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Genotoxicity of Atrazine, Avenoxan, Diuron and Quizalofop-P-ethyl Herbicides using the *Allium cepa* Root Chromosomal Aberration Assay

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ABSTRACT

Cytogenetic effects of different herbicides viz. Atrazine, Avenoxan, Diuron and Quizalofop-P-ethyl (QPE), were evaluated in the root tip meristem cells of *Allium cepa*. In the *Allium* root growth test, the effective concentration (EC_{50}) value was determined as approximately 0.5 ppm in the case of Atrazine and Avenoxan and 1.0 ppm in the case of Diuron and QPE herbicides with a control for each combination. Mitotic index decreased with increasing herbicide concentration at each exposure time. In anaphase-telophase cells, the total percentages of different chromosomal aberrations like stickiness, bridges, break(s), ring chromosomes, vagrant chromosomes, c-mitosis, delayed anaphase, laggard(s) and micronuclei at high concentration (1 ppm) were calculated as 31.85% (Atrazine), 29.94% (Avenoxan), 36.66% (Diuron) and 41.04% (QPE). The total number of chromosome aberrations increased as herbicide concentration increased. Micronucleated cells were observed at different stages of the cell cycle. The frequency of the micronucleus was markedly higher at 1 ppm than at other test concentrations.

Keywords: Atrazine, Avenoxan, Diuron, herbicides, mitotic index, QPE
Abbreviations: Bg, chromatin bridge; **Bk**, chromosomal break(s); **Cm**, c- mitosis; **Da**, delayed anaphase; **DPX**, dibutyl phthalate xylene; **EC_{50}**, effective concentration; **Lg**, laggard chromosome; **Mn**, micronuclei; **ppm**, parts per million; **QPE**, Quizalofop-P-ethyl; **Rg**, ring chromosome(s); **Sc**, stickiness; **Vg**, vagrant chromosome; **2,4-D,** 2,4-dichlorophenoxyacetic acid

INTRODUCTION

The indiscriminate use of pesticides and herbicides in agriculture justifies the evaluation of the toxicity of various chemicals/agents. Several studies have shown that chronic exposure to low to high level of pesticides can cause birth defects and that prenatal exposure is associated with carcinogenicity. Chromosomal aberrations can be accepted as an indicator of genotoxic damage induced by pesticides (Srivastava and Mishra 2009). For this purpose, the *Allium cepa* L. is one of the most frequently used higher plant species (Grant 1994). The *A. cepa* root chromosomal aberration assay for genotoxicity was introduced by Levan (1938) and has been used on pesticides in various studies (Mastrangelo *et al*. 2006; Mustafa and Arikan 2008; Srivastava and Mishra 2009; Çavuşoğlu *et al*. 2011). The *A. cepa* root chromosomal aberration assay was simple and just as reliable as the method where chromosomal aberrations were recorded in all types of mitotic cells (Rank and Nielsen 1997; Sharma *et al.* 2010). The test can be used to measure both toxicity (EC_{50}, where the treated root length are half the length of the control) and cytotoxicity. The rate of the root growth can be correlated with the mitotic index (Liu *et al*. 1992). The root chromosomal aberrations and micronucleus assays have been shown to be highly reliable in genotoxicity testing (SmakaKincl *et al*. 1996; Natarajan 2002, Abu and Mba 2011; Olorunfemi and Ehwre 2011). The micronucleus assay is traditionally performed in mice, where the bone marrow or peripheral blood is analyzed for the presence of micronuclei.

Quizalofop-P-Ethyl (QPE) is a phenoxy herbicide compound, commonly used in agriculture in controlling weeds. The indiscriminate use of herbicides in agriculture and the increase of pollution in ecosystems due to industrial development, results in the evaluation of the toxicity of these chemicals (Marcano *et al*. 2004; Elefsiniotis *et al*. 2007; Mustafa and Arikan 2008). Currently, the literature is unavailable on the cytological or genotoxical effects of QPE herbicide in plant systems or assays.

Atrazine [2-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazin-4,6-diamine] is a herbicide used for the control of weeds in agriculture. It has been classified as a restricted use pesticide due to its potential for contamination. It is slightly to moderately toxic to human and other animals. Symptoms of atrazine has been reported to cause cancer, reproductive abnormalities, poisoning include abdominal pain, diarrhea and vomiting, eye irritation, and skin reactions (Brusick 1994; Hayes *et al.* 2002; Bolle 2004). Atrazine is readily absorbed through the gastrointestinal tract.

