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Full Length Research Paper

Genotoxicity of Chlorpyrifos, Alpha-thrin, Efekto virikop and Springbok to onion root tip cells

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The pesticides, chlorpyrifos, Alpha-thrin, Efekto virikop and springbok were assessed for cytotoxicity and genotoxicity in the onion root tip assay. Onion seeds were germinated on moistened filter paper in petri dish at room temperature until radicles appeared. Germinated seeds were exposed to three concentrations of each pesticide for 20 h. About 1 – 2 mm length of root tip was cut, fixed in acetic alcohol, washed in ice cold water, hydrolyzed in warm 1 N HCl, stained with aceto-carmine and squashed on glass slide. For each treatment, about 3000 cells were scored and classified into interphase and normal or aberrant division stage. Cytotoxicity was determined by comparing the mitotic index (MI) of treated cells with that of the negative control. The MI of cells treated with chlorpyrifos, Alpha-thrin or springbok was half or less, that of the control at one or more doses and adjudged cytotoxic. Efekto virikop was not cytotoxic. Genotoxicity was measured by comparing the number of cells/1000 in aberrant division stages at each dose with the negative control using the Mann-Whitney test. Chlorpyrifos was genotoxic ($P < 0.05$), inducing chromosome lagging and bridges, pulverized and stick chromosomes, multipolar anaphase and telophase. Efekto virikop and springbok induced lagging chromosomes. Alpha-thrin was not genotoxic.

Key words: ***Allium cepa***, cytotoxicity, genotoxicity, mitotic index, pesticides, root tip cells.

INTRODUCTION

The use of pesticides has greatly improved agricultural yield through inhibition of disease causing organisms and by acting against pest in the fields and during storage of agricultural products (Taylor et al., 1997). Without their use, epidemic and endemic famine, soil erosion and immunocompromised animals are a distinct possibility (Beyersmann et al., 1994). However, pesticides, in addition to their intended effects, are sometimes found to affect non-target organisms, including humans (Chantelli-Forti et al., 1993; Chaudhuri et al., 1999). The mutagenic and carcinogenic action of herbicides, insecticides and fungicides on experimental animals is well known and several studies have shown that chronic exposure to low levels of pesticides can cause mutations and/ or carcinogenicity (IARC, 1990, 1991; Bull et al., 2006; Karabay and Gunnehir, 2005).

Pesticide residues can be present in fruit and vegetables and represent a risk for human health. Several studies have shown that chronic exposure to low levels of pesticides can cause birth defects and that prenatal exposure is associated with carcinogenicity (Feretti et al., 2007).

Genotoxicity and mutagenicity of pesticides for non-target organisms and their influence on ecosystems are of worldwide concern (Pimentel et al., 1998). Over the past decade, issues of animal use and care in toxicology research and testing have become one of the fundamental concerns for both science and ethics. Emphasis has been given to the use of alternatives to mammals in testing, research and education (Mukhopadhyay et al., 2004).

The ***Allium cepa*** assay is an efficient test for chemical screening and in situ monitoring for genotoxicity of environmental contaminants and has been widely used to study genotoxicity of many pesticides revealing that these compounds can induce chromosomal aberrations in root meristems of ***A. cepa*** (Thais et al., 2007; Cabrera et al., 1994). Because of the potential environmental impact connected with the introduction and heavy use of pesti-

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cides, we have assessed the pesticides, chlorpyrifos, Alpha-thrin, efekto virikop and springbok for inhibition of cell division (toxicity) and genotoxicity in the ***A. cepa*** chromosome aberration assay to add to the data on ***in vitro*** and ***in vivo*** genotoxic studies that are necessary to assess the possible health risks associated with the extensive use of pesticides.

Chlorpyrifos is an emulsifiable broad-spectrum Organophosphate contact and stomach poison with a long residual action for the control of flies, mosquitoes, cockroaches, bedbugs and ants on a wide variety of crop types (EPA, 1984). The acute systemic toxicity of organophosphates reflects cholinergic hyperstimulation due to inhibition of cholinesterase; in the case of chlorpyrifos, this action is produced by its active metabolite chlorpyrifos oxon (Gennady et al., 2001). It is not known whether chlorpyrifos can cause cancer in humans. Animal studies have not shown that chlorpyrifos causes cancer (ATSDR, 1997; Yano et al., 2000).

