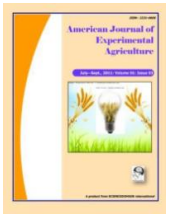

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Effects of Petroleum Products in Soil on α-Amylase, Starch Phosphorylase and Peroxidase Activities in Cowpea and Maize Seedlings

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Authors' contributions

This work was carried out in collaboration between all authors. Author FIA designed the study, wrote the protocol and wrote the first draft of the manuscript. Author PNO reviewed the experimental design and all drafts of the manuscript. Author FIA managed the analyses of the study and performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine the effect of petroleum products (kerosene, diesel, engine oil and petrol) contaminated soil at various concentrations on the activities of α-amylase, starch phosphorylase in the cotyledons of cowpea and maize seedlings as well as peroxidase activity in the leaves of both seedlings.
Place and Duration of Study: This study was conducted in Delta State University, Abraka, Nigeria between April 2007 and August 2011.
Methodology: Improved varieties of maize (*Zea mays* L) and *Vigna unguiculata* (L) Walp were planted in soil contaminated at different concentrations comprising six groups. Each group was replicated five times. Groups 1 to 5 contained 0.1%, 0.25%, 0.5%, 1.0% and 2.0% (v/w) respectively of each of the petroleum products while group six served as control (0.0%). Three

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seeds were planted in each bag and watered daily. Four days after germination the activities of α-amylase, starch phosphorylase in the cotyledons of the cowpea and maize seedlings were analysed. This was followed by the determination of peroxidase activity in the leaves of cowpea and maize seedlings four, eight and twelve days after germination.

Results: The results showed that the petroleum products caused metabolic perturbations in the seedlings. This is indicated by the significant ($P<0.05$) decrease in the activities of starch degrading enzymes: α-amylase and phosphorylase as well as peroxidase activity compared to their respective control values.

Conclusion: Kerosene decreased the activities of the enzymes more than the other petroleum products. The effect of petroleum products contaminated soil was more severe in cowpea seedlings relative to maize seedlings.

Keywords: α-amylase; starch phosphorylase; peroxidase; cowpea seedlings; maize seedlings; soil.

1. INTRODUCTION

Plant growth and development depend on resources present in soil and air, which consists of external and internal growth factors [1]. Presence of petroleum in the external environment leads to changes in the growth and development pattern of the plant. Petroleum compounds are highly toxic to plants and are detrimental to their growth and development. Petroleum is toxic to higher plants [2-5]. Since seed germination is the first physiological process affected by petroleum, the ability of a seed to germinate in a medium containing petroleum would be indicative of its level of tolerance to this chemical. One toxic effect of petroleum on plant is the depression of seed germination. Various reports hinted that crude oil [6-9], water soluble fraction of crude oil [10] and spent engine oil [11] inhibited seed germination. The retardation in seed germination due to exposure to petroleum had been attributed to decrease in available air and water [12,13]. Vwioko and Fashemi [14] indicated that soaking the seed in water before sowing reduced drastically the effect of spent engine oil on seed germination. Moreover, the inhibitory effect of petroleum on germination was equally attributed to hydrocarbon mediated decrease in nutrient mobilizing enzymes in germinating bean seed [4].

Eriyamremu et al. [6] reported that Bonny light crude oil alters protease activity in cowpea seedlings. In addition, Achuba [4] reported that exposure to whole crude oil inhibited the activities of **α** -amylase and phosphorylase in the cotyledon of germinating cowpea seeds. Petroleum stress can induce three possible types of metabolic modification in plants. These include alteration in the production of pigments such as chlorophyll [4,5,15], increased production of metabolites such as glucose, total carbohydrate as well as proteins and amino acids [4] and alterations in plant enzyme activities [4,6] An increase in lipid peroxidation product was observed in plant exposed to petroleum [16,17]. The activity of peroxidase was lower in plant exposed to pollution [18].

