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Poaceae with PGPR Bacteria and Arbuscular Mycorrhizae Partnerships as a Model System for Plant Microbiome Manipulation for Phytoremediation of Petroleum Hydrocarbons Contaminated Agricultural Soils

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Abstract: Total petroleum hydrocarbons (TPHs) are a persistent environmental organic contaminant. The possibility of obtaining synergistic effects between plants and microorganisms has further increased the possibility of alternative techniques for bioremediation. Oloptum miliaceum (L.) Röser & H.R. Hamasha and Pennisetum setaceum (Forssk.) Chiov. are, undoubtedly, good model plants for phytoremediation because they have large roots, leaf biomass, and a fast and effective renewal capacity, also, they have a great capacity to host endophytes in their roots. Gas chromatography-mass spectrometry (GC-MS) based on carbon fraction number was a basic technique used to determine the hydrocarbon degradation, and microorganism’s population was identified by high-throughput sequencing of 16s rRNA. The microbial consortium used allows the plant to increase overall biomass, adapt more in terms of redox biology (Superoxide dismutase SOD, catalase CAT, ascorbate peroxidase APX, guaiacol peroxidase GPX), and stress markers (Glutathione S-transferase GST, Phenylalanine Ammonia Lyase PAL, Proline content, and lipid peroxidation MDA). In addition, the photosynthetic efficiency and the soil dehydrogenase activity were monitored. After 240 days, the percentage of TPHs removed in Group 2 was 94%, whereas in Group 1, it was 78% in Oloptum miliaceum and Pennisetum setaceum. The removal of aliphatic hydrocarbons (C13–C36) was observed in Oloptum miliaceum and Pennisetum setaceum inoculated with the consortium of indigenous bacteria selected from rhizosphere soil and mycorrhizae strains. Our data demonstrate that, the Poaceae, in relation to its great ecological and vegetative potential, could be a great candidate for extensive remediation of soils contaminated by TPHs.

Keywords: Poaceae; Oloptum miliaceum; Pennisetum setaceum; Total petroleum hydrocarbons; contaminated agricultural soils

1. Introduction

Total petroleum hydrocarbons (TPHs) are a recalcitrant environmental organic pollutant [1–3]. They provoke consistent detriment to ecosystems and their buildup of in animal and plant tissues can result in significant gene mutations [4–7]. Recently, uses of plants to remove pollutants have significantly increased, considering that the use of plants was also a good remediation approach with regard to cost-containment. Moreover, the possibility to obtain synergistic effects between plants and microorganisms useful for the growth and development has increased the possibility of alternative
techniques [8–12]. Therefore, bacteria associated with plants, such as endophytic bacteria and fungi that live in cortical tissue of roots of plants and rhizosphere bacteria that live on/near the roots of plants, contribute to biodegradation of organic contamination in soil, and improve phytoremediation. These endophytes, because of intimate contact with their host plants, play vital roles in plant development, growth, and fitness, as well as decontaminating polluted soil [13,14]. Endophytic microorganisms are a highly varied group that stay within the tissues of plants for at least part of their life cycle [15]. Endophytic bacteria and fungi host a large amount of genes that degrade pollutants [16]. These benefits from endophytic microorganisms could improve the usage of endophytes in the phytoremediation of organic contaminants in soils. They produce phytohormones [17,18] such as indole-3-acetic acid [19,20], cytokinins, and gibberellins. Once a plant-endophyte association has been established, this partnership can generate new phytoremediation targets for polluted land [21]. Many species have been tested for eliminating TPHs, such as, ryegrass (Lolium multiflorum) [22], tall fescue (Festuca arundinacea) [1], alfalfa (Medicago sativa) [23], maize (Zea mays) [2,24,25], poplar (Populus spp.) [26], and red weed (Cynodon dactylon) [27]. Many studies have focused attention to the Poaceae, cleaning up from TPHs, since they are very resistant to stress TPHs, have good root spread (colonize large areas), and do not need special care and nutrients [28–30]. Grasses generally have these alternatives reproductive characteristics [31].

Oloptum miliaceum and Pennisetum setaceum are bushy plants with numerous upright or ascending culms, wrapped in sheaths and with the appearance of tight tufts. They are perennial plants cultivated by means of gems placed at ground level [32]. They have strong ecological value as they resist drought and live in poor and exposed land. They live well even on disturbed soils. This species is very resistant to dryness because it does not need any special cultural practices nor is it subject to plant diseases [32].

The aims of this study are to assess soil TPHs remediation by plants and microorganisms to use this technology in the field. The mesocosms were carried out to evaluate the capacity of two species of Poaceae (Oloptum miliaceum and Pennisetum setaceum) for TPHs removal by a mechanism of phytodegradation. Poaceae are a good candidate for rhizodegradation because of their large and collated roots supplying a great root surface for the development of bacterial communities.

