

Assesment of the Genotoxic Effect of the Diazinon on Root Cells of *Allium cepa* (L.)

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ABSTRACT

Organophosphorous pesticides (OPs) posses a great potential of acute toxicity for exposed animals and men. To evaluate the toxic potential of the organophosphate diazinon on root meristematic cells of *Allium cepa* L., was created two groups: In group 1 (control group), was not given any chemical. In group 2 (diazinon-treatment group), different doses (10, 40, 80 and 160 ppm) and times periods (24, 48 and 72 h) were administered. After exposure, cell death, effective concentration (EC_{50}), mitotic index, cellular /chromosome aberrations, DNA damage by comet assay and RAPD-PCR were assessed at exposure times. EC_{50} value of diazinon was detected approximately 80 ppm. Hyperchromasia, later segregation, micronucleus, pulverised nucleus, nuclear cytoplasmic shrinkage and cell death, cytoplasmic vacuolation were detected in meristem cells as chromosome/celular aberrations for 72 h at 80 ppm. DNA damage was identified using tail DNA%, tail lengths and tail moment from these cells. Increasing exposure doses of diazinon caused increasing tail DNA% and tail lengths at 72 h. DNA bands of increasing concentrations treated groups were more distant to compare with the control group according to RAPD-PCR method. Diazinon cause cytotoxic and genotoxic on *A. cepa* root and could be considered for further toxicological evaluations.

Key words: diazinon, *Allium* test, DNA damage, genotoxicity, RAPD-PCR



INTRODUCTION

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INTRODUCTION

Organophosphorus pesticides (OPs), such as diazinon, are one of the most part used in agriculture as pesticides. To control of soil insects and pests, on ornamental plants, and on fruits, vegetables and field crops, it has been used during the past decade. OPs were caused inhibition of acetyl cholinesterase activity in paralysis and death insects, disorder of nervous system of humans and animals. Previous studies have shown that OPs are mutagenic, carcinogenic, cytotoxic, genotoxic, teratogenic, and immunotoxic¹.

Diazinon(O,O-diethyl-O-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl]phosphorothioate) (C₁₂H₂₁N₂O₃PS) is widely used especially in agriculture but one of great environmental toxic compounds can be absorbed by soil and surface waters. Reaching the living diazinon has decomposed to form diazoxon, hydroxydiazoxon and hydroxydiazinon in microsome of cell².

Many scientific researchers have revealed that diazinon caused histopathological, biochemical and physiological alterations³. Diazinon have toxic effects on blood cells, spleen, thymus and lymph nodes of rats⁴ and other organisms⁵. Mitochondrial membrane of rat liver and cytochrome P450 system of human liver were damaged by diazinon⁶. Diazinon caused changes in liver enzymes and biochemical indices and swelling of mitochondria and levels of several enzymes of glucose metabolism in hepatocytes^{7,8}. Al-Attar³ showed that diazinon causes several severe the hematobiochemical and histopathological alterations in liver, kidney and testis in rats. The *Allium cepa* (L.) root chromosomal aberration assay, introduced by Levan⁹, is used to detect toxicity of pesticides¹⁰, herbicides¹¹, and other xenobiotics. It is a simple and reliable assay used to record all chromosomal aberrations in mitotic cells^{12, 13}. The test can be employed to measure both cytotoxicity and genotoxicity; the rate of root growth can be correlated with the mitotic index¹⁴. Bianchi et al.¹⁵ evaluated imidacloprid insecticide, sulfentrazone herbicide and to the mixture of them the cytotoxic and genotoxic effects of low concentrations of pesticides on *A. cepa* for 24 h. Kumar et al.¹⁶ evaluated the cytogenetic effects of chromium (III) oxide nanoparticles on the root cells of *A. cepa*. Prajitha and Thoppil¹⁷ found that possible genotoxic effects of *A. spinosus* leaf extracts on *A. cepa* root meristematic cells and its antigenotoxic effects against H₂O₂-induced genetic damage in *A. cepa*.

Polymerase chain reaction (PCR) is fundamental to generate a sufficient number of molecular markers with smaller amounts of less pure DNA¹⁸. The random amplified polymorphic DNA (RAPD) technique determines the DNA sequence for the targeted genome of different vegetables and animal varieties species specific DNA comparison and to evaluate the interaction of toxic material with DNA resulting in damage¹⁹. Many researchers were used RAPD molecular analysis for determining toxic effect of many chemicals on studies about animal foods and plant¹⁸. Aksakal et al.²⁰ were used the herbicide on root of maize with RAPD assay because of its genotoxic property. Tedeschi et al.¹⁸ shown that systematic studies could contain RAPD analyses to obtaining characteristic properties of *Tropea* red onion.

