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Evaluation of the Effects of Bioaugmentation and Biostimulation on the Vegetative Growth of *Zea mays* Grown in Crude Oil Contaminated Sandy Loam Soil

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Abstract
Crude oil pollution of arable lands with consequent loss of soil fertility is a major problem associated with the exploration and exploitation of this natural resource. In this study the effect of crude oil on seed germination and shoot growth of maize crop as well as the efficacy of some bioremediation techniques for crude oil contaminated soil were evaluated. Bonny light crude oil (specific gravity : 0.81; API gravity : 43.2^0) 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 of soil) for the controlled pollution of pristine soil samples. Thereafter, seeds of *Zea mays* (maize) were sown and the soil samples treated with either NPK compound fertilizer or bacterial inoculant. Control soil samples were polluted with crude oil but not treated with either fertilizer or bacterial inoculant. Two levels of compound fertilizer were used at carbon to nitrogen ratios of 10.0:1.0 and 10.0: 2.0. Bacterial inoculant was also used at two levels consisting, $5x10^8$ cells/mL and 10 $x10^8$ cells/ml of *Bacillus subtilis*. Soil bioremediation was assessed by the growth response of the maize plants. Results obtained showed biostimulation with compound fertilizer enhancing growth in maize up to 10% crude oil level whereas bioaugmentation with *B. subtilis* could only produce growth up to 5% crude oil pollution. Plant growth response increased with increase in inoculum level but decreased with increase in fertilizer level, suggesting a possible toxicity of thefertilizer to hydrocarbonoclastic microorganisms at high concentrations.

1. Introduction
Crude oil pollution has evoked much anxiety in different parts of the world today owing to the ecological havoc associated with it. This natural resource often referred to as “black gold” has however produced a dramatic turn-around in the economies of many nations as well as world transportation technology and industry. Even though the merits associated with the use of petroleum are deemed by many to outweigh the demerits one cannot over-emphasize the monumental damage caused by petroleum on the biotic and abiotic components of the ecosystem [1-10]

The release of crude oil and its products into the environment is a threat to agricultural land. Petroleum hydrocarbons (PHCs) sterilize’ the soil and prevent crop growth and yield for varying periods of time [11]. For example, a good percentage of oil spills that occurred on dry land, between 1978 and 1979 in Nigeria affected farms in which crops such as rice, maize, yams, cassava and plantain were cultivated [12]. The effect of petroleum on the germination and growth of some plants have been reported [13]. The recovery of soil fertility after an oil spill depends on several factors including quantity spilled [14]. Restoration of the fertility of agricultural land previously contaminated by oil is of great importance.

The deleterious effects of crude petroleum on many components of the ecosystem create the need for clean-up techniques to restore polluted environments. Consequently, different governments, research groups and environmental managers are constantly devising approaches and strategies to reclaim contaminated environments [15-20]. Physical and chemical clean-up methods include burning, sinking, mechanical removal and use of detergents. However these methods have their side-effects. Majority of them are expensive while some only transfer the oil to a different location or even pollute the environment further. Fortunately there exist in nature, microorganisms which can degrade petroleum hydrocarbons without any inimical after-effects. These organisms, known as hydrocarbonoclastic microorganisms are widely distributed in many ecosystems including soils, water and marine sediments [21,22]. The hydrocarbonoclastic microorganisms are key players in the biological method of crude oil removal.

This method, also called bioremediation, is both ecofriendly and cost-effective but itself has the shortcoming of being too slow under normal conditions. This is why the focal point of current bioremediation researches is on strategies to speed up the reclamation of polluted environments. The paper evaluates the relative efficacies of two bioremediation strategies namely, bioaugmentation and biostimulation on the vegetative growth of *Zea mays* (maize plant) grown in crude oil contaminated sandy loam soil.

2. Materials and Methods

2.1. Materials Used

The major materials used in the study were:

Bonny light crude oil which was obtained from the Nigerian National Petroleum Corporation (NNPC) Port Harcourt Refinery, Alesa-Eleme, Rivers State, Nigeria

Pristine sandy loam soil (pH=6.01) collected from Botanical Garden, University of Nigeria, Nsukka

Viable seeds of maize (*Zea mays*) purchased from Ogige Market, Nsukka

Compound fertilizer (NPK 15:15:15) purchased from Ogige Market, Nsukka

Stock culture of a hydrocarbonoclastic strain of *Bacillus subtilis* (obtained from the author's collection) used for bioaugmentation study.