Alpha-thrin 100 SC is a pyrethroid suspension contact and stomach insecticide, the active ingredient being alpha-cypermethrin (Kegley et al., 2007). Cypermethrin use caused significant increases in chromosome aberration (CA) and micronucleated (MN) erythrocytes frequency in farm workers (Carbonell et al., 1995; Lander et al., 2000). DNA damage was detected in tissue of workers involved in the production of cypermethrin (Grover et al., 2003). A dose-related increase in sister chromatid exchanges and inhibition of mitotic cell division was observed in the bone marrow cells of 3-month-old mice that were injected subcutaneously with 0.75, 1.5, or 3 mg/kg body weight of Cypermethrin (Seehy et al., 1983). Cypermethrin has been classified as a possible human carcinogen/evidence of non-carcinogenicity for humans (EPA, 2002).

Efekto virikop with the active ingredient as copper oxychloride is a wettable powder fungicide and bactericide with protective properties for the control of certain diseases on fruit, vegetables, flowers and ornamentals. Copper oxychloride was found to cause chromosomal aberration in the Italian population exposed to it while working in the fields (De Ferrari et al., 1991). Copper oxychloride was not classified as a human carcinogen (EPA, 2002).

Springbok with the active ingredient as glyphosate is a soluble concentrate non-selective foliar, systemic herbicide for the control of a wide range of annual and perennial grasses, broadleaf weeds and certain woody perennials. Glyphosate kills plants by inhibiting the plant enzyme enolpyruvylshikimate-phosphate synthase (EPSPS), required for synthesis of essential amino acids required for growth (Kegley et al., 2007). An international panel of toxicology experts concluded that “multiple lifetime feeding studies have failed to demonstrate any tumorigenic potential for glyophosate. Accordingly it was concluded that glyophosphate is noncarcinogen” (Williams et al., 2000).

MATERIALS AND METHODS

Onion seeds

Varieties of Texas Grano 502 P.R.R. packed by Starke Ayres (Pty) Ltd at Gauteng, South Africa, were purchased from Maseru garden centre, Lesotho, Southern Africa.

Alpha-thrin is a product of Villa Crop Protection Company; chlorpyrifos and springbok are products of Agro-Serve (Pty) Ltd; Efekto virikop is a product of Manro Chemicals. Their concentrations were as follows: Chlorpyrifos (480 g/L); Alpha-thrin 100 SC (alpha-cypermethrin 100 g/L); Efekto virikop (copper oxychloride 850 g/kg); Springbok (glycine 360 g/l and as glyphosate isopropylamine salt 480 g/l).

Ethanol (absolute) is a product of Associated Chemical Enterprises (PTY) LTD of The Republic of South Africa; hydrochloric acid and glacial acetic acid are products of UNILAB of The Republic of South Africa; methyl methane-sulfonate (MMS, 99%) CAS 66-27-3 is a product of ALDRICH, USA; aceto-carmine stain CAS number 84-1423 is from Carolina Biological Supply Company, USA.

Preliminary dose selection experiment

Preliminary dose selection experiment was conducted for each chemical with concentration ranges between ten times above and below the manufacturers recommended dose. However, in cases where no inhibition of germination was observed, higher doses were tested.

For each test, 50 onion seeds were spread on a filter paper moistened with a specific concentration of the pesticide in a petri dish and kept at room temperature to germinate, about 3 days. The number of seeds that produced a radicle were recorded at the end of the three days and compared to the number of seeds that germinated in the concurrent water treated control to derive the percentage germinating at each concentration. The LC50 for each pesticide was determined from the curve of percentage of seeds that germinated against dose.

Genotoxicity assay

The method used was similar to the method of Matsumoto et al. (2006). ***A. cepa*** (onion) seeds were germinated in petri dishes containing pesticide-soaked filter paper (test), water-soaked filter paper (negative control) or on filter paper soaked in aqueous solution of 1% methyl methanesulfonate (positive control). In this project, a discontinuous treatment protocol was used. Seeds were first soaked in distilled water until the radicles reached a length of about 2 cm. Germinated seeds were transferred to petri plates containing chemicals at different doses in which they were left for 20 h (acute treatment) at room temperature. At the end of the 20 h exposure, some seeds were collected at random and assessed. The concentrations of each pesticide tested were; ¼ LC50, ½ LC50 and LC50, as determined in the preliminary dose selection experiments.

