Hydrocarbon degradation, plant colonization and gene expression of alkane degradation genes by endophytic *Enterobacter ludwigii* strains

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Abstract

The genus *Enterobacter* comprises a range of beneficial plant-associated bacteria showing plant growth-promotion. *Enterobacter ludwigii* belongs to the *Enterobacter cloacae* complex and has been reported to include human pathogens but also plant-associated strains with plant beneficial capacities. To assess the role of *Enterobacte*r endophytes in hydrocarbon degradation, plant colonization, abundance and expression of CYP153 genes in different plant compartments, three plant species (Italian ryegrass, birdsfoot trefoil and alfalfa) were grown in sterile soil spiked with 1% diesel and inoculated with three endophytic *Enterobacter ludwigii* strains. Results showed that all strains were capable of hydrocarbon degradation and efficiently colonized the rhizosphere and plant interior. Two strains, ISI10-3 and BRI10-9, showed highest degradation rates of diesel fuel up to 68% and performed best in combination with Italian ryegrass and alfalfa. All strains expressed the CYP153 gene in all plant compartments, indicating an active role in degradation of diesel in association with plants.

Capsule:

Enterobacter ludwigii strains belonging to the *E. cloacae* complex are able to efficiently degrade alkanes when associated with plants and to promote plant growth.

Keywords: *Enterobacter ludwigii*, endophytes, hydrocarbon degradation, gene abundance, gene expression

Introduction

Plants interact with a great diversity of microorganisms, including enteric bacteria. These interactions, which are lined by the characteristics of both, host plant and bacteria, result in associative, commensal, symbiotic, or parasitic relationships between both partners. Members of the *Enterobacteriaceae* are distributed in many environments, with some being saprophytes and others being parasites of plants and animals. Several studies have shown that *Enterobacteriaceae* may have beneficial effects on plant development when they are associated with plants (Lodewyckx et al., 2002; Taghavi et al., 2009). They may improve plant growth via nitrogen fixation, suppression of plant pathogens and production of phytohormones and enzymes involved in the metabolism of growth regulators such as ethylene, 1-aminocyclopropane 1-carboxylic acid (ACC), auxins and indole-3-acetic acid (IAA) (Gyaneshwar et al., 2001; Kämpfer et al., 2005; Taghavi et al., 2009). Organisms such as *Enterobacter radicincitans*, *E. arachidis*, *E. oryzae*, and *Enterobacter* sp. CBMB30, which were isolated from the wheat phyllosphere, groundnut rhizosphere, poplar and rice endosphere, respectively, are known as plant growth-promoting bacteria (Lee et al., 2006; Peng et al., 2009; Taghavi et al., 2009; Madhaiyan et al., 2010).

In previous experiments we repeatedly isolated *Enterobacter*-related strains from the rhizosphere and endosphere of plants (Italian ryegrass and birdsfoot trefoil) grown in diesel-contaminated soils (Yousaf et al., 2010a). Further characterization revealed that several strains belong to *Enterobacter ludwigii*. This species is known for its clinical relevance as most isolates have been isolated from clinical specimens (Hoffmann et al., 2005). *E. ludwigii* belongs to the *E. cloacae* complex, which has been frequently isolated

from nosocomial infections; however, it is not clear whether *E. ludwigii* is a true pathogen or has a rather commensal character (Paauw et al., 2008). Generally, few studies on *E. ludwigii* are available, but it has been reported as a plant-associated bacterium with plant growth-promoting and biocontrol capacities (Shoebitz et al., 2009).

Global industrialization over the past years has resulted in numerous sites with strong contamination of the soil with persistent organic and inorganic contaminants. Aliphatic hydrocarbons (e.g. diesel fuel and engine oils) make up a substantial proportion of substances found at contaminated sites (Stroud et al., 2007). The use of plants and their associated microorganisms for the treatment of hydrocarbon-contaminated soils has attained increasing acceptance as a viable clean-up technology (Lelie et al., 2001). The efficiency of a phytoremediation process depends mainly on the presence and activity of plant-associated microorganisms carrying degradation genes required for the enzymatic break-down of contaminants. The rhizosphere and plant endosphere have been reported to host pollutant-degrading bacteria (Siciliano et al., 2001; Andria et al., 2009;) and highly diverse alkane degrading bacteria containing alkane degrading genes have been isolated from the plant environment (Kaimi et al., 2007). Expression analysis of alkane monooxygenase (*alkB*) and a cytochrome P450 hydroxylase (CYP153 gene) indicated degradation in the rhizosphere as well as in the plant interior (Powell et al., 2006; Andria et al., 2009; Afzal et al., 2011).

In this study we characterized in detail selected alkane degrading *Enterobacter* strains, which were previously isolated from Italian ryegrass and birdsfoot trefoil (Yousaf et al., 2010a) and identified as *E. ludwigii.* In plant experiments, we studied in detail the hydrocarbon degradation and plant colonization capacities of these strains.

Materials and Methods

Isolation and characterization of bacterial strains

Three strains, IRI10-4, BRI10-9 (root endophytes) and ISI10-3 (shoot endophyte), were isolated from Italian ryegrass (IRI10-4, ISI10-3) and birdsfoot trefoil (BRI10-9) (Yousaf et al., 2010a). At harvest, plants were shaken to dislodge the soil loosely attached to roots and shoots were cut 2 cm above soil. Roots and shoots were carefully washed and surface-sterilized with 70% ethanol (IT: 3 min, BT: 5 min), then treated with 1% NaOCl (IT: 5 min, BT: 6 min), followed by washing 3 times with sterile distilled water (1 min each time). For the isolation of strains, surface-sterilized roots and shoots were homogenized with a pestle and mortar in NaCl solution (0.9%, w/v). After settling of plant material, serial dilutions were spread on minimal basal medium (MBM) containing 1% diesel followed by incubation at 30^{o}C for 4 days. These strains have the capacity to degrade alkanes and contain a cytochrome P450 type alkane hydroxylase (CYP153) gene (Yousaf et al., 2010a).

Almost complete 16S rRNA sequences were determined for selected strains using the primers and conditions described by Coenye et al. (1999). Based on 16S rRNA gene phylogenetic analysis these strains were considered to belong to the *Pantoea* – *Enterobacter* clade. In order to provide stronger support for the description of these strains, *rpoB* gene sequence analysis was performed using the primers and conditions as described by Brady et al. (2008).

Phylogenetic analysis was done as described by Brady et al. (2008). Briefly, the sequences were aligned using CLUSTAL_X (Thompson et al., 1997) and overhangs were trimmed. The program MODELTEST 3.7 (Posada and Crandall, 1998) was then applied

to the datasets to determine the best-fit evolutionary model. Maximum-likelihood and neighbour-joining analyses were performed using Phyml (Guindon and Gascuel, 2003) and PAUP 4.0b10 (Swofford, 2000), respectively, by applying the models and parameters determined by MODELTEST.

