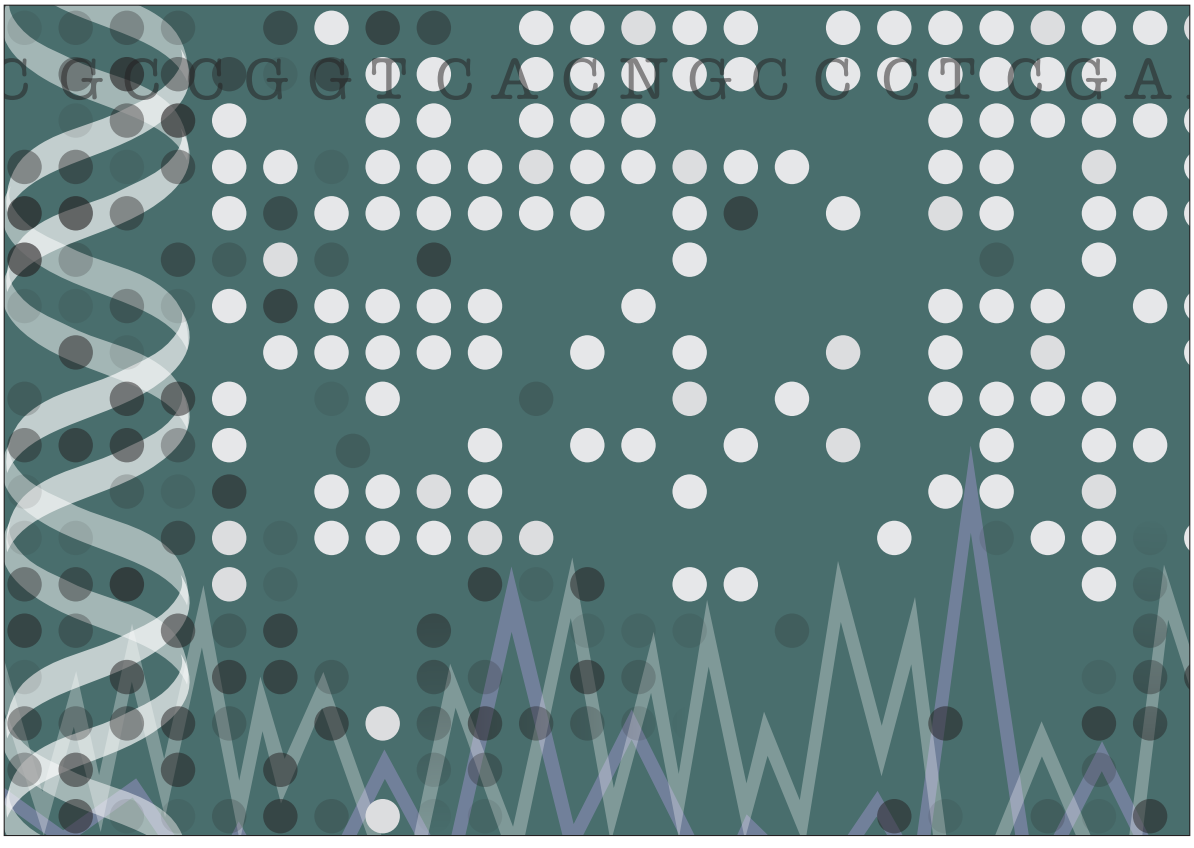

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Cytotoxic and Genotoxic Effects of Diphenyl-ether Herbicide GOAL (Oxyfluorfen) using the *Allium cepa* test

[1]Asya Dragoeva, [1]Vanya Koleva, [1]Nurzhihan Hasanova and [2]Stoicho Slanev
[1]Department of Biology, University of Shumen, Bulgaria
[2]Agricultural Institute, Shumen, Bulgaria

Corresponding Author: Asya Dragoeva, Department of Biology, University of Shumen, Bulgaria

