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Rhizostimulation of Hydrocarbon Degrading Microorganisms Using *Chrysopogon zizanioides* and *Eucalyptus camaldulensis* Species During Bioattenuation of Hydrocarbon Polluted Soils

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Abstract:
Background: *This study was carried out to compare the rhizostimulation potentials of C. zizanioides and E. camaldulensis on microorganisms cfu during the phytoremediation of hydrocarbon contaminated potted soils. The objectives were to: i). determine the best treatment for the rhizostimulation of the microbial population in soils during the bioattenuation process. ii). examine the most efficient plant species in the Phytostimulation of hydrocarbon degrading microorganisms during the bioattenuation of hydrocarbon contaminated soils. iii). identify the various bacterial and fungal colonies associated with the bioattenuation of hydrocarbon.*
Materials and Method*: The design was the Split-Split Plot experiment (4 x 4 x 2). The main plot factors were the crude oil contamination (4 levels), the sub-Plot factors were the soil amendments (4 levels) while the sub-sub-Plot factors were the plant species (2 levels).*
Results: *Results indicated that there were significant differences ($p < 0.05$) in rhizostimulation potentials of the plant species in different crude oil contamination levels and in the different soil amendments for both bacteria and fungi. Although E. camaldulensis species was involved in biostimulation of microbial cfu, C. zizanioides was found better efficient (16.13 x 10^6 soil bacteria cfu g g^{-1} at 9 WAT and 10.04 x 10^6 soil fungal cfu g g^{-1} at 12 WAT respectively. Microorganisms found involved in bioattenuation of hydrocarbon include Bascillus spp., Staphylococcus spp., and Streptococcus spp for bacteria and Aspergillus spp., Rhizopus spp., and Penicillium spp for fungi. The most frequently occurred genus of microorganisms were Bacillus and Rhizopus.*
Conclusion: *In conclusion, C. zizanioides was recommended as efficient rhizostimulating plant species. It was concluded that the mixture of both the grass and woody plant species are required for optimum growth and stimulation of hydrocarbon degrading microorganisms during phytoremediation procedure in the field. Additionally, bacterial and fungal cfu was observed to have increased with increasing hydrocarbon concentration under the influence of the two plant species and the applied landfarming treatments of a mixture of NPK (g g-1) and Cowdung (3:1 v/v) fertilizer. The most frequently occurred genus of microorganisms were Bacillus and Rhizopus.*
Keywords*: Rhizostimulation, Phytoremediation, Colony Forming Units (cfu), Chrysopogon zizanioides, Eucalyptus camaldulensis.*

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I. Introduction

Population growth and anthropogenic activities especially but not limited to oil exploration, deforestation, urbanization and industrialization among others has resulted in the depletion of natural resources and its subsequent environmental degradation leading to food shortages and health hazards[1,2]. Additionally, the rapid urbanization and industrialization leads to serious environmental pollution by heavy metals, metalloids, radionuclides and organic compounds during oil spills and use of Agrochemicals[3]. Mining activities, disposal of wastes effluents from industries and residential area, heavy use of fertilizers and pesticides including irrigation of contaminated water has also led to contaminated soils[4]. Consequently, elevated concentrations of heavy metals such as Lead (Pb), Nickel (Ni), Cadmium (Cd), Iron (Fe), Copper (Cu), Zinc (Zn), Chromium (Cr), Manganese (Mn) among others and organic pollutants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyl (PCBs), trinitrotoluene (TNT), phenols and pesticides have been reported by several scientists[4, 5, 6].

The presence of these pollutants, especially organic pollutants that include pesticides such as: atrazine, dieldrin, hexachlorobenzene, dichlorodiphenyltrichlorethane (DDT), and 1,2,4- trichlorobenzene that often contaminate soils seriously affect the soil biota, nutrients availability and soil productivity because of their persistence and toxicity in soil[6,7,4].

Plants and their associated rhizosphere organisms have been found to be useful in pollutant decontamination from soil and groundwater aquifer. While some plants are found to facilitate biodegradation of organic pollutants indirectly by stimulating soil microbes in the rhizosphere zone through the process of phytostimulation *or* rhizodegradation[8,9], other plants are found to degrade organic pollutants directly through enzymatic activities in a process called phytodegradation[8,10].

