Petroleum hydrocarbon rhizoremediation and soil microbial activity improvement via cluster root formation by wild Proteaceae plant species

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Highlights

- Wild plant species varied in tolerance to total (aliphatic) petroleum hydrocarbon (TPH)
- Rhizosphere microbial activity was strongly associated with plant growth status
- Poaceae and Proteaceae plants exhibited high TPH rhizoremediation potential
- The TPH removal rates were associated with root biomass production
- Overall microbial abundance was not significantly correlated with TPH removal rates

GRAPHICAL ABSTRACT

Eight native wild plant species

Screening process

Suitable candidates for rhizoremediation of TPH
Abstract

Rhizoremediation potential of different wild plant species for total (aliphatic) petroleum hydrocarbon (TPH)-contaminated soils was investigated. Three-week-old seedlings of Acacia inaequilatera, Acacia pyriformia, Acacia stellaticeps, Banksia seminuda, Chloris truncata, Hakea prostrata, Hardenbergia violacea, and Triodia wiseana were transplanted in a soil contaminated with diesel and engine oil as TPH at pollution levels of 4,370 (TPH1) and 7,500 (TPH2) mg kg\(^{-1}\), and an uncontaminated control (TPH0). After 150 days, the presence of TPH negatively affected the plant growth, but the growth inhibition effect varied between the plant species. Plant growth and associated root biomass influenced the activity of rhizomicrobiome. The presence of B. seminuda, C. truncata, and H. prostrata significantly increased the TPH removal rate (up to 30% compared to the unplanted treatment) due to the stimulation of rhizosphere microorganisms. No significant difference was observed between TPH1 and TPH2 regarding the plant tolerance and rhizoremediation potentials of the three plant species. The presence of TPH stimulated cluster root formation in B. seminuda and H. prostrata which was associated with enhanced TPH remediation of these two members of Proteaceae family. These results indicated that B. seminuda, C. truncata, and H. prostrata wild plant species could be suitable candidates for the rhizoremediation of TPH-contaminated soil.

Key words: Petroleum hydrocarbon remediation; Soil dehydrogenase activity; Proteaceae plants; Rhizosphere; Soil respiration; Wild plant species
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1. Introduction

Soils contaminated with hydrocarbons have been of great concern worldwide due to the ever-growing usage of petroleum fossil fuels. In Australia alone, nearly 80,000 sites were reported to be seriously contaminated by petroleum hydrocarbons, according to the National Environment Protection Council (NEPC 1999). Total petroleum hydrocarbon (TPH) is the measurable amount of any mixture of hydrocarbons that have originated from crude oil.

There are several hundred of these compounds which are made up of two types: volatile petroleum hydrocarbons and extractable petroleum hydrocarbons. With respect to chemical structure, TPH can be classified as either aliphatic or aromatic hydrocarbon compounds (Kuppusamy et al. 2020). Although aliphatic petroleum hydrocarbons are significant pollutants in the soil environment, this class of chemicals have been receiving much less research attention compared to the aromatic counterparts, which only equates to less than 5% of TPH by volume (Stroud et al. 2007). Similar to aromatic hydrocarbons, the toxicity and persistence of aliphatic compounds in the environment adversely affect not only the human health but also ecosystems (Abbasian et al. 2015). Consequently, it is necessary to develop cost-effective and eco-friendly remediation technologies for the sustainable management of aliphatic hydrocarbon-contaminated soils.

Among various bioremediation approaches, rhizoremediation (i.e., the use of plants and their associated microorganisms in the root zone) is an emerging method, and especially effective for degrading organic contaminants, offering both ecological and engineering advantages (Ahkami et al. 2017; Gupta et al. 2020; Hoang et al. 2020). The rhizoremediation method examined in this research work could be applicable to remove degradable organic contaminants such as aliphatic hydrocarbons and aromatic hydrocarbons from contaminated soils (Gaskin and Bentham 2010; Hoang et al. 2020; Sivaram et al. 2018). The proposed method is suitable for the remediation of especially derelict field sites contaminated with
aliphatic hydrocarbons within plant tolerance levels. In contrast to conventional methods (e.g., excavation, incineration), rhizoremediation is non-intrusive and protects soils from any functional and structural damage while reducing the mass flux of the contaminants to receptors (Cundy et al. 2016). Plants produce varying quantity and quality of root exudates which may fuel an initial substrate-driven microbial community establishment followed by a shift in rhizosphere dynamics (chemistry and biology) (Bulgarelli et al. 2013). As a result, even closely related plant genotypes may harbour notably diverse microbial populations in the rhizosphere, which are able to degrade contaminants with different rates (Dagher et al. 2019). Owing to the cooperative evolution and adaptation of microorganisms in the soil-plant-microbe systems, the use of native plants is generally preferred over introduced plant species for hydrocarbon biodegradation (Hatami et al. 2019). Native plants offer better interactions with local beneficial soil microbiota than invasive plant species, enabling efficient rhizoremediation of contaminants (Bolan et al. 2013; Chowdhury et al. 2017). Studies on the use of wild plant species for rhizoremediation of TPH is limited, and mostly focused on grass species belonging to the Poaceae family (Gaskin and Bentham 2010; Kiamarsi et al. 2020; Steliga and Kluk 2020). However, shallow root systems of grasses cannot reach deep soil layers, and hence become ineffective to remediate contaminants residing at a depth (Cook and Hesterberg 2013). Plants outside grasses (Poaceae family) also showed rhizoremediation potential, such as shrubs and trees (e.g., Chromolaena odorata, Haematosylum campechianum) (Anyasi and Atagana 2018; Pérez-Hernández et al. 2017), legumes (e.g., Cajanus cajan, Lotus corniculatus) (Allamin et al. 2020; Hussain et al. 2019), ornamental plants (e.g., Hylotelephium spectabile) (Cheng et al. 2019), and even agronomic crops (e.g., Zea mays, Vicia faba) (Baoune et al. 2019; Ghalamboran et al. 2020). Hence, it is important to test the suitability of non-Poaceae plant species for TPH biodegradation under different contamination scenarios and biomes. In this context, testing the tolerance of wild
plant species to various levels of hydrocarbon contaminations, and the plants’ degree of stimulation of the rhizosphere microbial activity are a need of the hour for achieving successful rhizoremediation of TPH contaminants. Because wild plants have developed synergistic relationships with other plants, soil microorganisms and the local environment, the proposed rhizoremediation process can be considered as an in-situ ‘rhizo-engineering’ technique which do not require intensive and intrusive engineering techniques involving excavation and off-site treatments such as incineration and soil washing. These special adaptations of wild plants have evolved over long periods of time and they allow wild plants to thrive under extreme conditions (e.g., low nutrient concentration, low soil moisture) (Bolan et al. 2011). Consequently, wild plants are easier to cultivate and manage, and therefore they have been utilized effectively for rhizoremediation and phytostabilization of contaminants including TPH in different studies (Abbaspour et al. 2020; Bolan et al. 2011; Cheng et al. 2017).

