Plant-Microbe Interactions for the Remediation of Hydrocarbon Contaminated Soil

Dissertation

Submitted to obtain the Doctoral degree at the University of Natural Resources and Life Sciences, Vienna

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Vienna, November, 2010

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. Angela Sessitsch for her constant encouragement, support, understanding, patience, critical inputs and continuous guidance throughout this project. She provided me an excellent guidance and I am extremely grateful for having had the opportunity to do my thesis under her supervision. My sincere thanks go to my co-supervisor Dr. Thomas G. Reichenauer for his support during the whole my research work and for reviewing this. I would like to thank Sohail Yousaf for his valuable help throughout my study.

I am grateful to every person in the Bioresources Unit of the AIT Austrian Institute of Technology GmbH for their kind support, help and valuable advices. My sincere thanks go to my close friends and Ph.D. fellows Yao Pan, Stefan Pfeiffer, Naveed Muhammad, Katharina Fallmann, Souzan-Chrysanthi Sanoussi, Claudia Fenzl and Michaela Prischl for their feedback, moral support and for being great friends throughout. I am indebted thanks to Alexandra Weilharter, Branislav Nikolic, Birgit Mitter, Tanja Kostic, Marlies Czetina, Günter Brader and Melanie Kuffner for their scientific help, support and encouragement. I am also thankful to Anton Grahsl, who assisted in performing the greenhouse experiments.

I would like to acknowledge my colleagues at the National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan for their endless support and Higher Education Commission, Govt. of Pakistan for funding. I particularly would like to give my thanks to Dr. Zafar M. Khalid who always encouraged me for pursuing a Ph.D. in foreign country and supported me and strengthened my self-confidence. I am also thankful to Dr. Qaiser M. Khan and Dr. Samina Iqbal for their help, support and encouragement for this study.

Finally, I dedicate this work for my beloved parents (Mother Shareefan Bibi and Father Muhammad Ashiq) for their love, support and encouragement throughout my endeavor. I am thankful to my brothers and their families for their support to my family during my Ph.D. study period. My sincere thanks are for my beloved wife Robina Sharif, who will always be an important person in my life. I am also thankful to my pretty daughter Zunash Afzal and lovely son Muhammad Umaid Afzal for their love and prayers for my PhD and safe home return.

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SUMMARY

A promising field to exploit plant-microbe interactions is the remediation of contaminated soils. Many plant growth-promoting bacteria can assist their host plant to overcome contaminated-induced stress responses, thus improving plant growth. During phytoremediation of organic contaminants, plants can further benefit from associated bacteria possessing appropriate degradation pathways and metabolic capabilities, leading to more efficient contaminant degradation and reduction of both phytotoxicity and evapotranspiration of volatile contaminants. Apart from environmental conditions the inoculation method and physicochemical properties of the soil are the main factors influencing the survival and activity of an inoculated strain as well as the growth of plants. This Ph.D. research attempted to examine the effect of the inoculation method (seed imbibement and soil inoculation) and soil types (sandy, loamy sand and loam) on plant-microbe interactions in the terms of bacterial colonization, gene abundance and gene expression in the rhizosphere and plant interior as well as hydrocarbon degradation.

The performance of three alkane degrading bacteria, which were inoculated by seed imbibement and soil inoculation, was tested. Italian ryegrass was grown in non-sterilized soil contaminated with diesel and inoculated with strains *Pantoea* sp. ITSI10, *Pantoea* sp. BTRH79 and *Pseudomonas* sp. MixRI75 individually as well as in combination. There was significantly more plant biomass and hydrocarbon degradation in inoculated treatments as compared to control treatments, however, strains inoculated in soil performed better than those applied by seed imbibement. Bacterial colonization was higher in the plant interior than in rhizosphere soil and by soil inoculation as compared to seed imbibement. Both *Pantoea* sp. strains showing ACC-deaminase activity performed better than *Pseudomonas* sp. strain lacking this activity. Most importantly, maximum hydrocarbon degradation was obtained by the addition of mixture of three bacteria as compared to by single strain application. The application of bacteria individually as well in combination by soil inoculation method resulted in significantly higher hydrocarbon degradation than the inoculation of bacteria by seed imbibement. Results indicated that besides the degradation potential of inoculant strains the inoculation method influences microbial colonization and activities during phytoremediation of contaminated soil.

The second part of the thesis aimed to assess the role of the soil type on colonization of two inoculated bacterial strains as well as on gene abundance and expression in the rhizosphere and plant interior. Italian ryegrass was grown in sterile soil containing 1% diesel and inoculated with endophytic bacteria *Pseudomonas* sp strain ITRI53 and *Pantoea* sp. strain BTRH79. Strains showed high colonization, gene abundance and expression in loamy soils. However, in sandy soil low colonization, gene abundance and absence of gene expression was observed indicating that the inoculated bacteria decreased in population and were also inactive. The soil type affected the abundance and expression of genes (*alkB* and CYP153) related to hydrocarbon degradation. Highest plant biomass, hydrocarbon degradation and gene expression were observed in loamy soil that had been inoculated with BTRH79. However, for both strains, *alkB* and CYP153 gene abundance and expression were the highest in the rhizosphere. Nevertheless, average activities (*alkB* and CYP153 transcripts / numbers) were higher in the shoot than in the rhizosphere. A positive relationship (r = 0.73) was observed between gene expression and hydrocarbon degradation indicating that catabolic gene expression is necessary for contaminant degradation. These results revealed that the soil type affects bacterial survival and catabolic activity and ultimately the degradation of pollutants.

Keywords: *hydrocarbons, phytoremediation, seed imbibement, soil inoculation, ACC deaminase, soil type, gene abundance, gene expression, degradation, alkB,* CYP153*.*

Pflanzen-Mikroben-Interaktionen für die Sanierung von Kohlenwasserstoff-kontaminierten Böden

ZUSAMMENFASSUNG

Kontaminierte Böden können mit Hilfe von Pflanzen und ihrer assoziierten Mikroflora saniert werden. Viele Mikroorganismen, die mit Pflanzen vergesellschaftet sind, helfen der Pflanze den durch die Kontaminationen verursachten Stress besser zu überwinden und unterstützen somit das Pflanzenwachstum. Viele mit Pflanzen assoziierten Mikroorganismen sind jedoch auch in der Lage toxische, organische Substanzen abzubauen und reduzieren somit die Phytotoxizität. Abbauende Mirkoorganismen können appliziert werden um den Abbauprozess zu unterstützen. Der Erfolg einer solchen Inokulation hängt von verschiedensten Umweltbedingungen ab, die das Überleben und die Aktivität der Bakterienzellen beeinflussen. In dieser Dissertation wurden der Einfluss der Inokulationsmethode sowie des Bodentyps auf die Besiedelung und Aktivität von inokulierten, Alkan-abbauenden Mikroben sowie auf den Abbau von Diesel in kontaminierten Böden untersucht.

Im ersten Versuch wurde der Abbau von drei Alkan-abbauenden Bakterien untersucht, die in Kombination mit Welschem Weidelgras zur Sanierung von kontaminierten Boden getestet wurden. Die Bakterienstämme, *Pantoea* sp. ITSI10, *Pantoea* sp. BTRH79 und *Pseudomonas* sp. MixRI75, wurden einzeln oder in Kombination auf Samen bzw. direkt in den Boden auf- bzw. eingebracht und das Welsche Weidelgras wurde in kontaminiertem und sterilisiertem Boden gepflanzt. Die inokulierten Pflanzen produzierten mehr Biomasse und bauten mehr Diesel-Kontamination als die nicht-inokulierten Behandlungen ab. Die Stämme, die direkt in den Boden eingebracht wurden, zeigten mehr Erfolg und auch eine höhere Besiedelung im Wurzelraum und im Pflanzeninneren als jene, die auf die Samen aufgebracht wurden. Beide *Pantoea* Stämme, die auch ACC Deaminase Aktivität zeigten, bewirkten besseren Abbau sowie größeres Pflanzenwachstum. Am effizientesten war die Kombination der verschiedenen Stämme.

Im zweiten Teil der Dissertation wurde der Einfluss des Bodentyps auf die Besiedelung von zwei inokulierten Stämmen untersucht. Weiters wurden die Abundanz und die Aktivität der applizierten Bakterien mittels molekularbiologischer Methoden untersucht. Es wurde wiederum das Welsche Weidelgras in sterilem und kontaminierten Boden gepflanzt, wobei hier drei verschiedene Bodentypen (Sand, lehmiger Sand und Lehm) zur Anwendung kamen. Es wurden die

Alkan-abbauenden Stämme *Pseudomonas* sp strain ITRI53 und *Pantoea* sp. strain BTRH79 inokuliert. In den beiden lehmigen Böden konnten beide Bakterienstämme gut besiedeln, zeigten hohe Abundanzen und eine hohe Expression der am Abbau beteiligten Gene, während im Sandboden sowohl die Besiedlung als auch die Aktivität der inokulierten Stämme niedrig war. Der Stamm BTRH79 bewirkte das höchste Pflanzenwachstum und die höchste Aktivität. Obwohl generell die Besiedelung im Wurzelraum am höchsten war, zeigten jene Bakterien, die das Pflanzeninnere besiedelten die höchste Aktivität per Zelle. Eine positive Korrelation zwischen Genexpression und Kohlenwasserstoffabbau indizierte, dass eine Expression der am Abbau beteiligten Gene für den Abbau essenziell ist.

Stichworte: *Kohlenwasserstoffe, Phytoremediaton, Samen-Inokulation, Boden-Inokulation, ACC-Deaminase, Bodentyp, Genabundanz, Genexpression, biologischer Schadstoffbbau, alkB, CYP153.*

CHAPTER 1

General Introduction

1.1. Background, objectives and scope of the research

Anthropogenic activities aimed at industrial and agricultural advancement have resulted in the non-judicious production and usages of chemical compounds. Although petroleum is one of the dominant components of our modern industrial society, the extraction, transport, and use of these fuels, however, poses inevitable environmental risks (Hall et al., 2003; Peña-Castro et al., 2006). The presence of petroleum hydrocarbons (PHCs) in soils is not only an adverse factor for human health, but also a negative impetus for plant growth (Joner et al., 2004; Meudec et al., 2007; Euliss et al., 2008).

In European soils, total PHCs concentrations range between 0.042 and 11.20 mg kg^{-1} (Nam et al., 2008). The majority of the urban soil concentrations fall in the 0.6-3.0 mg kg^{-1} range. Areas with heavy transportation networks and industrialization are likely to have much higher levels of total hydrocarbons (Germaine et al., 2009). Among hydrocarbon pollutants, diesel oil is a complex mixture of alkanes and aromatic compounds that frequently are reported as soil contaminants leaking from storage tanks and pipelines or released in accidental spills (Wang et al., 2005; Ciric et al., 2009), and found to be toxic to soil microorganisms and to plants (Tesar et al., 2002; Lapinskienė et al., 2006). Compared to other medium distillate fuels, diesel has the highest content of environmentally persistent hydrocarbons, which are often highly toxic and are regulated due to their mutagenicity and carcinogenicity (CCME, 2001; Farrel-Jones, 2003).

Therefore, the remediation of PHCs contaminated soils is becoming one of the hot topics in the field of environmental sciences and engineering (Chaudhry et al., 2005; Euliss et al., 2008). At present, common remediation methods for disposing contaminated soils include mainly physical remediation, chemical remediation and bioaugmentation (addition of biodegradative bacteria to contaminated soils). However, traditional physical and chemical remediation methods are costly and prone to a second contamination (Zhou and Song, 2004). Bioaugmentation is commonly employed as a remediation technology. However, numerous studies indicate that introduced microorganisms often do not survive in the environment and thus do not increase contaminant remediation (Gentry et al., 2004; Pandey et al., 2009). A novel potential remediation

treatment for these contaminated sites is phytoremediation, which is a cost-effective technique defined as the use of plants and their associated microorganisms to remove, transform, or assimilate toxic chemicals located in soils, sediments, groundwater, surface water, and even the atmosphere (Susarla et al., 2002; Reichenauer, 2008; Glick, 2010). At the same time, phytoremediation is a low input approach depending on natural attenuation by biodegradation and physicochemical mechanisms that decrease the contaminant concentration where sowing plants may be the only intervention (Cuevas et al., 2008). While some success has been reported using plants alone in bioremediation (Pilon-Smits, 2005; Gerhardt et al., 2009; Peng et al., 2009), the use of plants in conjunction with plant-associated bacteria offers much potential for bioremediation (McGuinness and Dowling, 2009; Weyens et al., 2009; Glick, 2010). The importance of plant-microbe partnerships in the remediation of organic contaminants was confirmed in studies at the level of rhizosphere (Ho et al., 2007; Kidd et al., 2008), the phyllosphere (Sandhu et al., 2007) and inside the plant (Siciliano et al., 2001; Barac et al., 2009). Phytoremediation systems for PHCs rely on a synergistic relationship between plants and their root-associated microbial communities. Degradation is facilitated through a rhizosphere effect; plants exude organic compounds through their roots, increasing the density and activity of potential hydrocarbon degrading microorganisms in the zone surrounding the roots (Anderson et al., 1993; Siciliano and Germida, 1998).

The selection of specific microorganisms in the rhizosphere has potential advantages for bioaugmentation (Kuiper et al., 2002; Singer et al., 2003). Specific rhizosphere-competent microorganisms that degrade a given contaminant can be added to soil along with a plant that supports the growth of these microorganisms (Normander and Hendriksen, 2002).

The biodegradation abilities of the bacteria and the expression and maintenance of bacterial genes in the rhizosphere are extremely important for the effective removal of contaminants in phytoremediation. However, several studies have shown that the application of specific microbes fails in efficient degradation of contaminants, probably due to poor survival in the rhizosphere (Rentz et al., 2005; Gilbertson et al., 2007; Gunderson et al., 2007). There are several explanations for the poor survival of inoculated microorganisms in the rhizosphere, including the inoculation method or soil properties. The way to inoculate can influence the efficiency of soil colonization and subsequent survival (Cunliffe et al., 2006; Weyens et al., 2009). Traditionally, seed imbibement and soil inoculation are frequently used for applying specific bacteria in phytoremediation studies; the efficiency of these methods in terms of colonization and

biodegradation has been rarely compared. Soil properties such as pH, texture, particle size and low nutrient availability, can also affect bacterial survival in soil (Kaimi et al., 2007; Sun et al., 2010) and plant growth (Kaakinen et al., 2007). There are many studies showing the effect of soil properties on bioremediation and phytoremediation, but the effect of soil type on bacterial colonization and gene abundance and expression was not studied.

The studies undertaken in this thesis address the bacterial survival and colonization in the rhizosphere soil and plant interior by culture-dependent and -independent methods, with the primary objective of contributing to our understanding of the specific plant-microbe interactions that facilitate hydrocarbon degradation in the plant environment. This objective was pursued through two studies designed to address the following hypotheses:

1) The inoculation method affects colonization and performance of bacterial inoculant strains in the phytoremediation of soil contaminated with diesel.

2) Soil type affects plant colonization, activity and catabolic gene expression of inoculated bacterial strains during phytoremediation of diesel.

This thesis is structured in four chapters. **Chapter 1** describes the background and objectives of the research and provides a general introduction on phytoremediation processes, combined use of plants and specific bacteria, factors effecting phytoremediation, hydrocarbon degradation and monitoring of inoculated bacteria in soil. **Chapter 2** presents results from an experiment addressing the manner of the inoculation method affects colonization and performance of bacterial inoculant strains in the phytoremediation of soil contaminated with diesel oil. To facilitate monitoring of the colonization processes inoculated strains were labelled with the *gusA* marker gene. We found that inoculation generally had a beneficial effect on plant biomass production and hydrocarbon degradation, however, strains inoculated in soil performed better than applied by seed imbibement. Performances correlated with the colonization efficiency of inoculated strains. The highest hydrocarbon degradation was observed in the treatment, in which all three strains were inoculated in combination into soil. In **Chapter 3** the colonization behaviour and gene abundance and gene expression of two strains, *Pseudomonas sp*. ITRI53 (an endophyte) and *Pantoea sp*. BTRH79 (a rhizosphere strain), were investigated after applying these strains to Italian ryegrass vegetated in three different types of soil. We aimed to assess whether soil type affects colonization and expression of degrading *alkB* and CYP153 genes in plant and rhizosphere. Culture-dependent and culture-independent analysis showed high colonization, gene abundance and expression in loamy soils after eight weeks of inoculation. By contrast, low

colonization, gene abundance and absence of gene expression in sandy soil were found. Soil type had a strong effect on the expression of genes (*alkB* and CYP153) specific to hydrocarbon degradation. The highest levels of genes expression and hydrocarbon degradation were seen in loamy soil that had been inoculated with BTRH79, and were significantly higher compared to those in other soils. A positive correlation (r = 0.73) was observed between gene expression and hydrocarbon degradation indicating that catabolic gene expression is necessary for contaminant degradation. These results suggest that soil type influences the bacterial colonization and microbial activities and subsequently the efficiency of contaminant degradation. Finally, general conclusions on the research performed are summarized in **Chapter 4**.

Phytoremediation has been proposed as an attractive strategy to achieve the efficient removal of pollutants. Plants are easy to monitor, they can be used to eliminate a wide range of pollutants, and agriculture techniques are available to minimize the costs of the treatment. Phytoremediation is usually more cost effective because it is an inherently naturally driven process. Once established, sites usually require little financial input with regards to maintenance. As contaminants remain on site, transportation costs associated with moving the contaminated material to a secondary treatment facility are eliminated. Practitioners estimate that, on average, phytoremediation is a minimum of ten times less expensive than other remediation technologies (Pilon-Smits, 2005). Elimination of transportation also reduces the possibility of spreading contaminants to other environments. Less site disturbance, contaminant containment via hydraulic gradients, erosion prevention, increased soil health and productivity, and a favourable public reaction to the “green aesthetics” of plant-based technologies all contribute to the growing popularity of phytoremediation. However there are some disadvantages and limitations of phytoremediation.

Bioaccumulation of metals within plant tissues may necessitate further treatment of harvested plants themselves. High contaminant concentrations may be toxic to plants, necessitating the pre-treatment of the site. The zone of effectiveness of phytoremediation is generally dictated by the depth of root growth, except in those cases where soluble contaminants are transported towards the root system by the evapotranspirative hydraulic gradient. Contaminants which are highly sorbed to soil particles may not be treatable due to limited bioaccessibility or bioavailability. Finally, the effectiveness of phytoremediation varies with

environmental conditions, including soil physicochemical properties and seasonal temperature fluctuations (Segura et al., 2009).

1.2. Phytoremediation

Phytoremediation is defined as the use of plants and their associated microorganisms to sequester, degrade, or stabilize xenobiotic contaminants (Figure 1.1). The past two decades have produced an impressive body of research in the field of phytoremediation for organic contamination. In 1990, Aprill and Simms (1990) reported that eight prairie grasses enhanced the removal of four different hydrocarbons. Since then, numerous studies have noted the positive effect of vegetation on the degradation or dissipation of hydrocarbons. Plants are autotrophic organisms capable of using sunlight and carbon dioxide as sources of energy and carbon. However, plants rely on the root system to take up water and other nutrients, such as nitrogen and minerals, from soil and groundwater. As a side effect, plants also absorb a diversity of natural and man-made toxic compounds for which they have developed diverse detoxification mechanisms (Eapen et al., 2007).

