Genotoxicity testing of quizalofop-P-ethyl herbicide using the *Allium cepa* anaphase-telophase chromosome aberration assay

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Abstract — Cytogenetic effects of phenoxy herbicide viz. quizalofop-P-ethyl (QPE, ethyl (R)-2-[4-[(6-chloro-2-quinoxalinyl)oxy]phenoxy] propionate) were evaluated in the root meristem cells of *Allium cepa*. In the *Allium* root growth test, the effective concentration (EC_{50}) value was determined as approximately 1.5 ppm. Cytological experiments were carried out using QPE concentrations of 0.75 (EC_{50}/2), 1.5 (EC_{50}) and 3 ppm (EC_{50}x2) at 24, 48, 72, and 96 h, with a control for each combination. Mitotic index decreased with increasing the herbicide concentration at each exposure time. In anaphase-telophase cells, the total percentages of stickiness, bridges, vagrant chromosomes, c-anaphase, multipolarity, and fragments according to total cells with chromosome aberrations were calculated as 38.57, 28.42, 16.67, 14.10, 1.60, and 0.64%, respectively. The total chromosome aberrations increased with an increasing the QPE concentration. Micronucleated cells were observed at interphase. The frequency of the micronucleus was markedly higher at 3 ppm compared to other test concentrations.

Key words: *Allium cepa*; chromosome aberration; herbicide; mitotic index; quizalofop-P-ethyl; root growth.

INTRODUCTION

Pesticides which are used in the modern agricultural practices for disease control have some dangerous effects (PANDY *et al.* 1994). Higher plants provide valuable genetic assay systems for screening and monitoring environmental pollutants. For this purpose, the *Allium cepa* is one of the most frequently used higher plant species (GRANT 1994). The *Allium* test for genotoxicity was introduced by LEVAN (1938) and has been used on pesticides in other studies (RANK and NIELSEN 1997; CHAUHAN *et al.* 1999; ATEEQ *et al.* 2002; YÜZBAŞIOĞLU *et al.* 2003; BOLLE *et al.* 2004; MARCANO *et al.* 2004; CHANDRA *et al.* 2005; KAYMAK and MURANLI 2005; MASTRANGELO *et al.* 2006). The *Allium test* was simple and just as reliable as the method where chromosome aberrations were recorded in all types of mitotic cells (RANK and NIELSEN 1997). The test can be used to measure both toxicity (effective concentration, EC_{50}, where the root bundles are half the length of the control) and genotoxicity. The rate of the root growth can be correlated with the mitotic index (LIU *et al.* 1992). The chromosome aberration and micronucleus assays have been shown to be highly reliable in genotoxicity testing (SMAKA-KINCL *et al.* 1996; NATARAJAN 2002).

Quizalofop-P-Ethyl (QPE) is a phenoxy herbicide compound. The herbicides, one of the pesticides, are used in agriculture in controlling weeds. The indiscriminate use of herbicides in agriculture, as well as the increase of pollution in ecosystems due to industrial development, justifies the evaluation of the toxicity of these chemicals (MARCANO *et al.* 2004). Currently, the literature is unavailable on the cytological effects of QPE herbicide in plant systems. The purpose of this study was to investigate the effects of QPE herbicide on root growth, mitotic index, chromosome aberrations, and micronucleus formation in the root meristem cells of *Allium cepa.*

MATERIAL AND METHODS

Test organism/Growth conditions - Equal-sized bulbs (25-30 mm in diameter) of a commercial variety of *Allium cepa* L. (2n=16) were chosen. The onions were kept cool and dry until cytotoxicity testing. Just before use, the outer scales of the bulbs were carefully removed and the brownish bottom plates were scraped away without destroy-

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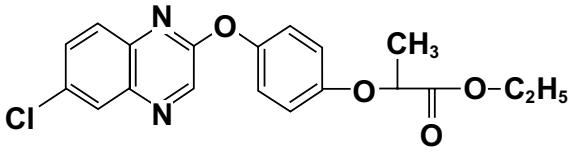
ing the root primordia. The experiments were maintained in laboratory conditions at 22±2 °C. The roots were protected from direct sunlight in order to minimize fluctuation of the rate of cell division (EVANS *et al*. 1957).