This study aims to evaluate eight wild plant species for the purpose of rhizoremediation of TPH, with a particular focus on aliphatic hydrocarbon contaminants. For the first time, members belong to Proteaceae family (B. seminuda and H. prostrata), which can develop cluster roots as an underground adaption for tolerance in extreme environments, were investigated for their potential for the rhizoremediation of TPH. The specific objectives are to determine the plant height and dry biomass parameters, soil respiration, soil dehydrogenase activity, and TPH removal rate under different plant and TPH concentration treatments. The provision of suitable wild plant species would offer an economically feasible and environmentally sustainable option for the remediation of TPH-contaminated sites.

2. Materials and methods

2.1. Experimental design
A factorial randomized block design with five replications was adopted to investigate the effect of TPH-contaminated soils (two pollution levels of 4,370 and 7,500 mg kg\(^{-1}\) denoted as TPH1 and TPH2, respectively, and an uncontaminated control) on the growth of eight wild plant species from three families, namely Fabaceae, Poaceae and Proteaceae (Supplementary Information; SI Table 1). These plants were selected due to their habitation of different climates and soil conditions, diverse taxa, and varied root morphologies. Furthermore, some of these plant species have been found to be effective in the phytoremediation of organic contaminants (Khan et al. 2018). The experiment also investigated the plants’ potential for enhanced removal of TPH in contaminated soils. The plant growth experiment was conducted under controlled glasshouse conditions (25°C for 16 h (daytime), and 18°C for 8 h (night), typical of the New South Wales State of Australia). The glasshouse received natural lights, and plants were irrigated with 50 mL of deionized water on alternate days over 150 days of the experiment. No external nutrients were applied to the soil during the plant growth experiment to mimic uncropped Australian soils and considering the nature of the native wild plants tested.

### 2.2. Preparation of TPH-contaminated soils

An uncontaminated soil (Red Kandosol; Australian Soil Classification) without any hydrocarbon contamination history was collected from an area in Cobar, New South Wales (-31.502085, 145.781435). The soil was transported to the glasshouse, air-dried, sieved through a 4-mm screen (mesh No. 5), and identified as a clay loam soil in texture (with 35, 37, and 28% of clay, silt, and sand, respectively). Selected physical and chemical properties of the soil determined following standard methods (Burt 2004) are shown in SI Table 2. Pro Quip Platinum Diesel and Castrol GTX Engine Oil 20W-50 were obtained commercially at a local service station in Newcastle, New South Wales. The sieved soil was artificially contaminated
with the mixture of diesel and engine oil (1:1, w/w) at target concentrations of 0.5% (5,000 mg kg\(^{-1}\)) and 1% (10,000 mg kg\(^{-1}\)), which resulted in initial pollutant levels named TPH1 and TPH2, respectively, as mentioned above. The mixture was chosen to represent aliphatic hydrocarbons covering a wide range of carbon chains, likely to show different degrees of biodegradation. In addition, by artificially contaminating a ‘clean’ site soil with known TPH concentrations, a greater experimental control was ensured in terms of the ability to evaluate growth and TPH remediation at a range of TPH levels for individual plant species. Therefore, data could be compared with growth performance in the uncontaminated controls and literature data.

The 0.5% contamination level was chosen representing the highest level in the majority of hydrocarbon-contaminated mine sites worldwide (Gaskin et al. 2008; Sun et al. 2004). The 1% pollution level was chosen to increase the hydrocarbon-derived stress on plants but without causing any serious damage (Sun et al. 2004). To achieve homogeneity in the TPH-soil mixtures, the soil with each TPH concentration was mixed thoroughly using a dual tumbler bin (15 kg soil at a time) for 30 min, then using a concrete pan mixer for 15 min, and left for 14 days before starting the plant growth experiment. After mixing, three sub-samples from the top (0 – 10 cm), middle (10 – 20 cm), and bottom (> 20 cm) layers of the soil at each pollution level (totally nine samples) were analysed for initial TPH concentrations to ensure the contamination homogeneity (SI Table 2).
2.3. Seedling emergence in Petri dishes and plant growth in micro-pots

Seeds of the selected wild plant species were commercially obtained from Nindethana Seed Service Pty, Ltd, (Western Australia, Australia). Seeds were soaked in 1% sodium hypochlorite for 5 min and rinsed thoroughly to eliminate any fungal pathogen. Preliminary tests (data not shown) showed that germination of all seeds was compromised at the TPH1 pollution level. Therefore, a standard germination test was applied for all plant seeds (Groves et al. 1982), with the exception of the species belonging to Poaceae (i.e., C. truncata and T. wiseana). To 120 x 120 x 17 mm (square) polystyrene Petri dishes, two layers of filter papers were placed, and evenly soaked with 3 mL of ultrapure water (Milli-Q®, 18.2 MΩ.cm). To each dish, 25 seeds of each selected plants (A. inaequilatera, A. pyrifolia, A. stellaticeps, B. seminuda, H. prostrata, and H. violacea) were added in triplicate maintaining 5 x 5 grid spaces, and sealed with Parafilm (Lamb et al. 2012). Seeds of A. pyrifolia were boiled at 100°C for 1 min to break dormancy and allowed to cool for 15 min at room temperature before adding to the Petri dishes.

All the plant species were germinated at 25°C and monitored regularly for seven days. Once radicles emerged to a certain length (approx. 5 to 10 mm), the germinated seeds were placed in micro-plant pots (one seedling per pot) filled with autoclaved propagation sand (obtained from Bunnings, Australia). For C. truncata and T. wiseana, ten seeds were directly sown in each micro-plant pot (twenty replicated pots, 200 seeds in total) to obtain good “seed to soil” contact, which is considered as the key to grass seed germination (Chauhan et al. 2018b).

Nutrient solution (20 mL) was added to every pot daily. The nutrient solution was prepared according to Asher and Loneragan (1967), with a modified phosphorus concentration. Particularly, the following concentrations of nutrients were achieved (in µM) in the solution: calcium 250, magnesium 100, potassium 250, sulfur 100, nitrogen (as NO₃⁻) 750, nitrogen (as...
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NH₄⁺ 100, phosphorus 1, chlorine 100, iron 2 (as Iron Ethylenediaminetetraacetic acid, FeEDTA), copper 0.1, manganese 1, zinc 0.5, cobalt 0.04, boron 3, and molybdenum 0.02. This nutrient solution was successfully tested for growing native plant species previously (Lamb et al. 2010).