The southern region of Nigeria is rich in petroleum resources. The exploitation, processing, transportation as well as disposal of waste oil have resulted in the contamination of the environment [19]. This exposes the biota to the deleterious effects of petroleum pollution. The major occupation of the inhabitants of this region is fishing and farming and some of the main cultivated crops are cowpea and maize [6]. Previous studies have reported that petroleum products penetrate the pore spaces of soil thereby affecting terrestrial vegetation and subsequently impede photosynthesis and other plant physiological processes [16,17,20]. Environmental consequences of refined petroleum products such as kerosene, diesel, engine oil and petrol have not been given the proper recognition they deserve. The aim of the current investigation was to monitor the effects of refined petroleum products on amylase, starch phophorylase and peroxidase activities in cowpea and maize seedlings.

2. MATERIALS AND METHODS

2.1 Refined Petroleum Products and Planting Materials

The refined petroleum products of known specific gravities (kerosene = 0.81; diesel = 0.85; engine oil=0.87; petrol = 0.75) were obtained from Warri Refining and Petrochemical Company, Warri, Nigeria. Improved varieties of maize *(Zea mays L)* were obtained as single batch from Delta Agricultural Development Project (DTADP) Ibusa Delta State, Nigeria. Improved varieties of *Vigna unguiculata* (L*)* Walp were obtained from International Institute of Tropical Agriculture IITA,

Ibadan, Nigeria. The soil (sand 84%, silt 5.0%, clay 0.4% and organic matter 0.6%, pH 6.1) was obtained from a fallow land in Delta State University, Abraka. The nutrient content of the soil used is shown in Table 1. The experiment was carried out under laboratory conditions (temperature 28ºC and 12hr day/ night).

Table 1. Physicochemical properties of test soil

Parameters	Value
pH	6.09
Total organic carbon, %	2.90
Phosphorus, mg/kg	<0.01
Nitrogen, mg/kg	8.47
Nitrate, mg/kg	9.86
Cation exchange capacity, meq /100g	0.74
Sodium, mg/kg	9.06
Potassium, mg/kg	6.72
Calcium, mg/kg	2.98
Magnesium, mg/kg	0.31

2.2 Soil Treatment and Planting of Seeds

One thousand six hundred grams of soil was added to each small size planting bags (1178.3 cm^3, 15 cm deep) and divided into six groups of five replicates. Groups 1 to 5 contained 0.1%, 0.25%, 0.5%, 1.0% and 2.0% (v/w) respectively of each of the petroleum products while group six served as control (0.0%).To the first bag, 1.6 ml of kerosene, corresponding to 0.1%, was added. The petroleum product treated soil sample was mixed vigorously with hand to obtain homogeneity of the mixture. The procedure was repeated for all the concentrations and the petroleum products. Each treatment including control was replicated five times. The treatments were watered every day in order to keep the soil moist. The design of the experiment was completely randomized design (CRD) [4].

Damaged seeds were determined by floatation. All seeds that floated on water were discarded and others that remained at the bottom of water were deemed potentially plantable. Three seeds were sown in each test bag to an approximate depth of 2 cm immediately after pollution and kept under partial shade. During the experiment 80 cm^3 of water was supplied to the set up as at when needed to keep the soil moist. Germination [which is indicated by the appearance of epicotyls (for cowpea) and hypocotyls (for maize) above the soil level] records was taken at 4 days interval up to 12 days. Seeds, which failed to sprout after 12 days were regarded as not germinable. At the end of each experimental period, the seedlings were carefully removed from the bags by destroying the bags while the bulk soil containing the seedling was placed under slow running tap water to wash off the soil particles.

2.3 Preparation of Extract and Determination of α-Amylase Activity

A 10 ml of 0.05M phosphate buffer, pH 8.0 (prepared in the laboratory) was added to the cotyledon, stored in ice, (0.5g) and homogenized manually by grinding in a ceramic mortar and pestle with 0.5g of acid wash sand. The homogenate was then centrifuged at 3000 g for 15minutes. The supernatant containing the crude enzyme was used to determine the level of activity of α-amylase.

