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Full Length Research Paper

Genotoxicity of hormoban and seven other pesticides to onion root tip meristematic cells

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Plants are direct recipients of agro-toxics and therefore important materials for assessing environmental chemicals for genotoxicity. Three doses, representing ¼, ½ and EC_{50} of hormoban, storm killer, villa, fungi-nil, bexadust, aphicide, karbadust and basagran were assessed for cytotoxic and genotoxic effects to onion root tip cells in the root tip chromosome aberration assay after 24 h exposure. Cytotoxicity was inferred when the Mitotic index (dividing cells/1000 scored) of treated cells was ≤ ½ negative control. All the pesticides were toxic. Genotoxicity was measured by analyzing 30 to 100 anaphase-telophase cells per dose of chemical for, chromosome fragments, bridges, vagrant chromosome, c-anaphase, multipolarity and stick chromosomes and comparing the percentage of aberrant cells at each dose with that of the negative control using the Chi-squared test. With the exception of basagran, the pesticides were genotoxic ($P < 0.05$). The C-anaphase and Stick chromosomes types of aberrations predominated which was evidence of the action of the pesticides on the mitotic spindle and the coiling of chromosomes during anaphase to telophase.

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

INTRODUCTION

The use of pesticides in modern agriculture has greatly improved 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; Mackenzie et al., 1998).

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; Yu, 2005; Bull et al., 2006).

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). Pesticides residues are known to persist in soil water and food and have posed problems all over the world (Subbarao, 1999).

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). Plant genotoxicity assays are relatively inexpensive, fast, give reliable results and chemicals which cause chromosomal aberration (CA) in plant cells also produce CA in cultured animal cells that are frequently identical (Grant, 1978; Ma et al., 1994).

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* (Ma et al., 1994; Fernandes et al., 2007).

In the present study, seven pesticides were assessed for inhibition of cell division (toxicity) and genotoxicity in the *A. cepa* root tip chromosome aberration assay

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Abbreviations: MI, Mitotic index; **CA,** chromosomal aberrations; **EC_{50},** half maximal effective concentration.

namely: homorban, storm killer, villa, fungi-nil, bexadust, aphicide, karbadust and basagran.

Hormoban contains both dicamba (3,6-dichloro-2-methoxybenzoic acid) and MCPA (4-chloro-2-methyl-phenoxy) acetic acid as active ingredients. Dicamba was classified as slightly toxic and not a potent human carcinogen (Extension Toxicology Network (EXTOXNET), 1996). There has been no demonstration of carcino-genicity by MCPA (Walker and Lawrence, 1992). MCPA works by concentrating in the actively growing regions of a plant (meristematic tissue) where it interferes with protein synthesis, cell division and ulti-mately the growth of the plant (EXTOXNET, 1993).

Storm killer is a rodenticide, highly active anticoagulant containing the active ingredient - flucoumafen (Pribilla, 1966) and a human taste deterrent (bitrex).

Villa is a liquid insecticide with the active ingredient as the synthetic pyrethroid, alpha-cypermethrin. Evidence for the carcinogenic potential of cypermethrin has not been demonstrated (van Heemstra-Lequin and van Esch, 1992).

Fungi-nil is a fungicide with the active ingredient as chlorothalonil. Chlorothalonil acts primarily as a fungicide and mildewicide, but also has some activity as a bac-tericide, microbiocide, algaecide, insecticide and acaricide. It is a broad spectrum, non-systemic pesticide (US EPA, 1999). Chlorothalonil has been classified as a likely human carcinogen (US EPA, 1999) and a carcinogen (Kegley et al., 2009).

Bexadust (Gamma-HCH/Lindane) is an organochlorine insecticide that while banned in many countries is still used in some countries (Li, 1999; Breivik et al., 1999). Life-time feeding studies in mice revealed that lindane increases hepatocellular tumors (IARC, 1987).

Aphicide is a systemic emulsifiable concentrated insec-ticide containing dimethoate as the active ingredient (EPA, 2007). Mice treated with dimethoate developed carcinomas in the adrenal, thyroid and pituitary glands (Nehez, 1983). Dimethoate induced significant development of neoplasms in treated rats (Degraeve et al., 1983).