Numerous xenobiotics and some of these free radicals have been given for DNA cell damage. This damage is caused by the disruption of the structure of DNA. It can be measured with comet assay also called Single Cell Gel Electrophoresis (comet) assay. This method is a so fast and sensitive for showing harmful effect of chemicals on DNA of alive cells²¹. This damage can turn into cancer or chromosomal deformity that cause to sister chromatid exchanges, micronuclei, cell death and mutations²².

The toxic effects of insecticides, especially diazinon, on *A. cepa* have not been studied until now. In this study, it was used for the first time six parameters, concentrations of EC₅₀, chromosome/cellular aberrations, cell death, DNA damage, mitotic index and RAPD-PCR in *A. cepa* with diazinon-treatment. Each of the selected parameters

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showed a toxic effect on tissue from a different angle. So, this work was intended to investigate the cytologic, genotoxic and mutagenic changes induced by diazinon effect in this species' roots.

MATERIAL AND METHODS

Chemicals

Diazinon, purity 99%, was obtained from Agricultural Struggle Center, Ankara, Turkey. Onions (*A. cepa* L., 2n=6) were obtained from a market for *Allium* test. Used other chemicals in experiment were purchased from Sigma-Aldrich.

Determination of EC₅₀ with Inhibition Test

The onions (*Allium cepa*, 2n= 16) were determined according to whether they received the chemicals.

They were transferred to test tubes containing pure water for 24 h. The root growth inhibition was determined via this method (% inhibition = changing in growth/Total growth X 100)²³. After one day, the EC₅₀ values of diazinon were determined at room temperature with different concentrations. After treatment period for 4 day, ten roots were obtained from each onion and took from measuring on them. EC₅₀ concentration was determined as one of concentration decreased the root growth by about 50 % compared with the control group. 40 ppm (1/2x EC₅₀), 80 ppm (EC₅₀) and 160 ppm (2x EC₅₀) concentrations of diazinon were determined and performed for the *Allium* test.

Determination of Cytogenetic Effect and Mitotic Index (MI)

1/2x EC₅₀, EC₅₀ and 2x EC₅₀ of concentrations were added into test tubes for 24, 48 and 72 h when six onion bulbs germinate for 24 h. Tip of roots were collected from control and treatment groups at the high level of maximum mitotic activity on sunny days and used Carnoy's fixative in ethanol: glacial acetic acid (3:1) for 24 h. 1 N HCL was used for hydrolyses at 60 °C for 7 mins²³.

After acetocarmine staining for cytogenetic analysis and destaining with 45% acetic acid, total and damaged cells were detected on six different slides with 40x of light microscope (Olympus BX53 DP72, Japan) .

Mitotic index (MI) was calculated on 5,000 cells for five slides for determining concentrations and applied followed formula for MI%. MI% = Divided cell number/Total cell number X 100 formulation²³.

Determination of Cell Death In Situ Conditions

Cell death was determined from equal 10 mm length roots of control and treatment groups using 3 ml of N,N-dimethylformamide for 1 h at room temperature. Evans blue was prepared 0.25 % (w/v) aqueous solution and used for 15 min and roots were washed with tap water half hour, according to the Baker and Mock²⁴. Dead and alive cells were determined according to the characteristics of holding the paint. photos were taken to show their vitality.

Determination of DNA Damage with Comet Assay

Growing ten onion roots were cut and finely chopped with the help of lancet at the end of 72 h. Roots centrifuged with magnetic stirrer at 500 rpm for 5 min and then rested for 20 min in PBS. Low melting point agarose (0.65%) was mixed with supernatant of treatment and control groups and then 75 µl of suspension was immediately

transferred on slides which have normal melting point agarose (0.05%). The slides were kept at +4 °C for 30 min. Slides leaving from coverslip were immersed into cold lysing solution for 1 h. Lysing solutions have 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, in which 10% DMSO, 1% Triton X-100. Horizontal gel electrophoresis platform was filled with freshly made pH>13 electrophoresis buffer (300 mM NaOH, 1 mM EDTA) until the liquid level completely covers the slides. To unwinding of the DNA, slides were waited for 20 min²⁵. Turn on power supply to 25 V for 20 min. The slides were lifted gently from the buffer and placed on a drain tray and then washed three times for 5 min with neutralizing buffer (0.4 M Tris–HCl buffer, pH 7.5). Slides was stained with 80 µl of ethidium bromide (10 mg in 50 ml of distilled water) for 5 min and then dipped in chilled distilled water to remove excess stain. The slides were covered with coverslip and analysed immediately using BS 200 ProP with software image (BS 200 ProP, BAB Imaging System, Ankara, Turkey). DNA damage was showed a 40x objective on a fluorescent microscope. The tail DNA% (100 - Head%DNA), tail length and tail moment of 50 comets were identified and calculated differences between groups.