The herbicide Avenoxan is commercial form of 2,4-dichlorophenoxyacetic acid (2,4-D) and it is widely used in agriculture. 2,4-D is a herbicide, applied mainly in low concentrations to eliminate broad leaf species, where it initiates the action of natural plant hormone indole-3-acetic acid but in high concentrations it induces chromosome abnormalities (Kallak and Javekylg 1971; Bushra *et al.* 2002). It may cause Non-Hodgking's Lymphoma and other cancers (Holland *et al.* 2002). 2,4-D causes chromosomal damage in root cells of plants (Khalatkar and Bharagava 1985).

Diuron [3-(3,4 dichlorophenyl)-1,1-dimethylurea] is a herbicide adequately used for weed control in agricultural and non-agricultural areas through soil application. It persists in soil for several years and cause toxicity to plants. These toxic compounds are absorbed through the roots and translocated to the aerial parts of the plant and induce phytotoxic effect by chlorophyll degradation (Ashton and Craft 1981; Saxena 2004).

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MATERIALS AND METHODS

Test organism

Healthy bulbs 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 destroying the root primordial with the help of sharp forcep. The experiments were maintained in laboratory conditions and the roots were protected from direct sunlight in order to minimize fluctuation of the rate of cell division.

Chemicals

The tested chemicals were Atrazine, Avenoxan, Diuron, QPE herbicides. HCl, Orcein, glacial acetic acid, and other chemicals were bought from Thomas Baker (Chemicals) Pvt. Ltd., Mumbai, India; LOBA Chemie Pvt. Ltd, Mumbai, India and SD Fine-Chem. Ltd., Mumbai, India.

Allium root growth test and determination of EC_{50}

Clean and healthy onion bulbs were allowed to produce roots in distilled water. After 2-3 days, the onion bulbs with freshly emerged roots were placed on coupling jars filled with different concentrations of each herbicide for 4 days. The root lengths from the control and experimental sets were measured (lengths of 10 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. The effective concentration (EC_{50}) value was determined as the effective concentration for 50% growth inhibition. Experiments were carried out in triplicate.

Cytogenetic parameters

Different concentrations (0.1, 0.5, 0.75 and 1 ppm) of Atrazine, Avenoxan, Diuron, QPE were used in the treatment of *Allium cepa* L. the solution were prepared in distilled water. Onion bulbs were rooted in distilled water for 24 h. The five bulbs which have approximately same root length were transferred to each test solutions. After the completion of 3 h treatment, the root tips of each bulb were cut carefully and fixed in fixative (ethanol: glacial acetic acid (3: 1, v/v)) for 24 h. After fixation, the roots were hydrolyzed in 1 part of 1N HCl for 1 min and squashed in aceto-orcein and 1N HCl (9:1) after intermittent heating for 3-5 min. After removing the root caps from well-stained root tips, they were immersed in a drop of 45% acetic acid on a clean slide, squashed under a cover slip with match stick and sealed with DPX or nail polish and examined microscopically (Nikon fluorescent microscope and camera). Ten root tip squashes were prepared for each treatment and a minimum 400 cells were examined for each concentration. The mitotic index was calculated using the method of (Mousa 1982). Chromosomal aberrations in each treatment were also recorded.

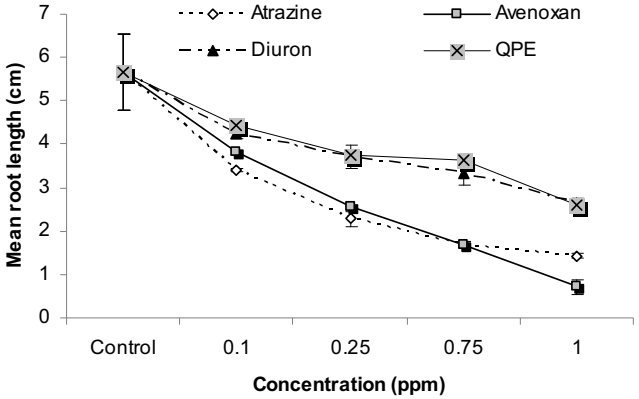

Fig. 1 Effect of different concentrations of herbicides on root length of *Allium cepa*.

