sp.) and 19 (*Streptomyces turgidiscabies*), both rhizosphere bacteria associated with common ash, showed a 100% toluene degradation capacity and a maximum TCE degradation capacity of 22% and 14% respectively over a 3-day testing period in Schatz medium with addition of 100 mg l^{-1} TCE (figure 5.8). The other bacterial strains (27, 34 and 37) did not show any TCE or toluene degradation capacity.

Table 5.3: Growth characteristics of cultivable bacterial strains isolated in association with English oak and common ash. The numbers refer to the strain numbers used in figure 5.5 and the 5 strains that were selected for testing TCE and toluene degradation using head space gas chromatography are highlighted in gray. +: growth (few colonies); ++: very good growth (many colonies)

Strain	284 + cmix	284	284 + tol	284 + TCE	284 + cmix + Ni	284+ cmix + Cd	284 + cmix + Zn	Strain	284 + cmix	284	284 + tol	284 + TCE	284 + cmix + Ni	284+ cmix + Cd	284 + cmix + Zn	Strain	284 + cmix	284	284 + tol	284 + TCE	284 + cmix + Ni	284+ cmix + Cd	284 + cmix + Zn
1	++	++	++	++	-	-	-	20	++	++	-	++	+	-	-	39	++	++	-	-	-	-	-
2	+	-	-	-	-	-	-	21	++	++	++	++	-	-	-	40	++	++	+	+	+	-	-
3	++	++	++	++	-	-	-	22	+	-	-	-	-	-	-	41	++	++	++	+	++	-	-
4	+	+	++	++	-	-	-	23	++	+	-	-	-	+	+	42	++	++	++	++	-	-	-
5	++	++	++	++	-	-	-	24	++	++	++	++	-	-	-	43	+	+	+	+	-	-	-
6	++	++	++	++	++	-	-	25	++	++	++	++	-	-	-	44	++	++	++	++	-	-	-
7	++	++	++	++	-	-	-	26	++	+	+	+	-	-	-	45	+	+	+	+	-	-	-
8	++	++	++	++	-	-	-	27	+	+	++	+	-	-	-	46	++	++	++	++	-	-	-
9	++	++	++	++	-	-	-	28	++	++	++	++	-	-	-	47	++	++	++	++	-	-	-
10	++	+	+	+	-	-	-	29	++	++	++	++	-	-	-	48	++	++	++	++	-	-	-
11	++	++	++	++	-	-	-	30	++	++	++	+	-	-	-	49	++	++	++	+	++	-	-
12	++	++	++	+	-	-	-	31	++	++	++	-	-	-	-	50	++	++	++	+	-	-	-
13	++	++	++	++	-	-	-	32	+	+	+	+	-	-	-	51	++	++	+	++	-	-	-
14	++	++	++	++	-	-	-	33	++	+	-	+	-	-	-	52	++	++	++	++	-	-	-
15	+	+	-	-	-	-	-	34	+	+	-	++	-	-	-	53	++	++	++	++	-	-	-
16	+	+	+	+	-	-	-	35	+	-	-	-	-	-	-	54	++	++	++	+	-	-	-
17	++	+	-	-	-	-	-	36	++	++	++	++	+	-	-	55	++	++	++	++	-	-	-
18	+	+	-	-	-	-	-	37	++	+	++	++	-	-	-	56	+	+	-	+	-	-	-
19	+	+	++	++	-	-	-	38	++	++	++	++	-	-	-								

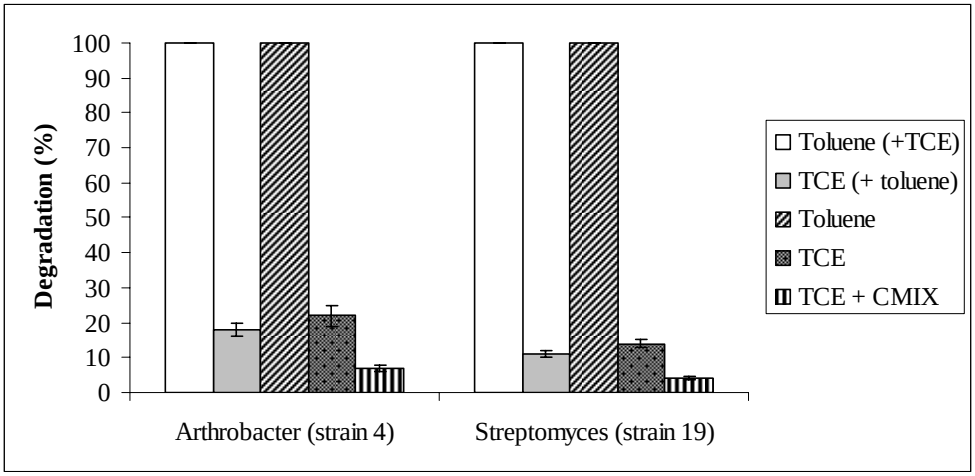


Figure 5.8: Toluene and TCE degradation tested by head space chromatography. Bacteria were grown in Schatz medium with addition of 100 mg l^{-1} toluene, 100 mg l^{-1} TCE or 100 mg l^{-1} toluene and 100 mg l^{-1} TCE, and in Schatz medium supplemented with C-mix (per liter medium: 0.52 g glucose, 0.35 g lactate, 0.66 g gluconate, 0.54 g fructose and 0.81 g succinate) and 100 mg l^{-1} TCE. Samples were taken at the beginning of the experiment and after 3 days. The volatilization of toluene and TCE was taken into account by measuring control samples (without addition of bacteria) and degradation was calculated as a percentage of the non-volatilized fraction.

5.3.4 In situ evapotranspiration

In common ash $10.84*10^{-3} \pm 1.17*10^{-3}$ ng TCE $cm^{-2}h^{-1}$ was evapotranspired to the atmosphere. The amount of transpired TCE from the leaves of English oak was $6.35*10^{-3} \pm 0.18*10^{-3}$ ng $cm^{-2}h^{-1}$. Since (a) the most common TCE degradation products in plant tissues are trichloroethanol, trichloroacetic acid, dichloroacetic acid and trichloroethanol glycoside (Burken and Ma, 2006) and (b) aerobic degradation of TCE has been reported for bacterial strains possessing the toluene ortho-monooxygenase (TomA) genes with TCE-epoxide, dichloroacetate, glyoxylate, and formate as metabolites, these oxidative metabolites were also analyzed. Although they have been identified in controlled cell culture experiments, whole-plant laboratory experiments, and full-scale field settings, none of these degradation products could be detected in these evapotranspiration measurements.

5.3.5 Discussion and conclusions

Poplar trees are frequently used for phytoremediation of groundwater contaminated with organic solvents (Schnoor *et al.*, 1995; Ferro *et al.*, 1997). Their fast growth and large transpiration potential make them 'trees of choice' for phytoremediation purposes (Schnoor *et al.*, 1995; Shim *et al*., 2000). In this study, the potential of English oak and common ash and their associated microorganisms for the phytoremediation of a TCE-contaminated site was investigated. Since they are both widely distributed native tree species in Europe possessing a relatively high transpiration capacity, it seemed very interesting to explore the diversity and remediation potential of their associated bacterial community. Since this work is part of a larger study on the potential role of traceable plant-associated bacteria to enhance *in situ* phytoremediation with as ultimate aim *in situ* (re-)inoculation of plant-associated bacteria equipped with the required degradation pathways, only the cultivable bacteria were characterized.

Cultivable bacteria were isolated from bulk soil, rhizosphere, root, stem, twig and leaf from English oak and common ash (table 5.2). For both tree species, the total amount of bacteria in the rhizosphere was one order of magnitude higher than in the bulk soil which can be explained by the 'rhizosphere effect'. The number of cultivable endophytes was highest in roots and stems and was lower in leaves, suggesting that colonization was mainly taking place through the roots.

