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Cytotoxic and Genotoxic Potency Screening of WIDE-SPEC Pesticide on *Allium cepa* L. Root Meristem Cells

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Abstract

Pesticides possess biological activity including genotoxic influence and can affect non target organisms. The cyto- and genotoxicological potency of wide-spec (Abamectin+Emaamectin benzoate) pesticide was screened using *Allium cepa* L. test. Onion roots were exposed for 24 h to ten concentrations (0, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1ml.L^{-1}) of pesticide and 8 gm.L^{-1} of cyclophosphamide as positive control. Some microscopic endpoints as: mitotic indices and chromosomal aberration types were evaluated. The results showed obvious concentration-dependence. Mitotic index decreased from 21.77% (negative control) to 11.97% (dose of 1.00). Abnormal cell's frequency was considerably increased, too. Root growth retardation was significant due to different doses. Physiological and clastogenic types of chromosomal aberrations, as: lagging, fragments, granule, bridges, polyploidy, stickiness, and micronuclei chromosomes were observed in all corresponding concentrations. Data of this study showed that analyzed pesticide (commonly used in Iraqi agriculture) can potentially induce cytotoxic and genotoxic effects on crops and ultimately damage biota and human health.

Key words: *Allium cepa*, cytotoxicity, genotoxicity, pesticides.

1. Introduction

Large amounts of chemicals are released into the environment and many of them have deleterious effects on non-target organisms, being a potential hazard to human health (Taylor *et al*., 1997). Pesticides are used in modern agriculture practices for efficient crop production and to improve the shelf life of agricultural yields through inhibition of diseases causing organisms in the field and during storage (Taylor *et al.,* 1997).

The pesticides possess biological activities including genotoxic and cytotoxic influences (Asita and Matebesi 2010, Paul *et al*., 2013). pesticides residues are known to persist in soil , water , and food , so constant use of these chemicals with mutagenic potential may result in changing the hereditary constitution of an organism being mutagenic and or carcinogenic (Drageova *et al.*, 2012 ; Bolognesi *et al*., 2003) .

Abamectin is a mixture of avermectin B1a and avermectin B1b (Dybas et al. 1989) . The avermectin represent a novel class of naturally occurring macrocyclic lactones with potent nematicidal , acaricidal and insecticidal activities. These components avermectin B1a and avermectin B1b are produced by fermentation of soil actinomycetes, *Streptomyces avermititilis* (Siddque *et al*., 2013). The benzoate salt emamectin benzoate is also used as an insecticide, in fruits, vegetables and crops fields (Jansson *et al.*, 1996). In Iraq WIDE-SPEC (Abamectin and Emamectin) insecticide is widely used in agriculture for disease control .

The *Allium cepa* assay is an efficient test for chemical screening and *in-situ* monitoring for genotoxicity of environmental contaminants (Firbas and Amon, 2013) due to the fact that data obtained with this plant show correlation with mammalian and non-mammalian test systems (constantin and Owen, 1982).

The studies made by many authors pointed out that the *Allium* test has been a useful tool for the detection of potentially genotoxic substances in water screening programmes (Sabti, 1989). Using *Allium* test a cytogenetic study was conducted on heavy metals and cyanide contaminated river water in south Bulgaria (Ivanova, *et al*., 2005).

Sifa (2009) reported the cytogenetic effects of food additives on the root meristem cells of *A. cepa*.

Allium test has been widely used to study genotoxicity of many pesticides revealing that these compounds can induce chromosomal aberrations in root meristems of *A. cepa* (Yekeen *et al.,* 2013; Thais *et al*., 2007; Cabrera *et al*., 1996).

The purpose of this study is to investigate the effects of WIDE-SPEC pesticide on root growth, mitotic index, chromosome aberrations and micronucleus formation in the meristem cells of *Allium cepa.*

2. Materials and Methods

The insecticide used in this study is a mixture of 10 gr.L^{-1} abamectin and 18 gr.L^{-1} emamectin benzoate , whose trade name is WIDE-SPEC ; batch no. 20080891 . The chemical was a product of Agrichem Manufacturing Industries Pty Ltd. Austria.

