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ORIGINAL ARTICLE

Screening of cytotoxic and genotoxic potency of two pesticides (malathion and cypermethrin) on *Allium cepa* L.

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Abstract

Background Malathion (organophosphate) and cypermethrin (pyrethroid) are widely in use to facilitate protection of major food crops in agriculture, due to which it is important to understand their toxic potential. *Allium cepa* L. has been considered as a reliable genetic model to detect the toxicity of all sorts of pollutants.

Objective The objective of the present work is to determine the cytotoxic and genotoxic effects of two widely used pesticides (malathion and cypermethrin) using *A. cepa* assay. *Allium* root growth inhibition test showed 0.5% concentration as the EC_{50} value in both the pesticides. In the toxicity experiment, $1/10 \times EC_{50}$; $1/5 \times EC_{50}$; EC_{50}; $2 \times EC_{50}$ and $3 \times EC_{50}$ concentrations of both the pesticides along with a control were employed in *Allium* assay.

Results Cytotoxic study showed mitotic index decreased with increasing the pesticides concentrations and exposure time. A series of mitotoxicity was observed under the influence of malathon and cypermethrin. Most types of chromosome aberrations observed in high percentage were stickiness, disturbance, c-metaphase, chromosome bridges in anaphase, lagging chromosome, and binucleate lesions. It was observed that malathion and cypermethrin are highly genotoxic to the onion, causing aberration at different phases of mitosis which can arrest cellular growth and may lead to senescence.

Conclusion In conclusion, the present results showed that malathion and cypermethrin can get absorbed in the exposed plant parts or other non-target organisms in the vicinity, and may adversely affect their genomes, thus cause significant harm to crop plants and the environment as well.

Keywords *Allium cepa* assay · Malathion · Cypermethrin · Chromosome aberrations · Mitotic index

Introduction

Pesticides are a group of chemicals, which are used to exterminate pest to increase yield and improve the shelf life of agricultural products. Besides, they are used in public health to reduce morbidity and mortality from pest-related disease. In recent years, there has been a tremendous increase in use of these chemicals without paying much attention to the adverse effects they may have due to the toxic ingredients (Badr and Ibrahim 1987; Anis et al. 1998). The use of the pesticides increased the production of food, increased profits for farmers and helped in the eradication of diseases, but this great achievement has resulted in injury and death of a variety of forms of life. Use of enormous quantities of pesticides also spoils the ecosystem directly or indirectly (Mozumder et al. 2013). There exists a direct relationship between the extant of pesticides used and signs and symptoms of illness due to exposure among farmers (Kishi et al. 1995). In general, excessively applied pesticides are not fully utilized to target pests; thereby may escapes to environment or accumulates in crops causing hazardous effects in non-target organisms. According to Zhang et al. (2011), about 4.6 million tons of chemical pesticides with about 500 different types is annually used across the world. Most pesticides used in agriculture today are synthetic organic chemicals that act by interfering with a vital metabolic process in the organism to which they are targeted (Mathur et al. 2005). Levan (1938) reported the classical test for the effect of chemicals

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on plant chromosome where he used the root tips from the onion bulbs of Liliaceae in an assay system.

Higher plants such as *Pisum sativum*, *Vicia faba*, *Hordeum vulgare*, *Crepis capillaries*, *Tradescantia paludosa*, and *Allium cepa* are proven bioindicators used to analyze the biological effects of chemical substances from diverse sources (Kluge and Podlesak 1985; Ma et al. 1995; Amer et al. 1999; Sang and Li 2004; Majer et al. 2005; Misik et al. 2006; Gadeva and Dimitrov 2008; Enan 2009; Ozkara et al. 2011). Among them, *A. cepa* assay has been the most efficient and reliable techniques for in situ monitoring and assessing the genotoxic effects of environmental contaminants including many pesticides, which revealed the growth inhibition and chromosomal aberrations induction potential of these compounds in root meristems of onion (Fiskesjo 1985; Cabrera et al. 1994; Saxena et al. 2005; Thais et al. 2007; Konuk et al. 2007; Liman et al. 2010). Onion is widely used to determine cytotoxic, genotoxic, and mutagenic effects of various environmental contaminants due to simple storage and handling requirements, also easy observation of parameters. Fiskesjo (1988) reported the validation of the obtained data from onion test system with data form other eukaryotic and prokaryotic test systems. In *Allium* assay, physiological and cytological parameters are considered for checking the deleterious effects of chemical substances, where these may block mitosis or produce mitotic and meiotic chromosome abnormalities.