The 16S rDNA identification of all isolated bacteria that were found to be different after ARDRA analysis resulted in 56 different bacterial strains comprising 26 strains exclusively associated with English oak, 15 strains exclusively associated with common ash and 15 strains associated with both tree species (figure 5.5). This indicates that both tree species were associated with both a specific bacterial population and a non-specific bacterial population.

In case of English oak, the bacterial diversity (number of different genera) in soil and rhizosphere seemed similar to the endophytic bacterial diversity, except that the fraction of Proteobacteria was higher and the fraction of Firmicutes was lower in the endophytic community (figure 5.6A and B). However, a closer look at the results shows that some taxa indeed were occurring in the bulk soil and the rhizosphere as well as inside the plant (e.g., *Arthrobacter*, *Streptomyces*,

Bacillus, *Paenibacillus*, *Pseudomonas*). Nevertheless, other taxa were exclusively found inside the plant (*Frigobacterium*, *Okibacterium*, *Curtobacterium*, *Aeromicrobium*, *Enterobacter* and *Erwinia*). This suggests that the endophytic community of English oak might be composed of both facultative endophytes colonizing the tree via the roots and obligate endophytes transferred from one generation to the other through the seeds. Furthermore, the endophytes exhibited a marked spatial compartmentalization, suggesting that in English oak some taxa of bacteria only occur in a specific plant part (e.g., *Paenibacillus*), while other did not show any compartment specificity (e.g., *Pseudomonas*) (figure 5.7A).

As to common ash, a clear difference could be noticed in the composition of the bacterial community in the soil and rhizosphere and the endophytic bacterial community, since Bacteroidetes represented 36.1% of the soil and rhizosphere bacteria, while they were completely lacking in the endophytic bacterial community (figure 5.6 C and D). Moreover, species of Proteobacteria dominated the endophytic bacterial community, while they only represented 10.1% of the soil and rhizosphere bacteria. In contrast with the bacterial endophytes associated with English oak, the endophytic bacterial community associated with common ash contained almost no species exclusively present inside the plant, which might indicate that the endophytic population consists mainly of facultative endophytes. This hypothesis is supported by the compartmentalization of the common ash associated endophytes (figure 5.7B), since most of the endophytes were localized in the roots and the stems, which is typical for facultative endophytes (Mastretta *et al.*, 2006). Given that obligate endophytes have to be transferred through the seeds, this difference in endophyte-type between English oak and common ash can possibly be explained by both the size and the structure of the seeds. Firstly, the seed is distinctly bigger and more robust in case of English oak. In addition, in seeds of English oak, the embryo possesses large cotyledons filling up the entire space within the seed coat while in case of common ash the cotyledons are tiny and the embryo's food reserve is almost entirely located in the extra-embryonic endosperm. Due to these clear differences, survival sites for endophytes might be different. Therefore, in a future experiment, the seed endophytes of English oak and common ash growing on the TCE-contaminated field site will be isolated and

characterized. The importance of seed endophytes as a vector for beneficial bacteria has already been demonstrated by Cankar *et al.* (2005) and Mastretta *et al.* (2006). Beside plant species-specific bacteria (figure 5.5), several taxa (e.g., *Pseudomonas*, *Bacillus*, *Paenibacillus* and *Arthrobacter*) that were found in association with both English oak and common ash were found in association with hybrid poplar (*Populus trichocarpa x deltoides*; Porteous-Moore *et al*. 2006). Moreover, we will characterize the bacterial community associated with the hybrid poplar trees newly planted on the TCE-contaminated site and compare this with the populations associated with English oak and common ash growing on the same site and with the bacterial community associated with hybrid poplar growing on a BTEX-contaminated site (Porteous-Moore *et al.* 2006).

In order to determine whether there was selection for specific bacterial phenotypes in the presence of TCE, representatives of all ARDRA types were tested for TCE and toluene tolerance. It is obvious that TCE concentrations in the groundwater up to 100 mg l^{-1} resulted for both tree species in a bacterial population that was dominated by TCE-tolerant strains (table 5.3). This enrichment of TCE-tolerant strains in response to the TCE contamination is consistent with the observations of Siciliano *et al.* (2001) and Becerra-Castro *et al.* (2009). Beside TCE-tolerant bacteria, there was also an enrichment of toluene-tolerant bacteria. The combined occurrence of TCE and toluene tolerance might be due to common mechanisms, which have been demonstrated for aerobic degradation of these compounds. Aerobic degradation of both toluene and TCE has been reported for bacterial strains possessing the toluene ortho-monooxygenase (TomA) genes of *Burkholderia cepacia* G4 (Mars *et al.,* 1996; Yee *et al.,* 1998), and for toluene-o-xylene monoxygenase (TomO) of *Pseudomonas stutzeri* OX1 (Ryoo *et al.,* 2000, 2001; Shim *et al.,* 2000, 2001). The fact that some strains (e.g., strains 4, 19, 34, 37) showed better growth on the 284 medium in the presence of TCE (and toluene) (table 5.3) suggests that TCE (and toluene) can be used as a substrate. The results of the degradation experiments indeed showed TCE (and toluene) degradation capacity (figure 5.8), which was already suggested by the remarkable decrease in TCE concentration through the transects at the field site (figure 5.4). Although these results support the hypothesis of a causal relationship between the strong decrease in

TCE concentration through the transects (figure 5.4) and the TCE (and/or toluene) degradation capacity of the bacterial population associated with the English oak and common ash, still a significant amount of TCE (English oak: $6.35*10^{-3} \pm 0.18*10^{-3}$ ng $cm^{-2}h^{-1}$; common ash: $10.84*10^{-3} \pm 1.17*10^{-3}$ ngTCE $cm^{-2}h^{-1}$) was evapotranspired from the leaves to the atmosphere. This implies that the natural bacterial community has insufficient capacity to degrade all the TCE taken up by the roots before it reaches the leaves. Therefore it might be worth attempting to inoculate the TCE-degrading bacteria that were isolated from oak and ash growing on this field, in order to enrich the quantity of degrading strains resulting in an improved remediation capacity. Furthermore, the newly planted poplar trees could be inoculated with TCE-degrading poplar endophytes, such as *Pseudomonas putida* W619-TCE (Taghavi *et al.* 2005), to improve the degradation capacity of the endogenous endophytic populations through horizontal gene transfer (see chapter 5.4).
For these inoculation experiments, it is favorable to work with bacteria that can easily be re-isolated, such as bacteria possessing heavy metal resistance. Such strains may allow an easier traceability for bioaugmentation of polluted sites. For this reason, all bacteria that were found to be different after ARDRA analysis were also tested for heavy metal resistance. Only a very limited number of strains were found to be resistant to the heavy metals Ni, Cd and/or Zn, which can be explained by the absence of selection pressure. Metal concentrations measured were indeed within the range of background values.

The results obtained in this study show that the bacterial community associated with English oak and common ash growing on a TCE-contaminated groundwater plume, was strongly enriched with toluene and/or TCE-tolerant strains, but that this was not sufficient to degrade all TCE before it reaches the leaves. Although both tree species were exposed to the same type and level of contamination and were growing side by side in the same woodland, their associated bacterial populations clearly differed in composition. The endophytic bacterial community associated with English oak contained significantly more species-specific, most likely obligate endophytes. This might be related to the seed type. Furthermore, the remediation capacity of English oak and common ash possibly might be improved by *in situ* inoculation of the TCE-degrading, plant-associated bacteria

that were isolated. Since English oak and common ash are both common native and widely spread species in Europe, this *in situ* inoculation strategy could have a large application potential. In addition, the newly planted poplar trees can be inoculated with *Pseudomonas putida* W619-TCE (Taghavi *et al.,* 2005), to improve the degradation capacity of the endogenous endophytic populations through horizontal gene transfer (see chapter 5.4).