DNA Extraction

Qiagen DNeasy plant mini kit (Qiagen, Hilden, Germany) was used for the genomic DNA extraction after diazinon treatment for 72 h. All stages was made according to the procedure given by the producer firm.

The optical density (OD) measurements were carried out for sampled DNA at 260 nm and 280 nm with a spectrophotometer (ACTGene Micro-Spectrophotometer) and OD260/OD280 was used for the purity of DNA. The A260/A280 ratio demonstrate the DNA purity, 1.8-2.0 values suggest “pure DNA”²⁶.

RAPD-PCR

RAPD-PCR protocol and genomic DNA extraction from control and treatment-groups of diazinon was done with respect to Per and Ercan²⁶. 2 µl of DNA template were used with 0.5 µl dNTPs (10 mM stock solution), 1.5 µl PCR buffer [10X buffer with (NH₄)₂ SO₄, Fermentas), 2 µl random primer (10 µM, Opc5; Sequence 5'→3' GATGACCGCC according to Tedeschi¹⁸), 1.5 µl MgCl₂ (25 mM stock solution, Fermentas), 0.25 µl Taq Polymerase (5 u/µl, Fermentas), 6.05 µl of sterile distilled water and 1.2 µl BSA (10 mg/ml). PCR conditions were chosen as one cycle at 94 °C for 2.5 min, followed by 45 cycles of 45 s at 94 °C, 45 s at 35 °C and 45 s at 72 °C, with a final extension step of 5 min at 72 °C. Electrophoresis applied with 1% agarose gel for the PCR products and then done staining with ethidium bromide. Photos of all images were taken.

Statistics

All results of experiment was compared each other by one-way analysis of variance (ANOVA) with Tukey test. Mean values were given as Standard deviation (SD). p < 0.05 was used for showing significant.

RESULTS

Cytotoxic effect of diazinon was showed with Allium test system on commercial onion bulbs. The effects of diazinon appeared at the end of 72 hours. Results of all treatment groups showed that 80 ppm concentration of diazinon was affected decreasing the root length by about 50%, versus the treatment of DMSO (1%) group. Increasing doses of diazinon have been shown to reduce root growth of bulbs. There were statistically

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significant differences between the groups in root growth receiving 10, 40, 80 and 160 ppm of diazinon ($p < 0.05$) (Table 1).

Table 1- Root growth inhibition test treated with increasing doses of diazinon

Test materials	Concentrations (ppm)	Time	Mean of root length (cm) \pm SD	% inhibition
DMSO (1%)	-	96 h	4.70 \pm 0.32	-
Diazinon	10		4.01 \pm 0.02	14.7
	40		3.11 \pm 0.12*	33.83
	80		2.36 \pm 0.45*	49.08
	160		2.04 \pm 0.24*	56.59

Asterisks indicate significant differences among exposed to different concentrations of used diazinon in *A. cepa* cell. Significance at $p < 0.05$.

Evans blue was used for viability testing due to its spread in dead cells. Control and diazinon treatment groups were separated with this process in this study according to Panda et al.²⁷. The dye was applied to the roots for a few minutes and the density in the roots was determined. Non-alive cell take up evans blue staining deeply but alive cell don't take up stain intensity (Figure 1). Dyed colors explained the proportional of cell death in the treatment and control groups.