Generally, plants are found to decontaminate pollutants from soil and ground water aquifer using different phytotechnology such as: rhizofilters[11,12], hydraulic barrier in rhizosphere zone to serve as phytostabilizers of pollutants in soil[13], as phytoaccumulators of contaminants[9,14,15], and as phytoextraction systems[16,17]. Other phytotechnology include the phytovolatilization process[18,19]. It is vital to note that these various phytotechnology are not mutually exclusive[9,20].

Microbial degradation of organic contaminants normally occurs because of microorganisms using the contaminant for their growth and reproduction. Organic contaminants do not only provide microorganisms with a source of carbon; but it provides electrons that the organisms use to obtain energy. Basic microbial metabolism of contaminants involves aerobic respiration but variations like anaerobic respiration, co-metabolism, fermentation, reductive dehalogenation, and the use of inorganic compounds as electron donors may occur. Interestingly, bacteria are found capable of quickly distributing genetic information to each other and this enable consortium of bacteria to adapt quickly to environmental changes, such as exposure to new contaminants[21]. Evidence suggests that the degradation of certain contaminants may take place only if a specific consortium of microbes occur at the contaminated site[22].

Although scientists have attempted various Ex-Situ and In-Situ bioremediation strategies in the remedy of polluted sites[23], depending on the chemical properties of the given site, type of soil, depth of the contamination and natural processes occurring on the site[24], the choice of remediation procedure to employ depends on the short-term and long-term effectiveness at meeting remediation goals, effective reduction of the volume of contaminants, reduction in contaminants toxicity and cost effectiveness of procedure[25]. For instance, tilling (in Land farming) can aerate the soil and bring organic contaminants to the soil surface, thereby promoting oxidation and photo-oxidation respectively[26]. Nevertheless, this method tends to remove only a little of volatile organics.

To this extent, phytoremediation method which is the use of green plants and their associated rhizosphere microorganisms for in-situ treatment of contaminated soil and groundwater is preferred to other methods although non-biological remediation technologies and bioremediation are not mutually exclusive[25,27,28,29]. This is because pollutant distribution and concentrations are heterogeneous for many sites and the most efficient and cost-effective remediation solution may be a combination of different technologies, such as excavation of the most contaminated spots followed by polishing the site with the use of plants in addition to soil amendments. Such an integrated remediation effort requires a multidisciplinary team of knowledgeable scientists[30, 31].

Although many organic compounds are observed to be metabolized by microbes found in or added to bulk soil, microbial biodegradation is rarely exploited successfully under field conditions because microbial populations in contaminated sites do not achieve enough biomass for acceptable rate of remediation[32]. To this extent, it is important to identify the most efficient phyto-stimulator plants that could be possibly involved in phytodegradation of organic pollutants for more efficient and effective phytoremediation of hydrocarbons in soils. The selected plant species were initially identified as phytoaccumulator of organic contaminants in a study by[1]. It is believed that the outcome will impact positively on the environment and improve the socioeconomic conditions of the Lake Chad Basin pruned hydrocarbon contamination, the Niger delta and perhaps wherever oil exploration is contemplated in Nigeria.

To this end, the objectives were to: i). determine the best treatment for the rhizostimulation of the microbial population in soils during the bioattenuation process. ii). examine the most efficient plant species in the Phytostimulation of hydrocarbon degrading microorganisms during the bioattenuation of hydrocarbon contaminated soils. iii). identify the various bacterial and fungal colonies associated with the bioattenuation of hydrocarbon.

This study is limited to contaminated soils of the Lake Chad Basin that is targeted for oil exploration and similar to soils of the Niger Delta where oil exploration and spillage is mostly reported, using mixtures of in-situ bioremediation methods to achieve decontamination of Total Petroleum Hydrocarbon (TPH) but carried out in Dutse, Jigawa State, Nigeria. The phytoremediation procedure, in addition to some soil amendments such as tillage, watering and fertilizer (organic and inorganic fertilizers such as: Cow-dung and NPK) application, was considered to enhance remediation of the soil in question.

II. Material And Methods

The Study Area

The study was carried out at a site near the Federal University Dutse, Jigawa State, Nigeria. Dutse is the state capital. Dutse lies between Latitude 11° N to 13° N and Longitude 8°E to 10° 15' E. Its semi-arid climate is characterized by long erratic dry season (October - May) and a short-wet season (June - September) heralded by violent dust storms followed by tornado and lightening. Its total annual rainfall ranges from 600 mm to 1000 mm. The mean annual temperature is about 25°C. Evapotranspiration is very high and relative humidity is highest in August (up to 80 per cent) and low in January through March (20 - 30). Most of the state falls within the Sudan Savannah vegetation belt, but traces of Guinea savannah vegetation are found in parts of the southern districts.

