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GROWTH ENHANCEMENT OF *PHRAGMITES AUSTRALIS*, *EICHHORNIA CRASSIPES* AND *SACCHARUM OFFICINARUM* FOR RHIZOREMEDIATION OF CRUDE OIL CONTAMINATED SOILS

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Abstract

P. australis, *E. crassipes* (in mangrove swamp) and *S. officinarum* (in rainforest) are capable of tolerating some levels of crude oil in soil. However, some important growth characteristics such speedy growths, extensive root system and increased biomass desirable for efficient rhizoremediation are depressed. To cushion this suppressive effects, plants were subjected to the following treatments: Plant + Soil (PS) (Control); Plant + Soil + Oil (PSO); Plant + Soil + Oil + Fertilizer (PSOF); Plant +Soil + Oil + Fertilizer + Microorganisms (PSOFM); and Plant + Soil + Fertilizer + Microorganisms + Solarization (PSOFMS). Treatments were monitored for 120 days to determine their effects on the following growth parameters: Germination, germination percentage, height, root length, dry weight, and leaf area. Results indicated that treatments PSOF, PSOFM and PSOFMS enhanced all growth parameters over contaminated untreated soil (PSO) with the exception of germination in *P. australis* and *S. officinarum*; while root length, leaf area in *E. crassipes* were statistically the same for PS, PSO, PSOFM and PSOFMS ($P < 0.05$). Overall, growth enhancement efficiencies of the applied treatments were in the order: PSOFM > PSOF > PSOFMS. Thus, growth of these plants can be enhanced in crude oil contaminated soil by the above treatments for efficient rhizoremediation.

Keywords: Crude oil, Rhizoremediation, *P. australis*, *E. crassipes, S. officinarum,* Fertilizer, Microorganisms, Contaminated soils

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Introduction

Rhizoremediation, an evolving bioremediation technique involves pollutant degradation and removal from the contaminated site through the cooperative interaction of plant roots and associated root zone microflora. Microbial and plant collaboration at the root zone (rhizoremediation) provides an elevated speed of hydrocarbon pollutants breakdown than either of phytoremediation (phytodegradation) or microbial remediation alone (Escalante Espinosa *et al*., 2005; Gurska *et al*., 2009; Xin *et al*., 2008). The nomenclature, rhizoremediation in place of phytoremediation is carefully chosen to stress the significant function of rhizosphere-competent microorganisms and root exudates (Shukla *et al*., 2010).

There are several successful studies and reports on the rhizoremediation of petroleum hydrocarbon and other polluting compounds in the environment. These include benzene, toluene, ethyl benzene and xylene (BTEX), Polyaromatic hydrocarbons (PAH), linear and branched petroleum hydrocarbon, polychlorinated hydrocarbons, chlorinated phenol, chlorinated solvent, and pesticides (Shukla *et al*., 2010; Kuiper *et al*., 2004; Pivetz, 2001; Tang *et al*., 2010). In comparison to other traditional remediation techniques, rhizoremediation is economical, less intrusive and environmentally friendly, presenting no damaging effects on soil structure and fertility (Pivetz, 2001).

Speedy growth, extensive root system which facilitate the growth of microorganisms, increased biomass generation, robustness, competitiveness and ability to withstand pollution are the ideal characteristics required for a plant to be selected for rhizoremediation (Pilon-Smits, 2005). Generally, the presence of crude oil in soil has been reported to impede these ideal characteristics for a large number of plants (Anoliefo and Vwioko, 1995; Sharifi *et al*., 2007; Omosun *et al*., 2008 and Ogbo *et al*., 2009; Vwioko and Fashemi, 2005). Rhizoremediation requires human mediation to institute a suitable microbe-plant association to improve on the natural process of attenuation of the polluted site (Frick *et al*., 1999). Not minding the fact that *S. officinarum* in rainforest, *P. australis* and *E. crassipes* in mangrove swamp have been demonstrated to tolerate some levels of crude oil in soil (Odokuma and Ubogu, 2014b; Ubogu, 2017); reduction in some of the afore mentioned ideal growth parameters (speedy germination, height, root length, biomass, and leaf area growth) in these plants occasioned by the presence of crude oil in soil (Odokuma and Ubogu, 2014b; Ubogu, 2017) is a major concern. It is in the context of engaging simple agronomical procedures in addressing some of these shortcomings, through plants growth enhancement for maximum exploitation of their rhizoremediation potentials that this study was conducted.

Materials and Methods

Selection of plants and crude oil concentration

Selection of *P. australis, E. crasipes* and *S. officinarum* for growth enhancement for the purpose of rhizoremediation at 6.0 % w w^{-1} crude oil concentration was based on earlier rhizosphere effect and tolerance studies of these plants to crude oil contamination in the rainforest and mangrove swamp (Odokuma and Ubogu, 2014a,b,c; Ubogu, 2017).

Analysis of soil for baseline physicochemical properties

Rainforest and mangrove swamp soil for plant propagation were analyzed for reference physicochemical data before treatments and plant propagation. The hydrometer method as stated by Aliyu and Oyeyiola (2011), was engaged for the determination of soil textural component, while micro-Kjeldahl procedure (Van Reeuwijk, 2002) for analysis of soil nitrogen (N) content. Soil pH, porosity, total organic carbon (TOC), phosphorus and total petroleum hydrocarbon (TPH) were determined through the methods of Hendershot *et al*. (2006), Ezzati *et al*. (2012), Skjemstand and Baldock (2006), Bray and Kurtz No.1(Van Reeuwijk, 2002), and US EPA - Method 8015C (2007) respectively.

Soil preparation and treatments for plant propagation

Soils from rainforest (with unknown history of crude oil pollution) and mangrove swamp (collected at ebb tide) for plant propagation were obtained within 0-15 cm soil vertical profile. Crude oil (specific gravity of 0.818 g cm^{-3}) was then incorporated into 4000.0 g soil in plastic pots at the rate of 0.0 g (0.0 ml) and 240.0 g (293.4 ml) to attain contamination levels of 0.0 (control) and 6.0 % w w^{-1} concentrations of oil in soil respectively.

With the exception of the control (0.0 % w/w crude oil concentration in soil) (S), soils containing 6.0 % w w^{-1} oil were subjected to the following treatments: Soil + Oil (SO); Soil + Oil + Fertilizer (SOF); Soil + Oil + Fertilizer + Microorganisms (HUB and HUF) (SOFM); and Soil + Oil + Fertilizer + Microorganisms (HUB and HUF) + Solarization (SOFMS). All treatments in pots including control were made in triplicate.

Isolation, identification and scale-up of hydrocarbon utilizing bacteria and fungi

Soils from *P. australis, E. crasipes* and *S. officinarum* rhizosphere for the isolation of hydrocarbon utilizing bacteria and fungi were collected using the method of Abdel-Rahim *et al.* (1983) as adapted by Ikediugwu and Ubogu (2012), Odokuma and Ubogu (2014a,b,c). Uprooted plants were shaken lightly to free loosely adhering soil; further shaking was done in a sterile polythene bag to obtain 5.0 - 10.0 g of soil.

For mangrove swamp plants, uprooted roots along with attached soil were permitted to drain-off water for a period of 3.0 to 5.0 minutes. Soil adhering to roots loosely were shaken off gently, thereafter the remaining soil together with tightly adhering roots were taken to the laboratory in polyethylene bag that had been previously sterilized. In the laboratory, the soils collected from the field were left for a period of 5.0 to 6.0 hours to air-dry (28.0 ± 2.0 ºC) after which soil was separated from roots.

The spread plate method was employed in the isolation of HUB and HUF on oil mineral salt agar medium (OMSA). The OMSA was constituted as outlined by Dutta and Singh (2016). The constituents of the OMSA includes, agar 20 g; distilled water, 1 L; Na_2HPO_4, 1.25g ; NaCl, 10.0 g; KH_2PO_4, 0.83 g; KCl, 0.29 g; $MgSO_4.7H_2O$, 0.42; $NaNO_3$, 0.42 g, and pH of 7.2. Filter-sterilized crude oil (Millipore filter with pore size 0.22 µm) was introduced into the above constituents at 1 % v v^{-1}after sterilization for 15.0 minutes at 121.0 ºC, 15 psi in an autoclave, cooled to 45.0 ºC, homogenized and dispensed into petri dishes for solidification under sterile state with the selective inclusion of tetracycline for fungi plates.

From the mixed culture of HUB and HUF growing on OMSA plates, pure culture isolates were obtained. This was done via sub-culturing of microbial isolates onto NA and PDA plates, and maintaining these at incubation temperature of 28.0 ± 2.0 ^{0}C for a period of 3 to 4 days and 7 to 10 days for HUB and HUF respectively.

