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Antioxidant Enzymes and Crude Mitochondria ATPases in the Radicle of Germinating Bean (*Vigna unguiculata*) Exposed to Different Concentrations of Crude Oil

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***Abstract*—**The study examined the effect of Bonny Light whole crude oil (WC) and its water soluble fraction (WSF) on the activities of antioxidant enzymes (catalase (CAT) and superoxide dismutase (SOD)) and crude mitochondria ATPases in the radicle of germinating bean (*Vigna unguiculata*). The percentage germination, level of lipid peroxidation, antioxidant enzyme and mitochondria Ca^{2+} and Mg^{2+} ATPase activities were measured in the radicle of bean after 7, 14 and 21 days post germination. Viable bean seeds were planted in soils contaminated with 10ml, 25ml and 50ml of whole crude oil (WC) and its water soluble fraction (WSF) to obtain 2, 5 and 10% v/w crude oil contamination. There was dose dependent reduction of the number of bean seeds that germinated in the contaminated soils compared with control ($p<0.001$). The activities of the antioxidant enzymes, as well as, adenosine triphosphatase enzymes, were also significantly ($p<0.001$) altered in the radicle of the plants grown in contaminated soil compared with the control. Generally, the level of lipid peroxidation was highest after 21 days post germination when compared with control. Stress to germinating bean caused by Bonny Light crude oil or its water soluble fraction resulted in adaptive changes in crude mitochondria ATPases in the radicle.

***Keywords*—**Antioxidant enzymes, Bonny Light crude oil, Radicle, Mitochondria ATPases.

I. INTRODUCTION

THE composition and function of soils are affected by the microbiota which is in turn affected by mixtures of hydrocarbons present in the soils. Metabolic imbalances occur in plants under stressful conditions which may lead to cell death. Events that occur in plants under stress soon lead to oxidative damage, chlorosis and cell death [1]. Studies have shown that crude oils can expose plants to stress because it can hinder water from spreading homogenously in soil, affect the microbiota and the nutrient level of soils [2]-[4]. Several studies have reported the varying effects of crude oils on plants.

References [5]-[7] reported that oil polluted soil inhibits plant growth, affect plant height, leaf area, moisture content and number of leaves plants in a dose dependent manner. Reference [8] reported that oil polluted soil inhibits the chlorophyll contents of *Corchorus olitorius.* Some of these observations of the effects of crude oils in plants have been linked to improved production of reactive oxygen species (ROS) and other free radicals in the plants which induce oxidative stress and cause peroxidation of lipid and several macro-molecules such as proteins [1], [9].

These effects of crude oils are attributed to its complex mixture of hydrocarbons, hydrocarbon like chemicals, some heavy metals like lead and cadmium. These chemicals not only harm the environment, it can pose serious threats to farmland which is linked to a complex food chain that include humans [10], [11]. Nigeria is an established crude oil exporting nation producing medium and light crude oil, such as Bonny Light. The activities of crude oil exploration, exploitation, transportation and storage leads to spillage that can seriously impact on the environment [12], [13]. Over 2,000 oil spillages have been reported in Nigeria within 1976-1990, releasing more than 5 million barrels of crude oil into the environment [14].

Bean (*Vigna unguiculata*) is one of the commonest and cheapest foods, in the Niger-Delta area of Nigeria from where most of Nigeria's crude oil is derived. There are several plant species that are capable of growing in soils polluted with hydrocarbons and they participate in their degradation (phytoremediation) through the rhizosphere which favours the growth of several microorganisms. Studies have shown that various plants such as jack pine [15], grasses, oat and wheat [16] and other agricultural crops like soybean, pea, and carrot [17] can tolerate crude oil pollutants in soil. Anti-oxidation and mineral uptake directly affects the health of plants. Uptake and transport of minerals are carried out by ion transporters many of which are active in nature and require ATPases.

However, investigations on the effect of crude oil on the activities of some of these ATPases involved in mineral metabolism are still under investigation. This study is thus aimed at assessing the role of Bonny Light Crude Oil and its water soluble fraction on lipid peroxidation, the antioxidant status and some ATPases in the radicle of bean (*Vigna unguiculata*).