ABSTRACT

The pesticides possess biological activity including genotoxic influence and can influence non target organisms. Oxyfluorfen (2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl) benzene) is classified as a possible human carcinogen. In present study cytotoxic and genotoxic effects of commercial formulation of GOAL (oxyfluorfen) were evaluated using *Allium cepa* assay. The effective concentration (EC50) value determined in root growth inhibition test using *T. aestivum* L. was 1.2 ml L^{-1}. *Allium cepa* root tips were exposed to 1.2 ml L^{-1} (EC50), 0.6 ml L^{-1} (1/2 EC50) and 0.3 ml L^{-1} (1/4 EC50) for 24 or 48 h. Mitotic index decreased substantially at 1/2 EC50 and EC50 at both treatment times. The herbicide caused a change in the frequencies of the different mitotic phases. GOAL induced various mitotic abnormalities revealing clastogenic and turbagenic activities. Anaphases and telophases with vagrant chromosomes and fragments were the most frequent kinds of abnormalities. GOAL caused spindle disturbances and produced C-mitotic effect and multipolar anaphases. We also noticed bridges in anaphase and telophase. The percent of chromosome aberrations increased with increasing the concentration and the duration of treatment. Micronucleated cells were observed in interphase cells. The frequency of the micronuclei was markedly higher after 48 h compared to 24 h exposure. In conclusion, the results indicate cytotoxic and genotoxic effect of GOAL in *A. cepa* root tip cells after exposure for 24 and 48 h at concentrations below commercially recommended.

Key words: GOAL, oxyfluorfen, EC50, *Allium cepa* assay, chromosome aberrations, mitotic index

INTRODUCTION

The environmental pollution has increased tremendously over the last few decades due to the various human activities. Agrochemicals necessary for efficient crop production are significant pollution source. The pesticides possess biological activity including genotoxic influence (Dimitrov *et al*., 2006; Lamsal *et al*., 2010; Mustafa and Arikan, 2008; Yuzbasioglu *et al*., 2009). So, pesticides with mutagenic potential can influence non target organisms and adversely affect human health (Bolognesi, 2003).

Oxyfluorfen (trade name GOAL) is a diphenyl-ether herbicide used for pre and post-emergent control of annual broadleaf and grassy weeds. It is applicated not only in a variety of crops but also in non-agricultural ornamental, forestry and residential areas (USEPA, 2002). Pesticides are registered on the basis of scientific studies showing their safety. Some tests of GOAL on rats, mice and on bacterial cell cultures and unscheduled DNA synthesis tests

have been negative, while other tests on mice and bacterial cell cultures have been positive (USEPA, 1992). However, oxyfluorfen is classified as a possible human carcinogen (USEPA, 2002). These data raise the necessity for further evaluation of potential adverse effect of oxyfluorfen.

The most commonly used toxicological tests were carried out on mammals. Recently one of the fundamental concerns for both science and ethics has become use of animals in toxicity studies (Mukhopadhyay *et al.*, 2004; Purchase, 1997). The principles of humane animal experimentation-replacement, reduction and refinement (known as the Three Rs) were defined by Russell and Burch (Russell and Burch, 1959). According to these principles alternative test objects have been searched. Fiskesjo (1985) underlined that "a standard test for toxicity must be easy to perform and the results should be rapidly obtained and reproducible". Higher plants provide valuable genetic assay systems and fulfill these demands (Grant, 1994; Leme and Marin-Morales, 2009). Moreover, many chemicals that have no genotoxic effect can be converted into true mutagens in plant cells. After metabolic activation, involving a chemical modification, mutagens would be introduced into the human food chain (Plewa and Wagner, 1993).

Among the plant species, *Alium cepa* is considered to be a suitable test system for evaluation of the genotoxic potential of pesticides (Asita and Matebesi, 2010; Bolle *et al.*, 2004; Chauhan *et al.*, 1998; Fernandes *et al.*, 2007; Mustafa and Arikan, 2008; Yuzbasioglu *et al.*, 2009). According to Fiskesjo (1988) "results from the *Allium* test have shown good agreement with results from other test systems, eukaryotic as well as prokaryotic".