Confirmation of the capacity of pure isolates to utilize hydrocarbon was performed further via the Vapor Phase Transfer (VPT) technique as stated by Chaudhry *et al*. (2014). The respective bacterial isolates were streaked, while fugal were implanted on Mineral Salt Agar (MSA) devoid of crude oil. Vapor of crude oil which served as the only carbon source emanated from sterile crude oil saturated filter paper (Whatman, No. 1) put on the cover of Petri dish. Incubation was carried out as described previously except that plates were placed in an upside position. Plates not inoculated were used as control. Colonies of bacteria and fungi obtained from the VPT technique were re-purified and maintained on NA and PDA slants respectively from where future studies were made.

Identification of HUB isolates were made based on their morphological, cultural and biochemical characteristics, using Bergey's Manual of Determinative Bacteriology (John *et al*., 1994) and Cheesebrough (2006). That of HUF was based on cultural and morphological characteristics in line with the identification schemes of Barnett and Hunter (1998), Humber (2005), Ellis *et al*. (2007).

The HUB and HUF obtained from rhizospheres of *S.officinarum, P. australies* and *E.crasssipes* were scaled up adapting the method of Odokuma and Dickson (2003). Eight bacterial and 12 fungal species were employed in the scale-up process. Young pure culture of each microbial species from their respective

slant was scrapped using a wire loop into 200.0 ml of Oil Mineral Salt broth in a 500.0 ml flask. One microbial species per flask making a total of 20 flasks. Flasks containing bacteria and fungi were incubated in a shaker at 28.0 ± 2.0 ºC for 10 and 14 days respectively. The content of each flask was then transferred separately into a four-liter jerry can that has been previously sterilized (using 70 % alcohol) containing 800.0 ml of freshly prepared Oil Mineral Salt broth. Jerry cans were incubated as earlier described with regular vigorous hand shaking for aeration.

Soil solarization

Soil solarization was carried out by modifying the method described by Elmore *et al*. (1997). This was done in mid-January (period of intense sunlight) by placing a transparent polyethylene sheets (0.025 mm thick) over crude oil contaminated rainforest and mangrove swamp soil in pots. Soil was first watered before laying the polyethylene sheets. Sheets were allowed to touch directly the surface of soil in a complete coverage pattern and fastened properly to avoid heat escape so as to achieve maximum attainable temperature. Pots were placed in direct sunlight for a period of two weeks. Soil temperature was monitored during the period of solarization by inserting thermometer into soil to a depth of 10.0 cm. Thermometer was sealed to sheet to avoid heat escape using transparent sealing tape. The average temperature attained within the solarization period was 42.5 ± 3.2 ºC.

Application of fertilizer to soil

Fertilizer was applied to soil after solarization. Twelve grams (12.0 g) of inorganic fertilizer (NPK-15:15:15) were applied to crude oil contaminated soils at 6.0 % w w^{-1} with the various treatments except for treatments (S) and (SO) in the plastic pots to obtain the typical 1.0 – 5.0 % nitrogen by weight of crude oil required for the best nutrient optimization amendment (Head and Swannell, 1999). Fertilizer was then worked into the soil using a sterile hand trowel.

Microbial inoculation of soil

Three hundred and fifty milliliters (350.0 ml) of normal physiological saline containing hydrocarbon utilizing bacteria and fungi at the rate of 3.2 x 10^{10} cfu ml^{-1} and 5.0 x 10^{8} cfu ml^{-1} of bacteria and fungi respectively was dispensed into contaminated mangrove swamp and rainforest soils in each plastic pots. This was then worked into the soil to mix properly using a sterile hand trowel.

Plant propagation

Plants were propagated in greenhouse for 120 days period. Each set of plant was propagated in the above treated soils (*S. officanarum* in rainforest, while *P. australis* and *E. crassipes* in mangrove swamp) to obtain the following sets of treatments: PS, PSO, PSOF, PSOFM and PSOFMS.

Stems of *S. officinarum* measuring 22.0 cm in length, 3.5 ± 0.1 cm in diameter without leaves were propagated, one/pot in in rainforest soil. Similarly, *P. australies* stem size of 30.0 cm length and diameter of 0.8 ± 0.1 cm devoid of leaves were propagated two/pot in triplicate in mangrove swamp soil. On the other hand, young and tender *E. crasssipes* were collected from the wild and transplanted into respective pots with whole plants (shoots consisting of five bulb-leaves, measuring 12.5 ± 1.5 cm in height, together with roots 7.5 ± 1.5 cm) one set per pot.

With the exception of *E. crasssipes,* all plants were propagated in plastic pots with the dimension of 25.0 cm width and 14.0 cm depth. Plants were regularly watered while keeping mangrove swamp soil in perpetual flooded state with tap water. *E. crasssipes* was planted in plastic pots with the dimension of 24.0 cm width and 30.0 cm depth. The increased pot size was to make for adequate allowance for water in pots in order for plants to maintain constant buoyancy. Water level was maintained by regularly topping up water loss by evaporation.

Determination of time and percentage sprouting of plants

Time (days) was accounted for plant shoots to emerge from soil surface regarding the germination period. However, the sprouting time in the case of *P. australies,* was taken as the emergence time of leaves on aerial nodes or tips of cutting stems. Percentage sprouting of propagated plants on the other hand was calculated for the respective treatments thus:

$$\text{Percentage sprouting} = \frac{\text{Sum of entire sprouted stems}}{\text{Sum of entire propagated stems}} \text{ x } 100$$

Height, leaf area growth measurement of plant

At designated interval, 30, 60, 90 and 120 days, plant growth measurements of terms height and leaf area were measured. Height measurement was performed using the adopted method of Omosun *et al*. (2008). With the aid of a graduated meter rule, height measurements were taken from the tip of the tallest leaf to plant base (surface level). The method of Pearce *et al*. (1975), was used to determine leaf area. This was done by

plotting the perimeter of leaf against length x breadth measurements. The slop obtained from this was subsequently used as multiplying factor for length x breadth measurements.

However, for *E.crasssipes* measurements were taken at day-0 of transplant. Plant height, and leaf area growth at day 30, 60, 90 and 120 were therefore taken as additional height, and leaf area gain within the period.

Thus:

$$\text{Gain in height, and leaf area growth} = \text{Measurements at the designated time intervals} - \text{Measurments at day 0}$$

Determination of root growth and weights (dry) of plants

Growth in root lengths and increase in the weights of plant were measured at day 30, 60, 90 and 120 of planting. Root length measurements were taken with aid of a graduated meter rule from the longest root tip to its base.

Weights (dry) were measured with aid of an electronic weighing balance. Weight readings were made after adhering soil particles were washed off completely from roots and the entire plant air-dried for an hour (all detached roots during washing were recovered through mesh and weighed along). Dry weight reading was determined after plant had been dried to a constant weight at 60.0 ºC in a hot-air oven.

Data analysis

Triplicate data collected for each of the parameters in this study were analyzed using the following statistical tools: Measure of central tendency, dispersion, Student t-test and analysis of variance ($P < 0.05$).

Results

Baseline physicochemical properties of experimental soil

Soil physicochemical baseline properties showed a low TPH (70 and 395 mg kg^{-1}), TOC (0.02 and 0.07 %) and acidic pH (6.1 and 5.08) for rainforest and mangrove swamp soils respectively. While the rainforest soil may be classified as loamy sand, that of mangrove swamp is sandy clay loam. Rainforest soil had a moderate porosity (61.0 %) as against mangrove swamp with low porosity level (33.0 %). The nitrogen and phosphorus content of both soils were similar (0.17 and 0.14 %) and (42.6 and 43.03 mg kg^{-1}) for rainforest and mangrove swamp soils respectively (Table 1).

Table 1: Baseline physicochemical properties of experimental soil

Characteristic	Value	
	Rainforest	Mangrove Swamp
TPH (mg kg^{-1})	70	395
TOC (%)	0.02	0.07
Nitrogen (%)	0.17	0.14
Phosphorus (mg kg^{-1})	42.6	43.03
Porosity (%)	61.0	33.0
Ph	6.1	5.08
Sand (%)	89.0	46.5
Silt (%)	6.58	24.7
Clay (%)	4.42	28.8

Culturable hydrocarbon utilizing bacteria and fungi isolated from the rhizospheres of S. officinarum, P. australis and E. crassipes

A total of eight different culturable species of HUB were isolated from the rhizospheres of the three plants. Out of these, *P. australies* haboured three, *E. crassipes* five and *S. officinarum* five. Similarly, 12 culturable species of HUF existed overall in the rhizospheres of the three plants. *P. australis* haboured four of these while *E. crassipes* and *S. officinarum* had five and eight respectively (Table 2). In terms of species diversity, hydrocarbon utilizing microorganisms in the rhizosphere were in the order: *S. officinarum* > *E. crassipes* > *P. australis*.

