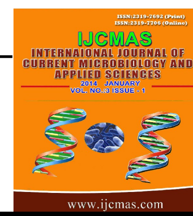

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Original Research Article

Evaluation of germination, shoot growth and rhizofungal flora of *Zea mays* and *Sorghum bicolor* in soil contaminated with varying levels of Bonny light crude oil

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A B S T R A C T

Bonny light crude oil was used at eight different levels (0.5%, 1.0%, 2.0%, 2.5%, 5.0%, 10.0%, 15.0% or 20.0% v/w) for the controlled pollution of soil samples to determine its effects on the germination, shoot growth and rhizofungal flora of two vascular plants (maize and sorghum) grown in potted sandy loam soil samples in Nsukka, Nigeria. At low to moderate levels (2% - 2.5%), crude oil suppressed germination of seeds of both crops. At 2.5% level germination time was lengthened by 24h in both maize and sorghum. Complete cessation of germination occurred in sorghum at 5% crude oil level, and in maize at 10% level. Phytochemical analysis showed seeds of both plants lacking saponin and maize seeds having higher percentage of flavonoids and alkaloids, and lower percentage of tannins and phytate. Crude oil significantly ($p< 0.05$) retarded the shoot growth of the crops in a dose-dependent manner. In maize, at the 7th week, the mean maximum shoot lengths of the control plant (0% crude oil) and test plants grown in soils with 1%, 2% and 2.5% crude oil levels varied from 60.6cm to 50.7cm, 32.1cm and 11.3cm respectively. For sorghum at the same period, the shoot lengths of the control and test plants varied from 45.5cm to 19cm, 15cm and 7cm respectively. The ability of maize to germinate in crude oil level that was lethal to sorghum makes it better adapted for growth in oil-polluted soils and consequently a better candidate for the phytoremediation of such soils than sorghum. The growth response of rhizosphere and bulk soil fungi was also crude oil- dose dependent.

Keywords

Crude oil; phyto-remediation; phyto-chemical; pollution; soil; rhizosphere.

Introduction

Crude oil and its products are deemed by many as leading causes of environmental pollution in the world today. Even though petroleum is one of the prime movers of civilization, urbanization and mechanization, the ravage it wreaks on the ecosystem marks it out as a potent pollutant. It negatively affects the physical environment as well as all species of flora and fauna (Atlas and Bartha, 1973; Chaineau *et al*., 2000; Wyszkowski *et al.,* 2001; Adoki and Orughani, 2007; Debojit

et al., 2011; Eze *et al*., 2013). The toxicity of crude and refined oil products to living organisms is an issue that needs urgent attention, especially in oil-rich nations of the world.

Pollution of the environment by crude oil and its products stems from both natural seeps and anthropogenic causes such as oil exploration, refining and transportation and lack of maintenance of oil pipelines and storage tanks leading to leakages into the terrestrial and aquatic environments. Spilled crude oil and crude oil products enter the soil environment and modify the physicochemical properties of the soil and its structure (Wyszkowki *et al*, 2004). This alteration destroys the biological activity of soils leading to a reduction in soil fertility and consequently seed germination, shoot growth, and productivity (Debojit *et al*., 2011; Bamidele and Igiri, 2011; Lin and Mendelsshohn, 2009). Even though it is already established that crude oil negatively affects seed germination and shoot growth, no two plants or their seeds manifest the same degree of sensitivity to the oil. The difference is usually caused by the innate property of each plant, among which is seed phytochemistry.

Another index of loss of biological activity of soils as a result of crude oil pollution is the reduction or inhibition of microbial activity. Microorganisms of particular interest in this study are the rhizofungal population (rhizosphere fungi) due to their many beneficial roles.

