Title: Comparison of Two Inoculation Methods of Endophytic Bacteria to Enhance Phytodegradation Efficacy of an Aged Petroleum Hydrocarbons Polluted Soil

Author: Małgorzata Pawlik, Tomasz Płociniczak, Sofie Thijs, Isabel Pintelon, Jaco Vangronsveld, Zofia Piotrowska-Seget

agronomy

MDPI

Article

Comparison of Two Inoculation Methods of Endophytic Bacteria to Enhance Phytodegradation Efficacy of an Aged Petroleum Hydrocarbons Polluted Soil

Małgorzata Pawlik [1,*], Tomasz Płociniczak [1], Sofie Thijs [2], Isabel Pintelon [3], Jaco Vangronsveld [2] and Zofia Piotrowska-Seget [1]

1 Faculty of Natural Sciences, Institute of Biology, Biotechnology and Environmental Protection, University of Silesia in Katowice, Jagiellonska 28, 40-032 Katowice, Poland; tomasz.plociniczak@us.edu.pl (T.P.); zofia.piotrowska-seget@us.edu.pl (Z.P.-S.)

2 Centre for Environmental Sciences, Hasselt University, Agoralaan building D, 3590 Diepenbeek, Belgium; sofie.thijs@uhasselt.be (S.T.); jaco.vangronsveld@uhasselt.be (J.V.)

3 Laboratory of Cell Biology and Histology, University of Antwerp Campus Drie Eiken, Universiteitsplein 1, Wilrijk, 2610 Antwerp, Belgium; isabel.pintelon@uantwerpen.be

* Correspondence: malgorzata.pawlik@us.edu.pl; Tel.: +48-32-2009-442

Received: 23 June 2020; Accepted: 10 August 2020; Published: 14 August 2020

Abstract: Endophyte-enhanced phytodegradation is a promising technology to clean up polluted soils. To improve the success rate of this nature-based remediation approach, it is important to advance the inoculation method as this has been shown to strongly affect the final outcome. However, studies evaluating inoculation strategies and their effect on hydrocarbon degradation are limited. This study aims to investigate two different manners of endophyte inoculation in *Lolium perenne* growing in an aged petroleum hydrocarbon polluted soil: (1) direct soil inoculation (SI), and (2) pre-inoculation of the caryopses followed by soil inoculation (PI). Different endophytic bacterial strains, *Rhodococcus erythropolis* 5WK and *Rhizobium* sp. 10WK, were applied individually as well as in combination. Depending on the method of inoculation, the petroleum hydrocarbon (PHC) degradation potential was significantly different. The highest PHC removal was achieved after pre-inoculation of ryegrass caryopses with a consortium of both bacterial strains. Moreover, both strains established in the aged-polluted soil and could also colonize the roots and shoots of *L. perenne*. Importantly, used endophytes showed the selective colonization of the environment compartments. Our findings show that the method of inoculation determines the efficiency of the phytodegradation process, especially the rate of PHC degradation. This study provides valuable information for choosing the most cost-effective and beneficial means to optimize phytodegradation.

Keywords: endophytic bacteria; phytoremediation; petroleum hydrocarbon polluted soil; inoculation; pre-inoculation; green fluorescent protein; plant growth-promoting bacteria

1. Introduction

Accidental spills and rapid industrialization have resulted in substantial amounts of petroleum hydrocarbons (PHC) ending up in soils and (ground) water. These pollutants are harmful, carcinogenic and/or mutagenic to humans, animals and plants [1,2], causing severe environmental and economic losses. Consequently, the worldwide demand for efficient and low ecological footprint remediation techniques to clean up petroleum polluted sites is increasing. Among the most effective methods to combat organic pollutants such as PHC, among the most promising is phytoremediation, especially

phytodegradation in the context of organic contaminants [3,4]. Phytodegradation is based on the application of plants and their associated microorganisms to remove pollutants from the environment which may be degraded [5–8]. Except the unquestioned number of economic and environmental advantages of this technique, many efforts have been made to enhance phytodegradation efficiency, and to predict the final result and/or time needed for clean-up [9–12]. To increase the performance of phytodegradation, it should be combined with the introduction of plant growth-promoting (PGP) and organic pollutant-degrading microorganisms [6,7,12]. Due to the close associations of endophytic bacteria with plants and their abilities to reside within the living tissues, they appear to be key candidates for optimizing the phytodegradation process [7,9–11,13]. Knowledge generated during the last two decades indicates that endophytic bacteria possess the abilities to mineralize hydrocarbons in soil and/or plant parts, consequently considerably reducing phytotoxicity and minimizing the stress these pollutants can cause to the plants [14–19].

Despite these known positive interactions during phytodegradation, experimental factors often significantly limit the beneficial effects of endophytic bacteria on the effective removal of organic pollutants from soil and the efficiency is far below expectations [20]. The combination of the most appropriate plant species and the selection of bacterial or fungal strains to enhance phytodegradation of PHC has been primarily examined on a laboratory scale [21,22]. The success of microbe-assisted remediation techniques depends to a large extent on the survival and activities of indigenous microbial communities and their interaction with introduced microorganisms. Intriguingly, even though promising results have been obtained in several plant-endophytic bacteria combinations, in some studies, the survival of the introduced strains was not adequate, their activity was lower than expected and/or hydrocarbon removal was unsatisfactory [15,18,23–28].

Notably, one of the phenomena that may explain such failures is connected with the existence of viable but nonculturable (VBNC) states in pollutant-degrading bacteria [24,29,30]. Recent studies have pointed out that strains with PGP and degradation capabilities can enter a VBNC-like state when they encounter harsh environmental conditions. Bacteria in this state are alive but have lost their ability to reproduce. Therefore, after inoculation, the number of bacteria strongly declines which may be a result of a transition of free-living bacteria into a VBNC-like state [24,30].

In this respect, there exists a wide divergence of phytodegradation outcomes that might be due to differences in inoculation techniques (seeds/soil spreading, leaf/shoot injection, seeds coating) and dosage of inoculates that were introduced into the soil. Due to the fact that different volumes of soil per pot were used, the effective bacterial densities in soils were highly different. Few studies are available specifically on the potential importance of bacterial densities and methods of application of endophytic strains during phytodegradation [18,20,24,31].

The influence of introduced bacteria on soil microbial communities and their effects on biological functions are still unclear and difficult to predict. A better understanding of the interactions/competition between the autochthonous microbiota and the introduced strains is very important to allow a better assessment of the phytodegradation processes and their outcomes. In addition, phytodegradation efficiency may be considered a result of a combination of these factors. A carefully considered selection of the inoculation method can assist to improve the processes of bacterial colonization, hydrocarbon degradation, seed germination and plant development. In addition, the deployment of complementary analyses methods may generate crucial information for the selection of the mode of application and the dosage of bacteria, which is of paramount importance. Therefore, finding appropriate methods of bacterial inoculation remains crucial.

The main objective of this study was to evaluate the effects of the application method on (1) PHC removal efficiency using *Lolium perenne*; (2) soil and ryegrass interior colonization by endophytic bacteria *Rhodococcus erythropolis* 5WK and *Rhizobium* sp. 10WK; (3) plant biomass production; (4) total numbers of bacteria with the potential to degrade aliphatic hydrocarbons in soil (real-time PCR). For this purpose, two inoculation methods were tested and compared: direct soil inoculation (SI) and pre-inoculation of grains followed by soil inoculation (PI).

2. Materials and Methods

2.1. Soil Properties

Petroleum hydrocarbon polluted soil was obtained from an industrial area near to the oil refinery in Czechowice-Dziedzice, Silesia, Southern Poland (49°54'49.0" N, 19°01'01.9" E). Soil was sampled from the top 0–20 cm around a waste lagoon where acidic, highly weathered petroleum sludges were deposited and stored. Details concerning the chemical and physical parameters of this soil were previously described [31,32]. The soil was air-dried at room temperature, sieved, and subsequently, soil moisture content was adjusted to 20%. The concentration of PHC in the soil was 12,853.34 ± 322.62 mg PHC kg^{-1} dry weight (d.w.).

Due to the fact that during the phytodegradation experiment, rifampicin resistant strains were used, the presence of autochthonous bacteria possessing rifampicin resistance was evaluated. Ten grams of soil was suspended in 90 mL 0.9% NaCl with 1% Tween 80 and shaken at 120 rpm for 30 min at 28 °C. The serial dilutions were prepared and plated onto a solid Luria-Bertani (LB) medium supplemented with 20–150 μg mL^{-1} of rifampicin and 50 μg mL^{-1} of nystatin. After 7 days of incubation at 28 °C, no bacterial colonies were observed.

