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Genotoxicity of glyphosate (pesticide) using the Allium cepa L. assay.

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Abstract- ***The use of pesticides because of their importance cannot be overemphasized. Although there are warnings of the health implications posed by pesticides to lives due to their uncontrolled use. It is expedient that, glyphosate (a commonly used pesticide in Nigeria) be investigated for its toxic effects. This study investigated the effect of glyphosate using the Allium cepa assay. The roots of onion were exposed for 24 hours to varying treatment concentrations (0g/L, 0.75g/L, 1.5g/L, 3.0g/L, 6.0g/L, and 8g/L of glyphosate). Microscopic end points of different chromosomal aberrations were evaluated and it was observed that glyphosate induced genotoxic effects such as stickiness, micro nuclei, lagging chromosomes, C - mitosis, and nuclear lesion. Macroscopic parameters such as root length and number of roots were also evaluated. Obtained data were statistically analyzed at 0.05 (5%) level of significance using analysis of variance and the Student's t-test. Root growth was retarded with increase in concentrations of glyphosate. Statistically, significant (P<0.05) differences were established between root lengths exposed to glyphosate when compared with the control treatment. However, number of roots showed no significant statistical difference when compared with the control treatment. The findings showed that glyphosate can pose adverse effects on lives and the environment at large. It is recommended that increased awareness and enlightenment of the public on the dangers associated with the use of pesticides and the need for adherence to safety measures be encouraged by the regulatory agencies saddled with responsibilities to do so.***

Keywords: Genotoxicity, Pesticide, Allium cepa Assay, Glyphosate.

I. INTRODUCTION

The wide use of pesticides for agricultural, domestic and industrial purposes due to their importance is indispensable despite the fact that these pesticides (chemicals) are known to have harmful effects (EEA, 2019). The harmful effects may result from the exposure of living cells to these pesticides which contaminate living cells (Lorenz, 2009). Pesticides can cause both cytotoxic and genotoxic effects in living organisms (Paul *et al.,* 2013). The continuous use of these pesticides which might remain in water, soil and food with mutagenic potentials may cause mutation and cancer in organism (Drageova *et al.,* 2012).

Irrespective of the benefits of pesticide, the effects of pesticides on the ecosystem and their mutagenicity for non-target organisms have attracted global interest (EC, 2023; James and Adeleke, 2016). WHO and UNEP reported that poisoning induced by pesticides to lives globally was more than 26 million people, rendering about 220,000 people dead every year (Richter 2002). Other cases that has been reported as adverse effects to health induced by pesticides includes effects on nervous system (Kamel *et al.,* 2007). It is therefore expedient that glyphosate a commonly used pesticide by farmers in Nigeria be tested (evaluated) for its toxicity.

In the past, Toxicological tests were mostly done using mammals. Currently the use of these mammals for toxicological test has generated worries globally among scientist and ethical believers (Mukhopadhyay *et al.,* 2004). Russell and Burch (1959) explained the need for humane animal experimentation replacement, reduction and refinement. These Principles are usually referred to as three Rs (Russell and Burch, 1959: Russel, 2005). Researchers over the years have always sought for alternative test objects. These demands are fulfilled by the use of higher plants in toxicity studies because it provides valuable genetic assay systems (Leme & Marin-Morale, 2009). The *Allium cepa* test is seen as an important and suitable test system used to assess the cytotoxic potentials of pesticides among plant species (Bolle *et al.,* 2004; Asita and Matebesi, 2010). Sifa (2009) also reported the cytogenetic effects of food additives on *Allium cepa* root meristem cells.

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II. MATERIALS AND METHODS

A. Method of Preparing the Onion Bulbs and Rootlets

Onion bulbs of an average weight in grams of 52g ± 1.8 were purchased. The onion bulbs were then dried in the sun for a period of 2 weeks. Their dead scales were removed and they were scraped at the base to promote the growth of new rootlets. The bulbs were each placed initially in 50ml plastic cups containing tap water for 48 hours for new roots to grow. They were then transferred to containers with the treatment solutions for 24 hours. These experiments were carried out in the dark as reported by some other researchers (Fachinetto *et al.,* 2008; Solange & Haywood, 2012; Firbas, 2013).

The roots of the onion bulbs were collected and fixed immediately in 3:1 ethanol –acetic acid for 24 hours. The rootlets were then removed from the fixative and transferred to70% ethanol and kept in the refrigerator (4^{o}C) until used. All samples were properly labeled to avoid mistakes.