2.2. Determination of the Effects of Crude Oil on Seed Germination and Vegetative Growth of Zea mays

Sandy loam soil samples were air-dried, sieved and dispensed in 3kg weights into twenty-seven (27) plastic buckets (20cm x 20cm) arranged in triplicates and perforated at their bases to prevent water-logging. Eight triplicate buckets (24) were the test samples while the remaining triplicate was the control. Each triplicate in the test group was polluted with one of eight levels (0.5%, 1.0%, 2.0%, 2.5%, 5.0%, 10.0%, 15.0% or 20.0% v/w) of Bonny light crude oil. The control was not polluted. Two seeds of maize were sown in each bucket immediately after pollution and buckets kept in a greenhouse and watered every four days. Germination time was recorded for seeds in every bucket and measurement of plant shoot length was done initially fourteen days after seed sowing and subsequently done weekly for seven weeks.

2.3. Simulated Bioremediation Experiment

The efficacies of two bioremediation techniques (biostimulation and bioaugmentation) in the remediation of crude oil contaminated soils were investigated. The parameter used to evaluate the degree of remediation was the growth response of the maize plants used.

Two bioremediation agents were used for the study: NPK compound fertilizer (15% nitrogen) and a hydrocarbonoclastic strain of *Bacillus subtilis.* Two levels of each soil amendment were prepared, and for the fertilizer the levels consisted of carbon to nitrogen ratios (C: N ratio) of 10:1 and 10:2. The inoculum levels were 5ml and 10ml of cells of *B. subtilis* suspended in normal saline to a level of 0.5 McFarland standard which contained approximately 1×10^8 cells/ml.

The pristine sandy loam soil was air-dried, sieved and dispensed in 3kg weights into one hundred and twenty (120) plastic buckets (20 x 20cm) divided into five groups with each group comprising a total of twenty-four (24) buckets. One group was the control while the remaining four groups were the biostimulation and bioaugmentation test groups. Each group of twenty-four was arranged in eight triplicates and each triplicate was polluted with one of eight levels (0.5%, 1.0%, 2.0%, 2.5%, 5.0%, 10.0%, 15.0% or 20.0% V/W) of Bonny light crude oil. Similarly each triplicate bucket in a test group was treated with one of two levels of either NPK fertilizer or bacterial inoculum. The control samples were also grouped in triplicates but without fertilizer or inoculum. The buckets were perforated at their bases to prevent water-logging. Two seeds of maize were sown in each bucket seven days after treatment. Thereafter all the buckets were kept in a greenhouse and watered every four days. Measurement of plant shoot length was done every week for seven weeks.

3. Results

3.1. Effects of Crude Oil on Seed Germination and Vegetative Growth of Maize Plants

The effect of crude oil on seed germination is presented in Table 1. Crude oil suppressed germination of the maize seeds at 2.5% and 5.0% pollution and as from 10.0% germination was totally inhibited (Table 1).The effect of crude oil on the shoot length of the crop is presented in Fig. 1.

Table 1: Effect of Different Levels of Crude Oil on Germination Time of the Maize Seeds