2. Materials and Methods

2.1. Selection of the Native Bacteria of Contaminated Soil

The bacterial strains were isolated from contaminated soil that has been withdrawn from a peri-urban cultivated area (Naples) near a fuel station, with contamination by petroleum hydrocarbons, mostly diesel. The isolation was as described previously by Guarino et al. [8]. Indolacetic acid production, siderophores release, exopolysaccharides production, and ammonia production were determined in vitro, as described by Guarino et al. [8].

2.2. Mesocosm Experiment

One thousand seeds of Italian multifloral millet (O. miliaceum (L.) Röser & H.R. Hamasha and one thousand seeds of fountain grass (P. setaceum (Forssk.) Chiov.) collected in abandoned peri-urban areas of Naples near the industrial complex of the refineries were sterilized in commercial surface and sterile distilled water for 1 h. Subsequently, twenty seeds of two species for each pot were selected, and seeded mesocosms were filled with about 29 kg of contaminated soil. The soil was sieved through a 2 mm sieve mesh. The main chemical and physical characteristics of contaminated soil are showed in Supplementary Table S1. Six of the forty-six isolated endophyte bacteria (Table 1) that showed different activity of Plant growth-promoting rhizobacteria (PGPR) (Comamonas koreensis, Ochrobactrum anthrophi, Pseudomonas fluorescens, Pseudomonas putida, Bacillus cereus, and Paenibacillus mucilaginosus) were selected for the mesocosms experiment.

Besides, a consortium of endomycorrhize (Glomus versiforme, Funneliformis mosseae, Rizosphagus irregular, Rizosphagus intraradicans) obtained from the repository of the Department of Science and Technology of the University of Sannio was added to isolated endophyte bacteria.
The experimental design included three different experimental conditions for each specie:

1. The first set of mesocosms was formed by 50 pots of *O. miliaceum* (20 seeds per pot) in contaminated soil without the inoculum of endophytic consortium strains selected (Group 1).

2. The second set of mesocosms consisted of 50 pots of *O. miliaceum* (20 seeds per pot) in contaminated soil supplemented by the endophytic consortium strains selected (4.0 × 10^{11}–1.0 × 10^{12} CFU ml\(^{-1}\) g of soil) (Group 2).

3. The control set consisted of 50 pots of *O. miliaceum* (20 seeds per pot) with selected endophytic consortium strains selected (4.0 × 10^{11}–1.0 × 10^{12} CFU ml\(^{-1}\) g of soil) in commercial non-contaminated soil (control group).

The pots, which were watered daily, were placed in the controlled greenhouse according to the following protocol: (i) First month (T1: 30 days): 14 h of light at 22 °C and 10 h of dark at 17 °C, (ii) Second month (T2: 60 days): 15 h of light at 24 °C and 9 h of dark at 19 °C, (iii) Third month (T3: 90 days): 16 h of light at 26 °C and 8 h of dark at 21 °C, and (iv) Eighth month (TF: 240 days): 16 h of light at 26 °C and 8 h of dark at 21 °C. The photosynthetic photon flux density (PPFD) was the same as described above. Each treatment was formed by three replicates.

Plants were removed from pots, and roots and leaves were divided. Fresh leaves were kept for subsequent analyses. Roots were washed with distilled water and blotted with tissue paper. Soil samples were kept at 4 °C before analysis.

2.3. Plant Analysis

2.3.1. Stress Marker and Antioxidant Enzyme Activity

For enzyme analysis, thirty samples of fresh leaves (250 g) of *O. miliaceum* and *P. setaceum* were homogenized under liquid nitrogen into a fine powder. The homogenates were centrifuged at 19,000g for 30 min at 4 °C. The supernatant was used to evaluate the activity of stress markers (Glutathione S-transferase, phenylalanine ammonia lyase, Proline content, and lipid peroxidation), as described by Guarino et al. [33].

The antioxidant enzyme activity (Superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase) was determined as described by Guarino et al. [33].

2.4. Chlorophyll Content

The contents (µg mL\(^{-1}\)) of Chl a, Chl b, and Chl a + b in the leaves of *O. miliaceum* and *P. setaceum* (0.5 g) were measured spectrophotometrically in accordance with the study by Lichtenthaler and Wellburn [34].

2.5. Biomass

The root system of *O. miliaceum* and *P. setaceum* was divided from the leaves. The roots were washed with deionized water and then with 10 mM Ethane-1,2-diyldinitrilo-tetraacetic acid (EDTA) to eliminate the soil particles. The fresh weight of a sample of leaves was determined, followed by flotation on water for up to 4 h. The turgid weight was then recorded, and the leaves were oven-dried to a constant weight, at about 85 °C.

2.6. Quantification of Petroleum Hydrocarbons

Thirty-five samples of *O. miliaceum* and *P. setaceum* of each set of mesocosms were divided into two sections (roots and leaves) and were carefully washed with deionized water. Two grams of leaves and roots were ground with anhydrous Na\(_2\)SO\(_4\) using the method described by Guarino and Sciarillo [9].

