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Full Length Research Paper

Crude oil degrading potential of *Pennisetum glaucum* (L.) R. Br

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Pollution by crude oil and its products is one of the most prevalent environmental problems that cause greenhouse effects and global warming. The crude oil-degrading potentials of *Pennisetum glaucum* was investigated using 0.2, 0.9, 5.0 and 6.0% v/w concentrations of crude oil, which were employed to pollute soil planted with the seeds of the plant. These treatments were repeated in soil without seeds and the control had no crude oil pollution. Total petroleum hydrocarbons (TPH) were determined for all soil samples using gas-liquid chromatography. Microbial count was carried out on soil rhizosphere using standard methods. The results show that percentage TPH degraded in soil planted with *P. glaucum* was 100, 99.53, 99.44 and 99.47 for 0.2, 0.9, 5.0 and 6.0% v/w concentrations, respectively. *P. glaucum* alone degraded 0.56, -0.29, 0.39 and 0.31% for the same treatments. The total viable count of microorganisms from the polluted, vegetated soil samples was significantly ($P < 0.05$) higher than that of the unvegetated ones. *P. glaucum* might have enhanced the biodegradation of crude oil by stimulating the proliferation of microorganisms in the soil and hence may be used for phytoremediation of crude oil polluted soils.

Key words: *Pennisetum glaucum*, crude oil, total petroleum hydrocarbons (TPH), microorganisms.

INTRODUCTION

The environmental impact of crude oil spillage has become a global problem since it produces greenhouse gases such as carbon dioxide, methane, oxides of nitrogen and sulphur, particulate matter and other substances which contribute to global warming. Carbon dioxide concentrations have increased by 40% since pre-industrial times, primarily from fossil fuel emissions and secondarily from net land use change emissions. The ocean has absorbed about 30% of the emitted anthropogenic CO_2, causing ocean acidification (IPCC, 2013).

Oil spills have caused destruction to plants, animals, arable and uncultivated lands as well as the entire ecosystem. Total petroleum hydrocarbons (TPHs) are some of the most common groups of persistent organic contaminants found in crude oil (Huang et al., 2005).

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People who live in oil producing areas are exposed to polluted food and water. Crude oil polluted soil may remain unsuitable for plant growth for years. Natural restoration of polluted land takes time and as such several methods such as bioremediation and phytoremediation have been evolved to increase the rate of hydrocarbon degradation in polluted sites.

Bioremediation is the use of microorganisms to degrade or transform toxic contaminants into non toxic substances, while phytoremediation is the use of higher terrestrial plants for the same degradation or transformation. These methods are economically viable, environmentally friendly, non-invasive and deliver intact, biologically active soil (Wenzel, 2009).

The phytodegradation of organic compounds can take place inside the plant or within the rhizosphere of the plant. Many different compounds can be removed from the environ-ment by this method, including solvents in ground water, petroleum and aromatic compounds in soils and volatile compounds in the air (Newman and Reynolds, 2004). Removal of petroleum hydrocarbons from soil in phytoremediation is often attributed to the microorganisms living in the rhizosphere under the influence of plant roots (Luepromchai et al*.,* 2007). The stimulation of microbial activity brought about by the interaction between microorganisms and root exudates is known as rhizosphere effect. Root exudates mediate interaction between plants and microbes. Plants with extensive rooting system explore large volumes of soil, support larger bacterial population in the rhizosphere and produce exudates which can directly affect the activity of the rhizobacterial population.

Millet (*P. glaucum* (L.) R. Br. (Clayton and Renvoize, 1982) belongs to the Poaceae family and is native to tropical and warm temperate regions of the world. It is an annual grass with an extensive fibrous root system. Among the four grasses selected to rehabilitate the degraded ecosystem of an oil shale mined land of Maoming Petro - chemical company, China, *P. glaucum* × *P. purpureum* had the lowest survival rate of 62%, while *Vetiveria zizanoides* had the highest survival rate of up to 99% (Xia, 2004).

