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Assessment of the Phytoremediation Activity of the Rhizobacterial Flora of *Arachis hypogaea* (Groundnut) on Hydrocarbon Contaminated Soil

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Abstract

Over the years, Nigeria's development has accelerated due to crude oil exploitation. In spite of its enormous benefits, it has wreaked much havoc and damage on the ecosystem due to its toxicity. The study evaluated hydrocarbon degradation potentials by the rhizobacterial flora of the legume *Arachis hypogaea* (Groundnut) grown in potted sandy-loamy soil samples in the green house of Kebbi State University of Science and Technology, Aliero, Nigeria. Crude oil concentrations of 0.5, 1.0, 2.0, 2.5, 5.0, 10.0, 15.0, and 20.0mls were used to contaminate the soil samples respectively. All soil samples apart from the control were polluted. Groundnut germinated after seven days at concentration of 0.0 to 2.5% but at higher concentration of 5.0% of the contaminant, the germination time increased to nine days and at concentration 20%, it increased to ten (10) days. Even though groundnut germination was observed in all concentrations of crude oil tested, significant shoot retardation still occurs in both legumes consequent on crude oil toxicity. Rhizobacterial population also diminished with increase in crude oil concentration. The rhizobacterial population diminished with increase in crude oil concentration. The rhizobacteria isolated from the soil sample include *Bacillus subtilis*, *Clostridium tetani*, *Staphylococcus aureus*, *Proteus vulgaris*, and *Enterobacter aerogenes*. Rhizobacterial population also diminished with increase in crude oil concentration. The study revealed the resistance of groundnut to crude oil ($p < 0.05$), marking groundnut out as a promising phytoremediation plant.

Keywords: *Arachis hypogaea*, Bacteria flora, Hydrocarbon-Contaminated Soil, Oil Spillage, Phytoremediation.

INTRODUCTION

A major problem facing oil-producing countries in the developing world is the issue of oil spillage (Plohl *et al.* 2002). This can have devastating effects on the biota of an environment. Oil spills and oil wastes discharged into the sea or land from refineries, factories or ships contain poisonous compounds that constitute potential danger to plants and animals (Amanda, 2006). The poisons can pass through the food web of an area and may eventually be eaten by humans (Gibson and Parales, 2000). Environmental contamination by hydrocarbons and petroleum products constitutes nuisance to the environment due to their persistent nature and tendency to spread into ground and surface water. This has attracted much attention in recent decades. Husaini *et al.* (2008) and Plohl *et al.* (2002) reported that used motor oils such as diesel or jet fuel contaminate natural environment with hydrocarbon.

The hydrocarbons may spread horizontally on the groundwater surface thereby causing

extensive groundwater contamination. Aromatic hydrocarbons are considered to be the most acute, toxic component of petroleum products, and are also associated with chronic and carcinogenic effects (Amanda, 2006). Lighter mono aromatics compounds include benzene, toluene, ethyl benzene and xylenes (BTEX). Aromatics with two or more rings are referred to as polycyclic aromatic hydrocarbons (PAHs) (Anderson *et al.*, 1974). Baker *et al.* (2002) reported that motor oil had concentrations of benzene up to 29 to 66 mg/L but those of other BTEX compounds were higher, typically 500 to 2000 mg/L. Hydrocarbon contamination of the air, soil, freshwater (surface and groundwater) especially by PAHs has drawn public health concerns because many PAHs are toxic, mutagenic and carcinogenic (Clemente *et al.*, 2001). Clinical studies have shown that exposure of a mixture of highly concentrated PAHs may cause skin, lung, stomach and liver cancers (ATSDR, 1990).