The ancient Pre Cambrian rocks of the basement complex comprising granites, schists and gneisses are separated from the younger sediment of the Chad Formation by a hydrological divide, which runs through Kiyawa, Dutse and Yankwashi. The Chad formation occupies the north-eastern parts of the state. However, the basement complex rocks have undergone weathering to give rise to fairly deep soils which are often covered by a sheet of laterite which has been exposed by denudation in some places. The Chad sediments are concealed by sand dunes and its sandy beds, formed over the impervious clays of the Chad formation, form the main source of water supply in the dry season. The soils are generally sandy at the top and compact at depth with often hardpans. Aeolian deposits from the Sahara Desert form substantial part of soils in the state especially towards the northern parts. The mixing of the subsoil in these deposits has given rise to clayey subsoil, which dominates the northern parts of the state[33].

Materials

Plant material

Seedlings of *Chrysopogon zizanioides* (L) Roberty and *Eucalyptus camaldulensis* (Dehnn) were obtained from a farm in Kiyawa town about 70 km from Dutse, Jigawa state capital. The plants were selected for their possible hydrocarbon remedial capabilities.

Crude oil

Bonny light crude oil was obtained from the Kaduna Refining and Petroleum Limited (A subsidiary company of the NNPC).

Experimental Technique

Seedlings of *E. camaldulensis* and *C. zizanioides* were cultivated in an uncontaminated soil at the nursery for two (2) months (November - December) to enable the seedlings to acclimatize before they were transplanted into crude oil contaminated potted soil. The experimental plots were plastic basins of known capacity (5 L). The pot soils were contaminated using three crude oil contamination levels: C1 (Control), C2 (0.3 L/4.0 kg soil), C2 (0.5 L/4.0 kg soil) and C3 (0.7 L/4 kg soil) as modified from the study of a scientists[41]. Seedlings of the plant species were then transplanted early morning into the contaminated plastic medium and left for three months (January - March). The basic experiment described was then replicated three times.

Experimental Design and Treatments

The design was the Split - Split Plot (4 x 4 x 2). This design was selected to ensure more precision to the selected plant species. The main plots were the Crude oil contamination (4 levels), Sub plot were the soil amendments (4 levels) while the sub-sub plot factor were the plant species (2 levels). The following were the soil amendments that served as treatments: T1=Control (tilled and watered daily), T2 =NPK (g kg^{-1}soil), T3= Cow-dung (3:1 v/v), T4= NPK (g kg^{-1}soil) + Cow-dung (3:1 v/v). Note that all experimental units were watered and tilled daily to ensure aeration.

Samples and Sampling Technique

Soil samples

Soil was sampled randomly at depth of 0 - 10 cm (the surface and at middle) using soil auger from each sampling unit. The sampled soil was then homogenized and composite soil sample was obtained from the experimental pots for each species every 21 days for a period of three months. All sampled soil for microbiological analysis were collected as quickly as possible to prevent exposure to the environment and subsequent error in measurements.

Microbiological Samples and Analysis

The dilution plate method[34] was used for counting colony forming units (CFU) for both bacteria and fungi using the colony counter. Nutrient agar (Oxoid) medium supplemented with 0.4 % (w/w) soluble starch was used for counting bacteria. On the other hand, potato dextrose agar was used for counting fungi colonies.

The colonies that appeared on the various plates was then counted and expressed as CFU/g soil. Plates for counting bacterial forming units was incubated for 24 hours at 37 ^{0}C while that of fungi was incubated at 25 ^{0}C for a period of 3-5 days. The genus of bacterial isolates was kept on nutrient agar at 4 ^{0}C and re-cultured every four weeks. Gram stain test was performed for each isolate. The bacterial isolates were identified on the basis of classification schemes published by Bergey's Manual of Systematic Bacteriology[35].

