DOCTORAATSPROEFSCHRIFT

2004

Faculteit Wetenschappen

Role of plant associated bacteria to improve phytoremediation of organic pollutants

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Wetenschappen, richting Biologie, te verdedigen door

Tanja Topalović Barac

Promotor: Prof. Dr. J. Vangronsveld

Co-promotor: Prof. Dr D. van der Lelie

Thank you

I have found myself in a world I would like to stay in. I have discovered what draws me ahead to continue this exciting journey of research. My doctorate is, I hope, only the beginning of everything I wish to discover.

I would like to express my special thanks to the people who made it possible for me to step into this world of research, Jaco Vangronsveld and Roland Valcke. Without their faith in me, I would not be here today.

I would also thank Jaco Vangronsveld for his scientific and human support during past four years.

Further, I would like to thank my colleagues: Chiara, Brahim, Ann R, Ann C, Wouter, Kristin, Karen, Araceli, Jan, Marc and Ludo for their readiness to hear me out and listen to different problems, for interesting discussions and their continuous support. I also would like to thank Carine, and especially Ann W, without whom I would have never succeeded achieving my goals.

Niels and Safiyh also deserve my thanks. Without their understanding and support, the most interesting and exciting parts of my research would not have happened.

I would like to thank Brigitte who kept an eye on my work and bravely encountered all the bacteria that came out of my “kitchen”.

My coworkers at the Endegrade Project: Uli, Stefan, David D, David R, Kieran, Fiona, Ed, Colin and Gunther have all been great to work with. I will remember their terrific cooperation for a long time.

I would like to send special thanks to the Dr. H. Clysters, D. Dubin, G. Vaes, H. Lemmens and R. Rooyackers from the Ford Factory in Genk for overseeing and financially supporting my work. Also I would like to thank to Ingenieur betrieb Dr. –Ing. W. Pütz for the monitoring data concerning the field experiment.

I would also like to thank my friends Tanja and Dejan for all their help and support.

A tremendous contribution to the success of my work belongs to my tragically deceased colleague and friend Licy Oeyen.

Last, but not the least, I must not forget those who suffered the most throughout my ups and downs, and who, along with me attended to the plants, even on weekends and in the middle of the night: my three sweethearts: Filip, Andrej and Zoran.

My doctorate is dedicated to the loving memory of my wonderful father Topalovic Miodrag.

Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
BSA	Bovine Serum Album
BTEX	Benzene, Toluene, Ethylbenzene, Xylene
C	Carbon
CFU	Colony Forming Units
DDT	Dichlorodiphenyltrichloroethane
DDE	Dichlorodiphenyldichloroethane
DNT	2,4-Dinitrotoluene
dNTP	Deoxyribonucleotide Triphosphate
2,4-D	2,4-dichlorophenoxyacetic acid
EDB	Ethylene Dibromide
EDTA	Ethylenediaminetetraacetic acid
EMBL	The European Molecular Biology Laboratory
GC-MS	Gas Chromatography Mass Spectrometry
Gfp	Green Fluorescent Protein
GMO	Genetically Modified Microorganisms
GST	Glutathione S-transferaze
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
HCN	Hydrogen Cyanide
Km	Kanamycin
K_{ow}	Octanol-water partitioning coefficient
MTBE	Methyl Tertiary-Butyl Ether
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
OD_{660}	Optical Density at 660nm
PAHs	Polyaromatic Hydrocarbons
PAR	Photosynthetically active radiation

PCA	Plant count agar
PCBs	Polychlorinated biphenyl
PCR	Polymerase Chain Reaction
PGPB	Plant Growth Promoting Bacteria
pK_a	Acidity constant
RCF	Root Concentration Factor
REP	Repetitive Extragenic Palindromic
SA	Sucrose aspargine
SDS	Sodium Dodecyl Sulphate
TCA	Trichloro Acetic Acid
TCE	Trichloroethylene
TPH	Total Petroleum Hydrocarbons
TSCF	Transpiration Stream Concentration Factor
TNT	2,4,6-Trinitrotoluene
Tris	Tris (hydroxylmethyl)-aminoethane
Triton X-100	Octyl Phenol Ethoxylate
VOC’s	Volatile Organic Compounds
PGPB	Plant Growth Promoting Bacteria

Table of contents

Summary

Chapter I Introduction

Chapter II Aim and outline of the thesis

Chapter III Prof of the concept: engineered endophytic bacteria improve phytoremediation of water-soluble, volatile organic pollutants

Chapter IV Potential of poplar cell cultures to degrade toluene

Chapter V Isolation, characterization and identification of endophytic and rhizosphere bacteria from *Populus x trichocarpa* cultivars "Hoogvorst" and "Hazendans"

Chapter VI Re-colonization of poplar cuttings by *gfp* expressing endophytic bacteria

Chapter VII The potential use of poplar cuttings, inoculated with endophytic bacteria possessing pTOM, for improved phytoremediation of toluene

Chapter VIII Application of poplar trees and their associated microorganisms for the in situ remediation of a BTEX contaminated groundwater plume

Chapter IX General discussion and perspectives

Bibliography

References

SUMMARY

Phytoremediation, the use of plants for the *in situ* treatment of contaminated soils and sediments, is an emerging technology that promises effective and inexpensive clean-up of certain contaminated sites. Phytoremediation is most suited for sites with shallow contamination (<5m depth), moderately hydrophobic pollutants such BTEX compounds, chlorinated solvents, nitrotoluene ammunition wastes or excess nutrients (nitrate, ammonium and phosphate) (Schnoor *et al.*, 1995). Although many organic pollutants are metabolized in plants, xenobiotics or their metabolites can be toxic to plants, and this could limit the applicability of phytoremediation. Alternatively, in case of volatile pollutants, plants can release the compounds or their metabolites, through the stomata, which could question the merits of phytoremediation (Schwitzguebél *et al.*, 2002).

A possible solution for these problems could be the use of endophytic bacteria, equipped with the necessary degradation pathway(s), which reside within living plant tissue, without doing substantive harm to the plant.

Yellow lupine (*Lupinus luteus* L.) and their associated endophytic bacteria (*Burkholderia cepacia*), equipped with a toluene degradation pathway, were used as a laboratory and greenhouse test species. Toluene was chosen as a model of a highly water soluble and volatile organic compounds. Inoculation with the endophytic bacterial strain *B. cepacia* VM1330 resulted in a drastic decrease of toluene phytotoxicity and a 3-4 times reduction of toluene evapotranspiration through the leaves.

Poplar cells cultures were used to demonstrate that poplar cells do not posses the capability for toluene degradation. Those results supported our hypothesis that for successful phytoremediation collaboration between plants (in our case poplar trees) and their associated microorganisms, with appropriate degradation pathways is necessary.

Subsequently, two poplar cultivars (*Populus trichocarpa* x *deltoides* cv. “Hoogvorst” and “Hazendans”) have been studied as representative model plants for phytoremediation in the field, with emphasis on the role of their associated bacteria. Plant material was prepared aseptically and the natural rhizosphere and endophytic bacterial communities were characterised and identified by comparative sequence analysis of 16S rDNA. One hundred and forty six morphologically, cultivable distinct isolates were obtained, belonging to twenty one different genera. The most dominant endophytic strains were chosen for further investigations.

Re-colonization capability of four different natural endophytic strains, all belonging to *Pseudomonas* genus, was studied showing that three of them could colonize poplar rhizosphere and roots, two were active colonizers of stem and only one could colonize the leaves.

Furthermore, poplar cuttings were inoculated with a chosen endophytic strain (*B. cepacia* VM1468) equipped, via natural gene transfer, with the TOM plasmid, which is able to degrade toluene. The effect of a 10 weeks application of toluene on the growth of poplar cuttings, inoculated with *B. cepacia* VM1468, was studied. Toluene evapotraspiration from the aerial parts of the poplar cuttings was investigated. Horizontal transfer of pTOM between introduced strain and naturally occurring endophytic bacteria in poplar cuttings was observed in this experiment. These bacteria were capable to assist their host plant in overcoming toluene toxicity, and at the same time lowered toluene phyto-volatilization.

The effect of poplar trees and their associated bacteria was monitored on a field site contaminated with BTEX compounds. During five years of study it was observed that poplar trees, together with their associated rhizosphere and endophytic bacteria, can play an active role in the phytoremediation process. After reaching the groundwater with their roots they stopped further dispersion of the pollutants plume.

Fytoremediatie of het gebruik van planten om vervuilde bodems en sedimenten *in situ* te bekantelen, is een beloftevolle technologie om effectief en goedkoop bepaalde vervuilde sites aan te pakken. Fytoremediatie kan het best gebruikt worden op plaatsen met een ondiepe contaminatie (<5m diepte), vervuild met matig hydrofobe polluenten zoals BTEX componenten, gechloreerde solventen, nitrotolueen akomstig van munitie-afval en verhoogde concentraties aan nutriënten (nitraten, ammonium en fosfaten) (Schnoor *et al.*, 1995). Hoewel vele organische polluenten kunnen gemetaboliseerd worden door planten, kunnen xenobiotica of hun afgeleide metabolieten toxisch zijn voor planten, wat de toepasbaarheid van fytoremediatie kan beperken. Anderzijds kunnen planten deze vluchtige componenten of hun metabolieten vrijstellen door de huidmondjes, waardoor het nut van fytoremediatie in vraag kan gesteld worden (Schwitzguebél *et al.*, 2002).

Een mogelijke oplossing voor deze problemen kan het gebruik van endofytische bacteriën zijn. Deze kunnen enerzijds uitgerust zijn met de nodige afbraakroutes en anderzijds in de plant verblijven zonder deze schade te berokkenen.

De gele lupine (*Lupinus luteus* L.) en zijn endofytische bacterie *Burkholderia cepacia*, uitgerust met een tolueen afbraakroute, werd gebruikt als testorganisme in het laboratorium en in de serre. Tolueen werd gekozen als voorbeeld van een polluent met twee belangrijke eigenschappen. Enerzijds heeft het een hoge wateroplosbaarheid, en anderzijds is het een vluchtige organische component. Inoculatie met de endofytische bacteriestam *B. cepacia* VM1330 resulteerde in een zeer sterke afname van de fytotoxiciteit van tolueen alsook een 3-4 maal reductie van de tolueen evapotranspiratie via de bladeren.

Gebruik van celculturen van populier toonde aan dat deze cellen niet de mogelijkheid hebben om tolueen af te breken. Deze resultaten steunen onze hypothese dat een efficiënte fytoremediatie, de samenwerking vereist tussen

planten (in dit geval populieren) en hun geassocieerde micro-organismen, uitgerust met de nodige afbraakroutes.

Vervolgens werden twee populier cultivars (*Populus trichocarpa* x *deltoides* cv. “Hoogvorst” and “Hazendans”) onderzocht als representatieve modelplanten voor fytoremediatie in het veld, met de nadruk op de rol van hun geassocieerde bacteriën. Het plantenmateriaal werd aseptisch voorbereid en de natuurlijke rhizosfeer- en endofytische bacteriële gemeenschappen werden gekarakteriseerd en geïdentificeerd met behulp van vergelijkende sequentie-analyse van het 16S rDNA. Honderdvierenzestig morfologisch verschillende, cultiveerbare isolaten werden bekomen, behorende tot 21 verschillende genera. De dominantste endofytische stammen werden gebruikt voor verder onderzoek.

De mogelijkheid tot re-kolonizatie van vier verschillende natuurlijke endofytische stammen, allemaal behorend tot het *Pseudomonas* genus, werd bestudeerd. Er werd aangetoond dat drie van hen de rhizosfeer alsook de wortels van populieren konden koloniseren, waarvan twee eveneens de stengel actief konden koloniseren en slechts één ervan de bladeren kon koloniseren.

In een verdere studie, werden populierstekken geïnoculeerd met een gekozen endofytische stam (*B. cepacia* VM1468), die uitgerust werd, via natuurlijke gentransfer, met het TOM plasmide, dat in staat is tolueen af te breken. Gedurende 10 weken werd tolueen toegediend aan populierstekken, geïnoculeerd met *B. cepacia* VM1468, waarna het effect op de groei van deze populierstekken werd bestudeerd. Horizontale transfer van het pTOM plasmide tussen de geïntroduceerde stam en de natuurlijke voorkomende endofytische bacteriën in populierstekken werd geobserveerd in dit experiment. Deze bacteriën konden de gastheerplant beschermen tegen tolueentoxiciteit en tegelijkertijd verlaagde ze de fytovolatilizatie van tolueen.

Het effect van populieraanplanten en hun geassocieerde bacteriën werd opgevolgd in een veldstudie op een site gecontamineerd met BTEX componenten. In deze studie reeds vijf jaar lopende werd waargenomen dat de populieren,

samen met hun geassocieerde rhizosfeer- en endofytische bacteriën, een actieve rol kunnen spelen in het fytoremediatieproces. Vanaf het stadium dat de wortels de grondwatertafel hadden bereikt werd een verdere verspreiding van de vervuilingspluim tegengehouden.

CHAPTER I
INTRODUCTION

1. PHYTOREMEDIATION

Remediation options currently applicable to contaminated soils and ground water are frequently expensive, environmentally invasive and do not make cost-effective uses of existing biological resources. As a result, they can not be applied for the treatment of large contaminated areas with diffuse pollution problems. The conventional technologies for soil remediation are based on physical and chemical approaches, both of which can be used *in situ* and *ex situ* (Table 1.1). These techniques rely upon civil engineering methodologies, involving either 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 (Vangronsveld and van der Lelie, 2003). Some of these treatment methods possess the risk of further dispersing contaminants into the environment. For instance, incineration can result in air pollution, leachates from landfills in the form of water and gases can reach ground water and drinking water wells, whereas excavation of soil can lead to the generation of toxic air emissions (Kuiper *et al.*, 2004). Landfilling also simply moves the contaminated soil from one location to another, and requires replacement soil to fill in the excavated site.

Method	**In situ**	**Ex situ**
Physical	Soil vapor extraction Thermally enhanced soil vapor extraction Containment systems and barriers	Landfilling Incineration Thermal desorption Soil vapor extraction
Chemical	Soil flushing Solidification Stabilization	Soil washing Solidification Stabilization Dehalogenation Solvent extraction Chemical reduction and oxidation

Table1.1: Classic soil remediation technologies (source: European Institute for Environmental Education and Training)

The choice of the remediation technology depends on the degree and the impact of the pollution, the future land use of the contaminated site, its economic value, and other economic, political and social issues. As a complement to traditional methods, especially for sites with diffuse, low pollution levels or with large volumes of contaminated water containing relatively low pollutant concentrations, the currently emerging phytoremediation technologies might provide a cost effective remediation approach (Schröder *et al.*, 2002).

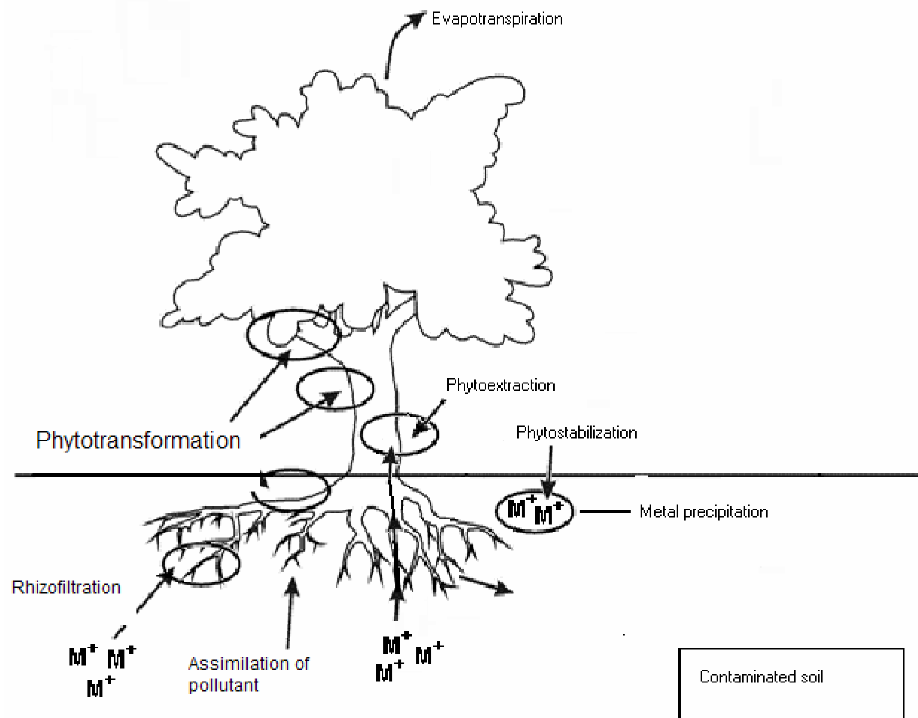


Figure 1.1: Schematic presentation of phytoremediation process

Phytoremediation is the use of living plants and their associated microorganisms for *on site* remediation of contaminated soil, sludge, sediments and groundwater primarily through stabilization, degradation or removal of the contaminants. More specifically, the term phytoremediation or phytotechnology encompasses application of plants for hydraulic control, degradation, sorption, translocation, transpiration, and rhizosphere-enhanced degradation of contaminants. The term also captures vegetative landfill caps, constructed treatment wetlands and the restoration / revegetation of damaged mine lands (EPA, 1998; Flathman and Lanza, 1998). Phytoremediation combines the Greek word *phyton*, “plant”, with the Latin word *remediare*, “to remediate”, “to correct or remove an evil”. The

basic idea that plants can be used for environmental remediation is very old and cannot be traced to any particular source (Salt *et al.*, 1998). Cunningham and Berti (1993) first used the term phytoremediation in the open technical literature, noting, however, that the use of plants in land farming of wastewaters has been occurring for at least 300 years (McCutcheon *et al*., 2003).

The contaminants that potentially can be managed using phytoremediation are quite diverse, including heavy metals, metalloids, radionuclides, salts, nutrients, xenobiotic organic chemicals, sewage and air pollutants (McCutcheon *et al.,* 2003). All require that the appropriate plant species is available that can tolerate the levels of contaminant present in the polluted environment.

Phytoremediation is an emerging technology that should be considered for remediation of contaminated sites because of its cost effectiveness, aesthetic advantages, and long-term applicability (Glick, 2003). Phytoremediation is well suited for use at extended field sites with diffuse contamination where other remediation methods, are not cost-effective or practicable. Use of phytoremediation is suited for sites with low concentrations of contaminants where only “polishing treatment” is required over long period of time and in conjunction with technologies where vegetation is used as a final cap and closure of the site (Dietz *et al.*, 2001). Plants used in phytoremediation are redefined as “solar-driven pumping and filtering systems” (Cunningham *et al.*, 1995). Sometimes, the phytoremediation sites can serve as wildlife sanctuary, nature parks or recreational area. In some cases the site can serve a valuable economic purpose.

Phytoremediation technology has some limitations and is not applicable for treatment of all contaminated sites. Although the cost associated with phytoremediation is generally lower than conventional methods, labor is required to oversee and implement planting, maintenance (water, fertilizer, pest and weed control) and sometimes harvest and disposal of the plants. Soil texture, water regime, pH, salinity and pollutant concentrations must be within the limits of

plant tolerance (Cunningham *et al.*, 1995). Bioavailability and biodegradation of contaminants are also very important in the phytoremediation process. As with all biological remediation processes, only the bioavailable fraction of the contaminant can be treated, while only those contaminants will be degraded for which the proper biodegradation mechanisms exist. The success of phytoremediation may be seasonal, depending on location, as the plant's activity will drop once the plant loses its leaves. This has e.g. to be considered when phytoremediation is being used to contain a contaminated groundwater plume. Other factors, such as the climate, will also influence its effectiveness of phytoremediation. Some forms of phytoremediation transfers contamination across media, e.g., from soil to air, as is the case for phytoremediation of selenium or volatile organic solvents. Phytoremediation is not effective for strongly sorbed contaminants such as PCBs, which will only move very slowly to the root zone where their degradation can occur. Also animals may damage the plants as they feed on them, creating need to replant, as well as the potential risk of transferring contaminants to the food chain.
However phytoremediation remains a promising technology for the remediation of many polluted sites with diffuse forms of contamination that are present in the soil within the reach of the plant root system.

Depending on the type of pollution, different phytoremediation strategies are available to treat contaminated soil, water and even air. These strategies fall into the following categories:

1. Phytoextraction is the use of plants for the uptake and transport of metals or organics contaminants from the soil into the roots and aboveground plant biomass, which can subsequently be harvested with conventional agricultural methods. Most plants that grow on metal contaminated soils can, to some extent, accumulate and store heavy metals in their roots and shoots. However, some

plants species, referred to as excluders, have developed strategies to exclude heavy metals from uptake, and are thus not suitable for phytoextraction (although they can be used for revegetation purposes). Metal hyperaccumulators are plants that can actively accumulate and concentrate heavy metals in their shoots to levels of at least 0.1% metal/dry biomass (100-1000 times more then normally accumulated by plants). These hyperaccumulators can be used to reduce certain levels of contamination to levels that are environmentally acceptable and meet regulatory requirements. The list of hyperacumulating plants is expanding rapidly and the largest numbers collected in the temperate zone belong to the *Brassicaceae* while in the tropic regions the *Euphorbiaceae* are the best represented (Baker and Brooks, 1989) The largest number of them hyperacumulate Ni, followed by Zn. Cd, Pb and even Tl. Recently, a hyperaccumulator fern was discovered for As (Ma *et al.*, 2001). Table 1.2 shows some hyperaccumulators and their shoot metal concentration. For a complete overview see Baker and Brooks, 1989; Reeves and Baker, 2000.

Heavy metal	**Plant species**	**Concentration in shoots (per dry weight)**
Cd	*Thlaspi caerulescens*	2130 mg/kg dry weight (0.2%)
Zn	*Thlaspi caerulescens*	43710 mg/kg dry weight (4.3%)
Cu	*Ipomoea alpina*	12300 mg/kg dry weight (1.23%)
Co	*Haumaniastrum robertii*	10232 mg/kg dry weight (1%)
Pb	*Thlaspi rotundifolium*	8200 mg/kg dry weight (0.8%)
Mn	*Vaccinium myrtillus*	5270 mg/kg dry weight (0.5%)
Ni	*Psychiotria dauarrei*	19990 mg/kg dry weight (1.9%)
As	*Pteris vittata*	4980 mg/kg dry weight (0.5%)

Table 1.2: Hyperaccumulator species and their shoot metal content (Reeves and Baker, 2000; Ma *et al.*, 2001)

Extensive research has been performed on the mechanisms of hyperaccumulation within the family of the *Brasicaceae*. One member of this family that has been

thoroughly studied for its Zn hyperaccumulation phenotype is *Thlaspi caerulescens* J&C Presl. As certain populations of this species have been shown to accumulate and tolerate up to 40000 µg/g Zn in their shoots (Chaney, 1993), it was considered as a very interesting experimental system for studying mechanisms of hyperaccumulation.

A major advantage of phytoextraction over classical remediation methods is that it removes hazardous compounds from soil without destroying the soil structure and with a limited impact on soil fertility (Dushenkov, 2003). However, a disadvantage of the method is that it can take decades before metal contamination will be reduced to acceptable levels. Therefore the acceptance of phytoextraction and any other phytoremediation based remediation strategies strongly depends on the possibilities to make it part of a general land management strategy.

2. Phytodegradation is the use of plants and associated microorganisms to degrade organic pollutants into simple molecules and incorporation of these molecules into plant tissue (Burken, 2002). Munitions (TNT, DNT, nitrotolene, nitrobenzene), atrazine, halogenated compounds (tetrachloromethane, trichloromethane, hexachloroethane, carbon tetrachloride, TCE), DDT and other chlorine and phosphorus based pesticides and phenols can be degraded in phytodegradation processes.

Often, organics are partially metabolised and/or modified before being stored into vacuoles. Glutathione and glutathione S-transferase isoenzyme family (GST) play crucial roles in the degradation of several herbicides (Gullner *et al.*, 2001). GSTs are able to catalyse conjugation reactions between a number of xenobiotics and glutathione. The herbicide-glutathione conjugates are generally much less toxic and more water-soluble than the original herbicide molecules (Edwards *et al.*, 2000). Phytodegradation will be described in detail in the next section, on phytoremediation of organic pollutants (section 1.1).

3. Phytostabilization is the use of plants to reduce the bioavailability of pollutants in the environment, this with the aim to prevent secondary contamination and exposure. Phytostabilization techniques are most appropriate for relatively immobile materials and large surface areas, and may work better with heavier textured soils. Phytostabilization can provide a dense vegetative ground cover which can greatly reduce soil erosion and human exposure to contaminants via dermal contact and inhalation (Cunningham *et al.*, 1997). Many organic contaminants are lipophilic and have a high affinity for the hydrophobic surfaces of organic mater. Such compounds may bind to living or dead plant root tissue and become immobilized, where they are thought to be less bioavailable, less mobile and less toxic than the free species (Chard *et al.*, 2001). Phytostabilization is a workable strategy for phenols, chlorinated solvents (tetrachloromethane and trichloromethane), hydrophobic organic compounds and even heavy metals (EPA 2000) which can be complexed in the root zone. Also, by consuming rain water plants will reduce leaching of heavy metals to the groundwater, while a vegetation cover can reduce or fully stop erosion, including wind erosion and erosion by runoff water (Vangronsveld and Cunningham, 1998). Successful phytostabilization was carried out on metalliferous mine wastes in the UK, where local metal tolerant plant species (*Agrostis tenuis* cv. Parys for copper waste, *Agrostis tenuis* cv. Goginan for acid lead and zinc wastes and *Festuca rubra* cv. Merlin for calcareous lead and zinc wastes) were used to stabilise the site and establish an excellent vegetation cover (Smith and Bradshow, 1979). Also on the highly toxic bare soil of and old zinc smelter site in Belgium, phytomobilization was proven to be very successful (Vangronsveld *et al.,* 1995, 1996).

4. Phytovolatilization is the use of plants to volatilize pollutants (Terry *et al.*, 1995). Many water soluble organic contaminants are readily taken up by the plant and transported to their above ground parts. However, some of these contaminants (Trapp *et al.*, 1994) are not easily degraded, resulting in the

accumulation of toxic compounds or their degradation intermediates. In some cases the plants might release the contaminants in the environment via volatilisation through stomata of the leaves or lenticels of the stem. Some recalcitrant organic compounds are more easily degraded in the atmosphere (photodegradation). Chemicals as chlorinated solvents (TCE, tetrachloromethane and trichloromethane), organic VOC's, BTEX, MTBE, EDB can be treated via phytovolatilization. Mercury is the primary metal contaminant that this process has been used for (Pilan-Smits and Pilan, 2000). The advantage of this method is that the mercuric ion [Hg(II)], may be transformed into its less elemental metallic form, Hg(0), which is volatile. The disadvantage to this approach is that mercury, after being released into the atmosphere, is likely to be recycled by precipitation and then redeposited back into lakes and oceans, repeating the production of methyl-mercury by anaerobic bacteria (USEPA, 2000). Plants also have the ability to absorb and sequester selenium and to convert inorganic selenium to volatile forms of organic compounds that are released harmlessly into the atmosphere (Banuelos *et al.*, 2002).

5. Rhizofiltration is the use of plant roots to precipitate and concentrate pollutants, mainly metals, from water and aqueous waste streams. Rhizofiltration using sunflower has been used in the remediation of radionuclides (strontium ^{90}Sr and caesium ^{137}Cs) from surface water near Chernobyl (International Groundwater technology). Hydrophobic organic chemicals, that tend to bind to the root biomass, can also be concentrated by rhizofiltration processes.

6. Rhizodegradation aims at stimulating the degradation of organic contaminants in the plant rhizosphere. Plants can stimulate the activity of their associated rhizosphere communities by secreting exudates (such as phenolic compounds, organic acids, alcohols...) from their roots, which can serve as carbon and nitrogen sources for growth, metabolic activities, and long-term

survival of diverse fungal and bacterial communities in the rhizosphere (Alkorta and Garbisu, 2001). Root necrosis and other processes provide a rich source of organic carbon and nutrients to stimulate growth of soil bacteria. In addition to providing nutrients, plants will also affect the local redox conditions in the rhizosphere: living roots can provide zones with higher oxygen levels that will stimulate the activity to aerobic bacteria, while the presence of abundant carbons sources such as dead root material will rapidly deplete oxygen levels, thus supporting anaerobes. Due to stimulation by the plant through diverse mechanisms, the density, diversity and activity of rhizosphere microorganisms is much greater than the microbial populations in the surrounding bulk soil (Anderson *et al.,* 1994). Some rhizosphere microorganisms are capable of degrading organic pollutants, and can be stimulated in their activity by the plant through secretion of specific exudates. For instance, plant phenolics such as catechin and coumarin may serve as co-metabolites for PCB-degrading bacteria (Bedard *et al.,* 1987). In addition to secreting organic compounds that support the growth and activities of rhizospheric microorganisms, plants also release a number of enzymes (laccases, dehalogenases, nitroreductases, nitrilases and peroxidases) into soil and water and these enzymes can degrade organic contaminants (Boyajian and Carreira, 1997; Wenzel *et al.*, 1999). Overall, rhizodegradation is an important strategy to obtain phytoremediation of organic contaminants. It is only limited by the presence of appropriate degradation pathway in the microbial rhizosphere community, and the retention time of the contaminant in the rhizosphere before it is taken up by the plant. Rhizodegradation is extremely valuable for the efficient degradation of soils contaminated with biodegradable organics such as BTEX, PAH’s, PCB’s, TPH and pesticides.

7. **The use of plants to remove pollutants from air**: the aerial parts of a plant, including the foliage, are covered by a cuticle, which acts as a barrier to reduce

water loss from the plant and also serves to retard or prevent penetration of foliar applied chemicals or environmental pollutants. The leaf surface contains small pores, or stomata, which open and close according to environmental conditions. The stomata provide an entry for carbon dioxide and other atmospheric gases required by the plant and an exit for the transpiration stream and O_2. Chemicals sprayed or deposited on leaves may partition into the cuticle, where they often bind tightly, but from which they may eventually be translocated to other plant parts, or diffuse into the plant through the stomata. It is likely that chemicals possessing a high water solubility are more readily transported through the plant via the phloem (the stream which carries nutrients and organic matter form the leaves to the stem and roots), while those that are more hydrophobic tend to be sorbed to and remain in the waxy cuticle of the leaves (Paterson *et al.*, 1990). A number of chemicals have been found to be accumulated via the aerial-foliar route: PCBs (Bacci and Gaggi, 1985), HCH, HCB, DDT (Bacci and Gaggi, 1986). The use of plants to remove pollutants form air is described as the “green lung” concept.

1.1 Phytoremediation of organic compounds

Soil and water contaminated with different organic xenobiotics pose a major thread for the environment and human health, and thus requires an effective and affordable technological solution. Phytoremediation offers advantages of stabilizing contaminated soil, slowing contaminant leaching into the groundwater and reducing wind and water erosion of soil (Anderson *et al.*, 1993). As indicated above plants are able to remediate organic compounds via three mechanisms (Schnoor *et al.*, 1995):

1. Direct uptake of contaminants and subsequent degradation, modification and/or accumulation of non-phytotoxic metabolites into plant tissue, or evapotranspiration of those contaminants.

2. Release of exudates and enzymes in the rhizosphere that stimulate microbial activity and biochemical transformations.

3. Overall enhancement of mineralization in the rhizosphere, which can be attributed to increased densities of mycorrhizal fungi and/or microbial consortia.

Chemicals may enter plants through the root system from the soil and its component soil solution, or from the atmosphere through aerial plant parts such as foliage, fruit, stem or bark.

Root uptake of organic compounds from soil and groundwater is affected by three factors:

1. environmental conditions
2. the physicochemical properties of the compound, and
3. plant properties

1.1.1 Environmental conditions

The bioavailability of organic contaminants for plant uptake is primarily under control of environmental soil factors such as organic and mineral matter content, pH, temperature and moisture. Climatic conditions determine plant transpirations rates, which in turn control rates of water movement to the root surface and in the xylem. The use of synthetic (Triton X-100, SDS) and naturally produced biosurfactants (rhamnolipids) to enhance the apparent water solubility and bacterial degradation of organic contaminants is well documented (Bragg *et al.*, 1994; Desai and Banat, 1997).

1.1.2 Physicochemical properties affecting plant uptake of organic pollutants

Physicochemical properties of the compound such as its water solubility, vapour pressure, molecular weight and octanol/water partition coefficient are important for plant uptake of organic xenobiotics. Organic chemicals may sorb to roots and be taken up, translocated, metabolised, or transpired (volatilised) by plants. The first step is sorption to roots. When chemical contaminants in soil, water or groundwater come into contact with roots, they may sorb or bind to the root structure and cell walls. Hemicellulose in the cell wall and the lipid bilayer of plant membranes can bind hydrophobic organic chemicals efficiently. Such sorption should be relatively reversible and can be measured using standard sorption isotherms (Dietz and Schnoor, 2001) A major criterion in assessing the probability that a target chemical will be taken up by plants is its lipophilicity. Lipophilicity is the most important property of a chemical in determining its movement into and within a plant. Lipophilicity is related to the partition coefficient of the pollutant between 1-octanol and water (K_{ow}). The K_{ow} is one of the most widely available experimental parameters for xenobiotics (Cunningham *et al.*, 1996). Chemicals most likely to be taken up by plant are moderately hydrophobic compounds with octanol-water partition coefficients ranging form 0.5-3.5 (Salt *et al.,* 1998; Trapp *et al.*, 1990). With a few notable exceptions (i.e. uptake of some polyaromatic hydrocarbons (PAHs) and herbicides from the vapor phase), movement of organics into plants occurs via the liquid phase.

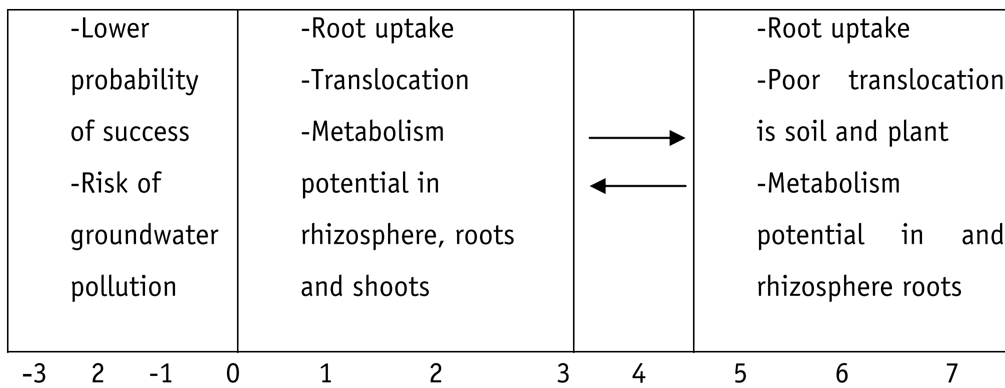


Figure 1.2: The role of the octanol-water partitioning coefficient (K_{ow}) in phytoremediation of soil organics

The relative ability of a plant to take up a chemical from the soil or groundwater and translocate it in its shoots is described by the Root Concentration Factor (RCF) and Transpiration Stream Concentration Factor (TSCF) for the chemical. Higher RCF and TSCF values are an indication of enhanced contaminant uptake by plants.

Both factors RCF and TSCF vary directly with the Partition coefficient P_{ow} (or K_{ow}) of the chemical; contaminants in solution with the highest RCF and TSCF posses a log K_{ow} of 1 to 3.5.

Contaminants with a log K_{ow}>3.5, that are highly hydrophobic, such as PCBs and DDT, show high sorption to the roots, but slow or no translocation to the stems and leaves (Trapp *et al*, 2001). They are bound to lipids in membrane organelles and cell walls (Paterson *et al.*, 1990). For example a log K_{ow} for dioxin is 6.14, for PCBs 4.12-6.11, for PAHs 4.07-7.66; In table 1.3 log K_{ow} values for different contaminants are presented.

Compounds with a log K_{ow} <1 cannot penetrate the lipid containing root epidermis; they are considered to be very water-soluble, and plant roots do not generally accumulate them at a rate surpassing passive influx into the transpiration stream (Cunningham and Berti, 1993; Collins *et al.*, 2002).

However, there are few exceptions, suggesting that not all plants use a passive uptake process for lipophilic compounds (Hulster *et al.*, 1994). In addition, Aitchison *et al*. (2000) showed that the very water-soluble compound 1,4-dioxane, with a log K_{ow} value of -0.27, was rapidly taken up and translocated by hybrid poplar cuttings, suggesting uptake via hydrogen bonding with water molecules into the transpiration stream.

2-Butanone	0.3	3-Chlorobenzoic acid	2.7
4-Acetylpyridine	0.5	Toluene	2.7
Aniline	0.9	1-Naphthol	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	Naphthalene	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	Phenathrene	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		

Table 1.3: log K_{ow} values for different contaminants

The transpiration stream concentration factor TSCF has been developed to predict the behaviour of a chemical compound in plants, and is based upon its log K_{ow}. The TSCF relates the concentration of the compound in the transpiration stream (xylem) to the exposure concentration (Briggs *et al.*, 1982) and is determined as following:

TSCF= µg compound per ml H_2O transpired / µg compound per ml ambient solution

A TSCF of 1.0 indicates unrestricted passive uptake of the compound into the plant. TSCF values lower than 1.0 indicates exclusion (restricted passive uptake) of the compound by the plant, while TSCF values greater than 1.0 infer active uptake.

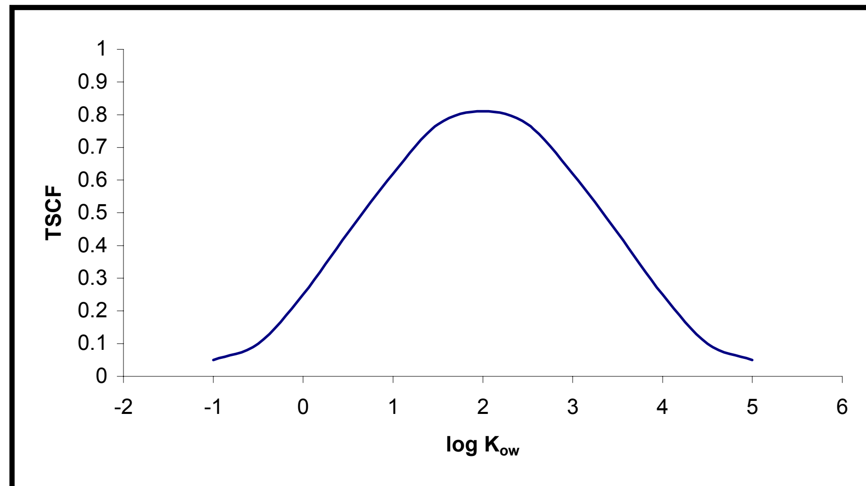


Figure 1.3: Graphic relationship between TCSF and Log K_{ow} in poplar trees, at 20°C (Schnoor *et al.*, 1995)

1.1.3 Plant properties affecting plant uptake of contaminants

Plant characteristics such as root surface area can substantially alter absorption of contaminants. Surface area may be increased in plants with an extensive root system, or in those with a high number of fine root hairs. As water mediates the transfer of solutes to the root, a plant characteristic that affects evapotranspiration could also influence the potential for uptake of organic contaminants (Cunningham *et al.,* 1997). Root lipid content is also important for contaminant uptake by plants (Chard *et al.*, 2001).

Water is absorbed from soil solution into the cortex or outer tissue of the root, which contain the apparent free space, occupying 10-20% of the root volume. As

it moves to the centre of the root, it encounters the endodermis, which contains the water impermeable Casparian strips, impregnated with suberin. To reach the xylem, the principal vessel for conducting water and solution upwards from the root, chemicals must pass the Casparian strips into the endodermis and hence to the tracheids and vessels of the vascular tissue. This movement through the endodermis is dependent on the chemical polarity and molecular configuration of the chemical. Organic pollutants may become sorbed, bound or metabolised in the endodermis before reaching the xylem. Chemicals that reach the xylem are transported throughout the plant via the transpiration stream or sap. They may also be transported in conjugated forms. For example, benzene is readily taken up, translocated to the leaves and transpired trough the leaves. Toluene, ethylbenzene, m-xylene and TCE can be also transpired throughout the plant, but their release time is longer than found for benzene (Burken and Schnoor, 1998).

Degradation of organic contaminants

Following plant uptake, organic contaminants may be metabolically transformed. Generally, plants enzymatically oxidize, reduce or hydrolyse organic compounds. The products of these transformation reactions are covalently attached (conjugated) to water soluble moieties and the conjugated compounds are removed from the cytoplasm, either by transport into vacuoles or by conversion into insoluble complexes that are frequently covalently bound to the cell wall matrix (lignification). Certain reports suggest that mineralization of some organic chemicals within plant tissues may be possible. Work with axenic cultures of *Myriophyllum spicatum* has shown that TNT is transformed reductively by intracellular activity to low levels of aminated nitrotoluenes and other soluble but unidentified metabolites through mineralization (Hughes *et al.*, 1997). Di- and trichlorinated biphenyls were metabolized via hydroxylation and glycosylation by the action of mixed function oxidases in rose cell cultures (Lee and Fletcher, 1992; Butler *et al.*, 1992). However, mineralization would be

extremely difficult to measure in whole plant systems because the CO_2 resulting from mineralization, would be assimilated by the plant during photosynthesis. The results of several studies indicate that PAHs can be degraded directly by plants. Edwards (1988) documented the metabolism of ^{14}C anthracene and ^{14}C benz(a)anthracene in bush bean grown in a nutrient solution containing the two PAHs. Edvards *et al*. (1982) also reported that soybean was capable of degrading ^{14}C-anthracene. Evidence of the degradation was given by measuring the $^{14}CO_2$ given off from plants placed in ^{14}C-anthracene contaminated soil.

Once an organic chemical is taken up and translocated, it undergoes one or more phases of transformation and detoxification (Lamoureux *et al.*, 1986; Trapp and McFarlane, 1994; Marss, 1996; Coleman *et al.*, 1997; Ohkawa *et al.*, 1999):

-Phase I- Conversion or chemical modification by the introduction of functional groups such as –OH, -NH_2 or –SH, which can occur via oxidation, reduction or hydrolysis reactions. Plant enzymes that typically catalyse hydrolysis reactions are esterases and amidases, but the major conversion reactions that take place are oxidations reactions catalysed by the cytochrome P450 system. The P-450 cytochromes are five-liganded hemo-proteins located in the endoplasmic reticulum, and they exist as many isoforms (in *Arabidopsis* more than 60 genes have been identified) (Bolwell *et al.*, 1994). Because the reactions in phase I are activation reactions, making the compound more prone to chemical transformation, these reactions do not always result in products with a decreased phytotoxicity; in some cases the metabolite is as toxic as the parent compound, and in others even a considerable increase in toxicity occurs (Coleman *et al.,* 1997).

-Phase II. – Xenobiotic or a phase I-activated metabolite will subsequently conjugate with glutathione, D-glucose, malonate or amino acids. This results in the formation of soluble, polar compounds, which have in general a decreased toxicity compared to their parental compound. These phase II reactions are

catalysed by glucosyl-, malonyl- and glutathione-transferases. The inactive, water soluble conjugates formed in this phase are exported from the cytosol by membrane-located transport proteins, which initiate phase III of the detoxification process. In addition to active translocation processes, insoluble conjugates can also be formed with cell wall components, this either through non-selective reactions with free radicals used in lignin synthesis or by more selective incorporation into hemicellulose.

-Phase III – Compartmentation: plants do not have a way to excrete unwanted compounds so conjugates from phase II are deposited in plant vacuoles or bound to cell wall and lignin complexes.

Sometimes, transformation of contaminants by the plant may cause additional problems due to the production of toxic metabolites (Siciliano and Germida, 1998). For example, a number of hydroxylated intermediates can be found during PCBs degradation, which may play role in a temporal increased toxicity, mutatoxicity and genotoxicity (Wilken *et al.*, 1995; Meier *et al.*, 1997). Plant can transform TCE into TCA (trichloro-acetate), which is toxic to the plant and tends to accumulate (Doucete *et al.*, 1998). Therefore a need exists to improve the metabolic properties of plants for certain classes of organic xenobiotics.

1.2 Poplar model species for phytoremediation of organic contaminants

Hybrid poplars can grow under a wide variety of conditions. These fast growing trees have been adapted to a number of uses including stream bank restoration, filtration of agricultural runoff, decontamination of polluted soil and ground water, cleaning municipal wastewater, biomass for bioenergy production, fixation of CO_2 and providing an alternative source of wood fibre for the wood products

industry. Homeowners are using hybrid poplars for windbreaks, privacy screens and firewood.

More than 30 species of poplar encompass their worldwide distribution. Different species can be found growing in a variety of habitats and locations. In addition, most poplar species seem to be inherently variable and different ecotypes can vary greatly in morphological and physiological characteristics. Besides the variability within species, cross-ability among many of the species is very high, allowing the creation of simple and complex hybrids. The use of hybrids potentially allows desirable traits from different species to be combined, and hybrids may also demonstrate heterosis (hybrid vigor), which surpasses the parent species in one or more measurable characteristics. Many of the poplar species and hybrids are also easy to propagate vegetatively; desirable clones can be easily multiplied to create large, uniform stands through the use of cuttings. Large trees can be grown on cleared land in a relatively short period of time, and specific clones can be found, which are adapted to many different soil and climate types.

Hybrid poplars are deciduous hardwood trees created by hybridising or crossing various poplar species. Native species used to create fast growing hybrids are:

1. *Populus deltoides* – Eastern Cottonwood
2. *Populus nigra* – European Black Poplar
3. *Populus trichocarpa* – Western Black Cottonwood and
4. *Populus maximowiczii* – Asian Poplar

When the idea to utilize a tree species to clean contaminated soil and groundwater came into being, hybrid poplars were the ideal choice for further investigation. This is because small poplar trees, or even non-rooted whips planted on a site, will quickly increase in biomass while utilizing local moisture and nutrients. The breakdown and /or translocation of contaminants are a desirable physiological side effect. Trees are also perennial and therefore do not need frequent replanting and have relatively large and deep root systems to reach

contaminants. In fact, poplars are considered phreatophytic. That is, they are water-lowing and their roots will seek out groundwater. In nature, poplars are generally associated with sites that have an abundant or continuous groundwater/moisture supply, such as stream banks. Reciprocally, it would appear, by their preferred habitat and measured stomatal conductance, that poplars transpire relatively large amount of water. These characteristics are also beneficial in their application to phytoremediation, where greater use of groundwater translates to a greater probability of contact and uptake of contaminants, or even curtailing (or possibly reversing) lateral movement of groundwater and associated further spread of contaminants of concern (Kassel *et al.*, 2002).

A major resource for research on poplar and its many applications is that the genome of the native species *Populus trichocarpa* is being sequence at the Joint Genome Institute (US Department of Energy). Sequence information can be downloaded at the following web page:http://genome.jgi-psf.org/Poptr1/Poptr1.home.html. With a genome of just over 500 million nucleotides, *Populus trichocarpa* was sequenced eight over to attain the highest quality standards. Poplar was chosen as the first tree DNA sequence decoded because of its relatively compact genetic complement, some 50 times smaller than the genome of pine, making the poplar and ideal model system for trees. The poplar genome, divided into 19 chromosomes, is four times larger than the genome of the first plant sequenced four years ago, *Aradopsis thaliana*. Thus far, researchers have revealed poplar's genome to be about one-third heterochromatin, that is, regions of chromosomes thought to be genetically inactive, which should provide shortcuts to important regulatory features.

Hybrid poplars have, in general, fulfilled their goals as the trees of choice for phytoremediation. Suitable hybrid clones grow rapidly on most sites provided that their basic needs are met. Pot, lysimeter, and field studies have demonstrated the uptake of organic contaminants such as TCE and have provided

solid evidence of their breakdown within the plant tissue (Newman 1997; Burken and Schnoor, 1998, 1999).

Poplar was chosen for phytoremediation applications for a variety of reasons. Poplar has a very wide geographical distribution and can be grown from southern Alaska into Central America. Members of the species can be easily crossed sexually. Propagation by cuttings is simple yielding clones of a given individual. Poplars can be grown axenically in culture and exogenous genes can be incorporated into the poplar genome. Thus there is the potential of incorporating genes that will more completely mineralise TCE or other organicpollutants. The absorption surface of roots in a stand of poplars is enormous and can approach 300,000 km/ha (Newman *et al.*, 2004).

Hybrid poplars can grow 3 meter and more per season, they live about 40 years, they can be harvested to make pulp logs after 6-7 years, and saw logs after 8-10 years, and they are inexpensive to cultivate and maintain. All these characteristics make them the ideal choice for phytoremediation of organic contaminants.

Burken and Schnoor (1998) demonstrated that a wide variety of organic chemicals are taken-up and translocated in hybrid poplars using a series of radio-labelled chemicals in the laboratory.

Newman *et al*. (1997) and Gordon *et al*. (1997) were used poplar trees for phytoremediation of TCE contaminated soil. The investigators found that over 95% of the TCE was removed from the stream water in the plots with trees.

Table 1.4 presented phytokinetics' remediation projects in which poplar trees were employed (Ferro *et al*., 1997).

Location of site	Groundwater contaminant	Date of installation
Ogden, UT	TPH	1995
Limon, CO	TPH	1995
Tacoma, WA	TPH	1995
Joplan, MO	NH4NO3	1996
Ogden, UT	TPH	1996
Salt Lake City, UT	TPH	1996
Moonachie, NJ	Toluene	1997
Salem, OR	TPH	1997
Southington, CT	PCE/DCE	1998
Genk, BE	BTEX	1999

Table 1.4: PhytoKinetics' remediation projects in which poplar tree were employed (Ferro *et al.*, 1997).

It is also important to note that poplar is not a good species for accumulation of heavy metals, as it is showing exclusion properties.

