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Genotoxicity effects of Flusilazole on the somatic cells of *Allium cepa*

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ABSTRACT

The aim of this study was to evaluate the effects of the fungicide flusilazole on somatic cells of *Allium cepa*. For evaluation of cytogenetic effects, root meristem cells of *A. cepa* were treated with 10, 20, 30 and 45 ppm (EC_{50} concentration) for 24, 48 and 72 h. The mitotic index and different types of chromosomal abnormalities such as bridges, stickiness and laggards were determined in both control and test groups. Acridine orange/Ethidium bromide double staining and fluorescence microscope was used to determine the stability of chromosome structure. Data obtained from staining process indicated that ratio of necrotic cells significantly increased by the flusilazole presoaking. The RAPD-PCR method was used and the higher doses treated-group (45 ppm) was more distant to the control group compare with others.

1. Introduction

Pesticides are extensively used to increase agricultural production by preventing losses due to pests [1]. Because of their persistence and longevity in the environment, they can accumulate in its different compartments and may even turn up in areas where they have never been used. In addition, they are bio-accumulated in plant and animal tissues [2].

Flusilazole is a fungicide that is a member of the triazole family of compounds. Flusilazole is the ISO approved name for 1-[[bis(4-fluorophenyl)methyl]silyl]methyl]-1*H*-1,2,4-triazole (CAS No. 85509-19-9). General structure of flusilazole is shown in Fig. 1. It is a broad-spectrum fungicide that belongs to the triazole subclass of ergosterol biosynthesis inhibitors and used to control fungal disease caused by pathogens of the ascomycetes, and basidiomycetes [3]. Flusilazole exhibits curative and preventative activities and is recommended for use in agriculture, horticulture and viticulture [4]. The LD_{50} of flusilazole in rat is 674 mg/kg, indicating slightly toxicity to mammals.

Higher plants provide excellent models for the genotoxicity assessment of environmental chemicals because plant chromosomes are easy to analyze in terms of size, morphology and number, and respond to treatment with toxins in a similar way to mammals and other eukaryotes [5]. In addition, they are simple, cost effective and can be applied to detect wide range of genetic damage. *Allium cepa* and *Allium sativum* are routinely used for the studying the effects of toxic substances on chromosomes and cell division. Further, *A. cepa* has been approved as indicator organisms for biomonitoring of environmental pollutants [6–8]. Plant bioassays also have been validated in international collaborative studies under the United Nations Environment Program (UNEP), World Health Organization (WHO) and US Environmental Protection Agency (US EPA), and proven to be efficient test for genotoxic monitoring of environmental pollutants [9,10].

No manuscript was reported on the genotoxic effects of flusilazole on the somatic cell of *A. cepa.* The main purpose of this manuscript was to investigate the potential cytogenetic effects of flusilazole on the root meristem cells of *A. cepa* using mitotic index, chromosomal and mitotic aberrations. To evaluate the interaction of flusilazole with DNA resulting in damage Acridine orange/Ethidium bromide staining was used.

2. Material and Method

2.1. Chemicals

Flusilazole was purchased from DuPont Corporation. For the present study, the other chemicals were used Sigma and Merck. Doubly distilled water was used throughout experiments. All experiments were performed at room temperature.

2.2. Methods

2.2.1. Stock solution

A stock solution of 100 ppm flusilazole was prepared by dissolving an appropriate amount of the insecticide in ethanol. This stock solution was stored in the dark and under refrigeration. Working

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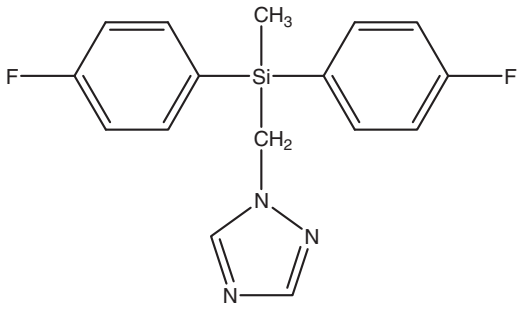

Fig. 1. Structural formula of Flusilazole.

solutions of flusilazole were prepared by diluting the stock solution with ethanol.