2.4. Plant growth test in TPH-contaminated soils

For each pollution level, 1 kg of soil was filled in each plastic grow pot (13.3 cm in diameter and 14 cm tall) which was previously lined with 0.1 mm textile fabric to promote drainage and to avoid soil loss. A saucer was placed under each pot to collect the leachate, which was reused for irrigation. For each plant species, when the seedlings grew approx. 5 to 10 cm height in the micro-pots, plants with similar height were transplanted to the TPH contaminated (TPH1 and TPH2) and control (TPH0) soils. Unplanted control pots received identical treatments as the planted pots. After 2 weeks, all pots with C. truncata and T. wiseana were uniformly thinned out to one individual plant per pot to ensure homogeneity amongst all plant species.

One day prior to harvesting the plants (day 150), shoot height (length between shoot base and apical tip) was measured. After harvesting, shoot and root biomasses were gently washed with deionized water to remove soil particles. Root and shoot biomasses (g dry weight) were determined for each pot after drying at 70°C for 48 h. In addition to plant height and dry biomass, relative growth performance (R) of TPH-contaminated soil (TPH1 and TPH2) as a percentage of growth of uncontaminated control soil (TPH0) within each plant species were calculated. The R values were used as an indicator of plant tolerance to TPH contamination (Kulakow et al. 2000). Plants whose R values were less than 25%, between 25 and 50%, and higher than 50% were respectively considered as strongly susceptible, moderately tolerant, and considerably tolerant plants (Sun et al. 2004). Furthermore, the relative growth of root to
whole plant of all pots were calculated by root: shoot ratios (i.e., the dry mass of roots
divided by the dry mass of shoots) for the estimation of the extent of root/soil contact, which
might play an important role in the degradation of TPH in the rhizosphere (Shahsavari et al.
2013).
Soils adhered to plant roots were collected by gentle hand tapping (Rovira 1974). For TPH
measurement, all soil samples were stored in a freezer (- 20°C) until extraction. For soil
dehydrogenase activity determination, subsamples were stored at 4°C until analysis.
Unplanted control soils were processed and stored the same way as the treatment samples.

2.5. Soil respiration
Soil respiration (CO₂ flux) was measured in the glasshouse by an automated soil respirometer
(Li-8100A, LICOR, Lincoln, NE, USA) within 30 min after harvesting the plant shoots from
pots (with the roots still remaining in the soil) (Zainul et al. 2017). Measurements of CO₂
fluxes (µmol CO₂ m⁻² s⁻¹) were made with a cylindrical metal column (20 cm in diameter and
20 cm tall) which was sealed at the bottom. The collar was perfectly fitted with a 20 cm
survey chamber (8100-103, LICOR) used in combination with the respirometer. Pots were
placed inside the metal column during soil respiration measurement. The offset (height
between the soil surface and column top) and soil area of each pot was measured, and entered
into the Li-8100A software for calculation of the system volume, which was used for
calculating the carbon flux (Zainul et al. 2017). In addition, a soil moisture probe (8150-204,
LICOR) fitted with the respirometer was used for soil moisture measurement to ensure no
significant difference of the soil moisture content between different treatments.
The SoilFluxPro™ software from LICOR was used to compute data files for chamber
measurements. This software used the ideal gas law (Eq. 1) and linear regression (R² > 0.99)
to calculate soil CO₂ fluxes.
PV = nRT  (Eq. 1)

where, p is the pressure of the gas (N m\(^{-2}\)), V is the volume of the gas (m\(^3\)), n is the number of moles of gas, R is the ideal gas constant (8.3145 J mol\(^{-1}\) K\(^{-1}\)), and T is the absolute temperature (K).

2.6. Soil dehydrogenase activity (DHA)

Soil DHA for rhizosphere samples was determined according to the method described by Casida Jr et al. (1964), where 2,3,5-triphenyltetrazolium chloride (TTC) was reduced to triphenyl formazan (TPF). Field moist soil (3 g) was added to a 50 mL centrifuge tube, along with 0.5 mL of 3% aqueous TTC solution, 30 mg CaCO\(_3\), and 1.25 mL of ultrapure water (Milli-Q\textsuperscript{®}, 18.2 M\(\Omega\).cm), successively. The samples were incubated for 24 h at 37\(^\circ\)C. After incubation, 10 mL methanol was added to the sample, and thoroughly mixed using a vortex mixer. The content was then centrifuged at 4000 rpm for 15 min. After filtering through a syringe filter (0.2 \(\mu\)m), the absorbance of the clear supernatant was measured at 485 nm wavelength by a spectrophotometer (Microplate Reader; Ensign\textsuperscript{TM}, Multimode, Perkin Elmer, USA). The result was presented as \(\mu\)g TPF per g\(^{-1}\) dry soil day\(^{-1}\). Assays without any soil but CaCO\(_3\) and TTC served as the controls. Additionally, a set of the contaminated soil was sterilized using an autoclave, which received the same procedure to determine DHA value to evaluate the interference of TPH itself to the DHA measurement.

2.7. Sample preparation and extraction of TPH

Concentration of TPH in soil was analysed according to Richter (2000). For sample extraction, 3 g of moist soil was taken into a 50 mL glass beaker. Diatomaceous earth (1.0 g) was added to the beaker and mixed thoroughly. The mixture was placed into extraction cells through a screw-on funnel to ensure that the entire soil was removed from the beaker. Glass fibre filters were fitted in the outlet before loading cells to the extraction system.
All extractions were carried out using a Dionex ASE 350 Accelerated Solvent Extractor (Thermofisher, USA) in Dionex ASE 150/350 Stainless Steel cells. Extractions were performed at 175°C and 1500 psi, with hexane: acetone (1:1, v/v). Heating and static time were 8 and 5 min, respectively. The flush volume was 75%, and purge time was 60 s, with 2 cycles. After extraction, the extracts were collected in clean 40 mL glass vials. The vials were placed into a Turbo Vap II evaporator until the solvent fully disappeared. Subsequently, 5 mL hexane was added to every vial, and 1 mL aliquot of sample was transferred into 2 mL autosampler vial for analysis by gas chromatography. In preliminary tests, the recoveries achieved were > 84% for the accelerated solvent extraction method on a model soil containing high clay content (50%, representing a hard case scenario for extraction) spiked with 5,000 and 10,000 mg TPH kg\(^{-1}\) soil.