2.7. Soil Analysis
2.7.1. Quantification of Petroleum Hydrocarbons

Soil samples were exposed to a pre-treatment comprising air-drying until continuous weight followed by sieving to 2 mm. Guarino and Sciarrillo [9] described the procedure of quantification of TPH. TPH content was quantified in low (C < 12) and higher molecular weight (C > 12) according to the method used for the SimDist analysis (Guarino and Sciarrillo, [9]).

2.7.2. Dehydrogenase Activity

Soil dehydrogenase activity (DHA) was determined by Hayano and the iodo nitrotetrazoliumformazan was measured spectrophotometrically at 485 nm.

2.8. Statistical Analysis

The data are the means of three replicates. The Dixon’s test was used to test the presence of outliers in the dataset. Differences between groups were tested using Duncan’s test.

3. Results

3.1. Screening of the Isolated Hydrocarbon Degraders’ Bacteria

Forty-six native bacteria were designated from contaminated soils. The isolates are Proteobacteria phylum, with more than 60% being part of the γ-subclass, about 25% being part of the b-subclass, and two strains being part of the a-subclass. *Pseudomonas* genus is the most represented (Table 1). The obtained results showed that 56% of the isolates produce the phytohormones Indole acetic acid (IAA), with 45% of isolates being able to produce Exopolysaccharides (EPSs), and 60% of isolates releasing siderophores. Ammonia-producing bacteria can act as biofertilizers in depleted soils, such as those which are contaminated, and more than half of the 56% of isolates (36%) showed this property (Table 1). Based on the results that established that the bacteria isolates are good applicants to support and improve a promising phytoremediation stage, we have selected the isolates strains for the mesocosms experiment.

**Table 1.** Primary screening of the assessment of potential Plant growth-promoting rhizobacteria (PGPR) by bacteria isolates recovered from contaminated soil from a peri-urban cultivated area (Naples). The isolates were categorized into three groups according to the produced amount: + low concentrations (<1 µg/mL), ++ moderate concentrations (1–2.99 µg/mL), and +++ high concentrations (>3 µg/mL).

| Bacterial isolates                 | Production IAA | Siderophores release | Exopolysaccharides production | Production of ammonia |
|-----------------------------------|----------------|----------------------|-------------------------------|----------------------|
| *Acetobacterium woodii*           | -              | -                    | -                             | -                    |
| *Achromobacter marplatensis*      | -              | -                    | -                             | -                    |
| *Achromobacter spanius*           | +              | -                    | -                             | -                    |
| *Achromobacter xylosoxidans*      | ++             | -                    | -                             | ++                   |
| *Comamonas koreensis*             | +++            | +++                  | +++                           | +++                  |
| *Comamonastesto steroni*          | ++             | +                    | ++                            | ++                   |
| *Comamonas aquatica*              | ++             | +                    | ++                            | +                    |
| *Ochrobactrum anthropi*           | +++            | +++                  | +++                           | +++                  |
| *Pseudomonas aeruginosa*          | +++            | +++                  | +++                           | +++                  |
| *Pseudomonas aeruginosa PA7*       | ++             | +                    | ++                            | +                    |
| *Pseudomonas stuzeri*             | ++             | +                    | +                             | +                    |
| *Pseudomonas mendocina NK-01*      | +              | -                    | +                             | -                    |
| *Pseudomonas resinovorans*        | -              | -                    | -                             | -                    |
3.2. Plant Analysis: Antioxidant Enzyme Activity and Stress Marker

Our results showed that rhizosphere microorganisms and exposure time to different treatments influence activities of the antioxidative enzymes. The activity of SOD in *O. miliaceum* and *P. setaceum* from 30 to 90 days rose significantly (*p < 0.05*) in plants with the rhizosphere microorganism in comparison to the plants without microorganisms (Group 1) and the control group (Figure 1). The highest SOD activity in leaves of *O. miliaceum* and *P. setaceum* with microflora at T1 and T3 could be a better response for plants to protect themselves from induced oxidative damage from hydrocarbons exposure in the presence of microorganisms that promote the growth of the plants. In this work, the CAT, GPX, and APX activities in leaves of *O. miliaceum* and *P. setaceum* showed parallel patterns to SOD activity and increased from T1 to T3 treatments compared with the control group and mesocosms with microorganisms (Group 2). For assessment of the degree of oxidative stress, the malandialdehyde content (MDA) was tested, such as Glutathione S-Transferase, Phenylalanine Ammonia Lyase, and Proline content (Figure 2). An increased level of MDA content was observed in the leaves of *O. miliaceum* and *P. setaceum* inoculated in response to hydrocarbons in the present study. After a longer exposure time (T5 240 days), decreased MDA content in the leaves of *O. miliaceum* was found, with no significant variance between the Control Group and Group 2. The