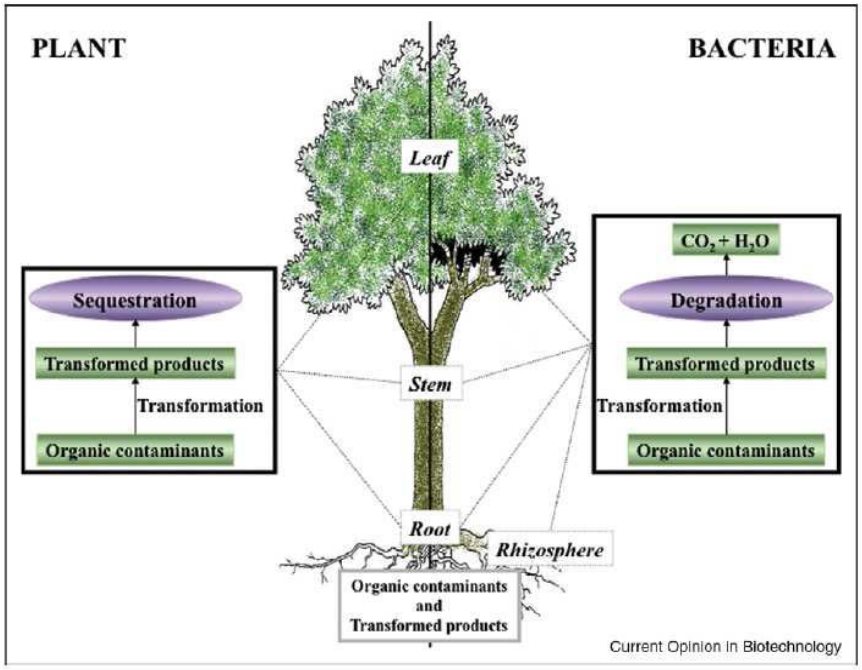


Figure 1.1. Contribution of plant and their associated bacteria to phytoremediation of organic contaminants (Weyens et al., 2009).

Pollutant-degrading enzymes in plants probably originate from natural defense systems against the variety of allelochemicals released by competing organisms, including microbes, insects and other plants (Singer 2006). From this viewpoint, plants can be seen as natural, solar-powered pump-and-treat systems for cleaning up contaminated environments, leading to the concept of phytoremediation (Pilon-Smits, 2005). First developed for the removal of heavy metals from soil, the technology has since proven to be efficient for the treatment of organic compounds, including chlorinated solvents, polyaromatic hydrocarbons and explosives (Salt et al., 1998; Pilon-Smits, 2005). Beyond the removal of contaminants from soil, phytoremediation involves different processes, such as enzymatic degradation, that potentially lead to contaminant detoxification (Salt et al., 1998; Dietz, 2001; Pilon-Smits, 2005). The degradation of both low molecular weight PAHs such as naphthalene and phenanthrene and high molecular weight PAHs such as pyrene, benzo(a)anthracene, and benzo(k)fluoranthene is reported to be enhanced in rhizosphere soil (Aprill and Sims, 1990; Binet et al., 2000). Similarly, the degradation of complex mixtures of hydrocarbons including diesel fuel and weathered crude oil is also enhanced by plants (Banks et al., 2003; Kaimi et al., 2006; Liste and Prutz, 2006). Phytoremediation studies have examined single plant species (Parrish et al., 2005; Kaimi et al., 2006; Yousaf et al., 2010a) or single and mixed plant species (Siciliano et al., 2003; Kirk et al., 2005; Phillips et al., 2006).

Although most studies find that specific plants are able to increase the degradation potential in hydrocarbon-contaminated soil there is little consensus as to the overall effectiveness of different treatments. There is little doubt that plant species vary greatly in their ability to increase hydrocarbon degradation (Siciliano et al., 2003; Parrish et al., 2004), with reported degradation varying from as little as 5% to greater than 50%. Contradictory results have been found in separate phytoremediation studies with regards to whether a specific plant species promotes hydrocarbon degradation. For example, the results of a recent study by Rezek et al. (2008) show little influence of *Lolium perenne* (perennial rye grass) on PAH degradation, while both Yousaf et al. (2010a) and Binet et al. (2000) reported a significant rhizosphere effect. However, considering the differences in degradation potential even within clones of plant cultivars (Schwab et al., 2006), comparable results between plant species and soil properties cannot be expected. There are numerous factors apart from the plant itself that will influence whether a specific plant will enhance hydrocarbon degradation. The nature of the hydrocarbon itself, contamination history and age, soil physico-chemical characteristics, soil and external environmental parameters, and the nature of the indigenous soil microbial communities will all

influence degradation outcomes (Anderson et al., 1993). What works in one soil and for one type of contamination often will not work under different conditions. Ultimately, for all situations, the efficiency of a given phytoremediation treatment is linked to the impact that the plant has on associated microbial communities.

The use of plants to remediate recalcitrant compounds in soil represents a low-cost alternative when contrasted with expensive, and often destructive, mechanical methods (Sung et al., 2003). Soil organic compounds can be directly degraded and completely mineralized by plant enzymes through phytoremediation (Alkorta and Garbisu, 2001; Wild et al., 2005); many plants produce, and often secrete to the environment, enzymes that can degrade a wide range of organic compounds. However, inorganic pollutants can not be degraded. Inorganic pollutants must either be stabilized in the soil to make them less bioavailable (i.e. phytostabilization); extracted, transformed, and accumulated in plant tissues (i.e. phytoextraction); or transformed into volatile forms (i.e. phytovolatization) (Pilon-Smits, 2005). Phytoremediation efficiency for metals is often limited by the bioavailability of the metal in soil, plant root development, and the level of tolerance of the plant to each particular metal (Pilon-Smits, 2005). In addition to stabilizing soils and improving aeration, plants can stimulate microbial activity and biochemical transformations in the rhizosphere through the release of root turnover and exudates (Leigh et al., 2002). Plants themselves have a positive effect on the microbial degradation of organic contaminants. This increased degradation potential is the result of higher microbial densities and metabolic activities in the rhizosphere due to microbial growth on root exudates and cell debris originating from the plant roots.

Some contaminants in soil and groundwater may be taken up by plant roots. One important plant uptake mechanism for some compounds is the potential absorption from the gas phase by the roots or leaves. However, the movement of contaminant in the subsurface is extremely affected by the flow of water to the roots. Plants transport water through the xylem and solutes through the phloem. Transport of an organic compound into roots is a function of the lipophilicity of the compound. Lipophilicity can be measured by its relative partitioning between an octanol phase and a water phase (Briggs et al., 1982). Lipophilicity also implies an equal propensity to partition into soil organic matter. Root absorption becomes difficult with heavily textured soils and soils higher in organic matter. Compounds that are the most water soluble will be most available for mass transport and diffusion into roots. With compounds having lower partitioning values, plant uptake is dependent on active plant processes, especially water

transpiration rates. Unlike root absorption, translocation to aboveground plant tissues via the transpiration stream is predominant for compounds having lower partitioning value. Plants with higher water transpiration rates can take up more of a compound with a low partitioning value than plants with reduced water requirements (Burken and Schnoor, 1998; Karthikeyan and Kulakow, 2003).

Once moved into plant, these compounds may have multiple fates; however, many compounds are bound to plant tissues. Some compounds can be accumulated in root tissue, some can be metabolized in the root, and some can be translocated via the phloem and subsequently volatilized from leaf surfaces. Sequestration of organic compounds is a common fate, often associated with cell wall materials such as lignin. Furthermore, plant parameters such as the mean daily water uptake rate have been shown to play an important role in determining the extent of contaminant and water absorption by the root, which, in turn, is related to the contaminant retardation (McFarlane, 1995). Volatile compounds or their metabolic products also can be released to the atmosphere through plant transpiration. Many organic contaminants that are recalcitrant in the surface environment react and degrade rapidly in the atmosphere with hydroxyl radicals, an oxidant formed in the photochemical cycle.

1.2.1. Phytoremediation of organics facilitated by exogenous bacteria

Although phytoremediation has potential as a viable remediation strategy for persistent organics, pollutants above a certain level can be toxic to both the plants and the associated microorganisms and, subsequently, low remediation (Glick, 2003; Peng et al., 2009; Germaine et al., 2009). To increase plant biomass in contaminated soils, plant growth-promoting and degrading bacteria can be used to mitigate plant stress responses, and enhance plant growth and degradation of contaminants (Weyens et al., 2009; Glick, 2010). Degradation of toxic organic compounds in environmental soil by plant-associated bacteria can involve endophytic and rhizobacteria. Bacteria that occur naturally in the internal tissues of plants (called endophytes) may promote plant growth, the beneficial to the plant host by producing a range of natural products, and contribute to enhanced biodegradation of environmental soil pollutants (Weyens 2009). Similarly bacteria that are associated with the rhizosphere of plants can benefit the plants by synthesizing compounds that protect plants by decreasing plant stress hormone levels, delivering key plant nutrients, protecting against plant pathogens, and degrading contaminants (McGuinness and Dowling, 2009; Glick, 2010). To date, the combined use of plants and

rhizobacteria or endophytic bacteria has been successfully exploited/applied to remove petroleum products (Table 1.1) from contaminated soils. The use of bacteria capable of degrading toxic organic compounds in combination with specific plants could offer an efficient, economic and sustainable remediation technology for the twenty one century.

1.2.2. Rhizosphere bacteria and phytoremediation

In the last 15 years scientists have successfully used plant together with plant growth-promoting and /or degrading bacteria to remove organic toxicants from the soil (Table 1.1). Plant growth-promoting bacteria may facilitate plant growth either indirectly or directly (Glick, 1995).

The ability of plant growth-promoting bacteria to act as biocontrol agents against phytopathogens and thus indirectly stimulate plant growth may result from any one of a variety of mechanisms including antibiotic production, depletion of iron from the rhizosphere, induced systemic resistance, production of fungal cell wall lysing enzymes, and competition for binding sites on the root (Glick, 1995). There are several ways in which plant growth promoting bacteria can directly facilitate plant growth. They may fix atmospheric nitrogen and supply it to plants - usually a minor component of the benefit that the bacterium provides to the plant; synthesize siderophores which can sequester iron from the soil and provide it to plant cells which can take up the bacterial siderophore-iron complex; synthesize phytohormones such as auxins, cytokinins and gibberelins, which can act to enhance various stages of plant growth; solubilize minerals such as phosphorous, making them more readily available for plant growth; and synthesize the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which can lower ethylene levels (Glick et al., 2007). A bacterium may directly affect plant growth and development using any one or more of these mechanisms. Since many plant growth-promoting bacteria posses several of these traits, a bacterium may utilize different traits at various times during the life cycle of the plant. Typically plant growth-promoting bacteria have little or no measureable effect on plant growth when the plants are cultivated under optimal and stress free conditions.

Degrading microorganisms may enhance a plants' adaptation to contaminants such as petroleum hydrocarbons by detoxifying contaminated soils through direct mineralization of these organic contaminants (Escalante-Espinosa et al., 2005) (Figure 1.2). Shaw and Burns (2004) showed that 2,4-dichlorophenoxyacetic acid mineralization was enhanced in the rhizosphere of *Trifolium*, due to the increased population sizes of 2,4-dichlorophenoxyacetic acid degraders within the rhizosphere.

Table 1.1. Examples of successful phytoremediation cases of different contaminants using rhizobacteria (RH) or endophytic bacteria (EN)

Compound	Plant used	Microbe used	Strategy	Reference
Tetrachlorophenol	Wheat (*Triticum* spp.)	*Herbaspirillum* sp K1	EN	Mannisto et al., 2001
Chlorobenzoic acids	Wild rye (*Elymus dauricus*)	*Pseudomonas aeruginosa* R75, *Pseudomonas savastanoi* B35	EN	Siciliano et al., 1998
2,4-dichlorophenoxyacetic acid	Pea (*Pisum sativum*)	*Pseudomonas putida* VM1450	EN	Germaine et al., 2006
Toluene	Yellow lupine (*Lupinus luteus* L.)	*Burkholderia cepacia* G4	EN	Barac et al., 2004
Toluene	Poplar (*Populus*)	*Burkholderia cepacia* Bu61	EN	Taghavi et al., 2005
Benzene, toluene, ethylbenzene and xylene	Popular (*Populus cv. Hazendans*)	*Pseudomonas sp.*	EN	Germaine et al., 2004; Moore et al., 2006
Hydrocarbons	Pea (*Pisum sativum*)	*Pseudomonas putida*	EN	Germaine et al., 2009
Explosives	Popular tissues (*Populus deltoidesnigra*)	*Methylobacterium populi*	EN	van Aken et al., 2004;a van Aken et al., 2004b
Polychlorinated biphenyls	Alfalfa (Medicago sativa), Sugar beet (*Beta vulgaris* L.)	*Pseudomonas fluorescens*	RH	Brazil et al., 1995
2,4-dichlorophenoxyacetic acid	Barley (*Hordeum Sativum* L.) Ryegrass (*Lolium perenne* L.)	*Burkholderia cepacia* Indigenous degraders	RH	Jacobsen, 1997; Shaw and Burns, 2004
Pentachlorophenol	Ryegrass (*Lolium perenne* L.)	Indigenous degraders	RH	He et al., 2005
Trichloroethylene	Wheat (*Triticum* spp.)	*Pseudomonas fluorescens*	RH	Yee et al., 1998
Petroleum products	White mustard (*Sinapsis alba* L.)	Indigenous degraders	RH	Liste et al., 2006
Crude oil	Wheat (*Triticum* spp.)	*Azospirillum lipoferum* sp.	RH	Muratova et al., 2005; Shaw and Burns, 2004
Polycyclic aromatic hydrocarbons	Tall fescue grass (*Festuca arundinacea*)	*Azospirillum lipoferum* sp. *Enterobacter cloacae* CAL2 *Pseudomonas putida* UW3	RH	Huang et al., 2004
Naphthalene	Barmultra grass (*Lolium multiflorum*)	*Pseudomonas putida* PCL1444	RH	Kuiper et al., 2001; Kuiper et al., 2004
Phenanthracene	Barley (*Hordeum sativum* L.)	Degrading rhizosphere colonizing *Pseudomonas*	RH	Ankohina et al., 2004
Chrysene	White Clover (*Trifolium repens* L.)	PAH tolerant *Rhizobium leguminosarum*	RH	Johanson et al., 2004

The absence of a population of degrading microorganisms can be overcome by the inoculation of the plant rhizosphere with pollutant degrading strains. This approach successful in reducing the levels of benzene, ethylene, toluene xylenes, hydrocarbons, polychlorinated biphenyls and pesticides in polluted environments (Johanson et al., 2004; Yousaf et al., 2010a; Germaine et al., 2006).

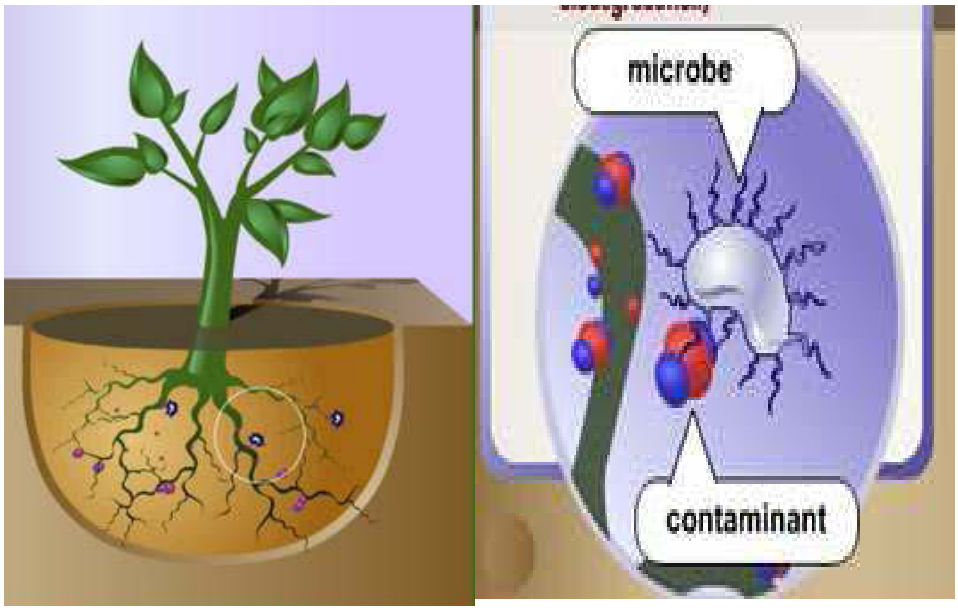


Figure 1.2. Plant-assisted microbial biodegradation of organic pollutants, modified from http://deoracle.org/learning-objects/phytoremediation-organic-contaminants.html.

The rhizosphere is defined as the volume of the soil over which roots have influence, and which is shared with soil bacteria. Plants release exudates in the rhizosphere likely to serve as carbon source for microbes (Gregory, 2006). As a result, high microbial build up of 1 – 4 orders of magnitude occur in the rhizosphere compared to bulk soil (Olson et al., 2003). Consequently, rhizosphere microbes can promote plant health by stimulating root growth via production of plant growth regulators, enhance mineral and water uptake. Remediation of contaminants in rhizosphere may be a passive process. Pollutants can be phytostabilized simply by erosion control and hydraulic control. There is also passive control of organic pollutants and inorganic cations to plant surface. Organic pollutants may be degraded in the rhizosphere by root-released plant enzymes or through phytostimulation of microbial degradation. Organics such as PAHs and other petroleum hydrocarbons have successfully been remediated in the rhizosphere by microbial activity (Olson et al., 2003; Hutchinson et al., 2004).

Successful rhizosphere colonization depends not only on interactions between the plants and the microorganisms of interest, but also on interactions with other rhizospheric microorganisms and the environment. Molecular techniques, such as denaturing or temperature gradient gel electrophoresis have allowed researchers to follow the modifications in bacterial communities after environmental perturbations, including the introduction of plants or biodegradative bacteria, changes in temperature, or the addition of contaminants (Kielak et al., 2008). Several techniques to follow seed and root colonization by bacteria have been developed during the last 15 years, which mainly include in situ hybridization assays using fluorescent probes and the visualization of bacteria that carry *luxAB* genes encoding bacterial luciferase, the green fluorescent protein, *gusA* gene encoding β-glucuronidase (GUS), or other reporter genes (Wilson et al., 1995; Segura et al., 2009). These techniques have been used to illustrate that introduced microorganisms are often unable to compete with indigenous microorganisms or are unable to establish high numbers in the rhizosphere (Lübeck et al., 2000; Afzal et al., 2010). Some bacteria have developed strategies to out-compete other microorganisms by delivering toxins, using extremely efficient nutrient utilization systems, or by physical exclusion (Lugtenberg and Dekkers, 1999). However, many other factors involved in successful colonization, under non-sterilized conditions, remain unknown.

Mounting evidence indicates that plants are able to select the bacteria living in their rhizosphere by different mechanisms, including root architecture, the modification of soil conditions, or the exudation of specific compounds. Each plant exudes specific compounds, which are dependent on the plant's particular secondary metabolism. Some plants can promote the growth of bacteria that are able to degrade certain compounds, while others secrete toxic compounds that select for tolerant bacteria, and some plants are able to secrete hydrolases that degrade acyl homoserine lactones, thus inhibiting bacterial quorum sensing (Hartmann et al., 2009).

Bacterial attachment to plant roots in an early step in plant root colonization. Initial approaches for identifying and studying genes involved in root colonization were based on the use of random or directed mutagenesis to isolate mutants impaired for colonization. Bacterial attachment has been extensively studied in rhizobacteria and although the molecular basis is still not completely understood, the general mechanism seems to be mediated by surface proteins, capsular polysaccharides, flagella and chemotaxis (Lugtenberg and Dekkers, 1999; Capdevila et al., 2004; Rodríguez-Navarro et al., 2007).

Traditionally, the mechanism most often involved to explain the various direct effects of plant growth-promoting bacteria on plants is the production of phytohormones, and most of the attention has focused on the role of the phytohormone auxin (Werner et al., 2001). However, in the last few years it has been found that a number of plant growth-promoting bacteria contain the enzyme ACC deaminase (Shaharoona et al., 2006; Nadeem et al., 2007; Yue et al., 2007) and that this enzyme can cleave the plant ethylene precursor ACC, and thereby lower the level of the phytohormone ethylene in a developing or stressed plant. During periods of environmental stress, plants produce high levels of "stress ethylene." Moreover, much of the growth inhibition that occurs as a consequence of an environmental stress is the result of the response of the plant to the increased levels of ethylene which exacerbates the response to the stress. However, ACC deaminase-containing bacteria can lower plant ethylene levels in plants and thereby provide some protection against the inhibitory effects of various stresses (Glick, 2003; Huang et al., 2005; Gurska et al., 2009).

