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Endophytic Bacteria and their Potential Application to Improve the Phytoremediation of Contaminated Environments

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Introduction

Endophytic bacteria seem to have a ubiquitous existence in most, if not all, higher plant species without causing disease symptoms. Therefore, they seem to be ideal to complement certain metabolic properties of their host plant, such as the fixation of nitrogen or the detoxification of contaminants. This review describes the ecology of endophytic bacteria and a number of natural beneficial interactions between endophytic bacteria and their plant host, such as diazotrophy. It also exploits the possibilities of using endophytic bacteria as carriers of new metabolic properties, which should assist the host plant in degrading organic contaminants or detoxifying heavy metals that presently cause a problem for phytoremediation. Finally, we will discuss the potential of horizontal gene transfer as a tool to adapt the metabolic capability of the endogenous endophytic community to better deal with specific environmental contaminants.

The associations of endophytic organisms with their hosts are varied and complex.

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AFLP, amplified fragment restriction polymorphism; ARDRA, amplified rDNA restriction analysis; BTEX, benzene, toluene, ethylbenzene and xylene; DGGE, denaturing gradient gel electrophoresis; IAA, indole-3-acetic acid; log K_{ow}, octanol/water partitioning coefficient; MtBE, methyl-t-butyl ether; PGPR, plant growth-promoting rhizobacteria; RLGS, restriction landmark genome scanning; rRNA, ribosomal RNA; SAM, S-adenosyl-L-metionine; SARST, serial analysis of ribosomal sequence tags; TCA, trichloroacetate; TCE, trichloroethylene; T-RFLP, terminal-restriction fragment length polymorphism.

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and we are only just beginning to understand these interactions. Bacterial and fungal symbionts exist across all areas of life, from the bacteria that colonize the human intestinal tract, to the ancient cells that became the plastids in plant cells, and the mitochondria that are present in almost all eukaryotic cells. *Rhizobium* and other bacteria are known to colonize root nodules and enter into a symbiotic relationship with a plant whereby the plant protects and supplies nutrients to the bacteria, and the bacteria provide nitrogen to the plant (Schultze and Kondorosi, 2002). However, we now know that plant endophytes go far beyond the well-studied root nodules, and exist within the leaf, root, and vascular tissue of the plant (Lodewyckx *et al.*, 2002b; Zinniel *et al.*, 2002). The large number of reports on these so-called endophytic bacteria clearly indicates that such bacteria exist in a variety of tissue types within numerous plant species, suggesting a ubiquitous existence in most, if not all, higher plants.

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

Ecology of endophytic bacteria

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

MOST COMMON SPECIES OF ENDOPHYTIC BACTERIA

In general, the highest endophytic densities are observed in the roots, and decrease from the stem to the leaves (Lamb *et al.*, 1996; Quadt-Hallman and Kloepper, 1996; Porteous-Moore *et al.*, 2006). This is consistent with the fact that many endophytic bacteria can also exist in the rhizosphere, and that their preferred path of entering the plant is via the lateral roots (Sprent and de Faria, 1988; Sturz and Nowak, 2000). It should be noted that attempts to evaluate total populations of cultivable bacteria in plants may produce varied results according to the growth medium used for isolation, the method of surface disinfection used, variations in growth conditions of the host plant, and the state in which the plant tissue was used, as is also the case for plant-associated rhizosphere bacteria (Kloepper and Beauchamp, 1992; Porteous-Moore *et al.*, 2006). In general, *Pseudomonaceae*, *Burkholderiaceae*, and *Enterobacteriaceae* are among the most common genera of cultivable endophytic species found. This was first observed by Gardner and colleagues, who identified bacteria present in the xylem fluid from the roots of the rough lemon rootstock of the Florida citrus tree (Gardner *et al.*, 1982). Among the thirteen genera found, the most frequently occurring genera were *Pseudomonas* (40%) and *Enterobacter* (18%).

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

For practical applications, where endophytic bacteria are engineered and subsequently used as a carrier to introduce new properties into their host plant, we are interested only in the subset of cultivable microorganisms. The advantage of the

above-mentioned bacterial families is that many of their members can be manipulated easily to express new properties by either natural gene transfer or transformation. The application of these engineered endophytic strains for improved phytoremediation will be discussed below.

CULTURE-DEPENDENT AND -INDEPENDENT METHODS TO ENUMERATE AND CHARACTERIZE ENDOPHYTIC COMMUNITIES

The research on endophytic bacteria has concentrated on the cultivable members of different endophytic communities. These are, in general, isolated after surface sterilization of the plant material. However, no surface disinfection protocols exist that result in the complete killing of surface bacteria on 100% of samples without penetrating interior tissues and thereby killing internal colonists, thus influencing the recoverable endophytic population. Therefore, comparisons between different studies should be carefully evaluated, taking into account the different surface sterilization methods and conditions used. We optimized sterilization protocols for the recovery of endophytic bacteria from different parts of poplar (Porteous-Moore *et al.*, 2006), willow, yellow lupine (Lodewyckx *et al.*, 2001), *Thlaspi caerulescens* (Lodewyckx *et al.*, 2002a), Brassica (Lodewyckx, 2001), and tobacco (Mastretta *et al.*, unpublished).

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

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

have been employed to analyse composition of endophytic communities. Sessitsch and colleagues amplified and subsequently sequenced 16S rRNA genes to analyse the populations of endophytic bacteria in three potato cultivars (Sessitsch *et al.*, 2002). Conn and Franco (2004) used T-RFLP to study the effect of microbial inoculants on the indigenous actinobacterial endophyte population in the roots of wheat. They observed that the application of a commercial, mixed inoculum in the soil reduced the endophytic actinobacterial diversity from 40 genera to 21 genera, and reduced the detectable root colonization by approximately half. Their results indicate that the addition of a non-adapted microbial inoculum to the soil disrupted the natural actinobacterial endophyte population, reducing diversity and colonization levels. This finding was in contrast to the results obtained after the addition of a single actinobacterial endophyte to the wheat plant, where an increase in colonization level could be confirmed, even though the indigenous endophyte population was not adversely affected. Tan and colleagues used 16S-23S ribosomal DNA intergenic spacer-targeted PCR for the differentiation of closely related rhizobial taxa and for the development of PCR protocols allowing the specific detection of strains to follow up the colonization of rice (*Oryza sativa*) roots by *Bradyrhizobium* and *Rhizobium* strains (Tan *et al.*, 2001).

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

INOCULATION OF PLANTS WITH ENDOPHYTIC BACTERIA

The fact that endophytic colonization appears especially abundant in root tissue (Lamb *et al.*, 1996; Quadt-Hallman and Kloepper, 1996; Porteous-Moore *et al.*, 2006) may reflect that the root is the primary site where endophytes gain entry into plants. With the exception of seed-transmitted bacteria, which are already present in the plant, potential endophytes must first colonize the root surface prior to entering the plant. This might explain the close relationship between endophytic and rhizosphere colonizing bacteria: many facultative endophytic bacteria can also survive as rhizosphere bacteria.

Under natural conditions, endophytic bacteria find their host by chemotaxis, electrotaxis, or accidental encounter. The main entry for endophytic bacteria appears to be through wounds that naturally occur as a result of plant growth, or through root hairs and at epidermal conjunctions (Sprent and de Faria, 1988). This was confirmed by several microscopic studies (Wiehe *et al.*, 1994; Benhamou *et al.*, 1996a,b; Pan *et al.*, 1997). Artificial wounding was shown to contribute to increased endophytic colonization in roots of plants compared to intact roots (Gagné *et al.*, 1987). Besides providing entry avenues, wounds also create favourable conditions for the approaching bacteria by allowing leakage of plant exudates, which serve as a source of nutrients for the bacteria.

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

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

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

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

Inside the plant, endophytic populations have been observed to grow to between 10^{+2} and 10^{+7} cells per gram of fresh tissue (Shishido *et al.*, 1999; Taghavi *et al.*, 2005). The major difference between beneficial endophytic bacteria and pathogenic species is that they do not cause disease symptoms, this despite their high numbers. For *Xylella fastidiosa* (which causes Pierce's disease of grapevine (*Vitis vinifera*) as

well as several other major agricultural diseases, but is a benign endophyte in most host plants), a direct correlation seems to exist between pathogenic behaviour and blockage of vessels by increased levels of colonization (Newman *et al.*, 2003). Using a *gfp*-labelled *X. fastidiosa* strain, it was observed that, in symptomatic leaves, the fraction of vessels colonized by *X. fastidiosa* was fivefold higher than in nearby asymptomatic leaves. The fraction of vessels completely blocked by *X. fastidiosa* colonies increased 40-fold in symptomatic leaves, and was the feature of colonization most dramatically linked to symptoms. Therefore, the extent of vessel blockage by bacterial colonization is highly likely to be a crucial variable in symptom expression. Intriguingly, the authors also observed that a high proportion (>80%) of colonized vessels were not blocked in infected leaves and instead had small colonies or solitary cells, suggesting that vessel blockage is not a colonization strategy employed by the pathogen but, rather, a by-product of endophytic colonization.

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

The fact that bacteria seem to be capable of colonizing the internal tissues of plants could confer an ecological advantage over bacteria that can only colonize plants epiphytically. The internal tissues of plants are thought to provide a more uniform and protective environment for microorganisms than plant surfaces, where exposure to extreme environmental conditions such as temperature, osmotic potentials, and ultraviolet radiation are major factors limiting long-term bacterial survival (Reinhold-Hurek and Hurek, 1998). However, there are probably other limiting factors that must be overcome when establishing populations in the internal tissues of plants. Thus, establishing and maintaining an introduced bacterial population would still be limited and influenced by the same factors that affect plant health.

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

the green fluorescent protein (*gfp*) gene, whose expression has been optimized for actinobacteria (Sun *et al.*, 1999) and has been coupled to a constitutively expressed promoter, *ermEp* (Schmitt-John and Engels, 1992). Using this *egfp* labelled derivative, the authors observed endophytic colonization from a very early stage of plant development, with colonization of the embryo, endosperm, and emerging radicle.

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

ENDOPHYTIC BACTERIA FROM METAL HYPERACCUMULATOR PLANTS

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

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

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

Beneficial effects of endophytic bacteria

Endophytic bacteria that provide a beneficial effect to their host plant generally may be organized into two different systems: either they are forming a symbiotic relationship through the construction of specialized structures such as nodules, where nitrogen fixation occurs, or they are free-living in the plant's vascular system. The latter is very similar to plant growth-promoting rhizobacteria (PGPR) that are generally found near the roots in the rhizoplane or on plant roots (Frommel *et al.*, 1991). This is not surprising, as most of the endophytes isolated are capable of surviving outside their host plant as rhizospheric bacteria (di Fiori and del Gallo, 1995), and many endophytic bacterial taxa, such as those isolated from sweet corn or cotton, were reported to be common soil bacteria (McInroy and Kloepper, 1994). Therefore, it is not unexpected that the mechanisms used by endophytic bacteria and plant growth-promoting rhizobacteria (PGPR) to beneficially affect their host plants seem to be similar (Kloepper *et al.*, 1991; Höflich *et al.*, 1994).