B. Microscopic Parameters

Slide Preparation

The methodology used was described by Nuffield foundation (2015). The stain used is acetocarmine solution. The solution used to hydrolyze the root tips was 1N HCL (Asita & Mokhobo 2013; Faizah, 2014; Nuffield Foundation 2015).

The following steps were taken:

The roots were taken out of the 70% ethanol. The root tips were cut and hydrolyzed in 1N HCL for 5 minutes in a water bath at 60^{o}C. They were then rinsed in distilled water and stored in water with 5 drops of 1% Ferric Chloride for at least 24 hours before slide preparation.

Squashes were made in a drop of acetocarmine stain, and viewed under a compound light microscope.

Viewing of the Slides

The slides were viewed using a binocular microscope (Biosphere-B 2005B) with the magnification 40x and pictures taken. Thereafter, the pictures were transferred to the computer for labeling.

C. Macroscopic Parameters

After the 24 hours growth of the onion root in the various test solutions (treatments), measurements of the root lengths were taken and noted. The number of roots were also counted (Rank, 2003; Firbas, 2013).

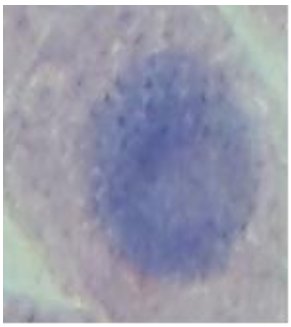
D. Statistical Analysis

The data obtained were statistically analyzed at 5% level of significance using analysis of variance and student's t-test with the jmp 4 statistical software.

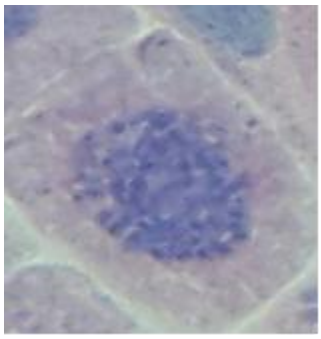
III. RESULT

A. Microscopic

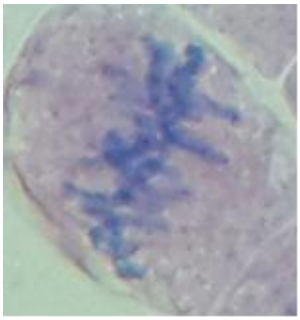
i. Control treatment



A



B



C

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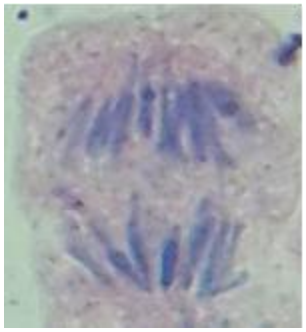
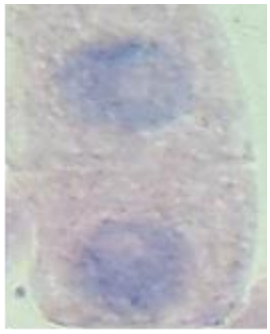


D

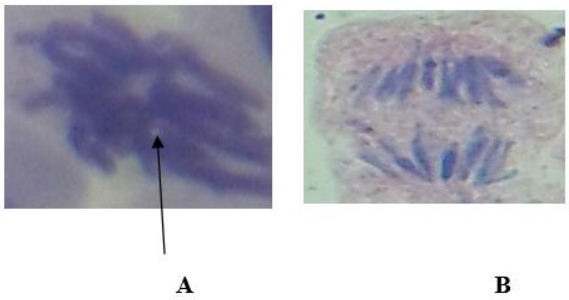


E

Interphase (A), Prophase (B), Metaphase (C)
Anaphase (D) Telophase (E)

Plate 1: Normal mitotic division phases in T_1 (Control treatment).