Data Analysis

Data collected were analysed using Analysis of Variance (ANOVA), the split-split plot model, using GenStat Discovery Edition 4 software but due to limitation of ranking the Generalized Linear Model (GLM) procedure of SAS (Statistical Analysis System, 1999) was also used. The probability level of certainty in the research was at 95 % confidence limit or $\alpha = 0.05$ although, $\alpha = 0.01$ was also used. Statistical means were compared using the Fisher's Least Significant Difference (LSD) at $p \leq 0.05$ and $p \leq 0.01$.

III. Result

Rhizostimulation of Microorganisms in during Bioattenuation of Total Petroleum Hydrocarbon (TPH)

The Bacterial and Fungal colony forming units (cfu) obtained during the course of this study indicated the ability of the studied plant species to stimulate hydrocarbon degrading microorganisms in their rhizosphere.

Bacteria colony forming units (cfu)

The results of mean squares extracted from the analysis of variance (ANOVA) for the number of bacteria cfu were presented in Table 1. The analysis indicated that there were highly significant differences ($p < 0.05$ and $p < 0.01$) in the number of bacterial cfu among the levels of crude oil contamination, the different soil amendments (treatments) and among the two tested plant species throughout the various sampled periods shown in weeks after transplant (WAT). In addition, the analysis further indicated that there were highly significant interactions ($p < 0.05$ and $p < 0.01$) for the number of bacteria cfu between the levels of crude oil contamination, soil amendments and the two plant species throughout the sampled periods.

Results of Table 2 indicated that at 3WAT, the highest number of bacterial cfu was obtained in the soil without crude oil contamination (C1 - Control) with 1499 X 10^6 cfu g^{-1} soil. The least bacteria cfu was observed in the highest crude oil contamination level (C4 – 07L) with 6.40 X 10^6 cfu g g^{-1} soil. The result further indicated that the bacteria cfu of C2 (0.3 L) and C3 (0.5L) that did not differ significantly yielded the second highest bacteria cfu. The general trend observed was that the number of bacteria cfu in crude oil contamination levels C2, C3 and C4 increases continuously at 3 WAT and 9 WAT until the number of bacteria cfu became almost equal to that of the control at the end of the experiment at 12 WAT. The result also showed that at 12 WAT, the bacteria cfu observed in C1 (control) and C3 (0.5L) that does not differ significantly contains the highest bacteria cfu with 17.80 x 10^6 cfu g g^{-1} soil and 17.44 x 10^6 cfu g g^{-1} soil respectively. This was closely followed by the bacteria cfu observed in C2, C3 and C4 that does not differ significantly with 17.11 x 10^6 cfu g g^{-1} soil, 17.44 x 10^6 cfu g g^{-1} soil and 17.10 x 10^6 cfu g g^{-1} soil respectively.

The soil amendment observed to yield the highest bacteria cfu was T4 (NPK g^{-1} Kg^{-1} + Cowdung 3:1 v/v) at 3 WAT, 6 WAT and 12 WAT with 13.62 x 10^6 cfu g^{-1} soil, 18.15 x 10^6 cfu g g^{-1} soil and 17.72 x 10^6 cfu g g^{-1} soil respectively. The trend observed indicated that while the bacteria cfu increased at 6 WAT, there was a general decrease in the number of bacteria cfu at 9 WAT for all treatments. However, at the end of the experiment, (12 WAT), there was increase in the number of bacteria cfu for T2, T3 and T4 that did not differ significantly and also produce the highest number of bacteria cfu with 17.85 x 10^6 cfu g g^{-1} soil, 18.03 x 10^6 cfu g g^{-1} soil and 17.72 x 10^6 cfu g g^{-1} soil respectively. The treatment that yielded the least bacteria cfu was T1 (control) with 15.86 x 10^6 cfu g g^{-1} soil.

Table 1: Mean Squares from the Analysis of Variance for number of Bacteria Colony Forming Units (cfu) in g g^{-1}soil at different sampling period

		Bacterial cfu (000,000) g g^{-1} Soil			
Source of variation	**df**	**3 WAT**	**6 WAT**	**9 WAT**	**12 WAT**
Crude Oil Conc.					
REP	2	5.758	0.2814	0.5095	3.1376
Crude Oil Conc. (A)	3	303.916**	21.0583**	4.8506**	2.6320**
Error	6	2.580	0.2539	0.2425	0.2704
Soil Amendments					
Treatment (B)	3	295.906**	4.8897**	1.1708**	24.4073**
A x B	9	66.304**	11.7086**	1.9732**	33.8103**
Error	24	1.393	0.2980	0.2257	0.3137
Plant Species					
Plant Species (C)	1	20.535**	98.4150**	140.6504**	21.9459**
A x C	3	9.857**	17.9350**	45.3249**	151.2970**