| Pseudomonas resinovorans NBRC 106553 | - | - | - | - |
| Pseudomonas fluorescens | +++ | +++ | +++ | +++ |
| Pseudomonas fluorescens P70-1 | ++ | ++ | ++ | ++ |
| Pseudomonas fluorescens SBW25 | ++ | ++ | ++ | ++ |
| Pseudomonas fluorescens F113 | + | + | + | + |
| Pseudomonas poae | - | ++ | - | - |
| Pseudomonas putida | +++ | +++ | +++ | +++ |
| Pseudomonas putida H8234 | - | ++ | - | - |
| Pseudomonas putida NBRC 14164 | - | - | - | - |
| Pseudomonas putida H8236 | + | - | - | - |
| Pseudomonas fulva 12-X | - | + | - | - |
| Pseudomonas stutzeri DSM 10701 | + | - | - | ++ |
| Pseudomonas stutzeri DSM 4166 | + | - | - | - |
| Pseudomonas stutzeri A1501 | - | - | - | - |
| Pseudomonas syringae pv. tomato | - | - | ++ | ++ |
| Pseudomonas brassicacearum | + | + | - | - |
| Pseudomonas putida group | ++ | ++ | - | - |
| Rhodococcus opacus B4 | - | ++ | - | - |
| Rhodococcus jostii | - | +++ | - | - |
| Paenibacillus mucilaginosus K02 | ++ | ++ | - | - |
| Paenibacillus mucilaginosus 3016 | ++ | ++ | - | - |
| Paenibacillus polymyxa | +++ | - | - | - |
| Bacillaceae group | +++ | +++ | +++ | +++ |
| Deinococcus proteolyticus | - | - | - | - |
| Bradyrhizobium japonicum USDA6 | - | ++ | ++ | - |
| Bradyrhizobium japonicum | - | ++ | ++ | - |
| Bradyrhizobium diaeconomum | - | + | - | - |
| Bradyrhizobium oligotrophicum | - | + | ++ | - |
| Bradyrhizobium oligotrophicum S58 | - | + | ++ | - |
| Nitrobacter hamburgensis | - | - | - | - |
| Nitrobacter hamburgensis X14 | - | - | - | - |
| Rhizobium leguminosarum | +++ | - | - | - |
increase of MDA content from T\textsubscript{1} to T\textsubscript{3} suggests a strong internal detoxification mechanism inside plant cells, while the decrease of MDA content during T\textsubscript{f} indicates that the plants need some time to tolerate the hydrocarbons' stress and therefore, respond to activate the antioxidative defense system against higher levels of Reactive oxygen species (ROS). The role of Glutathione S-transferase (GST) is very evident and is in line with other antioxidant enzymes, such as Phenylalanine Ammonia Lyase (PAL) (Figure 2).
Figure 1. Antioxidant activity of SOD (Umg/protein), CAT (nmol H$_2$O$_2$ mg$^{-1}$ protein min$^{-1}$), GPX (nmol guaiacol mg$^{-1}$ protein min$^{-1}$) and APX (nmol ascorbate mg$^{-1}$ protein min$^{-1}$) in leaves of Olopum miliaceum and Pennisetum setaceum to Group 1, 2 and Control Group after 30(T1), 30 (T2), 90 (T3) and 120 (TF) days. Values represent mean ± SE. Different letters indicate means that are significantly different from each other (Ducan’s test, p < 0.05).
Figure 2. Antioxidant activity of GST (µM/min/µg protein), PAL (µg t-cinnamic acid/h/µg protein), Proline content (µmol g fw) and MDA (µmol/g fw) in leaves of Oloptum miliaceum and Pennisetum setaceum to Group 1, 2 and Control Group after 30(T1), 30 (T2), 90 (T3) and 120 (TF) days. Values represent mean ± SE. Different letters indicate means that are significantly different from each other (Ducan’s test, p < 0.05).
3.3. Biomass

Phenological characteristics of *O. miliaceum* and *P. setaceum* (roots’ and leaves’ length, root biomass, leaves biomass) were determinate. In Table 2, there is the variation in plants’ growth parameters under the influence of contaminated soil. Growth depression was observed for the plants in Group 1, in fact, the non-inoculated plants exposed to contaminated soil showed a marked decrease of length of roots and leaves. However, the contamination of TPHs on plant growth was more pronounced after 90–120 days. The maximum root and leaf length were achieved in plants in Group 2, with inoculum of autochthonous bacteria after 240 days in *O. miliaceum* (285 ± 15 cm and 118 ± 4 cm, respectively) and *O. setaceum* (291.6 ± 15 cm and 97.6 ± 5 cm, respectively).

