Dichlorophen and Dichlorovos mediated genotoxic and cytotoxic assessment on root meristem cells of *Allium cepa*

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ABSTRACT

Plants are direct recipients of agro – toxics and therefore important materials for assessing environmental chemicals for genotoxicity. The meristematic mitotic cell of *Allium cepa* is an efficient cytogenetic material for chromosome aberration assay on environmental pollutants. Onion root tips were grown on moistened filter paper in petri dish at room temperature. Germinated root tips were then exposed to three concentrations of each pesticide for 24 h. About 1 – 2 mm length of root tip was cut, fixed in cornoy's fixative, hydrolyzed in warm 1 N HCL, stained with acetocarmine and squashed on glass slide. 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 Dichlorophen and Dichlorovos at one or more concentration was half or less than that of control are said to be cytotoxic. Genotoxicity was measured by comparing the number of cells/1000 in aberrant division stages at each dose with the negative control using Mann – Whitney U test. Both Dichlorophen and Dichlorovos are genotoxic at higher concentrations i.e. 0.001%, 0.002% and 0.028%, 0.056% inducing chromosome fragment, chromosome lagging and bridges, stick chromosome and multipolar anaphase.

Keywords: *Allium cepa*, cytotoxicity, genotoxicity, mitotic index, pesticides, root tip cells

INTRODUCTION

Pesticides have been used in modern agriculture to greatly improve the 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).

Pesticides form an important group of environmental pollutants and genotoxic effects of several chemical groups of pesticides have been shown by *in vivo* and *in vitro* experiments (Bolognesi, 2003; Abdollahi et al., 2004; Kaushik & Kaushik, 2007). However, genotoxicity data for few pesticides exist (Gandhi et al., 1995), while reports on majority of them are lacking.

Among pesticides, organophosphates and organochlorines are constantly a matter of worry because of their wide use. Both group of chemicals bear the potentiality to cause genotoxicity and carcinogenicity (Kaushik & Kaushik, 2007). However, in addition to intended effects of pesticides, they 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 animal is well known and several studies have shown that chronic exposure to low levels of pesticides can cause mutation and/or carcinogenicity (International Agency for Research on Cancer, 1990; International Agency for Research on Cancer, 1991; Karabay & Gunnehir, 2005; Bull et al., 2006).

Organophosphorus (OP) pesticides, which are widely used in agriculture as insecticides, leave residues to varying extent in agricultural produce such as vegetables and fruits (Iram et al., 2009). OP compounds exert acute toxic effect that are mainly due to suppression of neuronal acetylcholinesterase activity (Sachanaa et al., 2003). The widespread uses of OP insecticides indicate the extensive availability and potential for accidental and intentional human exposure (El-Behissy et al., 2001).

Dichlorovos (2, 2 – dichlorovinyl dimethyl phosphate – DDVP) is an OP compound used to control household, public health and stored product insects. It is effective against mushroom flies, aphids, spider, mites, caterpillars, thrips and white flies in greenhouse, outdoor fruits and vegetable crop (Lotti, 2001).

Organochlorine pesticides are endocrine disrupting chemicals, meaning they have subtle toxic effect on the body's hormonal system (Lemaire et al., 2004). Endocrine disrupting chemicals often mimic the body's hormones, disrupting normal functions and contributing to adverse health effects.

Dichlorophen (2, 2 – methylene bis 5 – chlorophenol – DDDM) is an organochlorine compound that is incompatible with strong oxidizing agents and can be slowly oxidized in air. It is hazardous in case of eye contact or ingestion, but less severe in case of skin contact or inhalation (Kintz et al., 1997).

No studies have been carried out, to our knowledge, on the cytotoxic and genotoxic effects of Dichlorophen and Dichlorovos on onion root tips, despite the fact that it is commonly used. The use of plant test systems for the evaluation of the mutagenic potential of pesticides is particularly important, since they largely enter the human food chain.

MATERIALS AND METHODS

Onion (*Allium cepa*, 2n = 16) bulbs equal in size 1.5 – 2.0 cm diameter were chosen from a population of locally available commercial variety, Nasik Red (N-53).

Dichlorovos CAS No. 62 – 73 – 7 and Dichlorophen CAS No. 97 – 23 – 4 are products of Sigma.