II. MATERIALS AND METHODS

A. Experimental Design

Sandy loam soil was weighed into 210 polythene bags such that each bag contains 500g soil. These were divided into four (4) groups. The test group was subdivided into two (2) groups such that the test group each contained 90 bags while the

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control group contained 30 bags. The set of soil filled bags which served as control were not contaminated with whole crude oil or its water soluble fraction. The other sets of bags served as tests. In one test group, the soil was contaminated with 2%, 5% or 10% (v/w, oil/soil concentrations) of the whole crude (WC) while the second test group the soil was contaminated with 2%, 5% or 10% (v/w, oil/soil concentrations) of water soluble fraction (WSF) and planted with bean as shown in Table I. In each bag, three (3) viable seeds of bean were planted and watered with distilled water. The study lasted for 21 days post germination. Germinated plants from 10 bags in each group were harvested at 7, 14 and 21 days. The radicle was recovered for biochemical assays.

B. Fractionation of Crude Oil

Bonny light crude oil that was obtained from Warri Refinery and Petrochemical Company in Delta State, Nigeria, was fractionated by the method of [18] into water soluble fraction (WSF) and water insoluble fraction (WIF). For the fractionation, a 1:2 dilution of 200ml of crude oil was put in a 1 litre conical flask and constantly stirred with a magnetic stirrer for 48h. The WSF then separated from the WIF in a separating funnel.

C. Thiobarbituric Acid Reactive Substances (TBARS) Assay

Thiobarbituric acid reactive substances were assayed according to the method of [19]. Values for TBARS are quantified using molar extinction coefficient (0.156 $\mu M^{-1}cm^{-1}$) and expressed in terms of malondialdehyde (MDA) units/g tissue and each unit represents one micromole of MDA.

D. Catalase (CAT) Activity Determination

The enzyme activity was assayed according to the method described by [20] and was expressed as units/g wet tissue. Dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2 with the formation of perchromic acid as an unstable intermediate. The amount of perchromic acid formed was taken as an activity unit.

E. Superoxide Dismutase (SOD) Activity Determination

The enzyme activity was assayed according to the method described by [21] and was expressed as units/mg tissue weight. One unit of enzyme was defined as the amount of the enzyme required for 50% inhibition of oxidation of epinephrine to adrenochrome in one minute.

F. Isolation of Crude Mitochondria

A modification of [22] was used for the isolation of mitochondria from the germinating bean and maize seedling. The radicle was homogenized in ice-cold extraction medium (Sucrose 0.25M, EDTA 5mM, EGTA 1mM, dithioerythritol 1mM, BSA 0.1%, polyclar-AT 0.6%, in HEPES-TRIS 10mM pH.7.4). The homogenate was filtered with a clean white cloth and the mitochondria immediately separated from the cytoplasmic fraction by centrifugation at 15,000g for 10mins. The resulting crude mitochondrial pellet was resuspended in a solution (Sucrose 0.25M, EDTA 5mM, EGTA 1mM, BSA 0.1% in HEPES-TRIS 10mM pH.7.4) and centrifuged at 600g for 5 minutes to remove nuclei and heavy cell debris. This washing procedure was repeated twice. The washed crude mitochondria were resuspended in another solution (Sucrose 0.25M, EGTA 30mM, in HEPES-TRIS 10mM pH.7.4) and was stored in ice. The samples obtained were subsequently used for the determination of ATPases.

G. Adenosine Triphosphatase (ATPase) Activity Determination

ATPases (Na^+/K^+, Ca^{2+} and Mg^{2+}) were measured by the method of [23]. Activity of the ATPases was assigned by measuring the amount of inorganic phosphate liberated following incubation with 25mM disodium ATP. The inorganic phosphate liberated was estimated by the method of [24]. Total protein was estimated by the method of [25] using BSA as a standard.

H. Statistical Analysis

The results of the study were expressed as mean plus or minus standard error of mean (SEM). Analysis of variance was used to test for differences in the groups while Duncan's multiple comparisons test was used to determine significant differences between means [26].