Chemical material - The tested chemical was phenoxy herbicide viz. quizalofop-P-ethyl (QPE, ethyl (R)-2-[4-[(6-chloro-2-quinoxalinyl)oxy]ph enoxy] propionate; CAS No. 100646-51-3). Molecular weight is 372.80 g/mol. The structural formula is as following:



Allium root growth test/Determination of EC_{50} - Clean and healthy onion bulbs were set up and allowed to produce roots in distilled water for 24 h, where after the homogeneously rooted five bulbs transferred to the control (distilled water) and different QPE herbicide solutions (5-50, 1-10 and 1-3 ppm) for 96 h. During the experiment, the test solutions were changed every 24 h instead of aeration. The root lengths from the control and experimental sets were measured (lengths of ten roots from each bulb) at the end of exposure time. The relative reduction of root length was calculated as the percentage of the deviation from the control (T/C%). The effective concentration (EC_{50}) value was determined as approximately 1.5 ppm. EC referees to Effective Concentration and the number 50 indicate the effective concentration for 50% growth inhibition. Experiments were carried out in triplicate.

Cytogenetic parameters - The onions were rooted in distilled water for 24 h. The five bulbs which have approximately same root length were transferred to the control and test solutions. Cytological experiments were carried out using QPE concentrations of 0.75 (EC_{50}/2), 1.5 (EC_{50}) and 3 ppm (2xEC_{50}) at 24, 48, 72, and 96 h, with a control for each combination. The root tips were sampled between 07.00-08.00 h, as the highest mitosis frequency in the onion is recorded between 06.00-09.00 h (SHARMA 1983). After completion of exposure, roots from 5 bulbs were immediately cut and fixed in solution of ethanol (99%) and glacial acetic acid (3:1) for 24 h. The roots were transferred to 70% alcohol and stored in refrigerator until use. The root tips were macerated in a solution of 1N HCl at 60 °C for 7 min. Then, the roots were washed with distilled water three times. Chromosomes were stained with Feulgen for 1 h at dark followed by squashing in 45% acetic acid. One slide prepared for per onion.

All slides were coded and examined blindly. The mitotic index, micronucleus in interphase, and chromosome aberrations in anaphase-telophase were investigated in cytogenetic analysis for each concentration and exposure time. The mitotic index was determined by scoring more than 5000 cells (more than 1000 cells per slide). Mitotic index was calculated as the percent ratio of dividing cells and total numbers of cells scored. Micronucleus frequency was determined by examination of more than 1000 interphase cells per slide (totally more than 5000 for each treatment). In chromosome aberrations test, 100 cells in anaphase or telophase were examined for aberrations per slide. Chromosome aberrations were examined in 500 anaphase-telophase cells for each treatment. The chromosome aberrations scored were stickiness, bridges, vagrant chromosomes, c-anaphase, multipolarity and fragments.

Statistical analysis - Data were analyzed using SPSS, ver. 10.0. The analysis of variance (ANOVA) was used to assess the significant differences between control and each treatment. If there was a significant differences ($P < 0.05$), the experimental data analyzed using Duncan's multiple range test.

RESULTS

Effects on Allium root growth and mitotic index - The root growth decreased with increasing the QPE herbicide concentration ($P < 0.05$). Above 3 ppm, there was no root growth during 96 h. After 96 h of growth in the control, the average length of roots was 5.34 ± 0.18 cm. Dose-response curves obtained between the concentrations of QPE herbicide and *Allium* root growth determined the effective concentration (EC_{50}) value which retards 50% root growth as 1.55 ppm. The root length after 96 h in EC_{50} was 2.75 ± 0.06 cm. However, for experimental purposes we used 1.5 ppm as EC_{50} for QPE herbicide.

The effect of QPE herbicide on the mitotic index (%) of *Allium cepa* root meristem cells is determined (Table 1). There were significant differences between QPE herbicide concentrations and the control ($P < 0.05$). Mitotic index significantly decreased in the herbicide concentrations