α-amylase assay was carried out by the method of Gupta et al. [21] and the activity calculated by using a formula proposed by Xiao et al. [22]. Assay reaction was initiated by adding 0.5 ml of starch solution (prepared in the laboratory) and 0.5 ml of enzyme in 0.1M phosphate buffer at pH 8.0 and incubated at 37ºC for 15 minutes. The reaction was then terminated by adding 1ml of 1 NH_4Cl. Then the mixture was then diluted to nearly 9ml with water followed by the addition of 1ml of iodine reagent. Finally the volume was adjusted to 10 ml with distilled water and the intensity of colour development was determined by measuring the absorbance at 620 nm with SP 1800 UV/SP spectrophotometer.

2.4 Preparation of Cotyledonary Extract and Determination of Starch Phosphorylase Activity

To the cotyledon sample (1.0g), 10ml of ice cold water was added and homogenized until the formation of thick slurry. The homogenate was filtered through a cheese cloth, then through a Watman filter paper followed by filtration using Buchner funnel and suction. The filtrate was placed in a water bath at 50ºC for 5 minutes to inactivate amylase and other enzymes. It was then cooled, followed by the addition of 2 g of cold NH_4SO_4 and centrifuged 15000 g to remove precipitated proteins. The supernatant was then decanted and used as the enzyme source.

2.5 Determination of Phophorylase Activity

A 5.0 ml of starch solution was placed in each of the 5 cuvettes along with 1 ml of potassium phosphate buffer, pH 5.5. Then 2 ml of the

enzyme extract was added to each tube as quickly as possible and allowed to stand for 60 seconds. This was followed by the addition of1.0M potassium iodide solution (1MKI) and the absorbance of each tube read at 660 nm. The amount of starch in each tube was calculated based on the standard curve obtained earlier.

2.6 Determination of Preoxidase Activity

The assay was carried out by the method reported by Rani et al, [23]. The reaction mixture consisted of 3ml of buffered pyrogallol (0.05M pyrogallol in 0.1M phosphate buffer (pH 7.0) and 0.5ml of 1% H_2O_2. To this was added 0.1ml enzyme extract and O.D change was measured at 430 nm for every 30 seconds for 2 minutes. The peroxidase activity was calculated using an extinction coefficient of oxidized pyrogallol (4.5 liters/Mol).

2.7 Statistical Analysis

The results were expressed as mean $\pm$ SEM. All results were compared with respect to the control. Comparisons between the test and control were made by using Analysis of Variance (ANOVA), Least Significant Difference (LSD) was used to conduct Post Hoc test for the significant difference. Differences at $p<0.05$ were considered as significant.

3. RESULTS

Cotyledons of both cowpea and maize seedlings grown in soil treated with kerosene, diesel, engine oil and petrol showed a reduction in α-amylase activity when tested four days after germination (Fig. 1). The reduction in α-amylase activity generally increased with increasing concentrations of petroleum products in soil and was greater for cowpea than in maize seedlings. It is evident that kerosene treatment of soil affected both seedlings more than the other petroleum products (Fig. 1).

Cotyledons of both cowpea and maize seedlings grown in soil treated with kerosene, diesel, engine oil and petrol showed a reduction in starch phosphorylase activity when tested four days after germination (Fig. 2). Depression of starch phosphorylase activity generally increased with concentration and was lesser in cowpea than in maize seedlings relative to the other petroleum products.

The activities of peroxidase in the leaves of cowpea and maize seedlings grown in kerosene, diesel, engine oil and petrol treated soils after four, eight and twelve days of germination are shown in Fig. 3. Generally, peroxidase activity significantly ($p<0.05$) decreased relative to the control, Moreover, kerosene was more toxic than the other petroleum products and affected cowpea seedlings more than maize.