Rhizomicroorganisms (fungi inclusive) are important in the degradation of pollutants, biofertilization through nitrogen fixation, phytostimulation and biocontrol of soil-borne plant pathogens (Chin-A-Woeng *et al.,* 1998). This area of soil around plant roots, known as the rhizosphere contains higher population and greater diversity of microorganisms than soil with no plants (Nichols *et al.,* 1997). This is probably because plant roots exude chemical substances into the soil that increase microbial activity by supplying enzymes, aliphatics, aromatics, amino acids, sugars and low molecular weight carbohydrates (Burken *et al.,* 1996).

The two plants used in this study were *Zea mays* (maize) and *Sorghum bicolor* (sorghum or guinea corn), both of which are staple cereals in different parts of Nigeria.

In the face of the fast-growing oil production and exploration activities in Nigeria, and considering the beneficial roles played by the two crops (maize and sorghum), this study was undertaken to evaluate their degrees of sensitivity and tolerance to crude oil by assessing the effects of the oil on their germination and shoot growth. The study also checked the effects of crude oil on the rhizofungal population and finally assessed the phytoremediation potentials of the two cereals using their respective degrees of resistance to petroleum hydrocarbon toxicity.

Materials and Methods

Sources of Materials

Crude oil

Bonny light crude oil (specific gravity = 0.81; API gravity = 43.2^0) was obtained from Nigerian National Petroleum corporation (NNPC) Port Harcourt Refinery Alesa-Eleme, Rivers state, Nigeria. The crude oil was unweathered, having been obtained fresh from the production plant.

Plant seeds

Viable seeds of *Zea mays* (maize) and *Sorghum bicolor* (sorghum) were purchased at Ogige market, Nsukka and stored at room temperature (25 – 30°C) for not more than 24 hours.

Soil Sample

Pristine sandy loam soil was collected from the Botanical Garden, University of Nigeria, Nsukka.

Soil Pollution with Crude oil and Sowing of Plant Seeds

The soil sample was air-dried, sieved and dispensed in 3kg weights into thirty-six (36) plastic pots (20cm deep x 20cm diameter) perforated at their bases. The pots were divided into two groups of eighteen each, and each group was used for one of the crop plants. Each pot in a group, apart from the control, was contaminated with one of eight different levels of Bonny light crude oil (0.5%, 1.0%, 2.0%, 2.5%, 5.0%, 10.0%, 15.0% or 20.0% v/w). All control samples were not polluted. Thereafter, seeds of the plants (maize and sorghum) were sown, which consisted of three seeds of each plant sown in duplicate pots. All pots were kept in a green house at the Botanical Garden, University of Nigeria, Nsukka and watered every four days by spraying.

Phytochemical Analysis of Plant Seeds

Determination of Phytate

Procedure

The plant material was extracted with 0.2N HCl. A 0.1ml volume of the extract was pipetted into a test tube. Two millilitres of solution A (as shown below) was added and the test tube covered. The tube was heated in boiling water for 30mins and later cooled to room temperature. Four millilitres of solution B was added and mixed very well. Absorbance was measured at 519nm (Onwuka, 2005).

Solution A = 0.2g of ammonium iron III sulphate mixed with 100ml of 2N HCl and the volume made up to 1000ml with distilled water.

Solution B = 10g of bipyridine and 10ml thioglycolic acid in distilled water and the volume made up to 1000ml.

Alkaloid Determination (Habourne, 1973)

A 5.0g weight of the plant material was measured into a 250ml beaker. Two hundred millilitres of 10% acetic acid-ethanol was added, covered and stood for 4h.

The extract was filtered and to it was added NH_4OH drop-wise until the precipitation was completed. The whole solution was allowed to settle and afterwards the precipitate was collected, washed with dilute NH_4OH solution and then filtered.

The residue was weighed and reported as the crude alkaloid.

Saponin Determination (Obadori and Ochuko, 2001)

A 20g weight of the sample was put into a flask containing 200ml of 20% ethanol. The mixture was heated in a water bath for 4hrs with continuous stirring at about 55°C.Filtration and re-extraction of the

residue were carried out using another 200ml of 20% ethanol. The combined extract was reduced to 40ml over water bath at about 90°C and the concentrate transferred into a 250ml separator funnel and washed with 20ml of diethyl ether. The aqueous layer was recovered while the other layer was discarded and the purification process repeated. Sixty milliliters of 17-butanol was added and the extract washed twice with 10ml of 5% aqueous NaCl. The remaining solution was heated in a water bath, evaporated and oven-dried to a constant weight.