A large number of variables can affect the phytoremediation of contaminated soils. These variables include differences in: plant type, soil composition, endogenous bacteria, the nature, concentration and range of the contaminants, the temperature range, and the type (and physiological state) of added bacteria. Recently, some considerations were suggested by Glick, (2010) that may facilitate the phytoremediation of organic contaminants from soil. These include: (i) inclusion of bacteria that are to be able to both promote plant growth (e.g. through the provision of IAA) and degrade soil contaminants are generally superior to bacteria that can only promote plant growth or degrade soil contaminants; (ii) plant growth-promoting activity that is based on the activity of ACC deaminase appears to be universally effective in promoting plant growth under stressful conditions and is therefore a key determinant in facilitating phytoremediation; and (iii) endophytic bacteria appear to be more effective than bacteria that bind exclusively to the plant rhizosphere.

Plants depend on their associated microorganisms to achieve an efficient degradation of organic compounds. Moreover, dense populations of diverse heterotrophic microorganisms live in the rhizosphere, the phyllosphere and inside the plant. These microbial associations increase the capacity for a stepwise transformation of organic contaminants by consortia and provide a habitat that is conducive to genetic exchange and gene rearrangements. The emerging picture suggests that plants draw pollutants, including PAHs, into their rhizosphere to varying extents via the

transpiration stream. Subsequent degradation can occur in plant itself, or in the rhizosphere, or both (Weyens et al., 2009).

In addition to degrading hydrocarbons per se, it has been observed that some bacteria may make these relatively insoluble compounds more bioavailable. This could occur through the formation of a bacterial biofilm directly on the surface of some crystal-like polycyclic aromatic hydrocarbons. This mechanism is likely to exist for only a limited number of bacteria that contain hydrophobic external surfaces. Bacteria with more hydrophobic surfaces would be dependent upon the (limited) concentration of hydrocarbons in the bulk liquid (Glick, 2010).

The easiest assays to probe for microbial activity against pollutants are those where it is possible to monitor CO_2 evolution when the chemical under scrutiny is available as a labeled compound (Bending et al., 2001). More sophisticated experiments can be done if substrates are labeled with a stable isotope to study the incorporation of a heavier C- or N-source into cell components (Madsen, 2006). The utilization of reporter genes to study the expression of catabolic genes in the rhizosphere is another technique that has been proven useful. The successful expression of *bph* [genes involved in the degradation of polychlorinated biphenyl (BCB)] in sugar beet using the recombinant strain *P. fluorescens* was reported (Brazil et al., 1995). A reporter strain that detected 3-chlorobenzoate, an intermediate in PCB-2 degradation, has been used to monitor the *in vivo* production of 3-CB on alfalfa roots. The authors used *gfp* fused with the meta-pathways pm promoter from *P. putida*, which is strongly induced by 3-CB (Boldt et al., 2004).

Among root exudates, numerous aromatic compounds (i.e. terpernes, flavonoids or lignin-derived components) with chemical structures similar to those of the contaminant are released and some can act as inducers of contaminant-degradation pathways (Singer et al., 2003). L-carvone, one of the components of spearmint root exudates, has been identified as an indicator of the genes involved in PCB degradation in *Arthrobacter* sp. strain B1B (Gilbert and Crowley, 1997). Other secondary plant metabolites, such as *p*-cymene, limonene, and the non-aromatic compound isoprene, can also induce the PCB-degradation pathways in *Arthrobacter*. Although the specific role of flavonoids as inducers of the degradation of organic pollutants has not been well established, it is known that several flavonoids sustain the growth of PCB degraders. Donnelly and colleagues (1994) reported the growth of *Ralstonia eutropha* H850 on 11 different flavonoids. The degradation of flavonoids also fostered the degradation of several PCB congeners. The degradation of flavonoids by rhizospheric bacteria leads to the formation of intermediates, including resorcinol, phloroglucinol phenylacetic acid, substituted cinnamic acids and

protocatechuic acid (Shaw and Burns, 2004). These compounds are likely to be mineralized through the β-ketoadipate pathway (Parke et al., 2000), which is active in the catabolism of several aromatic contaminants. Protocatechuate is an intermediate in the degradation of polycyclic aromatic hydrocarbons (PAHs) in some microorganisms (Kim et al., 2008). Salycilate, which induces systemic acquired resistance in plants, is a good inducer of the PAH-degradation pathways (Chen and Aitken, 1998). Non-aromatic plant compounds, such as linoleic acid, have also been shown to be responsible of the stimulation of pyrene and benzo[a]pyrene degradation by Gram-positive bacteria (Yi and Crowley, 2007). Although there are several reports about enhanced PAH degradation by rhizobacteria, Aprill and Sims (1990) and Rentz et al. (2004), reported that the phenanthrene-degrading activity of *P. putida* ATCC 17484 was repressed after incubation with root extracts from six different plants. Catabolic repression was the most probable cause for this repression; analysis of the root exudates indicated a minor proportion of phenolic compounds relative to other easily degradable substrates (acetates, amino acids and glucose). The apparent discrepancies between the enhanced PAH biodegradation in the rhizosphere and the inhibition of PAH degrading activity by root exudates can be explained because the rhizosphere can sustain greater numbers of degradative strains than bulk soil.

The list of contaminant-degrading bacteria associated with plant rhizospheres is very extensive. In a recent study done in our laboratory, several rhizosphere-isolated bacteria have been characterized by their ability to degrade hydrocarbons (Yousaf et al., 2010b). Many other rhizospheric bacteria have previously been described as able to degrade a wide variety of contaminants (Daane et al., 2001; Kuiper et al., 2001; Johnson et al., 2004). For the efficient removal of soil contaminants, not only do microbes with the appropriate catabolic genes have to be maintained in the rhizosphere, but the genes have to be conveniently expressed (Juhanson et al., 2009).

1.2.3. Endophytic bacteria and phytoremediation

The majority of plant-associated bacteria derives from the soil environment. They may migrate to rhizosphere and subsequently the rhizoplane of their host before they are able to show beneficial effects. Some rhizoplane colonizing bacteria can also penetrate plant roots, and some strains may move to aerial plant parts (Compant et al., 2010). Because the complex plant-rhizobacteria interactions, the use of endophytic bacteria for biodegradation has been extensively explored in the last years (Doty, 2008; Ryan et al., 2008; Weyens et al., 2009). Endophytic bacteria

that colonize the internal tissues of the plant without causing a negative effect (Sessitsch et al., 2002) face less competition for nutrients and are physically protected from adverse changes in the environment (Schulz et al., 2006) and enhanced remediation of contaminants. However, successful remediation by endophytic bacteria requires the transport of the contaminant to the plants' internal tissues. Contaminant transport and its distribution in plants have been reported to depend on soil and plant properties and on the physicochemical properties of the contaminant. Although the biodegradative capacity of the endophytic bacteria has not been extensively investigated, reports on the ability of several endophytic bacteria to degrade some pollutants (i.e. explosives, herbicides or hydrocarbons) have been published (van Aken et al., 2004a; Germaine et al., 2006). Also, endophytic bacteria resistant to high concentrations of heavy metals, BTEX (benzene, toluene, ethyl-benzene and xylenes), TCE or PAHs have been identified (Moore et al., 2006; Doty, 2008). Some plants can accumulate bacterial endophytic genotypes for the degradation of contaminants (Siciliano et al., 2002).

When an efficient rhizodegradation seems possible, compounds with a lipophilicity in the optimum range seem to enter the root xylem before the soil and rhizosphere microflora can degrade them (Trapp et al., 2000). Since the residence time of contaminants in the xylem ranges from several hours to up to two days (Mc Crady et al., 1987), degrading endophytes colonizing the xylem are perfect candidates to reduce phytotoxicity and to avoid evapotranspiration of contaminants or their degradation intermediates into the environment (Figure 1.3). If no naturally occurring endophytes with the desired metabolic properties are available, endophytic bacteria can be isolated, equipped with the appropriate degradation pathways and subsequently re-inoculated in their host plant. The general idea behind the use of engineered endophytes to improve phytoremediation is to complement the metabolic properties of their host plant. Whereas soil bacterial-assisted phytoremediation has been studied (Johnson et al., 2004; Peng et al., 2009; Cai et al., 2010), there is little information on the potential of endophytic bacteria isolated from plants grown in PAH-contaminated soils on the phytoremediation of PAH-contaminated soils (Toledo et al., 2006). Endophytic bacteria may be of particular interest as they have the advantage of being relatively protected from the competitive, high-stress environment of the soil (Sturz and Nowak, 2000). Endophytic microorganisms can have the capacity to promote plant establishment under adverse conditions and enhance plant growth and development. A better understanding of the characteristics of degrading endophytic bacteria is a critical prerequisite for the development of effective phytoremediation of PAH-contaminated soils.

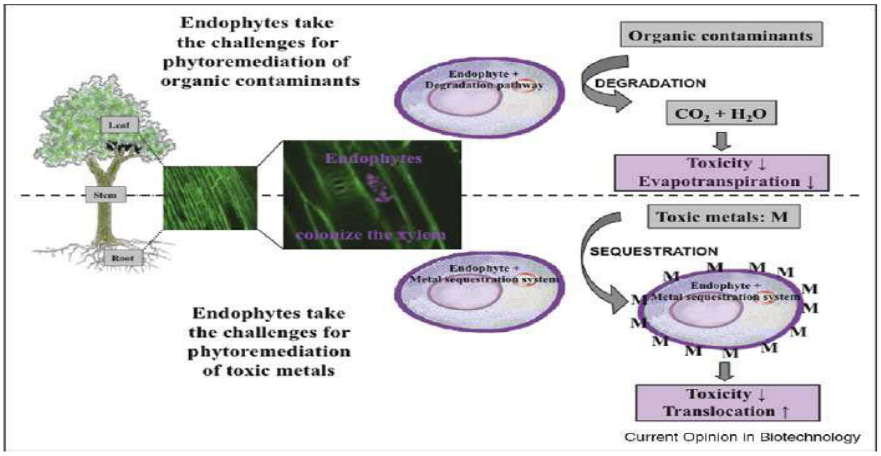


Figure 1.3. Role of endophytes in the phytoremediation of soil contaminants (Weyens et al., 2009a)

Several recent studies have found that plants may harbour diverse endophytic communities which include bacteria capable of degrading hydrocarbons. As with other endophytes, the diversity of these degrader populations is influenced by plant-specific factors. Yousaf et al. (2010b), isolated hydrocarbon degraders *Pseudomonas*, *Arthrobacter*, *Enterobacter*, and *Pantoea* species from the root and stem tissues of Italian ryegrass and birdsfoot trefoil vegetated in hydrocarbon contaminated soil. Similarly, Siciliano et al. (2001) found that endophytic hydrocarbon degraders in tall fescue (*Festuca arundinacea*) and rose clover (*Trifolium fragiferum*) at an aged-hydrocarbon contaminated site, with up to 4% of culturable endophytes possessing genes involved in hydrocarbon degradation. There is increasing interest in the role of endophytes in phytoremediation application as they can serve as vector delivering biodegradative capacities inside the plant (Barac et al., 2004). However, it is not fully understood; whether potentially degrading endophytes are active once inside the plant and thereby contribute to the detoxification of the pollutant and better survival of the plant under toxic conditions.

A few studies reported that some endophytic bacteria colonize flowers, fruits and seeds. However, under natural conditions the majority of flowers and fruits does not contain endophytic bacteria at all or only very low densities. It is likely that only specialized endophytic strains are able to colonize and survive in reproductive plant organs. Few species were isolated from sterilized rice seeds (Compant et al., 2010). Strains belonging to *Pseudomonas* and *Rahnella* genera were additionally isolated from Norway spruce (Canker et al., 2005) as well as yellow lupine seeds (Barac et al., 2004), and from some other plants, providing some information on the type of microorganisms colonizing plant reproductive organs.

1.2.4. Application of microbial mixtures in phytoremediation

Much research has been carried on the use of seed- or soil-applied microorganisms to promote plant growth, in many cases applying individual microbial strains, with varying success. However, the application of several strains in combination may be better able to perform than single isolates. A broad range of environmental conditions may be tolerated by microorganisms in a mixture (Raupach and Kloepper, 1998), and multiple actions of mode may be exhibited by microorganisms in combination, microbial mixtures may more effectively target plant stress due to contaminants (Bennett and Whipps, 2008; Afzal et al., 2010).

1.2.5. Transgenic plants and phytoremediation

Phytoremediation is the use of plants to clean up environmental pollution. However, detoxification of organic pollutants by plants is often slow, leading to the accumulation of toxic compounds that could be later released into the environment. An exciting alternative to the use of plant-associated bacteria to degrade toxic organic compounds in soil is the use of recombinant DNA technology to generate transgenic plants expressing bacterial enzymes resulting in improved plant tolerance and metabolism of toxic organic compound in soil. A recent study describes the development of transgenic poplars (*Populus*) over expressing a mammalian cytochrome P450, a family of enzymes commonly involved in the metabolism of toxic compounds (Doty, 2008). The engineered plants showed enhanced performance with regards to the metabolism of trichloroethylene and the removal of a range of other toxic volatile organic pollutants, including vinyl chloride, carbon tetrachloride, chloroform and benzene. This work suggests that transgenic plants might be able to contribute to the wider and safer application of phytoremediation. Typically, transgenic plants exhibiting new or improved phenotypes are engineered by the over-expression and/or introduction of genes from other organisms, such as bacteria or mammals. Heterotrophs possess the enzymatic machinery necessary to achieve a complete mineralization of organic molecules; bacterial and mammalian degradative enzymes can therefore be used to complement the metabolic capabilities of plants (Eapen et al., 2007). Transgenic plants for phytoremediation were first developed in an effort to improve heavy metal tolerance; for example, tobacco plants (*Nicotiana tabacum*) expressing a yeast metallothionein gene for higher tolerance to cadmium, or *Arabidopsis thaliana* over expressing a mercuric ion reductase gene for higher tolerance to mercury (Misra and Gedamu, 1989; Rugh et al., 1996). The first attempts to transform plants for phytoremediation of organic compounds targeted explosives and

halogenated organic compounds in tobacco plants (French et al., 1999; Doty et al., 2000). A number of studies have shown that transgenic plants are capable of detoxifying herbicides, organic explosives, TCE, and PCBs, using bacterial genes encoding enzymes involved in the detoxification of the target organic contaminant (McGuinness and Dowling, 2009).

1.2.6. Effect of inoculation method on phytoremediation

For several decades bacteria have been introduced into soils to improve the growth of crops through soil inoculation or bacterial coatings on seeds, roots and tubers. Even now, there is a still growing interest in the application of beneficial bacteria to soil, in particular given the vast possibilities of strain improvement offered by modern biotechnology. Among the main objectives are the enhancement of plant growth promotion, biological control of soil-borne pathogens and the degradation of xenobiotic compounds. Although many studies have reported success in the introduction of specific bacteria into the soil, the results of bacterial colonization have been extremely variable, which is a major obstacle in analyzing a reliable and effective inoculation method (van Elsas and Heijnen, 1990; van Elsas et al., 1992; Albajes et al., 2002; Taghavi et al., 2005). Most of the specific contaminant degraders and/or plant growth-promoting bacteria are applied onto seeds or soil, and rhizosphere competence is considered as a crucial factor in determining the successes of both plant-growth promotion and contaminant degradation by inoculated bacteria.

One of the least expensive techniques that can be used to introduce microorganisms into soil is to cover the seeds with the appropriate bacteria. For this, microbes need to adhere well to the seeds (Colleran, 1997). Adhesion to seeds has been studied using classical counts of viable cells, and more recently by taking advantage of reporter genes. These assays are frequently coupled to microscopy techniques in order to facilitate the identification of target microbes. Scanning electron microscopy has also been used to track bacteria adhered to seeds. One of most original approaches for studying the adhesion of bacteria to seeds was developed by Espinosa-Urgel et al. (2000) who designed a strategy based on the selection of mutants unable to adhere to seeds. To this end, the model microorganism *P. putida* KT2440 was mutagenized randomly with mini-Tn5. The pool of Km^R mutants was mixed with both corn seeds placed in a syringe. Following incubation, the seeds were washed to remove bacteria that failed to adhere or which adhered loosely. By repeating the process, the authors ended up with a number of enriched mutants with limited adherence to seeds. Some of the mutants were defective in the attachment to abiotic and

biotic surfaces, while others were only defective in attaching to biotic surfaces, suggesting that biofilm formation proceeds through two different mechanisms depending on whether the surface can be a source of nutrients or not. Motility and chemotaxis proteins were not detected during this screen, probably because under the conditions used (shaking) the bacteria do not need to move towards the seeds. In this work, as in others before, several outer membrane protein were shown to be involved in seed adhesion (Smit et al., 1992; Yousef-Coronado et al., 2008) and this is in agreement with the fact that outer surfaces are the first contact point between a bacterium and the seed.

The physiological characteristics of the inoculant organism determine to a great extent its fate and activity in soil. The physiological traits that play a role in the capacity of inoculant bacteria to colonize soil and survive are often not well known (van Veen et al., 1997). The inoculant formulation is also important in providing a suitable microenvironment to prevent a rapid decline in bacteria introduced in the soil. The importance of maintaining effective inoculum density was shown systematically in a study for bioremediation of atrazine-contaminated soil (Newcombe and Crowley, 1999). The contaminated field was divided into three plots that were inoculated once, four or eight times, respectively, with an atrazine-degrading bacterial consortium. After 12 weeks of inoculation, there was no degradation in soil that was inoculated only once. However, the other soils (inoculated four and eight times, respectively) had 38% and 72% atrazine mineralization. The use of multiple strains, such as different strains for contaminant-degradation and plant growth promotion, might represent a highly successful strategy.

Several delivery methods for introducing bacteria into plants have been reported, including seed inoculation, soil drench, foliar spray and pruned-root dip (Bressan and Borges, 2004; Rosenblueth and Martinez-Romero, 2006). An important research question that remains is how plants in the field can be inoculated more efficiently. Several options are possible: inoculation of seeds or cuttings and inoculation by spraying inoculum on the soil or directly onto growing plants (Weyens et al., 2009). Efficient use of specific microbial strains to seeds in a viable way for phytoremediation is only the first step towards applying beneficial microorganisms to improve plant survival and growth on a contaminating soil. It is also very essential that the introduced microorganisms sustain their viability, colonize the rhizosphere and developing roots. Generally, soil inoculation and seed imbibement are used to apply specific bacteria to plants in phytoremediation studies. Many studies repot the direct application of a microbial strains on plant seeds for pollutant degradation (Kuiper et al., 2001; Ronchel and Ramos, 2001; Germaine et al.,

2006; Germaine et al., 2009). Some studies have applied specific degraders to seedlings by soil inoculation (Villacieros et al., 2005; Andria et al., 2009; Yousaf et al., 2010a). However, the survival and colonization in the rhizosphere and plant interior of beneficial microorganisms applied to seed or soil has not been compared in term of colonization and degradation.

1.2.7. Effect of soil properties on phytoremediation

The environmental fate of an organic chemical in soil is dependent on both the physicochemical characteristics of the compound as well as the physical, chemical and biological properties of the soil. In an attempt to accelerate phytoremediation, exogenous microorganisms are often introduced into contaminated environment. Despite the general rhizosphere effect, an increasing number of reports have indicated that the bacterial composition in the rhizosphere is affected by complex interactions, including soil type, plant species and root zone localization (Marschner et al., 2001; Chen et al., 2007; Segura et al., 2009). However, in order to determine the outcome of any remedial strategy, an understanding of the interplay between the biotic and abiotic factors is important (Labana et al., 2005).

In general, population sizes of bacteria decline more or less rapidly following introduction in to natural soil, and growth of introduced populations in microbiologically undisturbed soils is a rare phenomenon (van Veen et al., 1997). It is evident that bioremediation/phytoremediation is influenced considerably by environmental factors including soil properties and physiological state of inoculated bacteria (Cunliffe et al., 2006; Kaakinen et al., 2007; Kaimi et al., 2007; Sun et al., 2010; Afzal et al., 2010a). Recently it has been reported that soil properties (e.g. soil organic matter, soil texture, particle size distribution, soil moisture, etc.) exert a significant influence on hexadecane removal (Jung et al., 2008). The above studies indicated that any prior information/understanding of the possible impacts of soil properties and physiological state of inoculated bacteria can be of greater significance for improving the overall efficiency and applicability of *in situ* bioremediation processes.