III. Results

The data on the percentage germination, lipid peroxidation and antioxidant enzyme activities of bean radicle germinated in soils contaminated with varying concentrations of Bonny Light whole crude oil (WC) is presented in Table II. Crude oil treatment of soil decreased the percentage germination in a dose dependant manner. This crude oil treatment increased lipid peroxidation, decreased SOD activity and increased catalase activity in the radicle of bean after 7 days of germination compared with the control. This effect of crude oil was dose dependent with the 50ml crude oil treatment resulting in the highest catalase activity and least SOD activity.

A similar trend was observed after 14 and 21 days of germination. However, lipid peroxidation was not significantly altered. The results in this study show that soils treated with whole crude oil affect germination of bean and alter CAT and SOD activities in germinating radicle.

Table III shows the percentage germination, lipid peroxidation, CAT and SOD activities of bean radicle germinated in soils contaminated with varying concentrations of water soluble fraction (WSF) of crude oil. Like whole crude oil treatment, WSF of crude oil also decreased percentage germination but the effect was not as much as WC. Also, after 7 days post germination, lipid peroxidation increased, CAT activity increased and the SOD decreased. This effect was elaborated after 14 and 21 days post germination.

The effect of varying concentrations of Bonny Light whole crude oil (WC) on the radicle mitochondria ATPase activity of bean is presented in Table IV. Varying effects of WC was observed in the activities of Ca^{2+} ATPase and Mg^{2+} ATPase in the mitochondria radicle. While WC significantly ($p<0.001$) increased Ca^{2+} ATPase after 7 days of germination compared

with control, it reduced Mg^{2+} ATPase at 5% dose. However, after 7, 14 and 21 days post germination, at doses higher than 5%, both ATPases were significantly raised in the bean grown on the WC treated soil compared with the control. The increase in the activities of these enzymes was elaborated as the concentration of WC treatment increased. This study reveals that Ca^{2+} ATPase and Mg^{2+} ATPase in the mitochondria radicle of bean are responsive to crude oil.

Table V shows the data on the effect of varying concentrations of water soluble fraction of crude oil (WSF) on the radicle mitochondria ATPase activity of bean. Statistical evaluation of the data reveals that WSF significantly ($p<0.001$) increased Ca^{2+} ATPase and Mg^{2+} ATPase compared with the control and the effect was dose dependent. This effect of WSF was evident after 14 and 21 days post germination. This study reveals that WSF like WC affect Ca^{2+} ATPase and Mg^{2+} ATPase of bean mitochondria radicle.

IV. Discussion

For plant to grow and develop properly, the soil must provide and make available the essential nutrients required for its uptake. Any disruption of this process will have a negative effect on the normal growth and development of the plants. Whole crude (WC) of Bonny light crude oil and its water soluble fraction (WSF) affected the percentage of the bean seeds that germinated (Tables II and III). There was a correlation between increasing WC and WSF concentrations and reduced percentage germination. This is not surprising since crude oil and other stress conditions have been shown to delay and reduce germination and in some studies, prevent germination [27]-[30].

This reduction and delay may be due to poor wettability and aeration of the soil and loss of seed viability [7]. On the basis of these results, it is suggested that elevated crude oil concentration can delay germination of bean with WC of Bonny Light crude oil having more effect on the plant. The relatively non-significant difference in lipid peroxidation observed in the radicle of plants grown on soils treated with WC relative to control may be due to termination of lipid peroxidation (LP) at an early stage or an interference of polyphenols with the assay [31]. The increase in lipid peroxidation observed in WSF of the crude oil may indicate breakdown of peroxidised lipids. It has been suggested that an increase in lipid peroxidation can generate severe cell damage due to increased production of toxic oxygen radicals [32], leading to reduced germination and growth. Plant tissues contains and produce several antioxidant enzymes scavenging reactive oxygen species (ROS) (such as SOD, catalase, peroxidases) and a network of low molecular mass antioxidants (ascorbate, glutathione, phenolic compounds, tocopherols) to control the level of ROS and to protect cells under stress conditions. Superoxide dismutase genes have been shown to be sensitive to increased ROS production and earlier report has shown a reduced production of the enzyme with increased thiobarbituric acid reactive substances [33]. The decrease in SOD in this study is in consonance with previous studies which shows a decrease in enzymatic activity being accompanied by LP in stressed plants [27], [31]. The increase in CAT activity could be metabolism mediated and an indication of toxic challenge or suicide reactions [34]. The increased level of CAT activity is an indication of increased production of free radicals occasioned by exposure of plants to crude oil. The observed increase in the activities of CAT suggests the development of adaptive or delayed response, which enables the plant cope with further crude oil impact on the radicle. These results demonstrate that any hydrogen peroxide (H_2O_2) formed as a result of SOD activity was consumed by CAT.