Table 2: Culturable hydrocarbon utilizing bacteria and fungi isolated from rhizospheres of *S. officinarum*, *P. australis* and *E. crassipes*

Isolate	Plant		
	P. australis	*E. crassipes*	*S. officinarum*
Bacteria			
Actinobacillus sp.	-	+	+
Bacillus sp.	-	+	+
Corynebacterium sp.	-	-	+
Klebsiella sp.	+	-	-
Micrococcus luteus	+	+	+
Nocardia sp.	-	-	+
Pseudomonas aeruginosa	+	+	-
Streptomyces sp.	-	+	-
Fungi			
Aspergillus flavus	+	-	-
A. fumigates	-	+	+
A. niger	+	-	+

Mucor sp.	+	+	-
Paecilomyces lilacinus	-	-	+
Penicillium chrysogenum	-	-	+
P. funiculosum	-	-	+
P. marneffei	-	-	+
P. oxalicum	+	+	-
T. viride	-	+	+
T. aureoviride	-	+	-
Verricillium sp.	-	-	+

Treatment effect on the sprouting of P. australis and S. officinarum in crude oil contaminated soil

The presence of crude oil delayed the sprouting time of *P. australis* and *S. officinarum* (PSO) as compared to the uncontaminated soil (PS). The sprouting time in the uncontaminated soils were 5.0 and 8.7 days as against 6.7 and 13.3 days in the contaminated untreated soils (PSO) for *P. australis* and *S. officinarum* respectively. The applied treatments: PSOF, PSOFM and PSOFMS did not enhance the sprouting time in these plants as compared to the control (PS) ($P < 0.05$) (Table 3).

While there were 100 % sprouting of propagated stems of *P. australis* and *S. officinarum* in the uncontaminated soil (PS), the contaminated untreated (PSO) and treated (PSOF, PSOFM and PSOFMS) soils witnessed reduction rate of sprouting save for PSOFM in *S. officinarum* were 100 % sprouting was recorded. Percentage sprouting for contaminated soils (untreated and treated) ranged between 80.3 to 86.7 % and 83.3 to 100 % for *P. australis* and *S. officinarum* respectively (Table 4).

Table 3: Treatment effect on the sprouting time of *P. australis, E. crasipes* and *S. officinarum* in crude oil contaminated soil.

	Sprouting Time (Days)		
Treatment	*P. australis*	*E. crasipes*	*S. officinarum*
PS(control)	5.0 ± 0.0^{a}	ND	8.7 ± 1.2^{a}
PSO	6.7 ± 0.6^{b}	ND	13.3 ± 1.2^{b}
PSOF	7.0 ± 1.0^{b}	ND	11.3 ± 0.8^{b}
PSOFM	6.7 ± 0.6^{b}	ND	11.3 ± 0.8^{b}
PSOFMS	7.0 ± 0.0^{b}	ND	13.7 ± 0.8^{b}

*Values with same alphabet along same column are statistically the same ($P < 0.05$)

ND - Not determined

Table 4: Treatments effect on the sprouting percentage sprouting of *P. australis, E. crasipes* and *S. officinarum* in crude oil contaminated soil.

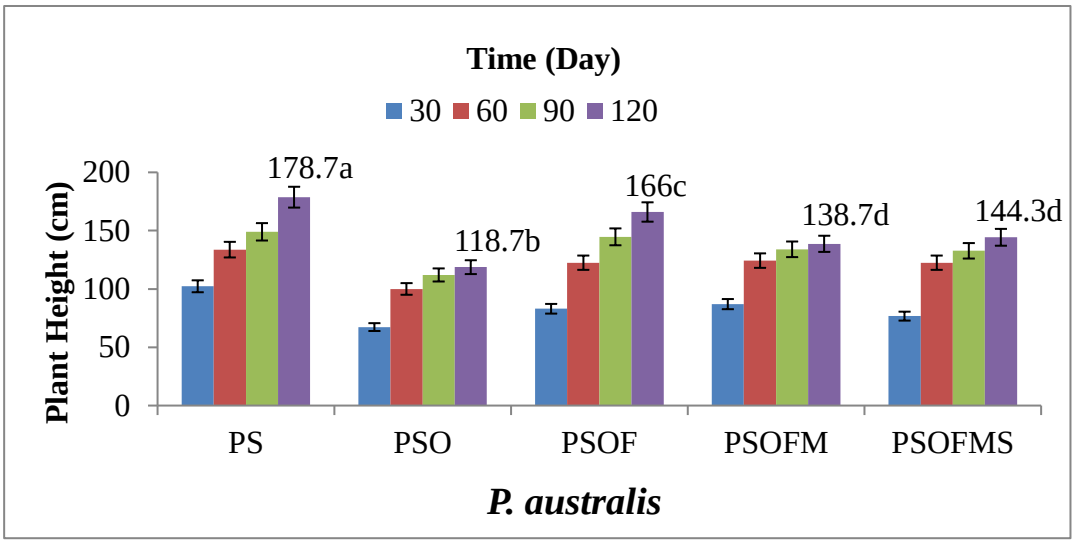
	% Sprouting		
Treatment	*P. australis*	*E. crasipes*	*S. officinarum*
PS(control)	100.0	ND	100.0
PSO	80.3	ND	83.3
PSOF	81.7	ND	91.2
PSOFM	86.7	ND	100.0
PSOFMS	80.3	ND	83.3

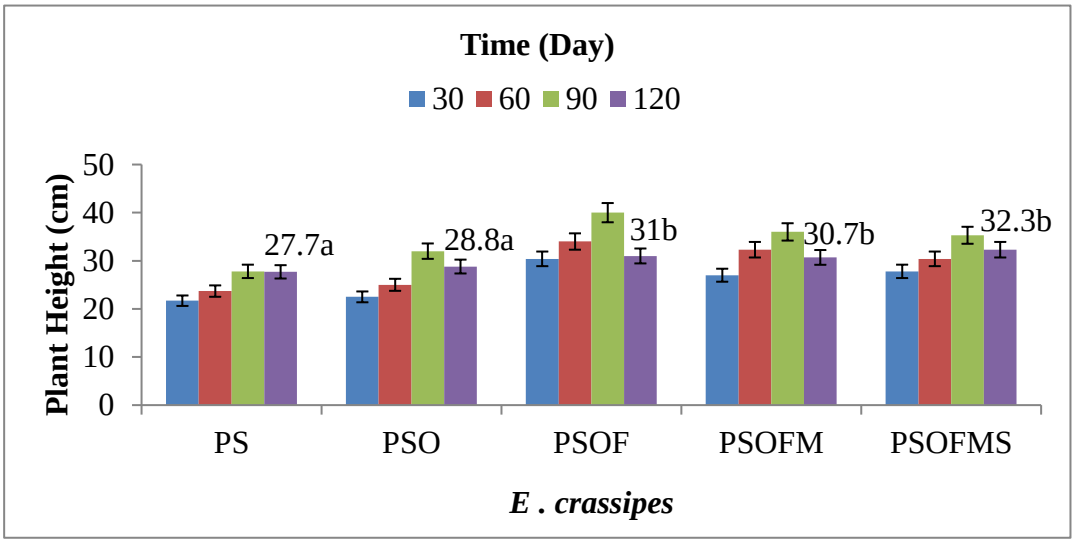
ND - Not determined

Treatment effect on height growth of P. australis, E. crassipes and S. officinarum in crude oil contaminated soil

Height growth measurements of *P. australis* and *S. officinarum* were measured at 30, 60, 90 and 120 days intervals showed a steady increase in plant heights with time in all of the treated and untreated soil including control. Although the highest heights growth for these two plants were recorded in uncontaminated soils (PS) (178.7 and 129.7 cm respectively), all the applied treatments (PSOF, PSOFM and PSOFMS) significantly enhanced plants height growth over contaminated untreated soils (PSO) (118.7 and 90.0 cm respectively) ($P < 0.05$) (Figure 1).

For *E. crassipes*, there was a progressive increase in plant height up to day 90 before undergoing decline in height growth for all propagated plants. Plant height analysis at day 90 and 120 showed that both untreated and treated contaminated soils yielded higher height growth than uncontaminated soil. However, while these height increases over the control were statistically significant at day 90, it was significant only for treated contaminated soils at day 120 ($P < 0.05$) (Figure 1).