1.3 BTEX

BTEX compounds are being produced on the scale of megatons per year as bulk chemicals for industrial use as solvents and starting materials for the manufacture of pesticides, plastics and synthetic fibres (Harwood and Gibson, 1997). One of the most common sources for BTEX contamination of soil and groundwater are spills involving the release of petroleum products such as gasoline, diesel fuel and lubricating and heating oil from leaking underground storage tanks, leakage from the subterranean portion of tanks at fuel storage facilities, are contributing to the volume of petroleum hydrocarbons contaminating the subsurface environment (Swoboda-Colberg, 1995). In the nature BTEX are found in volcanoes and wood fires. Gasoline as well other fuels

contain benzene, toluene, ethylbenzene, and the xylene isomers (collectively known as BTEX), which all are hazardous compounds regulated by the EPA. These BTEX compounds may comprise greater than 60% of the mass that goes into solution when gasoline is spilled into water (Barbaro *et al.*, 1992). Due to their relatively high solubility, BTEX compounds are the hydrocarbons that are the most frequently reported as groundwater contaminants, for instance at gas stations. Considering that gasoline leaks from underground storage tanks are a major source of groundwater contamination and that almost 50% of the drinking water supply in the United States comes from groundwater wells, there is high potential for drinking water contamination resulting from leaking underground storage tanks (Brauner and Killingstad, 1996). The reason why BTEX compounds, entering soil and groundwater system, are considered such a serious problem is that they all demonstrate some acute and long term toxic effects (Mehlman, 1993). In addition to toxicity, benzene is known to be a carcinogen, and strong evidence between long term exposure to benzene and leukaemia has been found (Bescol-Liversac *et al.*, 1982). Upon exposure to benzene, the benzene will move into the blood stream from where it can get into fatty tissues in which it can undergo reactions that produce phenol, an even more serious carcinogen than benzene. The inhalation of toluene and xylenes in concentrations of 0.4 mg/l causes headache, dizziness and irritation of the mucous membranes (Christensen and Elton, 1996). In higher concentrations toluene and xylenes can lead to a reduced ability of co-ordination. The exposure pathways via which BTEX compounds can enter the body include consumption of contaminated crops, inhalation of vapour from the soil, intake of contaminated drinking water and skin exposure.

In soil, once BTEX has entered an aquifer, its biological and abiotic transformation, volatilization and sorption are the 3 principal mechanisms of its removal. Recent studies indicate that BTEX migration is only slightly retarded by partitioning in low organic content aquifers and that the compounds are not

irreversibly sorbed. Biological transformation and volatilisation are the major removal mechanisms for BTEX contamination of soils and groundwater. Entry into the groundwater will be followed by rapid movement of a significant portion of the release. The adsorption of benzene and toluene has been determined to be significantly less than xylene isomers, which would contribute further to the greater mobility of these compounds. The xylene isomers also migrate, but at a slower rate than either benzene or toluene. Aerobic degradation of BTEX compounds can proceed by various pathways, involving either an activation of the aromatic ring by dioxygenation or monooxygenation reactions, or through a processing of the side chain(s) (Williams and Sayers, 1994).

The densities of all BTEX compounds are less than water, so that any BTEX present at levels above solubility would be dispersed in the soil pore structure or form a floating layer within the groundwater system. The high vapour pressure of benzene, and to a lesser extent, that of remaining BTEX compounds could produce a significant gas phase transfer within the vadose zone and possibly into the atmosphere.

Because of the health concerns surrounding soil and groundwater contamination from petroleum products, there is a high interest in researching this area. The continual release of petroleum products into the subsurface environment requires the need for monitoring and remediation techniques. Even though there has been a lot of research going on within this area the problem of how to set up actual remediation processes is still very complex.

	Benzene	**Toluene**	**Ethylbenzene**	**m-Xylene**	**o-Xylene**	**p-Xylene**
chemical formula	C_6H_6	C7H8	C8H10	C8H10	C8H10	C8H10
Molecular weight (g/mole)	78	92	106	106	106	106
Log K_{ow}	2.13	2.69	3.15	3.20	2.77	3.15
water solubility (logC_w^{sat}, 25°C (mg/l)	1750	515	152	_	175	198
Henry'law constant (25°C) (kPa*m^3/mole	0.55	0.67	0.80	0.70	0.50	0.71
Vapour pressure (-log P°, 25°C) (atm)	0.90	1.42	1.90	1.98	1.98	1.98
Specific density (at 20°C)	0.8787	0.8669	0.8670	0.8642	0.8802	0.8610
polarity	non-polar	non-polar	non-polar	non-polar	non-polar	non-polar
biodegradability	aerobic	anaerobic/aerobic	aerobic	aerobic	aerobic	aerobic

Table 1.5: BTEX physical-chemical properties

BTEX and TCE have an intermediate log K_{ow} values (see table 1.4) within the range that favours phytoremediation, whereby they are rapidly taken by the plants such as poplar tree and transported via the transpiration stream, and have a high transpiration stream concentration factor. Phytoremediation of water soluble and volatile organic compounds, such as BTEX and TCE, is however limited by an insufficient degradation of those pollutants by plants (due to a lack of metabolic pathways) and their associated rhizosphere organisms (due to a too short retention time in the rhizosphere as the result of the rapid plant uptake), resulting in poisoning of the plants and/or volatilization of the compounds through the leaves into the air, causing a new environmental problem.

2. PLANT BACTERIA INTERACTIONS

A method to improve degradation and enhance the transformation rate of xenobiotics is by providing (seeding) specific microbes that are able to degrade the xenobiotics of interest. This method is referred to as bioaugmentation. However, until now bioaugmentation has not found widespread application. Goldstein *et al.,* (1985) reports five possible reasons for the failure of bacterial inocula:

1. The concentration of the contaminants at a site can be too low to support growth of the inoculum;
2. The presence of certain compounds in the environment can inhibit the growth or activity of the inoculum;
3. The protozoan-grazing rates on the inoculum can be higher than the growth rates of the bacteria, resulting in a decline of the bacterial population;
4. The inoculum can prefer to use other carbon sources present in soil, instead of the contaminant, and;

5. The inocula may fail because of the inability of the microbes to spread through the soil and reach the pollutant.

In addition, nutrient additives, pH regulation and mechanical tilling to aerate soil and spread inoculated microbes through the soil make the use of bioremediation techniques economically less feasible, while they also alter the ecosystem (Heitkamp and Cerniglia, 1989). An option would be to use a combination of plants and specific microorganisms, which will help the plants to counteract the phytotoxic effects of the contaminants, this by improved degradation or by reducing the plant's stress response.

For example, Siciliano and Germida (1998) found that inoculating Daurian wild rye with two *Pseudomonas* strains allowed plant growth in a soil contaminated with a mixture of 2,3-dichlorobenzoic acid and 3-CBA, despite the fact that the microbial inoculums did not result in reduced levels of these contaminants in the soil.

Therefore, the use of plant growth promoting microorganisms in combination with plants offers a promising combination to establish plant growth at contaminated sites or to improve the overall degradation capabilities of plants and their associated microorganisms.

2.1 Rhizosphere bacteria

The rhizosphere soil has been described as the zone of soil under the direct influence of plant roots and usually extends a few millimetres from the root surface and is a dynamic environment for microorganisms (Ramaswami *et al.,* 2003). The overall effect of the plant-microbe interaction is an increase in microbial biomass by an order or magnitude or more compared with that of microbial populations in bulk soils, microbial population densities in the rhizosphere are typically measured at 10^8-10^9 cells per gram of soil or root (Curl and Truelove, 1986). This "rhizosphere effect" is often expressed quantitatively as

the ratio of the number of microorganisms in rhizosphere soil to the number of microorganisms in non-rhizosphere soil, the R/S ratio (Buyer *et al.,* 2002). Although R/S ratios commonly range from 5 to 20, they can run as high as 100 or greater. The rhizosphere microbial community is comprised of microorganisms with different types of metabolism and adaptive responses to variation in environmental conditions. The production of mucilaginous material and the exudation of a variety of soluble organic compounds by the plant root play an important part in root colonization and maintenance of microbial growth in the rhizosphere (Daane *et al.,* 2001).

Different root zones can also influence the heterogeneity of the rhizosphere community, which reflects microbial succession after the primary colonization of new roots. Early studies of the rhizosphere microbial community suggested that *Pseudomonas* were the predominant microorganisms associated with plant roots, comprising anywhere from 30-90% of the cultivable organisms isolated on agar media (Vancura, 1980). More recent studies using media that select for oligotrophic microorganisms or DNA probing methods show that there is tremendous diversity in the community of microorganisms associated with roots, and that these communities vary for different plant species, or even for the same plant species in different soils (Latour *et al.*, 1996). Microbial communities associated with individual plants may even differ from one root to the next, depending on stochastic events that lead to the successful colonization of a particular root by different microorganisms (Crowley *et al.,* 1997).

Rhizoremediation (the degradation of recalcitrant pollutants by bacteria in the rhizosphere) is an attractive process since plant roots provide a large surface area for significant population of bacteria and transport the root-colonizing, pollutant degrading microorganisms to the pollutants up to 10-15 m deep in the soil (Scott *et al*., 1995). Rhizoremediation has shown promise based on the use of wild-type bacteria in their native environments to degrade a variety of pollutants (Yee *et al*., 1998).

Plant growth promoting bacteria can positively influence plant growth and development by both indirect and direct way (Glick *et al.,* 1999; Whipps, 2001). PGPB can directly promote plant growth by providing the plant with a stimulating compound that is synthesized by the bacterium or by facilitating the uptake of nutrients from the environment by the plant (Brown 1974; Davison, 1988; Klopper *et al*., 1989, Lambert and Joos, 1989; Patten and Glick, 1996; Glick *et al*., 1998). Examples of this direct stimulation of plant growth include:

- Synthesis of siderophores, which can solubilize and sequester iron from the soil and provide it to the plant cells (see below).
- Fixation of atmospheric nitrogen, that is supplied to plants cells
- Synthesis of different phytohormones, including auxins and cytokinins, that can enhance various stages of plant growth.
-Microbial mechanisms for the solubilization of minerals that contain essential macronutrients, such as phosphorus, which then become more readily available for plant growth.
- Possess enzymes, such as 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, that can modulate plant growth and development (see below).

A particular bacterium may affect plant growth and development using only one, or more of these mechanisms and a bacterium may utilize different mechanisms under different conditions. For example, bacterial siderophore synthesis is likely to be induced only in soils that do not contain sufficient levels of bioavailable iron, although siderophore production in PGPB was also reported in the presence of elevated levels of heavy metals, such as Zn (Hofte *et al*., 1994). Also, bacteria do not fix nitrogen when sufficient nitrogen is available.

Indirectly, PGPB can stimulate plant growth by controlling fungal, bacterial and viral diseases (Liu *et al*., 1995; Maurhofer *et al*., 1998), as well as insects (Zehnder *et al*., 1997) and nematode pests (Sikora, 1988). PGPB provide different mechanisms for suppressing plant pathogens (Ramamoorthy *et al*., 2001). Those mechanisms include competition for nutrients and space (Elad and Baker, 1985;

Elad and Chet, 1987), antibiosis by producing antibiotics such as pyrrolnitrin, pyocyanine, and 2,4-diacetyl phloroglucinol (Pierson and Thomashow, 1992), the production of lytic enzymes such as chitinases and β-1,3-glucanases which degrade chitin and glucan present in the cell wall of fungi (Potgieter and Alexander, 1996;), HCN production (Defago *et al.,* 1990) and degradation of toxin produced by pathogens (Borowitz 1992; Duffy and Defago, 1997).

A way in which rhizosphere inhabiting PGPB can prevent the proliferation of phytopathogens, and thereby facilitate plant growth, is through the production of siderophores with a very high affinity for iron (Castignetti and Smarrelli, 1986). The secreted siderophore molecules bind most of the Fe^{3+} that is available in the rhizosphere, and as a result effectively prevent any pathogens in this immediate vicinity from proliferating because of a lack of iron (O'Sullivan and O'Gara, 1992). Evidence for this mechanism comes from several studies including a report where a mutant strain of *Pseudomonas aeruginosa* deficient in siderophore production no longer protected tomato plants against *Pythium* damping-off (Buysens *et al.*, 1994). Generally, *Pseudomonas* sp. have been considered to play a major role in disease inhibition through the production of siderophores and thanks to this efficient iron uptake mechanism, they can quickly colonize the rhizosphere and thus physically displace deleterious organisms (Lodewyckx, 2001). *Bacillus thuringiensis* occurs naturally in the soil and on plants. This bacterium produces a crystal protein that is toxic to specific groups of insects and has been available as a microbial insecticide since the 1960's. The primary insects controlled by *B. thyringiensis* are numerous moth and butterfly caterpillars, as well as, certain beetle and fly larvae (Aronson *et al*., 1986).

PGPB and heavy metal contamination

In the presence of high levels of heavy metals plants produce the stress hormone ethylene, which could result in inhibition of root elongation. The use of ACC (1-aminocyclopropane-1-carboxylic acid) deaminase-containing plant growth promoting bacteria have been shown to decrease the level of stress ethylene (Glick *et al.*, 1998), resulting in an increase of the plant fitness and thus can be considered to act as a plant growth promoting characteristic. In addition, plants are able to take up and utilize complexes between bacterial siderophores and iron. Plant siderophores bind to iron with a much lower affinity than bacterial siderophores so that in metal contaminated soils a plant is unable to accumulate a sufficient amount of iron unless bacterial siderophores are present (Burd *et al*, 1998; Glick *et al.*, 1998).

A special niche for plant-associated bacteria is constituted by heavy metal-accumulating plants. The presence of plant associated bacteria (rhizospheric or endophytic) will require specific adaptation of these bacteria, such as adaptation to high levels of heavy metals. Additionally, characterization of these bacteria and their heavy metals resistance phenotypes can also provide valuable information on the bioavailability of heavy metals in the rhizosphere and within the hyperaccumulator plant (Lodewyckx *et al*, 2002a). Whiting *et al*. (2001) demonstrated that the rhizosphere bacteria associated with *Thlaspi caerulescens* increased the amount of water-soluble zinc in the soil and, as such, helped to enhance the zinc accumulation in *T. caerulescens* shoots. Lodewyckx *et al*. (2002a) studied the bacterial populations associated with *T. caerulescens* subsp. *calaminaria* grown in soil collected from the abandoned Zn and Pb mining and smelter site of Plombières, Belgium. The rhizosphere population was well adapted to heavy metal in concentrations 1mM Zn and 0.8 mM Cd and showed a survival rate of 88% and 78% on the respective selective heavy metals, which indicates a difference in metal availability in the vicinity of the root compared to the nonrhizosphere soil.

PGPB and organic contaminants

When a site gets polluted with organic xenobiotics, the composition of the indigenous microbial population in the soil and groundwater will adapt to this new situation. Bacteria able to use the contaminant as a nutrient source will be able to proliferate and may become more dominant (Liu and Suflita, 1993), thus outcompeting other members of the endogenous population. However, microbes with the suitable catabolic genes are not always available on the site for all contaminants, which will result in an inefficient removal of the contaminant and accumulation of toxic and recalcitrant compounds in the environment (Pieper and Reineke, 2000).

Microbial activity is, compared to bulk soil, generally higher in the rhizosphere due to readily biodegradable substrates exuded from the plant. For example, corn exudates from hydroponic cultures added to columns with polluted soil have been used to demonstrate that exudates can increase the growth of microorganisms and enhance the mineralization of ^{14}C-labeled pyrene (Yoshitomi and Shann, 2001). Similarly, a heavily PAH contaminated soil was drenched with synthetic root exudates, with or without mineral N and P, demonstrating the individual role of easily assimilable sugars and mineral nutrients on growth of PAH degrading bacteria and biodegradation of twelve 3 - 6 ring PAH (Joner *et al.*, 2001).

Corgié *et al.* (2003) described a compartment device constructed to study interactions between rhizosphere carbon flow and PAH biodegradation in the rhizosphere. Microbial counts of culturable bacteria clearly reflected spatial distribution induced by the plants, as the numbers of heterotrophic and PAH degrading microorganisms were always the highest near the root mat, while decreasing with distance from the roots. In the unpolluted treatment, exudates inputs induced a gradient of heterotrophic and PAH degrading bacteria with cell numbers also decreasing with increasing distance from the roots. Without plants, no spatial variation in microbial numbers was observed.

Kunc (1989) studied the effect of continuously-supplied, synthetic root exudates on changes in the number and proportion of organisms capable of degrading 2,4-D. After 2 weeks on a 2,4-D contaminated soil, the proportion of 2,4-D (2,4-dichlorophenoxyacetic acid) degraders in the control soil had increased from 9.4% to 77% of the total microbial population, while the proportion in the stimulated rhizosphere soil increased slightly more from 9.4% to 93.7%.

A consortium isolated from the rhizosphere of wheat utilized the herbicide Mecoprop as a sole carbon and energy source (Lappin *et al.*, 1985), and bacteria from rhizosphere soils amended with succinic acid and formic acid produced 5 to 10 times more $^{14}CO_2$ from mineralization of radiolabelled pyrene than nonsterile control soils produced (Schwab *et al.*, 1995). Rhizoremediation of TCE was studied by Yee *et al.*, (1998), the relatively short-term microcosm experiments showed that TCE is effectively degraded by wheat roots colonized by *Pseudomonas fluorescens* recombinant strain expressing toluene *ortho*-monooxygenase constutivly. Plants and wild-type bacteria have been shown to increase TCE degradation in the rhizosphere; for example, the legume *Lespedeza cuneata* converted 30% of (^{14}C)TCE to (^{14}C)CO_2 (Anderson and Walton, 1995). Also, three different indices of microbial activity (plant biomass determination, disappearance of TCE from the headspaces of spiked soil slurries and mineralization of (^{14}C) TCE to (^{14}C) CO_2) all provided evidence that microbial activity is greater in rhizosphere soils and that TCE degradation occurs faster in the rhizosphere than in the bulk soil (Walton and Anderson, 1900).

Successive attempts to introduce beneficial bacteria into the rhizosphere of agricultural crops have generally met with varying degrees of failure due to the difficulties of incorporating non-resident bacterial components into established and acclimated microbial communities (Sturz and Nowak, 2000). For example, despite many years of attempting to modify naturally occurring soil populations of *Rhizobium*, such efforts have not been very successful (Brockwell *et al.,* 1998; Thies *et al.*, 1991).

Considering the biodiversity of indigenous soil bacteria and the population densities involved, it is not surprising that it has been proven difficult to make any long lasting structural changes to the composition of bacteria within any given soil-community. One strategy, which may help contributing to the establishment of pre-selected beneficial organisms in root zone soils, and which has until recently been excluded from the research, is through fostering the early establishment of selected communities of endophytic microorganisms within root systems (Sturz and Nowak, 2000).

2.2 Endophytic bacteria

Endophytic bacteria have been defined as prokaryotic organisms that reside internally in the plant tissues without causing visible harm to their hosts and which can be re-isolated from surface disinfected plant parts and internal plant extracts (Hallmann *et al.*, 1997; Asis and Adachi, 2003). They are ubiquitous in most plant species, residing latently or actively colonizing plant tissues locally as well systematically.

Endophytic bacteria have been isolated from both monocotyledonous and dicotyledonous plants. Over 129 species have been isolated from internal plant tissues, where *Pseudomonas*, *Bacillus*, *Enterobacter, Burkholderia, Enterobacter, Acetobacter,* and *Agrobacterium* have been found to be the most abundant bacterial genera (Hallman *et al*., 1997; Lodewyckx *et al*., 2002b).

Generally, the highest bacterial densities are usually observed in the roots and decrease progressively from the stem to the leaves (Lamb *et al*., 1996; Mocali *et al*., 2003). Typically, the average bacterial population density in the root interior is between 10^3 and 10^5 CFU/gram root, compared to population densities in soil 10^7 and 10^8 CFU/ gram soil (Hallmann *et al.*, 1997). The fact that colonization is

especially abundant in root tissue may reflect the fact that the root is the primary site where endophytes gain entry into plants (Lodewyckx *et al.*, 2002b). Endophytes colonize intercellular spaces and vascular tissues of plants, but rarely intracellular spaces (Sessitsch *et al.,* 2002; Reiter *et al.*, 2003). Initially, endophytic bacteria were regarded as latent pathogens or as contaminants from incomplete surface sterilization (Thomas and Graham, 1952), but since then various reports have demonstrated that bacterial endophytes are able to promote plant growth and health: increased disease resistance through the novo synthesis of structural compounds and fungi-toxic metabolites at sites of attempted fungal penetration (Benhamou *et al.*, 1996b), the induction and expression of general molecular-based plant immunity (Sticher *et al.*, 1997; Sturz *et al.*, 2000), or the simple exclusion of other organisms (phytopathogens or colonists) by niche competition (Sturz and Nowak, 2000; Nejad and Johnson, 2000), improvement of the plant's ability to withstand environmental stresses (e.g. drought, pollution), or enhanced N_2 fixation (Sturz and Mattheson, 1996; Benhamou *et al.*, 2000, Sturz and Nowak, 2000). Several factors favour endophytic bacteria compared with rhizosphere bacteria. Endophytes have a natural and intimate association with plants. The internal tissues of plants provide a relatively uniform and protected environment when compared with the rhizosphere, where ectophytic bacteria must compete for nutrients with other microbes and endure fluctuations of temperature and moisture, as well as exposure to ultraviolet radiation on above ground surfaces (Chen *et al.*, 1995). In plant tissues bacterial endophytes may originate from the rhizosphere, seeds, vegetative material and phylloplane (McInroy and Kloepper, 1995; Raaijmakers *et al.*, 1995). Entry of these bacteria into the plant can be through sites of emergence of lateral roots, other wounds, natural openings, including lenticels, stomata and germinating radicles (Hallman *et al.*, 1997). Besides gaining entrance to plants through natural openings or wounds, numerous endophytic bacteria including *Azoarcus* sp. (Hurek *et al.*, 1994), *Pseudomonas fluorescens* (Duijff *et al.*, 1997) and *Azospirillum irakense*

(Khammas and Kaiser, 1991) appear to actively penetrate plant tissues using hydrolytic enzymes like cellulases and pectinases. Enzymatic degradation of plant cell walls by these bacteria was only observed when they colonized the root epidermis but never after colonizing intercellular spaces of the root cortex. This suggests that endophytes induce the production of their cellulytic and pectinolytic enzymes specifically for the penetration into their host plant. Although these observations suggest the posibility of active penetration mechanisms for some endophytic bacteria, very little is known about the origin and regulation of these enzymes. It is assumed that these bacteria must possess some regulatory mechanism to specifically regulate their enzyme production in terms of quantity and time of expression (Lodewyckx *et al.*, 2002b). Since these enzymes are also produced by pathogens, more knowledge on their regulation and expression is needed to distinguish endophytic bacteria from plant pathogens. In general, endophytic bacteria occur at lower population densities than pathogens, and at least some of them do not induce a hypersensitive response in the plant, indicating that they are not recognized by the plant as pathogens. A completely different way of penetration is described by Ashbolt and Inkerman (1990) for sugar cane via the mealybug (*Maconellicoccus hirsutus*) and by Kluepfel (1993), via a range of different insects.

As the bacteria can proliferate inside the plant tissue, they are likely to interact more closely with the host, face less competition for nutrients, and are more protected from adverse changes in the environment than bacteria in the rhizosphere (Reinhold-Hurek and Hurek, 1998). The relationship between host and bacterial endophytes is not static. Once inside the plant, endophytic populations have been observed to grow to between 2.0 and 7.0 $\log_{10}$ cells per gram of fresh tissue (Shisido *et al.*, 1999). Communities of bacterial endophytes may not only be host specific, but also plant tissue sensitive, reacting and adapting at certain tissue sites and among certain tissue types within the host plant as it develops (Sturz *et al.*, 1999).

Application of endophytic bacteria for plant protection

Black rot, caused by *Xantomonas campestris pv. campestris*, is a serious disease of vegetable belonging to the family of the *Brassicaceae* in Zimbabwe (Mguni, 1996) and many other places of the world. Mguni (1996) isolated a number of strains of *Bacillus* spp. with antagonistic potential against black rot from cabbage and kale. One of the isolated strains (*Bacillus subtilis* strain BB) has shown promise as a control agent of black rot under field conditions in Zimbabwe (Mguni, 1996; Wulff, 2000).

Application of endophytic bacteria (*Pseudomonas fluorescens*) by stem injection in cotton plants reduced root rot caused by *Rhizoctonia solani* and vascular wilt caused by *Fusarium oxysporum* f. sp. *vasinfetum* (Chen *et al*., 1995). These bacteria move upward and downward from the point of application and by colonizing the internal tissues, they can exclude the entry of a pathogen in the vascular stele.

In pea, colonization of epidermis, cortex and vascular tissue in roots by endophytic bacteria prevented the entry of mycelial growth of a fungus or restricted the growth of mycelium to the epidermis (Benhamou *et al*., 1996a, 1996b).

Seed treatment of tomato with the endophytic bacterium *Bacillus pumilus* strain SE 34 prevented the entry of vascular wilt fungus *F. oxysporum* f. sp. *radicislycopersici*, into the vascular stele and the mycelial growth stayed restricted to the epidermis and outer root corex (Benhamou *et al.*, 1998).

Probably, Fahey (1988; 1991) described the first work directed at the introduction and expression of heterologous genes in an endophytic microorganism with the purpose of insect control. The endophyte *Clavibacter. xyli* subsp. *cynodontis*, a gram positive, xylem-inhabiting bacterium, capable of colonizing several plant species, was used as the expression host of the d-endotoxin gene of. *Bacillus thuringiensis*, whose gene product provides protection against insects in nature, especially against Lepidoptera and Coleoptera. The

genetically modified *Clavibacte. xyli* was shown to secrete the d-endotoxin inside the plant, protecting its host against attacks of target insects. The author described several advantages of inoculating this bacterium into corn plants as a way of protecting it against insects:

1. The product requires only one application in seeds or aspersion over young plants.
2. There is no need for a large amount of the product because the bacterium multiplies after inoculation.
3. The genetically modified bacterium stays restricted to the inner parts of the plant and thus, there is no dissemination to other plants.
4. The process does not yield toxic compounds.
5. This and other similar products require a shorter time to be technologically developed because it is easier to modify a microorganism than a plant.
6. The modified microorganism is not transmitted to progeny seed of inoculated plants and therefore, from a commercial point of view, it must be acquired constantly.
7. The process has wide applicability because it can be used for other plant species, and
8. Bacteria multiplication is high inside the plant, resulting in up to 10^8/gram of inoculated plant.

Application of endophytic bacteria in environmental remediation

Siciliano *et al.* (2001) showed that plants grown on soil contaminated with xenobiotics naturally recruited endophytes with the necessary contaminant degrading genes. The flexible and specific nature of the endophytic community composition under different environmental conditions, such as the presence of contaminants, suggest that the mechanism by which plants and their endophytic

microbial community interact is much more dynamic and responsive than previously believed.

Bacterial populations associated with the Zn hyperaccumulator *Thlaspi caerulesncens* subsp. *calaminaria* grown in a soil coming from Zn-Pb mine spoil in Plombièrs, Belgium, were investigated by Lodewyckx *et al*. (2002a). The bacterial population of the nonrhizospheric soil consisted of typical soil bacteria, some exhibiting multiple heavy metal resistance characteristics that often are associated with Zn and Pb polluted substrates: 7.8% and 4% of the population survived in the presence of elevated levels of Zn (1mM) and Cd (0.8mM), respectively. For the bacterial population isolated from the rhizosphere, the comparable survival rates were 88 and 78% when these bacteria were tested for their Zn and Cd resistance, respectively. This observation indicates a selective enrichment of the metal-resistant strains in the rhizosphere, most likely due to an increased availability of the metals in the soils near the roots compared to nonrhizospheric soil. The endophytic inhabitants of the roots and shoots were isolated, identified, and characterized. Although similar endophytic species were isolated from both compartments, those from the rhizoplane and roots showed lower resistance to Zn and Cd than the endophytic bacteria isolated from the shoots. In addition, root endophytic bacteria had additional requirements. Contrary to the root residing inhabitants, the shoot represented a niche rich in metal-resistant bacteria and even seemed to contain species that were exclusively abundant there. These differences in the characteristics of the bacterial communities associated with *T. caerulescens* might possibly reflect altered metal speciation in the different soils and plant compartments studied.

Lodewyckx *et al.,* (2001) showed that endophytes of yellow lupine (Lupinus *luteus*) were able to increase the nickel accumulation and nickel tolerance of the inoculated plants. The ncc-nre nickel resistance system of *Ralstonia metallidurans* 31A was efficiently expressed in *Burkholderia cepacia* L.S.2.4 (Taghavi *et al*., 2001) and *Herbaspirillum seropediacae* LMG2284. The authors hypothesized that

once the nickel resistance bacteria were inoculated into their host plants, they could possibly alter the nickel speciation and therefore decrease the levels of free nickel ions and their phytotoxic effect on the plant's metabolism. *Lupinus luteus*, when grown on a nickel enriched substrate and inoculated with *B. cepacia* L.S.2.4::*ncc-nre*, showed indeed a significant increase (30%) of nickel concentration in the roots, whereas the nickel concentration in the shoots remained comparable with that of the control plants. The inoculation of *Lolium perenne* (cv Atlas) with the nickel resistante 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.

In conclusion, endophytic bacteria have the potential to assist their host plant in overcoming constraints imposed by a contaminated environment.

CHAPTER II

AIM AND OUTLINE OF THE THESIS

Although phytoremediation of organic xenobiotics is a promising technology, our literature survey shows that phytoremediation of organic contaminants is not always a feasible option: in some cases the concentrations of the contaminants can be higher than the phytotoxicity levels for the plant, while in other cases water soluble organic pollutants, especially volatile organic solvents, can be transported through the plant's transpiration stream and be released into the air without any significant degradation. In order to make phytoremediation more broadly applicable and acceptable, for instance when zero emission levels are required, we hypothesized endophytic bacteria, if equipped with correct degradation pathway, can help their host plant to survive higher concentration of contaminants that are normally phytotoxic, and also can help to reduce the evapotranspiration of water soluble pollutants.

As a model system to test if endophytic bacteria, when equipped with the appropriate degradation pathway, can assist their host plant in the degradation of an organic contaminant, *Lupinus luteus* was used together with its associated endophytic bacterium *Burkholderia cepacia*. The results we obtained were very promising and showed that the endophyte was able to efficiently degrade the model compound toluene, by so reducing both the phytotoxicity and release of the compound. This prompted as to extend this concept to, poplar trees, which are known as plants suitable for phytoremediation, and their endophytic bacteria.

Chapter III shows the results which proved the feasibility of the concept. Using *Lupinus luteus* it is shown that endophytic bacteria equipped with the appropriate degradation pathway, can have a positive effect on plant growth and health in

the presence of increased levels of toluene, as well as on reducing the evapotranspiration of toluene through the leaves to the air.

In chapter IV the capability of poplar cell cultures to degrade toluene was tested. A poplar cell culture suspension was used in order to investigate if the plant possesses enzymes for toluene degradation. We could not demonstrate any degradation of toluene during this experiment, indicating that poplar cells are not capable for toluene degradation.

Chapter V describes the isolation, characterization and identification of rhizosphere and endophytic bacteria from different parts of poplar trees (*Populus trichocarpa* x *deltoides* cv. “Hoogvorst” and “Hazendans”) growing on a BTEX polluted site. The cultivable isolates were characterised and identified by comparative sequence analysis of their 16S rDNA. One hundred and forty six morphologically distinct isolates were obtained, belonging to twenty one distinct genera.

Chapter VI explores the re-colonisation capability of endophytic bacteria, marked with kanamycin and *gfp* (green fluorescent protein), into poplar cuttings. Three of the four tested strains, all belonging to the genus *Pseudomonas*, were capable to re-colonize poplar cuttings.

Chapter VII explores the possibility of endophytic bacteria, equipped with appropriate degradation pathway, to assist poplar cuttings in phytoremediation process of toluene. Horizontal gene transfer was observed between the inoculated bacteria and the endogenous population of poplar endophytes which resulted in a reduction of phytotoxicity and evapotranspiration of toluene.

Chapter VIII describes a field experiment in which is demonstrated the phytoremediation, where poplar trees are used to contain a BTEX contaminated groundwater plume. Special attention is paid to the natural occurrence of plant-associated bacteria which are able to use BTEX as a carbon source.

Chapter IX provides a summarising discussion and the perspectives of this research.

CHAPTER III

PROOF OF THE CONCEPT: ENGINEERED ENDOPHYTIC BACTERIA IMPROVE PHYTOREMEDIATION OF WATER-SOLUBLE, VOLATILE ORGANIC POLLUTANTS

Published in Nature Biotechnology 22, 583-588 (2004) Barac T., Taghavi S., Borremans B., Provoost A., Oeyen L., Colpaert J. V., Vangronsveld J. and van der Lelie D.

ABSTRACT

Phytoremediation of highly water soluble and volatile organic xenobiotics is often inefficient because plants do not completely degrade these compounds through their rhizospheres. This results in phytotoxicity and/or volatilisation of chemicals through the leaves, which can cause additional environmental problems. We demonstrate that endophytic bacteria equipped with the appropriate degradation pathway improve the *in planta* degradation of toluene. We introduced the pTOM toluene-degradation plasmid of *Burkholderia cepacia* G4 into *B. cepacia* L.S.2.4, a natural endophyte of yellow lupine. After surface-sterilized lupine seeds were successfully inoculated with the recombinant strain, the engineered endophytic bacteria strongly degraded toluene, resulting in a marked decrease in its phytotoxicity, and a 50-70% reduction of its evapotranspiration through the leaves. This strategy promises to improve the efficiency of phytremediating volatile organic contaminants.

3.1 INTRODUCTION

Emerging phytoremediation technologies have been applied at various scales to treat moderately hydrophobic pollutants, such as benzene, toluene, ethylbenzene and xylene (BTEX) compounds, chlorinated solvents, nitrotoluene ammunition wastes and excess nutrients (Schnoor *et al.*, 1995). Phytoremediation of organic xenobiotics is based on interaction between plants and their associated microorganisms in a process whereby plants draw pollutants, including polyaromatic hydrocarbons (PAHs), into their rhizosphere via the transpiration stream (Harvey *et al.*, 2002); subsequently, microorganism-mediated degradation occurs in the plant itself, in the rhizosphere or in both.

The fate of the contaminant in the rhizosphere-root system largely depends on its physicochemical properties. Organic xenobiotics with a log K_{ow} (octanol/water partition coefficient) < 1 are considered to be very water-soluble, and plant roots do not generally accumulate them at a rate surpassing passive influx into the transpiration stream (Cunningham and Berti, 1993). Contaminants with a log K_{ow} > 3.5 show high sorption to the roots, but slow or no translocation to the stems and leaves (Trapp *et al.*, 2001). However, plants readily take up organic xenobiotics with a log K_{ow} between 0.5 and 3.5, as well as weak electrolytes (weak acids and base or amphoteres as herbicides). These compounds seem to enter the xylem faster than the soil and rhizosphere microflora can degrade them, even if the latter is enriched with degrading bacteria (Trapp *et al.*, 2000). Once taken up, plants metabolise these contaminants, although some of them, or their metabolites, such as trichloroethene (TCE), which is transformed into trichloro acetic acid, can be toxic (Doucete *et al.*, 1998). Alternatively, plants preferentially release volatile pollutants, such as BTEX compounds and TCE and their metabolites, into the environment by evaporation via the leaves, which calls into question the merits of phytoremediation (van der Lelie *et al.*, 2001; Schwitzguebel *et al.*, 2002; Ma and Burken, 2003; Burken and Schnoor, 1999).

There is thus a need for different strategies to improve the phytoremediation of such contaminants.

In this study we aimed to increase the degradation of volatile, water-soluble organic contaminants during their transport in the plant's vascular system using engineered endophytic bacteria. Endophytic bacteria reside within the living tissue of a plant without substantively harming it (Misaghi and Donndelinger, 1990; James and Olivares, 1997). They are ubiquitous in most plant species, residing latently or actively colonizing the tissues. Bacterial endophytes are highly diverse with many different taxa colonizing a wide variety of plant species (Lodewyckx *et al*., 2002b). It has been reported (Frommel *et al*., 1991) that substantial numbers of endophytes (10^3-10^6) can colonize the vascular system (phloem, xylem). The highest densities usually are observed in the roots and decrease progressively from the stem to the leaves.

In this study we demonstrate that a genetically modified endophytic strain of *B. cepacia*, whose host plant is the yellow lupine (*Lupinus luteus* L.), improves *in planta* degradation and reduces evapotranspiration of toluene, a moderately hydrophobic (log K_{ow} = 2.69 at 20°C) volatile compound.

3.2 MATERIAL AND METHODS

3.2.1 Construction of a toluene-degrading endophyte

B. cepacia strain BU0072 (Ni^R, Km^R), a derivative of the endophytic bacterium *B. cepacia* L.S.2.4, was used (Lodewyckx *et al.*, 2002a; Taghavi *et al*., 2001). *B. cepacia* G4 (pTOM, Tol^+) (Shields *et al*., 1995) served as a donor strain for toluene degradation. After conjugation (Taghavi *et al*., 2001), transconjugants that were resistant to nickel plus kanamycin, and could grow on toluene as sole carbon

source were selected on '284' minimal medium (Schlegel *et al.*, 1961) supplemented with 1 mM $NiCl_2$ plus 100µg/ml kanamycin, while the plates were incubated under a toluene atmosphere to provide the carbon source. The 284 medium contains per liter distilled water: 6.6g Tris-HCl, 4.68g NaCl, 1.49g KCl, 1.07g NH_4Cl, 0.43g $NaSO_4$, 0.20g $MgCl_2 \cdot 6H_2O$, 0.03g $CaCl_2 \cdot 2H_2O$, 40mg $NaHPO_4 \cdot 2H_2O$, 10ml Fe(III)NH_4 citrate solution (containing 48mg:100ml), 1ml microelements solution, final pH 7. A carbon source, such as gluconate, is added at 0.2% (wt/vol). The microelement solution contains per liter distilled water: 1.3ml 25% HCl, 144mg $ZnSO_4 \cdot 7H_2O$, 100mg $MnCl_2 \cdot 4H_2O$, 62mg H_3BO_3, 190mg $CoCl_2 \cdot 6H_2O$, 17mg $CuCl_2 \cdot 2H_2O$, 24mg $NiCl_2 \cdot 6H_2O$ and 36mg $NaMoO_4 \cdot 2H_2O$. The presence of the *nre* Ni resistance marker and the pTOM plasmid in the transconjugants was confirmed with PCR using *nre* and pTOM specific primers, respectively. A representative transconjugant, *B. cepacia* strain VM1330, which had the correct genetic makeup and grew under the appropriate selective conditions with toluene as sole carbon source, was selected for further studies.

The stability of the pTOM plasmid in VM1330 was verified by growing the strain for 100 generations on nonselective 284 gluconate minimal medium in the absence of toluene. Following, 100 individual colonies were selected on nonselective medium, and subsequently replica-plated on 284 minimal medium supplemented with 1mM $NiCl_2$ plus 100µg/ml kanamycin, while the plates were incubated under a toluene atmosphere to provide the carbon source. All colonies tested thrived, demonstrating the stability of toluene-degradation properties located on the pTOM plasmid (less than 1% loss of the plasmid after growth for 100 generations under nonselective conditions), as well as the other resistance markers.

3.2.2 Inoculation of yellow lupine with *B. cepacia*

B. cepacia VM1330 was grown in 284 gluconate medium (250ml culture) at 22°C on a rotary shaker for approximately 7 days until a density of 10^9 CFU/ml was reached (OD_{660} of 1). The cells were collected by centrifugation, washed twice in 10 mM $MgSO_4$ and suspended in 1/10 of the original volume 10 mM $MgSO_4$ to obtain an inoculum with a cell density of 10^{10} CFU/ml.

Seeds of *L. luteus* L. were surface-sterilized for 30 minutes at 20°C in a solution containing 1% active chloride (wt/vol, added as a NaOCl solution) and 1 droplet Tween 80 per 100 ml solution. The seeds then were rinsed three times for 1 minute in sterile water and dried on sterile filter paper. To test the efficiency of sterilization, the seeds were incubated for 3 days at 30°C on 869 medium (Mergeay *et al.*, 1985), which contains per liter distilled water: 10g tryptone, 5g yeast extract, 5g NaCl, 1g D-glucose, 0.345g $CaCl_2 \cdot 2H_2O$ (pH 7). Seeds were considered sterile when no bacterial growth was observed. Five surface sterile seeds of *L. luteus* L. were planted in a sterile plastic jar (800ml), completely filled with sterilized perlite and saturated with 400ml of a half-strength sterile Hoagland's nutrient solution. Subsequently, the bacterial inoculum was added to each jar at a final concentration of 10^8 CFU/ml Hoagland's solution (three Hoagland's stock solutions are prepared; solution 1: macroelements, containing 102g KNO_3, 70.8g $Ca(NO_3)_2 \cdot 4H_2O$, 23g $NNH_4H_2PO_4$, 49g MgSO4 · 7H2O per 10 liters distilled water; solution 2: Fe solution containing 1.9g FeSO4 · $7H_2O$, 1.25g EDTA-di-Na-salt per 250ml distilled water; solution3: microelements: 2.86g H_3BO_3, 1.81g $MnCl_2 \cdot 4H_2O$, 0.08g $CuSO_4 \cdot 5H_2O$, 0.09g $H_2MoO_4 \cdot H_2O$, 0.22g $ZnSO_4 \cdot 7H_2O$; Hoagland's solution contains per 10 liters: 1 liter solution 1, 3ml solution 2 and 10ml solution 3, pH 6.5). The jars were covered with sterile tinfoil to facilitate bacterial colonization and prevent contamination and dispersion of the inoculated bacteria through the air. After the seeds had germinated, perforations were made in the tinfoil and plants were allowed to grow through them over 21

day in a growth chamber (constant temperature of 22°C, relative humidity 65% and 14/10 h light and dark cycle, PAR (photosynthetic active radiation) 165µmol/m^2s). The same procedure was used to inoculate *L. luteus* L. with the *B. cepacia* strains BU0072 and G4.

3.2.3 Recovery of endophytic bacteria

Plants were harvested after 21 days. Roots and shoots were treated separately. Fresh root and shoot material was vigorously washed in distilled water for 5 min, surface-sterilized for 5 min in a solution containing 1% active chloride (wv/vol, added as a NaOCl solution) supplemented with 1 droplet Tween per 100ml solution and rinsed three times in sterile distilled water. A 100µl sample of the third rinsing water was plated on 869 medium to verify the efficiency of sterilization. After sterilization, the roots and shoots were macerated in 10ml 10 mM MgSO4 using a Polytron PT1200 mixer (Kinematica A6). Samples (100µl) were plated on different selective and nonselective media to test for presence of the endophytes and their characteristics.

3.2.4 Toluene degradation and phytotoxicity tests on hydroponics

Three-weeks-old *L. luteus* L. plants (both controls and those inoculated with *B. cepacia* VM1330, BU0072 or G4) were used to evaluate the phytotoxicity of toluene and its *in planta* degradation. The lupine plants were carefully taken out from the jars and their roots were vigorously rinsed in sterile water to remove bacteria from the surface. Subsequently, plants were grown hydroponically, settled in a two-compartment glass cuvette system (29cm high; 9cm in diameter) (Figure 3.1). To avoid gas exchange between the upper and lower compartments, they were separated by a glass plate, with an insertion through which the stem of the plant was introduced. Each cuvette contained one plant and the space around

the stem was made gas-tight with a Polyfilla exterior mixture (Polyfilla), so that shoots in the upper compartment and roots in the lower compartment were completely separated, allowing no gas exchange between them, except through the stem. The upper compartment, the glass plate and the lower compartment were sealed with Apiezon (Apiezon Products M&I Materials LTD). The lower compartment was filled with 300ml of sterile, half-strength Hoagland's solution. Different toluene concentrations of 0, 100, 500 and 1000 mg l^{-1} were added to the Hoagland's solution at the beginning of the experiment. The cuvettes with plants were placed in a growth chamber with constant temperature 22°C and 14/10 hours light/dark cycle; photosynthetic active radiation 165 $mmol/m^2s$. Each compartment was connected with a synthetic air source (Air Liquide) with an inflow of 1 liter per hour. The phytotoxic effects of toluene under the different conditions were examined by determining the increase in plant biomass after 4 days.

We examined the effect of the different endophytic bacteria on toluene degradation and evapotranspiration using the following experimental setup. In the experiments where 100mg/l toluene was added, two-serial linked Tenax traps (Capped Sample Tubes (Perkin Elmer) and Tenax 60/80 (Alltech) were inserted in the outflow of each compartment to capture any transpired or volatilised toluene. The traps were changed regularly. A column filled with $CaCl_2$ was installed between the cuvettes and the Tenax traps as a trap to prevent condensation of water in the Tenax traps. To optimize the Tenax adsorption capabilities, the traps were cooled with dry ice. The whole experiment ran for 96 hours, and toluene concentration in the traps was determined by GC-MS (Automatic thermal Desorption System ATD400, Auto System XL Gas Chromatograph, Turbo Mass Spectrometer, Perkin Elmer). All experiments were done in triplicate to allow statistical analysis of the data using ANOVA.

3.2.5 Toluene toxicity test under green house conditions

After 3 weeks of growth under the above conditions, control plants and lupine plants inoculated with *B. cepacia* strains VM1330, BU0072 and G4 were transferred into half-liter pots filled with a nonsterile sandy soil, irrigated with half-strength Hoagland's solution. Plants were allowed to stabilize for 3 days. Subsequently, plants were irrigated every day with half-strength Hoagland's solution to which toluene was added at concentrations of 0, 100, 250 and 500mg l^{-1}. After two weeks plants were harvested and their biomass was determined. For each treatment five replicas were done.

3.3 RESULTS

3.3.1 Inoculation of yellow lupine with B. *cepacia*

We chose yellow lupine as our plant model. *B. cepacia* BU0072 (Lodewyckx *et al.*, 2001; Taghavi *et al.*, 2001), which is derived from the endophytic strain *B. cepacia* L.S.2.4, and its toluene-degrading derivate VM1330, were used to inoculate the plant. Both are resistant to kanamycin and nickel. In addition, we tested the endophytic characteristics of the toluene-degrading soil bacterium *B. cepacia* G4.

Twenty-one days after inoculating the bacteria, the plants were harvested; roots and shoots were separated, surface sterilized, rinsed and macerated. The total numbers of specific bacteria in the crushed materials, as well as their specific growth characteristics (resistance properties and toluene degradation) were determined on different selective media (Table 3.1). The number of CFUs (colony forming units) was calculated per gram of fresh weight of roots or shoots. We

found that three weeks after inoculation, all three *B. cepacia* strains could be isolated from yellow lupine. For control plants without inoculum, no bacteria were found on the selective media, except on nonselective medium (284+glucose), demonstrating that despite surface sterilization of the seeds, endogenous endophytic bacteria remained in the plants.

Inoculum[a]	Plant part	284+gluc[b]	284+Ni+Km +gluc[b]	284+Ni+Km+tol[b]	284+tol[b]
No	Shoot	$2.3 \cdot 10^2$ (2)[c]	0	0	0
No	Root	$1.7 \cdot 10^3$ (3)	0	0	0
VM1330	Shoot	$6.9 \cdot 10^3$ (1)	$3.8 \cdot 10^2$(1)	$5.8 \cdot 10^2$(1)	$4.3 \cdot 10^2$(1)
VM1330	Root	$9.5 \cdot 10^3$ (1)	$2.2 \cdot 10^2$(1)	$1.7 \cdot 10^2$(1)	$1.9 \cdot 10^2$(1)
BU0072	Shoot	$1.3 \cdot 10^4$ (2)	$2.2 \cdot 10^2$(1)	0	0
BU0072	Root	$1.5 \cdot 10^3$ (2)	$1.5 \cdot 10^3$(1)	0	0
G4	Shoot	$5.7 \cdot 10^4$(2)	0	0	$1.0 \cdot 10^4$(1)
G4	Root	$7.8 \cdot 10^4$ (2)	0	0	$1.5 \cdot 10^2$(1)

Table 3: Growth characteristics and number of bacterial colonies isolated from roots and shoots of *L. luteus* plants. [a]*L. luteus* plants were inoculated with *B. cepacia* strains VM1330, BU0072 and G4. As controls, plants without inoculum were analysed. [b]Ni: 1 mM nickel; Km: 100µg/ml kanamycin; tol: toluene vapor as C-source; gluc: gluconate as C-source. [c]The number of bacteria was determined 21 d after inoculation. The number of bacteria is expressed per g fresh weight. Data are the average of three experiments. Numbers in parentheses are the numbers of different morphological types of bacteria as observed visually.

As expected, bacteria isolated from the shoots and roots of yellow lupine inoculated with VM1330 (Km^R, Ni^R, Tol^+) grew on all three selective media. Plants inoculated with BU0072 (Km^R, Ni^R, Tol^-) harboured no toluene-degrading bacteria. From plants inoculated with strain G4 (Tol^+), isolated bacteria grew on a medium containing toluene, but only in the absence of nickel and kanamycin.

Using Repetitive Extragenic Palindromic (REP)-PCR we demonstrated that the bacteria isolated on the selective media had the same genetic finger printings as *B. cepacia* G4, BU0072 and VM1330, respectively (results not shown). In addition, the presence of *nre* (strain BU0072), pTOM (strain G4), and both *nre* and pTOM (strain VM1330) was determined by PCR, confirming that the three strains had

colonized the plants. Bacteria isolated from the control plants showed a different REP-PCR pattern from strains BU0072, G4 and VM1330. These endophytic bacteria, which were also found after inoculation with *B. cepacia* BU0072 and G4, were further characterized and found to belong to typical endophytic and rhizosphere-colonizing species. Based on their REP-PCR patterns, five distinct species were found and these were identified by the sequence of their 16S rDNA: (i) *Paenibacillus* sp., a typical plant-associated, nitrogen-fixing bacterium of the rhizosphere, which has been found as an endophyte in plants as diverse as pine and potato (Reiter *et al.*, 2003; Bent and Chanway, 2002); (ii) *Bacillus megaterium*, (iii) *Pantoea* sp. and (iv) a *Pseudomonas* sp., which all have been reported to be endophytic bacteria in pea (Elvira-Recuenco and van Vuurde, 2000), a member of the Fabaceae, like yellow lupine; and (v) a *Bacillus novalis* sp. nov., which was recently isolated form the Drentse A grasslands (Heyrman *et al.*, 2004). None of these bacteria was able to grow on toluene as a carbon source.

3.3.2 Selective enrichment of endophytic bacteria by toluene

We investigated how the presence and concentration of toluene affected colonization by the bacteria. Earlier results had indicated that growing plants on contaminated soil could selectively enhance the prevalence of endophytes containing the degradation pathways for specific pollutants (Siciliano *et al.*, 2001). After 21 days of growth without toluene, the plants were transferred to glass cuvettes containing toluene in the lower compartment at final concentrations of 0, 100, 500 and 1000mg l^{-1}. After 96 h the plants were harvested, and their shoots and roots examined for endophytic colonization. Samples and dilutions of 100µl were plated on different media. We could not discern a clear effect of the toluene concentrations on the efficiency of colonization by different endophytic strains, and therefore, differences in the toluene-degradation properties of the inoculated plants will not reflect

differences in the density of the endophytic population. An average of 10^5-10^6 CFU per gram fresh weight was found, and as in the experiment described in Table 3.1, growth on selective media was observed only after plants had been inoculated with the appropriate strains. Strain G4 was, in general, the most efficient colonizer of yellow lupine (10^6 CFU per gram fresh weight compared to 10^5 CFU for BU0072 and VM1330). In addition, the same endogenous endophytic bacteria were found as in the control plants after surface sterilization.

