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BIOREMEDIATION OF DIESEL FUEL CONTAMINATED SOIL BY *Podocarpus polystachyus* ENHANCED WITH ORGANIC WASTES

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ABSTRACT A greenhouse experiment was performed to evaluate the effectiveness of *Podocarpus polystachyus* in phytoremediation of soil contaminated with 1% and 2.5% w/w diesel fuel. This research was aimed at assessing the potential of 5% (w/w) of three organic waste amendments (biowastes)[tea leaf (TL), soy cake (SC) and potato skin (PS)] to enhance degradation of diesel in contaminated soils for a period of 270 days. Addition of biowastes, especially SC, to contaminated soil planted with P. *polystachyus* rapidly increased the rate of removal of diesel fuel by 90% and 84% in soil contaminated with 1% and 2.5% oil, respectively. Loss of diesel fuel at 53% and 43% were recorded in treatments without organic waste amendment and planted with P. *polystachyus* in 1% and 2.5% contamination, respectively. Diesel fuel degradation was more rapid in the soil amendment with SC than in other organic waste amendments. P. *polystachyus* roots did not accumulate oil from the contaminated soil, but the number of hydrocarbon utilizing bacteria (HUB) was high in the rhizosphere, thus suggesting that the mechanism of the oil degradation was via rhizodegradation or phytovolitilization. P. *polystachyus* with organic waste amendments has potential in restoring hydrocarbon-contaminated soil.

(Keywords: Phytoremediation, *Podocarpus polystachyus*, Diesel fuel, Organic waste amendments)

INTRODUCTION

Global production of oil is estimated at more than one trillion barrels and 1.7 to 8.8 million metric tons of oil is released into the world's water and soil every year [1]. About 90% of this emission is directly related to human activities including deliberate illegal waste disposal [1]. There are several remediation technologies currently being used, such as soil incineration to excavation, soil washing and solidification by electro kinetic systems [2, 3]. These engineering-based technologies are most appropriate for highly polluted sites and are often not suited for the treatment of widespread yet low levels of contamination found in many parts of the world. Phytoremediation of soil contaminated with organic chemicals is a challenging problem in environmental science and engineering [4]. Plant metabolism of organic compounds is a vital phytoremediation process for suitable waste management. In addition, the high nutrient content of most organic wastes can lead to enhanced bacterial breakdown and degradation of oil.

In this study P. *polystachyus* was selected due to its hardness and its characteristics as a non-edible plant that can grow in tropical areas. The aim of this study is to evaluate the potential of *P. polystachyus* in degradation of hydrocarbons from soil and to investigate the effects of different organic amendments on the ability of *Podocarpus* in removing diesel fuel.

MATERIAL AND METHODS

Sample collections

Soil was obtained from the Nursery section of the Asia–Europe Institute, University of Malaya, Kuala Lumpur. Soil samples were air-dried and mixed well, sieved through a 2 mm sieve for analysis. The diesel fuel was purchased from a petrol station in Petaling Jaya, Malaysia. As N and P are usually the limiting inorganic nutrients for oil-degrading bacteria, organic wastes were as a nutrient source. Organic wastes used in this study were collected from different locations, for example, tea leaf (TL) and potato skins (PS) were collected from the Institute of Graduate Studies (IGS) canteen, University of Malaya and soy cake (SC) was prepared in the laboratory. P. *polystachyus* were used for phytoremediation studies. It is more tolerant than most plants, in dry soil and irregular watering and is widely cultivated in India and Malaysia. Physico and chemical analysis of soil and organic wastes were carried out using standard methods. The experiments were set up in triplicate replication.

Soil preparation

Two kilograms of unsterilized, air-dried soil was placed into each plastic bag. Soils were artificially contaminated with 1 and 2.5% (w/w) diesel fuel and thoroughly mixed. 5% (w/w) of organic wastes (TL, SC and PS) were also mixed individually with the fuel-contaminated soil. The polluted soil with organic wastes, were allowed to stabilize for 5 days before transplanting the plants into the contaminated soil. Control treatments consisting bags of plants without diesel fuel or organic wastes were also set up. An additional control treatment comprising of autoclaved soil (at 121˚C and 15 psi for 1 h) containing 0.5% (w/w) NaN_3, was also set up to determine non-biological loss of diesel fuel from the soil (**Table 1**). In total, 108 microcosms were set-up at room temperature (30 ± 2˚C) with 24 h fluorescent light. The plants were watered moderately every two days with tap water.