The plant used as test material was *allium cepa* L. (2n=16). Equal-sized and healthy onion bulbs (25-30 mm in diameter) were chosen. Before starting the experiments onion bulbs were dried and the outer brownish scales were carefully removed .

Eight concentrations (0.3,0.4,0.5,0.6,0.7,0.8,0,0.9 and 1.00 ml.L^{-1}) of the pesticide were prepared with distilled water used as diluents as well as negative control . Cyclophosphamide 8g.L^{-1} was used as positive control.

Eight (8) onion bulbs were germinated per concentration with each bulb in culture tube filled with the freshly prepared concentration of the pesticide. After 48h the root number and length were counted . Two bulbs with the highest and poorest growth were neglected.

2.1 Genotoxicity Assay

The method used was similar to the method of Matsumoto *et al*. (2006). *Allium cepa* (onion) seeds were germinated in petri dishes containing pesticide-soaked filter paper (test), water-soaked filter paper (negative control) or on filter paper soaked in aqueous solution of 8% cyclophosphamide (positive control). In this experiment, a discontinuous treatment protocol was used. Seeds were first soaked in distilled water until the radicles reached a length of about 2cm. Germinated seeds were transferred to petri plates containing the pesticide at different doses in which they were left for 24 hours (acute treatment) at room temperature. At the end of exposure period, onion bulbs were assessed.

2.2 Root Harvest and Slide Preparation

Root tips 1-2 cm long were cut and placed in a watch glass and fixed in acetic alcohol (Ethanol: Glacial acetic acid in 3:1 ratio) at 4-6 C° for 3 hours. The root tips were washed twice with ice cold water for 10 minutes each and allowed to dry. A solution of 1N HCl pre-heated to 60° C was added to the root tips in the watch glass for 10 minutes and the HCl was discarded. The HCl treatment was repeated a second time. Two root tips were transferred to a clean microscope slide and were cut 2 mm from the growing tip. The tips were kept and the rest was discarded. Aceto-carmine stain was added to the slide to cover the root tips for 2-3 minutes. A cover glass was placed on the root tip and the root tip spread evenly to form a monolayer by gently tapping the cover glass with a pencil eraser to facilitate the scoring process for normal and aberrant cells in the different stages of the cell cycle.

2.3 Scoring of Slides

The slides were viewed under the light microscope (Olympus CX 21) using the 100x objective with oil immersion. A total of 3000 cells were scored on each slide. The cells were recorded as normal or aberrant in the different stages of the cell cycle namely: interphase, prophase, metaphase, anaphase or telophase. All cells with chromosomal aberrations were counted and the most representative ones for each abnormality were photographed using a Zeiss PrimoStar microscope mounted with Canon camera model, Power Shot A640.

3. Data Analysis

3.1 Cytotoxicity was Determined by the Mitotic Index

The Mitotic index was calculated as the number of cells containing visible chromosomes (i.e. cells in the division stages) divided by the total number of cells scored.

The mitotic indices of the treated cells at each dose of pesticide were compared with that of the negative control group. Any dose of a test substance was adjudged to be cytotoxic if the mitotic index of treated cells at that concentration was half or less, compared to the mitotic index of the concurrent water treated cells.

3.2 Genotoxicity Assessment

Dividing cells with any of the under listed abnormalities were recorded, namely;

a. Lagging chromosomes b. Cells with fragment chromosomes.
c. Granular chromosomes d. Bridges formation e. polyploids.
f. Cells with stick chromosomes g. Micronucleus.

The number of aberrant cells /1000 cells in each of the four division stages for pesticide treated cells were compared with the numbers for the water treated (negative control) cells by the Chi-Square test and linear regression equation using the SPSS 20.0 for statistical package. The calculated chi-sqaure value for each comparison was obtained. If the calculated value was more than the critical value at the 0.05 probability then a

statistically significant difference existed between the mean and the pesticide was adjudged to be genotoxic at the dose of the pesticide.