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

*No germination observed

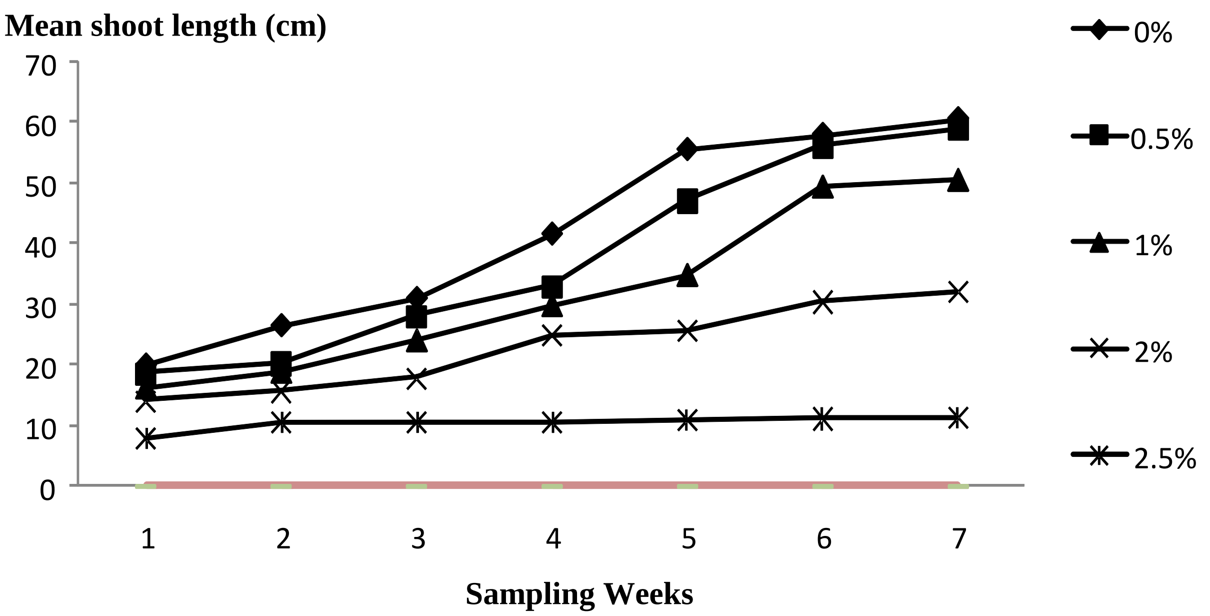


Fig 1: Effects of varying levels of crude oil pollution on the vegetative growth of maize

Crude oil significantly ($P < 0.05$) retarded shoot growth in the plant and the degree of retardation was crude oil dose-dependent. There was growth stagnation at 2.5% crude oil contamination whereas at 5% no growth occurred even though there was evidence of germination which was severely delayed (Table 1).

3.2. Effects of Biostimulation and Bioaugmentation on the Germination and Vegetative Growth of Zea mays.

The effects of the two bioremediation techniques namely, biostimulation and bioaugmentation on the recovery of crude oil polluted arable soil samples as evaluated by seed germination and subsequent vegetative growth of the maize plants in the polluted soils are shown in Fig. 2 and 3. The compound fertilizer, NPK produced germination and growth in the maize plant at 10% crude oil pollution level (Fig. 2). This is an improvement over the control (Table 1) where germination not only stopped at 5.0% crude oil pollution but the germinated seed failed to grow and died soon afterwards. The bioaugmentation agent, *B. subtilis* even though could not produce germination beyond 5.0% pollution was able to enhance growth at 5.0% pollution (Fig. 3).