This finding demonstrates that the plants inoculated with PGPR displayed greater growth response than non-inoculated control plants. No plant was dead, however, some of the plants presented signs of phytotoxicity, such as yellowing of leaves and slower growth, compared with the control, which is consistent with the findings of Wyszkowski and Ziółkowska [35] and Al-Baldawi et al. [36]. Therefore, the presence of microorganisms reduced the toxic effect produced by contaminated soil, because the rhizobacteria provide defense to the plants and at the same time, benefit from plant exudates and from the plant itself [3,37]. The rest of the phenological characteristics (root and leaf biomass) of *O. miliaceum* and *P. setaceum* inoculated with the microbial consortium were also improved (Table 2). The morphology of the *O. miliaceum* and *P. setaceum* plants in response to different levels of TPHs was observed throughout the 240-day experimental period. The hydrocarbons in the soil can inhibit and reduce plant growth [38]. Plants in soil polluted with TPHs exhibited signs of phytotoxicity, with the death of some *O. miliaceum* and *P. setaceum* plants verified after 30 days. The roots’ and leaves’ biomass of *O. miliaceum* and *P. setaceum* were influenced by the TPHs treatments compared with the corresponding control, but the plants survived after 60 days of treatment. The dry biomass of *O. miliaceum* and *P. setaceum* significantly increased for all treatments in Group 2, as shown in Table 2.

**Table 2.** Biomass (dry weight (g plant⁻¹)) in roots and leaves of *Oloptum miliaceum* and *Pennisetum setaceum* for Group 1, 2, and Control Group after 30 (T₁), 60 (T₂), 90 (T₃), and 240 (T₄) days. Values represent mean ± SE. Different letters indicate means that are significantly different from each other (Duncan’s test, p < 0.05).

| Biomass (dry weight (g plant⁻¹)) | Roots   | Leaves | Roots   | Leaves |
|----------------------------------|---------|--------|---------|--------|
| *Oloptum miliaceum*              |         |        |         |        |
| Group 1                          |         |        |         |        |
| T₁                               | 34.1± 9ₐ | 25 ± 5ₐ | 37.3 ± 3ₐ | 27.7 ± 4ₐ |
| T₂                               | 45.2 ± 5ₘ | 36 ± 10ₘ | 72.1 ± 5ₘ | 49.9 ± 5ₘ |
| T₃                               | 150 ± 10ₗ | 49.6 ± 5ₗ | 160.3 ± 1ₗₗ | 69.3 ± 3ₗ |
| T₄                               | 190 ± 5ₙ | 77.8 ± 1₀ₙ | 201.3 ± 1₀ₙ | 71.2 ± 5ₙ |
| Group 2                          |         |        |         |        |
| T₁                               | 34.2 ± 1₀ₖ | 36.4 ± 6ₖ | 38.5 ± 5ₖ | 41.3 ± 4ₖ |
| T₂                               | 71.8 ± 1₁ₖ | 52.6 ± 10ₖ | 88.4 ± 8ₖ | 73.7 ± 5ₖ |
| T₃                               | 234 ± 10ₗ | 71.9 ± 6ₗ | 189.8 ± 1₁ₗ | 91.1 ± 7ₗ |
| T₄                               | 285 ± 15ₙ | 118 ± 4ₙ | 291.6 ± 15ₙ | 97.6 ± 5ₙ |
| Control Group                    |         |        |         |        |
| T₁                               | 50.9 ± 5ₖ | 55 ± 5ₖ | 48.4 ± 5ₖ | 44.7 ± 5ₖ |
| T₂                               | 98.1 ± 5ₖ | 69 ± 5ₖ | 101.4 ± 1₀ₖ | 66.7 ± 4ₖ |
| T₃                               | 273 ± 10ₙ | 89 ± 1₀ₙ | 245.6 ± 1ₙₙ | 89.6 ± 5ₙ |
| T₄                               | 312 ± 10ₙ | 113 ± 8ₙ | 287.7 ± 1₂ₙ | 101.3 ± 10ₙ |
3.4. Chlorophyll Content

The *O. miliaceum* and *P. setaceum* of Group 1 exhibited a significant decrease of Chl a, Chl b, and Chl a + b content in leaves (Table 3). This significant decrease is caused by the inhibition of biosynthesis of precursors of chlorophyll, loss of pigments, and disorganization of thylakoid membranes [39]. *O. miliaceum* and *P. setaceum* inoculated with bacteria (Group 2) had greater Chl a, Chl b, and Chl a + b than the non-inoculated plants grown under the same stress (Table 3).