Mitotic index = (No. of dividing cells/Total No. of cells) × 100

Statistical analysis

Mitotic index (MI) was calculated by scoring dividing cells. The experimental data is presented as mean ± SE of triplicate experiments. To determine the significance among the mean values, one-way ANOVA was applied followed by the *F* test and represented by an HSD value ($P < 0.05$).

RESULTS

Morphological abnormalities such as stiffness and discoloration of roots were observed in higher concentrations (i.e., 1.0 ppm) along with root growth retardation (**Fig. 1**). The root growth decreased with increasing the concentration (0.1, 0.5, 0.75 and 1.0 ppm) of herbicides. Above 1 ppm, there was no root growth during four day treatment. After fourth day treatment of growth in the control, the average length of roots was 5.66 ± 0.882 cm. Dose-response curves obtained between the concentrations of herbicides and *Allium* root growth determined the effective concentration (EC_{50}) value which retards 50% root growth as 0.5 ppm in case of Atrazine and Avenoxan and 1.0 ppm in case of Diu-

Table 1 Effect of herbicides on mitotic cell divisions of *Allium cepa* L.

Treatment	Concentration (ppm)	No. of cells observed	No. of dividing cells	Mitotic index (% ± SE)
Control (distilled water)	-	2639	718	27.21 ± 0.017
Atrazine	0.1	2648	694	26.21 ± 0.022*
	0.5	2721	536	19.69 ± 0.02*
	0.75	2784	426	15.30 ± 0.1*
	1.0	2831	314	11.09 ± 0.009*
Avenoxan	0.1	2817	618	21.94 ± 0.025*
	0.5	2618	541	20.66 ± 0.065*
	0.75	2914	468	16.06 ± 0.009*
	1.0	2516	324	12.88 ± 0.044*
Diuron	0.1	2532	511	20.18 ± 0.012*
	0.5	2917	584	20.02 ± 0.009*
	0.75	2684	432	16.09 ± 0.023*
	1.0	2748	401	14.59 ± 0.072*
QPE	0.1	2533	534	21.08 ± 0.006*
	0.5	2616	469	17.93 ± 0.038*
	0.75	2717	423	15.57 ± 0.073*
	1.0	2819	346	12.27 ± 0.036*

One-way ANOVA
Atrazine treatment: (F ratio = 21320.84*; HSD = 0.22)
Avenoxan treatment: (F ratio = 21225.51*; HSD = 0.17)
Diuron treatment: (F ratio = 19110.82*; HSD = 0.16)
QPE treatment: (F ratio = 18437.77*; HSD = 0.19)