Root harvest and slide preparation

Root tips 1 - 2 cm long were cut and placed in a watch glass and fixed in acetic alcohol (ethanol : glacial acetic acid in 3:1 ratio) at 4 - 6^{o}C for 3 h. The root tips were washed twice with ice cold water for 10 min each and allowed to dry. A solution of 1 N HCl pre-heated to 60^{o}C was added to the root tips in the watch glass for 10 min and the HCl was discarded. The HCl treatment was repeated a second time. Two root tips were transferred to a clean microscope slide and were cut 2 mm from the growing tip. The tips were kept and the rest was discarded. Aceto-carmine stain was added to the watch glass to

cover the root tips for 2 - 3 min. A cover glass was placed on the root tip and the root tip spread evenly to form a monolayer by gently tapping the cover glass with a pencil eraser to facilitate the scoring process for normal and aberrant cells in the different stages of the cell cycle.

Scoring of slides

The slides were viewed under the light microscope (Olympus CX 21) using the 100X objective lens with oil immersion. A total of 3000 cells were scored on each slide. The cells were recorded as normal or aberrant in the different stages of the cell cycle namely: interphase, prophase, metaphase, anaphase or telophase. All cells with aberrations were counted and the most representative ones for each abnormality were photographed using a Zeiss PrimoStar microscope mounted with Canon camera model, Power Shot A640.

Data analysis

Cytotoxicity determination

Cytotoxicity was determined by the mitotic index method. The mitotic index (MI) was calculated as the number of cells containing visible chromosomes (i.e. cells in the division stages) divided by the total number of cells scored.

The mitotic indices of the treated cells at each dose were compared with that of the negative control group. Any dose of a test substance was adjudged to be cytotoxic if the mitotic index of treated cells was half or less, compared to the mitotic index of the concurrent water treated cells.

Genotoxicity determination

Dividing cells with any of the under listed abnormalities were recorded, namely; C-metaphase (no spindle fibres), pulverized chromosomes, stick chromosomes, chromosome bridges, lagging chromosomes or chromosome fragments at anaphase and telophase, multipolar anaphases and telophases.

The number of aberrant cells /1000 cells in each of the four division stages for pesticides treated cells were compared with the numbers in the aberrant division stages for the water treated (negative control) cells by the Mann-Whitney U test using the SPSS 10.0 for Windows statistical package. The calculated U value for each comparison (pesticide and negative control) was obtained. If the calculated U value was less than the critical value from the table at the appropriate degrees of freedom (in our own case, $n_1 = 4$ and $n_2 = 4$) at the 0.05 probability then a statistically significant difference existed between the medians and the pesticide was adjudged to be genotoxic at the dose of the pesticide.

RESULTS

Cytotoxicity of the pesticides

The results of the cytotoxicity determination are presented in Table 1. Cells treated with Alpha-thrin or springbok had reduced mitotic indices compared with cells treated with water which was indicative of inhibition of cell division by these pesticides. Alpha-thrin and springbok were therefore adjudged to be cytotoxic at all doses tested. The cytotoxicity of springbok increased with dose. The highest dose of Alpha-thrin was the most cytotoxic. Chlorpyrifos was very cytotoxic at the lowest concentration. Efekto virikop did not demonstrate any cytotoxicity to the onion root cells at any of the doses that was tested. The positive control chemical, methyl methane sulphonate at 1% concentration in water did not inhibit mitotic cell division of the onion root tip cells.

Genotoxicity of the pesticides

The mutagenic effects of the pesticides as, determined by comparing the number of aberrant cells in division stages for each dose of each pesticide with that of the concurrent control in the Mann-Whitney U test are presented in Table 2.

Significant differences were detected in the frequency of aberrant division stages when cells treated with Chlorpyrifos, Efekto virikop or springbok were compared with the water (negative control) treated group, at one or two doses ($P < 0.05$). The genotoxic effect increased with dose of each pesticide. The positive control chemical, methyl methane sulphonate, was genotoxic. Alpha-thrin was not genotoxic at any of the doses of the pesticide that was tested.

The types of aberrations observed in cells treated with the different pesticides are presented in Table 3. Chlorpyrifos induced the most types of mutation in treated cells.

The pictures of the different types of genotoxic effects of the pesticides on ***A. cepa*** cells observed on glass slides of squashes of root tips are presented in Figures 1 - 7.