4. DISCUSSION

Petroleum mediated alterations in the activity of plant enzymes were earlier reported [4,24]. In the present study, refined petroleum products inhibited starch degrading enzymes in the cotyledons of germinating cowpea and maize seeds. The dependence of the two starch degrading enzymes in cowpea and maize seedlings on concentration of petroleum products in soil is shown in Figs 1 and 2 respectively. The enzymes: α-amylase and starch phosphorylase are necessary for degrading polysaccharide in seeds. Starch phosphorylase act repeatively on the non reducing end of amylo pectin branches to give glucose-1-phosphate which, after conversion to glucose-6-phosphate, could enter the tricaboxylic acid cycle via glycolytic pathway for production of energy needed by germinating plants [4,25,26]. Therefore, inhibition of starch phosphorylase could disturb respiratory activities of germinating cowpea and maize seedlings as earlier reported [10,10,6,27]. Similarly, α-amylase in conjunction with β-amylase cleaves starch at α- (1→ 4) glycosidic bond from the non reducing end of amylopectin to produce maltose units that are degraded by α-glucosidase to form glucose [28] needed for cellular metabolism [26]. Generally, inhibition of the activities of starch phosphorylase and α-amylase by the refined petroleum products could disturb the production of glucose-1-phosphate and free glucose, thereby predisposing seedling to wide array of metabolic perturbations in cellular metabolism. Comparatively, kerosene seems to inhibit starch phosphorylase and α-amylase activities more than the other three refined petroleum products. The severe toxic effects of kerosene have been attributed to its effects on soil microorganisms [29]. The effect of these refined petroleum products was more pronounced in cowpea compared to maize seedlings. This is in agreement with the report of Coskun and Zihnioglu [30] who showed that monocotyledonous seeds are less affected by toxicants than dicotyledonous seeds.