Calculation:

$$\% \text{ Saponin} = \frac{\text{weight of residue}}{\text{weight of sample}} \times \frac{100}{1}$$

Flavonoid Determination (Bohan and Kocipia, 1974)

Ten grams of the sample were put in a flask and extracted with 100ml of 80% aqueous methanol at room temperature. The mixture was filtered using Whatman filter and the filtrate transferred into a weighed crucible. Finally, the content of the crucible was evaporated to dryness over a water bath and later weighed to a constant weight.

$$\text{Percentage flavonoid} = \frac{\text{weight of residue}}{\text{weight of sample used}} \times \frac{100}{1}$$

Determination of Tannins (Pearson, 1976)

Procedure

A 1g weight of the test sample was put into a flask and 10ml distilled water was added. This was allowed to stand for 30 minutes at room temperature with gentle shaking at 5-minute intervals, at the end of which the mixture was centrifuged. Exactly 2.5ml of the supernatant and 2.5ml of standard tannin solution were measured into separate 50ml volumetric flasks. One millilitre of Folin-Dennis reagent was added into each flask followed by 2.5ml of saturated Na_2CO_3 solution.

The solution was made up to the mark and later incubated for 90minutes at room temperature. The absorbance was read at 250nm.

Calculation:

$$\text{Percentage tannin} = \frac{An}{As} \times C \times \frac{100}{W} \times \frac{Vf}{Va}$$

Where:

An = Absorbance of test sample
As = absorbance of the standard
C = Concentration of standard
W = Weight of sample used
Vf = Total volume of extract
Va = Volume of extract analyzed

Analytical Techniques

Seed Germination

Germination of seeds was assessed daily for 56 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 recorded for seeds in every pot.

Plant Growth Evaluation

Plant shoot growth was measured 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.

Microbiological Analyses

Measurement of the population of fungi in the rhizosphere of the two cereals and that of the surrounding bulk soil was carried out using the total plate count technique (Wistreich, 1997). One gram of top soil sample (0-3cm deep) (Adoki and Orugbani, 2007) was collected from the rhizosphere of each plant and surrounding bulk soil at two-weekly intervals and put into sterile labeled polythene bags. The samples were immediately taken to the laboratory for analysis. One gram of each sample was serially diluted using sterile distilled water and the 10^{-8} dilution plated out on sterile Sabouraud dextrose agar plates further made selective by the incorporation of 50µg of chloramphenicol per millilitre; incubation was for 3 – 5 days at room temperature (25 – 30^{0}C).

Statistical Analysis

Data analysis was carried out using a two-way analysis of variance (ANOVA), which was done by comparing tests with $p< 0.05$.

Results and Discussion

Germination

Bonny light crude oil used in this study significantly ($P < 0.05$) suppressed germination of the seeds (Table 1). In maize and sorghum germination was totally inhibited at crude oil levels of 10% - 20% v/w and 5% - 20% v/w respectively. In the two crops, germination time was increased by 24h as from 2.5% crude oil contamination. One hundred percent (100%) germination occurred in sorghum only at the 0.5% crude oil contamination; in maize 100% germination occurred in soils that contained up to 1.0% crude oil contamination (Table 2). At 2.5% crude oil contamination, only half (50%) of the seeds of each plant germinated. Even though maize seeds germinated in crude oil level of up to 5%, only 33% of the seeds were able to germinate at that level (Table 2).

Quantitative phytochemical analysis of seeds of the crops (Table 3) revealed that both seeds lacked saponin but contained phytate, tannins, flavonoids and alkaloids. Maize had a lower percentage of phytate and tannins and higher levels of flavonoids and alkaloids than sorghum.

