



Evaluation of the Cytogenotoxic Effects of Emulsifiable Concentrate form of Amitraz Pesticide on *Allium cepa* L

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ABSTRACT: The cytogenotoxic effects of emulsifiable concentrate of amitraz pesticides was evaluated using *Allium cepa* L. test. The root meristems of *A. cepa* L. were treated with five concentrations (1%, 5%, 10%, 20% and 40%) of the chemical pesticide at 48 h for cytogenetic analyses and 96 h for root length inhibition. Pesticide doses affected root length significantly ($P < 0.05$) at 5% to 40%; with 50% effective concentration (EC_{50}) value of 18% while there was no significant difference between control and 1% ($p > 0.05$). The mean root length of the treated *A. cepa* for Amitraz pesticides in all concentrations was lower compared to the control showing the obvious mitodepressive effects of amitraz pesticides. A dose dependent reduction in the total mitotic dividing cells and mitotic index was observed in *A. cepa* treated with the pesticides. The values of mitotic index obtained for amitraz pesticides at 5% (5.20), 10% (4.0), 20% (2.30) and 40% (0.80) were lower than half of the negative control (7.25), which reflect its cytotoxicity. All the concentrations of the pesticides used in the present study induced important abnormalities during mitotic division. These aberrations were: chromosome stickiness, disturbed spindle, anaphase and telophase bridges, chromosome fragments, laggard chromosomes, and c-Mitosis. The highest abnormality number was observed in the root tips of *Allium cepa* (5%) while the least was at 40%. Frequencies of chromosome abnormalities were low at 20% and 40% concentration because of damaged cell and lower cell divisions. The present study, showed the inhibition of growth and induction of chromosomal aberrations by amitraz, this suggest their capability in inducing cytotoxicity and genome instability.

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A countless number of chemicals and toxic compounds, originating mostly from industrial and agricultural activities are being released in to our soil, water, and air environment constantly and continuously, and many of them are believed to have deleterious effects (Taylor *et al.*, 1997; Adesuyi *et al.*, 2015; Njoku *et al.*, 2016; Adesuyi *et al.*, 2016). The use of chemicals in modern agriculture has significantly increased productivity (Njoku *et al.*, 2018a). The world's limited croplands and growing population necessitated measures to increase crop and animal production in order to ensure food safety and security (Zhang *et al.*, 2007). This has led to significant increase in the concentration of insecticides, herbicides, pesticides and other associated chemicals in food and in our environment, with associated negative risks and effects on human health (Anderson *et al.*, 2014). A pesticide is any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest (insects, mites, ticks, nematodes, weeds, rats, etc.), including insecticide, herbicide, fungicide, and various other substances used to control pests (U.S

EPA, 2007; Adesuyi *et al.*, 2018; Njoku *et al.*, 2018b). Amitraz is a non-systemic acaricide and insecticide (Corta *et al.*, 1999) and has also been described as a scabicide. It was first synthesized by the Boots Co. in England in 1969 (Harrison *et al.*, 1973). Amitraz has been found to have an insect repellent effect, works as an insecticide and also as a pesticide synergist (Hayes and Laws, 1991). Besides its application as pesticide on plants, amitraz is also used as an animal ectoparasiticide on cattle, goats, sheep, pigs and dogs (Corta *et al.*, 1999). In these applications, it is exclusively applied externally (Peter *et al.*, 2006). It achieves special efficiency against mites (*Demodex canis*), but it also works against lice, flies, and all development stages of ticks (Peter *et al.*, 2006). In combination with additional agents it can be used against flea-infestation as well. For the treatment of dogs amitraz is available as a collar or as a spray- or wash-solution and has an immediate effect against tick infestation as well as a preventive effect. *Allium cepa* (Onion) has been considered as a most efficient test organism to indicate the presence of mutagenic chemicals due to its kinetic characteristics of