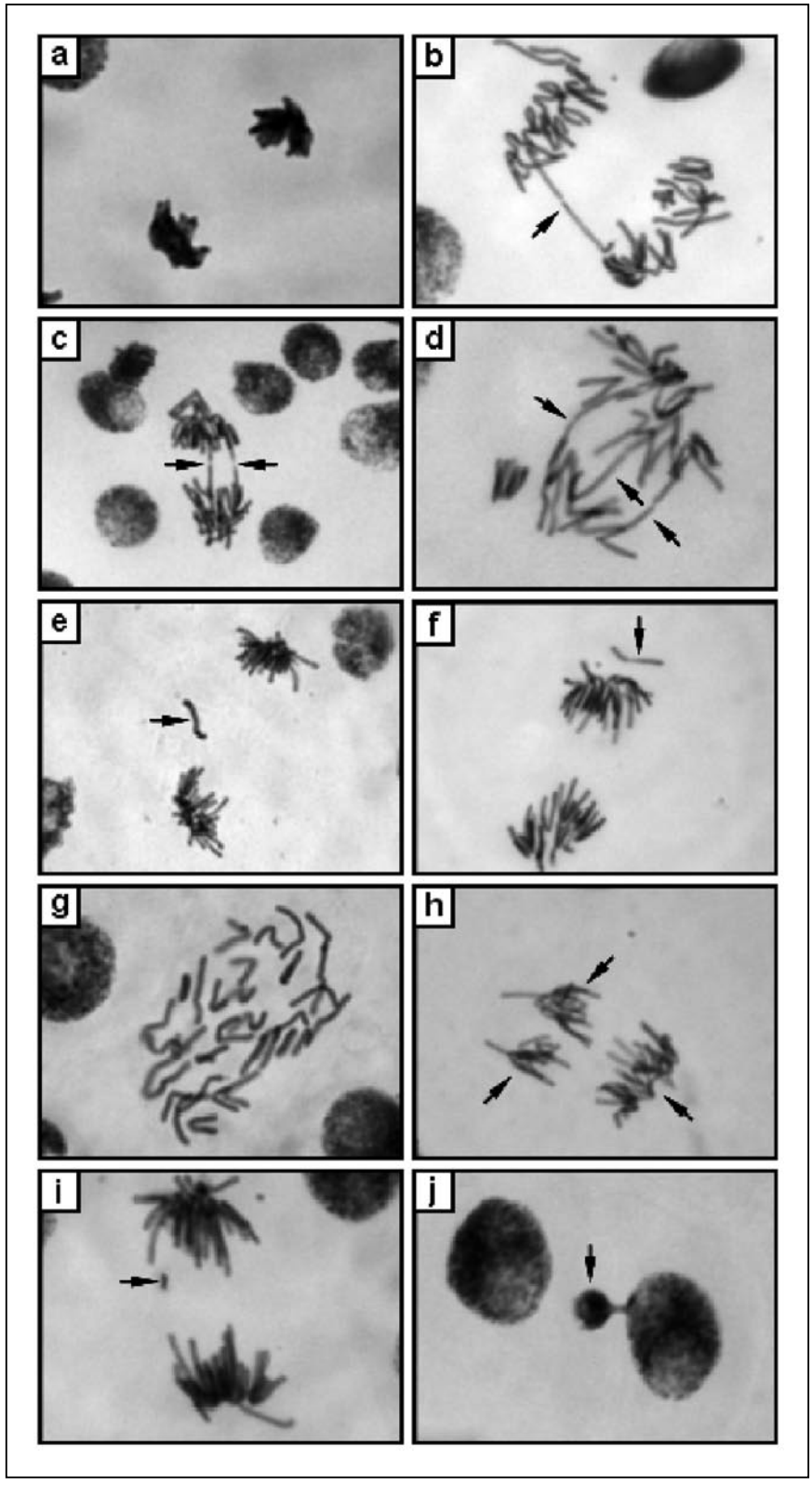


Fig. 1 — Chromosomal aberrations induced by QPE herbicide in root tip cells of *Allium cepa*. (a) Stickiness; (b, c, d) Bridges; (e, f) Vagrant chromosomes; (g) c-Anaphase; (h) Multipolarity; (i) Anaphase cell with a fragment; (j) Micronucleus in interphase cell.

compared to control at each exposure time. The percentage of mitotic index was significantly low at 3 ppm compared to other concentrations at 24, 72 and 96 h. In contrast, there were no significant differences between QPE herbicide concentrations at 48 h (Table 1).

Effects on chromosome aberrations and micronucleus formation - Results of genotoxicity tests with the *Allium* anaphase-telophase test were evaluated (Table 1). The highest QPE herbicide concentration (3 ppm) showed high toxicity on the root cells. Therefore, it was not possible to find 500 anaphase or telophase cells for the examination at 72 and 96 h periods. The changes in the organization and morphology of the chromosomes in the root tips exposed to the herbicide were observed (Fig. 1). Six types of chromosome aberrations were recorded in anaphase-telophase cells (Fig. 1 a-i). The total percentages of stickiness, bridges, vagrant chromosomes, c-anaphase, multipolarity, and fragments according to total cells with chromosome aberrations were calculated as 38.57, 28.42, 16.67, 14.10, 1.60, and 0.64%, respectively. The stickiness was the most frequently observed chromosome aberration, although, it was not observed in all the control treatments. The total chromosome aberrations increased with an increasing in the QPE herbicide concentration. However, there were no significant differences between 0.75 and 1.5 ppm QPE concentrations in all exposure times. The total chromosome aberrations (%) were significantly higher at the highest concentration (3 ppm) of QPE herbicide than the other herbicide concentrations. Micronucleated cells were observed at interphase (Fig. 1j). The induction of micronucleus formation was generally observed in all treatments. Micronucleus formation was markedly higher at 3 ppm than the other herbicide concentrations in all the exposure times (Table 2).