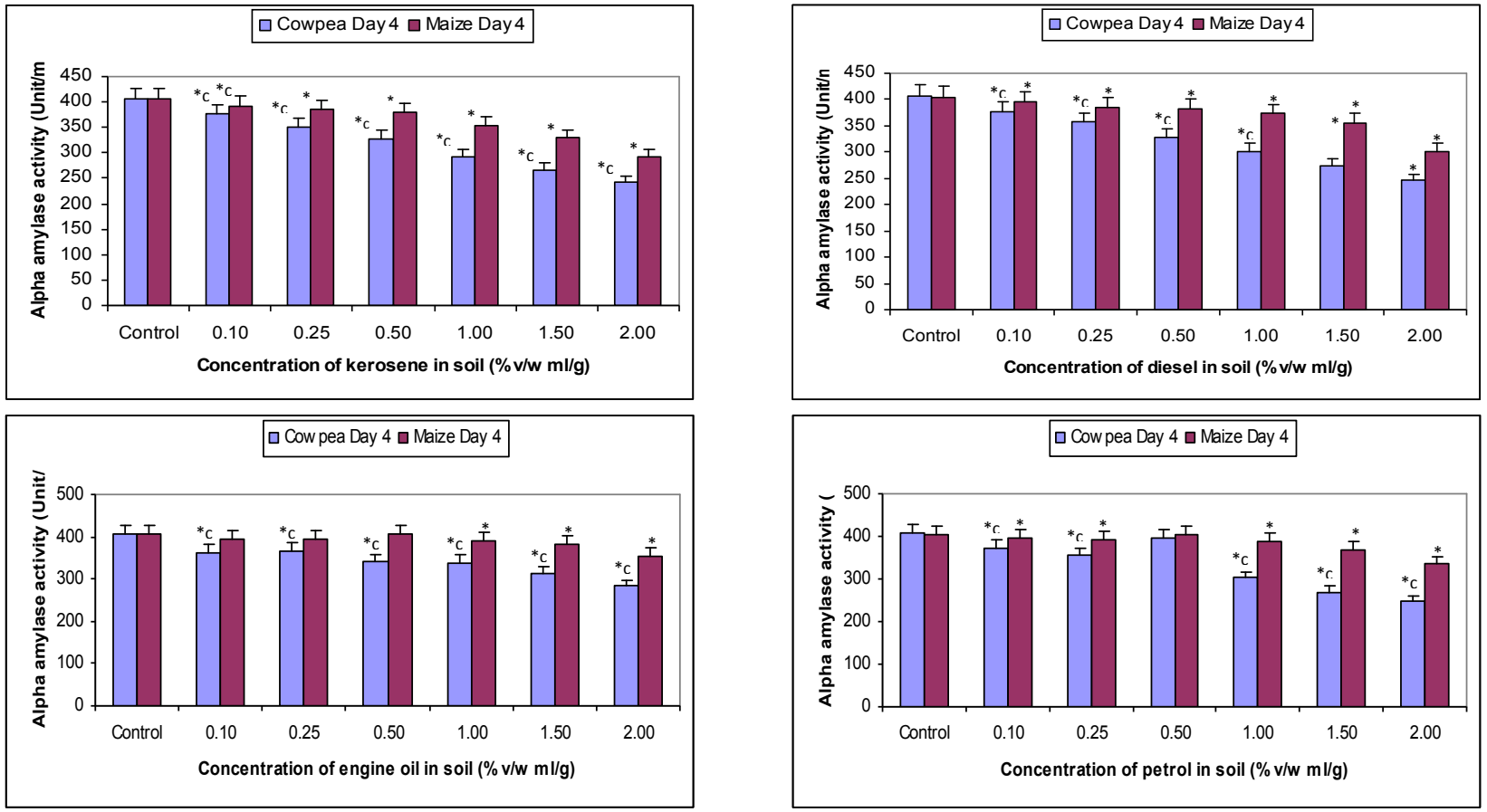

Fig. 1. Effect of concentration of petroleum products on α-amylase activities in cotyledom of cowpea and maize after four days of germination. *Significantly lower as compared to control; +Significantly lower as compared to engin oil; ++Significantly lower as compared to kerosene; ɵ Significantly higher relative to control; [a]Significantly lower relative to other petroleum products; [b]Significantly higher relative to other petroleum products; [c]Significantly lower in cowpea relative to maize seedlings; [d]Significantly higher in cowpea relative to maize seedlings