Analytical determination of TPH in soil samples was performed by gas chromatography fitted with flame ionization detector, GC-FID (Model No. 7697A, Headspace Sampler 7697A, Agilent Technology, USA). The following conditions were applied for all analyses: capillary column (30 m x 0.25 mm, ID = 0.25 mm); \(\text{H}_2\) carrier gas; FID at 330°C; injector temperature 300°C; oven temperature programmed from 40 to 300°C at 12°C min\(^{-1}\) after 2-min hold with a 15-min hold at the final temperature; column flow rate 1.5 mL min\(^{-1}\). External calibration standards were prepared from Hydrocarbon Window Defining Standard stocks in chloroform (Novachem, Australia). The linear standard curves were prepared with six concentrations (1, 5, 10, 20, 60 and 100 µg mL\(^{-1}\)) for three common TPH fractions: \(\text{C}_{10} - \text{C}_{14}\), \(\text{C}_{15} - \text{C}_{28}\), and \(\text{C}_{29} - \text{C}_{36}\) in the aliphatic hydrocarbons (diesel and engine oil) used in this study. Varian Star\textsuperscript{TM} Version 4.5 software was used to integrate the total chromatogram areas of the three TPH fractions. Sample concentrations were measured using the standard curves, and TPH values were calculated as the sum of \(\text{C}_{10} - \text{C}_{36}\) and presented as mg TPH kg\(^{-1}\) dry soil. The GC-FID system was purged by injecting \(n\)-hexane (solvent blank) to ensure the system was free of
contamination. In addition, a soil-free sample was subjected to the same extraction procedure mentioned above to detect any potential interferences. Limits of detection for C\textsubscript{10} – C\textsubscript{14}, C\textsubscript{15} – C\textsubscript{28}, and C\textsubscript{29} – C\textsubscript{36} were 35, 79, and 69 ng mL\textsuperscript{-1}, respectively, according to the calibration standard curve (Guideline 2005). In addition, accuracy was evaluated using the recovery for real soil samples spiked with 16.67 µg mL\textsuperscript{-1}, which was diluted from the stock standard solution of 500 µg mL\textsuperscript{-1}. The recovery rate was determined using the measured concentrations in the enriched samples and the added concentration. The average recovery was 89% (n = 3), which conformed satisfactory performance of the method, and the value was in accordance with ISO 16703 standard (Standardization 2004).

The TPH removal rates under different plant and soil treatments were calculated using Eq. 2 (Qi et al. 2015):

\[
\text{TPH removal rate (\%) = } \frac{(\text{Initial soil TPH} – \text{Final soil TPH})}{\text{Initial soil TPH}} \times 100 \quad \text{(Eq. 2)}
\]

2.8. Statistical analysis

All data were analysed for normality and homogeneity using SPSS Version 25 package for Windows. Analysis of variance (ANOVA) test was conducted at α = 0.05 to determine significant difference between treatments for plant height, dry biomass, soil respiration, DHA, and TPH removal rate. Treatment means showing significance were separated with Tukey’s test at 5% level of confidence.

3. Results

3.1. Effect of TPH on plant height and biomass

After 150 days, plant growth was evaluated by comparing plant heights and dry biomasses between TPH-untreated and TPH-treated plants within each plant species. Overall, the results from one-way ANOVA showed that there were significant differences (p < 0.05) in plant
height and dry biomass between TPH0 and TPH1- or TPH2-treated plants within each plant species (Tables 1 & 2).

Table 1. Plant height (in cm) of eight Australian wild plant species in different pollution levels. Mean values ± standard deviation (n = 3). * Significantly different at p = 0.05 for each plant species. Numbers within parenthesis represent R values.

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<th>Plant species</th>
<th>TPH concentration</th>
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<td>TPH0</td>
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<tr>
<td>A. inaequilatera</td>
<td>22.13 ± 1.90*</td>
</tr>
<tr>
<td>A. pyrifolia</td>
<td>22.87 ± 1.51*</td>
</tr>
<tr>
<td>A. stellaticeps</td>
<td>10.83 ± 0.17*</td>
</tr>
<tr>
<td>B. seminuda</td>
<td>14.90 ± 0.22</td>
</tr>
<tr>
<td>C. truncata</td>
<td>30.97 ± 3.20*</td>
</tr>
<tr>
<td>H. prostrata</td>
<td>29.13 ± 5.31*</td>
</tr>
<tr>
<td>H. violacea</td>
<td>20.37 ± 2.05*</td>
</tr>
<tr>
<td>T. wiseana</td>
<td>19.17 ± 2.05*</td>
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Seven of the tested plant species, i.e., A. inaequilatera, A. pyrifolia, A. stellaticeps, C. truncata, H. prostrata, H. violacea, and T. wiseana (except B. seminuda) were susceptible to TPH1 and TPH2 levels, although the extent varied among the plant species. For example, A. inaequilatera, A. stellaticeps, H. violacea, T. wiseana were strongly susceptible to TPH1 and TPH2 levels, according to their relative growth (Tables 1 & 2). In contrast, B. seminuda and C. truncata showed considerable tolerance to either of the contamination levels. Interestingly, despite significant reductions in plant height and biomass in the TPH-contaminated soils, all of the eight plant species were able to survive until the end of the experiment, and some
species produced considerably increased root biomass compared to those grown in the
uncontaminated soil (Figure 1).

Shoot length (plant height) values showed that the response varied among plant species and
TPH concentrations (Table 1). For almost all plant species, TPH-contaminated soils led to
considerable reductions in shoot lengths compared to the control soil ($p < 0.05$), although no
significant difference was observed between TPH1 and TPH2 pollution levels ($p > 0.05$). The
most significant growth inhibition was observed for *H. violacea* at TPH1 and TPH2
contamination levels (R values were 19 and 23% that of the control, respectively).

Conversely, shoot length of *B. seminuda* showed no significant difference between different
pollution levels ($p > 0.05$). This indicated that *B. seminuda* was the least affected plant by
TPH contamination with respect to shoot length development, regardless of TPH
concentrations.
Figure 1. Development of cluster roots in the members belong to Proteaceae family (A), and dry root biomass of the four tolerant plant species grown in different TPH levels after 150 days (B). The error bars indicate the standard deviation of the means (n = 3). Different lowercase letters indicate significant difference within the same plant species.
Similarly, within individual species, plant dry biomass from the contaminated soils was significantly different to that of the control in almost all plants tested. No significant difference was detected between TPH1- and TPH2-contaminated soils (p > 0.05) (Table 2).

After 150 days of growth, dry biomass of the eight plant species decreased in the contaminated soils (both TPH1 and TPH2). Compared with the control, > 75% reduction in dry biomass was found in *A. inaequilateral*, *A. stellaticeps*, *H. violacea*, and *T. wiseana* (Table 2). Interestingly, adding diesel/oil to the soil at TPH1 level caused no significant decrease in the biomass of *B. seminuda* plant. Nevertheless, plant biomass at TPH2-contaminated soil was significantly lower than in the control soil for this plant species (p < 0.01).

**Table 2.** Plant dry biomass (in g) of eight Australian wild plant species in different pollution levels. Mean values ± standard deviation (n = 3). * Significantly different at p = 0.05 for each plant species. Numbers within parenthesis represent R values.