DISCUSSION

Cabrera et al. (1994) have also validated the use of plants for evaluation of environmental pollutants such as pesticides because plants are direct recipients of agrotoxics, so they are important material for genetic test and for environmental monitoring of places affected by such pollutants. The higher plants ***A. cepa*** (onion), ***Tradescantia paludosa*** and ***Vicia faba*** have relatively 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; Kong and Ma, 1999; Moraes and Jordão, 2001; Patra and Sharma, 2002).

The mutagenic effects observed in cells treated with the pesticides included C-metaphase (no spindle fibres), pulverized chromosomes, stick chromosomes, chromosome bridges, lagging chromosomes or chromosome fragments at anaphase and telophase, multipolar anaphases and telophases.

A 1% solution of the positive control chemical, methylmethanesulfonate (MMS) was genotoxic but not cytotoxic to the onion root tip cells in the present study. This agreed with results of Rank and Nielsen (1998).

The broad spectrum Organo-phosphate insecticide,

Table 1. Cytotoxicity of pesticides to onion root tip cells.

Treatment	Conc. (%)	Number of cells in the different stages/1000 cells scored						MI	MI Control/ MI test
			Division stages						
		INTERP	PROP	METAP	ANAP	TELOP	TOTAL		
Water	100	814.77	66.51	64.52	34.25	19.95	185.23	0.185	1.00
MMS	1.000	764.84	111.12	49.42	42.45	32.17	235.16	0.235	0.79
CPF	0.017	991.01	1.66	2.33	2.66	2.33	8.98	0.009	20.61*
	0.034	865.00	24.97	28.35	45.56	36.11	134.99	0.135	1.37
	0.068	845.90	34.51	30.82	48.92	39.87	154.12	0.154	1.20
ALP-T	0.183	954.07	25.30	7.65	5.99	6.99	45.93	0.046	4.03*
	0.365	944.26	13.52	15.50	7.59	19.13	55.74	0.056	3.32*
	0.730	966.17	11.17	7.56	8.86	6.24	33.83	0.034	5.48*
EFV	1.250	876.08	99.67	7.64	7.64	8.97	123.92	0.124	1.49
	2.500	880.03	103.36	1.66	8.31	6.65	119.98	0.120	1.54
	5.000	886.38	100.33	6.64	2.32	4.32	113.61	0.114	1.63
SPR	0.036	917.22	38.56	20.94	12.96	10.31	82.77	0.083	2.24*
	0.073	926.87	32.58	19.28	12.64	8.65	73.15	0.073	2.53*
	0.146	946.54	24.90	13.28	9.30	5.97	53.45	0.053	3.47*

CPF = Chlorpyrifos; ALP-T = Alpha-thrin; EFV = Efekto virikop; SPR = Springbok; Con. = Concentration; MI = Mitotic Index; INTERP = Interphase; PROP = Prophase; METP = Metaphase; ANAP = Anaphase; TELOP = Telophase; * = Cytotoxic (MI control: MI test ≥ 2).

Table 2. Mutagenic potencies of the pesticides to onion root tip cells.

Treatment	Conc. (%)	Number of cells in the different division stages/1000 cells scored										M-W U Values (calculated)
		PROP		METAP		ANAP		TELOP		TOTAL		
		N	ABN	N	ABN	N	ABN	N	ABN	N	ABN	
Water	100	66.51	0.00	64.52	0.00	34.25	0.00	19.95	0.00	1000.0	0.00	8
MMS	1.000	90.22	20.90	33.17	16.25	30.51	11.94	26.20	5.97	914.43	85.57	0†
CPF	0.017	0.00	1.66	1.66	0.67	1.66	1.00	2.33	0.00	990.68	9.32	2
	0.034	23.62	1.35	23.96	4.39	40.16	5.40	35.10	1.01	968.28	31.72	0 †
	0.068	21.78	12.73	27.47	3.35	31.83	17.09	34.17	5.70	956.11	43.89	0 †
ALP-T	0.183	20.97	4.33	5.32	2.33	5.99	0.00	6.99	0.00	984.69	15.31	4
	0.365	13.52	0.00	13.85	1.65	6.27	1.32	18.80	0.33	996.70	3.30	2
	0.730	11.17	0.00	6.90	0.66	3.61	5.25	3.94	2.30	991.79	8.21	2
EFV	1.250	87.71	11.96	7.64	0.00	7.31	0.33	8.97	0.00	984.05	15.95	4
	2.500	90.73	12.63	1.66	0.00	5.98	2.33	6.65	0.00	980.72	19.28	4
	5.000	87.04	13.29	3.65	2.99	1.66	0.66	2.99	1.33	974.75	25.25	0 †
SPR	0.036	38.56	0.00	19.28	1.66	11.30	1.66	8.98	1.33	987.03	12.97	2
	0.073	32.58	0.00	16.29	2.99	8.98	3.66	6.32	2.33	978.06	21.94	2
	0.146	24.24	0.66	7.64	5.64	6.31	2.99	2.32	3.65	971.45	28.55	0 †