2.2. Bacterial Strains and Selection of Rifampicin-Resistant Mutants

The endophytic strains *Rhodococcus erythropolis* 5WK and *Rhizobium* sp. 10WK that were used in this study were previously isolated from the surface-sterilized roots of *Oenothera biennis* collected from a long-term polluted site located in Czechowice-Dziedzice, Silesia, Southern Poland. Strains were identified based on 16S rRNA gene sequencing and characterized according to their PGP mechanisms, hydrocarbon degradation potential, and genes involved in hydrocarbon degradation (*alkB, alkH, P450* (CYP153)) [13].

The spontaneous rifampicin-resistant mutants of *R. erythropolis* 5WK and *Rhizobium* sp. 10WK were selected by plating overnight cultures onto a solid LB medium containing 5 μg mL^{-1} of rifampicin. Subsequently, well-grown colonies were transferred from one to another plate with LB amended with increasing concentrations of rifampicin (20–150 μg mL^{-1}). The obtained rifampicin-resistant strains were tested to confirm their stability of resistance by subculturing them several times in an LB medium without antibiotics. Then, individual colonies growing in the nonselective medium were plated in LB supplemented with 150 μg mL^{-1} of rifampicin (LB+rif). The $5WK^{rif}$ and $10WK^{rif}$ mutants thrived, demonstrating the stability of rifampicin resistance, and had identical PGP abilities and hydrocarbon degradation potential to the parental endophytic strains.

2.3. Inoculum Preparation

Strains *R. erythropolis* $5WK^{rif}$ and *Rhizobium* sp. $10WK^{rif}$ were grown in a liquid LB+rif medium at 28 °C on a rotary shaker (120 rpm) for 14–16 h until the desired density (OD_{600nm} = 1.5–2.0) of the bacterial suspension. Bacterial cells were recovered by centrifugation (5000 rpm, 20 °C, 20 min), washed three times with sterile 0.9% NaCl and resuspended in 40 mL sterile 0.9% NaCl. The number of bacteria in the inoculum was estimated based on optical density and plating techniques. To standardize the dosage of bacterial inoculation, the amount of bacterial solution introduced into the soil in each pot was calculated to consist of 10^8 colony forming units (cfu) g^{-1} d.w. of soil. The bacterial strains were used as an inoculum in the form of each single strain, separately, ($5WK^{rif}$; $10WK^{rif}$) and as a consortium of both strains ($5WK^{rif}$ + $10WK^{rif}$).

2.4. Experimental Design and Inoculation

For the phytodegradation experiment, each pot (550 mL) was filled with 450 g of soil prepared as described above. Next, the ryegrass caryopses (0.40 ± 0.1 g per pot) were sown at a depth of 1 cm and covered with a thin layer of soil. The soil moisture was kept at a constant level of 20%. All pots were placed in a growth chamber under controlled conditions (14 h light photoperiod at 300 μmol m^{-2} s^{-1}

PAR; temperature 23/18 °C day/night). Plants were grown for 75 days. The fresh weight of the plants was determined at days 14, 31 and 75. Soil samples were collected from all treatments and stored at −20 °C.

Two inoculation methods were examined: (1) direct soil inoculation (SI); (2) pre-inoculation of caryopses followed by soil inoculation (PI). In the first method (SI), the ryegrass grains (0.40 ± 0.1 g) were sown into the soil and 40 mL of bacterial inoculum containing single strains ($5WK^{rif}$ or $10WK^{rif}$) or a consortium of both strains ($5WK^{rif}$ + $10WK^{rif}$) was poured on the soil.

In the second method (PI), caryopses were pre-inoculated with the bacterial inoculum. Ryegrass grains (0.40 ± 0.1 g) were surface sterilized by sequential washing for 5 min in 70% ethanol and 0.01% active sodium hypochlorite. Subsequently, three rinses with sterile water were used to remove the disinfectants. Sterility was verified by plating the last rinsing water onto an LB medium. The sterility was considered adequate when no bacterial growth was observed. Sterilized caryopses were incubated in the dark without shaking at 28 °C with 14 mL of bacterial suspension (10^8 cells mL^{-1} of medium) containing strain $5WK^{rif}$, $10WK^{rif}$ or a consortium of both strains mixed in equal proportions. After 24 h, the bacterial suspension was removed, caryopses were carefully rinsed three times with sterile water and transferred to the pots. Subsequently, 40 mL of fresh bacterial inoculum consisting of single strains ($5WK^{rif}$ or $10WK^{rif}$), or a consortium of both strains, was poured onto the soil.

The pot experiment was arranged in a completely randomized block design with three replications. Eight different treatments were assessed:

(1) Polluted soil (S);
(2) Polluted soil with ryegrass grains (R);
(3) Polluted soil with ryegrass grains inoculated with the $5WK^{rif}$ strain (SI + 5WK);
(4) Polluted soil with ryegrass grains inoculated with the $10WK^{rif}$ strain (SI + 10WK);
(5) Polluted soil with ryegrass grains inoculated with a consortium of the $5WK^{rif}$ and $10WK^{rif}$ strains (SI + 5WK + 10WK);
(6) Polluted soil with ryegrass grains pre-inoculated with the $5WK^{rif}$ strain followed by soil inoculation with the $5WK^{rif}$ strain (PI + 5WK);
(7) Polluted soil with ryegrass grains pre-inoculated with the $10WK^{rif}$ strain followed by soil inoculation with the $10WK^{rif}$ strain (PI + 10WK);
(8) Polluted soil with ryegrass grains pre-inoculated with a consortium of the $5WK^{rif}$ and $10WK^{rif}$ strains followed by soil inoculation with the consortium of $5WK^{rif}$ and $10WK^{rif}$ strains (PI + 5WK + 10WK).

2.5. Petroleum Hydrocarbons (PHC)

The concentrations of PHC were determined in soils before and after the phytodegradation experiment, according to the method previously described [31,32]. The hydrocarbons with carbon atom numbers between 10 and 40 were quantified in each pot according to the protocol ISO 16703:2011 using a gas chromatograph (Agilent 7820A) equipped with a flame ionization detector (FID) and a Rxi-5 ms capillary column (25 m × 0.2 mm ID × 0.33 µm).

2.6. Establishment of Inoculated Endophytic Bacteria

In order to detect and enumerate living 5WK and 10WK cells in soil, roots and shoots at 14, 31 and 75 days, the standard plate count method was applied. Ten grams of soil was suspended in 90 mL 0.9% NaCl with 1% Tween 80 and shaken at 120 rpm for 30 min at 28 °C. The serial dilutions were prepared and plated onto solid LB amended with 150 µg mL^{-1} of rifampicin and 50 µg mL^{-1} of nystatin (LB+rif+nyst). After 7 days of incubation at 28 °C, the numbers of bacterial cells were estimated and expressed as cfu g^{-1} d.w. of soil. Ryegrass roots and shoots were separated and washed with 1% Tween 80 to remove the adhering soil. The plant tissues were surface sterilized by submersing them for 5 min in 70% ethanol and 0.01% active sodium hypochlorite, followed by three times rinsing with

sterile distilled water. The surface sterilization efficiency was estimated by plating each final rinsing water onto LB+rif+nyst and a solid LB medium without antibiotics. If no colonies were observed after incubation for 7 days at 28 °C, the surface sterilization was considered as sufficient. Next, roots and shoots were macerated separately with 0.9% NaCl for 2 min. Serial dilutions of this macerate were spread on LB+rif+nyst followed by incubation at 28 °C for 7 days. The bacterial colonies were counted on each plate and expressed as cfu g^{-1} fresh weight (f.w.) of roots or shoots.

2.7. Plant Weight

The effects of the introduced strains on plant weight were determined after 14, 31 and 75 days. Ryegrass plants were removed from the soil, cleaned up under tap water, dried with a paper towel and weighted. At the end of the experiment, the growth efficiency index (EI) was calculated according the equation where M_1 is the total plant biomass at the end of the experiment (75 days) and M_0 is total plant biomass obtained at 14 days of phytodegradation.

$$EI\ (\%) = \left(\frac{M_1 - M_0}{M_0}\right) * 100\%$$

2.8. Real-Time PCR

Total DNA from the soil was extracted using the Power Soil DNA Isolation Kit (MoBio) as described by the manufacturer. Quantification of the 16S rRNA and alkane hydroxylase gene (*alkH*) copy number was carried out for all samples by real-time PCR (qPCR) using a LightCycler96 (Roche). The *alkH* gene was chosen because it allowed the estimation of the potential of soil microorganisms for aliphatic hydrocarbons, and the primers had an appropriate length for real-time PCR. Specific primers for 16S rRNA pE 5′ AAA CTC AAA GGA ATT GAC GG 3′ and pF 5′ ACG AGC TGA CGA CAG CCA TG 3′ were applied [33]. The *alkH* gene was amplified by primers alk-H1-F 5′ CIG IIC ACG AII TIG GIC ACA AGA AGG 3′ and alk-H3-R 5′ IGC ITG ITG ATC III GTG ICG CTG IAG 3′ [34]. Amplification was performed in 20 µL reaction mixtures containing FastStart Essential DNA Green Master (Roche Diagnostic, Germany), 0.2 mM of each primer and 0.2 mg of template DNA. The SYBR Green I signal intensities were measured. The reaction for 16S rRNA was performed according to the temperature profile 95 °C for 10 min followed by 28 cycles at 95 °C for 10 s, 57 °C for 20 s and 72 °C for 30 s. qPCR conditions for the *alkH* gene were as follows: 10 min at 95 °C, 30 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min. In order to avoid the detection of primer dimers at the end of each extension, step fluorescence was determined at 81 °C. A final melting curve analysis from 65 to 95 °C was performed. The number of copies of both genes was determined on the basis of standard curves prepared from serial dilutions of the standard. The standard was prepared according to the protocol of the InsTAclone PCR Cloning Kit (Thermo Scientific, Waltham, MA, USA), according to which, the PCR product of analyzed genes was cloned into the pTZ57R/T vector (Thermo Scientific). The purity and concentration of the standard DNA was determined spectrophotometrically (NanoDrop, Thermo Scientific).