Table 1: Experimental Design

Treatment	Details of Treatment
A	2 kg soil + 1% oil + 5% TL + *Podocarpus* plant
B	2 kg soil + 1% oil + 5% SC + *Podocarpus* plant
C	2 kg soil + 1% oil + 5% PS + *Podocarpus* plant
D	2 kg soil + 1% oil + *Podocarpus*
E	2 kg soil + 1% oil only
F	2 kg soil + 2.5% oil + 5% TL + *Podocarpus* plant
G	2 kg soil + 2.5% oil + 5% SC + *Podocarpus* plant
H	2 kg soil + 2.5% oil + 5% PS + *Podocarpus* plant
I	2 kg soil + 2.5% oil + *Podocarpus* plant
J	2 kg soil + 2.5% oil only

Sampling

Soil samples from the phytoremediation experiments were collected monthly for nine months. Soil samples were taken within the root zone of plants from each bag every month for analysis total petroleum hydrocarbon (TPH), pH, total organic carbon and HUB counts. At the completion of the experiment (270 days), the plants were uprooted to determinate total biomass. The root tissues were extracted with 1:1 hexane/acetone in a Soxhlet extractor for 10 h to determine if the roots had absorbed the hydrocarbon from the soil. To assess hydrocarbon content removal, the extracts were analyzed for hydrocarbons using gas chromatography with a mass-selective detector (QP2010A). The GC was equipped with cross-linked 5% phenyl methyl siloxane capillary column. Helium was used as the carrier gas. The temperature was set at 40 ◦C and raised by 10 ˚C min^{-1} until 300 ◦C, which was maintained for 8min.

First- order kinetics model used is expressed by the following equation [9]:

$$C_t = C_i e^{-kt}, \qquad (1)$$

Where C_t (mg/g), is the diesel fuel concentration in soil at instant t, C_i (mg/g) is the initial concentration of soil, k is the rate constants of the first order expressed in (day^{-1}), and t is the time. The model estimated the half-life of hydrocarbons and biodegradation rate in soil relative to treatments applied.

$$\text{Half life} = \ln(2)/ k \qquad (2)$$

Isolation and identification of bacterial diesel degraders

Three replicate samples from each oil polluted soil were withdrawn every two weeks for the enumeration both heterotrophic and hydrocarbon utilizing bacteria. In order to isolate and enumerate both heterotrophic and hydrocarbon utilizing bacteria, bacteria enrichment process used mineral salt medium (MSM). Bacterial colonies were picked and pure cultures were obtained by repeated sub-culturing on nutrient agar. The bacterial isolates were characterized based on culture parameters, microscopic techniques (gram straining reaction) and biochemical tests using Biolog® Microstation system method (Biolog Inc., CA, USA) for identification [10].

Analysis of variance (ANOVA) with SPSS (version 18) at $\alpha=0.05$ (95% confidence level) was used for statistical analysis.

RESULTS AND DISCUSSION

Low N content (0.24%) and P content (0.08%) was recorded for the soil used (**Table 2**). Of the organic wastes used, SC had higher amount of N (1.3%) compared to PS (1.1%) and TL (1.02%

Table 2: Physical and chemical properties of organic wastes and soil used for project

Parameters	Soil	TL	SC	PS
Total nitrogen (%)	0.2 ± 0.5	1.0± 0.1	1.3 ± 0.1	1.1±0.1
Phosphorus (%)	0.6 ± 1.5	0.7 ± 0.7	0.9 ± 0.9	0.7±0.1
Moisture content (%)	8 ± 0.6	34.3 ± 0.5	75.9 ± 1.6	62.1 ±2.0
Organic C (%)	0.7± 0.9	0.9±1.2	1.2± 0.9	1.1±1.1
pH	6.4 ± 0.6	6.5±1.2	6.8±1.2	6.9±0.5
Lead (mg/kg)	19.2 ± 1.8	-	-	-
Zinc (mg/kg)	18.1± 1.6	-	-	-

