GENOTOXIC ANALYSIS OF VEGETABLES EXPOSED TO PAH FROM SPENT ENGINE OIL

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Abstract. The study showed the genotoxic response of edible vegetables to PAH from spent engine oil in comparison to three PAH component (benzo(a)pyrene, benzo(k)fluoranthene and benzo(ghi)perylene). Molecular characterization of exposed plant samples was carried out to determine the level of toxicity on the DNA. The result of the genomic effect of these pollutants revealed alteration at genetic level through DNA insertions, deletions. There was significant difference at ($p < 0.01$) in plants samples transplanted in different concentrations of PAH and spent engine oil polluted soil. The DNA estimation of plant samples was ascertained using Nano drop spectrophotometer. The DNA quality band was revealed in gel pictures. Furthermore, polymorphism was observed at 65%, 58% and 19% of *A. hybridus* and *T. occidentalis* using the three primers. This demonstrates the impact of PAH toxicity on edibles crops as the occurrence of DNA polymorphism as seen in the form of DNA band breakage and alteration suggest the toxicity level exerted on edible crops by this PAH.

Key words: genotoxicity; phylogenetic; polymorphism; PCR.

INTRODUCTION

The continuous increasing demands of human for basic necessities has led to the growth of global industrialization and as such contribute tremendously to the accumulation of hazardous substances in the environment [10, 17]. However, this has brought about adverse changes in the ecosystem, as pollutants are capable of affecting all forms of life. The accumulation of some of these toxic pollutants such as crude oil products, polycyclic aromatic hydrocarbons (PAHs), phenols etc maybe serve as a hindrance to the growth of plant and microorganisms due to their hydrophobicity [18]. PAHs are known to be present in all environment due to their ability to exist in both gaseous and solid phase, and as such can cause serious damage in the environment depending on its toxicity. The increase in the atmospheric release of PAH due to incomplete combustion from human activities has left its residues in the soil and has brought about its accumulation in food crops and other organisms, thus allowing it entrance into the food chain [24]. Furthermore, the expression of PAH effect on living organisms which includes microorganisms, plants and humans can be mutagenic, carcinogenic, teratogenic and immunotoxic [2, 4]. PAH found within soil pores can cause severe loss of soil productivity as a result of its reduction of soil aeration and water infiltration. PAHs ability to accumulate in food serves as a threat to the existence of all life forms as its toxicity has the potential to alter plants DNA, thus leading to mutation, developmental disorder and formation of cancerous cells in animals [11]. The rate at which PAH is taken up by plant is dependent on the soil, plant species and PAH property, which plays an important role in their interactions.

PAH routes of exposure to human body is through ingestion, inhalation and dermal contact. Furthermore, the detoxification of PAH takes place in the liver of mammals by catalytic processes of cytochrome P450 in association with oxidative enzyme which produces epoxide glutathione conjugates that are water soluble [1, 26]. However, diolepoxides, quinones, hydroxyl alkyl derivatives which are reactive intermediates are formed during PAH metabolisms, and are not easily polarized to be excreted, as such forms covalent adducts with nucleic acid which can lead to genotoxic effects [1]. Furthermore, PAHs has been classified into four groups by the International Agency for Research on Cancer (IARC) i.e., 1 as carcinogenic to humans; 2 as probably carcinogenic to humans; 3 as possibly carcinogenic to humans; and 4 as non-carcinogenic to humans [8]. Schlüter and Harris [20], reported that exposure of humans to PAH above its acceptable limit can possibly lead to 45% carcinogenic risk. Exposure to PAH can lead to its bioavailability and accumulation in fatty tissues of internal organs due to its high lipophilicity [1, 12]. Studies have proven that dietary source plays a significant role through which humans are exposed to PAH. Furthermore, research records have shown that over 70% of PAH exposure in non-smokers is linked with food intake [11]. Also, dietary exposure to PAH has shown serious threat of cancer, mutation, and infertility [27]. Therefore, it is necessary to investigate the effect of spent engine oil and PAH exposure at the genetic level on some edible crops (fluted pumpkin and green), in order to ascertain its level of damage.