B x C	3	106.891**	24.1431**	1.7790**	32.4029**
A x B x C	9	23.423**	13.0208**	8.6268**	32.5762**
Error	32	1.624	0.1326	0.1943	0.7098
Total	95				

** = Highly Significant at $p < 0.01$; WAT = Weeks after Transplant in contaminated soil

Table 2: Bacterial cfu (000,000) g g^{-1} Soil in the Rhizosphere of *C. zizanioides* and *E. camaldulensis*

	Bacterial cfu g g^{-1} Soil			
Treatments	**3 WAT**	**6 WAT**	**9 WAT**	**12 WAT**
Crude Oil Contamination				
C1 (0 L)	14.99^{a}	17.00^{b}	14.44^{b}	17.80^{a}
C2 (0.3 L)	9.96^{b}	18.53^{a}	14.64^{b}	17.11^{b}
C3 (0.5 L)	9.49^{b}	17.03^{b}	15.22^{a}	17.44^{ab}
C4 (0.7 L)	6.40^{c}	18.73^{a}	15.37^{a}	17.10^{b}
Mean	10.21	17.82	14.92	17.36
p of f	0.001	0.001	0.002	0.010
s.e.d	0.464	0.1454	0.1422	0.1501
Soil Amendments				
T1	8.22^{c}	18.22^{a}	15.24^{a}	15.86^{b}
T2	6.30^{d}	17.66^{b}	14.77^{b}	17.85^{a}
T3	12.69^{b}	17.25^{c}	14.89^{b}	18.03^{a}
T4	13.62^{a}	18.15^{a}	14.78^{b}	17.72^{a}
Mean	10.21	17.82	14.92	17.36
p of f	0.001	0.001	0.007	0.001
s.e.d	0.341	0.1576	0.1371	0.1617
Plant Species				
C. zizanioides	10.67^{a}	16.81^{b}	16.13^{a}	16.89^{b}
E. camaldulensis	9.75^{b}	18.83^{a}	13.71^{b}	17.84^{a}
Mean	10.21	17.82	14.92	17.36
p of f	0.001	0.001	0.001	0.001
s.e.d	0.260	0.0743	0.0900	0.1720

T1 = Control; T2 = NPK (g kg^{-1}); T3 = Cow-dung (3:1 v/v); T4 = NPK (g kg^{-1}) + Cow-dung (3:1 v/v); WAT = Weeks after transplant in contaminated soil. Figures with same alphabets within columns do not differ significantly for Crude contamination, Soil amendments and Plant speccies respectively p of f = Probability value of F

In terms of the tested plant species, while *C. zizanioides* species proved to be the best bacterial cfu stimulating plant at 3 WAT and 9 WAT with 10.67 x 10^{6} cfu g g^{-1} soil and 16.13 x 10^{6} cfu g g^{-1} soil respectively, that of *E. camaldulensis* species yielded the highest bacterial colony at 6 WAT and at the end of the experiment at 12 WAT with 18.83 x 10^{6} cfu g g^{-1} soil and 17.84 x 10^{6} cfu g g^{-1} soil respectively. Additionally, the observed trend showed a continuous increase in the bacteria cfu in the sampling periods for all the tested plant species.

Fungi colony forming units (cfu)

The Mean squares extracted from ANOVA table for Fungi cfu was presented in Table 3. The analysis revealed similar trend with that of bacteria in that there were highly significant differences ($p < 0.05$ and $p < 0.01$) in the number of fungal cfu among the levels of crude oil contamination, different soil amendments and among the two tested plant species throughout the various sampled periods. In addition, there were highly significant interactions ($p < 0.05$ and $p < 0.01$) for the number of fungi cfu between the levels of crude oil contamination, soil amendments and the two plant species throughout the sampled periods.

