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CYTOTOXIC INVESTIGATION OF CRUDE OIL IMPACT ON SELECTED CROPS VIA PRE-PLANTING AND POST-PLANTING TREATMENTS

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Abstract. Oil spills destroy farmlands with detrimental impact on agricultural crops, thereby deteriorating the health of humans and other animals that consume the crops from the polluted regions. Assays for this study were conducted between two soil groups polluted with 100 mL crude oil (A - soil polluted before planting [PB] and B - soil polluted two weeks after planting [PA]) within a period of 28 days from their pollution time; at Federal University of Technology, Owerri, Nigeria. The impact of oil spill on three edible plants (*Allium cepa, Telfairia occidentalis* and *Zea mays*) were evaluated through plant growth measurement, total chlorophyll test done by spectrophotometry of leaf samples and histo-anatomical investigations. Results obtained at the end of the experiment showed a decrease in plant height, leaf area and leaf number for all the polluted plants compared with their controls. However, plants grown on group A soil experienced delayed emergence and retarded growth, but survived better than plants grown on group B soil. The photomicrographs revealed alterations and anomalies in leaf structures for both polluted groups as compared to their control samples. A notable decline in total chlorophyll contents occurred amongst the plants in group B when compared to plants in group A. Therefore, this study deduced an elevated cytotoxic effect of crude oil in all the polluted crop samples, except for mild anomalies observed in the leaves of *T. occidentalis* in group A (UPB); which also showed no physical sign of crude oil pollution based on the non-observance of leaf yellowing, deformity or defoliation.

Key words: oil spill; foliar; cytotoxicity; anomalies; chlorophyll; histology.

INTRODUCTION

Nigeria being a major oil-producing country has predominant oil polluted sites especially in the Niger Delta regions and other oil-producing states, Imo State inclusive. Furthermore, the hydrophobic nature of crude oil easily injures or kills plants by inducing stomata closure, chloroplasts damage, tissue infiltration, gaseous exchange obstruction, vascular bundles damage, inefficient water and nutrient uptake as well as disruption of photosynthesis [6]. Crude oil has acute and chronic impacts on human health as most of its components are carcinogens, mutagens or teratogens that cause harm even at low concentration [26]. In this regard, there is an increase on the nation's food insecurity, cultural conflicts, unemployment and death rates, thus drastically affecting the country's economy and population respectively [3, 4]. Crude oil exhibits herbicidal effects on direct contact with plants as well as prevents germination and development of plants. Its negative impact on plants is depicted by their poor yield and retarded growth features such as reduced leaf area, leaf number, plant height, stem girth, plant biomass, further to root length, resulting from impaired absorption of essential nutrients by plant roots and limited water supply [5, 24]. The quantity of crude oil and its duration in the soil or on plants (if sprayed) influence the rate of toxicity in plants, as no germination occur at high concentration of crude oil while stunted growth and reduced performance happen at low concentration [9]. All these are attributed to the ability of the volatile oil to penetrate into the seed integument or through plant cuticles, giving rise to suffocation of embryo in seeds or coalescence of cells at different tissues due to oxygen deprivation. A lot of experimental studies have been conducted to reveal the effects of petroleum contaminants on the genetic and morphological structures of some plants. For instance, Komolafe *et al.* [13] and Iheme *et al.* [11] in their respective works, reported cell division anomalies (sticky and vagrant chromosomes) in *Sorghum bicolor* at different phases of mitosis and higher percentage of chromosomal aberrations in the plant as a result of petroleum toxicity. Hence this study was geared to ascertain the cytotoxic effect of crude oil on selected crops' cells and tissues through chlorophyll content test and tissue sectioning of leaves for crystals.

MATERIALS AND METHODS

Soil Sample Collection

Soil samples were collected randomly using sterilized soil auger within the depth of 0-20 cm from the Teaching and Research farm, School of Agriculture and Agricultural Technology (SAAT) Research farm FUTO, Nigeria, as the site has no history of crude oil contamination. The samples were air-dried and seived using a 2 mm mesh sieve. Then, 5 kg soil each was weighed into perforated sterile seed bags and a quantity taken to the laboratory for analysis within 1 hour of collection. The crude oil used for this research is bonny light which was collected with sterile containers from Akiri in Oguta, Imo State, Nigeria.