For decades, people have believed that harmful chemical pesticides were the only true way to rid gardens and crops fields from pests. Soil pollution has occurred from the use of pesticides and it takes years and sometimes decades for some of these chemicals to break down. These pesticides are also harmful to animals, plants, as well as human health. Nitrogen fixation, which is required for the growth of higher plants, is hindered by pesticides in soil. Pesticides can kill bees and are strongly implicated in pollinator decline, the loss of species that pollinate plants. On the other side, pesticides have some direct harmful effect on plant including poor root hair development, shoot yellowing and reduced plant growth. Animal including humans may be poisoned by pesticides residues that remain on food. Pesticides caused various effects on human health such as asthma, birth defects, neurological effect, cancer, hormone disruptions.

The large-scale application of hazardous pesticides for the control of insects in agricultural practices has become a potential threat to the genetic constitution of economically important crops as well as to the human and environmental health in the world and especially in India. Hence, it is necessary to test the cytotoxic and genotoxic potency of commonly used pesticides to understand the extent of damage caused on plants and other systems, which certainly support their judicious use in agriculture. Therefore, in the present study, an attempt has been made to screen the cytotoxic and genotoxic effects of two widely applied pesticides, namely malathion (organophosphate) and cypermethrin (pyrethroid) using *A. cepa* root tip bioassay system.

Materials and methods

The uniformed size infection-free young onion bulbs of a commercial variety of *A. cepa* L. (2n = 16) were used in the assay. Onion bulbs were kept in contact with distilled water after carefully removing the lowermost base portion, keeping root primordia intact for root development. The experiment was carried out under laboratory conditions restricting direct exposure to sunlight for maintaining constant rate of cell division (Evans et al. 1957).

Chemicals

Two widely used insecticides malathion and cypermethrin were considered for the study. Dimethyl sulphoxide (DMSO), a biological tissue penetrant (Amin et al. 2015), was used to prepare test solutions. All the chemicals were procured from Sigma-Aldrich, USA. Test substances malathion and cypermethrin were dissolved in dimethyl sulphoxide (1% DMSO, purity 99%) for the preparation of stock solution. The chemical structural formulae of malathion and cypermethrin are shown in Fig. 1.

Determination of EC_{50}

EC_{50} for the test substances malathion and cypermethrin was determined following Ozkara et al. (2015). Uniform and healthy onion bulbs were allowed to grow roots placed onto test tubes filled with distilled water in the dark at room temperature for 24 h. Next, 14 best growing bulbs were picked for the trial and treated with different concentrations (0.01%; 0.05%; 0.1%; 0.5%; 1%; 1.5% and 2%) of malathion and cypermethrin each at room temperature (~ 22 ± 3 °C) for 96 h.

Additionally, one set of bulbs each was treated with 1% DMSO as negative control and ethyl methane sulfonate (EMS) as positive control. On the fifth day, the length of the entire root pack from control and exploratory sets (lengths of five roots from every bulb) were estimated. The half maximal effective concentration (EC_{50}) at 50% root growth inhibition was estimated based on effective control root lengths plotted against test concentrations.

Cytogenetic assay

For cytogenetic assay, $1/10 \times EC_{50}$; $1/5 \times EC_{50}$; EC_{50}; $2 \times EC_{50}$ and $3 \times EC_{50}$ doses were employed to determine the potency malathion and cypermethrin on onion root tip cells.

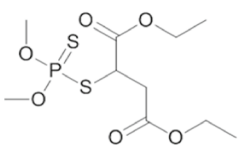
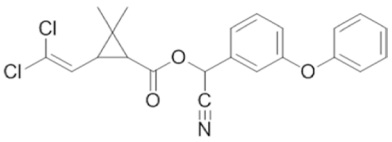
Class	Organophosphate	Pyrethroid
MF	$C_{10}H_{19}O_6PS_2$	$C_{22}H_{19}Cl_2NO_3$
Structure		
	a	b
IUPAC Name	diethyl 2-dimethoxyphosphinothioylsulfanyl butanedioate	[cyano-(3-phenoxyphenyl)methyl] 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate

Fig. 1 **a** Malathion and **b** cypermethrin

Initially, onion bulbs were placed in distilled water-filled test tubes in the dark at room temperature for 24 h. After that, a set of five bulbs with approximately equal root lengths was transferred to each of the test concentrations and one set in distilled water for control. Onion roots were exposed to different concentrations of malathion and cypermethrin for 12 and 24 h under the standard laboratory conditions. Finally, roots were collected separately maintaining proper exposure time during the morning hours between 6 and 7 a.m. Immediately after collection, roots were fixed in chilled Carnoy's solution (ethanol:glacial acetic acid = 3:1) for 24 h keeping at 4 ℃ overnight. After fixation the thoroughly washed root tips were preserved in 70% alcohol and kept in the refrigerator for further cytological studies.