ACC deaminase activity

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

Plant experiment

For the plant experiment three sets of pots were prepared in triplicate: (1) pots planted with Italian ryegrass (IT) (*Lolium multiflorum* var. Taurus), (2) pots planted with birdsfoot trefoil (BT) (*Lotus corniculatus* var. Leo) and (3) pots planted with alfalfa (AL) (*Medicago sativa* var. Harpe). Agricultural soil (agricultural top soil from Seibersdorf, Lower Austria, Austria; pH 7.4, 27 g sand kg^{-1}, 621 g silt $kg^{-1,}$ 352 g clay kg^{-1}, 2.4 g C_{org} kg^{-1}) was sterilized by 30 kGy γ-radiation and amended with 10% compost. The sterility of sterilized soil was checked by plating soil suspensions on Tryptic Soy Agar (Merck) plates, no growth was observed. Before sowing, soil was amended with filter-sterilized diesel fuel (10,000 mg kg^{-1} soil) and incubated at room temperature for one week. Pots with dimensions 13 x 13 x 13 cm were filled with spiked soils and subsequently placed in the greenhouse. Pots were arranged in a completely randomized block design. Seeds of IT, BT and AL were surface sterilized by soaking in 5% sodium hypochlorite solution for 2 min, then in 70% ethanol for 2 min, and were then washed with sterile water for 3

times. Surface-sterilized seeds (200 per pot) were sown. One week after seed germination, plants were thinned to 170 per pot and each pot was inoculated with 100 ml inoculant suspension (app. 10^9 CFU ml^{-1}, cultivated in Luria Bertani broth at 30°C, centrifuged and resuspended in 0.9% (w/v) NaCl) containing one of the strains described above. For control treatments, spiked soil was treated with 100 ml of 0.9% NaCl instead of inoculum suspension. Plants were grown at 25°C in the greenhouse (16 h light / 8 h dark) and watered with equal amounts when needed.

Plants were harvested at two growth stages. First harvest was done after 42 days of seed germination and second harvest at flowering (IT 102 days after germination, BT and AL 150 days after germination). Plants were cut 2 cm above ground and remaining plants were harvested to obtain root and rhizosphere samples. 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.

Hydrocarbon analysis of soil samples

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

Detection and enumeration of inoculant strains

The rhizosphere soil was collected by gently sampling the soil closely attached to root surface. Subsequently, roots and shoots were carefully washed and surface sterilized as described by Yousaf et al. (2010a), replacing distilled sterile water by DEPC-treated

water. The efficacy of surface sterilization was checked by plating shoots and roots, and aliquots of a final rinse on LB plates, no colonies were observed after 3 days of incubation, ensuring the surface sterilization efficiency.

For the isolation of alkane degrading rhizosphere bacteria, the soil slurry was prepared by mixing 5 g soil with 15 ml of 0.9% (w/v) NaCl solution, agitated (180 rpm) for 1 hour at 30^{o}C. After the settlement of soil particles, serial dilutions up to 10^{-4} were spread onto solid Minimal Basal medium (MBM) (Alef, 1994) containing 1% (v/v) filter-sterilized diesel. For the isolation of endophytes, 3 g of surface sterilized roots or shoots were homogenized with a pestle and mortar in 12 ml NaCl solution (0.9%, w/v). The homogenized material was agitated for 1 hour at 30^{o}C. After settling of solid material, serial dilutions up to 10^{-3} were spread on MBM containing 1% (v/v) filter-sterilized diesel. Bacterial colonies on each plate were selected randomly and transferred to solid MBM amended with 2% (v/v) filter-sterilized diesel followed by incubation at 30^{o}C for 4 days. Thirty colonies of each treatment were randomly selected and their identity with the inoculant strain was confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA intergenic spacer region (IGS) (Rasche et al., 2006a). Isolates and inoculant strains had identical restriction patterns.

Extraction of DNA and RNA

DNA from rhizosphere soil (0.5 g) was extracted by using FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, Ohio, USA), whereas RNA was isolated with RNA Power Soil Total RNA isolation Kit (MO Bio Laboratories) as described by the manufacturer, and was quantified photometrically (Nanodrop ND-1000, Nanodrop Technologies,

Wilmington, DE, USA). Roots and shoots were briefly ground in liquid N_2 and microbial cells were disrupted by bead-beating (Reiter et al., 2003). For isolation of DNA the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, Ohio, USA) was used. RNA was isolated by using RNEASY Plant Mini Kit (Qiagen). In RNA preparations genomic DNA was eliminated by DNase I enzyme (Ambion) digestion and potential presence of contaminating DNA was checked by PCR amplification of 16S rDNA (Rasche et al., 2006b).

Quantitative analysis of the abundance and expression of CYP153 genes

Reverse transcription (RT) was performed with 150-200 ng RNA, the specific primer P450rv3 (van Beilen et al., 2006) and Omniscript Reverse Transcriptase (Qiagen) according to the manufacturer's instructions. Abundance and expression of CYP153 genes were quantified by quantitative (real-time) (q)PCR using an iCycler IQ (Biorad) according to procedures described previously (Afzal et al., 2011). Standards for qPCR were generated by serial dilution of stocks containing purified CYP153 plasmid from a clone. The number of copies of the target gene in a ng plasmid DNA was determined, and then a serial dilution was prepared from 10^8 to 10^1 copies to use as an external standard curve ($r^2 > 0.95$), allowing determination of the number of copies of the gene in each sample of DNA and cDNA. Analyses were performed in triplicate and gene copy numbers were calculated as described by Powell et al. (2006). Reaction mixtures (25 µl) contained 5 µl of Q Mix (Evergreen), 2.5 µl 10mg/ml BSA, 1 µl DMSO, 2.6 µl 5 µM of each primer, 50-100 ng of DNA/cDNA template and RNase free water. Thermal cycling conditions were: 3 min 95°C followed by 40 cycles of 95°C for 25 s, 58°C for 25 s, 72°C

for 45 s followed by a melting curve from 50 to 100°C. Besides melting curve analysis, PCR products were examined on 2% agarose gels. No primer dimers were detected.

To test possible inhibitory effects on quantitative PCR amplification caused by co-extracted humic substances, the optimal dilution for each DNA/cDNA extract was determined by pre-experiments (data not shown). Serial dilutions of DNA and cDNA were spiked with 10^6 copies of amplified CYP153 genes to check for real-time PCR inhibition. Highly linear standard curves (r^2 values > 0.95, PCR efficiency > 98%) over the dilution range and a detection limit of 10^1 copies were obtained indicating no PCR inhibition. CYP153 gene copy numbers were quantified relative to a standard curve of a positive control and were normalized to the copy number of control plants. Statistical analysis was based on Duncan's multiple range test using SPSS software package (SPSS Inc., Chicago, IL).