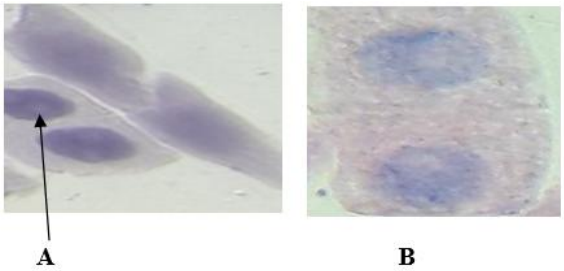
ii. Aberrations Induced by Glyphosate



A B

Lagging chromosome (A); Normal anaphase (B)

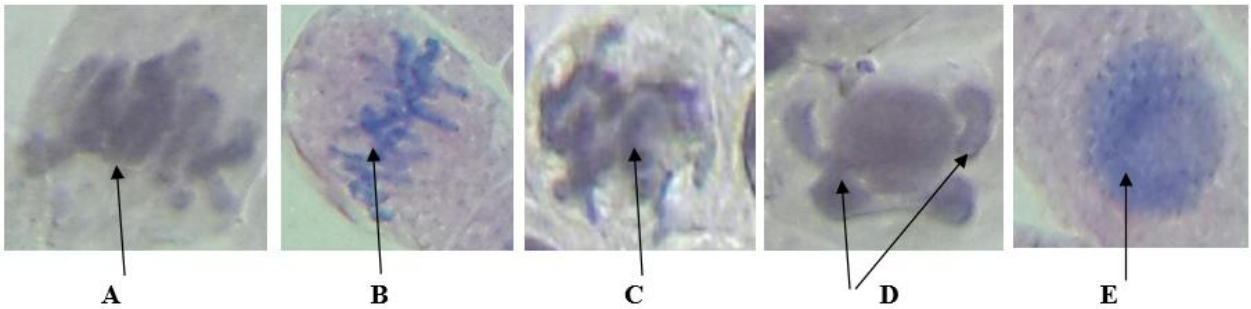
Plate 2: Lagging chromosomes induced by T_2 (0.75g/l glyphosate).



A B

Nuclear lesion (A) Normal telophase (B)

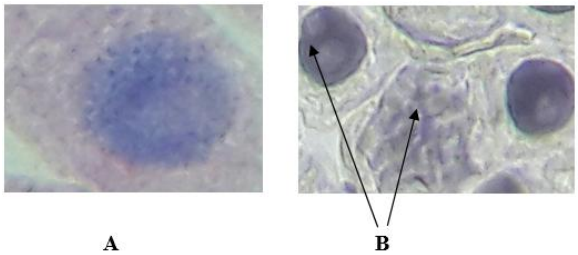
Plate 3: Nuclear lesion induced by T_3 (1.5 g/l glyphosate).



A B C D E

Sticky metaphase (A); Normal metaphase (B); C-mitosis (C); Micro nuclei (D); Normal Prophase (E)

Plate 4: Sticky metaphase, C-mitosis and Micro nuclei induced by T_4 (3.0g/l glyphosate)



A B

Normal Prophase (A); Nuclear lesion (B)

Plate 5: Nuclear lesion induced by T_5 (6.0 g/l glyphosate)

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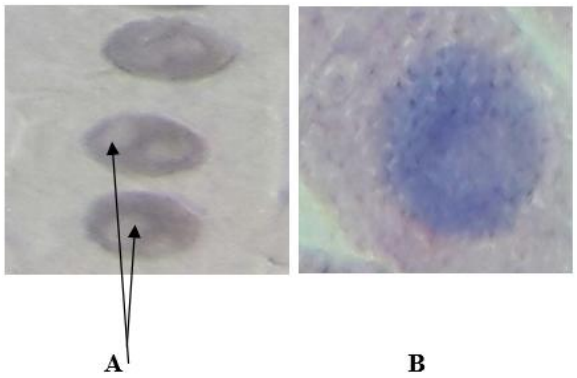

Nuclear lesion (A); Normal Prophase cells (B)

Plate 6: Nuclear lesions induced by T_6 (8.0 g/l glyphosate)

B. Macroscopic

Table 1: Mean Values of Measurable Macroscopic Parameters.

Replicates	Concentrations (g/L)	Average No. roots (cm) ± standard deviation	Average root length (cm) ± standard deviation
B_1T_1 B_2T_1 B_3T_1	CONTROL (0g/L)	21.00 ± 0.82	5.70 ± 0.14
B_1T_2 $B2T_2$ B_3T_2	0.75g/L. glyphosate	21.00 ± 0.82	5.00 ± 0.05
B_1T_3 B_2T_3 B_3T_3	1.5g/L. glyphosate	22.33 ± 0.47	4.60 ± 0.24
B_1T_4 B_2T_4 B_3T_4	3.0g/L. glyphosate	20.67 ± 0.94	3.90 ± 0.25
B_1T_5 B_2T_5 B_3B_5	6.0g/L. glyphosate	21.67 ± 0.94	3.30 ± 0.05
B_1T_6 B_2T_6 B_3T_6	8.0g/L. glyphosate	18.33 ± 5.25	2.90 ± 0.21