Staining and microscopic parameters

Cytological slides were prepared by following acetocarmine squashing technique (Sharma et al. 1965). Initially, the root tips were hydrolysed in 1 N HCl and 2% acetocarmine (1:9) with gentle warming for 5 min without boiling. After that, root tips were carefully washed with distilled water. Then roots were kept in 1% acetocarmine for 1–3 h with intermittent heating until the acetocarmine begins to boil. Next, a healthy hydrolysed root tip was taken on a clean slide and the root cap was cut off with a sharp razor blade. A drop of acetocarmine or 45% acetic acid was then added and the apical 2-mm root tip was carefully covered with a coverslip. Finally, the slide was heated quickly and squashed gently by applying hard and uniform pressure between a folded blotting paper without disturbing the position of the coverslip.

For the cytotoxicity test, mitotic index (MI) was calculated for each concentration and duration of treatment with the following formula (Balog 1982):

$$\text{Mitotic Index (\%)} = \frac{\text{Total number of dividing cells}}{\text{Total number of cells observed}} \times 100.$$

The data obtained from control as well as treatments were analyzed and compared to determine the cytotoxic effects of the insecticides on mitotic activities of onion root tip cells.

For genotoxicity test, chromosomal aberration frequency (CF) was obtained for each concentration and duration of treatment with the following formula:

$$\text{Chromosomal aberration frequency (CF)} = \frac{\text{Total number of aberrated cells}}{\text{Total number of diving cells}}.$$

The calculated CF values from control as well as treatments were subjected to a comparative study to establish the genotoxic effects of the insecticides on the genome of onion root tip cell.

IBM SPSS, version 20.0 was used for statistical analyses of the observed data on root length, MI and CF in different treatment conditions.

Results

Effects on onion root growth

The inhibition test based on root lengths at different treatment conditions indicated that the root growth decreased significantly with increasing concentrations (0.01%; 0.05%; 0.1%; 0.5%; 1%; 1.5% and 2%) of both the insecticides malathion and cypermethrin (Table 1). The negative and positive controls provided maximum and minimum root length range between 5.03 ± 0.19 and 1.52 ± 0.18, respectively. The effective concentration (EC_{50}) value determined based on 50% root growth inhibition (Table 1) and dose–response curve (Fig. 2) was found to be at 0.50% concentration approximately in both the test substances malathion (3.23 ± 0.13 cm) and cypermethrin (3.41 ± 0.10 cm). After determination of EC_{50} value,

Table 1 Allium root growth inhibition test

Test substance	Concentrations (%)	Mean[a] of root length (cm) ± SD
Negative control	–	5.03[a] ± 0.19
Positive control	–	1.52[h] ± 0.18
Cypermethrin	0.01%	4.94[a] ± 0.16
	0.05%	4.69[b] ± 0.11
	0.10%	4.35[c] ± 0.17
	0.50%	3.41[d] ± 0.10
	1.00%	3.24[d] ± 0.18
	1.50%	2.86[e] ± 0.08
	2.00%	2.38[g] ± 0.09
Malathion	0.01%	4.68[b] ± 0.14
	0.05%	4.36[c] ± 0.12
	0.10%	4.21[c] ± 0.25
	0.50%	3.23[d] ± 0.13
	1.00%	3.01[e] ± 0.07
	1.50%	2.59[f] ± 0.09
	2.00%	2.21[g] ± 0.14

SD standard deviation

[a]Means followed by the same letter is not different at the 5% level of significance, based on the Duncan multiple range test

$1/10 \times EC_{50}$; $1/5 \times EC_{50}$; EC_{50}; $2 \times EC_{50}$ and $3 \times EC_{50}$ and control group (dH_2O) were used at 12- and 24-h treatment durations in the experiment.