Nucleotide sequence accession numbers

The partial nucleotide sequences of *rpoB* gene determined in this study were deposited in GeneBank data base with accession numbers JF932310 to JF932312.

Results

Characterization of hydrocarbon-degrading strains

Fig. 1 shows the results from the phylogenetic analysis of the strains based on *rpoB* gene nucleotide sequence. The strains analyzed in this study were assigned to *E. ludwigii*. We used *rpoB* based sequences in order to provide stronger support for the description of

taxonomic position of these strains, because on the basis of 16S rDNA phylogenetic tree, the taxonomic position of these strains was not clear (data not shown).

Hydrocarbon degradation

The effect of plants and inoculation on diesel fuel degradation was determined 6 weeks (first harvest of IT, BT and AL), 14 weeks for IT and 21 weeks for AL and BT (second harvest) after germination (Table 1). The degradation of hydrocarbons in soil with inoculation was significantly higher ($p<0.05$) than in uninoculated controls at both harvest times. At the first harvest the maximum decrease in hydrocarbon content was observed with strain ISI10-3 in combination with IT (48%) and with AL (40%), followed by BRI10-9 in combination with AL (38%). At the second harvest strain BRI10-9 showed maximum hydrocarbon degradation in combination with IT (68%). Strain ISI10-3 showed 65% hydrocarbon decrease in association with IT and 60% with AL. Generally, strains ISI10-3 and BRI10-9 showed higher hydrocarbon removal at both harvest times and IT performed better than AL and BT.

Plant biomass production

Results for shoot and root biomass of IT, AL and BT grown in contaminated and non-contaminated soil are shown in Table 2. Diesel contamination in soil had an inhibitive effect on plant growth. All three plant species produced less shoot and root biomass in soil when grown in the presence of diesel. Plant biomass was generally lower at the first harvest compared to the second harvest. Biomass production was significantly higher in inoculated treatments than in uninoculated contaminated treatments. More shoot biomass

was produced in the inoculated treatments as compared to the control at the first harvest (56% compared to 34%) and second harvest (76% compared to 53%). Inoculation also led to significantly higher root biomass. Strains ISI10-3 and BRI10-9 led to significantly higher root and partly also shoot dry weight than strain IRI10-4, which correlates with the ACC deaminase activity found in the strains ISI10-3 and BRI10-9.

Cultivation-dependent analysis of colonization

Results from microbial plate counts are given in Table 3. The microbial numbers in rhizosphere soil were higher at the first harvest than at the second harvest for all strains and plant combinations with exception of IRI10-4 and BRI10-9 in association with birdsfoot trefoil, where microbial numbers were lower at the first harvest than at the second harvest. At the first harvest, strain ISI10-3 colonized best and showed highest colonization (2.3×10^8 cells g^{-1} dry soil) in the rhizosphere of IT followed by AL. At the second harvest, the highest microbial numbers (4.5×10^7 cells g^{-1} dry soil) were observed for BRI10-9 in combination with IT followed by ISI10-3. These results clearly showed that strain ISI10-3, originally isolated from the shoot interior of Italian ryegrass, better colonized the rhizosphere of IT, BT and AL at both harvest times. The second best rhizosphere colonizer was BRI10-9, originally isolated from the root interior of birdsfoot trefoil. The population size of inoculant strains in the rhizosphere ranged from 10^4 to 10^8 cells g^{-1} dry soil (first harvest) and from 10^5 to 10^7 cells g^{-1} dry soil at second harvest.

In the root interior, highest colonization was observed in the endorhiza of IT and BT. Strain IRI10-4, originally isolated from the root interior of Italian ryegrass, better colonized IT roots at the first harvest , whereas at the second harvest BT roots were better

colonized. Microbial numbers ranged from 10^3 to 10^7 cells g^{-1} dry root at the first harvest and 10^4 to 10^7 cells g^{-1} dry root at the second harvest. All strains were capable of colonizing the shoot interior. Strain ISI10-3 (a shoot endophyte) showed significantly higher shoot colonization than other strains. Highest colonization was observed in the shoot interior of IT. Microbial numbers gradually increased from the first harvest to the second harvest time.

Quantification and expression of CYP153 genes

Real-time PCR of the CYP153 gene was used to quantify the population size of alkane degrading bacteria by a cultivation-independent analysis (Tables 4 and 5). Generally and in agreement with cultivation-based results, bacterial CYP153 gene abundance in the rhizosphere was highest at the first harvest (up to 1.1 x 10^9 copies g^{-1} dry soil) and decreased in all treatments towards the flowering stage. CYP153 gene abundance was lower in the endosphere and increased towards the second harvest (Table 4). Among different treatments, IT hosted the highest abundance of alkane degrading bacteria. Overall, the highest gene abundance at both harvest points, in the rhizosphere and shoot interior, was observed with strain ISI10-3 and IT. However, in the root interior IRI10-4 showed significantly higher gene abundance with IT and BT at the first and at the second harvest time, respectively.

All strains principally expressed CYP153 genes in the rhizosphere and endosphere of all three plant species, indicating an active role in hydrocarbon degradation (Table 5). The differences between strains and plant species in regard to CYP153 gene expression followed essentially the same pattern as CYP153 gene abundance. The comparison

between samples taken at different harvest times showed that the total number of bacteria, measured via CFU count and real-time PCR, decreased with time especially in the rhizosphere and root interior. Even though the gene expression also decreased with time, higher CYP153 expression was still observed in all plant compartments. The results showed that bacterial abundance and gene expression was affected by strain, plant type and plant environment. In BT and AL average activities were higher in endosphere than in the rhizosphere. Highest activities (transcripts / abundance) were calculated for ISI10-3 in combination with IT and BT as compared to other strains (Fig. 2). However, activity was generally depended on the strain and was affected by the plant and the sampling time.

Discussion

Recently, several studies have reported that human pathogens belonging to the *Enterobacteriaceae* such as *Salmonella enterica* and *Escherichia coli* may colonize plants (reviewed by Holden et al., 2009). Plants frequently serve as hosts for many enteric bacteria including *Erwinia*, *Pectobacterium*, *Pantoea* and *Enterobacter*, which may colonize as epiphytes, endophytes and/or pathogens. The genus *Enterobacter* comprises a range of beneficial plant-associated bacteria showing plant growth promotion and/or biocontrol activity (Taghavi et al., 2009; Madhaiyan et al., 2010). However, various *Enterobacter* members, in particular bacteria belonging to the *E. cloacae* complex including *E. ludwigii*, are known for their potential pathogenicity to humans, although a commensal character for bacteria belonging to this complex except for *E. cloacae* has been suggested (Paauw et al., 2008). This is supported by the fact that *E.*

ludwigii has not been isolated only from clinical samples but also from plants, where these strains have shown plant growth promotion (Shoebitz et al., 2009). In this study we taxonomically characterized selected *Enterobacteriaceae* strains, which were isolated previously from Italian ryegrass and birdsfoot trefoil grown in a diesel-contaminated soil. Three strains (IRI10-4, ISI10-3 and BRI10-9) were characterized in detail and showed to belong to *E. ludwigii*.