Exposure to crude oil

The experiment was carried out in two (2) different groups as adopted from the work of Firi-Appah *et al.* [9]. Group A was polluted before planting (PB) using same quantity (100 mL) of crude oil poured into 5 kg soil which was mixed to homogeneity using a spatula

while Group B was polluted 2 weeks after planting (PA) using 100 mL of crude oil together with 50 mL of sterile water poured evenly to the 5 kg soil and around the plants (ring application). Unpolluted group served as control with zero amount of crude oil. The same procedure was repeated for all test plants in triplicates and the seed bags were labelled properly, making a total of 27 seed bags to be arranged in a completely randomized design.

Plant Growth Experiment

Three viable seeds of each test plant (*Zea mays*, *Allium cepa* and *Telfairia occidentalis*) were planted in each of the labelled perforated bag and kept under a shade away from excessive sunshine and rainfall while being watered every 48 hours. The growth characteristics including plant height, number of leaves and leaf area were calculated for each experimental group on fortnight basis for a period of 28 days after exposure to crude oil [20, 27].

Plant Height (cm). Plant height for each test plant, was measured using a meter rule. Measurement was from the soil surface to the tip of the plant for *Allium cepa* and *Telfaria occidentalis* but for *Zea mays*, it was from the soil surface to the highest point of the arch of the uppermost leaf.

Number of leaves. The number of healthy leaves on each test plant was counted on fortnight basis for each plant stand and the average number of leaves was calculated.

Leaf Area (cm^2). The leaf area of each test plant was calculated by multiplying the leaf's length by its breadth**.**

Anatomical analysis to determine crude oil toxicity on the leaves of test plants

Sectioning of leaves of test plants was done at the end of the experiment according to the procedures outlined by Ilodibia *et al.* [12] and Moghanm *et al.* [16] with some modifications. The samples (0.5 cm length) were dipped in formalin-acetic-alcohol solution (2:1:1v/v) for 1 week, in order to impede autolysis and fix the cells. After which, they were rinsed with water and dehydrated by passing through ascending series of ethanol (60%, 70%, 80% till absolute concentration) for 30 minutes each. Then, the samples were further subjected to two changes of chloroform for 30 minutes each and embedded in molten paraffin wax at 50°C for another 30 minutes. Subsequently, a sledge microtome was used to section the leaves into slices of about 5-10 micrometres thick before staining with a primary dye; safranin and counter-staining with methylene blue. In order to mount the specimens on slides, Canada balsam was utilized. A 22 mm × 22 mm cover-slip was used to meticulously cover each slide. Consequently, photomicrographs were obtained while the mounted specimens were examined under a bright-field light microscope.

Determination of chlorophyll content in test plants leaves

Using spectrophotometry on leaf samples extracted with 80% acetone as described by Liang *et al*. [14], the chlorophyll content of test plants was calculated at intervals of 14 days for each experimental group. Approximately 1 g of fresh leaves after being blended at room temperature using a laboratory homogenizer, were centrifuged for 5 minutes in a 1.5 mL tube with 1 mL of an 80% acetone solution. A double-beam spectrophotometer was used to measure the absorbance of the supernatant at wavelengths of 646 nm and 663 nm (A646 and A663). Samples with absorbances above one were diluted by half with 80% acetone and then reassessed. The following equation was used to estimate the concentration of chlorophyll:

$$\text{Chlorophyll a } (\mu g/mL) = -1.93 \cdot A646 + 11.93 \cdot A663$$

$$\text{Chlorophyll b } (\mu g/mL) = 20.36 \cdot A646 - 5.50 \cdot A663$$

$$\text{Total chlorophyll } (\mu g/mL) = 6.43 \cdot A663 + 18.43 \cdot A646$$

Statistical Analysis

All data samples from each experimental group in triplicates were analyzed statistically using Microsoft Excel 2013 and Mintab 2017 one-way analysis of variance (ANOVA). The means were separated with Dunnett's multiple test at $p<0.05$. Results were presented in mean ± standard deviation (SD).