Soils physically support plants, and act as reservoirs for the water and nutrients needed by plants. Soils are complex mixtures of mineral particles of various shape and sizes; living and dead organic materials including microorganisms, roots, and plant and animal residues; air; and water. The soil physical characteristics begin with the sizes (texture) and arrangement (structure) of individual soil particles, these two characteristics intimately affect the pore space between the particles. Soil texture is a term which describes the mixture of different sizes of mineral particles.

Soil texture components, sand, silt and clay, relate primarily to particles smaller than 2 millimeters in diameter. These particles are more active is soil processes which support plant growth. Coarser particle, gravel and stones, are either inert or detrimental to plant cultivation. Sandy soils usually have rapid water infiltration and good aeration but low water holding and nutrient storage capacity (Karthikeyan and Kulakow, 2003). However, there is a considerable range in these properties within the sand fraction. Silty soils (medium particle size) have a slower water intake but a higher water holding capacity. Silt is an essential component of the medium textured, versatile soil called loam. Clay, the finest size fraction, holds water and plant nutrient ions. Soil texture classes have been defined to describe the relative amounts of sand, silt and clay in soils. Soils of different texture differ in particle size composition and, thus, in pore size distribution (van Elsas et al., 1986). Pore size distribution strongly determines the fate of introduced microorganisms, and differences in the behavior of bacteria released into different-textured soils may be related to differences in the protective pore spaces present in these soils. In an earlier study it was found that soil particles had a great impact on seed germination and seedling growth (Anisuzzaman et al., 2001). Enhanced inoculant survival was observed in the finer-textured soil since the decline of the inoculant numbers was greater in sandy soil used than in silt loam soil (van Elsas et al., 1986; van Veen et al., 1997). Under similar prevailing conditions, the inoculant revealed higher survival levels in finer-textured (clayey) than in coarser (sandy) soils (van Elsas et al., 1986). Colonization of soil particles and aggregates is assumed to be vital to ultimate inoculant survival in soil (Hattori et al., 1976). The survival of *Azospirillum brasilense* in 23 soil types was found to correlate with soil clayey content, in addition to other factors (Bashan et al., 1995).

Organic matter is an extremely important component of soil. Small changes in organic matter content make large differences in the physical characteristics of a soil including sorption capacity, water retention, and structure formation. It also has a dramatic effect on chemical properties such as cation exchange and chelation of metals and other ions (Karthikeyan and Kulakow, 2003). The effect of the soil type on the fate of introduced bacteria also corroborates the known effect of soil type on microbe-mediated turnover of organic matter in soil (van Veen et al., 1985). Turnover of organic matter has been observed to be slower in finer-texture soils than in coarser ones. This effect has been explained by the different extents to which soils offer physical protection to organic matter against degradation by microbes and subsequent turnover of microbial biomass, in which protozoan grazing plays an important role (Kuikman and Veen, 1989).

Despite the fact that that there is an impressive number of publications reporting the isolation of microbes with the capacity to degrade contaminants (e.g. Cerniglia, 1993; Urbane et al., 2003; Parales and Haddock, 2004), many attempts to re-introduce these microorganisms into soils to remove pollutants have been unsuccessful. This is probably due to the lack of knowledge regarding the behavior of these microbes in the environment. Factors, such as soil type, soil moisture, temperature, limitation in microbial reactions to environmental stress conditions (i.e. the toxicity of the contaminant and the scarcity of nutrients), predators, and the inability of inoculated microbes to compete with autochthonous microflora, have been reported to influence the performance of microbes during bioremediation (Goldstein et al., 1985; van Veen et al., 1997; Head, 1998).

Environmental factors have been investigated and they have been placed in two subgroups: (1) biotic factors and (2) abiotic factors. Biotic factors largely exert their effect by reducing the survival, activity and migration of degradative microorganisms, wherein the above alterations are a direct outcome of 'predation by protists', 'competition with autochthonous microorganisms', 'protozoan grazing' and 'other eukaryotic interactions' (Rentz et al., 2005). Among the above factors, the reduced survival of degradative microorganisms has been found to be most detrimental mechanism involved in non-optional performance of the *in situ* bioremediation process. Abiotic factors such as physiochemical nature of the contaminant environment nutrient availability, presence, chemical nature and concentration of contaminants, soil properties, etc. can influence considerably the bioremediation process (Pandey et al., 2009).

The bioavailability of contaminants can be influenced by soil properties, including soil texture, particle size, organic matter content, water content, pH, and structure, in addition to properties of the contaminant and environmental factors, such as temperature and moisture (Sijm et al., 2000). Clayey soils hold more water than sandy soils, and have more binding sites for ions, especially cations-cation exchange capacity (CEC) (Shang et al., 2004). The concentration of organic matter in soil is also positively correlated with CEC, as well as with capacity to bind hydrophobic organic pollutants. This is because humus mainly consists of dead plant materials, and plant cell walls have negatively charged groups that bind cations as well as lignin that binds hydrophobic compounds (Winnike-McMillan et al., 2005).

Interactions between the hydrocarbons and soil organic matter are believed to be responsible for the decline in degradation over time. These interactions include partitioning, adsorption, and absorption, chemisorption, diffusion, dissolution and covalent binding which

result in an aged or bound contaminant that is readily available for biodegradation (Parrish et al., 2005). Aging is defined as the movement of a chemical into soil micropores or into the soil organic matrix and the transformation and incorporation of pollutants into stable soil solid phases. This process limits the released of hydrocarbons into bulk liquid phase, making them inaccessible to microorganisms, thus decreasing biodegradation (Parrish et al., 2005). Aged soil contaminants are increasingly unavailable for uptake and biodegradation by soil microorganisms. However, the percentage of the total contamination that is bioavailable varies among different soil types (Alexander, 2000). Soils with high clay and/or organic matter content strongly bind lipophilic compounds, making them less available (White et al., 1997). Microbial bioavailability also driven by mass transfer of the contaminant from soil solid phase to solution, relative to the inherent activity of the target organisms (Cuypers et al., 2002). Plant and microorganisms have been shown to secrete enzymes and exudates that act as surfactants and may increase available contaminant concentration in soil (Makkar and Rockne, 2003).

1.3. Hydrocarbons

Hydrocarbons contain hydrogen and carbon, and can be found in the environment as gasses, tiny particles, or droplets. Hydrocarbons, primarily measured as total petroleum hydrocarbons, are the majority of organic compounds in most crude oils and contain hundreds of individual components. Most hydrocarbons in the environment are associated with the use of petrol, diesel, crude oil, and oil products in vehicles used for transportation. Hydrocarbons can be gases (e.g. methane and propane), liquids (e.g. hexane and benzene), waxes or low melting solids (e.g. paraffin wax and naphthalene), or polymers (e.g. polyethylene, polypropylene and polystyrene). There are three major categories of aromatic hydrocarbons of concern as contaminants of environmental soil. These are: (i) polycyclic aromatic hydrocarbons (PAHs), (ii) heterocyclic aromatic hydrocarbons, and (iii) alkyl PAHs (McGuinness and Dowling, 2009).

1.3.1. Hydrocarbon-degrading bacteria

Hydrocarbon-degrading bacteria have been found in habitats ranging from polar soils to marine environments (Whyte et al., 2002). While these degraders are generally found at much lower concentrations in pristine environments (Margesin et al., 2003), the ubiquitous presence of hydrocarbons themselves, or of aromatic and aliphatic analogs, has resulted in the maintenance of degradation potential within most populations. Johnsen and Karlsen (2005) estimated the PAH

degradation capacity of 13 soils, from pristine to heavily contaminated, and found that the capacity to degrade pyrene and phenanthrene was ubiquitous except in the most pristine forest soils, which exhibited low pyrene mineralization. Although many sites were far from pollution sources, PAHs were present in all soils, likely due to atmospheric deposition of pyrogenic PAHs.

The phylogenetic diversity of hydrocarbon degraders is vast, with degraders found in most, if not all, branches of the microbial family tree. Several recurrent groups are found in most phytoremediation studies. *Pseudomonas*, *Sphingomonas*, *Burkholderia*, *Arthrobacter*, *Flavobacterium*, *Rhodococcus*, *Ralstonia*, *Stenotrophomonas*, *Acinetobacter*, *Mycobacterium*, *Micrococcus, Alcaligenes*, and *Nocardioides* species comprise hydrocarbon-degradering strains (Liste and Prutz, 2006; Aislabie et al., 2008; Yousaf et al., 2010b). There is some evidence that strategist groups become more prevalent in contaminated soils (Whyte et al., 2002; Margesin et al., 2003). These studies found that β- and γ-Proteobacteria groups, including numerous *Pseudomonas* spp. and *Acinetobacter* spp., were increased in relative prevalence in contaminated soils compared to pristine soils, while groups such as Actinobacteria were maintained in both soil types. Other studies however, have found that while hydrocarbon contamination does shift microbial populations, community responses differ with soil type. In soils ranging from clay to loam, Actinobacteria, in particular *Rhodococcus* sp. increased in four of seven soil types in response to contamination (Hamamura et al., 2006). The dominance of a given degrader group may be a function of time. Kaplan and Kitts (2004) found that during early phases of hydrocarbon degradation in a land farming facility, when degradation rates were rapid, *Pseudomonas* and *Flavobacterium* dominated. After this initial period, slower growing *Alcaligenes*, *Microbacterium*, and *Bacteroides* became dominant.

1.3.2. Hydrocarbon metabolism

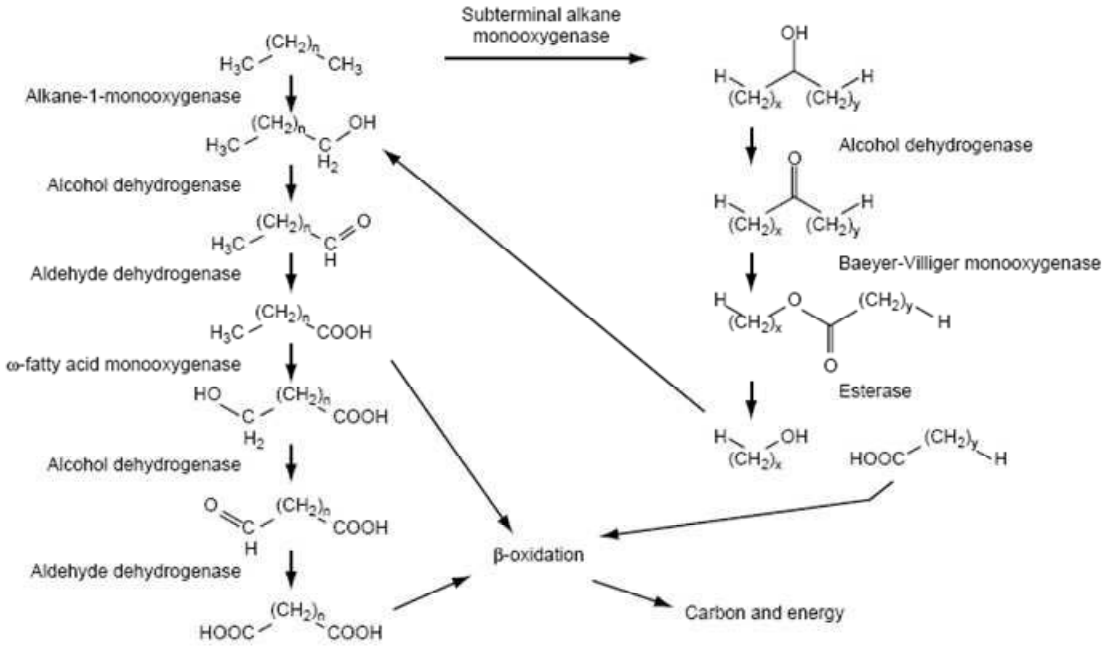
Crude oil and refined petroleum products contain complex mixtures of hydrocarbons, including aliphatic and aromatic hydrocarbons. Aliphatic hydrocarbons are linear, cyclic, or branched hydrocarbons which may be saturated (alkanes) or unsaturated (alkenes and alkynes). Aromatic hydrocarbons consist of one or more (polyaromatic) benzene rings. Soil bacterial communities degrade these hydrocarbons via numerous different catabolic pathways (van Beilen and Funhoff, 2007) which are subject to numerous regulatory mechanisms. In order to understand how plants may impact the degradation ability of soil microbial communities, these pathways of metabolism must be understood.

Aerobic metabolism of alkanes begins with the terminal or sub-terminal incorporation of oxygen into the hydrocarbon by a hydroxylase enzyme. The prevalence of naturally occurring alkanes, such as plant waxes, has led to the ubiquitous distribution of constitutive alkane hydroxylases. The best characterized system, common in *Pseudomonas* and *Rhodococcus* species, utilizes an integral membrane hydroxylase (encoded by *alkB*) to initiate metabolism of short to medium chain alkanes (C5 to C16). Once oxidized to a primary alcohol, subsequent oxidation steps by alcohol and aldehyde dehydrogenases convert the compounds to fatty acids that may then be further metabolized via β-oxidation and the citric acid cycle (Figure 1.4) (van Beilen and Funhoff, 2007). Recent studies have shown that some bacteria, including *Caulobacter* sp., *Acinetobacter* sp., and *Sphingomonas* sp., utilize an alternate or additional enzyme system, cytochrome P450 hydroxylase (van Beilen et al., 2006), to metabolize aliphatic hydrocarbons. Cytochrome P450 hydroxylases are inducible enzymes known to be up-regulated in the presence of alkanes (Sabirova et al., 2006). Although most alkane degraders contain several alkane hydroxylases which metabolize varying lengths of alkanes (van Beilen and Funhoff, 2007), *alkB* is commonly assessed to determine the degradation potential of microbial communities.

Aerobic degradation of polyaromatic hydrocarbons (PAH) is also initiated by the action of oxygenases, which introduce oxygen to the aromatic rings. PAH degradation has been best characterized in *Pseudomonas* species for phenanthrene and naphthalene (Habe and Omori, 2003; van Hamme et al., 2003; Ellis et al., 2006).

1.3.3. Hydrocarbon measurement

Among many analytical methods that have been used recently for assessment of the *in situ* biodegradation, the most effective ones are based on spectroscopic analyses for example UV-Vis, fluorescence and fourier transform infrared (FTIR) spectroscopy. Spectroscopic methods have also been used for analysis of chemical pollutants that were not identified previously (Weber et al., 2000). The major advantage of spectroscopic methods is their ability for rapid monitoring of the degradation process along with identification of degradation intermediates produced during the metabolic process. UV-Vis spectroscopy has also been integrated successfully with mathematical modeling for determination of the substrate utilization process, microbial activity products and biomass-associated products as indicators of the *in situ* load of different organic chemicals in a test sample (Carvallo et al., 2007).



Source: van Beilen *et al*., 2003

Figure 1.4. Metabolic pathway for degradation of alkanes by terminal and sub-terminal oxidation.

The specific ability of FTIR spectroscopy to distinguish even among very similar chemical structures was used along with the GC-MS studies of biotransformation of 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (DDE), a molecule that was earlier considered to be a dead-end product of dichloro-diphenyl trichloroethane (DDT) transformation. In this study, *Pseudomonas acidovorans* strain M3GY was identified for the ability to further transform DDE; however, the transformation products could only be identified using GC-MS-FTIR analysis (Hay and Focht, 1998). Such reports have clearly established the positive impact of different analytical methods on the bioremediation studies including *in situ* bioremediation studies/trials.

1.3.4. Impact of plants on microbial degradation of hydrocarbons

There are several ways in which plants may increase the degradative potential of rhizosphere microbial communities, including general increases in microbial population densities, specific increases in degrader population densities, increased catabolic gene expression, increased horizontal transfer of catabolic genes, and enhanced bioavailability of hydrophobic hydrocarbons. A primary factor impacting these degradation outcomes is plant exudation.

Some organic acids exuded by plant roots, including the citric acid cycle intermediates succinate, citrate and fumarate, are believed to primarily exert catabolite repression impacts on aromatic compound metabolism. This repression however, is neither a single nor a simple mechanism and the stage at which it occurs differs with organic acid, aromatic compound, and bacterial species (Tropel and van der Meer, 2004). For example, Kuiper et al. (2002) found that succinate actually promoted a higher level of naphthalene dioxygenase expression in *P. putida* PCL1444 than did other organic acids. However, in *Pseudomonas* spp. succinate decreases expression of another key enzyme, protocatechuate 3,4 dioxygenase, that catalyzes the metabolism of protocatechuate to β-carboxy-cis,cis-muconate (Collier et al., 1996). Fumarate has been found to have disparate impacts on genes involved in aromatic compound degradation. For example, in chloro-aromatic metabolic pathways fumarate competitively inhibits a key transcriptional activator, ClcR, in a concentration dependent manner (McFall et al., 1997). In aromatic pathways however, a related transcriptional activator for a catechol degradation operon, CatR, is not repressed by fumarate (McFall et al., 1997).

The organic acid acetate has also been shown to be a strong inhibitor of aromatic catabolic gene expression in bacterial isolates. Kamath et al. (2004) found that *nahG* expression in *P. putida* ATCC17484 was reduced approximately 40% relative to the control when the bacteria were exposed to naphthalene in the presence of 40 mg L-1 acetate. Similarly, acetate has been shown to be a strong repressor of *xyl* operon promoters on the *P. putida* TOL plasmid (Holtel et al., 1994; Collier et al., 1996). Degradation by other bacterial groups is also impacted, as Ampe et al. (1996) found that utilization of phenol and its metabolic intermediates by *Alcaligenes eutrophus* was inhibited by acetate. Catabolite repression also occurs during degradation of aliphatic hydrocarbons, with organic acids such as succinate repressing expression of the *alkB* operon (Dinamarca et al., 2003).

Several studies have shown that HGT of catabolic plasmids likely occurs in contaminated soil environments. Herrick et al. (1997) examined sequence homologies of *nahAc* genes (naphthalene dioxygenase) from numerous bacterial strains isolated from a coal tar contaminated site, and found evidence of that horizontal gene transfer had contributed to microbial adaptation to contamination. A later correlative study found similar evidence of HGT of the naphthalene dioxygenase gene *phnAc* between *Burkholderia* at a coal tar contaminated site (Wilson et al., 2003). Although no studies have directly related increased catabolic fitness in the rhizosphere to increased HGT, Jussila et al. (2007) found evidence for the transfer of the pWWO plasmid

(between *P. migulae* and *P. oryzihabitans* in the rhizosphere of the legume *Galega orientalis* (goat's rue)). Given the high proportion of catabolic genes found on plasmids, it seems likely that increases in HGT by specific plant species, perhaps stimulated by different amounts or proportions of exudates, contributes to the increased degradative fitness observed in many phytoremediation studies.

1.3.5. Fate of organic contaminant in the rhizosphere

Microorganisms act as catalysts for the mass transfer processes and in some cases act as individual bioreactors to degrade contaminants. An organic compound can be biodegraded by oxidation or reduction, by the removal of functional group, or by partial degradation of the core structure. Mineralization is a process where the original organic molecules are degraded to produce CO_2, microbial biomass, and inorganic compounds containing N, P, and S (Turco and Sadowsky, 1995). The following equation for heterotrophic microbial growth defines the primary nutritional requirements for aerobic biodegradation that occurs in the root zone of plant (Shimp et al., 1993). Under anaerobic conditions, intermediate materials such as fermentation product or CH_4 will be produced instead.

$$CH_mO_1 + aNH_3 + bO_2 \rightarrow y_cCH_pO_nN_q + zCH_rO_sN_t + cH_2O + dCO_2$$

Many organic contaminants are unable to these biological degradation processes in the aqueous phase. However, microbial degradation is usually substrate limited. It may be limited by nitrogen, phosphorous, or trace minerals. Under saturated conditions, biodegradation may be oxygen limited. Plants support microbial communities in the rhizosphere by providing microorganisms with nutrients via root exudation. Furthermore, nitrogen may be provided from decaying roots or from root nodules by nitrogen fixation. Oxygen may be supplied directly through the roots or by diffusion through root channels. Ultimately, plants can improve the rate of microbial degradation (Karthikeyan and Kulakow, 2003).