As our strains showed hydrocarbon degradation activities in preliminary plate assays, we tested in this study, whether these *E. ludwigii* strains are able to degrade hydrocarbons in a soil environment or to colonize plants efficiently. To the best of our knowledge this is the first report of hydrocarbon degradation by *E. ludwigii*. We were particularly interested in strains, which were isolated from the plant interior, as they have several advantages for phytoremediation applications. Facultative endophytes generally can colonize the rhizosphere soil as well as the plant endosphere (Weyens et al., 2009). Furthermore, endophytes may protect plants against the inhibitory effects of high concentrations of hydrocarbon and may promote plant growth by e.g. reducing ethylene levels with ACC deaminase activity (Glick, 2003; Sheng et al., 2008). All strains we tested showed substantial hydrocarbon degradation, however, strains showed different degradation capacities, although they all contained the same type of alkane hydroxylase gene. Generally, strains ISI10-3 and BRI10-9, showed higher degradation capacity than IRI10-4. The lower degradation activity correlated with a rather poor plant colonization and the comparably low degradation of strain IRI10-4 can be explained by its low abundance, particularly in the rhizosphere and the shoot interior. These results are in agreement with our previous findings (Yousaf et al., 2010a), where we observed that

those strains, which showed high hydrocarbon degradation rates, were also efficient colonizers.

Highest degradation was found with Italian ryegrass, although this plant was (due to its rapid growth) harvested seven weeks earlier than birdsfoot trefoil and alfalfa. This indicates that different plants stimulate degrading strains and degradation activity differently. The higher degradation with Italian ryegrass may be explained by enhanced stimulation of degradation activity by root exudates or a better aerated environment (Juhanson et al., 2007; Truu et al., 2007). Grasses have a fibrous root system, which can penetrate soils providing a large surface area for bacteria to colonize. Consequently, generally more bacterial cells were found to be associated with Italian ryegrass than with other plants. A higher degradation rate was found until the first harvest time, which then decreased until the second harvest time. This may be due to the degradation of easily degradable components of hydrocarbons, but might be also related to the fact that the number of degrading bacteria decreased with time, at least in some plant compartments.

Contaminating substances such as hydrocarbons generally inhibit plant growth (Yousaf et al., 2010b). The primary inhibiting factors are considered to be toxicity of low molecular weight compounds and hydrophobic properties that decrease the ability of plants to absorb water and nutrients (Kirk et al., 2005; Kechavarzi et al., 2007). Diesel is one of the most phytotoxic and persistent fuel types that contaminate soils and its negative influence on shoot and root biomass has been documented in several studies (Hou et al., 2001; Palmroth et al., 2002). In our study contamination led to a strong reduction in shoot and root biomass, however, inoculation significantly reversed this effect. Up to more than 76% shoot and up to 93% more root biomass was produced in

inoculation treatments as compared to the uninoculated controls. More biomass increase occurred between the first and second harvest than between inoculation and the first harvest. As the abundance of alkane degrading bacteria decreased with time, the most likely reason for the higher biomass production in the second stage in comparison to the control treatments is the lower hydrocarbon concentration leading to reduced toxicity for the plants. Inoculated bacteria might have promoted plant growth directly or indirectly by reducing hydrocarbon levels. Both strains (ISI10-3 and BRI10-9) showing ACC deaminase activity were more efficient in plant growth promotion as well as in hydrocarbon degradation. The bacterial enzyme ACC-deaminase can reduce ethylene levels produced by plants under stress and therefore may alleviate stress symptoms leading to better plant growth (Glick, 2003). Our results are in agreement with previous studies (Gurska et al., 2009; Afzal et al., 2011) reporting enhanced root growth and hydrocarbon degradation with strains having ACC-deaminase activity. Plant growth, especially root growth is important in the context of phytoremediation, as the rhizosphere plays an important role in catabolic activity and survival of associated microorganisms (Juhanson et al., 2009).

In phytoremediation, hydrocarbons are degraded mainly by soil and plant-associated microbial communities and it has been suggested that the phytoremediation potential correlates with the number of pollutant-degrading bacteria in the plant environment (Glick, 2003; Liste and Prutz, 2006; Muratova et al., 2008). Successful application of plant-microbe systems for rhizoremediation relies on in-situ establishment of a high number of degrading bacteria (Liu et al., 2007). The results from our study showed that *E. ludwigii* strains were able to efficiently colonize the rhizo- and

endosphere of Italian ryegrass, birdsfoot trefoil and alfalfa over a period of 150 days. The best hydrocarbon degrading strains, i.e. ISI10-3 and BRI10-9, colonized all plants well, however, microbial numbers decreased with time. Strain IRI10-4 (a root endophyte) showed higher colonization in the root interior than other strains, whereas strain ISI10-3 (a shoot endophyte) showed higher colonization in the shoot interior. Similar observations were also previously observed (Rosenblueth and Martinez-Romero, 2006; Andria et al., 2009), who postulated that endophytes are generally better able to colonize plant interior.

Our results revealed that the abundance and expression of CYP153 genes of all *E. ludwigii* strains involved in hydrocarbon degradation varied distinctly between different strains, plants species, plant developmental stages and plant compartments (Tables 4 and 5). Bacterial CYP153 gene abundance and expression was highest in the rhizosphere at the first harvest in all treatments. This can be related to enhanced root exudation and high amounts of nutrients in the rhizosphere for bacterial growth and co-metabolism of alkane degradation (Olson et al., 2003; Bürgmann et al., 2005; Hai et al., 2009). The gene abundance and expression was lower in the endosphere at initial stages but increased with time. This indicates that inoculated bacteria first establish in the rhizosphere and then reach the plant interior at a later stage. Strain ISI10-3 showed highest abundance and expression in rhizosphere and shoot interior, however, in the root interior IRI10-4 showed significantly higher gene abundance and expression than other strains. This might be because IRI1-4 was originally isolated from the root interior and ISI10-3 from the shoot interior. All strains principally expressed alkane degrading genes in all plant compartments, indicating an active role in degradation of diesel in various plant

compartments. The average activities (transcripts / abundance) were variable and depended on the inoculant strain, plant species and time of analysis. Some strains generally showed high activity in the shoot interior, which was also previously reported by Andria et al. (2009).