TL: Tea Leaf, SC: Soy Cake, PS: Potato Skin

Response of Plants to the Oil

The appearance of the plants to 1% and 2.5% concentration of diesel were monitored throughout the 270 days of the experiment. No plant death was recorded in the 1% diesel fuel; however some of the plants in the 2.5% fuel showed signs of phytotoxicity such as yellowing of leaves compared with the control, the signs are in similar to the finding of Vouillamoz and Mike [11]. Plants in soil contaminated with 2.5% diesel oil showed high symptoms of phytotoxicity with death of at least one *Podocarpus* plant recorded in each treatment (data not shown). These results show that *Podocarpus* plants can tolerate minimum degree of exposure to hydrocarbons. At the end of 270 days, biomass of the *Podocarpus* plants in each treatment was determined as shown in **Table 3**.

Loss of diesel fuel in Soil Contaminated with 2.5% and 1% Oil

The rate of diesel fuel loss in soil treatment contaminated with 1% and 2.5% oil are shown in Figures 1 and 2. The loss of diesel fuel at the end of 270 days in soil contaminated with 2.5% and 1% oil

Table 3. Dry mass of *Podocarpus* plant parts at the end of experiment (270 days).

	Dry weight (g)		
Treatment	Leaves	Stem	Roots
A	2.5 ± 0.3	1.9 ± 0.4	1.1 ± 0.5
B	4.3 ± 0.8	6.6 ± 0.4	2.9 ± 0.3
C	2.9 ± 0.6	2.7 ± 0.5	2 ± 0.6
D	1.1±0.4	0.9 ± 0.7	0.6 ± 0.5
E	1.0 ± 0.7	0.5 ± 0.2	0.3 ± 0.4
F	3.6 ± 0.8	3.3 ± 0.7	1.8 ± 0.2
G	2.0 ± 1.1	1.7 ± 0.9	0.8 ± 0.3
H	0.7 ± 0.4	0.6 ± 0.1	0.3 ± 0.2
M	4.2 ± 0.4	4 ± 1.1	2.2 ± 0.2

A, soil + 1% oil + TL; B, soil + 1% oil + SC; C, soil + 1% oil + PS; D, soil + 1% oil only; E, soil + 2.5% oil + TL; F, soil + 2.5% oil + SC; G, soil + 2.5% oil + PS; H, soil + 2.5% oil only; M, control soil i.e. without oil contamination.

ranged from 12– 84% and 13 – 91%, respectively in all the different treatments. Contaminated soil treated with SC recorded the highest loss of oil (84% and 91%) in 270 days followed by soil treated with PS (72% and 79%) in 2.5% and 1% contaminated soil respectively. The contaminated soil containing only *Podocarpus* plant, without organic wastes treatment recorded 43% and 53% oil loss while control soil without *Podocarpus* plant showed 23% and 26% oil loss in 2.5% and 1% contaminated soil respectively at the end of 270 days. 12% and 13% oil loss in soil contaminated with 2.5% and 1% oil may be due to non-biological factors like evaporation; this was recorded in autoclaved soil treated with sodium azide after 270 days. High loss of oil in soil treated with SC and *Podocarpus* plants may be due to the presence of appreciable nitrogen (1.3%) and phosphorus (0.9 %[1]) contents in SC (Table 2), this was recorded also in our previous works, where soil amended with SC recorded 78% loss of diesel fuel in soil [12]. *Podocarpus* plant amended with SC grew better and taller (about 30% than other treatments) with lots of fibrous roots than other treatments in the experimental set up. The result is in agreement with that of Palmroth et al. (2002), who recorded 60% loss of diesel fuel during one month in diesel polluted soil amended with NPK fertilizer and planted with pine tree [13]. ANOVA results did not show any significant difference between the soils treated with SC, PS and TL at P < 0.05 confidence level, whereas significant difference was observed between the soil treated with different organic wastes, soil with only *Podocarpus* plants and soil without *Podocarpus* plants. These results pointed out that addition of organic wastes into the contaminated soil planted with *Podocarpus* increased the loss of oil in the soil by at almost 30%; this is in line with the findings of Vouillamoz and Milke (2009), who demonstrated that compost addition combined with phytoremediation, increases the rate of removal of diesel fuel in soil [11]. A significant difference was observed between soil treated with different organic wastes, unplanted contaminated soil and contaminated soil planted with *Podocarpus* at both concentrations ($P < 0.05$). Whereas, statistical analysis does not show any significant difference in biodegradation of diesel fuel between soils amended with PS and SC but there was a significant difference in the results between soils amended with TL and two other different organic waste amendments. However, results indicated that in all treatments amended with organic wastes the rate of oil loss was significantly higher than those of unamended and unplanted treatments.