Bioremediation is the application, stimulation, or enhancement of microorganisms to decontaminate soils or ground water. Considerable research has focused on evaluating the aerobic and anaerobic biodegradation and transformation processes that play an important role in the ultimate fate of a contaminant in the subsurface (Mihelcic and Luthy, 1988; Zhang and Young, 1997). Furthermore, these contaminant reduction pathways can be stimulated under naturally occurring conditions and through actively engineered remediation processes, including phytoremediation. The possibility of microbial degradation of xenobiotics in and near the root

zone is promising. Many studies demonstrate the degradation of organic contaminants in the root zone (Aprill and Sims, 1990; Anderson et al., 1993; Karthikeyan and Kulakow, 2003).

1.4. Bioremediation monitoring techniques

The main difficulty associated with specific enriched bacterial inoculation is ensuring that the introduced degraders are effective and can survive the harsh environmental conditions of soil for long periods of time. The survival of the introduced highly efficient microbial degraders at the contaminated site is an essential attribute in the reduction of contamination levels by the introduced organisms (MacNaughton et al., 1999; Margesin et al., 2003). In the case of phytoremediation, efficient plant colonization by inoculated bacteria is likely to enhance their survival and hydrocarbon degradation (Andria et al., 2009; Afzal et al., 2010; Yousaf et al., 2010a). The enumeration and detection of inoculated strain in soil can not be correctly attained by conventional procedures such as dilution plating and microscopy of unmarked strains (Männistö et al., 2001; Park et al., 2004; Baek et al., 2009). To assist this research, genetically marked microorganisms may be used. The use of biomarkers as tag for monitoring the survival and efficacy of specific microbe in soil and plant shows great promise (Germaine et al., 2004; Kristensen et al., 2006). For example, *gusA* marker gene encoding the enzyme β-gulucuronidase (Wilson et al., 1995) is unique markers that can be detected in Gram-negative bacteria by non-destructive methods and have potential environmental application in soil and plant (Wilson et al., 1995; Sessitsch et al., 1997; Wang et al., 2004). Use of *gusA* marker gene is particular appropriate for studies on plant-microbe interactions, as GUS activity is not detected in plants or in many soil bacteria (Wilson et al., 1995). The specific strains tag with *gusA* genes, are providing a unique, visual phenotype; namely, blue colony, on X-glcA containing medium (Wilson et al., 1995). Analysis is extremely simple, fast, and permits a high data throughput (Sessitsch et al., 1997), and expensive equipments are not required for detection of individual *gusA* marked cells or colonies. It was reported that tagging of bacteria with *gusA* not disturbing their competitiveness and survival in soil and plant (Sessitsch et al., 1997; Wang et al., 2004). The labeling of strain with *gusA* gene cassette simplify the recognition of the microbes from soil and plant extract under controlled laboratory provisions by either plating on spectinomycin/streptomycin containing agar plates or PCR with designed primers (Wilson et al., 1995).

The limitations of culture-dependent methods have resulted in increased use of culture-independent methods to determine the ecological fate and activity of microorganisms under

natural environments (Salminen et al., 2008; Yergeau et al., 2009; Panicker et al., 2010; Yousaf et al., 2010a). Advances in molecular biology and gene technology have exciting possibilities for rapidly detecting and identifying specific microorganisms. One of the most commonly used methods for the detection of microorganisms in environmental samples is through PCR amplification of the bacterial small subunit of ribosomal RNA gene (16S rRNA) (Lane et al., 1985). For bioremediation studies, the 16S RNA gene from the added microorganism(s) can be PCR amplified and detected by gel electrophoresis. However, if organisms similar to inoculant are present at the target site, it may be necessary to analyze the 16S rRNA PCR product by additional techniques, such as terminal fragment length polymorphism (T-RFLP) analysis or to fully sequence the PCR product, in order to increase the detection specificity (Hendrickson et al., 2002). Recent studies have demonstrated the possibility of detecting very low concentration of microorganisms in contaminated environment using quantitative PCR. This development has also been fueled by the advent of molecular methodologies for isolation of the DNA directly from the environmental sample without the need for culturing and isolation of bacterial strains. Apart from the survival of the degrading strain, it is also extremely important that the microorganism(s) should remain active for carrying out the desired degradation reaction. This realization has resulted in an increased emphasis on the need for monitoring the degradation activity during *in situ* bioremediation (Young and Phelps, 2005). By assessing the abundance or the expression of key genes in environmental samples one can get a potential measure of the degradation activity. New advances in realtime PCR have improved the applicability of catabolic gene assessment during bioremediation (Siciliano et al., 2001; Whyte et al., 2002; Yergeau et al., 2009). Functional gene abundance can be by extraction of all DNA in e.g. a soil samples at different stages of a bioremediation action. The extracted DNA can then be exposed to a single gene PCR assay. The expression of functional genes in environmental samples can be asses by the reverse transcription of whole community mRNA, and this is of interest in bioremediation studies when assessing the actual activity of the gene on the site (Powell et al., 2006; Panicker et al., 2010).

Further improvement in the quantification of the DNA sample has been achieved using real-time monitoring of the PCR reaction using real-time (RT), quantitative PCR. The methodology is based on the multiple amplification cycles in which template DNA is initially denatured, followed by annealing of oligonucleotide primers targeting specific sequences, followed by subsequent extension of a complementary strand from each annealed primer by a thermostable DNA polymerase, resulting in an exponential increase in amplicon numbers during the PCR. The

increase in amplicon numbers is recorded in 'real-time' during the PCR via detection of a fluorescent reporter that indicates amplicon accumulation during every cycle. Two reporter systems are commonly used, namely, the intercalating SYBR green assay (Wittwer et al., 1997) and the *Taq*Man probe system (Livak et al., 1995). The working methodology of RT-PCR is well established and has been reviewed thoroughly (Kubista et al., 2006; Cupples, 2008). The selective advantage of this method is its ability to quantify the DNA amount in real time during the amplification process. Unlike other quantitative PCR methods that measure the end-point amplicon quantities (which may be affected by quite a few PCR parameters other than the template concentration), RT-PCR tracks the comparative release of fluorophore units as an indicator of the number of template DNA molecules (Valasek and Repa, 2005). Several recent bioremediation studies have used RT-PCR as a conclusive method for determination of *in situ* survival of degradative strain(s) (van Raemdonck et al., 2006; Baek et al., 2009; Baldwin et al., 2010).

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CHAPTER 2

The inoculation method affects colonization and performance of bacterial inoculant strains in the phytoremediation of soil contaminated with diesel oil

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Running Head: Effects on inoculation method on phytoremediation

Keywords: bacterial inoculants, seed imbibement, soil inoculation, alkane degradation, ACC deaminase, *gusA*

***Submitted to International Journal of Phytoremediation in August 2010**

Abstract

Plants in combination with their associated microflora can remediate soils, which are contaminated with organic pollutants such as petroleum hydrocarbons. Inoculation of plants with degrading bacteria is one approach to improve remediation processes, but is often not successful due to the competition with the resident microflora. It is therefore of high importance to address the persistence and colonization behavior of inoculant strains. The objective of this study was to determine whether the inoculation method (seed imbibement and soil inoculation) influences bacterial colonization, plant growth promotion and hydrocarbon degradation. Italian ryegrass was grown in non-sterilized soil polluted with diesel and inoculated with different alkane-degrading strains *Pantoea* sp. ITSI10, *Pantoea* sp. BTRH79 and *Pseudomonas* sp. MixRI75 individually as well as in combination. Strains were labeled with the *gusA* gene to follow their colonization behavior. Two strains (ITSI10 and BTRH79) showed 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. Inoculation generally had a beneficial effect on plant biomass production and hydrocarbon degradation, however, strains inoculated in soil performed much better than applied by seed imbibement. Performance correlated with the colonization efficiency of the inoculated strains. The highest hydrocarbon degradation (79%) was observed in the treatment, in which all three strains were inoculated in combination into soil. Strains with ACC-deaminase activity performed better than the strain without ACC-deaminase activity. Our study revealed that besides the degradation potential and competitive ability of inoculant strains the inoculation method plays an important role in determining the success of microbial inoculation.

Introduction

Diesel oil is a pollutant that frequently is reported as soil contaminant (Wang et al., 2005; Ciric et al., 2009). Above a certain level diesel pollution can be toxic to plants and microorganisms (Tesar et al., 2002; Adam and Duncan, 2003; Lapinskienė et al., 2006). Phytoremediation, i.e. the use of plants and associated microorganisms, provides an ecologically and economically attractive technique for remediating soils contaminated with hydrocarbons (Pilon-Smits, 2005; Dzantor, 2007; Olson et al., 2008). One of the major limitations of phytroremediation is that many plant species are sensitive to contaminants (Huang et al., 2001; Chaudhry et al., 2005) and even plants that can tolerate the pollutant usually produce less biomass and cannot effectively support degradation (Glick, 2003; Peng et al., 2009; Cébron et al., 2009). To overcome this problem, plant growth-promoting bacteria (Johnson et al., 2004; Huang et al., 2005) and bacteria showing the capacity to degrade contaminants may be applied to protect the plants from toxic effects due to the presence of the pollutant (Weyens et al., 2009a; McGuinness and Dowling, 2009; Gerhardt et al., 2009). However, several studies have shown that the application of specific microbes often fails in efficient degradation of contaminants, probably due to poor survival in the rhizosphere (Rentz et al., 2005; Gilbertson et al., 2007; Gunderson et al., 2007).

Successful application of selected microorganisms in a viable way is the first step towards improving phytoremediation of a contaminated soil. It is equally important that the microorganisms remain viable and can colonize roots and rhizosphere in order to continue improving plant growth and potentially degrade soil contaminants (Ramos et al., 2010). An important question is how plants in the field can be inoculated more efficiently for phytoremediation (Weyens et al., 2009a). Seeds or cuttings may be inoculated by spraying the inoculum onto the soil or directly onto growing plants. Traditionally, seed imbibement and soil inoculation are frequently used for delivery, transport and distribution of bacteria (Huang et al., 2004; Villacieros et al., 2005; Germaine et al., 2009; Yousaf et al., 2010a), however, the efficiency of these procedures in terms of colonization has been rarely compared.

Recently, it has been suggested that certain considerations may facilitate the phytoremediation of organic environmental contaminants (Glick, 2010). These include the use of: (1) bacteria that are either engineered or selected to be able to both promote plant growth and degrade soil contaminants are generally superior to bacteria that can only promote plant growth or degrade soil contaminants; (2) plant growth-promoting bacteria with 1-aminocyclopropane-1-caroxylic acid (ACC) deaminase activity, which improves plant stress tolerance and could be

therefore a key determinant in facilitating phytoremediation; and (3) the use of endophytic bacteria, which are likely to be more effective in plant colonization than rhizosphere bacteria (Doty, 2008; Weyens et al., 2009b; Weyens et al., 2009c). Although several microorganisms can completely degrade a specific organic contaminant, individual species generally do not contain entire degradation pathways (Ueno et al., 2006; Ueno et al., 2007; Gerhardt et al., 2009). Therefore, microbial consortia or strain combinations have been often found to be more effective in pollutant degradation than a single strain (Yateem et al., 2007; Alarcón et al., 2008).

In this study we used bacteria, which were previously isolated from plants and which proved to degrade hydrocarbons efficiently. As host plant we chose Italian ryegrass, which is able to tolerate diesel pollution, and strains were either applied individually or in combination. The aim was to assess the effect of the inoculation method on plant growth, diesel degradation and bacterial survival and colonization in the rhizosphere and plant interior. Furthermore, we compared the effects of bacteria showing ACC deaminase as well as of a degrading strain lacking ACC deaminase activity.

Materials and methods

Bacterial strains

Three bacterial strains, which were previously isolated from the rhizosphere and endosphere of Italian ryegrass (*Lolium multiflorum* var.Taurus) and birdsfoot trefoil (*Lotus corniculatus* var. Leo) (Yousaf et al., 2010b), were used in this study. These included two *Pantoea* sp. strains, ITSI10 (an Italian ryegrass endophyte; carrying an unknown gene responsible for alkane degradation) and BTRH79 (a rhizosphere strain from birdsfoot trefoil carrying a cytochrome P450 alkane hydroxylase), and one *Pseudomonas* sp. strain, MixRI75 (a root endophyte found in Italian ryegrass as well as birdsfoot trefoil; carrying an alkane monooxygenase (*alkB*) gene). Both *Pantoea* sp. strains showed ACC deaminase activity.

Tagging of bacterial strains with the *gusA* gene

To facilitate monitoring of the colonization process all strains were labelled with the *gusA*10 gene as described by Wilson et al. (1995).

ACC deaminase activity

ACC deaminase activity of *gusA*10-marked and parental strains were tested on the minimal medium containing 0.7 g ACC L^{-1} as sole nitrogen source, as described by Kuffner et al. (2008).

Inoculation of seeds by imbibement

Strains of ITSI10::*gusA*10, MixRI75::*gusA*10 and BTRH79::*gusA*10 were cultivated in LB broth amended with 1% (v/v) filter-sterilized diesel at 30ºC. Cells were harvested by centrifugation, washed and re-suspended in sterile 0.9% NaCl solution. Seeds of Italian ryegrass (IT) were surface-sterilized by soaking them in 5% sodium hypochlorite solution for 2 min, then in 70% ethanol for 2 min, and then seeds were thoroughly rinsed with sterilized distilled water. Suspensions containing 10^{11} bacterial cells mL^{-1} in 0.9% NaCl solution were used for seed imbibement (12 hours at room temperature), which was performed as described previously (Wang et al., 2004). Ten seeds of each treatment were analysed to assess the survival of the inoculated bacteria. Seeds were shaken in 0.9% NaCl solution, and serial dilutions were plated on M9 medium (Alef, 1994) containing succinate, acetate and citrate (SAC), each at a concentration of 2 g L^{-1}. Seeds contained about 10^{+9} cfu per seed. For the treatment using the combination of strains ITSI10::*gusA*10, MixRI75::*gusA*10 and BTRH79::*gusA*10, cells of each strain were mixed in equal proportions and it was confirmed that the final cell concentration was 10^{11} cells mL^{-1}. For control and soil inoculation treatments, sterilized seeds were soaked in 0.9% NaCl solution.

Plant experiment

A greenhouse experiment was performed to compare the efficiencies of different inoculation procedures and strains. Italian ryegrass (IT) (*Lolium multiflorum* var. Taurus) was shown to tolerate diesel contamination in previous experiments and was therefore chosen as host plant. For the experiment an agricultural soil (agricultural top soil Seibersdorf, Lower Austria, Austria; pH 7.4, 27 g sand kg^{-1}, 621 g silt kg^{-1}, 352 g clay kg^{-1}, 2.4% C_{org}) was used. The soil was air-dried and passed through a 2-mm stainless steel mesh, and amended with 30% sand and 10% compost. Then it was spiked with filter-sterilized commercially available diesel fuel (7.5 g kg^{-1} dry soil). Commercially available diesel fuel is composed of ~64% saturated aliphatic hydrocarbons (alkanes), ~1 to 2% unsaturated aliphatic hydrocarbons, and ~35% aromatic hydrocarbons (including polycyclic aromatic hydrocarbons) (Risher and Rhodes, 1995). Pots were filled with

spiked soils (1.5 kg pot^{-1}) and subsequently placed in the greenhouse. The treatments included a diesel-contaminated soil (control), diesel-contaminated soil with IT (vegetated control), diesel-contaminated soil with IT and bacterial inoculation by seed imbibement, and diesel- contaminated soil with IT and soil inoculation. Inoculants contained strains ITSI10::*gusA*10, MixRI75::*gusA*10 and BTRH79::*gusA*10 individually or in combination. Three replicates of each treatment were used and pots were arranged in a completely randomized block design. Hundred inoculated and non-inoculated seeds were planted in each pot.

For soil inoculation, 100 mL of a bacterial suspension (washed with 0.9% NaCl solution) containing 10^9 cells mL^{-1} was poured onto the soil surface immediately after seed sowing. For the seed inoculation treatment, seeds inoculated by seed imbibement (see above) were planted. Pots were watered with tap water from below when needed. Temperature and light were allowed to fluctuate with ambient conditions (26 June to 26 September 2009, Seibersdorf, Austria) and the average day/night temperatures were 22ºC /15ºC. One week after seed germination, seedlings were counted and poor growing removed to 75 per pot.

Plants were harvested 93 days after sowing. Shoots were cut 2 cm above ground and plant biomass was determined. After the plants were removed from the pots and roots separated from bulk soil, the soil from each pot was thoroughly mixed to obtain homogenized samples for hydrocarbon extraction. These soil samples were then stored at -80°C until further analysis.

Detection and enumeration of inoculated bacteria

Rhizosphere soil was obtained by agitating roots and sampling the soil still attached to the roots. Subsequently, shoots, roots and seeds were carefully washed, surface-sterilized with 70% ethanol (3 min), treated with 1% NaOHCl (5 min), followed by washing 3x with sterile distilled water (1 min each time). The efficacy of surface sterilization was checked by plating shoot, root and seeds, and aliquots of the final rinse onto LB plates, and no colonies were observed after 3 days of incubation, ensuring the surface sterilization efficiency.

For the isolation of rhizosphere bacteria, a soil slurry was prepared by mixing 3 g rhizosphere soil with 9 mL of 0.9% (w/v) NaCl solution and agitation (180 rpm) for 1 hour at 30°C. After the settlement of soil particles, serial dilutions up to 10^{-3} were plated onto selective M9 medium containing SAC, 5-bromo-4-chloro-3-indolyl-*β*-D-glucuronide (XGlcA) (100 µg mL^{-1}), isopropyl-*β*-D-galactopyranoside (IPTG) (100 µg mL^{-1}) and spectinomycin (100 µg mL^{-1}). Cycloheximide (100 mg L^{-1}) was added to prevent fungal growth. For the isolation of endophytes, 1 g of surface-sterilized

shoots and roots were homogenized in 6 mL 0.9% NaCl solution by using a mortar and pestle. Similarly, 0.2 g seeds of each treatment were homogenized in 2 mL 0.9% NaCl solution. The homogenized material was shaken for 1 hour at 30°C. After settling the solid material, serial dilutions up to 10^{-3} were spread on selective M9 medium. The plates were incubated at 30°C for 48 hours and then transferred to 4°C for three days. Blue colonies were counted on each plate. Thirty blue colonies of each treatment were randomly picked and the identity of isolates with the inoculant strain was confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA intergenic spacer region (IGS) (Andria et al., 2009). Isolates and inoculant strains had identical restriction patterns.

Cultivation-independent analysis

Cultivation-independent analysis was performed to confirm the presence of inoculated strains in soil and plant interior. For this, DNA from rhizosphere soil and sterilized plant material was extracted and terminal restriction fragment length polymorphism (T-RFLP) analysis was performed as described earlier (Yousaf et al., 2010a).

Hydrocarbon analysis of soil samples

Total hydrocarbon content (THC) of the soil was measured employing infrared spectroscopy as described previously (Yousaf et al., 2010a).

Statistical analysis

Data analyses for plant biomass, total hydrocarbon content (THC) in soil, and bacterial densities were done by using SPSS software package. Comparisons between treatments were carried out by one-way analysis of variance (ANOVA). Duncan's test was applied for ANOVA after testing homogeneity of variance.

Results

Plant biomass

Growth parameters (seed germination, root, shoot and seed dry weight) were determined to evaluate the effect of both, the inoculation and the method of inoculation, on plant

development (Table 2.1). Seed germination as well as seed production were not influenced by bacterial inoculation. Biomass (shoot and root) production was significantly enhanced due to inoculation. Soil inoculation resulted in higher biomass production than seed imbibement. Highest biomass production was achieved with the strain combination followed by strains BTRH79::*gusA*10, ITSI10::*gusA*10 and MixRI75::*gusA*10. Strains with ACC deaminase activity (BTRH79::*gusA*10, ITSI10::*gusA*10) induced higher shoot and particularly root production than the strain lacking this activity.