Chapter 5.4 *Bioaugmentation with engineered endophytic bacteria improves phytoremediation*

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Bioaugmentation, the introduction of natural or genetically modified microorganisms to improve the remediation of contaminated groundwater, has successfully been applied to remediate groundwater polluted with chlorinated solvents (Steffan *et al*., 1999; Ellis *et al*., 2000) and so has become a widely accepted practice. However, this technology requires maintaining hydraulic control of the groundwater and continuously injecting nutrients, inducers, or oxygenating compounds. Phytoremediation is a more cost-efficient alternative for the remediation of such contaminated groundwater, but the evapotranspiration of volatile contaminants and their partial degradation products still undermine its merits (Ma and Burken, 2003). Laboratory studies show that the *in planta* metabolism of organic compounds can be strongly improved by inoculating plants with endophytic bacteria that can decompose the contaminants (Barac *et al*., 2004; Taghavi *et al.*, 2005; Germaine *et al.*, 2006; Germaine *et al.*, 2009). Bioaugmentation with endophytic bacteria would have several benefits over traditional bioaugmentation, that is, simply enriching the soil with a consortium of pollutant-degrading bacteria. In the former case, the bacteria reside in a contained environment, making them less susceptible to predation; the plant provides nutrients to the bacteria, thus supporting their growth and establishment. However, several remaining problems must be resolved before endophytes can be successful in field-scale phytoremediation projects (Newman *et al.*, 2005; van der Lelie *et al.*, 2005). For example, during bioaugmentation, the inoculated bacteria would have to compete against the endogenous, well-adapted microbial population. Here, horizontal gene-transfer could play an important role in enhancing the metabolic capabilities of the endogenous endophytes (Taghavi *et al.*, 2005); rather than integrating a new

bacterium into a stable community, the degradation pathway is transferred among members of the community.

To test whether the *in planta* bioaugmentation of phytoremediation with endophytic bacteria will enhance the remediation of organic solvents, we chose the same TCE-contaminated site as in chapter 5.3. At this site, a mixed woodland of English oak (*Quercus robur*) and common ash (*Fraxinus excelsior*) was already present on the contaminated groundwater plume (figure 5.4). The sharp decrease in TCE concentrations along transects under this mixed woodland suggested that phytoremediation was already taking place (chapter 5.3). To further control the groundwater TCE plume, hybrid poplar trees [*Populus deltoides x (trichocarpa x deltoides) cv. Grimminge*] were planted in the spring of 2006 perpendicularly to the plume (figure 5.4). Two years later, *in situ* evapotranspiration measurements on 6 independent poplar trees (taken in May 2008) revealed that a significant amount of TCE was evaporating through the poplar leaves (an average of $8.0 \times 10^{-2} \pm 0.3 \times 10^{-2}$ ng TCE cm^{-2} h^{-1}; table 5.4). As a case study to lower this amount, we inoculated three trees with *P. putida* W619-TCE. We selected this strain as the ideal candidate because it is a root endophyte of poplar that was equipped, via natural gene transfer, with the pTOM-Bu61 plasmid coding for constitutive TCE degradation (Taghavi *et al.*, 2005). According to European legislation, the constructed *P. putida* W619-TCE is considered as a non-Genetic Modified Organism and so can be used in field applications. Its parental strain, the non-pathogenic *P. putida* W619 of which the genome was recently sequenced (http://genome.jgi-psf.org/finished_microbes/psepw/psepw.home.html), is closely related to *P. putida* KT2440, an organism that is Generally Recognized as Safe (GRAS certified) (Timmis *et al.*, 2002). Furthermore, inoculating poplar with *P. putida* W619 under greenhouse conditions promoted plant growth fourfold, decreased the activities of glutathione reductase in the roots, and superoxide dismutase in the roots and the leaves, and considerably lowered stomatal resistance, all indicative of the poplar's improved fitness (chapter 4.1). Beside this plant growth-promoting effect, the TCE-degrading *P. putida* W619-TCE is capable of reducing TCE phytotoxicity and evapotranspiration (chapter 4.2).

5.4.1 In situ evapotranspiration

P. putida W619-TCE was cultured in 869 medium (Mergeay *et al.*, 1985) and 40 liter of this bacterial solution (10^8 cfu ml^{-1}) was added to the roots of three experimental trees via drainage tubes, installed when the trees were planted. At the same time, the three control trees received 40 liter of 869 medium without bacteria. Three months after inoculation (mid August), the *in situ* evapotranspiration of TCE was determined from the leaves of the control and inoculated poplar trees; the respective values had an average of $7.2 \times 10^{-2} \pm 0.3 \times 10^{-2}$, and $0.8 \times 10^{-2} \pm 0.1 \times 10^{-2}$ ng TCE cm^{-2} h^{-1}, representing a nine-fold reduction ($p = 0.0002$) in TCE evapotranspiration for the inoculated trees (table 5.4). In contrast, TCE evapotranspiration through the leaves of the non-inoculated control trees was similar to that seen before inoculation (table 5.4), indicating that reduced evapotranspiration was not related to differences in the plant's growth cycle.

Table 5.4: TCE evapotranspiration of the experimental trees before and after inoculation with *P. putida* W619-TCE.

	TCE-evapotranspiration (10^{-2} ng cm^{-2} h^{-1})	
Tree	Before inoculation (May)	3 months after inoculation (August)
1	8.9 ± 0.8	7.8 ± 1.8
2	7.1 ± 1.2	7.2 ± 1.0
3	8.4 ± 1.5	6.8 ± 0.5
4[a]	7.9 ± 0.9	0.9 ± 0.1
5[a]	8.1 ± 1.3	0.9 ± 0.4
6[a]	7.5 ± 0.8	0.5 ± 0.1

[a] *P. putida* W619-TCE inoculated trees are highlighted in grey

In situ TCE evapotranspiration was determined for three independent non-inoculated control poplar trees and three poplar trees inoculated with *P. putida* W619-TCE. For each tree, three TCE evapotranspiration measurements were performed simultaneously. The amount of evapotranspired TCE was calculated per hour and unit of leaf area. Values are mean ± standard error of 3 replicates.

5.4.2 Genotypic characterization of the poplar-associated bacteria

To verify that the inoculated *P. putida* W619-TCE had been integrated into the plant-associated microbial community, cultivable bacteria from the rhizosphere-soil, roots, stems, twigs, and leaves of the three inoculated and three non-inoculated experimental trees were isolated and identified via BOX- and ARDRA-DNA fingerprinting and 16S rRNA gene sequencing.

In the rhizosphere-soil, the bacterial communities of inoculated and non-inoculated poplars were characterized by common species (*Pseudomonas* spp., *Arthrobacter* spp., *Bacillus* spp., *Acinetobacter* spp., and *Acidovorax* spp.), as well as species exclusively found in non-inoculated trees (*Agromyces* spp., *Microbacterium* spp., *Chromobacterium* spp. and *Aeromonas* spp.) or the inoculated ones (*Flavobacterium* spp., *Chryseobacterium* spp., *Paenibacillus* spp., *Ralstonia* spp. and *Enterobacter* spp.) (figure 5.9a). The total numbers of cultivable isolates found in the rhizospheres of the non-inoculated trees (3.5 x 10^7 cfu per g rhizosphere-soil) and the inoculated trees (5.0 x 10^7 cfu per g rhizosphere-soil) were similar and were dominated by *Pseudomonas* spp. (figure 5.10a). Interestingly, the *Pseudomonas* spp. in the rhizospheres are phylogenetically distinct from *P. putida* W619-TCE (figure 5.9a), indicating that after 3 months the inoculated strain was not established in the rhizosphere. Most of the remaining cultivable rhizospheric bacteria were represented by *Acinetobacter* spp. and *Flavobacterium* spp., respectively, for the non-inoculated and inoculated poplar trees. This difference suggested that inoculation with *P. putida* W619-TCE changed the composition of the rhizospheric community.