RESULTS

Plant Growth Analysis

The plant height, leaf area and leaf number as clearly depicted correspondingly in figures 4, 5, 6, 7, 8 and 9; where the plant growth parameters were measured at 2 week intervals for both experimental groups (A - polluted before planting and B - polluted 2 weeks after planting), beginning from their pollution time till 28 days after each group exposure to crude oil.

Results obtained showed a decrease in plant height, leaf area and leaf number for both polluted groups as compared to their control samples at day 14 and day 28 respectively. As shown in figure 4 for group A plants, huge significant differences ($p<0.05$) were recorded between the plant heights of treated and untreated *Allium cepa* and *Zea mays* at both day 14 and day 28 but, the values for *Telfairia occidentalis* were insignificantly different at day 14 (UC = 38.60^a, UPB = 22.27^a) and day 28 (UC = 53.47^a, UPB = 31.23^a) respectively. However, the height values for all plants in group B as expressed in figure 5, indicated that the polluted plants were statistically different from their controls at days 14 and 28. Figures 6 and 7 showed that with the exception of *Telfairia occidentalis* in group A, which showed no significant change at day 14, there were substantial differences ($p<0.05$) in the leaf area of all group A and group B plants. According to figure 8 and figure 9, which graphically depicted the number of leaves for all the plants in groups A and B, there were significant differences between OC and OPB, OC and OPA, MC and MPB, MC and MPA, and UC and UPA

at days 14 and 28 while UC and UPB were statistically the same at those times.

Anatomical Evaluation of the leaves of test plants

The cross-sectional leaves of the crude oil treated and untreated test plants (maize, onion and fluted pumpkin) were represented in photomicrographs as shown correspondingly in figures 1, 2 and 3. Figure 1 compared the leaf structures of contaminated samples (maize polluted before planting - MPB and maize polluted 2 WAP - MPA) with an uncontaminated sample (maize control - MC). Observations from MC revealed the upper and lower epidermis of the leaf, the stoma, vascular bundles (xylem and phloem) with their vessels encircled by chloroplast-filled bundle sheath cells and layers of mesophyll cells. MPB showed enlarged vascular bundles and coalesced mesophyll cells while MPA exhibited total cell coalescence as well.

The sectioned leaves of onion polluted before planting (OPB) and onion polluted 2 WAP (OPA) were presented in figure 2, together with the unpolluted plant (onion control - OC). The photomicrograph for OC showed the leaf epidermis, stoma, vascular bundle and parenchyma in normal condition whereas OPB revealed distorted cells. Then, the vascular bundles and leaf epidermis of OPA were shrivelled.

Fluted pumpkin leaves exposed to crude oil (fluted pumpkin polluted before planting - UPB and fluted pumpkin polluted 2 WAP - UPA) were displayed in figure 3 with the untreated fluted pumpkin leaves (fluted pumpkin control - UC). Healthy forms of spongy parenchyma, vascular bundles and epidermis were observed in UC but, UPB unveiled enlarged vascular bundles and slightly enlarged spongy cells. However, deformed spongy parenchyma and shrivelled vascular bundles were discovered in the photomicrograph for UPA.