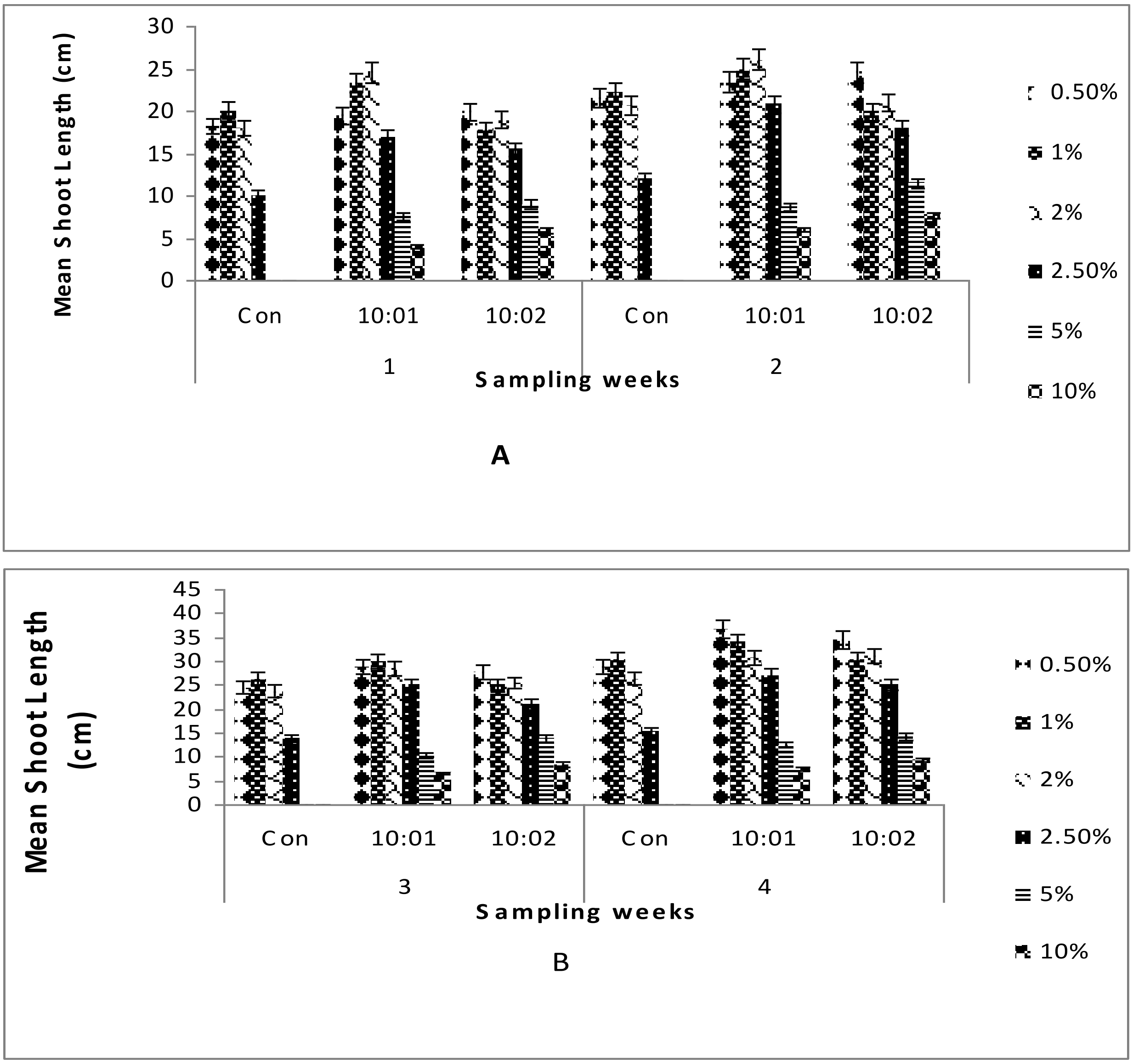


Fig 2: Effects of Compound Fertilizer (NPK- 15:15:15) Supplementation on the Vegetative Growth of Maize in Sandy Loam Soil with Varying Levels of Crude Oil Contamination. Con = control10:01, 10:2 = Carbon to Nitrogen (C: N) ratios.

The NPK fertilizer enhanced growth in maize more than the bacterial inoculant *Bacillus subtilis* as evidenced by the higher shoot lengths of the plants seen in the biostimulated samples. The first level

(C:N ratio of 10:1) of fertilizer enhanced soil recovery more than the second level (C:N ratio of 10:2) whereas the bioaugmentation agent caused better growth enhancement at the second level (10ml) than at the first level (5ml) in the plants. Comparatively, the biostimulation agent used enhanced soil recovery more than *B. subtilis*.