Catalase is one of the most important plant enzymes catalyzing the dismutation of hydrogen peroxide into oxygen and water [35]. Catalase activity ensures the maintenance of cell integrity and functional performances [36]. Increased activity of antioxidant enzymes could contribute to better cell protection from chemical toxins, thereby improving the adenosine triphosphate (ATP) production during photosynthesis [32]. This result suggested that increased CAT activity in bean might be sufficient to protect proteins, chlorophyll and lipids of the radicle of the plant against ROS attack.

To adapt to stress produced by crude oil, the plant can cause a change in energy charge and other processes which participate in signaling. The study showed increased mitochondria Mg^{2+} and Ca^{2+} ATPase activities (Tables IV and V) in the values obtained as the concentrations of the crude oil increased in bean and this was found to be significant ($p<0.001$) between (2-10%) oil in soil contamination relative to the control value. The pattern of result for mitochondria ATPase activities obtained in this study appears to be at variance with that reported by [37] for heavy metals (cadmium and lead). The increased mitochondria Mg^{2+} ATPase (the ATPase concerned mostly with ATP generation in the mitochondria) activities ($p<0.001$) in the radicle of bean grown in contaminated soils (Tables IV and V) infers improved production of ATP.

As the plants grew older, the ATPase activity also increased. The increase in mitochondria Mg^{2+} ATPase will increase transport of Mg^{2+} into the plant. The increase influx may also protect the plant against the damaging effect of lipid peroxidation by maintaining membrane integrity as well as stimulating sodium/potassium (Na^+/K^+) ATPase activity for carbohydrate metabolism, hence, providing energy for metabolic processes in the growing plant and facilitating osmotic adjustment [38]. Potassium has been reported to increase carbohydrate metabolism by translocating sugars, help to increase stomata opening, regulate the water in plant cell by preventing lose of water that may physiologically dry the plant [38].

Plant mitochondrial functions are not limited to only energy supply under environmental stress, but have been shown to also contain a permeability transition pore (PTP) [39] which may be a possible link between the sensing of the stress signal and the adaptive response.

The physiological function of PTP, which, when fully open, permits free diffusion of solutes with a low molecular mass

[40], is not fully understood, but circumstantial evidence suggests that it is involved in Ca^{2+} homeostasis [41], [42] and linked to stress sensing through the programmed cell death. High matrix Ca^{2+}- concentrations and inorganic phosphate promote pore opening, whereas ADP and H^+ cause inhibition. The increase in Ca^{2+}-ATPase activity will lead to increase Ca^{2+} absorption or transport into the plant. Ca^{2+} uptake by mitochondria have been shown to promote pore opening and this may infer an open mitochondria PTP. Ca^{2+} has also been reported to maintain membrane integrity and protect the plant from the injurious effects of hydrogen ion (H^+), high salts and other potentially toxic ions present in the contaminated environment [38].

This increase transport of Ca^{2+} occasioned by the increased Ca^{2+}-ATPase activities will help to cushion the damaging effect of the increase in lipid peroxidation observed during the study.