3.3.3 Effect on plant growth during hydroponic cultivation

We examined the effect of toluene on the hydroponic growth of plants inoculated with *B. cepacia* VM1330, BU0072 and G4 and compared it with the effect on non-inoculated controls. The experiments were carried out in the glass cuvette system schematically presented in Figure 3.1. The growth indexes were calculated as the difference in plant's fresh weight between the onset of the experiment and after 96h exposure to different concentrations of toluene. In the absence of toluene, plants inoculated with *B. cepacia* G4 produced significantly (P=0.05) less biomass than the control plants and those inoculated with *B. cepacia* BU0072 and VM1330 (Figure 3.2). This indicates that high numbers of the environmental *B. cepacia* G4 strain, which is not known to be a natural endophyte of yellow lupine, has a negative effect on plant development.

For plants and bacteria incubated in the presence of toluene, the growth indices suggested that increasing levels of toluene resulted in greater phytotoxicity (Fig. 3.2). However, plants inoculated with the endophytic strain *B. cepacia* VM1330, which can efficiently metabolize toluene, showed no difference in growth compared to controls without toluene. Even at concentrations of 1000mg l^{-1} toluene in the growth medium, these plants showed no signs of phytotoxicity, in contrast to the control plants that experienced phytotoxicity at levels above 100mg l^{-1}. This shows that strain VM1330 efficiently assists its host plant in

overcoming toluene's phytotoxicity. Plants inoculated with *B. cepacia* BU0072 and G4 show phytotoxicity to toluene that is similar to that of the non-inoculated control plants, suggesting that the combination of natural endophytic behaviour plus toluene degradation capacity is required to protect the plant. The statistical significance of the results was confirmed at 5% level using a two-way ANOVA model, separately exploring treatment (bacterial inocula) and toluene doses.

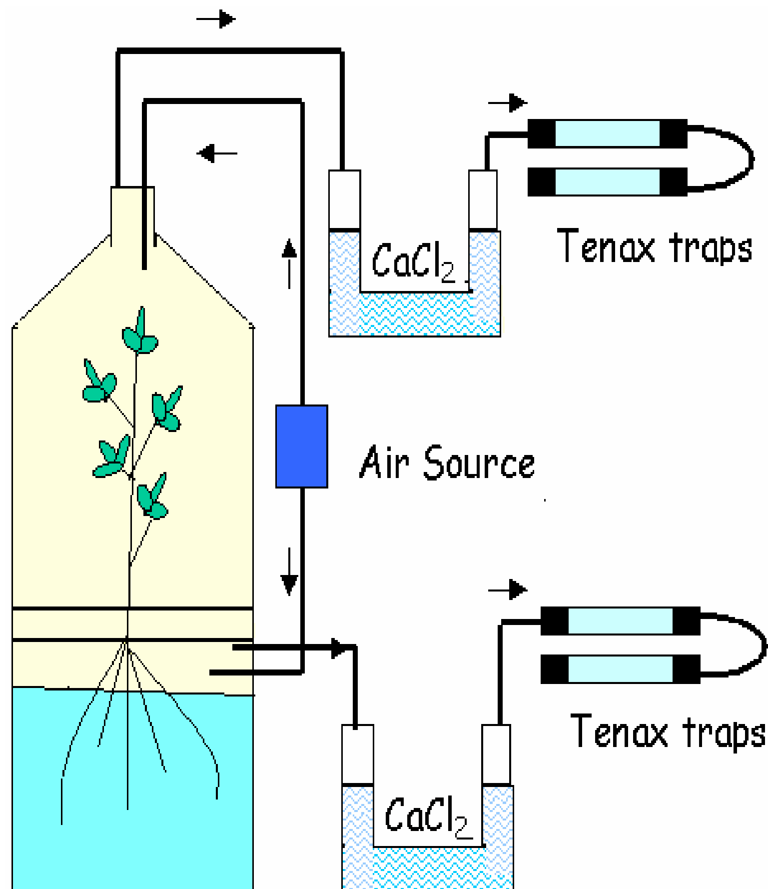


Figure 3.1: Schematic representation of the experimental setup for measuring toluene evapotranspiration. Yellow lupine plants are grown as hydroponics in a two-compartment glass cuvette system. Continuous airflow allows toluene from the gas phases from both compartments to collect on Tenax traps. A $CaCl_2$-filled column was installed to prevent condensation of water in the Tenax traps. Tenax traps were cooled with dry ice to optimize adsorption capacities..

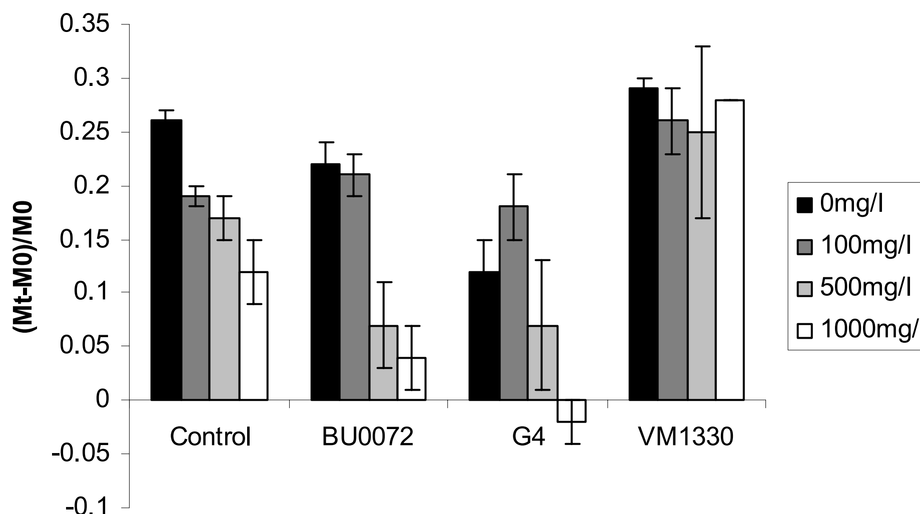


Figure 3.2 Difference in biomass (g) between inoculated and control yellow lupine plants, before and after adding toluene. Plants were grown as hydroponics in the glass cuvette system described in Figure 3.1. Non-inoculated control plants and lupine plants inoculated with *B. cepacia* strains VM1330, BU0072 and G4 were used. M0, plant weight (g) before addition of toluene; Mt, plant weight (g) after 96h after toluene addition. The concentrations of toluene were 0, 100, 500 and 1000 mg/l, respectively. Standard deviations are indicated as bars. The statistical significance of the results was confirmed at the 5% level using a two-way ANOVA model, separately exploring treatment (bacterial inocula) and toluene doses.

3.3.4 Toluene degradation and evapotranspiration

After adding toluene at a sub-phytotoxic concentration of 100 mg l^{-1}, we measured the amount of toluene that is evapotranspirated through the aerial parts of the plant (upper compartment) as well as its disappearance from Hoagland’s nutrient solution (lower compartment) using gas chromatography/mass spectrometry (GC-MS) (Figure 3.3).

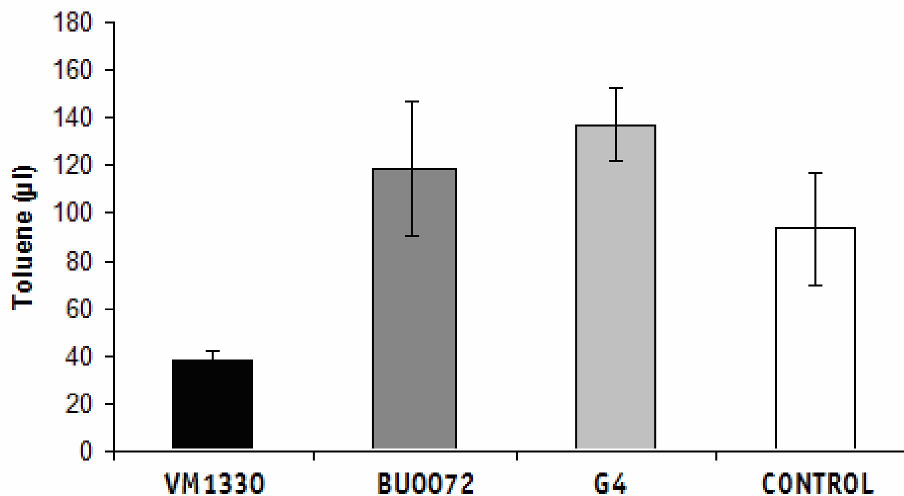


Figure 3.3 Total amount of toluene (µg) detected in Tenax traps connected with the upper compartment (containing the aerial part of L. luteus plant) determined by GC-MS. For this experiment, non-inoculated control plants and lupine plants inoculated with *B. cepacia* strains VM1330, BU0072 and G4 were used. The statistical significance of the results was confirmed at the 5% level using a one-way ANOVA model.

Compared to control plants and plants inoculated with *B. cepacia* BU0072 or G4, those inoculated with *B. cepacia* VM1330 released 50-70% less toluene in the upper compartment (Fig. 3.3). This result shows this toluene-degrading endophytic strain not only protects its host plant against phytotoxicity, but also significantly (P=0.05) lowers toluene evapotranspiration through the aerial parts, even at levels that are not toxic to control plants. No significant (P=0.05) differences in the concentrations of evapotranspirated toluene were observed between plants inoculated with BU0072 or G4, and the non-inoculated controls. The statistical significance of the reduced toluene release in the presence of strain VM1330 was confirmed at 5% level using a one-way ANOVA model.

To further examine the fate of the toluene, we also analyzed the amount of that evaporated from the Hoagland's solution in the gasphase of the lower compartment. The smallest amount of evaporated toluene, 2.523 (±853) µg, was obtained from plants inoculated with *B. cepacia* VM1330, compared to 3.378

(±987)µg, 4.362 (±733)µg and 7.367 (±298)µg for the control plants and the plants inoculated with BU0072 or G4, respectively. These results show that together the endophytic strain *B. cepacia* VM1330 and its host plant, yellow lupine, improve the degradation of toluene, lowering both its phytotoxicity and release by evapotranspiration.

3.3.5 Effect on plant growth during greenhouse studies

We further examined the protective effect of the endophytic strain *B. cepacia* VM1330 on its host plant in greenhouse studies growing the plants on a nonsterile sandy soil. It is clear that control plants suffer from phytotoxic effects when irrigated for two weeks with water that contains toluene at a concentration of 100mg l^{-1}, and that they cannot survive higher toluene concentrations (Figs. 3.4 and 3.5).

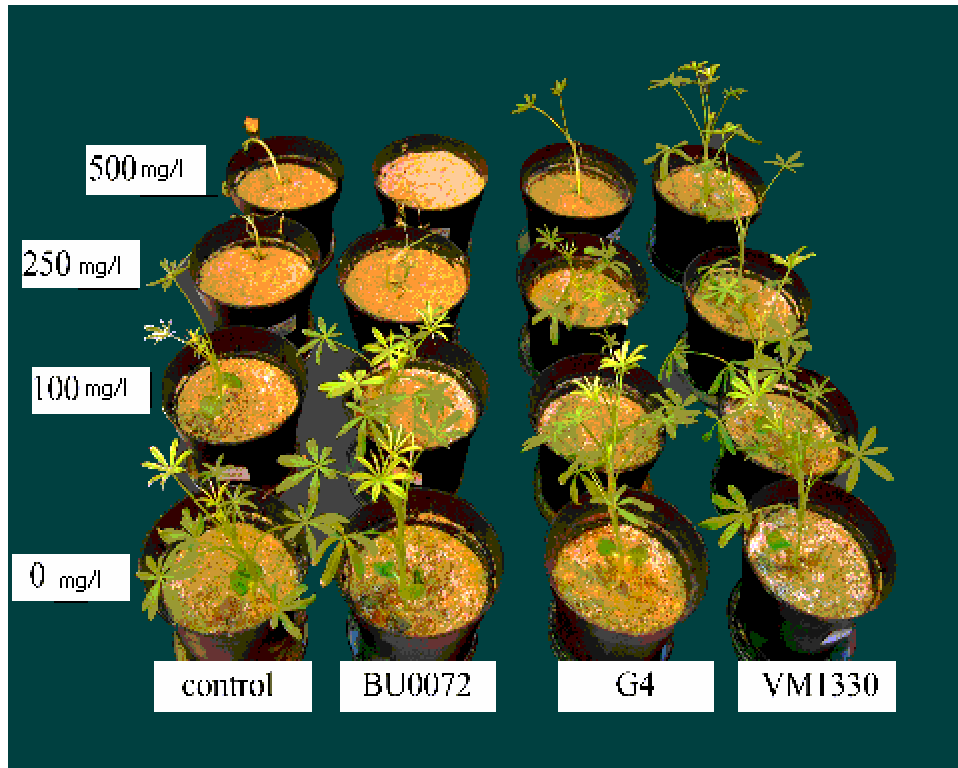


Figure 3.4: Phytotoxic effect of toluene on yellow lupine plants in non-sterile sandy soil under the greenhouse conditions. The labels indicate the control plants and lupine plants inoculated with *B. cepacia* strains VM1330, BU0072 and G4. Plants were irrigated every other day with half-strength Hoaglad's solution to which toluene was added in concentrations of 0, 100, 250 and 500mg l^{-1}. A picture of representative plants was taken after 14 days of irrigation with toluene containing solutions.

Inoculation of yellow lupine with *B. cepacia* BU0072 had a slightly positive effect, as it reduced toluene phytotoxicity in the group treated with 100mg/l. Plants exposed to the higher toluene concentrations died within one week. Inoculation with toluene-degrading *B. cepacia* G4 strain resulted in a partial reduction of toluene phytotoxicity, which can be explained by degradation of toluene in the rhizosphere

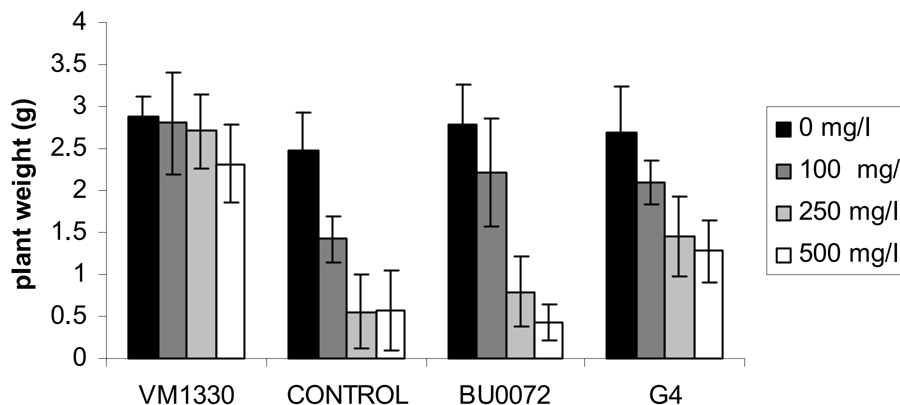


Figure 3.5: Biomass (g) of yellow lupine plants, grown in nonsterile sandy soil under the greenhouse conditions, after 14 days exposure to different toluene concentrations. For this experiment, noninoculated control plants and lupine plants inoculated with *B. cepacia* strains VM1330, BU0072 and G4 were used. Plants were irrigated every other day with half-strength Hoagland's solution to which toluene was added at concentrations of 0, 100, 250 and 500mg l^{-1}. Standard deviations are indicated as bars. The statistical significance of the results was confirmed at the 5% level using a two-way ANOVA model, separately exploring treatment (bacterial inocula) and toluene doses.

A clear protective effect of *B. cepacia* VM1330 was observed on the growth of yellow lupine at all toluene concentrations tested, including the 500mg l^{-1} doses. The statistical significance of the results was confirmed at the 5% level using a two-way ANOVA mode, separately exploring treatment (bacterial inocula) and toluene doses. These results confirm our hypothesis that endophytic bacteria, when equipped with the appropriate degradation pathway, can help plants survive under conditions of elevated levels of toluene.

3.4 DISCUSSION AND CONCLUSIONS

In this chapter we describe how an engineered endophytic bacterium can improve the phytoremediation of an organic contaminant. We demonstrate that an endophytic bacterium equipped with the appropriate degradation pathway not only protects its host plant against the phytotoxic effect of an environmental contaminant, but also improves the overall degradation of contaminant resulting in its decreased evapotranspiration to the environment.

The protective effect of the endophytic bacteria was demonstrated for plants grown either as hydroponics or in non-sterile soil under greenhouse conditions. The results also show that the presence of *B. cepacia* VM1330, a toluene-degrading endophyte, provides much better protection against the phytotoxic effects of toluene that obtained when the rhizosphere of the plant is inoculated with *B. cepacia* G4, a toluene degrading soil bacterium (Figs 3.4 and 3.5).

The introduction of the pTOM plasmid into *B. cepacia* BU0072, which resulted in strain *B. cepacia* VM1330, was done through natural gene transfer (conjugation). Although for practical purposes we used a marked recipient strain, which allows us to monitor the colonization of yellow lupine, this experiment can easily be repeated using a naturally-occurring endophytic recipient, such a *B. cepacia* L.S.2.4, and an autotrophic mutant of *B. cepacia* G4. Accordingly, the transconjugants obtained by conjugation using a naturally occurring endophytic recipient should not be considered genetically modified microorganisms (GMO), a factor that may facilitate their application and public acceptance. It will be relatively straightforward to construct, by natural gene transfer, a collection of endophytic bacteria with *a la carte* degradation properties because many endophytic bacteria are closely related to environmental strains that carry degradation pathways for a broad spectrum of organic xenobiotics on mobile DNA elements. For example, we are constructing derivates of *B. cepacia* L.S.2.4 that constitutively express *tom*A toluene-ortho-monooxygenase of pTOM (Sharp *et al*.,

1998; Shields and Reagin, 1992). This strain will be applied to improve the phytoremediation-based containment of TCE-contaminated groundwater plumes. Other applications we envisage include the use of engineered endophytic bacteria to degrade pesticide and herbicide residues in crop plant, addressing important food safety related issues.

The next step will be to expand and apply the work at various levels to poplar (*Populus* sp.) and willow (*Salix* sp.) to phytoremediate groundwater contaminated with water-soluble organics. A collection of approximately 150 different endophytic bacteria from poplar and willow is available, and these strains have been identified and characterized (Chapter 5).

We have also shown the inoculation of poplar with a *gfp*-marked endophytic *Pseudomonas* sp., demonstrating the generalizability of the approach (Germaine *et al.*, 2004; Chapter 6). We can reasonably hypothesize that endophytic bacteria, possessing the genetic information required to efficiently degrade an organic contaminant, promote its breakdown as it moves through the plant's vascular system. In trees, the time between the uptake of the pollutant by the roots and its arrival in the leaves can take several hours to days (McCrady *et al.*, 1987), allowing sufficient time for efficient degradation by endophytic bacteria in the xylem.

We expect that the application of genetically engineered endophytic bacteria will become a general strategy to improve the efficiency of phytoremediating volatile organic contaminants and other water soluble organic xenobiotics, and that the technique will gain regulatory and public acceptance.

CHAPTER IV

POTENTIAL OF POPLAR CELL CULTURES TO DEGRADE TOLUENE

ABSTRACT

Toluene degradation was investigated in cells suspension cultures of poplar (*Populus trichocarpa* x *deltoides* cv. “Hoogvorst”). To distinguish between degradation, volatilization and/or absorption of toluene to the cell walls, living cell suspensions, dead cell suspensions, as well as a MS (Murashige and Skoog) liquid medium control were used. The process was followed over 96 hours, during which toluene concentrations were measured by Head space GC MS (Gas Chromatograph, Mass Spectrometer). For all three conditions toluene completely disappeared from the medium within 24 hours, however, no differences in the kinetics of disappearance of toluene were observed between the living cells, dead cells or medium only, indicating that poplar cell cultures were unable to efficiently degrade toluene.

3.1 INTRODUCTION

To study the interactions of organic contaminants such as BTEX with a system as complex as a poplar tree, with its associated microorganisms, and to obtain information about the toluene degrading capacity of the plant cells, it might be relevant to use tissue and cell culture techniques in order to combine part of the biological complexity of the intact organism with a degree of experimental control that may not be obtained *in vivo*. Primarily, the environmental conditions (temperature, light, medium composition) can be well controlled and as microbial

activity is excluded from these sterile cultures, any metabolic activity measured should be derived from plant cell activity (Nepovim *et al.*, 2004). Secondly, biological variability can be well controlled and the use of genetically defined clones of cells may simplify the analysis of experimental data. Conversely, results obtained with specialized cell-based systems might be unrepresentative for a broader range of cell types and may be more difficult to interpret in terms of the entire plant. Freshly isolated cells (primary culture) are more likely to reflect biochemical activities of cells *in vivo*, whereas the results obtained with continuous cell lines are more reproducible. In the cell suspension cultures the pollutant will be homogenously distributed in the growth medium allowing each cell to be in contact with the contaminant. The results obtained in cell culture experiments can, to some degree, be extended to whole plants, because it is generally known that the spectrum of resulting metabolites in plants and plant cell cultures is in principle identical, although quantitative differences may occur (Harms, 1992).

3.2 MATERIAL AND METHODS

3.2.1 Physiological requirements for plant cell growth

Nutritive requirements

The composition of the culture medium is an important factor for a successful establishment of a tissue culture. MS medium (Murashige and Skoog, 1962) is a balanced salt mixture of the principal inorganic ions, plus 7 trace element compounds, 3 vitamins, inositol, glycine and sucrose as the major carbon source.

Macro-nutrients: (chemical elements required in large amounts by living organisms for proper growth and development, being major constituents of living

matter, including carbon, nitrogen, oxygen, phosphorus and calcium) are supplied at millimolar concentrations. Per liter medium content: 332.02 mg $CaCl_2$, 170 mg $KHPO_4$, 1990 mg KNO_3, 180.54 mg $MgSO_4$ and 1650 mg NH_4NO_3. Nitrogen is usually applied as NO_3^- or NH_4^+ or both. Plant cells can grow in a medium containing exclusively nitrate as a nitrogen source, while NH_4^+ in excess may be toxic.

Micro-nutrients: (chemical elements or organic compounds required in low amounts by living organisms for proper growth and development, e.g. trace elements such as zinc, iron, copper are supplied at micromolar concentrations) per liter medium: 0.025 mg $CoCl_2 \cdot 6H_2O$, 0.025 mg $CuSO_4 \cdot 5H_2O$, 36.7 mg FeNaEDTA, 6.2 mg H_3BO_3, 0.83 mg Kl, 16.9 mg $MnSO_4 \cdot H_2O$, 0.25 mg $NaMoO_4 \cdot 2H_2O$, 8.6 mg $ZnSO_4 \cdot 7H_2O$. Iron is furnished as a chelated compound, usually with EDTA.

The macro- and micro-elements present in plant tissue culture media can be used by plant cell as building blocks for the synthesis of organic molecules, or as catalysts in enzymatic reactions. The ions of the dissolved salts play an important role as counterion in the transport of ionized molecules by the plant, in the osmotic regulation and in maintaining the electrochemical potential of the plant. Nitrogen, sulphur and phosphorus are components of proteins and nucleic acids. Magnesium and many microelements form essential parts of enzymes and cell organelles and are therefore important in the catalyzation of various reactions. Calcium and boric acid are mainly found in the cell wall and especially calcium has an important task in the stabilization of biomembranes. Potassium and chloride, on the contrary, are important in the osmotic regulation, for maintenance of the electrochemical potential and for the activation of a large number of enzymes.

Carbon source: sucrose is one of the most readily assimilated carbon sources. When all the sucrose added to the medium is consumed, cells hydrolyze their endogenous stock of carbohydrates. Vacuolar sucrose is first consumed. When 60-70% of starch is hydrolyzed, the cell respiration rates decline concomitantly with autolysis of portions of cytoplasm, accompanied by a decline in the number of mitochondria per cell and by the accumulation of phosphorylcholine in the cytoplasm. The effects of a sucrose starvation during 2-4 days are reversed within a few hours by addition of sucrose. In this medium 30 g per liter sucrose is added.

Vitamins: Gamborg B5 (Gamborg *et al.*, 1968): myoinositol (100 mg l^{-1}) (cyclic hexahydric alcohol, occurring in various forms, of which myo-inostol is the most important; it is a constituent of phospho-lipids and also of phytic acid and phytin in plants); nicotinic acid (0.5 mg l^{-1}) (niacin, a member of the vitamin B complex, vitamin B7, found in all living cells as the nicotinamide moiety of the enzyme cofactors NAD and NADP. Blood, liver, legumes and yeast are particularly rich sources); pyridoxine HCl (0.5 mg l^{-1}) (a form of vitamin B6, a phenolic alcohol derived from pyridine. It is a precursor to the coenzyme pyridoxal phosphate); thiamine HCl (0.1 mg l^{-1}) (vitamin B1, water-soluble vitamin, a member of the vitamin B complex, found especially in embryos and yeast. It is a precursor of the coenzyme thiamine pyrophosphate required for carbohydrate metabolism).

Phytohormones: 2.4-dichlorophenoxyacetic acid (1125 µg l^{-1}) (auxins are involved in cellular elongation and differentiation, in root growth, the development of vascular tissue, phototropism, the development of fruits and the normal suppression of the growth of lateral buds) and kinetine (1075 µg l^{-1}) (cytokinines act in concert with auxin to promote rapid cell division. They are used to induce the formation of plantlets from callus tissue in culture). Auxins and cytokinins

are two phytohormones controlling cell division in plants. The ratio auxin/cytokinine determines differentiation of the cells into different plant organs. If the ratio auxin/cytokinine is high, root formation will be induced. When the ratio is low, shoot formation will be favored, whereas both auxins and cytokinines are supplied at high concentrations the newly formed cells will not differentiate and callus will be formed (Skoog and Miller, 1957).
Antimicrobial agents: amfotericine B, added in concentration 250 µg l^{-1}, acts by binding to steroidal alcohols (specifically ergosterol) in the cell membrane of susceptible fungal, algal, protozoal and animal cells resulting in an increase of membrane permeability that allows leakage of such essential components as glucose and potassium.

3.2.2 Physical factors

pH: initially the pH of freshly prepared medium is acidic (5.9). It must be noted that there is a pH drop of 0.5 units after autoclaving. During the culture cycle, pH values vary from 5.5 to 7 because culture media are poorly buffered. These variations are attributed to the assimilation of NO_3^- and NH_4^+.

Temperature: the cell cultures are maintained in climatized room at a temperature of 22°C (+/- 2°C).

Oxygenation: the cell suspension cultures are conveniently oxygenated by using platform shakers with orbital movement (60-120 rpm). Movement of the liquid medium facilitates fragmentation of tissue leading to smaller units and promotes gaseous exchange.

Light: at atmospheric CO_2 concentrations the light compensation point is about 5-10 µmol photons $m^{-2}s^{-1}$ and photosynthesis is usually saturated between 500 and

2000 µmol $m^{-2}s^{-1}$, depending on plant type. In our cell cultures the axenic tissue cultures were incubated under 12 x 18W light tubes (6 x blue and 6 x red) separated 20cm one from another, with photosynthetic active radiation 165 µmol$m^{-2}s^{-1}$.

3.2.3 Aseptic preparation of plant material for culture

The tissue cultures from poplar, *Populus trichocarpa* x *deltoides* var. “Hoogvorst”, were established by growing explants of surface sterilized leafs on the surface of an agar-based MS medium. Initial sterilization was achieved by washing the leaf in a saturated $Ca(OCl)_2$ solution during 10 minutes. Following, leaves were rinsed in sterile distilled water, dried on sterile filter paper and sterile cutted in a patch’s of approximately 1cm^2. Leaf explants were placed on an agar-based MS medium and incubated at 22°C, under 16/8 day/light conditions until callus tissue was grown from the wounded plant tissue.

Cell suspension cultures were obtained by transferring fragments of the growing callus to a sterile Erlenmeyer flask (250ml) containing liquid MS medium (50ml). These flasks were sealed with cellulose stopper and shaken for 7 days on a rotary shaker (80rpm) at 22°C, under 16/8 day/light conditions. The gentle agitation causes fragmentation of the callus tissue into individual cells and cell aggregations. It is rare for plant suspension cultures to be entirely unicellular, due to the presence of plasmodesmata and cell walls.

After 7 days the cell suspension was sieved through a 500µm (40 mesh) sieve, into a sterile measuring cylinder and left to precipitate for 10 minutes. Supernatant was removed and the recipient refilled with MS medium untill a volume of 50ml was reached. Subsequently, the cell suspension was transferred to sterile Erlenmeyer flasks and incubated at the same conditions. This procedure was repeated 3 times. Cell suspension cultures often require a minimum inoculum size on subculture. An inoculum volume of 10% v/v (10% v/v: 10% cell

suspension up to 100 ml medium; 10% w/v 10g cells in 100ml medium) may be necessary to ensure successful subcultures. It may also be helpful to add a small amount of "conditioned" medium from a previous culture to permit cell division. This could be due to unknown compounds secreted by the cells or to compounds present at toxic concentrations in fresh culture media containing cells at very low density (threshold 10^3cell/ml). To exclude any microbial contamination all handling was performed in a laminar flow under sterile conditions. Each time when cell suspensions were subcultured, 100µl of culture was plated on 869 medium (Mergeay *et al.*, 1985) as a sterility control.

Cell culture in liquid medium avoids some of the disadvantages inherent to tissue cultures on agar. Movement of cells in the liquid medium facilitates gas exchange, suppresses any polarity due to gravity and eliminates the gradients of nutrients within the medium and from cell to cell (Bligny and Leguay, 1987).

3.2.4 Toluene toxicity for poplar cell culture

Before measuring the toxicity of toluene for poplar cells the exponential growth phase was determined. After subculturing, 100µl of cell culture suspension was sampled during 8 days, always at the same time. 100µl 0,8M manitol, 2% cellulase and 0.2% pectolyase were added to an eppendorf tube with cell suspension in order to separate cells from each order. After an incubation period of 30 minutes at 30°C, 0.4% trypan blue was added to the cell suspension and carefully mixed. 10µl was placed in a Fuchs-Rosenthal counting chamber (hemacytometer) and living and dead cells were counted (trypan blue colors only dead cells) using a light microscope.

To determine the effect of different toluene concentrations on culture growth, 48 hours after subculturing in Erlenmeyer flasks filled with 50ml cells suspension, toluene was added in concentration of 0, 50, 100, 150, 200 and 250mg l^{-1}. The Erlenmeyer flasks were closed with cellulose stops and placed on a rotary shaker

(80 rpm), temperature 22°C and under 16/8 day/light conditions. Cell culture samples were taken immediately and after 24 hours. Samples were treated as described earlier and after counting of dead and living cells, the percent of viability was calculated (% viability = number of living cells / number of total cell number). For each concentration three replicates were used.

3.2.5 Degradation of toluene by plant cells

During this experiment the volatilization of toluene from the medium to the gas phase as well adsorption to the cell walls was taken into account. Volatilization of toluene was determined by using only MS medium, while the adsorption of toluene to the cells was accounted by analysing a dead cell suspension (cell suspension autoclaved during 1 minute at 121°C and 15psi). Living cell suspensions were used to determine metabolizaton of toluene. In 9 sterile Erlenmeyer flasks (3 with 50ml MS medium, 3 with 50ml medium + 48 hours old dead cells and 3 with 50ml medium + 48 hours old living cells) toluene was added in a concentration of 100mg l^{-1}. Erlenmeyers were sealed with sterile cellulose stops and placed on a rotary shaker (80rpm). One milliliter of samples each were taken after 5′, 30′, 1, 2, 3, 4, 5, 6, 9, 24, 31, 48, 55, 72 and 96 hours and placed in 20ml headspace vials (Alltech Art. Nr. 6636), closed with a 20mm septum and fixed with 20mm aluminium caps. To each sample 20µl (2%) of 80% H_3PO_4 was added in order to stop all possible enzymatic reactions. 10µg of the d6-benzene (Janssen Chimica) was added as internal standard in each vial using a Hamilton syringe. Samples were analyzed by headspace -GC-MS (Headspace apparatus HS40XL, Auto System XL Gas Chromatograph, Turbo Mass Spectrometer, Perkin Elmer).

4.3 RESULTS

4.3.1 Toxicity of toluene for poplar plant cells

The increase in cell material in the cell cultures of poplar (cv. "Hoogvorst") reaches a saturization level after 48 hours, beyond which no further increase in biomass occurs. If such a saturated cell suspension is subcultured by diluting it back to the initial cell content, the same growth cycle is repeated.

The viability of the cells exposed to toluene can be observed in function of concentrations (Figure 4.1). The addition of toluene at concentration higher than 100 mg l^{-1} has significant inhibiting effects on the growth of poplar cells cultivated in liquid MS medium. The LD50 which is the concentration of chemical compounds that causes death of 50% of organisms tested in certain period of time (Newman *et al*., 1997), was found to be around 175 mg l^{-1} toluene for cells under conditions used in our experimental setup (Figure 4.1). 100 mg l^{-1} of toluene was chosen for further degradation experiments because this concentration affects the growth of the poplar cell culture, but with a toxicity effect that is less then LD50 value.

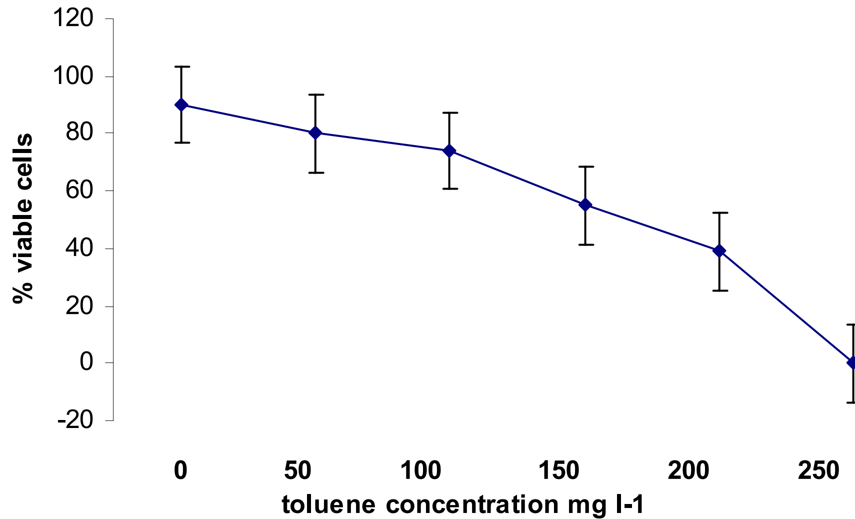


Figure 4.1: Viability of the poplar cells exposed during 24 hours to toluene concentrations of 0, 50, 100, 150, 200 and 250 mg l^{-1}. % living cells (viability = number of living cells / total cell number x 100). All experiments were performed in triplicate; Standard error indicated as bars.

4.3.2 Toluene degradation by a poplar cell culture

To explore if poplar cells possess an active metabolic route to counteract toluene phytotoxicity, we examined the disappearance of 100 mg l^{-1} toluene from the active cell culture. In addition, dead cells and medium controls were used to account for abiotic factors assisting in the removal of toluene from cell culture. No significant differences were found between volatilization (medium only), absorption (dead cells) and degradation (living cells). The results presented in Figure 4.2 indicated that a poplar cell cultures were unable to significantly degrade toluene under the conditions tested.

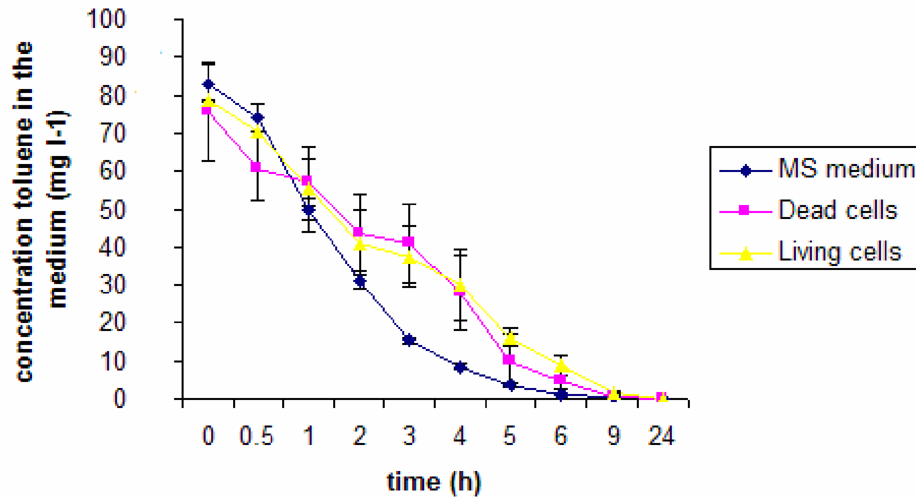


Figure 4.2: Degradation of toluene in living cell suspension (enzyme reaction), dead cell suspension (absorption) and in medium only (volatilization).

4.4 CONCLUSIONS

Results obtained in the present study show that toluene has an inhibiting effect on the growth of poplar cells (Figure 4.1) with an LD50 value of 175 mg l^{-1} toluene. At the same time it was not possible to observe any stimulating effect of living cells on the disappearance of toluene from the medium. This suggests that poplar cells do not possess the capacity to degrade toluene. Twenty four hours after addition all toluene disappeared from the medium but it was not possible to detect any difference between the media containing living cells and media with only dead cells and MS medium without cells (Figure 4.2).

Therefore we concluded that poplar trees can take up and transport toluene (described and modeled by Trapp *et al.*, 1990) from the roots to the shoots, but that during this transport process no significant *in planta* degradation of toluene

occurs. Since mass balance analysis of toluene on intact plants indicates that toluene degradation is occurring in the plant, this in addition to volatilization via the stomata and the stem lenticels and location to plant biomass, we hypothesize that also in poplar endogenous endophytic bacteria play a significant role in the observed toluene degradation. A similar observation was made by Siciliano *et al*. (2001) who showed that the percentage of endophytic bacteria able to degrade specific organic compounds significantly increased when plants were grown in an environment contaminated with this compound.

CHAPTER V
ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF ENDOPHYTIC BACTERIA FROM *POPULUS TRICHOCARPA* X *DELTOIDES*

Most of this chapter is part of the manuscript "Endophytic bacterial diversity in poplar trees growing on a BTEX contaminated site and the identification of isolates with potential to enhance phytoremediation" by Porteous Moore F, Barac T, Borremans B, Oeyen L, Vangronsveld J, van der Lelie D, Campbell CD and Moore ERB submitted for Plant Soil.

ABSTRACT

Endophytic bacteria were isolated from the root, stem and leaf of two cultivars of *Populus trichocarpa* x *deltoides* growing on a site contaminated with BTEX compounds. After isolation these bacteria were characterized genotypically by comparative sequence analysis of partial 16S rRNA genes and BOX-PCR genomic DNA fingerprinting, and phenotypically by the tolerance to a range of target pollutants, heavy metals and antibiotics. One hundred and forty six stable, morphologically-distinct rhizosphere and endophytic isolates were obtained, belonging to twenty one different genera; 6 isolates could not be identified with confidence to a genus. The endophytic bacteria exhibited marked spatial compartmentalization within the plant suggesting the existence of species-specific and non-specific associations between bacteria and plants. A number of isolates demonstrated the ability to degrade BTEX compounds, or to grow in the presence of TCE. This endophyte bacterial diversity was investigated as part of a larger study to assess the possibility and practicality of using endophytic bacteria to enhance *in situ* phytoremediation. Our study has demonstrated that within the diverse bacterial communities found in poplar trees there are several endophytes which are worth further investigation concerning mechanisms of bacterial-plant interaction, re-colonisation potential and degradation kinetics, could potentially be used to improve phytoremediation strategies.

5.1 INTRODUCTION

Bacteria have been known to reside within plant tissue for over 50 years (Tervet and Hollis, 1948; Hollis 1951). Historically thought to have been pathogenic, many have recently been discovered to have beneficial effects on their host plant (Davison 1988; Hoflish *et al.*, 1994; Lodewyckx *et al.*, 2002b) or to have no observable effects (Kado, 1992). These bacterial endophytes are known to reside in the shoots, roots and leaves of a wide range of plants. Bacteria from 82 genera have been found in a broad range of plants (Lodewyckx *et al.*, 2002b) including both woody plants (Brooks *et al.*, 1994) and arable crops (Ficher *et al.*, 1992; McInroy and Kloeper, 1995).

Endophytic bacteria may act as plant growth promoters (Kloepper *et al.*, 1991; Holfich *et al*, 1994) by fixing atmospheric nitrogen (Davison, 1988), sequestering iron from the soil (Kloepper *et al.*, 1986) and synthesizing phytohormones and enzymes (Lambert and Joos, 1989). They have also been shown to exhibit strong anti-fungal activity (Brooks *et al.*, 1994); Hinton and Bacon 1995; Mukhopadhyay *et al.*, 1996), antagonize bacterial pathogens (Van Buren *et al.*, 1993) and control plant parasitic nematodes (Hallmann *et al.*, 1995).

Endophytic bacteria have been used for biotechnology applications such as plant-growth promotion or enhanced pest control (Hallmann *et al.*, 1995) but have only recently been considered in relation to degradative capacity (Siciliano *et al.*, 2001; van der Lelie *et al.*, 2001; Barac *et al.*, 2004, chapter 3). Phytoremediation is an emerging bioremediation technology encompassing the use of plants to stabilize or clean contaminated soil (Burd *et al.*, 1998) and is gradually becoming accepted as a commercial alternative to the more conventional physico-chemical and civil-engineering methods. Phytoremediation of organic contaminants is, however, still limited for certain classes of compounds that are either phytotoxic (e.g. phenols and chlorophenols: Pfleeger 1991; TNT and dinitrotoluene Thompson *et al.*, 1998) or that are volatilized through the plant (e.g. MTBE,

BTEX, TCE: Trapp *et al.*, 1994). The concept of using endophytic bacteria to enhance phytoremediation by actively degrading pollutant compounds as they are translocated through the plant has been proposed relatively recently (van der Lelie *et al.*, 2001). Enhanced *in planta* degradation could potentially reduce phytotoxicity, enhance rates of pollutant uptake and removal, as well reduce volatile losses to the atmosphere (Barac *et al.*, 2004, Chapter 3). A primary consideration for the success of such applications is characterization and selection of suitable endophytic bacteria, in appropriate candidate plants. Quantifying the diversity, distribution, physiology and ecology of endophytes is also a prerequisite to determine the potential of endophytes for phytoremediation applications. Furthermore, potential to enhance degradation by introducing selected endophytic degrader bacteria, requires that cultivable species need to be isolated and characterized to ensure they are appropriate and do not carry risks such as pathogenicity. The majority of information on the diversity of endophytic bacteria is based on those isolated from agriculturally important plant species (Lodewyckx *et al.*, 2002b). For phytoremediation fast growing, deep-rooting plant species, such poplar, are appropriate, but little is known about their associated endophyte populations. Consequently, in this chapter we describe the diversity of the endophytes found in the two cultivars of poplar: *Populus trichocarpa* x *deltoides* cv. “Hoogvorst” and “Hazendans”, growing in a phytoremediation field trial, to identify potential suitable candidates bacteria for enhancing that phytoremediation. Given the wide range of bacterial species reported as endophytes in the literature, we hypothesized that similar levels of diversity would be present in the trees but that different compartments of the plant might contain different assemblages of species, and that the bacterial species found may vary between different tree cultivars. The isolated endophytic bacteria were characterized by comparative sequence analysis of partial 16S rRNA genes, BOX PCR profiling of the genomic DNA, and physiological

characterization, in relation to a range of substrates, and antibiotics and heavy metal sensitivity.

5.2 MATERIALS AND METHODS

5.2.1 Comparative evaluation of plant surface sterilization techniques

The aim of this experiment was to explore the optimal conditions for plant surface sterilization. For successful isolation of endophytes it is necessary that the plant part surface becomes sterile while the interior of the organ remains unaffected. Stems, leaves and roots were sampled from *Populus trichocarpa* x *deltoides* cv. "Hoogvorst" cuttings grown under greenhouse conditions. Those cuttings originated from poplar trees growing near the Ford factory in Genk (Belgium) (Chapter 8). All samples were collected using ethanol sterilized secateurs and processed immediately. All methods were tested in triplicate.

Stem surface sterilization

From the fresh twigs, the leaves were removed and the stem was cut into 3cm long pieces and soaked in 95% ethanol, saturated calcium-hypo-chloride $(CaOCl)_2$, or in solutions containing 0.1, 0.3 or 1% active chloride (NaOCl, Sigma) (all Cl solutions were supplemented with 1 droplet Tween 80 per 100ml) for 0/5/10/15/30 min. After sterilization, stem pieces were washed in sterile water, dried on sterile filter paper and incubated on 869 (Mergeay *et al.*, 1985) medium for 5 days at 30°C. 100µl of third rinsing water was plated on the same medium and incubated at the same conditions.

Leaf surface sterilization

The leaves were carefully removed from the stem and washed in sterile water, then a whole leaf was shaken in saturated calcium-hypo-chloride, 1 or 2% active chloride for 0/1/3/5/10/15 min. The leaf was then removed from the sterilization solution with a sterile forceps, rinsed 3 times in sterile water, dried on filter paper and incubated as described above. 100µl of the third rinsing water was incubated as well.

Root surface sterilization

After the roots had been cut with sterile scissors they were thoroughly washed three times in sterile water. Subsequently, they were soaked in 0.1, 1 or 2% active chloride solution for 1/3/5/10/15 min., rinsed in sterile water and dried on sterile filter paper. A root section was then incubated as described above, as was 100µl of the final rinsing water.

Sterility check

Plate counts were made after 5 days, compared with PCR results, and the most efficient sterilization method was applied to all samples taken for the extraction.

PCR

PCR amplification was carried out on each rinse solution targeting the 16S-23S ITS region gene using primers M16F1195 (5′ –AGAGTTTGATCKTGGCTCAG- 3′) and M23R458 (5′ –CGGYTACCTTGTTACGAC-3′) and using 1 µl of the rinse water in 50 µl PCR reaction (consisting 10 µl Q-solution, 5 µl Q-buffer, 2.5mM $MgCl_2$, 1.25U Taq polymerase (all Qiagen, Crawley, U.K.), 0.8 µg/µl BSA (Roche, Switzerland), 1µl 10 mM dNTP’s and 1 µl of each primer (50pmol) (Qiagen-Operon, Germany) (prepared as a master mix prior to DNA addition). Cycling conditions were: 1 cycle of 95°C for 5 min and 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and then 1 cycle of 72°C for 10 min. The resulting product was then

checked for presence of a PCR product by gel electrophoresis at 100 V for 30 min on 1% agarose gel.

5.2.2 Isolation and identification of endophytic and rhizosphere bacteria of poplar trees from the field site

Samples of shoot, leaf, root and rhizosphere soil were collected from *Populus trichocarpa* x *deltoides* cultivars ("Hazendans" and "Hoogvorst") grown on the Ford factory site, in Genk, Belgium in June 2001, where poplar mediated phytoremediation is being used for the containment and *in situ* treatment of a BTEX contaminated groundwater plume. However, at the time of sampling the plant materials, the plant roots had not yet reached the BTEX contaminated groundwater plume (planted in 1999, see Chapter 8).

Two cm sections of shoot were taken in triplicate from each plant and surface sterilized by shaking for 5 min in solution containing 1% active chloride (NaOCl), before being rinsed 3 times in sterile water. A 100 µl sample of the 3rd rinse water was plated out as a sterility check in all cases onto 869 media (Mergeay *et al.*, 1985) plates. Xylem sap was extracted by means of a Scholander pressure bomb (Schurr, 1998) using N_2 gas at 25 bar, under aseptic conditions. As in all cases, 100 µl of sample was spread over 4 different media (869, 1/10 869, Schatz medium (Sz) and Sz + C mix) (Schatz and Bovel, 1956) and incubated for 7 days at 30°C; all visually different bacterial colonies were selected and sub-cultured 3 times to ensure purity.

Leaf samples were taken in triplicate from the same trees in September 2001 and surface sterilized by shaking in a solution containing 2% active chloride (NaOCl) for 10 min, followed by 3 rinses in sterile distilled water. Again, the 3rd rinsing water was plated out to check sterility. A 0.6 g fresh weight sample of each leaf

was macerated in 10 ml 10 mM $MgSO_4$ for 90 sec using a Polytron PT1200 mixer (Kinematica A6), then 100µl of this blend was plated out as described above.

Root samples were also taken in September 2001 from two poplar cultivars, by excavation to a depth of 1.5 m. Rhizosphere soil was removed by vortexing 1.9 g fresh weight of root material in 20 ml sterile 10 mM MgSO4, and collecting the precipitated soil slurry. This soil slurry was serially diluted to 10^{-5} and plated out. Subsequently, the roots were washed thoroughly, shaken in 2% active chloride solution (NaOCl) for 10 min to sterilize the surface, and then rinsed three times in sterile distilled water, before being macerated in 5 ml of 10 mM $MgSO_4$ and the slurry spread on different agar plates for culture.

DNA extraction and PCR amplification

DNA was extracted from individual colonies, suspended in 100 µl of sterile water using the BIO101 FastDNA® Spin Kit for Soils, with the FastPrep® instrument and the protocols of the manufacturer (Q-Biogene, U.K.). Briefly, the cells were disrupted by beating with glass beads, the DNA was bound to a matrix, washed and eluted from the matrix and recovered in the provided buffer.

PCR amplification was carried out, targeting the 16S gene using primers M16F63 (5′ – GGCCTAACACATGCAAGTC), hybridizing at the complement of nucleotide positions 45-63 (*Escherichia coli* 16S rRNA gene sequence numbering), and M16R1494 (5′ – TACGGYTACCTTGTTTACGAC) hybridizing at positions 1494-1514. The extracted DNA (1 µl) was used in triplicate 50 µl PCR's, each reaction, consisting of: 10µl Q-solution, 5 µl Q-buffer (10x), 2.5 mM $MgCl_2$, 1.25U *Taq* (All Qiagen, U.K.), 0.8 µg/µl BSA (Roche, Switzerland), 1 µl 10mM dNTP's (Bioline, U.K.) and 1 µl of each primer (50 pmol, Qiagen-Operon, Germany), prepared as a master mix prior to DNA addition. Cycling conditions were: 1 cycle of 95°C for 5 min, and 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and then 1 cycle of 72°C 10 min (using DNA engine PTC-200, GRI, U.K.). The resulting PCR-DNA product was then checked by gel electrophoresis at 100 V for 30 min on a

1% agarose gel. PCR products were cleaned, and the triplicate reactions concentrated into 1 elution of 50 µl using Qiagen Qiaquick PCR kit (Qiagen, U.K.), and quantified spectrophotometrically, using the Eppendorf Biaphotometer (Cambidge, U.K.).

