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Genotoxicity evaluation of the insecticide Chlorpyrifos Using Chromosomal Behaviour of Root Meristem in *Allium cepa* L.

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ABSTRACT
The genotoxic potential of organophosphate insecticide Chlorpyrifos 20% EC was evaluated by using chromosomal aberration in root meristem of Allium cepa L. The rooted bulbs of Allium cepa were treated with different concentration of insecticide (0.2%, 0.4%, 0.6%, 0.8% and 1.0%) for different time period (24, 48 and 72h). Results revealed that mitotic index gradually decreases in all sets of treatments and minimum (5.13%) in highest concentration and treatment period which is lower than control plants (15.69%). The cytogenetic end points like mitotic depression, relative abnormality rate and chromosomal abnormality frequency were gradually increased from low dose and duration (0.2%,24 h) to high dose and duration (1.0%, 72 h) and have maximum value at 1.0%, 72 h is 67.3%, 55.76% and 2.86% respectively. It shows significant difference ($p \leq 0.05$) when compared with control plants. These abnormalities appeared in various degree depending on treatment duration and concentration of chlorpyrifos. Among various chromosomal aberration, chromatin bridge, breaks, stickiness, laggard, vagrant, fragments, C-mitosis, multipolarity, ring chromosome, as well as micronuclei were observed in mitotic preparation. The cytogenetic biomarker are very efficient and non-expensive tool to screening the potential of toxicity at chromosomal as well as DNA level. Toxicity assessment of the pesticide chlorpyrifos shows that it was highly toxic to plant cell.
Pesticide
Keywords: *Chlorpyriphos, Cytotogenotoxicity, Mitotic Index, Chromosome abnormality, Allium cepa L.*

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INTRODUCTION

Insecticide is used in agricultural areas to avoid losses from insect and increasing agriculture output to feed over growing population and control vector born disease. Many cytological studies have been carried out to detect the harmful effect of pesticide on different plants [1,2,3]. In some studies, insecticides inhibited the root growth and shows detrimental effects on meristematic cells and cellular structure or rapturing cell membrane [4,5]. However, potential harmful effects of pesticide considered. The main problem arising from the uncontrolled use of insecticide in agriculture is environmental contamination caused by these agents or their by product which can have negative impact on ecosystem including human health. [6,7].
This pesticide belongs to the group Chlorinated organophosphate insecticide. The organophosphate are esters of phosphoric acid, thiophosphoric acid and other phosphoric acid [8] and are precursors of many insecticides, herbicide and nerve agent. Chlorpyrifos [diethyl o-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is one of the most used organophosphate insecticide on field crops vegetables and fruit crops. This is moderately hazardous as class-II classified by WHO. Extensive use of organophosphate pesticide it has created many negative influences on environment because of their toxicity. Several investigators had studied the adverse effect of the pesticide on genetic material of plant cell [9, 10]. *Allium cepa* test assay is used in screening and monitoring of genotoxic agents according to the standard protocol for the plant assay established by the international program on chemical safety (IPCS) and the world health organization [9].
The *Allium cepa* is an efficient test material for chemical screening and in situ monitoring for cytogenotoxicity due to meristematic nature of plant root [11] and low chromosome number (2n=16) and is large in terms of structure. Onion are inexpensive and easy to obtained as they can be grown any season around the year [12] for these reason, *A. cepa* was chosen as the test material in this research.
There is no such information about cytogenetic abnormality caused by chlorpyrifos 20 EC in *Allium cepa* L. plant. Therefore, the objective of the present study was to evaluate the possible genotoxic effects of

insecticide by using *A. cepa* as a biological system. To accomplished these, mitotic index and chromosomal aberration in different mitotic phases in mitotic cell.

MATERIAL AND METHODS

A fresh healthy onion bulb of *Allium cepa* of approximately same size collected from local market. The old roots were removed from the reduced stem and exposed root disc were suspended into plastic cups containing distilled water for three days to facilitate root growth. The onion bulbs were transferred from the plastic cups containing the distilled water to those containing the different concentration of Chlorpyrifos 20% EC (0.2%, 0.4%, 0.6%, 0.8% and 1.0% respectively) while some of the bulbs were transferred into new plastic cups containing distilled water to serve as control. The duration of treatment ranged from 24, 48, 72 hours respectively for each of the concentrations including the control. After completion of treatment period the root tips were cut and fixed in carnoy's fixative (Absolute alcohol, glacial acetic acid – 3:1) for 24 hrs. and washed three times with distilled water. After fixation, the root tips were hydrolyzed with 1N HCl at 60^{o} C for 10 minutes in order dissolve cell wall [13] and washed with distilled water thrice and stored. The root was transferred on a glass slide and cut the root tip (1-2mm) with surgical blade and then dipped in a drop of 2% acetocarmine for 2 minutes. The cover slip was carefully placed over the slide by avoiding the entry of air bubble. Finally, pressed the section of slide containing stained root tip by thumb pressure by wrapping the slide with blotting paper which help to absorbed extra stained. The edge of cover slide was sealed with clear nail varnish for preservation [14].