In conclusion this study revealed, that *E. ludwigii* strains efficiently interact with various plant species, efficiently colonize the rhizosphere as well as the plant interior, at least under the conditions tested, and are able to promote plant growth. Furthermore, all strains efficiently degraded hydrocarbons, especially strains ISI10-3 and BRI10-9 performed best, both in terms of plant growth promotion and hydrocarbon degradation. The close interaction with plants and hydrocarbon degradation activities suggest a potential for phytoremediation applications, however, issues such as potential pathogenicity towards animals or humans require further testing.

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References

Afzal, M., Yousaf, S., Reichenauer, T.G., Kuffner, M., Sessitsch, A., 2011. Soil type affects plant colonization, activity and catabolic gene expression of inoculated

bacterial strains during phytoremediation of diesel. Journal of Hazardous Materials 186, 1568-1575.

Alef, K., 1994. Biologische Bodensanierung: Methodenbuch. Wiley VCH Verlag GmbH.

Andria, V., Reichenauer, T.G., Sessitsch, A., 2009. Expression of alkane monooxygenase (*alkB*) genes by plant-associated bacteria in the rhizosphere and endosphere of Italian ryegrass (*Lolium multiflorum* L.) grown in diesel contaminated soil. Environmental Pollution 157, 3347-3350.

Brady, C., Cleenwerck, I., Venter, S., Vancanneyt, M., Swings, J., Coutinho, T., 2008. Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). Systematic and Applied Microbiology 31, 447-460.

Bürgmann, H., Meier, S., Bunge, M., Widmer, F., Zeyer, J., 2005. Effects of model root exudates on structure and activity of a soil diazotroph community. Environmental Microbiology 7, 1711-1724.

Coenye, T., Falsen, E., Vancanneyt, M., Hoste, B., Govan, J., Kersters, K., Vandamme, P., 1999. Classification of *Alcaligenes* faecalis-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. International Journal of Systematic and Evolutionary Microbiology 49, 405-413.

Glick, B.R., 2003. Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotechnology Advances 21, 383-393.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52, 696-704.

Gurska, J., Wang, W., Gerhardt, K.E., Khalid, A.M., Isherwood, D.M., Huang, X.D., Glick, B.R., Greenberg, B.M., 2009. Three year field test of a plant growth promoting rhizobacteria enhanced phytoremediation system at a land farm for treatment of hydrocarbon waste. Environmental Science and Technology 43, 4472-4479.

Gyaneshwar, P., James, E., Mathan, N., Reddy, P., Reinhold-Hurek, B., Ladha, J., 2001. Endophytic colonization of rice by a diazotrophic strain of *Serratia marcescens*. Journal of Bacteriology 183, 2634-2645.

Hai, B., Diallo, N., Sall, S., Haesler, F., Schauss, K., Bonzi, M., Assigbetse, K., Chotte, J., Munch, J., Schloter, M., 2009. Quantification of key genes steering the microbial nitrogen cycle in the rhizosphere of sorghum cultivars in tropical agroecosystems. Applied and Environmental Microbiology 75, 4993-5000.

Hoffmann, H., Stindl, S., Ludwig, W., Stumpf, A., Mehlen, A., Heesemann, J., Monget, D., Schleifer, K., Roggenkamp, A., 2005. Reassignment of *Enterobacter dissolvens* to *Enterobacter cloacae* as *E. cloacae* subspecies *dissolvens* comb. nov. and emended description of *Enterobacter asburiae* and *Enterobacter kobei*. Systematic and Applied Microbiology 28, 196-205.

Holden, N., Pritchard, L., Toth, I., 2009. Colonization outwith the colon: plants as an alternative environmental reservoir for human pathogenic enterobacteria. FEMS Microbiology Reviews 33, 689-703.

Hou, F.S., Milke, M.W., Leung, D.W., MacPherson, D.J., 2001. Variations in phytoremediation performance with diesel-contaminated soil. Environmental Technology 22, 215-222.

Juhanson, J., Truu, J., Heinaru, E., Heinaru, A., 2007. Temporal dynamics of microbial community in soil during phytoremediation field experiment. Journal of Environmental Engineering and Landscape Management 15, 213–220.

Juhanson, J., Truu, J., Heinaru, E., Heinaru, A., 2009. Survival and catabolic performance of introduced *Pseudomonas* strains during phytoremediation and bioaugmentation field experiment. FEMS Microbiology Ecology 70, 446-455.

Kaimi, E., Mukaidani, T., Tamaki, M., 2007. Screening of twelve plant species for phytoremediation of petroleum hydrocarbon-contaminated soil. Plant Production Science 10, 211-218.

Kämpfer, P., Ruppel, S., Remus, R., 2005. Enterobacter radicincitans sp. nov., a plant growth promoting species of the family *Enterobacteriaceae*. Systematic and Applied Microbiology 28, 213-221.

Kechavarzi, C., Pettersson, K., Leeds-Harrison, P., Ritchie, L., Ledin, S., 2007. Root establishment of perennial ryegrass (*L. perenne*) in diesel contaminated subsurface soil layers. Environmental Pollution 145, 68-74.

Kirk, J.L., Klironomos, J.N., Lee, H., Trevors, J.T., 2005. The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil. Environmental Pollution 133, 455-465.

Kuffner, M., Puschenreiter, M., Wieshammer, G., Gorfer, M., Sessitsch, A., 2008. Rhizosphere bacteria affect growth and metal uptake of heavy metal accumulating willows. Plant and Soil 304, 35-44.

Lee, H., Madhaiyan, M., Kim, C., Choi, S., Chung, K., Sa, T., 2006. Physiological enhancement of early growth of rice seedlings (*Oryza sativa* L.) by production of

phytohormone of N_2-fixing methylotrophic isolates. Biology and Fertility of Soils 42, 402-408.

Lelie, D., Schwitzguébel, J., Glass, D., Vangronsveld, J., Baker, A., 2001. Peer Reviewed: Assessing phytoremediation's progress in the United States and Europe. Environmental Science and Technology 35, 446-452.

Liste, H., Prutz, I., 2006. Plant performance, dioxygenase-expressing rhizosphere bacteria, and biodegradation of weathered hydrocarbons in contaminated soil. Chemosphere 62, 1411-1420.

Liu, L., Jiang, C., Liu, X., Wu, J., Han, J., Liu, S., 2007. Plant–microbe association for rhizoremediation of chloronitroaromatic pollutants with *Comamonas* sp. strain CNB 1. Environmental Microbiology 9, 465-473.

Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E., Taghavi, S., Mezgeay, M., van der Lelie, D., 2002. Endophytic bacteria and their potential applications. Critical Reviews in Plant Sciences 21, 583-606.

Madhaiyan, M., Poonguzhali, S., Lee, J., Saravanan, V., Lee, K., Santhanakrishnan, P., 2010. *Enterobacter arachidis* sp. nov., a plant growth-promoting diazotrophic bacterium isolated from rhizosphere soil of groundnut. International Journal of Systematic and Evolutionary Microbiology 60, 1559-1564.