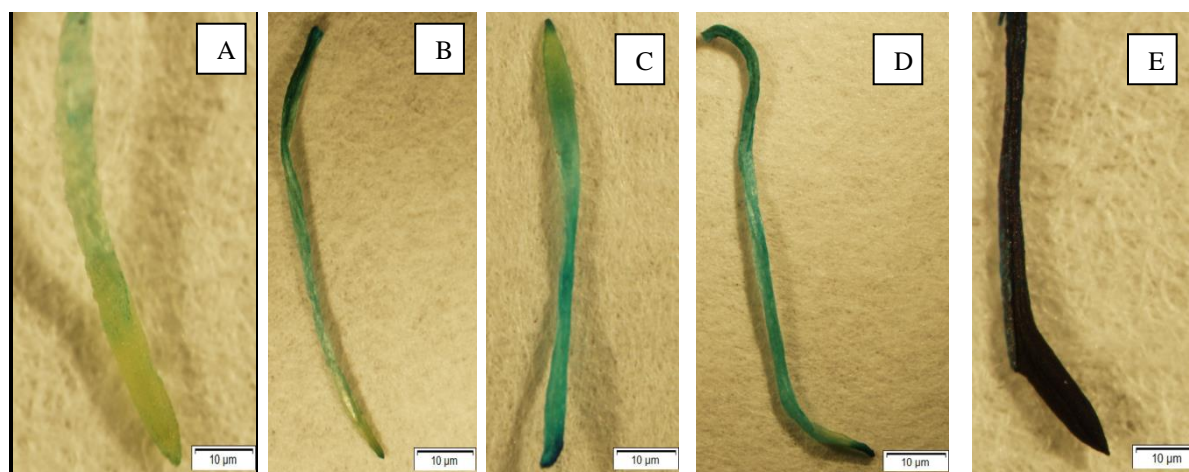


Figure 1. Histochemical staining of cell death of *A. cepa* roots control and treated with increasing doses of diazinon (A) control group, (B) 10 ppm, (C) 40 ppm, (D) 80 ppm, (E) 160 ppm of diazinon.

Chromosome anomalies were showed as hyperchromasia, later segregation, micronucleus, chromosome loss, chromosomal adherence, chromatin globules, pulverised nucleus in anaphase-telophase phase for 72 h treatment groups compared to control cells (Figures 2 A-H). While 13.5% anomalies were obtained at the 10 ppm of diazinon at 24 h, 99.9% anomalies were observed at the 160 ppm at 72 h (Table 2). Cytoplasmic changing were obtained from roots of *A. cepa* such as cell fragmentation and receding of cell contents, nuclear cytoplasmic shrinkage and cell death, cytoplasmic vacuolation, cellular breakage (Figures 2I-L).