CPF = Chlorpyrifos; ALP-T = Alpha-thrin; EFV = Efekto virikop; SPR = Springbok; N = Normal cell; ABN = Abnormal cell; Con. = Concentration; M-W = Mann-Whitney; PROP = Prophase; METP = Metaphase; ANAP = Anaphase; TELOP = Telophase; † = Mutagenic (U ≤ 0; P < 0.05, Mann-Whitney U – test)

Chlorpyrifos induced all the different types of damages to the cell division apparatus at 0.068% and 0.034% (680 and 340 ppm) concentrations but not at the lower concentration of 0.017% (170 ppm). The wide range of genotixic effects induced by chlorpyrifos is indicative of the wide range of targets in the cell that are susceptible to its effects including, spindle fibres, chromosomes, kinetochore, centriols and enzymes. Gennady et al. (2001) also found chlorpyrifos to be toxic to sea urchin embryo and larvae at higher concentration (> 600 ppm). Chlorpyrifos damaged DNA and induced micronuclei in fish (Ali et al., 2008). However, Chlorpyrifos was not mutagenic in the reverse mutation assay with ***Salmonella typhimurium*** TA98, TA100, TA1535, TA1537, TA1538

Table 3. Types of mutation induced by the different pesticides.

Pesticide	Mutation induced
CPF	Pulverized chromosomes, Stick chromosomes, decondensed chromosomes, Multipolar anaphases and telophases, chromosome lagging, chromosome fragments, chromosome bridge,
ALP-T	Nill
EFV	Chromosomes lagging
SPR	Chromosome lagging

CPF = Chlorpyrifos; ALP-T = Alpha-thrin; EFV = Efekto virikop; SPR = Springbok.

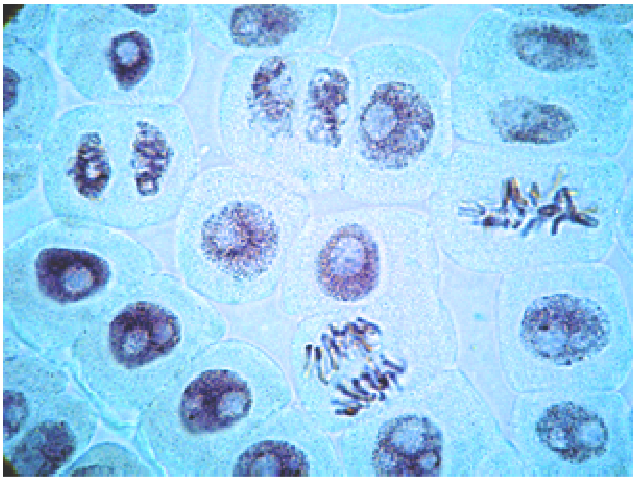

Figure 1. ***A. cepa*** root tip cells not treated with pesticide showing normal metaphase cell (A) and normal anaphase cell (B).

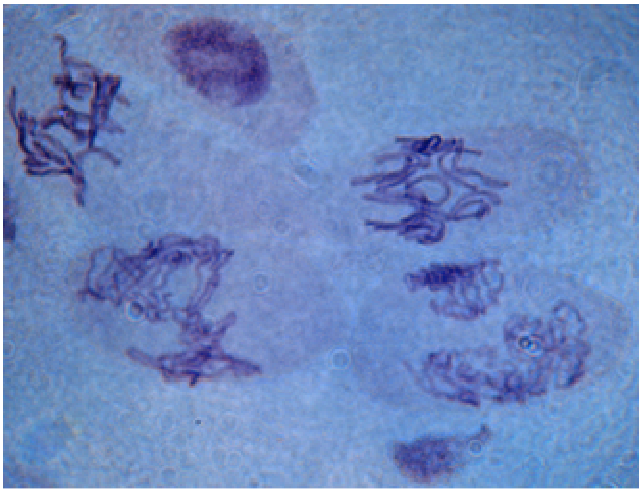

Figure 3. ***A. cepa*** root tip cells treated with chlorpyrifos showing cells with stick chromosomes.