Muratova, A., Dmitrieva, T., Panchenko, L., Turkovskaya, O., 2008. Phytoremediation of oil-sludge–contaminated soil. International Journal of Phytoremediation 10, 486-502.

Olson, P., Reardon, K., Pilon-Smits, E., 2003. Ecology of rhizosphere bioremediation. Phytoremediation: Transformation and control of contaminants, 317-353.

Paauw, A., Caspers, M., Schuren, F., Leverstein-van Hall, M., Delétoile, A., Montijn, R., Verhoef, J., Fluit, A., 2008. Genomic diversity within the *Enterobacter cloacae* complex. PLoS One 3, 3018-3028.

Palmroth, M.R., Pichtel, J., Puhakka, J.A., 2002. Phytoremediation of subarctic soil contaminated with diesel fuel. Bioresource Technology 84, 221-228.

Peng, G., Zhang, W., Luo, H., Xie, H., Lai, W., Tan, Z., 2009. *Enterobacter oryzae* sp. nov., a nitrogen-fixing bacterium isolated from the wild rice species *Oryza latifolia*. International Journal of Systematic and Evolutionary Microbiology 59, 1650-1655.

Posada, D., Crandall, K., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817-818.

Powell, S.M., Ferguson, S.H., Bowman, J.P., Snape, I., 2006. Using real-time PCR to assess changes in the hydrocarbon-degrading microbial community in Antarctic soil during bioremediation. Microbial Ecology 52, 523-532.

Rasche, F., Velvis, H., Zachow, C., Berg, G., Van Elsas, J., Sessitsch, A., 2006a. Impact of transgenic potatoes expressing anti bacterial agents on bacterial endophytes is comparable with the effects of plant genotype, soil type and pathogen infection. Journal of Applied Ecology 43, 555-566.

Rasche, F., Hodl, V., Poll, C., Kandeler, E., Gerzabek, M.H.,van Elsas, J.D., Sessitsch, A., 2006b. Rhizosphere bacteria affected by transgenic potatoes with antibacterial activities compared with the effects of soil, wild type potatoes, vegetation stage and pathogen exposure. FEMS Microbilogy Ecology 56, 219-235.

Reiter, B., Wermbter, N., Gyamfi, S., Schwab, H., Sessitsch, A., 2003. Endophytic Pseudomonas spp. populations of pathogen-infected potato plants analysed by 16S rDNA- and 16S rRNA-based denaturating gradient gel electrophoresis. Plant and Soil 257, 397-405.

Rosenblueth, M., Martinez-Romero, E., 2006. Bacterial endophytes and their interactions with hosts. Molecular Plant-Microbe Interactions 19, 827-837.

Sheng, X., Chen, X., He, L., 2008. Characteristics of an endophytic pyrene-degrading bacterium of *Enterobacter* sp. 12J1 from *Allium macrostemon* Bunge. International Biodeterioration and Biodegradation 62, 88-95.

Shoebitz, M., Ribaudo, C., Pardo, M., Cantore, M., Ciampi, L., Curá, J., 2009. Plant growth promoting properties of a strain of *Enterobacter ludwigii* isolated from *Lolium perenne* rhizosphere. Soil Biology and Biochemistry 41, 1768-1774.

Siciliano, S.D., Fortin, N., Mihoc, A., Wisse, G., Labelle, S., Beaumier, D., Ouellette, D., Roy, R., Whyte, L.G., Banks, M.K., Schwab, P., Lee, K., Greer, C.W., 2001. Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Applied and Environmental Microbiology 67, 2469-2475.

Stroud, J., Paton, G., Semple, K., 2007. Microbe aliphatic hydrocarbon interactions in soil: implications for biodegradation and bioremediation. Journal of Applied Microbiology 102, 1239-1253.

Taghavi, S., Garafola, C., Monchy, S., Newman, L., Hoffman, A., Weyens, N., Barac, T., Vangronsveld, J., Van Der Lelie, D., 2009. Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. Applied and Environmental Microbiology 75, 748.

Thompson, J., Gibson, T., Plewniak, F., Jeanmougin, F., Higgins, D., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25, 4876-4882.

Truu, J., Heinaru, E., Vedler, E., Juhanson, J., Viirmäe, M., Heinaru, A., 2007. Formation of microbial communities in oil shale chemical industry solid wastes during phytoremediation and bioaugmentation. Bioremediation of Soils Contaminated with Aromatic Compounds 76, 57-66.

van Beilen, J.B., Funfhoff, E.G., van Loon, A., Just, A., Kaysser, L., Bouza, M., Holtackers, R., Röthlisberger, M., Li, Z., Witholt, B., 2006. Cytochrome P450 alkane hydroxylases of the CYP153 family are common in alkane-degrading eubacteria lacking integral membrane alkane hydroxylases. Applied and Environmental Microbiology 72, 59-65.

Yousaf, S., Ripka, K., Reichenauer, T., Andria, V., Afzal, M., Sessitsch, A., 2010a. Hydrocarbon degradation and plant colonization by selected bacterial strains isolated from Italian ryegrass and birdsfoot trefoil. Journal of Applied Microbiology 109, 1389-1401.

Yousaf, S., Andria, V., Reichenauer, T., Smalla, K., Sessitsch, A., 2010b. Phylogenetic and functional diversity of alkane degrading bacteria associated with Italian ryegrass (*Lolium multiflorum*) and birdsfoot trefoil (*Lotus corniculatus*) in a petroleum oil-contaminated environment. Journal of Hazardous Materials 184, 523-532.

Figure legends

Fig. 1. Neighbor joining tree of *Enterobacter* species based on *rpoB* sequences showing the phylogenetic position of strains IRI10-4, BRI10-9 and ISI10-3.

Fig. 2. Mean values of ratio of CYP153 gene expression / abundance in the rhizosphere (RH), root interior (RI), shoot interior (SI) of A) Italian ryegrass (IT), B) birdsfoot trefoil (BT) and C) alfalfa (AL). 1st harvest: 6 weeks after germination, 2nd harvest at flowering stage, IT: 14 weeks after germination; BT and AL: 21 weeks after germination. Data are means (n=3), error bars indicate standard deviation.

Table 1. Hydrocarbon concentrations in soils vegetated with Italian ryegrass (IT), birdsfoot trefoil (BT) and alfalfa (AL). Data are means (n=3), standard deviations are presented in parentheses. Means with different letters are significantly different at a 5 % level of significance in each column.