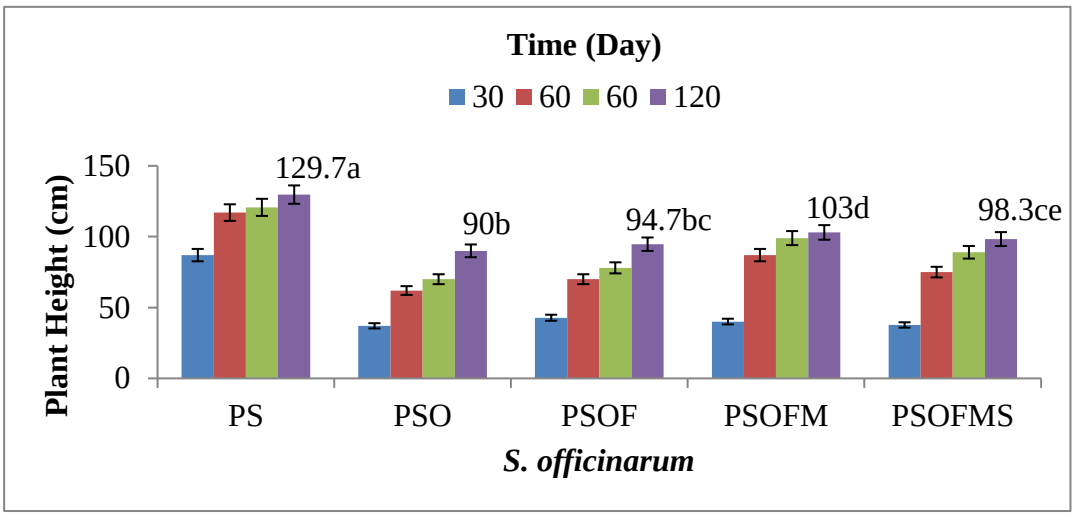


Figure 1: Treatment effect on height growth of *P. australis*, *E. crassipes* and *S. officinarum* at 6 % v w^{-1} crude oil concentration

*Values with same alphabet are statistically the same (n= 15, ANOVA, $P < 0.05$)

Treatment effect on root growth and dry weights of P. australis, E. crassipes and S. officinarum in crude oil contaminated soil

There was a steady increase in the root lengths of the three plants tested (for control, contaminated untreated and treated soils) within the study period except for *E. crassipes* which only recorded a steady root growth up to day 60 after which further root increases were not observed. Root growth analysis at day 120 showed that in *P. australis*, while all applied treatments enhanced root length growth over contaminated untreated soil, there were no differences in the root length growth in the treated contaminated soils with that of the control ($P < 0.05$) (Figure 2).

On the other hand, in *E. crassipes*, no significant differences were observed in the root length growth between control, contaminated untreated and treated soils ($P < 0.05$) (Figure 2). Furthermore, treatment PSOFMS did not enhance root growth in *S. officinarum* as this was statistically the same with contaminated untreated soil. However, while treatment PSOF and PSOFM enhanced root growth over contaminated untreated soil, the growth enhancements were far less than that of the control ($P < 0.05$) (Figure 2).

Generally, the dry weights of *P. australis, E. crassipes* and *S. officinarum* witnessed steady increases throughout the study period save *E. crassipes* where there were some form of weight decreases as from day 90 for control (PS), and treatments PSOFM and PSOFMS. This notwithstanding, analysis at day 120 in all three plants, revealed that the applied treatments produced higher dry weights measurements than contaminated untreated soils. Furthermore, while there was no difference in the dry weights between PSOF and PS in *P. australis*, PSOFM had higher dry weight than PS (control).

Similarly, while all the treated soils yielded higher dry weight growth over contaminated untreated soil and control, contaminated untreated soil also yielded higher dry weight measurement than the control in *E. crassipes* ($P < 0.05$) (Figure 3). On the other hand, in *S. officinarum* while all the applied treatments yielded higher dry weights than the untreated contaminated soil, these weight increases were significantly less than the control ($P < 0.05$) (Figure 3).

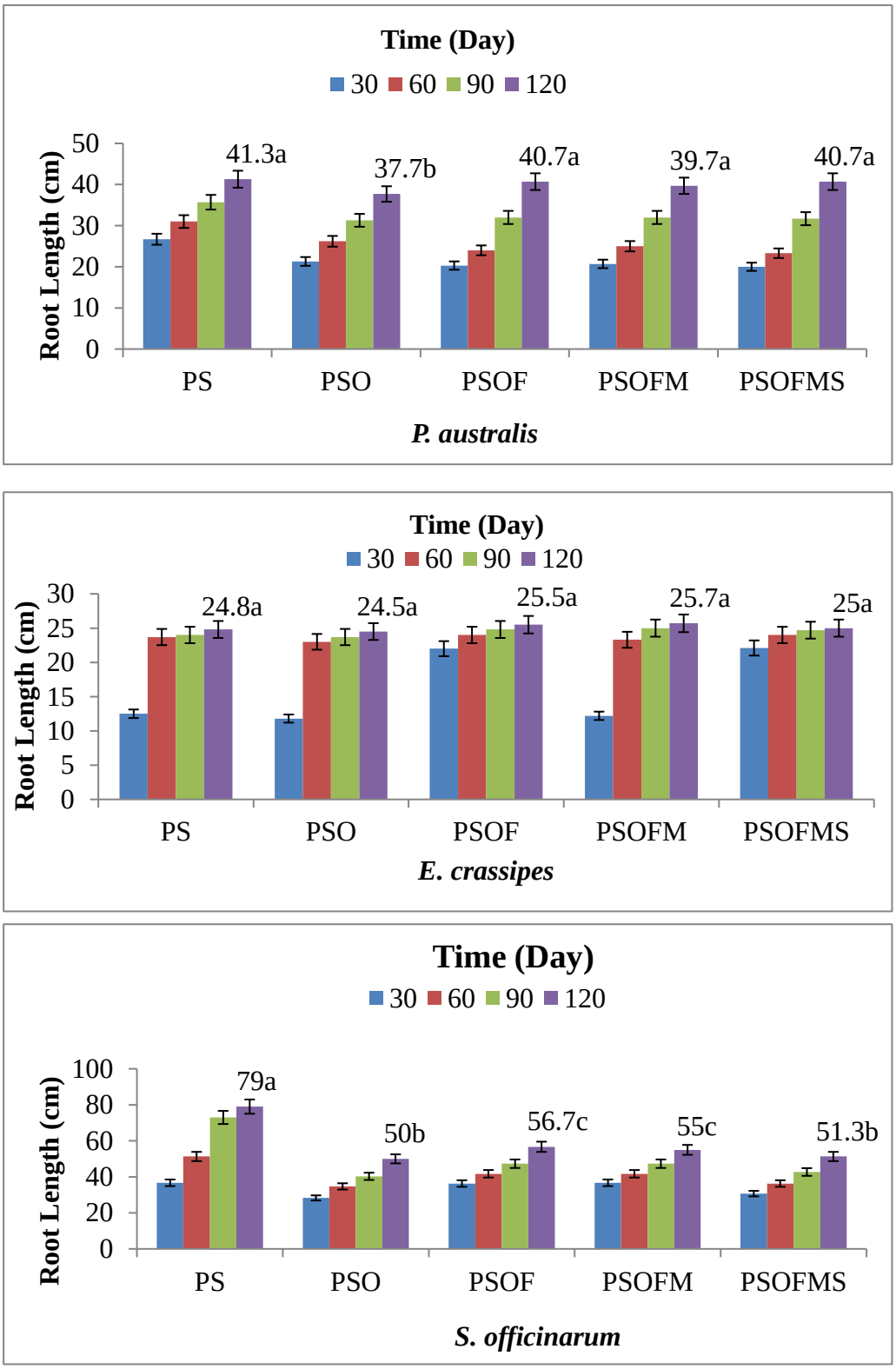
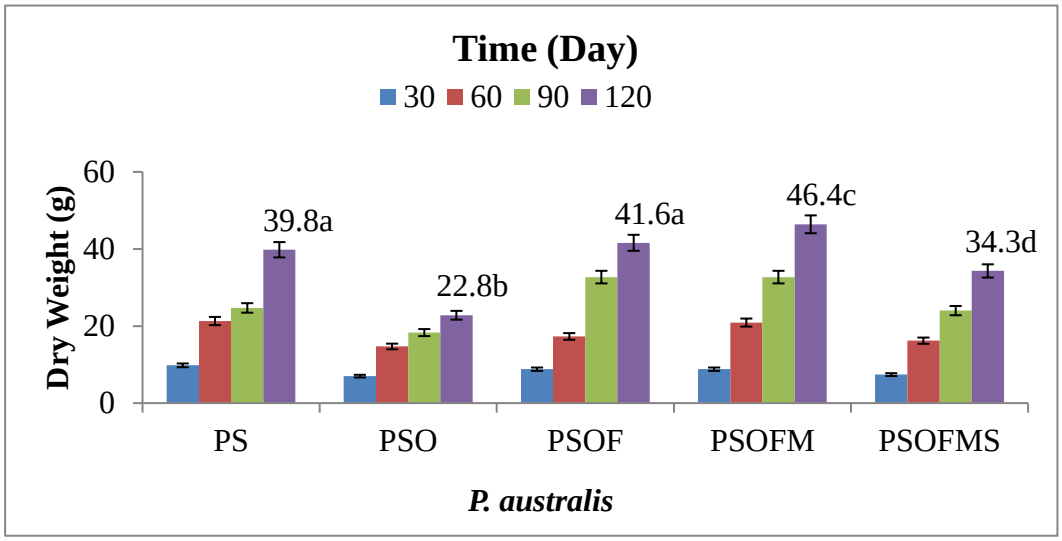
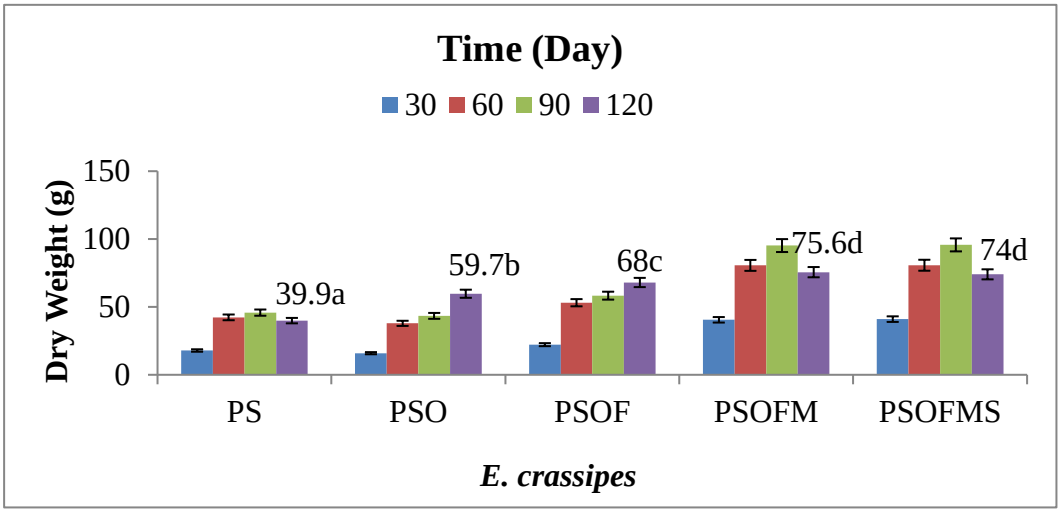