Karbadust is another trade name of Carbaryl (alongside with adios, servin and dicarbam). Carbaryl is a member of the n-methylcarbamate class of pesticides and can cause cholinesterase inhibition in humans. Carbaryl is classified as a likely human carcinogen based on vascular tumors in mice (US EPA, 2004).

Basagran is an herbicide containing bentazon (3-isopropyl-1H-benzo-2,1,3-thiadiazin-4-one-2,2-dioxide) (Kegley et al., 2008). Available studies on human exposures have not shown any evidence of a carcino-genic response (U.S. EPA, 1998).

MATERIALS AND METHODS

Onion seeds: variety of Texas Grano 502 P.R.R. Product of Sakata seeds Lanseria 1748, Republic of South Africa, were purchased from Maseru garden centre, Lesotho, Southern Africa.

Pesticides: All the eight pesticides namely, Hormoban [(3,6 - dichloro-2-methoxybenzoic acid, 100 g/l; (4-chloro-2-methylphenoxy) acetic acid (MCPA), 250 g/l], Storm killer (flocoumafen, 0.05 g/kg), Villa (Alpha-cypermethrin, 100 g/l), Fungi-nil (Dicarboximide, 500 g/kg), Bexadust (Gamma BHC, 6 g/Kg), Aphicide (Dimethoate, 400 g/l), Karbadust (Carbaryl, 50 g/Kg) and Basagran (Benzothiadiazinone, 480 g/l) were products of BASF Agro-serve (Pty) Ltd, Republic of South Africa and were purchased from the Maseru garden centre, Lesotho, Southern Africa.

Reagents: Ethanol (Absolute) was a product of Associated Chemical Enterprises (PTY) LTD of the Republic of South Africa; Hydrochloric acid and Glacial acetic acid were products of UNILAB of the Republic of South Africa; Aceto-carmine stain from Carolina Biological Supply Company, USA.

Preliminary seed germination experiment to select doses of pesticide

Preliminary dose selection experiment was conducted for each chemical with concentration ranges between ten times above and below the manufacturers recommended dose (% solution in water). However, in cases where no inhibition of germination was obser-ved, higher doses were tested.

For each test, 100 onion seeds were spread on a filter paper moistened with a specific concentration of the pesticide in a petri dish and kept for 3 days at room temperature to germinate. 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 negative control to derive the percentage germinating at each concentration. The EC_{50} for each pesticide was determined from the curve of percentage germination 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) and water-soaked filter paper (negative control). In this project, a discontinuous treatment protocol was used. Seeds were spread on water moistened filter paper in a petri dish until they germinated and the radicles reached a length of about 5 cm. Germinated seeds were transferred onto filter paper kept moistened in a petri dish with specific concentration of pesticide for 24 h (acute treatment) at room temperature. At the end of the 24 h exposure, two root tips from two seeds per dose were collected at random and assessed. Three concentrations of each pesticide representing the ¼ EC_{50}, ½ EC_{50} and EC_{50}, as determined in the preliminary dose selection experiments were tested, together with a concurrent negative control which was water.

Root harvest and slide preparation

Root tips 1 - 2 cm long were cut from the germinated seeds and placed in a small glass specimen bottle and fixed in acetic alcohol (ethanol : glacial acetic acid in 3:1 ratio) for 24 h in a fridge at 4 - 6°C. The root tips were washed twice with ice cold water for 10 min each and allowed to dry in a watch glass. A solution of 1 N HCl pre-heated to 60°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 singly to a clean microscope slides and cut 2 mm from the growing tip. The tips were kept and the rest was discarded. Aceto-carmine stain was added to each slide to cover the root tip for about 10 min. A glass cover slip

was placed on the root tip and tapped gently with a pencil eraser to spread the cells evenly to form a monolayer to facilitate the scoring process for normal and aberrant cells in the different stages of the cell cycle.

Scoring of slides and data analysis

The slides were viewed under the light microscope (Olympus CX 21) using the 100X objective lens with oil immersion. The most representative ones for each structural aberration were photographed using a Zeiss PrimoStar microscope mounted with Canon camera model, Power Shot A640.

Mitotic index: On one slide for each treatment, a total of 2000 cells, classified into interphase or dividing cell (Prophase, Metaphase, Anaphase or telophase) were scored. The mitotic index (MI) was expressed as the number of dividing cells per 1000 cells scored.