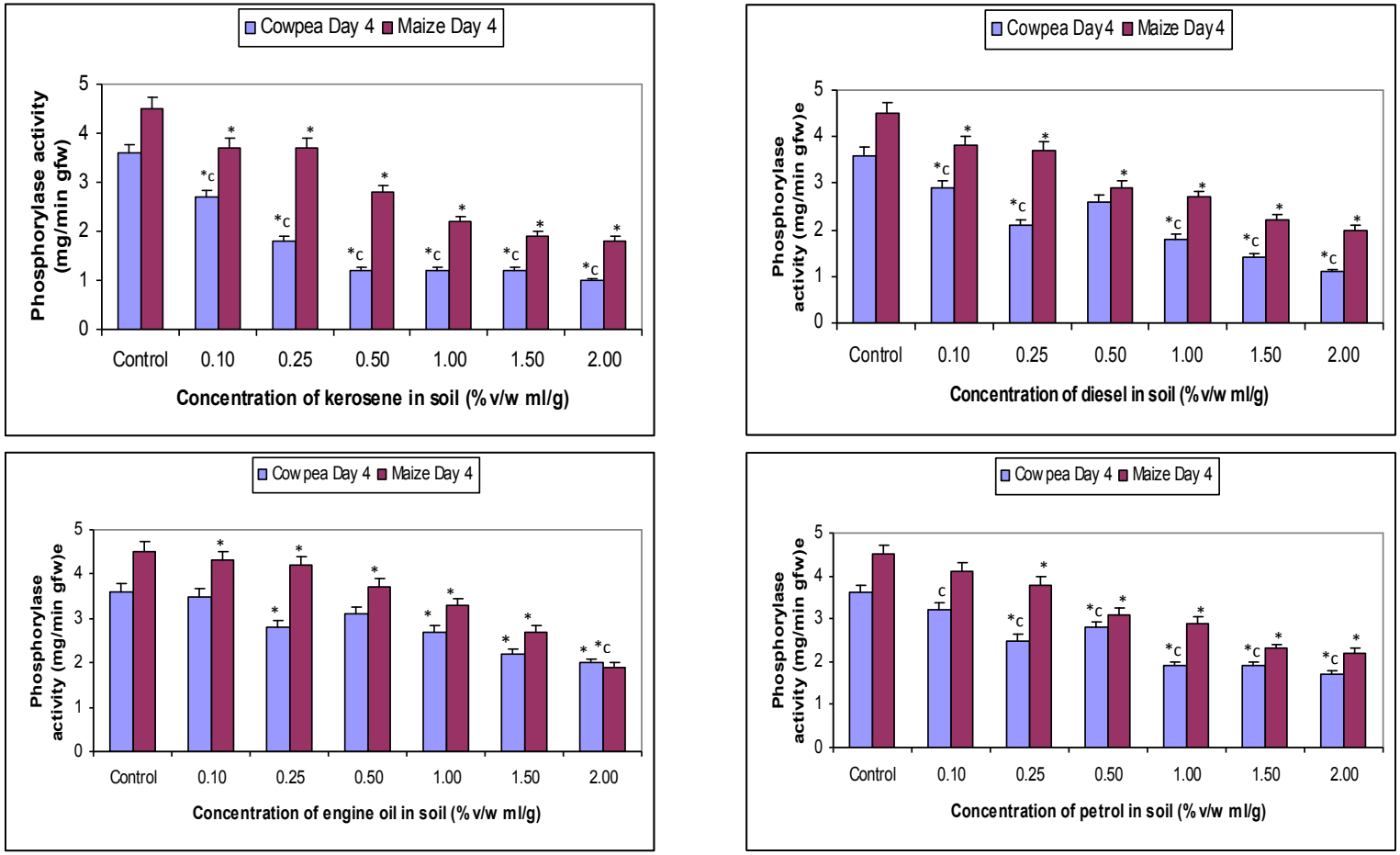

Fig. 2. Effect of concentration of petroleum products on phosphorylase activities in cotyledom of cowpea and maize after four days of germination. *Significantly lower as compared to control; [+]Significantly lower as compared to engin oil; [++]Significantly lower as compared to kerosene; [e]Significantly higher relative to control; [a]Significantly lower relative to other petroleum products; [b]Significantly higher relative to other petroleum products; [c]Significantly lower in cowpea relative to maize seedlings; [d]Significantly higher in cowpea relative to maize seedlings