MATERIALS AND METHODS

Growth Experiment

Spent engine oil was obtained from Chukwuma Nwoha mechanic village Owerri while, the three PAH components used was procured from chemi-science laboratory ltd, Imo state, Nigeria. The seeds of *A. hybridus* and *T. occidentalis* were procured from Ekeonuwa market, Douglas Owerri. All the materials

were obtained from owerri, Imo state Nigeria. Each concentration of the pollutants was mixed separately in each perforated plastic bucket containing 10kg of soil as shown in table 1 before transplantation was carried out. Plant growth was conducted in a sreenhouse at IITA, Ibadan Oyo State, Nigeria as was shown in figure 1 (a & b). Seedlings were allowed to grow on the nursery for a period of 2weeks. Thereafter, seedlings of equal length (10cm) were transplanted into spent engine oil polluted soil, and soil spiked with benzo[a]pyrene, benzo[k]fluoranthrene and benzo(ghi)perylene and soil without the pollutants as control respectively. They were done in replicate and the growth duration was four weeks. The leaves of these plants sample were harvested for ISSR amplification. The temperature and relative humidity of the screen house was maintained at 20 ± 0.25°C and 79.79 ± 4.07%.

Figure 1. **A** - *A. hybridus* in polluted soil; **B** - *T. occidentalis* in polluted soil.

Table 1. Soil treatment with different concentration of pollutants.

Treatments	Measurement
Treatment 1(spent engine oil)	100mL SEO in 10kg Soil
	200mL SEO in 10kg Soil
Treatment 2(PAH)	300mL SEO in 10kg Soil
	20mg/L B(a)P in10kg Soil
	20mg/L B(k)F in 10kg Soil
	20mg/L B(ghi)P in 10kg Soil

DNA Extraction

The leaves of *A. hybridus* and *T. occidentalis* of the plant spent engine oil and PAHs were collected from the green house and transferred to the laboratory in an ice box and stored at -20°C. Samples were prepared by putting two steel balls and approximately 100 mg of lyophilized tissues into 2ml Eppendorf tube. Freeze-dried plant tissues were grinded into fine powder by using genogrinder-2000 for 30-40 seconds at 1500 rpm. 700 µL of SDS extraction buffer was added into the samples and mixed gently to homogenize. Incubation in water bath was done at 65°C for 20 minutes and mixed gently by rocking intermittently. Tubes were removed from water bath and allowed to cool for 3-5 minutes and 200 µL of 5 M ice cold potassium acetate was added and mixed gently. Incubation on ice was done for 20 minutes and 700 µL of chloroform isoamyl alcohol (24:1) was added and mixed gently by continuous inversion. Centrifugation was done at 10.00 0rpm for 10 minutes with micro-centrifuge of 7cm radius. Supernatant was transferred into a new Eppendorf tube. Ice cold isopropanol of 700 µL was added into each sample and mixed gently. Samples were incubated at -80°C for 15 minutes and then centrifuged at 10.000 rpm for 10 minutes. Supernatants were discarded and 500 µL of ethanol was added and centrifuged at 10.000 rpm for 10 minutes. Ethanol was discarded and samples were air dried. DNA was then resuspended with 5% RNase and 95% autoclaved water [5].

DNA Quantification

DNA quantification was done according to the method of Garcia-Alegria *et al.* [6]. It was carried out using Nanodrop 2000 spectrophotometer. It was calibrated with sterile water before utilization. The concentration and purity of the DNA was determined by measuring the absorbance of the diluted DNA solution at 260/280nm.