Preparation of slides and Microscopic examination

For the determination of mitotic index (MI) and the frequencies of chromosomal aberration (CA). All prepared slides were subjected to microphotography with the help of microscope with camera attachment. Objective lenses taken into consideration were of 10X, 40X and 100 X magnification. Cider oil emulsion was applied for the lens of 100 X magnification. Five slides were prepared from each set of treatment and on every slide almost 1000 cells were observed.

Cytogenotoxic assessment

Cytogenotoxicity was determined by Mitotic index. The mitotic index and chromosomal aberration were calculated according to the standard method described by Bakare *et al.,* [15] The total number of cells of each set of treatment was calculated by this method-

$$Mitotic\ index = \frac{Number\ of\ dividing\ cell}{total\ number\ of\ cells} X\ 100$$

In this study, at least 1000 cell were counted in each slide and mitotic index was calculated by determining the cells undergoing mitosis within 1000 cells and stage of their division.

$$Mitotic\ depression\ = \frac{MI(Control) - MI(Treated)}{MI(Control)} X\ 100$$

The percentage of aberrant cells can be calculated by eq-

$$\%\ of\ aberrant\ cells = \frac{Number\ of\ aberrant\ cells}{Total\ number\ of\ cells}\ X\ 100$$

The percentage of aberrant cells can be calculated by eq-

$$\%\ of\ aberrant\ cells\ (PAC) = \frac{Number\ of\ aberrant\ cells}{Total\ number\ of\ cells}\ X\ 100$$

$$\%\ of\ Relative\ abnormality\ rate\ (RAR) = \frac{Number\ of\ aberrant\ cells}{Total\ number\ of\ \ diving\ cells}\ X\ 100$$

The data was analyzed using SPSS 16.0 software with a single significance level of $p \leq 0.05$. the mitotic index (MI) and chromosomal abnormalities were compared using one-way ANOVA.

RESULTS AND DISCUSSION

Cytogenetic biomarker

Mitotic index and mitotic depression in root meristems of *Allium cepa* L.

It is evident from the results the mitotic index decreased considerably in the different treatment groups. Microscopic examination shows that squashes of *Allium cepa* L. root tip meristem cells shows that Chlorpyrifos treatments induced number of chromosome abnormalities when compared with control plants. The increase of mitotic abnormalities was depending on the increasing treatment period and concentration. Increased mitotic index induce the cell proliferation in root meristematic zone and screening tool for all cytogenotoxicity of toxicants [16]. Increased mitotic index also impacts on sub-phases of mitotic cycle. In this study, prophase index declined from concentration 0.2% to 1.0% at 24, 48 and 72h in dose and duration dependent manner. At 24, 48 and 72h it ranged from 43.33% to 38.23%, 41.89% to 36.84% and 41.42% to 36.53% respectively. Similarly, metaphase index also inhibited in all test system. It also declined from lowest dose and duration (24.66%) to highest dose and duration of treatments (19.23%). Anaphase indices also followed same trend and decreases in all sets of treatments (Table 1). Unlike to these indices, telophase index increased in all set of treatments. It stimulated from 12.66% to

26.47% at 24 h, 11.48% to 29.82% at 48 h and 15% to 30.76% at 72h in 0.2% to 1.0% respectively. Obtained data shows different phase indices is attribute to response of meristematic cells towards organophosphate pesticide.

Mitotic index reliable biomarker to determine the cytogenotoxicity [17]. MI is the basic criteria to analyze cytogenotoxicity for all living organism [18]. At all treatment period the mitotic index decreasing in dose and duration dependent manner and have minimum value at 1.0%, 72 h (5.13). (Figure: 3A)

Mitotic depression (MD) significantly ($p \leq 0.05$) increased in all sets of treatments when compared to control. It increased from 6.25 to 56.67% at 24 h, 10.72 to 63.53% at 48 h and 15.04 to 67.3% at 72 h in all concentration applied. These finding on root meristems cells of onion are suggestive of their mito-inhibitory property and this inhibition was more pronounced as well as dose and duration dependent. Mitotic index is directly proportional to mitotic depression [19,20] (Figure: 3D)

Relative abnormality and chromosomal aberration in root meristem of *Allium cepa* L.