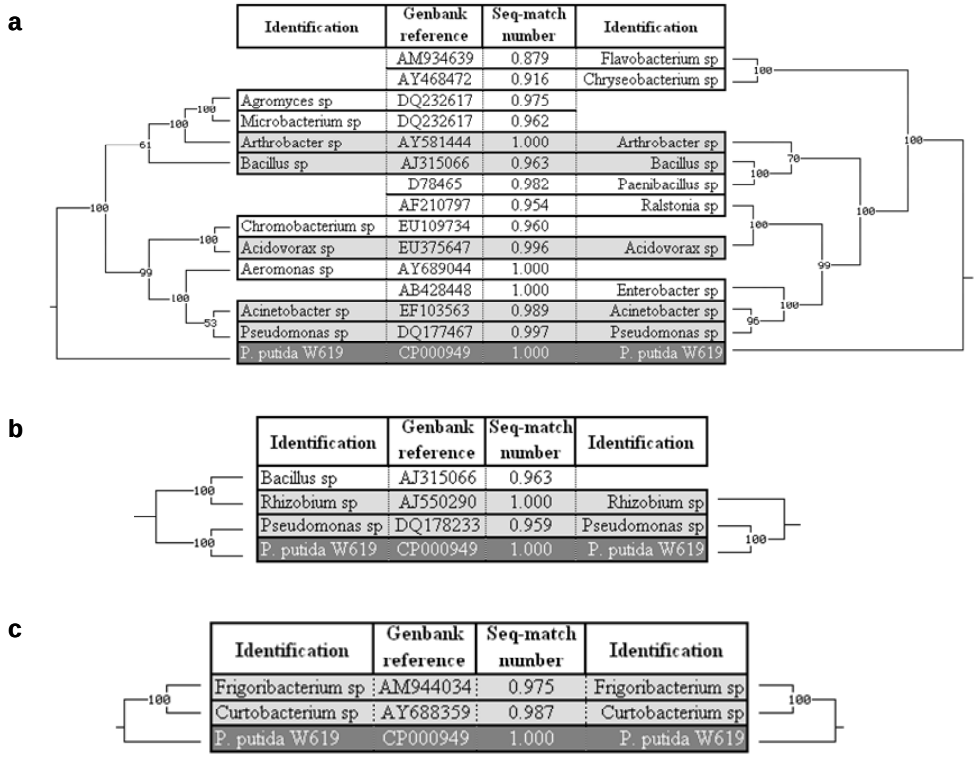

Figure 5.9: Neighbour-joining trees of 16S rRNA genes of the isolated bacteria. (a) the rhizosphere-soil bacteria, (b) the root endophytes and (c) the stem endophytes associated with non-inoculated (left) and inoculated (right) poplar were isolated and identified. The 16S rRNA gene-based identification, the accession number of the closest related strain, and the sequence match number are shown. For comparison, strain *P. putida* W619-TCE (denoted by dark grey) was included. Bacterial strains that are present in both inoculated and non-inoculated control plants are highlighted in light grey. To construct neighbour-joining trees, data from the three inoculated trees were combined, as were the data from the non-inoculated trees.

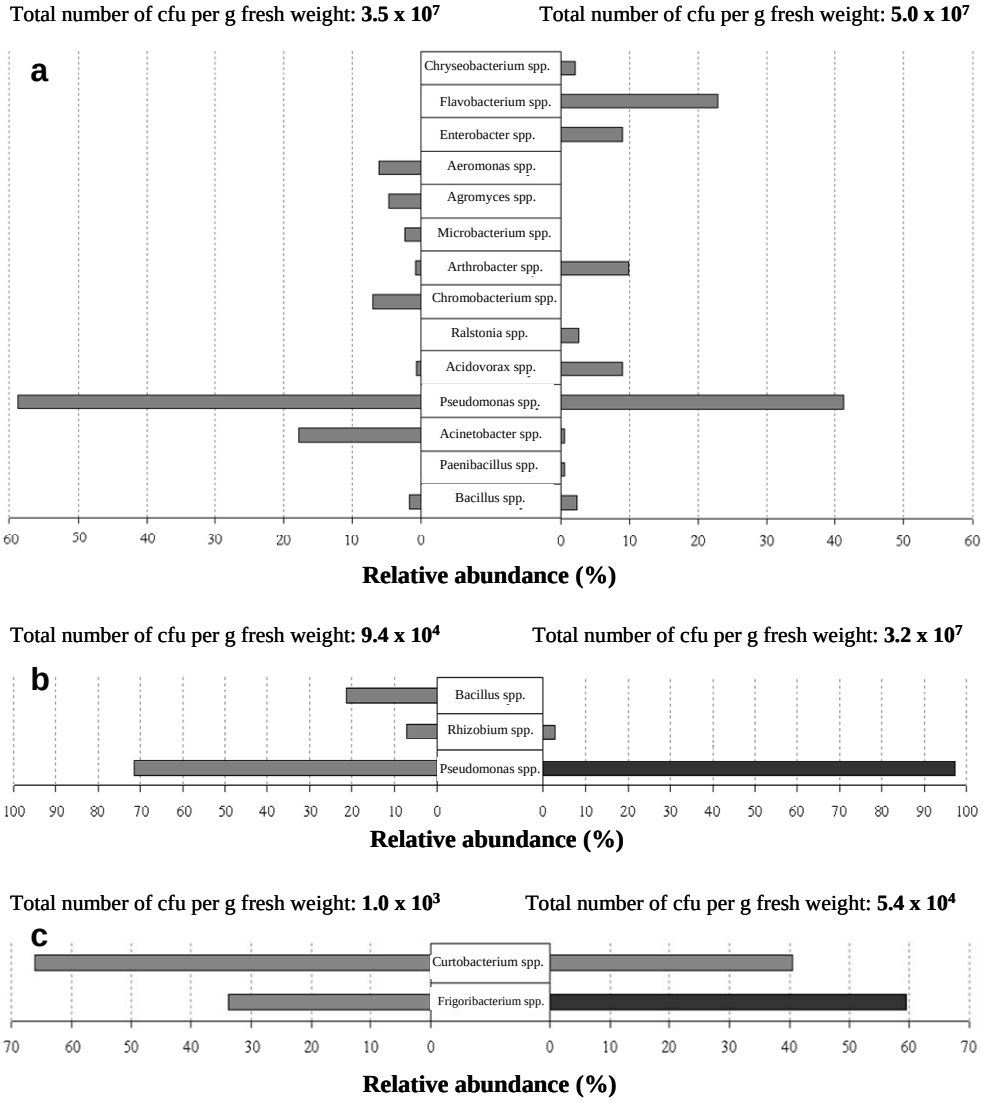

Figure 5.10: Relative abundance of cultivable bacteria isolated from poplar.
The relative abundance of (a) the rhizosphere-soil bacteria, (b) the root endophytes and (c) the stem endophytes of non-inoculated (left) and inoculated (right) poplar is expressed as a percentage of the total number of cultivable isolates per gram fresh weight present in the rhizosphere-soils (a), the roots (b) or the stem (c). The black bars represent the bacterial strains that possess the pTOM-Bu61 plasmid and can degrade TCE. To analyze the diversity, data from the three inoculated trees were combined, as were the data from the non-inoculated trees.

The cultivable members of the endophytic community in the roots of non-inoculated poplar trees encompass *Bacillus* spp., *Rhizobium* spp., and *Pseudomonas* spp., while roots from the inoculated poplars contained only *Rhizobium* spp. and *Pseudomonas* spp. (figure 5.9b). With 16S rRNA gene sequencing, the *Pseudomonas* spp. from both endophytic communities were undistinguishable from *P. putida* W619-TCE. However, BOX-PCR DNA fingerprinting analysis revealed that the *Pseudomonas* spp. isolated from the roots of the three inoculated trees indeed corresponded to the inoculated *P. putida* W619-TCE, while the *Pseudomonas* spp. isolated from the roots of three non-inoculated trees showed different BOX-PCR patterns (figure 5.11).