Plant growth-promoting bacteria, under nutrient-imbalanced conditions, have been reported to be key elements for plant establishment. For this reason, their use can support eco-friendly crop production favouring a reduction in the use of agrochemicals (Herrera *et al.*, 1993; Glick, 1995; Requena *et al.*, 1997). However, at present, there are fewer than 20 different commercially available biocontrol PGPR strains (Penrose and Glick, 2003).

DIRECT PLANT GROWTH-PROMOTING ACTIVITY OF ENDOPHYTIC BACTERIA

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

DIAZOTROPHY

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

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

Azorhizobium caulinodans, suggested that in order to fully exploit the possible benefits of this association, the inoculum should be used in a low-input, nutrient-deficient system (van Nieuwenhove *et al.*, 2001). Nitrogen-fixing bacteria associated with the rhizosphere have been isolated from several tropical grasses such as *Paspalum* and *Digitaria* (e.g. *Azotobacter*, *Azospirillum*, and *Herbaspirillum* species) (Döbereiner and Day, 1976; Vose, 1983). A novel nitrogen-fixing bacterium, an *Azoarcus* species (Reinhold-Hurek *et al.*, 1993), was isolated from Kallar grass (*Leptochloa fusca*), a non-domesticated plant from Pakistan.

N_2-fixing bacteria were also isolated from plant tissues. In these tissues, the fight for C-sources is reduced and the quantity of free oxygen that inhibits the nitrogenase activity is almost zero. Dalton and colleagues showed that endophytic, nitrogen-fixing bacteria were present in a symbiotic relationship within plant tissues of *Ammophila arenaria* (Dalton *et al.*, 2004). Another studied association is the one of sugar cane (*Sorghum officinarum*), in which endophytic nitrogen fixation is carried out by *Gluconoacetobacter diazotrophicus* and *Herbaspirillum seropedicae*, which live in the intercellular spaces and xylem (Cavalcante and Döbereiner, 1988; Sevilla *et al.*, 2001). *G. diazotrophicus* was also isolated from other sucrose-rich plants that are propagated vegetatively, such as sweet potato and Cameroon grass (Cavalcante and Döbereiner, 1988; Paula *et al.*, 1991; Döbereiner *et al.*, 1998). There are also numerous accounts of other grass species, some of agricultural importance such as maize, wheat, oats, barley, and rice, that may be associated with endophytic nitrogen-fixing bacteria, although it is unclear whether or not significant amounts of nitrogen are fixed in these systems (Vose, 1983; Engelhard *et al.*, 2000; Riggs *et al.*, 2001). On the other hand, many studies have proved a direct contribution of endophytic bacteria to plant nitrogen fixation, such as in sugar cane (Boddey *et al.*, 1995a,b), rice (Ladha and Reddy, 1995; Yanni *et al.*, 1997), and wheat (Webster *et al.*, 1997). According to a review from Boddey and colleagues, certain Brazilian cultivars of sugar cane, after ^{15}N nitrogen balance studies, obtained over half their needs for nitrogen from biological nitrogen fixation (>150 kg N ha^{-1} $year^{-1}$) (Boddey *et al.*, 1995a). In rice plants, inoculation with *Azospirillum* contributed to 66% of the total ammonium in the plants (Malik *et al.*, 1997). It was also shown that azotobacterization is beneficial in raising vigorous seedlings of mangroves in coastal wetlands (Ravikumar *et al.*, 2004). The results of the azotobacters, which were inoculated in *Rhizophora* seedlings, revealed a direct effect on the host plant: inoculation significantly increased the root biomass by an average of 98%, the root length by 48%, the leaf area by 278%, and the shoot biomass by 29%, as compared to non-inoculated controls. Inoculation also resulted in increased levels of total chlorophylls and carotenoids by 151% and 159%, respectively.

PHOSPHATE SOLUBILIZATION

Phosphorus is one of the most import plant nutrients. The vast majority of soil P is found as insoluble forms. Plants can absorb P when it is in its monobasic ($H_2PO_4^-$) or dibasic (HPO_4^{2-}) soluble form (Glass, 1989). Even when P is applied to the soil as a fertilizer, it is rapidly immobilized and becomes unavailable to plants (Nautiyal, 1999; Rodriguez and Fraga, 1999). Solubilization of P in the rhizosphere is the most

common way used by PGPR to increase nutrient availability to their host plant (Richardson, 2001). Phosphate-solubilizing bacteria are common in the rhizosphere (Nautiyal *et al.*, 2000; Vazquez *et al.*, 2000) and they use secretion of organic acids and phosphatases to convert phosphate to a plant available form (Kim *et al.*, 1998). Recent studies focusing on the association of phosphate-solubilizing rhizospheric bacteria and their host plant included: *Azotobacter chroococcum* and wheat (Kumar and Narula, 1999); *Bacillus circulans* and *Cladosporium herbarum* with wheat (Singh and Kapoor, 1999), *Bacillus* sp. and five crop species (Pal, 1998); *Enterobacter agglomerans* and tomato (Kim *et al.*, 1998); *Pseudomonas chlororaphis* and *P. putida* with soybean (Cattelan *et al.*, 1999); *Rhizobium* sp. and *Bradyrhizobium japonicum* with radish (Antoun *et al.*, 1998); and *Rhizobium leguminosarum* bv. *phaseoli* and maize (Chabot *et al.*, 1998).

Endophytic bacteria have also been reported to solubilize immobilized mineral phosphate. Rodriguez and Fraga, and Verma and colleagues suggested that during initial colonization, endophytic bacteria could enhance phosphate availability to soybean plants (Rodriguez and Fraga, 1999; Verma *et al.*, 2001). Results from Kuklinsky-Sobral and colleagues supported this suggestion (Kuklinsky-Sobral *et al.*, 2004), showing that 52% of the endophytic bacteria isolated from soybean could solubilize mineral phosphate.

PHYTOHORMONES

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

IAA production is common in plant-associated bacteria and, depending on the bacteria, the IAA production seems to follow different pathways. In the case of phytopathogenic bacteria, IAA is, in general, produced from tryptophan via the intermediate indoleacetamide. In plant beneficial bacteria, IAA is predominantly

synthesized via indolepyruvic acid (Manulis *et al.*, 1998; Patten and Glick, 2002).

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

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

COUNTERACTING STRESS-INDUCED ETHYLENE

Ethylene is a phytohormone involved in different plant processes ranging from seed germination, cell differentiation, stress responses, flowering induction, fruit ripening, and senescence. It induces decreased membrane fluidity, influences the turnover of phospholipids in membranes, enhances the leakage of solutions from plant cells, and decreases root elongation (Mayak *et al.*, 2004). Ethylene release is known to be increased in plants exposed to both abiotic and biotic environmental stress conditions, such as heavy metals, flooding, drought, infection by fungal or bacterial pathogens, and is synthesized from L-methionine via S-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC), which is the immediate precursor of ethylene.

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

Ethylene is also a component of the signalling pathway controlling the rhizobial infection in legumes. A correlation was observed between decreased ethylene production and increased nodulation levels (Ma *et al.*, 2004). Low ethylene levels are required for entry of infection threads into the outermost layer of cortical cells by allowing proper disposition of cytoskeleton and formation of cytoplasmic bridges. On the other hand, high ethylene concentration will induce the abortion of infection threads. For instance, in alfalfa, about 95–99% of infection thread progressions abort in the cortex (Vasse *et al.*, 1993). The infection threads containing ACC-deaminase-producing bacteria can better suppress the defence signals in the plant cells by

decreasing ethylene levels, thus increasing the persistence of infection threads. ACC-deaminase activity has been found in several rhizobial strains, including *Rhizobium leguminosarum* bv. *viciae* strains, *Rhizobium hedysari* (Ma *et al.*, 2003), and *Mesorhizobium loti* (Kaneko *et al.*, 2000; Sullivan *et al.*, 2002). Ma and colleagues found a 40% increase in nodulation numbers in alfalfa plants inoculated with an engineered ACC-deaminase overproducing *S. meliloti* strain (Ma *et al.*, 2004), compared to plants inoculated with the wild-type strain. An investigation conducted by Donate-Correa and colleagues on the rhizosphere microbial population associated with *Chamaecytisus proliferus* (tagasaste) reported that 50% of the isolated *Pseudomonas* strains possessed ACC-deaminase activity; moreover, they observed an increase in nodule numbers in co-inoculation experiments run with the *Bradyrhizobium* strain BTA-1 (Donate-Correa *et al.*, 2004). These experiments resulted in a positive effect on root elongation, plant growth, and nodulation levels, and underline the importance of the bacterial influence on plant growth and yield production. An alternative strategy to overcome high ethylene levels was found for *Bradyrhizobium elkanii*, which produces rhizobitoxine, an ethylene biosynthesis inhibitor: rhizobitoxine production by *B. elkanii* decreases ethylene production in plant roots and enhances nodulation on *M. atropurpureum* Urb. cv. Siratro (Yuhashi *et al.*, 2000).

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

INDIRECT PLANT GROWTH-PROMOTING ACTIVITY

PGPR, as well as endophytic bacteria, seem to be able to lessen or prevent the deleterious effects of phytopathogenic organisms through niche competition, antibiosis, and induced systemic resistance (ISR) in plant. This ability can be considered as an indirect way to promote plant growth. For instance, treatment of maize seeds with a culture of *Burkholderia cepacia*, isolated from the rhizosphere of maize, resulted in disease suppression and growth promotion (Bevivino *et al.*, 1998). Similar observations were made by Hebbar and colleagues, who described an antag-

onistic effect of *B. cepacia* against *Fusarium moniliforme*, a soil-borne fungal pathogen of maize (Hebbar *et al.*, 1992a,b). From healthy wheat, Coombs and Franco could isolate and characterize endophytic actinobacteria, organisms responsible for the production of two-thirds of the microbially derived antibiotics involved in plant pathogen defence (Coombs and Franco, 2003a).