2.2.2. Ec_{50}

EC_{50} of flusilazole against the *A. cepa* root growth was determined according to the method described earlier studies [11]. We treated root meristem of *A. cepa* with different concentrations of flusilazole for different durations. Clean and healthy onion roots, commercially obtained, were placed over the test tubes filled with test concentrations of flusilazole (10, 20, 30, 40, 45, 50, 60, 80, and 100 ppm). A set of bulbs was also exposed to 0.12% ethanol and considered as negative control. Taking root lengths of control groups as 100% lengths of experimental groups were plotted against test concentrations and the point showed 50% growth was designated as EC_{50} concentration.

2.2.3. Cytogenetic assay

In order to demonstrate possible concentration-dependent effect of flusilazole and the relative sensitivity of *A. cepa*, 10, 20, 30 and 45 ppm (EC_{50} concentration) were tested. Clean and healthy onion bulbs rooted in tap water and roots 1–2 cm long were exposed to aqueous concentrations (10 bulbs or cloves/concentration) of flusilazole for 24, 48 and 72 h. At the end of exposure, few root tips from each test group were immediately placed in a chilled Carnoy's fixative (ethanol: acetic acid = 3:1) and remaining bulbs with intact roots were transferred to tap water for a further 24 h recovery. Sets of 10 bulbs were also incubated with solvent control, untreated control and positive control. Fixation and staining of the root tip cells were carried out as reported earlier [12].

2.2.4. Acridine orange/Ethidium bromide staining

Cells were assessed by Acridine orange/Ethidium bromide staining. Cells were stained with 0.01% (w/v) acridine orange and 0.01%(w/v) ethidium bromide in 10 mM PBS buffer (pH 7.0) for 30 min. Slides were examined using a Olympus BX-51 light microscope equipped with fluoresce filters and photographed by Olympus C-5050 digital camera. Experiments were repeated three times and hundreds of cells were analyzed.

2.2.5. Mitotic index/chromosomal aberrations analysis

The mitotic index (MI) and the frequencies of chromosomal aberrations (CA) were determined out as described earlier studies [12]. For each test group, five slides were prepared by root tips with 45% acetic acid. Slides were randomly coded and scored blindly. For MI, the different stages of mitosis were counted in a total of 2000 interphase cells per concentration, and expressed as a percentage. CA was analyzed in 400–500 dividing cells for each test group.

2.2.6. Isolation of DNA

After Flusilazole treatment for 72 h, DNA was isolated from *A. cepa* samples by Invitrogen Plant DNA Isolation Kit.

2.2.7. Rapd-pcr

Amplification of genomic DNA was performed in a 50 μl reaction mixture containing 18.5 Milli Q water, 1.75 μl of 10× Taq polymerase buffer, 1.2 μl of $MgCl_2$, 1.5 μl of dNTPs, 0.6 μl of each primer and 0.6 μl Taq polymerase before addition of DNA.

RAPD-PCR was performed with 7 different RAPD primers (supplemental data), following control of DNAs by agarose gel electrophoresis. PCR bands were checked again by agarose gel electrophoresis following PCR analysis. PCR analysis was performed three times for control.

Amplification was performed in Techne Thermal Cycler with 5 cycles for denaturation at 94 °C for 1 min, annealing at 27 °C for 30 s and extension 72 °C for 1 min, and then 45 cycles for denaturation at 94 °C for 45 s, annealing at 39 °C for 1 min and extension at 72 °C for 45 s, final extension at 72 °C for 15 min. Amplified products were kept in 4 °C.

Nine microlitre each PCR product was electrophoresed on agarose gel (%2) in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) at 50 V for 2 h. The gel was stained ethidium bromide and photographed.

2.2.8. Estimation of genomic template stability

Each change that was observed in RAPD profiles was given as + 1 arbitrary score. The average was then calculated for each experimental group exposed to Flusilazole. Template genomic stability (%) was calculated as "100-(100*a*/*n*)" where "*a*" is the average number of changes in DNA profiles and "*n*" the number of bands that were selected in control DNA profiles.

2.2.9. Statistical analysis

The data were expressed in percent and the prevalence of significance was determined by One-Way ANOVA test. The statistical analysis submitted in Table 2 and Table 3 indicates significant difference in mitotic cells.