Ethanol (Merck) is of analytical grade. Glacial acetic acid CAS No. 64 – 19 – 7 and hydrochloric acid are products of Fisher scientific. Methyl methane – sulfonate (MMS, 99%) CAS No. 66 – 27 – 3, a product of *Super Religare Laboratories* Limited (Formerly *SRL* Ranbaxy Ltd.), was used as positive control. Acetocarmine CAS No. 64 – 19 – 7 is a product of Loba Cheme.

Determination of LC50 and dose selection

Fifty (50) onion seeds were spread on filter paper moistened with different concentrations of pesticide in a petri dish and left to germinate at room temperature for about three days. The number of seeds which produced radicle were recorded at the end of 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 both pesticides was determined from the curve of percentage of root length that germinated against dose.

As per above procedure the LC50 for Dichlorophen and Dichlorovos was determined to be 0.004 % and 0.224 %, respectively. On the basis of determined LC50, three random concentrations in increasing order within the LC50 were selected for both pesticides. The three concentrations taken in this study do not correlate with the concentration used in the field, as the concentrations used in the field vary from place to place.

Genotoxicity assay

The method used was similar to the method of Asita & Matebesi (2010). *A. cepa* (onion) seeds were germinated in petri dishes containing pesticide – soaked filter paper (test), water – soaked filter paper (negative control) and on filter paper soaked in aqueous solution of 1% methyl methanesulfonate (positive control). In this study, a discontinuous treatment protocol was used. *A. cepa* seeds were first soaked in distilled water until the radicles reached a length of about 1 – 3 cm. Germinated seeds were transferred to petri plates containing chemicals at different concentration in which they were left for 24 h at room temperature. At the end of the 24 h exposure, some seeds were collected at random and assessed.

Root harvest and slide preparation

Root tips 1 – 3 cm long were cut and placed in a watch glass and fixed in acetic alcohol (ethanol: glacial acetic acid in 3:1 ratio) for 12 h at room temperature. After this the root tips were hydrolyzed in 1 N HCL at 60 ^{o}C for 10 minutes and stained with Acetocarmine for 20 minutes, then squashed on glass slide under 45% acetic acid to determine the mitotic index and the presence of chromosomal aberrations.

Scoring of slides

The slides were viewed under the light microscope (Olympus CH 20 *i*) 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 an Olympus U – PMTV microscope mounted with optical zoom camera.

DATA ANALYSIS

Cytotoxic determination

Mitotic index method was used for determination of cytotoxicity, which was similar to the method of Asita & Matebesi (2010). 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 said 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-mitosis (no spindle fibres), stick chromosomes, chromosome bridges, lagging chromosomes or chromosome breaks.

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 pesticides

Table 1 presents the cytotoxicity of pesticides on onion root tips. Cells treated with Dichlorophen and Dichlorovos had reduced mitotic indices compared with the cell treated with water, which indicate inhibition of cell division by these pesticides. Dichlorophen and Dichlorovos are therefore said be cytotoxic at one or more doses. However, the decrease of the MI was not dose dependent. Significant levels of inhibition were apparent at lower concentrations of both pesticides, which means that they are cytotoxic at lower concentration. In addition to lower concentration Dichlorovos was also cytotoxic at higher concentration. The positive control chemical, methyl – methane sulfonate at 0.2% concentration in water did not inhibit mitotic cell division of the onion root tip cells.

Genotoxicity of pesticides

Table 2 presents the genotoxic effect of pesticides on onion root tips.

Significant differences were detected in the frequency of aberrant division stages in Dichlorophen and Dichlorovos treated cells when compared with water (negative control) treated group, at two higher doses ($P < 0.05$), while at lower dose of both compounds the genotoxic effect was not observed. In comparison with the positive control (MMS) used in the present investigation, a known genotoxic compound.