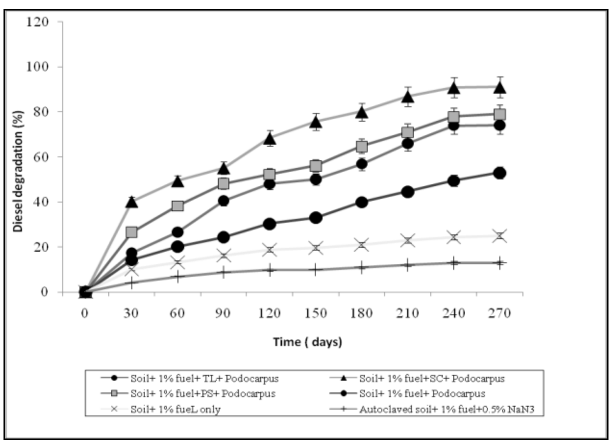


Figure1. Biodegradation of 1% diesel fuel in contaminated soil with P. polystachyus

Uptake of Oil by *Podocarpus*

Gas chromatographic mass spectrometry analysis of the extract did not show accumulation of diesel oil in all the treatments. This is in contrast with the finding of Palmroth et al. (2002), who found accumulation of diesel oil by grass root, but result is in line with Chaineau et al. (1997) who did not observe uptake of oil by maize root [14]. The result suggests that the mechanism of phytoremediation process may be through rhizodegradation or phytovolitilization that has been well documented [15, 16, 17]. In addition, the removal of the oil may be because of root exudates produced by the *Podocarpus* plant, which enhance the oxidation and mineralization of soil oil in the soil.

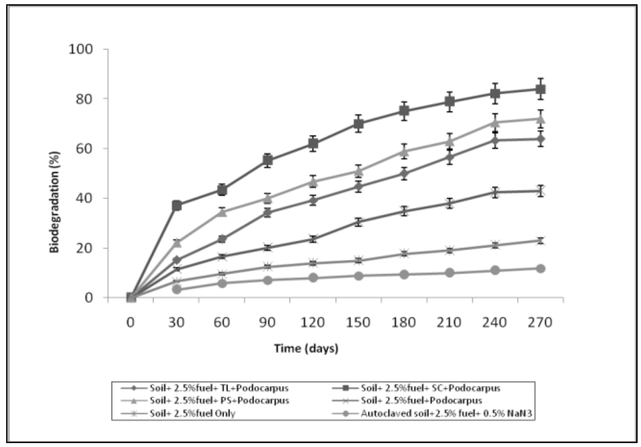


Figure 2. Biodegradation of 2.5% diesel fuel in contaminated soil with P. polystachyus.