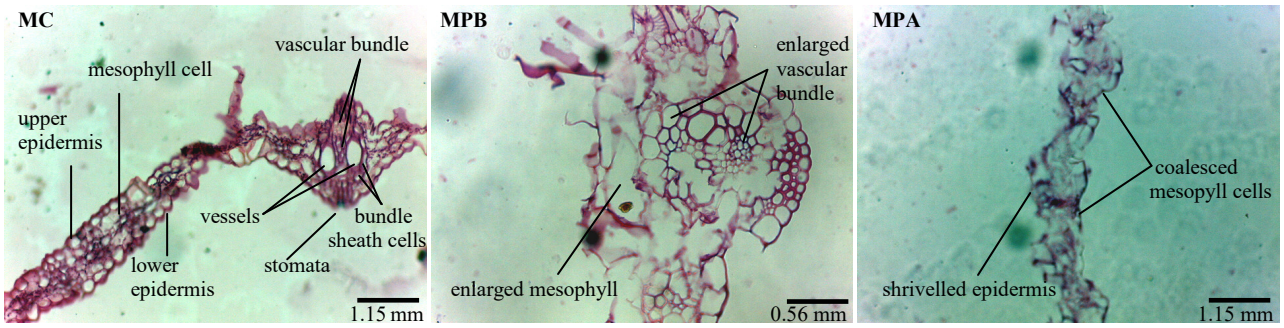

Figure 1. Photomicrographs of polluted and unpolluted maize leaves. **Note:** MC - Maize Control, MPB - Maize Polluted before Planting, MPA - Maize Polluted 2 WAP.

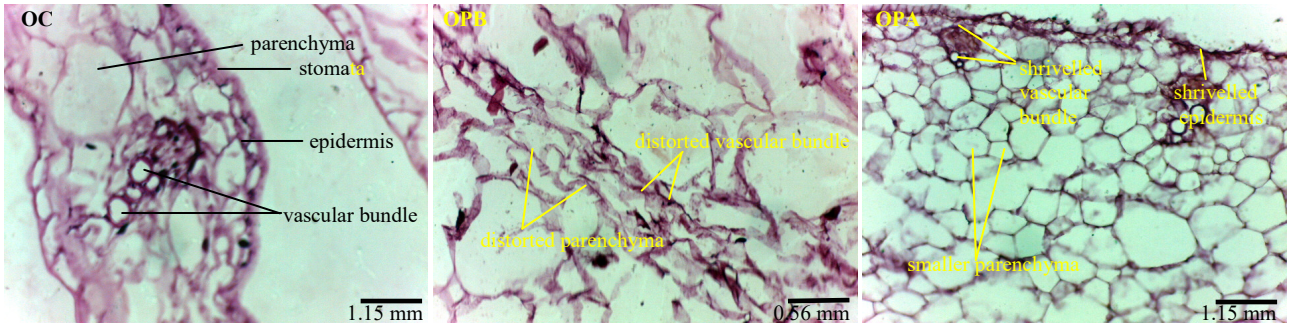

Figure 2. Photomicrographs of polluted and unpolluted onion leaves. **Note:** OC - Onion Control, OPB - Onion Polluted before Planting, OPA - Onion Polluted 2 WAP.

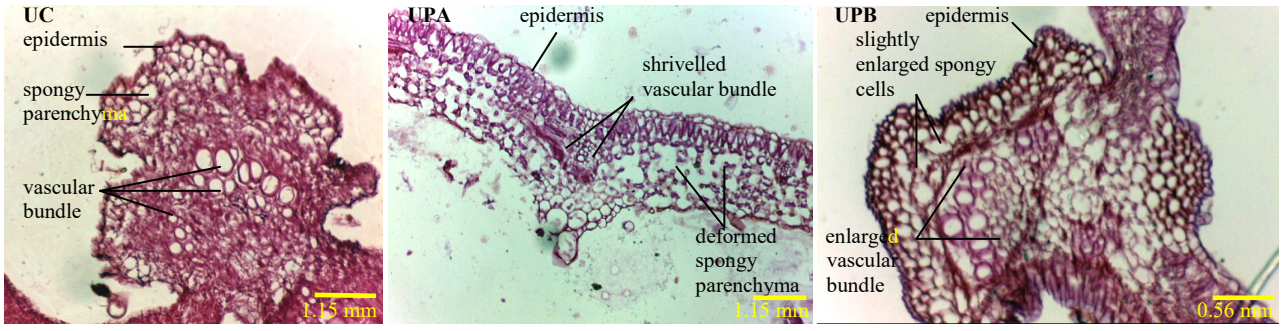

Figure 3. Photomicrographs of polluted and unpolluted fluted pumpkin leaves**. Note:** UC - Fluted pumpkin Control, UPB - Fluted pumpkin Polluted before Planting, UPA - Fluted pumpkin Polluted 2 WAP