Plant shoot growth and rhizofungal flora

The shoot lengths of the crops were significantly ($P < 0.05$) retarded by the oil (Figs. 1 and 2). This can be seen when the shoot lengths of the control crops (those with 0% crude oil pollution) are compared with those of the test plants. For instance, in maize, the mean maximum shoot lengths at the seventh week, of the control and test plants grown in soils with 1%, 2% and 2.5% crude oils varied from 60.6cm to 50.7cm, 32.1cm and 11.3cm respectively. For sorghum at the same period, the shoot lengths of the control and test plants varied from 45.5cm to 19cm, 15cm and 7cm respectively. The growth response of the two plants indicates that maize had higher tolerance for the crude oil than sorghum even though it failed to grow at 5.0% crude oil level after germination.

The effects of crude oil on fungi in maize and sorghum rhizospheres and bulk soils are presented in figures 3 and 4.Growth of rhizofungal and bulk soil fungal

populations were enhanced by low levels (0.5% and 1.0%) of crude oil (Figs.3 and 4). This pattern was also observed in our earlier study on the effects of crude oil on groundnut and cowpea rhizobacteria (Eze *et al*., 2013). High levels of crude oil (2.5% or beyond) however depressed the growth of rhizosphere and bulk soil fungi.
Petroleum hydrocarbons adversely affect plants in a variety of ways including coating of plant parts with hydrophobic substances thereby inhibiting transpiration and respiration, reducing cell membrane permeability, disrupting metabolic conversions leading to changes in chemical composition and through their toxicity (Pezeshki *et al.,* 2000). In addition, it has also been shown that high doses of petroleum hydrocarbons can depress germination in seeds (Sparrow and Sparrow, 1988; Malek-Hossein *et al.,* 2007; Amadi *et al.,* 1996). This has been confirmed by this study.

The lower phytate content of maize seeds could have enhanced their germination in soils polluted with crude oil level of up to 5.0%. This is because high levels of phytate (an anti-nutrient) have been reported to inhibit mineral nutrient absorption in organisms (Raboy, 2002; Urbano *et al.,* 2000). Some of these mineral elements (e.g. calcium, phosphorus and magnesium) are needed for seed germination. It is therefore reasonable to expect that when seeds with low phytate level are sown in crude oil polluted soils they may germinate better than those with higher phytate level because they probably will have only the external crude oil factor to contend with during germination. Results of this study show a remarkable retardation of shoot growth in the two plants as a result of crude oil pollution. This growth retardation effect of crude oil was also observed in our earlier work with cowpea and groundnut (Eze *et al*., 2013) and reported by other researchers that worked on other vascular plants (Bamidele and Igiri, 2011; Debojit *et al*., 2011; Lin and Mendelsshohn, 2009; Adoki and Orugbani,2007). When plant parts are coated with crude oil, the oil reduces respiration and cell membrane permeability in the affected parts due to hydrophobicity leading to reduction in nutrient absorption, metabolism and growth in the plants.

Crude oil had a dose-dependent suppressive effect on fungal populations in the rhizospheres of maize and sorghum and bulk soils adjacent to them. The rhizosphere fungi among others play very significant roles in the biodegradation of pollutants (Chi-A-Woeng, 1998). For this reason, every effort should be made to preserve them in the soil.

Even though marked reductions were observed in the populations of fungi in the rhizosphere and bulk soil as a result of crude oil pollution, higher populations occurred in the rhizosphere soil than in the adjacent bulk soil. This agrees with previous reports that there is increased microbiological activity within the rhizosphere (Nichols *et al.,* 1997; Clegg *et al.,* 2002; Kuiper *et al.,* 2003). This increase was probably caused by sloughed-off tissues and exudates from the plants, which served as nutrients to the microorganisms.