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<tr>
<td></td>
<td>TPH0</td>
<td>TPH1</td>
<td>TPH2</td>
</tr>
<tr>
<td><em>A. inaequilateral</em></td>
<td>2.99 ± 0.38*</td>
<td>0.61 ± 0.30 (0.20)</td>
<td>0.64 ± 0.06 (0.21)</td>
</tr>
<tr>
<td><em>A. pyrifolia</em></td>
<td>2.80 ± 0.05*</td>
<td>1.36 ± 0.43 (0.49)</td>
<td>1.24 ± 0.38 (0.44)</td>
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<tr>
<td><em>A. stellaticeps</em></td>
<td>1.44 ± 0.04*</td>
<td>0.26 ± 0.04 (0.18)</td>
<td>0.20 ± 0.03 (0.14)</td>
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<tr>
<td><em>B. seminuda</em></td>
<td>2.17 ± 0.13</td>
<td>2.00 ± 0.28 (0.92)</td>
<td>1.09 ± 0.22 (0.50)*</td>
</tr>
<tr>
<td><em>C. truncata</em></td>
<td>10.87 ± 1.27*</td>
<td>5.56 ± 0.50 (0.51)</td>
<td>5.27 ± 0.20 (0.49)</td>
</tr>
<tr>
<td><em>H. prostrata</em></td>
<td>9.40 ± 1.52*</td>
<td>3.53 ± 0.29 (0.38)</td>
<td>2.90 ± 0.10 (0.31)</td>
</tr>
<tr>
<td><em>H. violacea</em></td>
<td>8.97 ± 1.28*</td>
<td>0.22 ± 0.01 (0.02)</td>
<td>0.22 ± 0.01 (0.02)</td>
</tr>
<tr>
<td><em>T. wiseana</em></td>
<td>5.39 ± 0.56*</td>
<td>1.04 ± 0.11 (0.19)</td>
<td>0.24 ± 0.06 (0.04)</td>
</tr>
</tbody>
</table>
Plant height and dry biomass were not compared among the different plant species because their initial heights and biomass as well as growth rates were different. With respect to plants’ tolerance to TPH via R values in this study, the growth rates were significantly lower in plants grown in the contaminated soils than in the control soil for almost all species tested and varied among the plant species. These results indicated phytotoxicity of TPH at varying extents to the plant species (Tables 1 & 2). *A. inaequilatera, A. stellaticeps, H. violacea, and T. wiseana* were strongly susceptible to both TPH1 and TPH2 pollution levels (R values below 25%). Since these four plant species were strongly sensitive to both TPH contamination levels, they were not used for further experimentation.

For further assessment of plant growth in the TPH-contaminated soils, the root: shoot dry biomass ratio was determined. In this study, the root: shoot ratio varied significantly between the four tolerant plant species in the contaminated and control soils. Of these, *A. pyriformia* and *C. truncata* showed no significant difference in root: shoot ratios under both levels of TPH and control soils. In contrast, considerable differences were detected among soil treatments in the case of *B. seminuda* and *H. prostrata*. The two Proteaceae species showed significantly increased root: shoot ratio ($p < 0.05$) in response to TPH contamination (Figure 2).
Figure 2. Root: Shoot ratio of the four tolerant plant species grown in different TPH levels after 150 days. The error bars indicate the standard deviation of the means (n = 3).

*Significantly different within the same plant species at $p = 0.05$. Different lowercase letters indicate significant difference between plant species at the same TPH level.

3.2. Soil respiration

Overall, soil respiration was higher in the planted compared to unplanted pots at all pollution levels (Figure 3). For each plant species and unplanted control, no significant difference was detected between TPH1 and TPH2-contaminated soils ($p > 0.05$). At the end of the experiment, *A. pyrifolia* and *C. truncata* showed higher (nearly double) soil respiration relative to those in *B. seminuda*, *H. prostrata*, and unplanted control treatments. Interestingly, there was no significant difference among plant treatments and unplanted control at TPH1 and TPH2 pollution levels for *B. seminuda* and *H. prostrata* ($p > 0.05$). While the presence of TPH tended to decrease soil respiration in all plant species, an opposite pattern was
observed in unplanted treatment, although no significant difference was observed in the latter case (Figure 3).

Figure 3. Soil carbon flux from unplanted pots and the four plants grown under different TPH concentrations. The error bars indicate the standard deviation of the means (n = 3).

*Significantly different within the same plant species at $\rho = 0.05$. Different lowercase letters indicate significant difference between plant species at the same TPH level.

3.3. Soil DHA

Tests confirmed that TPH did not interfere with the colour development step of the DHA assay. Although the presence of plants stimulated DHA in all the soil treatments, both B. seminuda and H. prostrata showed no significant difference in DHA values at TPH1 and TPH2 pollution levels compared to those in the unplanted control ($\rho > 0.05$) after 150 days (Figure 4). This result suggests that although these species had a rhizosphere effect on the
microbial activity in the uncontaminated soil, the effect was not evident in the presence of TPH.

At both TPH pollution levels, DHA values in the rhizosphere of the four plant species and unplanted control after 150 days tended to be higher than those in the uncontaminated soil. However, at TPH2 pollution level, soil DHA in *C. truncata* decreased significantly compared to that in the uncontaminated soil (*p* < 0.05). This decline indicated that any TPH pollution level higher than the TPH1 level used in this study may be the critical level for the change in the activity of dehydrogenase enzyme for this plant species. The TPH1 pollution level enhanced soil DHA of *C. truncata*, while the TPH2 pollution level inhibited the DHA (Figure 4).

**Figure 4.** Effect of TPH contamination on soil DHA in the rhizosphere of tested plants and unplanted control. The error bars indicate the standard deviation of the means (*n* = 3).

*Significantly different within the same plant species at *p* = 0.05. Different lowercase letters indicate significant difference between plant species at the same TPH level.
3.4. Hydrocarbon removal rate

Figure 5 shows that with the exception of *A. pyrifolia*, the removal rates (%) of the four tested tolerant plants at TPH1 and TPH2 contamination levels in the rhizosphere soils had higher values than those in the unplanted control treatment (*p* < 0.05), at the end of the experiment. A 10 to 30% enhancement in TPH removal rate in the planted soils was observed relative to the unplanted control. In addition, as the concentration level increased from TPH1 to TPH2, the removal rate did not change significantly for any plant species and unplanted treatment. At TPH1 and TPH2 pollution levels, only three plants (*B. seminuda*, *C. truncata*, and *H. prostrata*) showed significant removal rates relative to the unplanted control (*p* < 0.05). The highest removal rate was observed for *C. truncata*, around two times than that in the unplanted control. In contrast, no significant difference was detected in the presence of *A. pyrofolia* compared to unplanted control (*p* > 0.05). In the rhizosphere soil, *B. seminuda* and *H. prostrata* had the second highest removal rates (about 1.5 times that of the unplanted control), with no significant difference between the two plant species (*p* > 0.05).
4. Discussion