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proliferation and possession of chromosomes suitable for cytotoxic study (Soumya *et al.*, 2016). Different parameters of *Allium cepa* such as root shape, growth, mitotic index, chromosomal aberrations etc can be used to estimate the cytotoxicity and mutagenicity of environmental contaminants and pollutants (Ahmed, 2014; Soumya *et al.*, 2016). Several authors have pointed out the *Allium* test has been a useful tool for the detection of potentially genotoxic substances. Many genotoxic studies have been carried out to detect the harmful effects of different pesticides which reveal their hazardous effects in addition to benefits. A non-significant induction of sperm cell aberrations in mice was reported for emulsifiable concentrate form of deltamethrin (Yekeen *et al.*, 2007). The cytogenetic effects of lambda-dacyhalothrin were investigated in humans and various animal species using different endpoints such as micronucleus (MN) formation, induction of chromosomal aberrations and sister chromatid exchange (Fahmy and Abdalla, 2001; Celik *et al.*, 2005). The cytogenotoxic effects of emulsifiable concentrate of cypermethrin, deltamethrin, lambda-dacyhalothrin and endosulfan on *Allium cepa* root cells, and the pesticides induced growth inhibition and caused cytogenotoxic effects on the meristematic cells of *Allium cepa* (Yekeen and Adeboye, 2013). Chromosomal anomalies produced by pesticides therefore can be regarded as a reliable evidence for the evaluation of genotoxicity (Grant, 1982). The analysis of these chromosomal alterations serves as a mutagenicity test and is one of the few direct methods to measure damages in systems exposed to possible mutagens or carcinogens (Tedesco and Laughinghouse (IV), 2012). Data gap exists in the information available on the genotoxicity of the emulsifiable concentrate form of amitraz pesticides. The purpose of this study is to evaluate the cytotoxic effects of amitraz pesticide on root growth, mitotic index, and chromosome aberrations in the meristem cells of *Allium cepa*.

MATERIALS AND METHODOLOGY

Test organisms and chemicals: Healthy and equal sized of common onion (*Allium cepa*), of an average size of 15-20 mm in diameter were purchased from Agege market, Lagos state, Nigeria. Amitraz was procured in the emulsifiable concentrate form from Caam Vet, Agro-Allied Chemical store, Old Agege motor road, Tabon-Tabon, Agege, Lagos State, Nigeria. All other chemicals used were of analytical grade.

***Allium cepa* viability test:** The onion bulbs (*Allium cepa* L.) used for experiment were sundried for three weeks, and the outer scales and brownish bottom plates were carefully removed, leaving the root ring

primordial intact. The onions that sprouted well above 0.5 cm long were used for the experiment while those that didn't grow well were discarded.

***Allium cepa* assay:** The *Allium* test for macroscopic as well as microscopic evaluations adopted in this study was as previously described by Fiskesjo (1997). The outer scales of the onion bulbs and brownish bottom plate were carefully removed thereby leaving the ring of fresh root primordial intact. The peeled bulbs were transferred into distilled water during the cleaning procedure to prevent the primordial from drying. This followed with the bulbs being exposed directly in five concentrations (1, 5, 10, 20, and 40 %) of each pesticide were prepared with distilled water used as diluents as well as the control. For 1%, 2 ml of pesticide to 198 ml of distilled water, for 5%, 10 ml of pesticide to 190 ml of distilled water, for 10%, 20 ml of pesticide to 180 ml of distilled water, for 20%, 40 ml of pesticide to 160 ml of distilled water, and for 40%, 80 ml of pesticides to 120 ml of distilled water. Twenty onion bulbs were set up in each series for each sample, out of which the best ten with good root growth were selected for analysis of root growth inhibition. Distilled water was used as negative control. The experiment was set up in the dark at 28 °C for 72 h. Test pesticides concentrations were changed daily. Photographs of test materials were taken with Nikon Digital Camera D80 (Nikon Corp., Japan) and special note was taken of change of the morphology. After 48 h, one root tip was removed from each bulb, fixed in ethanol: glacial acetic acid (3:1, v/v) and hydrolysed with a solution of 1 N HCl at 65 °C for 3 min. After staining the tissue, the specimen on the slide was gently covered with a cover slip, allowing the stain to spread evenly over the square parts of the cover slip to eliminate air bubble. The slide with the specimen was then placed in between two folds of the filter paper and using the blunt end of a pen, gentle tapping and pressure was applied around the square area of the cover slip for even squashing of the specimen. Finally, the square edges of the cover slip of the squashed onion roots was sealed with white transparent nail hardener to prevent drying out of the preparation by the heat of the microscope (Grant, 1982). Three slides were prepared for each concentration and control. After 96 h, mean length of root bundles were obtained as described by Fiskesjo (1985) and the EC₅₀ values was extrapolated from the graph of percentage root growth relative to control (inhibition) against pesticides concentrations. The slides were viewed under the microscope to observe mitotic stages and chromosomal aberrations to produce photomicrographs. The mitotic index (MI) was calculated as the ratio of number of dividing cells to number of observed cells (Fiskesjo, 1997). The

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frequency of aberrant cells (%) was calculated based on the number of aberrant cells per total cells scored at each concentration of each effluent.