The purpose of present study was to evaluate the cytotoxicity and genotoxicity of herbicide oxyfluorfen (trade name GOAL) using *Allium cepa* assay.

MATERIALS AND METHODS

Compound tested: The commercial form of the test chemical (GOAL, active substance 240 g L^{-1} oxyfluorfen, "Rohm and Haas", USA, 90 mL dka^{-1}) was used in this study, because in agriculture land oxyfluorfen is used in this way. Oxyfluorfen (2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene) (Fig. 1) belongs to the diphenyl ether group of herbicides. Its chemical formula is $C_{15}H_{11}ClF_3NO_4$, molecular weight 361.7.

Root growth inhibition test and determination of Effective Concentration for 50% growth inhibition (EC50) using *T. aestivum* L.: Dose selection experiment was conducted with different concentrations below the manufacturers recommended dose of 3 ml L^{-1} (3.0, 1.5, 0.3, 0.15, 0.03 mL L^{-1}). The water solutions were prepared just before each treatment using commercial product GOAL. For toxicity study seeds of *T. aestivum* L. obtained from local market were used. Seeds were thoroughly rinsed with tap water and after that with distilled water. After cleaning the seeds were placed between two sheets of filter paper and dried at 25°C. Twenty seeds with one and the same shape and size were placed on filter paper in each of 6 Petri dishes (11 cm in diameter). Five milliliter of each of five tested solutions or distilled water, as a control, was applied to the

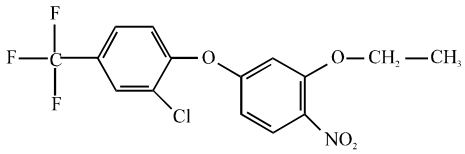

Fig. 1: The structural formula of oxyfluorfen

seeds. The dishes were sealed and incubated at 25±1°C for 96 h. The length of the roots of germinated seeds was measured in mm. A growth curve was drawn based on the obtained values in a diagram (abscissa: treatment concentration; ordinate: root length in percent of control). From the growth curve EC50 value was obtained: The effective concentration causing 50% growth inhibition in relation to control (Fiskesjo, 1985). Three replications were done.

Cytogenetic analysis (*Allium cepa* test): We choose to test the cytotoxic and genotoxic effects of three concentrations of herbicide GOAL, representing ¼ EC50, ½ EC50 and EC50 after 24 and 48 h exposure. Thirty *Allium cepa* L. cv. Stuttgarter Riesen seeds (2n = 16) purchased from a local market were placed on filter paper in Petri dishes containing 5 mL of distilled water. The dishes were sealed and incubated at 25±1°C for 72 h. Twenty germinated seeds with equal length of roots (~ 1 cm) were removed and placed on filter paper in each of another four Petri dishes. Five mL of each water solution were added to one dish and incubated at 25±1°C for 24 h. Distilled water was used as a negative control. The same procedure was repeated in the second experiment but the duration of exposure was 48 h.

Chromosomal aberrations in *Allium cepa* root cells were assessed by light microscopy (Rank, 2003). At the end of the 24 and 48 h exposure the roots were fixed in Clarke's fixative (95% ethanol: acetic acid glacial, 3:1) for 90 min, hydrolysed in 3N HCl for 8 min and in 45% acetic acid (CH_3COOH) for 30 min at room temperature and stained for 90 min in 4% acetocarmine. After staining, the terminal root tips (1-2 mm) were cut off and squashed in 45% CH_3COOH. The endpoints measured were mitotic index, index of each phase of mitotic division, chromosomal aberrations and cells with micronuclei. Each sample consisted of six root meristems. At least 1000 cells of each root meristem were analysed. The mitotic index was determined as a ratio between the number of cells in mitosis and the total number of analysed cells. The index of each phase of mitotic division was calculated as a ratio between the cell number in the respective period and the number of dividing cells. The categories of aberrations scored in mitotic cells included chromosomal bridges and fragments, vagrant chromosomes, aberrant metaphases and anaphases in dividing cells. The percent of micronuclei in interphase cells were also detected.