2.9. Plant Colonization by Gfp-Labelled Endophytic Bacteria

The *Rhizobium* sp. 5WK strain was labelled with the enhanced green fluorescent protein (eGFP) as previously described [35–37]. The 5WK strain was equipped with the plasmid using a triparental conjugation with *Escherichia coli* DH5α as a donor strain, and *E. coli* PRK 2013 as a helper bacterium. The plasmid with a constitutive expression of *egfp* under control of the lac promoter was introduced to the *Rhizobium* sp. 5WK strain.

A similar experiment was carried out to monitor the colonization and distribution of the 5WK strain to confirm its abilities to be endophytic. The labeled strain was inoculated to *L. perenne* seedlings near to the roots zone (10^9 cells mL^{-1}). After 24–48 h, the colonization was examined with a spinning disk confocal laser microscope Ultra VIEW VoX, PerkinElmer (Zaventem, Belgium). An excitation

wavelength of 485 nm (green) was used for *egfp*, and 405 (Dapi) for plant cell wall structures. Lenses used for image acquisition included a 40× CFI Plan Fluor lens (numerical aperture of 0.75; working distance of 0.72 mm) and a 20× CFI Plan Apochromat VC lens (numerical aperture of 0.75, and working distance of 0.72 mm). Images were taken using a Hamamatsu C9100-50 camera (Hamamatsu Photonics K.K., Hamamatsu, Japan). The confocal pictures were analyzed using ImageJ software and Amira 3D visualization software version 6.1.0 (FEI Visualization Sciences Group, Hillsboro, OR, USA) as described previously [36,37].

2.10. Statistical Analysis

STATISTICA 13.1 PL software (StatSoft, Tulsa, OK, USA) was used for a statistical analysis of the data. Relevant data tables and charts were obtained using Microsoft Excel (2010). Analysis of Variance (ANOVA) followed by a post-hoc Least Significant Difference (LSD) test were carried out to identify any significant effects of PHC removal among all treatments. The significant differences in the copy numbers of the 16S rRNA and *alkH* gene (qPCR) in the soil during the pot experiment, plant weight and the comparison of the numbers of living 5WK and 10WK cells in soil and ryegrass tissues, were performed using one-way ANOVA followed by a post-hoc Tukey's test. The data are presented as the mean ± standard deviation (SD) of three replicates.

3. Results

3.1. PHC Concentration

The initial concentration of PHC in the soil used in this experiment was 12,853.34 ± 322.62 mg kg^{-1} dry soil. At the end of the experimental period of 75 days, we found out that the inoculation of planted soils with selected endophytic bacteria increased the effectiveness of phytodegradation as compared to uninoculated soil and plants (S, R) (Table 1).

Table 1. Petroleum hydrocarbon (PHC) removal efficiency (%) after 75 days of phytodegradation in different treatments. The initial concentration at the beginning of experiment was 12,853.34 ± 322.62 mg kg^{-1} d.w. of soil.

	Treatment	PHC Removal (%)
Polluted soil with ryegrass grains inoculated with strain	SI + 5WK	2.2 ± 0.4cd
	SI + 10WK	1.6 ± 0.6d
	SI + 5WK + 10WK	2.1 ± 1.8d
Polluted soil with ryegrass grains pre-inoculated with strain followed by soil inoculation with strain	PI + 5WK	8.9 ± 2.6b
	PI + 10WK	9.7 ± 1.3b
	PI + 5WK + 10WK	19.1 ± 2.5a
Polluted soil	S	1.7 ± 0.5d
Polluted soil with ryegrass grains	R	4.7 ± 1.4c

(5WK) The *Rhodococcus erythropolis* 5WK strain; (10WK) the *Rhizobium* sp. 10WK strain; (5WK + 10WK) the consortium of *Rhodococcus erythropolis* 5WK and *Rhizobium* sp. 10WK. Mean ± standard deviation of three replicates; comparisons between treatments with regard to the initial concentration of PHC in the soil were carried out by one-way ANOVA and an LSD test $p < 0.05$, and values marked by different letters (a, b, c, d) are significant.

The highest removal of PHC (19.1% ± 2.5%) was observed for the pre-inoculation of seeds with a consortium of both strains (Table 1). Obtained results showed that only in PI + 5WK + 10WK treatment, we observed the statistically significant decrease in PHC concentration (Table 1). The lowest PHC reduction was observed in SI treatments and for soil without bacterial inoculation and plants, where removal of PHC was within the range 1.6–2.2%.

3.2. Detection and Quantification of Inoculated Endophytic Bacteria

The establishment of the inoculated bacterial strains in soil and plant tissues was investigated at the end of the experimental period (75 days; Table 2). The colonization pattern of ryegrass tissues seemed to be connected with the mode of bacterial inoculation and preferences of the strains. In SI treatments, the counts of the 5WK^{rif} strain was higher in roots than in shoots; unexpectedly, this strain was not detected in the soil. The 10WK^{rif} strain did not colonize plant roots and shoots effectively and maintained a high abundance in the soil. After inoculation of the consortium, the numbers of endophytic bacteria were two times higher in roots than in soil. A different pattern of colonization was observed when pre-inoculation was applied. The presence of 5WK^{rif} was confirmed only inside the roots. However, 10WK^{rif} colonized not only roots but also shoots. Pre-inoculation of the grains with a consortium of both strains led to a higher number of colony-forming units in soil than in roots.

Table 2. The numbers of endophytic bacteria in soil and ryegrass tissues after 75 days of phytodegradation.

		Numbers of Endophytic Bacteria in Soil and Ryegrass Tissues		
	Treatment	**SOIL (log cfu g^{-1} d.w.)**	**ROOT (log cfu g^{-1} f.w.)**	**SHOOT (log cfu g^{-1} f.w.)**
Polluted soil with ryegrass grains inoculated with strain	SI + 5WK	0	5.84 ± 0.08bc	4.84 ± 0.18cde
	SI + 10WK	8.34 ± 0.12a	0	0
	SI + 5WK + 10WK	4.56 ± 0.28e	6.55 ± 0.07b	0
Polluted soil with ryegrass grains pre-inoculated with strain followed by soil inoculation with strain	PI + 5WK	0	5.94 ± 0.52b	0
	PI + 10WK	0	4.72 ± 0.12de	4.8 ± 0.17de
	PI + 5WK + 10WK	5.7 ± 0.05bcd	4.48 ± 0.21e	0

(5WK) The *Rhodococcus erythropolis* 5WK strain; (10WK) the *Rhizobium* sp. 10WK strain; (5WK + 10WK) the consortium of *Rhodococcus erythropolis* 5WK and *Rhizobium* sp. 10WK. Mean ± standard deviation of three replicates; means followed by different letters (a, b, c, d, e) are significantly different according to one-way ANOVA and a Tukey's test $p < 0.05$.

The post-hoc Tukey's test ($p < 0.05$) indicated statistically significant differences between the numbers of bacterial strains detected in soil and in roots when the consortium of both strains was applied. Post-hoc analysis did not indicate differences in the number of bacterial cells inside shoots (Table 2).

3.3. Plant Biomass Production

With regard to soil inoculation (SI), a significant increase in the growth efficiency index (13.3%) was only observed in soil inoculated with 5WK. In respect of pre-inoculation of the grains (PI), an increased plant biomass was detected after inoculation with the single strains (PI + 5WK and PI + 10WK) (Figure 1). The value of growth indices calculated for these treatments was 106.1% and 28% EI, respectively. In turn, the value of ryegrass growth index in the treatment without inoculation (R) was 36.4% EI. In general, plant biomasses were influenced by the inoculum applied (single strains or consortium). Nevertheless, plant growth and biomass production were lower when the PI method was used (Figure 1). Moreover, the statistical analysis did not show the differences between plant weights in all treatments (Figure 2).