Total chlorophyll content of the leaves of test plants

Total chlorophyll content of plants in group A was determined at day 14 and day 28 as shown in figure 10. Results obtained showed higher chlorophyll values for UC, UPB, OC, OPB and MC at day 28 compared to their values at day 14. Meanwhile, the total chlorophyll content values for MPB had no noticeable rise at days 14 and 28 respectively. For the control samples, UC had the highest total chlorophyll value, followed by MC and finally OC whereas the total chlorophyll values for the polluted samples were highest in UPB, then MPB and least in OPB. However, a notable decrease in total chlorophyll values were recorded between OC and OPB as well as MC and MPB at both day 14 and 28 but, no significant change ($p<0.05$) was observed between UC and UPB at days 14 and 28 respectively.

Figure 11 portrayed the total chlorophyll contents of group B plants at days 14 and 28. All the control samples had higher chlorophyll values than their polluted counterparts on both days. An obvious decline in total chlorophyll contents occurred statistically ($p<0.05$) between UC and UPA, OC and OPA as well as MC and MPA even though; total chlorophyll content values were not detected for UPA and MPA at day 28.

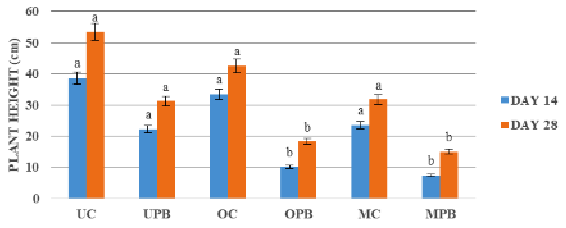

Figure 4. Plant Height of plants in group A at Day 14 and Day 28 respectively. **Note:** MC - Maize Control, MPB - Maize Polluted before Planting, OC - Onion Control, OPB - Onion Polluted before Planting, UC - Fluted pumpkin Control, UPB - Fluted pumpkin Polluted before Planting. Values are mean ±standard deviation from triplicates. Means not labelled with the letter a are significantly different from the control level mean of each test plant at ($p<0.05$).

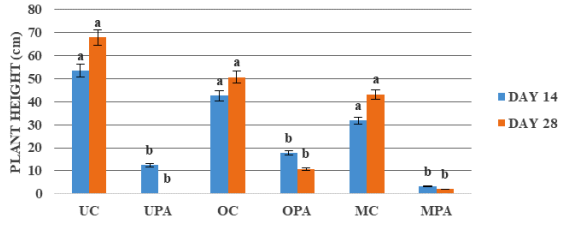

Figure 5. Plant Height of plants in group B at Day 14 and Day 28 respectively**. Note:** MC - Maize Control, MPA - Maize Polluted 2 WAP, OC - Onion Control, OPA - Onion Polluted 2 WAP, UC - Fluted pumpkin Control, UPA - Fluted pumpkin Polluted 2 WAP. Values are mean ±standard deviation from triplicates. Means not labelled with the letter a are significantly different from the control level mean of each test plant at ($p<0.05$).

DISCUSSION

Results on growth performance of test plants showed luxuriant growth of the control samples but a decrease in plant height, leaf area and leaf number for both polluted groups as compared to their control samples. All these can possibly be attributed to the ability of the volatile oil to penetrate into the seed integument or through plant cuticles, giving rise to suffocation of embryo in seeds or coalescence of cells at different tissues due to oxygen deprivation.