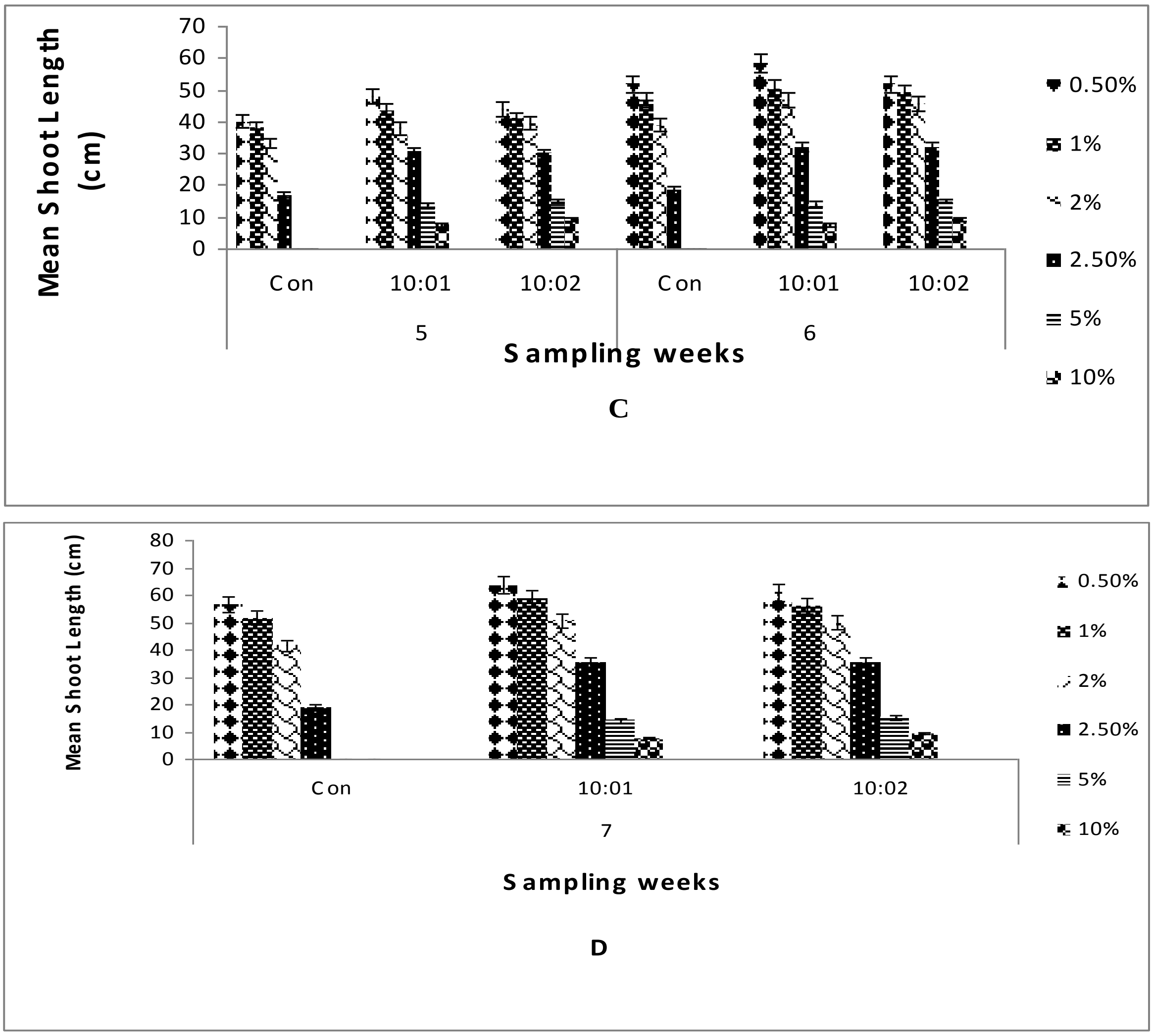


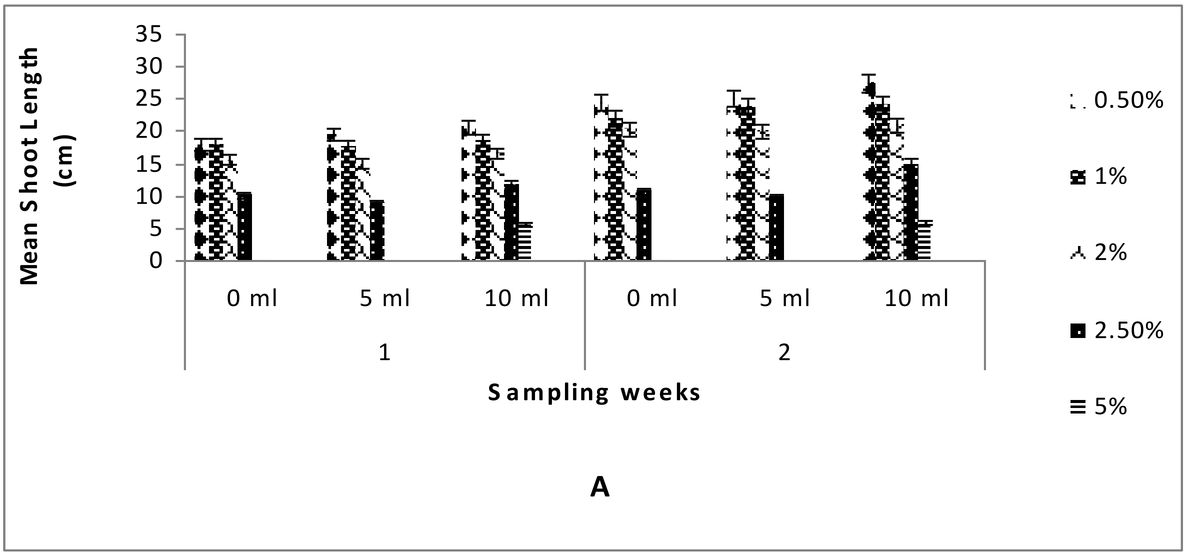
Fig 2: Effects of Compound Fertilizer (NPK- 15:15:15) Supplementation on the Vegetative Growth of Maize in Sandy Loam Soil with Varying Levels of Crude Oil Contamination. Con = control 10:01, 10:2 = Carbon to Nitrogen (C: N) ratios.

4. Discussion

4.1. Effects of Crude Oil on Seed Germination and Vegetative Growth of the Maize Plants.

Crude petroleum at high concentrations had a significant ($p<0.05$) negative effect on the germination (Table 1) and shoot growth (Fig 1) of the maize plants. As from 10% crude oil, there was complete cessation of germination in the maize seeds. This inhibition of germination observed in maize at high doses of crude oil is in line with the finding of Malek-Hossein et *al.* [23] who studied the effect of light crude oil on the germination of alfalfa (*Medicago sativa*) and observed germination inhibition at high doses. Their observation confirmed the report of Amadi *et al.* [24] that high doses of petroleum hydrocarbon can inhibit germination in some plants. This is because crude oil being hydrophobic lowers cell membrane permeability and in consequence impedes water and mineral nutrient absorption necessary for seed germination [4].

The shoot lengths of the plants were significantly ($P<0.05$) retarded by the oil. This is easily apparent when the shoot lengths of the test samples are compared to those of the control crops. For instance, at the eighth week, the mean maximum shoot lengths of plants in the control and 2.5% polluted soils were 60.0 cm and 10.5cm (Fig. 1) respectively. In a related study by Adoki and Orugbani [25], it was also reported that growth of fluted pumpkin and okro were retarded by crude oil.