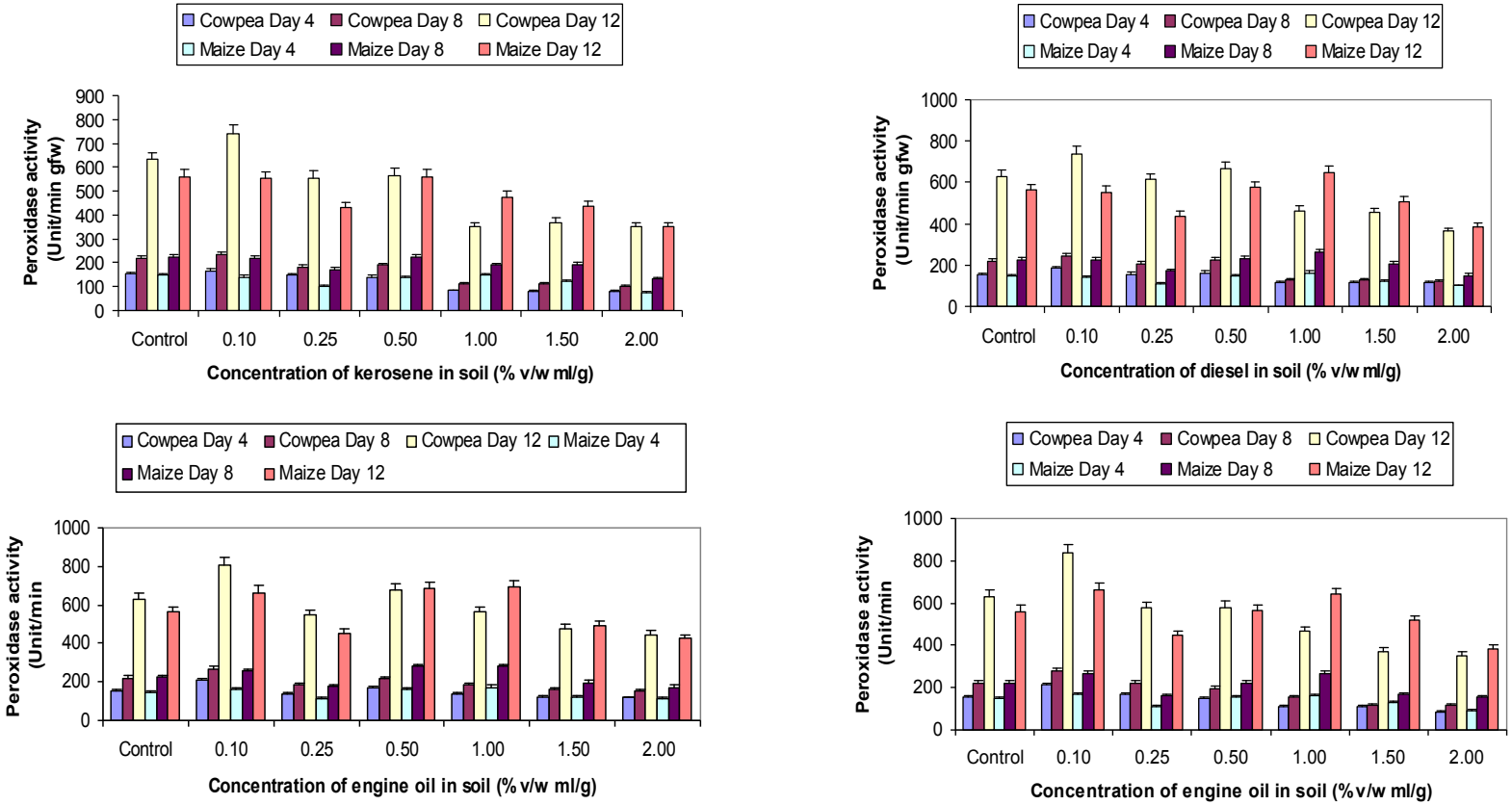

Fig. 3. Effect of concentration of petroleum products on peroxidase activities in leaves of cowpea and maize after four, eight and twele days of germination. No significant difference in peroxidase activity between cowpea and maize (P= 0.87); time had a significant effect on peroxidase activity (P= 0.00); significance difference in activity between control and other concentrations (P= 0.00); significance difference in peroxidase activity among the petroleum products (P= 0.00); time and plant species had significant interaction with peroxidase activity (P= 0.00); time, concentration and petroleum product had significant effect on peroxisae activity (P= 0.00); concentration, plant species, petroleum product had significant effect on peroxidase activity (P= 0.00); concentration, plant species, petroleum product had significant interaction (P= 0.03)

Peroxidase activity was lower in the leaves of cowpea and maize seedlings grown in refined petroleum products treated soil after four, eight and twelve days of germination (Fig. 3). This is in contrast to the increase in the enzyme activity in seedlings grown in cotton wool tainted with heavy metals [31]. The decrease in peroxidase activity observed in this study, which was more pronounced at higher concentrations (Fig. 3) of petroleum products in soil, could indicate the initiation of disruption in the biochemical process that precedes the appearance of apparent symptoms of toxicity. That the cowpea and maize seedlings are under metabolic perturbations is further highlighted by the inhibition of peroxidase activity after 12 days of exposure to petroleum product treated soil (Fig 3). Like starch phosphorylase and α-amylase, peroxidase activity was affected more by kerosene compared to other three refined petroleum products. The effect is more pronounced in cowpea compared to maize seedlings (Fig. 3). Peroxidases are a family of enzymes, which are involved in a variety of cellular function such as lignifications, suberization, cell wall elongation, growth, regulation of cell wall biosynthesis and plasticity [32-35]. This explains why seedling exposed to petroleum hydrocarbon exhibited a retarded growth as was observed by earlier reports [4,5]. It has been speculated that peroxidase restricts elongation growth by the formation of diphenyl cross-links. This study, therefore, in part, seems to suggest that one of the mechanisms of petroleum mediated growth retardation is via inhibition of peroxidase activity.

5. CONCLUSION

It is pertinent to state that petroleum products mediated toxicity in exposed plants is achieved via inhibition of starch degrading enzymes in the cotyledon of germinating seeds as well as inhibition of peroxidase activity in the leave of the seedling. Moreover, the toxicity of kerosene is more severe than the other petroleum products studied.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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