Plant	Treatment	Hydrocarbon concentration (g kg^{-1} soil)				
		Initial value	1st harvest[a]	% decrease	2nd harvest[b]	% decrease
IT						
	Control (+D)	10	9.48^{fg} (0.33)	5	8.04^{h} (0.26)	20
	ISI10-3	10	5.23^{a} (0.31)	48	3.51^{ab} (0.33)	65
	IRI10-4	10	6.98^{d} (0.40)	30	5.07^{ef} (0.44)	49
	BRI10-9	10	6.56^{bcd} (0.34)	34	3.24^{a} (0.30)	68
BT						
	Control (+D)	10	8.90^{f} (0.44)	11	8.05^{h} (0.28)	20
	ISI10-3	10	6.81^{cd} (0.46)	32	4.29^{cd} (0.41)	57
	IRI10-4	10	8.13^{e} (0.28)	19	7.09^{g} (0.58)	29
	BRI10-9	10	6.87^{cd} (0.40)	31	4.36^{cd} (0.56)	56
AL						
	Control (+D)	10	9.76^{g} (0.32)	2	8.08^{h} (0.34)	19
	ISI10-3	10	5.96^{b} (0.34)	40	4.03^{bc} (0.33)	60
	IRI10-4	10	6.78^{cd} (0.50)	32	5.69^{f} (0.43)	43
	BRI10-9	10	6.16^{bc} (0.44)	38	4.78^{de} (0.29)	52

[a] 6 weeks after germination
[b] at flowering stage; IT 14: weeks after germination; BT and AL 21: weeks after germination

Table 2. Shoot and root dry weight of Italian ryegrass (IT), birdsfoot trefoil (BT) and alfalfa (AL). Data are means (n=3), standard deviations are presented in parentheses. Means with different letters are significantly different at a 5 % level of significance in each column.

Treatment	IT		BT		AL	
	1st harvest [a]	2nd harvest [b]	1st harvest	2nd harvest	1st harvest	2nd harvest
shoot biomass (g dry weight)						
Control (-D)	11.6^{a} (1.3)	21.0^{a} (0.6)	2.0^{a} (0.3)	14.2^{a} (0.5)	4.0^{a} (0.3)	15.4^{a} (1.1)
Control (+D)	3.4^{c} (1.1)	6.1^{d} (0.5)	0.9^{b} (0.4)	2.4^{c} (0.7)	0.7^{c} (0.2)	2.0^{c} (1.0)
ISI10-3	7.1^{b} (1.0)	13.1^{b} (0.6)	1.3^{b} (0.2)	6.0^{b} (0.6)	1.6^{b} (0.3)	8.1^{b} (1.1)
IRI10-4	6.4^{b} (1.2)	10. 9^{c} (0.7)	1.1^{b} (0.2)	5.5^{b} (0. 7)	1.5^{b} (0.3)	3.0^{c} (1.2)
BRI10-9	6.9^{b} (1.2)	13.0^{b} (0.6)	1.1^{b} (0.4)	6.6^{b} (0.7)	1.6^{b} (0.3)	7.9^{b} (1.3)
root biomass (g dry weight)						
Control (-D)	6.3^{a} (0.6)	16.4^{a} (0.9)	0.7^{a} (0.1)	5.0^{a} (0.4)	0.3^{a} (0.1)	7.0^{a} (0.2)
Control (+D)	2.9^{c} (0.6)	4.7^{d} (0.9)	0.2^{b} (0.2)	0.5^{c} (0.2)	0.1^{b} (0.1)	0.2^{d} (0.1)
ISI10-3	6.1^{a} (0.8)	12.4^{b} (0.8)	0.6^{a} (0.2)	1.9^{b} (0.3)	0.3^{a} (0.1)	2.8^{b} (0.2)
IRI10-4	4.6^{b} (0.7)	5.7^{cd} (0.7)	0.3^{b} (0.1)	1.6^{b} (0.2)	0.1^{b} (0.1)	0.4^{d} (0.2)
BRI10-9	5.3^{ab} (0.6)	6.5^{c} (0.9)	0.6^{a} (0.1)	1.8^{b} (0.3)	0.2^{ab} (0.1)	1.2^{c} (0.2)

[a] 6 weeks after germination
[b] at flowering stage; IT 14: weeks after germination ; BT and AL 21: weeks after germination

Table 3. Colony forming units (CFU) in the rhizosphere (RH), root interior (RI), shoot interior (SI) of Italian ryegrass (IT), birdsfoot trefoil (BT) and alfalfa (AL). Data are means (n=3), standard deviations are presented in parentheses. Means with different letters are significantly different at a 5 % level of significance in each column.

Treatment	CFU/g dry weight RH			CFU/g dry weight RI			CFU/g dry weight SI		
	IT	BT	AL	IT	BT	AL	IT	BT	AL
				1st harvest [a]					
ISI10-3	2.27E+08 [a] (3.08E+06)	1.09E+07 [g] (2.67E+05)	1.02E+08[b] (2.19E+06)	2.89E+07[b] (1.05E+06)	1.08E+03[d] (1.33E+02)	1.33E+05[d] (7.36E+03)	1.90E+05[a] (2.72E+04)	8.61E+04[b] (3.36E+04)	5.42E+04[c] (7.09E+03)
IRI10-4	4.14E+07 [f] (1.72E+06)	5.95E+04 [h] (7.60E+04)	5.52E+07[d] (2.72E+06)	4.96E+07[a] (5.99E+06)	7.68E+04[d] (2.62E+03)	1.46E+07[c] (7.36E+05)	1.61E+03[e] (3.87E+02)	3.85E+04[cd] (4.70E+03)	1.75E+04[de] (4.79E+02)
BRI10-9	4.76E+07 [e] (2.51E+06)	4.70E+04 [h] (6.09E+04)	6.68E+07[c] (2.50E+06)	1.90E+04[d] (8.89E+02)	8.10E+04[d] (1.74E+03)	2.58E+06[d] (1.29E+05)	9.72E+03[de] (3.06E+03)	ND	2.86E+04[cde] (6.16E+02)
				2nd harvest [b]					
ISI10-3	4.18E+07[ab] (6.94E+06)	2.36E+07[c] (2.04E+06)	3.96E+07[b] (1.49E+06)	2.10E+06[e] (1.53E+05)	9.21E+06[c] (1.14E+06)	8.89E+04[f] (1.54E+04)	1.07E+06[a] (1.23E+05)	2.46E+05[c] (3.45E+04)	9.98E+05[b] (3.83E+04)
IRI10-4	2.76E+06[d] (2.02E+05)	1.82E+06[d] (5.22E+05)	3.80E+06[d] (2.65E+05)	1.11E+05[f] (1.14E+04)	6.57E+07[a] (2.56E+06)	2.09E+07[b] (1.39E+06)	6.79E+04[de] (1.03E+03)	4.67E+04[de] (5.16E+03)	1.16E+05[d] (8.17E+03)
BRI10-9	4.46E+07[a] (2.90E+06)	3.02E+06[d] (3.16E+05)	3.02E+06[d] (2.46E+05)	7.33E+05[ef] (1.15E+05)	5.22E+06[d] (3.09E+05)	9.10E+05[ef] (1.54E+04)	3.19E+04[e] (5.12E+03)	1.38E+04[e] (1.46E+03)	2.52E+04[e] (7.36E+02)

[a] 6 weeks after germination

[b] at flowering stage; IT 14: weeks after germination ; BT and AL 21: weeks after germination

Table 4. CYP153 gene abundance in the rhizosphere (RH), root interior (RI), shoot interior (SI) of Italian ryegrass (IT), birdsfoot trefoil (BT) and alfalfa (AL). Data are means (n=3), standard deviations are presented in parentheses. Means with different letters are significantly different at a 5 % level of significance in each column.