Table 3. Chlorophyll content (mg g⁻¹ fw) in leaves of *Oloptum miliaceum* and *Pennisetum setaceum* for Group 1, 2, and Control Group after 30 (Ti), 60 (Tj), 90 (Tj), and 240 (Tr) days. Values represent mean ± SE. Different letters indicate means that are significantly different from each other (Duncan’s test, *p* < 0.05).

| Chlorophyll content (mg g⁻¹ fw) | *Oloptum miliaceum* | *Pennisetum setaceum* |
|---------------------------------|---------------------|----------------------|
|                                 | Group 1              | Group 2              |
|                                 | T1                   | Tj                   |
|                                 | 0.85 ± 0.01<sup>a</sup> | 0.15 ± 0.02<sup>a</sup> | 1.00 ± 0.03<sup>a</sup> | 0.79 ± 0.01<sup>a</sup> | 0.19 ± 0.02<sup>a</sup> | 0.98 ± 0.01<sup>a</sup> |
|                                 | T2                   | 0.82 ± 0.01<sup>a</sup> | 0.13 ± 0.01<sup>a</sup> | 0.95 ± 0.02<sup>a</sup> | 0.76 ± 0.02<sup>a</sup> | 0.16 ± 0.01<sup>a</sup> | 0.92 ± 0.02<sup>a</sup> |
|                                 | Tj                   | 0.79 ± 0.02<sup>a</sup> | 0.11 ± 0.01<sup>a</sup> | 0.90 ± 0.01<sup>a</sup> | 0.84 ± 0.01<sup>a</sup> | 0.18 ± 0.01<sup>a</sup> | 1.02 ± 0.04<sup>a</sup> |
|                                 | Tr                   | 0.81 ± 0.01<sup>a</sup> | 0.18 ± 0.01<sup>a</sup> | 0.99 ± 0.01<sup>a</sup> | 0.86 ± 0.01<sup>a</sup> | 0.16 ± 0.01<sup>a</sup> | 1.02 ± 0.02<sup>a</sup> |
|                                 | T1                   | 1.23 ± 0.04<sup>a</sup> | 0.19 ± 0.01<sup>a</sup> | 1.42 ± 0.02<sup>a</sup> | 0.97 ± 0.02<sup>a</sup> | 0.22 ± 0.01<sup>a</sup> | 1.19 ± 0.05<sup>a</sup> |
|                                 | Tj                   | 1.37 ± 0.01<sup>a</sup> | 0.23 ± 0.02<sup>a</sup> | 1.60 ± 0.05<sup>b</sup> | 1.18 ± 0.05<sup>b</sup> | 0.26 ± 0.02<sup>a</sup> | 1.44 ± 0.04<sup>b</sup> |
|                                 | Tj                   | 1.45 ± 0.02<sup>b</sup> | 0.31 ± 0.01<sup>b</sup> | 1.76 ± 0.05<sup>c</sup> | 1.27 ± 0.04<sup>c</sup> | 0.26 ± 0.01<sup>b</sup> | 1.53 ± 0.03<sup>c</sup> |
|                                 | Tr                   | 1.59 ± 0.01<sup>c</sup> | 0.33 ± 0.01<sup>c</sup> | 1.92 ± 0.05<sup>d</sup> | 1.36 ± 0.03<sup>d</sup> | 0.30 ± 0.01<sup>c</sup> | 1.66 ± 0.05<sup>d</sup> |

3.5. The Adsorption of TPHs by *O. miliaceum* and *P. setaceum* Tissue (Roots and Leaves)

The levels of TPHs in the plant tissue (roots and leaves) along with a summary of the statistical analysis are presented in Figure 3. The value of TPHs of *O. miliaceum* in Group 1 (plants without bacteria) ranged between 15 and 28 mg/kg in roots and 9 and 14 mg/kg in leaves, from 30 to 240 days. These results improve significantly with the inoculation of the consortium (Group 2), since there is a concentration of TPH in the roots of 48 and 28 mg/kg in the leaves referred to 240 days.

The value of TPHs in the leaves of *P. setaceum* in Group 1 (plants without bacteria) ranged between 19 and 31 mg/kg in roots and 8 and 12 mg/kg in leaves from 30 to 240 days. These results improve significantly with the inoculation of the consortium (Group 2) since there is a concentration of TPH in the roots of 45 and 19 mg/kg in the leaves referred to 240 days (Figure 3).

This result shows that the consortium also plays an important role in increasing the uptake of TPHs in the plant.

Mostly, the concentration of TPHs in plant samples was observed in the order: root > leaves. The content of TPHs in the plant samples shows that there is a good uptake of TPHs across root and leaf samples of *O. miliaceum* and *P. setaceum*. In rhizodegradation, contaminants will be degraded in the soil through the bioactivity exuded by plants or from soil organisms such as bacteria. The lower level of TPHs in leaves may have been caused by phytodegradation or phytotransformation of petroleum hydrocarbons, which occurs within the leaves of the plant itself [40].
3.6. Soil Analysis: Quantification of Petroleum Hydrocarbons and Dehydrogenase Activity