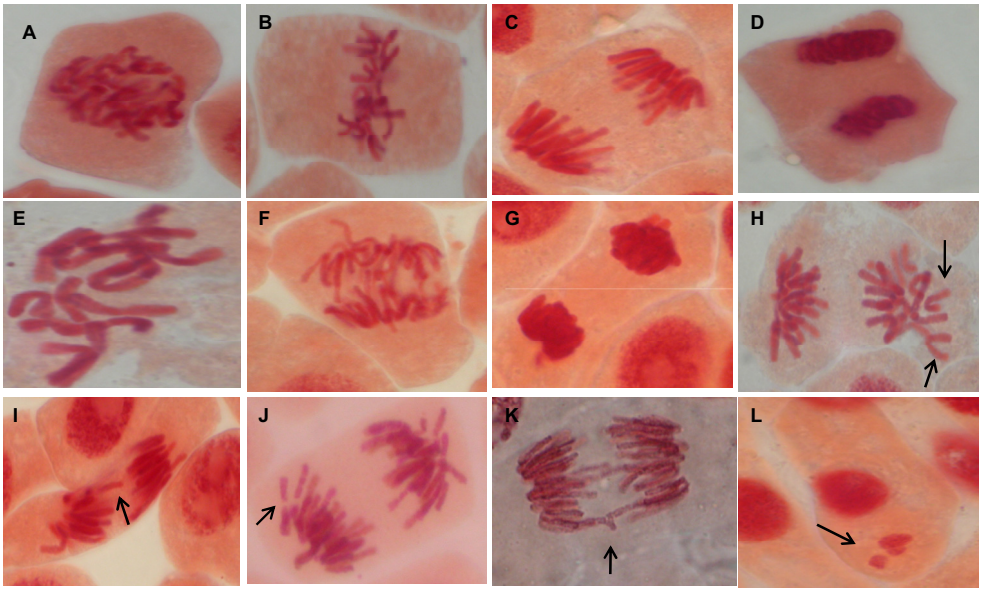

Fig. 2 Control stages of mitosis (**A-D**). (**A**) Prophase, (**B**) metaphase, (**C**) anaphase, (**D**) telophase. Disturbed stages of mitosis in root tip cells of *A. cepa* (**E-L**). C-mitosis (**E**), delayed anaphase (**F**), stickiness (**G**), vagrant (**H**), laggard (**I**), chromosomal break(s) (**J**), chromatin bridge(s) (**K**), micronuclei (**L**). Arrows indicate particularly, the specific aberration.

ron and QPE herbicides (**Fig. 1**). The root length after treatment in EC_{50} was 2.287 ± 0.162 cm (Atrazine), 2.543 ± 0.007 cm (Avenoxan), 2.633 ± 0.047 cm (Diuron) and 2.6 ± 0.16 cm (QPE).

Exposure to different concentrations (0.1 to 1.0 ppm) of herbicides significantly and dose dependently inhibited the mitotic index (**Table 1**) in the root tip cells of *Allium cepa*. The table indicates that there was an exponential relationship between the percentage of aberrations and concentrations of herbicides. There were significant differences between herbicide concentrations and the control ($P < 0.05$). Mitotic index significantly decreased in the herbicides concentrations as compared to control at each treatment. The percentage of MI was significantly low at 1 ppm compared to other concentrations. In contrast, Atrazine was found to be more toxic at a high concentration as compared to other herbicides.

Effects on chromosome aberrations and micronucleus formation

Results of genotoxicity tests with the *Allium* anaphase-telophase test were evaluated (**Table 2**). The highest herbicides concentration (1 ppm) showed high toxicity on the root cells. The changes in the organization and morphology of the chromosomes in the root tips exposed to the herbicide were observed (**Fig. 2**). Nine types of chromosomal aberrations were recorded in anaphase-telophase cells. The total mean percentages of physiological aberrations (c- mitosis, delayed anaphase, laggard(s), vagrant chromosome(s), stickiness) and clastogenic aberrations (chromatin bridge(s), break(s), ring chromosome and micronuclei) according to total cells with chromosomal aberrations were calculated as 1.39 (Control), 15.91 (Atrazine), 14.62 (Avenoxan), 20.58 (Diuron) and 22.81% (QPE), respectively. The stickiness, vagrant chromosomes and c-mitosis were the most frequently observed chromosomal aberrations, although, c-mitosis and stickiness were not observed in the control

Table 2 Results from genotoxicity testing of different herbicides in the *Allium cepa* root chromosomal aberration assay.

Treatment	Conc. (ppm)	No. of cells	Chromosomal aberrations									Total aberrations (% ± SE)
			Cm	Da	Lg	Vg	Sc	Bg	Bk	Rg	Mn	
Control	-	718	0	2	1	3	0	3	1	-	-	1.39 ± 0.012*
Atrazine	0.1	594	3	6	3	4	3	2	2	1	2	4.38 ± 0.026*
	0.5	536	9	12	6	7	6	2	4	2	2	9.33 ± 0.02*
	0.75	426	13	18	8	7	13	4	6	4	4	18.07 ± 0.075*
	1.0	314	17	21	9	9	14	7	9	8	8	31.85 ± 0.071*
Avenoxan	0.1	618	4	3	4	4	4	6	3	2	3	5.34 ± 0.026*
	0.5	541	7	4	5	6	6	7	4	3	6	8.87 ± 0.031*
	0.75	468	9	6	6	8	12	7	6	4	9	14.32 ± 0.026*
	1.0	324	18	9	8	8	16	9	7	8	14	29.94 ± 0.029*
Diuron	0.1	584	3	2	6	4	9	4	4	4	3	6.68 ± 0.023*
	0.5	511	6	3	9	12	13	7	8	8	9	14.68 ± 0.02*
	0.75	432	9	9	14	18	14	9	9	11	12	24.3 ± 0.234*
	1.0	401	14	19	16	21	21	12	16	14	14	36.66 ± 0.06*
QPE	0.1	534	4	6	5	6	8	4	5	2	4	8.24 ± 0.01*
	0.5	469	9	9	7	9	9	6	8	4	5	14.07 ± 0.094*
	0.75	423	12	18	13	18	12	9	14	6	6	27.89 ± 0.023*
	1.0	346	14	19	19	19	16	13	18	12	12	41.04 ± 0.102*