The increase that occurred in rhizofungal and bulk soil fungal counts in samples with low levels (0.5% and 1.0%) of crude oil was probably because low crude oil levels create a more favourable carbon -

Table.1 Effects of different levels of crude oil on germination time of the plants

	Crude oil levels								
Crop plants	**Control, 0.0%**	**0.5 %**	**1.0 %**	**2.0 %**	**2.5 %**	**5.0 %**	**10.0 %**	**15.0 %**	**20.0%**
	Germination time (days)								
Maize	4 ±0.5	4 ±0.5	4 ±0.5	4 ±0.5	5 ±0.5	5 ±0.6	*	*	*
Sorghum	4 ±0.5	4 ±0.5	4 ±0.5	4±0.5	5 ±0.6	*	*	*	*

* No germination

Table.2 Percentage germination of the plants in sandy loam soil polluted with varying concentrations of Bonny light crude oil.

	Crude oil levels								
	0%	0.5%	1.0%	2.0%	2.5%	5.0%	10.0%	15.0%	20.0%
	Germination (%)								
Maize	100	100	100	67	50	33	*	*	*
Sorghum	100	100	67	67	50	*	*	*	*

* No germination

Table.3 Phytochemical Contents of Seeds of the Crop Plants

Crop plants	Phytate (%)	Tannin (%)	Flavonoid (%)	Alkaloid (%)	Saponin (%)
Maize	1.90 ±0.19	0.023 ±0.003	5.0 ±0.70	7.5 ±0.42	–
Sorghum	2.09 ±0.64	0.030 ±0.007	3.0 ±0.35	7.0 ±0.35	–

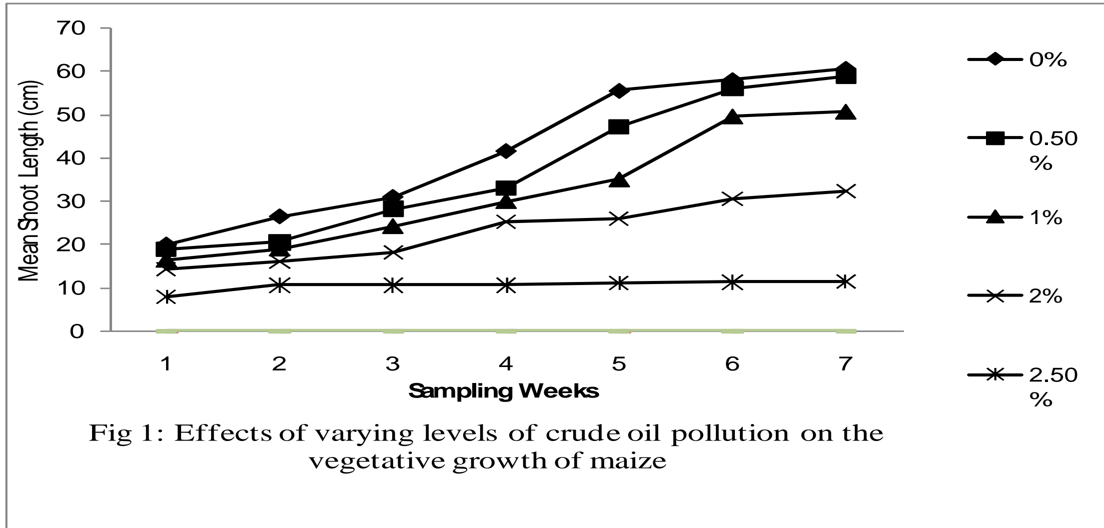

Fig 1: Effects of varying levels of crude oil pollution on the vegetative growth of maize