Another way that endophytic bacteria can control pathogen proliferation is via iron control. They can excrete organic compounds (siderophores) that are able, with very high affinity, to bind the Fe^{3+} that is available in the rhizosphere, and as a result, prevent the proliferation of pathogens (O'Sullivan and O'Gara, 1992). Evidence for this mechanism comes from several studies, including a report where a mutant strain of *Pseudomonas aeruginosa*, deficient in siderophore production, no longer protected tomato plants against *Pythium* (Buysens *et al.*, 1994). Generally, *Pseudomonas* sp. have been considered to play a major role in disease inhibition through the production of siderophores and, thanks to this efficient iron uptake mechanism, they can quickly colonize the rhizosphere, and thus physically displace deleterious organisms. Strains of *Pseudomonas fluorescens* were investigated recently for biocontrol against tomato spotted wilt virus (TSWV) in tomato. Analyses indicated a reduction in viral antigen concentration in *P. fluorescens*-treated tomato plants corresponding with a reduced disease rating. Moreover, all *P. fluorescens*-treated tomato plants showed enhanced growth and yield, compared to the control plants (Kandan *et al.*, 2005). A more general effect was observed after application of dialysates from *Bacillus cereus* isolated from the rhizosphere of healthy tomato plants. The plants treated in this way were protected against leaf fungal and bacterial pathogens, emphasizing that macromolecules synthesized by PGPR and released into the environment can act as elicitors of systemic resistance (Romeiro *et al.*, 2004). Interesting results were also achieved with rust-affected *Pinus taeda* L. *fusiform*. This infection is caused by *Cronartium quercuum* f. sp. *fusiform* and it is the most damaging stem disease for *Pinus* spp. in the southern United States. After treating *Pinus* seeds with three different bacterial strains, the seedlings analysed at the end of one season showed reduced rust gall infections and also a higher biomass production compared to untreated control plants (Enebak and Carey, 2004).

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

Application of endophytic bacteria to improve phytoremediation

The potential that endophytes have to offer for agricultural gains has been realized beyond their use as natural biocontrol agents. The inherent nature of certain endophytes to potentially colonize plants in a systemic manner provides a novel

approach as a delivery system to plants for various beneficial traits. This paper specifically addresses the potential of endophytic bacteria to improve phytoremediation of heavy metals and organic contaminants.

PHYTOREMEDIATION

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

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

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

ENDOPHYTE-ASSISTED PHYTOREMEDIATION OF ORGANIC CONTAMINANTS

The fate of contaminants in the rhizosphere–root system largely depends on its physicochemical properties. Organic xenobiotics with a log K_{ow} <1 are considered to be very water-soluble, and plant roots generally do not accumulate them at a rate surpassing passive influx into the transpiration stream (Cunningham and Berti, 1993). Contaminants with a log K_{ow} >3.5 show high sorption to the roots, but slow or no translocation to the stems and leaves (Trapp *et al.*, 2001). However, plants readily

take up organic xenobiotics with a log K_{ow} between 0.5 and 3.5, as well as weak electrolytes (weak acids and bases or amphoteres as herbicides). These compounds seem to enter the xylem faster than the soil and rhizosphere microflora can degrade them, even if the latter is enriched with degrader bacteria (Trapp *et al.*, 2000). Once taken up, plants metabolize these contaminants, although some of them, or their metabolites, such as TCE that is transformed into TCA, can be toxic (Doucette *et al.*, 1998). Alternatively, plants preferentially release volatile pollutants, such as BTEX compounds and TCE and their metabolites, into the environment by evaporation via the leaves, which questions the merits of phytoremediation (Burken and Schnoor, 1999; van der Lelie *et al.*, 2001; Schwitzguébel *et al.*, 2002; Ma and Burken, 2003).

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

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

HORIZONTAL GENE TRANSFER TO THE ENDOGENOUS ENDOPHYTIC COMMUNITY

Although the application of engineered endophytic bacteria to improve phytoremediation of volatile organic contaminants has several obvious advantages over the application of engineered rhizosphere bacteria or the genetic engineering of the plant's metabolism, several obstacles have to be overcome before this technology can move towards application (Newman and Reynolds, 2005). One major point of concern is the persistence and stability of the engineered organisms and their degradation capabilities in field-grown plants, as phytoremediation projects can conceivably last decades. As long as a selection pressure is present, there will be an advantage for those endophytic community members possessing the appropriate degradation characteristics. However, this is no guarantee that strains of the inoculum will become an integrated part of the endogenous endophytic community. While marked endophytic strains, isolated from the endogenous endophytic community

from poplar, were successfully used to re-inoculate their original host plants (Germaine *et al.*, 2004), attempts to inoculate poplar with a toluene-metabolizing *Burkholderia cepacia* strain that has yellow lupine as its natural host seemed unsuccessful: ten weeks after inoculation, the inoculum no longer could be detected among the cultivable bacteria (Taghavi *et al.*, 2005). However, despite the absence of the inoculum, improved toluene degradation and a reduction of toluene phytotoxicity and release via evapotranspiration were observed. Horizontal gene transfer of the pTOM-Bu61 encoded toluene-*ortho*-monooxygenase had occurred from *Burkholderia cepacia* to different members of the endogenous endophytic community, both in the presence and absence of toluene, allowing the endogenous endophytic communities to adapt and to deal with the toluene contamination.

Horizontal gene transfer has been shown to play an important role in rapidly adapting a microbial community to a new environmental stress factor (Dong *et al.*, 1998), including rhizosphere communities (van Elsas *et al.*, 1998; Ronchel *et al.*, 2000; Devers *et al.*, 2005), and we had speculated that it could play an important role in adapting the endogenous endophytic community (van der Lelie *et al.*, 2005): rather than integrating a new bacterium in a stable community, the degradation pathway is transferred among the members of the community. It can be hypothesized that horizontal gene transfer also played a role in the selective enrichment of catabolic genotypes observed by Siciliano and colleagues (Siciliano *et al.*, 2001). This enrichment in the root interior was found to be both plant and contaminant dependent: selective enrichment for endophytic bacteria containing the appropriate catabolic genes was found for plants grown on petroleum hydrocarbon contaminated sites, on field sites contaminated with nitroaromatics, and after the addition of petroleum to sediment. Similarly, the numbers of *alk*B- or *ndo*B-positive endophytes in *Festuca arundinacea* were correlated with the concentration of creosote in the soil, but not with the numbers of *alk*B- or *ndo*B-positive bacteria in the bulk soil.

BACTERIAL ENHANCED PHYTOREMEDIATION OF HEAVY METALS

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

Metal availability in soils plays an important role in the efficiency of the

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

Since, in many cases, metal uptake by plants is limited by low metal solubility, it is necessary that the efforts for selection of appropriate rhizosphere manipulation be continued. There is a need to find cheaper, environmentally benign chemical compounds with chelating properties, as well as to better understand the role of rhizospheric bacteria in metal solubility, plant uptake, and tolerance. The plant–rhizosphere interactions controlling metal uptake by roots are, indeed, of primary interest. It is necessary to identify which are the main limiting factors and to find appropriate solutions to overcome them. There is some evidence that diffusion of metals is such a limitation that, even in moderately contaminated soils, mass flow contribution is less than 10% of total metal uptake (McGrath *et al.*, 2001). The diffusion rate in soil generally depends on metal availability in soil solution and, on the other hand, on the concentration gradient driven by metal ions' uptake by roots. It was found that roots of *T. caerulescens* responded positively to Zn and Cd supply (Whiting *et al.*, 2000), but not to enhanced metal solubility by changes in rhizosphere pH (Knight *et al.*, 1997). To what extent root exudates can mobilize metals (as was shown for Fe and possibly Zn: Marschner, 1995) or if microbial rhizosphere communities stimulated by these root exudates (Anderson, 1997) can contribute to metal phytoavailability, remains to be further examined. As certain plants can use microbial siderophores to improve their iron uptake, it has been hypothesized that bacterial metal chelators, such as siderophores, can eventually improve the uptake of heavy metals by plants (van der Lelie *et al.*, 1999). Bacterial siderophores can be considered as natural chelators, the bacterial production of which is in tight equilibrium with plant activity, thus improving heavy metal uptake and translocation as part of the phytoextraction process. Another possibility that should be considered is the use of plant growth-promoting bacteria that stimulate root formation by plants. A better-developed root system can lead to increased metal uptake.

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

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

Other studies conducted on non-hyperaccumulator plant species and their associated bacteria confirmed the possibility of an enhanced plant metal uptake in the case of plant–bacteria interactions. When grown on soil contaminated with lead, Indian mustard (*Brassica juncea*), a high-biomass-producing, metal-tolerant plant species, showed hyperaccumulation of the metal in its roots. At the same time, enrichment of Pb- and Cu-resistant *Arthrobacter* species occurred in the rhizosphere (Roy *et al.*, 2005). Chen and colleagues isolated Cu tolerant bacteria from the rhizosphere of *Elsholtzia splendens*, a copper accumulator plant generally growing on copper mines (Chen *et al.*, 2005). The re-inoculation of these strains in the plant rhizosphere resulted in an increased water copper concentration and facilitated Cu accumulation by the plants. This role of the bacteria was confirmed using ampicillin, an antibiotic that inhibits microbial activity: in the presence of the antibiotic, the added bacteria failed to exert their mobilizing effect on Cu. It was concluded that the strains used as inoculum were able to excrete low molecular weight compounds that increased heavy metal availability to their host plants.

Conclusions

Endophytic bacteria are a very challenging field of research, both from a fundamental and applied perspective. The first attempts to use endophytic bacteria for the improvement of pest control or phytoremediation processes are promising, but considerable research efforts are required to optimize the practical applications. More knowledge is required on the population dynamics and activity of endophytic bacteria in their host plants. Also, the effects of contaminants on these processes should be further evaluated. For the construction of endophytic strains with new catabolic functions, natural gene transfer offers great potential. Many catabolic functions are found in soil bacteria, where they are encoded on self-transferrable plasmids or transposons. These can be transferred easily using natural gene transfer. Heterologous expression of the catabolic functions might not be a major problem, especially when the donor strain and the recipient endophytic strain are closely related, as is frequently the case.

A considerable research effort will be required also to design strategies for the reinoculation of endophytic bacteria under field conditions. In order to guarantee reproducibility, reliable methods of inoculum delivery should be developed. This is especially the case for the inoculation of trees such as poplar with endophytic bacteria. Intense testing of different delivery systems indicated that the application method for introducing endophytic bacteria into plant tissue is strain specific (Musson *et al.*, 1995). Some methods that proved to be successful include the infusion of a bacterial suspension into imbibed seeds (Turner *et al.*, 1993) or bacterial application via alginate beads (Bashan, 1986), which have the advantage of adding bacteria-specific nutrients to the alginate to improve bacterial survival

rates. It should be noted that the development of successful application technologies would fully depend on improving our understanding of how bacterial endophytes enter and colonize plants. This remark could be applied to all aspects of the ecology of bacterial endophytes and only under those circumstances can the potential use of bacterial endophytes for plant beneficial purposes be fully evaluated.