Mitotic index (MI)

Tables 2 and 3 show the data of total cell count of microscopic observations. The mitotic index of root tip cells that were treated with different concentrations of malathion and cypermethrin (0.05%, 0.1%, 0.5, 1%, and 1.5%) for different periods of time (12 and 24 h) decreased compared to that of the control (root tips treated with distilled water only). Root tip treated with malathion showed highest MI (31.51%) at 24-h duration for 0.05% concentration and lowest MI (21.05%) at 12-h duration for 1.5% concentration (Table 2). Similarly, root tip treated with cypermethrin showed highest MI (35.21%) at 24-h duration for 0.05% concentration whereas lowest MI (20.22%) at 24 h for 1.5% concentration (Table 3). Overall, average MI was found to be maximum at 24 h in case of both malathion (26.2%) and cypermethrin (28.2%) (Fig. 3).

Chromosomal aberration frequency (CF)

Cytological aberration frequency estimation indicates that all the tested concentrations of the two insecticides (0.05%, 0.1%, 0.5, 1%, and 1.5%) induced chromosome abnormalities mostly at metaphase and anaphase stages of mitotic cell division (Figs. 4 and 5). Most types of chromosome aberrations observed in high percentage were stickiness, disturbance, c-metaphase, chromosome bridges in anaphase, lagging chromosome, binucleate lesions, etc (Fig. 5). Treatment

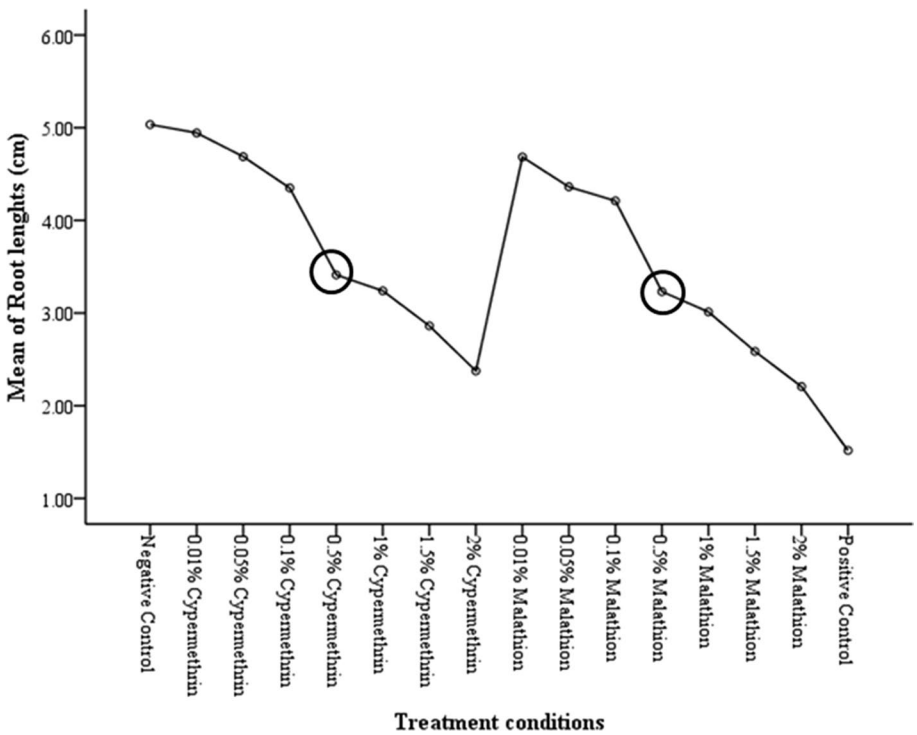


Fig. 2 Dose–response curve between test concentrations and control groups with EC_{50} value

Table 2 Mitotic indices and chromosomal aberrations observed in *A. cepa* root meristem treated with malathion

Treatment	Duration (h)	Conc. (%)	Total no. of cells analysed	No. of cells showing division	No. of cells showing aberrations	Mitotic Index (%)	MI mean ± SE	Chromosomal aberration frequency (CF)	CF mean ± SE
Control	12	DW	279	116	–	41.58	41.58 ± 0.87	–	–
	24		281	113	–	40.21	40.21 ± 0.92	–	–
Malathion	12	0.05	273	76	20	27.84	24.4 ± 1.28	0.26	0.23 ± 0.102
		0.10	310	80	13	25.81		0.16	
		0.50	302	77	25	25.50		0.32	
		1.00	344	75	13	21.80		0.17	
		1.50	342	72	16	21.05		0.22	
	24	0.05	311	98	28	31.51	26.2 ± 1.69	0.29	0.30 ± 0.132
		0.10	291	82	24	28.18		0.29	
		0.50	262	66	17	25.19		0.26	
		1.00	250	61	21	24.40		0.34	
		1.50	231	50	15	21.65		0.30	