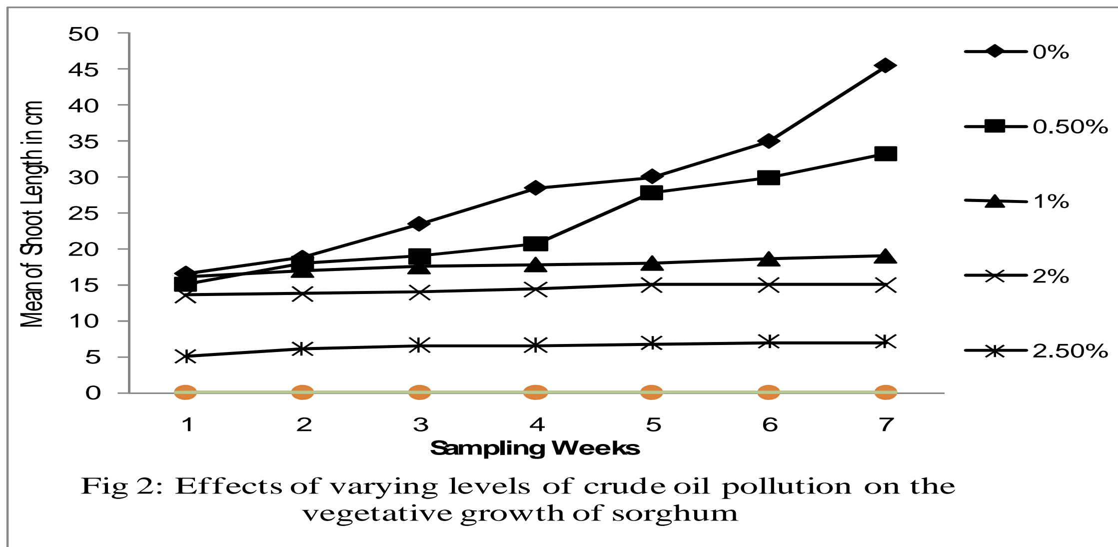

Fig 2: Effects of varying levels of crude oil pollution on the vegetative growth of sorghum

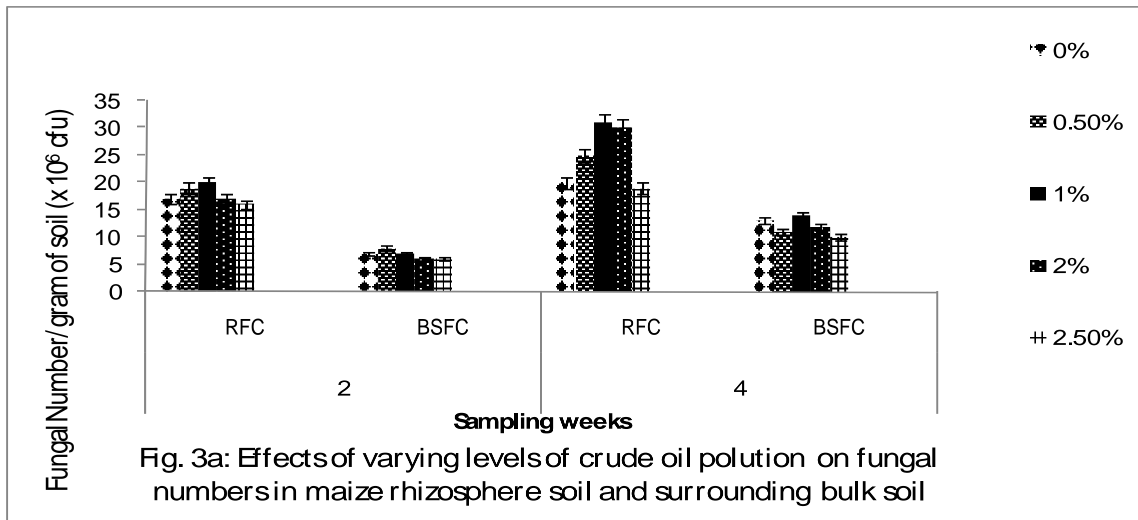

Fig. 3a: Effects of varying levels of crude oil polution on fungal numbers in maize rhizosphere soil and surrounding bulk soil