3. Result and discussion

The genotoxic effect of the different concentrations of flusilazole on the mitotic cell division of the root tip cells of *Allium* are given in Table 1. Increasing flusilazole concentration and exposure time resulted in a corresponding decrease in mitotic index. At 48 and 72 h presoaking duration the significantly inhibition mitotic index was observed at 30 and 45 ppm concentrations of flusilazole treated root meristem cells of *A. cepa*.

The various chromosomal aberrations like stickiness, chromosomal bridge and laggards were observed in flusilazole treated root meristem cells (Table 2 and Fig. 3). Stickiness and chromosomal bridge were the most frequent chromosomal abnormalities. The highest percentage (14.52%) of chromosomal aberration was seen at 72 h presoaking duration 45 ppm concentration of flusilazole (Table 2).

The genotoxicity of flusilazole has been studied by few authors using different assay in mammalian [13–15]. Toxicity on short-term exposure to flusilazole was investigated in feeding studies in rats, mice, and zebrafish [13–16]. The data of the present study showed that high concentration of flusilazole (30 and 45 ppm) diminished the mitotic index and induced the chromosomal aberrations at all presoaking durations (Table 1 and Table 2).

The significant decline of mitotic index with higher concentrations at 48 and 72 h presoaking duration is the outcome of inhibition of cell division which reflects the cytotoxic potential of

Table 1
Inhibition of mitotic index and ratio of necrotic cells in the root tip cells of *Allium cepa*.

Test Concentrations (ppm)	Exposure time	Mitotic index	Necrotic cells%
Control	24	11.52 ± 0.56	0.51 ± 0.18
	48	11.30 ± 1.14	0.87 ± 0.11
	72	12.05 ± 0.75	0.33 ± 0.23
10	24	11.04 ± 0.62	0.73 ± 0.25
	48	10.71 ± 1.13	1.40 ± 0.40
	72	10.37 ± 0.96	1.27 ± 0.11*
20	24	10.96 ± 0.77	1.01 ± 0.20**
	48	9.96 ± 0.80	3.07 ± 0.64*
	72	11.30 ± 0.76	3.27 ± 0.11**
30	24	9.41 ± 0.53*	4.87 ± 0.81*
	48	8.08 ± 0.61**	10.67 ± 0.83**
	72	6.41 ± 0.53**	13.67 ± 1.53**
45	24	8.00 ± 0.72*	7.00 ± 1.73*
	48	7.25 ± 0.08*	12.60 ± 1.39**
	72	4.67 ± 0.72**	34.47 ± 2.33**

n = Number of replicates,
x = Mean values,
SD = standard deviations.
* $p < 0.001$.
** $p < 0.05$.

flusilazole in *A. cepa*. The reduction of mitotic index may be either due to the inhibition of DNA S-phase or blocking of G_1 suppressing DNA synthesis or effect of test compound at G_2 phase of the cell cycle [17–19]. Saxena also showed that exposing the root tips of *Allium sativum* to cypermethrin lead to the destabilization of the DNA structure and unwinding of the DNA helix, thereby inducing chromosomal damage [12]. In the present study, these citations suggest that flusilazole could have the same effect on mitotic cell division in *A. cepa*.

Chromosomal bridge was very frequent chromosomal abnormality observed in somatic cells of *A. cepa* (Table 2). This chromosomal bridge may be the result of dicentric chromosomal formation due to the breaking and reunion of chromosomal. Chromosomal stickiness was another most frequent chromosomal aberration induced by flusilazole (Table 2). This stickness may be attributed to intermingling of chromatin fibers which lead to subchromatid connections between chromosomes [20]. In previously data, similar abnormalities were also reported in plant cells exposed to other chemicals [21,22].

Table 3
Changes of total bands in control and polymorphic bands in UV-A treated samples. a: appearance of new bands, b: disappearance of normal bands, c: decrease in band intensities, d: increase in band intensities.