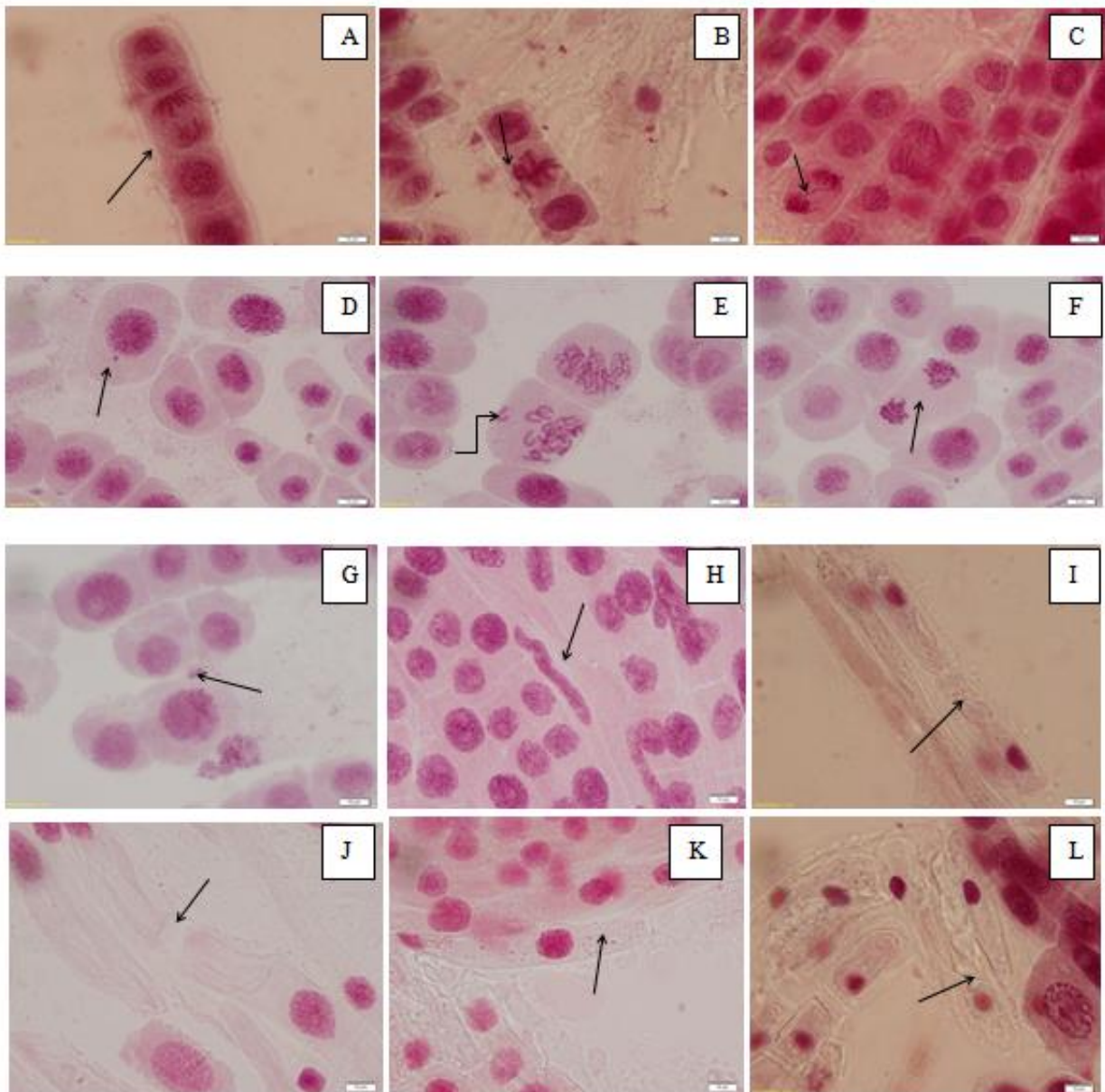


Figure 2. Chromosome and cellular abnormalities. Bars = 10 μm (A) normal anaphase, (B) hyperchromasia, (C) later segregation, (D) micronucleus, (E) chromosome loss, (F) chromosomal adherence, (G) chromatin globules, (H) pulverised nucleus, (I) cell fragmentation and receding of cell contents, (J) nuclear cytoplasmic shrinkage and cell death, (K) cytoplasmic vacuolation, (L) cellular breakage.

Effect of diazinon on *Allium cepa* root cells**Table 2.** Percentage of chromosome aberrations of diazinon at different doses and times

SD: standard deviation

*Significantly different from control ($p < 0.05$)

Concentration (ppm)	Counted cell	Mitotic index%±SD	hyperchromasia	later segregation	micro nucleus	c-mitosis	chromosome loss	chromosomal adherence	chromatin globules	pulverised nucleus	Total anomalies (%)±SD
Control-24	4515	12.23±2.98	-	-	-	-	-	-	-	1	1
10 ppm	4523	12.65±3.65	3.4	0.1	-	2.8	1	1	0.2	5	13.5
40 ppm	4525	12.45±2.81	5.3	0.1	0.1	4	3	5	0.4	10*	27.9
80 ppm	4522	10.54±1.45*	18.2*	0.4*	0.3	15.2*	5	9*	0.6	12*	60.5
160 ppm	4532	10.05±1.42*	25.4*	0.5*	0.5*	19.2*	7*	13*	1*	18*	84.6
Control-48	4188	12.85±2.45	1.2	-	-	2	1	1	0.1	3	8.3
10 ppm	4370	10.20±1.17	7.2	0.8	0.1	5.4	2	6	0.3	8	29.8
40 ppm	4315	9.85±3.45*	11.3*	0.9	0.2	10.2*	5*	12*	0.5	15*	55.1
80 ppm	4550	8.65±1.85*	22.4*	1.1*	0.3	20.1*	6*	15*	0.9*	19*	84.8
160 ppm	4387	7.80±2.45*	26.5*	1.6*	0.6*	21.2*	6*	18*	1.3*	15*	90.2
Control-72	4218	12.35±2.14	3.5	-	-	1	-	2	1	5	12.5
10 ppm	4451	12.4±3.20	12	1.5	-	7.2	1	7	3	14*	45.7
40 ppm	4280	9.55±1.40*	16.2*	1.5	0.4	11.4*	4	10*	5	19*	67.5
80 ppm	4270	8.20±1.25*	25.8*	1.7	0.6*	18.2*	5*	13*	6*	25*	98
160 ppm	4280	5.25±0.45*	27*	1.9*	0.8*	19.2*	6*	14*	6*	27*	99.9

Level of MI of root meristem cell of roots were showed in diazinon treatment group and control groups (Table 2). There were statistically significant differences between the groups in MI (%) receiving 10, 40, 80 and 160 ppm of diazinon at each exposure time versus to control. The lowest MI% values were obtained from 72 h applications of 160 ppm with a score of 5.25 ± 0.45

Table 3 and Figure 3 showed the tail DNA%, tail length and tail moments in meristem cells of *A. cepa* root subjected to diazinon 72 h. Evaluated were significantly ($p < 0.05$) increased in diazinon treatment with increasing the treatment concentrations (10, 40, 80 and 160 ppm). The % tail DNA values ranged from 65.70 ± 2.18 to 92.55 ± 2.78 and tail length values ranged from 26.00 ± 40.24 to 60.00 ± 16.97 (Figures 3A-E). There is no statistically significance between treatment groups in DNA damage in control and 10 ppm of diazinon compared with 80 and 160 ppm of diazinon ($p < 0.05$).

