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THE INOCULATION METHOD AFFECTS COLONIZATION AND PERFORMANCE OF BACTERIAL INOCULANT STRAINS IN THE PHYTOREMEDIATION OF SOIL CONTAMINATED WITH DIESEL OIL

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Plants in combination with microorganisms can remediate soils, which are contaminated with organic pollutants such as petroleum hydrocarbons. Inoculation of plants with degrading bacteria is one approach to improve remediation processes, but is often not successful due to the competition with resident microorganisms. It is therefore of high importance to address the persistence and colonization behavior of inoculant strains. The objective of this study was to determine whether the inoculation method (seed imbibement and soil inoculation) influences bacterial colonization, plant growth promotion and hydrocarbon degradation. Italian ryegrass was grown in non-sterilized soil polluted with diesel and inoculated with different alkane-degrading strains* Pantoea *sp. ITSI10,* Pantoea *sp. BTRH79 and* Pseudomonas *sp. MixRI75 individually as well as in combination. Inoculation generally had a beneficial effect on plant biomass production and hydrocarbon degradation, however, strains inoculated in soil performed better than applied by seed imbibement. Performance correlated with the colonization efficiency of the inoculated strains. The highest hydrocarbon degradation was observed in the treatment, in which all three strains were inoculated in combination into soil. Our study revealed that besides the degradation potential and competitive ability of inoculant strains the inoculation method plays an important role in determining the success of microbial inoculation.

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

INTRODUCTION

Diesel oil is a frequently reported soil contaminant (Wang et al. 2005; Ciric et al. 2009) and above a certain level diesel pollution can be toxic to plants and microorganisms (Tesar et al. 2002; Adam and Duncan 2003; Lapinskienė et al. 2006). Phytoremediation, that is, the use of plants and associated microorganisms, provides an ecologically and economically attractive technique for remediating soils contaminated with hydrocarbons

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(Pilon-Smits 2005; Dzantor 2007; Olson et al. 2008). One of the major limitations of using this approach is the fact that many plant species are sensitive to contaminants (Huang et al. 2001; Chaudhry et al. 2005) and even plants, which tolerate the pollutant usually do not grow well and cannot effectively support degradation (Glick 2003; Peng et al. 2009; Cébron et al. 2009). To overcome this problem, plant growth-promoting bacteria (Johnson et al. 2004; Huang et al. 2005) and bacteria showing the capacity to degrade contaminants may be applied to protect the plants from toxic effects due to the presence of the pollutant (Weyens et al. 2009b; McGuinness and Dowling 2009; Gerhardt et al. 2009). However, several studies have shown that the application of specific microbes often fails in efficient degradation of contaminants, probably due to poor survival in the rhizosphere (Rentz et al. 2005; Gilbertson et al. 2007; Gunderson et al. 2007).

Successful application of selected microorganisms in a viable way is the first step towards improving phytoremediation of a contaminated soil. In addition, it is equally important that the microorganisms remain viable and colonize the plant environment (Ramos et al. 2010). An important question is how plants in the field can be inoculated to allow efficient colonization and degradation (Weyens et al. 2009b). Seeds or cuttings may be inoculated by spraying the inoculum onto the soil or directly onto growing plants. Traditionally, seed imbibement and soil inoculation have been frequently used for delivery, transport and distribution of bacteria (Huang et al. 2004; Villacieros et al. 2005; Germaine et al. 2009; Yousaf et al. 2010b), however, the efficiency of these procedures in terms of colonization has been rarely compared.

Recently, it has been suggested that certain aspects may facilitate the phytoremediation of organic environmental contaminants (Glick 2010). These include the use of: (1) bacteria that are either engineered or selected to be able to both promote plant growth and degrade soil contaminants are generally superior to bacteria that can only promote plant growth or degrade soil contaminants; (2) plant growth-promoting bacteria with 1-aminocyclopropane-1-caroxylic acid (ACC) deaminase activity, which improves plant stress tolerance and could be therefore a key determinant in facilitating phytoremediation; and (3) the use of endophytic bacteria, which are likely to be more effective in plant colonization than rhizosphere bacteria (Doty 2008; Weyens et al. 2009a, 2009c). Although several microorganisms can completely degrade a specific organic contaminant, individual species generally do not contain entire degradation pathways (Ueno et al. 2006, 2007; Gerhardt et al. 2009). Therefore, microbial consortia or strain combinations have been often found to be more effective in pollutant degradation than single strains (Yateem et al. 2007; Alarcón et al. 2008).