Gel Electrophoresis

This was done using the method of Lee *et al.* [13]. Agarose was dissolved in 100 mL of TBE buffer to final concentration of 1%. The gel was then cooled down under running tap water. The DNA bands were stained with Ethidium bromide of 14µL. DNA of 6 µL concentration from each sample were gently loaded into the wells. The gel was ran at 100 V for 1hr 30minutes and the DNA bands was photographed under aplegen UV light transilluminator.

PCR Amplification Using ISSR Marker

PCR amplification was done using ISSR marker according to Lee *et al.*; Moubasher *et al.* [13, 15]. PCR reaction was carried out in a volume of 25 µL containing 3 µL template DNA, 2.5 µL PCR buffer, 2.0 µL of dNTPs, 1.0 µL $MgCl_2$, 1.0 µL $DMSO_4$, 2 µL of ISSR primer, 0.06 µL of Taq DNA polymerase and 13.44 µL of nuclease free water. The primers used for the PCR amplification include UBC 811, UBC 827 and UBC 808 respectively. The PCR amplification was performed as follows: initial denaturation (at 94°C for 1 mins), annealing (at melting temperature of 45°C -

55^0C for 1 min), extension (extension at 72^0C for 3mins and then cool down to 4^0C. Amplification was performed in a mycycler (Biorad). Each reaction was repeated 30X. The PCR product was separated by electrophoresis on 1.5% agarose gel containing 14 µg ethidium bromide in a 1xTBE buffer. The molecular ladder used was 50bp DNA ladder (N3236S) which was purchased from England biolab through international institute of tropical agriculture(IITA).The fragmented DNA was visualized and photographed under aplegen UV light using transilluminator.

The bands from the PCR products were scored using 1 and 0 to indicate its presence and absence which was used in the formulation of binary matrix. Polymorphism in the band was calculate using the formula of Sampaio *et al.* [19]:

$$P = [(a+b) / c] \times 100$$

where: P = polymorphism
a = number of newly appeared bands
b = number of disappeared bands
c = total number of bands

Statistical analysis

The analysis of molcular variance (AMOVA) and UPGMA Dendrogram was performed utilizing FAMD 1.23 β. AMOVA was carried out to obtain population variation from molecular data of polluted plants sample [20].

RESULT

DNA Quality

The gel electrophoresis picture of DNA bands of *A. hybridus* and *T. occidentalis* exposed to PAHs and different concentrations of spent engine oil polluted soil were presented on Figure 1. The gel picture shows the quality of the DNA of *Amaranthus hybridus* and *Telfairia occidentalis* isolated from plants exposed and unexposed to these different pollutants. It was observed that 1 (control), 2, 6, 10, 12 showed line high band intensity compared to others. The light intensity shows the state and integrity of the DNA.

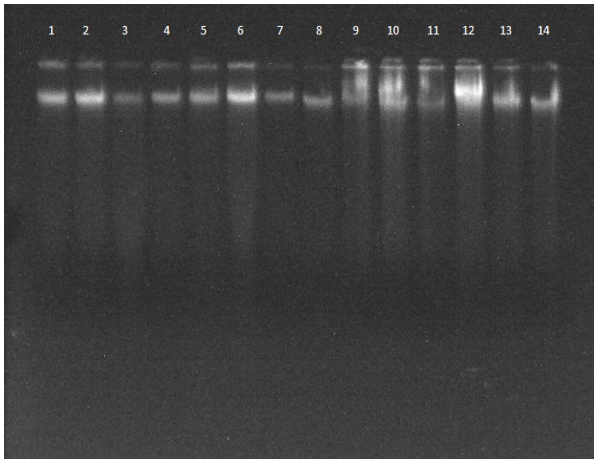

Figure 2. Gel pictures, depicting band intensity of DNA bands and estimation of DNA from crop plants exposed to B(a)P, B(k)F, B(ghi)P and spent engine oil polluted soil. lane 1 and 8: Control (*A. hybridus* and *T. occidentalis*), Lanes 2, 3, 4 (*A. hybridus*)/9, 10, 11 (*T. occidentalis*): plant treated with B(a)P, B(k)F and B(ghi)P. Lanes 5, 6, 7 (*A. hybridus*)/12, 13, 14 (*T. occidentalis*) plant treated with different concentrations of spent engine oil.