Phytoremediation describes the treatment of environmental problems by using plants without the need to excavate the contaminant material and dispose of it elsewhere (Rui *et al.*, 2012). One of the indices of loss of biological activity of soils as a result of crude oil pollution is the reduction or inhibition of microbial activity (Amanda, 2006). Microorganisms of particular interest in this study are the rhizobacterial flora (rhizosphere bacteria), due to their beneficial roles. They have been shown to be important in the degradation of pollutants, biofertilization through nitrogen fixation, phytostimulation and biocontrol of soil-borne plant diseases (Chin-A-Woeing *et al.*, 1998). The area of soil around plant roots, known as the rhizosphere contains higher populations and greater diversity of microorganisms than soil with no plant (Nichols *et al.*, 1997). This is because plant roots release exudates into the soil that increase microbial activity by supplying nutrients to the organism (Eze *et al.*, 2013). The exudates consist of enzymes, aliphatics, aromatics, amino acids, sugars and low molecular weight carbohydrates (Burken and Schnoor, 1996). Phytoremediation is an eco-friendly approach for remediation of contaminated soil and wastewater using plants (Nichols *et al.*, 1997). It consists of two components, one by the root colonizing microbes and the other by plants themselves, which accumulate the toxic compounds to further change to non-toxic metabolites. Various compounds viz: organic synthetic compounds, xenobiotics, pesticides, hydrocarbons, heavy metals and radionuclides are among the contaminants that can be effectively remediated by plants (Suresh and Ravishankar, 2004; Schroder *et al.*, 2002). Different mechanisms are employed in phytoremediation of petroleum hydrocarbons. Both plants and microorganisms are involved directly or indirectly in the degradation or transformation of petroleum hydrocarbons into products that are generally less toxic and less persistent in the environment than the parent compound (Nwadinigwe and Onyeidu, 2012). The primary mechanisms for plant-mediated remediation of soils contaminated with petroleum hydrocarbons as outlined by Amanda (2006) are: phytodegradation (rhizodegradation), phytostabilization, phytoextraction (phytoaccumulation), phytovolatilization and rhizofiltration. The success of phytoremediation at a given site cannot always be attributed to just one of these mechanisms because a combination of mechanisms may be at work (Eze *et al.*, 2013).

The present study is therefore aimed at evaluating the phytoremediation potential of the *Arachis hypogaea* on hydrocarbon contaminated soil.

MATERIALS AND METHODS

Sample Collection

Crude oil (specific gravity = 0.81; API gravity = 43.2°) was obtained from the Nigerian National Petroleum Corporation (NNPC), Port Harcourt Refinery, Alesa - Eleme, Rivers State, Nigeria, on 2nd July, 2017. The crude oil was unweathered, having been obtained fresh from the production plant.

Plant Seeds

Seeds of *Arachis hypogaea* (groundnut) were purchased at Kebbi Central Market, Birnin Kebbi, Kebbi State, Nigeria, and stored at ambient temperature

Soil Sample

Fifty kilogram (50kg) of sandy loam soil was collected from Fadama Teaching and Research Centre Jega, Kebbi State, Nigeria, by clearing the top soil to reduce contaminants. It was dug to the depth of 2m and collected using clean polythene bags in the morning.

Soil Processing and Sowing of Plant Seeds

Completely randomized design (CRD) was adopted in this study. This is because the test plants (*Arachis hypogaea*) were allocated randomly to the hydrocarbon-contaminated soils (treatment) and uncontaminated soils (control). This study lasted for twelve (12) weeks.

The soil sample was air-dried, sieved and dispensed in 3 kg weights into eighteen (18) plastic pots (20 cm deep × 20 cm diameter) perforated at their bases for aeration with three replicates. Each pot in a group, apart from the control, was contaminated with one of eight different levels of crude oil (0.5, 1.0, 2.0, 2.5, 5.0, 10.0, 15.0 or 20.0% v/w) (Eze *et al.*, 2013). All control samples were not contaminated. Thereafter, seeds of the plants (groundnut) were sown, which consisted of three seeds of each plant sown in triplicate pots. All pots were kept in a Green house at the Faculty of Agriculture, Kebbi State University of Science and Technology, Aliero, Kebbi State, Nigeria and watered every twenty four hours by spraying.

Enumeration of Bacteria

Enumeration of bacteria population in the rhizosphere of the contaminated samples and that of the control was carried out using the standard plate count technique (Wistreich, 1997).

Soil samples (0 to 3 cm deep) were collected as described by Adoki and Orugbani (2007) both from the contaminated samples and control at two-weekly intervals from each pot and put into sterile labeled polythene bags. A sterilized spatula was used to dig the soil to collect soil sample from the root area. The samples were immediately taken to the laboratory for analysis. One gram of each sample was serially diluted using 9ml of sterile distilled water and up to 10⁻⁸ dilution to reduce the bacterial load. Using a sterile micropipette, 0.1ml was inoculated by spread plating on sterile nutrient agar plates for 24 hours at 37°C. After 24hrs, the plates that had 30-300 colonies were counted and recorded.