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

MATERIALS AND METHODS

Bacterial Strains

Three bacterial strains, which were previously isolated from the rhizosphere and endosphere of Italian ryegrass (*Lolium multiflorum* var. Taurus) and birdsfoot trefoil (*Lotus*

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

Tagging of Bacterial Strains with the *gusA* gene

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

ACC Deaminase Activity

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

Inoculation of Seeds by Imbibement

Strains of ITSI10::*gusA*10, MixRI75::*gusA*10 and BTRH79::*gusA*10 were cultivated in LB broth amended with 1% (v/v) filter-sterilized diesel at 30°C. Cells were harvested by centrifugation, washed and re-suspended in sterile 0.9% NaCl solution. Seeds of Italian ryegrass (IT) were surface-sterilized by soaking them in 5% sodium hypochlorite solution for 2 min, then in 70% ethanol for 2 min, and then seeds were thoroughly rinsed with sterilized distilled water. Suspensions containing 10^{11} bacterial cells mL^{-1} in 0.9% NaCl solution were used for seed imbibement (12 hours at room temperature), which was performed as described previously (Wang et al. 2004). Ten seeds of each treatment were analyzed to assess the survival of the inoculated bacteria. Seeds were shaken in 0.9% NaCl solution, and serial dilutions were plated on M9 medium (Sambrook et al. 1989) containing succinate, acetate and citrate (SAC), each at a concentration of 2 g L^{-1}. Seeds contained about 10^9 cfu per seed. For the treatment using the combination of strains ITSI10::*gusA*10, MixRI75::*gusA*10 and BTRH79::*gusA*10, cells of each strain were mixed in equal proportions and it was confirmed that the final cell concentration was 10^{11} cells mL^{-1}. For control and soil inoculation treatments, sterilized seeds were soaked in 0.9% NaCl solution.

Plant Experiment

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

hydrocarbons) (Risher and Rhoads 1995). Pots were filled with spiked soils (1.5 kg pot^{-1}) and subsequently placed in the greenhouse. The treatments included a diesel-contaminated soil (control), diesel-contaminated soil with IT (vegetated control), diesel-contaminated soil with IT and bacterial inoculation by seed imbibement, and diesel-contaminated soil with IT and soil inoculation. Inoculants contained strains ITSI10::*gusA*10, MixRI75::*gusA*10 and BTRH79::*gusA*10 individually or in combination. Three replicates of each treatment were used and pots were arranged in a completely randomized block design. One hundred inoculated and non-inoculated seeds were planted in each pot.

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

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

Detection and Enumeration of Inoculated Bacteria

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

For the isolation of rhizosphere bacteria, a soil slurry was prepared by mixing 3 g rhizosphere soil with 9 mL of 0.9% (w/v) NaCl solution and agitation (180 rpm) for 1 hour at 30°C. After the settlement of soil particles, serial dilutions up to 10^{-3} were plated onto selective M9 medium containing SAC, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (XGlcA) (100 μg mL^{-1}), isopropyl-β-D-galactopyranoside (IPTG) (100 μg mL^{-1}) and spectinomycin (100 μg mL^{-1}). Cycloheximide (100 mg L^{-1}) was added to prevent fungal growth. For the isolation of endophytes, 1 g of surface-sterilized shoots and roots were homogenized in 6 mL 0.9% NaCl solution by using a mortar and pestle. Similarly, 0.2 g seeds of each treatment were homogenized in 2 mL 0.9% NaCl solution. The homogenized material was shaken for 1 hour at 30°C. After settling the solid material, serial dilutions up to 10^{-3} were spread on selective M9 medium. The plates were incubated at 30°C for 48 hours and then transferred to 4°C for three days. Blue colonies were counted on each plate. Thirty blue colonies of each treatment were randomly picked and the identity of isolates with the inoculant strain was confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA intergenic spacer region (IGS) (Andria et al. 2009). Isolates and inoculant strains had identical restriction patterns.