Assessment of Genotoxic effect of Different pollutants on the Genomic DNA of *A. hybridus* and *T. occidentalis*

The electropherosis gel picture showing the DNA polymorphism of *Amaranthus hybridus* and *Telfairia occidentalis* plant exposed to the different PAH components (benzo(a)pyrene, benzo(k)fluoranthrene, benzo(ghi) perylene) and various concentrations of spent engine oil (SEOPS) is shown on figure 3 (A, B & C). However, band lane 1 and 8 are controls for the plants sample though, all bands generated were primer dependent. In figure 2, the band lane 1-7 represents *A. hybridus*, while, lane 8-14 represent *T. occidentalis*. In figure 3A lane 3 & 4 had the same number of DNA band fragments. Lane 2 & 5 had band appearance and disappearance respectively. The total DNA band scored in figure 3A is 26. Though, the number of newly appeared band was 8 and the disappeared band was 9. The percentage polymorphism that occurred is 65%. Furthermore, band generated using UBC 827 primer in figure 3B, total number of bands present is 25. However, the number of new bands that appeared is 9 while, the ones that disappeared was 6. The percentage polymorphism detected is 58%. In addition, figure 3C band has percentage polymorphism of 19%. The total number of DNA fragments were recorded in Table 2. However, the ISSR band profile of the plants sample was shown in table 3.

Genetic variation obtained from polluted plants samples using AMOVA at ($p < 0.01$) was presented on Table 4. It was observed that genetic variation among sample group was less than the variation within the sample. The percentage variation of plants sample (WS) response to toxicity groups was 71% while, plants sample (AS) response to toxicity groups was 29% demonstrating the impact of toxicity on genetic differentiation, though, it was more within the populations of plants sample than among the populations groups. However, the populations were all significant at ($p < 0.01$) indicating the impact of toxicity on the genetic level though, the Phi-stat recorded 0.31 revealing a weak positive relationship between the pollutants and the plants samples.

The distance matrix dendrogram showing hierarchical clustering of the plants sample was constructed. Unpolluted *Amaranthus hybridus* (1A) were closely related to *Amaranthus hybridus* grown with benzo(ghi)perylene pollutant (4A) than other pollutants. *Amarathus hybridus* grown in Benzo(a)pyrene (2A), spent engine oil polluted soil A (5A), and spent engine oil polluted soil B (6A) has the most distant relationship when compared to control plant (1A). However, *T. occidentalis* grown in spent engine oil polluted soil C (14T) were the most distantly clustered and *T. occidentalis* plant grown in spent

engine oil polluted soil B (13T) shares the most similarities when compared to control plant (8T) while, *T. occidentalis* grown in spent engine oil polluted soil A (12T) are inter-related with *T. occidentalis* grown in spent engine oil A(12T). Hence, this dendrogram illustrate the relationship of plants response grown in different polluted soil.