B= Bulb; T= treatment

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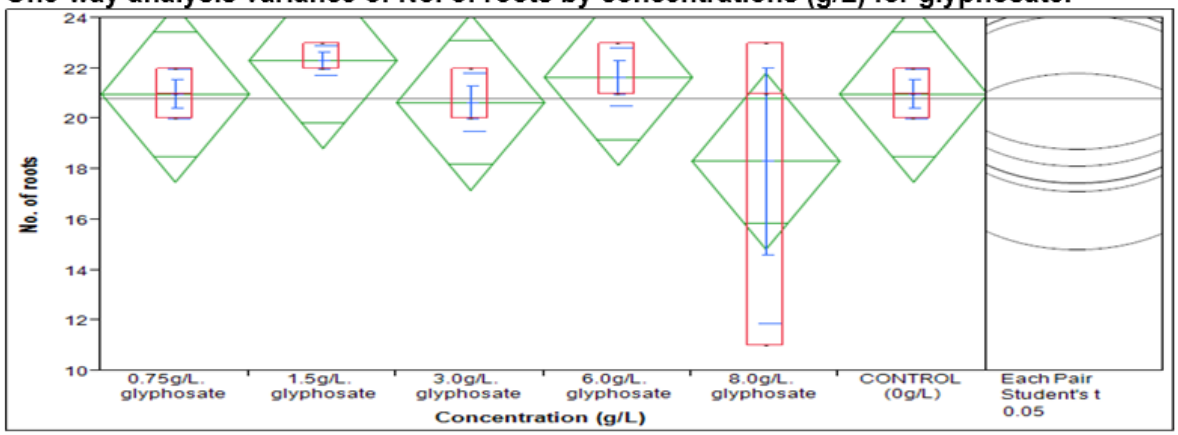
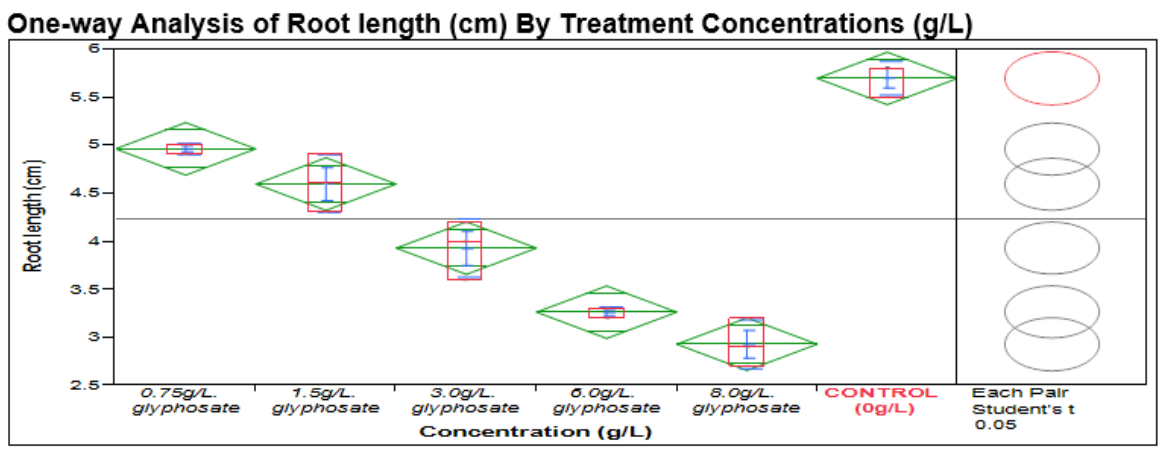


Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob> F
Concentrations (g/L)	5	27.83333	5.56667	0.7209	0.6204
Error	12	92.66667	7.72222		
C. Total	17	120.50000			

Figure 1: One-way Analysis of Variance for Root Number by Concentrations (g/l) for Glyphosate



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob> F
Concentrations (g/L)	5	16.613333	3.32267	70.3624	<.0001*
Error	12	0.566667	0.04722		
C. Total	17	17.180000			

Figure 2: One-way Analysis of variance for root length (cm) by concentration (g/L) for glyphosate.