Table 3 Mitotic indices and chromosomal aberrations observed in *A. cepa* root meristem treated with cypermethrin

Treatment	Duration (h)	Conc. (%)	Total no. of cells analysed	No. of cells showing division	No. of cells showing aberrations	Mitotic Index (%)	MI mean ± SE	Chromosomal aberration frequency (CF)	CF mean ± S.E
Control	12	DW	279	116	–	41.58	41.58 ± 0.87	–	–
	24		281	113	–	40.21	40.21 ± 0.92	–	–
Cypermethrin	12	0.05	240	71	33	29.58	24.6 ± 1.29	0.46	0.38 ± 0.171
		0.10	250	60	26	24.00		0.43	
		0.50	210	50	15	23.81		0.30	
		1.00	312	72	18	23.08		0.25	
		1.50	233	52	24	22.32		0.46	
	24	0.05	267	94	11	35.21	28.2 ± 2.86	0.12	0.22 ± 0.099
		0.10	351	118	19	33.62		0.16	
		0.50	280	80	21	28.57		0.26	
		1.00	254	60	13	23.62		0.22	
		1.50	267	54	19	20.22		0.35	

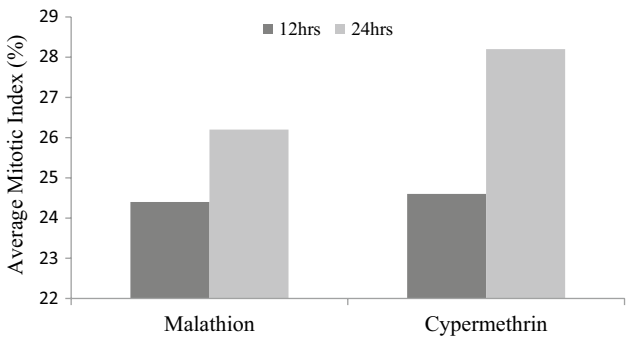


Fig. 3 Average effect of malathion and cypermethrin at different treatment durations on mitotic index of root tip cells of *A. cepa*

with malathion at 1% (24 h) showed highest (0.34) chromosomal aberration frequency and lowest (0.16) at 0.1% (12 h) concentration (Table 2). Treatment with cypermethrin 0.05% and 1.5% at 12-h duration showed the highest (0.46) chromosomal aberration frequency and lowest aberration frequency at 0.05% at 24-h duration (Table 3). Overall, average chromosomal aberration frequency was observed to be maximum at 24-h treatment duration in malathion whereas at 12-h treatment duration in cypermethrin (Fig. 6).