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

We also need to consider the potential role of naturally occurring endophytes, and what their role in phytoremediation may be. We know that endophytes have multiple roles in plants, but understanding what that role may be in remediation has not been explored in any depth. Work by van Aken and colleagues showed that an endophytic *Methylobacterium populum* sp. isolated from poplar had the ability to degrade TNT in the laboratory (van Aken *et al.*, 2004a,b). However, the role of this bacterium, and the extent of its contribution to TNT degradation within the intact plant system, is still being studied.

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

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References

ABOU-SHANAB, R.I., DELORME, T.A., ANGLE, J.S. *ET AL.* (2003). Phenotypic characterization of microbes in the rhizosphere of *Alyssum murale*. *International Journal of Phytoremediation* **5**, 367–379.

AMANN, R.I., LUDWIG, W. AND SCHLEIFER, K.H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiology Ecology* **59**, 143–169.

ANDERSON, T. (1997). Development of a phytoremediation handbook: considerations for enhancing microbial degradation in the rhizosphere. Internet communication. http://es.epa.gov/nceya/ru/index.html

ANTOUN, H., BEAUCHAMP, C.J., GOUSSARD, N., CHABOT, R. AND LALANDE, R. (1998). Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: effect on radishes (*Raphanus sativus* L.). *Plant and Soil* **204**, 57–67.

ARAÚJO, W.L., MARCON, J., MACCHERONI, W., JR., VAN ELSAS, J.D., VAN VUURDE, J.W.L. AND AZEVEDO, J.L. (2002). Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. *Applied and Environmental Microbiology* **68**, 4906–4914.

ARSHAD, M. AND FRANKENBERGER, W.T. (1991). Microbial production of plant hormones. *Plant Soil* **113**, 1–8.

ASHBOLT, N.J. AND INKERMAN, P.A. (1990). Acetic acid bacterial biota of the pink sugar cane mealybug, *Saccharococcus sacchari*, and its environs. *Applied and Environmental Microbiology* **57**, 707–712.

BAKER, A.J.M. (1981). Accumulators and excluders: strategies in the response of plants to heavy metal. *Journal of Theoretical Biology* **3**, 643–654.

BAKER, A.J.M., MCGRATH, S.P., REEVES, R.D. AND SMITH, J.A.C. (2000). Metal hyperaccumulator plants: a review of the ecology and physiology of a biochemical resource for phytoremediation of metal-polluted soils. In: *Phytoremediation of contaminated soil and water*. Eds. N. Terry and G. Banuelos, pp 85–107. Boca Raton, USA: Lewis Publishers.

BARAC, T., TAGHAVI, S., BORREMANS, B. *ET AL.* (2004). Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. *Nature Biotechnology* **22**, 583–588.

BARAZANI, O. AND FRIEDMAN, J. (1999). Is IAA the major root growth factor secreted from plant-growth-mediating bacteria? *Journal of Chemical Ecology* **25**, 2397–2406.

BASHAN, Y. (1986). Alginate beads as synthetic inoculant carriers for slow release of bacteria that affect plant growth. *Applied and Environmental Microbiology* **51**, 1089–1098.

BASHAN, Y. AND HOLGUIN, G. (1997). *Azospirillum*–plant relationships: environmental and physiological advances (1990–1996). *Canadian Journal of Microbiology* **43**, 103–121.

BASTIÁN, F., COHEN, A., PICCOLI, P., LUNA, V., BARALDI, R. AND BOTTINI, R. (1998). Production of indole-3-acetic acid and gibberellins A1 and A3 by *Acetobacter diazotrophicus* and *Herbaspirillum seropendicae* in chemically-defined culture media. *Plant Growth Regulation* **24**, 7–11.

BELIMOV, A.A., HONTZEAS, N., SAFRONOVA, V.I. *ET AL.* (2005). Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). *Soil Biology and Biochemistry* **37**, 241–250.

BENHAMOU, N., KLOEPPER, J.W., QUADT-HALLMANN, A. AND TUZUN, S. (1996a). Induction of defense related ultrastructural modifications in pea root tissues inoculated with endophytic bacteria. *Plant Physiology* **112**, 919–929.

BENHAMOU, N., BÉLANGER, R.R. AND PAULITZ, T.C. (1996b). Pre-inoculation of Ri T-DNA-transformed pea roots with *Pseudomonas fluorescens* inhibits colonization by *Phythium ultimum* Trow: an ultrastructural and cytochemical study. *Planta* **199**, 105–117.

BENT, E., TUZUN, S., CHANWAY, C.P. AND ENEBAK, S. (2001). Alterations in plant growth and root hormone levels of lodgepole pines inoculated with rhizobacteria. *Canadian Journal of Microbiology* **47**, 793–800.

BEVIVINO, A., SARROCCO, S., DALMASTRI, C., TABACCHIONI, C., CANTALE, C. AND CHIARINI, L. (1998). Characterization of a free-living maize-rhizosphere population of *Burkholderia cepacia*: effect of seed treatment on disease suppression and growth promotion of maize. *FEMS Microbiology Ecology* **27**, 225–237.

BODDEY, R.M., DE OLIVEIRA, O.C., URQUIAGA, S. *ET AL.* (1995a). Biological nitrogen fixation associated with sugar cane and rice: contribution and prospects for improvement. *Plant and Soil* **174**, 195–209.

BODDEY, R.M., REIS, V.M., URQUIAGA, S. *ET AL.* (1995b). N_2 fixation in sugar cane: the role of *Acetobacter diazotrophicus*. In: *Nitrogen fixation: fundamentals and applications*. Eds. I.A. Tikhonovich, N.A. Rovorov and W.E. Newton, pp 641–646. Dordrecht, The Netherlands: Kluwer Academic.

BROOKS, D.S., GONZALEZ, C.F., APPEL, D.N. AND FILER, T.H. (1994). Evaluation of endophytic bacteria as potential biological control agents for oak wilt. *Biological Conservation* **4**, 373–381.

BURKEN, J.G. AND SCHNOOR, J.L. (1999). Distribution and volatilization of organic compounds following uptake by hybrid poplar trees. *International Journal of Phytoremediation* **1**, 139–151.

BUYSENS, S., POPPE, J. AND HÖFTE, M. (1994). Role of siderophores in plant growth stimulation and antagonism by *Pseudomonas aeruginosa* 7NSK2. In: *Improving plant productivity with rhizosphere bacteria*. Eds. M.H. Ryder, P.M. Stephens and G.D. Bowen, pp 139–141. Adelaide, Australia: Commonwealth Scientific and Industrial Research Organization.

CANKAR, K., KRAIGHER, H., RAVNIKAR, M. AND RUPNIK, M. (2005). Bacterial endophytes from seeds of Norway spruce (*Picea abies* L. Karst). *FEMS Microbiology Letters* **244**, 341–345.

CATTELAN, A.J., HARTEL, P.G. AND FUHRMANN, J.J. (1999). Screening for plant growth-promoting rhizobacteria to promote early soybean growth. *Soil Science Society of America Journal* **63**, 1670–1680.

CAVALCANTE, V.A. AND DÖBEREINER, J. (1988). A new acid-tolerant nitrogen-fixing bacterium associated with sugar cane. *Plant and Soil* **108**, 23–31.

CHABOT, R., BEAUCHAMP, C.J., KLOEPPER, J.W. AND ANTOUN, H. (1998). Effect of phosphorus on root colonization and growth promotion of maize by bioluminescent mutants of phosphate-solubilizing *Rhizobium leguminosarum* biovar *phaseoli*. *Soil Biology and Biochemistry* **30**, 1615–1618.

CHEN, C., BANSKE, E.M., MUSSON, G. AND KLOEPPER, J.W. (1994). Biological control potential and population dynamics of endophytic bacteria in a cotton/*Fusarium* wilt system. In: *Improving plant productivity with rhizosphere bacteria*. Eds. M.H. Ryder, P.M. Stephens and G.D. Bowen, pp 191–193. Australia: CSIRO.

CHEN, Y.X., WANG, Y.P., LIN, Q. AND MING, Y. (2005). Effect of copper-tolerant rhizosphere bacteria on mobility of copper in soil and copper accumulation by *Elsholtzia splendens*. *Environment International* **131**, 861–866.

CONN, V.M. AND FRANCO, C.M.M. (2004). Effect of microbial inoculants on the indigenous actinobacterial endophyte population in the roots of wheat as determined by terminal restriction fragment length polymorphism. *Applied and Environmental Microbiology* **70**, 6407–6413.

COOMBS, J.T. AND FRANCO, C.M.M. (2003a). Isolation and identification of actinobacteria from

surface-sterilized wheat roots. *Applied and Environmental Microbiology* **69**, 5603–5608.

COOMBS, J.T. AND FRANCO, C.M.M. (2003b). Visualization of an endophytic *Streptomyces* species in wheat seed. *Applied and Environmental Microbiology* **69**, 4260–4262.

CUNNINGHAM, S.D. AND BERTI, W.B. (1993). Remediation of contaminated soils with green plants: an overview. *In Vitro Cellular and Developmental Biology* **29**, 207–212.

DALTON, D., KRAMER, S., AZIOS, N., FUSARO, S., CAHILL, E. AND KENNEDY, C. (2004). Endophytic nitrogen fixation in dune grasses (*Ammophila arenaria* and *Elymus mollis*) from Oregon. *FEMS Microbiology Ecology* **49**, 469–479.

DE SALAMONE, I.E.G., HYNES, R.K. AND NELSON, L.M. (2001). Cytokinin production by plant growth promoting rhizobacteria and selected mutants. *Canadian Journal of Microbiology* **47**, 404–411.

DELL'AMICO, E., CAVALCA, L. AND ANDREONI, V. (2005). Analysis of rhizobacterial communities in perennial *Graminaceae* from polluted water meadow soil, and screening of metal-resistant, potentially plant growth-promoting bacteria. *FEMS Microbiology Letters* **52**, 153–162.

DELORME, T.A., GAGLIARDI, J.V., ANGLE, J.S. AND CHANEY, R.L. (2001). Influence of the zinc hyperaccumulator *Thlaspi caerulescens* J. & C. Presl. and the non-metal accumulator *Trifolium pratense* L. on soil microbial populations. *Canadian Journal of Microbiology* **47**, 773–776.

DEVERS, M., HENRY, S., HARTMANN, A. AND MARTIN-LAURENT, F. (2005). Horizontal gene transfer of atrazine-degrading genes (atz) from *Agrobacterium tumefaciens* St96-4 pADP1::Tn5 to bacteria of maize-cultivated soil. *Pest Management Science* **61**, 870–880.