4.1. Plant growth performance

It was reported the addition of 0.5% and 1% (w/w) of TPH to soil caused significant decrease in plant growth (Robson et al. 2003; Wei et al. 2019). These results were in agreement with our experimental data that the growth of all the plant species were inhibited in TPH1- and TPH2-treated soils. The inhibition of plant growth caused by TPH contamination could be explained by the fact that plant roots were in direct contact with the contaminants. On the one hand, low-molecular-weight (lighter, C_{10} - C_{19} ) fractions of TPH could enter the plant body through roots causing direct toxic effects on the plant cells (Basumatary et al. 2012; Bell et al. 2014). On the other hand, high-molecular-weight (heavier, > C_{19} ) fraction could hinder the
absorption of nutrients and water from the soil into the roots by forming a water-repellent
film of oil covering the root surface, which in turn also would affect the respiration of roots
(Jonker et al. 2006; Lin and Mendelssohn 2009). The TPH might also negatively affect soil
properties such as water holding capacity and soil aeration, further hindering the plant growth
(Chen et al. 2015; Mena et al. 2016). The freshly contaminated clay loam soil with low
organic content coupled with low-molecular-weight aliphatic hydrocarbons in diesel used in
the current study could be expected to allow considerable bioavailability and contribute to the
observed outcomes of plant growth. In addition, high-molecular-weight aliphatic
hydrocarbon compounds, mainly found in the engine oil, could have negative effects on soil
properties, thereby impeding plant performance in the contaminated soil.

Increased root biomass could be an adaptive strategy to reduce phytotoxicity in plants (Qi et
al. 2019; Shahsavari et al. 2013). In this study, the members belonging to Proteaceae family
(i.e., *B. seminuda* and *H. prostrata*) increased root biomass in the TPH-treated soils by
forming cluster roots. Exudates of cluster roots are characterized with relatively high
proportion of readily available substrates for enhancing rhizosphere microorganism
abundance and activities, which is advantageous in rhizoremediation of the TPH-
contaminated soil (Martin et al. 2014; Shane et al. 2004).

Root: shoot ratio is an indicator of plant stress and it could allow comparison of different soil
treatments within each plant species and also amongst various plants (Agathokleous et al.
2019; Husáková et al. 2018). Higher root: shoot ratio in *B. seminuda* and *H. prostrata* under
TPH1 and TPH2 levels suggested that the plant species increased biomass allocation to roots
in the TPH-contaminated soil. Plants with a higher proportion of roots were considered to
perform better in rhizoremediation approach (Huang et al. 2005; Nie et al. 2010). Regarding
root biomass as the lone determinant of rhizoremediation, *B. seminuda* and *H. prostrata*
showed the potential to remediate soil up to TPH2 contamination level in this study.
4.2. Soil respiration

Soil respiration, which is related to plant root and microbial respiration, has been recommended to be a quick and accurate assessment of metabolically active microbial communities in contaminated soils, and it gives an idea of the quantity of easily mineralizable substrates present in the soil (Gielnik et al. 2019; Kim et al. 2018; Wang et al. 2014). Therefore, any change in soil respiration observed in the TPH-contaminated soils compared to the corresponding uncontaminated soil could reflect the metabolic state of soil microbial communities, and the abundance of metabolically active microbes in the soil (Salazar et al. 2019).

Increased soil respiration in the planted compared to the unplanted treatments in this study indicated that all the four plant species enhanced their rhizosphere microbial activity, a phenomenon termed as the rhizosphere effect (Barati et al. 2018). The positive effect of plants on soil respiration with and without TPH addition was similar to that found by Muratova et al. (2012). The authors reported an increased CO$_2$ flux in 1% TPH-contaminated soil cultivated by Lolium perenne L. (ryegrass) compared to soil without plants after three weeks of plant growth. Similarly, the soil respiration rate was significantly higher in barley-cultivated soils than in uncultivated treatment at 0, 4, 6, and 8% TPH contamination rates, indicating that plant roots stimulated microbial activity in the rhizosphere in all the pollution levels (Barati et al. 2018). However, in the present study, the CO$_2$ fluxes were not observed to increase at the end of the experiment for two plant species (i.e., B. seminuda and H. prostrata) compared to unplanted controls at both TPH pollution levels (Figure 3). Since this is the first time the two plant species have been assessed for rhizoremediation of TPH-contaminated soils, the reason for this disparity is not fully understood. It could be explained in part by the true extent of rhizosphere effect to the associated microorganisms in these plant...
species, and also in terms of their exudation patterns (Gaskin and Bentham 2010). Further investigations are therefore warranted to elucidate the role of root exudates of these two plant species in influencing the rhizosphere microbiota.

Within each plant species, the presence of TPH at both the pollution levels showed a negative effect on the CO₂ flux (Figure 3). Similar observation was reported in the literature. For example, Khan et al. (2018) found that hydrocarbons significantly decreased the diversity and abundance of rhizosphere microorganisms. The microbial activity decreased with the addition of diesel in the rhizosphere of *C. truncata* and *Triticum aestivum* (wheat) because of hydrocarbon toxicity (Khan et al. 2018). Additionally, any adverse effect on plant growth due to TPH contamination might have an additional inhibitory effect on soil microorganisms in the rhizosphere and plant root productivity (Dagher et al. 2019; Merkl et al. 2006; Saraeian et al. 2018). Increased competition between roots and rhizosphere microorganisms for nutrients such as nitrogen, phosphorus and potassium in such nutrient-poor soils, resulting from TPH contamination, could also be a possibility (Arslan et al. 2014; Kuzyakov and Xu 2013). These reasons altogether led to the decreased soil respiration in the contaminated than uncontaminated soils in this study. Nevertheless, an increased CO₂ flux (although not significant) in TPH1 and TPH2 pollution levels compared to uncontaminated soil in the unplanted treatment indicated that soil microorganisms might not suffer from TPH toxicity (Figure 3). Therefore, the reasons for the reduction of soil respiration in the TPH-contaminated planted soils compared to the control soil could be linked to plant root-driven decreases in the abundance of metabolically active rhizosphere microbes.