One thousand (1,000) cells per slide per concentration were scored for the frequency and occurrence of different types of chromosomal aberrations in the dividing cells (Bakare *et al.*, 2000). These are calculated as follows:

$$\text{FOA} = \frac{\text{No of aberrant cells}}{\text{No of cells scored}} \times 100$$

Where FOA = frequency of aberration

$$\% \text{ RI} = \frac{\text{MRLC} - \text{MRLT}}{\text{MRLC}} \times 100$$

Where RI = root inhibition; MRLC = mean root length control; MRLT = mean root length treatment

$$\text{MI} = \frac{\text{NCT}}{\text{Total number of cell}} \times 100$$

Where MI= mitotic index; NCT= number of dividing cell in the treatment

$$\text{M In} = \frac{\text{MIC} - \text{MIT}}{\text{MIC}} \times 100$$

Where M In = mitotic inhibition; MIC = mitotic index of control; MIT = mitotic index of treatment

Statistical analysis: The means with the standard errors for each of the concentrations per pesticide were calculated. The data obtained for the root length of the treated groups and the control was compared using t-test and considered significant at $P \leq 0.05$. Microsoft Excel and GraphPad 7.0 statistical packages were used.

RESULTS AND DISCUSSION

Mean root length, growth inhibition and mitotic indices of Amitraz 20 EC pesticide on Allium cepa: The Allium test is considered to be a standard procedure for quick testing and detection of toxicity and pollution levels in the environment. In *Allium cepa* test, there usually seems to be a relative decrease in root growth (cytotoxicity) and chromosomal deviations (genotoxicity). Length of *Allium cepa* roots treated with different pesticide doses are shown in Table 1. Pesticide doses affected root length significantly ($P < 0.05$) at 5%, 10%, 20% and 40% while there was no significant difference between

control and 1% ($p > 0.05$). The mean root length of the treated *A. cepa* for amitraz pesticides in all concentrations was lower compared to the control showing the obvious mitodepressive effects of emulsifiable concentrate form of amitraz pesticides. The pesticides induced significant growth inhibition at 10.0, 20.0 and 40.0%. The highest percentage root inhibition was observed at 40% concentration (61.45%) followed by 20% (54.22%). The cytotoxicity level of a test compound can be determined based on the increase or decrease in the mitotic index (MI), which can be used as a parameter of cytotoxicity in studies of environmental biomonitoring (Topcu *et al.*, 2013). The mitotic index reflects the frequency of cell division. Table 1 also shows the microscopic evaluation of the pesticides. A dose dependent reduction in the total mitotic dividing cells and mitotic index was observed in *A. cepa* treated with the pesticides. The values of mitotic index obtained for amitraz pesticide at 5% (5.20), 10% (4.0), 20% (2.30) and 40% (0.80) were lower than half of the negative control (7.25), which reflect its cytotoxicity. Cytotoxicity is defined as a decrease in mitotic index and as increase in the fraction of cells with c-Mitosis, multipolar anaphase and sticky, and laggards (Fiskesjo, 1995; Marcano *et al.*, 2004). Reduction in mitotic activity could be due to the inhibition of DNA synthesis (Sudhakar *et al.*, 2001) or due to a block in the G2-phase of the cell cycle, thus preventing the cell from entering mitosis (Yekeen and Adebayo, 2013). On the other hand the inhibition of certain cell cycle specific proteins remains as a possible herbicide target site. Thus, inhibition of enzyme DNA polymerase which is necessary for the synthesis of DNA precursors as well as other enzymes more directly involved with spindle production, assembly or orientation could explain the reported antimitotic effect (Tartar *et al.*, 2006). Therefore, these processes explained the inhibitory effect of amitraz pesticides induced in *Allium* plant-system in the present study. Also mitotic activity decreased as the concentration of the pesticides increases. Mitotic inhibition of *Allium cepa* by emulsifiable concentrate of amitraz was found significantly higher than for the control. As a result of these data, Amitraz can be called a mitotoxic agent that prevented entry into the cell cycle. Yıldız and Arıkan (2008) and Eren *et al.* (2015) reported that quizalofop-P-ethyl and dioxacarb pesticides inhibited mitosis in *Allium cepa*. Inhibitions of mitosis by several other pesticides have also been reported (Liman *et al.*, 2011; Popescu *et al.*, 2013) The EC_{50} value of 18% concentration was extrapolated for amitraz pesticide. The growth inhibitory effect of the pesticide is indicated by the significant reduction of root length compared to the control.

Table 1: Mean root length and root inhibition of *Allium cepa* L. exposed to different concentrations of amitraz pesticides

Conc. (%)	Mean root length	Percentage Root Inhibition (%)	No of dividing cells	Mitotic Indices	Mitotic Inhibition
0 Control	3.32±0.05	-	145	14.50	-
1	2.88±0.05	13.25	94	9.40	35.17
5	2.42±0.10*	27.11	52	5.20	64.14
10	2.01±0.16*	39.46	40	4.00	72.41
20	1.52±1.25*	54.22	23	2.30	84.14
40	1.28±0.08*	61.45	8	0.80	94.48

1000 cells were scored per concentration; *t-test show significant difference compared to control (P ≤ 0.05).