DI FIORI, S. AND DEL GALLO, M. (1995). Endophytic bacteria: their possible role in the host plant. In: Azospirillum *VI and related organisms*. Eds. I. Fendrik, M. del Gallo, J. Vanderleyden and M. de Zamaroczy. Berlin, Heidelberg, Germany: Springer Verlag.

DÖBEREINER, J. AND DAY, J.M. (1976). Associative symbioses in tropical grasses: characterization of microorganisms and nitrogen-fixing sites. In: *Proceedings of the 1st International Symposium on Nitrogen Fixation*. Eds. W.E. Newton and C.J. Nyman, pp 513–537. Pullman, USA: Washington State University Press.

DÖBEREINER, J., REIS, V.M. AND LAZARINI, A.C. (1998). New nitrogen-fixing bacteria in association with cereals and sugar cane. In: *Nitrogen fixation: hundred years after*. Eds. F.J. de Bruijn and W.E. Newton, pp 717–722. Stuttgart, Germany: Gustav Fischer.

DONATE-CORREA, J., LEÚN-BARRIOS, M. AND PÉREZ-GALDONA, R. (2004). Screening for plant growth-promoting rhizobacteria in *Chamaecytisus proliferus* (tagasaste), a forage tree-shrub legume endemic to the Canary Islands. *Plant and Soil* **266**, 261–272.

DONG, Q., SPRINGEAL, D., SCHOETERS, J., NUYTS, G., MERGEAY, M. AND DIELS, L. (1998). Horizontal transfer of bacterial heavy metal resistance genes and its applications in activated sludge systems. *Water Science and Technology* **37**, 465–468.

DOUCETTE, W.J., BUGBEE, B., HAYHURST, S. *ET AL.* (1998). Phytoremediation of dissolved phase trichloroethylene using mature vegetation. In: *Bioremediation and phytoremediation: chlorinated and recalcitrant compounds*. Eds. G.B. Wickramanayake and R.E. Hinchee, pp 251–256. Columbus, USA: Battelle Press.

DUIJFF, B.J., GIANINAZZI-PEARSON, V. AND LEMANCEAU, P. (1997). Involvement of the outer membrane lipopolysaccharides in the endophytic colonization of tomato roots by biocontrol *Pseudomonas fluorescens* strain WCS417r. *New Phytologist* **135**, 325–334.

EGAMBERDIYEVA, D. AND HÖFLICH, G. (2004). Effect of plant growth-promoting bacteria on growth and nutrient uptake of cotton and pea in a semi-arid region of Uzbekistan. *Journal of Arid Environments* **56**, 293–301.

ENEBAK, S.A. AND CAREY, W.A. (2004). Plant growth-promoting rhizobacteria may reduce fusiform rust infection in nursery-grown loblolly pine seedlings. *Southern Journal of Applied Forestry* **28**, 185–188.

ENGELHARD, M., HUREK, T. AND REINHOLD-HUREK, B. (2000). Preferential occurrence of diazotrophic endophyte, *Azoarcus* sp., in wild rice species and land race of *Oryza sativa* in comparison with modern races. *Environmental Microbiology* **2**, 131–141.

FALLIK, E., SALIG, E. AND OKON, Y. (1994). Morphology and physiology of plant roots inoculated with *Azospirillum*. In: Azospirillum/*plant associations*. Ed. Y. Okon, pp 77–86. Boca Raton, FL, USA: CRC Press.

FISHER, P.J., PETRINI, O. AND LAPPIN, S.H.M. (1992). The distribution of some fungal and bacterial endophytes in maize (*Zea mays* L.). *New Phytologist* **122**, 299–305.

FRANKE, I.H., FEGAN, M., HAYWARD, C., LEONARD, G. AND SLY, L.I. (2000). Molecular detection of *Gluconacetobacter sacchari* associated with the pink sugar cane mealybug *Saccharicoccus sacchari* (Cockerell) and the sugar cane leaf sheath microenvironment by FISH and PCR. *FEMS Microbiology Ecology* **31**, 61–71.

FROMMEL, M.I., NOWAK, J. AND LAZAROVITS, G. (1991). Growth enhancement and developmental modifications of *in vitro* grown potato (*Solanum tuberosum* ssp. *Tuberosum*) as affected by a nonfluorescent *Pseudomonas* sp. *Plant Physiology* **96**, 928–936.

GAGE, D.J., BOBO, T. AND LONG, S.R. (1996). Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). *Journal of Bacteriology* **178**, 7159–7166.

GAGNÉ, S., RICHARD, C., ROUSSEAU, H. AND ANTOUN, H. (1987). Xylem residing bacteria in alfalfa roots. *Canadian Journal of Microbiology* **33**, 996–1000.

GARDNER, J.M., FELDMAN, A.W. AND ZABLOTOWICZ, M. (1982). Identity and behavior of xylem-residing bacteria in rough lemon roots of Florida citrus trees. *Applied and Environmental Microbiology* **43**, 1335–1342.

GERMAINE, K., KEOGH, E., BORREMANS, B. *ET AL.* (2004). Colonization of Poplar trees by *gfp* expressing bacterial endophytes. *FEMS Microbiology Ecology* **48**, 109–118.

GLASS, A.D. (1989). *Plant nutrition: an introduction to current concepts*. Boston, MA, USA: Jones and Bartlett.

GLICK, B.R. (1995). The enhancement of plant growth by free-living bacteria. *Canadian Journal of Microbiology* **41**, 109–117.

GUTIERREZ-MANERO, F.J., RAMOS-SOLANO, B., PROBANZA, A., MEHOUACHI, J., TADEO, F.R. AND TALON, M. (2001). The plant growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillis licheniformis* produce high amounts of physiologically active gibberellins. *Physiologia Plantarum* **111**, 206–211.

GUTIERREZ-ZAMORA, M.L. AND MARTINEZ-ROMERO, E. (2001). Natural endophytic association between *Rhizobium etli* and maize (*Zea mays* L.). *Journal of Biotechnology* **91**, 117–126.

HALLMANN, J., KLOEPPER, J.W., RODRIGUEZ-KABANA, R. AND SIKORA, R.A. (1995). Endophytic rhizobacteria as antagonists of *Meloidogyne incognita* on cucumber. *Phytopathology* **85**, 1136.

HALLMANN, J., QUADT-HALLMANN, A., MILLER, W.G., SIKORA, R.A. AND LINDOW, S.E. (2000). Endophytic colonization of plants by the biocontrol agent *Rhizobium etli* G12 in relation to *Meloidogyne incognita* infection. *Phytopathology* **91**, 415–422.

HASNAIN, S. AND SABRI, A.N. (1996). Growth stimulation of *Triticum aestivum* seedlings under Cr-stress by non-rhizospheric pseudomonas strains. *Environmental Pollution* **97** (3), 265–273.

HEBBAR, K.P., DAVEY, A.G., MERRIN, J. AND DART, P.J. (1992a). Rhizobacteria of maize antagonistic to *Fusarium moniliforme*, a soil-borne fungal pathogen: colonization of rhizosphere and roots. *Soil Biology and Biochemistry* **24**, 989–997.

HEBBAR, K.P., DAVEY, A.G., MERRIN, J., MCLOUGHLIN, T.J. AND DART, P.J. (1992b). *Pseudomonas cepacia*, a potential suppressor of maize soil-borne diseases—seed inoculation and maize root colonization. *Soil Biology and Biochemistry* **24**, 999–1007.

HENDRICKX, B., JUNCA, H., VOSAHLOVA, J. *ET AL.* (2006). Alternative primer sets for PCR detection of genotypes involved in bacterial aerobic BTEX degradation: distribution of the genes in BTEX degrading isolates and in subsurface soils of a BTEX contaminated industrial site. *Journal of Microbiological Methods* **64**, 250–265.

HERRERA, M.A., SALAMANKA, C.P. AND BAREA, J.M. (1993). Inoculation of woody legumes with selected arbuscular mycorrhizal fungi and rhizobia to recover desertified Mediterranean ecosystems. *Applied and Environmental Microbiology* **59**, 129–133.

HINTON, D.M. AND BACON, C.W. (1995). *Enterobacter cloacae* is an endophytic symbiont of corn. *Mycopathologia* **129**, 117–125.

HIROTSUNE, S., HATADA, I., KOMATSUBARA, H. *ET AL.* (1992). New approach for detection of amplification in cancer DNA using restriction landmark genomic scanning. *Cancer Research* **52**, 3642–3647.

HÖFLICH, G., WIEHE, W. AND KÜHN, G. (1994). Plant growth stimulation by inoculation with symbiotic and associative rhizosphere mechanisms. *Experientia* **50**, 897–905.

HOLLIS, J.P. (1951). Bacteria in healthy potato tissue. *Phytopathology* **41**, 350–366.

HONG, Y., PASTERNAK, J.J. AND GLICK, B.R. (1991). Biological consequences of plasmid transformation of the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. *Canadian Journal of Microbiology* **37**, 796–799.

HUREK, T., REINHOLD-HUREK, B., VAN MONTAGU, M. AND KELLENBERGER, E. (1994). Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. *Journal of Bacteriology* **176**, 1913–1923.

JACOBS, M.J., BUGBEE, W.M. AND GABRIELSON, D.A. (1985). Enumeration, location, and characterization of endophytic bacteria within sugar beet roots. *Canadian Journal of Botany* **63**, 1262–1265.

JAMES, E.K., REIS, V.M., OLIVARES, F.L., BALDANI, J.I. AND DÖBEREINER, J. (1994). Infection of sugar cane by the nitrogen fixing bacterium *Acetobacter diazotrophicus*. *Journal of Experimental Botany* **45**, 757–766.

JAMES, K. AND OLIVARES, F.L. (1997). Infection and colonization of sugar cane and other *Graminaceous* plants by endophytic diazotrophs. *Critical Reviews in Plant Sciences* **17**, 77–119.

KADO, C.I. (1992). Plant pathogenic bacteria. In: *The prokaryotes*. Eds. A. Ballows, H.G. Trüper, M. Dworkin, W. Harder and K.-H. Schleifer, pp 660–662. New York, USA: Springer-Verlag.

KANDAN, A., RAMIAH, M., VASANTHI, V.J. *ET AL.* (2005). Use of *Pseudomonas fluorescens*-based formulations for management of tomato spotted wilt virus (TSWV) and enhanced yield in tomato. *Biocontrol Science and Technology* **15**, 553–569.

KANEKO, T., NAKAMURA, Y., SATO, S. *ET AL.* (2000). Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. *DNA Research* **7**, 331–338.