4.3. *Dehydrogenase activity*

Dehydrogenase activity (DHA) is a measure of total microbial activity in soil, and can indicate the onset of biodegradation process (Kaimi et al. 2007; Maila and Cloete 2005). In
this study, increased DHA was observed in the presence of plants in comparison to the unplanted treatment. Similar observations were reported by Zamani et al. (2018) that DHA was higher in planted than in unplanted soils during TPH treatment. The exact role of plants in stimulating microbial activity might result from the release of root exudates which supply nutrient and carbon sources for soil microorganisms (Herz et al. 2018). Furthermore, the physical effect of roots in improving soil aeration and harbouring microorganisms through the soil, is also a possibility (Jacoby et al. 2017). However, that trend was not observed in the presence of B. seminuda and H. prostrata compared to the unplanted treatment at both TPH pollution levels (Figure 4). The reason for this disparity might be related to root exudate characteristics of these plant species as the rhizosphere microbial community is greatly affected by root exudates that depend on plant species (Dagher et al. 2019; Gaskin and Bentham 2010). In this study, the two Proteaceae species (i.e., B. seminuda and H. prostrata) formed cluster roots in TPH-contaminated soils regardless of TPH concentrations, but not in the control soil (Figure 1). The rhizosphere of cluster roots is often characterised by high concentrations of readily available carbon sources (mainly low molecular weight organic anions and phenolics) that mobilize soil nutrients (de Britto Costa et al. 2016). Additionally, acid phosphatase enzyme is exuded at high rates in the rhizosphere of cluster roots (Lambers et al. 2018; Shane et al. 2004). The enzyme is hypothesised to not only enhance phosphorus supply to the plants but also provide an easily degradable energy source for rhizosphere microorganisms, and increase the bioavailability of TPH (Martin et al. 2014). Consequently, the development of strong nutrition depletion zones around the roots of these species caused the reduced soil DHA at the end of the experiment (Kaimi et al. 2006; Luo et al. 2015). Dehydrogenase is an enzyme that occurs in all viable microbial cells (Järvan et al. 2014). In the current study, DHA was not affected by the presence of TPH in a similar way to soil respiration. This is possibly because, a part of the microflora (i.e., plant root–associated
microorganisms) was in a dormant state in the rhizosphere due to the plant root-derived growth-substrate deficiency in the TPH-contaminated soil (Blagodatskaya et al. 2014). As a result, the presence of TPH in planted treatment decreased soil respiration via plant root (Jiang et al. 2017; Yu et al. 2015). On the other hand, the increased DHA in contaminated soils might be due to the adaptation of microorganisms by secreting enzymes in the stressful environment. In addition, TPH compounds such as saturated and aliphatic (n-alkanes) compounds in the mixture could serve as available carbon source, which would enhance soil microbial activity (Ikeura et al. 2016; Stroud et al. 2007). The stimulatory effect of TPH on soil microbial activity in planted treatments was reported earlier too (Dhote et al. 2017; Ebadi et al. 2018). In the present study, the relatively low DHA values were resulted from the absence of glucose in the measurement method as a growth substrate for microorganisms. Indeed, Petrisor et al. (2004) reported that the value with glucose addition was able to evaluate the activity of whole DHA in the soil, and can be 10 times higher than that without glucose.

4.4. Hydrocarbon removal rates

The level of soil microbial activity (measured from soil respiration and/or DHA) could reflect the soil microorganisms’ ability to degrade petroleum pollutants, consequently TPH removal from polluted soils (Rodríguez-Rodríguez et al. 2016; Zamani et al. 2018). However, that trend was not clear in this study. For example, high values of soil respiration and DHA in *A. pyrifolia* did not lead to high TPH removal rates (Figures 3, 4 & 5). Therefore, an enhancement of soil respiration and/or DHA did not necessarily relate to the increase of TPH removal rate in the rhizosphere, compared to the unplanted treatment. The true extent of TPH rhizodegradation, in fact, might not depend on the whole microbial community as indicated by soil DHA, but the activity of specific hydrocarbon-degrading microorganisms (Bastida et
These microorganisms, which are selectively enriched under the combined selective pressure of TPH and rhizosphere, could utilize TPH compounds as carbon and energy sources in order to protect their roots from the toxic effects of petroleum contaminants (Afzal et al. 2011; Khan et al. 2013). The exploration of shifts in the rhizosphere microbial community composition and hydrocarbon-degrading gene expression would shed more light on the efficiency of TPH rhizoremediation, could be a future study.

Different plant species, and even closely related plant genotypes, vary in their potential for rhizoremediation of TPH (Dagher et al. 2019; Ikeura et al. 2016). Grasses have been shown as suitable candidates for the elimination of TPH in soils in numerous studies (Gaskin and Bentham 2010; Hussain et al. 2019; Kiamarsi et al. 2020; Steliga and Kluk 2020). *C. truncata* is a perennial grass with fibrous roots, which provide sufficient surface for microbial growth and plant nutrient absorption (Chauhan et al. 2018a). Similarly, *B. seminuda* and *H. prostrata*, from Proteaceae family, developed cluster roots in the nutrient-poor soils resulting from the TPH contamination (Abbasian et al. 2016a; Andreoli et al. 2015; Ávila-Valdés et al. 2019), thereby enhancing root biomass relative to that in the control soil (Figure 1). This pattern might be the main reason for the relatively high removal rates of TPH in the soil by the three plants compared to the unplanted control and *A. pyrifolia*, which developed much less root biomass than *B. seminuda*, *C. truncata*, and *H. prostrata* (Figures 1 & 5). It is worth noting that *A. pyrifolia* is a member belonging to Fabaceae family, which is able to fix nitrogen directly from the atmosphere through nodules on the plant roots. The use of legumes could generally increase soil enzymatic activity more than non-legumes (Maseko and Dakora 2013; Zhou et al. 2017); therefore, it could explain the high soil respiration and DHA observed in the *A. pyrifolia* plant treatment, which did not result in high TPH removal in the contaminated soil.
Aliphatic hydrocarbons (e.g., \(n\)-alkane) are readily degraded by aerobic microorganisms that use molecular oxygen for the initial activation (Abbasian et al. 2015). Three possible peripheral pathways for aerobic aliphatic hydrocarbon biodegradation are proposed (Varjani 2017) (SI Figure 1). However, the most frequently encountered mechanism pathway is terminal oxidation (Zhang et al. 2011). The process starts with oxidation of the substrate molecules which introduces an oxygen atom inside the terminal methyl group to form an alcohol group (Meng et al. 2017). Alkane hydroxylases, which mainly consists of the integral-membrane alkane monooxygenase (AlkB) and the cytochrome P450 CY153 family, are the key enzymes for the first and foremost oxidation step in aerobic degradation of \(n\)-alkanes (Smits et al. 2002). Generally, these enzymes could hydroxylate \(n\)-alkanes with short- and medium-chain length (up to \(C_{16}\)). However, \(n\)-alkanes with the chain length up to \(C_{32}\) could be hydroxylated by some Actinomycetes whose AlkB-type alkane hydroxylases were fused with rubredoxin protein (Nie et al. 2011; Piccolo et al. 2011). The alcohol can be further oxidized to the corresponding aldehyde and then to fatty acid prior to entering the \(\beta\)-oxidation and tricarboxylic acid cycles (Abbasian et al. 2016b). The terminal oxidation of aliphatic TPH contaminants can be described as follows:

\[
R-CH_3 \xrightarrow{\text{Alkane hydroxylases}} R-CH_2OH \xrightarrow{\text{Alcohol dehydrogenase}} R-CHO \xrightarrow{\text{Aldehyde dehydrogenase}} R-COOH \xrightarrow{\beta\text{-oxidation}} \text{Microbial biomass + CO}_2 + \text{H}_2\text{O}
\]

Microorganisms present at the rhizosphere of plants growing in a TPH-contaminated soil are equipped with metabolic machinery to use the contaminants as a carbon and energy source (Van Hamme et al. 2003; Yergeau et al. 2014). The extent of aliphatic hydrocarbon degradation, indeed, is connected with the abundance and activity of hydrocarbon-degrading microorganisms with functional enzymes encoded in functional genes (Gielnik et al. 2020).
Therefore, studies concerning the genes, such as \( \textit{alkB} \), involved in the degradation of aliphatic hydrocarbons, could give a better indication of the “soil bioremediation potential”, which warrants future investigation.