Results of the fungal cfu for the four sampled period was presented in Table 4. The result had shown that while C4 (0.7L) level of crude oil contamination yielded the highest number of fungal cfu at 3 WAT, 6 WAT and 9 WAT with 9.83 x 10^{6} cfu g g^{-1} soil, 11.03 x 10^{6} cfu g g^{-1} soil and 11.68 x 10^{6} cfu g g^{-1} soil respectively, that of the control (C1 – no contamination) yielded the highest at the end of the experiment at 12 WAT with 11.96 x 10^{6} cfu g g^{-1} soil. The least fungal cfu count was observed in C3 (0.5L) with 7.85 x 10^{6} cfu g g^{-1} soil; th at of C4 (0.7L) was observed to be the second least in fungal cfu at the 12 WAT with 8.60 x 10^{6} cfu g g^{-1} soil. The general trend observed showed a significant reduction of fungal cfu for C1, C2, and C3 crude oil contamination levels at 6 WAT and 9 WAT. The fungal cfu was later observed to continue to increase significantly. However, reverse was the trend for C4 that showed reductrion in the fungal cfu at the end of the experiment (12 WAT).

In terms of the soil amendments during the bioattenuation procedure, the best observed treatment that stimulates fungal growth at the end of the experiment (12 WAT) was T3 and T4 that did not differ significantly with 10.45 x 10^{6} cfu g g^{-1} soil and 10.23 x 10^{6} cfu g g^{-1} soil respectively.

C. zizanioides was observed to be the best species for the rhizostimulation of hydrocarbon degrading fungal cfu in all the four sampled periods of 3WAT, 6 WAT, 9 WAT and 12 WAT with 11.03 x 10^6 cfu g g^{-1} soil, 9.81 x 10^6 cfu g g^{-1} soil, 8.90 x 10^6 cfu g^{-1} soil and 10.04 x 10^6 cfu g g^{-1} soil respectively.

Table 3: Mean Squares from Analysis of Variance for number of Fungal Colony Forming Units (cfu) in g g^{-1}soil at different sampling period

		Fungal cfu g g^{-1} Soil			
Source of Variation	**df**	**3 WAT**	**6 WAT**	**9 WAT**	**2 WAT**
Crude Oil Conc.					
REP	2	0.5691	0.05542	0.23656	0.4754
Crude Oil Conc. (A)	3	37.0059**	115.61750**	141.98028**	76.9707**
Error	6	0.1245	0.10250	0.03351	0.1907
Soil Amendments					
Treatment (B)	3	9.4195**	56.00361**	80.78528**	28.2026**
A x B	9	17.1614**	26.59407**	44.05704**	10.1866**
Error	24	0.1027	0.06115	0.07108	0.1702
Plant Species					
Plant Species (C)	1	367.7751**	169.60167**	47.32042**	39.5267**
A x C	3	3.9812**	61.61750**	21.14014**	10.2742**
B x C	3	5.8670**	21.05139**	72.59736**	6.9878**
A x B x C	9	24.5524**	22.28389**	21.03375**	43.2231**
Error	32	0.1032	0.06396	0.06917	0.1844
Total	**95**				

** = Highly Significant at $p < 0.01$; WAT = Weeks after Transplant in contaminated soil

Table 4: Fungal cfu (000,000) g g^{-1} Soil in the Rhizosphere of *C. zizanioides* and *E. camaldulensis*

	Fungal cfu g g^{-1} Soil			
Treatments	**3 WAT**	**6 WAT**	**9 WAT**	**12 WAT**
Crude Oil Contamination				
C1 (0 L)	9.55^{b}	6.39^{d}	6.88^{c}	11.96^{a}
C2 (0.3 L)	7.22^{c}	9.58^{b}	8.02^{b}	9.20^{b}
C3 (0.5 L)	9.69^{ab}	6.94^{c}	6.23^{d}	7.85^{d}
C4 (0.7 L)	9.83^{a}	11.03^{a}	11.68^{a}	8.60^{c}
Mean	9.07	8.48	8.20	9.40
p of f	0.001	0.001	0.001	0.001
s.e.d	0.1018	0.0924	0.0528	0.1261
Soil Amendments				
T1	8.81^{b}	8.46^{b}	5.77^{d}	8.47^{b}
T2	8.65^{b}	10.65^{a}	10.10^{a}	8.47^{b}
T3	10.00^{a}	7.28^{d}	8.94^{b}	10.45^{a}
T4	8.82^{b}	7.55^{c}	7.99^{c}	10.23^{a}
Mean	9.07	8.48	8.20	9.40
p of f	0.001	0.001	0.001	0.001
s.e.d	0.0925	0.0714	0.0770	0.1191
Plant Species				
C. zizanioides	11.03^{a}	9.81^{a}	8.90^{a}	10.04^{a}
E. camaldulensis	7.12^{b}	7.15^{b}	7.50^{b}	8.76^{b}
Mean	9.07	8.48	8.20	9.40
p of f	0.001	0.001	0.001	0.001
s.e.d	0.0656	0.0516	0.0537	0.0876