KAUSHIK, R., SAXENA, A.K. AND TILAK, K.V.B.R. (2000). Selection of Tn*5*::lacZ mutant isogenic to wild-type *Azospirillum brasilense* strains capable of growing at sub-optimal temperatures. *World Journal of Microbiology and Biotechnology* **16**, 567–570.

KENNEDY, I.R. AND TCHAN, Y.T. (1992). Biological nitrogen fixation in non-leguminous field crops: recent advances. *Plant and Soil* **141**, 93–118.

KHAMMAS, K.M. AND KAISER, P. (1991). Characterization of a pectinolytic activity in *Azospirillum irakense*. *Plant and Soil* **137**, 75–79.

KIM, K.Y., JORDAN, D. AND MCDONALD, G.A. (1998). Effect of phosphate-solubilizing bacteria and vesicular-arbuscular mycorrhizae on tomato growth and soil microbial activity. *Biology and Fertility of Soils* **26**, 79–87.

KLOEPPER, J.W. AND BEAUCHAMP, C.J. (1992). A review of issues related to measuring colonization of plant roots by bacteria. *Canadian Journal of Microbiology* **38**, 1219–1232.

KLOEPPER, J.W., TIPPING, E.M. AND LIFSHITZ, R. (1991). Plant growth promotion mediated by bacterial rhizosphere colonizers. In: *The rhizosphere and plant growth*. Eds. D.L. Keister and P.B. Cregan, pp 315–326. Dordrecht, The Netherlands: Kluwer Academic Publishers.

KLOEPPER, J.W., SCHIPPERS, B. AND BAKKER, P.A.H.M. (1992). Proposed elimination of the term endorhizosphere. *Phytopathology* **82**, 726–727.

KLUEPFEL, D.A. (1993). The behaviour and tracking of bacteria in the rhizosphere. *Annual Review of Phytopathology* **31**, 441–472.

KNIGHT, K., ZHAO, F., MCGRATH, S.P. AND SHEN, Z. (1997). Zinc and cadmium uptake by the hyperaccumulator *Thlaspi caerulescens* in contaminated soils and its effects on the concentrations and chemical speciation of metals in soil solution. *Plant and Soil* **197**, 71–78.

KOVTUNOVYCH, G., LAR, O., KAMALOVA, S., KORDYUM, V., KLEINER, D. AND KOZYROVSKA, N. (1999). Correlation between pectase lyase activity and ability of diazotrophic *Klebsiella oxytoca* VN13 to penetrate into plant tissue. *Plant and Soil* **215**, 1–6.

KUKLINSKY-SOBRAL, J., ARAÚJO, W.L., MENDES, R., GERALDI, I.O., PIZZIRANI-KLEINER, A.A. AND AZEVEDO, J.L. (2004). Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. *Environmental Microbiology* **6**, 1244–1251.

KUMAR, V. AND NARULA, N. (1999). Solubilization of inorganic phosphates and growth

emergence of wheat as affected by *Azotobacter chroococcum* mutants. *Biology and Fertility of Soils* **28**, 211–219.

KYSELA, D.T., PALACIOS, C. AND SOGIN, M.L. (2005). Serial analysis of V6 ribosomal sequence tags (SARST-V6): a method for efficient, high-throughput analysis of microbial community composition. *Environmental Microbiology* **7**, 356–364.

LADHA, J.K. AND REDDY, P.M. (1995). Extension of nitrogen fixation to rice—necessity and possibilities. *GeoJournal* **35**, 363–372.

LALANDE, R., BISSONNETTE, N., COUTLÉE, D. AND ANTOUN, H. (1989). Identification of rhizobacteria from maize and determination of their plant-growth promoting potential. *Plant and Soil* **115**, 7–11.

LAMB, T.G., TONKYN, D.W. AND KLUEPFEL, D.A. (1996). Movement of *Pseudomonas aureofaciens* from the rhizosphere to aerial plant tissue. *Canadian Journal of Microbiology* **42**, 1112–1120.

LEIFERT, C., MORRIS, C.E. AND WAITES, W.M. (1994). Ecology of microbial saprophytes and pathogens in tissue culture and field grown plants: reasons for contamination problems *in vitro*. *Critical Reviews in Plant Sciences* **13**, 139–183.

LEVANOY, H., BASHAN, Y., ROMANO, B. AND KLEIN, E. (1989). Ultrastructural localization and identification of *Azospirillum brasilense* on and within wheat roots by immunogold labelling. *Plant and Soil* **117**, 207–218.

LODEWYCKX, C. (2001). A potential role for bacterial endophytes in phytoremediation of heavy metal contaminated soils. Ph D thesis. Limburgs Universitair Centrum, Diepenbeek, Belgium.

LODEWYCKX, C., TAGHAVI, S., MERGEAY, M., VANGRONSVELD, J., CLIJSTERS, H. AND VAN DER LELIE, D. (2001). The effect of recombinant heavy metal resistant endophytic bacteria on heavy metal uptake by their host plant. *International Journal of Phytoremediation* **3**, 173–187.

LODEWYCKX, C., MERGEAY, M., VANGRONSVELD, J., CLIJSTERS, H. AND VAN DER LELIE, D. (2002a). Isolation, characterization and identification of bacteria associated to the zinc hyperaccumulator *Thlaspi caerulescens* subsp. *calaminaria*. *International Journal of Phytoremediation* **4**, 101–115.

LODEWYCKX, C., VANGRONSVELD, J., PORTEOUS, F., MOORE, E.R.B., TAGHAVI, S. AND VAN DER LELIE, D. (2002b). Endophytic bacteria and their potential applications. *Critical Reviews in Plant Sciences* **21**, 583–606.

LOIRET, F.G., ORTEGA, E., KLEINER, D., ORTEGA-RODÉS, P., RODÉS, R. AND DONG, Z. (2004). A putative new endophytic nitrogen-fixing bacterium *Pantoea* sp. from sugar cane. *Journal of Applied Microbiology* **97**, 504–511.

MA, W., SEBESTIANOVA, S.B., SEBESTIAN, J., BURD, G.I., GUINEL, F.C. AND GLICK, B.R. (2003). Prevalence of 1-aminocyclopropane-1-carboxylate deaminase in *Rhizobia* spp. *Antonie van Leeuwenhoek* **83**, 285–291.

MA, W., CHARLES, T.C. AND GLICK, B.R. (2004). Expression of an exogenous 1-aminocyclopropane-1-carboxylate deaminase gene in *Sinorhizobium meliloti* increases its ability to nodulate alfalfa. *Applied and Environmental Microbiology* **70**, 5891–5897.

MA, X. AND BURKEN, J.G. (2003). TCE diffusion to the atmosphere in phytoremediation applications. *Environmental Science and Technology* **37**, 2534–2539.

MAHAFFEE, W.F., KLOEPPER, J.W., VAN VUURDE, J.W.L., VAN DER WOLF, J.M. AND VAN DEN BRINK, M. (1997). Endophytic colonization of *Phaseolus vulgaris* by *Pseudomonas fluorescens* strain 89B-27 and *Enterobacter asburiae* strain JM22. In: *Improving plant productivity in rhizosphere bacteria*. Eds. M.H. Ryder, P.M. Stevens and G.D. Bowen, p 180. Melbourne, Australia: CSIRO.

MALIK, K.A., RAKHSHANDA, B., MEHNAZ, S., RASUL, G., MIRZA, M.S. AND ALI, S. (1997). Association of nitrogen-fixing, plant growth promoting rhizobacteria (PGPR) with Kallar grass and rice. *Plant and Soil* **194**, 37–44.

MALINOWSKI, D.P., ZUO, H., BELESKY, D.P. AND ALLOUSH, G.A. (2004). Evidence for copper binding by extracellular root exudates of tall fescue but not perennial ryegrass infected with *Neotyphodium* spp. endophytes. *Plant and Soil* **267**, 1–12.

MANULIS, S., HAVIV-CHESNER, A., BRANDL, M.T., LINDOW, S.E. AND BARASH, I. (1998).

Differential involvement of indole-3-acetic acid biosynthetic pathways in pathogenicity and epiphytic fitness of *Erwinia herbicola* pv. *gypsophilae*. *Molecular Plant–Microbe Interactions* **11**, 634–642.

MARSCHNER, H. (1995). *Mineral nutrition of higher plants*, p 889. London, UK: Academic Press.

MARSH, T.L. (1997). Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Current Opinion in Microbiology* **2**, 323–327.

MAYAK, S., TIROSH, T. AND GLICK, B.R. (2004). Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant Physiology and Biochemistry* **42**, 565–572.

MCCULLEY, M.E. (2001). Niches for bacterial endophytes in crop plants: a plant biologist's view. *Australian Journal of Plant Physiology* **28**, 983–990.

MCGRATH, S.P. AND ZHAO, F.J. (2003). Phytoextraction of metals and metalloids from contaminated soils. *Current Opinion in Biotechnology* **14**, 277–282.

MCGRATH, S.P., ZHAO, F. AND LOMBI, E. (2001). Plant and rhizosphere processes involved in phytoremediation of metal-contaminated soils. *Plant and Soil* **232**, 207–214.

MCINROY, J.A. AND KLOEPPER, J.W. (1994). Novel bacterial taxa inhabiting internal tissue of sweet corn and cotton. In: *Improving plant productivity with rhizosphere bacteria*. Eds. M.H. Ryder, P.M. Stephens and G.D. Bowen, p 190. Australia: CSIRO.

MCINROY, J.A. AND KLOEPPER, J.W. (1995). Survey of indigenous bacterial endophytes from cotton and sweet corn. *Plant and Soil* **173**, 337–342.

MEHNAZ, S., MIRZA, M.S., HAURAT, J. *ET AL.* (2001). Isolation of 16S rRNA sequence analyses of the beneficial bacteria from the rhizosphere of rice. *Canadian Journal of Microbiology* **472**, 110–117.

MENGONI, A., BARZANTI, R., GONNELLI, C., GABBRIELLI, R. AND BAZZICALUPO, M. (2001). Characterization of nickel-resistant bacteria isolated from serpentine soil. *Environmental Microbiology* **3**, 691–698.

MENGONI, A., GRASSI, E., BARZANTI, R. *ET AL.* (2004). Genetic diversity of bacteria communities of serpentine soil and rhizosphere of the nickel-hyperaccumulator plant *Alyssum bertolonii*. *Microbiology Ecology* **48**, 209–217.

MIRZA, M.S., AHMAD, W., LATIF, F. *ET AL.* (2001). Isolation, partial characterization, and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugar cane *in vitro*. *Plant and Soil* **237**, 47–54.

MISAGHI, I.J. AND DONNDELINGER, C.R. (1990). Endophytic bacteria in symptom free cotton plants. *Phytopathology* **80**, 808–811.