4. Results

4.1 Number and Length of *A. cepa* Roots

Number and length of *Allium cepa* roots treated with different pesticide doses are shown in Table 1. Pesticide doses affected root length significantly ($P<0.05$), whereas, root number didn't show any differences due to different doses. Concentrations from 0.6-8.0 showed same length, which was higher than that of the doses of 0-0.5. About 98.3% of variation in root length was determined by pesticide dose as regression equation describing this relation is:

Root length= 9.584-6.67*dose (R^2=98.3%).

Table (1) Root number and length of different doses (±standard error)

Dose	Root number	Root length (cm)
Negative control (0)	15.28±2.66	9.65±0.64 a
0.3	21.57±4.34	7.41±0.80 a
0.4	20.14±3.38	7.22±0.55 a
0.5	15.29±2.29	6.03±0.22 a
0.6	13.57±2.62	5.90±0.65 b
0.7	14.57±2.37	4.54±0.40 b
0.8	16.14±3.44	3.96±0.46 b
0.9	14.00±2.90	3.88±0.59 b
1.00	10.85±1.88	3.00±0.37 c
Positive control (8)	12.86±2.34	3.19±0.46 c

Means with different subscripts differ significantly at p≤ 0.05

4.2 Cytotoxicity of the Pesticide

The results of the cytotoxicity determination are presented in Table 2 and Fig. 1. Cells treated with the pesticide had reduced mitotic indices compared with cells treated with water which was indicative of inhibition of cell division by these pesticides. For all pesticide levels, the cytotoxic effects were observed in cells exposed to all doses (0.3-8.0). There were significant ($P<0.05$) differences in mitotic index among all doses (table, 2). The cytotoxic effects were therefore dose dependent. The association (linear regression equation) between mitotic index and doses was:

Mitotic index= 16.858-1.12*dose (R^2=51.0%).

Dose can change around 51% of mitotic index as it decreased from 21.77% (0 dose) to 8.83% (at dose of 8). Doses of either (0.3 or 0.4) or (0. 5 or 0.6) or (0.7or 0.8) or (0.9 or 1.0) showed similar mitotic index. In comparison of mitotic index of different doses with that of negative control, a dose of 0.3 and 1.00 showed 86.49% and 54.9% of negative control respectively.

On the other hand, mitotic indices of different mitotic division showed fluctuated percentages due to different doses of the pesticide (fig. 1). Index decreased significantly ($p<0.05$) as division phase proceed. It recorded highest values at prophase and the lowest values at telophase. Mitotic index decreased at a rate of 13.37% each phase toward telophase and different phase describe 89.80% of the total variation of mitotic index. Regression equation describing the relation between mitotic index and division phases is:

Mitotic index= 58.42-13.37*phase (R^2=89.80%). Table (3) showed the association between mitotic index and stage of division according to different doses of the pesticide. Different level of significance is related to different dose of wide-spec pesticide.

Table (2) Number of observed and dividing cells and mitotic index (±standard error)

Dose	No. of cells observed	No of dividing cell	Mitotic index	Mitotic index as % of 0 dose
Negative control (0)	3000	653	21.77±0.03 a	100
0.3	3000	565	18.83±0.05 b	86.49
0.4	3000	535	17.83±0.08 b	81.90
0.5	3000	506	16.87±0.04 c	77.49
0.6	3000	477	15.90±0.02 c	73.03
0.7	3000	447	14.90±0.09 d	68.44
0.8	3000	418	13.93±1.00 d	63.98
0.9	3000	389	12.97±0.08 e	59.58
1.0	3000	359	11.97±0.06 e	54.98
Positive control (8)	3000	265	8.83±0.09 f	40.56