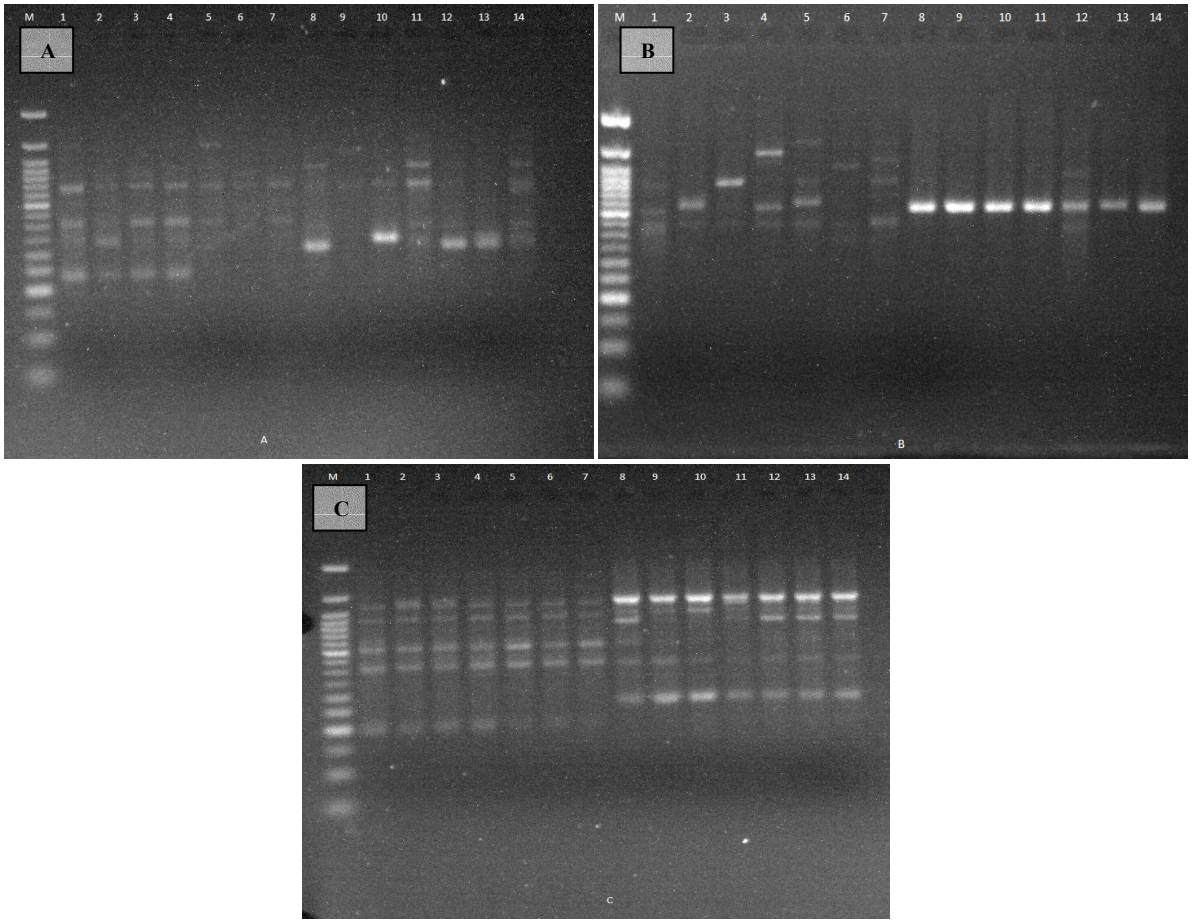

Figure 3. ISSR PCR amplification products for *A. hybridus* and *T. occidentalis* treated with three components of PAH and various concentrations of SEOPS using UBC-811, 827 and 808 primer. Lane M: 50bp DNA ladder; lane 1 and 8: control (*A. hybridus* and *T. occidentalis*): Lanes 2, 3, 4 (*A. hybridus*)/9, 10, 11 (*T. occidentalis*): were treated with B(a)P, B(k)F and B(ghi)P. Lanes 5, 6, 7 (*A. hybridus*)/12, 13, 14 (*T. occidentalis*) were treated with various concentrations of SEOPS; **A** - Primer UBC 811 generated bands, **B** - primer UBC 827 generated bands, **C** - Primer UBC 808 generated bands.

Table 2. Number of bands obtained from different ISSR Marker of Plant Samples.