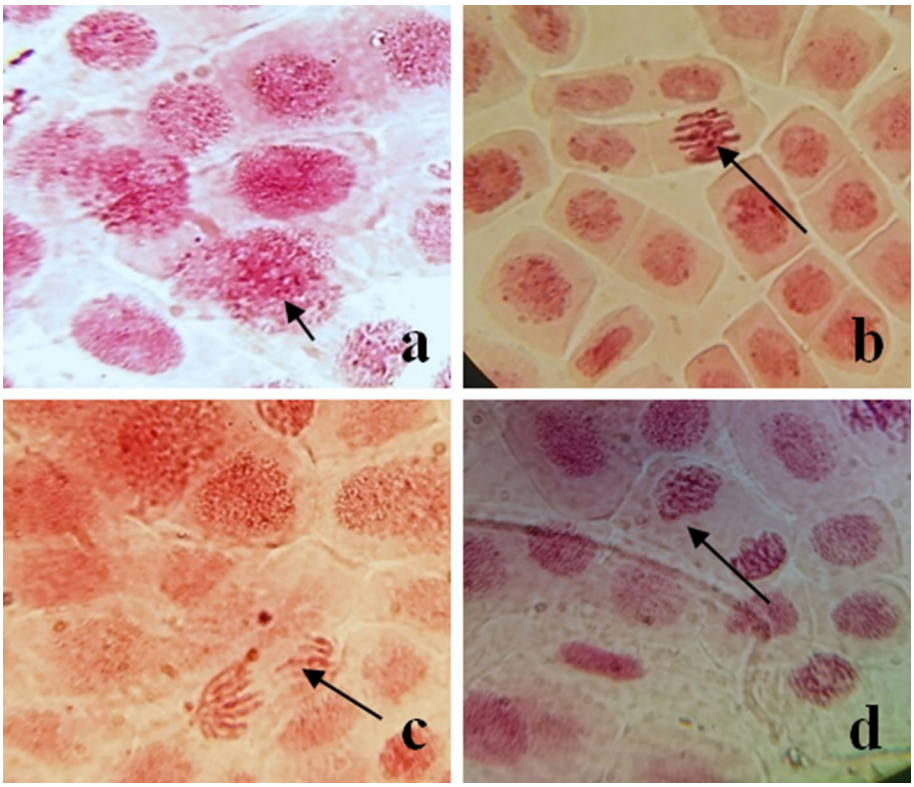


Fig. 4 Photoplates showing normal stages of onion root tip cells in control condition. **a** Prophase, **b** metaphase, **c** anaphase, **d** telophase

Discussion

Cellular abrasion, cytotoxicity, and genotoxicity are the new orders of physioanalytic paradigm observed in dividing plant cells to monitor the toxicological effects of chemical-based biocides. Here, the toxicological effects of malathion and cypermethrin were checked and analyzed on *A. cepa* plant model. Malathion is an OP herbicide or pesticide which is generally regarded as neurotoxic as it irreversibly inactivates acetylcholinesterase (AChE) enzyme rendering neuro-associated disorders eventually leading to fatality. Plants are always regarded as good and safe study models for the assessment of various biocides used in agriculture, domestic and forest-like environments and their immediate settings. Plants play a crucial role in every biogeochemical cycle as they are both direct and indirect co-associates, in scavenging these used biocides along with the soil micro-flora. These agrotoxic compounds applied to ward off pests get accumulated in the plant systems and this property can be used to monitor the cytotoxic effects of the biocides. On the other hand, cypermethrin is a synthetic pyrethroid used as an insecticide in large-scale commercial agricultural applications as well as in consumer products for domestic purpose. It behaves as a fast-acting neurotoxin in insects. It is easily degraded in soil and plants but can be effective for weeks when applied to indoor inert surfaces.

In the present study, we have reported the potential cytotoxic effect or inhibitory effect on the dividing meristematic cells of onion by malathion and cypermethrin. Through this experiment, it was observed that there was a gradual increase in mitotic depression in the root cells treated with increased malathion and cypermethrin concentrations. In the preliminary EC_{50} study, the adverse biological effect of malathion and cypermethrin was observed to be dose dependent and linear with significant decrease in growth parameter with increasing concentrations; a similar result was also reported by Ozkara et al. (2015). The results on mitotic index % revealed the cytotoxic effect of malathion and cypermethrin increases with increasing concentrations and treatment durations. Treatment with malathion at 0.5% (12 h) and 1% (24 h) showed higher chromosomal aberration frequency and lower at 1% (12 h). Treatment with cypermethrin at 0.05% and 1.5% (12 h) showed higher chromosomal aberration frequency and lower at 0.05% (24 h).

The genotoxic effect of malathion and cypermethrin estimated based on chromosomal aberration frequencies showed variations at different concentrations. The observations on treatment durations showed independent effects where longer exposure cannot be generalized as higher mitotic aberrations. This may be due to the different saturation levels at different concentrations of malathion and cypermethrin. Cypermethrin showed higher average aberration frequencies at 12-h treatment duration while