Table 3. Scored DNA damage (±SD) in cells from *A. cepa* roots exposure to increasing concentrations with mean values of tail DNA%, tail length and tail moment of comets by image analysis

Different exposure concentrations of diazinon	Tail DNA% Mean±SD	Tail length Mean±SD	Tail moment Mean±SD
Control	65.70±2.18 ^a	26.00±40.24 ^a	17.27±3.36 ^a
10 ppm	66.63±2.00 ^a	27.50±5.30 ^a	18.54±4.08 ^a
40 ppm	88.78±2.45 ^b	45.50±1.06 ^b	40.37±0.24 ^b
80 ppm	92.06±2.28 ^c	57.00±14.14 ^c	53.12±14.32 ^c
160 ppm	92.55±2.78 ^c	60.00±16.97 ^c	56.47±17.37 ^c

Superscript letters indicate significant differences among exposed to different concentrations of diazinon on roots cell. Significance at $p < 0.05$.

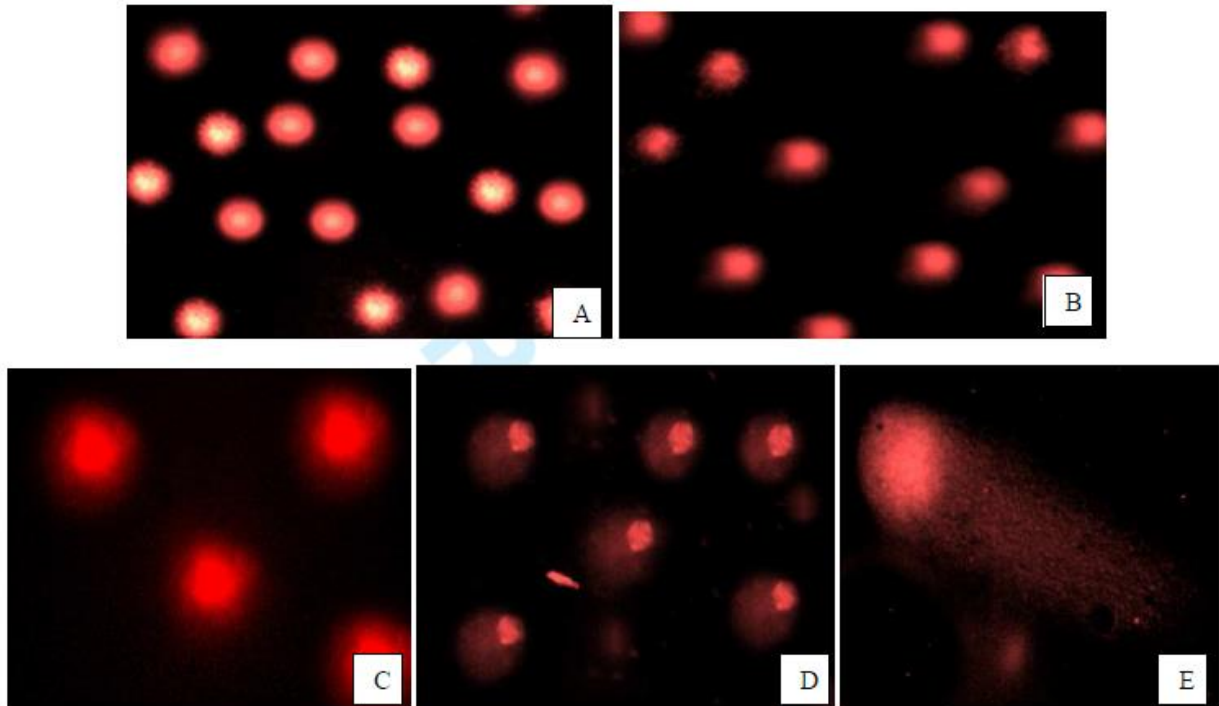


Figure 3. Comet assay micrograph of root tissues of *A. cepa* treated with increasing doses of diazinon for 72 h. (A) control (B) 10 ppm, (C) 40 ppm, (D) 80 ppm (E) 160 ppm.