For the analysis of the TPHs levels in the three Groups of treatment, a specific analytical procedure was developed in order to identify five different classes on the basis of the number of carbon atoms in the hydrocarbon chain: low molecular weight hydrocarbons (C ≤ 12), aliphatic (C_{13–C_{18}}), aliphatic (C_{19–C_{36}}), aromatics (C_{11–C_{22}}), and high molecular weight hydrocarbons (C > 12). Low molecular weight hydrocarbons (C ≤ 12) were absent in all samples. The presence of aromatic compounds C_{11–C_{22}} was never detected (Figure 4). As is already known, long-chain hydrocarbons are less biodegradable and consequently, the residual observed contamination was reasonably made by the TPHs fraction most recalcitrant to biological actions. Moreover, a high number of carbon atoms leads to a reduction of both volatility and solubility in water, with a resulting decrease of natural mitigation of contamination. Reasonably, a fraction of branched chain compounds was also present, characterized by very slow biodegradation. All of this experimental evidence allowed for the identification of the residual TPHs fraction as particularly recalcitrant and difficult to be removed by a simple chemical–physical action. After 240 days, the plants of *O. miliaceum* and *P. setaceum* supplemented by the endophytic consortium (Group 2) had removed 94% of TPHs from soil, the plants of only *O. miliaceum* and *P. setaceum* removed 78% (Group 1). These results demonstrate that a successful strategy for overcoming the challenge of plant stress in phytoremediation of TPHs is to use plant growth-promoting rhizobacteria (PGPR) [4,8,41–45]. Soil dehydrogenase activity was strongly related to removal of TPHs, indeed, the maximum value (67.8 ± 1.15 in *O. miliaceum* and 70.1 ± 0.05 in *P. setaceum*) was measured in Group 2 after 90 days (Table 4).

These results demonstrate a hold relationship between DHA and biodegradation of petroleum-contaminated soil [46–49]. Moreover, between 60 and 90 days, there was a positive relationship between DHA and root lengths; in other words, the root growth contributes to TPHs’ degradation by stimulating the microbial activity of rhizosphere.
Figure 4. Total amount of TPHs, Aliphatic C13–C18 and Aliphatic C19–C36 (mg/kg) in soil to Groups 1 and 2 after 30 (T1), 60 (T2), 90 (T3), and 240 (TF) days in soil mesocosms of (A) Oloptum miliaceum and (B) Pennisetum setaceum. Values represent mean ± SE. Different letters indicate means that are significantly different from each other (Duncan’s test, p < 0.05).
Agronomy 2020, 10, 547

Table 4. Soil DHA in Group 1, 2, and Control Group after 0, 30 (T1), 60 (T2), 90 (T3), and 240 (T4) days in mesocosms of Oloptum miliacum and Pennisetum setaceum. Values represent mean ± SE. Different letters indicate means that are significantly different from each other (Duncan’s test, p < 0.05).

| Groups             | DHA (mg formazan 100 g soil dw⁻¹ hr⁻¹) |
|--------------------|---------------------------------------|
|                    | T0         | T1         | T2         | T3         | T4         |
| Oloptum miliacum   |            |            |            |            |            |
| Group 1            | 3.3 ± 0.05 | 15.8 ± 0.09 | 26.3 ± 1.08 | 60.8 ± 2.21 | 46.3 ± 1.35 |
| Group 2            | 3.5 ± 0.10 | 23.3 ± 1.01 | 32.8 ± 1.11 | 77.5 ± 2.10 | 67.8 ± 1.15 |
| Control Group      | 3.5 ± 0.05 | 14.3 ± 0.05 | 35.1 ± 0.05 | 69.3 ± 0.05 | 75.7 ± 0.05 |

| Pennisetum setaceum|            |            |            |            |            |
| Group 1            | 3.2 ± 0.05 | 7.6 ± 0.05  | 15.9 ± 0.02 | 64.9 ± 0.03 | 56.7 ± 0.05 |
| Group 2            | 3.6 ± 0.04 | 26.4 ± 0.02 | 30.1 ± 0.02 | 79.3 ± 0.04 | 70.1 ± 0.05 |
| Control Group      | 3.5 ± 0.05 | 14.3 ± 0.05 | 35.1 ± 0.05 | 69.3 ± 0.05 | 75.7 ± 0.05 |

4. Discussion

The phytoremediation of soil contaminated by TPH, assisted by consortia, is based on the potential of the plant and on the degree of association with endophyte microorganisms able to change organic contaminants into harmless final products. Endophytes improve the efficacy of phytoremediation of organic pollutants, and they may also manifest inherent biodegradation abilities around or within host plants, increasing metabolic activities in the rhizosphere and in the endosphere. Thus, the integrated actions between host plants and endophytes are critical factors in their ability to clean up polluted soil [11,12,16]. Apart from the above-mentioned benefits, endophytes also promote plant growth by enhancing nutrient acquisition and absorption of water, bringing significant improvements to the development and growth, and causing less stress, as evidenced by the results of the analysis of the stress markers. These characteristics of increased plant growth by endophyte microorganisms facilitate adaptation to abiotic stress factors and increase the biomass of Oloptum and Pennisetum, making it suitable for phytoremediation. The absorption of TPH by two species of Poaceae is controlled by the hydrophobicity of these organic pollutants [50]. The plant roots before their degradation discreetly absorb hydrophobic complexes by microflora rhizosphere. In this context, the functional bioavailability grade of hydrophobia is important. It can become the real limiting factor of phytoremediation of recalcitrant organic contaminants in soils [51]. The biosurfactants released by microorganisms or plants can help in the removal of organic pollutants in soils by rising their bioavailability and uptake by plants or microbial degradation, and thus, improve the effectiveness of phytoremediation in situ [52,53]. In this work, we have magnified the effectiveness of phytoremediation of TPH in soil by increasing inoculation within the rhizosphere with endophytes makers of biosurfactants. For example, the root with the endophytes that belong to the genus Bacillus, secretes ecocurfactants and show a good emulsification activity, which are excellent biosurfactant properties for bioremediation of soil contaminated by TPH.