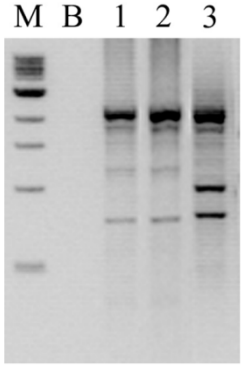

Figure 5.11: BOX-PCR fingerprints of the different *Pseudomonas* strains.
1: *P. putida* W619-TCE, 2: *Pseudomonas* spp. isolated from roots of poplar trees inoculated with *P. putida* W619-TCE, and 3: *Pseudomonas* spp. isolated from roots of non-inoculated poplar trees. M: 1kB DNA marker; B: blank

We noticed a strong increase in the total number of cultivable root isolates from 9.4×10^4 cfu g^{-1} fresh weight for the non-inoculated trees, to 3.2×10^7 cfu g^{-1} fresh weight for the inoculated ones (figure 5.10b). Since *Pseudomonas* spp. dominate both endophytic root communities, this increase most likely reflects an enrichment of *P. putida* W619-TCE in the roots of inoculated trees. This conclusion was confirmed by the presence of the pTOM-Bu61 plasmid among the *Pseudomonas spp*. in the roots of all inoculated trees (see below), while the plasmid was absent from the *Pseudomonas spp*. residing in the roots of the control poplars.

For all trees tested, the cultivable members of the endophytic communities in the stems of non-inoculated and inoculated trees consisted of *Frigoribacterium* spp. and *Curtobacterium* spp. (figure 5.9c); no *P. putida* W619-TCE was isolated. However, we gained good evidence that inoculation with *P. putida*

W619-TCE alters community composition, even in parts of the plant where it is not established. Thus, along with an increase in the total number of isolates from 1.0 x 10^3 cfu g^{-1} fresh weight for the non-inoculated poplar trees, up to 5.4 x 10^4 cfu g^{-1} fresh weight for the inoculated poplar trees, there was a shift to *Frigoribacterium* spp. as the dominant species (figure 5.10c).
No cultivable endophytic bacterial strains were isolated from the twigs and the leaves of the control or inoculated trees.

5.4.3 TCE degradation capacity of the poplar-associated bacteria

To verify that the reduced TCE evapotranspiration observed after inoculation was related to bioaugmentation with *P. putida* W619-TCE, the TCE degradation capacities of representative members of all isolated strains were determined using head-space gas chromatography (see 5.1.4), and compared to that of *P. putida* W619-TCE. Bacteria were grown in Schatz medium (Schatz and Bovell, 1952) with and without adding a carbon-mix (per liter medium: 0.52 g glucose, 0.35 g lactate, 0.66 g gluconate, 0.54 g fructose and 0.81 g succinate) and supplemented with 100 mg l^{-1} TCE. Only the endophytic *Pseudomonas* spp. and *Frigoribacterium* spp. (black bars in figures 5.10b and 5.10c) isolated, respectively, from the roots or stems of the inoculated trees, could constitutively degrade TCE with efficiencies similar to *P. putida* W619-TCE (figure 5.12).
For all three inoculated trees, both species became the dominant members of their respective endophytic communities, suggesting that the capacity to degrade TCE offers a selective advantage. For all individual isolates of both *Pseudomonas* spp. and *Frigoribacterium* spp. the presence of the pTOM-Bu61 plasmid was confirmed by PCR (against its *tomA4* gene). Since no TCE degradation phenotype nor pTOM-Bu61-like plasmids were found among the cultivable members of the different endophytic and rhizosphere communities of the non-inoculated poplar trees, we attribute the TCE degradation capacity of endophytic communities from the roots and stems of the inoculated trees directly to bioaugmentation with *P. putida* W619-TCE. This strain becomes well-established and dominates the roots' endophytic community. Furthermore, the *Frigoribacterium* spp. (figures 5.9c and 5.10c) present as an endogenous endophyte in the stem, received the pTOM-Bu61 plasmid via horizontal gene-

transfer, and thereafter became the dominant member of its endophytic community. This was generally observed for the endophytic communities of all three inoculated poplars.

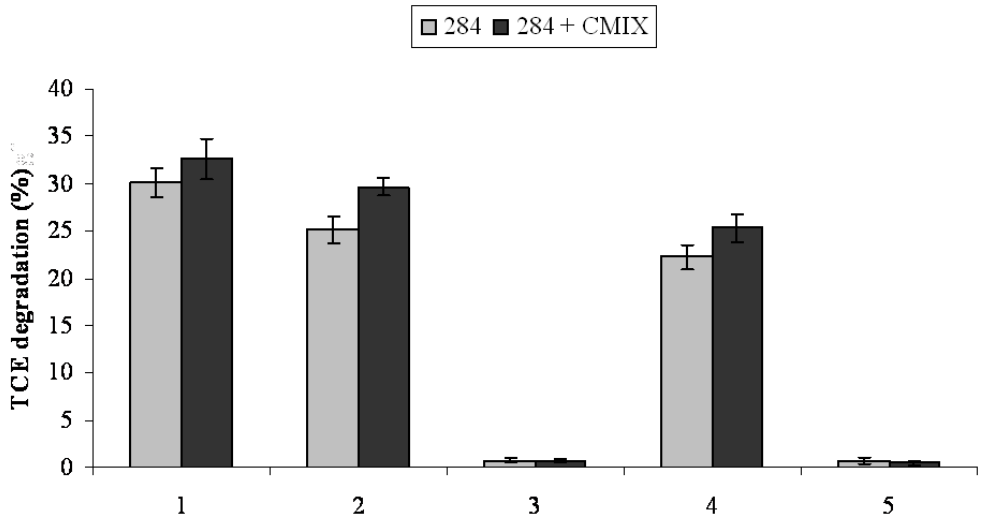

Figure 5.12: TCE degradation of bacteria 1-5 tested by head-space chromatography.
1: *P. putida* W619-TCE; 2: *Pseudomonas* spp. isolated from the roots of poplar trees inoculated with *P. putida* W619-TCE; 3: *Pseudomonas* spp. isolated from the roots of non-inoculated poplar trees; 4: *Frigoribacterium* spp. isolated from the stems of poplar trees inoculated with *P. putida* W619-TCE; and 5: *Frigoribacterium* spp. isolated from the stems of non-inoculated poplar trees. Bacteria were grown in Schatz medium with addition of 100 mg l^{-1} TCE and in Schatz medium supplemented with C-mix (per liter medium: 0.52 g glucose, 0.35 g lactate, 0.66 g gluconate, 0.54 g fructose and 0.81 g succinate) and 100 mg l^{-1} TCE. Samples were taken at the beginning of the experiment and after 3 days. The volatilization of TCE was taken into account by measuring control samples (without addition of bacteria)

However, the *Pseudomonas* spp. and *Frigoribacterium* spp. lost their TCE degradation capacity within 20 generations when cultivated under non-selective conditions. This loss of degradative capacity was also demonstrated in chapter 5.2 for a toluene phytoremediation site; thus once the contaminants' concentration decreased below the detection limit and no longer exerted selection pressure, the endophytic community is restored to its original situation and loses its degradation potential.

To test if *P. putida* W619-TCE became a stable, TCE-degrading member of the root endophyte population, one year after inoculation, cultivable endophytes were isolated and characterized again. The TCE-degrading *P. putida* W619-TCE was still dominating the root endophyte population and the amount of TCE evapotranspired through the leaves was stabilized at the same level as one year before. These observations indicate that the concept of using endophytes with the appropriate degradation pathway is also working in the field. However, monitoring needs to be continued in the following years.