Cytotoxicity: The mitotic indices of the treated cells at each dose of each pesticide were compared with that of the negative control group. A dose of pesticide was adjudged cytotoxic if the mitotic index of treated cells was ≤ ½ of the mitotic index of the concurrent water treated cells.

Genotoxicity test: A total of 30 to 100 anaphase and telophase cells were examined for chromosome aberration per dose of each pesticide from one slide. The following categories of aberrations were observed and scored: Chromosome fragments, bridge, vagrant chromosomes, C-anaphase, multipolar anaphases and telophases and stick chromosomes.

The percentage of Anaphase-Telophase cells with aberrations at each dose of each pesticide was compared with that of the negative control using the Chi-squared test (SPSS 10.0 for Windows statistical package). A dose of pesticide was considered to be genotoxic if the Chi-squared test was significant at P = 0.05.

RESULTS

Cytotoxicity of the pesticides

The results of the cytotoxicity determination are presented in Table 1. All eight pesticides were toxic at one or more of the three concentrations tested.

Genotoxicity of the pesticides

The result of the determination of the genotoxic effects of the pesticides are presented in Table 2. Hormoban, storm killer, villa, fungi-nil, bexadust, aphicide and karbadust were genotoxic at one or more doses of pesticide tested. Basagran however, was not genotoxic as the cells observed were in late telophase. It has to be noted that aphicide and basagran were very toxic such that it was impossible to score 30 anaphase-telophase cells on a slide. For the pesticides that induced genotoxic effects, the C-Anaphase and Stick chromosomes classes made up 75% and above, of the total CA with the exception of one dose each of Storm killer and villa where the C- Anaphase and Stick chromosomes classes made up 50% of the total CA observed. The most common types of aberrations observed were therefore C-anaphase and stick chromosomes.

Hormoban, storm killer and villa induced bridges ≥ twice the control value at one dose each. The formation of chromosomal bridges was not accompanied by the occurrence of chromosomal fragment. Only Bexadust and Karbadust induced multipolar anaphases and telophases.

Figures 1 - 6 are the pictures of the different types of genotoxic effects of the pesticides on *A. cepa* root tip meristematic cells.

DISCUSSION

The mitotic indices of onion root tips treated with all eight pesticides, hormoban, storm killer, villa, fungi-nil, bexadust, aphicide karbadust and basagran were reduced to half or less than half compared with the negative control at one or more doses and were adjudged as toxic to the onion root tip cells. A depression of the mitotic index has been recorded by many investigators as a result of treatment with pesticides (Amer and Farah, 1974; Panda and Sahu, 1985; Asita and Makhalemele, 2008). In addition, seven of the pesticides namely, hormoban, storm killer, villa, fungi-nil, bexadust, aphicide and karbadust also exhibited genotoxic effects to onion root tip cells exposed for 24 h.

The commonest types of genotoxic effects observed were C-anaphase and Stick chromosomes which together accounted for 50% and above of the total CA observed for all the genotoxic pesticides. The presence of c-metaphase cells was evidence of the action of the pesticides concerned on the mitotic spindle (Matsumoto et al., 2006). The stick chromosomes have resulted in the abnormal uncoiling of chromosomes during anaphase to telophase (Qian et al., 2006). The seven pesticides are thus more likely to be aneugenic than clastogenic.

The active ingredients in hormoban, dicamba induced sister chromatid exchanges in mammalian cells *in vitro* (González et al., 2006). MCPA, the other ingredient, was only weakly mutagenic to bone marrow and ovarian cells of hamsters and negative results were reported for all other mutagenic tests (Walker and Lawrence, 1992).

The active ingredient in storm killer, flocoumafen, did not induce reverse gene mutation in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538 nor in *Escherichia coli* WP2uvrA pkm 101 either with or without metabolic activation and at concentrations ranging from 31 to 2000 µg/plate, beyond which precipitation from suspension occurred (Brooks et al., 1984). When incubated at concentrations ranging from 5 to 25 mg/liter for 24 h in monolayer cultures of rat liver RL4 cells, flocoumafen did not induce *in vitro* chromosomal damage (Brooks et al., 1984). Oral administration of flocoumafen to rats at doses of 0.25 or 1000 mg/kg body weight did not produce chromosomal damage (Allen et al., 1986).