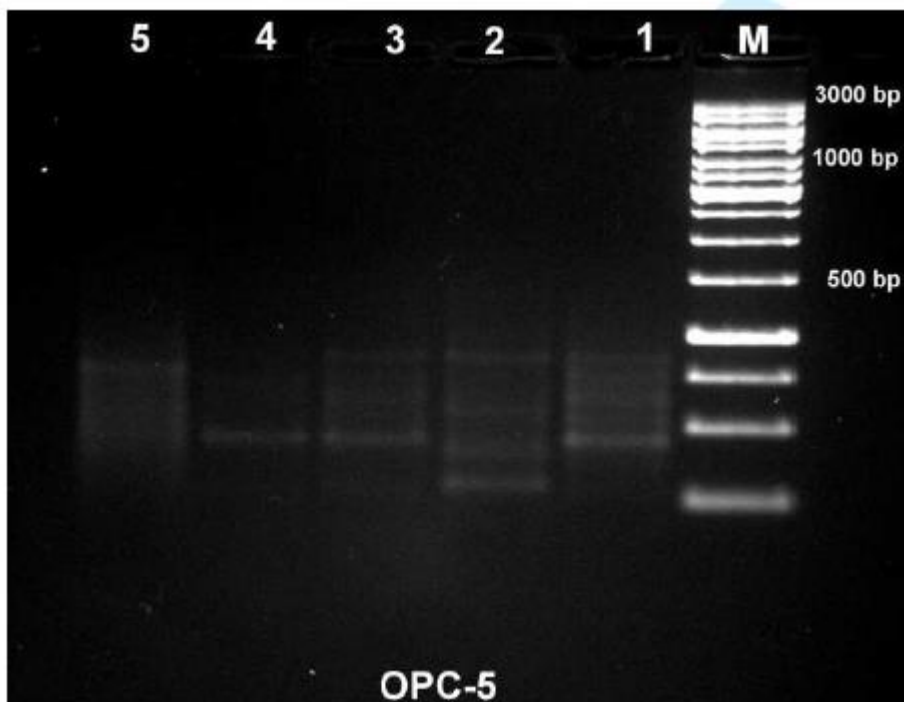


Figure 4. OPC-5 RAPD primers used in (1) control group, and diazinon treatment groups (2) 10 ppm, (3) 40 ppm, (4) 80 ppm, (5) 160 ppm for 72 h.

Extraction and purification of DNA were obtained from *A. cepa* roots and 260 and 280 nm of optical density (OD) was obtained between 1.8-2.0. It means that DNA is quite free of

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protein²⁸. Figure 4 seen toxic effect of different concentrations of diazinon on the structures of RAPD profiles of bulbs. Appearance of new bands or/and disappearance of normal bands were obtained from increasing concentrations of the diazinon compared with the control samples.

DISCUSSION

Effect of OPs insecticides were determined in terms of human health. Previous study demonstrated that there is important increase in accumulate and damage to non-target species²⁹. Diazinon is also an OP which provide controlling insects in crops, ornamentals, lawns, fruit and vegetables and as a pesticide in domestic and agricultural³⁰. In this study, several effects were observed in the *A. cepa* cells analyzed in accordance with the concentrations magnitude of diazinon. Concentration of EC₅₀ is important parameter for determining the toxic effect. It was obtained EC₅₀ (80 ppm), 2x EC₅₀ (160) and 1/2x EC₅₀ (40 ppm) value according to length of each root for diazinon. To show the possible harmful effects on tips, determined concentrations of diazinon were used in this study.

Diazinon causing cytotoxic and genotoxic effect has not been used on *A. cepa* roots until now. To determinate of these effects of chemicals, *Allium* test system is used because of sensitive and best system^{27,31}. Also, *A. cepa* has a great deal of correlation with many cells^{23, 32}. In this study, diazinon was showed that it has toxic effect on alive cells. Especially, diazinon determined toxic effect on *A. cepa* meristematic cell, root growth and also possibly lead to DNA damage.

Bhagyanathan and Thoppil³³ were used *Cynanchum sarcomedium* for determining viable and non-viable cells of *A. cepa* roots. Non-alive cells of this plant stained with different level according to treatment period. Different level of plant extract showed high cell death and this situation is similar method of Evans blue staining. In this work, diazinon caused to highly toxic effect and because of showing strong and weak staining in root tips. Obtained roots from six bulbs were used for defining of death of cells. The root color was found to be at a very high density after 72 hours.