5.4.4 Conclusions

We demonstrated that *in situ* inoculation into poplars of endophytic bacteria with the appropriate degradation pathway remarkably lowers TCE evapotranspiration and that endophytic bacteria capable of degrading TCE have a selective advantage, are able to dominate the community, and once this pathway is no longer utilized, it is rapidly lost by the bacteria. The concept of using natural or engineered endophytes for remediation purposes is broadly applicable. Many endophytic strains are related closely to environmental strains that carry, on mobile DNA elements, degradation pathways for a broad spectrum of organic contaminants; hence, it may be relatively straightforward to construct, via conjugation, non-GMO endophytic bacteria with *a la carte* degradation properties.

Considering that one of the major obstacles to the implementation of phytoremediation is the evapotranspiration of volatile contaminants, we believe that improved phytoremediation via bioaugmentation will offer a safe, break-through approach to the large-scale application of phytoremediation of organic contaminants. Another point making this work very special and original is that we introduced a bacterial strain, that was modified under laboratory conditions, back into the environment, which is in general very difficult to achieve.

Section 6

General discussion, conclusions and perspectives

Introduction

Conventional techniques used for remediation of contaminated soils and groundwater, frequently are costly and often cause drastic changes in soil structure and biological activity. In contrast, phytoremediation is an *in situ*, solar powered remediation technology that requires minimal site disturbance and maintenance resulting in a low cost and a high public acceptance. For this reason, phytoremediation is a very promising alternative remediation technology, however, (a) phytotoxicity, (b) insufficient degradation of volatile organic contaminants leading to evapotranspiration and (c) a limited contaminant uptake and translocation are still constraints that have to be conquered. Although in many studies the role of plant-associated bacteria during phytoremediation is underestimated, it was already demonstrated by Barac *et al.* (2004) and Lodewyckx *et al.* (2001) that these plant-associated bacteria can be exploited to improve phytoremediation efficiency. When an endophyte, possessing the genetic information required for the efficient degradation of toluene, was inoculated in lupine plants to promote the *in planta* toluene degradation, this resulted in a decreased toluene phytotoxicity and evapotranspiration (Barac *et al.*, 2004). Lodewyckx *et al.* (2001) demonstrated that inoculation of yellow lupine with endophytic bacteria equipped with the ncc-nre Ni resistance could reduce Ni phytotoxicity and improved Ni uptake in the roots. Although these results were good indications that endophytes can be exploited to overcome the constraints mentioned above and thus can contribute to bring phytoremediation towards field application, there still remained some critical issues that needed closer investigation. At first, at most sites plants and their associated microorganisms have to be able to grow on mixes of organic contaminants and toxic metals which are often very phytotoxic. Further, the successful colonization of yellow lupine plants by inoculated strains on laboratory scale is no guarantee that (a) *in situ* inoculation of endophytes will lead to a stable establishment and enrichment of the inoculated strain and that (b) inoculation in the field can result in a stable reduced phytotoxicity and evapotranspiration and in an improved contaminant uptake. In this work, these critical issues were investigated with the purpose to apply endophyte-enhanced phytoremediation in the field.

General discussion

In this work, a first step forward towards field-scale application of phytoremediation was to extend the concept of endophyte-enhanced phytoremediation to mixed contaminations of toxic metals and organic contaminants. As described in the first experiment with lupine exposed to a mixture of Ni and toluene (chapter 3.3) and in the second experiment with lupine exposed to a mixture of Ni and TCE (chapter 3.4), inoculation with the toluene- and TCE-degrading yellow lupine endophyte *Burkholderia cepacia* VM1468 can reduce phytotoxicity of the host plant and evapotranspiration of toluene and TCE.

In both cases, inoculation with *B. cepacia* VM1468 resulted in a good establishment and, after exposure to the contaminants, even in an enrichment of the inoculated strain in the roots (table 3.1 and 3.2). This selective enrichment was also observed several times in the field (Siciliano *et al.*, 2001; Becerra-Castro *et al.*, 2009; Porteous-Moore *et al.*, 2006; chapter 5.2: table 5.3; chapter 5.3: figure 5.10).

Only in the experiment with lupine and mixed contamination of Ni and toluene, *B. cepacia* VM1468 not only colonized the root but also the shoot (table 3.1). Probably this difference in colonization efficiency was due to the different inoculation strategy used (see 3.1.1).

During both experiments, the phytotoxicity caused by the mixed contaminations was evaluated by determining root and shoot biomasses and the activities of stress related enzymes in the roots and the leaves. In the first experimental set up (combination of Ni and toluene), inoculation with *B. cepacia* VM1468 resulted in increased root and shoot biomasses (figure 3.4) and strongly reduced stress related enzyme activities in the roots and the shoots (figure 3.5), all indicative for a highly reduced phytotoxicity. In the experiment with lupine and mixed contamination of Ni and TCE, a similar reduction in phytotoxicity was observed, though only in the roots (figures 3.8 and 3.9). A possible explanation for the absence of the reduced phytotoxicity in the shoot can be that the inoculated *B. cepacia* VM1468 only colonized the roots and was not detected in the shoot in the second experiment.

To evaluate if inoculation with the toluene- and TCE-degrading, Ni-resistant *B. cepacia* VM1468 could improve the phytoremediation efficiency of mixed

contaminations of Ni and toluene or of Ni and TCE, toluene and TCE evapotranspiration, and Ni uptake were determined. The amounts of respectively evapotranspired toluene or TCE were slightly reduced after inoculation with *B. cepacia* VM1468 (figures 3.6 and 3.10). A similar reduction in toluene evapotranspiration was achieved after inoculation with the toluene-degrading *B. vietnamiensis* BU61, though this strain did not have any plant growth-promoting effect (figures 3.4 and 3.6). However, in both experiments, the observed reductions in evapotranspiration were not significant due to the high degree of variability in the obtained results. The Ni uptake was not affected by inoculation with the toluene-degrading, Ni-resistant *B. cepacia* VM1468 when yellow lupine was exposed to a mixture of Ni and toluene (figure 3.7). Only after inoculation with the Ni-resistant *B. cepacia* BU72, an increased uptake of Ni in the roots was observed (figure 3.7). Interestingly, in the second experiment with lupine exposed to Ni and TCE, inoculation with *B. cepacia* VM1468 resulted in a strongly increased Ni uptake in the roots (figure 3.11). This different effect of *B. cepacia* VM1468 on Ni uptake in both experiments can be due to the different inoculation procedure (see 3.1.1) or the different way of Ni exposure (see 3.1.1 and 3.1.3) but also by the different organic contaminant Ni exposure was combined with.

Although the results of both experiments indicate that endophytes equipped with the appropriate degradation pathway and a metal resistance/sequestration system can be exploited to improve phytoremediation efficiency, the differences between the results of both experiments make us aware of the relatively high impact that rather small changes in experimental set up can have on the result. Consequently, these results (a) are only strong indications that endophytes can improve phytoremediation of mixed contaminations in the field and (b) are a good illustration of the emerging need to perform field experiments since only this type of experiments can deliver the final proof that the concept can be applied for large-scale field applications.

In a next step towards implementation of endophyte-enhanced phytoremediation on field scale, yellow lupine, our model test plant, was replaced by poplar cuttings, but the experiments were still performed on laboratory scale. Poplar was chosen as host plant because it is an excellent

candidate for both phytoremediation as well as feedstock for biofuel production; the poplar endophyte *Pseudomonas putida* W619 equipped with a pTOM plasmid coding for toluene and TCE degradation was selected for inoculation. In this section of the work, (a) the colonization process and the plant growth promotion capacity (chapter 4.1) and (b) the potential to reduce TCE phytotoxicity and evapotranspiration (chapter 4.2) of *P. putida* W619-TCE were explored.