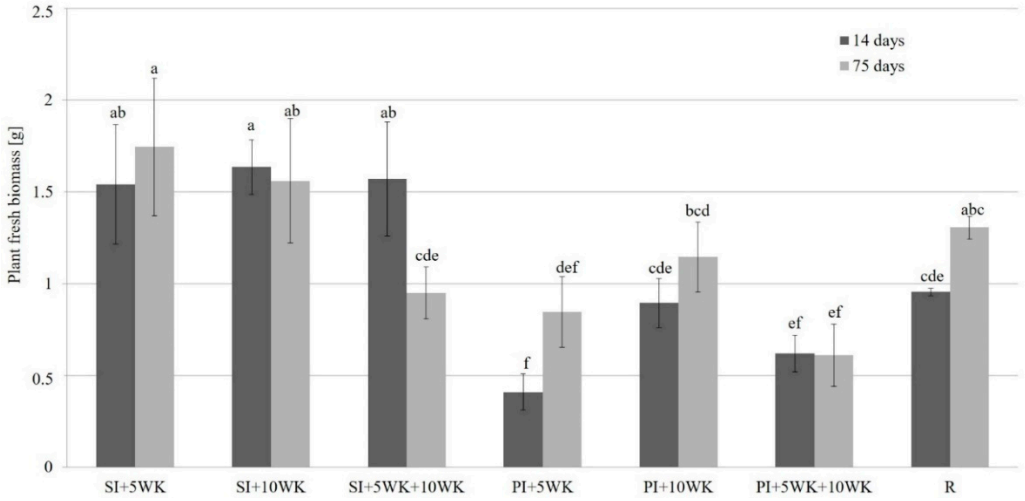

Figure 1. Effect of bacterial inoculation and pre-inoculation on biomass production (g) of ryegrass during the phytodegradation experiment. Presented are the means and standard deviations (SD) of plants (n = 20) from three replicate pots. Means followed by different letters (a, b, c, d, e, f) are significantly different (one-way ANOVA, Tukey's test $p < 0.05$). Abbreviations: (R) polluted soil with ryegrass; (SI) polluted soil with ryegrass grains inoculated with strain; (PI) polluted soil with ryegrass grains pre-inoculated with strain followed by soil inoculation with strain; (5WK) the *Rhodococcus erythropolis* 5WK strain; (10WK) the *Rhizobium* sp. 10WK strain; (5WK + 10WK) the consortium of *Rhodococcus erythropolis* 5WK and *Rhizobium* sp. 10WK.

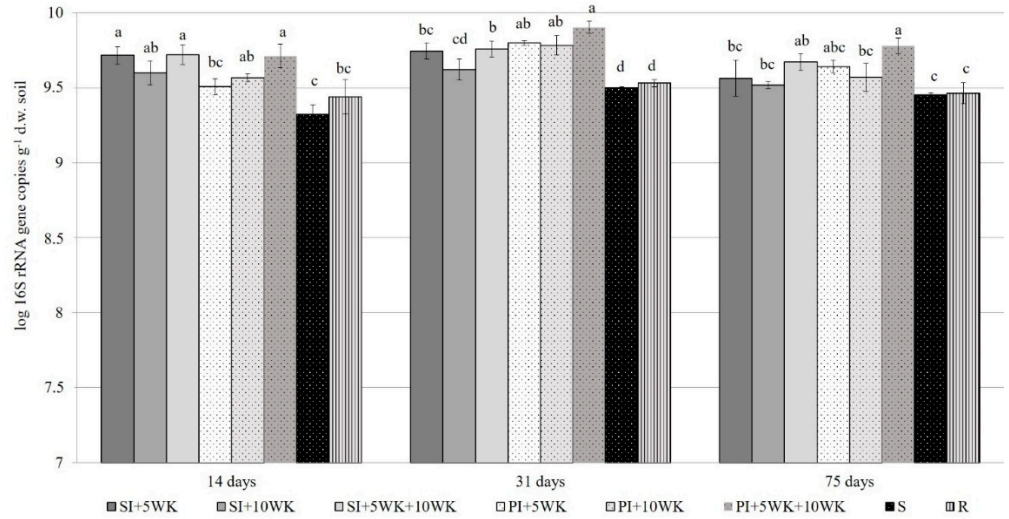

Figure 2. Numbers of 16S rRNA gene copies in soil during the phytodegradation experiment. Presented are the means and standard deviations (SD) of plants (n = 20) from three replicate pots. Means followed by different letters (a, b, c, d) are significantly different and calculated for each day separately (one-way ANOVA for each day, Tukey's test $p < 0.05$). Abbreviations: (S) polluted soil; (R) polluted soil with ryegrass; (SI) polluted soil with ryegrass grains inoculated with strain; (PI) polluted soil with ryegrass grains pre-inoculated with strain followed by soil inoculation with strain; (5WK) the *Rhodococcus erythropolis* 5WK strain; (10WK) the *Rhizobium* sp. 10WK strain; (5WK + 10WK) the consortium of *Rhodococcus erythropolis* 5WK and *Rhizobium* sp. 10WK.

3.4. Abundance of Autochthonous Bacteria and Alkane-Degrading Bacteria in the Soil

Real-time PCR of the 16S rRNA and *alkH* gene was used to quantify the abundance of all autochthonous bacteria and the alkane-degrading bacteria in the soil (Figures 2 and 3).

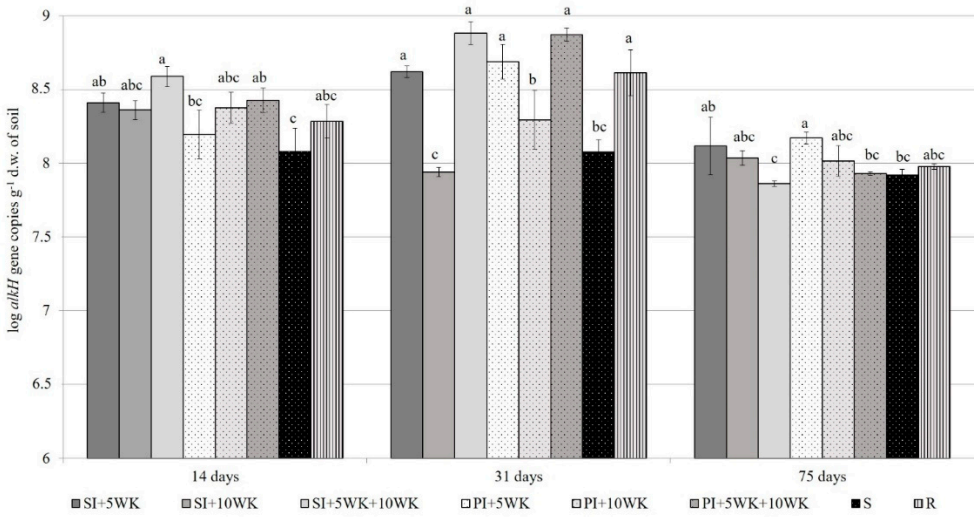

Figure 3. Number of *alkH* gene copies in soils during the phytodegradation experiment. Presented are the means and standard deviations (SD) of plants (n = 20) from three replicate pots. Means followed by different letters (a, b, c) are significantly different and calculated for each day separately (one-way ANOVA for each day, Tukey's test, $p < 0.05$). Abbreviations: (S) polluted soil; (R) polluted soil with ryegrass; (SI) polluted soil with ryegrass grains inoculated with strain; (PI) polluted soil with ryegrass grains pre-inoculated with strain followed by soil inoculation with strain; (5WK) the *Rhodococcus erythropolis* 5WK strain; (10WK) the *Rhizobium* sp. 10WK strain; (5WK + 10WK) the consortium of *Rhodococcus erythropolis* 5WK and *Rhizobium* sp. 10WK.

The identify numbers of 16S rRNA gene copies allowed the determination of the effects of the introduced strains 5WK and 10WK on the autochthonous community in the polluted soil (Figure 2). In order to assess the differences in the numbers of copies of the 16S rRNA gene in the soil between the different treatments on days 14, 31 and 75, one-way ANOVA and post-hoc Tukey's tests $p < 0.05$ were performed (Figure 2). On days 14, 31 and 75, no significant differences in the average numbers of copies of the 16S rRNA gene were detected between the noninoculated soils (R) with or without plants (S) growing on it. On day 14, the content of the 16S rRNA gene was significantly ($p < 0.05$) higher in soil where a consortium of the strains was applied (SI + 5WK + 10WK; PI + 5WK + 10WK) compared to the noninoculated soil (R, S). A significantly higher amount of 16S rRNA genes was observed in SI and PI treatments compared to S and R treatments. It is worth noting that at the end of the experiment, statistical differences were observed between the treatments where a consortium of the strains was introduced (SI + 5WK + 10WK; PI + 5WK + 10WK) compared to the noninoculated soil with (R) or without plants (S).