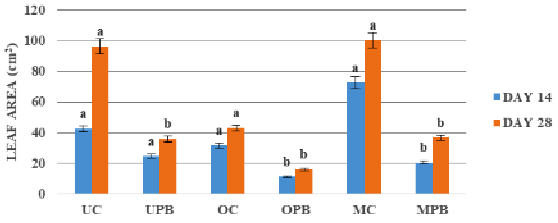

Figure 6. Leaf Area of plants in group A at Day 14 and Day 28 respectively. **Note:** MC - Maize Control, MPB - Maize Polluted before Planting, OC - Onion Control, OPB - Onion Polluted before Planting, UC - Fluted pumpkin Control, UPB - Fluted pumpkin Polluted before Planting. Values are mean ±standard deviation from triplicates. Means not labelled with the letter a are significantly different from the control level mean of each test plant at ($p<0.05$).

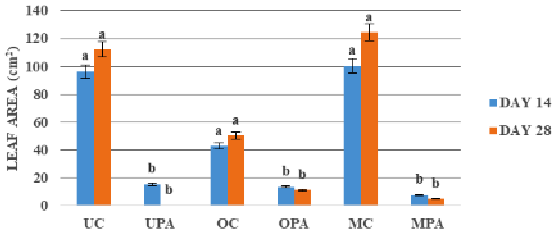

Figure 7. Leaf Area of plants in group B at Day 14 and Day 28 respectively. **Note:** MC - Maize Control, MPA - Maize Polluted 2 WAP, OC - Onion Control, OPA - Onion Polluted 2 WAP, UC - Fluted pumpkin Control, UPA - Fluted pumpkin Polluted 2 WAP. Values are mean ±standard deviation from triplicates. Means not labelled with the letter a are significantly different from the control level mean of each test plant at ($p<0.05$).

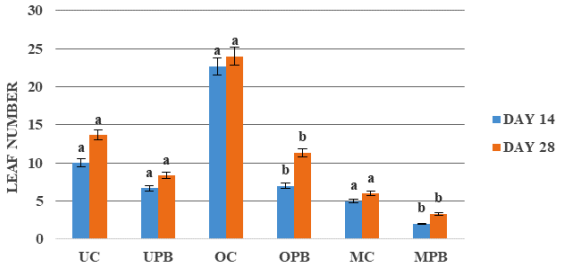

Figure 8. Leaf Number of plants in group A at Day 14 and Day 28 respectively. **Note:** MC - Maize Control, MPB - Maize Polluted before Planting, OC - Onion Control, OPB - Onion Polluted before Planting, UC - Fluted pumpkin Control, UPB - Fluted pumpkin Polluted before Planting. Values are mean ±standard deviation from triplicates. Means not labelled with the letter a are significantly different from the control level mean of each test plant at ($p<0.05$).

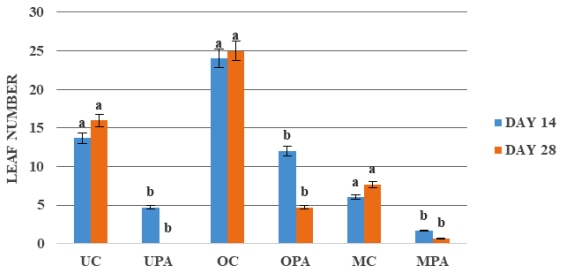

Figure 9. Leaf Number of plants in group B at Day 14 and Day 28 respectively. **Note:** MC - Maize Control, MPA - Maize Polluted 2 WAP, OC - Onion Control, OPA - Onion Polluted 2 WAP, UC - Fluted pumpkin Control, UPA - Fluted pumpkin Polluted 2 WAP. Values are mean ±standard deviation from triplicates. Means not labelled with the letter a are significantly different from the control level mean of each test plant at ($p<0.05$).