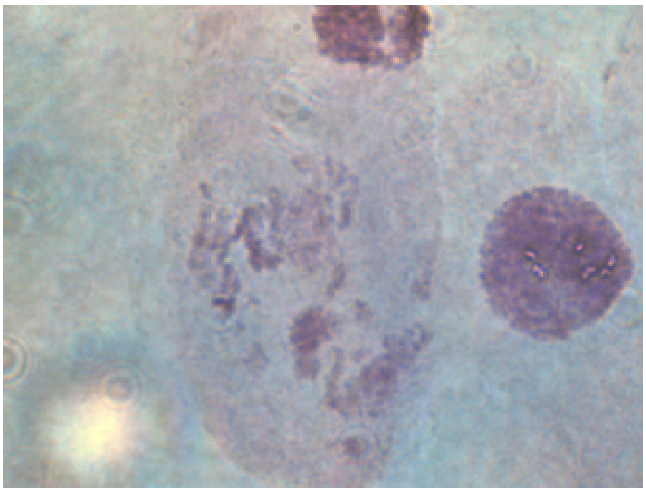

Figure 2. ***A. cepa*** root tip cells treated with pesticide showing cell with pulverized chromosomes.

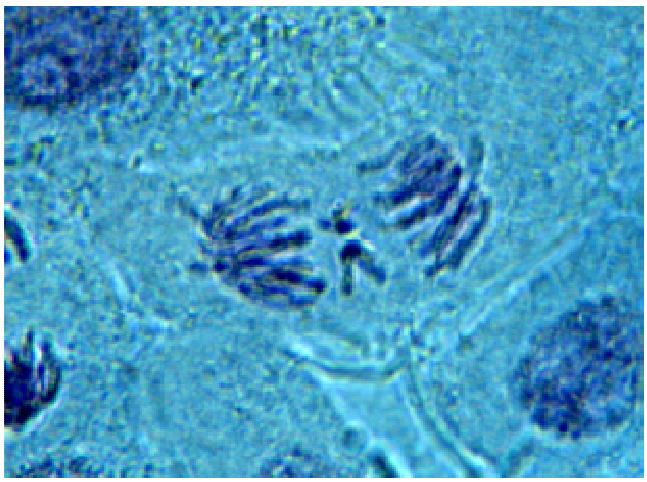

Figure 4. ***A. cepa*** root tip cells treated with pesticide showing a cell at anaphase with lagging chromosomes.

and in the forward mutation of the hprt locus of Chinese hamster ovary cells (Gollapudi et al., 1995).

The pyrethyroid contact and stomach insecticide, alpha-thrin was cytotoxic but not genotoxic at 0.730, 0.365 and 0.183% in the present study with onion root tip cells. Seehy et al. (1983) found that in mice, both technical and formulated products of alpha cypermethrin showed a dose dependent sister chromatid exchanges in

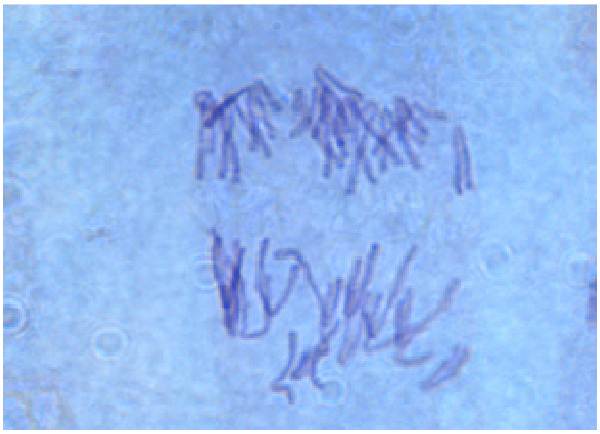

Figure 5. ***A. cepa*** root tip cells treated with pesticide showing a cell at anaphase with multipolar spindle.