Table 2.1. Effect of bacterial inoculation method on shoot and root fresh weight (FW) and dry weight (DW) of Italian ryegrass (*Lolium multiflorum* var.Taurus) harvested 93 days after seed germination

Treatment	Shoot		Root	
	FW (g)	DW (g)	FW (g)	DW (g)
Control (uninoculated)	23.7^{e} (1.23)	6.4^{d} (0.43)	5.7^{h} (0.43)	1.9^{g} (0.28)
Seed imbibement				
Pantoea sp. ITSI10::*gusA*10	30.2^{cd} (1.35)	8.4^{c} (0.40)	8.0^{f} (0.54)	2.6^{ef} (0.30)
Pseudomonas sp. MixRI75::*gusA*10	27.7^{d} (1.12)	7.3^{d} (0.69)	6.7^{g} (0.52)	2.1^{fg} (0.35)
Pantoea sp. BTRH79::*gusA*10	30.6^{cd} (1.48)	8.5^{c} (0.49)	8.6^{e} (0.75)	2.7^{de} (0.41)
Strain combination	33.5^{bc} (1.54)	9.3^{abc} (0.60)	9.9^{d} (0.71)	3.2^{cd} (0.37)
Soil inoculation				
Pantoea sp. ITSI10::*gusA*10	33.1^{bc} (1.34)	9.2^{bc} (0.57)	11.2^{c} (0.57)	3.7^{bc} (0.25)
Pseudomonas sp. MixRI75::*gusA*10	29.4^{d} (1.58)	8.4^{c} (0.54)	8.3^{ef} (0.63)	2.6^{ef} (0.32)
Pantoea sp. BTRH79::*gusA*10	34.9^{b} (1.47)	9.7^{ab} (0.40)	12.1^{b} (0.59)	3.9^{b} (0.22)
Strain combination	38.7^{a} (1.85)	10.2^{a} (0.55)	13.4^{a} (0.73)	4.6^{a} (0.37)

Means in the same column followed by the same letter are not significantly different at a 5% level of significance, n = 3; the standard error of three replicate is presented in parentheses.

Persistence of inoculated strains

Isolation and enumeration of the three inoculated strains applied by both inoculation methods showed that they were able to efficiently colonize in the rhizosphere and plant interior (Table 2.2). Generally, soil inoculation led to better colonization in the rhizosphere as well as in the plant interior than inoculation by seed imbibement. In the rhizosphere strain BTRH79::*gusA*10, originally isolated from the rhizosphere of Birdsfoot trefoil, colonized better than the endophyte strains, whereas in the root interior strain MixRI75::*gusA*10, a root endophyte, performed best. The shoot endophyte, strain ITSI10::*gusA*10 colonized the shoot

interior better than other strains applied individually. Generally, more bacterial cells were found when strains were applied in combination. By soil inoculation strain ITSI10::*gusA*10 was dominant in the rhizosphere (57%) and root interior (52%), while BTRH79*::gusA*10 was more abundant in the shoot interior (62%). Strain MixRI75::*gusA*10 showed lowest abundance in all three compartments (11-23%). When strains were applied in combination by seed imbibement only BTRH79::*gusA*10 was able to colonize.

Table 2.2. Effect of bacterial inoculation method on bacterial persistence in rhizosphere (RH), root interior (RI), shoot interior (SI) and in seed interior (SeI) of Italian ryegrass (*Lolium multiflorum* var.Taurus)

Treatment	RH (cfu g^{-1} soil) * 10^4	SI (cfu g^{-1} shoot) * 10^4	RI (cfu g^{-1} root) * 10^4	SeI (cfu g^{-1} seed) * 10^4
Control (uninoculated)	0	0	0	0
Seed imbibement				
Pantoea sp. ITSI10::*gusA*10	4.2^{c} (2.3)	13^{d} (0.6)	4.4^{f} (1.0)	0
Pseudomonas sp. MixRI75::*gusA*10	0.6^{d} (0.4)	0.6^{g} (0.2)	10^{e} (2.2)	0
Pantoea sp. BTRH79::*gusA*10	4.5^{c} (2.4)	1.1^{f} (0.3)	3.2^{f} (1.8)	0.2
Strain combination	41^{b} (2.8)	28^{c} (0.7)	11^{e} (2.4)	0
Soil inoculation				
Pantoea sp. ITSI10::*gusA*10	51^{b} (2.1)	300^{b} (0.5)	16^{d} (3.5)	0
Pseudomonas sp. MixRI75::*gusA*10	4.2^{c} (0.6)	1.2^{f} (0.3)	36^{b} (3.1)	0
Pantoea sp. BTRH79::*gusA*10	76^{a} (3.7)	3.1^{e} (0.7)	32^{bc} (2.4)	0
Strain combination	72^{a} (2.8)	570^{a} (0.5)	400^{a} (1.2)	0

Means in the same column followed by the same letter are not significantly different at a 5% level of significance, n = 3, the standard error of three replicate is presented in parentheses.

Hydrocarbon degradation

In vegetated soil hydrocarbon degradation was more efficient than in non-vegetated soil, however, inoculation further significantly improved hydrocarbon degradation (Table 2.3). Generally, plants inoculated by soil inoculation showed significantly lower hydrocarbon concentrations than those inoculated by seed imbibement. The maximum reduction (5.92 g kg^{-1} dry soil) was observed with plants inoculated with the combination of the three inoculant strains by soil inoculation. By both inoculation methods, higher hydrocarbon removal was observed with

ACC-deaminase - producing bacteria ITSI10::*gusA*10 and BTRH79::*gusA*10 than by bacteria lacking this activity (MixRI75::*gusA*10).

Table 2.3. Effect of bacterial inoculation method on hydrocarbon degradation in diesel-contaminated soil vegetated with Italian ryegrass (*Lolium multiflorum* var.Taurus)

Treatment	Hydrocarbon concentration in soil (g kg^{-1} soil)	
	0 day	After 90 days
Control (unvegetated)	7.50 (0.62)	6.20^{h} (0.50)
Control (uninoculated)	7.50 (0.62)	5.27^{g} (0.25)
Seed imbibement		
Pantoea sp. ITSI10::*gusA*10	7.50 (0.62)	3.82^{ef} (0.31)
Pseudomonas sp. MixRI75::*gusA*10	7.50 (0.62)	4.20^{f} (0.38)
Pantoea sp. BTRH79::*gusA*10	7.50 (0.62)	3.72^{ef} (0.32)
Strain combination	7.50 (0.62)	2.95^{cd} (0.41)
Soil inoculation		
Pantoea sp. ITSI10::*gusA*10	7.50 (0.62)	2.31^{bc} (0.28)
Pseudomonas sp. MixRI75::*gusA*10	7.50 (0.62)	3.52^{de} (0.37)
Pantoea sp. BTRH79::*gusA*10	7.50 (0.62)	2.10^{ab} (0.46)
Strain combination	7.50 (0.62)	1.58^{a} (0.33)

Means in the same column followed by the same letter are not significantly different at a 5% level of significance, n = 3; the standard error of three replicate is presented in parentheses.

Cultivation-independent analysis

The persistence of ITSI10, BTRH79 and MixRI75 in the rhizosphere and endosphere was confirmed by using T-RFLP analysis of the 16S rRNA, *cyp153* and *alkB* genes, respectively. This analysis showed the presence of all inoculant strains in the rhizosphere and endosphere of IT. Bacteria containing the *cyp153* gene were also found in the rhizosphere and endosphere of uninoculated plants, but different subtypes of alkane-degrading genes were encountered. Indigenous bacteria containing the *alkB* gene were not detected in the rhizosphere and endosphere of uninoculated plants.

Discussion

The application of degrading microorganisms in phytoremediation applications is a promising approach to accelerate the clean-up of polluted soils. Degrading microorganisms may

enhance a plant's adaptation to contaminants such as petroleum hydrocarbons by detoxifying contaminated soils through direct mineralization of these organic contaminants (Escalante-Espinosa et al., 2005; Alarcón et al., 2008) leading also to better plant growth. Additionally, inoculant strains or the native plant-associated microflora may show plant growth-promoting activities, e.g. through ACC deaminase activity, further promoting plant growth. Enhanced plant growth may in turn stimulate the associated microflora and degradation processes. In this study, plant biomass was enhanced by 12% to 44% by inoculation as compared to non-inoculated plants. Increased plant biomass production by bacterial inoculation would effectively lower the ratio of phytotoxic contaminants to the amount of plant tissue, lowering plant stress (Alkorta and Garbisu, 2001; Gurska et al., 2009). This particular tendency suggests that inoculation of hydrocarbon degrading-microorganisms decreased the potential toxic effect due to the bioavailable spiked hydrocarbons. The two strains ITSI10 and BTRH79 exhibiting alkane degradation as well as ACC-deaminase activities were highly efficient in enhancing plant biomass (especially root biomass) and consequently hydrocarbon degradation and performed better than strain MixRI75 lacking ACC deaminase activity. The bacterial enzyme ACC deaminase can reduce ethylene levels produced by plants under stress and therefore alleviates stress symptoms leading to better plant growth (Glick, 2003).

The maximum hydrocarbon reduction (79%) was achieved with inoculated plants, which was far higher than that obtained with non-inoculated plants. This increased degradation potential was probably the result of higher microbial densities and metabolic activities of the inoculant strains in the plant environment. In an earlier study, where Italian ryegrass was grown for 152 days in a diesel-spiked soil (1.8%), hydrocarbons contents were decreased to 58% of the initial value (Kaimi et al., 2006). In our study, highest dissipation (79%) of hydrocarbons was seen after 93 days. Best degradation was achieved with the combination of all three strains as well as the two ACC-deaminase producing *Pantoea* strains. Degradation correlated with efficient colonization of the rhizosphere and root biomass production providing support for colonizing microorganisms.

For the application of microbial inoculants different practices are used. Seed imbibement or coating and soil inoculation represent commonly applied practises, however, the effects on plant growth, bacterial colonization or hydrocarbon degradation have been rarely investigated. In this study, bacteria applied by soil inoculation significantly enhanced plant biomass, hydrocarbon degradation and colonization to a higher degree than bacteria applied by seed imbibement. Already in earlier studies, soil inoculation similarly resulted in better crop yields (Schiffmann and

Alper, 1968; Habish and Ishag, 1974) and more efficient colonization (Höfte et al., 1990) than direct inoculation of seeds. In contrast, there are several studies showing that seed inoculation is better suited than other inoculation methods (Müller et al., 2009; Müller and Berg, 2008). Seed imbibement is a well established method especially for vegetables and is applied for obtaining a fast and uniform germination of seeds (Gray, 1994). In addition, seed inoculation was used to enhance the phytoremediation of naphthalene (Germaine et al., 2009) and total petroleum hydrocarbons (Gurska et al., 2009). Seed imbibement is practicable and implementable into commercial seed production. However, various optimization steps are required to obtain optimal efficiencies (Müller and Berg, 2008). One reason for the observed differences between soil and seed inoculation may be that Italian ryegrass seeds released exudates before germination in soil, which favoured the growth of other seed-residing microorganisms and out-competed the inoculated bacteria on the seed coat (Sturz and Christie, 1996). Furthermore, cells may have been stressed or injured during seed plantation and germination. Seed exudates can consist of many different molecules including sugars, amino acids, organic acids, and phenolic compounds among others, but little is known about the specific response of microorganisms to these (Nelson, 2004). Additionally, various sulphur-containing compounds are produced by seeds and some of these have been found to have anti-microbial effects (Lanzotti, 2006), which might reduce the number of inoculated cells. The fact that only strain BTRH79 was able to survive on seeds when the three inoculant strains were applied in combination by seed imbibement, whereas all three strains survived when applied by soil inoculation, further indicates that seeds provide a specific micro-environment to which inoculant strains need to be adapted.

In the present study, significantly more plant biomass and hydrocarbon degradation were recorded for plants inoculated with consortium of three bacteria. A broader range of environmental conditions may be tolerated by inoculated microorganisms applied as a consortium and due to multiple modes of action a mixture of microorganisms may more effectively target pollutants and improve the plant growth (Haung et al., 2004; Chaudhry et al., 2005; Yateem et al., 2007). Escalante-Espinosa et al., (2005) applied a combination of ten bacterial and three fungal strains, which degraded 2.83 g kg^{-1} dry soil total petroleum hydrocarbons extracted from weather contaminated soil during 60 days. Similarly, in another study, degradation of medium crude oil hydrocarbons was highest (59%) after 80 days in a treatment, in which a mixture of microorganisms was inoculated to Italian ryegrass (Alarcón et al., 2008). However, although strain

combinations have proven in many cases to be successful, also incompatibility among inoculated strains may occur leading to antagonism in the plant environment (Lutz et al., 2004).

All three *gusA*10 marked inoculated strains were successfully recovered from the rhizosphere (10^3 to 10^5 cfu g^{-1} dry soil) and plant interior (10^3 to 10^6 cfu g^{-1} dry plant) 93 days after inoculation. Bacterial survival and colonization are necessary for efficient degradation of hydrocarbons. Rhizosphere bacteria directly encounter the soil contaminant, however, face high competition with other microorganisms. Endophytic bacteria can proliferate within plant tissues and therefore face less competition for nutrients and are better protected from adverse changes in the environment than rhizosphere bacteria (Beattie, 2006). The fact that all the three strains efficiently colonized root and shoot interior further supports the theory that endophytic bacteria have to be rhizosphere-competent (Compant et al., 2010) and then may enter roots and translocate to various internal plant tissues. Among the three inoculant strains, BTRH79::*gusA*10 was the only strain, which was detected within seeds (10^3 cfu g^{-1} dry weight). A few studies reported that some endophytic bacteria colonize flowers, fruits and seeds (Compant et al., 2010). It was observed that strain ITSI10 (a shoot endophyte), MixRI75 (a root endophyte) and BTRH79 (a rhizosphere strain) showed better colonization in shoot, root and rhizosphere, respectively, when inoculated on soil as a single strain. These observations are in agreement with Rosenblueth and Martínez-Romero (2006) and Andria et al. (2009), who concluded that endophytes are generally better able to colonize the plant tissues than rhizosphere isolates. However, when these bacteria were applied in combination, ITSI10 dominated the rhizosphere (57%) and root interior (52%), and BTRH79 the shoot interior (62%). Mounting evidence indicates that plants are able to select the bacteria living in their rhizosphere by different mechanisms, including the exudation of specific compounds, root architecture, or the modification of soil conditions (Hartmann et al., 2009).

Cultivation-independent analysis confirmed the presence and survival of the inoculated strains in the rhizosphere and plant environment. Alkane-degrading genes were observed in inoculated as well as uninoculated soil despite the fact that an agricultural soil was used, which was spiked with diesel, and not a soil which has been contaminated for a long time. Other studies also reported that alkane-degrading bacteria are present in uncontaminated soil (Kloos et al., 2006; Yousaf et al., 2010a).

In conclusion, this study demonstrates the potential of applying strain combinations to improve degradation processes, although the mechanisms of enhanced effects are poorly understood. Furthermore, our results showed that the inoculation procedure may influence

bacterial colonization and consequently degradation efficiency. Inoculation procedures have to be cheap and easy to apply, however, it is rarely considered that they may have an impact on bacterial establishment in the plant environment. As bacterial colonization is a key requirement for efficient remediation, it is recommended to select inoculation procedures, which warrant highly efficient bacterial colonization.

Acknowledgements

M. Afzal would like to thank NIBGE and Higher Education Commission, Govt. of Pakistan, for funding. We are thankful to A. Grahsl, who assisted in performing the greenhouse experiment.

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CHAPTER 3

Soil type affects plant colonization, activity and catabolic gene expression of inoculated bacterial strains during phytoremediation of diesel

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Keywords: soil type, *Pseudomonas, Pantoea, alkB*, CYP153, gene abundance, gene expression

***Submitted to Journal of Hazardous Materials in November 2010**

Abstract

The combined use of plants and associated microorganisms has great potential for cleaning up soils contaminated with petroleum hydrocarbons (PHC). This so called rhizodegradation can in principle be enhanced by inoculation with microorganisms that show a high ability of PHC-degradation. Apart from environmental conditions the physicochemical properties of the soil are the main factors influencing the survival and activity of an inoculated strain as well as the growth of plants. This study examined the effect of different soil types (sandy, loamy sand and loam) on the survival, gene abundance and catabolic gene expression of two inoculated strains (*Pseudomonas* sp. strain ITRI53 and *Pantoea* sp. strain BTRH79) in the rhizosphere and shoot interior of Italian ryegrass vegetated in diesel contaminated soils. Culture-dependent and culture-independent analysis showed high colonization, gene abundance and expression in loamy soils after eight weeks of inoculation. By contrast, low colonization, gene abundance and absence of gene expression in sandy soil were found. Soil type had a strong effect on the expression of genes (*alkB* and CYP153) specific to hydrocarbon degradation. The highest levels of genes expression and hydrocarbon degradation were seen in loamy soil that had been inoculated with BTRH79, and were significantly higher compared to those in other soils. A positive correlation (r = 0.73) was observed between gene expression and hydrocarbon degradation indicating that catabolic gene expression is necessary for contaminant degradation. These results suggest that soil type influences the bacterial colonization and microbial activities and subsequently the efficiency of contaminant degradation.

Introduction

Petroleum hydrocarbons are frequently occurring pollutants and an increasing number of sites is seriously polluted by these contaminants world-wide (Margesin et al., 2003; Heiss-Blanquet). Phytoremediation is a promising technology for the removal of pollutants from contaminated environments. It employs plants and their associated microorganisms to degrade, transform, assimilate, metabolize, or detoxify hazardous pollutants from soils (Reichenauer and Germida, 2008; Agamuthu et al., 2010; Cai et al., 2010). Although the use of plants alone in bioremediation was successful in some cases (Olson et al., 2008; Peng et al., 2009; Gerhardt et al., 2009), the combined use of plants and biodegradative and/or plant growth-promoting bacteria is particularly promising for the decontamination of polluted soils (Alarcón et al., 2008; Al-Awadhi et al., 2009; Glick).

The degradation of toxic organic compounds in soil by plant-associated bacteria can be mediated by endophytic and/or rhizosphere bacteria. Endophytic bacteria colonize in the internal tissues of plants and do not confer pathogenic effects to their host (Compant et al., 2010). They have been reported to have a high potential to promote plant growth and contribute to enhanced biodegradation of pollutants (Sessitsch et al., 2002; Germaine et al., 2009; Weyens et al., 2009). Rhizosphere bacteria colonize the root environment, where root exudates act as substrate for microorganisms supporting also the degradation of organic contaminants (Alkorta, and Garbisu, 2001; Palmroth et al., 2002; Glick, 2003). Many plant-associated bacteria have the ability to deaminate 1-amino-cyclopropane-1-carboxylic acid (ACC), a precursor of ethylene in plants, which is particularly produced under stress conditions. Bacteria exhibiting ACC deaminase activity have the potential to promote plant growth and alleviate plant stress such as that induced by the presence of toxic contaminants (Glick, 2003).

For efficient phytoremediation of organic soil contaminants, the soil has to provide the appropriate environment for both, the plant and the associated microorganisms. Soil properties including texture, organic matter content, pH, particle size, cation exchange capacity and structure have been shown to affect not only plant growth and microbial colonization (Labana et al., 2005; Kaimi et al., 2007; Sun et al., 2010), but also the degradation of organic contaminants (Kaakinen et al., 2007; Jung et al., 2008; Haritash, and Kaushik, 2009). Moreover, the bioavailability of hydrocarbons may be influenced by soil properties (Sijm et al., 2000; Chung and Alexander, 2002; Parrish et al., 2005). Hydrocarbons strongly bind to humic substances and clay minerals (Richnow et al., 1995). However, it has been also shown that sandy soils may bind hydrocarbons by

adsorption despite the absence of silty material or significant amounts of organic matters. This was explained by high microporosity (Löser et al., 1999).

In bioremediation studies, the quantitative analysis of functional genes such as alkane hydroxylase genes has provided a valuable tool for studying the relationship between specific microbial populations or strains and the performance of degradation processes (Piskonen et al., 2005; Panicker et al., 2010; Salminen et al., 2008). The abundance of alkane degrading genes has been assessed in the soil and plant interior (Siciliano et al., 2001; Whyte et al., 2002; Yergeau et al., 2009), but the activity of alkane degrading bacteria in situ and under natural conditions by e.g. gene expression has been rarely addressed (Panicker et al., 2010; Powell et al., 2006). However, for efficient degradation not only the abundance of plant-associated bacteria and/or applied inoculants strains but also the degrading activity is highly important (Juhanson et al., 2009). Characteristics of the soil environment such as soil organic matter or particle sizes may influence the colonization process but may also have pronounced influence on the expression of degrading genes. Therefore, the objective of this study was to determine the effect of the soil type and the associated properties on the performance of hydrocarbon degrading inoculants strains.