DNA sequencing

Cleaned PCR products were cycle sequenced using Applied Biosyshoots Big Dye Terminator V.2 chemistry and processed with an ABI 310 Capillary DNA Sequencer (Applied Biosyshoots Inc., U.K.) and the M16R518 primer, described previously (Hauben *et al.*, 1997). The sequence data were checked manually (i.e., for incorrect "calling" of nucleotides), the PCR primer sequence was removed, and the sequences were submitted to the EMBL Nucleotide Sequence Database (Kulikova *et al.*, 2004), using the FastA3 algorithm (Pearson, W.R. 1994) (http://www.ebi.ac.uk/fasta3/), and compared to reference 16S rRNA gene sequence data, including those of the type strains of species with validly published names. The partial 16S rRNA gene sequence data from a selected group of isolates thought to be of importance for future research were submitted for deposit into EMBL.

BOX PCR genomic DNA profiling

Extracted DNA was amplified by PCR using the BOX1 primer (5'-CTACGGCAAGGCGACGCTGACCG-3'), ExTaq™ polymerase and TaKaRa dNTP's (BioWhittaker Europe), according to the manufacturer's protocols, but otherwise using the same mastermix as described previously. Thermocycling conditions were: 95°C for 5 min, 35 cycles of 1 min at 95°C, 1 min at 50°C and 8 min at 65°C, and 1 cycle of 8 min at 65°C. The obtained PCR products were separated by gel electrophoresis in 2% agarose gel (1:1 SeaKem LE agarose: Metaphor agarose FMC BioProducts BioWhittaker, Europe) run for 16 h at 2.5V/cm gel. Gel images were analyzed, using the Pearson correlation coefficient and UPGMA clustering

algorithm of the Gel Compar II Bionumeric program (Bionumerics Version 1.01, Applied Maths, Belgium).

Phenotypic profiling

The isolates were cultured on various media types containing a range of BTEX, antibiotics and metals to evaluate their natural tolerance to these compounds as an initial method of screening them for future use as potential endophytic strains. The abilities of the bacterial isolates to grow in the presence of BTEX or TCE compounds were assessed by plating on Shatz agar medium (Schatz and Bovel, 1956) and incubating at 30°C for 7 days in sealed 10 liter vessels with 600 µl of the test compound added. No other C-source was included.

Heavy metal resistance of the isolates was tested, using 284 agar (Schlegel *et al.*, 1961) with the addition of 1mM nickel, 2mM zinc or 0.8mM cadmium. Gluconate (0.2%, w/v) was added, as a carbon source, and cultures were incubated at 30°C for 7 days. Antibiotic resistance was tested using 284 agar containing kanamycin (100 µg/ml), tetracycline (20 µg/ml), amplicillin (100 µg/ml) or chloramfenicol (25 µg/ml), which were added aseptically to the medium after autoclaving. Gluconate (0.2%, w/v) was added, as a carbon source, and cultures were incubated at 30°C for 7 days.

5.3 RESULTS

5.3.1 Evaluation of plant surface sterilization techniques

The surface sterilization is an important step in the isolation of endophytic bacteria. The plant surface should be as sterile as possible, in order to make sure

that isolated bacteria are real endophytes, according to definition in chapter 1. At the same time, the inner part of the plant should remain unaffected.

For surface sterilization of stems the solutions with 1% active chloride and saturated calcium-hypo-chloride gave satisfactory results; a sterilization time of 5 min was sufficient (Table 5.1a). Finally, for isolation of endophytic bacteria from the stem (xylem sap), the 5 min stem surface sterilization with a solution containing 1% active chloride was chosen.

For the leaves, surface sterilization with NaOCl solution containing 2% active chloride gave more satisfactory results than the surface sterilization with solution with 1% active chloride or $Ca(OCl)_2$ (Table 5.1b). Therefore, a 10 min treatment with a solution containing 2% chloride was used to isolate endophytic bacteria from leaves of poplar.

From all root surface sterilization techniques the 10 min sterilization in a solution with 2% active chloride proved to be the most effective (Table 5.1c). The surface of the root was no longer contaminated, as indicated by the controls, while the inner part of the root was still viable.

	Ethanol	$Ca(OCl)_2$	0.1% Cl	1% Cl	2% Cl
0 min	bac+fun	bac+fun	fun	bac+fun	bac+fun
5 min	fun	/	bac	/	/
10 min	bac	/	/	/	/
15 min	/	/	fun	/	/
30 min	fun	/	/	/	/

Table 5.1a: Evaluation of different sterilization procedures for surface disinfection of poplar stem. Bac –bacteria observed on medium, fun – fungi observed on medium, bac+fun – bacteria and fungi observed on medium, / - medium was sterile

	$Ca(OCl)_2$	0.1% Cl	1% Cl	2% Cl
0 min	bac+fun	bac+fun	bac+fun	bac+fun
5 min	bac+fun	bac+fun	bac+fun	bac
10 min	bac+fun	bac+fun	bac+fun	/
15 min	bac+fun	bac	/	/
20 min	fun	/	/	/

Table 5.1b: Evaluation of different sterilization procedures for surface disinfection of poplar leaf. Bac –bacteria observed on medium, fun – fungi observed on medium, bac+fun – bacteria and fungi observed on medium, / - medium was sterile

	$Ca(OCl)_2$	0.1% Cl	1% Cl	2% Cl
0 min	bac+fun	bac+fun	bac+fun	bac+fun
5 min	bac+fun	bac+fun	bac+fun	/
10 min	bac+fun	bac+fun	bac+fun	/
15 min	bac+fun	bac+fun	bac+fun	/
20 min	bac+fun	bac+fun	bac+fun	/

Table 5.1c: Evaluation of different sterilization procedures for surface disinfection of poplar root. Bac –bacteria observed on medium, fun – fungi observed on medium, bac+fun – bacteria and fungi observed on medium, / - medium was sterile

5.3.2 Isolation and identification of endophytic and rhizosphere bacteria of poplar trees from the field site

The selection of morphologically different colonies from cultures inoculated with plant tissue material resulted in 51 isolates being obtained form *Populus trichocarpa* x *deltoides* cv. “Hoogvorst” (root 12, stem 8, leaves 18, rhizosphere 13) and 95 from *Populus trichocarpa* x *deltoides* cv. “Hazendans” (root 33, stem 17, leaves 10, rhizosphere 35). Some isolates proved difficult to maintain on media and, therefore, were removed from further study, on the grounds that an isolate with potential for biotechnology applications would need to be readily cultivable.

16S rDNA sequence analysis

The cultivable, stable, isolates were characterized by comparative sequence analysis of 16S rDNA generated by PCR, obtaining determinations of approximately 500 nucleotide positions upstream from the 5' – terminus and representing one-third of the complete gene sequence. The results of the analyses of the partial 16S rRNA gene sequence, including the most similar overall matches with sequence data in the EMBL Nucleotide Database (Kulikova *et al.*, 2004), and matches with the most similar sequences of type strains of species with validly published names are presented in Table 5.2a and 5.2b. Identifications of endophyte isolates were limited to tentative allocation to genera, based upon 16S rRNA gene sequence similarities falling within similarity spectra observed for species of the respective genera. Approximately 86% of the total number of endophyte isolates could be assigned with confidence to a bacterial genus. Approximately 63% of the endophytes isolated possessed 16S rDNA sequence similarities below the range of interspecies similarities of any genus.

Table 5.2a: Partial 16S rDNA sequence identification of bacterial isolates from poplar cv. "Hazendans" tissues. (HZD= Hazendans; prefixed by W = root isolate , no prefix = stem isolate, B = leaf isolate, R = rhizosphere soil isolate.

Isolate no.	***EMBL most similar strain***	**Accession no.**	**% sim**	***EMBL most similar type strain***	**Accession no.**	**% sim**
WHZD14	*Pseudomonas putida*	AF094737	99.2	*Pseudomonas plecoglossicida*	AB009457	98.4
WHZD19	*Pseudomonas putida*	AF094737	98.6	*Pseudomonas plecoglossicida*	AB009457	98.4
WHZD24	*Pseudomonas oryzihabitans*	FOIAM03	99.4	*Pseudomonas oryzihabitans*	FOIAM03	99.4
WHZD6	*Pseudomonas syringae*	AB001448	99.2	*Pseudomonas frederisksbergensis*	PFR249382	98.6
WHZD11	*Pseudomonas aeruginosa*	AE004844	95.7	*Pseudomonas rhodesiae*	AF064459	96.1
WHZD2	*Pseudomonas tolaasii*	AF320990	95.0	*Pseudomonas tolaasii*	AF320988	95.0
WHZD5	*Pseudomonas sp.*	AF013254	99.8	*Pseudomonas tolaasii*	AF320988	99.6
WHZD10	*Pseudomonas sp.*	AF013254	99.8	*Pseudomonas tolaasii*	AF320988	99.6
WHZD7	*Pseudomonas sp.*	AF013254	99.2	*Pseudomonas tolaasii*	AF320988	99.2
WHZD9	*Pseudomonas sp.*	AF013254	99.4	*Pseudomonas tolaasii*	AF320988	99.2
WHZD3	*Pseudomonas sp.*	AF013254	99.3	*Pseudomonas tolaasii*	AF320988	99.1
WHZD12	*Pseudomonas sp.*	AF013254	98.1	*Pseudomonas tolaasii*	AF320988	97.8
WHZD35	*Pseudomonas sp.*	AF408905	99.4	*Pseudomonas oryzihabitans*	FOIAM03	99.4
WHZD36	*Pseudomonas sp.*	AF408905	99.4	*Pseudomonas oryzihabitans*	FOIAM03	99.4
WHZD4	*Pseudomonas sp.*	PSPAJ2813	98.6	*Pseudomonas jessenii*	AF068259	97.6
WHZD18	*Pseudomonas sp.*	AF013254	97.3	*Pseudomonas jessenii*	AF068259	96.8
WHZD16	*Acinetobacter lwoffi*	AF188302	98.8	*Acinetobacter lwoffi*	AL16SRRNB	98.9
WHZD17	*Acinetobacter lwoffi*	AF188302	98.8	*Acinetobacter lwoffi*	AL16SRRNB	98.9
WHZD20	*Acinetobacter lwoffi*	AF188302	98.8	*Acinetobacter lwoffi*	AL16SRRNB	98.9
WHZD23	*Enterobacter cloacae*	U65720	99.0	*Enterobacter cloacae*	U65720	99.0
WHZD29	*Enterobacter cloacae*	U65720	99.0	*Enterobacter cloacae*	U65720	99.0
WHZD26	*Herbaspirillum seropedicae*	HS16SRRN	97.4	*Herbaspirillum seropedicae*	HS16SRRN	97.4
WHZD27	*Herbaspirillum seropedicae*	HS16SRRN	97.0	*Herbaspirillum seropedicae*	HS16SRRN	97.0
WHZD25	*Enterobacter aerogenes*	AF395913	99.6	*Klebsiella pneumoniae sub.sp.rhino*	KP16S3010	98.9
WHZD31	*Arthrobacter histidinolovorans*	AHRNA16S	99.4	*Arthrobacter histidinolovorans*	AHRNA16S	99.4
WHZD37	*Arthrobacter histidinolovorans*	AHRNA16S	99.4	*Arthrobacter histidinolovorans*	AHRNA16S	99.4
WHZD22	*Arthrobacter histidinolovorans*	AHRNA16S	98.3	*Arthrobacter histidinolovorans*	AHRNA16S	98.3
WHZD32	*Arthrobacter illicis*	AIRNA16S	98.9	*Arthrobacter illicis*	AIRNA16S	98.9
WHZD21	*Arthrobacter illicis*	AIRNA16S	98.7	*Arthrobacter illicis*	AIRNA16S	98.7
WHZD28	*Arthrobacter sp.*	AB017650	98.9	*Arthrobacter pascens*	APRDNA16	96.4
WHZD1	*Bacillus megaterium*	AF142677	99.6	*Bacillus megaterium*	BMEG16S	97.3

Isolate no.	***EMBL most similar strain***	**Accession no.**	**% sim**	***EMBL most similar type strain***	**Accession no.**	**% sim**
HZD1	*Pseudomonas veronii*	AF064460	99.8	*Pseudomonas veronii*	AF064460	99.8
HZD2	*Pseudomonas veronii*	AF064460	99.8	*Pseudomonas veronii*	AF064460	99.8
HZD5	*Pseudomonas veronii*	AF064460	99.8	*Pseudomonas veronii*	AF064460	99.8
HZD8	*Pseudomonas veronii*	AF064460	99.3	*Pseudomonas veronii*	AF064460	99.3
HZD7	*Pseudomonas veronii*	AF064460	97.4	*Pseudomonas veronii*	AF064460	97.4
HZD10	*Pseudomonas pavonaceae*	PPIAM18	99.4	*Pseudomonas fulva*	PFIAM14	98.9
HZD17	*Pseudomonas pavonaceae*	PPIAM18	99.3	*Pseudomonas jessenii*	AF068259	99.0
HZD18	*Pseudomonas pavonaceae*	PPIAM18	99.3	*Pseudomonas jessenii*	AF068259	99.0
HZD14	*Pseudomonas pavonaceae*	PPIAM18	99.1	*Pseudomonas fulva*	PFIAM14	98.7
HZD15	*Pseudomonas pavonaceae*	PPIAM18	98.2	*Pseudomonas fulva*	PFIAM14	98.9
HZD16	*Pseudomonas pavonaceae*	PPIAM18	97.8	*Pseudomonas fulva*	PFIAM14	97.4
HZD6	*Pseudomonas veronii*	AF064460	98.7	*Pseudomonas migulae*	AF074383	99.3
HZD4	*Pseudomonas fulva*	PFIAM14	97.0	*Pseudomonas fulva*	PFIAM14	97.0
HZD3	*Bacillus horikoshii*	BS16SRR06	99.8	*Bacillus horikoshii*	BS16SRR06	99.8
HZD11	*Bacillus megaterium*	AF142667	98.6	*Bacillus megaterium*	BMEG16S	96.7
HZD9	*Paenibacillus amylolyticus*	PAD396	98.2	*Paenibacillus amylolyticus*	PAD396	98.2
HZD13	*Micrococcus luteus*	AF057289	99.1	*Arthrobacter nicotianae*	ANRDNA16	96.0
BHZD12	*Xanthomonas cynarae*	AF208315	99.6	*Xanthomonas cynarae*	AF208315	99.6
BHZD3	*Xanthomonas cynarae*	AF208315	99.4	*Xanthomonas cynarae*	AF208315	99.4
BHZD1	*Xanthomonas cynarae*	AF208315	99.4	*Xanthomonas campestris*	AF123092	99.4
BHZD6	*Xanthomonas gardneri*	AF123093	99.5	*Xanthomonas campestris*	AF123092	99.3
BHZD8	*Pseudomonas extremorientalis*	AF405328	98.4	*Pseudomonas veronii*	AF064460	98.2
BHZD9	*Pseudomonas maltophila*	SMA293473	98.4	*Pseudomonas beteli*	AB021406	98.2
BHZD10	*Sphingomonas suberifaciens*	RS16SSRNA	98.8	*Sphingomonas adhaesiva*	SA16ASRD3	99.0
BHZD11	*Sphingomonas suberifaciens*	RS16SSRNA	94.4	*Sphingomonas adhaesiva*	SA16ASRD3	91.5
BHZD7	*Sphingomonas sp*	AF395031	93.0	*Blastomonas ursincola*	AB024289	91.5
BHZD2	*Variovorax paradoxus*	VSP313017	97.3	*Xylophilus ampelinus*	AF078758	96.5

Isolate no.	*EMBL most similar strain*	Accession no.	% sim	*EMBL most similar type strain*	Accession no.	% sim
RHZD94	*Pseudomonas rhodesiae*	AF064459	99.5	*Pseudomonas rhodesiae*	AF064459	99.5
RHZD1	*Pseudomonas rhodesiae*	AF064459	98.9	*Pseudomonas rhodesiae*	AF064459	98.9
RHZD3	*Pseudomonas rhodesiae*	AF064459	98.4	*Pseudomonas rhodesiae*	AF064459	98.4
RHZD79	*Pseudomonas veronii*	AF064460	99.0	*Pseudomonas veronii*	AF064460	99.0
RHZD74	*Pseudomonas veronii*	AF064460	97.5	*Pseudomonas veronii*	AF064460	97.5
RHZD2	*Pseudomonas tolaasii*	AF320990	99.6	*Pseudomonas tolaasii*	AF320988	99.5
RHZD95	*Pseudomonas fluorescens*	AF134704	98.1	*Pseudomonas lini*	AY035996	98.1
RHZD56	*Burkholderia phenazinium*	AB021394	97.4	*Burkholderia glathei*	BG16SRRNA	97.4
RHZD49	*Burkholderia phenazinium*	AB021394	96.8	*Burkholderia glathei*	BG16SRRNA	96.6
RHZD53	*Burkholderia phenazinium*	AB021394	96.0	*Burkholderia glathei*	BG16SRRNA	95.6
RHZD86	*Burkholderia phenazinium*	AB021394	96.0	*Burkholderia glathei*	BG16SRRNA	95.2
RHZD33	*Burkholderia phenazinium*	AB021394	95.6	*Burkholderia glathei*	BG16SRRNA	95.4
RHZD83	*Ralstonia eutropha*	AF501357	98.1	*Ralstonia basilensis*	RBSJ2302	98.6
RHZD91	*Ralstonia basilensis*	AF312021	97.8	*Ralstonia basilensis*	RBSJ2302	97.4
RHZD81	*Ralstonia solanacearum*	AL646073	81.5	*Ralstonia basilensis*	RBSJ2302	81.5
RHZD28	*Sinorhizobium meliloti*	SME591782	96.8	*Rhizobium rhizogenes*	AT16SRD6	96.5
RHZD27	*Sinorhizobium meliloti*	SME591782	93.5	*Rhizobium rhizogenes*	AT16SRD6	93.0
RHZD87	*Rhizobium tumefaciens*	ATRGD	98.6	*Rhizobium vitis*	AV45329	95.3
RHZD10	*Herbaspirillum seropedicae*	HS16SRRN	95.2	*Herbaspirillum seropedicae*	HS16SRRN	95.2
RHZD68	*Aquaspirillum autotrophicum*	AB074524	96.7	*Herbaspirillum seropedicae*	HS16SRRN	94.0
RHZD85	*Bosea thiooxidans*	BTH250800	99.0	*Bosea thiooxidans*	BTH250800	98.5
RHZD98	*Sphingomonas witflariensis*	SSP416410	98.3	*Sphingomonas macrogoltabidus*	D84530	96.6
RHZD37	*Variovorax paradoxus*	AF451851	96.0	*Pseudomonas lanceolata*	AB021390	89.6
RHZD12	*Paenibacillus amylolyticus*	PAD396	97.4	*Paenibacillus amylolyticus*	PAD396	97.4
RHZD4	*Paenibacillus amylolyticus*	PAD396	97.3	*Paenibacillus amylolyticus*	PAD396	97.3
RHZD5	*Paenibacillus amylolyticus*	PAD396	97.1	*Paenibacillus amylolyticus*	PAD396	97.1
RHZD9	*Arthrobacter agilis*	AF511518	96.6	*Arthrobacter agilis*	MARDNA16	96.6
RHZD70	*Arthrobacter agilis*	AF511518	95.4	*Arthrobacter agilis*	MARDNA16	95.5
RHZD64	*Arthrobacter ilicis*	AIRNA16S	98.8	*Arthrobacter ilicis*	AIRNA16S	98.8
RHZD19	*Arthrobacter aurescens*	AF501369	98.1	*Arthrobacter ilicis*	AIRNA16S	98.8
RHZD93	*Arthrobacter histidinolovorans*	AF501356	94.6	*Arthrobacter histidinolovorans*	AHRNA16S	93.9
RHZD42	*Staphylococcus caprae*	AB009935	99.4	*Staphylococcus capitis*	SE16SRRNA	99.1
RHZD17	*Staphylococcus epidermidis*	AF269927	92.9	*Staphylococcus epidermidis*	SE16SRRNA	93.0
RHZD76	*Micrococcus luteus*	MLU409096	99.5	*Arthrobacter nicotiniae*	ANRDNA16	96.0
RHZD78	*Micrococcus luteus*	MLU409096	99.0	*Arthrobacter nicotiniae*	ANRDNA16	95.1

Table 5.2b: Partial 16S rDNA sequence identification of bacterial isolates from poplar cv. "Hoogvorst" tissues. (HV= Hoogvorst; prefixed by W= root isolate , no prefix = stem isolate, B = leaf isolate, R = rhizosphere soil isolate.

Isolate no.	*EMBL closest match*	Accession no.	% sim	*Closest type strain*	Accession no.	% sim
WHV5	*Burkholderia phenazinium*	AB021394	96.7	*Burkholderia glathei*	BG16SRRNA	95.4
WHV6	*Burkholderia phenazinium*	AB021394	96.7	*Burkholderia glathei*	BG16SRRNA	95.3
WHV1	*Burkholderia phenazinium*	AB021394	94.7	*Burkholderia glathei*	BG16SRRNA	94.3
WHV3	*Burkholderia phenazinium*	AB021394	94.7	*Burkholderia glathei*	BG16SRRNA	94.3
WHV22	*Burkholderia multivorans*	AF097531	98.7	*Burkholderia multivorans*	BMU18703	99.0
WHV17	*Burkholderia multivorans*	AF097531	98.0	*Burkholderia cepacia*	AF097530	97.8
WHV21	*Burkholderia ambifaria*	AY028444	98.6	*Burkholderia cepacia*	AF097530	98.3
WHV2	*Burkholderia glathei*	BG16SRRNA	97.9	*Burkholderia glathei*	BG16SRRNA	97.9
WHV4	*Burkholderia carophylii*	U91570	97.1	*Burkholderia glathei*	BG16SRRNA	96.3
WHV19	*Pseudomonas putida*	AF094737	99.6	*Pseudomonas plecoglossicida*	AB009457	99.6
WHV20	*Pseudomonas putida*	AF094737	98.5	*Pseudomonas plecoglossicida*	AB009457	98.3
WHV16	*Pseudomonas putida*	AF094737	96.8	*Pseudomonas plecoglossicida*	AB009457	96.6
HV5	*Pseudomonas veronii*	AB056120	99.8	*Pseudomonas veronii*	AF064460	99.8
HV4	*Pseudomonas veronii*	AB056120	99.4	*Pseudomonas veronii*	AF064460	99.4
HV9	*Pseudomonas veronii*	AB056120	98.6	*Psuedomonas putida*	AF094736	98.6
HV8	*Pseudomonas putida*	AF094737	99.8	*Pseudomonas putida*	AF094736	99.8
HV10	*Pseudomonas pavonaceae*	PPIAM18	99.4	*Pseudomonas fulva*	PFIAM14	99.4
HV6	*Pseudomonas asplenii*	AB021397	95.0	*Pseudomonas asplenii*	AB021397	95.0
HV7	*Pseudomonas syringae*	PSZ76669	90.6	*Pseudomonas syringae*	PSZ76669	90.6
HV11	*Bacillus macroides*	MB16RRNA	92.7	*Bacillus macroides*	MB16RRNA	92.7

Isolate no.	*EMBL closest match*	Accession no.	% sim	*Closest type strain*	Accession no.	% sim
BHV17	*Pseudomonas graminis*	PGY11150	99.8	*Pseudomonas graminis*	PGY11150	99.8
BHV20	*Pseudomonas graminis*	PGY11150	99.6	*Pseudomonas graminis*	PGY11150	99.6
BHV26	*Pseudomonas oleovorans*	POIAM17	99.4	*Pseudomonas stutzeri*	AF094748	95.2
BHV23	*Pseudomonas oleovorans*	POIAM17	99.2	*Pseudomonas stutzeri*	AF094748	95.5
BHV3	*Sphingomonas adhaesiva*	SA16SRD3	88.3	*Sphingomonas adhaesiva*	SA16SRD3	88.3
BHV13	*Sphingomonas sp.*	AF395031	98.8	*Sphingomonas adhaesiva*	SA16SRD3	97.2
BHV28	*Sphingomonas sp.*	AF395031	98.6	*Sphingomonas adhaesiva*	SA16SRD3	97.2
BHV19	*Sphingomonas sp.*	AF395031	98.2	*Sphingomonas adhaesiva*	SA16SRD3	96.3
BHV5	*Moraxella osloensis*	MO16RR	98.3	*Moraxella lincolnii*	MLI247229	90.9
BHV25	*Moraxella osloensis*	MO16RR	98.3	*Moraxella lincolnii*	MLI247229	90.9
BHV27	*Zoogloea ramigera*	ZR16SRRNB	98.4	*Duganella zoogloeoides*	ZRR16S3	95.1
BHV6	*Zoogloea ramigera*	ZR16SRRNB	94.3	*Duganella zoogloeoides*	ZRR16S3	95.1
BHV10	*Xanthomonas cynarae*	AF208315	99.2	*Xanthomonas cynarae*	AF208315	99.2
BHV32	*Salmonella enterica*	AL627266	88.9	*Enterobacter cloacae*	ECL251469	90.2
BHV34	*Clavibacter sp.*	CSP310417	96.2	*Agreia bicolorata*	AF159363	92.6
BHV24	*Clavibacter sp.*	CSP310417	94.2	*Agreia bicolorata*	AF159363	92.6
BHV12	*Frigoribacterium sp.*	AF157479	99.6	*Agreia bicolorata*	AF159363	93.0
BHV2	*Curtobacterium flaccumfacien*	CFL310414	91.4	*Curtobacterium luteum*	CL16SR	90.4
RHV33	*Pseudomonas stutzeri*	PSU65012	99.1	*Pseudomonas graminis*	PGY11150	98.4
RHV24	*Pseudomonas veronii*	AB056120	97.4	*Pseudomonas rhodesiae*	AF064459	98.2
RHV40	*Sphingomonas suberifasciens*	RS16SSRNA	95.6	*Sphingmonas mali*	SM16SR	95.9
RHV48	*Sphingomonas suberifasciens*	RS16SSRNA	86.6	*Sphingmonas mali*	SM16SR	86.2
RHV3	*Ralstonia basilensis*	AF312021	100.0	*Ralstonia basilensis*	RBSJ2302	99.0
RHV76	*Ralstonia basilensis*	AF312021	96.7	*Ralstonia basilensis*	RBSJ2302	96.1
RHV2	*Bosea thiooxidans*	BTH250800	98.4	*Bosea thiooxidans*	BTH250800	97.7
RHV82	*Burkholderia phenazinium*	AB021394	95.9	*Burkholderia glathei*	BG16SRRNA	95.1
RHV61	*Rhizobium loti*	AP003001	91.8	*Mesorhizobium amorphae*	AF041442	91.1
RHV69	*Frateuria aurantia*	AF376025	89.4	*Frateuria aurantia*	FAU010841	89.4
RHV73	*Aquaspirillum autotrophicum*	AB074524	86.3	*Herbaspirillum frisingense*	HFR238358	85.5
RHV71	*Kocuria rhizophila*	KR16SRRNA	85.5	*Kocuria rhizophila*	KR16SRRNA	85.5
RHV75	*Paenibacillus amylolyticus*	PAD386	98.1	*Paenibacillus amylolyticus*	PAD386	98.1

Bacteria of the Gammaproteobacteria (Ludwig and Klenk, 2001) dominated the collection of isolates, comprising 59% of the total of isolated strains; including 42% *Pseudomonas* spp., with smaller percentage numbers of *Xanthomonas* spp., *Acinetobacter* spp. and *Enterobacter* spp. representing the majority of the remainder of the Gammaproteobacteria. The Betaproteobacteria (Ludwig and Klenk, 2001) made up 18% of the isolate collection, with *Burkholderia* spp. (10%) and *Herbaspirillum* spp. representing the majority of the group. The Alphaproteobacteria (Ludwig and Klenk, 2001) formed 10% of the total number of isolates, and were largely represented by *Sphingomonas* spp. (9%). Gram-positive bacteria comprised 13% of the total number of isolates, and were represented largely by *Arthrobacter* spp. (10%), *Bacillus* spp., *Paenibacillus* spp. and *Agreia* spp. The endophytic bacteria exhibiting 16S rDNA sequence similarities matching most closely the sequences of species of the genus *Pseudomonas* were numerically predominant among the isolates collected. The 16S rDNA sequences indicated that majority of these isolates are closely related phylogenetically to *P. veronii* (17%), *P. tolaasii* (17%), *P. plecoglossicida* (12%), *P. fulva*, (12%), *P. jessenii* (7%), with fewer number of isolates being related to *P. graminis, P. putida P. migulae*, and *P. rhodesiae*. The root material of *P. trichocarpa* x *deltoides* cv. "Hazendans" yielded a larger diversity of isolates than the root material of *P. trichocarpa* x *deltoides* cv. "Hoogvorst", with *Pseudomonas* spp. comprising 36% of all root isolates. Also isolated from the root material were *Arthrobacter* spp, *Enterobacter* spp. and *Acinetobacter* spp. The observed prevalence of *Pseudomonas* spp. within *P. trichocarpa* x *deltoides* cv. "Hazendans" continued into the stem of the tree, with 77% of isolates showing >=99% similarity to the type strain of a known species of *Pseudomonas*. From the samples of leaf material, 10 morphologically distinct isolates were obtained, including *Xanthomonas* spp. and *Pseudomonas* spp.

Within *P. trichocarpa* x *deltoides* cv. "Hoogvorst" root material 12 morphologically distinct isolates were identified, and 7 of these were *Burkholderia* spp. and 3

were *Pseudomonas* spp. Within the stem, diversity was lower, with 6 of 8 morphologically distinct isolates being assigned to the genus *Pseudomonas*. Within samples of the leaf material, however, 18 distinct morphotypes were isolated, although 8 of these could not be identified to >= 95% similarity to a known type strain. Of the remainder, 4 isolates were attributed to *Pseudomonas* spp., 3 to *Sphingomonas* spp. and 2 to *Duganella* spp.

A large number of distinct colony morphotypes were isolated from the rhizosphere soil of *P. trichocarpa* x *deltoides* cv. “Hazendans” (Table 5.2a). Of the 35 isolates that were analysed by partial 16S rDNA sequence analysis, a diversity of sequence types was observed from 6 different genera, including *Pseudomonas* spp., *Burkholderia* spp., *Ralstonia* spp., *Rhizobium* spp., *Arthrobacter* spp., and *Paenibacillus* spp. Thirteen morphologically-distinct isolates were collected from the rhizosphere soil of *P. trichocarpa* x *deltoides* cv. “Hoogvorst” (Table 5.2b). These isolates comprised 5 different genera, with 1 or 2 different sequence types within each. Five isolates could not be reliably assigned to a genus, based upon the 16S rDNA sequence similarities.

BOX-PCR genomic DNA fingerprinting

Identity to the genus level was conferred on those isolates which showed 16S rRNA gene sequence similarities falling within the similarity spectra observed for species of the respective genera. Likewise species was assigned for those falling within the range of interspecies sequence similarities. Therefore, those endophyte isolates listed with no identification in Figure 5.1a-d had sequence similarities with the type strain of that genera <95%.

Figure 5.1a shows the clustering of BOX PCR fingerprints for the root isolates from *P. trichocarpa* x *deltoides* cv. “Hazendans” compared to those of *P. trichocarpa* x *deltoides* cv. “Hoogvorst”, with several groups which exhibit similar banding patterns and sequence identity. Endophyte isolates WHZD 2, 3, 5 and 7 all show similar banding patterns and are identified as *Pseudomonas tolaasii*.

Isolates WHZD 26 and 27 are both identified as *Herbaspirillum seropedicae*, and give similar fingerprints. Strains WHV 16, 17, 19 and 20 also clustered together and were identified by sequence analysis as being closely related to *Pseudomonas plecoglossicida* (96.6%, 99.6% and 98.3%), with the exception of strain WHV17 which is closer to *Burkholderia cepacia* (97.8%, basonym *Pseudomonas*). This suggests that the sequence identification may be correct to the genus level in this cluster, but not to the species. Isolate WHV1 and WHV22 also have similar banding patterns and are both related to genus *Burkholderia* but sequence data showed WHV22 to be more related to *B. multivorans* (99%) and WHV1 as *B. glathei* (94.3%), suggesting that the species might be incorrect for the latter.

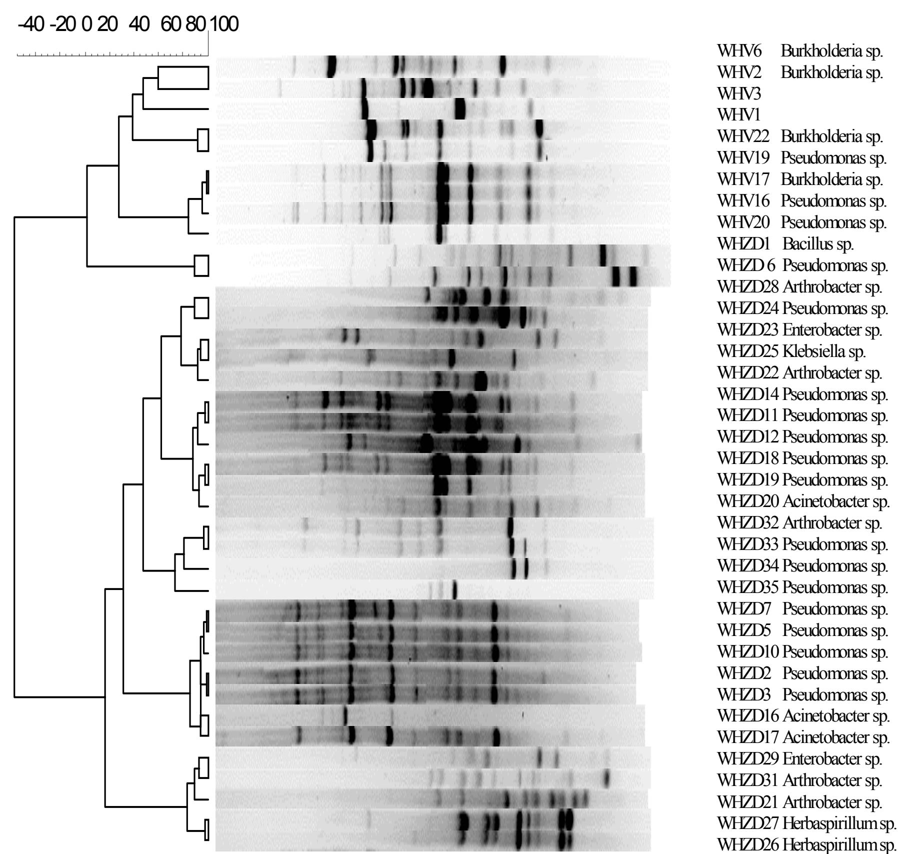


Figure 5.1a: Dendrogram of BOX-PCR fingerprint of bacterial isolates from the root, of poplar cv. “Hazendans” and “Hoogvorst”

Figure 5.1b shows two main clusters for the BOX PCR data for the stem isolates of *Populus trichocarpa* x *deltoides* cv. "Hazendans" compared to those of *Populus trichocarpa* x *deltoides cv*. "Hoogvorst". The cluster comprising HZD 1, 2, 5, 6 and 7 were all closely related by sequence data to *Pseudomonas veronii* (99.3-99.8%). The strains in the remaining BOX PCR cluster comprising HZD 10, 15, 16 and 17 are closely related to *Pseudomonas fulva* (97.4-98.9%), with the exception of HZD 17 which is closer to *P. jessenii* (99%).

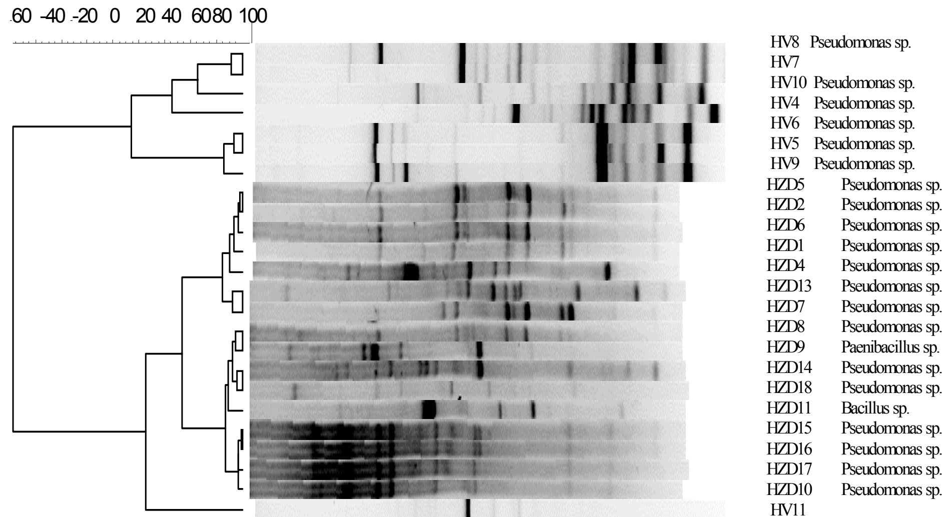


Figure 5.1b: Dendrogram of BOX-PCR fingerprint of bacterial isolates from the stem of poplar cv. "Hazendans" and "Hoogvorst"

Figure 5.1c shows the dendogram of BOX-PCR fingerprints for the leaf bacterial isolates. BHV24 and BHV25 clustered together but were unable to be reliably identified by sequence data. BHZD11 and BHZD9 also clustered together, and BHZD9 was identified as *Pseudomonas* spp. but a reliable identification was not achieved for BHZD11. Two isolates for the two different poplar varieties also clustered together BHV10 and BHZD12, both identified as being closely related to *Xanthomonas* spp. Figure 5.1d shows the BOX-PCR fingerprint of the rhizosphere

bacteria isolated from both cultivars of poplar. RHZD81 and RHZD91 cluster by their BOX-PCR fingerprint are closely related to *Ralstonia basilensis*. RHZD4 and 12 also cluster and are related to *Paenibacillus amylolyticus*.

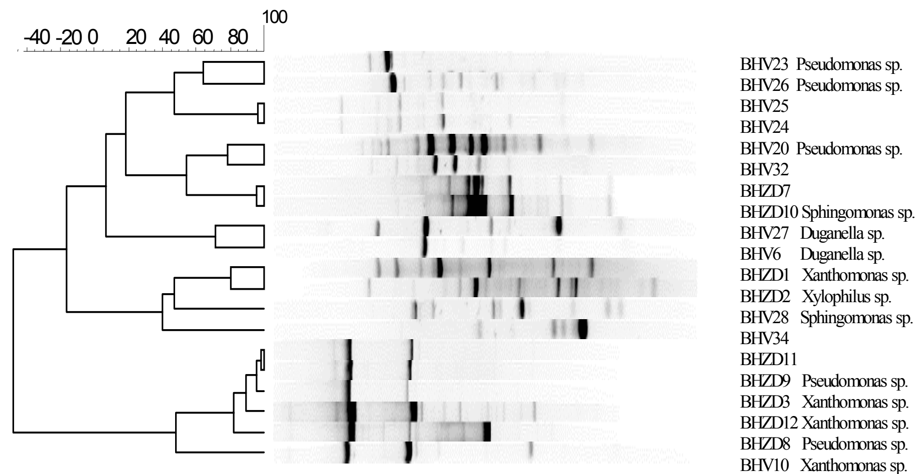


Figure 5.1c: Dendrogram of BOX-PCR fingerprint of bacterial isolates from the leaf of poplar cv. “Hazendans” and “Hoogvorst”

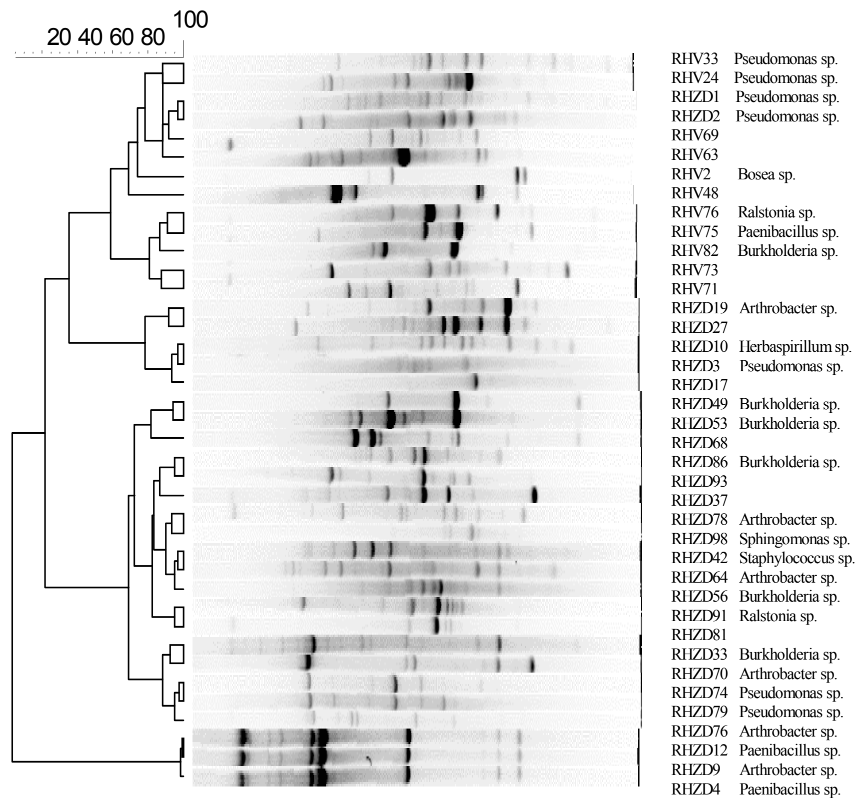


Figure 5.1d: Dendrogram of BOX-PCR fingerprint of bacterial isolates from the rhizosphere soil of poplar cv. “Hazendans” and “Hoogvorst”

Substrate tolerance

Of the isolates from the root of *Populus* cv. “Hazendans (Table 5.3a), nine could both grow on BTEX and in the presence of TCE (WHZD7, WHZD11, WHZD14, WHZD16, WHZD17, WHZD20, WHZD24, WHZD35, WHZD36), while 3 others could grow in the presence of a heavy metal only (WHZD6, WHZD21, WHZD22) and 1 on BTEX only (WHZD23). Many of the isolates from the stem material (Table5.3b) were able to grow in the presence of a range of BTEX or TCE containing compounds (HZD5, HZD8, HZD9, HZD10, HZD11, HZD13, HZD17) and 3 could grow solely on O-xylene as a carbon source (HZD14, HZD15, HZD18). Of these isolates, only HZD5, HZD14, HZD15 and HZD18 showed any tolerance to an antibiotic, and

none showed any tolerance to a heavy metal. Leaf isolates (Table 5.3c) were unable to metabolize or tolerate any BTEX related compound, antibiotic or heavy metal, with exception of BHZD8. Of the bacteria isolated from the rhizosphere soil (Table 5.3d), 8 were able to grow in the presence of any of the BTEX related or TCE compounds to which they were exposed. (RHZD4, RHZD5, RHZD12, RHZD27, RHZD28, RHZD53, RHZD56, RHZD76), and 2 to more limited range (RHZD49 and RHZD78).

Seq. ID	isolate	869	Sz	BTEX	B	T	EB	m-X	p-X	o-X	TCE	Ni	Zn
P. tolaasii	WHZD12	+	+	-	-	-	-	-	-	-	-	-	-
P. tolaasii	WHZD2	+	+	-	-	-	-	-	-	-	-	-	
P. tolaasii	WHZD3	+	+	-	-	-	-	-	-	-	-	-	-
P. tolaasii	WHZD7	+	+	+	+	+	+	+	+	+	+	-	-
P. tolaasii	WHZD9	+	+	-	-	-	-	-	-	-	-	-	-
P. tolaasii	WHZD10	+	+	-	-	-	-	-	-	-	-	-	-
P. tolaasii	WHZD5	+	+	-	-	-	-	-	-	-	-	-	-
P. plecoglossicida	WHZD14	+	+	+	+	+	+	+	+	+	+	-	-
P. plecoglossicida	WHZD19	+		-	-	-	-	-	-	-	-	-	-
P. jessenii	WHZD4	+	+	-	-	-	-	-	-	-	-	-	-
P. jessenii	WHZD18	+	+	-	-	-	-	-	-	-	-	-	-
P. frederisksbergensis	WHZD6	+	+	-	-	-	-	-	-	-	-	-	+
P. rhodesiae	WHZD11	+		+	+	+	+	+	+	+	+		
A. illicis	WHZD21	+	+	-	-	-	-	-	-	-	-	+	+
A. illicis	WHZD32	+	+	-	-	-	-	-	-	-	-	-	-
A. histidinolovorans	WHZD22	+	+	-	-	-	-	-	-	-	-	-	+
A. histidinolovorans	WHZD37	+	+	-	-	-	-	-	-	-	-	-	-
A. pascens	WHZD28	+	+	-	-	-	-	-	-	-	-	-	-
F. oryzihabitants	WHZD33	+		-	-	-	-	-	-	-	-	-	-
F. oryzihabitants	WHZD34	+	+	+	+	+	+	+	+	+	+	+	+
F. oryzihabitants	WHZD35	+	+	+	+	+	+	+	+	+	+	+	+
F. oryzihabitants	WHZD36	+	+	+	+	+	+	+	+	+	+	+	+
F. oryzihabitants	WHZD24	+	+	+	+	+	+	+	+	+	+	+	+
A. iwoffi	WHZD16	+	+	+	+	+	+	+	+	+	+	+	+
A. iwoffi	WHZD17	+	+	+	+	+	+	+	+	+	+	+	+
A. iwoffi	WHZD20	+	+	+	+	+	+	+	+	+	+	+	+
E. cloacae	WHZD29	+	-	-	-	-	-	-	-	-	-	-	-
E. cloacae	WHZD31	+	+	+	-	-	-	-	-	-	-	-	-
H. seropediacae	WHZD26	+	+	+	-	-	-	-	-	-	-	-	-
H. seropediacae	WHZD27	+	+	+	-	-	-	-	-	-	-	-	-
K. pneumoniae	WHZD25	+	-	-	-	-	-	-	-	-	-	-	-
E. cloacae	WHZD23	+	-	+	+	+	+	+	+	+	+	-	-
B. megaterium	WHZD1	+	-	-	-	-	-	-	-	-	-	-	-

Table 5.3a: Growth characteristics of different bacterial strains isolated from poplar cv. “Hazendans” root material. (+ growth, -no growth; B-benzene, T-toluene, EB-ethylbenzene, m- p- o-X- xylene)

Seq. ID	isolate	869	Sz	BTEX	B	T	EB	m-X	p-X	o-X	TCE	Ni	Zn	Cd	Km	Te	Am	Ch
P. veronii	HZD1	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
P. veronii	HZD2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. veronii	HZD5	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+
P. veronii	HZD7	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. veronii	HZD8	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. fulva	HZD4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. pavonaceae	HZD14	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
P. pavonaceae	HZD15	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
P. pavonaceae	HZD16	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
P. pavonaceae	HZD10	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
P. pavonaceae	HZD17	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
P. pavonaceae	HZD18	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
P. veronii	HZD6	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+
B. megaterium	HZD11	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
B. horikoshii	HZD3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M. luteus	HZD13	+	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-
P. amylolyticus	HZD9	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-

Table 5.3b: Growth characteristics of different bacterial strains isolated from poplar cv. "Hazendans" stem material. (+ growth, -no growth; B-benzene, T-toluene, EB-ethylbenzene, m- p- o-X- xylene;)

Seq. ID	isolate	869	Sz	BTEX	B	T	EB	m-X	p-X	o-X	TCE	Ni	Zn
X. campestris	BHZD1	+	+	-	-	-	-	-	-	-	-	-	-
X. cynarae	BHZD12	+	+	-	-	-	-	-	-	-	-	-	-
X. cynarae	BHZD3	+	+	-	-	-	-	-	-	-	-	-	-
X. campestris	BHZD6	+	-	-	-	-	-	-	-	-	-	-	-
Ps. veronii	BHZD8	+	+	+	+	+	+	+	+	+	+		
Ps. beteli	BHZD9	+	+	-	-	-	-	-	-	-	-	-	-
S. adhaesiva	BHZD10	+	+	-	-	-	-	-	-	-	-	-	-
S. adhaesiva	BHZD11	+	+	-	-	-	-	-	-	-	-	-	-
X. arrpelinus	BHZD2	+	+	-	-	-	-	-	-	-	-	-	-
B. ursincola	BHZD7	+	+	-	-	-	-	-	-	-	-	-	-

Table 5.3c: Growth characteristics of different bacterial strains isolated from poplar cv. “Hazendans” leaf material. (+ growth, -no growth, B-benzene, T-toluene, EB-ethylbenzene, m- p- o-X- xylene)

Seq. ID	isolate	869	Sz	BTEX	B	T	EB	m-X	p-X	o-X	YCE
Ps. Rhodesiae	RHZD1	-	-	-	-	-	-	-	-	-	-
Ps. rhodesiae	RHZD94	-	-	-	-	-	-	-	-	-	-
Ps. rhodesiae	RHZD3	-	-	-	-	-	-	-	-	-	-
Ps.veronii	RHZD79	-	-	-	-	-	-	-	-	-	-
Ps. lini	RHZD95	-	-	-	-	-	-	-	-	-	-
Ps tolaasii	RHZD2	-	-	-	-	-	-	-	-	-	-
Ps lanceolata	RHZD37	-	-	-	-	-	-	-	-	-	-
A. agilis	RHZD9	-	-	-	-	-	-	-	-	-	-
A. agilis	RHZD70	-	-	-	-	-	-	-	-	-	-
A. nicotiniae	RHZD76	+	+	+	+	+	+	+	+	+	+
A. nicotiniae	RHZD78	+	-	+	+	+	+	-	-	-	-
A. ilicis	RHZD19	-	-	-	-	-	-	-	-	-	-
A. ilicis	RHZD64	-	-	-	-	-	-	-	-	-	-
A. histidinolovorans	RHZD93	-	-	-	-	-	-	-	-	-	-
B. glathei	RHZD49	+	+	+	-	-	-	+	+	+	+
B. glathei	RHZD53	-	+	+	+	+	+	+	+	+	+
B. glathei	RHZD56	+	+	+	+	+	+	+	+	+	+
B. glathei	RHZD86	-	-	-	-	-	-	-	-	-	-
B. glathei	RHZD33	-	-	-	-	-	-	-	-	-	-
R. rhizogenes	RHZD27	+	+	+	+	+	+	+	+	+	+
R. rhizogenes	RHZD28	+	+	+	+	+	+	+	+	+	+
R. vitis	RHZD87	-	-	-	-	-	-	-	-	-	-
R. basilensis	RHZD81	-	-	-	-	-	-	-	-	-	-
R. basilensis	RHZD83	-	-	-	-	-	-	-	-	-	-
R. basilensis	RHZD91	-	-	-	-	-	-	-	-	-	-
P. amylolyticus	RHZD4	+	+	+	+	+	+	+	+	+	+
P. amylolyticus	RHZD5	+	+	+	+	+	+	+	+	+	+
P. amylolyticus	RHZD12	+	+	+	+	+	+	+	+	+	+
H. seropedicae	RHZD68	-	-	-	-	-	-	-	-	-	-
H. seropedicae	RHZD10	-	-	-	-	-	-	-	-	-	-
S. capitis	RHZD42	-	-	-	-	-	-	-	-	-	-
S. epidermis	RHZD17	-	-	-	-	-	-	-	-	-	-
B. thiooxidans	RHZD85	-	-	-	-	-	-	-	-	-	-
S. macrogoltabidus	RHZD98	-	-	-	-	-	-	-	-	-	-

Table 5.3d: Growth characteristics of different bacterial strains isolated from poplar cv. “Hazendans” rhizosphere soil material. (+ growth, -no growth, / not tested; B-benzene, T-toluene, EB-ethylbenzene, m- p- o-X- xylene)

Bacteria isolated from the root of *Populus* cv. “Hoogvorst” (Table 5.4a) showed no metabolization or tolerance to any BTEX or heavy metal compound with the exception of WHV17, which could grow in the presence of TCE. The isolates from the stem material (Table 5.4b) showed no tolerance to BTEX compounds with the exception of isolate HV11, which tolerated no heavy metals, antibiotics or alternative C-sources. Three isolates (HV5, HV8, HV9) were tolerant to Ni and Cd, and most stem isolates were able to grow in the presence of ampicillin, chloramphenicol and a range of C-substrates. The isolates from the leaf material of *Populus* cv. “Hoogvorst” were all able to grow on 869 and Schatz media, except isolate BHV32 which could not grow on Schatz medium. Only strains BHV25, BHV32 and BHV34 were able to tolerate TCE, and most BTEX related compounds. Bacteria isolated from the rhizosphere soil of *Populus* cv. “Hoogvorst” (Table 5.4d) were able to tolerate a range of BTEX and TCE containing compounds (RHV40, RHV48, RHV61).