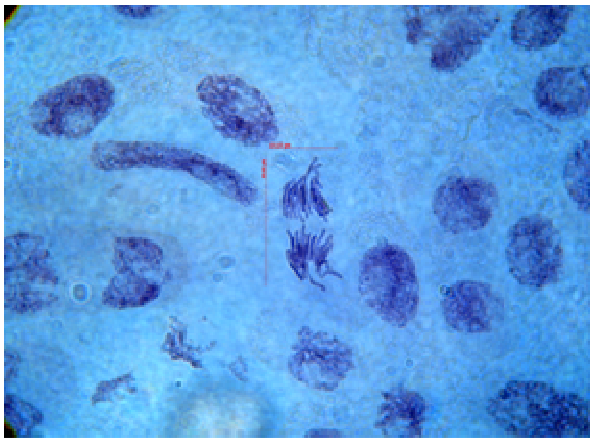

Figure 6. ***A. cepa*** root tip cells treated with pesticide showing a cell at anaphase with chromosome bridge.

dividing cells at all dose levels but the highest doses inhibited mitotic division. Cypermethrin dissolved in dimethyl sulphoxide DMSO and added to standard ***Drosophila*** food induced a significant dose-dependent increase in DNA damage in the cells of brain ganglia and anterior midgut of ***D. melanogaster*** (Mukhopadhyay et al., 2004). Cypermethrin and Alphamethrin elicited varying degrees of cytotoxic, turbagenic (toxicity to spindle) and clastogenic effects but generally more turbagenic and weak clastogenic (Rao et al., 2005).

This pesticide's cytotoxicity might have masked its potential genotoxicity in the onion root cells used in this assay.

The powder fungicide and bactericide, efekto virikop was not cytotoxic at any of the doses tested but was genotoxic at the highest dose of 5%. In a study of the long term effects of copper oxychloride, DeFerrari et al. (1991) found that it caused chromosomal aberration in the Italian population exposed to it while working in the fields.

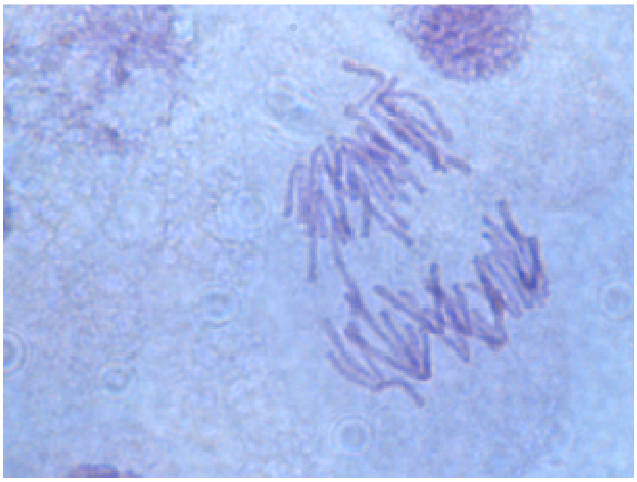

Figure 7. ***A. cepa*** root tip cells treated with pesticide showing a multipolar cell with chromosome bridge and fragment.

The non-selective foliar, systemic herbicide, springbok (active ingredient is glyphosphate) was cytotoxic at all the doses tested but exhibited a genotoxic effect at 0.146% only. Glyphosate alone has rarely caused genetic damage in laboratory tests, including studies of mutations in hamster ovary cells, bacteria, and mouse bone marrow cells (EPA, 1993). Glyphosate was also not mutagenic in other studies of rats, mice, (WHO, 1994) and onion cells (Rank et al., 1993). The absence of mutagenic effect of glyphosphate in bacterial tests was attributed to the fact that the shikimic acid pathway enzymes that are essential for growth and survival of most plants but inhibited by glyphosate is not very important in bacteria (Pluymen et al., 1984; Kegley et al. 2007). In one study in 1997, however, glyophosphate was genotoxic in tests with tadpole (Clements et al., 1997). Glyophosphate products such as Roundup demonstrated mutagenicity. In ***Salmonella*** bacteria, Roundup was weakly mutagenic at two concentrations. In onion root cells, Roundup caused an increase in chromosome aberrations, also at two concentrations (Rank et al., 1993). These results are in agreement with our findings because springbok is a glyophosphate product.

Conclusion

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

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