- ***Means with different subscripts differ significantly at 0.05***

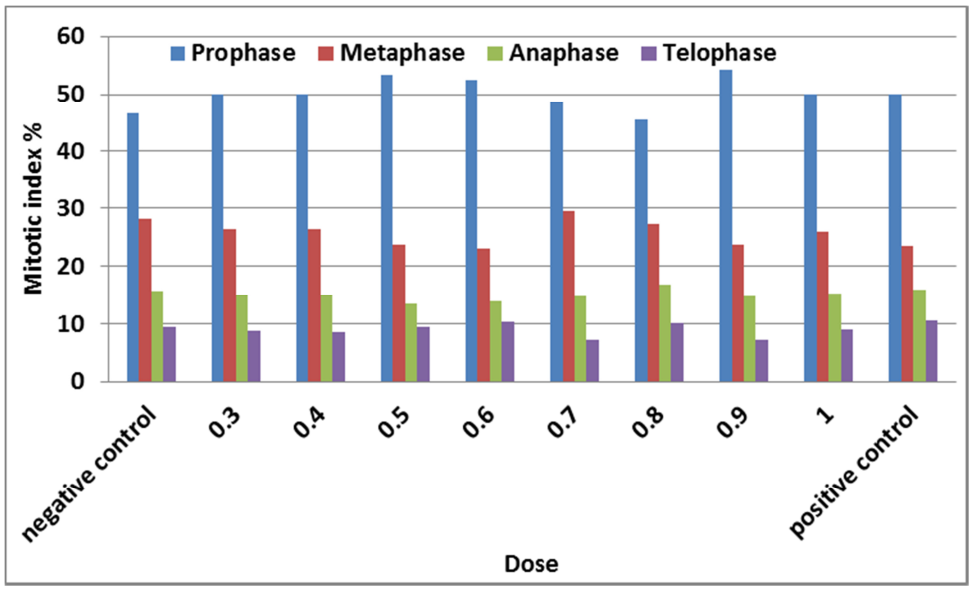


Figure (1) Mitotic index of *A. cepa* root tips exposed to different doses pesticide

Table (3) Rate of deterioration of mitotic index due to different dose of pesticide

Dose	Rate of deterioration of mitotic index%	Level of significant
Negative control	12.38	0.024
0.3	13.55	0.040
0.4	13.57	0.040
0.5	14.21	0.075
0.6	13.51	0.085
0.7	13.89	0.017
0.8	11.69	0.024
0.9	14.95	0.062
1.0	13.37	0.044
Positive control	12.55	0.072

4.3 Genotoxicity of the Pesticide

The genotoxic effects of the pesticide as, determined by comparing the number of aberrant cells in division stages for each dose of each pesticide with those of the concurrent negative control in Chi-Square test are presented in Figure 2. The genotoxic effects that were observed in the present study included different chromosomal aberrations (lagging, bridges, fragments, granular, stick, polyploidy and micronucleus).
Representative pictures of different types of chromosomal abnormalities are presented in Figure 4.