SeqID	isolate	869	Sz	BT-EX	B	T	E-B	mX	pX	oX	TCE	Ni	Zn
B.glathei	WHV1	+	+	-	-	-	-	-	-	-	-	-	-
B.glathei	WHV2	+	+	-	-	-	-	-	-	-	-	-	-
B.glathei	WHV3	+	+	-	-	-	-	-	-	-	-	-	-
B.glathei	WHV4	+	+	-	-	-	-	-	-	-	-	-	-
B.glathei	WHV5	+	+	-	-	-	-	-	-	-	-	-	-
B.glathei	WHV6	+	+	-	-	-	-	-	-	-	-	-	-
B.cepacia	WHV21	+	+	-	-	-	-	-	-	-	-	-	-
B.cepacia	WHV17	+	+	-	-	-	-	-	-	-	+	-	-
P. plecoglossicida	WHV19	+	-	-	-	-	-	-	-	-	-	-	-
P. plecoglossicida	WHV20	+	+	-	-	-	-	-	-	-	-	-	-
P. plecoglossicida	WHV16	+	+	-	-	-	-	-	-	-	-	-	-

Table 5.4a: Growth characteristics of different bacterial strains isolated from poplar cv. “Hoogvorst” root material. (+ growth, -no growth, / not tested; B-benzene, T-toluene, EB-ethylbenzene, m- p- o-X- xylene)

Table 5.4b: Growth characteristics of different bacterial strains isolated from poplar cv. “Hoogvorst” stem material. (+ growth, -no growth, / not tested; B-benzene, T-toluene, EB-ethylbenzene, m- p- o-X- xylene)

Seq. Id	**isolate**	**869**	**Sz**	**BTEX**	**B**	**T**	**EB**	**m-X**	**p-X**	**o-X**	**TCE**	**Ni**	**Zn**	**Cd**	**Km**	**Te**	**Am**	**Ch**
Ps. veronii	HV4	+	+	/	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Ps. veroni	HV5	+	+	/	-	-	-	-	-	-	-	+	-	+	-	+	+	+
Ps. asplenii	HV6	+	+	/	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Ps. siringae	HV7	+		/	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Ps. putida	HV8	+	+	/	-	-	-	-	-	-	-	+	-	+	-	-	+	+
Ps. putida	HV9	+	+	/	-	-	-	-	-	-	-	+	-	+	-	-	+	+
Ps. fulva	HV10	+	+	/	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Ps. macroides	HV11	+	+	/	+	+	+	+	+	+	-	-	-	-	-	-	-	-

Table 5.4c: Growth characteristics of different bacterial strains isolated from *Populus* cv. “Hoogvorst” leaf material. (+ growth, -no growth, / not tested; B-benzene, T-toluene, EB-ethylbenzene, m- p- o-X- xylene)

Seq. Id	**isolate**	**869**	**Sz**	**BTEX**	**B**	**T**	**EB**	**m-X**	**p-X**	**o-X**	**TCE**	**Ni**	**Zn**
Ps. graminis	BHV17	+	-	-	-	-	-	-	-	-	-	-	-
Ps. graminis	BHV20	+	-	-	-	-	-	-	-	-	-	-	-
Ps. stutzeri	BHV23	+	-	-	-	-	-	-	-	-	-	-	-
Ps. stutzeri	BHV26	+	+	-	-	-	-	-	-	-	-	-	-
S.adhaesiva	BHV3	+	+	-	-	-	-	-	-	-	-	-	-
S.adhaesiva	BHV13	+	+	-	-	-	-	-	-	-	-	-	-
S.adhaesiva	BHV19	+	-	-	-	-	-	-	-	-	-	-	-
S.adhaesiva	BHV28	+	+	-	-	-	-	-	-	-	-	-	-
A. bicolorata	BHV12	+	-	-	-	-	-	-	-	-	-	-	-
A. bicolorata	BHV24	+	-	-	-	-	-	-	-	-	-	-	-
A. bicolorata	BHV34	+	+	+	+	+	+	+	+	+	+	+	+
D. zoogloeoides	BHV27	+	-	-	-	-	-	-	-	-	-	-	-
D. zoogloeoides	BHV6	+	+	-	-	-	-	-	-	-	-	-	-
M. lincolnii	BHV25	+	+	-	+	-	-	+	+	+	-	-	-
M. lincolnii	BHV5	+	+	-	-	-	-	-	-	-	-	-	-
C. luteum	BHV2	+	+	-	-	-	-	-	-	-	-	-	-
X. cynarae	BHV10	+	+	-	-	-	-	-	-	-	-	-	-
E. cloacae	BHV32	+	+	+	+	+	+	+	+	+	+	-	-

Seq. Id	isolate	869	Sz	BTEX	B	T	EB	m-X	p-X	o-X	TCE
Ps. rhodesiae	RHV24	-	-	-	-	-	-	-	-	-	-
Ps. graminis	RHV33	-	-	-	+	+	+	+	-	-	+
S. mali	RHV40	+	+	+	+	+	+	+	+	+	+
S. mali	RHV48	-	-	-	-	-	-	-	-	-	-
R. basilensis	RHV3	-	-	-	-	-	-	-	-	-	-
R. basilensis	RHV76	-	-	-	-	-	-	-	-	-	-
M. amorphae	RHV61	+	+	+	+	+	+	+	+	+	+
F. aurantia	RHV69	-	-	-	-	-	-	-	-	-	-
K. rhizophila	RHV71	-	-	-	-	-	-	-	-	-	-
H. frisingense	RHV73	-	-	-	-	-	-	-	-	-	-
P. amylolyticus	RHV75	-	-	-	-	-	-	-	-	-	-
B. glathei	RHV82	-	-	-	-	-	-	-	-	-	-
B. thiooxidans	RHV2	-	-	-	-	-	-	-	-	-	-

Table: 5.4d: Growth characteristics of different bacterial strains isolated from poplar cv. "Hoogvorst" rhizosphere soil material. (+ growth, -no growth; B-benzene, T-toluene, EB-ethylbenzene, m- p- o-X- xylene)

5.4 DISCUSSION

Previous studies of endophytic bacteria have relied upon cultivation, and morphological and phenotypic characterizations for their identification. In the study presented here, the endophytic bacterial isolates have been analyzed and characterized genotypycally, including partial 16S rRNA sequence analyses. Comparative sequence analysis of 16S rRNA genes are able to estimate the identification of bacteria with good reliability to the genus level and with good approximation to the species level. The sequence similarities and closest matches to the reference sequence listed in Table 5.2a and 5.2b provide an overview of the level of similarity to described species. It should be pointed out that, although no formal identification of bacteria based on 16S rRNA gene sequence is accepted, these data provide the best overview of the phylogenetic placements of

the isolates that can be obtained with a single analytical method. While the 16S rDNA sequence analyses provide reliable phylogenetic placements of the isolates, BOX-PCR (Rademaker *et al.*, 1998) genomic profiling is able to differentiate closely related bacteria. While this study has focused upon the analysis of the bacterial diversity associated with selected poplar cultivars used for phytoremediation applications, the biotechnological potential of using endophytic bacteria to enhance phytoremediation requires that the bacterial strains can be resolved, detected and monitored at the strain level. Thus, a rapid genomic DNA fingerprinting technique, such a BOX-PCR, has proven to be a valuable tool for the high-resolution differentiation of the isolates.

5.4.1 Diversity assessment

Twenty one putative genera were observed from a collection of 146 isolates. Gram negative bacteria, in particular Gammaproteobacteria, dominated this collection, with the genus *Pseudomonads* comprising 42% of the total isolate collection. This was not unexpected, as it has been previously observed that in many cases *Pseudomonas* are abundant in both the soil environment (Spiers *et al.*, 2000) and the plant interior (Samish *et al.*, 1963; Gardner *et al.*, 1982; Hallmann *et al.*, 1997; Tanprasert and Reed, 1997).

Both poplar varieties were studied in detail with their root, stem, leaf and rhizosphere soil samples being processed. Rhizosphere soil was isolated from a 1.5 below the surface as poplar trees are deep rooting in order to access a large volume of groundwater, and are therefore ideal for phytoremediation applications. In the case of poplar cv. “Hazendans” twenty-seven different sequence types were detected in the rhizosphere soil, twenty in the root and twelve in the stem. However, there were only seven potentially different morphotypes in the leaf and none of these matched those found in the stem, when BOX profiles were compared. It is hypothesized that endophytic bacteria

colonise plants primarily through the root network via natural and artificial wound sites, root hairs and at epidermal junctions (Sprent and de Faria, 1988; Pan *et al.*, 1997). Therefore, it is to be expected that endophyte diversity will decrease with distance from these entry points. However, leaf endophytic populations are likely to be a combination of those translocated from the stem and those entering through leaf wounds or stomata, the secondary proposed colonization route for endophytic bacteria (Lodewyckx *et al.*, 2002b).

In poplar cv. "Hoogvorst" there were eleven different sequence types in the rhizosphere soil, five each in the root and stem based on the BOX PCR fingerprint and the sequence data. However, in the leaf of "Hoogvorst" there were potentially seventeen different morphotypes. This may be accounted for by penetration of bacteria through leaf stomata or leaf wounds.

In general it appears that the diversity of endophytic bacteria found within these trees is higher than we might have expected from previous plant studies. The two varieties of poplar, although growing intermingled on the same site, do seem to host different endophytic populations in terms of diversity, abundance and activities, suggesting that each plant species has an association with a specific bacterial population. It is likely that the total endophytic bacterial population is composed of both these plant-specific bacteria, and a non-specific population.

It is noticeable within this isolate collection that there are no *Streptomyces* or *Archaea* present, which one would expect to find in the soil environment, and thus potentially within a plant as well. The genera isolated here in fact largely match those previously isolated from a range of plant tissues (Lodewyckx *et al.*, 2002b).

This study also highlights a useful analysis strategy for differentiating between strains that may appear similar when partially identified by sequence analysis, but demonstrate differing whole genome fingerprints and physiological characteristics.

5.4.2 Compartmentalization

The data presented here provide an insight into the extent of compartmentalization occurring in bacteria residing within poplar. It is very important to assess the location of a bacterium that is intended for an enhanced phytoremediation application as different target compounds are translocated through the plant at varying rates, in direct relation to their log K_{ow} value (Trapp *et al.*, 1994). Organic xenobiotics with a log K_{ow} between 0.5 – 3.5 in particular are readily taken up and translocated through plants (Trapp *et al.*, 1994; Trapp *et al.,* 2001) and therefore a successful bacterially enhanced phytoremediation strategy will partner a bacterium located in the plant tissue where the pollutant residence time is longest. There seems to be a strong effect of compartmentalization in the various plant tissues, illustrated by Figures 5.2a and 5.2b, in both poplars cv. “Hazendans” and “Hoogvorst”. In both of the tree cultivars there seems to be no isolates found in adjacent plant/soil compartments that have the same total genome fingerprint. It has been shown that the composition of xylem sap varies qualitatively at different points within the plant, as well as seasonally, and this would explain the formation of niches specific to certain bacteria (Rennenberg *et al.*, 1994; Sagisaka S, 1974; Schneider *et al.*, 1994; Weber *et al.*, 1998).

Figure 5.2a: Schematic representation of endophyte isolates locations, with respect of genera, within the compartments of poplar cv. “Hazendans”

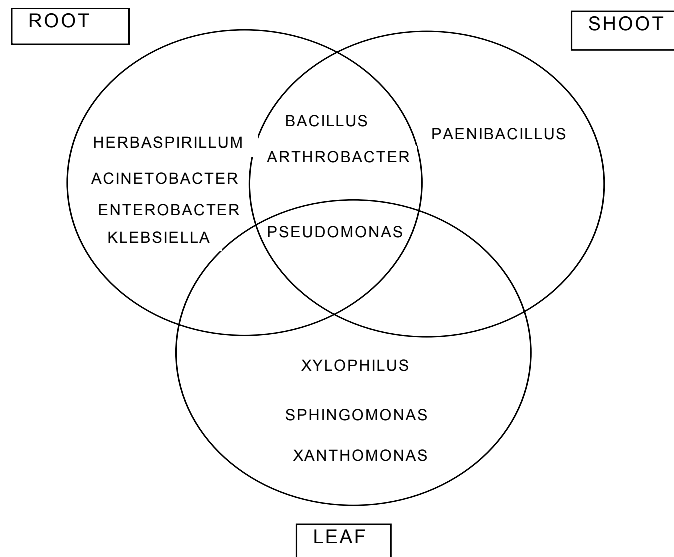
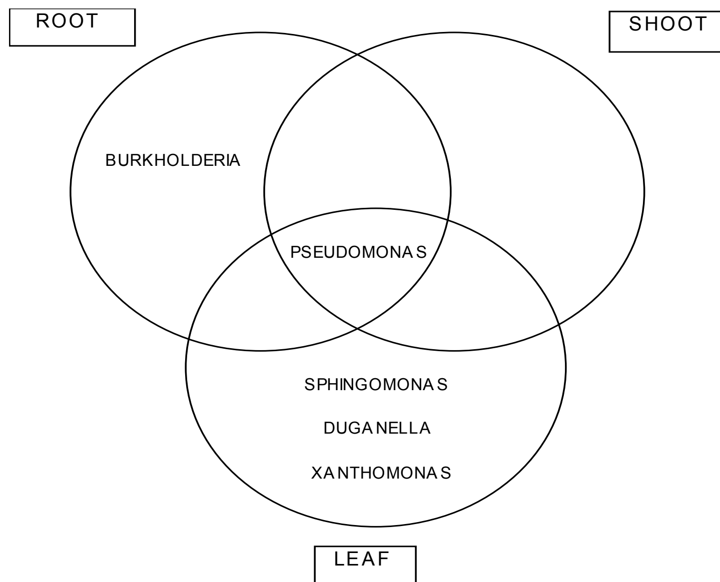


Figure 5.2b: Schematic representation of endophyte isolates locations, with respect of genera, within the compartments of poplar cv. “Hoogvorst”.



5.4.3 Application potential

Assessment of the catabolic properties of these isolates allows selection of those most suitable for further study. Those with an inherent ability to grow on a BTEX-related compound or in the presence of TCE may be able to be used directly as endophytic degraders. We identified a considerable number of rhizosphere and endophytic bacteria that were able to tolerate and/or degrade BTEX compounds, despite the fact that the tree roots had not yet reached the BTEX contaminated groundwater. A quantitative assessment of their representation within the plant community can then be made, to assess whether sufficient numbers exist to effectively enhance the plants degradation capacity, or whether their numbers will go up when a selective pressure under the form of BTEX compound is applied

to the system. Tolerance to antibiotic or heavy metal would be advantageous here for re-isolation in controlled condition experiments, or for the introduction of new properties using natural gene transfer. Accordingly, these microorganisms will not be considered as genetically modified microorganisms (GMO), a factor that will facilitate their application and public acceptance. It will be relatively straightforward to construct, by natural gene transfer, a collection of endophytic bacteria with *a la carte* degradation properties. The ability of three of the isolates described here to recolonize poplar root and stem material has been verified, thereby demonstrating their potential for use in field scale trials (Germaine *et al.,* 2004; Chapter 6). Bacterially assisted phytoremediation has recently been demonstrated by Barac *et al*. (2004; Chapter 3), where natural endophytic bacteria isolated form yellow lupine plants had the pTOM plasmid introduced, and were re-inoculated into yellow lupine. These plants were then able to degrade toluene to a much greater degree, and a reduction in phyto-volatilization of 50-70% was observed.

Table 5.5 shows a collection of 34 isolates, with potential for pollutant tolerance/degradation that could be studied further with the objective of enhancing phytoremediation in mind.

Tree	Location	Isolate no's
Populus cv. Hazendans	shoot	HV 5,8,9,11
	leaf	BHV 25
	rhizo. soil	RHV40, 48
Populus cv. Hoogvorst	root	WHZD 6,11,14,21,22
	shoot	HZD 5,6,8,9,10,11,13,14,15,17,18
	leaf	BHZD 8
	rhizo. soil	RHZD 4,5,12,27,28,49,53,56,76,78

Table 5.5: Endophytic isolates showing potential for future study for use in enhanced phytoremediation

5.5 CONCLUSIONS

The information gathered in this study allows for the selection of isolates that are cultivable, non-pathogenic, plant specific, and located within the plant compartment most suitable for the biodegradation of the target compound. Further studies are most needed to ascertain the seasonal dynamics of the endophytic population, with particular emphasis on quantitative information on the dominance of a target isolate in relation to the whole population. This would potentially allow modeling of the potential degradation kinetics of a plant-bacteria combination and maximize enhanced phytoremediation potential to when bacteria are in their greatest numbers. It seems clear however that there exist both intra- and inter-specific variations in the endophytic bacterial diversity of the two poplar cultivars studied here. It also appears that some of these bacteria have inherent degradation capacities that could be further exploited in a remediation technology.

From an assessment of the molecular and physiological characteristics of these bacteria, a short list of isolates suitable for future study can be drawn up, and used to develop partnerships of plant and bacteria ideally suited for tackling particular contaminated soil problems, where phytoremediation is currently limited due to phytotoxicity.

CHAPTER VI

RE-COLONISATION OF POPLAR CUTTINGS BY *gfp* EXPRESSING ENDOPHYTIC BACTERIA

Most of these results were published in FEMS Microbiology Ecology 48 (2004) 109-118; Germaine K, Keogh E, Garcia-Cabellos G, Borremans B, van der Lelie D, Barac T, Oeyen L, Vangronsveld J, Porteous Moore F, Moore ERB, Campbell CD, Ryan D and Dowling DN.

ABSTRACT

Until now little is known about the re-colonization patterns or population sizes of endophytic bacteria in deciduous trees. This study describes the construction and re-colonisation patterns of four green fluorescent protein (*gfp*):kanamycin^R labelled bacterial endophytes when re-introduced into poplar trees, their host plant. Two of these endophytes showed considerable colonization in the roots and stems of inoculated plants. *gfp* expressing cells of three strains were observed to colonise the xylem tissue of the root. Three strains proved to be efficient rhizosphere colonisers, supporting the theory that the rhizosphere can serve as a source of bacterial endophytes.

6.1 INTRODUCTION

Bacterial colonisation of the internal tissues of plants has been described in almost all plant species examined so far. Although many of these bacteria are phytopathogenic, a considerable number have also been found that colonise the plant without causing disease (Sessitch *et al*., 2002). Such bacteria are referred to as endophytic bacteria. Colonisation may take place at the local tissue level or throughout the plant, with bacterial colonies and biofilms residing latently in the

intercellular spaces and inside the vascular tissues (Sessitch *et al.*, 2002; Gage *et al.*, 1996; Gopalaswamy *et al.*, 2000; Hinton and Bacon, 1995). A diverse array of bacterial species have been reported to be endophytic, including *Acetobacter*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Herbaspirillum* and *Pseudomonas* (Elbeltagy *et al.*, 2001; Mcinroy and Kloepper, 1994; Misko and Germida, 2002; Salgado *et al.*, 1997; Tapia-Hernandez *et al.*, 2000), see Lodewyckx *et al.* (2002b), for a full review. Indeed many bacterial genera have been isolated from a given tissue within a single plant (Sturz *et al.*, 1997). Sturz and Nowak (2000) proposed that these endophytes originate from the rhizosphere or phylloplane micro-flora, and observed that many rhizosphere bacteria could penetrate and colonise root tissue, providing a route into the xylem. In this vascular tissue, the bacteria could transport themselves throughout the plant and hence colonise it systematically. Once inside the plant, endophytic populations have been observed to grow to between 2.0 and 7.0 $\log_{10}$ colony forming units (CFU) per gram of fresh tissue (McInroy and Kloepper., 1994; Shishido *et al.*, 1999)

Certain endophytic bacteria have been shown to enhance plant growth, increase plant resistance to pathogens, drought and even herbivores, such that their commercial potential has received much study (Sturz and Nowak, 2000; Azedvedo *et al.*, 2000; Bacilio-Jimenez *et al.*, 2001; Chanway *et al.*, 1989; James, 2000; Reiter *et al.*, 2002). Siciliano *et al.* (2001) showed that plants grown on soil contaminated with xenobiotics naturally recruited endophytes with the necessary contaminant degrading genes. In addition Lodewyckx *et al.*, (2001), showed that endophytes of yellow lupine were able to increase the nickel accumulation and nickel tolerance of the inoculated plant. Particular endophytes can confer an increased level of resistance to the plant against specific xenobiotics (Siciliano *et al.*, 2001). Consequently, it may be necessary or advantageous to introduce bacteria expressing degradative capacity into a plant species intended for such applications. However, the use of microbial inoculations for biocontrol, growth

promotion or plant-assisted bioremediation requires an efficient level of re-colonisation and competence of the introduced microbe.

Assessing colonisation efficiency and population size requires an ability to track and identify the inoculated strain within the host plant. Introduction of antibiotic resistance genes into the strain provides a simple method of tracking colonisation. However, strains inoculated into plants may lose their antibiotic resistant phenotype (Narin and Chanway, 2002). This problem can be overcome by coupling antibiotic resistance with genes for expressing green fluorescent protein (*gfp*), which provides a unique, visual phenotype and is a simple, stable method of studying population dynamics of the organisms within the plant tissues. The *gfp* polypeptide is 27 kDa and when irradiated with blue or near UV light (A_{395}) produces a green fluorescence (A_{508}). It is a useful marker in environmental microbial studies because it is expressed in most Gram-negative bacteria, does not require any exogenous substrates and is extremely stable (Errampalli *et al.*, 1999). The *gfp* marker gene has proved to be very useful in colonisation studies and has been used to visualise the infection and root nodulation events of both *Rhizobium* spp. and *Agrobacterium tumefaciens* (Gage *et al.*, 1996; Li *et al.*, 1999; Stuurman *et al.*, 2000). Elbeltagy *et al.* (2001), successfully showed the colonisation of the shoots of wild rice plants by a *gfp* labelled version of the nitrogen fixer *Herbaspirillum*, while Ramos *et al.* (2000), used *gfp* to assess the physiological status of *Pseudomonas putida* cells within the rhizosphere of barley seeds.

Here we describe the isolation, identification and re-colonisation efficiency of four poplar tree endophytes when re-introduced into their host poplar tree. The bacterial strains were genetically marked with a kanamycin:*gfp* cassette inserted into their chromosome, allowing the visualisation of colonising cells and the estimation of population sizes within the various tissues of the host plant.

6.2 MATERIALS AND METHODS

6.2.1 Isolation and identification of endophytic bacteria from poplar trees.

The endophytic bacteria used in this study were isolated from xylem sap of poplar trees (*Populus trichocarpa* x *deltoides* cv. "Hoogvorst"). The poplar trees were growing on a phytoremediation site near a Ford car factory in Genk, Belgium (see Chapter 8). The groundwater from this site contains increased concentrations of zinc, nickel and BTEX compounds. The extraction of xylem sap was carried out by means of Scholander pressure bomb instrument (Schuur, 1998), which is a pressure chamber connected to a bottle containing nitrogen gas. After surface sterilization (5 min in a solution containing 1% active chloride), the young twig was sealed in the pressure chamber with the cut end exposed through a hole in the chamber cover. The chamber pressure was slowly increased (5-25 bar) until xylem sap was forced back to the cut surface. Xylem sap (100 µl) was extracted and spread over different solid isolation media, 869 medium (Mergeay *et al.*, 1985), 1/10 strength 869 medium, and Schatz medium (Schatz and Bovel, 1956) supplemented with a carbon source mix (1.3 ml/l glucose (40%), 0.7 ml/l lactate (50%), 2.2 ml/l gluconate (30%), 2.7 ml/l fructose (20%) and 3 ml/l succinate (1 M). After an incubation period of 7 days at 30°C, eight different morphotypes were isolated and identified by 16S rDNA analysis (chapter 5). HV4 was identified as *Pseudomonas veronii*, HV6 as *P. aspleni*, HV9 as *P. putida* and HV10 as *P. fulva* (see Chapter 5).

6.2.2 Selection of endophytic strains for re-inoculation studies

Four endophytic strains were chosen for the recolonisation studies based on their putative endophytic features, other potentially useful biocontrol/bioremediation traits (outlined in Table 6.1) and the 16S rDNA sequence characterisation, which indicated that these strains were unrelated to known phytopathogenic bacteria.

To assess their bioremediation potential, the endophytic strains were grown on minimal media plates containing various organic chemicals as the sole carbon source. Growth of these plates after 48 h was considered an indication of the biodegradation of the targeted compound. Putative endophytic traits (cellulase activity and motility) were determined by the methods of Verma *et al*. (2001). Heavy metal resistance was assessed by growing the endophytic strains on nutrient agar supplemented with various concentrations of heavy metals. Biocontrol properties of the endophytes were determined by streaking the test strain in the centre of sucrose asparagine (SA) agar plates containing high and low iron concentrations. Streaks of either *Pythium ultimum* or *Bacillus subtilis* were then made approximately one from either side of the test strain streak. The plates were incubated for 48 h and examined for growth inhibition of *P. ultimum* and *B. subtilis* (Russo *et al*., 1996).

Trait/strain		HV4 (VM1449)	HV5	HV6 (VM1450)	HV7	HV8	HV9 (VM1453)	HV10 (VM1454)	HV11
Endophytic features	**Motility**	+	+	+	+	+	+	+	ND
	Cellulase activity	-	+	+	+	+	+	+	ND
Plant growth prompting features	**Phosphate solubilization**	+	+	+	+	+	+	-	ND
	Indole production	-	+	+	+	-	+	-	ND
Biodegrad. ability	**2.4-D***	-	-	+	-	-	+	-	ND
	Toluene**	-	+	-	-	-	-	-	ND
	Naphtalene**	-	-	-	-	-	-	-	ND
Bio-control	***B. subtilis***	-	-	-	-	-	+	-	ND
	P. ultimum	-	-	-	-	-	-	-	ND
Heavy metal resistance	**Zinc**	Re2	Re5	Re5	Re2	Re2	Re5	Re5	Re2
	Copper	Re2	Re2	Re2	Re2	Re2	Re5	Re2	Re5
	Arsente	Re2	Re5	Re5	Re5	Re5	Re5	Re2	Re5
Id. Based on 500bp 16S rDNA sequence		*P. veronii*	*P. veroni*	*P. asplenii*	*P. syringe*	*P. putida*	*P. putida*	*P. fulva*	*B. macroides*
% similarity to database strains		99.4	99.8	95	90.6	99.8	98.6	99.4	92.7

Table 6.1: Potentially useful traits of selected strains. Re2 resistant to 2mM concentrations; Re5 resistant to above 5 mM concentrations; ND not determined. * 1 mM concentration; ** supplied in vapour phase.

6.2.3 Bacterial strains, plasmids and culture conditions

Strains and plasmids are listed in Table 6.2.

The endophytic strains were maintained on Lauria-Bertani (LB) agar (Merck) or 284 Tris-minimal medium (Schlegel *et al.*, 1961) at 30°C. The *Escherichia coli* strain CM2780 carrying the pFAJ1819 plasmid was grown in LB broth (Miller, 1972) supplemented with 50 µg/ml kanamycin (Km) at 37°C.

Bacterial strain	Characteristic	Source/reference
P. veronii **HV4**	Poplar tree endophyte	This study
P. aspleni **HV6**	Poplar tree endophyte	This study
P. putida **HV9**	Poplar tree endophyte	This study
P. fulva **HV10**	Poplar tree endophyte	This study
E. coli **CM2780**	pFAJ1819	Herrero *et al.*, 1990
VM1449	HV4 with a mini-Tn5 insertion of *gfp*,Km^R	This study
VM1450	HV6 with a mini-Tn5 insertion of *gfp*,Km^R	This study
VM1453	HV9 with a mini-Tn5 insertion of *gfp*,Km^R	This study
VM1454	HV10 with a mini Tn5 insertion of *gfp*,Km^R	This study
Plasmid pFAJ1819	A transposon/suicide plasmid containing a pUT mini Tn5 transposon carrying a kanamycin resistance gene and two copies of the gfp gene under the regulation of a strong constitutive promoter	Herrero *et al.*, 1990; Xi *et al.*, 1999,

Table 6.2: Bacterial strains used in this study (P – *Pseudomonas*).

6.2.4 Introduction of the gfp:kanamycin cassette into selected endophytic strains

The gfp donor strain, CM2780 carrying the pFAJ1819 plasmid was grown overnight in LB medium supplemented with 50 µg/ml Km and the endophytic recipient strains were grown overnight in LB medium, washed in 10^{-2} M $MgSO_4$ and aliquots of 100 µl were added to a sterile filter (0.45µm) and incubated overnight at 30°C on solid LB medium. The mating mixture was plated out on 284 Tris-minimal medium supplemented with 50 µl/ml Km and incubated at 30°C for 4-5 days. The transconjugants were purified and the presence of the *gfp* gene was confirmed by PCR using the following primers: gfp-F5'-CCCCCCCGGGCTAGATTTAAGAAGG-3' and gfp-R5'-TTTTCCCGGGTTATTTGTATAGTTCATCCATGCC-3'. Individual colonies were resuspended in 100 µl of 10^{-2} M $MgSO_4$ and 5 µl was taken as a template for PCR. Amplification was performed in a Trio-Thermoblock (Biometra). 100 µl reaction mixtures, containing 0.5 U TaKaRa Ex *Taq* polymerase (Cambrex Bio Science, Verviers), each of the nucleotides at 200 µM, and each of the primers at 1 µM, were subjected to a preliminary denaturation step at 94°C for 10 min, followed by 35 cycles of incubation at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min and terminated with one step of 8 min at 72°C. This growth rates, Biolog[R] profiles, motility and cellulase activities of the transconjugants were tested and compared to the wild type strains to ensure the gfp cassette had not been randomly inserted into genes involved in colonisation. Obtained strains were named VM1449 (HV4), VM1450 (HV6), VM1453 (HV9) and VM1454 (HV10) (see table 6.2).

6.2.5 Plant re-inoculation

Fresh cultures of the endophytic *gfp*:Km^R derivative strains (VM1449, VM1450, VM1453 and VM1454) were grown in LB broth, at 30°C, 200 rpm, to an approximate absorbance (A_{600}) value of 1.0. Cells were harvested by centrifugation, washed in 0.85% sterile saline or in 10 mM $MgSO_4$ and resuspended in 100 ml modified ISO 8692 (1997) (or for VM1454 in ½ saturated Hoagland's solution) plant nutrient solution containing 5% LB broth. These inocula contained between 10^8 and 10^9 cells/ml as determined by standard plate counts. Woody stem cuttings (approximately 35cm long) were harvested from mature poplar trees and surface disinfected using 70% ethanol. These cuttings were cultivated hydroponically in the inoculum suspension for six weeks (or for inoculum with strain VM1454 for 72 hours) at 20-25°C under a 16 h light/8 h dark regime. After the inoculation period the trees were transferred to pots containing a compost/vermiculite substrate (3:1 ratio) that had been previously sterilised by autoclaving for 2 h at 121°C but which was not maintained under sterile conditions throughout the experiment. The trees were cultivated under the same conditions as detailed above. Un-inoculated trees served as controls.

6.2.6 Enumeration of cultivable endophytic populations within plant tissues

Trees were destructively sampled 10 weeks after inoculation. Healthy samples of leaf, fleshy stem, sap from the woody stem, root and rhizosphere tissues were taken from each plant. Leaves and stems were surface sterilised by swabbing with 70% ethanol. Roots were surface sterilised by placing in a solution containing 2% active chloride for 2.5 min. Plants inoculated with VM1454 were treated in a different way. Leaves and roots were sterilized during 10 min in solution

containing 2% active chloride. Both, fleshy and woody stem, were sterilized during 5 minutes in solution containing 1% active chloride. Both sterilization solutions containing chloride (1 and 2%) were supplemented with 1 droplet Tween 80 per 100 ml. The sterilising agents were removed from tissues by rinsing three times in sterile water. To check for sterility, surface sterilised tissues were pressed against a plate count agar (PCA) plate (Merck) and samples of the third rinsing were plated onto PCA or 869 medium. Excess water was removed from tissues using sterile paper towels. Sap was collected from woody stems by vacuum extraction and collected in sterile Eppendorf tubes. 1 g samples of the surface sterilised tissues were homogenised using sterile pestle and mortars, serially diluted in 0.85% sterile saline and 100 µl samples were spread plated onto PCA and PCA containing 100 µg/ml kanamycin. Plant material from plants inoculated with VM1454 was macerated in 10ml mM $MgSO_4$ using a Polytron PT1200 mixer (Kinematica A6). Serial dilutions were made and plated on 284 medium with gluconate and 284 with 100µg/ml kanamycine and gluconate as a carbon source. Sap and rhizosphere samples were serially diluted and plated in the same manner. Plates were incubated at 30°C and examined for growth after 72 h. The number of colony forming units per gram (CFU g^{-1}) of fresh tissue was calculated.

Bacteria isolated on selective media from rhizosphere, root, stem and twig of poplar “Hoogvorst” inoculated with VM1454, were plated on 869 medium (Mergeay *et al.*, 1985). The inoculated strain VM1454 was also plated on the same media as a control. Those bacteria were also tested for presence of *gfp* using primers gfp-F5’-CCCCCCCGGGCTAGATTTAAGAAGG-3’ and gfp-R5’-TTTTCCCGGGTTATTTGTATAGTTCATCCATGCC-3’ and fingerprinted using BOX PCR.

6.2.7 *In planta* visualisation of *gfp* expressing endophytes, using epi-fluorescent microscopy

Hand cut sections of surface sterilised leaf, stem and root tissues were examined under blue light (395 nm) using an Nikon E400 epi-fluorescent microscope equipped with a 100 W mercury short arc photo-optic lamp. Lucia® imaging software (version 4.6) was used to capture and process microscopic images. Visualisation of *gfp* expression proved difficult due to auto-fluorescence from the plant tissue itself. *gfp* visualisation was achieved by counter staining the tissue section in 0.05% methyl violet for 30 s, which caused the plant cells to fluoresce red under near UV light.

6.3 RESULTS

6.3.1 Isolation and identification of endophytic bacteria from poplar trees

A collection of endophytic bacteria was isolated from xylem sap of poplar trees (see Chapter 5). Eight of these were picked according to different morphotypes, designated HV4-HV11 and were identified by sequence analysis of a 500 bp sequence of their 16S rDNA gene, with reference to the 16S rDNA genes sequences of described bacteria with validly published names in the EMBL Nucleotide Sequence database (Kulikova *et al.*, 2004). Seven strains were identified as species of *Pseudomonas* and one as a *Bacillus* sp. Four Gram-negative isolates, designated as HV4, HV6, HV9 and HV10 were selected for the re-colonisation study. These four strains were further identified by sequence analysis of 1500 bp of their 16S rDNA gene. The nearly complete 16S rDNA gene

sequences of HV4, HV6, HV9 and HV10 (chapter 5, table 5.1b) data indicate that all four strains are certainly species of *Pseudomonas*. The 16S rDNA sequence analyses indicated that none of the strains are closely related to any known phytopathogenic bacterium.

6.3.2 Construction of *gfp* expressing endophytic strains

After 5 days the mating between CM2780 (pFAJ1819) and the selected endophytes, HV4, HV6, HV9 and HV10 resulted in transconjugants that were Km^R. The presence of *gfp* gene in these strains was confirmed by PCR analysis. *E. coli* strain CM2780 was used as control. All transconjugants tested showed the *gfp* specific amplicon of 750 bp corresponding to the *gfp* gene. A representative transconjugant of each conjugation was chosen and named, respectively, as VM1449, VM1450, VM1453 and VM1454. These transconjugants were compared with their wild type parent strains for specific growth rate, Biolog™ metabolic profiles and for cellulase activity. For VM1449, VM1453 and VM1454 all parameters were similar to those of the wild type strains. This was also the case for VM1450 except that Biolog™ profile showed a minor difference (one substrate out of 95 tested) to that of the wild type. These data suggest that mini-Tn5-*gfp* cassette did not disrupt any key trait required for the survival of the marked strains and that they could be used in re-colonisation experiments.

6.3.3 Colonisation and enumeration of endophytic population within plant tissues

Inoculated poplar trees were allowed to grow for 10 weeks before sampling took place. Total bacterial populations and Km^R, *gfp* expressing populations were determined for each of the tissues examined. Endophytic bacteria are considered to be those isolated from the internal tissues of surface sterilised plants.

However, it is difficult to determine whether an organism is truly endophytic or merely a survivor of the surface sterilisation process (Zamora and Romero, 2001). To ensure that the sterilisation processes were adequate, the sterilised tissues were pressed against the surface of a sterile PCA plate and samples of the third water rinsings were also plated onto PCA plates. Bacterial counts of these plates were always between 0-10^1 CFU g^{-1}, which was considered to be a good indication that the surface was successfully sterilised. No *gfp* expressing, kanamycin resistant cells were isolated from non-inoculated plants. A number of indigenous endophytic strains were also isolated on the kanamycin plates. To ensure that only the inoculated strains were counted, these plates were examined under the epi-fluorescent microscope and only those colonies expressing *gfp* were enumerated.

Pseudomonas sp. strain VM1449 was detected only in the rhizosphere and the interior root tissues of inoculated trees (Table 6.3). The total cultivable aerobic rhizosphere population was determined to be between 10^7 and 10^8 CFU g^{-1} and the numbers of strain VM1449 accounted for as much as 3.2% (Table 6.3) of the total culturable population. No colonisation of VM1449 was detected in the stems or leaves.

Trees inoculated with strain VM1450 showed notable colonisation in all tissues including the leaves (Table 6.3). As with VM1449, the samples from the rhizosphere showed the greatest level colonisation rates, followed by the root and then by the woody stem. Inoculum populations within the root where in the order of 10^4 CFU g^{-1} tissue. VM1450 populations in the rhizosphere accounted for up to 7.8% of the total bacterial community during the course of the experiment. Inside the root, VM1450 cells comprised as much as 2.7% of the total root endophytic population, while in the stem (tissue and sap), VM1450 numbers were between 10^3 and 10^4 CFU g^{-1} which represents 10% of the total cultivable population.

After 10 weeks, plants inoculated with strain VM1453, showed a similar colonisation pattern to VM1450, with the exception of the leaf. The rhizosphere population was stable at approximately 10^7 CFU g^{-1} tissue throughout the experiment. VM1453 population comprised 7.1% (Table 6.3) of the total rhizosphere population while internal root communities were as high as 1.0% of the total root cultivable endophyte population. Within the stem, VM1453 numbers were on average 10^4 CFU g^{-1}.

	Leaf			Stem			Root			Rhizosphere		
Strain	1	2	3	1	2	3	1	2	3	1	2	3
VM 1449	$2.3 \cdot 10^{3}$	0	0	$7.2 \cdot 10^{3}$	0	0	$1.8 \cdot 10^{4}$	$5.4 \cdot 10^{2}$	0.3	$2.3 \cdot 10^{8}$	$7.4 \cdot 10^{6}$	3.3
VM 1450	$2.9 \cdot 10^{3}$	$5.0 \cdot 10^{2}$	1.7	$1.3 \cdot 10^{4}$	$2.4 \cdot 10^{3}$	18.0	$6.4 \cdot 10^{5}$	$1.7 \cdot 10^{4}$	2.7	$2.8 \cdot 10^{8}$	$2.2 \cdot 10^{7}$	7.8
VM 1453	$3.4 \cdot 10^{3}$	0	0	$1.2 \cdot 10^{4}$	$1.3 \cdot 10^{3}$	11.7	$1.2 \cdot 10^{6}$	$1.2 \cdot 1^{4}$	1.0	$3.5 \cdot 10^{7}$	$2.5 \cdot 10^{6}$	7.1
VM 1454	$4.0 \cdot 10^{2}$	0	0	$2.0 \cdot 10^{4}$	0	0	$1.1 \cdot 10^{5}$	0	0	$1.2 \cdot 10^{7}$	0	0

Table 6.3: Total number of colony forming units, number of Km resistant – *gfp* expressing colony forming units and relative inoculum percentage within the plant tissues. 1 - Total bacterial population (cfu/g); 2 - Km^{R}, *gfp* expressing bacterial population (cfu/g); 3 - Inoculum as a % of total population.

Bacterial strain VM1454 was not recovered from any plant part, even not from the rhizosphere. No *gfp* expressing colonies were observed on plates with bacterial population isolated form leaf, twig, stem, root or rhizosphere samples of "Hoogvorst" cuttings inoculated with VM1454. All strains grown on selective media were purified and plated on 869 medium together with the control strain VM1454 (Figure 6.1). Photography taken under UV light shows that none of the bacteria is fluorescent.

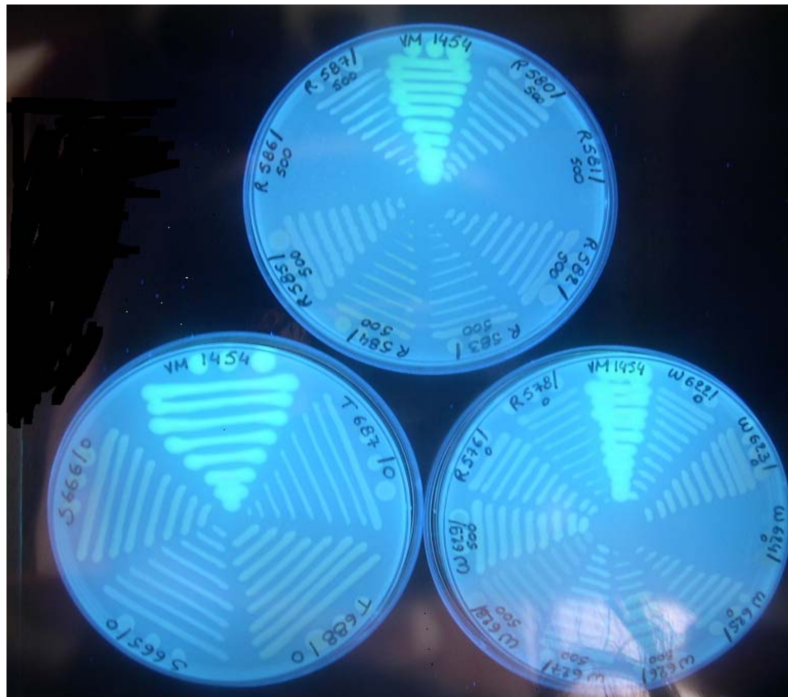


Figure 6.1: Bacterial isolates form rhizosphere (plate 1), stem and twig (plate 2) and root (plate 3) of poplar cv. "Hoogvorst" cuttings inoculated with VM1454, grown during 10 weeks under greenhouse conditions. No *gfp* expressing bacteria, except control VM1454 strain.

To verify colonization capability of strain VM1454 all isolates strains were analysed by BOX PCR (Figure 6.2). None of the isolated bacteria shows the same

fingerprinting as the control VM1454 strain. Therefore we may conclude that VM1454 was not capable to re-colonize poplar cuttings.

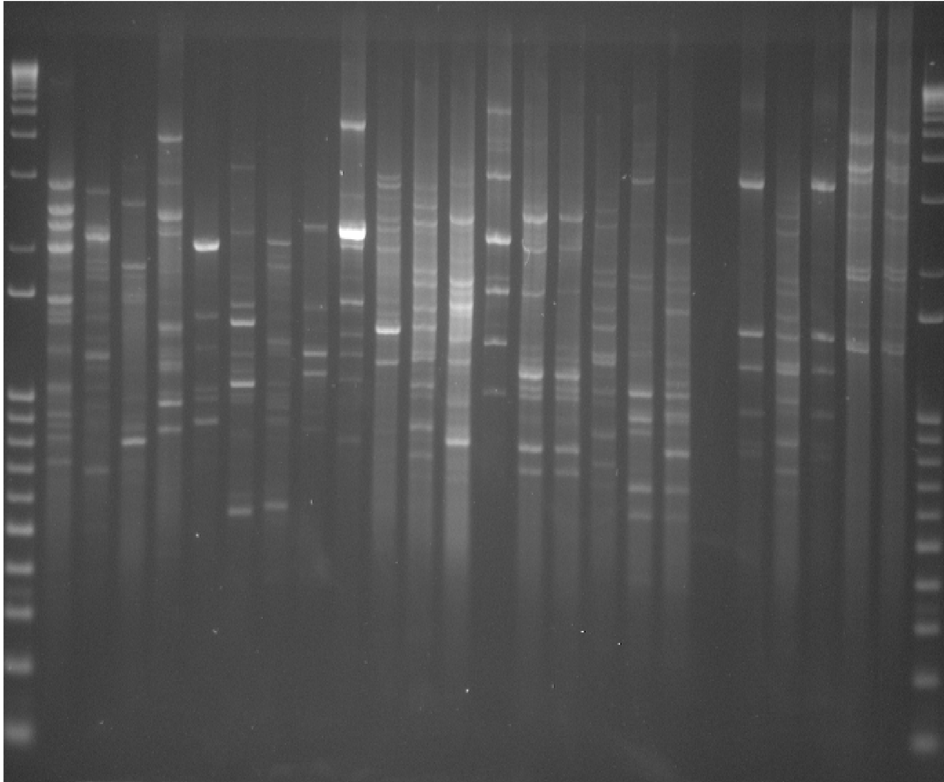


Figure 6.2: M-marker; 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 bacterial strains isolated from rhizosphere of poplar plants inoculated with VM1454; 10, 11, 12, 13, 14, 15, 16, 17 and 18 strains isolated from roots of poplar plants inoculated with VM1454; 19 and 20 bacteria isolated from stem of poplar plants inoculated with VM1454; 21 and 22 bacteria isolated from twig of poplar plants inoculated with VM1454; 23 and 24 control VM1454.

6.3.4 *In planta* visualisation of *gfp* expressing endophytes

Methyl violet counter staining proved useful when examining plant tissues and the rhizosphere for endophyte colonisation. However, when using this dye, some masking of the *gfp* expression was noted. Adjusting the staining time markedly reduced this masking.

Strain VM1449 expressing *gfp* was visible in longitudinal section of surface sterilised roots particularly just beneath the epidermal surface and between the intercellular spaces of xylem tracheid cells (Figs. 6.3a and 6.3b). Cells expressing *gfp* were also clearly visible in the rhizosphere (Fig. 6.3g) of non-sterilised roots. At no time were VM1449 cells visualised in the stem or leaves of the plants sampled.

Colonies of *gfp* expressing VM1450 cells were observed in the rhizosphere (Fig. 6.4h) and in the intercellular spaces of the xylem tracheid cells of the root and woody stem (Figs. 6.3c and 6.3d) No *gfp* expressing cells were observed in fleshy new stems or in the leaf although the strain could be recovered on plates.

Visualisation of VM1453 showed cells residing between the intercellular spaces of the outer-cortex and extensive colonisation within the cellular pits of xylem tracheids in the roots (Figs 6.3e and 6.3f). However, micro-colony formation in the stem and leaves was not observed but individual of pairs of cells were visualised residing near the stem vascular tissues. *gfp* expressing cells within the plant tissues were noticeably larger and more spherical than expected.