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Table 2: Root Number Comparison for Each Pair Using Student's t - test for Glyphosate

Abs(Dif)-LSD	1.5g/L. glyphosate	6.0g/L. glyphosate	0.75g/L. glyphosate	CONTROL (0g/L)	3.0g/L. glyphosate	8.0g/L. glyphosate
1.5g/L. glyphosate	-4.9436	-4.2770	-3.6103	-3.6103	-3.2770	-0.9436
6.0g/L. glyphosate	-4.2770	-4.9436	-4.2770	-4.2770	-3.9436	-1.6103
0.75g/L. glyphosate	-3.6103	-4.2770	-4.9436	-4.9436	-4.6103	-2.2770
CONTROL (0g/L)	-3.6103	-4.2770	-4.9436	-4.9436	-4.6103	-2.2770
3.0g/L. glyphosate	-3.2770	-3.9436	-4.6103	-4.6103	-4.9436	-2.6103
8.0g/L. glyphosate	-0.9436	-1.6103	-2.2770	-2.2770	-2.6103	-4.9436

*Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level		Mean
1.5g/L. glyphosate	A	22.333333
6.0g/L. glyphosate	A	21.666667
0.75g/L. glyphosate	A	21.000000
CONTROL (0g/L)	A	21.000000
3.0g/L. glyphosate	A	20.666667
8.0g/L. glyphosate	A	18.333333

Levels not connected by same letter are significantly different.

Table 3: Root Length Comparison for Each Pair using Student's t- test for Glyphosates.

Abs(Dif)-LSD	CONTROL (0g/L)	0.75g/L. glyphosate	1.5g/L. glyphosate	3.0g/L. glyphosate	6.0g/L. glyphosate	8.0g/L. glyphosate
CONTROL (0g/L)	-0.3866	0.3467	0.7134	1.3801	2.0467	2.3801
0.75g/L. glyphosate	0.3467	-0.3866	-0.0199	0.6467	1.3134	1.6467
1.5g/L. glyphosate	0.7134	-0.0199	-0.3866	0.2801	0.9467	1.2801
3.0g/L. glyphosate	1.3801	0.6467	0.2801	-0.3866	0.2801	0.6134
6.0g/L. glyphosate	2.0467	1.3134	0.9467	0.2801	-0.3866	-0.0533
8.0g/L. glyphosate	2.3801	1.6467	1.2801	0.6134	-0.0533	-0.3866

*Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level		Mean
CONTROL (0g/L)	A	5.7000000
0.75g/L. glyphosate	B	4.9666667
1.5g/L. glyphosate	B	4.6000000
3.0g/L. glyphosate	C	3.9333333
6.0g/L. glyphosate	D	3.2666667
8.0g/L. glyphosate	D	2.9333333

Levels not connected by same letter are significantly different.

IV. DISCUSSION

A. Microscopic Parameters

The usefulness of the *Allium cepa* assay in assessing the cytotoxicity, genotoxicity and mutagenicity of environmental chemicals like other researchers is clearly demonstrated in our findings. The microscopic parameters analyzed by this test system showed different types of abnormalities observed in cells as well as chromosomal aberrations (CAs). CAs are characterized by change in either total number of chromosomes or in chromosomal structure, which may occur due to exposure of cells to chemicals. In this study, different abnormalities were evaluated at different stages of mitotic cell division which includes prophase, metaphase, anaphase, and telophase. Chromosomal aberrations and other forms of cytotoxic effects were observed from cells exposed to the pesticide (glyphosate) for all tested treatment concentrations adopted in this study. The aberrations observed include: stickiness, presence of micro nuclei, lagging, C- mitosis, nuclear lesion and damaged cells. This is in conformity with the work of some other investigators who showed that several other pesticides induced some level of abnormalities in the cell using the *Allium cepa* assay (Asita *et al.,* 2010; Yekeen 2013; Faizah, 2014).

The chromosomal aberrations that were detected must have resulted from chromatin dysfunctions (stickiness and fragments) or as a result of the toxic effects posed by the pesticides on the formation of the spindle apparatus which then resulted in the disturbances of the cell division (Faizah, 2014).