Isolation and Identification of the Test Bacteria

The colonies observed were sub-cultured onto nutrient agar and were incubated at 37°C for 18hours in order to obtain pure cultures of the bacteria cell. From the colonies that developed, a smear was made on a clean glass slide using sterile wire loop. It was dried and heat fixed. The smear was flooded with crystal violet solution for 60 seconds and rinsed, tipped off and covered with Lugol’s iodine for 2 minutes. The stain was decolorized with acetone and washed off immediately with distilled water. It was counter stained with safranin for 2 minutes and rinsed with distilled water. The back of the slide was wiped clean; the smear was placed on a draining rack and allowed to air dry. The smear was viewed under the microscope using oil immersion objective x100. Further biochemical tests such as (catalase, coagulase, oxidase, indole, motility and urease test) to confirm the isolates to species level was carried out as described by Oyeleke and Manga (2008) as well as Cheesbrough (2000).

Biochemical Characterization of the Bacterial Isolates

Using standard methods adopted by Cheesbrough (2000), the following test were carried out: Catalase, Coagulase, Citrate, Motility, Indole, Urease, Triple sugar iron, Methyl red, Voges-Proskauer, Mannitol, Spore formation, Oxidase tests.

Seed Germination

Germination of seeds was observed daily for 60 days as positive or negative; it was positive if there was a visible cracking of the seed coat with measurable root or shoot production (Maila and Cloete, 2002). The germination time (in days) was observed and recorded for seeds in every pot.

Plant Growth Evaluation

Plant shoot growth was measured with meter ruler (cm) with initially fourteen days after seed sowing and subsequently done weekly throughout the eight-week experiment. Measurement was carried out using a calibrated 30 cm transparent plastic rule.

STATISTICAL ANALYSIS

Data analysis was carried out using a one-way analysis of variance (ANOVA), and the difference was done to determine statistical significance differences (p< 0.05).

RESULTS

Total Bacteria Count of the Soil Samples

Table 1 represent the total bacteria count of the soil sample polluted with crude oil where groundnut was grown. The result indicated that at the concentration of 0.0%, the number of bacterial count were high but gradually decreases as the concentration of crude oil increases. At the concentration 5.0% to 20%, the number of bacteria count from the soil declined significantly.

Table 1: Mean Bacteria Counts (log₁₀ cfu/g) of Soil Samples were Groundnut was Grown.

Replication	Concentration								
	GO	G 0.5	G 1.0	G 2.0	G 2.5	G 5.0	G 10.0	G 15.0	G20.0
1 st	9.88	9.70	9.60	9.54	9.48	9.43	9.40	9.30	9.18
2 nd	9.90	9.65	9.54	9.40	9.36	9.30	9.28	9.26	9.00
3 rd	9.93	9.60	9.48	9.43	9.40	9.36	9.32	9.23	9.00
Mean	9.90 ^a ±0.01	9.65 ^f ±0.05	9.54 ^e ±0.06	9.46 ^{de} ±0.07	9.41 ^{cd} ±0.06	9.36 ^{bcd} ±0.07	9.33 ^{bc} ±0.06	9.26 ^b ±0.04	9.06 ^a ±0.10

GO = Control sample

Means with the same superscript are not significantly different at (P<0.05)

Bacteria Identification

Table 4 indicates the bacteria isolated from the contaminated soil where groundnut was grown. After the isolates has been subjected to various morphological and biochemical tests, the

following bacteria genera were identified: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Clostridium tetani*, *Bacillus subtilis*,