Table 3 presents the types of aberration observed in the cells treated with Dichlorovos and Dichlorophen. The different types of mitotic abnormalities due to genotoxic effects of the pesticides and the normal anaphase in negative control on *A. cepa* cells observed on glass slides are presented in plate 1 (a – i)

Table 1. Cytotoxicity of pesticides on onion root tip cells

Treatment	Conc (%)	MI	Mean±SE	MI Control/MI Test
Water	100	0.158	39.55±9.08	1.00
MMS	0.2	0.244	61.03±1.64	0.65
DDDM	0.0005	0.020	4.97±0.80	7.9*
	0.001	0.145	36.14±5.89	1.09
	0.002	0.172	43.07±1.50	0.92
DDVP	0.014	0.050	12.47±3.39	3.16*
	0.028	0.136	34.08±1.02	1.61
	0.056	0.056	14.04±3.21	2.82*

DDM = dichlorophen; DDVP = dichlorovos; MMS = Methyl methane sulfonate; SE = Standard error, Conc = concentration; MI = mitotic index;* =cytotoxic (MI control: MI test > 2)

DISCUSSION

Organophosphorus and organochlorine pesticides are widely used by farmers in India and in other countries because of their high efficiency towards the target organisms (Levine, 1991; Mineau, 1991). Extensive use of these pesticides in crop protection and for household purposes has resulted in their widespread distribution in the environment (Mineau, 1991). While they contribute greatly to the animal and human prevention of vectors of diseases, their use also creates many problems because of their toxicity to non– target organisms, persistence and combined effects with other agro – biochemicals and environmental factors (Levine, 1991; Mineau, 1991; U.S. Environmental Protection Agency, 1999).

The *Allium cepa* assay is an efficient test for chemical screening and *in situ* monitoring for genotoxicity of many pesticides. Results have shown that these compounds can induce chromosomal aberration in root meristem of *A. cepa* (Feretti et al., 2007). Pesticides residues can be present in fruit and vegetables and represent a risk for human health.

Table 2. Mutagenic potencies of the pesticides on onion root tip cell

Treatment	Conc (%)	Number of cells in different division stages/1000 cells scored										
		PROP		METAP		ANAP		TELOP		TOTAL		M – WU Value (Calculated)
		N	ABN	N	ABN	N	ABN	N	ABN	N	ABN	
Water	100	58.42	0.00	51.25	0.00	28.17	0.00	20.39	0.00	1000.0	0.00	20
MMS	0.2	105.11	6.01	41.77	7.65	37.42	5.03	31.27	0.90	971.45	28.55	0**
DDDM	0.0005	1.25	2.66	4.12	0.87	4.66	1.00	2.33	0.00	992.48	7.52	6
	0.001	23.62	1.35	23.96	4.39	40.16	5.40	35.10	1.01	978.28	21.72	0**
	0.002	32.64	1.87	26.05	4.77	43.12	5.80	38.75	1.12	976.28	23.72	0**
DDVP	0.014	19.24	5.66	9.64	3.64	4.31	4.99	1.32	4.65	984.63	15.37	10
	0.028	104.37	5.96	11.61	1.03	9.80	0.51	7.25	0.06	996.70	3.30	0**
	0.056	23.55	1.35	11.22	2.06	8.20	1.10	6.80	0.17	993.58	6.42	0**

DDDM = dichlorophen; DDVP = dichlorovos; N = normal cells; ABN = abnormal cells; Conc = concentration; M – WU = Mann – Whitney U; PROP = prophase; METAP = metaphase; ANAP = anaphase; TELOP = telophase. ** = mutagenic ($U \leq 0$; $P < 0.05$, Mann – Whitney U - test)

Table 3. Types of mutation induced by pesticides

Pesticides	Mutation type					
	CB	MA	CM	CB'	SC	LC
DDVP	+	+	+	+	-	+
DDDM	-	-	-	+	+	+

DDVP = dichlorovos; DDDM = dichlorophen; CB = chromosome break; MA = multipolar anaphase; CM = C – mitosis; CB' = chromosome bridge; SC = stick chromosome; LC = lag chromosome

The findings of the present study indicate that DDVP and DDDM can induce cytotoxic and genotoxic effect on the meristematic cells of *Allium cepa*. The MI inhibition and induction of chromosomal aberration in plant cells by several pesticides have been reported earlier by different researchers (Chauhan et al., 1999; Sudhakar et al., 2001). The observations of the present study are in accord with the earlier report, wherein a clear indication of the mitoclastic and clastogenic actions are seen, which are evident from the lowering of the MI and manifestation of spindle abnormalities (Chauhan et al., 1999; Sudhakar et al., 2001). The lower chemical concentration stimulates the rate of cell division.