TABLE I
CONCENTRATION OF CRUDE OIL CONTAMINATION IN SOIL

Group	% Contamination	Number of bags
Control	0	30
Whole crude (WC)	2%	30
WC	5%	30
WC	10%	30
Water soluble fraction (WSF)	2%	30
WSF	5%	30
WSF	10%	30

TABLE II
PERCENTAGE GERMINATION, LIPID PEROXIDATION AND ANTIOXIDANT ENZYME ACTIVITIES OF BEAN (*VIGNA UNGUICULATA* L.) RADICLE AFTER GERMINATION IN SOIL CONTAMINATED WITH WHOLE CRUDE OIL

Group/Parameter	Control	2% WC	5% WC	10% WC
No of seeds planted	100	100	100	100
% germination	100	60	47	37
7 days after germination				
Lipid Peroxidation	0.10 ± 0.01^{a}	0.20 ± 0.01^{b}	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}
Catalase	0.11 ± 0.04^{a}	0.86 ± 0.01^{b}	1.62 ± 0.03^{c}	2.06 ± 0.04^{d}
SOD	0.78 ± 0.02^{a}	0.83 ± 0.06^{b}	0.71 ± 0.01^{a}	0.19 ± 0.10^{c}
14 days after germination				
Lipid Peroxidation	0.20 ± 0.01^{a}	0.20 ± 0.01^{a}	0.20 ± 0.01^{a}	0.30 ± 0.01^{b}
Catalase	0.94 ± 0.07^{a}	1.04 ± 0.02^{ab}	1.25 ± 0.04^{b}	1.73 ± 0.03^{c}
SOD	1.30 ± 0.01^{a}	0.86 ± 0.02^{b}	1.24 ± 0.01^{a}	0.38 ± 0.20^{c}
21 days after germination				
Lipid Peroxidation	0.20 ± 0.01^{a}	0.20 ± 0.01^{a}	0.20 ± 0.01^{a}	0.30 ± 0.01^{b}
Catalase	1.22 ± 0.10^{a}	1.26 ± 0.10^{a}	1.04 ± 0.07^{a}	1.36 ± 0.02^{a}
SOD	1.82 ± 0.06^{a}	1.08 ± 0.03^{b}	1.36 ± 0.07^{c}	0.89 ± 0.02^{d}

Values are Means ± S.E.M. For % contamination, n=4. Means of the same row carrying different notations are statistically different at P<0.001. Catalase activity is expressed as unit/g wet tissue. S.O.D =Superoxide dismutase activity expressed as unit/mg protein. Lipid peroxidation is presented in µmole MDA/g tissue.

V. CONCLUSION

In conclusion, the increase in lipid peroxidation observed in the germinating bean radicle as a result of crude oil, may be cushioned by increased mitochondria Mg^{2+} -ATPase activities which increases mitochondria energy production and Ca^{2+}-ATPase activities which increases transport of Ca^{2+}. Also, the decrease in SOD which may infer susceptibility of the bean radicle to crude oil may have been compensated for by the increase in CAT.

TABLE III
PERCENTAGE GERMINATION, LIPID PEROXIDATION AND ANTIOXIDANT ENZYME ACTIVITIES OF BEAN (*VIGNA UNGUICULATA* L.) RADICLE AFTER GERMINATION IN SOIL CONTAMINATED WITH WATER SOLUBLE FRACTION OF CRUDE OIL

Group/Parameter	Control	2% WSF	5% WSF	10% WSF
No of seeds planted	100	100	100	100
% germination	100	72	59	42
7 days after germination				
Lipid Peroxidation	0.10 ± 0.01^{a}	0.10 ± 0.01^{ac}	0.10 ± 0.01^{ac}	0.10 ± 0.01^{ac}
Catalase	0.11 ± 0.04^{a}	0.53 ± 0.10^{b}	1.45 ± 0.02^{c}	2.51 ± 0.06^{d}
SOD	0.78 ± 0.02^{a}	0.50 ± 0.03^{b}	0.37 ± 0.03^{a}	0.10 ± 0.03^{c}
14 days after germination				
Lipid Peroxidation	0.20 ± 0.01^{a}	0.20 ± 0.01^{ab}	0.20 ± 0.01^{ab}	0.40 ± 0.01^{c}
Catalase	0.94 ± 0.07^{a}	1.19 ± 0.1^{ab}	1.41 ± 0.10^{bc}	1.55 ± 0.06^{c}
SOD	1.30 ± 0.01^{a}	0.98 ± 0.04^{ab}	0.89 ± 0.10^{b}	0.57 ± 0.02^{c}
21 days after germination				
Lipid Peroxidation	0.20 ± 0.01^{a}	0.20 ± 0.01^{ab}	0.20 ± 0.01^{ab}	0.50 ± 0.01^{c}
Catalase	1.22 ± 0.10^{a}	1.56 ± 0.10^{a}	1.33 ± 0.07^{a}	1.45 ± 0.10^{a}
SOD	1.82 ± 0.06^{a}	1.53 ± 0.01^{b}	0.91 ± 0.06^{c}	0.87 ± 0.02^{d}