Table 2: Bacteria Identified from Soil Samples where Groundnut was Grown

Gram React.	Shape	Cat	Coa	Man	Cit	Ure	MR	VP	Oxi	Ind.	Trp	Mot	Spo	Bacteria Isolates
+	Rod	+	-	+	+	-	-	+	-	-	-	+	+	<i>Bacillus subtilis</i>
-	Rod	+	-	+	+	-	-	-	+	-	+	+	-	<i>Pseudo. aeruginosa</i>
-	Rod	+	-	+	+	-	+	+	-	-	-	+	-	<i>Entero. aerogenes</i>
+	Rod	-	-	-	-	-	-	-	-	-	-	+	+	<i>Clostridium tetani</i>
+	Cocci	+	+	+	+	-	+	-	-	-	-	-	-	<i>Staphy. aureus</i>

KEY: Cat = catalase test, Coa = Coagulase test, Man = Mannitol test, Cit = Citrate Test, Ure = Urease Test, Vp = Voges-Proskauer, Oxi = Oxidase test, Ind = Indole test, Trp = Triple Sugar test, MR = Methyl Red Test, Mot = Motility test, Spo = Spore formation

Germination of Groundnut Seeds on the Crude Oil Contaminated Oil

Table 3 represents the germination of groundnut. From the table, groundnut

germinated at different level of contamination with crude oil but the table indicates that at concentration 5.0 to 20%.

Table 3: Germination of Groundnut Seeds on the Crude Oil Contaminated Soil

Crop Plant	Crude Oil Level (%)									
	0.0	0.5	1.0	2.0	2.5	5.0	10.0	15.0	20.0	
Groundnut	+	+	+	+	+	+	+	+	+	+

Key: + = There is germination
- = No germination

Germination in Days of Groundnut on Crude Oil Contaminated Soil

Table 4: Represent the germination time in days of legume. The table indicates that groundnut germinated after seven days (7) of sowing at concentration 0.0 to 2.5% but at higher concentration of 5.0% of the contaminant, the germination time increase to

nine days and at concentration 20.0% it increased to 10 days. The table indicates that cowpea germinated on the fourth day at concentration 0.0 to 2.0%. At concentration 2.5, it germinated around the fifth day but cease to germinate as a concentration increase to 5.0.

Table 4: Germination Time (days) of Seeds at Different Level of Crude Oil Contaminated soil

Crop Plant	Crude Oil Level (%)									
	0.0	0.5	1.0	2.0	2.5	5.0	10.0	15.0	20.0	
Groundnut	7±0.6	7±0.58	7±0.25	7±1.10	7±0.45	9±0.60	9±0.58	9±0.25	10±0.4	

Shoot Growth of Groundnut at Weekly Interval on Crude Oil Contaminated Soil

Table 8 indicates the shoot growth of groundnut measured with a metre rule from the second week to eight week. From the table, the groundnut in the control test (GO) shows a progressive increase in the shoot growth as the week progresses, but as the contaminant were introduced at different concentration there was a progressive decline in the shoot growth as the percentage of the crude oil contamination

increases. In groundnut, the mean maximum shoot lengths at the 8th week, of the control plants and plants grown in soils with 2.5, 5, 10, 15 and 20% crude oil contamination varied from 17cm to 15 cm, 13 cm, 12cm and 10 cm respectively.

Even though groundnut germinated and grew in all the levels of crude oil pollution, there was growth depression and subsequent stagnation at high doses.

Table 5: Shoot Growth of Groundnut at Weekly Interval on Crude Oil contaminated Soil

Test Sample	Sampling weeks							Mean total (cm) \pm SD
	2	3	4	5	6	7	8	
Go	14	15	17	19	22	24	26	20 \pm 1.15
0.5	13	13	15	17	20	22	23	18 \pm 1.10
1.0	11	11	13	16	17	19	20	15 \pm 0.78
2.0	9	10	11	14	15	17	18	13 \pm 1.15
2.5	9	10	11	13	14	15	17	13 \pm 0.58
5.0	8	9	10	11	12	13	15	11 \pm 0.45
10.0	7	7	9	10	11	12	13	10 \pm 0.60
15.0	6	7	8	9	10	11	12	9 \pm 1.10
20.0	4	5	6	7	8	9	10	7 \pm 0.78

Go = Control

DISCUSSION

A total of six bacteria species were identified in this study. The dominant bacterium was *Bacillus subtilis*. Several reports of bioremediation of petroleum contaminants by the action of *Bacillus subtilis* in extreme environments have been recorded (Adriano *et al.*, 2007). Ijah and Antai (1988) reported *Bacillus subtilis* as being the dominant bacteria with 35% of all the petroleum oil utilizing bacteria characterized from highly polluted soil samples. This is consistent with the present study since *Bacillus* was the most dominant bacteria isolated. There is growing evidence that isolates belonging to the *Bacillus subtilis* could be effective in cleaning oil spills (Ghazali *et al.*, 2004).