T1 = Control; T2 = NPK (g kg^{-1}); T3 = Cow-dung (3:1 v/v); T4 = NPK (g kg^{-1}) + Cow-dung (3:1 v/v); WAT = Weeks after transplant in contaminated soil. Figures with same alphabets within columns do not differ significantly for Crude contamination, Soil amendments and Plant species respectively p of f = Probability value of F

Microorganisms involved in the Bioattenuation of Total Petroleum Hydrocarbon

The results presented in Table 5 had indicated the list of the genus of microorganisms (both bacteria and fungi) identified to be actively involved in the degradation of total petroleum hydrocarbon. Bacteria identified include gram positive (+ ve) and gram negative (- ve) genus of *Bacilli spp.*, gram + ve genus of *Staphylococci spp.*, as well as gram + ve genus of *Streptococci spp.* In addition, the genus of fungi identified in the course of the study were both septate and non - septate fungi which includes: *Aspergillus spp.*, *Rhizopus spp.*, and *Penicillium spp*.

Table 5: Genus of Microorganisms identified during Bioattenuation of Total Petroleum Hydrocarbon

Bacteria	Fungi
Gram + ve and - ve *Bacilli spp*	*Aspergillus spp*

Gram + ve *Stphaylococcus spp*	*Rhizopus spp*
Gram + ve *Streptococcus spp*	*Penicillium spp*

Biostimulation of Microorganisms in Rhizosphere during Bioattenuation of TPH

The varying number of bacterial and fungal cfu among levels of crude oil contamination and in the different soil amendments under the influence of the tested plant species throughout the various sampled periods implied that plant exudates do encourage microbial populations growth and hydrocarbon degradation in the contaminated soils. This is as supported in a report[36,37] that plant - microorganism interaction do exist during remediation as a means for biodiversity conservation in hydrocarbon contaminated soils within the rhizosphere of plants.

Since the least number of bacterial cfu was observed in the highest crude oil contamination level (C4 – 0.7 L) at the 3 WAT which was contrary to the control (C1 – no contamination), it indicated that the consortium of hydrocarbon degrading bacteria found in soil is drastically affected by the level of crude oil contamination. This is in consonance with the report that microorganism count, especially fungi, decreases with increasing crude oil contamination except where soils augmented with plants and poultry dung is used as treatments[37,38]. This research also indicated that crude oil contamination between the levels of 0.3 L/ 4 kg soil to 0.5 L/ 4 kg soil can fairly be tolerated by the consortium of native microorganisms at 3 WAT. This means that to maintain the best result of remediation with native microorganisms during phytoremediation process, treatment of the contaminated soil with the mixture of NPK fertilizer with Cowdung (NPK g g^{-1} + Cowdung 3:1 v/v respectively) improves the soil condition for the optimum growth of the hydrogen degrading microorganisms. This is in consonance with the report[39, 40] that the addition of organic materials does improve the chemical properties of hydrocarbon contaminated soil (such as pH, OC, total nitrogen, available P, Ca, K, and Mg) thereby enhancing the solubility and removal of the contaminants and improve hydrocarbon degradation rate by microorganism. It was further reiterated[39] that the addition of NPK fertilizer do restore carbon to nutrient ratios to the optimum required for the growth of petroleum utilizing organisms, especially bacteria in contaminated soils.

Although the best rhizostimulating plant species for bacteria cfu was *C. zizanioides* species, it was observed that *E. camaldulensis* was the best at the end of the experiment (12 WAT) for fungal cfu. This indicated that the mixture of both grass and woody plant species during phytoremediation process is better. This is so due to the fact that while the grass stimulates the growth of degrading microorganisms especially bacteria at the beginning of the phytoremediation process, on the long-run, fungal consortium improves because of the heavy exudation of woody plants. As bioattenuation continues in the contaminated soils, the pH tends to increase and this led to the increase in the colonies of bacteria now actively involved in hydrocarbon degradation. This is in consonance with the result reported[41] that crude oil pollution leads to increase in soil pH and since soil bacteria thrive better in neutral than in acidic soils, the increase in soil pH during bioattenuation means that degradation of organic contaminants by especially bacteria is taking place. In addition, it indicates that bacterial colonies were at a specific or non-specific interactions with exudate secretion of specific compounds or chemically related compounds similar to the contaminants as suggested[42].