The copy numbers of the *alkH* gene detected in the soils allowed the estimation of the influence of the introduced strains on the numbers of bacteria able to degrade aliphatic hydrocarbons (Figure 3). One-way ANOVA and Tukey's tests $p < 0.05$ were performed to evaluate the differences between the numbers of the *alkH* gene on days 14, 31 and 75. Nonsignificant differences were observed between noninoculated soils without (R) and with plants (S) on days 14 and 75. On day 14, similar to 16S rRNA gene numbers, the significant differences were observed in the amount of the *alkH* gene between soil with consortium inoculation (SI + 5WK + 10WK; PI + 5WK + 10WK) and soil without bacterial strain introduction (S). On day 31, no significant differences were observed between SI + 5WK + 10WK; PI + 5WK + 10WK and S treatment. Furthermore, nonstatistically important results were observed with single strain introduction (SI + 5WK; SI + 10WK; PI + 5WK; PI + 10WK) compared to planted soil (R). Moreover, the numbers of the *alkH* gene in all treatments at the end of the experimental period (75 days) were similar.

3.5. Plant Colonization by Gfp-Tagged Endophytic Bacteria

The main objective of this part of the study was to confirm the endophytic nature of the 5WK strain. This experiment allowed us to detect the site and the route of colonization by *Rhizobium* sp. 5WK. Twenty-four hours after inoculation, the *gfp*-tagged 5WK abundantly colonized the root surface of *L. perenne* seedlings (Figure 4A,C). Interestingly, *gfp-Rhizobium* sp. 5WK was also detected inside root hairs of *L. perenne* (Figure 4D). Furthermore, it is important to mention that the root conductive tissues were also colonized with significant numbers of tagged-bacteria (Figure 4B).

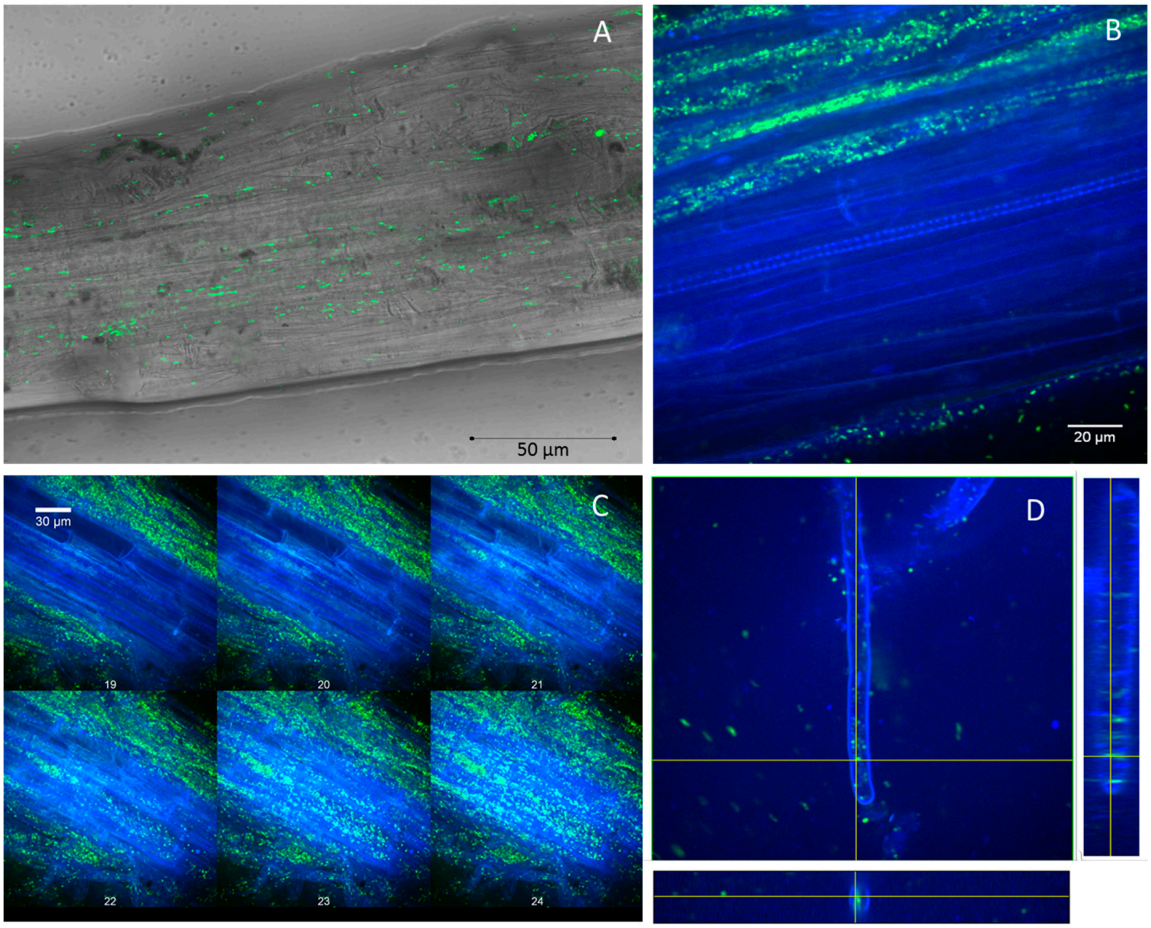

Figure 4. Confocal laser scanning micrographs of the *gfp*-tagged *Rhizobium* sp. 5WK strain colonizing the root surface (**A**,**C**), root conductive tissues of *L. perenne* (**B**) and ortho-image of the root hair (**D**), showing bacterial cells (green) inside plant cells (in blue). Numbers represent different tiles of the stack.

4. Discussion

Despite the importance of remediating polluted sites and studies on factors influencing the microbial assisted phytoremediation, still, little is known about the most appropriate method of bacterial inoculation that may significantly increase the efficiency of phytodegradation.

Results of our experiments confirm that the inoculation of ryegrass planted soils with specific bacterial strains can significantly improve the remediation of PHC polluted soils (Table 1). The observed effect depended on the method of inoculation (SI or PI) as well the strains used (single strain or consortium). All endophytes used in the study possessed genes encoding enzymes involved in the degradation of aliphatic and aromatic hydrocarbons and a broad suite of physiological properties thought to be responsible for the promotion of plant growth [13]. The application of such endophytic bacteria may be more advantageous compared to bioaugmentation with free-living bacteria, as endophytes have a potential to colonize some niches inside an autochthonous community

due to their close association with a plant host. It has also been found that genes responsible for biodegradation may transfer from inoculants to native endophytes and thus speed up the removal of contaminants [37,38].

Pre-inoculation of ryegrass seeds followed by soil inoculation regardless of treatment significantly increased the efficiency of PHC degradation as compared to the soil inoculation, planted soil and soil without strain introduction. The highest rate of PHC removal (19.1% of the initial PHC concentration) was observed in soil inoculated with a consortium of strains (5WK + 10WK). This is in agreement with results of other studies, in which the combined use of plants and a consortium of endophytic bacteria increased the loss of PHC, even up to 78% [3,23,24]. In another study, the PHC removal using phytoremediation supported by a bacterial consortium was shown to be 7.3% more efficient compared to plants without inoculation and 5.3% more than just bioaugmentation of unplanted soil [39]. The higher usefulness of consortia than single strains is attributed to the fact that intermediates of a catabolic pathway of one strain may be further degraded by other strains equipped with a suitable catabolic pathway [38,40].

However, we did not observe a positive effect on PHC removal of the consortium as compared to single strain when only soil inoculation was employed. At the end of the experimental period, the PHC concentrations in the nonplanted soil (S) and soil planted with ryegrass (R) were reduced by 1.7% and 4.7%, respectively. In contrast, in the study by Afzal et al. [23], the amount of PHC in various soils planted with *Lolium multiflorum* was lower—up to 20% of the initial PHC concentration. The observed differences might be related to the high initial PHC concentration in the soil used in our study. The high concentration of PHC inhibited the growth and development of the plants, and this might affect the microbial activity in the rhizosphere and finally the phytoremediation efficiency. Moreover, in soils heavily contaminated with hydrocarbons, the poor endophytic colonization and low rate of hydrocarbon degradation was noted [26]. This might explain why the remediation rate in our experiment was not as high as expected.