Figure 2: Treatment effect on root length growth of *P. australis*, *E. crassipes* and *S. officinarum* at 6 % v w^{-1} crude oil concentration

*Values with same alphabet are statistically the same (n=15, ANOVA, $P < 0.05$)





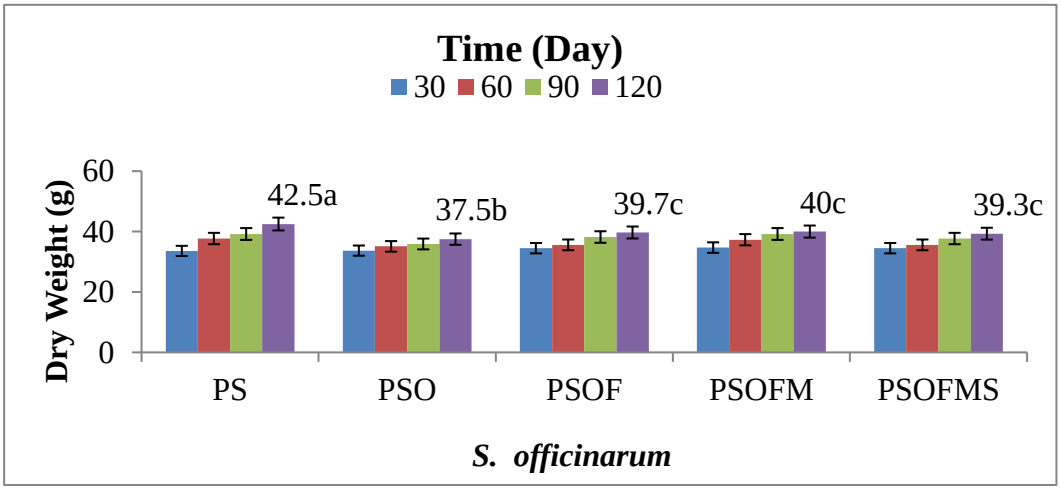
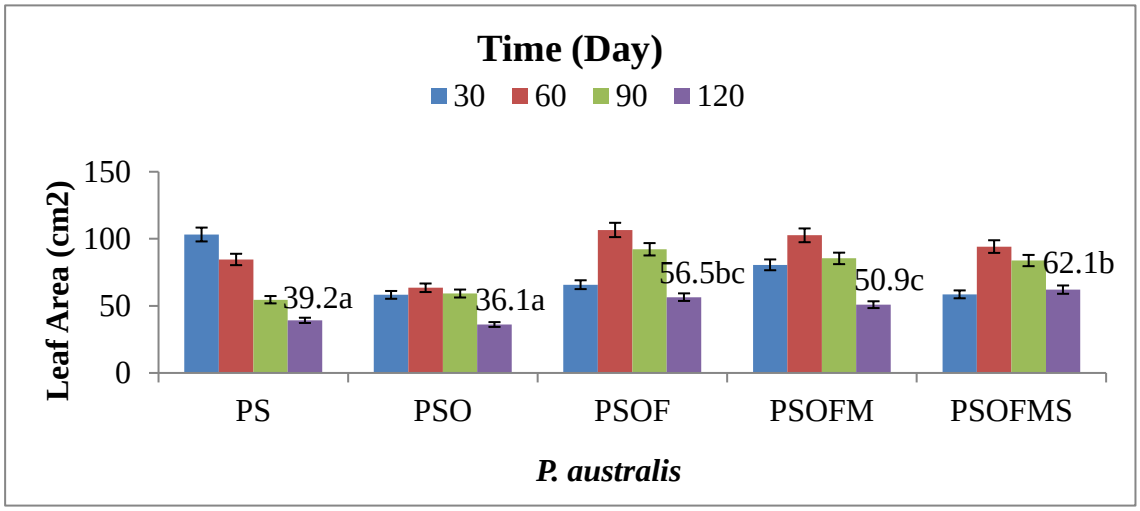


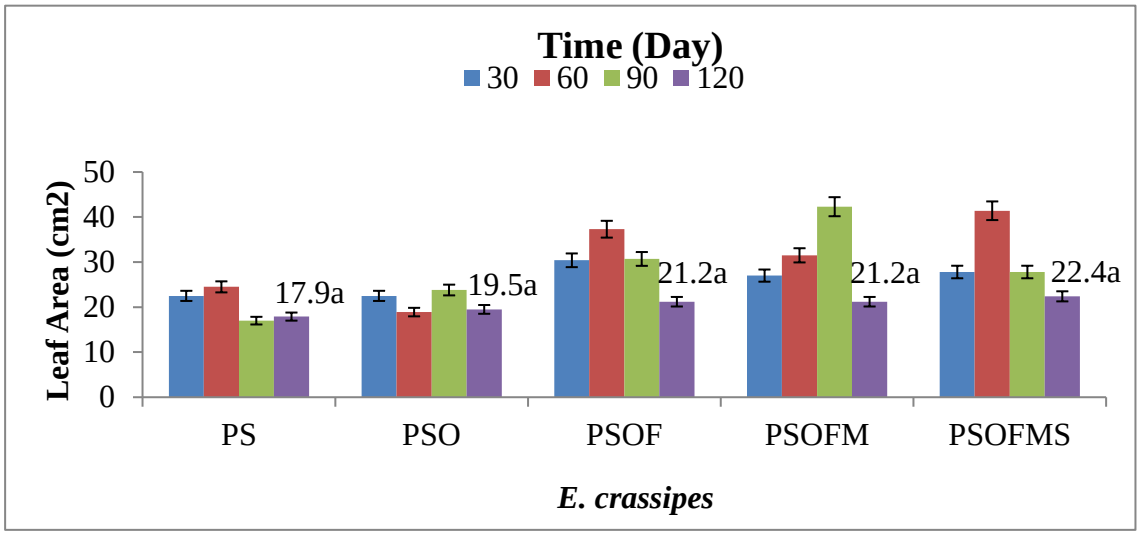
Figure 3: Treatment effect on dry weights of *P. australis*, *E. crassipes* and *S. officinarum* at 6 % v w^{-1} crude oil concentration.