MUKHOPADHYAY, N.K., GARRISON, N.K., HINTON, D.M. *ET AL.* (1996). Identification and characterization of bacterial endophytes of rice. *Mycopathologia* **134**, 151–159.

MUSSON, G., MCINROY, J.A. AND KLOEPPER, J.W. (1995). Development of delivery systems for introducing endophytic bacteria into cotton. *Biocontrol Science and Technology* **5**, 407–416.

MUYZER, G., DE WAAL, E.C. AND UITTERLINDEN, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**, 695–700.

NAUTIYAL, C.S. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiology Letters* **170**, 265–270.

NAUTIYAL, C.S., BHADAURIA, S., KUMAR, P., LAL, H., MONDAL, R. AND VERMA, D. (2000). Stress induced phosphate solubilization in bacteria isolated from alkaline soils. *FEMS Microbiology Letters* **182**, 291–296.

NEUFELD, J.D., YU, Z., LAM, W. AND MOHN, W.W. (2004). Serial analysis of ribosomal sequence tags (SARST): a high-throughput method for profiling complex microbial communities. *Environmental Microbiology* **6**, 131–144.

NEWMAN, K.L., ALMEIDA, R.P.P., PURCELL, A.H. AND LINDOW, S.E. (2003). Use of a green fluorescent strain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. *Applied and Environmental Microbiology* **69**, 7319–7327.

NEWMAN, L.A. AND REYNOLDS, C.M. (2005). Bacteria and phytoremediation: new uses for endophytic bacteria in plants. *Trends in Biotechnology* **23**, 6–8.

NOEL, T.C., SHENG, C., YOST, C.K., PHARIS, R.P. AND HYNES, M.F. (1996). *Rhizobium leguminosarum* as plant growth-promoting rhizobacterium: direct growth promotion of canola and lettuce. *Canadian Journal of Microbiology* **42**, 279–283.

OKON, Y. (1985). *Azospirillum* as a potential inoculant for agriculture. *Trends in Biotechnology* **3**, 223–228.

O'SULLIVAN, D.J. AND O'GARA, F. (1992). Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiological Reviews* **56**, 662–676.

PAL, S.S. (1998). Interactions of an acid tolerant strain of phosphate solubilizing bacteria with a few acid tolerant crops. *Plant and Soil* **198**, 169–177.

PAN, M.J., RADEMAN, S., KUNER, K. AND HASTINGS, J.W. (1997). Ultrastructural studies on the colonization of banana tissue and *Fusarium oxysporum* f. sp. *cubense* race 4 by the endophytic bacterium *Burkholderia cepacia*. *Journal of Phytopathology* **145**, 479–486.

PANDEY, P., KANG, S.C. AND MAHESHWARI, D.K. (2005). Isolation of endophytic plant growth promoting *Burkholderia* sp. MSSP from root nodules of *Mimosa pudica*. *Current Science* **89**, 177–180.

PATRIQUIN, D.G. AND DÖBEREINER, J. (1978). Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. *Canadian Journal of Microbiology* **24**, 734–742.

PATTEN, C.L. AND GLICK, B.R. (2002). Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Applied and Environmental Microbiology* **68**, 3795–3801.

PAULA, M.A., REIS, V.M. AND DÖBEREINER, J. (1991). Interactions of *Glomus clarum* with *Acetobacter diazotrophicus* in infection of sweet potato (*Ipomoea batatas*), sugar cane (*Saccharum* spp.) and sweet sorghum (*Sorghum vulgare*). *Biology and Fertility of Soils* **11**, 111–115.

PENROSE, D.M. AND GLICK, B.R. (2003). Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiologia Plantarum* **118**, 10–15.

PORTEOUS-MOORE, F., BARAC, T., BORREMANS, B. *ET AL.* (2006). Endophytic bacteria in poplar trees growing on BTEX-contaminated site: the characterization of isolates with potential to enhance phytoremediation. *Systematic and Applied Microbiology* (in press).

QUADT-HALLMANN, A. AND KLOEPPER, J.W. (1996). Immunological detection and localization of the cotton endophyte *Enterobacter asburiae* JM22 in different plant species. *Canadian Journal of Microbiology* **42**, 1144–1154.

QUADT-HALLMANN, A., BENHAMOU, N. AND KLOEPPER, J.W. (1997). Bacterial endophytes in cotton: mechanisms entering the plant. *Canadian Journal of Microbiology* **43**, 577–582.

QUISPEL, A. (1992). A search for signals in endophytic microorganisms. In: *Molecular signals in plant–microbe communications*. Ed. D.P.S. Verma. pp 471–490. Boca Raton, USA: CRC Press.

RADEMAKER, J.L.W., LOUWS, F.J. AND DE BRUIJN, F.J. (1998). *Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. Molecular microbial ecology manual*. Dordrecht, The Netherlands: Kluwer Academic Publishers.

RAO, V.R., RAMAKRISHNAN, B., ADHYA, T.K., KANUNGO, P.K. AND NAYAK, D.N. (1998). Current status and future prospects of associative nitrogen fixation in rice. *World Journal of Microbiology and Biotechnology* **14**, 621–633.

RAVIKUMAR, S., KATHIRESAN, K., IGNATIAMMAL, S.T.M., SELVAM, S.B. AND SHANTHY, S. (2004). Nitrogen-fixing azotobacters from mangrove habitat and their utility as marine biofertilizers. *Journal of Experimental Marine Biology and Ecology* **312**, 5–17.

REINHOLD-HUREK, B. AND HUREK, T. (1998). Interactions of Gramineous plants with *Azoarcus* spp. and other diazotrophs: identification, localization, and perspectives to study their function. *Critical Reviews in Plant Sciences* **17**, 29–54.

REINHOLD-HUREK, B., HUREK, T., GILLIS, M. *ET AL.* (1993). *Azoarcus gen.* nov., nitrogen fixing proteobacteria associated with root of Kallar grass [*Leptochloa fusca* (L) Knuth], and the description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. *International Journal of Systematic Bacteriology* **43**, 574–584.

REQUENA, B.N., JIMENEZ, I., TORO, M. AND BAREA, J.M. (1997). Interactions between plant

growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi and *Rhizobium* spp. in the rhizosphere of *Anthyllis cytisoides*, a model legume for revegetation in Mediterranean semi-arid ecosystems. *New Phytologist* **136**, 667–677.

RICHARDSON, A. (2001). Prospects for using soil microorganisms to improve the acquisition of phosphorous by plants. *Australian Journal of Plant Physiology* **28**, 897–906.

RIGGS, P.J., CHELIUS, M.K., INIGUEZ, A.L., KAEPPLER, S.M. AND TRIPLETT, E.W. (2001). Enhanced maize productivity by inoculation with diazotrophic bacteria. *Australian Journal of Plant Physiology* **28**, 829–836.

RODRIGUEZ, H. AND FRAGA, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology* **17**, 319–339.

ROMEIRO, R.S., FILHO, L., VIEIRA, J.R., SILVA, H.S.A., BARACAT-PEREIRA, M.C. AND CARVALHO, M.G. (2004). Macromolecules released by a plant growth-promoting rhizobacterium as elicitors of systemic resistance in tomato to bacterial and fungal pathogens. *Journal of Phytopathology* **153**, 120–123.

RONCHEL, M.C., RAMOS-DIAZ, M.A. AND RAMOS, J.L. (2000). Retrotransfer of DNA in the rhizosphere. *Environmental Microbiology* **2**, 319–323.

ROY, S., LABELLE, S., MEHTA, P. *ET AL.* (2005). Phytoremediation of heavy metal and PAH-contaminated brownfield sites. *Plant and Soil* **272**, 277–290.

SALISBURY, F.B. (1994). The role of plant hormones. In: *Plant–environment interactions*. Ed. R.E. Wilkinson, pp 39–81. New York, USA: Marcel Dekker.

SANDERMANN, H., JR. (1994). Higher plant metabolism of xenobiotics: the 'green liver' concept. *Pharmacogenetics* **4**, 225–241.

SCHLEGEL, H.G., COSSON, J.P. AND BAKER, A.J.M. (1991). Nickel-hyperaccumulating plants provide a nickel for nickel-resistant bacteria. *Botanica Acta* **194**, 18–25.

SCHLESINGER, W.H. (1991). *Biogeochemistry: an analysis of global change*. San Diego, USA: Academic Press.

SCHMITT-JOHN, T. AND ENGELS, J.W. (1992). Promoter constructions for efficient secretion expression in *Streptomyces lividans*. *Applied and Environmental Microbiology* **36**, 493–498.

SCHULTZE, M. AND KONDOROSI, M. (2002). Regulation of symbiotic root nodule development. *Annual Review of Genetics* **32**, 33–57.

SCHWITZGUÉBEL, J.P., VAN DER LELIE, D., GLASS, D.J., VANGRONSVELD, J. AND BAKER, A.J.M. (2002). Phytoremediation: European and American trends, successes, obstacles and needs. *Journal of Soils and Sediments* **2**, 91–99.

SESSITSCH, A., REITER, B., PFEIFER, U. AND WILHELM, E. (2002). Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and Actinomycetes-specific PCR of 16S rRNA genes. *FEMS Microbiology Ecology* **39**, 23–32.

SEVILLA, M., BURRIS, R.H., GUNAPALA, N. AND KENNEDY, C. (2001). Comparison of benefit to sugar cane plant growth and $^{15}N_2$ incorporation following inoculation of sterile plants with *Acetobacter diazotrophicus* wild-type and Nif- mutant strains. *Molecular Plant–Microbe Interactions* **14**, 358–366.

SHARROCK, K.R., PARKES, S.L., JACK, H.K., REES-GEORGE, J. AND HAWTHORNE, B.T. (1991). Involvement of bacterial endophytes in storage roots of buttercup squash (*Cucurbita maxima* D. hybrid *'Delicia'*). *New Zealand Journal of Crop and Horticultural Science* **19**, 157–165.

SHISHIDO, M., BREUIL, C. AND CHANWAY, C. (1999). Endophytic colonization of spruce by plant growth-promoting rhizobacteria. *FEMS Microbiology Ecology* **29**, 191–196.

SICILIANO, S.D., FORTIN, N., MIHOC, A. *ET AL.* (2001). Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. *Applied and Environmental Microbiology* **67**, 2469–2475.

SIMONET, P., GROSJEAN, M.C., MISRA, A.K., NAZARET, S., COURNOYER, B. AND NORMAND, P. (1991). Frankia genus-specific characterization by polymerase chain reaction. *Applied and Environmental Microbiology* **57**, 3278–3286.

SINGH, S. AND KAPOOR, K.K. (1999). Inoculation with phosphate-solubilizing microorganisms and a vesicular arbuscular mycorrhizal fungus improves dry matter yield and nutrient uptake by wheat grown in a sandy soil. *Biology and Fertility of Soils* **28**, 139–144.