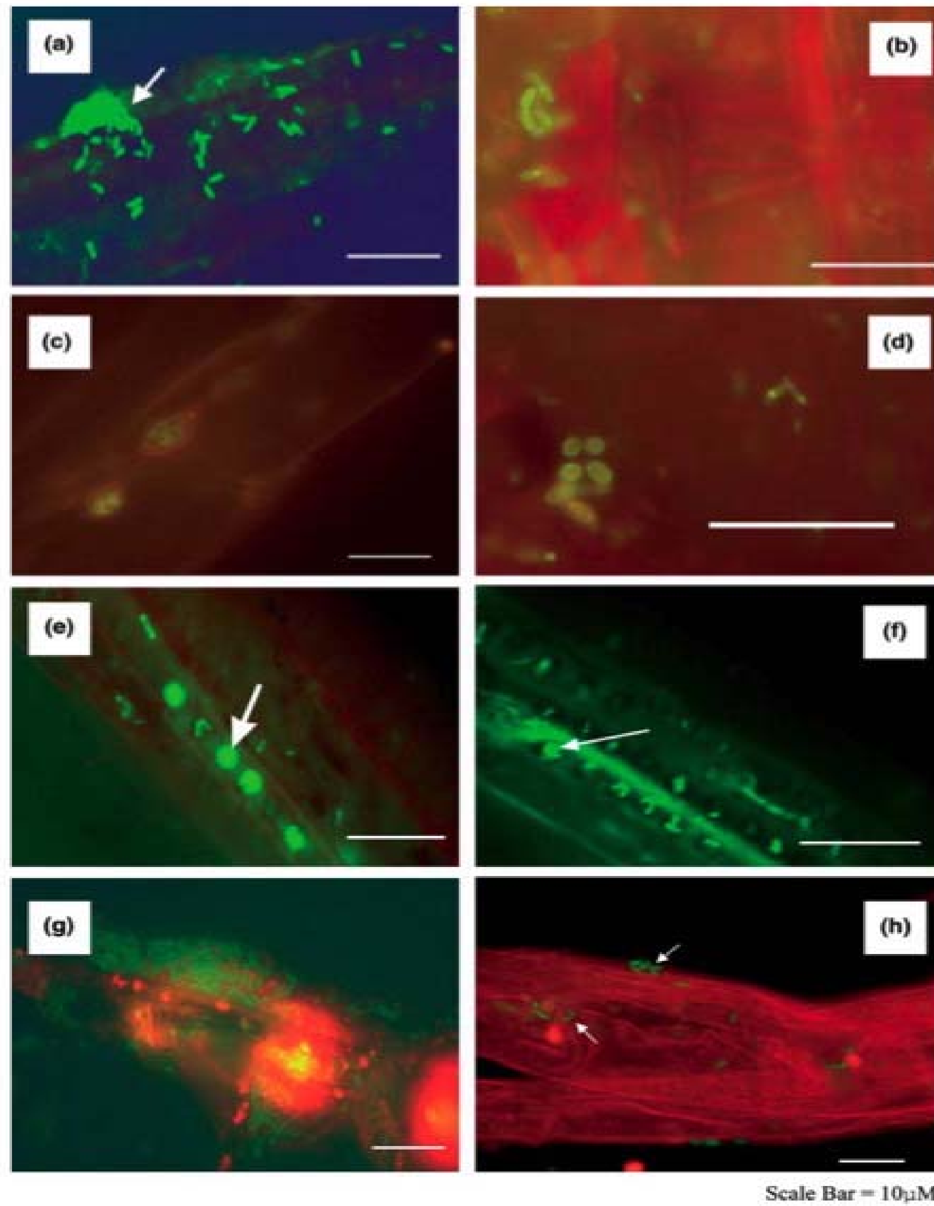


Figure 6.3: Visualization of inoculated endophytes within plant tissues (1000x). (a) VM1449 macro-colony residing on the surface of inner-cortex cells on the root. (b) Individual VM1449 cells on xylem tracheid cells in the root. Note enlarged cells. (c) VM1450 micro-colonies inhabiting the intercellular spaces of root xylem cells. (d) VM1450 cells in intercellular colonies showing enlarged cellular morphology. (e,f) Xylem traceid pits showing colonisation by VM1453 cells. (g) VM1449 cells colonising the rhizosphere of a lateral root (14 weeks after inoculation). (h) Micro-colonies of VM1450 residing on the root surface (1000x). Scale bar = 10 µM.

6.4 DISCUSSION

Most studies that investigated bacterial endophytic re-colonisation of plants focused on plants of agricultural importance. Although there have been studies on the isolation of bacterial endophytes from trees (Chanway *et al.*, 1989), to our knowledge this is the first reported study of an endophytic recolonisation of a hard wood deciduous tree. Four endophytes designated as HV4, HV6, HV9 and HV10 were isolated from the xylem sap of *Populus trichocarpa* x *deltoides* cv. “Hoogvorst” and were characterised by 16S rDNA sequencing. From these sequence data it is clear that these isolates are members of the genus *Pseudomonas*. However, a definitive identification to the species level is not possible based on 16S rDNA gene sequence comparisons alone. These isolates have close similarities to other isolates reported to degrade xenobiotic compounds, such as *Pseudomonas* sp. EK1, a 1,3-dichloropropene degrader, *Pseudomonas* SN-1, a naphthalene degrader, *Pseudomonas* sp. A2, a PAH degrader and *Pseudomonas* 1pA-2 which degrades diterpenoid compounds produced by some species of trees. The isolates also exhibit similarities to bacteria associated with plants, such as *Pseudomonas* sp. Fa3, an epiphyllic bacterium isolated from the leaves of strawberry plants, as well as *Pseudomonas* sp. ND9L, isolated from rhizosphere soil, this bacterium inhibits fungal (*Cercospora beticola*) infections on sugar beet. Comparative analysis of the 16S rDNA sequences of the strains indicated that they are not closely related to any known plant pathogens. These strains were the tagged with a *gfp*:kanamycin random insertion transposon and their respective derivates (VM1449, VM1450, VM1453 and VM1454) were re-inoculated into cuttings of their original host plant. The presence of the marker genes appeared to have no negative effect on the ability of the strains to colonise the rhizosphere and the interior tissues of the plant. However, although these derivatives had similar metabolic profiles and growth properties to that of

the wild type strains, it is a remote possibility that the transposon insertion affected their colonisation ability with respect to wild type strain.

Strains VM1449, VM1450 and VM1453 could be re-isolated from the interior tissues of surface sterilised roots. Two of the strains were detected in the stems and occasionally in the leaves of inoculated plants. However, population sizes in these tissues did vary greatly between replicates. This may have been an artefact of the surface sterilization protocol or simply evidence of differing rates of colonisation within individual plants. The effectiveness of the sterilisation protocol varied according to the thickness of the sample. Thin samples were prone to over-sterilisation. Thus, where possible, throughout the experiment, tissue sections of similar thickness were sampled.

Population size of VM1449, VM1450 and VM1453 strains decreased markedly from the rhizosphere to the root interior. The fact that the three strains were efficient colonisers of the rhizosphere further supports the theory that endophytes can originate from the rhizosphere (Misko and Germida, 2002) and from there, move into the internal plant tissues. Although the strains were inoculated into autoclaved vermiculite, the poplar plants were not sterile and a large population of non-inoculated bacteria were co-isolated from the rhizosphere, suggesting that some of these were derived from the autochtonous endophytic community within the poplar plants. This may help to re-inoculate and replenish the rhizosphere microbial flora when the growing season begins after the winter decline. The populations of inoculated strains comprised on average only 1-4% of the total cultivable microbial population in the rhizosphere and as much as 18% of the internal root population. The survival of these strains 10 weeks after inoculation, despite the fact that there was no observable selective pressure, suggests that they are good competitors. Populations of inoculated strains in the root and stem (including the sap) were on average, in the same range, up to 10^4 CFU g^{-1} fresh weight, which is consistent with reports (McInroy and Kloepper, 1994; Shishido *et al.*, 1999) in other plants.

Interior colonisation by VM1449 was detected only in the root. Population sizes were in the order of 10^2 CFU g^{-1} of plant tissue. This suggests that VM1449 is not active coloniser, but it may colonise the tissues at a slower rate or through accidental disposition. This is further supported by the fact that the strain did not show any cellulase activity (possibly required for endophytic colonisation), whereas both VM1450 and VM1453, which did show cellulase activity, were seen to be active colonisers of the stem and leaves. VM1449 cells expressing *gfp* were seen to randomly colonise the surface of cells in the root cortex and was also observed to form micro-colonies intrecellularly within the inner margin of the pericycle, adjacent to root xylem cells. At no time were VM1449 cells visualised in the stem or leaves, which corresponds with the results of the enumeration analysis.

Strain VM1450 was the only inoculated strain to be detected in the leaves suggesting that it is an efficient systemic coloniser. Its motility, cellulase activity and its ability to colonise the xylem (as shown by *gfp* detection) are probably contributing factors to the spread of this strain throughout the plant. Colonies of VM1450 cells were visualised mainly in vascular tissues, with a proliferation of cells on and within the intercellular spaces between adjacent xylem tracheid cells. The rapid spread of this strain from the root to aerial tissues suggests that it uses the vascular system as a route for systemic colonisation.

VM1453 appeared to be an efficient coloniser of the root and stem but colonisation in the leaves was not found in this period of study. The observed colonisation pattern of VM1453 was markedly different from these of the other two strains. VM1453 cells were almost exclusively located in the vascular system and specifically within the pits of xylem cells. Large fluorescent colonies could be clearly seen residing in these cell pits along the length of the plant cell wall. These pits are typically between 1 and 14 µm wide (depending on their location in the plant) and facilitate the lateral transport of water and minerals throughout the plant (Fahn, 1982). It is likely that this strain also uses the xylem to

transport itself into the stem, where it was recovered in high numbers. The fact that the three endophytes were found to colonise the vascular tissue is not surprising as the literature details numerous endophytic strains with this ability (Gopalaswamy *et al.*, 2000; Reinhold-Hurek and Hurek, 1998). At no time during the microscopic examination of the plant tissues was intracellular colonisation observed nor did there appear to be any cellular damage caused by the colonisation of inoculated endophytes.

There was a noticeable change in the cellular morphology of the inoculated strains when visualised within the plant tissues. The cells appeared to be larger and more spherical than when grown on LB. Changes in cellular shape dependent on environmental conditions have been reported previously (Li *et al.*, 1999; Ramos *et al.*, 2000). Li *et al.* (1999), also observed this phenomenon with *Agrobacterium tumefaciens* cells when inoculated into plants. It was proposed that bacterial cells are better nourished upon successful colonisation, but this paper also cited reports that cell shape is related to the growth rates of strains within a particular environment and that slower growth rates yielded excessively large cells. Interestingly, there was no observed change in the morphology of bacteria colonising the rhizosphere. These observations have been supported by Ramos *et al.* (2000), who showed that *P. putida* colonising the rhizosphere of barley had high growth rates under sterile conditions during day 1, however, potential low growth rates were detected under non-sterile conditions.

6.5 CONCLUSIONS

This study has shown the successful recolonisation of poplar trees by three endophytic bacterial strains under controlled conditions. Two of the strains, VM1450 and VM1453, demonstrated efficient colonisation resulting in high population numbers within the plants tissues. None of the introduced stains

showed any signs of pathogenicity towards their host plant and others tested. Many studies have shown that the colonisation levels in field trials are less successful than those in laboratory trials. This is probably due to increased microbial competition and less favourable environmental conditions (Zinniel *et al*., 2002). Therefore, additional long-term field trials need to be carried out in order to gain a better understanding of the colonisation pattern and population dynamics of endophytic bacteria in poplar trees in the field.

CHAPTER VII

THE POTENTIAL USE OF POPLAR CUTTINGS, INOCULATED WITH ENDOPHYTIC BACTERIA POSSESSING pTOM, FOR IMPROVED PHYTOREMEDIATION OF TOLUENE

ABSTRACT

Phytoremediation of highly water soluble and volatile organic xenobiotics, such as BTEX, often is limited due to an insufficient degradation of the pollutants by plants and their rhizosphere. This can result in toxic effects on the plants by these compounds and their metabolites, or in volatilization of compounds through the leaves, which potentially causes new environmental problems.

Poplar cuttings (*Populus trichocarpa* x *deltoides* cv. "Hoogvorst") were inoculated with the endophytic bacterial strain *Burkholderia cepacia* VM1468, which possesses the TOM plasmid encoding for toluene degradation. We subsequently studied the effect of this inoculation on the phytotoxicity of toluene. As controls, either non-inoculated plants or plants inoculated with *B. cepacia* (pTOM), a soil isolate, were used. After exposure to 500 mg l^{-1} toluene all plants showed signs of toluene phytotoxicity: however, plants inoculated with the endophytic strain VM1468 formed more biomass and showed reduced evapotranspiration of toluene compared to non inoculated plants or plants inoculated with *B. cepacia* strain BU61, with the latter plants suffering less from toluene phytotoxicity than with the non-inoculated control plants. By analyzing the microbial communities associated with the non-inoculated control plants and plants inoculated with VM1468 or BU61, we were able to demonstrate that horizontal gene transfer of pTOM efficiently occurred to the endogenous endophytic bacteria, both in the presence and absence of toluene. However, neither strain VM1468 nor BU61, none of them having poplar as their natural host plant, was able to establish itself within the poplar associated microbial

community: after 10 weeks, neither VM1468 nor BU61 could be isolated from rhizosphere of plant tissue samples. This work demonstrates for the first time the potential of natural gene transfer to adapt an endophytic microbial community to deal with the stress imposed by a new environmental insult.

7.1 INTRODUCTION

Phytoremediation is a technology that uses green plants and their associated microorganisms to remediate soils, sediments, surface and groundwater contaminated with heavy metals, metalloids, radionuclides, organics and nutrients (Cunningham *et al.*, 1996). The fast growth of poplar trees and their large transpiration potential make them “trees of choice” for phytoremediation purposes (Schnoor *et al.*, 1995; Shrive *et al.*, 1994). However, phytoremediation can only work at sites where the concentration of pollutants is not toxic to the plants. Also, evapotranspiration of pollutants via the plant into the air can cause a new environmental problem (Schwitzguebel *et al.*, 2002). We already proved that phytoremediation of volatile and water soluble organic xenobiotics can be improved by using recombinant endophytic bacteria (Chapter 3; Barac *et al.*, 2004). Endophytic bacteria equipped with a toluene degradation pathway, inoculated in yellow lupine were reducing toluene phytotoxicity and evapotranspiration. Endophytic bacteria are prokaryotes that colonize internal plant tissues of healthy plants without causing symptoms of disease (Wilson 1995). They have been found in numerous plant species (Chanway 1998), with most being members of common soil bacterial genera such as *Pseudomonas*, *Bacillus*, and *Azospirillum* (Chanway 1996). It seems reasonable to hypothesize that endophytic bacteria, possessing the genetic information required for the efficient degradation of a pollutant, can promote degradation as the pollutant moves through the plant vascular system. Especially in trees, such as poplar or

willow that are currently used to develop phytoremediation strategies for organics, the time period between uptake of molecules by the roots and their arrival in the leaves takes several hours to days (Mc Crady *et al.*, 1987) as the compounds travel through the vascular system.

For this study, which aims at investigating the potential of endophytic bacteria to improve the phytoremediation of volatile organic contaminants by poplar, toluene was chosen as a model compound. Toluene (log K_{ow} 2.69, moderately hydrophobic and volatile), which is a very common environmental pollutant, is part of the BTEX family and can be easily taken up by the plants, including poplar (EPA, 2000). However, poplar cells are lacking the metabolic pathway for toluene degradation (see Chapter 4), and as a result a substantial amount of the toluene may be released in the environment via evapotranspiration (Burken and Schnoor, 1999). In addition, microbial degradation pathways for toluene are well characterized and often located on mobile elements, such as plasmids, facilitating their transfer into endophytic bacteria.

In order to test the potential of endophytic bacteria, possessing a toluene degradation pathway, to improve toluene phytoremediation process poplar (*Populus trichocarpa* x *deltoides* cv. "Hoogvorst") cuttings were inoculated with the bacterial strain *Burkholderia cepacia* VM1468, which is an endophyte from yellow lupine, and with *B. cepacia* BU 61, which is an environmental strain. Both strains possess the TOM plasmid and can degrade toluene. Poplar cuttings were exposed to toluene in concentrations of 0 or 500 mg l^{-1}. After 10 weeks, plants were harvested and their root, shoot and leaf biomass were determined and growth index calculated. During harvesting, plants samples were surface sterilized and bacteria were isolated on selective media. These bacteria were then purified, characterized and identified, with emphasis on their potential to use toluene as a carbon source. Evapotranspiration of toluene was measured 7 weeks after inoculation of the poplar cuttings, and was found to be significantly lower for the plants inoculated with the endophytic strain VM1468.

7.2 MATERIAL AND METHODS

7.2.1 Strain construction

A nickel and kanamycin resistant *Burkholderia cepacia* strain L.S.2.4 named BU0072 (Taghavi *et al*., 2001) was equipped with a toluene degradation pathway. As the donor strain for this degradation pathway *B. cepacia* BU61 (Shields *et al*., 1992) was used. *B. cepacia* BU61 is a derivative of *B. cepacia* G4 and possesses a mutated TOM plasmid, which renders the strain's toluene degradation capacity constitutive. Donor strain and receptor strain were grown overnight in LB (luria broth) medium, washed in 10 mM $MgSO_4$ and aliquots of 100µl were added to a sterile filter (0.45µl). After incubation of the filter on non-selective medium, transconjugants were harvested and washed in a 10 mM $MgSO_4$ solution in order to release the bacteria. Transconjugants were selected by means of their acquired $N^+Km^+Tol^+$ phenotype on minimal Tris buffered medium supplemented with the appropriate concentration of each selective marker, i.e. 1 mM $NiCl_2$, 100 µg/ml Km and toluene as carbon source. A representative transconjugant, referred to as *B. cepacia* strain VM1468 was chosen for further studies.

7.2.2 Re-inoculation of poplar cuttings

Cuttings were taken from the *Populus trichocarpa* x *deltoides* cv. "Hoogvorst" growing at a BTEX polluted site near the Ford factory in Genk, Belgium. Cuttings were 40 cm long and had a diameter of approximately 1 cm. They were surface disinfected with 75% ethanol and kept in tap water for four weeks to establish roots. When the roots were sufficiently developed, cuttings were placed in two different inocula. The first inoculum contained *B. cepacia* VM1468 strain and the second *B. cepacia* BU61. Both strains were grown in 284 (Schlegel *et al*., 1961)

minimal liquid medium with the addition of 0.2% gluconate as carbon source, at temperature of 30°C on a rotary shaker for a 24 hours. When the inoculum reached a concentration of 10^9 CFU/ml (colony forming units) (OD~1), the cells were harvested by centrifugation at 6000 rpm during 15 min and washed twice with 10 mM $MgSO_4$. The inoculum was prepared by resuspending to a final concentration of 10^8 CFU/ml in 1 liter ½ concentrated Hoagland's solution to wich 200 ml 284 medium supplemented with 0.2% gluconate was added. Cuttings were immersed into the inoculum during 72 hours; control plants were settled for three days in the same solution without bacteria. Subsequently, they were planted in 4 liter pots filled with non sterile sandy soil and saturated with ½ concentrated Hoagland's solution. Before planting weight, root number and root length of cuttings were determined as well as the number of leaves. Poplar plants were irrigated with ½ strength Hoagland solution or distilled water every other day and allowed to stabilize during two weeks under greenhouse conditions, before being challenged with toluene.

7.2.3 Effect of toluene on growth of poplar cuttings

During 10 weeks poplar cuttings, either without inoculum or inoculated with *B. cepacia* VM1468 or BU61, were exposed to toluene concentrations of 0 or 500 mg l^{-1}. This high toluene concentration was used in order to cause, in a relatively short period of time, clear effects of toluene toxicity. The weight of the cutting together with the mass of its pot, and the sandy soil irrigated with Hoagland's solution was brought to a total mass of 4800 gram. Every other day, pots with cuttings were weighed and filled up, with Hoagland's solution or water, until the initial weight. Also every other day 460 µl of toluene (to obtain a concentration of 500 mg l^{-1}) was injected into the soil at six different places using a syringe (1ml) with needle (0.9 x 70 mm) in order to spread the toluene as homogenously

as possible and to avoid evaporation from the surface of the soil. In order to prevent growth of algae, the pots were covered with dark grey plastic foil. For each treatment 10 replicas were established. After 10 weeks of exposure to toluene the cuttings were harvested, their total biomass determined, as well as root, young twig and leaf weight, including the number and length of roots and total number of leaves as well as their surface area.

7.2.4 Recovery of inoculated bacteria

After 72 hours of inoculation and 12 weeks of growth under greenhouse conditions, from which 10 weeks in the presence of toluene (described in previous paragraphs), plants were harvested. In order to examine the success rate of the inoculation, rhizosphere, root, stem, twig and leaf samples were taken from non inoculated plants and plants inoculated with *B. cepacia* VM1468 or BU61, this from the plants exposed to 0 or 500 mg l^{-1} toluene. After surface sterilization plant parts were macerated in 10 mM $MgSO_4$ using a Polytron PT1200 mixer (Kinematica A6). Serial dilutions were made and 100µl samples were plated on non-selective and 3 different types of selective media to test for the presence of the inoculated strains as well as other cultivable bacteria. Roots and leaves were sterilized during 10 minutes in a solution containing 2% active chloride (wt/vol, added as a NaOCl solution, Fluka) and supplemented with 1 droplet Tween 80 (Merck) per 100 ml solution; stems and twigs were sterilized for 5 minutes in a solution containing 1% active chloride also supplemented with 1 droplet of Tween 80 as well. After surface sterilization leaves, roots, stems and twigs were rinsed in three times sterile water for 1 min in sterile water and dried on sterile filter paper. The third rinsing water was plated on 869 medium (Mergeay *et al.*, 1985) as a control for sterility. The rhizosphere samples were immediately serially diluted in 10 mM $MgSO_4$ and plated on the same media. After

7 days incubation at 30°C the number of CFU was determined and calculated per gram of plant tissue or rhizosphere soil.

The bacteria isolated on the different selective media that showed morphological differences, were purified and genetically fingerprinted using BOX-PCR (Rademaker *et al.*, 1998). Subsequently, bacteria which showed different genetic fingerprinting and that were able to grow on toluene as sole carbon source were tested for the presence of the pTOM plasmid using PCR technique with *tom*A4 specific primers (tomA4: 5'gTTgTTgCCCTCCAAACCCTA-3' en 5'TgAGAAATCggggTgATAgC-3'). Those which strains giving amplification of the correct fragment with the *tom*A4 primer set will be identified by sequencing of their 16S rDNA.

7.2.5 Toluene evapotranspiration

Poplar cuttings (*Populus trichocarpa* x *deltoides* cv. "Hoogvorst"), inoculated as described before, were planted in sterile perlite saturated with ½ strength Hoagland's solution. After seven weeks growth under greenhouse conditions cuttings were carefully removed out from the pots, their roots were vigorously rinsed with sterile water, and then they were placed in glass cuvettes. The cuvettes used in this experiment were 60 cm high and 25 cm in diameter (Figure 7.1).

Figure 7.1: Two compartments glass cuvettes system for measurement of toluene evapotranspiration.

Each cuvette compartment was inserted in a flow-through system using a synthetic air source (Air Liquide) with an in-flow of 3 liter per hour on one side and with two linked Chromosorb 106 traps (Capped Sample Tubes (Perkin Elmer) and Chromosorb 60/80 (Alltech)) on the out flow. Between the plants cuvettes and the Chromosorb traps a column with $CaCl_2$ was placed in order to prevent

water condensation in the traps. Chromosorb 106 has a much better adsorption capacity for toluene than Tenax (described in chapter 3), so that cooling with dry ice was not necessary for optimal toluene trapping. Upper and lower compartment were separated with a glass plate with an insertion through which the stem of the plant was introduced. This insertion was gas tight closed with Polyfila exterior mixture (Polyfilla), so that roots in the lower and shoots in the upper compartment were completely separated; no gas exchange was possible between those two compartments, except through the stem of the plant. The upper compartment, the glass plate and the lower compartment were sealed with Apiezon (Apiezon Products M&I Materials LTD). The lower compartment was filled with ½ strength, sterile Hoagland's solution to which toluene was added in a concentration of 100 mg l^{-1} at the beginning of experiment. Chromosorb traps were changed at regular intervals and they were analyzed by GC-MS (Automatic thermal Desorption System ATD400, Auto System XLL Gas Chromatograph, Turbo Mass Spectrometer, Perkin Elmer). Cuvettes with plants were placed in a growth chamber with a constant temperature of 22°C and 14/10 hours light/dark cycle; photosynthetic active radiation 165 $\mu molm^{-2}s^{-1}$ (see Chapter 3; Barac *et al.*, 2004). The experiment was running over 96 hours in triplicate for each group of plants. The amount of evapotranspired toluene was calculated per unit of leaf area.

7.3 RESULTS

Toluene phytotoxicity: effect on the growth of poplar cuttings

Non inoculated and inoculated cuttings (with *B. cepacia* VM1468 or BU61) were grown as described under 7.2.3. For all plants a growth index was calculated as the difference between the plant fresh weight at the start of the experiment and after 10 weeks (Figure 7.2). Figure 7.3 shows the growth indexes for the non-inoculated plants and the plants inoculated with *B. cepacia* VM1468 and BU61 after 10 weeks exposure to toluene.

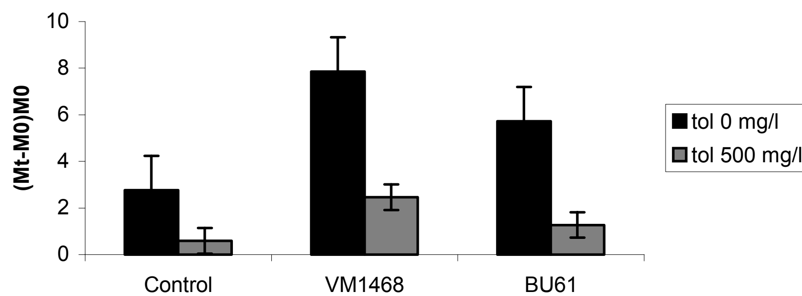


Figure 7.2: Difference in total biomass (g) after 10 weeks of growth of non-inoculated and inoculated (with *B. cepacia* VM1468 and BU61) poplar cuttings whether or not exposed to toluene (0 or 500 mg l^{-1}). Mt – plant weight 10 weeks after toluene edition; M0 – plant weight before toluene addition. Given are mean and standard error of at least five replicates.

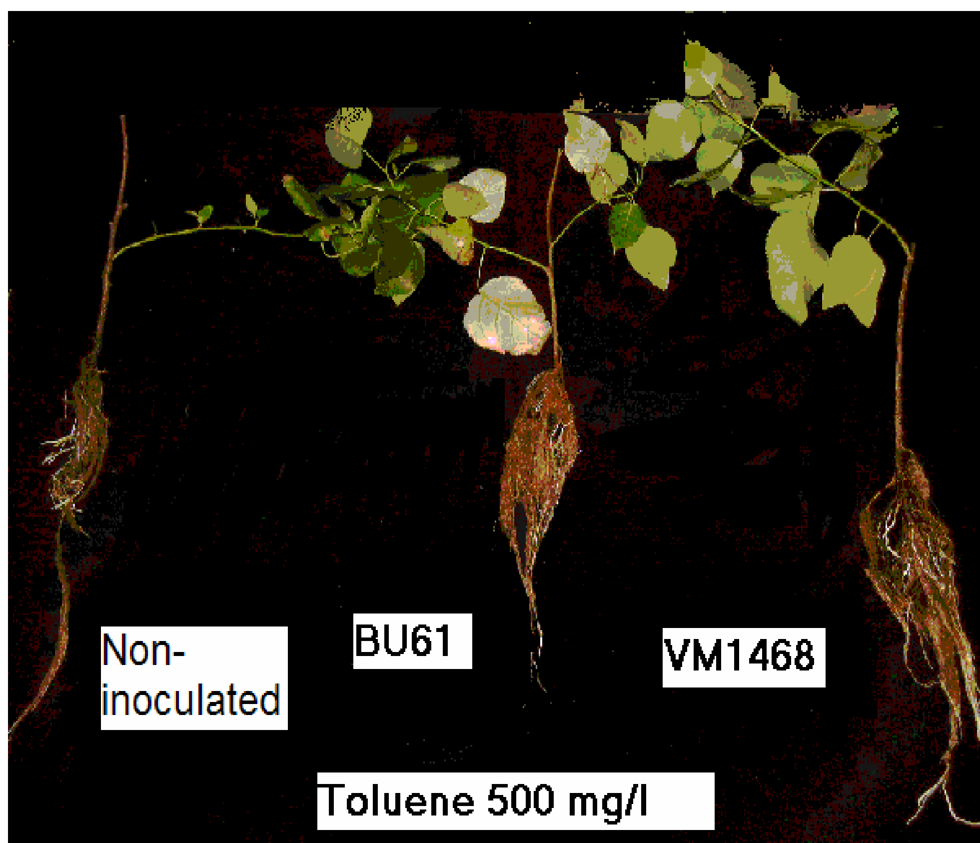


Figure 7.3: Poplar cuttings (*Populus trichocarpa* x *deltoides* cv. "Hoogvorst" after 10 weeks exposure to toluene in concentration 500 mg l^{-1}.

In general, plants inoculated with both bacterial strains produced more biomass than the non-inoculated plants, even in the absence of toluene. It is obvious that all plants that were every other day exposed to the high amount of toluene (500 mg l^{-1}) showed growth reduction. However, plants inoculated with the endophytic strain *B. cepacia* VM1468 produced a 2-3 times higher mean biomass compared with non-inoculated plants. Inoculation with the *B. cepacia* BU61 strain also had a positive effect on plant growth in the presence of toluene. Those plants produce double biomass compared with control plants, but still two-time less then plants inoculated with VM1468.

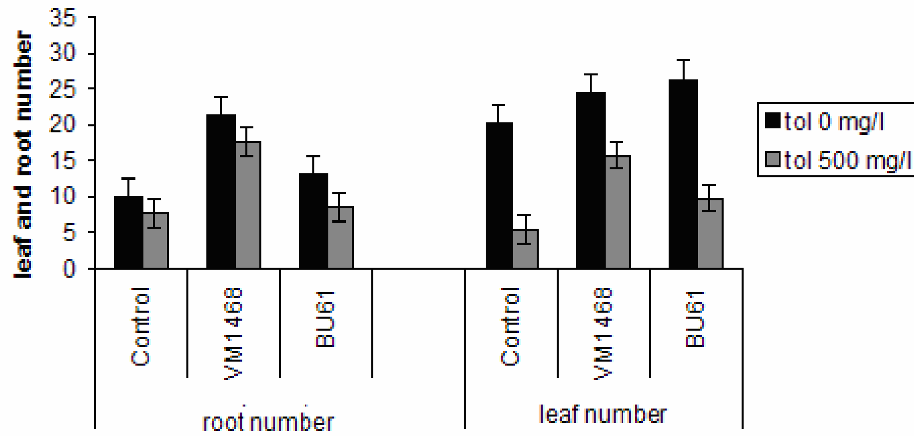


Figure 7.4: Difference in root and leaf number after 10 weeks of growth of non-inoculated and inoculated (with *B. cepacia* VM1468 and BU61) poplar cuttings whether or not exposed to toluene (0 or 500 mg l^{-1}). Given are mean and standard error of at least five replicates.

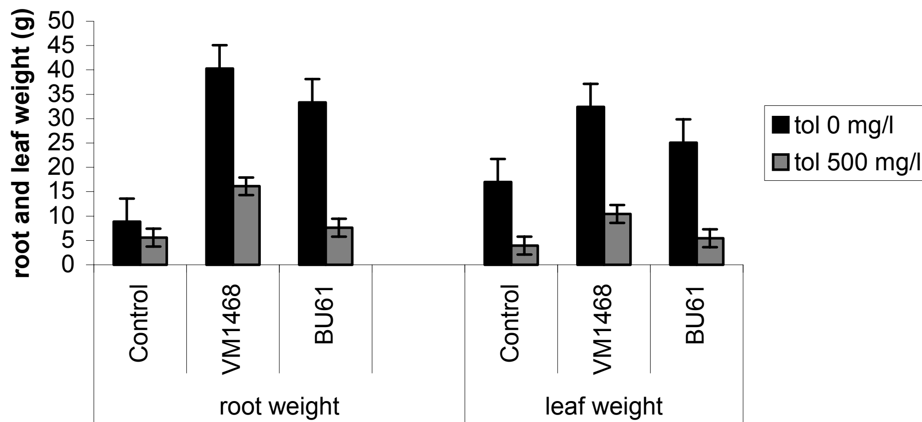


Figure 7.5: Difference in root and leaf weight after 10 weeks of growth of non-inoculated and inoculated (with *B. cepacia* VM1468 and BU61) poplar cuttings whether or not exposed to toluene (0 or 500 mg l^{-1}). Given are mean and standard error of at least five replicates.

All plants exposed to toluene formed less roots than plants grown in the absence of toluene (Figure 7.4). After toluene exposure the numbers of roots for the non-inoculated plants and plants inoculated with *B. cepacia* BU61 were not significantly different, while plants inoculated with *B. cepacia* VM1468 produced about twice as much roots (Figure 7.4). A similar trend was observed for their root weight (Figure 7.5).

From all plants, those inoculated with BU61 produced the most leaves if not exposed to toluene. After toluene treatment, plants inoculated with *B. cepacia* VM1468 formed 30-40% more leaves then plants inoculated with *B. cepacia* BU61 and about 60% more than non-inoculated plants (Figure 7.4). Without toluene treatment, leaves weight was highest for plants inoculated with *B. cepacia* VM1468, followed by BU61 and non-inoculated plants. The same trend was observed after the plants were exposed to toluene.

7.3.2 Recovery of inoculated bacteria

Poplar cuttings of *Populus trichocarpa* x *deltoides* cv. "Hoogvorst" were inoculated with the *B. cepacia* strains VM1468 or BU61, and grown as described under 7.2.4. After the plants were growing for 12 weeks under greenhouse conditions (from which some plants for 10 weeks in presence of toluene) rhizosphere, root, stem, twig and leaf were sampled to study the recovering of inoculated bacteria. After 7 days incubation at 30°C the total numbers of specific bacteria, their different morphology as well as their specific growth characteristics (resistance properties and toluene degradation) were determined (Table 1). The numbers of CFU were calculated per gram of fresh weight of plant material or rhizosphere soil.

Inoculated	**Plant part**	**Toluene**	**284+gluc**	**284+Km +gluc**	**284+Km +tol**	**284+tol**
No	Rhizos.	0mg/l	$25x10^6$(7)	$38.8x10^4$(2)	0	$21.9x10^7$(3)
No	Root	0mg/l	$95.8x10^8$(4)	$10.8x10^7$(1)	0	0
No	Stem	0mg/l	$11x10^8$(3)	0	0	0
No	Twig	0mg/l	$44.9x10^7$(4)	0	0	0
No	Leaf	0mg/l	$58.6x10^6$(1)	0	0	0
No	Rhizos.	500mg/l	$21.3x10^6$(6)	$13x10^5$(2)	0	$88.8x10^6$(3)
No	Root	500mg/l	$32.5x10^7$(6)	$29.3x10^6$(2)	0	0
No	Stem	500mg/l	$30.9x10^7$(3)	0	0	0
No	Twig	500mg/l	$32x10^7$(1)	0	0	0
No	Leaf	500mg/l	$28.5x10^5$(2)	0	0	0
VM1468	Rhizos.	0mg/l	$20.5x10^6$(6)	$21.3x10^5$(2)	$12.4x10^6$(2)	$12.4x10^6$(3)
VM1468	Root	0mg/l	$43.4x10^7$(5)	$10.6x10^7$(3)	$74.4x10^5$(2)	$37.9x10^7$(3)
VM1468	Stem	0mg/l	$51.7x10^7$(4)	$47.2x10^4$(2)	$12.7x10^6$(3)	$92.1x10^5$(4)
VM1468	Twig	0mg/l	$32.2x10^3$(3)	$37.3x10^3$(2)	$46x10^3$(2)	$36.8x10^3$(2)
VM1468	Leaf	0mg/l	$6.8x10^2$(1)	0	0	0
VM1468	Rhizos.	500mg/l	$17.4x10^6$(9)	$11.9x10^5$(2)	$13.7x10^5$(2)	$12.4x10^6$(4)
VM1468	Root	500mg/l	$55.8x10^7$(4)	$52.9x10^6$(2)	$10.4x10^6$(2)	$56.3x10^6$(3)
VM1468	Stem	500mg/l	$45.5x10^6$(5)	$10.9x10^6$(3)	$89.5x10^5$(2)	$21.9x10^7$(3)
VM1468	Twig	500mg/l	$36.4x10^7$(4)	$20.1x10^5$(2)	$55.9x10^5$(2)	$20.7x10^5$(2)
VM1468	Leaf	500mg/l	$12x10^7$(2)	0	0	0
BU61	Rhizos.	0mg/l	$54.3x10^5$(5)	$2.28x10^5$(3)	0	$32.3x10^6$(4)
BU61	Root	0mg/l	$48.2x10^7$(5)	$20.2x10^6$(3)	$18.7x10^6$(3)	$24.8x10^7$(3)
BU61	Stem	0mg/l	$65.8x10^7$(5)	0	0	$20.9x10^7$(4)
BU61	Twig	0mg/l	0	0	0	0
BU61	Leaf	0mg/l	$63.9x10^6$(3)	0	0	0
BU61	Rhizos.	500mg/l	$70.3x10^5$(6)	$64.4x10^5$(4)	0	$75.3x10^5$(5)
BU61	Root	500mg/l	$27.4x10^7$(8)	48.10^6(3)	$43.5x10^6$(2)	$15.8x10^7$(4)
BU61	Stem	500mg/l	$32.3x10^7$(4)	0	0	$29.1x10^7$(3)
BU61	Twig	500mg/l	$37.3x10^7$(1)	0	0	$40.6x10^7$(1)
BU61	Leaf	500mg/l	$27.6x10^6$(2)	0	0	0

Table 7.1: Recovery of bacteria from *Populus trichocarpa* x *deltoides* cv. “Hoogvorst” cuttings that were inoculated with *B. cepacia* VM1468 and BU61. As controls non-inoculated plants were analyzed. The number of bacteria is expressed per gram fresh weight. Numbers in parentheses are the numbers of different morphological types of bacteria as observed visually. Km – 100µg/ml kanamycin; gluc – gluconate as C- source; tol – toluene as C- source.

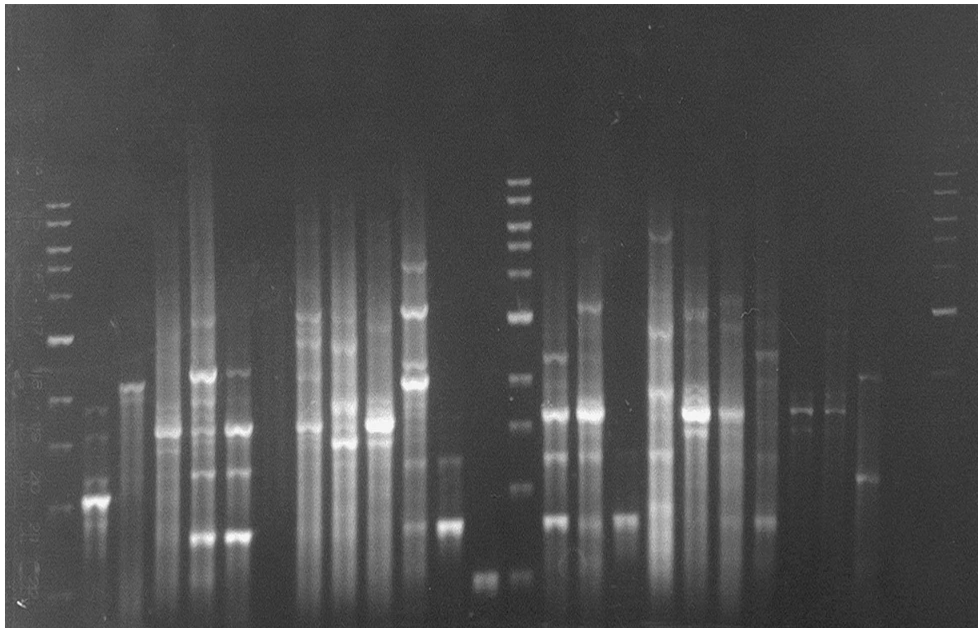
From non-inoculated plants only some bacteria isolated from rhizosphere were able to grow on toluene as carbon source. From plants inoculated with *B. cepacia*

VM1468, bacteria isolated from the rhizosphere, root, stem and twig could grow on all three selective media. Exceptions were bacteria isolated from leaves, which could only grow on the non-selective medium. From plants inoculated with *B. cepacia* BU61 bacteria isolated from the rhizosphere, root and stem (for plants exposed to toluene from young twig as well) were growing on selective medium, without kanamycin, with toluene as only carbon source (Table 7.1). Bacteria isolated from the rhizosphere and roots could also grow on the 284 gluconate medium supplemented with kanamycin (100 mg l^{-1}), while bacteria isolated from the roots where also able to grow on the combination of kanamycin and toluene as sole carbon source (Table 7.1). Those bacteria, growing on the selective media, were further characterized in the same way as bacteria isolated from plants inoculated with *B. cepacia* VM1468.

Finding different morphotypes of bacteria on the selective media, especially on the kanamycin containing medium with toluene as the sole carbon source, suggested transfer of antibiotic resistance and toluene metabolization properties from the donor strains BU1 and VM1468 to the endogenous plant associated communities, including the endophytes. This was supported by the observation that no toluene degrading endophytes could be found for those plants that did not receive a pTOM containing inoculum. To further test our hypothesis of gene transfer, especially transfer of the *tom* genes, all phenotypically different bacteria growing on selective media with toluene as sole carbon source were purified, their DNA was extracted and they were fingerprinted using BOX-PCR (Figures 7.7a, 7.7b, 7.7c and 7.7d), which allows to discriminate between bacteria up to the strain level (see also Chapter 5). All bacteria tested showed different fingerprints from the *B. cepacia* VM1468 and BU61 donor strains. BOX-PCR fingerprints also indicated that almost all bacteria isolated from the rhizosphere, roots, stem and twig of non inoculated and poplar cuttings inoculated with *B. cepacia* strains VM1468 or BU61 are different. The bacteria with different fingerprints were tested for the presence of the *tom* genes, using primers specific

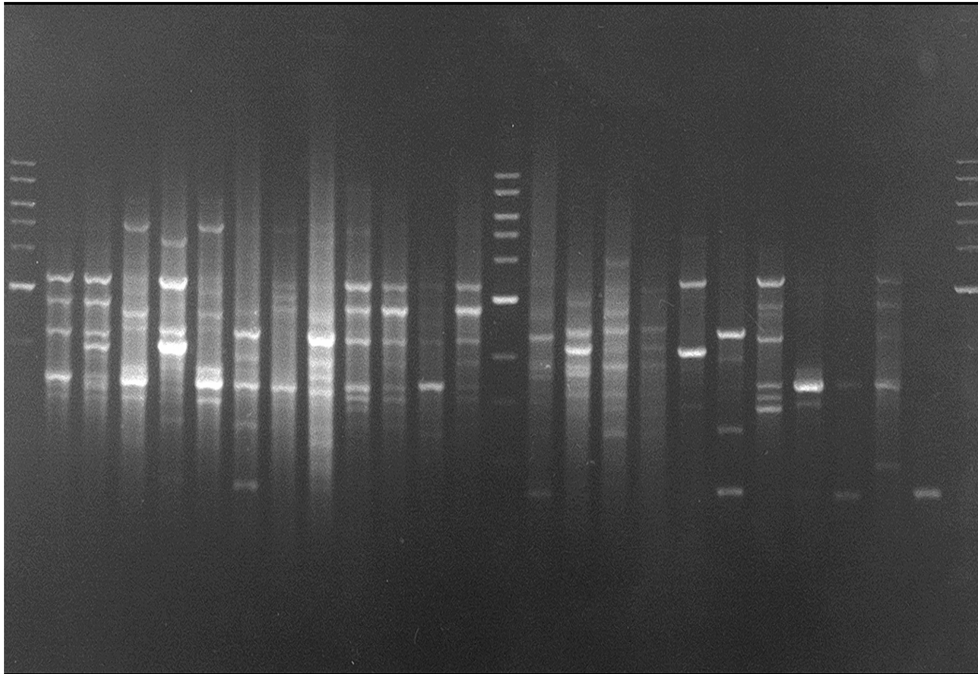
for the *tom*A4 gene. Most of the strains isolated from the rhizosphere, root, stem and twig of plants inoculated with *B. cepacia* VM1468 or BU61 showed the presence of the *tom*A4 gene.

From these results we may conclude that horizontal gene transfer occurred between the two donor strains, both having pTOM, and plant associated bacteria, including rhizosphere strains and endogenous endophytic bacteria. However, further identification of these bacteria is very important before we can arrive at our final conclusions. These bacteria will be identified by sequencing of their 16S rDNA.



M 1 2 4 5 6 7 8 9 10 12 13 14 M 15 16 17 20 21 22 23 25 26 27 28 M

Figure 7.6a: M-marker; 1 control strain BU61; 2 control strain VM1468; 4, 5, 6, 7, 8, 9, 10, 12, 13 and 14 – bacteria isolated from the roots of poplar cuttings inoculated with strain VM1468; 15, 16, 17, 20, 21, 22 and 23 – bacteria isolated from the roots of poplar cuttings inoculated with strain BU61; 25, 26, 27 and 28 – bacteria isolated from stem of poplar cuttings inoculated with strain VM1468



M 29 30 31 32 33 34 35 36 37 38 39 40 M 41 42 44 46 47 48 49 53 54 56 57 M

Figure 7.6b: M – marker; 29, 30, 31, 32, 33, 34, 35, 36 and 37 – bacteria isolated from the stem of poplar cuttings inoculated with strain VM1468; 38, 39, 40 and 41 – bacteria isolated from the stem of poplar cuttings inoculated with strain BU61; 42, 44, 46, 47 and 48 – bacteria isolated from twigs of poplar cuttings inoculated with VM1468; 49 – bacterium isolated from twigs of poplar cutting inoculated with BU61; 53, 54, 56 and 57 – bacteria isolated from rhizosphere of non inoculated poplar cuttings.

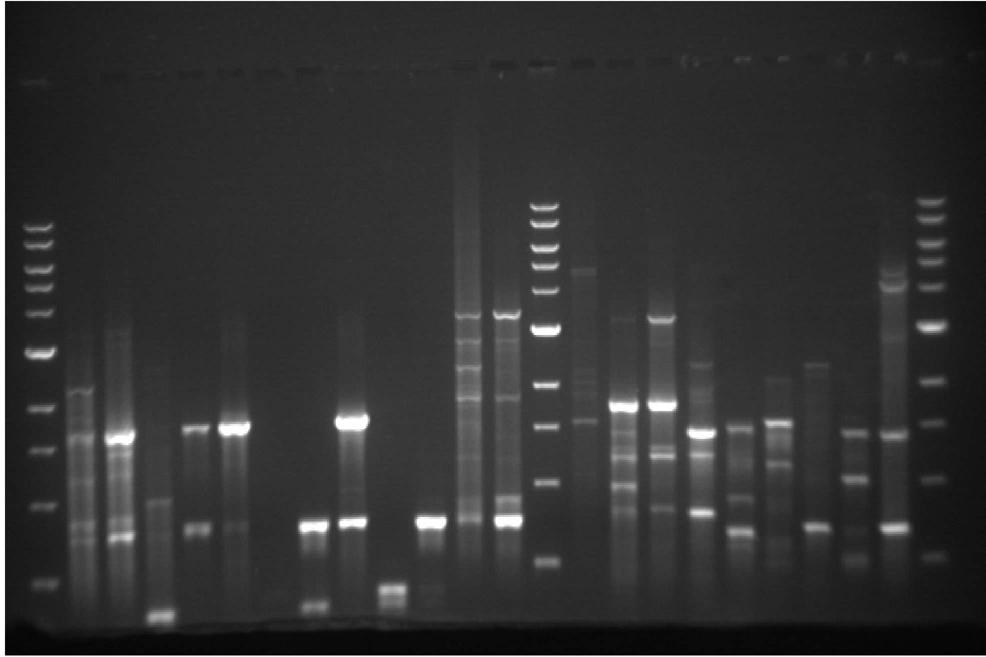


Figure 7.6c: M – marker; 58, 59 and 60 – bacteria isolated from the rhizosphere of non inoculated poplar cuttings; 63, 65, 66, 67, 68, 69, 70, 71, 73 and 74 – bacteria isolated from the rhizosphere of the poplar cuttings inoculated with strain VM1468; 75, 76, 77, 78, 79, 80, 81, 82, 83 84 and 85 – bacteria isolated form the rhizosphere of poplar cuttings inoculated with BU61

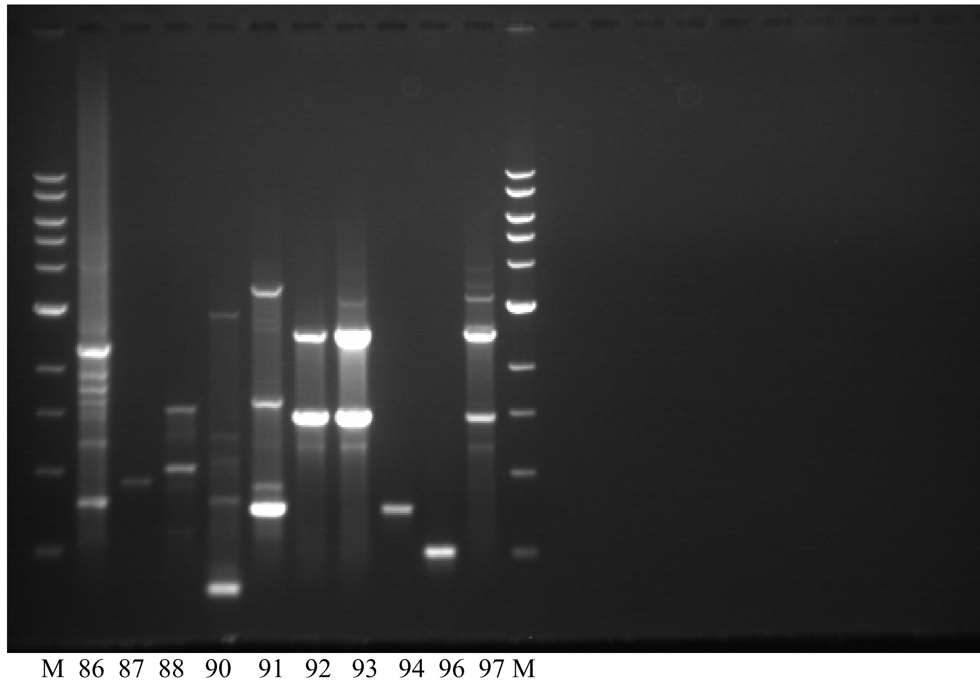


Figure 7.6d: M-marker; 86, 87, 88 and 90 bacteria isolated from the rhizosphere of poplar cuttings inoculated with strain BU61; 91, 92, 93, 94, 96 and 97 bacteria isolated in another experiment

7.3.3 Toluene evapotranspiration

After adding 100 mg l^{-1} toluene to the Hoagland's solution in the lower compartment evapotranspiration of toluene through the leaves was measured as described under 7.2.5.

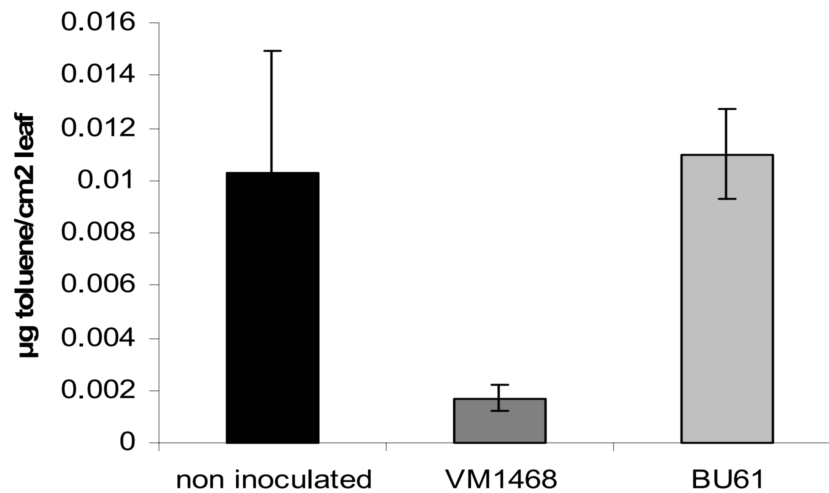


Figure 7.7: Total amount of toluene (µg) evapotranspirated through leaves into the upper cuvette compartment, adsorbed with Chromosorb traps, determined by GC-MS. Amount of evapotranspirated toluene is calculated per square centimeter of leaf area. Given are mean of standard deviation of 3 replicates.