Villa, with the active ingredient as alpha-cypermethrin

Table 1. Determination of the mitotic index among 2000 cells scored following 24 h exposure of onion root tip cells to three different concentrations each of different pesticides.

Test Compound	Concentration of solution (% w/v)	Interphase	Cells in division stages per 2000 cells scored					MI	MI as % of control
			Proph.	Metaph.	Anaph.	Teloph.	Total		
Water		1686	69	31	21	31	152	76	100
Hormoban	0.0115	1892	62	13	11	22	108	54	71
	0.023	1948	37	3	4	8	52	26	34†
	0.046	1970	16	2	4	8	30	15	20†
Storm Killer	0.025	1904	49	16	4	27	96	48	63
	0.05	1914	52	23	4	7	86	43	57
	0.1	1976	7	3	4	10	24	12	16†
Villa	1.67	1916	59	3	12	10	84	42	55
	3.38	1928	55	4	6	7	72	36	47†
	6.67	1966	25	2	2	5	34	17	22†
Fungi-nil	1.02	1896	58	12	12	22	104	52	68
	2.12	1944	30	3	9	14	56	28	37†
	4.24	1976	12	6	2	4	24	12	16†
Bexadust	15	1962	18	12	3	5	38	19	25†
	30	1968	16	4	4	8	32	16	21†
	60	1948	19	10	10	13	52	26	34†
Aphicide	0.046	1964	16	4	8	8	36	18	24†
	0.092	1982	10	2	2	4	18	9	12†
	0.18	1992	5	0	1	2	8	4	0.05†
Karbadust	5.91	1940	28	8	12	12	60	30	40†
	11.03	1958	23	3	7	9	42	21	28†
	22.06	1970	19	4	4	3	30	15	20†
Basagran	0.035	1924	45	22	3	6	76	38	50†
	0.07	1970	13	9	2	6	30	15	20†
	0.14	1994	4	0		2	6	3	0.04†

MI = Mitotic index (number of cells in division stages out of 1000 cells); Proph. = Prophase; Metaph. = Metaphase; Anaph. = Anaphase; Teloph. = Telophase; † = Toxic (MI test ≤ 1/2 of Control).

was genotoxic in the present study. However, α-cypermethrin was not mutagenic in the *S. typhimurium* reverse mutation assay with TA98, TA100, TA1535, TA1537, TA1538 and *E. coli* WP2 uvrA (Brooks and Wiggins, 1992) and was negative in the *in vivo* mouse micronucleus test (Vanderwaart, 1995). A commercial formulation of α-cypermethrin (Fastac 100 EC, containing 10% α-cypermethrin as the active ingredient) induced sister chromatid exchanges (SCEs), chromosomal aberrations (CAs) and micronucluei (MN) in human peripheral lymphocytes in a recent *in vitro* study (Kocaman and Topakta, 2008).

Fungi-nil was genotoxic to the onion root tip cells in the present study. The active ingredient in fungi-nil, Dicarboximide (captan), however, was an extremely weak genotoxin *in vivo* in mice (Provan et al., 1992). In the micronucleus test, captan (62.5 µ/l) was genotoxic to Xenopus but not genotoxic to Pleurodeles at all concentrations tested (Mouchet et al., 2006).

Bexadust with the active ingredient as gamma benzene hexachloride (BHC) was genotoxic to *A. cepa* root tip cells in the present study. Analysis of genotoxicity of BHC on *Salmonella* assay showed no mutagenic effects (Dubois et al., 1997). In *in vivo* analysis, BHC showed micronucleus formation in mice bone marrow (Bhunya and Jena, 1992). When human peripheral lymphocyte cells were treated with BHC for 24, 48 and 72 h, there was a dose-dependent increase in the frequency of chromosomal aberrations and sister chromatid exchanges and a significant decrease in mitotic index was observed at all concentrations and times of exposure. BHC did not show a significant effect on cell kinetics (Rupa et al., 1989).

Aphicide was genotixic at the two lower doses in the present study. In the single gel electrophoresis (comet) assay, the active ingredient, dimethoate alone at 100 µl/ml caused significant DNA damage on human peripheral lymphocytes incubated at 37°C for 30 min

Table 2. Genotoxic effects of pesticides to onion root tip cells after 24 h exposure.