Statistical analyses: We processed the experimental data by Student's t-test. In root inhibition test we choose as an experimental unit the root. The calculations were carried out on the assumption that roots used in each treatment made one sample and each sample was tested against the control sample. In *Allium cepa* test we choose as an experimental unit the cell, instead of the root. The calculations were carried out on the assumption that all the cells of the six root meristems made one sample and each sample was tested against the negative control.

RESULTS AND DISCUSSION

Root growth inhibition test and determination of EC50: The degree of toxicity of herbicide GOAL (240 g L^{-1} oxyfluorfen) to *T. aestivum* was assessed by means of the root length values (Fig. 2). The dose of 0.03 ml L^{-1} caused only a slight negative effect- inhibition of root elongation by 3.67% compared with the control. The growth inhibitory effect increased with concentrations- differences with the control were 13.54% (0.15 ml L^{-1}) and 23.55% (0.3 ml L^{-1}). We observed much stronger inhibition of root growth at the concentrations of 1.5 ml L^{-1} (differences with the control of 58.7%) and 3.0 ml L^{-1} (differences with the control of 91.15%). The effective concentration of the test chemicals that cause 50% of root length as compared to control (EC50) was determined-approximately 1.2 ml L^{-1}.

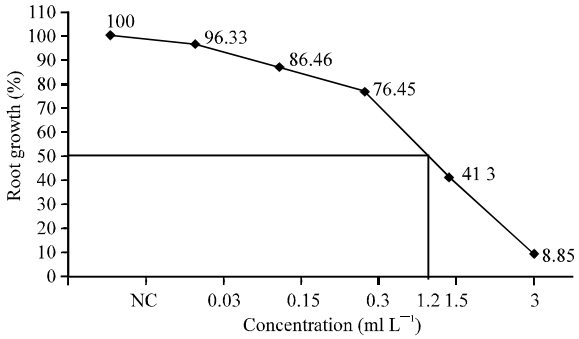

Fig. 2: Effect of treatment with GOAL (3.0, 1.5, 0.3, 0.15, 0.03 ml L^{-1}) for 96 h on root growth of *T. aestivum*

Table 1: Effect of treatment with GOAL (0.3, 0.6 and 1.2 ml L^{-1}) for 24 h and 48 h on mitotic index and phase indices in root tip meristems of *Allium cepa* L.

Time of treatment	Sample	No. of cells analysed	MI% (Mean±SD)	Prophase, PhI%	Metaphase, PhI%	Anaphase, PhI%	Telophase, PhI%
24 h	NC	6772	6.35±0.24	26.28±0.44	26.51±0.44	23.72±0.43	23.49±0.42
	0.03%	7465	5.81±0.23	17.51±0.38***	31.11±0.46	34.56±0.48***	16.82±0.37***
	0.06%	7922	5.06±0.22***	20.70±0.41*	35.41±0.48**	27.93±0.45	15.96±0.37**
	0.12%	7835	4.20±0.20***	17.93±0.38**	33.74±0.47*	29.79±0.46*	18.54±0.39
24 h	PC	6991	3.69±0.19***	20.54±0.40	31.78±0.47	26.74±0.44	20.93±0.41
48 h	NC	6680	6.54±0.25	27.23±0.45	33.41±0.47	21.28±0.41	18.08±0.39
	0.03%	7142	6.33±0.24	21.90±0.41*	32.74±0.47	24.56±0.43	20.80±0.40
	0.06%	6925	5.20±0.22***	15.28±0.36***	33.06±0.47	31.11±0.46***	20.56±0.40
	0.12%	7058	5.21±0.22***	35.33±0.48**	25.54±0.44**	19.57±0.40	19.57±0.40