Treatment	CYP genes abundance (copies/g dry weight) RH			CYP genes abundance (copies/g dry weight) RI			CYP genes abundance (copies/g dry weight) SI		
	IT	**BT**	**AL**	**IT**	**BT**	**AL**	**IT**	**BT**	**AL**
				1st harvest [a]					
ISI10-3	1.12E+09[a] (1.06E+08)	4.34E+08[c] (3.22E+07)	1.04E+09[a] (3.93E+07)	2.59E+07[c] (2.29E+06)	1.13E+04[d] (1.04E+02)	5.16E+05[d] (1.94E+05)	2.93E+06[a] (3.16E+05)	3.25E+05[b] (7.63E+04)	2.00E+05[bc] (8.40E+04)
IRI10-4	4.38E+08[c] (5.75E+07)	3.22E+06[d] (5.82E+05)	3.84E+08[c] (7.15E+07)	9.68E+07[a] (4.52E+06)	7.97E+04[d] (8.15E+04)	4.38E+07[b] (1.32E+07)	5.68E+03[c] (1.01E+03)	7.75E+04[c] (4.70E+03)	5.52E+04[c] (5.20E+03)
BRI10-9	6.76E+08[b] (4.72E+07)	3.57E+06[d] (5.34E+05)	1.05E+09[a] (8.13E+07)	4.01E+04[d] (1.27E+04)	3.64E+04[d] (2.47E+03)	2.62E+06[d] (5.78E+05)	1.54E+04[c] (3.20E+03)	ND	6.62E+04[c] (4.13E+03)
				2nd harvest [b]					
ISI10-3	9.68E+08[a] (4.26E+07)	1.92E+08[c] (4.18E+07)	9.04E+08[b] (3.66E+07)	2.66E+06[d] (1.29E+06)	9.52E+06[c] (3.29E+05)	1.39E+05[d] (3.15E+04)	2.96E+07[a] (5.56E+06)	3.49E+06[b] (3.47E+05)	3.20E+06[b] (1.28E+06)
IRI10-4	8.68E+07[d] (2.63E+06)	4.64E+06[e] (4.65E+05)	8.31E+06[e] (4.69E+05)	2.22E+05[d] (1.37E+05)	5.07E+07[a] (5.44E+06)	3.87E+07[b] (4.54E+06)	8.65E+04[b] (1.33E+04)	7.47E+04[b] (1.39E+04)	4.43E+05[b] (2.14E+04)
BRI10-9	9.36E+08[ab] (1.83E+07)	8.24E+06[e] (7.62E+05)	7.79E+06[e] (8.14E+05)	5.59E+05[d] (2.21E+04)	3.36E+06[d] (2.46E+05)	9.30E+05[d] (4.62E+04)	6.02E+04[b] (1.42E+04)	4.18E+04[b] (1.09E+04)	5.35E+04[b] (1.04E+04)

[a] 6 weeks after germination

[b] at flowering stage; IT 14: weeks after germination ; BT and AL 21: weeks after germination

Table 5. CYP153 gene expression in the rhizosphere (RH), root interior (RI), shoot interior (SI) of Italian ryegrass (IT), birdsfoot trefoil (BT) and alfalfa (AL). Data are means (n=3), standard deviations are presented in parentheses. Means with different letters are significantly different at a 5 % level of significance in each column.

Treatment	CYP genes expression (copies/g dry weight) RH			CYP genes expression (copies/g dry weight) RI			CYP genes expression (copies/g dry weight) SI		
	IT	**BT**	**AL**	**IT**	**BT**	**AL**	**IT**	**BT**	**AL**
				1st harvest [a]					
ISI10-3	6.66E+08[a] (3.07E+07)	8.89E+07[c] (6.19E+06)	1.14E+08[b] (8.42E+06)	5.47E+06[c] (5.04E+05)	6.38E+03[e] (3.56E+02)	2.07E+05[e] (4.16E+04)	1.17E+06[a] (6.71E+04)	1.07E+05[b] (4.24E+03)	1.13E+05[b] (4.98E+03)
IRI10-4	4.81E+07[c] (4.64E+06)	5.92E+05[d] (2.10E+04)	3.76E+07[c] (2.49E+06)	9.27E+06[a] (4.91E+05)	1.50E+04[e] (1.41E+04)	7.28E+06[b] (4.35E+05)	1.27E+03[c] (4.52E+01)	2.07E+04[c] (5.98E+02)	2.57E+04[c] (2.12E+04)
BRI10-9	9.51E+07[bc] (3.78E+06)	8.60E+05[d] (6.22E+04)	1.01E+08[bc] (6.58E+06)	1.48E+04[e] (3.06E+03)	5.20E+03[e] (3.99E+03)	7.73E+05[d] (4.33E+04)	9.58E+03[c] (2.95E+02)	ND	5.07E+04[c] (5.75E+02)
				2nd harvest [b]					
ISI10-3	4.17E+08[a] (5.93E+07)	4.98E+07[d] (1.56E+06)	1.30E+08[c] (5.86E+06)	5.72E+05[d] (5.72E+04)	1.19E+06[c] (4.42E+04)	4.78E+04[e] (4.00E+03)	1.04E+07[a] (4.78E+05)	1.05E+06[b] (8.67E+04)	1.13E+06[b] (4.62E+04)
IRI10-4	1.09E+07[d] (1.33E+05)	6.85E+05[e] (2.80E+05)	1.01E+06[e] (3.12E+05)	5.87E+04[e] (4.95E+03)	8.14E+06[a] (3.36E+05)	5.28E+06[b] (3.34E+05)	1.23E+04[c] (4.20E+02)	1.05E+04[c] (5.64E+02)	5.04E+04[c] (3.95E+03)
BRI10-9	1.66E+08[b] (4.21E+06)	2.85E+06[e] (2.80E+05)	2.37E+06[e] (1.35E+05)	8.94E+04[e] (7.23E+03)	6.87E+05[d] (4.47E+04)	4.49E+05[d] (4.75E+04)	1.32E+04[c] (5.40E+02)	7.55E+03[c] (2.95E+02)	1.17E+04[c] (3.93E+02)

[a] 6 weeks after germination

[b] at flowering stage; IT 14: weeks after germination ; BT and AL 21: weeks after germination