Sticky chromosomes are those chromosomes which did not completely condense during metaphase (Osterberg *et al.,* 1984). Here, chromatin masses that are not distinguishable as chromosomes are observed as clumps. Spindle fibres are absent in cells that possess sticky chromosomes (Asita & Mokhobo, 2013). Stickiness may also occur due to increased chromosomal contraction or from DNA depolymerization and partial nucleoproteins dissolution (Turkoglu, 2007). Occurrence of sticky chromosomes depicts toxic effect usually the type that is irreversible and can lead to the death of the cell (Khanna *et al.,* 2013). This is in conformity with the reports of Turkoglu (2007) and (Rencuzogullari *et al.,* 2001) who also observed the occurrence of sticky chromosomes when they worked on the effects of different chemicals on cells.

Micronuclei may occur as a result of the development of chromosomes that are isolated, caused by an unequal distribution of genetic materials. This finding is also in line with the report of Meng *et al.* (1992) who explained that the occurrence of micronuclei in a cell is used for the detection of genetic damages that originates from mutagenic chemical exposures. It was also explained in their work that micronuclei may also occur when acentric fragments/whole chromosomes are not incorporated into the main nucleus at the time of the cell division cycle. Hence, substances that promote the occurrence of micronuclei are seen to be aneugenic or clastogenic (Meng *et al.,* 1992).

Lagging chromosomes are the whole chromosomes that do no migrate to the opposite ends of the pole during the cell division stage called anaphase. This effect arises from the possible damages done to the kinetochores. This result is same with the work of Turkoglu (2007) when he worked on genotoxicity of five food preservatives using *Allium cepa* assay and reported that lagging chromosomes may have occurred because of the inability of the chromosomes to become attached to the spindle fibres and then migrate to the opposite ends of the poles during anaphase.

C-mitosis occurs when cells (mainly mitotic cells) do not have spindle fibres and so the chromosomes in the cell becomes scattered all through the cell because of the failure of the chromosome to get attached to spindle fibres. C-mitosis as a term was coined by Levan (1938) and explained that colchicine prevents

the assembly of the spindle fibres and results in scattering of the chromosomes over the cells.

In addition to the observed chromosomal aberrations, higher concentrations of both glyphosate induced whole cell damage.

B. Macroscopic Parameters

The toxic effects of chemicals released into the environment can also be evaluated by analyzing macroscopic parameters of root number and root length (growth retardation) of the *Allium cepa*. The results of the present study suggest that glyphosate is toxic, retarding the growth of roots in length (root lengths). It is well known that the utilization of oxygen, linked with respiration is greater in the meristematic regions. It is obvious that the reduction occurs in the region of elongation where oxidation reactions are not too favorable compared to the meristematic regions (Lerda *et al.,* 2010). It can be deduced that glyphosate act in the region of elongation and pose toxicity in the meristematic region. The effects of glyphosate is concentration-dependent. This is because root lengths exposed to the various treatments decrease with increase in the concentration of glyphosate. Significant statistical ($P<0.05$) differences were established between root lengths of onion roots exposed to varying treatment concentrations of glyphosate and control. However, there was no significant difference established between the number of roots of onions exposed to control treatment and glyphosate treatments tested at 0.05 level of significance. This is in conformity with the findings of Faizah (2014) who worked on WIDE-SPEC pesticide using *Allium cepa* assay and reported that statistically, significant differences in the root length of onion was established when comparison was made between roots exposed to control treatment and onion roots exposed to varying treatment concentrations of WIDE-SPEC pesticide, but he however observed no statistical significant difference in root number of onion roots exposed to control treatment and the varying concentration treatments of WIDE-SPEC pesticide.

V. CONCLUSION/RECOMMENDATIONS

In conclusion, the pesticide (glyphosate) is genotoxic. The induction of stickiness, presence of micro nuclei, lagging chromosomes, C- mitosis and nuclear lesion that were observed indicates that glyphosate can pose adverse effects on plants (and by extension, animals) as well as the environment at large. The disruption of the cell activities caused chromosomal aberrations and damaged cells especially with increase in concentration of glyphosate treatments.

The concentrations of glyphosate used in the field (by most farmers in Nigeria) is 6.0g/L. This concentrations which form part of the treatments for this work are harmful to the onion cells and could be harmful to animals and other plants that come in contact with them.

The evaluated macroscopic parameter such as root lengths (longitudinal growth) was also inhibited by glyphosate. It is recommended that increased awareness and enlightenment of the public on the dangers associated with the use of these chemicals and the need for adherence to safety measures by putting on PPE while making use of these chemicals be encouraged by the regulatory agencies.

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