Therefore, it is realistic to assume that endophytes could produce biosurfactants in vivo, which facilitate degradation of organic pollutants in plant, leading to a better rhizodegradation of organic contaminants in soils [54]. Our results show exactly this tendency of rhizodegradation, probably acting on the bioavailability of pollutants and the related rhizosphere environment that has been formed. The choice to use high EPS-producing endophytes bacteria is aimed at the possibility of creating ideal and effective conditions for the degradation of TPH [55]. Our system is responsible for the effectiveness of phytoremediation. Oloptum and Pennisetum, in relation to the morphological composition of roots, are generally free from secondary growth with roots that have little to no lignification and the ability to quickly explore the soil, and in relation to their biochemical activity of the production of exudates, mucilage allows for the specialization of a highly effective phytoremediation rhizoplane microbial architecture. Root development of these interesting Poaceae in partnership with endophytes has almost doubled, and the functional redox biology activity demonstrates the metabolic skills of combining, detoxifying, and/or rhizodegrading pollutants. Our
results demonstrate that the prospects of degrading hydrocarbons are concentrated at the rhizosphere level. In fact, the degradation of contaminants is extremely elevated at the rhizosphere level, where the production of a diversity of biomolecules produced at different levels from the endophyte-plant system plays a key role. The activated enzymatic processes and degraded genes produced are just one example of a co-metabolic perspective that trigger between plants and endophytes. The ability of the Poaceae, and especially Oloptum and Pennisetum, to develop an active and wide root and the capacity to host a high quantity of microorganisms both in the rhizosphere and in the endosphere, allow for effective activities of break down in hydrocarbons.

5. Conclusions

O. miliaceum and P. setaceum have never been studied for their phytoremediation characteristics of organic compounds nor is their great capacity for endophytic partnership known [54]. They were only once used in some heavy metal phytostabilization experiments in combination with biochar. These plants have a great ecological value, as they are a phytotype that naturally lives on poor and disturbed soils and stands up well, even in xerophytics environments. Also, in nature, they show a significant ecological value and an amazing ability to regenerate after the removal of leaves. They show a rapid and effective regenerative capacity (about 15 days from the cut) due to the considerable amount of meristems present at the base of the leaves, with a significant increase of biomass production. Our data also shows that O. miliaceum and P. setaceum have great adaptability to disturbed substrates and other physico-chemical composition.

The high plasticity and environmental adaptability are the foundation that will enable these plants to become an important model of bioremediation systems’ development in drought environments and in poor and disturbed soils. The ability to quickly change various eco-physiological parameters, such as PAL and GST, and rebalance the photosynthetic efficiency, demonstrates the great biological plasticity of these plants. In addition, the high and diverse microbial population that is hosting the rhizosphere contributes synergistically to the species adaptation speed and performance-specific response to stresses. These features, together with minimum maintenance incidence (little water, the absence of serious diseases), are a prerequisite for O. miliaceum and P. setaceum to become new candidate species for bioremediation TPHs. The ecosystem service and the physiological response of the plants during the endophytic-assisted phytoremediation demonstrate our experimental model: Oloptum and Pennisetum show a great resolute value. Choosing bacteria that largely produce EPs, ACC, and siderophores guarantee the possibility of different strategies of attack on recalcitrant molecules like TPH. The addition of mycorrhizae fungi that naturally colonize these roots have magnified the rhizodegradation activity. In other words, the plant-endophyte–microorganism association should not be seen in a perspective of a summation of effects but a rearrangement of co-metabolism to consider, together, one big organism that reorganizes itself through signals and metabolic adjustments. In fact, more effective degradation of contaminants requires interactions to support growth and catabolic collaboration between plants and hypothetically thousands of degradative microbial taxa that certainly colonize the host. This is ultimately controlled to the concept of “Meta-organism” or Microbiome vegetable, mentioning the total of a host plant and its connected microbial population. Omics tools’ accessibility has facilitated extensive surveys of biodiversity and functional characteristics of the endophytic community of Oloptum and Pennisetum. However, it still lacks studies in situ of endophyto-assisted phytoremediation at the metaorganism level.

Supplementary Materials: Table S1: The main chemical and physical characteristics of contaminated soil. Supplementary Materials is able online at www.mdpi.com

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