Materials and methods

Soils

The physicochemical properties of the three soils used for this study are presented in Table 3.1. Standard soils were purchased from LUFA Speyer, Germany that were characterized as sand (LUFA 2.1), loamy sand (LUFA 2.2) and loam (LUFA 2.4) according to USDA. Apart from their differences in particle size, these soils showed also different values in pH, organic carbon, and cation exchange capacity. They were used directly without any addition of macro- or micronutrients.

Bacterial strains

Two bacterial strains, which were previously isolated from the endosphere and rhizosphere of Italian ryegrass and birdsfoot trefoil (Yousaf et al., 2010a), were used in this study. These included *Pseudomonas* sp. strain, ITRI53 (a root endophyte isolated from Italian ryegrass carrying an alkane monooxygenase (*alkB*) gene), and *Pantoea* sp. strain BTRH79 (a rhizosphere

strain isolated from birdsfoot trefoil carrying a cytochrome P450 alkane hydroxylase gene (CYP153)). Both strains have the capacity to degrade alkanes (Yousaf et al., 2010a), while only the *Pantoea* sp. strain shows ACC deaminase activity. Strains were cultivated in 10% Luria Bertani broth amended with 1% (v/v) filter-sterilized diesel at 30°C. Cells were harvested by centrifugation and resuspended in sterile 0.9% NaCl solution.

Table 3.1. Physicochemical properties of three different types of soil (standard soils from LUFA, Speyer, Germany)

Parameters	Soil type (according to USDA)		
	Sand (LUFA 2.1)	Loamy sand (LUFA 2.2)	Loam (LUFA 2.4)
Organic carbon (%)	0.74±0.14	2.09±0.40	2.99
Particles < 0.02 mm (%)	7.9±1.1	13.5±1.1	51.5
pH-value (0.01 M $CaCl_2$)	5.1±0.5	5.5±0.1	7.2
Cation exchange capacity (meq/100g)	4.0±1.0	10.0±0.5	33.6
Particle sizes according to USDA (%)			
< 0.002 mm	2.9±0.8	6.4±0.9	27.2
0.002 – 0.05 mm	9.1±1.4	11.6±0.7	40.6
0.05 – 2.0 mm	88.0±1.0	82.0±0.7	32.2
Water holding capacity (g/100g)	31.8±3.0	46.5±6.0	45.7
Weight per volume (g/100 ml)	1430±57	1220±78	1310

Mean values of different batch analysis according to Good Laboratory Practice (GLP) ± standard deviation. All values refer to dry matter. Soil was collected, analyzed and characterized by LUFA Speyer, Germany.

Plant experiment

For the plant experiment Magenta boxes were filled with 300 g soil and sterilized by 30 kGy-rediation. Before sowing, the soil was amended with 1% (v/v) filter-sterilized diesel and then mixed with 50 ml inoculant suspension (app. 10^{10} cfu/ml) containing either strain ITRI53, strain BTRH79 or sterile 0.9% NaCl solution. Control treatments with and without the amendment of diesel but without bacterial inoculation were included. One hundred seeds of Italian ryegrass (*Lolium multiflorum*) were surface-sterilized in a 5% (v/v) NaOCl for 10 min, washed five times with sterilized water and were sown in each box and each treatment was triplicated. Plants were grown at 25 °C in a sterile environment and subjected to a cycle of 16 h light and 8 h dark for 2 months. Plants were watered with sterile distilled water when needed.

Sampling and extraction of DNA and RNA

After 1 week, 2 weeks and 8 weeks, shoots were cut from 2 cm above soil and weighed. The remaining plants were harvested to obtain root and rhizosphere samples. Rhizosphere soil was collected by gently sampling the soil closely attached to the root surface. The roots were washed, dried and weighed. The remaining soil was mixed and stored at -80°C for total hydrocarbon analysis. Shoots were surface-sterilized as described earlier (Andria et al., 2009). Sterility was checked by plating on Tryptic Soy Agar plates (TSA, Merck).

DNA from rhizosphere was extracted by using the FastDNA Spin Kit for soil (Qbiogene), whereas RNA was isolated with the FastRNA Pro Soil-Direct Kit (MP Biochemicals) as described by the manufacturers. Shoots were briefly ground in liquid N_2 and microbial cells were lysed by beat-beating (Reiter et al., 2003). For isolation of DNA and RNA the DNeasy Plant Mini Kit and RNeasy Plant Mini Kit (Qiagen) were used. In RNA preparations genomic DNA was eliminated by DNase I enzyme (Ambion) digestion and the potential presence of contaminating DNA was checked by PCR amplification of 16S rDNA (Andria et al., 2009).

Quantification of the inoculant strains by cultivation

Surface-sterilized shoots (1 g) were cut into small pieces. Rhizosphere soil as well as shoot were resuspended in 2 ml of 0.9% (w/v) NaCl solution and shaken at 180 rpm for 30 min. After plant and soil particles were settled, the aqueous phase (100 µl) of 10^{-3} dilutions were plated on 10% TSA in duplicates and incubated at 30 °C for two days to determine CFU/g dry soil or plant material. Ten colonies were randomly picked and the identity of isolates with the inoculant strain was confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA intergenic spacer region (IGS) (Rasche et al., 2006). Similarly, *alkB* and CYP153 genes were amplified, digested with *Alu*I (Invitrogen), electrophoresed on 1.5% agarose gels and compared with the profiles obtained from the inoculant strains. Isolates and inoculant strains had identical restriction patterns (Andria et al., 2009).

Quantitative analysis of the abundance and expression of *alkB* and CYP153 genes

Reverse transcription was performed with 10-20 ng RNA, the specific primers PpalkB-for and P450-for and Superscript II Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. Abundance and expression of both genes were quantified by real-time PCR using an iCycler (IQ) (Biorad) as described by Andria et al. (2009). Besides melting curve analysis, PCR

products were examined on 2% agarose gels. No primer-dimers were detected. Serial dilutions of DNA and cDNA were spiked with 10^6 copies of amplified *alkB* and CYP153 genes to check for real-time PCR inhibition (López-Gutiérrez et al., 2004). Highly linear standard curves (r^2 values > 0.95, PCR efficiency > 98%) over the dilution range and a detection limit of 10^1 copies were obtained indicating no PCR inhibition. The *alkB* and CYP153 gene copy numbers were quantified relative to a standard curve of positive control (Andria et al., 2009).

ACC deaminase activity

ACC deaminase activity of two strains were tested on the minimal medium containing 0.7 g ACC L^{-1} as sole nitrogen source, as described earlier (Kuffner et al., 2008).

Hydrocarbon analysis of soil samples

Total hydrocarbon content (THC) of the soil was measured employing infrared spectroscopy as described previously (Yousaf et al., 2010b).

Statistical analysis

All statistical analyses were performed using SPSS software package (SPSS In. U.S.A.) and Excel (Microsoft, U.S.A.). The data were subjected to analysis of variance, and significant differences between means were determined by Duncan's multiple range test ($p < 0.05$).

Results

Plant biomass

Growth parameters (seed germination, shoot length and shoot and root biomass) were determined to evaluate the effect of the soil type on plant development (Table 3.2 and 3.3). In the absence of the contaminant, there was significantly more seed germination, shoot length and biomass (shoot and root) in loamy soils than in sandy soil. In soils containing diesel, plants without bacterial inoculation displayed significantly less seed germination, shoot length and plant biomass than the plants grown in unpolluted soils. Soils inoculated with both, *Pseudomonas* sp. strain ITRI53 and *Pantoea* sp. strain BTRH79, exhibited a significantly higher percentage of seed germination, shoot length and plant biomass as compared to un-inoculated soils. The strain with

ACC deaminase activity (BTRH79) induced higher shoot and particularly root biomass compared to the uninoculated diesel soil than the strain (ITRI53) lacking this activity. In uninoculated as well as in inoculated sandy soil plant growth was very slow. Plant biomass production gradually increased with time.

Table 3.2. Effect of soil type on seed germination (SG) and shoot length (SL)

Soil type/treatment	SG (%)	SL (cm)		
		1st harvest	2nd harvest	3rd harvest
Sandy soil				
Control (vegetated) (- diesel)	81^{d} (3.6)	9^{cd} (0.7)	17^{d} (0.6)	20^{e} (0.7)
Control (vegetated)	65^{f} (2.3)	7^{e} (0.8)	12^{f} (0.4)	14^{g} (0.8)
Pseudomonas sp. ITRI53	68^{ef} (2.6)	7^{e} (0.6)	13^{ef} (0.3)	15^{g} (0.5)
Pantoea sp. BTRH79	72^{e} (3.3)	8de (0.5)	14^{e} (0.2)	17^{f} (0.3)
Loamy sand soil				
Control (vegetated) (- diesel)	89^{ab} (3.9)	12^{ab} (0.3)	25^{ab} (0.4)	30^{b} (0.8)
Control (vegetated)	78^{d} (2.9)	8de (0.2)	17^{d} (0.7)	21^{e} (0.6)
Pseudomonas sp. ITRI53	83^{cd} (3.3)	10^{bc} (0.4)	20^{d} (0.5)	26^{d} (0.4)
Pantoea sp. BTRH79	87^{bc} (2.6)	11^{b} (0.6)	23^{c} (0.8)	28^{c} (0.9)
Loamy soil				
Control (vegetated) (- diesel)	94^{a} (2.3)	13^{a} (0.7)	26^{a} (0.4)	32^{a} (0.6)
Control (vegetated)	81^{d} (2.9)	9^{cd} (0.5)	18^{d} (0.5)	21^{e} (0.4)
Pseudomonas sp. ITRI53	89^{ab} (2.6)	11^{b} (0.4)	23^{c} (0.9)	27^{d} (0.5)
Pantoea sp. BTRH79	91^{ab} (2.9)	12^{ab} (0.3)	25^{ab} (0.7)	30^{b} (0.3)

1st harvest, 2nd harvest and 3rd harvest were after one week, four weeks and eight weeks of seed germination, respectively. Each value is the mean of three replicates, means in the same column followed by the same letter are not significantly different at a 5% level of significance, the standard error of three replicate is presented in parentheses.

Abundance of inoculant strains and expression of degrading genes

The abundance of the two inoculant strains was followed by cultivation and by a cultivation-independent approach. Both approaches clearly showed that stain BTRH79 better colonized the rhizosphere of Italian ryegrass in all three soil types than strain ITRI53 (Table 3.4), whereas strain ITRI53 better colonized the shoot interior of Italian ryegrass (Table 3.5). Inoculated strains did not only colonize the rhizosphere and shoot interior of plants grown in loamy sand and loamy soils, but also expressed alkane degradation genes indicating an active role in the degradation of the pollutant. The highest gene abundances and gene expression levels were found in the loamy soil, where also plant growth and hydrocarbon degradation (compare section 3.3 and table 6) were the highest.

Table 3.3. Effect of soil type on shoot and root dry weight (DW)

Soil type/treatment	1st harvest		2nd harvest		3rd harvest	
	Shoot DW (g)	Root DW (g)	Shoot DW (g)	Root DW (g)	Shoot DW (g)	Root DW (g)
Sandy soil						
Control (vegetated) (- diesel)	1.9^{f} (0.14)	ND	2.2^{g} (0.12)	0.8^{f} (0.07)	2.8^{h} (0.21)	1.0^{f} (0.08)
Control (vegetated)	1.4^{g} (0.08)	ND	1.6^{h} (0.10)	0.6^{f} (0.05)	2.0^{i} (0.16)	0.7^{g} (0.03)
Pseudomonas sp. ITRI53	1.5^{g} (0.12)	ND	1.7^{h} (0.12)	0.6^{f} (0.09)	2.2^{i} (0.14)	0.8^{fg} (0.02)
Pantoea sp. BTRH79	1.6^{g} (0.13)	ND	1.8^{h} (0.15)	0.7^{f} (0.10)	2.4^{hi} (0.18)	0.9^{fg} (0.05)
Loamy sandy soil						
Control (vegetated) (- diesel)	2.9^{c} (0.18)	ND	6.8^{b} (0.23)	2.0^{bc} (0.13)	9.1^{c} (0.45)	3.1^{bc} (0.24)
Control (vegetated)	2.1^{f} (0.13)	ND	4.6^{f} (0.31)	1.4^{e} (0.08)	6.5^{g} (0.37)	2.3^{e} (0.14)
Pseudomonas sp. ITRI53	2.4^{e} (0.17)	ND	5.8^{d} (0.34)	1.6^{de} (0.06)	8.2^{e} (0.28)	2.6^{d} (0.19)
Pantoea sp. BTRH79	2.7^{cd} (0.23)	ND	6.4^{c} (0.36)	1.8^{cd} (0.12)	8.9^{cd} (0.31)	2.9^{c} (0.16)
Loamy soil						
Control (vegetated) (- diesel)	3.5^{a} (0.24)	ND	7.3^{a} (0.35)	2.7^{a} (0.16)	10.1^{a} (0.42)	3.5^{a} (0.23)
Control (vegetated)	2.6^{de} (0.16)	ND	5.2^{e} (0.26)	1.8^{cd} (0.11)	7.8^{f} (0.27)	2.5^{de} (0.16)
Pseudomonas sp. ITRI53	2.8^{cd} (0.13)	ND	6.0^{d} (0.30)	2.1^{bc} (0.13)	8.7^{d} (0.34)	2.9^{c} (0.15)
Pantoea sp. BTRH79	3.2^{b} (0.29)	ND	6.9^{b} (0.34)	2.4^{b} (0.14)	9.6^{b} (0.37)	3.3^{ab} (0.11)

1st harvest, 2nd harvest and 3rd harvest were after one week, four weeks and eight weeks of seed germination, respectively. Each value is the mean of three replicates, means in the same column followed by the same letter are not significantly different at a 5% level of significance, the standard error of three replicate is presented in parentheses. ND = not determined

However, in sandy soil both strains showed comparatively low survival and abundance in the rhizosphere and shoot interior, and expression of alkane degrading genes was only detected in the shoot but not in the rhizosphere. Similarly, hydrocarbon degradation was lower in sandy soil. The comparison between samples taken at different harvests showed that the total number of bacteria, measured via both CFU counting and real-time PCR, decreased with time. Maximum hydrocarbon degradation was observed at the second harvest, which correlated well (r = 0.7) with high *alkB* and CYP153 gene expression levels. These results showed that bacterial abundance and expression of alkane degrading genes was affected by the soil type and decreased with time. When the degradation potential was high, gene numbers were high as well. Control non-inoculated soils did not show colonies at the beginning of the experiment, however a few colonies were detected after eight weeks presumably due to cross-contamination (data not shown). In the same soil, none of the functional genes (*alkB* and CYP153) were detected, and the degradation of hydrocarbon was very low.

Table 3.4. Effect of soil type on colony forming unit (cfu), *alkB* and CYP153 genes abundance and gene expression in the rhizosphere of Italian ryegrass inoculated either with *Pseudomonas* sp. ITRI53 or with *Pantoea* sp. BTRH79, respectively

Soil type/treatment	Cfu/g dry weight * 10^5			Genes abundance (copies/g dry weight) * 10^5			Gene expression (copies/g dry weight) * 10^5		
	1st harvest	2nd harvest	3rd harvest	1st harvest	2nd harvest	3rd harvest	1st harvest	2nd harvest	3rd harvest
Sandy soil									
Pseudomonas sp. ITRI53	1.5^{d} (0.7)	0.61^{e} (0.08)	0.48^{e} (0.09)	0.68^{e} (0.1)	0.25^{d} (0.04)	0.19^{e} (0.04)	0	0	0
Pantoea sp. BTRH79	3.2^{d} (0.4)	0.72^{e} (0.08)	0.53^{e} (0.03)	0.79^{e} (0.12)	0.37^{d} (0.09)	0.24^{e} (0.037)	0	0	0
Loamy sandy soil									
Pseudomonas sp. ITRI53	560^{c} (45)	85^{d} (17)	46^{d} (2.2)	160^{d} (26)	34^{c} (4.9)	78^{c} (15)	53^{c} (4.7)	8.4^{c} (0.62)	17^{c} (5.7)
Pantoea sp. BTRH79	1600^{b} (830)	630^{b} (88)	140^{b} (31)	530^{b} (86)	270^{b} (87)	94^{b} (7.4)	240^{ab} (48)	79^{b} (4.2)	27^{b} (6.6)
Loamy soil									
Pseudomonas sp. ITRI53	550^{c} (34)	380^{c} (84)	86^{c} (23)	330^{c} (82)	34^{c} (8.2)	37^{d} (3.9)	94^{b} (6.8)	12^{c} (5.3)	6.9^{d} (1.6)
Pantoea sp. BTRH79	4700^{a} (790)	3400^{a} (680)	590^{a} (84)	570^{a} (79)	850^{a} (56)	460^{a} (4)	270^{a} (75)	460^{a} (86)	130^{a} (82)

Means in the same column followed by the same letter are not significantly different at a 5% level of significance, n = 3; the standard error of three replicate is presented in parentheses. 1st harvest, 2nd harvest and 3rd harvest were after one week, four weeks and eight weeks of seed germination, respectively; the standard error of three replicate is presented in parentheses.

Table 3.5. Effect of soil type on colony forming unit (cfu), *alkB* and CYP153 genes abundance and gene expression in the shoot of Italian ryegrass inoculated either with *Pseudomonas* sp. ITRI53 or with *Pantoea* sp. BTRH79, respectively

Soil type/treatment	Cfu/g dry weight * 10^5			Genes abundance (copies/g dry weight) * 10^5			Gene expression (copies/g dry weight) * 10^5		
	1st harvest	2nd harvest	3dr harvest	1st harvest	2nd harvest	3rd harvest	1st harvest	2nd harvest	3rd harvest
Sandy soil									
Pseudomonas sp. ITRI53	0.48^{c} (0.06)	7.1^{e} (0.83)	1.8^{e} (0.46)	0.06^{c} (0.02)	0.39^{e} (0.06)	0.037^{e} (0.01)	0.037^{c} (0.007)	0.24^{d} (0.06)	0.026^{e} (0.008)
Pantoea sp. BTRH79	0.25^{e} (0.05)	0.58^{e} (0.09)	0.22^{e} (0.06)	0.02^{d} (0.006)	0.52^{e} (0.07)	0.014^{e} (0.004)	0.008^{d} (0.003)	0.28^{d} (0.06)	0.009^{e} (0.007)
Loamy sandy soil									
Pseudomonas sp. ITRI53	3.2^{b} (0.38)	680^{b} (65)	420^{a} (84)	0.37^{b} (0.06)	8.1^{c} (0.75)	6.4^{a} (0.41)	0.29^{b} (0.08)	5.4^{b} (0.82)	3.7^{a} (0.06)
Pantoea sp. BTRH79	0.24^{e} (0.06)	25^{d} (6.2)	38^{d} (7.2)	0.023^{d} (0.004)	1.3^{d} (0.52)	0.86^{d} (0.09)	0.016^{d} (0.01)	0.75^{d} (0.07)	0.39^{d} (0.08)
Loamy soil									
Pseudomonas sp. ITRI53	5.2^{a} (0.83)	790^{a} (78)	250^{b} (51)	0.45^{a} (0.07)	76^{a} (7.2)	5.3^{b} (0.64)	0.31^{a} (0.07)	39^{a} (4.9)	3.2^{b} (0.8)
Pantoea sp. BTRH79	0.37^{d} (0.07)	81^{c} (9.6)	53^{c} (6.8)	0.056^{c} (0.01)	9.4^{b} (0.24)	1.8^{c} (0.35)	0.038^{c} (0.008)	3.8^{c} (0.64)	0.86^{c} (0.2.5)

Means in the same column followed by the same letter are not significantly different at a 5% level of significance, n = 3; the standard error of three replicate is presented in parentheses; 1st harvest, 2nd harvest and 3rd harvest were after one week, four weeks and eight weeks of seed germination, respectively.