Data are expressed as Means±SD. *p≤0.05, **p≤0.01; ***p≤0.001 (compared to NC), MI: Mitotic index, PhI: Phase index, NC: Negative control (distilled water), PC: Positive control (methyl methanesulfonate, 11 mg L^{-1})

We provided the growth inhibition test using *T. aestivum*, according to our previous study (Kalcheva *et al.*, 2009) and other data about suitability of this plant for evaluation of environmental pollutants (Kaymak and Muranli, 2006; Kumar, 2010). The results from present study revealed concentration-dependent and statistically significant inhibition of root growth of *T. aestivum* by the GOAL at concentrations above 0.03 ml L^{-1} when compared with the control.

***Allium cepa* test:** Table 1 shows the results of the influence of GOAL at concentrations representing ¼ EC50, ½ EC50 and EC50 after 24 and 48 h exposure on the mitotic index and on the frequency of mitotic phases. Treatment with 0.3 ml L^{-1} at both exposure times caused slight negative influence on mitotic index but the difference with the control was not significant. Upon all other treatments the mitotic index was significantly decreased in comparison with those exposed to water only.

Changes in mitotic activity show that GOAL at concentrations above ¼ EC50 depressed cellular proliferation in *Allium cepa* root tips after treatment for 24 and 48 h. Similar effects on mitotic index were described by many researchers following the treatment of *Allium cepa* roots with different pesticides (Asita and Matebesi, 2010; Aydemir *et al.*, 2008). Yuzbasioglu *et al.* (2009) in a study about genotoxic potential of commercial herbicide Illoxan comment a possible reason for significantly decreased mitotic index in plant cells: "blocking of G1 suppressing DNA synthesis

Table 2: Effect of treatment with GOAL (0.03, 0.6 and 1.2 ml L^{-1}) for 24 h and 48 h on abnormalities in mitotic cells in root tip meristems of *Allium cepa* L.

		Abnormalities in mitotic cells						
Time of treatment	Sample	Number of cells analysed	SA-A (n)	V (n)	SA-M (n)	Br (n)	Fr (n)	Total % (Mean±SD)
24 h	NC	430	–	1	2	1	3	1.63±0.13
	0.03%	434	5	10	6	1	4	5.99±0.24***
	0.06%	401	5	11	6	3	2	6.73±0.25***
	0.12%	329	3	10	6	3	8	9.12±0.29***
24 h	PC	258	–	14	1	4	25	17.05±0.38***
48 h	NC	437	–	1	–	1	6	1.83±0.13
	0.03%	452	5	13	3	3	19	9.51±0.29***
	0.06%	360	6	12	3	4	19	12.22±0.33***
	0.12%	368	9	15	8	8	10	13.59±0.34***

Data are expressed as Means±SD. ***$p \leq 0.001$ (compared to NC), NC: Negative control (distilled water), PC: Positive control (methyl methanesulfonate, 11 mg L^{-1}), SA-A: Spindle abnormalities in anaphase, SA-M: Spindle abnormalities in metaphase, V: Vagrant chromosomes, Br: Bridges, Fr: Fragments, n-number

(Schneiderman *et al*., 1971), blocking of G2 preventing the cell from entering mitosis (Van't Hof, 1968) or inhibition of DNA synthesis at the S-phase (Sudhakar *et al*., 2001)". The inhibition of mitotic index demonstrates the cytotoxic potential of GOAL. On the other hand, it is not recommended to score chromosome aberrations if the mitotic index is too low (Rank, 2003). Our data confirmed the statement, that EC50 value is useful parameter for selection of test concentrations for gecotoxcity assays (Mustafa and Ariken, 2008). In present study the highest concentration for the genotoxicity test was chosen as EC50 value and there were enough dividing cells for the microscopic analysis.