Primers	Control	10 ppm	20 ppm	30 ppm	45 ppm
		a b c d	**a b c d**	**a b c d**	**a b c d**
Primer 1	7	0 3 0 2	1 4 0 1	1 1 0 2	1 1 0 3
Primer 2	8	1 1 0 1	0 2 0 1	1 2 1 2	1 5 1 2
Primer 3	7	0 2 0 3	1 2 1 3	0 4 1 3	1 5 0 2
Primer 4	3	0 0 1 2	4 0 0 3	0 0 1 2	2 0 0 2
Primer 5	7	1 0 0 0	1 0 0 0	1 0 0 0	1 0 0 0
Primer 6	7	0 1 0 2	1 1 1 0	0 7 0 0	2 5 1 1
Primer 7	3	1 0 1 0	0 2 0 0	1 0 0 2	0 0 1 1
Primer 8	0	6 0 0 0	6 0 0 0	5 0 0 0	6 0 0 0
Total Bands	42	9 7 2 10	14 11 2 8	9 14 3 11	14 16 3 11
a + b		16	25	23	30
a + b + c + d		28	35	37	44

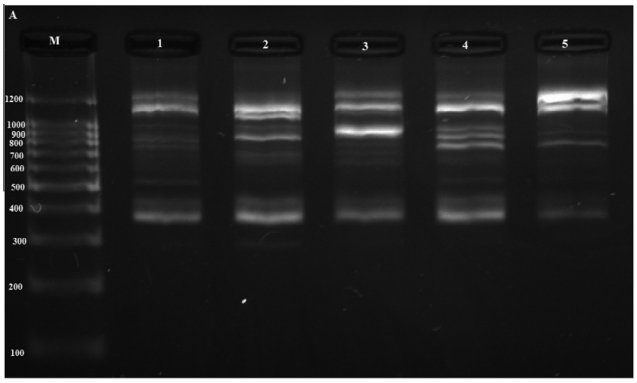

Fig. 2a. OPA9 RAPD primers used in control group (**1**) and Flusilozole exposed group, (**2**) 10 ppm (**3**) 20 ppm (**4**) 30 ppm (**5**) 45 ppm.

Acridine orange (AO) is a nucleic acid-specific ultraviolet fluorochrome stains DNA bright yellowish-green on a typically green cytoplasmic background and thus is way to detect the healthy chromosome structure. The fact that relatively strong AO signals were detected in the non-stressed or lower stressed root meristem cells of *A. cepa* implied that there was a low degree of DNA damage because AO tends to combine with relatively intact DNA molecules and produced a very strong signal. Ethidium bromide (EB) used

Table 2
Types and percentage of chromosomal abnormalities induced by flusilazole in the root tip cells of *Allium cepa*.

Test Concentrations (ppm)	Exposure time	Bridges (%)	Stickiness (%)	Laggards (%)	Total chromosomal aberrations (%)
Control	24	0.03 ± 0.02	ND	ND	0.02
	48	0.13 ± 0.06	0.13 ± 0.06	0.11 ± 0.01	0.34
	72	0.10 ± 0.02	0.02 ± 0.01	0.08 ± 0.01	0.2
10	24	0.63 ± 0.39	0.33 ± 0.15	ND	0.71
	48	0.63 ± 0.06*	1.00 ± 0.11**	0.87 ± 0.07**	2.51
	72	0.83 ± 0.11**	0.69 ± 0.32**	0.75 ± 0.13*	2.06
20	24	0.28 ± 0.10**	0.68 ± 0.28	0.14 ± 0.04	2.1
	48	1.03 ± 0.23*	1.07 ± 0.17*	0.92 ± 0.11**	3.01
	72	1.27 ± 0.47**	1.30 ± 0.56**	1.93 ± 0.15**	4.5
30	24	1.29 ± 0.51**	1.05 ± 0.09	1.47 ± 0.15	3.81
	48	1.66 ± 0.11**	1.88 ± 0.14**	1.26 ± 0.23*	4.8
	72	2.61 ± 0.42**	4.25 ± 0.87*	2.68 ± 0.38**	9.54
45	24	1.74 ± 0.53*	1.67 ± 0.14	1.77 ± 0.15	5.17
	48	1.95 ± 0.15**	2.50 ± 0.16**	1.91 ± 0.30**	6.37
	72	3.88 ± 0.35**	6.67 ± 0.52**	3.98 ± 0.15**	14.52

n = Number of replicates,
x = Mean values,
SD = standard deviations.
* $p < 0.001$.
** $p < 0.05$.