Bacteria belonging to the *Alcaligenes* and *Enterobacter* genera are also widely reported to be implicated in petroleum hydrocarbon utilization (Raboy, 2002). According to Chikere *et al.* (2009), bacteria of the genera *Alcaligenes* and *Enterobacter* had been isolated from petroleum oil contaminated soils. The presence of *Staphylococcus aureus* in the present study also agrees with the study conducted by Adriano *et al.* (2007) who isolated *Staphylococcus hominis* from petroleum oil contaminated soils and Gomes *et al.*, (2004) who also isolated *Staphylococcus aureus* from a diesel contaminated soil.

The ability of these bacteria to survive in crude oil contaminated soil agrees with previous reports that there is increased microbiological activity within the rhizosphere (Nichols *et al.*, 1997; Clegg and Murray, 2002; Kuiper *et al.*, 2002). This increase is caused by exudates and sloughed-off tissues from the plants, which served as nutrients to the microorganisms (Salanitro *et al.*, 2004).

In this research work, groundnut seed was able to germinate and grow at all level of crude oil contamination. A similar effect of petroleum on germination was reported by Adam and Duncan,

(2002) and Njoku *et al.*, (2009). They reported reduction in germination rate in several plant species caused by petroleum contamination. The decrease in germination as diesel concentration increased might not just be due to the contaminant concentration but also to the hydrocarbon type, plant species and reduction in oxygen transfer between the seed and the surrounding environment as reported by Salanitro *et al.* (2004). The negative effect of diesel oil on germination with increased diesel oil concentration might also be due to their hydrophobic properties as reported by Adam and Duncan (2002) and Ogboghodo *et al.* (2004). Hydrocarbons may coat the seed, preventing or reducing gas and water exchange; they may also enter the seeds and alter the metabolic reactions and/or kill the embryo by direct toxicity (Adam and Duncan, 2002; Labud *et al.*, 2007).

The depression of germination of seeds by crude oil is in line with previous reports on related research (Bamidele and Igiri, 2011; Debojit *et al.*, 2011; Malek-Hosseini and Gholamreza, 2007; Amadi *et al.*, 1996; Sparrow and Sparrow, 1988). Crude petroleum is able to interfere with seed germination by coating the seeds with oily substances thereby limiting water-air movement within the seed and directly through toxic actions (Gholamreza, 2007).

This study revealed that groundnut seeds had higher percentage germination at 1.0% crude oil level and above. This was probably caused by the innate resistant qualities of the groundnut seeds (Raboy, 2002). The remarkably low phytate content of groundnut seed might have enhanced its germination and growth at all the levels of crude oil used, since high levels of phytate (an anti-nutrient) inhibit mineral nutrients absorption in both plants and animals as reported by Raboy, (2002) and Urbano *et al.*, (2000).

Some of these mineral elements (for example, Ca, P and Mg) are needed for seed germination (Debojit *et al.*, 2011). When the phytate level is low, seeds sown in crude oil polluted soil will probably have only the external crude oil factor to contend with during germination.

Shoot growth retardation in plants due to petroleum pollution as observed in this work had been reported by different workers on related studies (Debojit *et al.*, 2011; Bamidele and Igiri, 2011; Bamidele, 2010; Lin and Mendelsshohn, 2009; Adoki and Orugbani, 2007). Adoki and Orugbani (2007) during their study with three vascular plants (fluted pumpkin, maize and okro) reported retardation in their shoot growth as a result of crude oil contamination. When crude oil coats plant parts with hydrophobic substances, it reduces respiration and cell membrane permeability in the affected parts. Reduction in cell membrane permeability consequently reduces nutrient absorption, metabolism and growth in the plants.

CONCLUSION

This study reveals that groundnut has higher remediation potential on soil sample polluted with crude oil at a specified concentration

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