The frequency of both Relative abnormality rate (RAR) and percentage aberrant cell (PAC) was induced as per increase in dose and duration of treatment. The RAR stimulated significantly in dose and duration dependent manner. It ranged from 0.62 to 55.76%. The maximum frequency of RAR was apparent at maximum concentration at 72 h. simultaneously PAC was also augmented as per treatment and exposure duration. The stimulation occurs from 0.09 to 2.86. (Table:2) & (Figure: 3B,3C)

Similarly, RAR, MD, PAC a prominent cytogenetic biomarker was significantly induce in dose and duration dependent manner all sets of treatments.

The most common abnormalities were stickiness, c-mitosis, and disturbed metaphase. In addition, at anaphase and telophase, fragments, bridges, lagging chromosome and irregular anaphase were also observed. (Fig. 2). The result obtained from this study reveal a concentration dependent decrease in the mitotic index in the cells of *Allium cepa*. This finding agreed with [21] who reported that pesticide induce a decrease in mitotic index in *Allium cepa*. Mitotic index is an acceptable measure of cytotoxicity for all living organism [22]. The cytotoxicity level can be determined by the decreased rate of mitotic index. A decrease below 50% usually has sublethal effects [23]. If mitotic index decreases below 22% of the control, that it causes lethal effect on test organism [24]. Generally cytotoxic substances inhibiting mitosis effect the microtubule configuration [25]. According to many investigators, abnormalities due to inhibition of spindle formation such as C-mitosis, multipolar anaphases, sticky and vagrant chromosome, reflects high toxicity of pollutants. [26,27,28,29]. The reduction of mitotic activity may result from a blocking of G1 stage suppressing DNA synthesis [30]. Among them, stickiness of chromosome was frequently observed. This is due to the inhibition of spindle formation [31]. Improper folding of chromosome fires that make the chromatid connected by subchromatid bridges as a result of sticky chromosome [32]. The frequency of chromosome stickiness significantly increased ($P < 0.05$) with increasing dose and duration of treatment and maximum at high concentration of 72 hours (20.68%). It reflects highly toxic to the cell, probably leading to cell death. Micronuclei formation in root meristem cell were also observed. It is an indication of mutagenic effect of certain physical and chemical factors [33]. Hence, MN is single method to assess the mutagenic effect of all test chemicals. Kirsch et al.,2011 [34] Stated that formation of micronuclei due to acentric fragments or laggard chromosome that are not able to incorporate in daughter nuclei formation. The maximum frequency of formation of micronuclei at high concentration at 72 hours (10.34%). It causes genomic loss to the organism.

The results from the present study indicates that chlorpyrifos can induce cytotoxic and genotoxic effects on the meristematic cells of *Allium cepa* L. Mitotic activity decreased due to inhibition of DNA synthesis [35] or due to arrest in G2 phase of cell cycle [36]. Several other pesticides have also been reported to induce genotoxic abnormalities by affecting mitotic spindle. [37]