Test compound	Concentration of solution (%)	MI	A - T scored	Aberrations observed in Anaphase-telophase cells scored						Total CA %
				Fragment %	Bridge %	Vagrant %	C-Anaphase %	Multipolarity %	Stick chromosomes %	
Water	100	76	83	0	1.205	0	0	0	0	1.205
Hormoban	0.0115	54	55	0	9	0	16.36	0	9.09	34.45**
	0.023	26	45	0	0	0	0	0	0	0
	0.046	15	32	0	0	0	0	0	50	50.00**
Storm killer	0.025	48	66	0	0	0	0	0	1.52	1.52**
	0.05	43	94	1.06	1.06	0	0	0	2.13	4.25*
	0.1	12	35	0	0	0	0	0	0	0
Villa	1.67	42	55	0	7.27	0	16.36	0	0	23.63**
	3.38	36	32	0	0	3.13	34.38	0	0	37.51**
	6.67	17	36	0	0	0	22.22	0	0	22.22**
Fungi-nil	1.02	52	54	0	0	0	22.22	0	0	22.22**
	2.12	28	45	0	0	0	0	0	0	0
	4.24	12	42	0	0	0	16.67	0	33.33	50.00**
Bexadust	15	19	60	0	0	0	25	10	35	70.00**
	30	16	42	0	0	0	16.67	0	16.67	33.34**
	60	26	66	0	0	0	54.55	12.12	9.09	75.76**
Aphicide	0.046	18	20	0	0	0	50	0	0	50.00**
	0.092	9	20	0	0	0	75	0	0	75.00**
	0.18	4	10	0	0	0	0	0	0	0
Karbadust	5.91	30	69	0	0	0	30.43	8.7	4.35	43.48**
	11.03	21	35	0	0	2	54.29	0	40	96.29**
	22.06	15	32	0	0	0	25	0	0	25.00**
Basagran	0.035	38	25	0	0	0	0	0	0	0
	0.07	15	18	0	0	0	0	0	0	0
	0.14	3	15	0	0	0	0	0	0	0

MI = Mitotic index (number of cells in division stages out of 1000 cells); A - T (anaphase and telophase cells); CA % = cells with chromosomal aberrations as % A - T cells examined; * $P < 0.05$; ** $P < 0.01$ in a chi-squared test.

(Basaran and Undeger, 2005). Dimethoate induced mutagenicity in the *Salmonella* reverse mutation assay (Ansari and Abdul, 2008).

The National Institute for Occupational Safety and Health labels carbaryl, the active ingredient in karbadust, as a mutagen and has identified over 20 studies conducted in the 1970s and 1980s documenting carbaryl's ability to cause genetic damage (NIOSH, 2004). A more recent study that analyzed the genotoxicity of carbaryl on human lymphoblastoid cell line by an *in vitro* DNA repair solid-phase assay showed that carbaryl stimulates the activity of an enzyme that transforms carbaryl into a compound that caused a severe DNA damage to the cells (Delescluse et al., 2001).

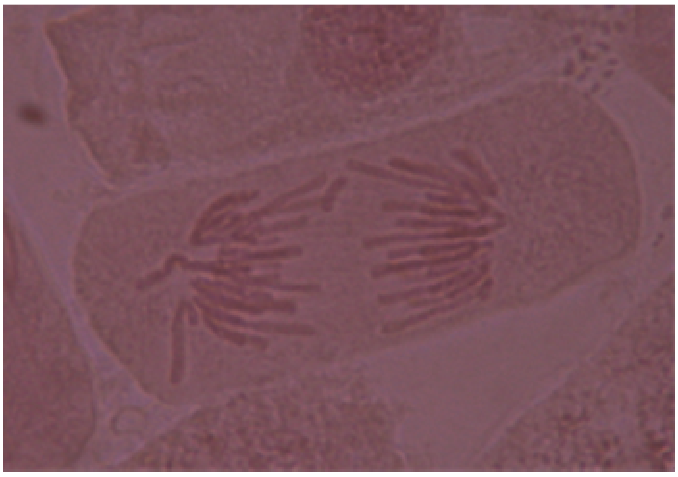

Figure 1. Pesticide treated onion cell with chromosome fragment.

Figure 2. Pesticide treated onion cell with chromosome bridge.