Wuana et al. (2013) reported that in a cadmium/lead contaminated soil, growth rates of *P. glaucum* were sigmoid, with growth rates appearing to decelerate with dose of cadmium and lead. They also added that soil to millet transfer factors showed that cadmium was more phytoavailable to millet than lead. The fibrous root structure of grasses is known to possess an extensive widely branched root system that provides a larger surface area for colonization by microorganisms than the tap root system (Diab, 2008).

Microorganisms have been reported to play major roles in bioremediation of crude oil contaminated soils (Rahman et al*.*, 2002; Isikhuemhen et al., 2003; Chikere et al., 2009; Fariba et al., 2010; Nwadinigwe and Onyeidu, 2012). Plant roots secret compounds that modulate underground microbial diversity (Baderi and Vivanco, 2009). The continued presence of plant roots and their exudates may be required for the degradation of hydrocarbons in crude oil polluted soil. Phytoremediation is important to oil producing nations where oil spillage is rampant and devastates the environment. Not much work has been carried out on hydrocarbon degrading potentials of *P. glaucum*. The objectives of this study therefore were, to investigate the role of *P. glaucum* in the degradation of crude oil in polluted soil, to determine the quantity of total petroleum hydrocarbon (TPH) degraded and to determine the microbial count of microorganisms in the soil rhizosphere of *P. glaucum* polluted with crude oil. The knowledge gained from this work may help affected nations and environmentalists in combating the menace of crude oil pollution, reduction of greenhouse emissions and in restoring the fertility of crude oil polluted land.

MATERIALS AND METHODS

Perforated black polythene bags (volume, 39.745 L) were filled, each with 16 kg of top soil collected at a depth of 10 cm, from the Botanic Garden, University of Nigeria, Nsukka. The set up was divided into parts A and B. Part A had no seed while part B had a seed planted in each bag. To simulate spillage, eight soil bags were polluted with 30 ml (0.2% v/w) of crude oil, 42 days after planting. The same was repeated with 150 ml (0.9% v/w), 750 ml (5.0% v/w) and 1000 ml (6.0% v/w) of crude oil separately, instead of 30 ml. Both parts A and B were polluted in the same manner. The control had no crude oil. The crude oil was obtained from Shell Petroleum Development Company, Oporoma, Bayelsa State, Nigeria. The experiment was completely randomized and carried out in three replicates. The bags were kept under the sun and were watered by rain fall since the experiment was carried out during the rainy season.

Determination of total petroleum hydrocarbons (TPH)

The unused crude oil was analyzed by gas-liquid chromatography (GLC) to determine the total petroleum hydrocarbon (TPH) composition. All the soil samples, vegetated and unvegetated, were collected 60 days after pollution (DAP) and also subjected to GLC to determine the TPH. The method used was the modified method of Shirdam et al. (2009). The soil samples were air dried at 25°C (room temperature) for 72 h. Two grams of the sample were weighed; 20 ml of hexane were added to weighed sample, stirred and left for 30 min. Approximately 1 cm of glass wool was passed into the column. Two grams of activated silica gel were heated in the oven at 130°C for 9 h and passed into the column to settle on the glass wool. Activated sodium sulphate (0.5 g) was added, 10 ml of dichloromethane (DCM) were poured into the column and the tap was opened to allow the DCM to run through. The sample was poured and immediately 10 ml of hexane were poured and allowed to run. The eluate was collected in a clean sampling bottle and labeled. In order to run in an Agilent GLC, the eluate was concentrated to 1 ml, poured into a GLC vial bottle and placed into the GLC to run. The GLC was equipped with a flame ionization detector (FID). For the unused crude oil, 2 ml were poured into a

Table 1. Total petroleum hydrocarbon distribution (ppm) in soil, unvegetated and vegetated with *Pennisetum glaucum,* polluted with different concentrations of crude oil.