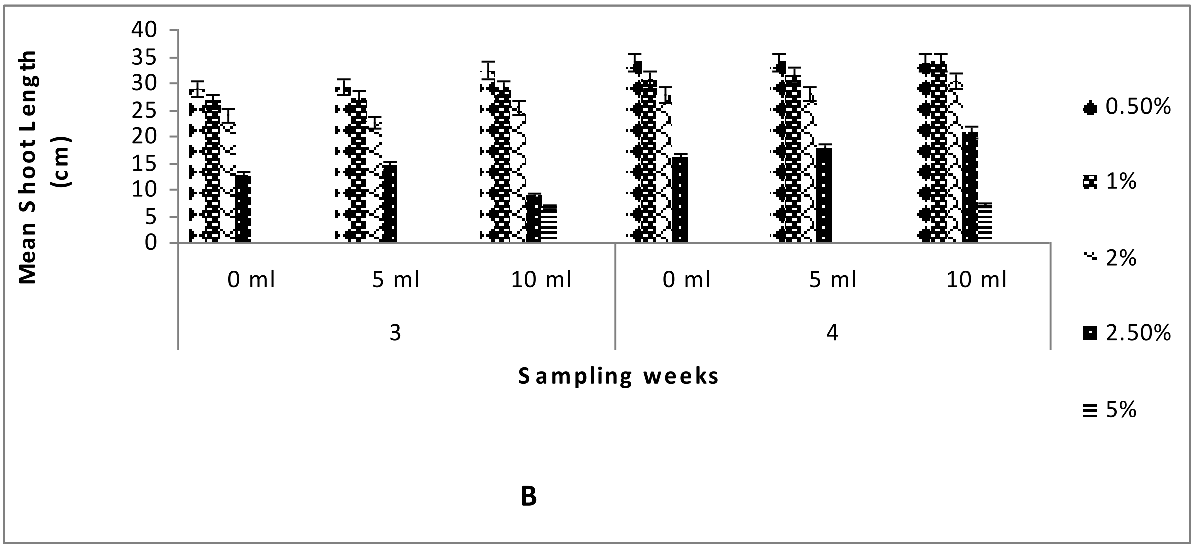
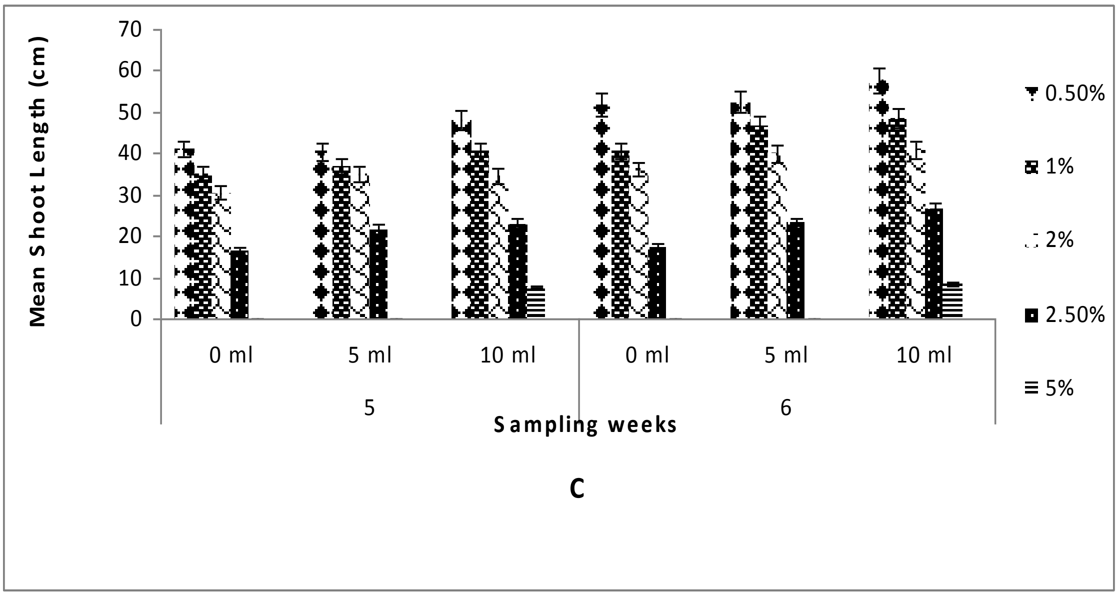


Fig. 3: Effects of bioaugmentation with a hydrocarbonoclastic strain of *Bacillus subtilis* on the vegetative growth of Maize in sandy loam soil with varying levels of crude oil contamination. 5mL = 5x10^8 cells of *B. subtilis/g*, 10mL = 10 x10^8 cells of *B. subtilis/g*

4.2. Simulated Bioremediation Experiment

The efficacies of the bioremediation agent, NPK compound fertilizer, and a hydrocarbonclastic strain of *Bacillus subtilis* in the restoration of crude oil-polluted soil samples were assessed in this phase of the study. The shoot lengths of the crop were used to evaluate the degree of soil remediation (Figures 2 and 3)

Generally, in all biostimulated samples, the lower levels of fertilizer (C:N ratio of 10:1) enhanced soil recovery more than the second level (C:N ratio of 10:2) which was a higher level suggesting that at that level the fertilizer might have slowed down microbiological activity. Biostimulation enhanced germination and shoot growth in maize up to 10% crude oil pollution. In non-biostimulated soil samples containing seeds of the plant, germination stopped at 2.5% pollution. Unlike biostimulation, the bioaugmentation agent, *Bacillus subtilis* caused better growth enhancement at the second level (10mL) than at the first level (5mL) in all the plants. This is contrary to what happened in biostimulation where there was better growth at the first level (C.N ratio of 10:1) than the second level (C:N ratio of 10:2).Comparatively, the biostimulation agent used promoted soil recovery more than *B. subtilis* as evidenced by the better growth performance it produced. The efficacy of biostimulation and bioaugmentation in the bioremediation of crude oil contaminated arable lands has been reported by other workers [26-28].