In the presented study, for phytodegradation purposes, *Lolium perenne* was used. Members of the *Lolium* genus are thought to be appropriate candidates for the phytoremediation of PHC [41,42]. Both 5WK and 10WK strains promoted the growth of ryegrass over the experimental period, as proved by the values of efficiency growth index (Figure 1). The highest value of this index was noted for SI + 5WK and SI + 10WK treatments. This could be associated with both the plant growth-promoting and hydrocarbon degradation abilities of these strains. It has been recognized that inoculation with endophytic bacteria may mitigate the phytotoxicity of PHC and that this may be exploited during the phytoremediation of aliphatic and aromatic hydrocarbon polluted soil [18,19,27,28,43]. However, in treatments with bacterial inoculated seeds, the inhibition of ryegrass was observed. This may be explained by the impact of the sterilization process on the rate of seed germination and plant development, especially at the beginning of the experiment [44,45]. The poor plant growth might have resulted in the alteration of rhizodeposition and might be reflected in the low percentage of PHC removal in SI treatments.

Apart from the selection of plants with increased pollutant tolerance, the production of sufficient root and shoot biomass, effective pollutant uptake and appropriate metabolic capabilities to degrade organic pollutants, an important prerequisite for successful remediation, is a survival of inoculants in PHC polluted soil and plants [16,18]. The strains we used can be designated as facultative endophytes because in addition to entering the plants, they were also able to establish in the soil. Furthermore, although ryegrass was not their parental host, it did not induce significant harmful effects. Indeed, endophytic strains are sometimes pathogenic to nonparental plant species [46]. Therefore, the selected endophytic may be used for phytoremediation purposes in combination with other plant species. In microbially supported phytodegradation, inoculation with the selected bacteria has to be accompanied with the monitoring of their survival in the soil and plant tissues. Therefore, inoculants should be tagged with a specific marker allowing the introduced cells to be identified and monitored among the populations of indigenous plant and soil microorganisms. Various markers have been used

to track the fates of inoculants following their reintroduction into the soil [47,48]. Among the more useful methods is labelling bacteria with the *gfp* gene, which encodes the green fluorescent protein (GFP) [49]. In our study, we labelled the 5WK strain with *gfp* to monitor its plant colonization and visualization in plant tissue (Figure 4). Confocal laser scanning micrographs of the *gfp*-tagged 5WK strain showed that this strain was able to colonize the root surface, enter from soil to root hairs and could migrate to the different plant tissues via conductive tissues (Figure 4). This confirms that the 5WK strain demonstrates endophytic behavior, and such a route of plant colonization by endophytic bacteria was reported earlier by Compant et al. [50], Truyens et al., [51] and was also supported by the results of Sanchez-Lopez et al. [36].

The colonization of plant tissue by inoculants depended on the method of inoculation. In the case of soil inoculation (SI), only the 5WK strain entered the plant tissues. In turn, the pre-inoculation (PI) method allowed both strains (5WK and 10WK) to colonize the interior of the plant. This suggests that endophytic strains may colonize the plant endosphere more easily when they attach to and eventually get into the caryopses. Application of PI allows the establishment of a beneficial, close association between the bacteria and host. It has already been published that this interaction might be more efficient for the plant to cope with stressful external environmental conditions (pollutants, low N content, drought) [52–54]. In general, endophytic bacteria are better plant colonizers than free-living microorganisms, which is related to the lifestyle of endophytes and their unique traits [17,55]. Interestingly, strains with high hydrocarbon degradation potential were reported to actively colonize specific plant niches and more successfully inhabit plants exposed to hydrocarbons [17,56,57]. This could be connected to their evolutionary adaptation to degrade plant metabolites, as they have a structure similar to aliphatic and/or aromatic hydrocarbons [58–60].

Results of our study showed that soil inoculation with SI and PI methods exerted a significant effect on the number of autochthonous bacteria expressed as the number of 16S rRNA gene copies (Figure 2). At the beginning and end of the experiment, the number of 16S rRNA genes in soils inoculated with the consortium PI + 5WK + 10WK and SI + 5WK + 10WK was higher compared to uninoculated and inoculated single strain soils. In turn, the soil inoculation in each treatment did not affect the number of *alkH* genes (Figure 3). Unexpectedly, at the end of the experiment period, the number of *alkH* gene copies was low, which demonstrated the dynamics of the inoculated bacterial strains and their aliphatic hydrocarbon degradation. A higher abundance of genes involved in hydrocarbon degradation was reported in the rhizosphere where root exudates provide co-metabolites. Furthermore, the availability of extra nutrients may enhance bacterial growth, survival, proliferation and metabolism of hydrocarbons [26,55]. It has been suggested that for phytoremediation, the vegetative stage of plant growth should be the principal phase. In general, obtained results in this research demonstrated that 5WK and 10WK strains have an endophytic nature. They were able to survive in soil but more often may colonize roots and shoots. The environmental studies with the phytoremediation subject very often demonstrated the difficulties with the exact enumeration of introduced strains. The variation in the number of inoculants could be the result of their transition to a VBNC-like state in harsh conditions (petroleum polluted soil). Moreover, the plant constantly reacts to changing environmental conditions, as a result of which it can affect one group of microorganisms supporting it or change the composition of its own microbiome. This could be a reason that we observed the reduction in the number of *alkH* genes when the number of 16S rRNA genes was at the same level. Not without significance are the effects of soil factors on microbial communities, which were comprehensive and not determined by a single factor. Moreover, the high petroleum pollution determines the presence of certain microorganisms and could be less favorable than lower concentrations [61]. This may be because high concentrations of petroleum adhere to the surface of the plant's roots, reducing the production of rhizosphere exudates, which makes it difficult to support the grow of important bacteria.

5. Conclusions

To better understand the factors influencing the efficiency of phytodegradation supported by endophytic bacteria, we compared methods of their introduction into soils. Our study indicated that the pre-inoculation of *Lolium perenne* grains followed by soil inoculation with a consortium of endophytes could increase phytodegradation efficiency. Furthermore, our results not only revealed high soil, roots and shoots endosphere colonization capabilities of the tested strains but also established a basis for the interaction between inoculated endophytes and autochthonic soil microbiota. These strains with multiple PGP traits, hydrocarbon degradation and plant tissue colonization capabilities can be a promising and important component of phytodegradation soils polluted by various classes of organic pollutants.

Complementary investigations in the lab and field should be performed on bacterial endophytes, their mechanisms and interactions with the plant, which determine the efficiency of PHC phytodegradation.

Author Contributions: Conceptualization, M.P. and Z.P.-S.; methodology, M.P., T.P., S.T., I.P.; validation, I.P., S.T., J.V., Z.P.-S.; formal analysis, M.P., T.P.; investigation, M.P., T.P.; data curation, M.P., T.P., S.T., I.P.; writing—original draft preparation, M.P.; writing—review and editing, Z.P.-S., S.T., J.V.; supervision, Z.P.-S., J.V. All authors have read and agreed to the published version of the manuscript.

Funding: The study was financed by the National Science Centre grant 2013/09/N/NZ9/01606. This research was supported by a BOF-BILA grant from Hasselt University, the UHasselt Methusalem project 08M03VGRJ. M. Pawlik was a scholarship holder within the DoktoRIS project—scholarship program for the innovation of the Silesia region, supported by the European Community from the European Social Fund.

Acknowledgments: We are grateful to Magdalena Pacwa-Płociniczak for introducing and explaining the qPCR method and Sławomir Sułowicz for clarifying the statistical analysis.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. USEPA (U.S. Environmental Protection Agency). Guidelines for carcinogen risk assessment. *Fed. Regist.* **1986**, *51*, 33992–34003.
2. USEPA (U.S. Environmental Protection Agency). Guidelines for mutagenicity risk assessment. *Fed. Regist.* **1986**, *51*, 34006–34012.
3. Balseiro-Romero, M.; Gkorezis, P.; Kidd, P.S.; Vangronsveld, J.; Monterroso, C. Enhanced degradation of diesel in the rhizosphere of *Lupinus luteus* after inoculation with diesel-degrading and plant growth-promoting bacterial strains. *J. Environ. Q.* **2016**, *45*, 924–932. [CrossRef] [PubMed]
4. Balseiro-Romero, M.; Gkorezis, P.; Kidd, P.S.; van Hammed, J.; Weyens, N.; Monterroso, C.; Vangronsveld, J. Use of plant growth promoting bacterial strains to improve *Cytisus striatus* and *Lupinus luteus* development for potential application in phytoremediation. *Sci. Total Environ.* **2017**, *581*, 676–688. [CrossRef] [PubMed]
5. Pilon-Smits, E. Phytoremediation. *Annu. Rev. Plant Biol.* **2005**, *56*, 15–39. [CrossRef]
6. Vangronsveld, J.; Herzig, R.; Weyens, N.; Boulet, J.; Adriaensen, K.; Ruttens, A.; Thewys, T.; Vassilev, A.; Meers, E.; Nehnevajova, E.; et al. Phytoremediation of contaminated soils and groundwater: Lessons from the field. *Environ. Sci. Pollut. Res. Int.* **2009**, *16*, 765–794. [CrossRef]
7. Khan, S.; Afzal, M.; Iqbal, S.; Khan, Q.M. Plant-bacteria partnerships for the remediation of hydrocarbon contaminated soils. *Chemosphere* **2013**, *4*, 1317–1332. [CrossRef]
8. Ma, Y.; Oliveira, R.S.; Freitas, H.; Zhang, C. Biochemical and molecular mechanisms of plant-microbe-metal interactions: Relevance for phytoremediation. *Front. Plant Sci.* **2016**, *7*, 918. [CrossRef]
9. Weyens, N.; van der Lelie, D.; Artois, T.; Smeets, K.; Taghavi, S.; Newman, L.; Carleer, R.; Vangronsveld, J. Bioaugmentation with engineered endophytic bacteria improves phytoremediation. *Environ. Sci. Technol.* **2009**, *43*, 9413–9418. [CrossRef]
10. Weyens, N.; van der Lelie, D.; Taghavi, S.; Newman, L.; Vangronsveld, J. Exploiting plant–microbe partnerships for improving biomass production and remediation. *Trends Biotechnol.* **2009**, *27*, 591–598. [CrossRef]