Straight chain group	Conc. of hydrocarbon in unused crude oil	Polluted, vegetated soil samples (% v/w)				Polluted, unvegetated soil samples (% v/w)			
		0.2	0.9	5.0	6.0	0.2	0.9	5.0	6.0
C_8	341.79	-	-	-	-	-	-	-	-
C_9	1070.02	-	-	-	-	-	-	-	-
C_{10}	1392.12	-	-	-	-	-	-	-	-
C_{11}	1949.83	-	-	-	-	-	-	-	-
C_{12}	2124.50	-	-	-	-	-	12.03	-	-
C_{13}	1991.34	-	-	13.15	-	-	-	-	-
C_{14}	2247.70	-	-	25.68	-	-	36.70	-	-
C_{15}	2542.92	-	-	33.70	-	-	19.08	14.03	13.36
C_{16}	2607.72	-	-	32.92	-	-	21.44	49.82	15.48
C_{17}	2832.61	-	-	31.51	27.40	-	17.48	158.70	40.77
C_{18}	3804.47	-	-	27.49	19.84	-	-	92.13	52.71
C_{19}	4245.75	-	-	32.86	26.80	-	-	60.50	99.99
C_{20}	3549.56	-	-	35.54	-	-	-	23.75	80.21
C_{21}	5985.27	-	-	-	-	-	-	-	-
C_{22}	5549.01	-	125.76	-	30.82	31.72	-	33.33	94.36
C_{23}	4221.22	-	61.58	-	28.29	25.68	-	30.67	28.07
C_{24}	4833.37	-	30.42	48.61	45.74	107.88	-	25.90	77.43
C_{25}	5523.29	-	61.70	63.11	123.27	172.44	-	78.56	-
C_{26}	1675.04	-	-	-	16.30	-	-	-	-
C_{27}	1064.24	-	-	-	-	-	-	-	-
C_{28}	228.85	-	-	-	-	-	-	-	-
C_{29}	58.68	-	-	-	-	-	-	-	-
C_{30}	40.15	-	-	-	-	-	-	-	-
C_{31}	110.76	-	-	-	-	-	-	-	-
C_{32}	93.35	-	-	-	-	-	-	-	-
Total TPH	60083.56	0.00	279.46	344.57	318.46	337.72	106.73	567.38	502.38

-, Means absence of hydrocarbons

separating funnel. Twenty-five milliliter of hexane were added to the sample for the extraction and the eluate was collected in a sampling bottle. The oil was poured back to the funnel and 25 ml hexane was added. The process was repeated and the eluate passed through 50 g of Na_2S0_4 to remove water and concentrated to 1 ml. One micro liter of the concentrate was injected into the GLC and the retention time was compared with those of the standard total petroleum hydrocarbon concentrations. The injector temperature was 280°C while that of FID detector was 340°C. The column used for analysis was DB-5 with 30 m length and 0.25 mm internal diameter. The initial column temperature was kept at 50°C for 5 min, increased to 250°C with 10°C min^{-1} slope and kept at 250°C for 40 min.

Determination of percentage TPH degraded

The total TPH obtained under each column (Table 1) is the sum of the remaining hydrocarbons after degradation, under the column. The total TPH under the unused crude oil is the standard and is regarded as 100%. The TPH degraded for each treatment is obtained by subtracting the