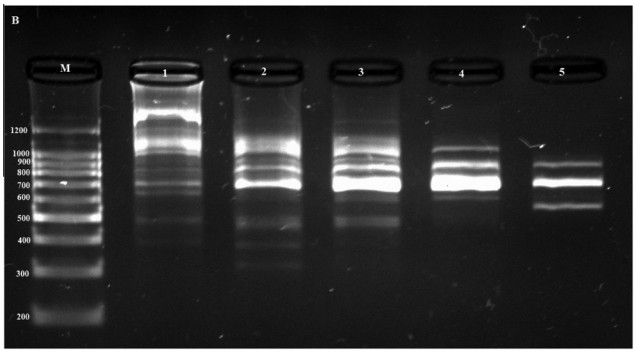

Fig. 2b. OPB14 RAPD primers used in control group (**1**) and Flusilozole exposed group, (**2**) 10 ppm (**3**) 20 ppm (**4**) 30 ppm (**5**) 45 ppm.

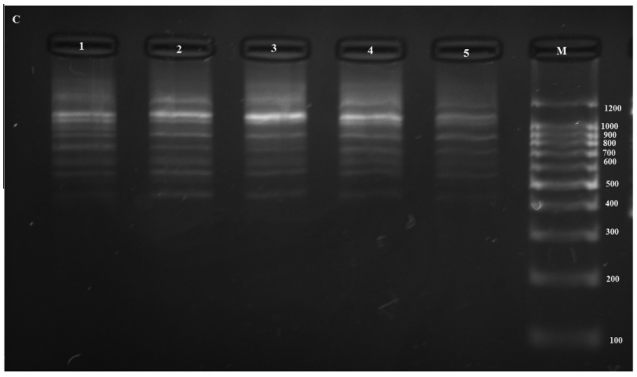

Fig. 2c. OPB5 RAPD primers used in control group (**1**) and Flusilozole exposed group, (**2**) 10 ppm (**3**) 20 ppm (**4**) 30 ppm (**5**) 45 ppm.

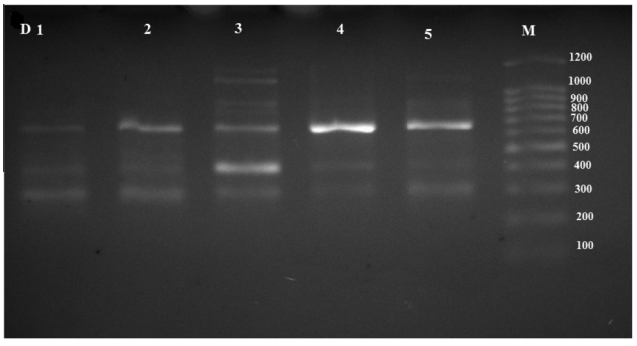

Fig. 2d. OPB6 RAPD primers used in control group (**1**) and Flusilozole exposed group, (**2**) 10 ppm (**3**) 20 ppm (**4**) 30 ppm (**5**) 45 ppm.

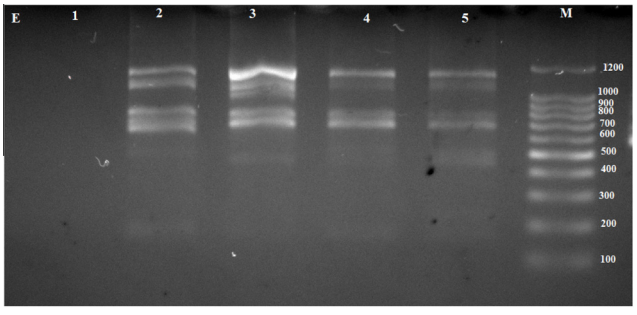

Fig. 2e. OPB7 RAPD primers used in control group (**1**) and Flusilozole exposed group, (**2**) 10 ppm (**3**) 20 ppm (**4**) 30 ppm (**5**) 45 ppm.