11. Weyens, N.; van der Lelie, D.; Taghavi, S.; Vangronsveld, J. Phytoremediation: Plant-endophyte partnerships take the challenge. *Curr. Opin. Biotechnol.* **2009**, *2*, 248–254. [CrossRef] [PubMed]
12. Thijs, S.; Sillen, W.; Rineau, F.; Weyens, N.; Vangronsveld, J. Towards an enhanced understanding of plant–microbiome interactions to improve phytoremediation: Engineering the metaorganism. *Front. Microbiol.* **2016**, *7*, 341. [CrossRef] [PubMed]
13. Pawlik, M.; Cania, B.; Thijs, S.; Vngronsveld, J.; Piotrowska-Seget, Z. Hydrocarbon degradation potential and plant growth-promoting activity of culturable endophytic bacteria of *Lotus corniculatus* and *Oenothera biennis* from a long-term polluted site. *Environ. Sci. Pollut. Res.* **2017**, *24*, 19640–19652. [CrossRef] [PubMed]
14. Barac, T.; Taghavi, S.; Borremans, B.; Provoost, A.; Oeyen, A.; Colpaert, J.V.; Vangronsveld, J.; van der Lelie, D. Engineered endophytic bacteria improve phytoremediation of water–soluble, volatile, organic pollutants. *Nat. Biotechnol.* **2004**, *22*, 583–588. [CrossRef] [PubMed]
15. Barac, T.; Weyens, N.; Oeyen, L.; Taghavi, S.; van der Lelie, D.; Dubin, D.; Spliet, M.; Vangronsveld, J. Application of poplar and its associated microorganisms for the in situ remediation of a BTEX contaminated groundwater plume. *Int. J. Phytorem.* **2009**, *11*, 416–424. [CrossRef]
16. Germaine, K.J.; Keogh, E.; Ryan, D.; Dowling, D.N. Bacterial endophyte-mediated naphthalene phytoprotection and phytoremediation. *FEMS Microbiol. Lett.* **2009**, *296*, 226–234. [CrossRef]
17. Yousaf, S.; Afzal, M.; Reichenauer, T.G.; Brady, C.L.; Sessitsch, A. Hydrocarbon degradation, plant colonization and gene expression of alkane degradation genes by endophytic *Enterobacter ludwigii* strains. *Environ. Pollut.* **2011**, *10*, 2675–2683. [CrossRef]
18. Afzal, M.; Khan, S.; Iqbal, S.; Mirza, M.S.; Khan, Q.M. Inoculation method affects colonization and activity of *Burkholderia phytofirmans* PsJN during phytoremediation of diesel-contaminated soil. *Int. Biodeterior. Biodegrad.* **2013**, *85*, 331–336. [CrossRef]
19. Peng, A.; Liu, J.; Gao, Y.; Chen, Z. Distribution of endophytic bacteria in *Alopecurus aequalis* Sobol and *Oxalis corniculata* L. from soils contaminated by polycyclic aromatic hydrocarbons. *PLoS ONE* **2013**, *8*, 12. [CrossRef]
20. Alvarez-Lopez, V.; Prieto-Fernandez, A.; Janssen, J.; Herzig, R.; Vangronsveld, J.; Kidd, P.S. Inoculation methods using *Rhodococcus erythropolis* strain P30 affects bacterial assisted phytoextraction capacity of *Nicotiana tabacum*. *Int. J. Phytorem.* **2016**, *18*, 406–415. [CrossRef]
21. Gkorezis, P.; Daghio, M.; Franzetti, A.; van Hamme, J.D.; Sillen, W.; Vangronsveld, J. The interaction between plants and bacteria in the remediation of petroleum hydrocarbons: An environmental perspective. *Front. Microbiol.* **2016**, *7*, 1836. [CrossRef] [PubMed]
22. Tardif, S.; Yergeau, É.; Tremblay, J.; Legendre, P.; Whyte, L.G.; Greer, C.W. The willow microbiome is influenced by soil petroleum-hydrocarbon concentration with plant compartment-specific effects. *Front. Microbiol.* **2016**, *7*, 1363. [CrossRef] [PubMed]
23. Afzal, M.; Yousaf, S.; Reichenauer, T.G.; Kuffner, M.; Sessitsch, A. Soil type affects plant colonization, activity and catabolic gene expression of inoculated bacterial strains during phytoremediation of diesel. *J. Hazard. Mater.* **2011**, *186*, 1568–1575. [CrossRef] [PubMed]
24. Afzal, M.; Yousaf, S.; Reichenauer, T.G.; Sessitsch, A. The inoculation method affects colonization and performance of bacterial inoculant strains in the phytoremediation of soil contaminated with diesel oil. *Int. J. Phytorem.* **2012**, *14*, 35–47. [CrossRef]
25. Andreolli, M.; Lampis, S.; Poli, M.; Gullner, G.; Biró, B.; Vallini, G. Endophytic *Burkholderia fungorum* DBT1 can improve phytoremediation efficiency of polycyclic aromatic hydrocarbons. *Chemosphere* **2013**, *92*, 688–694. [CrossRef]
26. Arslan, M.; Afzal, M.; Amin, I.; Iqbal, S.; Khan, Q.M. Nutrients can enhance the abundance and expression of alkane hydroxylase CYP153 gene in the rhizosphere of ryegrass planted in hydrocarbon-polluted soil. *PLoS ONE* **2014**, *9*, e111208. [CrossRef]
27. Sun, K.; Liu, J.; Jin, L.; Gao, Y. Utilizing pyrene-degrading endophytic bacteria to reduce the risk of plant pyrene contamination. *Plant Soil* **2014**, *374*, 251–262. [CrossRef]
28. Sun, K.; Liu, L.; Gao, Y.; Jin, L.; Gu, Y.; Wang, W. Isolation, plant colonization potential, and phenanthrene degradation performance of the endophytic bacterium *Pseudomonas* sp. Ph6-gfp. *Sci. Rep.* **2014**, *4*, 5462. [CrossRef]
29. Su, X.; Sun, F.; Wang, Y.; Hashmi, M.Z.; Guo, L.; Ding, L.; Shen, C. Identification, characterization and molecular analysis of the viable but nonculturable *Rhodococcus biphenylivorans*. *Sci. Rep.* **2015**, *5*, 18590. [CrossRef]