*Values with same alphabet are statistically the same (n= 15, ANOVA, $P < 0.05$)

Treatment effect on leaf area growth of P. australis, E. crassipes and S. officinarum in crude oil contaminated soil

For *P. australis,* with the exception of uncontaminated soil (PS) where plant attained its maximum leaf area at day-30, all other plants in contaminated untreated and treated soils attained their maximum leaf area at day-60, after which the leaf area began to decrease with age. In contrast to this, *E. crassipies,* did not show any consistent pattern of growth in terms of leaf area with time. However, *S. officinarum* witnessed progressive increases in the leaf area in both contaminated untreated and treated soils as well control. Leaf area growth analysis at day 120 revealed all applied treatments enhanced leaf area growth over untreated contaminated soil as well as control in *P. australis*. However, for *E. crassipes* no significant difference occurred in all propagated plants. On the other hand in *S. officinarum*, while all the treatments applied produced higher leaf area growth over contaminated untreated soil, treatments were significantly lower than the control ($P < 0.05$) (Figure 4).





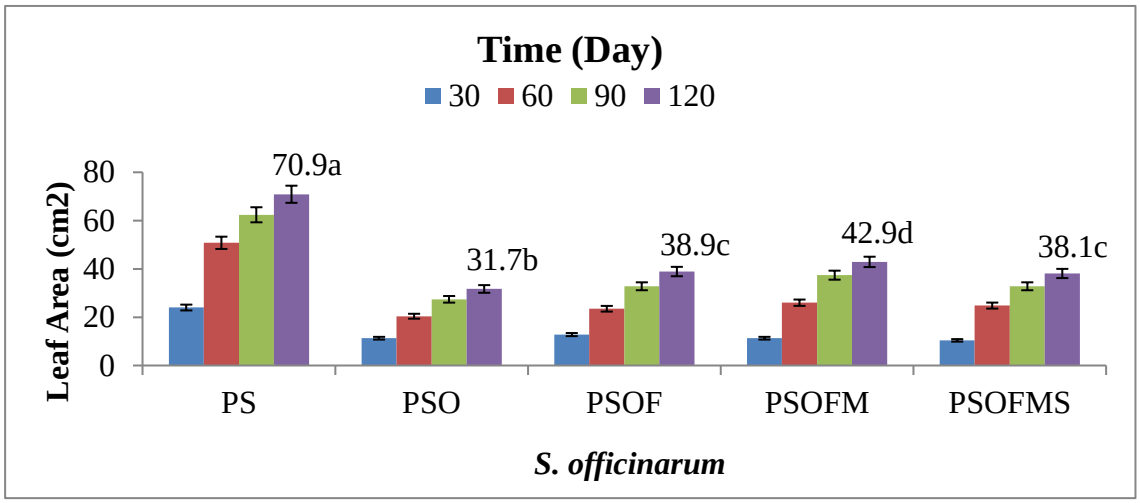


Figure 4: Treatment effect on leaf area of *P. australis*, *E. crassipes* and *S. officinarum* at 6 % v w^{-1} crude oil concentration.

*Values with same alphabet are statistically the same (n=15, ANOVA, $P < 0.05$)

Discussion

Baseline physicochemical data showed that the experimental soils are suitable for the growth of *P. australis, E. crassipes* (in mangrove swamp) and *S. officinarum* (in rainforest). The comparably low TPH and TOC in the soils suggest that there has not been any meaningful crude oil impact.

The following hydrocarbon utilizing bacteria (*Actinobacillus* sp., *Bacillus* sp., *Corynebacterium* sp., *Klebsiella* sp., *Micrococcus luteus, Nocardia* sp., *Pseudomonas aeruginosa*, and *Streptomyces* sp.) and fungi (*Aspergillus flavus, A. fumigatus, A.niger*, *Mucor* sp., *Paecilomyces lilacinus, Penicillium funiculosum, P.oxalicum* , *P. chrysogenum, P. marneffei, Verticillium* sp., *Trichoderma aureoviride* and *T.* viride) that were isolated from the rhizospheres of *E. crassipes P. australis* and *S. officinarum* and subsequently scaled-up for rhizospheres re-inoculation in this study, have also been identified and reported by other workers in previous studies as hydrocarbon degrading microorganisms in mangrove swamp and rainforest soils (Narajo *et al*., 2007; Obire and Anyanwu 2009; Chikere *et al*., 2009; Chuma, 2010; and Behera *et al.* , 2012).

In order to ascertain plant species suitability for rhizoremediation, germination test in the designated contaminant(s) is necessary (Gaskin, 2008). The result of this study indicates that the presence of crude oil in soil prolonged sprouting time in *P. australis* and *S. officinarum*. The presence of oil in soil has been reported to retard germination by hindering roots access to water and oxygen (Ogbo *et al*., 2009). The application of the various treatments failed to cushion this deceleration effect. Although, Jat *et al.* (2014) reported that soil solarization generally improves plant germination, the findings in this study did not show that. This disagreement may be attributed to the presence of the oil which may have reacted with some physicochemical components of the solarized soil to slow down germination. Germination rates of *P. australis* and *S. officinarum* in the crude oil contaminated soils were also reduced. The applied treatments did not improve germination rate save for PSOFM in *S. officinarum* were 100 % sprouting was recorded. While Soomroa *et al*. (2014) and Wains *et al.* (2012), reported the inability of inorganic fertilization (NPK) to enhance germination rate of *S. officinarum*, Asaolu *et al.* (2012), reported same for *Moringa oleifera.* On the contrary, Ramteke *et al*. (2013), reported that while NPK fertilization enhanced percentage germination in *Pisum sativum* it failed to do so for *Vigna radiata* and *V. Catjang*in. These apparent conflicting results of fertilization may possibly be related to species variation.

The presence of oil in soil depressed height growth in *P. ausralis* and *S. officinarum*. Decrease in plant height in the presence of oil over a range of concentrations in soil has been reported (Anoliefo and Vwioko, 1995; Sharifi *et al*., 2007; Omosun *et al*., 2008 and Ogbo *et al*., 2009). Reduction in height growth is attributed to increased stress imposed on the plants by a number of interacting factors: The toxic effect of the oil itself, microbe-plant competition for oxygen and nutrients during oil degradation, and microbial

formation of phytotoxic hydrogen sulfide due to oxygen limitation. Furthermore, the capacity of soil to retain air and moisture decreases with the presence of oil due to its effect on the physical structure (Dejong 1980). However, with the application of the following treatments: PSOF, PSOFM and PSOFMS, the depressive effect of oil on height growth of these plants was cushioned to a greater extent hence producing higher heights growth than the contaminated untreated soil, although this recorded growth enhancement were significantly lesser than the uncontaminated control. Soil inoculation with plant growth promoting rhizobacteria (Jeon *et al*., 2003; Egamberdieva, 2010); co-inoculation of plant growth promoting rhizobacteria (PGPR) and phosphorus solubilizing bacteria (PSB) with NPK fertilization (Abbas *et al*., 2013) or NPK fertilization alone (Rahman and Akter, 2012; Soomroa *et al.* ,2014), have been reported to enhance plant height growth. Bacteria species associated with plants rhizosphere are beneficial to plant growth (Bashan and de- Bashan, 2005). The importance of rhizosphere microbes particularly bacteria and mycorrhizal fungi in soil that are polluted can be great, sequel to the facts that they can help amplify the degree of plant tolerance to abiotic stress, encourage plant growth and consequently accelerating remediation of impacted soils (Hrynkiewicz and Baum, 2012). In the case of *E. crassipes*, contaminated treated and untreated soils yielded higher height growth than uncontaminated soil with treated soils performing better overall. Ochekwu and Madagwa (2013), also reported similar findings with *E. crassipes* in crude oil contaminated water. These findings suggest that, either the plant is capable of utilizing some levels of oil directly for its growth, or some inherent components of the oil act as growth stimulant to the plant. Some components of petroleum act as growth stimulating hormones (Fattah and Wort, 1970), at low levels this may produce growth stimulating effects on plants leading to increased crop yield (Pal and Overcash, 1978).