Numerous studies report that microbially-facilitated rhizoremediation is a principal pathway for TPH removal from soil (Correa-García et al. 2018; dos Santos and Maranho 2018; Thijs et al. 2016). Plants able to promote the degradation of TPH though the rhizosphere effect have been reported (Allamin et al. 2020; Cheng et al. 2019). Indeed, plant roots could directly absorb hydrocarbon compounds (Wei et al. 2019). Moreover, root exudates, whose amount are proportionally linked with root biomass, could enhance the degradation of TPH in the rhizosphere by specifically promoting efficient bioremediation communities (Bell et al. 2014; Eisenhauer et al. 2017). As a result, increased root biomass has been considered as the key to success in rhizoremediation technology (Huang et al. 2005; Saraeian et al. 2018; Wang et al. 2011; Wei et al. 2019).

A simple linear regression was carried out to investigate the relationship between plant dry biomass, soil respiration and DHA and TPH removal rate. However, there was only a strong positive linear relationship between dry root biomass (g) and TPH removal rate (%) (Figure 6), with Pearson’s correlation coefficient of 0.9034 \((p < 0.001)\). The relationship can be presented as follows (Eq. 3):

\[
\text{TPH removal rate (\%)} = 9.8696 \times \text{dry biomass (g)} + 19.263 \quad (R^2 = 0.8162, \quad N = 8) \quad (\text{Eq. 3})
\]
Figure 6. Correlation between TPH removal rate and dry root biomass across the four tolerant plant species at both TPH pollution levels

The finding that the production of root matter in TPH-contaminated soil across plant species was related to TPH removal rate confirms the importance of screening plant species that are able to tolerate TPH. In addition, to promote root biomass production, plant species adapted to local conditions is a requisiste for successful phytoremediation of TPH-degraded sites. In this study, the Proteacea species and *C. truncata* showed the greatest root production, and the increased root biomass was associated with the enhanced TPH removal at the end of the study. The stimulation of cluster roots in the presence of TPH contamination concurrently with enhanced TPH removal indicates the promise of the Proteaceae family plants for TPH rhizoremediation.

5. Conclusion
The tolerance of the eight wild plant species to two levels of TPH contamination were investigated in this study. The presence of aliphatic hydrocarbon compounds had negative effects on the plant growth, and inhibited soil respiration and dehydrogenase activity for almost all plant species tested, although to varying extents. *A. pyrifolia, B. seminuda, C. truncata, and H. prostrata* showed greater potential of TPH rhizoremediation than the other four species due to the higher tolerance of the former species to TPH contamination than the latter ones. The grass species (*C. truncata*) was the most efficient for the rhizoremediation of TPH-contaminated soil. *B. seminuda and H. prostrata* (tree and shrub species, respectively) also showed remarkable rhizoremediation potential due to their ability to form cluster roots in the contaminated soil. While selecting plants for rhizoremediation their ability to develop high root biomass reaching to contaminants in impacted soils should be considered, to maximize the removal rates. Further studies should consider the ways to enhance (e.g., rhizosphere engineering) the remediation efficiency of suitable/selected plant species, particularly species from Proteaceaee family, which has largely been neglected in rhizoremediation investigation. There is also scope for future work exploring rhizosphere microbiota alteration in composition and hydrocarbon-degrading gene expression, and plant-specific exudates being deposited in the rhizosphere during the remediation process.

References


Functional potential of sewage sludge digestate microbes to degrade aliphatic hydrocarbons during bioremediation of a petroleum hydrocarbons contaminated soil. J. Environ. Manage.: 11648.


Zhou Y, Zhu H, Fu S, Yao Q (2017) Variation in soil microbial community structure associated with different legume species is greater than that associated with different grass species. Front. Microbiol. 8: 1007.
Supplementary Information for:

**Petroleum hydrocarbon rhizoremediation and soil microbial activity improvement via cluster root formation by wild Proteaceae plant species**

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### SI Table 1. Characteristics of Australian wild plant species used for the rhizoremediation of TPH

<table>
<thead>
<tr>
<th>Plant type</th>
<th>Plant species</th>
<th>Common name</th>
<th>Family</th>
<th>Life cycle</th>
<th>Soil grown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tree</td>
<td><em>Acacia inaequilatera</em></td>
<td>Kanji Bush</td>
<td>Fabaceae</td>
<td>Perennial</td>
<td>Sandy loam</td>
</tr>
<tr>
<td></td>
<td><em>Banksia seminuda</em></td>
<td>River Banksia</td>
<td>Proteaceae</td>
<td>Perennial</td>
<td>Sandy</td>
</tr>
<tr>
<td>Shrub</td>
<td><em>Acacia pyrifolia</em></td>
<td>Ranji Bush</td>
<td>Fabaceae</td>
<td>Perennial</td>
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</tr>
<tr>
<td></td>
<td><em>Acacia stellaticeps</em></td>
<td>Poverty Bush</td>
<td>Fabaceae</td>
<td>Perennial</td>
<td>Clayey sand</td>
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<td></td>
<td><em>Hakea prostrata</em></td>
<td>Harsh Hakea</td>
<td>Proteaceae</td>
<td>Perennial</td>
<td>Sandy loam</td>
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<tr>
<td></td>
<td><em>Hardenbergia violacea</em></td>
<td>Purple Coral Pea</td>
<td>Proteaceae</td>
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<td>Grass</td>
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</tr>
<tr>
<td></td>
<td><em>Triodia wiseana</em></td>
<td>Hard Spinifex</td>
<td>Poaceae</td>
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<td>Sandy</td>
</tr>
</tbody>
</table>

### SI Table 2. Selected physicochemical characteristics of the experimental soil and initial TPH concentrations used in the rhizoremediation study

<table>
<thead>
<tr>
<th>pH (H₂O)</th>
<th>pH (CaCl₂)</th>
<th>EC (µS cm⁻¹)</th>
<th>Organic C (%)</th>
<th>Total N (%)</th>
<th>Available P (mg kg⁻¹)</th>
<th>Total S (%)</th>
<th>TPH1 (mg kg⁻¹)</th>
<th>TPH2 (mg kg⁻¹)</th>
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<tr>
<td>6.04</td>
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<td>3.0</td>
<td>0.002</td>
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<td>7,500±</td>
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<td>52</td>
<td>166</td>
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</table>
**Supplementary Figure**

**SI Figure 1.** Three possible pathways for aerobic aliphatic hydrocarbon biodegradation