30. Bodor, A.; Bounedjoum, N.; Vincze, G.E.; Kis, A.E.; Laczi, K.; Bende, G.; Szilagyi, A.; Kovacs, T.; Perei, K.; Rakhely, G. Challenges of unculturable bacteria: Environmental perspectives. *Rev. Environ. Sci. Biotechnol.* **2020**, *19*, 1–22. [CrossRef]
31. Płociniczak, T.; Fic, E.; Pacwa-Płociniczak, M.; Pawlik, M.; Piotrowska-Seget, Z. Improvement of phytoremediation of an aged petroleum hydrocarbon-contaminated soil by *Rhodococcus erythropolis* CD 106 strain. *Int. J. Phytorem.* **2017**, *19*, 614–620. [CrossRef] [PubMed]
32. Pacwa-Płociniczak, M.; Płaza, G.A.; Piotrowska-Seget, Z. Monitoring the changes in a bacterial community in petroleum-polluted soil bioaugmented with hydrocarbon-degrading strains. *Appl. Soil Ecol.* **2016**, *105*, 76–85. [CrossRef]
33. Edwards, U.; Rogall, T.; Blöcker, H.; Emde, M.; Böttger, E.C. Isolation and direct complete nucleotide determination of entire genes: Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res.* **1989**, *17*, 7843–7853. [CrossRef] [PubMed]
34. Phillips, L.A.; Germida, J.J.; Farrell, R.E.; Greer, C.W. Hydrocarbon degradation potential and activity of endophytic bacteria associated with prairie plants. *Soil Biol. Biochem.* **2008**, *12*, 3054–3064. [CrossRef]
35. Lagendijk, E.L.; Validov, S.; Lamers, G.E.M.; De Weert, S.; Bloemberg, G.V. Genetic tools for tagging Gram-negative bacteria with mCherry for visualization in vitro and in natural habitats, biofilm and pathogenicity studies. *FEMS Microbiol. Lett.* **2010**, *305*, 81–90. [CrossRef]
36. Sánchez-López, A.S.; Pintelon, I.; Stevens, V.; Imperato, V.; Timmermans, J.P.; González-Chávez, C.; Carrillo, R.; van Hamme, J.D.; Vangronsveld, J.; Thijs, S. Seed endophyte microbiome of *Crotalaria pumila* unpeeled: Identification of plant-beneficial methylobacteria. *Int. J. Mol. Sci.* **2018**, *19*, 291. [CrossRef]
37. Syranidou, E.; Thijs, S.; Avramidou, M.; Weyens, N.; Venieri, D.; Pintelon, I.; Vangronsveld, J.; Kalogerakis, N. Responses of the endophytic bacterial communities of *Juncus acutus* to pollution with metals, emerging organic pollutants and to bioaugmentation with indigenous strains. *Front. Plant Sci.* **2018**, *9*, 1526. [CrossRef]
38. Taghavi, S.; Barac, T.; Greenberg, B.; Borremans, B.; Vangronsveld, J.; van der Lelie, D. Horizontal gene transfer to endogenous endophytic bacteria from poplar improves phytoremediation of toluene. *Appl. Environ. Microbiol.* **2005**, *71*, 8500–8505. [CrossRef]
39. Chuluun, B.; Shah, S.H.; Rhee, J.S. Bio-augmented phytoremediation: A strategy for reclamation of diesel oil-contaminated soils. *Int. J. Agric. Biol.* **2014**, *16*, 624–628.
40. Heinaru, E.; Merimaa, M.; Viggor, S.; Lehiste, M.; Leito, I.; Truu, J.; Heinaru, A. Biodegradation efficiency of functionally important populations selected for bioaugmentation in phenol- and oil-polluted area. *FEMS Microbiol. Ecol.* **2005**, *51*, 363–373. [CrossRef]
41. Kirk, J.L.; Klironomos, J.N.; Lee, H.; Trevors, J.T. The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil. *Environ. Pollut.* **2005**, *133*, 455–465. [CrossRef] [PubMed]
42. Tang, J.; Wang, R.; Niu, X.; Zhou, Q. Enhancement of soil petroleum remediation by using a combination of ryegrass (*Lolium perenne*) and different microorganisms. *Soil Tillage Res.* **2010**, *110*, 87–93. [CrossRef]
43. Porteous-Moore, F.P.; Barac, T.; Borremans, B.; Oeyen, L.; Vangronsveld, J.; van der Lelie, D.; Campbell, C.D.; Moore, E.R. Endophytic bacterial diversity in poplar trees growing on a BTEX-contaminated site: The characterization of isolates with potential to enhance phytoremediation. *Syst. Appl. Microbiol.* **2006**, *7*, 539–556. [CrossRef] [PubMed]
44. Sen, M.K.; Jamal, M.A.H.M.; Nasrin, S. Sterilization factors affect seed germination and proliferation of *Achyranthes aspera* cultured in vitro. *Environ. Exp. Biol.* **2013**, *11*, 119–123.
45. Barampuram, S.; Allen, G.; Krasnyanski, S. Effect of various sterilization procedures on the in vitro germination of cotton seeds. *Plant Cell Tissue Organ Cult.* **2014**, *118*, 179–185. [CrossRef]
46. Hardoim, P.R.; van Overbeek, L.S.; van Elsas, J.D. Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol.* **2008**, *10*, 463–471. [CrossRef]
47. Ma, Y.; Rajkumar, M.; Freitas, H. Inoculation of plant growth promoting bacterium *Achromobacter xylosoxidans* strain Ax10 for the improvement of copper phytoextraction by *Brassica juncea*. *J. Environ. Manag.* **2009**, *90*, 831–837. [CrossRef]
48. Płociniczak, T.; Chodór, M.; Pacwa-Płociniczak, M.; Piotrowska-Seget, Z. Metal-tolerant endophytic bacteria associated with *Silene vulgaris* support the Cd and Zn phytoextraction in non-host plants. *Chemosphere* **2019**, *219*, 250–260. [CrossRef]

49. Errampalli, D.; Leung, K.; Cassidy, M.B.; Kostrzynska, M.; Blears, M.; Lee, H.; Trevors, J.T. Applications of the green fluorescent protein as a molecular marker in environmental microorganisms. *J. Microbiol. Methods* **1999**, *35*, 187–199. [CrossRef]
50. Compant, S.; Clement, C.; Sessitach, A. Plant growth–promoting bacteria in the rhizo–and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.* **2010**, *42*, 669–678. [CrossRef]
51. Truyens, S.; Weyens, N.; Cuypers, A.; Vangronsveld, J. Bacterial seed endophytes: Genera, vertical transmission and interaction with plants. *Environ. Microbiol. Rep.* **2015**, *7*, 40–50. [CrossRef]
52. Siciliano, S.D.; Fortin, N.; Mihoc, A.; Wisse, G.; Labelle, S.; Beaumier, D.; Ouellette, D.; Roy, R.; Whyte, L.G.; Banks, M.K.; et al. Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. *Appl. Environ. Microbiol.* **2001**, *6*, 2469–2475. [CrossRef] [PubMed]
53. Oliveira, A.L.M.; de Lima Canuto, E.; Reis, V.M.; Baldani, J.I. Response of micropropagated sugarcane varieties to inoculation with endophytic diazotrophic bacteria. *Braz. J. Microbiol.* **2003**, *34*, 59–61. [CrossRef]
54. Vargas, L.; Santa Brígida, A.B.; Mota Filho, J.P.; de Carvalho, T.G.; Rojas, C.A.; Vaneechoutte, D.; van Bel, M.; Farrinelli, L.; Ferreira, P.C.G.; Vandepoele, K.; et al. Drought tolerance conferred to sugarcane by association with *Gluconacetobacter diazotrophicus: A* transcriptomic view of hormone pathways. *PLoS ONE* **2014**, *9*, e114744. [CrossRef]
55. Andria, V.; Reichenauer, T.G.; Sessitsch, A. 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. *Environ. Pollut.* **2009**, *157*, 3347–3350. [CrossRef]
56. Yousaf, S.; Andria, V.; Reichenauer, T.G.; Smalla, K.; Sessitsch, A. 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. *J. Hazard. Mater.* **2010**, *184*, 523–532. [CrossRef]
57. Yousaf, S.; Ripka, K.; Reichenauer, T.G.; Andria, V.; Afzal, M.; Sessitsch, A. Hydrocarbon degradation and plant colonization by selected bacterial strains isolated from Italian ryegrass and birdsfoot trefoil. *J. Appl. Microbiol.* **2010**, *4*, 1389–1401. [CrossRef]
58. Han, J.I.; Choi, H.K.; Lee, S.W.; Orwin, P.M.; Kim, J.; Laroe, S.L.; Kim, T.G.; O'Neil, J.; Leadbetter, J.R.; Lee, S.Y.; et al. Complete genome sequence of the metabolically versatile plant growth-promoting endophyte *Variovorax paradoxus* S110. *J. Bacteriol.* **2011**, *193*, 1183–1190. [CrossRef]
59. Mitter, B.; Petric, A.; Shin, M.W.; Chain, P.S.; Hauberg-Lotte, L.; Reinhold-Hurek, B.; Nowak, J.; Sessitsch, A. Comparative genome analysis of *Burkholderia phytofirmans* PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants. *Front. Plant Sci.* **2013**, *4*, 120. [CrossRef]
60. Afzal, M.; Khan, Q.M.; Sessitsch, A. Endophytic bacteria: Prospects and applications for the phytoremediation of organic pollutants. *Chemosphere* **2014**, *117*, 232–242. [CrossRef]
61. Shen, Y.; Ji, Y.; Li, C.; Luo, P.P.; Wang, W.; Zhang, Y.; Nover, D. Effects of phytoremediation treatment on bacterial community structure and diversity in different petroleum-contaminated soils. *Int. J. Environ. Res. Public Health* **2018**, *15*, 2168. [CrossRef] [PubMed]