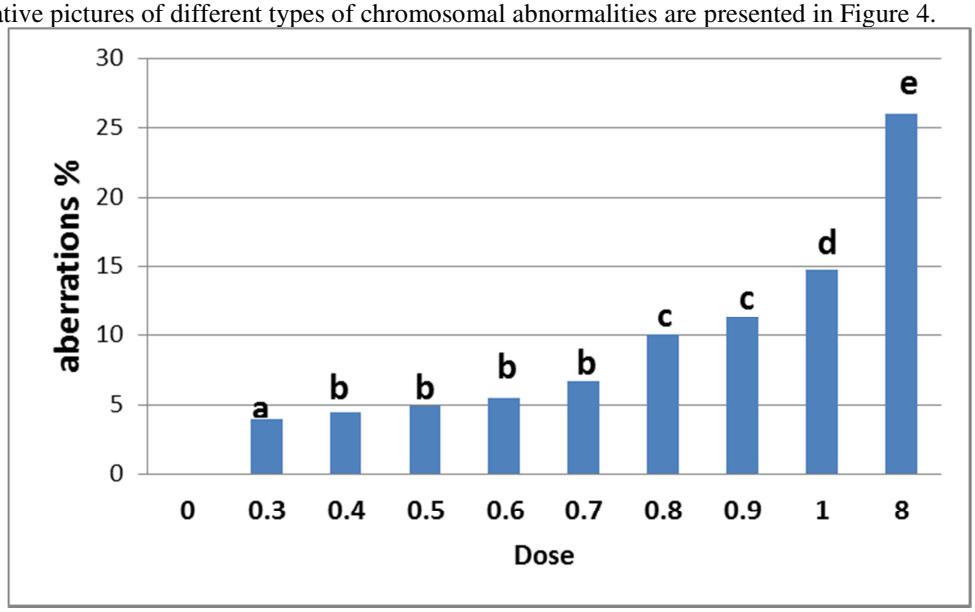
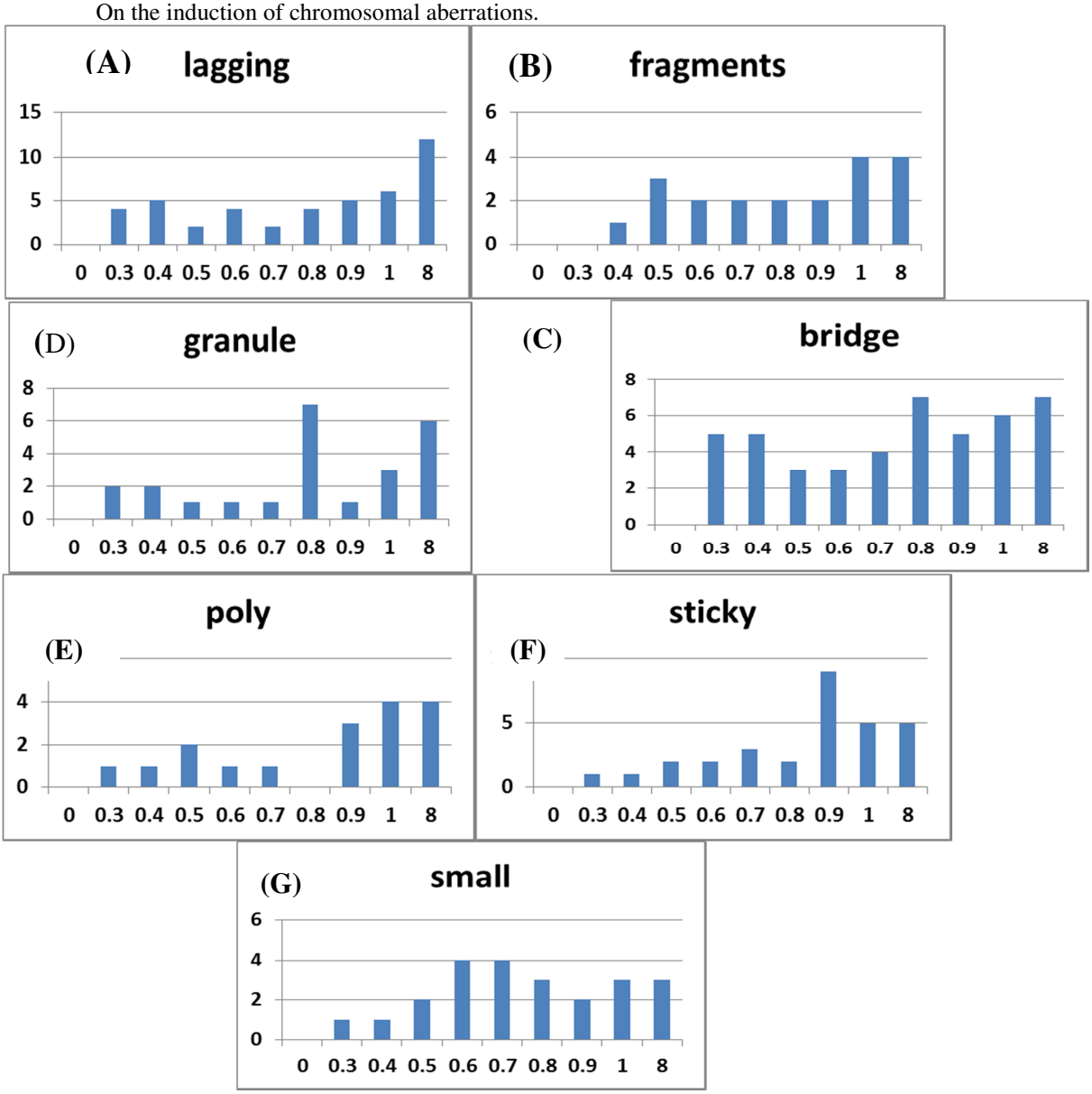