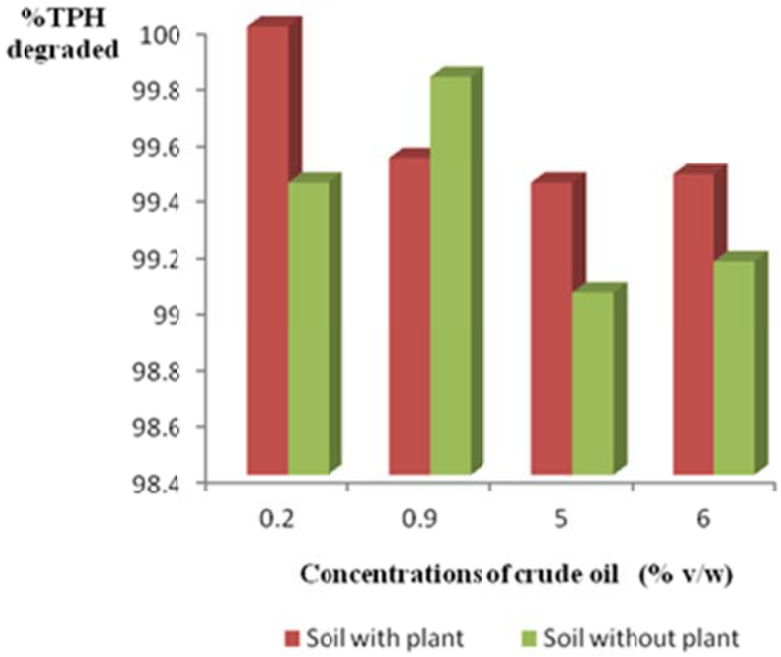

Figure 1. Percentage Total Petroleum Hydrocarbon degraded in the soil with and without *Pennisetum glaucum,* polluted with different concentrations of crude oil (% v/w).

remaining TPH under the treatment, from the standard. Percentage TPH degraded under each column is obtained by dividing the total TPH degraded under each treatment by the total TPH in the standard and multiplying by 100. Since vegetated soil contained both *P. glaucum* and microorganisms while unvegetated soil contained only microorganisms, it means that any degradation in vegetated soil was carried out by both the plant and microorganisms and any degradation in unvegetated soil is carried out by microorganisms. Therefore, the percentage TPH degraded by the plant alone is obtained by subtracting the percentage TPH degraded in unvegetated soil from the percentage TPH degraded in vegetated soil (This subtraction resulted in -0.29% found in the result, for 0.9% crude oil treatment).

Microbial count of rhizosphere

The total viable count (TVC) of microorganisms in the rhizosphere was carried out 60 days after pollution, in the Department of Pharmaceutical Microbiology, University of Nigeria, Nsukka, according to Henrik (1994). Sterilization was carried out by autoclaving at 121°C for 15 min. Each soil sample was serially diluted from 10^{-1} to 10^{-5}. For total viable count, 28 g of nutrient agar medium, which consisted of meat extract (1.0 g/L), yeast extract (2.0 g/L), peptone (5.0 g/L), sodium chloride (5.0 g/L) and agar (15.0 g/L), was dissolved in 1 L of distilled water. The inoculation was carried out by the pour plate method. One ml of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} of the sample was pipetted into sterile Petri-dishes, separately. After allowing the autoclaved media to cool to 45°C, 10 ml of molten agar medium was added to the plate containing the inoculums and was homogenized to ensure complete dispersal of the sample. The plates were allowed to set before incubating them in an oven at 37°C for 24 h. The results of the TVC were subjected to Analysis of Variance (ANOVA) and means were compared using Duncan's multiple range tests at $P < 0.05$ (Edafiogho, 2006).

RESULTS

Total petroleum hydrocarbons

The original unused crude oil sample which is the standard, showed the presence of high concentrations of straight chain hydrocarbons of C^8 - C^{32} (Table 1). Some other hydrocarbons like C^1 - C^7 and C^{33} - C^{40} were neither detected in the standard nor in any of the polluted soil samples, by the GLC. No straight chain groups, C^8 - C^{11}, C^{21} and C^{27} - C^{32} were detected in all the polluted soil samples (vegetated and unvegetated), even though they were found in the standard. No hydrocarbon was detected in the control and in the vegetated soil polluted with 0.2% v/w crude oil. Small quantities of hydrocarbons were detected in the vegetated and unvegetated soil when compared with the quantities detected in the standard. Percentage TPH degraded in vegetated soil, polluted with 0.2, 0.9, 5.0 and 6.0% v/w crude oil treatments were 100% (that is, all the hydrocarbons were degraded), 99.53, 99.44 and 99.47%, respectively, while the percentage TPH degraded in unvegetated soil polluted with the same quantities of crude oil were 99.44, 99.82, 99.05 and 99.16%, respectively (Figure 1). Therefore, *P. glaucum* alone degraded 0.56, -0.29, 0.39, and 0.31% for the same treatments, respectively (Percentage TPH degraded by the plant alone is obtained by subtracting the percentage TPH degraded in unvegetated soil from the % TPH degraded in vegetated soil).