The herbicide caused a change in the frequencies of the mitotic phases. The changes were not peculiar to any treatment. The exception was changes in prophase ratios. After all treatments, except EC50 for 48 h, we observed lower prophase ratios than the control. Only exposure to EC50 for 48 h resulted in an increase of the cells in prophase with a corresponding decrease in the percentage of metaphase compared with the control.

Similar accumulation of prophase cells was observed in other experiments (Lamsal *et al*., 2010). It can be speculated that the decisive effect of GOAL on mitotic phase distribution is caused by EC50 for 48 h.

Treatment with GOAL at all tested concentrations and both treatment times increased the percent of chromosome aberrations in mitotic cells (Table 2). This data is found to be statistically highly significant when compared to control ($p<0.001$). The treatment with 0.3 and 0.6 ml L^{-1} for 24 h increased approximately fourfold the percent of aberrations compared with the control. The effect of 1.2 ml L^{-1} for the same time was stronger-5.6-fold increase of aberrations compared with negative control. When duration of the treatment increased we observed much more aberrations. The effect of the lowest tested concentration of the herbicide (0.3 ml L^{-1}) for 48 h was almost the same as the highest concentration (1.2 ml L^{-1}) for 24 h. The treatment with 0.6 and 1.2 ml L^{-1} for 48 h increased the percentage of chromosomal aberrations in mitotic cells-by 6.7-fold and by 7.4-fold, respectively compared to the controls.

GOAL induced a variety of different chromosome aberrations (Fig. 3). The kind of abnormalities was not peculiar to any concentration or time after the treatment. Anaphases and telophases with fragments and vagrant chromosomes were the most frequent kinds of aberrations. Metaphases and anaphases with spindle abnormalities were the second. We scored bridges in anaphase and

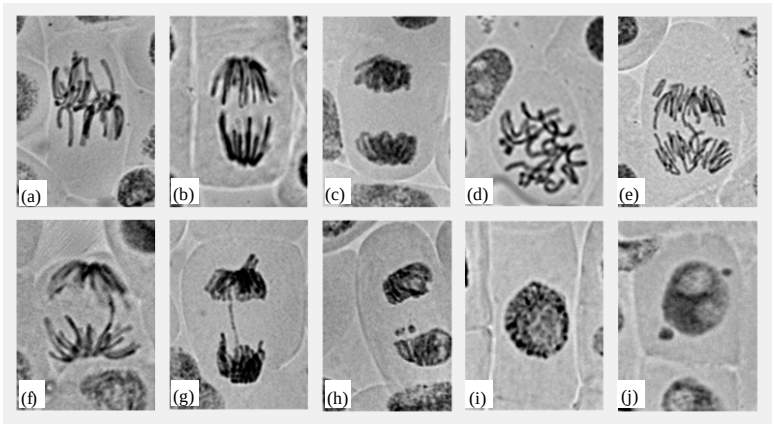

Fig. 3(a-j): Aberrations induced by GOAL in *Allium cepa* root tips: (a)-normal metaphase, (b)-normal anaphase, (c)-normal telophase, (d)-C-mitosis, (e)-spindle abnormalities in anaphase, (f)-anaphase bridge; (g)- anaphase-telophase bridge, (h)- telophase fragment, (i)-normal interphase cell and (j)-micronuclei in interphase cell

telophase cells but at a lesser frequency. There were also a few metaphases with a vagrant chromosome and metaphase plates with a fragment.