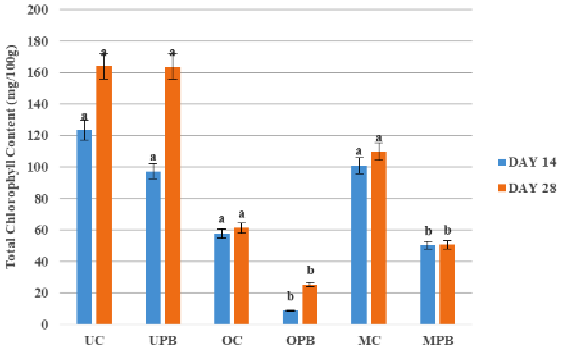

Figure 10. Total Chlorophyll content of plants in group A at Day 14 and Day 28 respectively. **Note:** MC - Maize Control, MPB - Maize Polluted before Planting, OC - Onion Control, OPB - Onion Polluted before Planting, UC - Fluted pumpkin Control, UPB - Fluted pumpkin Polluted before Planting. Values are mean ±standard deviation from triplicates. Means not labelled with the letter a are significantly different from the control level mean of each test plant at ($p<0.05$).

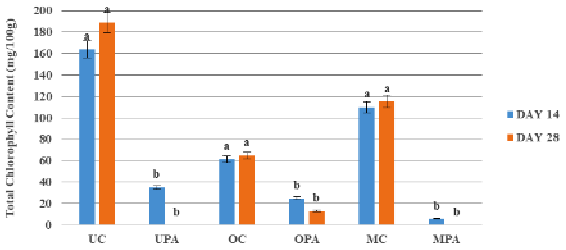

Figure 11. Total Chlorophyll content of plants in group B at Day 14 and Day 28 respectively. **Note:** MC - Maize Control, MPA - Maize Polluted 2 WAP, OC - Onion Control, OPA - Onion Polluted 2 WAP, UC - Fluted pumpkin Control, UPA - Fluted pumpkin Polluted 2 WAP. Values are mean ±standard deviation from triplicates. Means not labelled with the letter a are significantly different from the control level mean of each test plant at ($p<0.05$).

According to Gbadebo and Adenuga's experiment [10], cowpea growth rate decreased with increasing crude oil contamination, as did the number of leaves and the percentage of protein in the plant's stems. In 2016, Obute *et al.* [17] reported in their work that refined petroleum products caused varying anomalies in mitosis, germination and development of *Allium cepa*. Ogbo *et al*. [19] stated that crude oil caused a notable decrease in the biomass, height and leaf area of *Paspalum scrobiculatum*. Osuagwu *et al*. [24] also observed significant reduction of plant growth, yield and leaf chlorophyll content in air potato after crude oil pollution; hence the reduced leaf area, number and chlorophyll content interrupted its photosynthetic activity. Even so, a very mild decrease was observed in plant height, leaf area and leaf number for *Telfairia occidentalis* in group A (UPB) and this might probably be due to its phytoremediation capability, as was observed in *Arachis hypogaea* which showed better tolerance of crude oil due to the endophytic bacteria it harbors [11].

Based on the morphological evaluation of all the polluted plants, onion and maize plants in group A had delayed seed emergence for about a week and experienced retarded growth but later survived better than the plants in group B, which exhibited high level of leaf wilting, slimy shoots, chlorosis, necrosis and even death recorded at day 28 for maize plants. This precisely confirmed the results on morphological evaluation of *Telfaria occidentalis*, which showed restrained seed emergence in a pre-existing crude oil polluted soil and eventually leaf chlorosis, as well as plant death in soil samples polluted 3 WAP as described by Erhenhi and Ikhajiagbe [7].The way that crude oil was applied and how long it stayed in the soil or on the plants were found to have an impact on the rate of toxicity in our research groups.

Alterations and anomalies in leaf structures were mostly discovered in the polluted plants (MPB, MPA, OPB, OPA, UPB and UPA) but, the control plants (MC, OC and UC) had well-arranged and healthy leaf structures. The results clearly demonstrated coalesced mesophyll cells in MPB and MPA, distorted cells in OPB and deformed spongy parenchyma in UPA. As we recently reported in a companion study [15] where varying total hydrocarbon content levels were found in the leaves of same test plants utilised for this investigation, these defects were the result of crude oil bioaccumulating in plant cells. This hindered efficient photosynthesis and oxygen diffusion in the treated plants; thereby causing suffocation, stunted growth and necrosis in them [16]. On the other hand, an experiment conducted by Oluwanisola and Abdulrahaman [23] expressed the negative influence that spent engine oil had on the internal structures of okra plant.