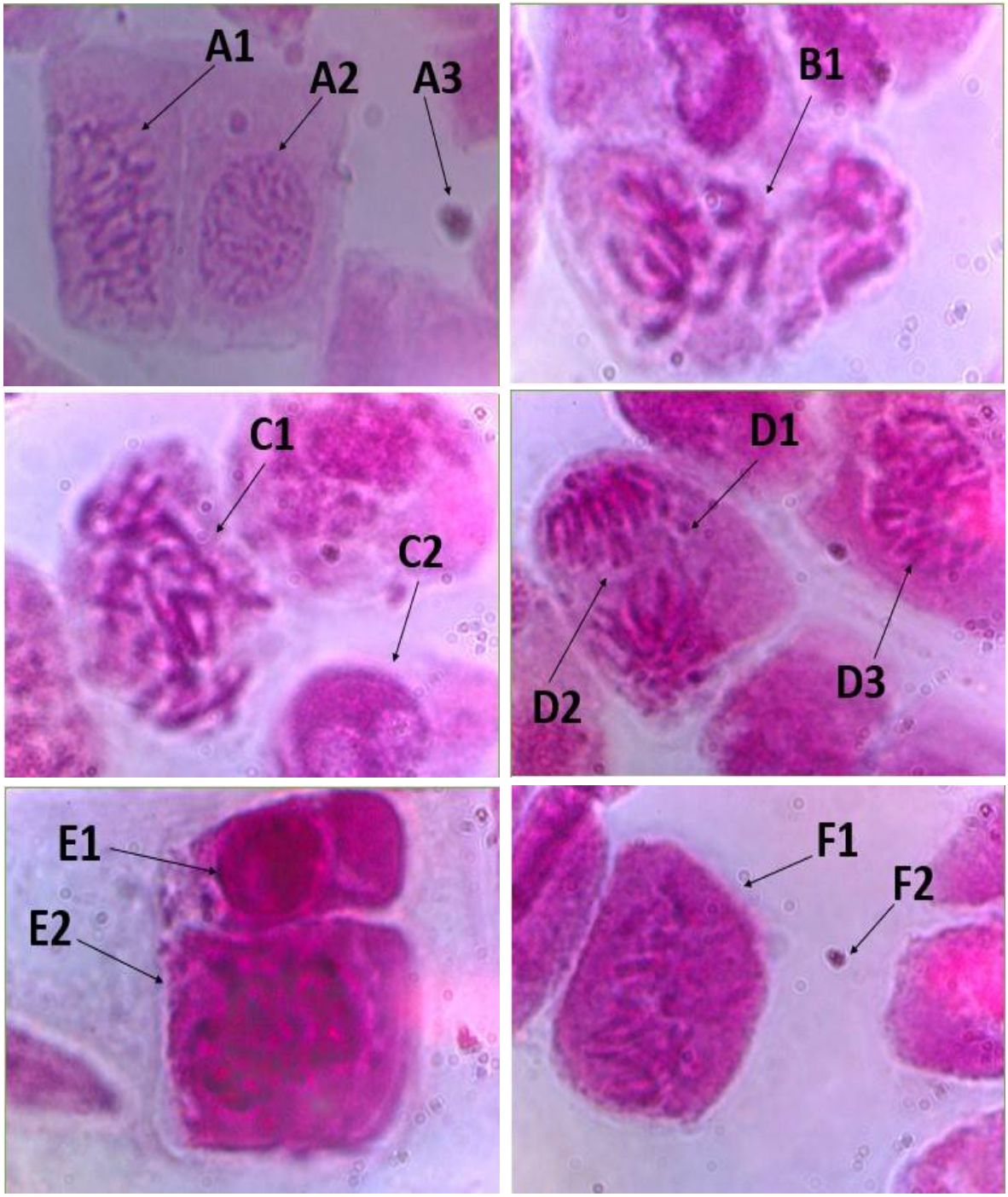


Figure 1 A1: Sticky metaphase, A2: Prophase, A3: Micronucleus; B1: Vagrant chromosome; C1: Irregular Anaphase, C2: Nuclear lesion; D1: Anaphase with bridge, D2: Anaphase with laggard, D3: C-Mitosis; E1: Prophase, E2: Stickiness; F1: Sticky metaphase, F2: Micronucleus.

Table 1: Effect of different concentration of pesticide Chlorpyrifos 20% EC on phase index (Prophase, Metaphase, Anaphase and Telophase) with Mitotic index in root meristem of Allium cepa L. exposed for 24, 48 and 72 h.