Pollutants	A(UBC 811)	B(UBC 827)	C(UBC 808)
UPS (*A. hybridus*)	3	3	5
B(a)P(*A. hybridus*)	2	2	5
B(k)P(*A. hybridus*)	3	1	5
B(ghi)P (*A. hybridus*)	3	3	5
SEOPS A (*A. hybridus*)	3	4	5
SEOPS B (*A. hybridus*)	0	1	5
SEOPS C (*A. hybridus*)	2	3	5
UPS (*T. occidentalis*)	1	1	6
B(a)P (*T. occidentalis*)	0	1	4
B(k)P (*T. occidentalis*)	1	1	4
B(ghi)P (*T. occindentalis*)	4	1	2
SEOPS A (*T. occindalis*)	1	3	4
SEOPS B (T. *occindetalis*)	1	1	4
SEOPS C (*T. occidentalis*)	4	1	4

Table 3. Summary of the ISSR band profile obtained from plants samples.

Primer	Sequence	Annealing temp	Polymorphic loci/ No. of loci	% polymorphic bands
UBC 811	(CA)8A	54°C	17/26	65%
UBC 827	(TC)8C	54°C	15/25	58%
UBC 808	(AG)8T	54°C	11/57	19%

ISSR: Inter-simple sequence repeat.

Table 4. Analysis of molecular variance of plant Samples.

Variation Source	DF	SS	VC	VP	P-Value
Among Sample(AS)	1	0.405	0.043Va	29%	<0.01
Within Sample(WS)	12	1.28	0.106Vb	71%	<0.01
Total	13	1.681	0.149Vt	100%	
Phi-statistics	Value	-	-	-	
PhiST	0.286	-	-	-	

DF: Degree of freedom, SS: Sum of square, VC: Variation components,
VP: variation percentage, Va: variance among population,
Vb: Variance of toxicity within group in population, Vt: Total variance.