The obvious absence of differential root growth between uncontaminated, contaminated untreated and treated soils in *E. crassipes* indicates that oil, NPK fertilization and microbial inoculants had no measurable influence on plant root growth at the tested concentration. In case of *S. officinarum*, only treatments PSOF and PSOFM enhanced root length growth over contaminated untreated soil, these enhancements were far less than the control. On the other hand, in *P. australis* all the applied treatments enhanced root length growth over contaminated untreated soil. Combined addition of biofertilizer (PGPR & PSB) with chemical fertilizers (NPK) has been reported to synergistically caused increase in plant roots length (Abbas *et al*., 2013). In this study, the root growth responses of *P. australis, E. crassipes* and *S. officinarum* were at variance to the applied treatments. These apparent differential responses may be ascribed to plant species variation. Ramteke *et al*. (2013), studied effect of NPK fertilizer on root growth of three plants and reported that while fertilization stimulated the root growth of *Vigna catjang* it failed to do so with *Pisum sativum* and *Vigna radiant*. Similarly, Egamberdieva (2010), reported that while the inoculation of two

species of Pseudomonas (*Pseudomonas spp. NUU1 and P. fluorescens NUU2*) stimulated the root growth of one of two cultivars of wheat (Turon) by 46.0 %, it failed to do so with the other wheat cultivar (Residence). Thus, the degree of beneficial influence of bacteria on the growth of plant depends on either the variety or species of the accommodating plant Chanway *et al*. (1988).

Plant biomass (dry weight) was significantly enhanced by all the applied treatments over the contaminated untreated soils for all the three plants investigated. Furthermore, these weight increases were so profound in *P. austalis* with treatment PSOFM and in *E. crassipes* for all applied treatments, resulting in higher plants biomass in the treated contaminated soils than the uncontaminated control. Although, there is paucity of information regarding plant biomass enhancement under crude oil contamination, previous reports in uncontaminated soils lend credence to the findings here reported that soil solarization, inoculation of plant growth promoting rhizobacteria and fungi as well as NPK fertilization enhances plant biomass (Stapleton and De Vay, 1981; Jeon *et al*., 2003; Egamberdieva, 2010; Asaolu *et al*., 2012; Strasil, 2012; Rahman and Akter, 2012; Abbas *et al*., 2013).

Similar to root responses, there were variations in the response of leaf area growth to the applied treatments among the three plants species. While *E. crassipes* showed no dissimilarity in leaf area growth to all the conditions investigated, *S. officinarum* showed improved leaf area growth for the applied treatments over contaminated untreated soil but not the uncontaminated control. Crude oil in soil has been reported to decrease leaf area growth (Ogbo *et al*., 2009; Omosun *et al*., 2008; Vwioko and Fashemi, 2005). The improved growth therefore suggests once again that the application of NPK fertilizer, inoculation of appropriate rhizosphere microorganisms and soil solarization may not only help to contain the negative effect of crude oil contamination but also stimulate leaf area growth in *P. australis*. Nitrogen fertilization has been reported to stimulate leaf area growth in reed canarygrass (*Phalaris arundinacea*) (Gomme, 1978).

Conclusions

With the exception of germination time and rate, the suppressive effects of crude oil on height, root length, biomass (dry weight), and leaf area in *P. ausralis, E. crassipes* (mangrove swamp) and *S. officinarum* (rainforest) were cushioned and enhanced by the application of fertilizer, microorganisms (rhizophere-competent) and soil solarization treatments, thus increasing their rhizomediation potentials. In general, treatment PSOFM showed higher growth enhancement efficiency than treatments PSOF and PSOFMS. For the optimization of plant growth for effective rhizoremediation, it is therefore recommended that simple

agronomical techniques such as the combined addition of fertilizer and hydrocarbon degrading microorganisms be applied to crude oil contaminated soil.

References

Abbas, Z., Zia, M.A., Ali, S., Abbas, Z., Waheed, A., Bahadur, A., Hameed, T., Iqbal, A., Muhammad, I., Roomi, S., Ahmad, M.Z. and Sultan. T., 2013. Integrated effect of plant growth promoting rhizobacteria, phosphate solubilizing bacteria and chemical fertilizers on growth of maize. *International Journal of Agriculture and Crop Science,* 6 (13), 913-921.

Abdel-Rahim, A.M., Baghadani, A.M. and Abdalla, M.H., 1983. Studies on the fungus flora inthe rhizosphere of sugar cane plants. *Mycopathologia,* 81, 183-186.

Aliyu, M.B. and Oyeyiola, G.P., 2011. Rhizosphere Bacterial Flora of Groundnut (*Arachis hypogeae*). *Advances in Environmental Biology,* 5(10), 3196-3202.

Anoliefo, G.O and Vwioko, D.E., 1995. Effects of spent lubricating oil on the growth of *Capsicum annum L.* and *Lycopersicon esculentum* Miller. *Environmental Pollution,* 88, 361-364.

Asaolu, V.O., Odeyinka, S.M. and Akinbamijo, O.O., 2012. The effects of four strains of mycorrhizal fungi and goat manure on fodder production by *Moringa oleifera* under rain-fed conditions in the Gambia. *Agriculture and Biology Journal of North America,* 3(10), 391-399.

Barnett, H.L. and Hunter, B.B., 1998. *Illustrated Genera of Imperfect Fungi*. 4th Edition. American Phytopathological Society Press, St. Paul, p.218.

Bashan, Y., de-Bashan, L.E., 2005. Bacteria/plant growth-promotion. In: Hillel, D. (Ed.), *Encylopedia of Soil in the Environment*. Elsevier, Oxford, p. 103-115.

Behera, B.C., Mishra, R.R. and Thatoi, H.N., 2012. Diversity of soil fungi from mangroves of Mahanadi delta, Orissa, India. *Journal of Microbiology Biotechnology Research,* 2 (3), 375-378.

Chanway, C.P., Nelson, L.M., Holl, F.B., 1988. Cultivar-specific growth promotion of spring wheat (*Triticum aestivum* L.) by coexistent *Bacillus* species. *Canadian Journal of Microbiology,* 34, 925–929.

Chaudhry, S., Luhach, J., Sharma, V. and Sharma, C., 2014. Assessment of diesel degrading potential of fungal isolates from sludge contaminated soil of petroleum refinery, Haryana. *Research Journal of Microbiology,* 7 (3), 182-190.

Cheesebrough, M., 2006. *District Laboratory Practice in Tropical Countries Part 2*. Cambridge University Press, UK, p. 434.

Chikere, C.B., Okpokwasili, G.C. and Chikere, B.O., 2009. Bacteria diversity in a tropical Crude-oil polluted soil undergoing bioremediation. *African Journal of Biotechnology,* 8(11), 2535-2540.

Chuma, C.O., 2010. Enhanced bioremediation of hydrocarbon contaminated mangrove swamp in the Nigerian oil rich Niger Delta using seawater microbial inocula amended with crude biosurfactants and micronutrients. *Nature and Science,* 8(8), 195-206.

Dutta, S. and Singh, P., 2016. Hydrocarbon degradation potential of indigenous fungal isolates from Indian oil refinery, Haldia, (W.B) India. *Science Research Reporter*, 6(1), 4-11.

Egamberdieva, D., 2010. Growth response of wheat cultivars to bacterial inoculation in calcareous soil. *Plant, Soil and Environment,* 56(12), 570–573.

Ellis, D., Davis, S., Alexiou, H., Handke, R. and Bartley, R., 2007. *Descriptions of Medical Fungi*. Mycology Unit Women's and Children's Hospital North Adelaide 5006 Australia, p. 204.

Elmore, C.L., Stapleton, J.J., Bell, C.E. and Devay, J.E., 1997. *Soil Solarization, a Nonpesticidal Method for Controlling Diseases, Nematodes, and Weeds*. University of California, Division of Agriculture and Natural Resources Publication, p. 17.

Escalante-Espinosa, E., Gallegos-Martinez, M.E., Favela-Torres, E., Gutierrez-Rojas, M., 2005. Improvement of the hydrocarbon phytoremediation rate by *Cyprus laxus* Lam. inoculated with a microbial consortium in a model system. *Chemosphere,* 59, 405-413.

Ezzati, S., Najafi, A., Rab, M.A. and Zenner, E.K, 2012. Recovery of soil bulk density, porosity and rutting from ground skidding over a 20-year period after timber harvesting in Iran. *Silva Fennica,* 46(4), 521–538.

Fattah, Q.H. and Wort, D.J., 1970. Effect of light and temperature on stimulation of vegetative and reproductive growth of bean plants by naphthenates. *Agronomy Journal*, 62, 576-577.

Frick, C.M., Farrell, R.E., and Germida, J.J., 1999. *Assessment of Phytoremediation as an In Situ Technique for Cleaning Oil–Contaminated Sites*. Petroleum Technology Alliance Canada: Calgary, Canada, p. 88.

Gaskin, B.S.E., 2008. *Rhizoremediation of Hydrocarbon Contaminated Soil Using Australian Native Grasses*. Ph.D Thesis, Flinders University of South Australia, p. 236.

Gomm, F.B., 1978. Growth and development of meadow plants as affected by environmental variables. *Agronomy Journal,* 7, 1061-1065.