Fig. (2) Aberrations % due to different doses of wide-spec pesticide

Doses of the pesticide induced significant frequencies of aberrant division stages compared with the water (negative control) treated group, ($P < 0.05$). They were thus genotoxic. The positive control chemical and the pesticide were genotoxic. Another method of estimating the genotoxic effect of the pesticide involves the calculation of the ratio of aberrant (ABN) to total dividing onion cells (ABN/ (N+ABN)) (Matsumoto et al, 2006). When that was applied to the data obtained in this study, it was found that, for the positive control chemical and at all concentrations of pesticide, the aberrant onion cell rate was higher than that recorded for the negative control (Fig. 3, A-G). This method of assessment the genotoxic effect was however not considered in the discussion in the present study. It has been included to highlight the need for standardization.

Figure 3: Effect of different concentrations of wide-spec pesticide
On the induction of chromosomal aberrations.



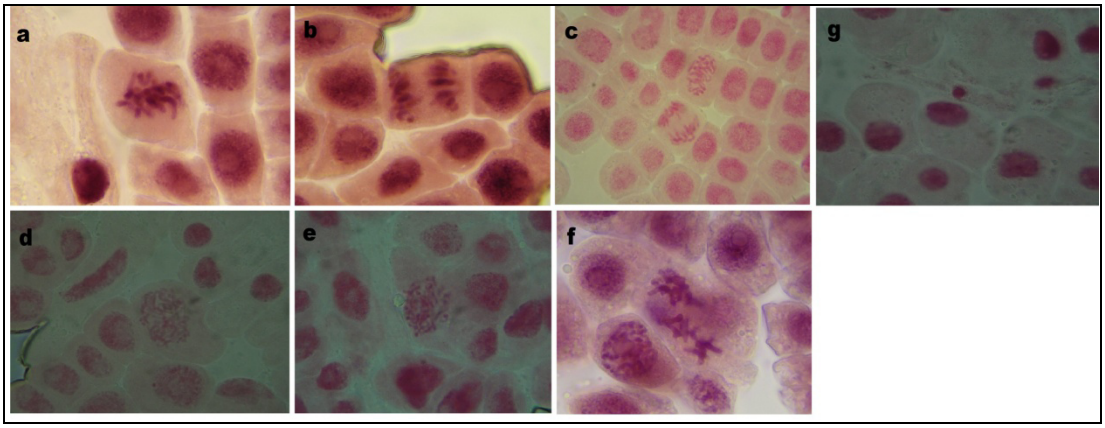


Figure 4: Different types of chromosomal aberrations induced by wide-spec pesticide in root tips of *Allium cepa*. (a) Lagging ; (b) Fragments ; (c) Granule ; (d) Bridges ; (e) polyploids ; (f) stickiness ; (g) micronucleus .

5. Discussion

The use of plants for the evaluation of environmental pollutants such as pesticides is becoming common practice because plants are direct recipients of agro toxics, so they are important material for genetic test and for environmental monitoring of places affected by such pollutants (Cabrera et al, 1994). The higher plants *Allium cepa* (onion), *Tradescantia paludosa* and *Vicia faba* have relatively large monocentric chromosomes in reduced numbers and are accepted as suitable test organisms for the study of cytogenotoxicity of environmental mutagenic substances and complex mixtures (Yekeen etal. 2013, Patra and Sharma 2002, Rank and Nielsen 1998).