Table 2. Microbial count (cfu /g) of soil polluted with different concentrations (%v/w) of crude oil, with or without *Pennisetum glaucum.*

Concentration of crude oil (% v/w)	Total viable count (cfu/g)
Vegetated, polluted soil samples	
Control (vegetated soil, without pollution)	$6.97 \times 10^6 \pm 11547^a$
0.2	$9.13 \times 10^7 \pm 88191^b$
0.9	$1.87 \times 10^7 \pm 81297^c$
5.0	$6.10 \times 10^7 \pm 57735^d$
6.0	$1.26 \times 10^7 \pm 81936^e$
Unvegetated, polluted soil samples	
Control (unvegetated soil, without pollution)	$5.60 \times 10^6 \pm 31797^f$
0.2	$8.82 \times 10^6 \pm 42702^g$
0.9	$3.71 \times 10^6 \pm 63333^h$
5.0	$1.44 \times 10^6 \pm 29059^i$
6.0	$8.54 \times 10^6 \pm 81742^g$

Values represent means ± standard error. Mean values with different letters in the column are significantly different at $p<0.05$.

Microbial count

The results of the total viable count (TVC) of microorganisms showed that the vegetated, polluted soil samples recorded a significantly ($P < 0.05$) higher TVC when compared with unvegetated, polluted soil samples (Table 2). For vegetated, polluted soil, the highest (significant at $P < 0.05$) TVC was recorded for 0.2% v/w crude oil treatment, followed by that of 5.0% v/w treatment, while the lowest was that of the control. For the unvegetated, polluted soil, the highest TVC was recorded for 0.2 and 6.0% v/w, followed by that of the control, while the lowest was that of 5.0% v/w treatment (significant at $P < 0.05$).

DISCUSSION

Some straight chain groups of hydrocarbons like C^1 - C^7 were volatile and so could not be detected by GLC both for the unused crude oil sample and the polluted soils. No hydrocarbons were detected in the vegetated soil polluted with 0.2% v/w crude oil, probably because all the hydrocarbons were phytodegraded by a combination of *P. glaucum* and microorganisms. The 0.2% crude oil spill was perhaps too small for the numerous microorganisms that had enough exudates from the plant. Hence, all the hydrocarbons (100%) were completely degraded. Since many hydrocarbons were not detected (examples, C^8 - C^{11}, C^{21} and C^{27} - C^{32}) or detected in smaller quantities in all the polluted soil samples, when compared with the unused crude oil sample, it showed that phytodegradation took place in the work. Hence, in the present experiment, the percentage TPH degraded in all the polluted soil samples, with or without *P. glaucum* ranged from 99.05 to 100%. Generally, more degradation of hydrocarbons took place in the presence of *P. glaucum* than in its absence, except for 5.0% polluted, unvegetated soil, where there was more phytodegradation for C^{15}, C^{20}, C^{24} as well as for C^{22}, C^{23}, C^{24}, C^{25} (for 0.9% v/w pollution) and for C^{26} (for 6.0% pollution). The reason for this unexpected behavior is not clear, although it may have to do with the concentration of the crude oil spilled and the type and quantity of microorganisms involved in the phytodegradation. In any case, degradation in the absence of the plant was high (99.05 to 99.82%). Therefore, microorganisms in the soil must have been responsible for this degradation of crude oil in the absence of the plant, to the extent that in unvegetated soil polluted with 0.9% v/w crude oil, there was so much degradation by the microorganisms that it appeared that the plant played no significant role in the phytodegradation, hence, it gave - 0.29% for the phytodegradation by the plant alone. Perhaps 0.9% oil spill is the right quantity which the microorganisms can degrade efficiently without the supply of exudates from the plant.