Table 2: Types of chromosomal aberrations caused by amitraz in root tips of *A. cepa*

Conc. (%)	No of Dividing cells	Cells with Aberrations	Frequency of aberrations (%)	Types of abnormalities					
				Stk	Dist	Brid	Frag	Lagg	C mit
Control	145	-	-	-	-	-	-	-	-
1	94	22	2.20	-	15	5	1	1	-
5	52	37	3.70	4	22	7	4	-	-
10	40	13	1.30	1	8	3	-	-	1
20	23	6	0.60	2	2	-	-	-	2
40	8	2	0.20	-	1	-	-	1	-

Stk-sticky chromosomes, Dist-disturbed spindle, Bri-chromosome bridge, frag-chromosome fragment, Lag-lagging chromosomes, C-mitosis

Chromosomal aberrations caused by amitraz on the root tips of Allium cepa L. Chromosomal aberrations in cells which are caused to the cytotoxic effects of pesticides on plants can be considered as an indicator of genetic damage. All the concentrations of amitraz pesticides used in the present study induced important abnormalities during mitotic division in *Allium cepa* L when compared to control. These abnormalities were: chromosome stickiness, disturbed spindle, anaphase and telophase bridges, chromosome fragments, laggard chromosomes, and c-Mitosis (Figure 1-5). The highest abnormality number was observed in the root tips of *Allium cepa* was 5% while the least was at 40%. Frequencies of chromosome abnormalities were low at 20% and 40% concentration because of damaged cell and lower cell divisions (Table 2).

The findings from this study is similar to earlier genotoxicity studies of Dursban 4 pesticides by Topcu *et al.* (2013) where *Allium cepa* showed concentration-related increase in the frequencies of chromosome aberrations. Chromosome breaks and fragments, vagrant chromosomes and double bridged formation were observed to be frequent aberrations in *Allium cepa*. Chromosome stickiness observed in this study may occur as a result of physical adhesion of the proteins of the chromosomes (chromatin material) (Ping *et al.*, 2012). Mercykutty and Stephen (1980) reported that this stickiness might be interpreted as a result of depolymerisation of DNA, partial dissolution of nucleoproteins, breakage and exchanges of the basic folded fiber units of chromatids, and stripping of the protein covering DNA in chromosomes. As it was reported, sticky chromosomes indicated the presence of a highly toxic substance, inducing irreversible effects in the physical state of the chromatin (Fiskesjö, 1985). Disturbed spindle was the highest and most

frequent aberration. It is likely that many of chromosomal aberrations induced by the action of various types of mutagenic agents might be due to the dysfunction of nuclear spindle. In this study, the disturbance of mitotic spindle could lead to C-metaphases and multipolar anaphase. The abnormal C-metaphases were formed as a result of the complete inactivation of division of the spindle (Fiskesjö, 1993; Sutan *et al.*, 2014). Consequently arrest of cells in metaphase stage might be one of the causes of mitotic inhibition.

Also, in this study occurrence of C-mitosis, lagging chromosomes and multipolar anaphases, clearly showed the accumulated effect of amitraz on spindle formation and function. In most studies, aberrations of mitotic cycle, change of mitotic index and chromosomal abnormalities observed after exposure to toxic metals, metalloids or organic pollutants were attributed to the disorganization and depolymerization of microtubules, which underlie these processes in higher plant cells (Adamakis *et al.*, 2013, Eleftheriou *et al.*, 2013; Yekeen and Adeboye, 2013).



Fig 1: Sticky chromosomes

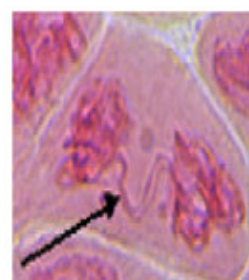


Fig 2: Chromosome Bridge at Telophase



Fig 3: C-Mitosis



Fig 4: Laggard Chromosomes

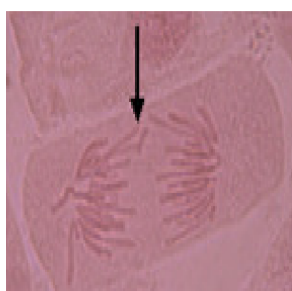


Fig 5: Chromosome fragment

Conclusion: The present study, showed the inhibition of growth and induction of chromosomal aberrations by the amitraz pesticides which indicates their cytogenotoxic potential. Our findings revealed that even at low concentration (5%) the pesticides was toxic for root tip cells of *A. cepa* with a high significant effect on mitotic process. The frequencies of all the mitotic anomalies showed a good correlation with the concentration of amitraz. In a long run, the use of this pesticide may have a negative impact on eukaryotic genome including plants and animals. This data provide more information on amitraz pesticides, the EC₅₀ of 18% concentration, and the likelihood of exposure to substantial concentration may constitute health risk to non-target organisms, and thus it will assist in future ecotoxicological evaluations.

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