Values are Means ± S.E.M., n=4. Means of the same row carrying different notations are statistically different at P<0.001. Catalase activity is expressed as unit/g wet tissue. S.O.D = Superoxide dismutase activity is expressed as unit/mg protein. Lipid peroxidation is presented in µmole MDA/g tissue.

TABLE IV
ADENOSINE TRIPHOSPHATASE ACTIVITY OF BEAN (*VIGNA UNGUICULATA* L.) RADICLE AFTER GERMINATION IN SOIL CONTAMINATED WHOLE CRUDE OIL

Group/Parameter	Control	2% WC	5% WC	10% WC
7 days after germination				
Mg^{2+} ATPase	0.17 ± 0.15^{a}	0.59 ± 0.15^{b}	0.53 ± 0.15^{c}	0.11 ± 0.24^{d}
Ca^{2+} ATPase	0.06 ± 0.21^{a}	0.05 ± 0.18^{a}	0.95 ± 0.30^{b}	0.10 ± 0.15^{c}
14 days after germination				
Mg^{2+} ATPase	0.21 ± 0.15^{a}	0.66 ± 0.40^{b}	1.06 ± 0.12^{c}	0.12 ± 0.44^{d}
Ca^{2+} ATPase	0.03 ± 0.06^{a}	0.06 ± 0.10^{b}	1.02 ± 0.35^{b}	0.11 ± 0.12^{c}
21 days after germination				
Mg^{2+} ATPase	0.22 ± 0.40^{a}	0.78 ± 0.42^{b}	1.12 ± 0.10^{c}	0.13 ± 0.12^{d}
Ca^{2+} ATPase	0.02 ± 0.17^{a}	0.13 ± 0.18^{b}	1.11 ± 0.30^{b}	0.12 ± 0.09^{c}

Values are Means ± S.E.M., n=4. Means of the same row carrying different notations are statistically different at P<0.001. ATPase = Adenosine triphosphatase activity is expressed as µmole of Pi released/min/mg protein.

TABLE V
ADENOSINE TRIPHOSPHATASE ACTIVITY OF BEAN (*VIGNA UNGUICULATA* L) RADICLE AFTER GERMINATION IN SOIL CONTAMINATED WITH WATER SOLUBLE FRACTION OF CRUDE OIL

Group/Parameter	Control	2% WSF	5% WSF	10% WSF
7 days after germination				
Mg^{2+} ATPase	0.17 ± 0.15^{a}	0.68 ± 0.24^{b}	1.02 ± 0.20^{c}	0.21 ± 0.15^{d}
Ca^{2+} ATPase	0.06 ± 0.21^{a}	0.16 ± 0.09^{b}	0.64 ± 0.12^{c}	0.37 ± 0.12^{d}
14 days after germination				
Mg^{2+} ATPase	0.21 ± 0.15^{a}	0.43 ± 0.40^{b}	2.84 ± 0.15^{c}	0.48 ± 0.35^{d}
Ca^{2+} ATPase	0.03 ± 0.06^{a}	0.16 ± 0.20^{b}	2.89 ± 0.55^{c}	0.52 ± 0.31^{d}
21 days after germination				
Mg^{2+} ATPase	0.22 ± 0.40^{a}	0.48 ± 0.37^{b}	0.30 ± 0.10^{c}	1.15 ± 0.20^{d}
Ca^{2+} ATPase	0.02 ± 0.17^{a}	0.19 ± 0.10^{b}	2.33 ± 0.10^{c}	1.15 ± 1.00^{d}

Values are Means ± S.E.M., n=4. Means of the same row carrying different notations are statistically different at P<0.001. ATPase = Adenosine triphosphatase activity is expressed as µmole of Pi released/min/mg protein.

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