Mitotic activity reduction could be due to the inhibition of DNA synthesis (Schneiderman et al., 1971; Sudhakar et al., 2001) or due to a block in the G_2 – phase of the cell cycle, thus preventing the cell from entering mitosis (Van't Hof, 1968). The mitotic activity suppression is often used to assess cytotoxicity (Smaka-Kincl et al., 1996). It has been reported by many investigators that a depression of the mitotic index is the result of treatment with pesticides (Amer & Farah, 1974; Panda & Sahu, 1985; Asita & Makhalemele, 2008).

The insecticides showed more effectiveness in the S – phase in comparison to the G_1 and G_2 phases of cell cycle (Srivastava et al., 2008). Several chromosomal aberrations (CAs) like stickiness, chromosomal break, chromosomal bridges, laggard, C-mitotic effect and multipolar anaphase have been formed.

C – mitosis was observed in root tips treated with DDVP. The occurrence of C – mitosis in *A. cepa* indicates that spindle formation was adversely affected (El-ghamery et al., 2000).

Chromosome break was one of the most frequent chromosome aberrations induced by DDVP. The induction of chromosome breaks by pesticides indicates the clastogenic potential of the test compounds (Chauhan & Gupta, 2005). Chromosome break induced by DDVP indicates that DDVP has more clastogenic activity compared to DDDM. Chemicals that induce chromosome breakage are known as clastogens and their action on chromosome is generally regarded to involve an action on DNA (Grant, 1978; Chauhan & Sundararaman, 1990). Breaks and unequal distribution were noticed in diverse materials as a result of treatment with various chemicals (Aly et al., 2002; Borah & Talukdar, 2002; Gomürgen, 2005).

Chromosome bridges were noticed in both DDVP and DDDM treated onion root tips, which were probably formed by breakage and fusion of chromosomes and chromatids. Gomürgen (2005) reported that potassium metabisulphite and potassium nitrate caused anaphase bridges in *A. cepa.* According to Gomürgen (2005), chromosome bridges may be due to the stickiness of chromosome and subsequent failure of free anaphase separation or may be attributed to unequal translocation or inversion of chromosome segments.

Laggards were observed after the treatment with both DDDM and DDVP, which are due to the failure of the chromosome to move to either of the poles. According to Permjit & Grover (1985), the lagging chromosomes can be attributed to the delayed terminalization, stickiness of chromosome ends, or because of the failure of chromosomal movement. Gomürgen (2005) reported that potassium sulphite and potassium nitrate, which are food preservatives, caused laggard chromosomes in *A. cepa*.

Chromosome stickiness was another frequent chromosomal abnormality induced by DDDM in meristematic cells of *A.cepa*. This stickiness is presumably due to the intermingling of chromatin fibers, which lead to subchromatid connection between chromosomes (McGill et al.,1974; Klasterska et al., 1976). According to Saxena et al. (2005), subchromatid connections were observed in plants cells exposed to pyrethrin insecticides. Stickiness can also be explained as physical adhesion of the proteins of the chromosome (Patil & Bhat, 1992). Stickiness is accepted as an indicator of toxicity, which results in cell death (El-Ghamery et al., 2000).

Anonymous (1980), reported that plant test systems among known test system are more sensitive for determining these effects of pesticides.

Rank & Nielsen (1994) shown that *Allium* test in some way is more sensitive than both the microscreen assay and the Ames test. It can even detect some carcinogenic substances that are not detected in the Ames test.

The *Allium* test has proved to be a reliable test for monitoring cyto – and genotoxicity of the different chemical substances. For *in situ* monitoring, meristematic cells of *Allium* and *Vicia* are very efficient cytogenetic materials for the detection of mutagenicity of the environmental chemicals (Ma et al., 1995).