Table 1 — Results from genotoxicity testing of quizalofop-P-ethyl herbicide in the *Allium cepa* anaphase-telophase chromosome aberration assay

Treatment		Mitotic index (% ± SE*)	No. of cells examined	Anaphase-telophase chromosome aberrations						Total aberration (% ± SE)
Time (h)	Conc. (ppm)			Stickiness	Bridge	Vagrant	c-Anaphase	Multipolarity	Fragment	
24	Control	9.70 ± 0.37 **a****	500	0	11	3	2	0	0	3.20 ± 0.37 **a**
	0.75	8.09 ± 0.41 **ab**	500	13	18	8	11	3	0	10.60 ± 1.96 **b**
	1.5	7.86 ± 0.33 **b**	500	23	16	20	9	1	1	13.60 ± 0.75 **b**
	3	3.48 ± 0.86 **c**	500	31	26	22	23	0	1	20.60 ± 2.01 **c**
48	Control	10.37 ± 0.92 **a**	500	0	8	0	3	1	1	2.60 ± 0.51 **a**
	0.75	7.27 ± 0.32 **b**	500	22	27	6	6	4	0	13.00 ± 0.45 **b**
	1.5	7.52 ± 0.36 **b**	500	25	19	7	17	1	0	13.80 ± 1.39 **b**
	3	7.01 ± 0.26 **b**	500	54	21	13	13	0	0	20.00 ± 1.22 **c**
72	Control	9.12 ± 0.46 **a**	500	0	9	1	2	0	0	2.40 ± 0.40 **a**
	0.75	8.66 ± 0.43 **a**	500	23	12	12	7	3	0	11.40 ± 0.51 **b**
	1.5	5.15 ± 0.44 **b**	500	23	20	13	13	0	0	13.80 ± 0.86 **b**
	3	3.35 ± 0.51 **c**	324	68	6	11	2	0	1	27.16 ± 1.35 **c**
96	Control	8.43 ± 0.26 **a**	500	0	6	4	2	1	1	2.80 ± 0.37 **a**
	0.75	5.84 ± 0.22 **b**	500	30	33	15	4	0	1	16.60 ± 0.87 **b**
	1.5	5.04 ± 0.47 **b**	500	25	32	21	16	1	0	19.00 ± 1.14 **b**
	3	2.19 ± 0.16 **c**	69	24	2	0	2	0	0	40.58 ± 1.59 **c**
		Percentage of aberrant cells (%)		38.57	28.42	16.67	14.10	1.60	0.64	

* SE = Standard error

** $P < 0.05$ in Duncan multiple range test

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DISCUSSION

Toxic effects of environmental pollutants may be evaluated by analyzing macroscopic (root

Table 2 — Results from genotoxicity testing of quizalofop-P-ethyl herbicide in the *Allium cepa* micronucleus assay

Treatment		No. of interphase cells examined	Micronucleus (%)
Time (h)	Conc. (ppm)		
24	Control	5120	0
	0.75	5145	0.10
	1.5	5522	0.06
	3	5463	0.26
48	Control	5134	0.02
	0.75	5086	0
	1.5	5281	0.09
	3	5217	0.21
72	Control	5060	0
	0.75	5115	0.06
	1.5	5136	0.04
	3	5198	0.12
96	Control	5528	0.02
	0.75	5170	0.06
	1.5	5101	0.02
	3	5193	0.08

growth decrease) as well as cytological parameters (types and frequencies of chromosome aberrations) (SMAKA-KINCL *et al.* 1996).