Maize and onion are monocotyledonous plants whose leaves are linear-shaped with stomata located on both upper and lower epidermis as shown in the photomicrographs labelled MC and OC whereas, ugu (UC) is a dicotyledonous plant with broader leaves whose stomata are located on either side of the epidermis. Conversely, the shrivelled epidermis seen in OPA confirmed leaf wilting and inadequate gaseous exchange caused by the hydrophobic and air impenetrable nature of crude oil polluted soil. Komolafe *et al*. [13] reported in their work that petrol

and spent oil, at higher concentration, negatively impacted the epidermal layer and stomata of guinea corn leaf.

The vascular bundles (xylem - for water and dissolved minerals dispersal and phloem - for sugar transportation) found in both monocot and dicot leaves are surrounded with bundle sheaths which aid in photosynthesis solely in C4 plants, such as maize and onion. Consequently, the shrivelled vascular bundles seen in OPA and UPA may have caused nutritional deprivation and plant death in these species. Similar description was made by Baruah *et al*. [2] about compressed vascular bundles obtained in *Cyperus brevifolius* planted in soil polluted with 50,000 ppm crude oil. Accumulation of potentially toxic elements (PTEs) in water hyacinth caused reductions in the plant epidermis, cortex and vascular bundles [11]. However, the enlarged vascular bundles and spongy cells shown in MPB and UPB could be traced to the plants adaptive response to poor supply of water, nutrients and oxygen; which to an extent reduced their growth performance when compared to the control plants. Results from this study is supported by the reports of Punwong *et al*. [25] and Olaranont *et al*. [21] which demonstrated the negative impacts of crude oil on the physiological and anatomical parameters of coastal plants (*Terminalia catappa* L. and *Ischaemum muticum* L.) respectively.

Chlorophyll concentration and light intensity both play important roles in photosynthesis because at greater concentrations, only limiting photon flux densites are required, whilst lower chlorophyll concentrations function effectively under high light intensities. The modified total chlorophyll contents observed in all the treated plants, strongly indicated the limited exposure of their leaves to sunlight due to the significant reduction in leaf area and leaf number. Hence, the damages in the plants cells and tissues were likely caused by nutrient unavailability as a result of crude oil toxicity. This corresponds with the report from Olubodun and Eriyamremu [22] on reduced total chlorophyll, glucose and starch contents of maize polluted with varying crude oil fractions. The hydrophobic nature of crude oil easily injures or kills plants by inducing stomata closure, chloroplasts damage, tissue infiltration, gaseous exchange obstruction, vascular bundles damage, inefficient water and nutrient uptake as well as disruption of photosynthesis. In addition, Odiyi *et al*. [18] stated a decline in leaf chlorophyll content of maize plant which adversely affected its yield.

Hazardous effects of petroleum products on the chlorophyll contents of *Barbula lambarenensis* was also reported by Fatoba *et al*. [8]. A study conducted at the amazon rain forest of Ecuador highlighted that decreasing chlorophyll contents in petroleum polluted plants indicate stress-response even at family-specific levels [1]. Also, results from the experiment carried out by Baruah *et al*. [2] demonstrated that chlorophyll content of *Cyperus brevifolius* reduced as the crude oil concentration increased.

The stress-adaptive ability of UPB was portrayed in this study due to its exceptional increase in total chlorophyll content on both day 14 and 28. However, UPA and MPA showed mottled leaves, necrosis and even mortality on day 28. In our study groups, it was found that the method of applying crude oil and the length of time it remained in the soil or on the plants affected the rate of toxicity. However, based on the absence of leaf yellowing, deformation, or defoliation; *Telfairia occidentalis* in group A (UPB) did not exhibit any outward symptoms of crude oil contamination.

Conflict of Interest. There is no actual or potential conflict of interest in relation to this article.

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