Figure 3. Pesticide treated onion cell with Vagrant chromosome

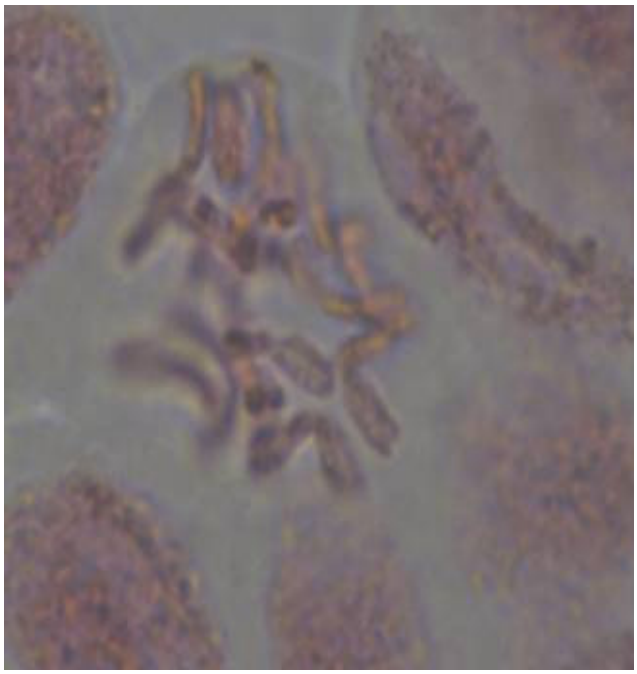

Figure 4. Pesticide treated onion cell showing c-anaphase type aberration.

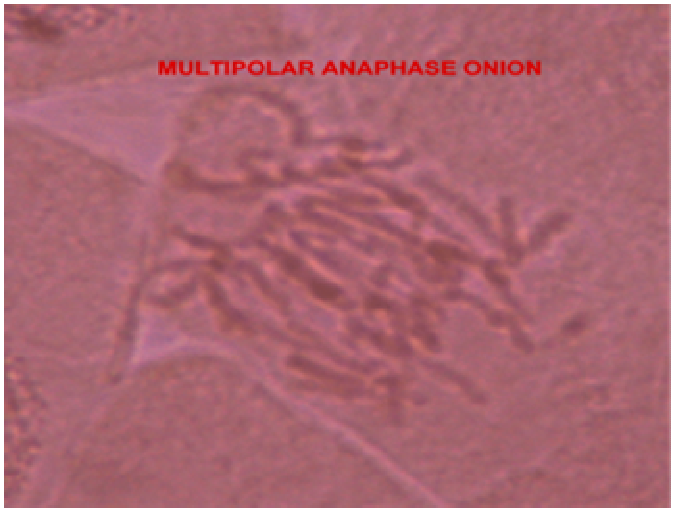

Figure 5. Pesticide treated onion cell showing multipolar anaphase.

Karbadust was genotoxic to the onion root tip cells in the present study also.

Basagran, which is the trade name of bentazon, was not mutagenic to the onion root tip cells used in this study. All the cells observed were in late telophase which was indicative of the high toxicity of basagran. Bentazon was not mutagenic in the reverse mutation test with *S. typhimurim* TA100, TA98, TA1535, TA1537, TA1538 and the reverse mutation test with *E. coli* WP2, with and without metaboloic activation (Moriya et al., 1983). Bentazon was also not genotoxic to spermatozoa and

Figure 6. Pesticide treated onion cell showing stick chromosomes.

bone marrow cells (Garagna et al., 2005). However the genotoxicity of basagran was demonstrated in the wing spot test of *Drosophila melanogaster*, an *in vivo* assay based on the loss of heterozygosity of the *mwh* and *flr* markers in the wing imaginal disk cells of larvae fed with chemical agents (Heres-Pulido et al., 2008).

Conclusion

In conclusion, hormoban, storm killer, villa, fungi-nil, bexadust, aphicide karbadust and basagran were toxic to onion root tip cells and with the exception of basagran, were also genotoxic, inducing mostly C-anaphase and Stick chromosomes types of aberration which was evidence of the action of the pesticides on the mitotic spindle and the coiling of chromosomes during anaphase to telophase.

The study has further demonstrated the usefulness of the *A. cepa* chromosome aberration assay in assessing the genotoxicity of environmental chemicals as mixtures or pure products.

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