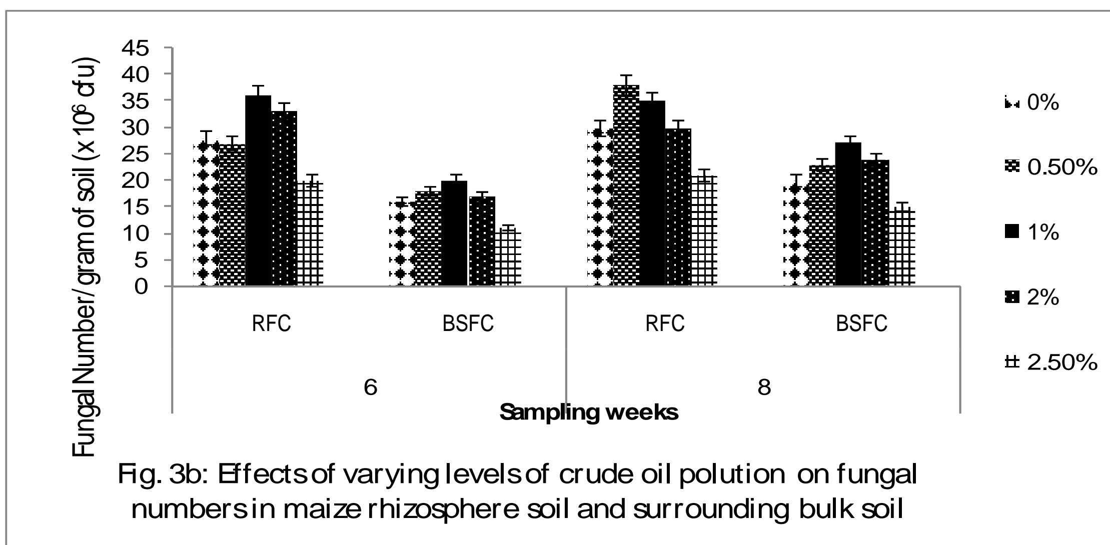

Fig. 3b: Effects of varying levels of crude oil polution on fungal numbers in maize rhizosphere soil and surrounding bulk soil

RFC = Rhizosphere fungal count; BSFC = Bulk soil fungal count

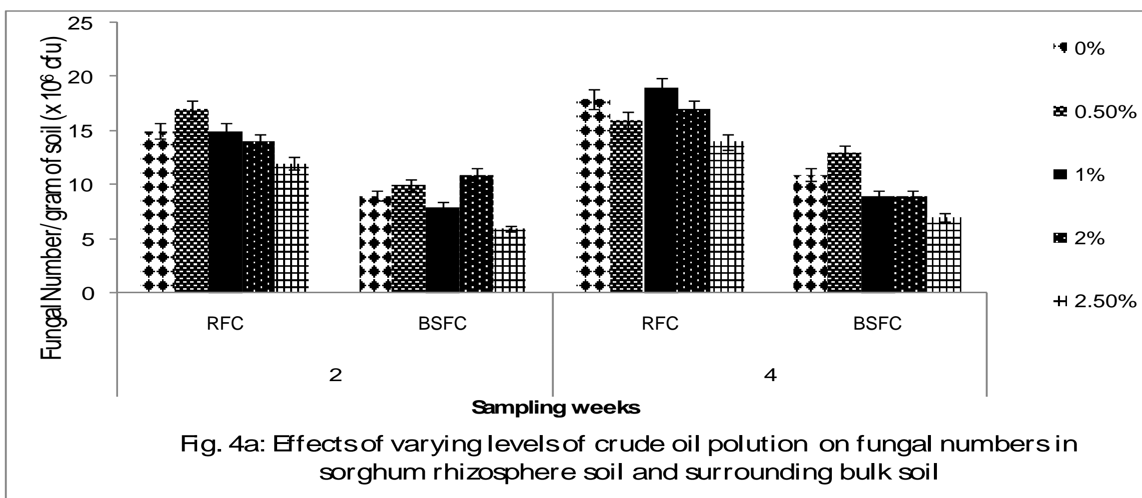

Fig. 4a: Effects of varying levels of crude oil polution on fungal numbers in sorghum rhizosphere soil and surrounding bulk soil