Cm: C-mitosis, Da: Delayed anaphase, Lg: Laggard chromosome(s), Vg: Vagrant chromosome(s), Sc: Stickiness, Bg: Chromatin bridge(s), Bk: Chromosomal break(s), Rg: Ring chromosome(s), Mn: Micronuclei.
One-way ANOVA
Atrazine treatment: (F ratio = 63179.83*; HSD = 0.22)
Avenoxan treatment: (F ratio = 188363.5*; HSD = 0.11)
Diuron treatment: (F ratio = 16648.68*; HSD = 0.50)
QPE treatment: (F ratio = 63795.49*; HSD = 0.29)

Table 3 Summary on use of *Allium cepa* root chromosomal aberration assay.

Agent(s) studied	Nature	Type of aberrations	References
Pharmaceutical effluents	Mixtures of toxic chemicals	RN, GI, MI, CA	Abu and Mba 2011
Cypermethrin	Insecticide	MI, CA	Cavusoglu *et al*. 2011
Contaminated soil	Industrial waste	CA	Katnoria *et al*. 2011
Squeezed *garri* extracts	*Cassava* roots	GI, CA	Olorunfemi and Ehwre 2011
Cassava effluents	Food species	GI, MI, CA	Olorunfemi *et al*. 2011
Curcumin and *Aloin*	Plant compounds	MI, CA	Palanikumar *et al*. 2011
Inula viscosa leaf extracts	Medicinal plant	MI, CA, MN	Çelik and Aslantürk 2010
Copper	Metal	GI, CA	Geremias *et al*. 2010
Urginea maritima (L.)	Bio-pesticides	MI, CA	Metin and Bürün 2010
Surface and wastewater	Complex environmental mixtures	GI, MI, CA	Radic *et al*. 2010
Nodularia moravica	Microalgae	GI, MI, CA	Staykova *et al*. 2010
Aqueous extract of bitter leaf	Medicinal plant	CA	Adegbite and Sanyaolu 2009
Coal fly ash	Mixture of chemicals	GI, MI, BN	Chakraborty *et al*. 2009
Cherry fruit	Fruit	MI, CA	Ranceliene *et al*. 2009
Chlorpyrifos, α-thrin, Efekto virikop and Springbok	Pesticides	MI, CA	Asita and Makhalemele 2008
Nano-silver	Anti-bacterial	MI, CA, MN	Babu *et al*. 2008
Magnesium sulphate	Fertilizers	CA	Bhatta and Sakya 2008
Maleic hydrazide	Metal	CA	Jabee *et al*. 2008
Petroleum hydrocarbon	Complex chemical mixture	MN, PN, CA	Leme *et al*. 2008
Quizalofop-P-ethyl	Herbicide	CA, MN	Mustafa and Arikan 2008

BN, binucleate; **CA**, chromosomal aberration(s); **GI**, growth inhibition; **MN**, micronuclei; **MI**, mitotic index; **PN**, polynucleate; **RN**, root number

treatment. The total chromosomal aberrations increased with an increase in the herbicides concentrations.

The total mean of chromosomal aberrations (%) were significantly higher at the highest concentration (1 ppm) of QPE and Diuron herbicides than the other herbicides concentrations. Micronucleated cells were observed at interphase (**Fig. 2**). The induction of micronucleus formation was generally observed in all treatments. Micronucleus formation was markedly higher at the concentrations of Diuron than the other herbicide concentrations in all the treatments (**Table 2**).