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

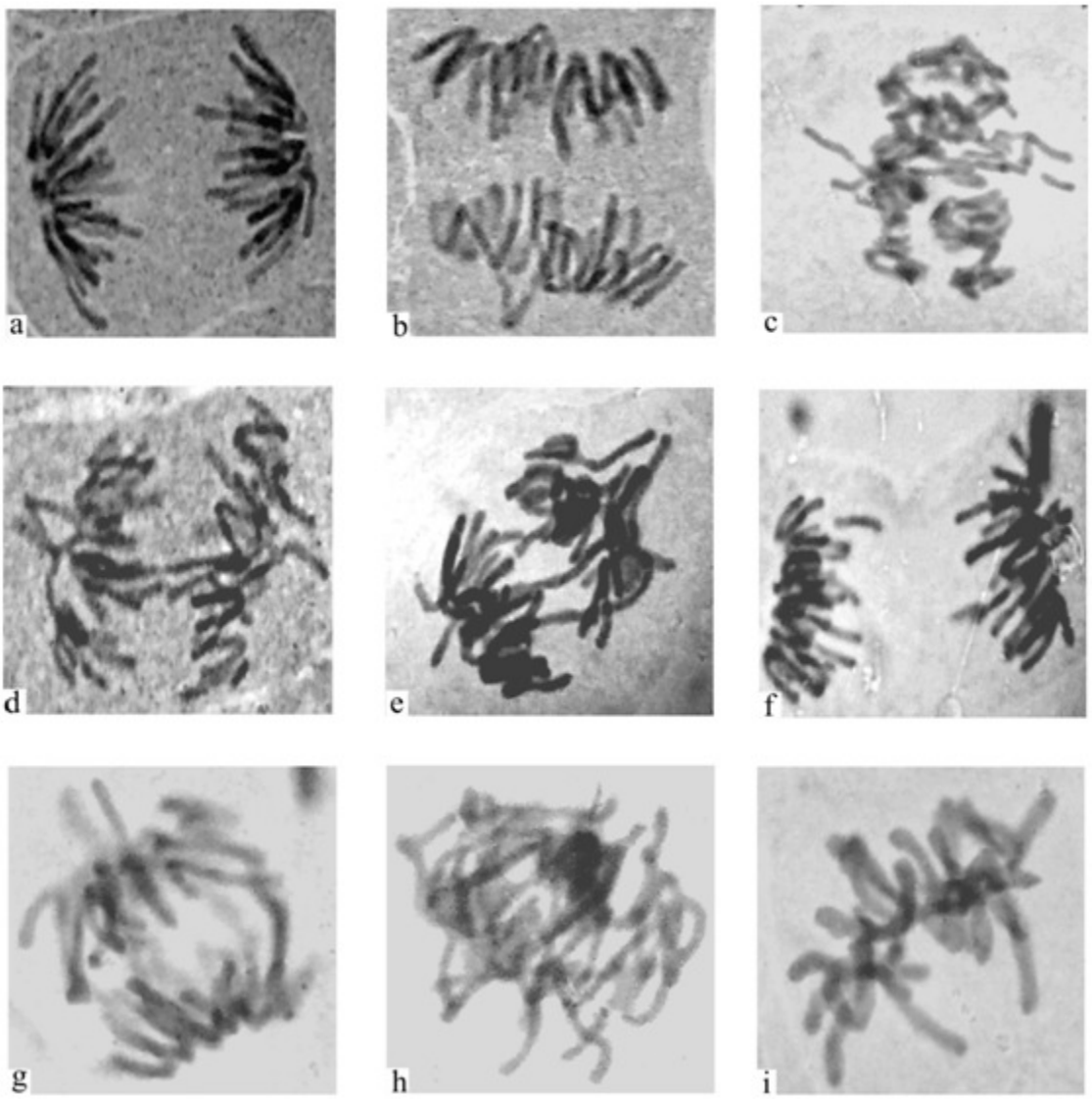

Plate 1. *Allium cepa* root tip cells showing normal anaphase (a) in negative control group; mitotic abnormalities induced by pesticide treated group showing – multipolar anaphase (b – c); chromosome bridge (d – e); chromosome break (f); lag chromosome (g); stick chromosome (h); and C – mitotic effect (i)

ACKNOWLEDGEMENTS

The authors especially want to mention the financial help provided by the UP-CST, Lucknow to Sibhghatulla; CST/D-598/2011, and UGC, New Delhi to Nazia Nazam; No.F.40-3 (M/S)/2009(SA-III-MANF) and Mohammad Iqbal Lone; 40-355/2011 (SR) for a broader research program. The authors also deeply acknowledge the Chairman, Department of Zoology, AMU, Aligarh for providing the necessary facilities.

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