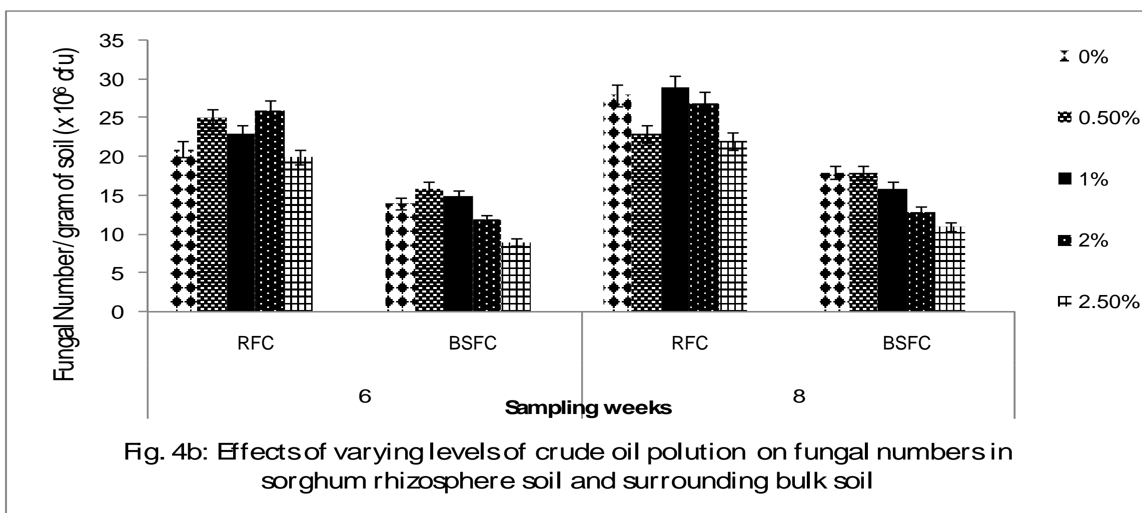

Fig. 4b: Effects of varying levels of crude oil polution on fungal numbers in sorghum rhizosphere soil and surrounding bulk soil

RFC = Rhizosphere fungal count BSFC = Bulk soil fungal count

nitrogen ratio (C:N ratio) for growth of hydrocarbonoclastic organisms than high oil levels. Microorganisms require about 10 parts of carbon to 1 part of nitrogen for good growth (Jobson *et al*.,1974). If the level of carbon is disproportionately increased bringing the C:N ratio to up to 100:1 or 1000:1 due for instance to oil spill, microbial growth and utilization of carbon will be hampered because there will be nitrogen deficiency in the polluted soil. Nitrogen and phosphorus are key nutrients needed by microbial degraders of petroleum hydrocarbons (Atlas and Bartha, 1973). Fungal population decreases observed in the rhizospheres and bulk soils at 2.5% crude oil occurred because high levels of crude oil in addition to causing nitrogen deficiency through C:N ratio imbalance, can retard the growth of organisms through toxicity and reduction in cell membrane permeability.

It can be deduced from the results (Tables 1-3; Figures 1 and 2) that maize resisted the toxic effects of crude oil more than sorghum. It had better germination and shoot growth as evidenced by its germination in soil sample with higher crude oil contamination (Tables 1 and 2) as well as its longer shoot lengths (figures 1 and 2). This suggests that maize may be a better phytoremediation candidate than sorghum since the major requirement for the use of any plant in phytoremediation of a polluted soil is its ability to grow and become established in the presence of the contaminant. Phytoremediation has been defined as the use of plants and their associated microorganisms to clean up contaminated habitats (Cunningham *et al.,* 1996).

The work also reveals the inhibitory effect of crude oil on the rhizosphere and bulk soil fungal flora of maize and sorghum (figure 3 and 4). These findings have not to our knowledge been reported in previous works.

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