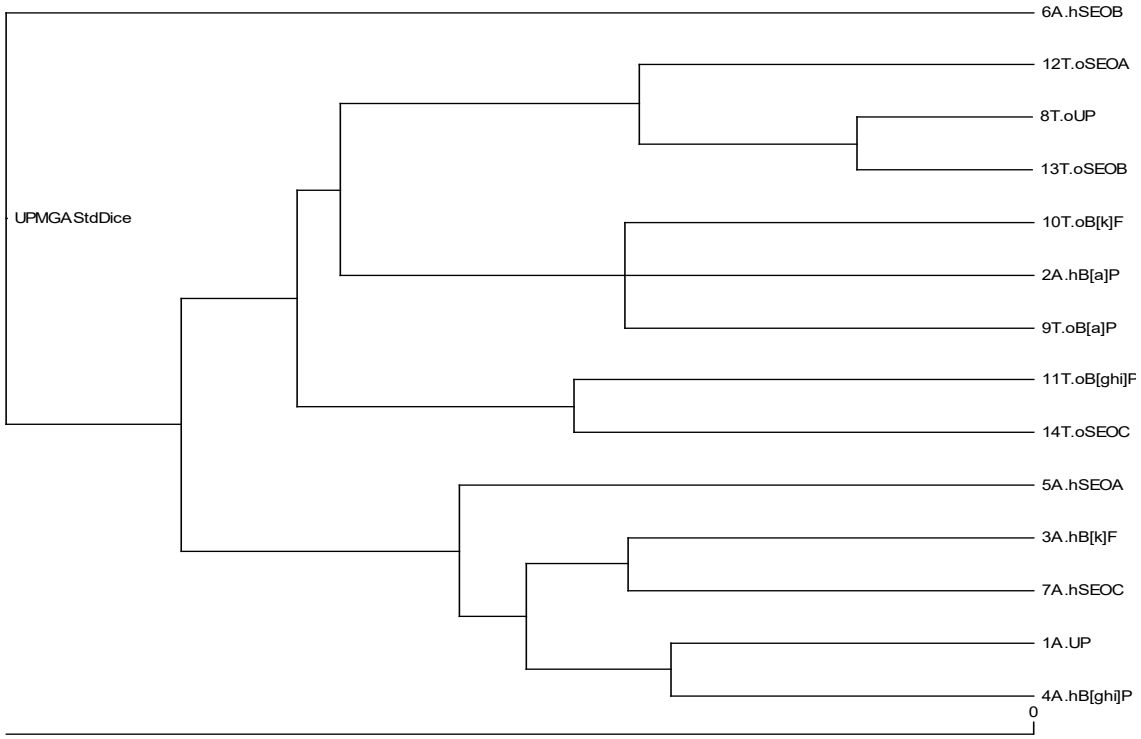

Figure 4. The UPGMA Dendrogram of DNA bands relationship of *A. hybridus* (1A-7A) and *T. occidentalis* plants (8T-14T) exposed to different PAH components.

DISCUSSION

Estimation of DNA extracted from unpolluted and polluted plants sample was to determine its purity and integrity when compared to the control. The occurrence of stunted growth and leaf chlorosis was observed in the plants. The *amaranthus hybridus* had more of stunted growth and yellowing of leaves than *Telfaria occidentalis*. The differences observed in the plants can be attributed to the plants morphology. The interference of the pollutant with soil nutrient may lead to nutrient immobilization and poor mineral uptake. Consequently, causing insufficient aeration of the soil which limit the transportation and respiration rate of plants sample. However, the ISSR marker used for molecular analysis shows that polymorphism occurred as disappearance, appearance was observed [6]. There was significant different at ($p < 0.01$) in the plants samples and this suggests the impact of the pollutants on the plants sample. Moreover, the dendrogram obtained from similarity matrix of plants response showed the relationship between plants response to treatments. The genetic polymorphism value from *Amaranthus hybridus* grown in spent engine oil polluted soil was more than the one grown in PAH polluted soil while, *Telfairia occidentalis* grown in PAH contaminated soil had more band disparity when compared to the control in response to polymorphism than the *Telfairia occidentalis* grown in spent engine oil soil. This reveals pollutant could have an effect on the DNA. These changes could be attributed to the stress induced by pollutants presence in the soil as it possesses the capability to modify structural DNA or alter the expression of regulatory genes [19]. The components of the pollutants involved can cause mutational changes in the DNA which could be inhibitive or change inherent expression. Hardonnier *et al*. [9] reported that DNA damage induced by PAH are through formation of adducts and reactive oxidative species. During PAH degradation, diol-epoxide enantiomers are formed which can bind to DNA thereby, causing mutagenicity and carcinogenicity. Benzo(a)pyrene possess the ability to generate reactive oxygen species in redox cycle leading to oxidative stress. The auto-oxidation of 6-OH-BaP which are precursors of benzo(a)Pyrene cations can generate BaP quinones [3]. PAH can also absorb ultraviolet rays which can induce reactive oxygen species also causing

DNA damage. However, DNA internal repair mechanism can revert or repair the genes saddled with cell control cycle but constant exposure to these pollutants can overwhelm the repair system which definitely will lead to DNA damage [25]. Mustapher *et al*. [16], reported the genotoxicity ability of PAH and heavy metals obtained from mechanic workshop and dumpsite to induce mutational changes. Zied *et al*. [27] also reported the toxic potentials of heavy metals in transcription of stress-induced genes and accumulation of their polypeptides. The mutational effect of pollutants was more from the spent engine oil polluted soil than from the PAH polluted soil and this was perceived in the DNA bands distortion [14].

Plants ability to take up harmful substances from their immediate surrounding into their tissues is dependent on the pollutant concentration as well as the plant characteristics. However, the severity of the pollutants was observed in the DNA of *A. hybridus* and *T. occidentalis* planted in two different pollutants. and may be utilize in assessing phyto-toxicity of these pollutants while, *T. occidentals.* This study may be beneficial for phyto-monitoring of these pollutants as it was adept in tolerating the stress exerted on it during the period of the experiment.

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