These results showed induction of aberrant mitotic cells and revealed dose and treatment-time dependent influence of GOAL. The low percentage of abnormal cells observed in the negative control may be due to an automutagenic effect reported also by other authors (Abu and Ezeugwu, 2008; Ivanova *et al*., 2005). Anaphase/telophase bridges and chromosome fragments suggest a clastogenic effect of GOAL (Bolle *et al*., 2004; De Campos *et al*., 2008). The abnormalities such as C-metaphase, disturbed metaphase and anaphase reflect the impairment of mitotic spindle altering the orientation of chromosomes. When C-mitotic effect is observed chromosomes were randomly distribute in the cell (Levan, 1938). Multipolar anaphases might be a result of partial or incomplete C-mitosis (Grant, 1978). The induction of vagrant chromosomes also is a consequence of spindle disturbances (Rank, 2003). Large number of abnormal metaphase and anaphase cells indicates that GOAL acts as potent spindle inhibitor. According to Grant (1978) turbagens should not be considered genetically insignificant because of the incorrect chromosome segregation. In the same paper Grant (1978) underlined, that turbagens generally caused mild clastogenic effect. In our study such regularity was observed only after the treatment with 0.3 and 0.6 ml L^{-1} for 24 h-breaks and bridges were below 20% of all aberrations. Exposure to GOAL at the same concentrations for 48 h induced almost equal clastogenic and turbagenic effect-about 50%. At the highest tested concentrations for both treatment times spindle abnormalities slightly predominated-about 60% of aberrant cells. From Table 2 it can be seen that compared to a positive control (MMS, 11 mg L^{-1}) GOAL had less genotoxic effect. However, the positive results from genotoxicity test might be a warning of the potential hazard of the tested herbicide.

The exposure to GOAL significantly increased the percent of micronuclei in interphase cells (Table 3). The induction of micronucleated cells in interphase increased with concentration and time of the treatment. After exposure for 24 h the frequencies of micronuclei were 4.3-fold (1/4 EC50), 5.7-fold (1/2 EC50) and 6.7-fold (EC50) higher than untreated control. These values was markedly higher after 48 h exposure compared to 24 h-5.6-fold (1/4 EC50), 6.4-fold (1/2 EC50) and 9.8-fold (EC50) compared with the control. Micronuclei are described as chromatin-containing

Table 3: Effect of treatment with GOAL (0.03, 0.6 and 1.2 ml L^{-1}) for 24 h and 48 h on abnormalities in interphase cells in root tip meristems of *Allium cepa* L.

Time of treatment	Sample	No. of interphase cells	Micronuclei (No.)	Cells with micronuclei, %(Mean±SD)
24 h	NC	6342	2	0.03±0.02
	0.03%	7031	9	0.13±0.04*
	0.06%	7521	13	0.17±0.05**
	0.12%	7506	15	0.20±0.04**
24 h	PC	6733	33	0.48±0.07***
48 h	NC	6243	3	0.05±0.02
	0.03%	6690	19	0.28±0.05***
	0.06%	6565	21	0.32±0.06***
	0.12%	6690	33	0.49 ±.07***

Data are expressed as Means±SD. *p≤0.05, **p≤0.01, ***p≤0.001 (compared to NC), NCL: Negative control (distilled water), PC: Positive control (methyl methanesulfonate, 11 mg L^{-1})

structures in the cytoplasm surrounded by a membrane originate from chromosome fragments or whole chromosome lagging at anaphase (Fenech, 2000). Their possible fate include expulsion from the cell, reincorporation into the nucleus or retention within the cytoplasm (Leach and Jackson-Cook, 2004). From Table 2, it can be seen a positive correlation between concentration and time of treatment and percent of micronuclei in interphase cells. Other studies also have shown that micronucleus formation is effective indicator of cytological damage (Ma *et al*., 1995). The induction of micronuclei confirmed the clastogenic potential of the test chemical.

The results showed that GOAL produced mitotic and interphase abnormalities in *Allium cepa* root tip cells at concentrations ¼ EC50-EC50 for 24 and 48 h. The analysis demonstrated a positive correlation between genotoxic effect and exposure time and concentration. The data obtained confirmed the statement of U.S. Environmental Protection Agency (USEPA, 2002) that oxyfluorfen must be considered a possible human carcinogen.

In conclusion, present study indicate cytotoxic and genotoxic effect of GOAL in *A. cepa* root tip cells after exposure for 24 and 48 h at concentrations below commercially recommended. As shown here, *A. cepa* may be a sensitive biosensor for screening the potential genotoxicity of pesticides.

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