From the results presented in chapter 4.1, it can be concluded that inoculation of the poplar (cv. Grimminge) cuttings with *P. putida* W619 resulted in good colonization of the rhizosphere, the root cortex and the root xylem vessels (figure 4.3); inoculation resulted in (a) remarkable plant growth promotion (figure 4.4), (b) decreased activities of glutathione reductase in the roots, and of superoxide dismutase in the roots and leaves (figure 4.6), and (c) a strongly reduced stomatal resistance (figure 4.5), all indicative of improved plant fitness in comparison with the non-inoculated control cuttings. On the long-term, the remarkably strong plant growth-promoting effect of wild-type *P. putida* W619 in this rather short-term experiment might be of direct importance for the use of poplar as a feedstock for biofuel production, and for carbon sequestration through biomass production. To confirm this, long-term follow up of field-grown poplar inoculated with plant growth-promoting strains is needed.

Since *P. putida* W619-TCE is also equipped with the pTOM plasmid, containing genes coding for toluene and TCE degradation, its potential to reduce TCE phytotoxicity and evapotranspiration and in this way to improve the phytoremediation efficiency of TCE was investigated in chapter 4.2. Non-inoculated poplar cuttings and cuttings inoculated with *P. putida* W619-TCE were supplied with 200 and 400 mg l^{-1} TCE in 2 different experimental systems. In the first system, cuttings were grown hydroponically and exposed to TCE for 3 days (short-term TCE exposure); in the second system, cuttings were grown in potting soil and exposed to TCE for 3 weeks (mid-term TCE exposure).

In the case poplar cuttings were exposed to TCE for 3 days and grown hydroponically, inoculation with the TCE-degrading *P. putida* W619-TCE significantly promoted plant growth and in addition resulted in a strongly decreased TCE phytotoxicity (figure 4.9). When poplar cuttings were exposed to TCE for 3 weeks and grown in potting soil, inoculation with *P. putida* W619-TCE only slightly promoted plant growth and reduced TCE phytotoxicity (figure 4.10).

A possible explanation for these different results might be the better balanced easily accessible pool of essential nutrients that is present in potting soil. Due to this, non-inoculated plants grown in potting soil might show almost optimal growth and by consequence, inoculation with *P. putida* W619-TCE could only slightly promote plant growth. Moreover, due to the nutrient rich quality of potting soil, it also might be a good substrate favouring growth and plant colonization of Plant Growth Promoting Bacteria (PGPB). Probably, in potting soil, PGPBs are already well represented without any inoculation and by consequence *P. putida* W619-TCE could only have a rather limited additional plant growth-promoting effect. This explanation for the different results in potting soil and hydroponics would imply that, on marginal land, endophytic bacteria can play a much more important role in plant growth promotion and even in improving phytoremediation. However, again field experiments need to be performed to support these hypotheses.

Beside the plant growth-promoting effect, *P. putida* W619-TCE could, by degrading TCE while it is transported through the xylem vessels, also reduce the TCE concentration in the roots and the leaves (figures 4.11 and 4.12) and the amount of TCE that was evapotranspired through the leaves (figure 4.13). The quantities of TCE that were extracted from leaves and roots of hydroponically grown cuttings were much higher than those of cuttings that were grown in potting soil (figures 4.11 and 4.12). The rather high amounts of TCE that were obtained from the roots of hydroponically grown cuttings might be due to a strong adsorption of TCE on the root apoplast. The higher amount of TCE found in the leaves of cuttings that were grown hydroponically can be explained by the fact that, in an hydroponic system, the roots are in full contact with the TCE-containing nutrient solution (in contrast with cuttings grown on potting soil), leading to a higher TCE uptake than for cuttings grown in potting soil. In potting soil, a major part of the TCE will get adsorbed on the high amount of organic matter. Since the second experiment, in which cuttings were grown in potting soil is more close to a field situation than the hydroponic experiment, the results obtained from the second experiment will probably be a better approximation for TCE concentrations in field grown poplar trees. For both experimental set ups, the decline in TCE concentration after inoculation was most prominent in the leaves (figures 4.11 and 4.12). Beside the fact that the amount of TCE that is

extracted from the roots also includes the TCE that is adsorbed on the root apoplast and in this way masks the effect of inoculation, the effect of *P. putida* W619-TCE can be much stronger in the leaves because *P. putida* W619-TCE residing in the xylem vessels can degrade TCE during its transport to the leaves.
Since the results of section 4 (exploration of poplar-*P. putida* W619 partnerships) demonstrate that *P. putida* W619 (a) possesses a good poplar colonization capacity (figure 4.3), (b) not only can promote plant growth and protect its host plant against TCE phytotoxicity (figures 4.4, 4.9 and 4.10), but (c) also improves TCE degradation leading to reduced amounts of both, TCE in the plant tissue (figures 4.11 and 4.12) and of TCE evapotranspired to the ambient air (figure 4.13), it can be concluded that *P. putida* W619-TCE and poplar are suitable candidates to test endopyte-enhanced phytoremediation in the field.

The final section describes the field trials that were performed to finally demonstrate the applicability of endophyte-enhanced phytoremediation in the field.
In the first field experiment, poplar and its naturally associated microorganisms were applied for the *in situ* remediation of a BTEX-contaminated groundwater plume (chapter 5.2). Analysis of the poplar-associated microorganisms demonstrated that, once the poplar roots got in contact with the BTEX-contaminated groundwater, a stimulating effect on the percentage of cultivable toluene-degrading endophytic and rhizosphere bacteria was observed (table 5.1a). This observation is in accordance with previous studies by Siciliano *et al*., (2001), and with the enrichment of toluene- and TCE-degrading lupine endophytes that was observed when lupine plants were exposed to Ni and toluene or Ni and TCE (chapters 3.3 and 3.4). Interestingly, once the BTEX concentrations below the poplar trees had decreased below detection limit, no rhizospheric nor endophytic bacteria able to grow on toluene as a sole carbon source were found any more (table 5.1b). This indicates that it concerns a selective enrichment due to the presence of the BTEX contamination and further demonstrates the plasticity of the endogenous microbial communities to assist their host plant in the phytoremediation of organic contaminants. Most likely, horizontal gene transfer played an important role in the adaptation of the

endogenous microbial communities, as previously reported by Taghavi *et al.*, (2005). During this field experiment, the *in situ* evapotranspiration of BTEX through the leaves could not be determined since the equipment to measure *in situ* evapotranspiration that we recently designed, was not yet available. In the mean time, the BTEX concentrations below the poplar trees decreased already below the detection limit; therefore it now is not possible anymore to perform *in situ* evapotranspiration measurements.

For the two other field experiments, a TCE-contaminated site was chosen where a mixed woodland of English oak (*Quercus robur*) and common ash (*Fraxinus excelsior*) was already present on the contaminated groundwater plume. Along transects under this mixed woodland, sharp decreases in TCE concentrations were observed, indicating the trees and their associated bacteria could have an active role of in the remediation process (figure 5.4). To investigate this, in chapter 5.3 (a) the bacteria associated with oak and ash were characterized and (b) the *in situ* TCE evapotranspiration was determined. The bacterial characterization revealed that, in accordance with previously observed selective enrichments (Siciliano *et al.*, (2001); Becerra-Castro *et al.*, 2009; chapter 3.3 and 3.4 and chapter 5.2), the majority of the isolated bacteria were tolerant to TCE, and TCE degradation capacity was observed in some of the strains (table 5.3). Although these results support the hypothesis of a causal relationship between the strong decrease in TCE concentration through the transects and the TCE (and/or toluene) degradation capacity of the associated bacteria, *in situ* evapotranspiration measurements revealed that a significant amount of TCE was evaporating through the leaves to the atmosphere. In conclusion, the natural, selective enrichment of TCE-tolerant and degrading strains probably had an active role in the phytoremediation process, however the acquired degradation capacity was not sufficient to avoid TCE evapotranspiration. A possible strategy to conquer this evapotranspiration to the ambient air is to increase the degradation capacity of the endogenous plant-associated bacteria by *in situ* inoculation with endophytic strains capable of degrading TCE. For that purpose, hybrid poplar trees [*Populus deltoides x (trichocarpa x deltoides) cv. Grimminge*] were planted perpendicularly on the contamination plume and were provided with a drainage tube around their roots to allow inoculation with the TCE-degrading poplar endophyte *P. putida* W619-TCE. *Pseudomonas putida* W619-