Toxic effects of environmental pollutants may be evaluated by analyzing macroscopic parameters of root number and root growth, while both genotoxicity and cytotoxicity can be monitored through cytological parameters (chromosomal aberrations, micronucleus and mitotic index). The results of the present study suggest wide-spec pesticide is toxic causing an inhibition of longitudinal growth of roots as a result of different concentrations applied. It is well known oxygen consumption associated with respiration is higher in the meristematic zone, where cell division is most active; it is plausible that the reduction occurs in the elongation zone, where oxidation reactions are less favourable than in the meristematic zone (Lerda et al. 2010). This could lead to a toxic effect of wide-spec and growth inhibition of the root, demonstrating that wide-spec acts in elongation zone and causes toxicity in the meristematic zone.

Mitotic index (MI) is considered as a parameter that allows one to estimate the frequency of cell division (Marcano et al. 2004). In the present study, the decrease of MI in *Allium cepa* was significant in all concentrations when compared to control, which were dose dependent as well as the pesticide caused a change in the frequency and fluctuations of the mitotic phases. A depression of the mitotic index has been recorded by many investigators as a result of treatment with pesticides. Fisun and Rasgel (2009) on *Allium cepa* by using fungicide raxil, herbicide illoxane (Yuzbasioglu et al. 2009), diphenyl-ether (Drageova et al. 2012), glycidal (Panneerselvan etal.2012) and blitox (Paul et al. 2013).

Changes in mitotic activity show that wide-spec pesticide at different concentrations depressed cellular proliferations in *Allium cepa* root tips after treatment for 24 h. Reduction in mitotic index was described by many authors following treatment of Allium cepa with a pesticide (Asita and Matebsi, 2010, Aydemir et al., 2008).

Several mechanisms were proposed for chemically decreased mitotic index in plant cells. The first is that a decrease in MI could be due to blocking of G1 suppressing DNA synthesis (Shcneiderman et al., 1971). The second possible mechanism is a blocking of G2 preventing the cell from entering mitosis (Van't, 1968). The lowering of the mitotic index might have been achieved by the inhibition of DNA synthesis at S- phase (Sudhakar et al., 2001).

The genotoxic effects, chromosomal aberrations (changes in the organization and morphology of the chromosomes) were observed in cells treated with the pesticide at all tested concentrations. According to Rank and Nielson (2003) chromosome aberrations analysis does not only allow estimation of genetic effects, but also enable evaluation of their clastogenic action. In this study seven types of aberrations were recorded; lagging, fragments, granules; bridges; polyploids; stickiness; and micronucleus. It has been shown by many investigators that several other pesticides induce chromosomal abnormalities in *Allium cepa* (Yekeen 2013, Asita et al. 2010). The detected chromosomal aberrations are due to chromatin dysfunction (stickiness, bridge, and fragments) or

due to the effect of the pesticide on spindle apparatus formation and thus resulted in cell division disturbances. Chromosome bridges result from chromosome and /or chromatid breakage and fusion or may be caused by stickiness of chromosomes which made their separation and free movements complete and thus they remained connected by bridges (Ping et al. 2012). Micronucleus can be spontaneously originated due to the development of the isolated chromosome that results from an unequal distribution of genetic material or as a result of acentric fragments or entire chromosome not incorporated to the main nucleus during cell cycle (Ping et al. 2012). However their induction is commonly used to detect genetic damages derived from exposure to mutagenic chemicals. However, all these changes may induce the formation of polyploidy when not reversed.
Aberrations of mitotic cycle, change of mitotic index and chromosomal aberrations observed after treatment or exposure to environmental pollutants such as toxic metals, pesticides were attributes to the disorganization and depolymerization of microtubules, which underlie these processes in higher plant cells (Adamakis et al., 2013, Eleftheriou et al., 2012, Dho, et al. 2010).

6. Conclusions

The results of this study showed that WIDE-SPEC pesticide causes decrease in the mitotic index and induced chromosomal abnormalities in *Allium cepa*, suggests the cytotoxic and genotoxic potential of this pesticide commonly used in Iraqi agriculture.
The concentrations used in the field are higher, and could be more harmful for animals and plants that come in contact with it. Hence, the use of this pesticide should be under control in agricultural fields.
The present study has, therefore, proved the usefulness of the *Allium cepa* chromosome aberration assay in assessing the genotoxicity of environmental chemicals.

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