In *Allium* root growth test, QPE herbicide was found toxic causing an inhibition in root growth of *Allium cepa*. The fact that the root growth decrease over 45% strongly indicates the presence of toxic substances (FISKESJÖ 1985) having sublethal effects on plants (HIDALGO *et al.* 1989). After 96 h of root growth in all concentrations, the root length was shown as a reliable indicator of toxicity of QPE herbicide. The results of the present study clearly indicate the utility of root meristem cells of *Allium cepa* in bio-monitoring environmental pollutants such as herbicides. On the other hand, the effective concentration (EC_{50}) value proved to be useful parameter for selecting the test concentrations for the genotoxicity assays (MA *et al.* 1995; CHAUHAN *et al.* 1999). Therefore, in this research, the EC_{50} value was detected as about 1.5 ppm, and the highest concentration for the genotoxicity test was chosen as 3 ppm ($2xEC_{50}$).

Mitotic index is considered a parameter that allows one to estimate the frequency of cellular division (MARCANO *et al.* 2004). Inhibition of mitotic activities is often used for tracing cytotoxic substances (LINNAINMAA *et al.* 1978). If the EC_{50} value is chosen as the highest concentration for the genotoxicity test, the mitotic index will never be below 50% of the control (RANK and NIELSEN 1997). In our research, mitotic index values in 1.5 ppm (EC_{50} value) concentration of QPE herbicide at all exposure times were above 50% of control. The reduction in cell division was more significant when the concentration of the herbicide increased at each exposure time. A ≥63% reduction of mitotic activity was detected in 3 ppm QPE herbicide treated *Allium* roots compared to their controls at 24, 72 and 96 h. The concentration-dependent inhibition of mitotic index illustrates the cytotoxic potential of QPE herbicide in *A. cepa*. Similar effects on mitotic index were described by many researchers following the treatment of *Allium cepa* roots with cypermethrin and fenvalerate insecticides (CHAUHAN *et al.* 1999), ceresan, agrosan and mercuric chloride fungicides (NANDI 1985), and pentachlorophenol, 2, 4-D, butachlor (ATEEQ *et al.* 2002), racer (YÜZBAŞIOĞLU *et al.* 2003) and maleic hydrazide (MARCANO *et al.* 2004) herbicides. On the other hand, the results of the analysis showed that the effect of QPE herbicide concentration and exposure time on mitotic index was significant level, but the effect of concentration was 1.68 times higher than the effect of the exposure times. The mitotic index value was 3.48% for 3 ppm at 24 h period, while it was 5.84% for 0.75 ppm at 96 h period. That is, subjecting the root tips to highest concentration for a short time is more deleterious than exposing them to low concentration for longer time. Once the herbicide penetrates

the cells and reaches a critical concentration, it remains in an active form, causing lesions during several following cellular cycles (RANK *et al.* 2002; MARCANO *et al.* 2004). The cytotoxicity level of environmental pollutants can be determined by the decreased rate of the mitotic index (SMAKA-KINCL *et al.* 1996). A mitotic index decrease below 22% of the negative control causes lethal effects on test organisms (ANTONSIEWICZ 1990), while a decrease below 50% usually has sublethal effects (PANDA and SAHU 1985) and is called cytotoxic limit value (SHARMA 1983). In our research, the sublethal effect was determined at 3 ppm compared to the control at 24, 72, and 96 h periods. These sublethal effect values for 24, 72, and 96 h were detected as 35.87, 36.73, and 25.98%, respectively. In contrast to this, neither lethal nor sublethal effects were observed at 48 h treatment, although mitotic index was significantly decreased at 0.75, 1.5, and 3 ppm compared to control. It may be suggest that a significant decrease in mitotic activity indicates mitodepressive effect of herbicide, and pesticides could (a) interfere with the normal development of mitosis, thus preventing a number of cells from entering the prophase and blocking the mitosis cycle during interphase (BADR 1986; BADR and IBRAHIM 1987; EL-GHAMERY *et al.* 2000), or (b) attribute to an increase in G_2 and S phases duration (VAN'T HOFF 1968; WEBSTER and DAVIDSON 1969; BADR and IBRAHIM 1987). In addition, such mitodepressive effect of environmental chemicals may also be due to the inhibition on DNA/protein synthesis of the biological systems (BADR 1986; BADR and IBRAHIM 1987; CHAUHAN *et al.* 1998; EL-GHAMERY *et al.* 2000). On the other hand, EPEL (1963) reported that the rate of mitosis was closely related to resultant level of ATP. In this sense, the pesticide treatment may disturb the respiratory pathways, resulting in the low production of energy containing and other essential compounds-ATP, sugars and protein molecules (JAIN and SARBHOY 1987).