Compared to non-inoculated plants or plants inoculated with *B. cepacia* BU61, poplar cuttings inoculated with *B. cepacia* VM1468 released about five times less toluene through the leaves. Those results illustrate that endophytic bacteria possessing the right degradation pathway not only protect their host plants against phytotoxicity, but also reduce evapotranspiration of the pollutant into the air.

7.4 CONCLUSIONS

A positive influence of inoculated strains *B. cepacia* strains BU61 and VM1468 on the growth of poplar cuttings (*Populus trichocarpa* x *deltoides* cv. “Hoogvorst”) was observed. Addition of toluene in a concentration of 500 mg l^{-1} affected biomass production of all these groups of plants. However, inoculation of cuttings with bacteria possessing pTOM resulted in a reduction of toluene phytotoxicity. The most positive effect on plants growth was observed in case when cuttings were inoculated with endophytic *B. cepacia* strain VM1468 (Figures 7.2, 7.3, 7.4 and 7.5).

Endophytic bacteria isolated from non inoculated poplar cuttings were not able to grow on selective media. On the contrary, different bacterial phenotypes isolated from cuttings inoculated with *B. cepacia* strains VM1468 and BU61 were growing on these selective media (Table 7.1). Those bacteria, capable for growing on toluene as only carbon source, showed different BOX-PCR fingerprints than the original inoculated strains (figure 7.6). This indicates that horizontal gene transfer can occur between inoculated bacteria and bacteria which natural colonizers of poplar cuttings. Furthermore, the mean evapotranspiration of toluene from the aerial plant parts was about 5 times lower when cuttings were inoculated with the endophytic *B. cepacia* strain VM1468 (Figure 7.7).

However, several questions still remain to be answered:

1. Why do plants inoculated with VM1468 grow better in the presence of toluene than plants inoculated with BU61, while in both plants an endophytic community with many members having the capability to degrade toluene was found?
2. Why was it possible that bacteria capable for growing on toluene were isolated from the rhizosphere of non-inoculated plants? Were toluene degrading bacteria present in the sandy soil used in this experiment? Does poplar roots release a phenolic type exudates that enriches for Tol+ bacteria in the rhizosphere?

To answer this question we will set up some editional experiments and also perform identification of the isolated bacteria.

CHAPTER VIII

APPLICATION OF POPLAR TREES AND THEIR ASSOCIATED MICROORGANISMS FOR THE IN SITU REDMEDIATION OF A BTEX CONTAMINATED GROUNDWATER PLUME

ABSTRACT

In 1999, 275 Poplar trees were planted on an experimental field near the Ford factory in Genk (Belgium) in order to install a bioscreen. This bioscreen combines the biodegradation activities of poplar trees and that of their associated rhizosphere and endophytic microorganisms and aims at containing a BTEX contaminated groundwater plume, which occurred as the result of leaking solvent and fuel storage tanks. Measurements, conducted over the 5 years period after the planting of the trees suggested that poplar trees and their associated microorganism have, once the plant roots reach contaminated groundwater zone, an active role in the remediation of the BTEX plume present, resulting in full containment of the plume. Analysis of the microbial communities associated with poplar showed that once the roots of the trees reached the BTEX contaminated groundwater, enrichment occurred of both rhizosphere and endophytic bacteria that were able to degrade BTEX contaminants.

8.1 INTRODUCTION

Leakage in the past of solvents and fuel from underground storage tanks at the site of the Ford car factory in Genk (Belgium) resulted in contamination of the groundwater with organic solvents (BTEX), fuel and heavy metals. BTEX is the acronym for benzene, toluene, ethylbenzene and xylenes. All BTEX compounds are toxic and have noticeable health effects at increased concentrations. Exposure to these compounds from groundwater systems is usually minimal but exposures can be persistent over a long period of time (long time effect) (Christensen and Elton, 1996). Pollutant migration is of high concern since pollutants can follow the flow of groundwater and migrate off-site.

Several initiatives were undertaken to stop further pollution of the groundwater. The source of the problem was removed: the storage tanks were excavated and replaced by above ground storage tanks so that the input of contaminants in the groundwater was stopped. Meanwhile, the pollution plume had spread south-east and different measures were taken in order to clean up the site. At the center of the pollution "pump and treat" (since 2003) and "air stripping" (since 2002) are applied under supervision of Dr. Ir. Pütz (Germany). Natural attenuation under aerobic and anaerobic conditions is investigated by VITO Mol, Belgium. Also phytoremediation is included in the restoration plan. The main aim was to 'cut off' the contamination plume and preventing off site migration, by creating a bioreactive barrier, at the south-east side of the Ford property.

Phytoremediation is the use of green plants to remove, contain, or render harmless environmental contaminants (Cunningham and Berti, 1993). It is a promising technology that addresses clean-up of organic solvents, PCBs, heavy metals, polyaromatic hydrocarbons, explosives, energetic compounds and nutrients. The phytoremediation process combines the activities and metabolic capabilities of plants and their associated microorganisms in a green

bioremediation process (Harvey *et al.*, 2002). On the Ford factory site hybrid poplar trees (*Populus trichocarpa* x *deltoid*s cv. "Hoogvorst" and "Hazendans") were chosen for phytoremediation purpose. Poplar trees are fast growing and deep rooting with a high water "pumping" capacity (average 420 liter/m^2 or 4200m^3 /ha after 6 months or 260 liter per tree per day during vegetation period). Poplar trees are phreatophytic (they can use water from the saturated zone) and they can, together with their associated microorganisms, reduce contaminant levels and prevent off-site migration (Ferro *et al.*, 1997) of the contaminants, this under prerequisite that the right conditions exist for implementing a phytoremediation strategy.

8.2 MATERIAL AND METHODS

8.2.1 Planting of the trees and monitoring of BTEX plume

In April 1999, 275 poplar trees were planted on the site near the Ford factory (see Figure 8.2). Planting was done to create a bioreactive zone perpendicular on the migration direction of the groundwater plume that was polluted with BTEX (concentration in the groundwater fluctuated between 0-1000µg l^{-1}) as well as with Nickel (0-100µg l^{-1}) and Zinc (0-1000µg l^{-1}). It was supposed that this concentration of pollutants was not too high (phytotoxic). Groundwater depth was 4-5 m, and thus within reach of the poplar roots. Two poplar cultivars, *Populus trichocarpa* x *deltoides* "Hoogvorst" and "Hazendans" were planted on this phytoremediation site which covers an area of about 20000m^2 (75 m width, 270 m length). The two different poplar clones were chosen in order to reduce the potential effects of fungal disease spreading. The 4 meter high trees with weakly developed roots were planted in 80 cm deep holes. In order to supply trees with sufficient nutrients, original soil was mixed with compost and then used to fill up

the planting holes. The trees were planted reciprocally ("Hazendans" – "Hoogvorst") with a distance of 7 meter between each other, in 9 rows and in each row 30 plants. Trees were planted perpendicularly on the direction of groundwater flow (Figure 8.1)

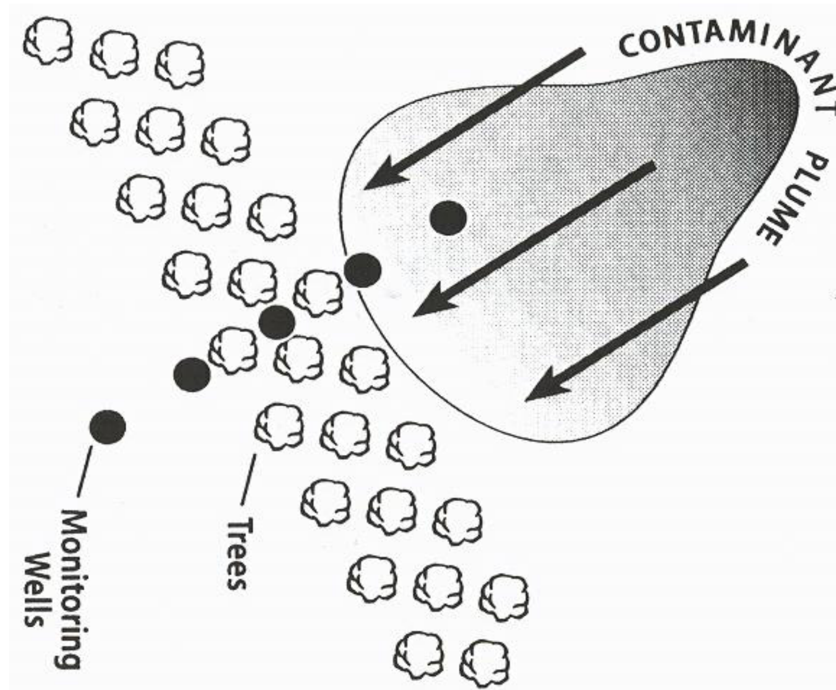


Figure 8.1: Rows of poplar trees planted perpendicularly on the BTEX plume.

Monitoring wells were installed up-and down-gradient from the poplar planting. Groundwater samples were taken by Ing. Dr. Pütz every six months in order to follow the dispersion of the contaminant plume.

8.2.2 Analysis of the microbial communities associated with *Populus trichocarpa* x *deltoides* cv. "Hoogvorst" growing within and outside the BTEX plume

Four years after planting of the trees both endophytic and rhizosphere bacteria were sampled from poplar trees growing within and outside the BTEX polluted area at the phytoremediation site near the Ford factory in Genk. For practical reasons in this study only microbial communities associated with *Populus trichocarpa* x *deltoides* cv. "Hoogvorst" were examined. Rhizosphere and root samples were taken at depth of 1.5m and placed in sterile falcon tubes (50ml) filed with 20ml sterile 10mM $MgSO_4$. Leaf and stem samples were collected and transported in separate plastic bags. All samples were taken from five different locations on the plants, from three trees situated inside and three outside the contamination plume. Samples were processed in the laboratory within two hours after sampling.

Rhizosphere samples were vortexed thoroughly, roots were removed and serial dilutions, up to 10^{-7} were prepared in 10 mM $MgSO_4$ solution. Different dilutions were spread over 1/10 strenght 869 solid media (Mergeay *et al.*, 1985). After autoclaving, 500 ml l^{-1} Amphotericin (Invitrogen Corporation) was added to the medium in order to prevent growth of fungi. After 7 days incubation at 30°C, colony forming units (CFU) were counted and calculated per gram of rhizosphere soil.

For the isolation of endophytic bacteria poplar roots were surface sterilized in solution containing 2% active chloride (NaOCl, Fluka) plus 1 droplet Tween 80 (Merck) per 100ml solution. Roots were submerged during 10 minutes in this solution and subsequently rinsed 3 times for 1 min in sterile distilled water. The third rinse solution was plated on 869 medium and used as check for sterility. Sterile roots were macerated during 60 sec in 10ml 10mM $MgSO_4$ using a Polytron PR1200 mixer (Kinematica A6). Serial dilutions were plated on Amphotericin

containing 1/10 strength 869 solid media and incubated for 7 days at 30°C before the CFU were counted and calculated per gram fresh weight.

Twigs of poplar trees were cut in 2 cm pieces and surface sterilized in a solution containing 1% active chloride and Tween 80 during 5 minutes. After rinsing in sterile water (3 x 1 min) samples were macerated during 90 sec with a Polytron PR1200 mixer and plated on Amphotericin containing 1/10 strength 869 solid media. The third rinsing solution was plated on 869 medium and used as check for sterility. Plates were incubated 7 days at 30°C before the CFU were counted and calculated per gram fresh weight.

Leaves were washed in distilled water and surface sterilized during 10 min in a sterilization solution containing 2% active chloride and Tween 80. Following surface sterilization, leaves were rinsed in sterile distilled water (3 x 1 min) and macerated for 60 sec in 10ml 10mM $MgSO_4$ using a Polytron PR1200 mixer (Kinematica A6). 100µl samples were plated on the Amphotericin containing 1/10 strength 869 solid media and incubated for 7 days at 30°C before the CFU were counted and calculated per gram fresh weight.

All phenotypicaly different bacteria from the different samples were purified and tested for there BTEX growth characteristics on Schatz medium (Schatz and Bovel, 1956) supplemented with C-mix (per liter medium: 1.3 ml glucose 40%, 0.7 ml lactate 50%, 2.2 ml gluconte 30%, 2.7 ml fructose 20% and 3 ml succinate 1M), Schatz medium without carbon source and Schatz medium with individual BTEX compounds. 600 µl of the test compound was added in an open vial to a 10 liter sealed containers in which Schatz plates, to which no carbon source was added, were placed for incubation at 30°C for 7 days period.

8.3 RESULTS

8.3.1 Monitoring of BTEX plume

Figure 8.2 presents the position of BTEX plume in October 1998, 6 months before trees were planted. In the center of the plume BTEX concentrations higher than 100000 µg l^{-1} were measured. The BTEX plume followed the groundwater flow in south-east direction. The BTEX concentration at the site where the trees were planted varied between 100 and 1000 µg l^{-1}.

Figure 8.3 shows the two poplar clones "Hoogvorst" and "Hazendans" in May 2000, 13 months after planting.

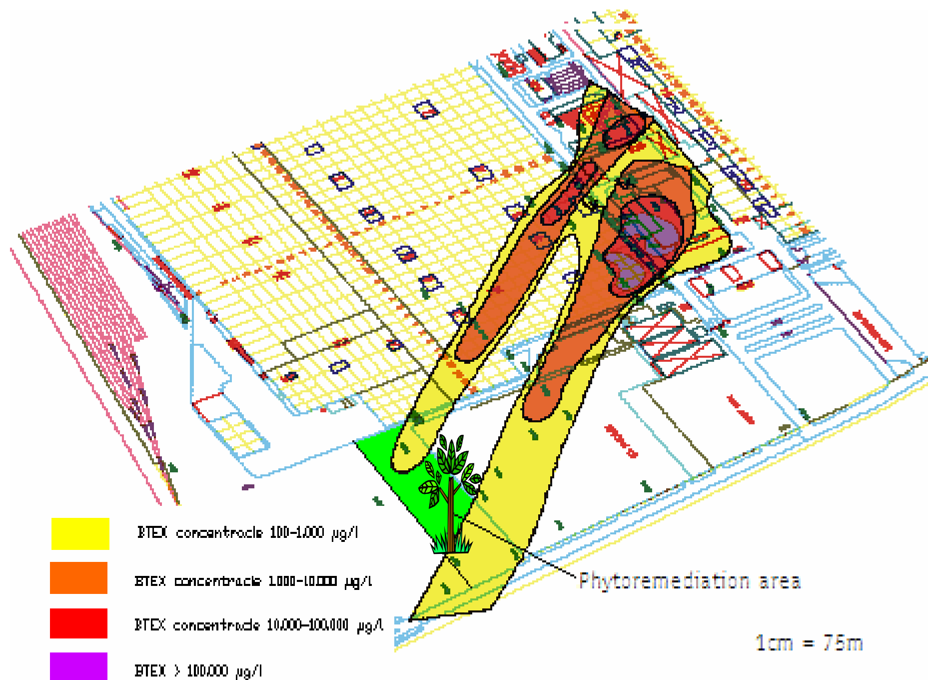


Figure 8.2: BTEX polluted site near the Ford factory in Genk (Belgium). Samples taken in October 1998, before planting of the poplar trees. Figure kindly provided by Ing. Dr. Pütz.

Figure 8.3: *Populus trichocarpa* x *deltoides* cv. “Hoogvorst” and “Hazendans” in May 2000, 13 months after planting at BTEX polluted site, near the Ford factory in Genk, Belgium.

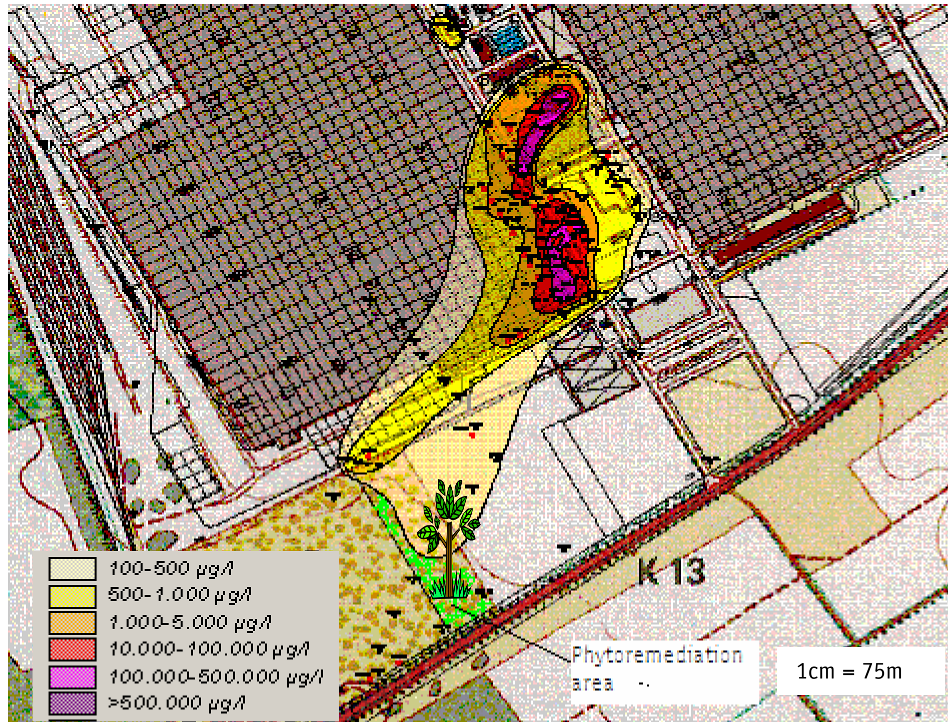


Figure 8.4: BTEX polluted site near the Ford factory in Genk (Belgium). Samples taken in October 2002, 30 months after planting of the poplar trees. Figure kindly provided by Ing. Dr. Pütz.

Monitoring performed in October 2002, 30 months (3 growing seasons) after planting of poplar trees (Figure 8.4) shows that concentration of BTEX in the center of plume is still higher than 500000 µg l^{-1}. BTEX concentrations at the poplar plantation site are still similar to these measured in 1998, but a very important observation is that the pollution plume is "cutted of" at this location. Formerly, the plume reached much far and even passed under the motorway.

Measurment from June 2003 (Figure not shown), 40 months after planting of the poplar trees confirmed the findings of October 2002: the pollutant plume is restricted to the site with poplar trees.

Figure 8.5 shows poplar trees growing at BTEX polluted site in November 2003, 45 months after planting the trees.

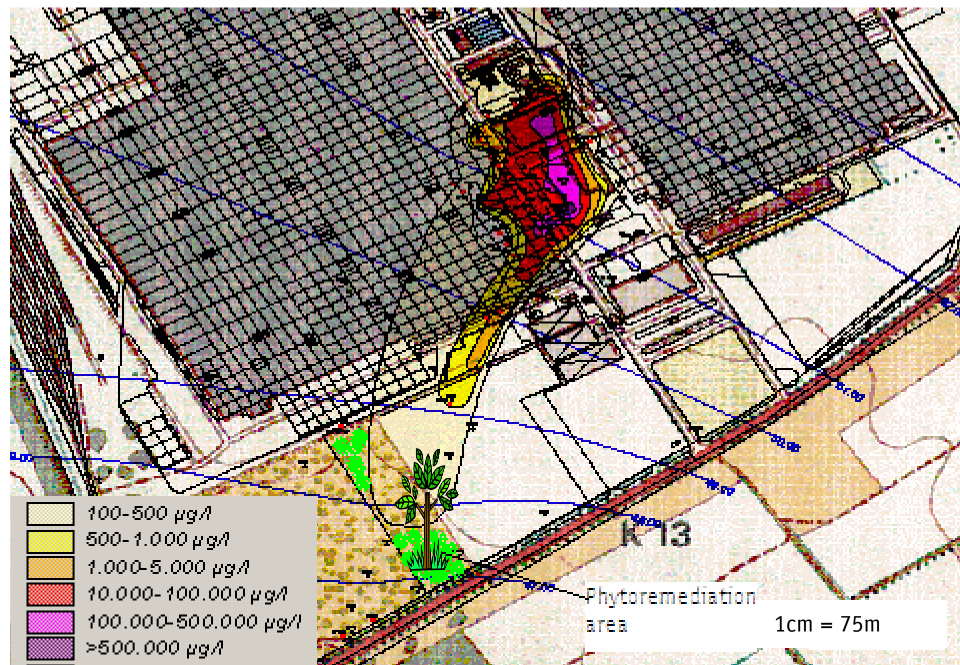


Figure 8.5: BTEX polluted site near the Ford factory in Genk (Belgium). Samples taken in November 2003, 45 months after planting of the poplar trees. Figure kindly provided by Ing. Dr. Pütz.

Figure 8.6: Poplar trees growing at BTEX polluted site near Ford factory in Genk, Belgium. June 2003, 38 months after planting of trees.

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

Plant associated microorganisms play an important role in the BTEX degradation, as poplar itself seems to be unable to degrade these compounds in an efficient way, as it was demonstrated with cell culture studies on cell lines of these cultivars (see chapter 4). The bacteria isolated from rhizosphere, roots, twigs and leaves of *Populus* trichocarpa x *deltoides* cv. "Hoogvorst" (planted four years ago) growing inside and outside BTEX plume were tested for their growth capacity on different BTEX. This was done by plating them on Schatz medium and by incubation in sealed 10 liter containers with 600 µl of the test compound added for 7 days at 30°C.
Results are presented in Tables 8.1 and 8.2

	Sz + B	Sz +T	Sz + Eb	Sz + m-X	Sz+ o-X	Sz+ p-X	Sz+ BTEX	Sz	Sz + Cmix
HV203	+	+	+	+	+	+	+	+	+
HV204	+	+	+	+	+	+	+	+	+
BHV201	+	+	+	+	+	+	+	+	+
BHV202	+	+	+	+	+	+	+	+	+
WHV200	+	+	+	+	+	+	-	-	+
WHV201	+	+	+	+	+	+	-	+	+
WHV202	+	+	+	+	+	+	+	+	+
WHV203	-	-	+	-	-	-	-	-	+
WHV204	+	+	+	+	+	+	-	+	+
WHV205	+	+	+	+	+	+	+	+	+
WHV206	+	+	+	+	+	+	+	+	+
WHV207	+	+	+	+	+	+	-	+	+
WHV208	+	+	+	+	+	+	-	-	+
WHV209	+	+	+	+	+	+	-	-	+
WHV210	-	-	+	-	-	-	-	-	-
WHV211	+	+	+	+	+	+	-	+	+
WHV213	+	-	+	+	+	+	-	-	+
WHV214	+	+	+	+	+	+	+	+	-
RHV200	-	-	+	-	+	-	-	-	+
RHV201	-	-	+	-	-	+	-	-	+
RHV202	+	-	+	-	+	+	-	+	+
RHV203	+	+	+	-	+	+	-	+	+
RHV204	+	+	+	+	+	+	-	+	+
RHV205	+	+	+	+	+	+	+	+	+
RHV206	+	+	+	+	+	+	+	+	+
RHV207	+	+	+	+	+	+	+	-	+
RHV208	+	+	+	+	+	+	+	-	+
RHV209	+	+	+	+	+	+	+	+	+
RHV210	-	+	+	+	+	+	-	+	+
RHV211	-	-	+	+	+	+	-	+	+
RHV212	+	+	+	+	+	+	-	+	+
RHV213	+	+	+	+	+	+	-	+	+
RHV214	+	+	+	+	+	+	-	+	+
RHV215	+	+	+	+	+	+	+	+	+
RHV216	-	-	+	+	-	+	-	+	+
RHV217	+	+	+	+	+	+	-	+	+
RHV218	+	+	+	+	+	+	-	+	+
RHV219	+	+	+	+	+	+	+	+	+
RHV220	-	-	+	+	+	+	-	+	+
RHV221	+	+	+	+	+	+	-	+	+
RHV222	-	-	+	+	+	+	-	+	+
RHV223	+	+	+	+	+	+	+	+	+

RHV224	+	+	+	+	+	+	-	+	+
RHV225	+	+	+	+	+	+	-	-	+
RHV226	+	+	+	+	+	+	+	+	+
RHV227	+	+	+	+	+	+	+	+	+
RHV228	+	+	+	+	+	+	+	+	+
RHV229	+	+	+	+	+	+	-	+	+

Table 8.1: Phenotypicaly different bacteria isolated from stem, leaf, root and rhizosphere soil of *Populus trichocarpa* x *deltoides* cv. "Hoogvorst", growing inside BTEX polluted area, were tested for growth on different BTEX compounds. HV- Hoogvorst; prefixed by B = leaf isolate, W = root isolate, R = rhizosphere soil isolate, no prefix = stem isolate. B = benzene, T = toluene, Eb = ethylbenzene, X = xylene. + = growth, - = no growth.

	Sz + B	Sz + T	Sz + E	Sz + m-X	Sz + o-X	Sz + p-X	Sz + BTEX	Sz	Sz + Cmix
HV205	+	+	+	+	+	+	+	+	+
HV206	+	+	+	+	+	+	+	-	+
HV207	+	+	+	+	+	+	+	+	+
HV208	+	+	+	+	+	+	+	+	+
HV209	+	+	+	+	+	+	+	+	+
HV210	+	+	+	+	+	+	+	+	+
WHV215	+	+	+	+	+	+	+	+	+
WHV216	+	-	+	+	+	-	-	+	+
WHV217	-	-	-	-	-	-	-	+	-
WHV218	-	-	-	-	-	-	-	-	-
WHV219	-	-	-	-	-	-	-	-	-
WHV220	+	-	+	+	+	+	-	+	+
WHV221	+	+	+	+	+	+	+	+	+
WHV222	+	+	+	+	+	+	+	+	+
WHV223	+	+	-	+	+	+	-	+	+
WHV224	+	-	-	+	+	+	-	+	-
WHV225	+	+	+	+	+	+	+	+	+
WHV226	+	-	-	-	-	-	-	-	+
WHV228	+	-	-	+	+	+	-	+	+
WHV229	-	-	-	+	+	+	-	+	+
WHV230	+	-	-	+	+	+	-	+	+
RHV231	+	+	-	+	+	+	-	+	+
RHV232	+	-	-	+	+	-	-	+	+
RHV233	+	+	+	+	+	+	+	+	+
RHV234	+	+	+	+	+	+	-	+	+
RHV235	-	-	-	-	-	-	-	+	-
RHV236	-	-	-	-	+	+	-	+	+
RHV237	+	+	+	+	+	+	-	+	+
RHV238	+	+	-	+	+	+	-	+	+
RHV239	-	+	-	+	+	-	-	-	+
RHV240	+	+	+	+	+	-	+	+	+
RHV241	-	-	-	-	-	-	-	-	+
RHV242	+	-	-	-	-	-	-	+	+
RHV243	-	-	-	-	-	-	-	-	-
RHV244	+	+	+	+	+	+	+	+	+
RHV246	+	+	+	+	+	+	+	+	+
RHV248	+	+	+	+	+	+	+	+	+
RHV249	+	+	+	+	+	+	+	+	+
RHV250	+	-	-	+	+	+	-	+	+
RHV251	+	+	+	+	+	+	-	+	+

Table 8.2: Phenotypicaly different bacteria isolated from stem, leaf, root and rhizosphere soil of *Populus trichocarpa* x *deltoides* cv. “Hoogvorst”, growing outside BTEX polluted

area, were tested for growth on different BTEX compounds. HV- Hoogvorst; prefixed by B = leaf isolate, W = root isolate, R = rhizosphere soil isolate, no prefix = stem isolate. B = benzene, T = toluene, EB = ethylbenzene, X = xylene. + = growth, - = no growth.

Tables 8.1 and 8.2 shows that from the twigs of plants growing outside the BTEX plume a higher number of phenotypicaly different cultivable strains could be isolated compared to the twigs of plants growing inside the polluted zone. All bacteria isolated from twig could grow in the presence of different BTEX compounds, on Schatz medium only and on Schatz medium with addition of C-mix. So, it cannot be concluded that those bacteria use BTEX as a carbon source, as they show autotrophic growth on plates without addition of a carbon source.
From the leaves of trees growing inside the plume no cultivable bacteria could be isolated. In the leaves of trees growing outside the plume 2 different cultivable phenotypes were observed, both growing on Schatz medium only, and Schatz with different BTEX compounds or C-mix.
From roots almost no difference in number of different isolated phenotypes was observed. However, 7 different phenotypes isolated from roots of plants growing inside the polluted area could not grow on Schatz medium only, but were growing in the presence of one or more BTEX compounds. This suggests that these bacteria can use BTEX compounds as a carbon source. Compared with this, in plants growing outside polluted area only one phenotype was observed with similar capabilities. Moreover, the total number of colony forming units, capable for degrading of BTEX, was more than three times higher in the roots of the plants growing inside the plume ($60{,}8 \cdot 10^5$ for plants growing inside plume, compared with $17{,}9 \cdot 10^5$ for plant growing outside the plume). This results suggest it that growth of trees in the BTEX plume zone results in an enrichment of endophytic bacteria in the root that are able to degrade these compounds. This in agreement with findings of Siciliano *et al* (2001).

From the rhizosphere soil of plants growing inside the BTEX plume 31 different phenotypes were isolated, from which 5 were not growing on Schatz medium, but could grow on Schatz with addition of BTEX compounds. From the rhizosphere soil of plants growing outside the BTEX plume, only one isolate which could not grow on Schatz was growing in presence of m- and o-xylene. The total number of CFU capable to grow on some or all of BTEX compounds from rhizosphere associated with plants growing inside polluted area was $12{,}8 \cdot 10^{6}$ CFU per gram, compared to $17{,}9 \cdot 10^{5}$ CFU for plants growing outside the pollution plume. This indicates that growth in the BTEX contaminated zone results over time in an enrichment of rhizosphere isolates able to use BTEX compounds as C-source.

8.4 CONCLUSIONS

Data of monitoring of the BTEX plume (Figures 8.2, 8.4 and 8.5) suggested that 5 years after planting the poplar trees and their associated microorganisms may play an active role in remediation of BTEX on the Ford site. From 2002 (the period when the poplar roots reached the groundwater), the BTEX plume is “cutted off” as the following of the poplar planting.

Growth of trees within the BTEX polluted zone has a positive effect on the number of rhizosphere and root colonizing endophytic bacteria able to use the contaminant as only carbon source. Increase in number of those bacteria achieved once the plants reach the plum with their roots, approximately 30 months after planting of the trees. This explains why during the examination of microbial community in the year 2001 (chapter 5) the numbers of bacteria capable for using BTEX as only C-source was much lower compared with samples collected and analyzed in the 2003 (this chapter).

Although the results of this study are very promising, evapotranspiration of BTEX through the leaves should be measured, to assure that BTEX compounds are

degraded by plants and their associated microorganisms, and not only removed from groundwater and transferred into the atmosphere.

CHAPTER IX
GENERAL CONCLUSIONS AND PERSPECTIVES

Phytoremediation is the use of plants for the remediation of contaminated soil and groundwater. During the phytoremediation process not only plants, but also their associated microorganisms play an active and important role. Very often phytoremediation of organic contaminants is limited due to insufficient degradation of targeted contaminants by the plant itself, which then results in the accumulation of toxic compounds and their incomplete degradation products in the plant, or their release into the environment. A good example is poplar, which is frequently used for the phytoremediation of organic contaminants, such as organic solvents like TCE and BTEX compounds. We tested poplar cells for their toluene degradation capabilities (chapter 4). Using *in vitro* growing poplar cell cultures, no degradation of toluene was observed, indicating that poplar cells do not posses the appropriate enzymes for toluene degradation. Since phytoremediation of BTEX compounds by poplar was shown to be efficient under field conditions (Collins *et al.*, 2002), this indicates that plant associated microorganisms present in the rhizosphere or as endophytes may play an important contribution in the phytoremediation process. Nevertheless, it was also reported that evapotranspiration of volatile organic compounds through the leaves of poplar cuttings can be important (Burken and Schnoor, 1999).

Microorganisms residing in the plant rhizosphere can play a very important role in degradation of organic contaminants. Due to the exudation of a variety of organic molecules, the bacterial population in the vicinity of the plant roots is often strongly enriched (often 10 – 100 and even 1000 times), as the bacteria are “feeding” on these root exudates. In case these rhizosphere populations are equipped with the appropriate degradation pathways, organic contaminants in the root zone can be degraded before they are eventually taken up by the plant

roots. This is especially the case for organic contaminants with a low or moderate mobility, such as the less-complex PAHs. However, in some cases, even if the plant rhizosphere is enriched with pollutant degrading microorganisms, water soluble pollutants are taken up by the plant faster then those microorganisms can degrade them (Trapp *et al.*, 2000), resulting in toxicity for the plants and/or volatilization of the contaminants. Therefore we hypothesized that endophytic bacteria, when equipped with the right degradation pathway, can as a result of the *in planta* degradation of the contaminant improve plant growth and health and also reduce release of contaminants, through the leaf stomata or stem lenticels, into the air. Thus, an active population of endophytic bacteria can potentially allow plant growth on sites contaminated with contamination concentration above the usual phytotoxicity threshold levels, and/or reduce contaminant emission levels.

As a test system to prove our hypothesis yellow lupine (*Lupinus luteus* L.) was chosen as a host plant for the endophytic species *Burkholderia cepacia* L.S.2.4, while toluene was chosen as a model compound representing water soluble, volatile BTEX contamination. During this study we successfully demonstrated that endophytic bacteria, when equipped with the right degradation pathway (in this case pTOM) can assist their host plant in overcoming the toxic effects of toluene when grown either hydroponically or on a sandy soil under greenhouse conditions (chapter 3). The naturally occurring yellow lupine endophyte, *Burkholderia cepacia,* was isolated from its host plant, marked with nickel and kanamycin and, via natural gene transfer (conjugation with the environmental *B. cepacia* G4 strain) equipped with the toluene degradation pathway, encoded on the pTOM plasmid. After successful inoculation with the resulting strain *B. cepacia* VM1330 plants stayed healthy even when they were exposed to toluene at high, normally growth inhibiting or even lethal, concentrations (Figure 3.2, 3.4 and 3.5). At the same time, the presence of this endophytic strain resulted in a 50-70% reduction of the evapotranspiration of toluene, this in comparison to non inoculated plants

or plants inoculated with the environmental *B. cepacia* G4 strain (Figure 3.3). This was the first prove that endophytic bacteria can ameliorate the phytoremediation process of water soluble and volatile organic compound. Bacteria used in this study were constructed by natural gene transfer and as such they are not considered as genetically modified microorganisms (GMO), a factor that can facilitate their application and public acceptance.

Furthermore, the endophytic strain *B. cepacia* VM1468, marked with nickel and kanamycin, and equipped with pTOM via conjugation with *B cepacia* BU61, (a constitutive tom A^+ mutant of *B. cepacia* G4, which is also able to oxidize TCE without of the need of induction by toluene), was used for inoculation of poplar cuttings (*Populus trichocarpa* x *deltoides* cv. "Hoogvorst"). Results obtained from a greenhouse study showed that, even when toluene was supplied at a high concentration (500 mg l^{-1}) plants inoculated with *B. cepacia* VM1468 were growing better (Figure 7.2, 7.3, 7.4 and 7.5) and that phyto-volatilization tended to be five times lower compared to non inoculated plants and plants inoculated with *B. cepacia* BU61 (Figure 7.7). It is very important to mention that after 10 weeks growth under greenhouse conditions, neither *B. cepacia* VM1468 nor BU61, could be recovered from rhizosphere, root, stem or leave samples of the inoculated plants, with or without toluene treatment (Chapter 7). However, significant numbers of naturally occurring bacteria capable of growing on toluene as their sole carbon source were isolated from the rhizosphere, root and stem of the plants (Table 7.1). Interestingly, although toluene degrading strains were recovered from the rhizosphere of the non-inoculated control plants, we failed to isolate endophytic bacteria able to grow on toluene, this in contrast to plants inoculated with either *B. cepacia* VM1468 or BU61: both rhizosphere and endophytic strains able to grow on toluene as sole carbon source could be isolated from the inoculated plants. Using PCR technique with *tom*A4 specific primers we proved that rhizosphere and endophytic bacteria, isolated from the root or stem, showed a positive reaction in the PCR assay. This strongly indicates

that members of the endogenous endophytic and rhizosphere population of poplar received via natural gene transfer the *tom* toluene degradation pathway from both *B. cepacia* strains. This conclusion is supported by observations that without inoculation no toluene degrading endophytes were found in poplar, and despite the fact that toluene degrading bacteria were observed in the rhizosphere of the non-inoculated control plants, no toluene degrading endophytic bacteria could be found. Therefore, it can be concluded that between endophytic bacteria, horizontal gene transfer can occur (chapter 7). Furthermore, we can speculate that horizontal gene transfer could play an important role in adapting the endogenous endophytic community rather than integrating a new bacterial strain in a stabile community. Thus, the fact that *B. cepacia* VM1468 and BU61 are no natural endophytic residents of poplar might have contributed to the transfer of the degradation pathway to the endogenous endophytic population. The degradation pathway is transferred among the members of the community where the inoculated endophytic strain acts as a starter culture. The bacteria isolated during this experiment will be further identified by sequencing of their 16S rDNA.

Poplar trees are very suitable for the phytoremediation of organic compounds with log K_{ow} values between 0.5 and 3.5 (Salt *et al.,* 1998; Trapp *et al.*, 1990). Therefore two poplar cultivars, *Populus trichocarpa* x *deltoides* cv. “Hoogvorst” and cv. “Hazendans” were chosen for a field experiment to contain and remediate a BTEX contaminated groundwater plume. For this purpose, 275 poplar trees (50 : 50 of each cultivar) were planted perpendicularly on the BTEX contaminated groundwater plume at the site of the Ford factory in Genk (Belgium) (chapter 8).
The bacterial, rhizosphere and endophytic, populations associated with poplar trees (*Populus trichocarpa* x *deltoides* cv. “Hoogvorst” and “Hazendans”) growing at the BTEX contaminated field site were investigated in order to obtain information’s concerning their diversity, compartmentalization, molecular and physiological characteristics (chapter 5). Twenty one different genera were

observed from a collection of 146 isolates. The predominant genus within the endophyte and rhizosphere populations associated with the two poplar cultivars was *Pseudomonas* (42%). This was not surprising, sinc it has been observed that members of the genus *Pseudomonas* are abundant in the soil environment (Spiers *et a*l., 2000) and in the plant interior (Tanpraseet and Reed, 1997). Population diversity decreased from the roots to the shoots. The high number of different morphotypes in the leaves of the "Hoogvorst" cultivar can be explained by penetration of bacteria through leaf stomata and wounds, specially do to the fact that leaf sampling for this study was performed almost at the end of vegetation period (chapter 5). Compared with this, in the leaf samples collected at the beginning of growth season (chapter 8) the numbers of different morphotypes were much lower. The two cultivars of poplar seem to host different endophytic populations, in terms of diversity, abundance and activities, suggesting that each plant species has an association with a specific bacterial population. It is likely that the total endophytic bacterial population is composed of both plant species specific and non specific populations, for bacteria isolated from the two poplar cultivars, about two years after planting of the trees and thus before the trees reached with their roots the contaminated groundwater plume; the number of isolates able to degrade one or more BTEX compounds was low (chapter 5). On the contrary, once the plants reached the BTEX contaminated groundwater table, the percentage of BTEX degraders among the total cultivable bacteria was much higher (chapter 8), indicating that the presence of contaminants can affect the structure of the plant associated bacterial populations and especially the presence of contaminants catabolic pathways. Similarly, Siciliano *et al*. (2001) showed that the genes encoding catabolic pathways increased within the root endophyte population in response to the presence of a given contaminant. A quantitative assessment of their representation within the plant community can then be made, to assess whether sufficient numbers exist to effectively enhance the plants degradation capacity.

Assessment of the catabolic properties and further characterization and identification of endophytic bacteria isolated from poplar trees should allows the selection of those species most suitable for further study. Those bacteria should be non pathogenic, cultivable, plant specific and localized within the plant compartment most suitable for biodegradation of the target compound. From our collection of over 150 endophytic bacteria isolated from the two poplar cultivars four strains were chosen for recolonizaton studies (chapter 6). All four strains belonged to genus *Pseudomonas* (*Pseudomonas veronii*, *P. asplenii*, *P. putida* and *P. fulva*) and three of them were shown to be capable to effectively re-colonize the poplar rhizosphere and the internal tissues (chapter 6). The population size of the strains capable for re-colonization decreased markedly from the rhizosphere to the root interior and further to the stem interior. Only one of the strains examinated was capable to colonize the leaf. The fact that those strains were efficient rhizosphere colonizers supports the theory that endophytes can originate from the rhizosphere (Misko and Germida, 2002) and from there colonize the internal plant tissues. Most probably their motility, cellulase activity and their ability to colonize the xylem are contributing factors to the successful spreading of strains throughout the plant. It is likely that these strains use the xylem to move into the above ground plant parts. At no time during the microscopic examination any cellular damage of the plant tissues was observed caused by the colonization of inoculated endophytes and none of the inoculated strains caused any signs of pathogenicity.

Five years after planting of the poplar "bioscreen" on the Ford site, the groundwater contamination plume was "cutted off" on the location of the poplar planting (chapter 8). This was observed after the plants roots reached the groundwater table. We will continue to monitor the dispersion of the BTEX plume. We also planned to continue our follow up of the bacterial community associated with two poplar cultivars growing on this site, and will address the seasonal

variations of the endophytic and rhizosphere populations associated with these plants. We also plan to monitor more in detail the evapotranspiration of the trees growing on this site. This work should result in a more complete picture of the ongoing phytoremediation process, and provide the data to further support the public acceptance of phytoremediation as a successful treatment option for BTEX contaminated groundwater.

All results obtained during this four years study are very promising and we expect that the application in phytoremediation of bacteria constructed through natural gene transfer (conjugation) will gain regulatory and public acceptance and will become a general strategy to improve the efficiency of phytoremediation of different organic contaminants. So far, the bacteria used for our laboratory and greenhouse studies were marked with nickel, kanamycin or green fluorescent protein, which allowed us to follow their colonization of yellow lupine or poplar cuttings. In order to be applied in the field, these experiments should be repeated with only naturally occurring endophytic recipients and naturally occurring environmental strains, such as a *B. cepacia* G4 or BU61, as donors of the catabolic properties. The resulting transconjugants are not considered genetically modified microorganisms (GMOs), a factor that may facilitate their application and public acceptance.
This work has a very broad potential of application. In this study, for practical reasons, only toluene was included as a test compound. In the future we expect to construct, via natural gene transfer, a collection of endophytic bacteria with *a la carte* degradation properties for a broad spectrum of organic xenobiotics on mobile DNA elements. Subsequently these strains can be applied for different purposes. It will be straightforward to develop endophytic bacteria to improve the phytoremediation of important environmental contaminants, including TCE and MTBE: *B. cepacia* VM1468, used as a model for improving the phytoremediation of toluene, is able to oxidize TCE without the need of

cometabolic induction by toluene. A logical continuity of our work will be to evaluate this strain for its potential to improve *in planta* degradation of TCE. In this case special attention has to be paid to the toxicity of Cl-radicals, which are a by-product of this degradation process. This shows the importance that during phytoremediation not only original compound, but also its potential degradation intermediates and products have to be considered.

The concept of improved degradation of organic contaminants by metabolically engineered endophytic bacteria can also be applied to improve food quality and safety: crop plants can be inoculated with endophytic bacteria able to degrade pesticide and herbicide residues, such as 2,4-D or atrazine, that can be taken up by plants. This has the potential to strongly reduce the transfer of these chemicals into the food chain.

Bibliography

International journals

Barac T, Taghavi S, Borremans B, Provoost, A, Oeyen L, Colpart JV, Vangronsveld J and van der Lelie D. (2004): Engeneered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. Nature Biotechnology 22, 583-588

Germaine K, Keogh E, Borremans B, van der Lelie D, Barac T, Oeyen L, Vangronsveld J, Porteus Moore F, Moore ERB, Campbel CD, Ryan D and Dowling D (2004) Colonisation of Poplar trees by *gfp* expressing endophytes, FEMS Microbiology Ecology, 48, 109-118.

van der Lelie D, Barac T, Taghavi S and Vangronsveld J (2005): Response to Newman: New uses of endophytic bacteria to improve phytoremediation. Trends in Biotechnology, January 2005

Porteous Moore F, Barac T, Borremans B, Oeyen L, Campbell CD, Vangronsveld J, van der Lelie D and Moore ERB (2004): Endophytic bacterial diversity in poplar trees growing on a BTEX contaminated site and the identification of isolates with potential to enhance phytoremediation. Plant Soil (submitted).

Porteous Moore F, Barac T, Borremans B, Oeyen L, Campbell CD, Vangronsveld J, van der Lelie D and Moore ERB (2004): Seasonal intra- and inter- tree variations in diversity of endophytic bacteria, as assessed by tRFLP, Journal of Applied Microbiology (submitted).

Porteous Moore F, Barac T, Oeyen L, Vangronsveld J, Campbell CD and Moore ERB (2004): Assessing a range of endophytic bacterial extraction techniques by molecular community profiling. Environmental Microbiology (submitted).

Abstracts of Oral Presentations

Barac T, Vangronsveld J, Oeyen L, Taghavi S, van der Lelie D and Borremans B (2004): Improved phytoremediation of water-soluble volatile organic pollutants: Use of engineered endophytic bacteria. International conference on bioremediation of soil and groundwater, Cracow, Poland.

Borremans B, Barac T, Oeyen L, Provoost A, Taghavi S, Vangronsveld J and van der Lelie D (2003): Phytoremediation of organic pollutants with help of endophytic bacteria. Proceedings of 8th International Conference on Contaminated Soil 2003 (Consoil 2003). International Congress Center, Gent, Belgium. Consoil 2003 Proceedings CD-ROM pp 1787-1799.

Borremans B, Barac T, Oeyen L, Provoost A, Taghavi S, Vangronsveld J and van der Lelie D (2004): Engineered endophytic bacteria improve phytoremediation of water soluble volatile organic products. Proceedings ESEB 2004, Oostende, Belgium.

Vangronsvald J, Ruttens A, Mastretta C, Oeyen L, Geebelen W and Colpaert J (2003): Use of plants for the sustainable management and remediation of contaminated soils and groundwater: field experiments in Belgium. Workshop “Achievements and prospects of phytoremediation in Europe”. Vienna, 2003

Germaine K, Keogh E, Borremans B, van der Lelie D, Barac T, Vangronsveld J, Ryan D and Dowling D (2004): Bacterial endegraders and development of herbicide resistant plants. Cost Action 859, Working Group 4, Leipzig, Germany

Ruttens A, Geebelen W, Mastretta C, Barac T, van der Lelie D and Vangronsveld J (2004): Short summary of the phytoremediation research in LUC (Belgium), with a focus on field experiments. Cost Action 859, Working Group 4, Leipzig, Germany

Moore E, Porteous F, Oeyen L, Barac T, Vangronsveld J, Wendler R, and Campbell CD (2002): Diversity of endophytic bacteria and applications in the phytoremediation of contaminated soils. XXIV Congresso Chileano de Microbiologia, 2002, El Quisco, Chile.

Porteous F, Oeyen L, Barac T, Vangronsveld J, Wendler R, Campbell CD, and Moore ERB (2002): Diversity of endophytic bacteria and applications in the phytoremediation of contaminated soils. XXIV Congresso Chileano de Microbiologia, 2002, El Quisco, Chile.

Posters

Barac T, Borremans B, Taghavi S, Provoost A., Oeyen L, Colpart J, Vangronsveld J, Carleer R, Yperman J, Czech J and van der Lelie, D. (2003): Phytoremediation of organic pollutants with help of endophytic bacteria. 24th Int. Seminarie voor Hetbeheer van afvalstoffen, VUB.

Borremans B, Barac T, Oeyen L, Provoost A, Taghavi S, Vangronsveld J and van der Lelie D (2003): Phytoremediation of organic pollutants with help of endophytic bacteria. Cost Action 837: 2nd scientific workshop on achievements and prospect of phytoremediation in Europe: Vienna, 2003.

Porteous F, Oeyen L, Trapp S, Borremans B, van der Lelie D, Vangronsveld J, Ryan D, Germain K, Dowling D, d'Haenes S, deBecker G, Campbell CD, Moore ERB and Karlson U (2002): "Endegrade" Endophytic bacteria for improving phytoremediations. 6th biennial ISEB Symposium, Veracruz, Mexico, 2002, ISEB Secretariat – International Society for Environmental Biotechnology, Veracruz.