TCE and poplar were chosen since in chapter 4.1 and 4.2 they were shown to be suitable candidates to test endophyte-enhanced phytoremediation in the field.
In chapter 5.4, the first successful *in situ* inoculation of these poplar trees with *P. putida* W619-TCE is reported. After *in situ* inoculation with the TCE-degrading *P. putida* W619-TCE, a 90% reduction of TCE evapotranspiration was achieved (table 5.4). This encouraging result could be realized through the establishment and enrichment of *P. putida* W619-TCE as a poplar root endophyte, and the further horizontal gene-transfer of TCE metabolic activity to members of the poplar's endogenous endophytic population (figures 5.10 and 5.11). The facts that one year after inoculation, (a) the inoculated strain still dominated the cultivable root endophyte community and (b) the amount of evapotranspired TCE was similar as the year before, indicate that the establishment and enrichment, and the TCE degradation capacity of *P. putida* W619-TCE is stabilized. Beside that this was the first successful *in situ* inoculation, another point making this work very original is that we introduced a bacterial strain, that was modified under laboratory conditions, back into the environment, which is in general very difficult to achieve.

Considering that (a) the major obstacles for implementation of phytoremediation in practice still are the mixed pollutions that are found at most contaminated sites and the evapotranspiration of volatile organic contaminants and that (b) the results of this work provide a solution for these constraints, endophyte-enhanced phytoremediation may contribute to a break-through for large-scale application of phytoremediation.

Conclusions and perspectives

The data obtained in the Section 3 of this work demonstrate that the concept of using endophytes equipped with the appropriate properties is also applicable for mixed contaminations, however, until now it was only tested on laboratory scale for Ni and toluene and for Ni and TCE. It can be assumed relatively straightforward to equip endophytes via horizontal gene transfer with the appropriate degradation pathways because many endophytic bacteria are closely related to environmental strains that carry pathways against a broad spectrum of organic contaminants on mobile DNA elements. Secondly, horizontal gene

transfer can also be used to improve heavy metal resistance of endophytic bacteria; for example, retrotransfer by broad host range plasmids such as RP4 can be used to acquire the desired heavy metal resistance (Top *et al.*, 1992). In this way, endophytes can be specifically tailored for a number of different mixed contaminations. Furthermore, it could be interesting in some cases to make use of consortia in order to be able to entirely complement the plant's metabolism. In addition, the concept of using endophytes to improve phytoremediation of mixed contaminations still has to be extended to field-scale application. Therefore, the same strategy as we applied to move endophyte-enhanced TCE phytoremediation towards field application can be used, meaning the concept should first be extended to poplar cuttings on laboratory scale before it can be tested in a field experiment.

The exploration of poplar cutting – *P. putida* W619 partnerships and the field experiments that were performed, finally led to a successful field-scale endophyte-enhanced TCE phytoremediation by *in situ* inoculation of a TCE-degrading poplar endophyte. Although monitoring one year after inoculation suggested that the establishment and enrichment, and the TCE degradation capacity of the inoculated *P. putida* W619-TCE was stable, further monitoring during the next years is needed to confirm these encouraging results. Although the obtained results were very promising, we have to be conscious of the fact that this was only a small-scale field application of endophyte-enhanced TCE phytoremediation. In order to move towards large-scale field applications, at first the strategy of inoculation needs to be optimized. Further, the concept should be extended to other tree/plant species and other contaminants, implying that other plant-associated bacteria need to be characterized and if necessary, equipped with the appropriate degradation pathways or metal resistance/sequestration systems. And even if we would succeed to manage all this, still, the application of endophyte-enhanced phytoremediation will be restricted to a limited number of contaminated sites. In case sites are contaminated with organic contaminants, only a limited plant-uptake of the contaminant and/or too phytotoxic contaminant concentrations can still hinder application of phytoremediation. In comparison with toxic metals, phytoremediation of organic contaminants is much more feasible since the time needed to phytoremediate a site contaminated with organic contaminants is in

general much shorter than for toxic metals. Phytoremediation of toxic metals, even if enhanced by plant-associated bacteria, is a slow process due to the mostly limited bioavailability of metals, the relatively low amounts of metals that are taken up by plants and the sometimes high metal concentrations present in the soil leading to phytotoxicity. Therefore, in case of metal-contaminated sites, "phytoremediation" will rather mean sustainable use and valorization of contaminated land and land management.

In addition, this concept of using endophytes to complement the plant's metabolic pathways, cannot only be applied with the purpose of remediation, but also has potential applications for protection of food chains. Germaine *et al.* (2006) demonstrated the successful use of bacterial endophytes for reducing levels of toxic herbicide residues in crop plants. Inoculation of pea plants (*Pisum sativum*) with a 2,4 dichlorophenoxyacetic acid (2,4-D)-degrading poplar endophyte resulted in an increased removal of 2,4-D from the soil; moreover, the plants did not show toxic responses and did not accumulate 2,4-D in their tissues.

Further, plant–microbe partnerships can be exploited to avoid the conflict between food and energy crops. By promoting plant growth, plant-associated bacteria can make it possible to produce biofuel feedstocks on marginal land that is not suited for agriculture. This marginal land comprises soils that either, lack nutrients, receive little rain, or have been contaminated due to previous industrial or agricultural activities. Short-term beneficial effects of plant growth-promoting microorganisms will result in improved plant establishment. These effects include accelerated root development thus resulting in better access to nutrients and water, and consequently a faster initial growth, which will allow the plants to outcompete weeds for available resources, thereby reducing the need for herbicides. Long-term beneficial effects of plant growth-promoting microorganisms might result in improved plant growth, health and survival, leading to environmentally and economically sustainable food, feed and biofuel feedstock production.

Although phytoremediation, the protection of food chains, biomass production and the increased demand for biofuel feedstocks has driven many studies on plant–microbe interactions, today's most important challenges to our environment are the result of climate changes caused by global warming, and the fate of carbon in the environment. One of the major contributing factors associated with climate change and global warming is the ever-increasing concentration of atmospheric CO_2. The increased CO_2 concentration in the atmosphere can enhance the carboxylation efficiency of Rubisco (Ceulemans and Mousseau, 1994) resulting in enhanced plant growth (Curtis and Wang, 1998), a more-than-proportional increase in C allocation to the roots (Zak *et al*., 1993; Hu *et al*., 2001), and a greater fine root production (Hungate *et al.*, 1997). The stimulated root growth has been attributed to nutrient limitation inducing plants to invest more carbohydrates into belowground growth and to release root exudates to utilize soil resources more effectively (Zak *et al.*, 2000).

Besides the direct effects that elevated CO_2-levels have on plants, indirectly, plant-mediated mechanisms caused by physiological changes will certainly impact plant-associated microbial communities. Lesaulnier *et al.* (2008) provided a detailed and deep branching profile of changes in the composition of the microbial community beneath trembling aspen (*Populus tremuloides*), induced by plant responses to elevated atmospheric CO_2 (560 ppm). Total microbial abundance did not change; however, increases in heterotrophic decomposers and ectomycorrhizal fungi were observed, supporting previously reported increases in fine root biomass turnover rates (Holmes *et al.*, 2003). Furthermore, Hu *et al.* (2001) noted that elevated CO_2 alters the interaction between plants and microbes in favor of plant N utilization, which is needed for sustaining the availability and translocation of the essential nutrients required for increased plant growth. Future investigations on the potential of asymbiotic and symbiotic root associations to sequester elevated levels of atmospheric CO_2, thus helping to counteract increased carbon emissions, should pay more attention to the impact that elevated atmospheric CO_2-levels have on the diversity of life, the complexity and functioning of microbial communities, and the cycling of essential elements.

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