Gurska, J., Wang, W.X., Gerhdta, K.E., Khalid, A.M., Isherwood, D. M., Haung, X.D., Glick, B.R., Greenberg, B.M., 2009. Three year field plant test of a plant growth promoting rhizobacteria enhanced phytoremediation system at a land in farm for treatment of hydrocarbon waste. *Environmental Science and Technology,* 43, 4472-4479.

Head I.M., Swanell, P.J.R., 1999. Bioremediation of petroleum hydrocarbon contaminants in marine habitat. *Current Opinion in Biotechnology*, 10, 234-239.

Hendershot, W.H., Lalande, H. and Duquette, M., 2006. Soil reaction and exchangeable acidity p. 3-8 In: Soon, Y.K. and Hendershot, W.H (ed.), *Soil Chemical Analysis*. Taylor and Francis Groups, LLC., p. 3-8.

Hrynkiewicz, K. and Baum, C., 2012. The potential of rhizosphere microorganisms to promote the plant growth in disturbed soils. In: Malik, A. and Grohmann, E. (Ed.), *Environmental Protection Strategies for Sustainable Development, Strategies for Sustainability.* Springer, Switzerland, p. 35-64.

Humber, R.A., 2005. *Entomopathogenic Fungal Identification*. USDA-ARS Plant Protection Research Unit US Plant, Soil & Nutrition Laboratory. Tower Road Ithaca, NY 14853-2901, USA, p. 32.

Ikediugwu, F.E.O., Ubogu, M., 2012 Root zone microflora is Responsible for suppressiveness of the white root rot disease in Akwete rubber plantations. *Journal of Plant Pathology and Microbiology,* 3, 151-156.

Jat, R.K., Jat, N., Jat, L., Jat, A.L. and Jat, R.L., 2014. Importance of plastics in horticulture. *Popular Kheti*, 2(1), 92-94.

Jeon, J., Lee1, S., Kim, H., Ahn, T. and Song, H., 2003. Plant growth promotion in soil by some inoculated microorganisms. *Journal of Microbiology*, 41 (4), 271-276.

John, G.H., Noel, R.K., Peter, H.A.S., James, T.S. and Stanley, T.W., 1994. *Bergey's Manual of Determinative Bacteriology*. The Williams and Wilkins Company Baltimore, p. 787.

Kuiper, I., Lagendijk, E.L., Bloemberg, G.V. and Lugtenberg, J.J., 2004. Rhizoremediation: A beneficial plant-microbes interaction. *Molecular Plant-microbe Interactions.* 17 (1), 6-15.

Narajo, L., Urbina, H., De Sisto, A. and Leon, V., 2007. Isolation of autochthonus non-white rot fungi with potential for enzymatic degrading of Venezuelan extra-heavy crude oil. *Biocatalysts and Biotransformation,* 25 (2), 341-349.

Obire, O. and Anyanwu, E.C., 2009. Impact of various concentrations of crude oil on fungal populations of soil. *International Journal of Environmental Science and Technology,* 6(2), 211-218.

Ochekwu, E., Madagwa, B., 2013. Phytoremediation potentials of water hyacinth (*Eichhornia crassipes* (mart.) Solms) in crude oil polluted water. *Journal of Applied Science and Environmental Management,* 17 (4), 503-507.

Odokuma, L.O. and Dickson, A.A., 2003. Bioremediation of a crude oil polluted tropical mangrove environment. *Journal of Applied Science and Environmental Management,* 7(2), 23-29.

Odokuma, L.O. and Ubogu, M., 2014a. Quantitative assessment of hydrocarbon utilizing microflora of the rhizosphere of some plants in the rainforest and mangrove swamp in the Niger Delta. *Australian Journal of Biology and Environment Res*earch, 1(2), 31-42.

Odokuma, L.O. and Ubogu, M., 2014b. Phragmitis australis growth and tolerance to crude oil contamination in mangrove swamp soil. *Bioremediation and Biodegradation,* 5 (7), 256-262.

Odokuma, L. O and Ubogu, M., 2014c. Rhizosphere hydrocarbon utilizing microflora of *Pharagmitis australis*. Foudation for African Development through International Biotechnology, Proceedings of the 20th Annual Conference/Workshop, Enugu, Nigeria, p. 12-21.

Ogbo, E.M., Zibigha, M. and Odogu, G., 2009. The effect of crude oil on growth of the weed (Paspalum Scrobiculatum L.)- phytoremediation potential of the plant. *African Journal Environmental Science and Technology*, 3(9), 229-233.

Omosun, G. Markson, A.A., Mbanasor, O., 2008. Growth and Anatomy of Amaranthus Hybridus as affected by diferrent crude oil concentrations. *American-European Journal of Science Research,* 3, 70-74.

Pal, D., Overcash, M. R., 1978. Plant-soil assimilative capacity for oils. Paper presented at the 85th National Meeting of the American Institute of Chemical Engineers. June, 1978. Philadelphia, Pennsylvania, p. 10-15.

Pearce, R. B., Mock, J.J., Bailey, T.B., 1975. Rapid method of estimating leaf area per plant in maize. *Crop Science,* 15, 691-694.

Pilon-Smits, E., 2005. Pytoremediation. *Annual Review of Plant Biology,* 56, 15-39.

Pivetz, B.E., 2001. Phytoremediation of contaminated ground water at hazardous waste sites. Ground water issue. EPA/540/S-01/500. p. 36.

Rahman, M. A., Akter, F., 2012. Effect of NPK fertilizers on growth, yield and yield attributesof okra (*Abelmoschus esculentus* (L.) Moench.). *Bangladesh Journal of Botany,* 41(2), 131-134.

Ramteke, A.A., Narwade, M.L., Gurav, A.B., Chavan, S.P. and Wandre, A.G., 2013. Study of germination effect of fertilizers like urea NPK and biozyme on some vegetable plants. *Der Chemica Sinica,* 4(3), 22-26.

Sharifi, M., Shadeghi, Y., Akbarpour, M., 2007. Germination and growth of six plant Species on contaminated soil with spent oil. *Environmental Science and Technology*, 4, 363-470.

Shukla, K.P., Singh, N.K. and Sharma, S., 2010. Bioremediation: Development, current practices and perspectives. *Genetic Engineering and Biotechnology Journal,* 3, 1-20.

Skjemstad, J.O. and Baldock, J.A., 2006. Total and organic carbon p. 3-8 In: Soon YK and Hendershot WH (ed.). *Soil Chemical Analysis*. Taylor and Francis Groups, LLC., 55-68.

Soomroa, A.F., Tuniob, S., Keeriob, M.I., Rajperb, I., Chacharb, Q. and Arainc, M.Y., 2014. Effect of inorganic NPK fertilizers under different proportions on growth, yield and juice quality of sugarcane (*Saccharum officinarum* L.). *Pure and Applied Biology,* 3(1), 10-18.

Stapleton, J.J., DeVay, J.E., 1981. Effect of soil solarisation on population of selected soilborne microorganisms and growth of deciduous fruit gtree seedlings. *Phytopathology,* 72, 323-326.

Strasil, Z., 2012. Evaluation of reed canary grass (*Phalaris arundinacea* L.) grown for energy use . *Research Agricultural Engineering,* 58(4), 119–130.

Tang, J.C., Wang, R.G., Niu, X.W., Wang, M., Chu, H.R. and Zhou, Q.X., 2010. Character-isation of petroleum-contaminated soil: effect of different influencing factors. *Biogeosciences,* **7**, 3961-3969.

Ubogu, M., 2017. *Analysis of the Efficiency of Enhanced Remediation of Crude Oil Contaminated Soils Using Rhizoremediation, Biostimulation and Bioaugumentation Techniques*. Ph.D Thesis, University of Port Harcourt, Nigeria. pp. 1-388.

US, EPA., 2007. Method 8015C. Nonhalogenated organics using GC/FID. Washington: US EPA. p.36

Van Reeuwijk, L.P., 2002. *Procedures for soil analysis: Technical paper 9*. International Soil Reference and Information Centre, Netherlands. P.101.

Vwioko, D.E., Fashemi, D.S., 2005. Growth Response of *Ricinus communis* L (Castor Oil) in spent Lubricating Oil Polluted Soil. *Journal of Applied Science and Envronmental Management,* 9, 73-79. 40.

Wains, G.M., Mudassir, M.A., Ghaffar, A., Fiaz, N., Sarwar, M.A., Ali, Z. and Malik, F.H.F., 2012. Performance of autumn planted sugarcane at varied fertilizer level. *Mycopathology,* 10(1), 21-23.

Xin, L., Li, X.J., Li, P.J., Li, F., Lei, Z. and Zhou, Q. X., 2008. Evaluation of plan- microorganisms synergy for the remediation of diesel fuel contaminated soil. *Bulletin of Environmental Contamination and Toxicology,* 81, 19-24.