- Aitchison E W, Kelly S L, Alvarez P J J and Schnoor J L (2000): Phytoremediation of 1,4-dioxane by hybrid poplar trees. Water Environ. Res. 72, 313-321
- Alkorta I and Garbisu C (2001): Review Paper: Phytoremediation of organic contaminants in soils. Elsevier Bioresource Technology 79, 273-276
- Anderson T A and Walton B T (1995): Comparative fate of (14C)trichloroethylene in the root zone of plants from a former solvent disposal site. Environ. Toxicol. Chem. 14, 2041-2047
- Anderson T A, Guthrie E A and Walton B T (1993): Bioremediation in the rhizosphere – plant roots and associated microbes clean contaminated soil. Environ. Sci. Technol. 27, 2630-2636
- Anderson T A, Kruger E L and Coats J R (1994): Enhanced degradation of a mixture of three herbicides in the rhizosphere of a herbicide-tolerant plant. Chemosphere 28, 1151-1157
- Aronson A I, Beckman W and Dunn P (1986): Bacillus thuringiensis and related insect pathogens. Microbiol. Rev. 50, 1-24
- Ashbolt N J and Inkerman P A (1990): Acetic acid bacterial biota of the pink sugar cane mealybug *Saccharococcus sacchari*, and its environs. Appl. Environ. Microbilo. 56, 707-712
- Asis C A and Adachi K (2003): Isolation of endophytic diazotroph *Pantoea agglomerans* and nondiazotroph *Enterobacter asburiae* from sweat potato stem in Japan. Lett. in App. Microbiol. 38, 19-23
- Azedvedo J, Maccheroni Jr W, Pereira J and Araujo W (2000): Endophytic microorganisms: a review on insect control and recent advances in tropical crops. Environ. Biotech. 3, 134-142
- Bacci E and Gaggi C (1985): Polychlorinated biphenyls in plant foliage: translocation or volatilization from contaminated soils. Bull. Environ. Contam. Tox. 35, 673-681
- Bacci E and Gaggi C (1986): Chlorinated pesticides and plant foliage: translocation experiments. Bull. Environ. Contam. Tox. 37, 850-857
- Bacilio-Jimenez M, Flores S, delValle M V, Perez A, Zepeda A and Zenteno E (2001): Endophytic bacteria in rice seeds inhibit early colonization of roots by *Azospirillum brasilense*. Soil Biol. Biochem. 33, 167-172
- Baker A J M and Brooks R R (1989): Terrestrial higher plants which hyperaccumulate metallic elements- A review of their distribution, ecology, and phytochemistry. Biorecovery 1, 81-126
- Banuelos G S, Lin Z Q, Wu L and Terry N (2002): Phytoremediation of selenium-contaminated soils and waters: fundamental and future prospects. Rev. Environ. Health 17, 291-306

- Barac T, Taghavi S, Borremans B, Provoost A, Oeyen L, Colpaert J V, Vangronsveld J and van der Lelie D (2004): Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. Nature Biotech. 22, 583-588
- Barbaro J R, Barker J F, Lemon L A and Mayfield C I (1992): Biotransformation of BTEX under anaerobic denitrifying conditions, field and laboratory observations. J. Contam. Hydrol. 11, 245-272
- Bedard D L, Wagner R E, Brennen M J, Haberl M L and Brown J F Jr (1987): Extensive degradation of aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutropphus* H850. Appl. Environ. Microbiol. 53, 1094-1102
- Benhamou N, Kloepper J W, Quadt-Hallman A and Tuzun S (1996a): Induction of defence-related ultrastructural modifications in pea root tissues inoculated with endophytic bacteria. Plant Physiol. 112, 919-929
- Benhamou N, Belnger R R and Paulitz T C (1996b): Induction of differential host responses by *Pseudomonas fluorescens* in ri T-DNA-transformed pea roots after challenge with *Fusarium oxysporum* f. sp. *Pisi* and *Pythium ultimum*. Phytopathology 86, 114-178
- Benhamou N, Kloepper J W and Tuzuns S (1998): Induction of resistance against Fusarium wilt of tomato by combination of chitosan with an endophytic bacterial strain: ultrastructure and cytochemistry of the host response. Planta 204, 153-168
- Benhamou N, Gagné S, Le Quéré D and Dehbi L (2000): Bacterial-mediated induced resistance in cucumber: beneficial effect of the endophytic bacterium *Serata plymuthica* on the protection against infection by *Pytium ultimum*. Phytopathology 90, 45-56
- Bent E and Chanway C P (2002): Potential for misidentification of a spore-forming Paenibacillus polymyxa isolate as an endophyte by using culture-based methods. Appl. Environ. Microbio. 68, 4650-4652
- Bescol-Liversac J, Chabaux C, Bolinard S and Guillam C (1982): A case of occupational benzene poisoning combined with tricholeukocytic leukemia. Toxicol. Eur. Res. 4, 329-335
- Bligny R and Leguay J-J (1987): Techniques of cell suspension culture. Methods in enzymology 148, 3-16
- Bolwell G P, Bozak K and Zimmerlin A (1994): Plant cytochrome P450. Phytochemistry 37, 1491-1506
- Borowitz J J, Stankie-Diez M, Lewicka T and Zukowska Z (1992): Inhibition of fungal cellulase, pectinase and xylanase activity of plant growth promoting florescent *Pseudomonas*. Bull. OILB/SROP 15, 103-106
- Boyaian G E and Carreira L H (1997): Phytoremediation: a clean transition from laboratory to marketplace? Na. Biotechnol. 15, 127-128

- Bragg J R, Prince R C, Harner E J and Atlas R M (1994): Effectveness of bioremediation for the exxon valdez oil spill. Nature 368, 413-418
- Brauner S J and Killingstad M (1998): In situ bioremediation of petroleum aromatic hydrocarbons. http://www.cee.vt.edu/program_areas/environmental/teach/gwprimer/btexbio/btexbio.htm
- Briggs G G, Bromilow R H and Evans A A (1982): Relationship between lipophilicity and root uptake and translocation of non-ionised chemicals by barley. Pestic. Sci. 13, 495-504
- Brockwwell J, Herridge D F, Morthorpe L J and Roughley R J (1998): Numerical effects of Rhizobium population of legume symbiosis. In: Nitrogen fixation by legumes in mediterranean agriculture. Beck D P and Materon L A. Eds. Martinus Nijhoff, Dordrecht, 178-193
- Brooks D S, Gonzales C F, Appel D N and Filer T H (1994): Evaluation of endophytic bacteria as potential biocontrol agents for oak wilt. Biol. Con. 4, 373-381
- Brown M E (1974): Seed and Root Bacterization. Ann. Rev. Phytopathol. 12, 181-197
- Burd G I, Dixon F G and Glick B R (1998): A plant growth promoting bacterium that Decreases Nickel Toxicity in Plant Seedlings. App. Environ. Microb. 64,3663-3668
- Burken J G and Schnoor J L (1998): Predictive relationships for uptake of organic contaminants by hybrid poplar trees. Environ. Sci. Technol. 32, 3379-3385
- Burken J G and Schnoor J L (1999): Distribution and volatilisation of organic compounds following uptake by hybrid poplar trees. Int. J. Phytorem. 2, 139-151
- Burken J G (2002): Uptake and metabolism of organic compounds: green-liver model. In: McCutceon S C and Schnoor J L, Phytoremediation: Transformation and Control of Contaminants. Wiley-interscience, New Jersey
- Butler J M, Groeger A W and Fletcher J S (1992): Characterization of monochlorinated biphenyl products formed by Paul's Scarlet Rose cells. Bull. Eviron. Contam. Toxicol. 49, 821-826
- Buyer J, Roberts D and Russek-Cohen E (2002): Soil and plant effects on microbial community structure. Canad. J. of Microbiol. 48, 955-964
- Buysens S, Poppe J and Höfte M (1994): Role of siderophores in plant growth stimulation and antagonism by Pseudomonas aeruginosa 7NSK2. In: Improving plant productivity with rhizosphere bacteria. Eds: Ryder M H, Stephens P M, Bowen G D. Commonwealth Scientific and Industrial Research Organisation, Adelaide, Australia, 139-141

- Castgnetti D and Smarelli J (1986): Siderophores, the iron nutrition of plants, and nitrate reductase. FEBS Lett. 209, 147-151
- Chanaway C P, Radley R A and Holl G B (1989): Bacterial inoculation of lodge wood pine, White Spruce and Douglas-fir grown in containers, Intermountain Forest Nursery Association Annual Meeting, Bismarck, North Dakota
- Chaney R L (1993): Zn phytotoxicity. In: AD Robinson, Eds Zinc in soil and plants. Kluwer academic Publishers, Dordrecht, the Netherlands
- Chanway C P (1996): Endophytes: they're not just fungi! Canad. J. of Botany 74, 321-322
- Chanway C P (1998): Bacterial endophytes: ecological and practical implications. Sydowia 50, 149-170
- Chard J, Ferro A M and Greene J (2001): Recent advances in phytoremediation of MTBE groundwater plumes. AEHS Magazine- Soil, Sediment & Groundwater; http://www.aehsmag.com/issues/2001/spring/phytoreadvances.htm
- Chen C, Bauske E M, Musson G, Rodriguez-Kabana R and Kloepper J W (1995): Biological control of *Fusarium* wilt on cotton by use of endophytic bacteria. Biol. Control 5, 83-91
- Christensen J S and Elton J (1996): Soil and groundwater pollution from BTEX. http://www.ce.vt.edu/enviro2/gwprimer/btex/btex.htm
- Coleman J O D, Randall R and Blake-Kalff M M A (1997): Detoxification of xenobiotics in plant cells by glutathione conjugation and vacuolar compartmentalization: a Fluorescent Assay Using Monochlorobimane. Plant Cell Environ. 20, 449-460
- Collins C, Laturnus F and Nepovim A (2002): Remediation of BTEX and trichloroethene. ESPR-Environ. Sci & Pollut. Res. 9 (1), 86-94
- Corgié S C, Joner E J and Leyval C (2003): Rhizospheric degradation of phenathrene is a function of proximity to roots. Plant and Soil 257, 143-150
- Crowley D E, Alvey S and Gilbert E S (1997): Rhizosphere ecology of xenobiotic-degrading microorganisms. In: Phytoremediation of soil and water contaminants, Kruger E L, Anderson T A and Coats J R (eds) ACS Symposium Series No 664. American Chemical Society, Washington, DC
- Cunningham S D and Berti W B (1993): Remediation of contaminated soils with green plants: an overview. In Vitro Cell. Dev. Biol. 29, 207-212
- Cunningham S D, Anderson T A, Schwab A P and Hsu F C (1996): Phytoremediation of soils contaminated with organic pollutants. Advances in Agronomy, vol 56, 56-113
- Cunningham S D, Shann J R, Crowley D E and Anderson T A (1997): Phytoremediation of contaminated water and soil. In: Phytoremediation of soil and water contaminants, Kruger E L, Anderson T A and Coats J R

(eds) ACS Symposium Series No 664. American Chemical Society, Washington, DC

- Cunningham S D, William R B and Jianwei W H (1995): Phytoremediation of contaminated soils. Trends Biotech. 13, 393-398
- Curl E A and Truelove B (1986): The Rhizosphere. Springer-Verlag, New York, Berlin 288
- Daane L L, Harjono I, Zylstra G J and Häggblom M M (2001): Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of salt marsh plants. App. and Environ. Microbiol. 67, 2683-2691
- Davison J (1988): Plant Beneficial Bacteria. Bio/technology 6, 282-286
- Defago G, Berling C H, Burger U, Haas D, Kahr G, Keel C, Voisard C, Wirthner P and Wüthrich B (1990): Suppression of black rot of tobacco and other root diseases by strains of *Pseudomonas fluorescens*: potential applications and mechanisms. In: Biological Control of Soil-borne Plant Pathogenes. Hornoby D. Eds; CAB International, Wallingford, Oxon, UK, 93-108
- Desai J D and Banat I M (1997): Microbial production of surfactants and their commercial potential. Microb. Mol Biol. Rev. 61, 47-64
- Dietz A C, and Schnoor J L (2001): Advances in phytoremediation. Environmental Health Perspectives vol 109, 163-168
- Doucete W J et al. (1998) Phytoremediation of dissolved phase trichloroethylene using mature vegetation. In Bioremediation and Phytoremediation: Chlorinated and Recalcitrant Compounds (eds. Wickraammanayakee G B and Hinchee R E), 251-256
- Duffy B K and Defago G (1997): Zinc improves biocontrol of *Fusarium* crown and root rot of tomato by *Pseudomonas fluorescens* and represses the production of pathogen metabolites inhibitory to bacterial antibiotic biosynthesis. Phytopathology 87, 1250-1257
- Duijff B J, Gianinazzi-Pearson V and Lemanceau P (1997): Involvement of the outer membrane lipopolysaccharides in the endophytic colonization of tomato roots by biocontrol *Pseudomonas fluorescens* strain WCS417r. New Phytologist 135, 325-334
- Dushenkov S (2003): Trends in phytoremediation of radionuclides. Plant and Soil 249, 167-175
- Edwards N T (1988): Assimilation and metabolism of polycyclic aromatic hydrocarbons by vegetation –an approach to this controversial issue for future research. Polycyclic Aromatic Hydrocarbons: A Decade of Progres - 10-th International Symposium, Columus, OH, Battelle Press.
- Edwards N T, Ross-Todd B M and Garver E G (1982): Uptake and metabolism of 14C anthracene by soybean (Glycne max). Environ. and Exper. Botany 22, 349-357

- Edwards R, Dixon D P and Walbot V (2000): Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends in Plant Science 5, 193-198
- Elad Y and Baker R (1985): The role of competition for iron and carbon in suppression of chlamydospore germination of *Fusarium oxysporum*. Phytopathology 75, 190-195
- Elad Y and Chet I (1987): Possible role of competition for nutrition in biocontrol of *Pythium* damping-off by bacteria. Phytopathology 77, 190-195
- Elbeltagy A, Nishioka K, Sato T, Suzuki H, Ye B, Hamada T, Isawa T, Mitsui H and Minamisaw K (2001): Endophytic colonisation and *in planta* nitrogen fixation by a *Herbaspirillum* sp. isolated from a wild rice species. Appl. Environ. Microbiol. 67, 5285-5293
- Elvira-Rucuenco M and van Vuurde J W (2000): Natural incidence of endophytic bacteria in pea cultivars under field conditions. Can. J. Microbiol. 46, 1036-1041
- EPA, U.S. Environmental Protection Agency, National Center for Environmental Research (2000): Final Report: Uptake of BTEX compounds by hybrid poplar trees in hazardous waste remediation
- EPA, U.S. Environmental Protection Agency, Office of Solid Waste and Emergency Response-Technology Innovation Office (1998): Technology Fact Sheet, EPA 542-F-98-011. Washington, DC
- Errampalli D, Leung K, Cassidy M B, Kostrzynska M, Blears M, Lee H and Trevors J T (1999): Applications of the green fluorescent protein as a molecular marker in environmental microorganisms. J. Microbiol. Methods 35, 187-199
- Fahey J W (1988): Endophytic bacteria for the delivery of agrochemicals to plants. In: Biologically active natural products (Chapter 9). American Chemical Society, 120-128
- Fahey J W, Dimock M B, Tomasino S F, Taylor J M and Carlson P S (1991): Genetically engineered endophytes as biocontrol agents: A case study in industry. In: Microbial Ecology of Leaves. Springer-Verlag, New York, 402-411.
- Fahn A (1982): Primary plant tissues. In: Plant Anatomy, 3rd Edn, Pergamon Press, Madrid, 129-138
- Ferro A M, Rieder J P, Kennedy J and Kjelgren R (1997): Phytoremediation of groundwater using poplar trees. In: Phytoremediation, Eds Thibeault C A, International Business Communications, Inc. 201-212
- Fischer P J, Petrini O and Lappin-Scott H M (1992): The distribution of some fungal and bacterial endophytes in maize (*Zea mays* L.). New Phytologist 122, 299-305

- Flathman P E and Lanza G R (1998): Phytoremediation: current views on an emerging green technology. Journal of Soil Contam. 7(4), 415-432
- Fromel M I, Nowak J and Lazarovits G (1991): Growth enhancement and developmental modifications of *in vitro* grown potato (*Solanum tuberosum* ssp. *Tuberosum*) as affected by a nonfluorescent *Pseudomonas* sp. Plant Physiol. 96, 928-936
- Gage D J, Bobo T and Long S R (1996): Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and Alfalfa (*Medicago sativa*). J. Bacteriol. 178, 7159-7166
- Gamborg O L, Miller R A and Ojima K (1968): Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50, 151-158
- Gardner J M, Feldman A W and Zablotowicz M (1982): Identity and behaviour of xylem-residing bacteria in rough lemon roots of Florida citrus trees. App. and Environ. Microbiol. 43, 1335-1342
- Germaine K, Keogh E, Garcia-Cabellos G, Borremans B, van der Lelie D, Barac T, Oeyen L, Vangronsveld J, Porteous Moore F, Moore E R B, Campbell C D, Ryan D and Dowling D N (2004): Colonisation of poplar trees by *gfp* expressing bacterial endophytes. FEMS Microbiol. Ecol. 48, 109-118
- Glick B R (2003): Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotech. Advances 21, 383-393
- Glick B R, Patten C L, Holguin G and Penrose DM (1999): Biochemical and genetic mechanisms used by plant growth promoting bacteria. London: Imperial College Press
- Glick B R, Penrose D M and Li J A (1998): A model for the lowering of plant ethylene concentrations by plant growth promoting bacteria. J. Theor. Biol. 190, 63-68
- Goldstein R M, Mallory L M and Alexander M (1985): Reasons for possible failure of inoculation to enhance biodegradation. Appl. Environ. Microbiol. 50, 977-983
- Gopalaswamy G, Kannaiyan S, O'Callaghan K, Kavey M and Cocking E (2000): The xylem of rice (*Oryza sativa*) is colonized by *Azorhizobium caulinodans*. Proc. R. Soc. Lond. B. 267, 103-107
- Gordon M, Choe N, Duffy J, Ekuan G, Heilman P, Muiznieks I, Newman L, Raszaj M, Shurtleff B, Strand S and Wilmoth J (1997): Phytoremediation of trichloroethylene with hybrid poplars. In: Phytoremediation of Soil and Water Contaminants. American Chemical Society, Washington, DC;
- Gullner G, Kömives T and Rennenberg H (2001): Enhanced tolerance of transgenic poplar plants overexpressing γ-glutamylcysteine synthetase towards chloroacetanilide herbicides. Exp. Botany 358, 971-979

- Hallmann J, Kloeper J W, Rodriguez-Kabana R and Sikora R A (1995): Endophytic rhizobacteria as antagonists of *Meloidogyne incognita* of cucumber. Phytopatology 85, 1136
- Hallmann J, Quadt-Hallmann A, Mahaffee W F and Kloepper J W (1997): Bacterial endophytes in agricultural crops. Can. J. Microbiol. 43, 895-914
- Harms H H (1992): *In vitro* systems for studying phytotoxicity and metabolic fate of pesticides and xenobiotics in plants. Pestic. Sci. 35, 277-281
- Harvey P J, Campanella B F, Castro P M, Harms H, Lichtfouse E, Schaffner A R, Smrcek S and Werck-Reichhart D (2002): Phytoremediation of poyaromatic hydrocarbons, anilines and phenols. Environ. Sci. Pollut. Res. Int. 9, 29-47
- Harwood C S and Gibson J (1997): Minireview Shedding light on anaerobic benzene ring degradation: a process unique to prokaryotes? J. of Bacteriology 179, 301-309
- Hauben L, Vauterin L, Swings J and Moore E R B (1997): Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. Int. J Syst. Bacteriol. 47, 328-335
- Heitkamp M A and Cerniglia C E (1989): Polycyclic aromatic hydrocarbon degrading by a *Myicobacterium sp.*in microcosms containing sediment and water from a pristine ecosystem. Appl. Environ. Microbiol. 55, 1968-19763
- Herrero M, de Lorenzo V and Timmis K N (1990): Transposon vectors containing non-antibiotic resistance markers for cloning and stabile chromosomal insertion of foreign genes in gram negative bacteria. J. Bacterio. 172, 6557-6567
- Heyrman J et al. (2004): *Bacillus novalis* sp. nov., *Bacillus vireti* sp., *Bacillus soli* sp. nov., *Bacillus bataviensis* sp. nov. and *Bacillus drentensis* sp. nov., from the Drentse A grasslands. Int. J. Syst. Evol. Microbiol. 54, 47-57
- Hinton D M and Bacon C W (1995): *Enterobacter cloacae* is an endophytic symbiont of corn. Mycopatholia 129, 117-125
- Hoflich G, Wiehe W and Kuhn G (1994): Plant growth stimulation by inoculation with symbiontic and associative rhizosphere mechanisms. Experientia 50, 897-905
- Hofte M, Dong Q, Kourambas S, Krishanapillair V , Sherratt D and Mergeay M (1994): The sss gene product which affects pyoverdin production in Pseudomonas aeruginosa 7NSK2, is a site-specific recombinase. Mol. Microbiol. 14, 1011-1020
- Hollis J P (1951): Bacteria in healthy potato tissue. Phytopatology 41, 350-366

- Hughes J B, Shanks J V, Vanderford M, Lauritzen J and Bhadra R (1997): Transformation of TNT by aquatic plants and plant tissue cultures. Environ. Sci. Tech. 31, 266-271
- Hulster A, Muller J F and Marschner H (1994): Soil-plant transfer of polychlorinated dibenzo-*p*-dioxins and dibenzofurans to vegetables of the cucumber family (*Cucurbitaceae*). Environ. Sci. Tech. 28, 1110-1115
- Hurek T, Reinhold-Hurek B, Van Montagy M and Kellenberger E (1994): Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. J. Bacteriol. 176, 1913-1923
- International Groundwater Technology (1996): Sunflower treat water near Chernobyl Vol 2, No 5, 20
- International Organization for Standardisation ISO (1997): Water Quality – Fresh Water Algal Growth Test With *Scenedesmus subspicatus* and *Raphidocelis subcapitata*. ISO8692
- James E K (2000): Nitrogen fixation in endophytic and associative symbiosis. Field Crops Res. 65, 197-209
- James K and Olivares F L (1997): Infection and colonization of sugar cane and other Graminaceous plants by endophytic diazotrophs. Crit. Rev. Plant Sci. 17, 77-119
- Joner E J, Johansen A, Loibner A P, de la Cruz M A, Szolar O H, Portal J M and Leyval C (2001): Rhizosphere effects on microbial community structure and dissipation and toxicity of polycyclic aromatic hydrocarbons (PAHs) in spiked soil. Environ. Sci. Technol. 35, 2773-2777
- Kado C I (1992): Plant pathogenic bacteria. In: The prokaryotes. Eds. Ballows H G, Dworkin M, Harder W and Schlieifer K-H. Springer-Verlag, New York, 660-662
- Kassel A G, Ghoshal D and Goyal A (2002): Phytoremediation of trichloroethylene using hybrid poplar. Physiol. Mol. Biol. Plants 8, 3-10
- Khammas K M and Kaiser P (1991): Characterization of a pectinolytic activity in *Azospirillum irakense*. Plant Soil 137, 75-79
- Kloepper J W, Lifshitz R and Zablotowicz R M (1989): Free-living bacterial inocula for enhancing crop productivity. Trends Biotech. 7, 39-43
- Kloepper J W, Scher F M, Laliberte M and Tipping B (1986): Emergence-promoting rhizobacteria: description and implications for agriculture. In: Iron, siderophores, and plant disease. Eds: Swinburne, T.R. Plenum Pres, New York, 155-164
- Kloepper J W, Tipping B and Lifshitz R (1991): Plant growth promotion mediated by bacterial rhizosphere colonizers. In: The Rhizosphere and plant growth. Eds: Keister D L, Cregan P B. Kluwer Academic Publishers, The Netherlands, 3156-326
- Kluepfel D A (1993): The behavior and tracking of bacteria in the rhizosphere. Ann. Rev. Phytopathol. 31, 441-472

- Kuiper I, Lagendijk E L, Bloemberg G V and Lugtenberg B JJ (2004): Rhizoremediation: A beneficial plant-microbe interaction. Mol. Plant-Micr. Int. Vol 17, No 1, 6-15
- Kulikova B *et al*. (2004): The EMBL Nucleotide Sequence Database. Nucl. Acids Res. 32, D27-D30
- Kunc F (1989): Degradation of 1-14C-2,4-dichlorophenoxyacetic acid in artificial rhizosphere soil. In: Vancura V. and Kunc F (eds) Interrelationships between microorganisms and plants in soil. Elsevier. New York, 329-334
- Lamb T G, Tonkyn D W and Kluepfel D A (1996): Movement of *Pseudomonas aureofaciens* from the rhizosphere to aerial plant tissue. Can. J. Microbiol. 42, 1112-1120
- Lambert B and Joos H (1989): Fundamental aspects of rhizobial plant growth promoting research . Trends Biotech. 215-219
- Lamoureux G L and Rusness D G (1986): Xenobiotic conjugation in higher plants. In Xenobiotics Conjugation Chemistry, ACS Symposium Series 299 (Paulson G D, Caldwell J, Hutson D H and Menn J J, eds). Washington, DC: American Chemical Society, 62-105
- Lane D J (1991): 16S/23S Sequencing. In: Nucleic Acid Techniques in Bacterial Systematics (Stackebrand E and Goodfellow M, Eds) Wiley, Chichster, UK, 115-175
- Lappin H M, Greaves M P and Slater J H (1985): Degradation of the herbicide mecoprop (2-(2-methyl-4-chlorophenxy) propionic acid) by a synergistic microbial community. App. Environ. Microbiol. 49, 429-433
- Latour X, Corberand T, Laguerre G, Allard F and Lemanceau P (1996): The composition of fluorescent Pseudomonad populations associated with roots is influenced by plant and soil type. Appl. Environ. Microbiol. 62, 2449-2456
- Lee I and Fletcher J S (1992): Involvement of mixed function oxidase systems in polychlorinated biphenyl metabolism by plant cells. Plant Cell Reports 11, 97-100
- Li L, Li Y, Meng L T and Pan S Q (1999): Gfp-aided confocal laser scanning microscopy can monitor *Agrobacterium tumefaciens* cell morphology and gene expression associated with infection. FEMS Microbiol. Lett. 179, 141-146
- Liu L, Kloepper J W and Tuzun S (1995): Induction of systemic resistance in cucumber against *Fusarium* wilt by plant growth promoting rhizobacteria. Phytopatology 84, 695-698
- Liu S and Suflita J M (1993): Ecology and evolution of microbial populations for bioremediation. Tibtech. 11, 344-315
- Lodewyckx C, Margeay M, Vangrosveld J, Clijsters H and van der Lelie D (2002a): Isolation, characterization and identification of bacteria

associated with the zinc hyperaccumulator *Thlaspi caerulescens* subsp. *calaminaria*. Int. J. Phytoremediation 4, 101-115

- Lodewyckx C, Taghavi S, Mergeay M, Vangronsveld J, Clijsters H and van der Lelie D (2001): The effect of recombinant heavy metal resistant endophytic bacteria in heavy metal uptake by their host plant. Int. J. Phytorem. 3, 173-187
- Lodewyckx C, Vangronsveld J, Porteous F, Moore E R B, Taghavi S and van der Lelie D (2002b): Endophytic bacteria and their potential applications. Critical Rev. in Plant Sciences 21, 583-606
- Lodewycx C (2001): PhD: A potential role for bacterial endophytes in phytoremediation of heavy metal contaminated soils
- Ludwig W and Klenk H-P (2001): Overview: a phylogenetic backbone and taxonomic framework for prokaryotic systematics. In: Bergey's Manual of Systematic Bacteriology, 2nd Ed, Vol 1, Archaea and the Deeply Branching and Phototrophic Bacteria. Boone D R, Castenholz R W and Garrity G M (eds.). Springer-Verlag, New York, 49-65
- Ma L Q, Komar K M, Tu C, Zhang W and Cai Y (2001): A fern that hyperaccumulate arsenic. Nature 409, 579
- Ma X and Burken J G (2003): TCE diffusion to the atmosphere in phytoremediation applications. Environ. Sci. Technol. 37, 2534-2539
- Marrs K (1996): The functions and regulation of glutathione S-transferases in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 127-158
- Maurhofer M, Reimmann C, Sasherer S P, Heeb S, Haas D and Defago G (1998): Salicyl acid biosythetic genes expressed in *Pseudomonas fluorescens* strain P3 improve the induction of systemic resistance in tobacco necrosis virus. Phytopathology 88, 678-684
- McCrady J, Mc Farlane C and Lindstrom F (1987): The transport and affinity of substituted benzenes in soybean stems. J. Exp. Botany 38, 1875-1890
- McCutcheon S C and Schnoor J L (2003): Overview of phytotransformation and control of wastes. Phytoremediation Transformation and Control of Contaminants 3-58 (John Wiley & Sons, Inc., Hoboken, New Jersey)
- McInroy J A and Kloepper J W (1994): Studies of the indigenous endophytic bacteria of sweet corn and cotton. In: Molecular Ecology of Rhizosphere Micro-organisms (O'Gara F, Dowling D N and Boesten B, eds) 19-28, VCH Press.
- McInroy J A and Kloepper J W (1995): Survey of indigenous bacterial endophytes from cotton and sweet corn. Plant Soil 173, 337-342
- Mehlman M A (1993): Dangerous and cancer-causing properties of products and chemicals in the oil refining and petrochemical industry: VIII Health effects of motor fuels: carcinogenicity of gasoline: scientific update. Environ. Res. 59, 238-249

- Meier J R, Chang L W, Jacobs S, Torsella J, Meckes M C and Smith M K (1997): Use of plant and earthworm bioassays to evaluate remediation of soil from a site contaminated with polychlorinated biphenyls. Environ. Toxicol. Chem. 16, 928-938
- Mergeay M, Nies D, Schlegel H G, Gerits J and Van Gijsegem F (1985): *Alcalignes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J. Bacteriol. 162, 328-334
- Mguni C M (1996): Bacterial black rot (*Xanthomonas campestris* pv. *campestiris*) of vegetable brassicas in Zimbabwe. PhD thesis, The Royal Veterinary and Agricultural University and Danish Government Institute of Seed Pathology. Pp 139
- Miller J H (1972): Experiments in Molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 354-358
- Misaghi I J and Donndelinger C R (1990): Endophytic bacteria in symptom free cotton plants. Phytopatol. 80, 808-811
- Misko A M and Germida J J (2002): Taxonomic and functional diversity of Pseudomonads isolated from the roots of field-grown canola. FEMS Microbol. Ecol. 42, 399-407
- Mocali S, Bertelli E, Di Cello F, Mengoni A, Sfalanga A, Viliani F, Cacitti A, Tegli S, Surico G and Fani R (2003): Fluctuation of bacteria isolated from elm tissues during different seasons and from different plant organs. Res. in Microbiol. 154, 105-114
- Mukhopadhyay N K, Garrison N K, Hinton D M, Bacon C W, Khush G S, Peck H D and Datta N (1996): Identification and characterization of bacterial endophytes of rice. Mycopathol. 134, 151-159
- Murashige T and Skoog F (1962): A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiology Plantarum 15, 473-497
- Nairn J D and Chanway C (2002): Temporary loss of antibiotic resistance by marked bacteria in the rhizosphere of spruce seedlings. FEMS Microbiol. Ecol. 40, 167-170
- Nejad P and Johnson P (2000): Endophytic bacteria induce growth promotion and wilt disease suppression in oilseed rape and tomato. Biol. Control 18, 208-215
- Nepovim A, Hubálek M, Podlipná R, Zeman S and Vanek T (2004): *In vitro* degradation of 2,4,6-trinitrotoluene using plant tissue cultures of *Solanum aviculare* and *Rheum palmatum*. Eng. Life. Sci. 1, 46-49
- Newman L A, Bod C, Choe N, Crampton R, Cortellucci, Domroes D, Doty S, Duffy J, Ekuan G, Fogel D, Hashomonay R, Heilman P, Martin D, Muiznieks L A, Newman T, Ruszaj M, Shang T, Shurleff B, Stanley S, Strand S E, Wang X, Wilmouth J, Yost M and Gordon M P (2004): Phytoremediation of trichloroethylene and carbon Tetrachloride: Results From Bench to Field. http://www.engg.ksu.edu/HSRC/97Proceed/Partner4/phytorem.html

- Newman L, Strand S E, Choe N, Duffy J, Ekuan C, Ruszaj M, Shurtleff B B, Wilmoth J, Heilman P and Gordo M P (1997): Uptake and biotransformation of trichloroethylene by hybrid poplars. Env. Scien. And Tech. 31, 1062-1067
- O'Sullivan D J and O'Gara F (1992): Traits of fluorescent *Pseudomonas* spp. Involved in suppression of plant root pathogens. Microbiol. Rev. 56, 662-676
- Ohkawa H, Imaishi H, Shiota N, Yamada T and Inui H (1999): Cytochrome P450s and other xenobiotics metabolizing enzymes in plants. In Food-Environment Challenge, Special Publications 233 (Brooks GT, Roberts T R, eds). Cambridge, UK: The Royal Society of Chemistry, 259-264
- Pan M J, Rademan S, Kuner K and Hastings J W (1997): Ultrastructural studies on the colonization of banana tissue and *Fusarium oxysporum* f.sp. *cubense* race 4 by the endophytic *Burkholderia cepacia*. J. of Phytopathology 145, 479-486
- Paterson S, Mackay D, Tam D and Shiu W Y (1990): Uptake of organic chemicals by plants: a review of processes, correlations and models. Chemosphere vol 21 nr 3, 297-331
- Patten C L and Glick B R (1996): Bacteria biosynthesis of Indole-3-aceric acid. Can. J. Microbiol. 42, 207-220
- Pearson W R (1994): Using the FASTA program to search protein and DNA sequence databases. Meth. Mol. Biol. 24, 307-331
- Pfleeger T (1991): A short-term bioassay for whole plant toxicity. In: Plants fro toxicity assessment, 2nd volume, ASTM STP 1115. Eds: Gorsuch J W, Lower R W, Wang W and Lewis M A. American Society for Testing and Materials, Philadelphia, 355-364
- Pieper D H and Reineke W (2000): Engineering bacteria for bioremediation. Curr. Opin. Biotechnol. 11, 262-270
- Pierson L S and Thomashow L S (1992): Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens*. Mol. Plant-Microbe Interact. 5, 330-339
- Pilan-Smits E and Pilan M (2000): Breeding mercury-breathing plants for environmental cleanup. Trends Plant Sci. 6, 235-236
- Potgieter H and Alexander M (1996): Susceptibility and resistance of several fungi to microbial lysis. J. Bacteriol. 91, 1526-1532
- Raaijmakers J M, Van der Sluis I, Van den Hout M, Bakker P A H M and Schippers B (1995): Dispersal of wild-type and genetically-modified *Pseudomonas* spp from treated seeds or soil to aerial parts of radish plants. Soil Biol. Biochem. 27, 1473-1478
- Rademaker J W L, Louws F J and de Bruijn F J (1998): Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. Molec. Microbial Ecology Manual 3.4.3, 1-27

- Ramamoorthy V, Viswanathan R, Raguchander T, Praksam V and Samiyappan R. (2001): Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pest and diseases. ELSEVIER Crop Protection 20, 1-11
- Ramaswami A, Rubin E and Banola S (2003): Non-significance of rhizosphere degradation during phytoremediation of MTBE. Int. J. of Phytor. 5, 315-331
- Ramos C, Molbak L and Molin S (2000): Bacterial activity in the rhizosphere analysed at the single cell level by monitoring ribosome contents and synthesis rates. Appl. Environ. Mirobiol. 66, 801-809
- Reeves R D and Baker A J M (2000): Metal-accumulating plants. In: Phytoremediation of toxic metals. Eds Raskin E, Ensley B D; John Wiley and Sons, Inc. New York
- Reinhold-Hurek B and Hurek T (1998): Interactions of gramineous plants with Azoarcus spp. and other diazotrophs: Identification, localization, and perspectives to study their function. Critical Reviews in Plant Science 17, 29-54
- Reinhold-Hurek B and Hurek T (1998): Life in grasses: diazotrophic endophytes. Trends Microbiol. 6, 139-144
- Reiter B, Burgmann H, Burg K and Sessitsch A (2003): Endophytic *nif*H gene diversity in African sweet potato. Can. J. Microbiol. 49, 549-555
- Reiter B, Pfeifer U, Schwab H and Sessitch A (2002): Response of endophytic bacterial communities in potato plants to infection with *Erwinia carotovora* subsp. *atroseptica*. Appl. Environ. Microbiol. 68, 2261-2268
- Reiter B, Werbter N, Gyamfi S, Schwab H and Sessitsch A (2003): Endophytic *Pseudomonas* spp. populations of pathogen-infected potato plants analyzed by 16S rDNA – and 16S rRNA – Based Denaturating Gradient Gel Electrophoresis. Plant and Soil 257, 397-405
- Renneberg H, Scupp R, Glavac V and Jochheim H (1994): Xylem sap composition of beech (Fagus sylvaticus L.) trees: seasonal changes in the axial distribution of sulphur compounds. Tree physiology 14, 541-548
- Rennenberg H, Scupp R, Glavac V and Jochheim H (1994): Xylem sap comosition of beech (Fagus sylvaticus L.) trees: seasonal changes in the axial distribution of sulphur compounds. Tree physiology 14, 541-548
- Russo A, Moenne-Loccoz Y, Fedi S, Higgins P, Fenton A, Dowling D N, O'Regan M and O'Gara F (1996): Improved delivery of biocontrol *Pseudomonas* and their antifungal metabolites using alginate polymers. Appl. Microbio. Biotechnol. 44, 740-745
- Sagisaka S (1974): Effect of low temperature on amino acid metabolism in wintering poplar. Plant Physiology 53, 319-327

- Sagosala S (1974): Effect of low temperature on amino acid metabolism in wintering poplar. Plant Physiology 53, 319-327
- Salgado T, Ramirez L, Hernandez A, Esparza M, Romero E and Mellado J C (1997): *Coffea arabica* L., a new host plant for *Acetobacter diazotophicus*, and isolation of other nitrogen fixing Acetobacteria. Appl. Environ. Microbiol. 63, 3676-3683
- Salt D E, Smith R D and Raskin I.(1998): Phytoremediation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 643-668
- Samish Z, Etinger-Tulczynska R and Bick M (1963): The microflora within the tissue of soft fruit and vegetables. Journal of Food Science 28, 259-266
- Scott, E.M., Rattray, E.A.S., Prosser, J.I., Killham, K., Glover, L.A., Lynch, J.M. & Bazin, M.J. (1995). A mathematical model for dispersal of bacterial inoculants colonising the wheat rhizosphere. *Soil Biology and Biochemistry* 27, 1307-1318.
- Schatz A and Bovel C (1956): Growth and hydrogenase activity of a new bacterium, *Hydrogenomonas facilis*. J. Bacteriol. 63, 87-98
- Schlegel H G, Kaltwasser H and Gottschalk G (1961): Ein Sumbersverfahren zur Kultur wasserstoffoxidierender Bacterien: Wachstum physiologische Untersuchngen. Arch. Mikrobiol. 38, 205-222
- Schneider A, Kreuzwieser J, Schupp R, Sauter J J and Rennenberg H (1994): Tiol and amino acid composition of the xylem sap of poplar trees (*Populus canadensis* "robusta"). Canad. J. of Botany 72, 347-351
- Schnoor J L, Licht L A, McCutceon, Wolfe N L and Carreira L H (1995): Phytoremediation of organic and nutrient contaminants. Env. Sc. and Tech. 29, 318-323
- Schuur U (1998): Xylem sap sampling – new approaches to an old topic. Trends in Plant Science 3, 293-298
- Schwab A P, Banks M K and Arunachalam M (1995): Biodegradation of polycyclic aromatic hydrocarbons in rhizosphere soils. In Hinchee R E, Anderson D B and Hoeppel R E (ed.), Bioremediation of Recalcitrant Organics,23-29. Battelle Memorial Institute, Columbus, Ohio.
- Schwitzguebel J-P, van der Lelie D, Glass D J, Vangronsveld J and Baker A (2002): Phytoremediation: European and American trends, successes, obstacles and needs. J. Soil Sed. 2, 91-99
- Sessitsch A, Reiter B, Pfeifer U and Wilhelm E (2002): Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and *Actinomycetes*-specific PCR of 16S rRNA genes. FEMS Microbiology Ecology 39, 23-32
- Sharp R R, Bryers J DD, Jones W G and Shields M S (1998): Activity and stability of a recombinant plasmid-borne TCE degraditive pathway in suspended cultures. Biotechnol. Bioeng. 57, 287-296

- Shields M S and Reagin M J (1992): Selection of a *Pseudomonas cepacia* strain constitutive for degradation of trichloroethylene. Appl. Environ. Microbiol. 58, 3977-3983
- Shields M S, Reagin M J, Gerger R R, Campbell R and Somerville C (1995): TOM, a new aromatic degradative plasmid from *Burkholderia* (*Pseudomonas*) *cepacia* G4. Appl. Environ. Microbiol. 61, 1352-1356
- Shishido M, Breuil C and Chanway C (1999): Endophytic colonization of spruce by plant growth promoting rhizobacteria. FEMS Microbiol. Ecol. 29, 191-196
- Shrive S C, McBride R A and Grodon A M (1994): Photosynthetic and growth responses of two broad-leaf tree species to irrigation with municipal landfill leachate. J. Environ. Qual. 23, 534-542
- Shröder P, Harvey P J and Schwitzguébel J-P (2002): Prospects for the phytoremediation of organic pollutants in Europe. ESPR – Environ. Sci. & Pollut. Res. 9 (1), 1-3
- Siciliano S D and Germida J J (1998a): Degradation of chlorinated benzoic acid mixtures by plant-bacteria associations. Environ. Toxicol. Chem. 17, 728-733
- Siciliano S D and Germida J J (1998b): Mechanisms of phytoremediation: biochemical and ecological interactions between plants and bacteria. Environ. Rev. 6, 65-79
- Siciliano S D, Fortin N, Mihoc A, Wisse G, Labelle S, Beaumier D, Oullette D, Roy R, Whyte L G, Banks M K, Schwab P, Lee K and Greer C W (2001): Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Appl. Environ. Microbiol. 67, 2469-2475
- Sikora R A (1988): Interrelationship between plant health promoting rhizobacteria, plant parasitic nematodes and soil microorganisms. Med. Fac. Landbouww. Rijksuniv. Gent. 53/2b, 867-878
- Skoog F and Miller C O (1957): Chemical regulation of growth and organ formation in plant tissues cultivated *in vitro*. In: Biological Action of Growth Substances 11th Symp. Sc. Exp. Biol. 11, 118-131
- Smith R A H and Bradshow A D (1979): The use of metal tolerant plant populations for the reclamation of metalliferous wastes. J. Appl. Ecol. 16, 595-612
- Spiers A J, Buckling A and Rainey P B (2000): Diversity and disturbance in experimental microcosmos. Nature 408, 961-964
- Sprent J I and de Faria S M (1988): Mechanisms of infection of plants by nitrogen fixing organisms. Plant and Soil 110, 157-165
- Sticher L, Mauch-Mani B and Métraux J P (1997): Systemic acquired resistance. Annu. Rev. Phytopathol. 35, 235-270

- Sturz A V and Matheson B G (1996): Populations of endophytic bacteria which influence host-resistance to Erwinia-induced bacterial soft rot in potato tubers. Plant Soil 184, 265-271
- Sturz A V and Nowak J (2000): Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops. Appl. Soil Ecology 15, 183-190
- Sturz A V, Christie B R and Nowak J (2000): Bacterial endophytes: potential role in developing sustainable systems of crop production. Critical Rev. in Plant Sciences 19, 1-30
- Sturz A V, Christie B R, Matheson B G, Arsenault W J and Buchanan N A (1999): Endophytic bacterial communities in the periderm of potato tubers and their potential to improve resistance to soil-borne plant pathogens. Plant Pathol. 48, 360-370
- Sturz A, Christie B R, Matheson B G and Nowak J (1997): Biodeversity of endophytic bacteria which colonize red clover nodules, root, stems and foliage and their influence on host growth. Biol. Fertil. Soils 25, 13-19
- Stuurman N, Bras C, Schlaman H, Wijfjes A, Bloemberg G and Spaink H (2000): Use of green fluorescent protein colour variants expressed on stable broad host range vectors to visualize *Rhizobia* interacting with plants. Mol. Plant-Microbe Interac. 13, 1163-1169
- Swoboda-Colberg N G (1995): Chemical contamination of the environment: sources, types and fate of synthetic organic chemicals. In: Young LY & Cerniglia E (eds) Microbial Transformations and Degradations of Toxic Organic Chemicals. Wiley-Liss inc., New York, 27-74
- Taghavi S, Delanghe H, Lodewyckx C, Mergeay M and van der Lelie D (2001): Nickel-resistance-based minitranspolons: new tools for genetic manipulation of environmental bacteria. Appl. Environ. Microbiol. 67, 1015-1019
- Tanprasent P and Reed B M (1997): Detection and identification of bacterial contaminants from strawberry runner explants. In Vitro cellular and Developmental Biology-Plant 33, 221-226
- Tapia-Hernandez A, Bustillos-Cristales M R, Jimenez-Salgado T, Mellado J C and Ramirez L E F (2000): Natural endophytic occurrence of *Acetobacter diazotrophicus* in pineapple plants. Microbiol. Ecol. 39, 49-55
- Terry N, Zayed A, Pilon-Smits E and Hansen D (1995): Can plants solve the selenium problem? Proceedings of the 14h Annual Symposium, Current Topics in Plant Biochemistry, Physiology and Molecular Biology: Will plants Have a role in Bioremediation? University of Missouri, Columbia, 63-64
- Tervet I W and Hollins J P (1948): Bacteria in the storage organs of healthy plants. Phytopatology 38, 960-967
- Thies J E, Singleton P W and Bohlool B B (1991): Influence of the size of indigenous rhizobial populations on establishment and symbiotic

performance of introduced rhizobia on field grown legumes. Appl. Environ. Microbiol. 57, 19-28

- Thomas W D and Graham R W (1952): Bacteria in apparently healthy pinto beans. Phytopathology 42, 214
- Thompson P L, Ramer L A, Guffey A P and Schnoor J L (1998): Decreased transpiration of poplar trees exposed to 2,4,6-trinitrotoluene. Environmental Toxicology and Chemistry 17, 902-906
- Trapp S and McFarlane J C (1994): eds Plant Contamination: Modeling and simulation of organic chemical processes. Boca Raton, FL: Lewis Publishers
- Trapp S, Köhler A, Larsen L C, Zambrano K C and Karlson U (2001): Phytotoxicity of fresh and weathered diesel and gasoline to willow and poplar trees. J. Soils Sed. 1, 71-76
- Trapp S, Matthies M, Scheunert I and Topp E M (1990): Modeling the bioconcentration of organic chemicals in plants. Environ. Sci. Technol. 24, 1246-1252
- Trapp S, McFarlane J C and Matthias M (1994): Model for uptake of xenobiotis into plants – validation with bromacil experiments. Environmental Toxicology and Chemistry 13, 413-422
- Trapp S, Zambrano K C, Kusk K O and Karlson U (2000): A phytotoxicity test using transpiration of willows. Arch. Environ. Contam. Toxicol. 39, 154-160
- USEPA – United States Environmental Protection Agency (2000): Introduction to phytoremediation, EPA 600/R-99/107. U.S. Environmental Protection Agency, Office of Research and Development, Cincinnati, OH
- Van Buren A, Andre C and Ishmaru C A (1993): Biological control of the bacterial ring rot pathogen by endophytic bacteria isolated form potato. Phytopatology 83, 1406
- van der Lelie D, D'Haene S, Dowling D N, Karlson U, Moore E R B, Taghavi S, Trapp S A J and Vangronsveld J (2001): Methods for improving phytoremediation treatment of a contaminated medium. Application nr. 60/291,344. U.S. Patent Office, 16 May, 2001. European Patent Office, EP 1 260 282 A2
- van der Lelie D, Schwitzguebel J-P, Glass D J, Vangronsveld J and Baker A (2001): Assessing phytoremediation's progress in the United States and Europe. Environ. Sci. Technol. 35, 446-452
- Vancura V (1980): Fluorescent Pseudomonads in the rhizosphere of plants and their relation to root exudates. Folia Microb. 25, 168-173
- Vangronsveld J, Van Assche F and Clijsters H (1995): Reclamation of a bare industrial area, contaminated by non-ferrous metals: in situ metal immobilization and revegetation. Environmental Pollution, 87, 51-59

- Vangronsveld J., Colpaert J., Van Tichelen K. (1996): Reclamation of a bare industrial area contaminated by non-ferrous metals: physico-chemical and biological evaluation of the durability of soil treatment and revegetation. Environmental Pollution, 94, 131-140.
- Vangronsveld J and Cunningham S eds. (1998): Metal-contaminated soils: In-situ inactivation and phytorestoration, Springer Verlag, ISBN: 1-57059-531-3
- Vangronsveld J and van der Lelie D (2003): The use of alternative techniques for remediation of polluted sites and groundwater in Flanders: Plant-based Strategies. JSS-J Soils & Sediments 3 (4), 250-251
- Verma S C, Ladha J K and Tripathi A KK (2001): Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. J. Biotechnol. 19, 127-141
- Walton B T and Anderson T A (1990): Microbial degradation of trichloroethylene in the rhizosphere: potential application to biological remediation of waste sites. App. and Environ. Microbiol. 56, 1012-1016
- Weber P, Stoermer H, Gessler A, Schnider S, Senbusch D, von Hanemann U and Rennenberg H (1998): Metabolic responses of Norway spruce (*Picea abies*) to long-term forest management practices and acute $(NH_4)SO_4$ fertilization: transport of soluble non protein nitrogen compounds in xylem and phloem. New Phytologist 140, 461-475
- Wenzel W W, Adriano D C, Sald D and Smith R (1999): Phytoremediation: a plant-microbe-based remediation system. In Bioremediation of Contaminated Soils, Agronomy Monograph no 37
- Whipps J M (2001): Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52, 487-511
- Whiting S N, de Souza M P and Terry N (2001): Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thlaspi caerulescens*. Environ. Sci. Tech. 35, 3144-3150
- Wilken A, Bock C, Bokern M and Harms H (1995): Metabolism of different PCB congeners in plant cell cultures. Environ. Toxicol. Chem. 14, 2017-2022
- Williams P A and Sayers J R (1994): The evolution of pathways for aromatic hydrocarbon oxidation in Pseudomonas. Biodegradation 5, 195-217
- Wilson D (1995): Endophyte: the evolution of a term, and clarification of its use and definition. Oikos 73, 274-276
- Wulff E G (2000): The use of antagonistic endophytic bacteria for controlling black rot of Brassica in Zimbabwe. PhD thesis, The Royal Veterinary and Agricultural University, Copenhagen, Denmark. Pp 134
- Xi C, Lambrechts M, Vanderleyden J and Michiels J (1999): Bi-functional gfp- and gusA-containing mini-Tn5 transposon derivatives for combined

gene expression and bacterial localization studies. J. Microbiol. Methods 35, 85-92

- Yee D C, Maynard J A and Wood T K (1998): Rhizoremediation of Trichloroethylene by a recombinant, root-colonizing *Pseudomonas fluorescens* strain expressing toluene *ortho*-monoxygense constitutively. App. and Environ. Microbiol. 64, 112-118
- Yelazhahan R, Samiyappan R and Vidhyasekaran P (1999): Relationship between antagonistic activities of *Pseudomonas fluorescens* isolates against *Rhizoctonia solani* and their production of lytic enzyme. J. Plant Dis. Prot. 106, 244-250
- Yoshitomi K J and Shann J R (2001): Corn (Zea mays L.) root exudates and their impact on 14C-pyrene mineralization. Soil Biol. Biochem. 33, 1769-1776
- Zamora M L G and Romero E M (2001): Natural endophytic association between *Rhizobium etli* and maize (*Zea mays* L.). J. Biotechnol. 91, 117-126
- Zehnder G, Kloepper J, Yao C and Wei G (1997): Induction of systemic resistance in cucumber against cucumber beetles (Coleoptera: Chrysomelidae) by plant growth promoting rhizobacteria. J. Econ. Entomol. 90, 391-396
- Zinniel D, Lambrecht P, Harris N, Feng Z, Kuczmarski D, Higley P, Ishimaru C, Arunakumari A, Barletta R and Vidaver A (2002): Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Appl. Environ. Microbio. 68, 21298-22208