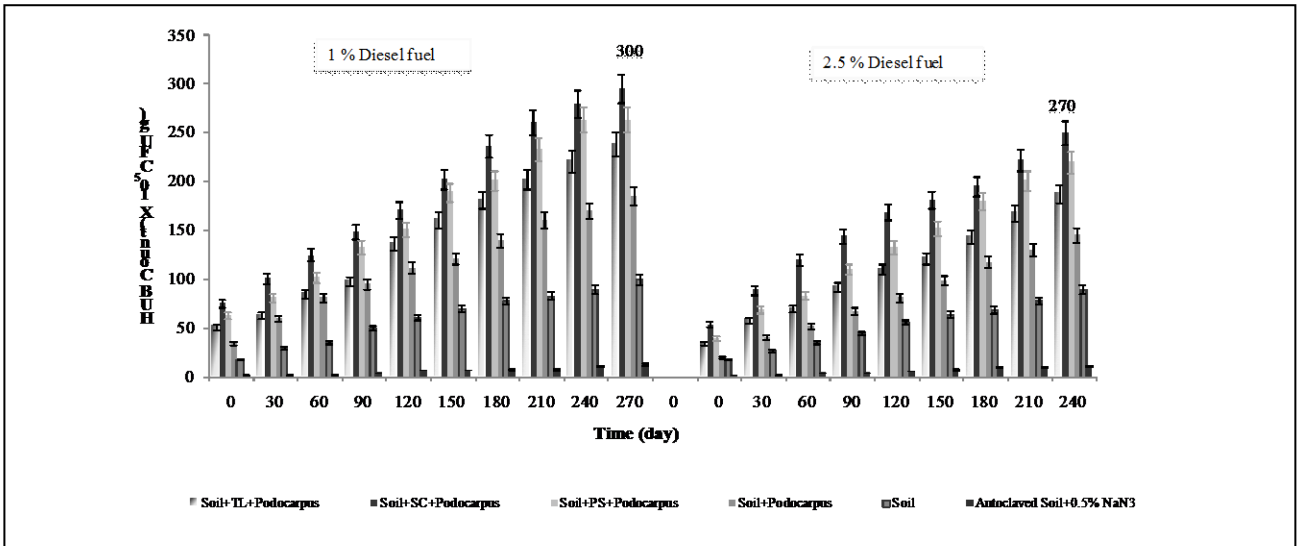
Bacterial enumeration

The counts of HUB in soil contaminated with 1% and 2.5% diesel oil contaminated soil are shown in Figures 3. Soil amended with SC and planted with *Podocarpus* shows the highest counts of HUB (300×10^5 CFU/g and 270×10^5 CFU/g) in both soil contaminated with 1% and 2.5% oil respectively. This is in line to the findings of Ijah and Antai (2003)[8], while the treatment without organic waste amendments with only *Podocarpus* plant recorded the lowest counts of HUB (180×10^5 CFU/g and 150×10^5 CFU/g) in 1% and 2.5% pollution respectively. The reason for the increase in counts of HUB in contaminated soil amended with organic wastes might be due to the quantity of nutrients in the organic wastes, especially N and P, that increased the proliferation of bacteria in the contaminated soil. The HUB isolated from the contaminated soil was identified by Biolog® Microstation system as species of *Pseudomonas sp.*, *Streptococcus sinensis* and *Bacillus amyloliquefaciens*. These bacterial species together with root exudates of *Podocarpus* plants probably help in the degradation of diesel fuel from the contaminated soil.

Kinetics of diesel removal and Half-life

Soil amended with SC had the highest degradation rate of 0.093 day^{-1} and half-life 7.38 days at 1% concentration oil in soil planted .There is significant difference in half life and degradation rate between control and treatments. The degradation rate of unamended control and autoclaved soil were 0.015 day^{-1} and 0.007 day^{-1} at 1% concentration in soil planted with Podocarpus. This result is similar to the findings of Namkoong et al., (2002) who reported higher degradation rate constant and low half-life in diesel contaminated soil and amended with 1:0.3 sewage sludge compared to those amended with 1:0.1 under the same conditions [19]. Adesodun and Mbagwu (2008) showed the highest biodegradation rate in oil contaminated soil amended with pig waste [20].

Figure3. Hydrocarbon Utilizing Bacteria (HUB) in the test soil by *P. Polystachyus*. Vertical bars indicate SE (*n*=3).



CONCLUSION

Podocarpus polystachyus shows a potential to tolerant minimum concentration of diesel fuel (2.5% and 1% w/w) in the contaminated soil. No accumulation of hydrocarbon was recorded in the plant tissues. Thus, suggesting that hydrocarbon loss mechanisms from the contaminated soil might be via rhizodegradation or phytovolatilization. Application of biowastes, especially SC, to the polluted soil is enhanced the growth of *Podocarpus* and population of microorganisms in the soil, also accounting for the additional degradation of oil by 41 % and 38% in soil contaminated with 1% and 2.5% oil respectively, compared with the treatment with *Podocarpus* alone. This study proves the ability of using *Podocarpus polystachyus* with SC amendment in remediating hydrocarbon contaminated soil.

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