The changes in the organization and morphology of the chromosomes were observed in the root tips exposed to the QPE herbicide. Six main types of chromosome aberrations were recorded in anaphase-telophase: Stickiness, bridges, vagrant chromosomes, c-anaphase, multipolarity and fragment. The percentage of total chromosome aberrations increased with increasing the test concentration at each exposure time. This result shows that the mitotic index was not blocked by effect of the herbicide even at 3 ppm. If the 3 ppm was too toxic, it would cause cell death, which might interfere with scoring of chromosome aberrations caused by the QPE herbicide. Therefore, the percentage of chromosome aberrations was not diminished. Among these aberrations, stickiness or sticky chromosomes, as a common type, were the most frequently observed aberration at anaphase-telophase stages of mitosis in root tips of *A. cepa* treated with QPE herbicide. Stickiness is considered to be a chromatid type aberration (BADR 1986). DARLINGTON and MC-LEISH (1951) suggested that stickiness might be due to degradation or depolymerization of chromosomal DNA. However, stickiness has been shown to be as a result of DNA condensation (ÖSTERBERG *et al.* 1984) and entanglement of inter-chromosomal chromatin fibers which led to subchromatid connections between chromosomes (CHAUHAN *et al.* 1986; PATIL and BHAT 1992). In the present research, the sticky chromosomes were observed in high frequency at all the QPE herbicide treatments. In contrast to, no sticky chromosomes were observed in control treatments. LIU *et al.* (1992) suggested that sticky chromosomes reflect highly toxic effects, usually of an irreversible type, and probably lead to cell death. The bridges involving one or more chromosomes were the most prominent and frequent type in addition to sticky chromosomes. The induction of bridges could be attributed to chromosome breaks, stickiness and breakage and reunion of the broken ends. The stickiness of chromosomes prevented the separation daughter-chromosomes and thus they remained connected by bridges (KABARITY *et al.* 1974; BADR *et al.* 1992). Sticky bridges might be also the result of incomplete replication of chromosomes by defective or less active replication enzymes (SINHA 1979) or late replicating DNA sequences of the telomeric heterochromatin (BENNET 1977). If heterochromatin blocks do not finish DNA replication when the nucleus is ready to divide, bridge formation would occur (KALTSIKES 1984). The spindle irregularities like vagrant chromosomes, c-anaphase and multipolarity were also observed, but the multipolarity was lesser extent. The induction of vagrant chromosomes leads to the separation of unequal number of chromosomes in the daughter nuclei and subsequently formation of daughter cells with unequal sized or irregularly shaped nuclei at interphase (EL-GHAMERY *et al.* 2003). LEVAN (1938) described colchicine mitosis (c-metaphase or c-anaphase) as an inactivation of the spindle followed by a random scattering of the condensed chromosomes in the cell. It is reported that the immediate effect of all the pesticide treatment was partial or entire inactivation of spindle mechanism followed by scattering of chromosomes (JAIN and SARBHOY 1987). Large number of vagrant chromosomes and c-anaphases indicates

that QPE herbicide acts as a potent spindle inhibitor. In the present study, a few cells with fragments were observed at anaphase-telophase in root tips of *A. cepa*. SAXENA *et al*. (2005) suggested that the induction of chromosome breaks by cypermethrin indicates the clastogenic potential of the test compound. The induction of these kinds of chromosome aberrations was determined at different concentrations and exposure times of environmental pollutants (CHAUHAN *et al*. 1999; AMIN 2002; ATEEQ *et al*. 2002; RANK *et al*. 2002; EL-GHAMERY *et al*. 2000; CHANDRA *et al*. 2005; SAXENA *et al*. 2005).

In addition to the types of chromosome aberrations induced in the anaphase-telophase cells, the formation of micronucleus in interphase cells was determined. The percentage of micronucleated cells was obviously higher at the highest QPE herbicide concentration (3 ppm) than other test concentrations. The induction of micronucleus in root meristem cells of *A. cepa* is the manifestation of fragments or vagrant chromosomes (NANDI 1985; DASH *et al*. 1988; GROVER and KAUR 1999; YI and MENG 2003). In some instances the vagrant chromosomes or fragments may have either dissolved in the cytoplasm or gradually clumped and may be surrounded by nuclear membrane to form micronucleus (EL-GHAMERY *et al*. 2003). On the other hand, recording of micronuclei in interphase cells showed that this endpoint does not give more information of clastogenicity than recording of chromosome aberrations in anaphase-telophase cells (RANK and NIELSEN 1997).

In conclusion, the test of chromosome aberration on plant systems constitutes a simple and reliable technique to detect the genotoxicity of pesticides. It also point to the importance of mutagenicity testing of the applied chemicals like pesticides before use.

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