DISCUSSION

Pesticides applied either to soil or crop plants are subjected to volatilization, leaching, which induce chromosomal aberrations, chemical modification and microbial degradation, which have higher risk of bringing about environmental pollution (Pandey 2007). The application of pesticides to the root meristems of *A. cepa* L. have revealed decrease in mitotic index with increasing concentrations of Atrazine, Avenoxan, Diuron and QPE. Similar effects on mitotic index were observed by many researchers following the treatment of *A. cepa* roots with insecticides like cypermethrin and fenvalerate (Chauhan *et al.* 1999), ceresan, agrosan and mercuric chloride fungicides (Nandi 1985) and pentachlorophenol, 2,4-D, butachlor (Ateeq *et al*. 2002), racer flurochloridone (Yuzbasioglu *et al*. 2003) and maleic hydrazide (Marcano *et al*. 2004), all herbicides. Plants are used for evaluation of environmental pollutants such as pesticides because they are direct recipients of agrotoxics, so they are important material for genetic test and for environmental monitoring of pollutants affected places (**Table 3**). The higher plants like *A. cepa*, *Tradescantia paludosa* and *Vicia faba* have large monocentric chromosomes in reduced numbers and are accepted as suitable test organisms for the study of environmental mutagenesis (Rank and Nielsen 1998; Grover and Kaur 1999; Moraes and Jordão 2001; Patra and Sharma 2002). Exposure of Atrazine (2000 mg/Kg) in mice through oral gavage has been demonstrated to induce lethal mutations and chromatid breaks in mice (Adler 1980). Ventura (2008) analyzed DNA damage by comet assay in Atrazine treated cells revealed mild effect on DNA at extremely high concentrations (6.25, 12.5 and 25 μg/L). Meisner *et al*. (1992) generated data from mammalian *in vivo* and *in vitro* test models show that higher doses (20 ppm) of Atrazine beyond the environmental concentrations can induce genotoxicity. Electron microscopic studies revealed that the Diuron treatment reduces the number of cortical microtubules in interphase and metaphase cells (Chauhan *et al*. 1998). The impairment of spindles function is due to the affinity of reactivity of Diuron with the tubulin-SH group (Kuriyana and Sakai 1974). In earlier studies concentrations of Diuron produced significant frequencies of aberrations in *A. sativum* and *A. cepa* root meristem cells (Chauhan 1989; Chauhan *et al*. 1998). Significant reduction in MI, noted in the present study may be due to the mitodepressive action of the chemicals indicating thereby the herbicides used interfere in the normal cell cycle resulting in decrease in number of dividing cells. Similar results had also been reported by (Badr 1986; Sadia and Vahidy 1994; Inceer *et al*. 2003; Yuzbasioglu *et al*. 2003) on various crop plants. The inhibition of specific proteins of cell cycle remains as possible herbicides target site which inhibit DNA polymerase and other enzymes resulting in antimitotic effect (Hidalgo *et al*. 1989). The mutagenic effects observed in cells treated with the herbicides included c-mitosis, stickiness, chromosomal bridge(s), lagging chromosomes or chromosomal breaks at anaphase and telophase, multipolar anaphases and telophases. These results are in agreement with *A. cepa* root tips with different herbicides, such as Sencorer and Gasegard (Haliem 1990; Mousa 1982), Cyanazine (Papes *et al*. 1989), Carbetamex and Paradone Plus (Badr 1983). Cytotoxic effects of the herbicides used in the present case were in conformity with that of (Saxena *et al*. 2004; Gul *et al*. 2006; Mustafa and Arikan 2008) with the treatment of toxic herbicides. Chemical abnormalities such as c-mitosis and lagging chromosome(s) were the result of disturbance in the spindle fiber formation caused by the pesticides or herbicides (Haliem 1990). Stickiness is induced either by the effect of herbicides on chromosomal protein attributed to the improper folding of chromosome fibers or may be due to the action of herbicides on the polymerization process, resulting in the fragmentation of chromosomes and bridges forms sticky chromosomes (El-Ghamery *et al*. 2000). Rank and Nielson in 1997 have reported that the chromosome bridges and fragments lead to structural changes in chromosomes of crop plants and in other organisms in the environment. Hence the higher concentrations of Atrazine, Avenoxan, Diuron and QPE may become genotoxic, chromotoxic and clastogenic in crop plants, therefore, its higher concentration is not suggestive. From the foregoing discussion, it is obvious that herbicides present in the environment can be absorbed by the plants, which may adversely affect the genetic systems, causing damage to chromosomes in crop plants and other organisms. Regular application of pesticides in agricultural practices is a potential threat to genetic constitution of crop plants and

animals. Therefore, judicial application of chemical pesticides is essential. Indiscriminate use of pesticides or herbicides should be discouraged as far as practicable.

CONCLUSION

The present study has demonstrated the usefulness of the *A. cepa* root chromosomal aberration assay in assessing the genotoxicity of environmental chemicals, even if they are not pure products.

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