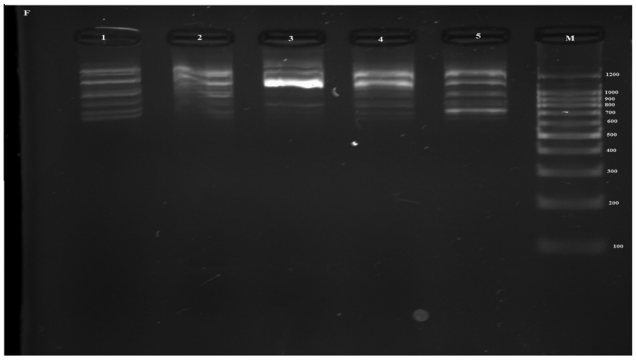

Fig. 2f. OPB8 RAPD primers used in control group (**1**) and Flusilozole exposed group, (**2**) 10 ppm (**3**) 20 ppm (**4**) 30 ppm (**5**) 45 ppm.

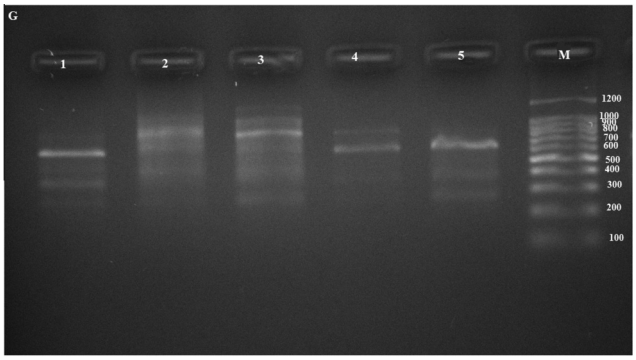

Fig. 2g. OPB10 RAPD primers used in control group (**1**) and Flusilozole exposed group, (**2**) 10 ppm (**3**) 20 ppm (**4**) 30 ppm (**5**) 45 ppm.

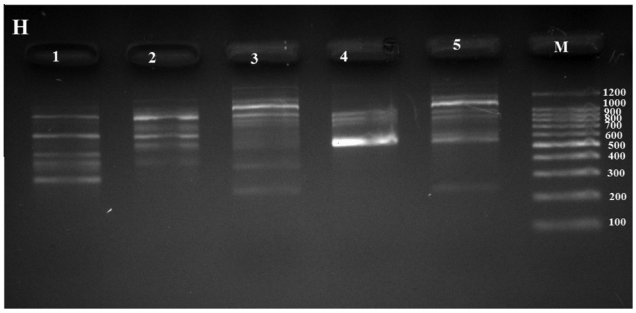

Fig. 2h. OPB18 RAPD primers used in control group (**1**) and Flusilozole exposed group, (**2**) 10 ppm (**3**) 20 ppm (**4**) 30 ppm (**5**) 45 ppm.

with low concentrations of acridine orange (AO) identifies normal (AO at high fluorescence level, EB low) and late apoptotic/necrotic (AO low, EB high) cells [23,24]. Ethidium bromide staining due to loss of membrane integrity identifies the population of necrotic cells; the mechanism of decreased AO staining may be related to as a result of destruction of DNA molecules into small fragments [23]. Our results obtained with acridine orange/ethidium bromide (AO/EB) double staining showed that number of necrotic cells significantly modified by the flusilazole presoaking (Fig. 3, Table 1). These results also indicated that rate of necrotic cells in root meristematic cells of *A. cepa* increased (12.60% and 34.47%) at 45 ppm flusilazole concentration at 48 and 72 h. These results showed that DNA damage induced by high concentration of flusilazole treatment (Fig. 3).

According to statistical analysis (t-test, $P < 0.05$) there is a significant difference between flusilazole treated groups at different