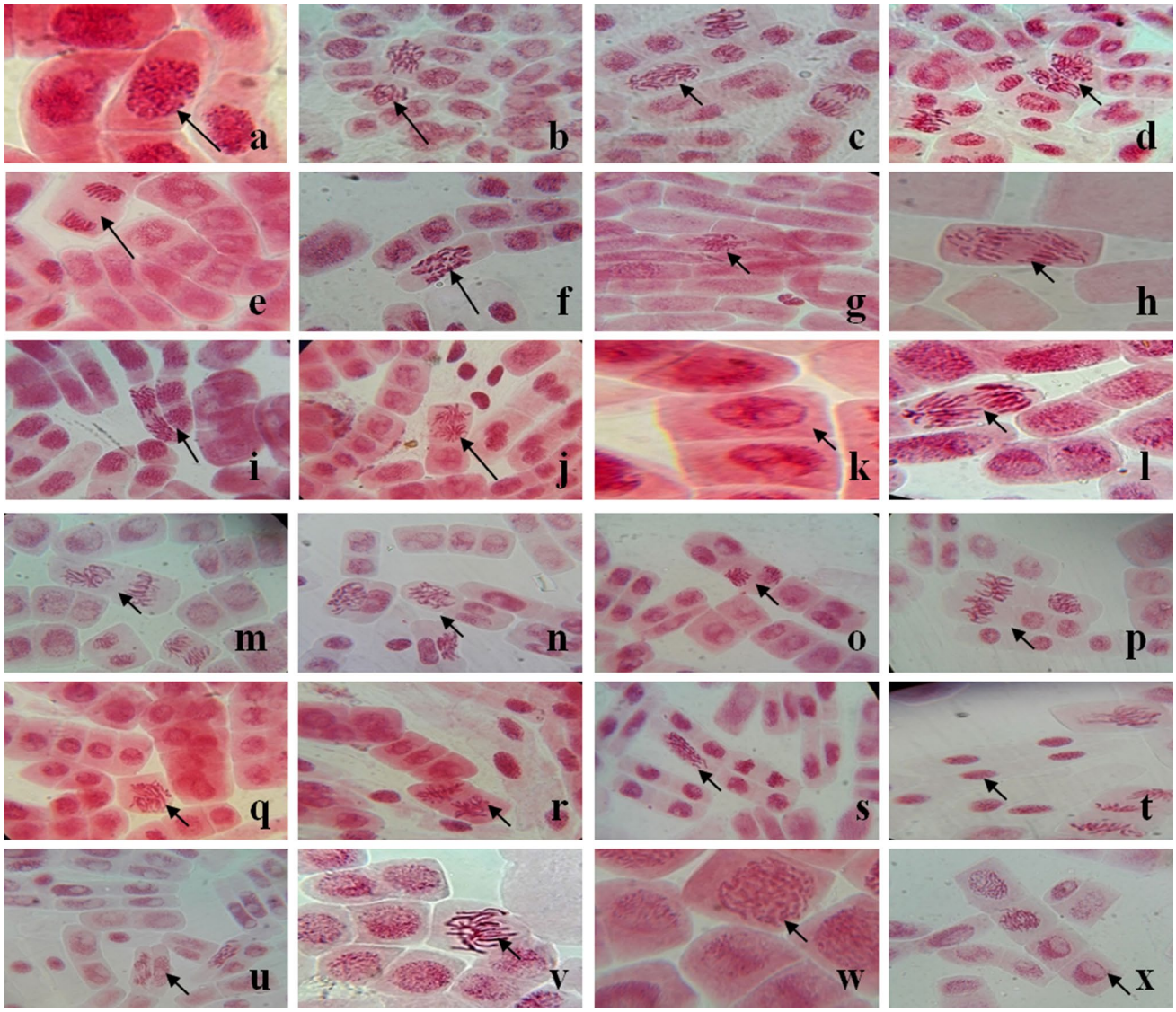


Fig. 5 Photoplates showing chromosomal aberration during 12- and 24-h treatment in different concentrations of malathion and cypermethrin. **a** Sticky prophase, **b** sticky metaphase, **c** chained metaphase in a polyploidy cell, **d** clumped metaphase, **e** sticky anaphase, **f** fragmented metaphase, **g** disturb metaphase, **h** laggard chromosome, **i** fragmented anaphase, **j** star-shaped anaphase with bridge, **k** multiple nuclear lesion, **l** anaphase bridge, **m** abnormal anaphase with breaks, **n** chromosome erosion at metaphase, **o** diagonal sticky anaphase, **p** exposure of chromosome scaffold, **q** clumped metaphase, **r** coagulated chromosome in hypoploid cell, **s** giant cell showing polyploidy, **t** hyperchromasia, **u** diagonal anaphase bridge, **v** vagrant chromosome, **w** late prophase, **x** nuclear lesion at prophase

malathion showed higher average aberration frequencies at 24-h treatment duration.

Effect of malathion and cypermethrin on chromosome behavior

In the control, the onion root tips revealed normal behavior of chromosomes comprising all the stages with normal metaphase 2n = 16. However, when the root tips were collected from 0.05%, 0.1%, 0.5, 1%, and 1.5% concentrations for 12 and 24 h of exposure time in malathion solution, various abnormalities were observed. Chromosome breakage, sticky anaphase, spiral nature of chromosome in telophase stage, normal metaphase with breakage, and disruptive nucleus were observed at different concentrations. Among the total number of cells, most were in the prophase stage followed by anaphase, metaphase, telophase, respectively. Stickiness may be produced by the physical adhesion of chromosomal proteins (Patil and Bhat 1992) or due to disturbances in the nucleic acid metabolism of the cell or the dissolution of protein covering the DNA in chromosomes (Mercykutty and Stephen 1980). Similar chromosomal aberrations such as chromosomal bridge, lagging chromosomes, unequal distribution of nucleus, chromosome divergence, uncoiling abnormality, and laggards were observed in various other studies (Chutia et al. 2012; Hore and Tanti 2014;