Although C. *zizanioides* was found to be effective in rhizostimulation of both hydrocarbon degrading bacteria and fungi, *E. camaldulensis* species encourages the growth of bacteria more at the first three weeks. The effectiveness of *C. zizanioides* (a perennial grass) in rhizostimulation could be due to the fact that the plant possesses massive root system and this tends to encourage aeration and the growth of microorganisms. Accordingly, the rhizosphere of plants improves the properties of soil through the injection of air and the introduction of nutrients that encouraged microbial diversity[43]. Also, it is buttressed that the interaction between the plant and microorganisms promotes the cometabolism of the contaminants that forces the activation of several metabolic options[15].

Genus of Microorganisms involved in Hydrocarbon Bioattenuation

The three genera of bacteria and fungi found within the contaminated soil during bioattenuation were gram positive and negative *Bacillus spp*, gram positive *Staphylococcus spp*, and gram-positive *Streptococcus spp*. Those of fungi includes: *Aspergillus spp*, *Rhizopus spp*, and *Penicillium spp*. The most frequently occurred genus of microorganisms were Bacillus and Rhizopus. This result indicated that both bacteria and fungus of these genus were responsible for the biodegradation of hydrocarbon within the contaminated soil. This result is in consonance with scientists[37, 38, 44] who found similar bacterial and fungal consortium of associated with hydrocarbon biodegradation in contaminated soils. They further stated that plants do not directly degrade contaminants but generate the proteins and enzymes that guarantees the growth of fungi and bacteria that degrade it.

IV. Conclusion

This research observed that the exudates of C. zizanioides and E. camaldulensis plant species did encourage rhizostimulation of Total Petroleum Hydrocarbon (TPH) degrading microorganisms (bacteria and fungi). The two tested plants were ab-initio identified as good Phyto accumulator of TPH in an earlier study[1,6]. Although C. zizanioides encouraged hydrogen degrading bacteria up to 9 WAT, E. camaldulensis woody species was the best rhizostimulating plant for fungal cfu at the end of the experiment at 12 WAT. To this end, it was concluded that the mixture of both the grass and woody plant species are required for optimum growth and stimulation of hydrocarbon degrading microorganisms during phytoremediation procedure in the field.

Bacterial and fungal cfu was observed to have increased with increasing hydrocarbon concentration under the influence of the two plant species and the applied landfarming treatments. Additionally, the best rhizostimulation was under the influence of the tested plant species was achieved with a mixture of NPK (g g-1) and Cowdung (3:1 v/v) fertilizer.

Hydrocarbon degrading bacterial cfu observed during the phytoremediation process are: gram positive and negative Bacillus spp, gram positive Staphylococcus spp, and gram-positive Streptococcus spp. Those of fungi includes: Aspergillus spp, Rhizopus spp, and Penicillium spp. The most frequently occurred genus of microorganisms were Bacillus and Rhizopus.

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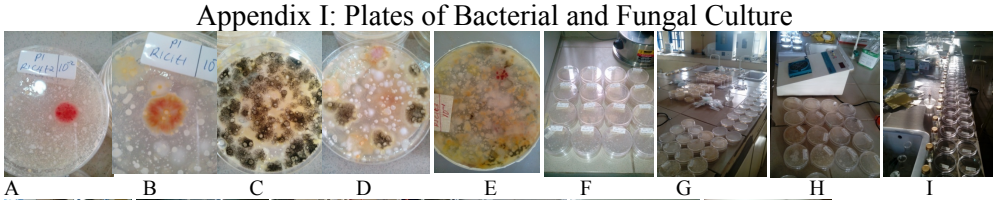
Appendix I: Plates of Bacterial and Fungal Culture



A B C D E F G H I

j k L M N O

A-E = Fungal cultures; F = Bacterial Culture; G and H = Bacterial and fungal cultures
I-L = Preparations for Culturing Microorganisms; M-O = Culture Media