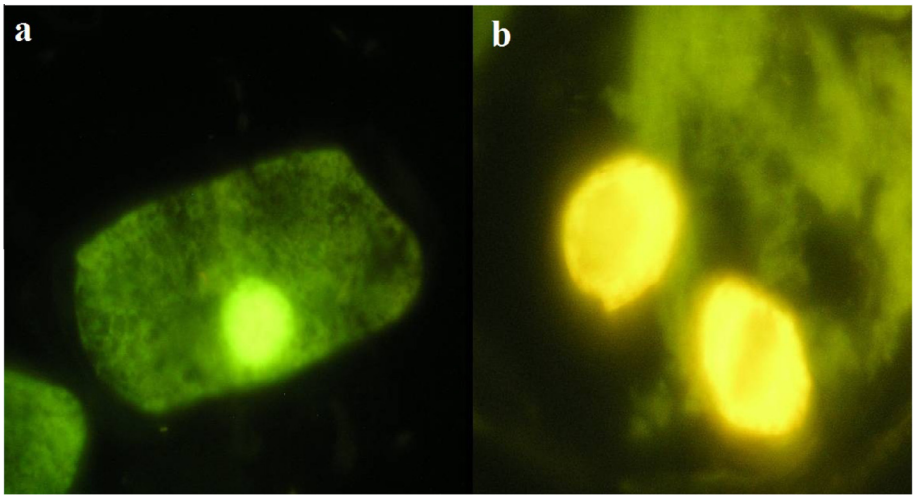

Fig. 3. The type of cell damage in onion roots treated with flusilazole revealed by AO/EB double staining test. **a** Bright gren undamaged nuclei with intact structure; **b** necrotic orange nucleus with intact structure.

concentrations and exposure times and the control group (Table 2 and 3).

Rank and Nielsen reported that if chemical is able to cause damage to the chromosomes in a reliable plant assay, then the chemical should be considered as having the potential to damage the chromosomes of other organisms in the environment [25]. In the present study, clastogenic types of flusilazole should be regarded as an agent that shows mutational activity in *A. cepa* which including decline of mitotic index, induced the chromosomal aberrations and the induction of DNA damage.

The RAPD-PCR method was successfully used to detect "DNA damages" induced by heavy metals, gamma, X-ray, UV radiation and pesticide [26,27]. Atienzar et al. [27,28] have shown that changes in band patterns observed in DNA fingerprint analyses reflect DNA alterations from single base changes to complex chromosomal rearrangements. Similarly, in the present study, Flusilazole of treatment to *A. cepa* caused almost a complete loss of different DNA fragments, suggesting severe inhibition of DNA amplification in RAPD (Fig. 2). The variation in band intensities and the disappearance of bands might be related with presence of DNA photoproducts which are produced by Flusilazole.

In present study, the genomic template stability decreased after flusilazole treatment. According to the RAPD assay, the genetic distance between the control group and the sample which is exposure to 10 ppm concentration of flusilazole group was found 39.02%. The genetic distance of the sample, exposure to 20 ppm and 30 ppm concentration of flusilazole, were found, respectively 60.97 and 56.10% and the distance of 45 ppm treated samples to the control group was found 73.17%. The higher doses treated-group (45 ppm) was more distant to the control group compare with others.

Different polymorphic bands were detected at each treatment concentration of flusilazole by six primers (Fig. 2). According to the results, polymorphysims were due to the loss or gain of amplified bands in treated samples compared with the control group. Table 3 explains the changes observed in RAPD profiles such as appearance/disappearance of bands and decreases/increases of band intensities. The numbers of new appearing bands were greater in higher dose of flusilazole and bands with approx. 900, 800 and 400 bp molecular sizes were shown to appear (Table 3). Results suggested that 3 bands were highly reproducible and stable for primers 1 and 3 (Fig. 2). Appearance of new PCR products occurred because some oligonucleotide priming sites could become accessible to oligonucleotide primers after structural changes or some changes in DNA sequence, occurred as a result of mutations. The appearance of new bands might be the result of genomic template instability of DNA repair mechanisms.

Pesticides can easily accumulate in plants, foods, water samples and food chain. Also unconscious applications of pesticides trigger this event. Some of pesticides are broken down or degraded by the action of sunlight, temperature, water or microorganisms but some of pesticides are resistant to degradation. So plants and environmental samples contain high levels of pesticides residues in their tissues and parts. As a result, present study suggests that flusilazole is capable of inducing genotoxicity in plants. Although the highest concentrations of flusilazole are not generally found in the environment, unconscious application of flusilazole may cause to genotoxic effect on crop. Therefore, it is essential to test the genotoxic effects of pesticide such as fungicide on plants and other systems before considering their applications for agricultural purposes.

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