SPRENT, J.I. AND DE FARIA, S.M. (1988). Mechanisms of infection of plants by nitrogen fixing organisms. *Plant and Soil* **110**, 157–165.

STURZ, A.V. (1995). The role of endophytic bacteria during seed piece decay and tomato tuberization. *Plant and Soil* **175**, 257–263.

STURZ, A.V. AND NOWAK, J. (2000). Endophytic communities of Rhizobacteria and the strategies required to create yield enhancing associations with crops. *Applied Soil Ecology* **15**, 183–190.

STURZ, A.V., CHRISTIE, B.R. AND MATHESON, B.G. (1998). Associations of bacterial endophyte populations from red clover and potato crops with potential for beneficial allelopathy. *Canadian Journal of Microbiology* **44**, 162–167.

SULLIVAN, J., TRZEBIATOWSKI, J.R., CRUICKSHANK, R.W. *ET AL.* (2002). Comparative sequence analysis of the symbiosis island of *Mesorhizobium loti* strain R7A. *Journal of Bacteriology* **184**, 3086–3095.

SUN, J., KELEMEN, G.H., FERNANDEZ-ABLOS, J.M. AND BIBB, M.J. (1999). Green fluorescent protein as a reporter for spatial and temporal expression in *Streptomyces coelicolor* A3(2). *Microbiology* **145**, 2221–2227.

TAGHAVI, S., BARAC, T., GREENBERG, B., VANGRONSVELD, J. AND VAN DER LELIE, D. (2005). Horizontal gene transfer to endogenous endophytic bacteria from poplar improves phytoremediation of toluene. *Applied and Environmental Microbiology* **71**, 8500–8505.

TAN, Z., HUREK, T., VINUESA, P., MÜLLER, P., LADHA, J.K. AND REINHOLD-HUREK, B. (2001). Specific detection of *Bradyrhizobium* and *Rhizobium* strains colonizing rice (*Oryza sativa*) roots by 16S-23S ribosomal DNA intergenic spacer-targeted PCR. *Applied and Environmental Microbiology* **67**, 3655–3664.

TERVET, I.W. AND HOLLIS, J.P. (1948). Bacteria in the storage organs of healthy plants. *Phytopathology* **38**, 960–967.

TIMMUSK, S., NICANDER, B., GRANHALL, U. AND TILLBERG, E. (1999). Cytokinin production by *Paenibacillus polymyxa*. *Soil Biology and Biochemistry* **31**, 1852.

TOMBOLINI, R., VAN DER GAAG, D.J., GERHARDSON, B. AND JANSSON, J.K. (1999). Colonization pattern of the biocontrol strain *Pseudomonas chlororaphis* MA 342 on barley seeds visualized by using green fluorescent protein. *Applied and Environmental Microbiology* **65**, 3674–3680.

TRAPP, S., ZAMBRANO, K.C., KUSK, K.C. AND KARLSON, U. (2000). A phytotoxicity test using transpiration of willows. *Archives of Environmental Contamination and Toxicology* **39**, 154–160.

TRAPP, S., KÖHLER, A., LARSEN, L.C., ZAMBRANO, K.C. AND KARLSON, U. (2001). Phytotoxicity of fresh and weathered diesel and gasoline to willow and poplar trees. *Journal of Soils and Sediments* **1**, 71–76.

TURNER, J.T., JEFFREY, L.K. AND CARLSON, P.S. (1993). Endophytes: an alternative genome for crop improvement. In: *International crop science*. Eds. D.R. Buxton, R. Shibles, R.A. Forsberg, B.L. Blad, K.H. Asay, G.M. Paulsen and R.F. Wilson, pp 555–560. Madison, USA: Crop Science Society of America.

VAN AKEN, B., YOON, J.M. AND SCHNOOR, J.L. (2004a). Biodegradation of nitro-substituted explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine by a phytosymbiotic *Methylobacterium* sp. associated with poplar tissues (*Populus deltoides* × *nigra* DN34). *Applied and Environmental Microbiology* **70**, 508–517.

VAN AKEN, B., PERES, C.M., DOTY, S.L., YOON, J.M. AND SCHNOOR, J.L. (2004b). *Methylobacterium populi* sp. nov., a novel aerobic, pink-pigmented, facultatively methylotrophic, methane-utilizing bacterium isolated from poplar trees (*Populus deltoides* × *nigra* DN34). *International Journal of Systematic and Evolutionary Microbiology* **54**, 1191–1196.

VAN DER LELIE, D. (1998). Biological interactions: the role of soil bacteria in the bioremediation of heavy metal-polluted soils. In: *Metal-contaminated soils. In situ inactivation and phytorestoration*. Eds. J. Vangronsveld and S.D. Cunningham, pp 31–50. Berlin, Heidelberg, New York: Springer-Verlag.

VAN DER LELIE, D., CORBISIER, P., DIELS, L. *ET AL.* (1999). The role of bacteria in the phytoremediation of heavy metals. In: *Phytoremediation of contaminated soil and water*. Eds.

N. Terry and G. Bañuelos, pp 265–281. Boca Raton, London, New York Washington D. C.: Lewis Publishers CRC Press LLC.

VAN DER LELIE, D., SCHWITZGUÉBEL, J.P., VANGRONSVELD, J. AND BAKER, A.J.M. (2001). Assessing phytoremediation's progress in the United States and Europe. *Environmental Science and Technology* **35**, 446A–452A.

VAN DER LELIE, D., BARAC, T., TAGHAVI, S. AND VANGRONSVELD, J. (2005). Response to Newman: new uses of endophytic bacteria to improve phytoremediation. *Trends in Biotechnology* **23**, 8–9.

VAN ELSAS, J.D., GARDENER, B.B., WOLTERS, A.C. AND SMIT, E. (1998). Isolation, characterization, and transfer of cryptic gene-mobilizing plasmids in the wheat rhizosphere. *Applied and Environmental Microbiology* **64**, 880–889.

VAN NIEUWENHOVE, C., MERCKX, R., VAN HOLM, L. AND VLASSAK, K. (2001). Dinitrogen fixation activity of *Azorhizobium caulinodans* in the rice (*Oryza sativa* L.) rhizosphere assessed by nitrogen balance and nitrogen-15 dilution methods. *Biology and Fertility of Soils* **33**, 25–32.

VANEECHOUTTE, M., ROSSAU, R., DE VOS, P. *ET AL.* (1992). Rapid identification of bacteria of the Comamonadaceae with amplified ribosomal DNA-restriction analysis (ARDRA). *FEMS Microbiology Letters* **72**, 227–233.

VASSE, J., DE BILLY, F. AND TRUCHET, G. (1993). Abortion of infection during the *Rhizobium meliloti*–alfalfa symbiotic interaction is accompanied by a hyper-sensitive reaction. *Plant Journal* **4**, 555–566.

VASSILEV, A., SCHWITZGUÉBEL, J.P., THEWYS, T., VAN DER LELIE, D. AND VANGRONSVELD, J. (2004). The use of plants for remediation of metal contaminated soils. *The Scientific World Journal* **4**, 9–34.

VAZQUEZ, P., HOLGUIN, G., PUNTE, M.E., LOPEZ-CORTEZ, A. AND BASHAN, Y. (2000). Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. *Biology and Fertility of Soils* **30**, 460–468.

VERMA, S.C., LADHA, J.K. AND TRIPATHI, A.K. (2001). Evaluation of plant growth promotion and colonization ability of endophytic diazotrophs from deep-water rice. *Journal of Biotechnology* **91**, 127–141.

VOS, P., HOGERS, R., BLEEKER, M. *ET AL.* (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**, 4407–4414.

VOSE, P.B. (1983). Development in non-legume N_2-fixing systems. *Canadian Journal of Microbiology* **29**, 837–850.

WAWRIK, B., KERKHOF, L., ZYLSTRA, G.J. AND KUKOR, J.J. (2005). Identification of unique type II polyketide synthase genes in soil. *Applied and Environmental Microbiology* **71**, 2232–2238.

WEBSTER, G., GOUGH, C., VASSE, J. *ET AL.* (1997). Interactions of rhizobia with rice and wheat. *Plant and Soil* **194**, 115–122.

WHITESIDES, S.K. AND SPOTTS, R.A. (1991). Frequency, distribution, and characteristics of endophytic *Pseudomonas syringe* in pear trees. *Phytopathology* **81**, 453–457.

WHITING, S., LEAKE, J., MCGRATH, S.P. AND BAKER, J. (2000). Positive responses to Zn and Cd by roots of the Zn and Cd hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* **145**, 199–210.

WHITING, S.N., DE SOUZA, M.P. AND TERRY, N. (2001). Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thlaspi caerulescens*. *Environmental Science and Technology* **35**, 3144–3150.

WIEHE, W., HECHT-BUCHHOLZ, C. AND HÖFLICH, G. (1994). Electron microscopic investigations on root colonization of *Lupinus albus* and *Pisum sativum* with two associative plant growth-promoting rhizobacteria, *Pseudomonas fluorescens* and *Rhizobium leguminosarum* bv. *trifolii*. *Symbiosis* **17**, 15–31.

YANNI, Y.G., RIZK, R.Y., CORICH, V. *ET AL.* (1997). Natural endophytic association between *Rhizobium leguminosarum* bv. trifolii and rice roots and assessment of its potential to promote rice growth. *Plant and Soil* **194**, 99–114.

YOU, C.B., LI, X., WANG, H.X., QIU, Y.S., MO, X.Z. AND ZHANG, Y.L. (1983). Associative nitrogen fixation of *Alcaligenes faecalis* with rice plant. *Biological N_2 Fixation Newsletter Sydney* **11**, 92–103.

YOU, C.B., SONG, W., WANG, H.X., LI, J.P., LIN, M. AND HAI, W.L. (1991). Association of *Alcaligenes faecalis* with wetland rice. *Plant and Soil* **137**, 81–85.

YUHASHI, K.I., ICHIKAWA, N., EZURA, H. *ET AL.* (2000). Rhizobitoxine production by *Bradyrhizobium elkanii* enhances nodulation and competitiveness on *Macroptilium atropurpureum*. *Applied and Environmental Microbiology* **66**, 2658–2663.

ZHAO, F.J., LOMBI, E. AND MCGRATH, S.P. (2003). Assessing the potential for zinc and cadmium phytoremediation with the hyperaccumulator *Thlaspi caerulescens*. *Plant and Soil* **249**, 37–43.

ZINNIEL, D.K., LAMBRECHT, P., HARRIS, N.B. *ET AL.* (2002). Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Applied and Environmental Microbiology* **68**, 2198–2208.