Cultivation-Independent Analysis

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

Hydrocarbon Analysis of Soil Samples

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

Statistical Analysis

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

RESULTS

Plant Biomass

Growth parameters (seed germination, root, shoot, and seed dry weight) were determined to evaluate the effect of both, the inoculation and the method of inoculation, on plant development (Table 1). Seed germination as well as seed production were not influenced by bacterial inoculation. Biomass (shoot and root) production was significantly enhanced due to inoculation. Soil inoculation resulted in higher biomass production than seed imbibement. Highest biomass production was achieved with the strain combination followed by

Table 1 Effect of bacterial inoculation method on shoot and root dry weight (DW) of Italian ryegrass (*Lolium multiflorum* var. Taurus) harvested 93 days after seed germination

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

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

Table 2 Effect of bacterial inoculation method on bacterial persistence in rhizosphere (RH), root interior (RI), shoot interior (SI) and seed interior (SeI) of Italian ryegrass (*Lolium multiflorum* var. Taurus)

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

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

strains BTRH79::*gusA*10, ITSI10::*gusA*10 and MixRI75::*gusA*10. Strains with ACC deaminase activity (BTRH79::*gusA*10, ITSI10::*gusA*10) induced higher shoot and particularly root production than the strain lacking this activity.

Persistence of Inoculated Strains

Isolation and enumeration of the three inoculated strains applied by both inoculation methods showed that they were able to efficiently colonize the rhizosphere and plant interior (Table 2). Generally, soil inoculation led to better colonization in the rhizosphere as well as in the plant interior than inoculation by seed imbibement.

In the rhizosphere strain BTRH79::*gusA*10, originally isolated from the rhizosphere of Birdsfoot trefoil, colonized better than the endophyte strains, whereas in the root interior strain MixRI75::*gusA*10, a root endophyte, performed best. The shoot endophyte, strain ITSI10::*gusA*10 colonized the shoot interior better than other strains applied individually. Generally, more bacterial cells were found when strains were applied in combination. By soil inoculation strain ITSI10::*gusA*10 was dominant in the rhizosphere (57%) and root interior (52%), while BTRH79*::gusA*10 was more abundant in the shoot interior (62%). Strain MixRI75::*gusA*10 showed lowest abundance in all three compartments (11–23%). When strains were applied in combination by seed imbibement only BTRH79::*gusA*10 was able to colonize.

Hydrocarbon Degradation

In vegetated soil hydrocarbon degradation was more efficient than in non-vegetated soil, however, inoculation further significantly improved hydrocarbon degradation (Table 3).

Table 3 Effect of bacterial inoculation method on hydrocarbon degradation in diesel-contaminated soil vegetated with Italian ryegrass (*Lolium multiflorum* var. Taurus)

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

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

Generally, plants inoculated by soil inoculation showed significantly lower hydrocarbon concentrations than those inoculated by seed imbibement. The maximum reduction (5.92 g kg^{-1} dry soil) was observed with plants inoculated with the combination of the three inoculant strains by soil inoculation. By both inoculation methods, higher hydrocarbon removal was observed with ACC deaminase - producing bacteria ITSI10::*gusA*10 and BTRH79::*gusA*10 than by bacteria lacking this activity (MixRI75::*gusA*10).

Cultivation-Independent Analysis

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

DISCUSSION

The use of degrading microorganisms in phytoremediation applications is a promising approach to accelerate the clean-up of polluted soils. Degrading microorganisms may enhance a plant's adaptation to contaminants such as petroleum hydrocarbons by detoxifying contaminated soils through direct mineralization of these organic contaminants (Escalante-Espinosa et al. 2005; Alarcón et al. 2008) leading also to better plant growth. Additionally, inoculant strains or the native plant-associated microflora may show plant growth-promoting activities, e.g., through ACC deaminase activity, further promoting plant growth. Enhanced plant growth may in turn stimulate the associated microflora and degradation processes. In this study, plant biomass was enhanced by 12% to 44% by inoculation

as compared to non-inoculated plants. Increased plant biomass production by bacterial inoculation would effectively lower the ratio of phytotoxic contaminants to the amount of plant tissue, lowering plant stress (Alkorta and Garbisu 2001; Gurska et al. 2009). This particular tendency suggests that inoculation of hydrocarbon degrading-microorganisms decreased the potential toxic effect due to the bioavailable spiked hydrocarbons. The two strains, ITSI10 and BTRH79, exhibiting alkane degradation as well as ACC deaminase activities were highly efficient in enhancing plant biomass (especially root biomass) and consequently hydrocarbon degradation and performed better than strain MixRI75 lacking ACC deaminase activity. The bacterial enzyme ACC deaminase can reduce ethylene levels produced by plants under stress and therefore alleviates stress symptoms leading to better plant growth (Glick 2003).

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

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

further indicates that seeds provide a specific micro-environment to which inoculant strains need to be adapted.

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

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

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

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

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

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