Previous studies have been showed mitotic index of roots' meristematic cells of *A. cepa* affected by some pesticides. Bianchi et al.³⁴ demonstrated that insecticide imidacloprid did not induce visible chromosomal changes in meristematic and F1 cells of *A. cepa* when exposed for 24 h but herbicide sulfentrazone caused a cytotoxic effect which could be visualized by total inhibition of cell division and nuclear fragmentation. Rodríguez et al.³⁵ showed the toxicity of imidacloprid on the DNA of *A. cepa* roots with exposure to different concentrations of this insecticide, demonstrating that chromosomal alterations and frequency of micronuclei increased in the tested concentrations of imidacloprid. Therefore, imidacloprid was genotoxic to the tested organisms. Few studies have examined its toxicity at the genetic level although diazinon is one of the most widely used worldwide. In this work, changing DNA structure show that increasing doses of diazinon have the possible toxic effect on the chromosomes of *A. cepa* roots cell and also mitotic index (MI) of tips was inhibited increasing doses of diazinon end of 24, 48 and 72 h.

According to Saxena et al.³⁶ carbofuran toxicity was showed that chromosomal and mitotic aberrations in the root meristem cells of *A. cepa* exposed to carbofuran for 24 h. Aqueous extracts of *C. sarcomedium* induced chromosomal aberrations on root of *A. cepa*³³. Hyperchromasia, later segregation, micronucleus, c-mitosis, chromosome loss, chromosomal adherence, chromatin globules, pulverised nucleus were determined as chromosomal disruption. According to results, the total chromosomal changing increased with increasing diazinon concentrations and exposure time as compared to the control. In this study, cytological changing were determined intracellular levels. Results presented here confirm the high sensitivity of root with

significant increases in cell damage after treatment of cell with doses of 24-72 h of increasing doses of diazinon. Major cellular changing were obtained as cytoplasmic shrinkage, cell fragmentation, cell membrane damage, cytoplasmic vacuolation, cellular breakage on root cells. The increasing treatment concentrations and times of diazinon cause statistically significant ($p < 0.05$) damage in root cells in this study. All these changing result from membrane damage, vacuolar disintegrations and reducing of cell contents.

To detect changing of DNA structure of living cell, comet assay can utilize for determining toxic effect of various chemicals²¹. Genotoxicity of AgNP was detected with comet assay on *A. cepa* cell for obtaining DNA damage²⁷. AgNP significantly caused broken strand of DNA in a dose-dependent manner on the root tips of *A. cepa*³⁷. Comet assay showed that DNA damage was significantly higher in 20 and 40 ppm of IM compared to the control. Herbicide has cytotoxic activity but not genotoxic activity (except 10 ppm) according to dose dependent manner in *A. cepa* root meristematic cells. In another study, genotoxic effects of chlorfenvinphos and fenbuconazole were used for comet assay on the root meristem cells of *A. cepa*. Increasing concentrations were used on the roots for 24 and 48 h and DNA damage was demonstrated in treatments chemical groups compared to the control³⁸. In this study, after the exposure period, single strand DNA breaks by comet assay were used. Single strand DNA breaks were measured as tail DNA%, tail length and tail moment of comet. The effects of diazinon on the roots cells of *A. cepa* with regard to tail DNA% and tail length were determined. Its clearly demonstrated, particularly tail DNA% and tail length increased in treatment group of diazinon for 72 h.

RAPD markers are also used to show DNA damage and mutations²⁰. Ozakca and Silah¹⁹ were shown that toxic effect of fungicide flusilazole on somatic cells of *A. cepa* using randomly amplified polymorphic DNA method. The RAPD method was used to demonstrate the toxic effect of different doses of diazinon on root tips. Diazinon application induced band loss and changing band density in DNA due to presence of DNA photoproducts and severe DNA inhibition. The genetic differences was obtained in most exposure to 80 ppm and 160 ppm concentrations of diazinon group end of 72 h. In terms of the RAPD method, all the changes in the DNA structure, the disappearance of the bands, the formation of new bands, the changes in the band density have shown that diazinon may be a toxic substance for root tips.

CONCLUSION

The effects of used insecticide on the *A. cepa* roots cytogenetic and mutagenic have not previously been described. These results show that the effect of diazinon on roots cell depends on the concentrations and times. Root cells are apparently more sensitive to pesticide and show increased cellular and chromosomal disorder, DNA damage after increasing exposure time. Therefore, it would be important to further toxic work and cytological evidences at molecular level to demonstrate the possible toxic effect of diazinon.

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