Hydrocarbon

In order to determine the effect of soil type on degradation of diesel, the residual amount of hydrocarbon in soil was determined. In soils that had been planted without inoculation, hydrocarbon degradation was very low and bacterial inoculation enhanced hydrocarbon degradation (Table 3.6). Generally, both un-inoculated and inoculated plants in loamy soil displayed more efficient hydrocarbon degradation than in sandy and loamy sandy soils. Soils inoculated with *Pantoea* sp. strain BTRH79 showed significantly more hydrocarbon degradation than those inoculated with *Pseudomonas* sp. strain ITRI53. Among inoculated soils, highest hydrocarbon degradation (62%) was observed in loamy soil inoculated with BTRH79. This was significantly more than the 20 % in the uninoculated control and also significantly more than the degradation rate in loamy sand (57% after 8 weeks) and in the sandy soils (24% after 8 weeks). In sandy soil the degradation was generally smallest with 12% in uninoculated treatment and 22% in the treatment with strain ITRI53. In vegetated, inoculated soils 11-29%, 20-51% and 22-62% hydrocarbon degradation was observed at the first, second and third harvest time, respectively.

Table 3.6. Effect of soil type and bacterial inoculants on hydrocarbon content at the tree dates of harvest

Soil type/treatment	Hydrocarbon concentration (g kg^{-1} soil)			
	Initial value	1st harvest	2nd harvest	3rd harvest
Sandy soil				
Control (vegetated)	10.0	9.4^{d} (0.52)	9.2^{f} (0.46)	8.8^{f} (0.47)
Pseudomonas sp. ITRI53	10.0	8.9^{c} (0.37)	8.0^{de} (0.50)	7.8^{d} (0.38)
Pantoea sp. BTRH79	10.0	8.7^{c} (0.46)	7.9^{d} (0.35)	7.6^{d} (0.35)
Loamy sand soil				
Control (vegetated)	10.0	8.8^{c} (0.48)	8.5^{e} (0.42)	8.3^{e} (0.41)
Pseudomonas sp. ITRI53	10.0	7.8^{b} (0.35)	6.4^{c} (0.55)	5.2^{c} (0.30)
Pantoea sp. BTRH79	10.0	7.6^{b} (0.43)	5.8^{b} (0.38)	4.3^{b} (0.60)
Loamy soil				
Control (vegetated)	10.0	8.5^{c} (0.49)	8.2^{de} (0.35)	8.0^{de} (0.48)
Pseudomonas sp. ITRI53	10.0	7.5^{b} (0.38)	5.5^{b} (0.36)	4.4^{b} (0.37)
Pantoea sp. BTRH79	10.0	7.1^{a} (0.33)	4.9^{a} (0.41)	3.8^{a} (0.28)

Means in the same column followed by the same letter are not significantly different at a 5% level of significance, n = 3; the standard error of three replicate is presented in parentheses. 1st harvest, 2nd harvest and 3rd harvest were after one week, four weeks and eight weeks of seed germination, respectively.

Discussion

Optimal plant growth is an important factor influencing rhizodegradation of hydrocarbons by supporting the colonization of degrading microorganisms and increasing oxygen availability. In the present study we obtained reduced seed germination and biomass (shoot and root) production of Italian ryegrass grown in sandy soil than in both loamy soils. Loamy soil provided the best habitat for plant growth, and sandy soil the worst. This may be due to differences in organic carbon content and cation exchange capacity. High cation exchange capacity generally indicates high nutrient levels potentially leading to better plant growth (Weissenhorn, 1996; Eason et al., 1999; Carrenho et al., 2007). Sandy soils are usually more porous, warmer, drier, and less fertile than soils with a finer texture thus limiting plant growth (Carrenho et al., 2007; Sylvia and Williams, 1992; Kirk et al., 2005). Seed germination, shoot length and biomass of plants grown in soils containing 1% diesel were significantly lower than of those grown in non-polluted soils. Hydrocarbons are known to inhibit plant growth (Sun et al., 2010; Andria et al., 2009; Yousaf et al., 2010b). The primary inhibiting factors are considered to be the toxicity of low molecular weight compounds and the hydrophobic properties that limit the ability of plants to absorb water by decreasing the field capacity of soils (Reichenauer, personal communication) and nutrients (Kirk et al., 2005). Inoculation appears to have protected plants from the phytotoxic effects of diesel. In inoculated soils, shoot height and plant biomass increased by 8% and 41% and 7% and 38%, respectively, as compared to non-inoculated soils. Particularly strain BTRH79 exhibiting alkane degradation capacity as well as ACC-deaminase activity was highly efficient in enhancing plant biomass (especially root biomass) and hydrocarbon degradation and performed better than strain ITRI53 lacking ACC deaminase activity. This is in line with previous reports showing that the bacterial enzyme ACC-deaminase alleviates plant stress symptoms (Glick, 2003; Huang et al., 2004; Huang et al., 2005). Similarly, Gurska et al. (2009) previously showed that inoculation with ACC deaminase - containing rhizobacteria enhanced root growth and hydrocarbon degradation.

Very low reduction (12-20% after eight weeks) of hydrocarbon was observed in all three types of non-inoculated, vegetated soil. As the soil was initially sterilized adsorption of the hydrocarbons to soil organic matter and possibly to roots is likely to have been the reason for the measured reduction of the contaminant. Among inoculated soils, total hydrocarbon reduction was higher in loamy soil (62%) and loamy sandy soil (57%) than in sandy soil (24%). This indicates that mostly the inoculated bacteria were responsible for the degradation of hydrocarbons. Better

developed roots and more root exudates, particularly in loamy soils, probably contributed to better colonization of the inoculants strains and more efficient degradation. In an earlier study, more hydrocarbons were degraded in a loamy and a clayey soil than in a sandy soil (Molnár et al., 2002). The concentration of basic nutrients are low in sandy soils (Kaimi et al., 2007) and no biodegradation of hydrocarbon was observed after 30 days without fertilization. Furthermore, Davis and Madsen (Davis and Madsen, 1996) reported that degradation of toluene was affected by soil type, soil organic matter content and inorganic nitrogen availability. They observed very slow toluene degradation in sandy soil due to low organic content (0.8%) as compared to sandy loam and clay soils containing high organic content, 4% and 5.5%, respectively.

Strain BTRH79 showing ACC deaminase activity in vitro was more efficient in hydrocarbon degradation than strain ITRI53 lacking this activity. Strain BTRH79 favoured shoot and root growth, the latter provided an increased surface area for bacterial colonization resulting also in higher degradation. This strain was also previously reported to efficiently colonize plants and degrade hydrocarbons (Yousaf et al., 2010b).

The ability of *Pseudomonas* sp. strain ITRI53 and *Pantoea* sp. strain BTRH79 to colonize the rhizosphere and shoot interior of Italian ryegrass was assessed using cultivation and DNA-based methods. In contrast to the high abundance of the inoculant strains in loamy sand and loamy soils, rather poor survival was observed in sandy soil. Similarly, bacterial gene abundance and expression were higher in loamy soils than in sandy soil. In sandy soil, low organic carbon, low cation exchange capacity and a limited surface area available for bacterial attachment might be underlying the lower bacterial colonization as well as lower or no activity. However, genes involved in the degradation of hydrocarbons were expressed in loamy soils throughout the experiment. The abundance of degrading genes (*alkB* and CYP153) showed positive correlations with gene expression (r = 0.82) and hydrocarbon degradation (r = 0.74). Catabolic genes may serve as markers of actual function: in the case of hydrocarbon degrading communities, strong positive correlations have previously been found between gene copies and transcripts (Sanseverino et al., 1993; Fleming et al., 1993; DeBruyn et al., 2007) indicating that the presence of genes is related to their activity. In contrast to the relationship between gene abundance and gene expression, it seems that inoculated bacteria were inactive in the sandy soil. In loamy sandy and loamy soils, a rapid decrease in soil hydrocarbon concentrations was observed within only four weeks of treatment (loamy sand, 32%; loamy soil, 40%). This could partly be due to the release of root

exudates enhancing bacterial growth and hydrocarbon degradation. Gene expression results also showed that this decrease in hydrocarbons in loamy sand and loam was caused by bacterial degradation, since the expression level of measured biodegradation genes (*alkB* and CYP153 genes) was higher at the first and second harvest than at the third harvest. The fact that the abundance and expression of inoculated bacteria / genes decreased throughout the study period may result from insufficient nutrient availability and sub-optimal environmental conditions such as matric water potential, pH and ionic strength (Van Elsas and Van Overbeek, 1993). The competition between plant roots and microbes for nutrients may have influenced microbial activities, especially in sandy soil characterized by a low organic matter content (Kaakinen et al., 2007). Secondly, the decrease in gene abundance and expression of hydrocarbon degraders may be due to a decline in easily biodegradable hydrocarbons (Powell et al., 2006).

Cultivation-dependent and cultivation-independent analysis showed that the inoculated strains, ITRI53 and BTRH79, well colonized the rhizosphere and shoot interior of Italian ryegrass. However, strain BTRH79, a rhizosphere bacterium, better colonized the rhizosphere than strain ITRI53, whereas strain ITRI53, a root endophyte, was more successful in colonizing the shoot interior. Furthermore, the endophyte ITRI53 was more active (higher transcript numbers / gene abundance) in the shoot interior, whereas the rhizosphere strain BTRH79 was constantly more active in the rhizosphere. Similar observations were made by Rosenblueth and Martínez-Romero (Rosenblueth and Martínez-Romero, 2006) and Andria et al. (2009), who postulated that endophytes are generally better able to colonize plant interior than the rhizosphere. Both strains also expressed functional genes in the plant interior indicating an active role in this environment. However, for both strains, *alkB* and CYP153 gene abundance and overall expression were the highest in rhizosphere indicating that root exudates provide nutrients for bacterial growth and co-metabolites for alkane degradation (Olson et al., 2003). Nevertheless, as the average activities (*alkB* /CYP153 transcripts / numbers) were higher in the shoot interior than in the rhizosphere (Fig. 3.1), we assume that a larger cell fraction of this population was active inside the plant than in the rhizosphere. This suggests that growth conditions were more favourable in the plant interior and more nutrients and/or activating substances might have been present. Similarly Andria et al. (2009) reported higher activity levels in the shoot interior.

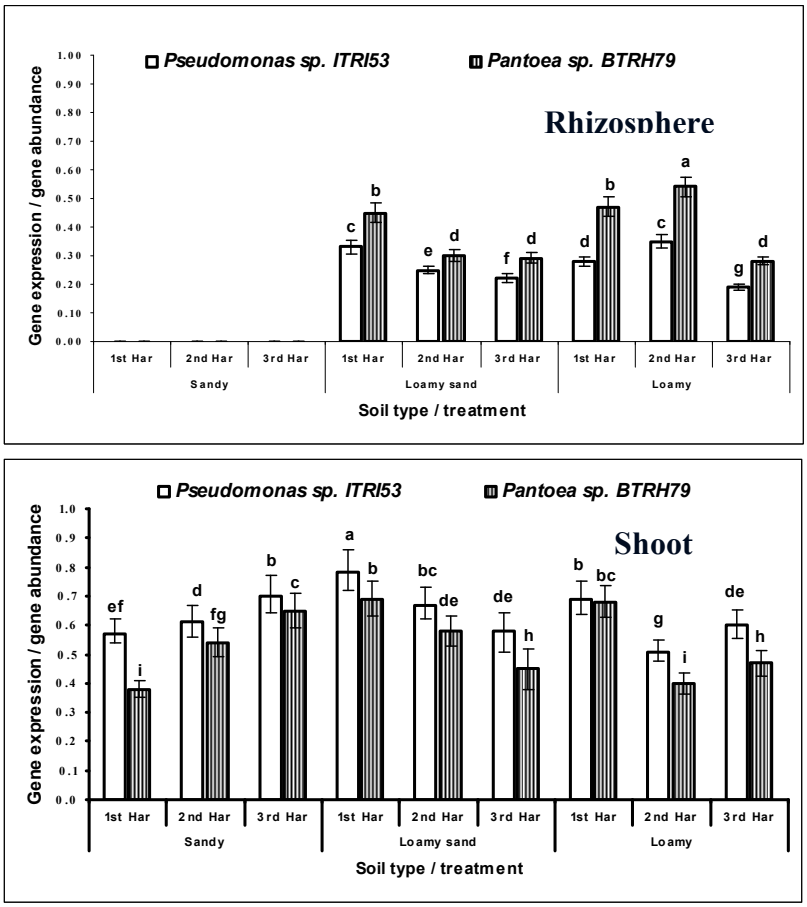

Figure 3.1. Ratio of gene expression and gene abundance in the rhizosphere and shoot interior of Italian ryegrass vegetated in three different types of soil spiked with 1% diesel.

In conclusion we showed that inoculation with suitable bacterial strains has the potential to support plant growth and enhance phytoremediation of hydrocarbons, however, the process is strongly influenced by soil characteristics such as the soil type, particle sizes or organic matter content. The soil type did not only affect plant growth but also substantially influenced microbial colonization and activities. The importance of such parameters should be considered in the design of bioremediation applications.

Acknowledgements

M. Afzal would like to thank NIBGE and Higher Education Commission, Govt. of Pakistan, for funding. We are thankful to A. Grahsl, who assisted in performing the greenhouse experiment.

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CHAPTER 4

GENERAL CONCLUSIONS

For beneficial plant-microbe interactions it is of primary importance that the inoculated microbes sustain viable and can colonize the rhizosphere and developing root in order to mediate their effects on plant growth and contaminant degradation. In addition to environmental factors, plant and bacterial genotype, the viability and colonization efficiency of an applied strain depend on the inoculation modes as well as on physicochemical properties of the soil environment. One of the aims of this thesis was to study the impact of the inoculation method on bacterial survival, activity and hydrocarbon degradation of inoculated degrading bacterial strains. The second aim was to investigate to which extent and in which way the soil type affects the colonization and activity of inoculants strains and consequently also on hydrocarbon degradation. Based on the results obtained in this thesis the following conclusions can be made:

- Two commonly used inoculation methods (seed imbibement and soil inoculation) in phytoremediation studies were compared in terms of plant growth, bacterial survival, and hydrocarbon degradation. Application of bacteria by soil inoculation method was found to be more efficient in enhancing plant biomass as compared to seed imbibement. Similarly, better bacterial colonization and hydrocarbon degradation was observed by soil inoculation. Highest performance was achieved with soil application of a consortium containing three strains. Bacteria showing plant growth promoting ACC deaminase activity were found to be effective in enhancing plant growth and subsequently hydrocarbon degradation. There was significantly more bacterial colonization in the plant interior as compared to rhizosphere soil and only BTRH79 could colonize seeds.
- The effect of soil types on plant growth, colonization and gene expression of two inoculant strains was studied. Generally, the soil type affected plant growth, bacterial colonization and hydrocarbon degradation. Culture-dependent and culture-independent analysis showed high colonization, gene abundance and expression in loamy soils. However, in sandy soil low colonization, gene abundance and absence of gene expression were found. The soil type had a strong effect on the expression of genes (*alkB* and CYP153) specific to hydrocarbon degradation. The highest levels of gene expression and hydrocarbon degradation was observed in loamy soil and significantly higher compared to those in

other soils. A positive correlation (r = 0.73) was observed between gene expression and hydrocarbon degradation indicating that the catabolic gene expression is necessary for contaminant degradation.

On the basis of our findings it can be concluded that inoculation method and soil type can affect plant growth, bacterial survival and activity and hydrocarbon degradation. The importance of inoculation procedures and soil type should be considered in the design of efficient phytoremediation applications.

Future prospects

Soil contamination might increase in future owing to rapid population growth and consequently a boost in industrialization, urbanization and intensive agriculture world-wide. To mitigate the ill-effects of soil contamination by organic chemicals and heavy metals, phytoremediation might be a effective and affordable approach to clean-up polluted soils. However, various aspects still need to be investigated to make this technology successful. In particular, attention should be paid to following aspects:

- The successful use of rhizobacteria in phytoremediation will much depend on the ability to establish the desired strains in the already existing community of soil microorganisms. Certain rhizobacteria may show beneficial effects on plant and/or a plant's tolerance to biotic or abiotic stress in greenhouse experiments under controlled conditions; however, these effects may not be evident when strains are applied in the field. The ability of plants to interact with beneficial bacteria should be considered in order to promote specific beneficial plant-bacterial combinations. In addition, studies on the practicability of the application of beneficial microbes *in situ* are needed.
- The inoculant formulation is important in providing a suitable microenvironment to prevent a rapid decline of inoculated bacteria. The use of bacterial combinations, such as different strains for alkane degradation, nitrogen fixation, root growth promotion and induced resistance among others, might represent a highly successful strategy, but additional research is required to further elucidate this approach. Concerted efforts should focus on increasing the numbers and diversity of beneficial microbial populations, such as those showing ACC deaminase activity or pollutant degradation. Genetic manipulation of plants expressing ACC deaminase and other pollutants degrading genes might be a new

breakthrough in improving remediation of polluted soils with heavy metals and organic chemicals.

- The exploitation of plant-endophyte partnerships for the remediation of contaminated soils and groundwater is a promising area. In the case of phytoremediation of organic contaminants, endophytic bacteria possessing the appropriate degradation pathway(s) can assist their host plant by degrading contaminants that are readily taken up by plants. In the case of phytoremediation of toxic metals, endophytes equipped with a metal-sequestration system and/or able to produce natural metal chelators can reduce metal toxicity for their host plant and/or increase metal translocation to the aerial parts. One promising area of research for future studies is the selection of native or engineered endophytes expressing both ACC deaminase and other contaminant specific genes to promote the sustainable production of biomass and bioenergy crops in conjunction with phytoremediation of soil contamination.
- Because the scope of combined use plant and specific microorganisms is to decrease the concentration of organic pollutants to levels undetectable or, if measurable, lower than the limits established as safe or tolerable by regulatory agencies, several criteria must be met for it to be seriously considered as a practical method for treatment. These criteria can be summarized as follows: (a) the plant have to grow well, (b) microorganisms have to survive, (c) those organisms must have the capacity to express catabolic genes, (d) chemicals with toxic effects to the plant and microorganisms must be absent at the site, (e) the target compound must be bioavailable, (f) conditions like soil properties, moisture, temperature etc. at the site should be optimized to sustain plant growth and microbiological growth or activity, (g) a proper bacterial inoculation method must be adopted to ensure high bacterial colonization in the plant environment, (h) the technology should be less expensive than alternative technologies.

To meet all of these criteria and successfully implement of phytoremediation technology, a multidisciplinary approach and basic knowledge in microbiology, biochemistry, physiology, ecology and genetics are required. Moreover, much information about the biotic and abiotic factors controlling the growth and metabolic activities of plant and microorganisms in polluted environments is necessary because several of the above criteria are highly empirical rather than knowledge based.

Examining the presence and the expression of the key genes involved in the degradation of target contaminants in the rhizosphere and plant interior can yield more information on microbial activity of rhizobacteria and endophyte than the simple presence of strains. Genes involved in degradation can be present but not be expressed due to disadvantageous soil properties and environmental factors. Increased expression levels of degradation genes are frequently correlated with the rate of contaminant degradation. Highly sensitive methods that can quantify gene expression are now available and the evaluation of the catabolic state of degrading microorganisms could help to identify the effect of environmental conditions, soil properties and over time on the metabolic activity of inoculant strains.

Furthermore, it is important to understand the ecology of the microbial community as whole, because microbes, which are not involved in degradation could influence the behavior of the degrading bacteria. Exploiting the molecular communication between plants and microbes, and exploiting this combination to achieve better results in the elimination of contaminants, is a fascinating area of research. Further research may reveal the mechanisms underlying microbe-plant interactions and we predict this approach will now be adopted to study the induction of catabolic pathways in polluted soils undergoing rhizoremediation. The signals that plant and microbes exchange when they recognize each other will have to be interpreted and the molecular basis of the specific interactions between certain plant genotypes with specific bacteria will need to be dissected. Information that can be derived from these studies may provide further insights on how to design a successful rhizoremediation strategy.

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