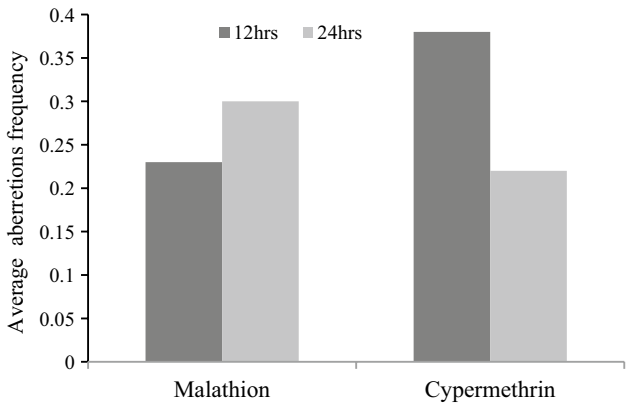


Fig. 6 Average effect of malathion and cypermethrin at different treatment durations on chromosome aberration frequency in root tip cells of *A. cepa*

Sarma and Tanti 2014, 2015; Tanti et al. 2009, 2012; Toijam et al. 2013). When the root tips were collected from 0.05%, 0.1%, 0.5, 1%, and 1.5% concentration for 12- and 24-h exposure time in cypermethrin solution, here also various abnormalities were observed. In all the above concentrations for 24 h, laggard chromosome, multiple nuclear lesion, vagrant chromosome, clumped metaphase, sticky prophase, anaphase bridge, and fragmented metaphase were observed. Chromosome bridge is formed by breakage and fusion of chromosomes and chromatids, the stickiness of chromosome and subsequent failure of free anaphase separation, and unequal translocation or inversion of chromosome segments (Gomórgen 2005). Permjit and Grover (1985) attributed laggard chromosomes to the delayed terminalization, stickiness of chromosome ends or the failure of chromosomal movement. Stickiness observed in the pyrethroid-treated onion roots may be due to physical adhesion of the proteins of the chromosome (Patil and Bhat 1992). Cypermethrin and alphamethrin were reported to elicit varying degrees of cytotoxic, turbagenic (toxicity to spindle) and clastogenic effects but generally more turbagenic and weak clastogenic (Rao et al. 2005). However, Asita and Makhalemele (2008) reported that alpha-thrin (active ingredient of alpha-cypermethrin) was only cytotoxic but not genotoxic at various concentrations in treated *A. cepa*. Cypermethrin has been classified as a possible human carcinogen (EPA 1992). A similar genotoxic effect of cypermethrin was also observed in the root meristem cells of sunflowers (Inceer et al. 2009). Rank and Nielsen (1997) attested that any chemical which found to be causing deleterious chromosomal changes in a dependable plant assay should be considered as a hazardous substance to damage the chromosomes of other organisms in the environment. Hence, the results indicate that the insecticides malathion and cypermethrin should be regarded as cytotoxic and genotoxic agents that show clastogenic activity in *A. cepa* assay.

In the present study, inhabitation of growth, decrease in mitotic index and induction of chromosomal aberrations in *A. cepa* assay substantiated that malathion and cypermethrin have significant cytotoxic and genotoxic potency in biological system. As malathion and cypermethrin are among the widely used pesticides, the results revealed their possible harmful effects on the exposed crops, which also includes major food crops like rice, wheat, etc. Also, the accumulations of these chemicals in the plant parts may be detrimental to human health, while direct applications may affect useful soil microbes. In conclusion, malathion and cypermethrin were found to be toxic in the plant test systems; thus, appropriate optimization or safer alternatives are necessary for future agricultural sustainability. Indeed, there is an urgent need for further awareness about the environmental and health problems associated with the use of synthetic pesticides in agriculture among the farmers and plant breeders, especially in the northeast region of India, to ensure the complete replacement currently used synthetic products with much safer alternatives sourced from nature.

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Author contributions NS and HP: contributed to design and performance the experiments, and analysis and interpretation of observations and data; RAL: contributed to the conception, drafting and revision of the manuscript. All authors reviewed the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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