These findings are similar to the work of Diab (2008) who reported that 30% reduction of total petroleum hydrocarbons (TPH) was observed in the soil rhizosphere of *Vicia faba,* as compared to 16.8 and 13.7% reduction in *Zea mays* and *Triticum aestivum*, respectively. Dominiguez-Rosado and Pichtel (2004) found that the used motor oil (1.5% w/w) they employed to contaminate soil seeded with mixed clover was completely degraded after 150 days. They further reported that 67% of the oil

was removed with a mixture of sunflower/mustard, but with the addition of NPK fertilizers, the oil was completely degraded. In addition, the grass/maize treatment resulted in a 38% oil degradation, which increased to 67%, with fertilizer application. In the present work, the percentage hydrocarbons degraded by the plant alone was quite small compared with the percentage hydrocarbons degraded by a combination of the plant and its associated microorganisms. Therefore, a combination of microbial degradation (bioremediation) and phytodegradation may perhaps make phytoremediation more efficient. This agrees with the report of Wenzel (2009) who confirmed that the efficiency of phytoremediation relies on the establishment of vital plants with sufficient shoot and root biomass growth, active root proliferation and / or root activities that can support a flourishing microbial consortium assisting phytoremediation in the rhizosphere.

In the present work, the TVC of microorganisms of the vegetated, polluted soil was higher than that of the unvegetated polluted soil. The observed increase in microbial activity in vegetated soils may be attributable to root exudates and oxygen input from roots of the plant as it was observed by Escalante-Espinosa et al. (2005). This is in agreement with the work of Odokuma and Inor (2002), who reported that bioaugumentation using bacteria (*Bacillus* and *Azotobacter*) improved the growth of *Phaseolus* sp. in crude oil-polluted soil. Chikere et al. (2009) also reported that bacteria contributed during bioremediation of crude oil - polluted soils. In the present work, 0.2% polluted, vegetated soil gave the highest (100%) TPH degradation and had the highest microbial load, while the 5% polluted, unvegetated soil gave the lowest (99.05%) TPH degradation and had the lowest microbial load. Therefore, it may be assumed that the higher the microbial load, the higher the hydrocarbon degradation. Comparatively less degradation took place for 5 and 6% oil spills, perhaps because the microorganisms had to tackle with higher quantities of crude oil spills, in the absence of the plant and its exudates, in the case of unvegetated soil.

Johnson et al. (2005) and Mueller and Shann (2006) reported that microbial communities in planted soils are greater and more active, than in unplanted soils. Fariba et al. (2010) indicated that fungal strains played the main role in the degradation of petroleum polluted soils but the roots of plants enhanced the process. Plants can enhance the biodegradation of hydrocarbons by stimulating the rhizosphere microbes into greater activity (Nie et al., 2009) through the supply of oxygen (Escalante-Espinosa et al., 2005), root exudates, enzymes that are capable of transforming organic pollutants and by altering the biotic, physical and chemical conditions of the soil (Nie et al., 2009). Hence, in the present work, both the plant and microorganisms are involved directly and indirectly in the degradation of petroleum hydrocarbons into less toxic products that are less persistent in the environment than the parent compounds. Therefore, in phytoremediation, the emission of CO_2, methane, oxides of nitrogen and sulphur, aerosols, as well as particulate matter, etc., which are released into the environment in an oil spill, are mitigated, thereby helping to reduce greenhouse effect and global warming. The roots of plants loosen the soil and transport nutrients and water to the rhizosphere, thus additionally enhancing the microbial activity. In conclusion, therefore, *P. glaucum* contributed to the phytodegradation of the crude oil polluted soil. Although the actual percentage degradation of hydrocarbons contributed by the plants alone, was quite small compared with the contributions made by a combination of the plant and soil microorganisms, yet the plant phytostimulated the activities of microorganisms in their bioremediative work by means of the rhizosphere activities.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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