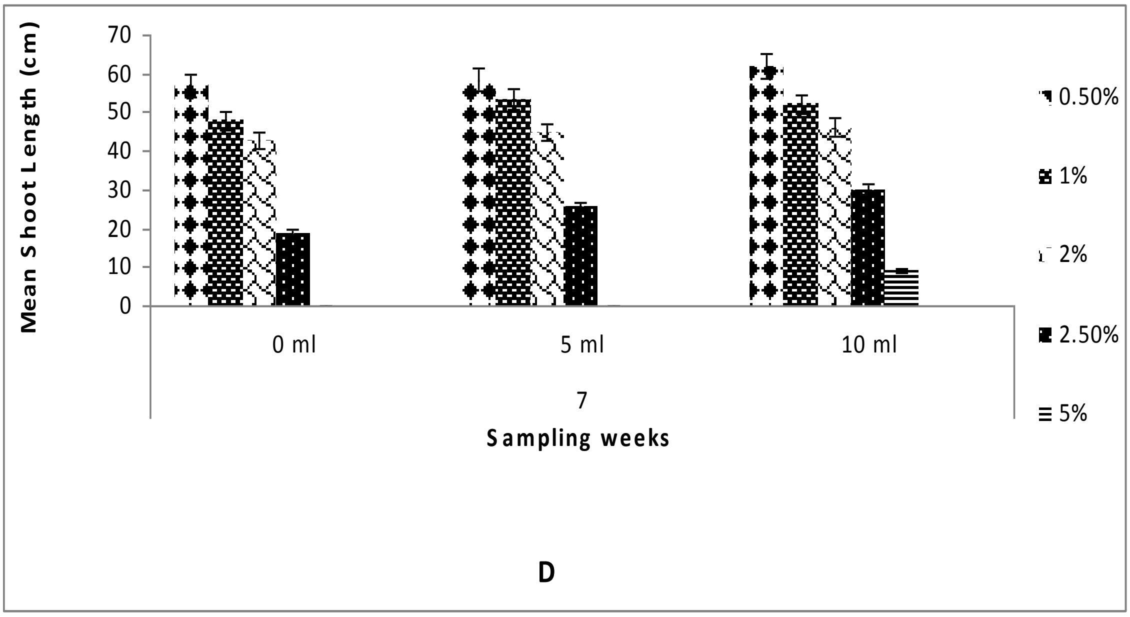


Fig. 3: Effects of bioaugmentation with a hydrocarbonoclastic strain of *Bacillus subtilis* on the vegetative growth of Maize in sandy loam soil with varying levels of crude oil contamination 5ml = 5x10^8 cells of *B. subtilis/g*, 10ml = 10 x10^8 cells of *B. subtilis/g*

Suja *et al*. [27] observed an accelerated degradation of petroleum hydrocarbons in polluted soil bioaugmented with a bacterial consortium and biostimulated with NPK compound fertilizer. However, in their own study they did not use plant growth response as index of remediation. Additionally, they combined biostimulation and bioaugmentation and did not do an evaluation of the relative efficacies of biostimulation and bioaugmentation which is one of the objectives of the present study.

Conclusion

This study has shown that high levels of the inorganic biostimulation agent (NPK 15:15:15) in the soil could inhibit soil recovery from crude oil pollution whereas high levels of hydrocarbonoclastic inoculum could promote soil recovery. Also, high levels of crude oil pollution impede germination in maize seeds and subsequent growth of the plants. Further researches in plant breeding and genetic engineering are necessary to select maize cultivars with increased resistance to crude oil toxicity and also to enhance the innate abilities of microorganisms to degrade crude oil.

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