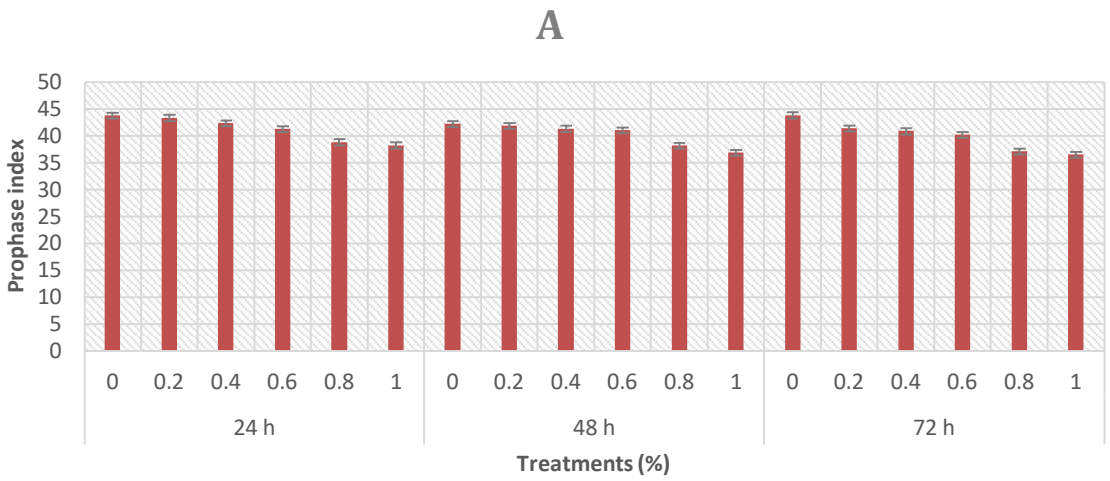
Duration of treatments	Concentration of test pesticide (%)	Total no. of dividing cell	Total no. of cell in prophase	Prophase index	Total no. of cell in metaphase	Metaphase index	Total no. of cell in anaphase	Anaphase index	Total no. of cell in telophase	Telophase index	Mitotic index
24 h	0	160	70	43.75	40	25	32	20	18	11.25	15.51 ± 0.47
	0.2	150	65	43.33	37	24.66	29	19.33	19	12.66	14.54 ± 0.21
	0.4	130	55	42.3	30	23.07	24	18.46	21	16.15	12.4 ± 0.23
	0.6	109	45	41.28	25	22.93	18	16.51	21	19.26	10.4 ± 0.25
	0.8	85	33	38.82	18	21.17	14	16.47	20	23.52	8.5 ± 0.54
	1.0	68	26	38.23	14	20.58	10	14.7	18	26.47	6.72 ± 0.36
48 h	0	161	68	42.23	42	26.08	37	22.98	14	8.69	15.66 ± 0.63
	0.2	148	62	41.89	36	24.32	33	22.29	17	11.48	13.98 ± 0.04
	0.4	121	50	41.32	27	22.31	22	18.18	22	18.18	11.19 ± 0.10
	0.6	100	41	41	22	22	16	16	21	21	10.12 ± 0.42
	0.8	76	29	38.15	16	21.05	12	15.78	18	23.68	7.44 ± 0.24
	1.0	57	21	36.84	11	19.29	8	14.03	17	29.82	5.71 ± 0.33
72 h	0	162	71	43.82	40	24.69	32	19.75	19	11.72	15.69 ± 1.15
	0.2	140	58	41.42	34	24.28	27	19.28	21	15	13.33 ± 0.23
	0.4	115	47	40.86	26	22.6	20	17.39	22	19.13	11.16 ± 0.41
	0.6	92	37	40.21	20	21.73	15	16.3	20	21.73	8.28 ± 0.13
	0.8	70	26	37.14	14	20	11	15.71	19	27.14	6.71 ± 0.16
	1.0	52	19	36.53	10	19.23	7	13.46	16	30.76	5.13 ± 0.59

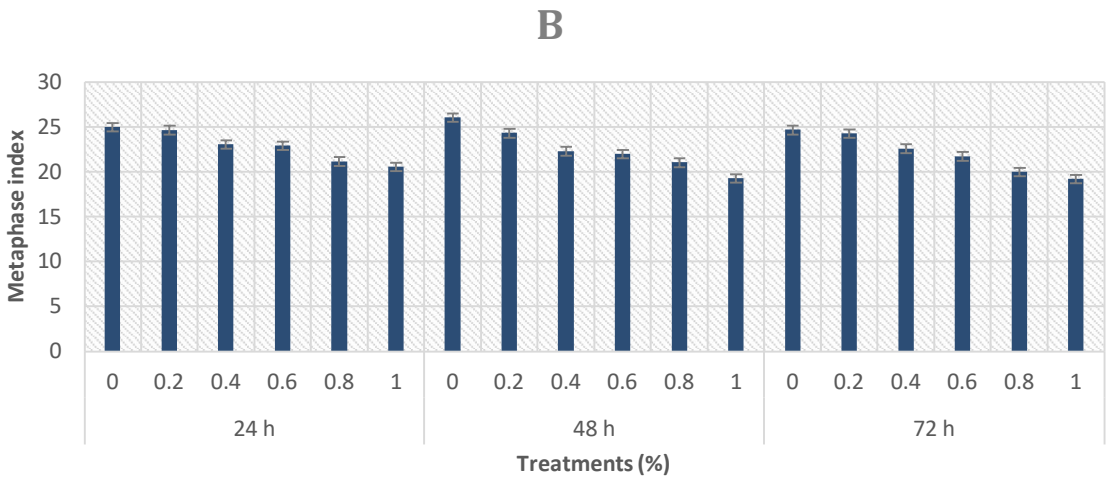
All values are the mean of triplicates. ± S.D. (n=3), different from the control ($P \leq 0.05$)

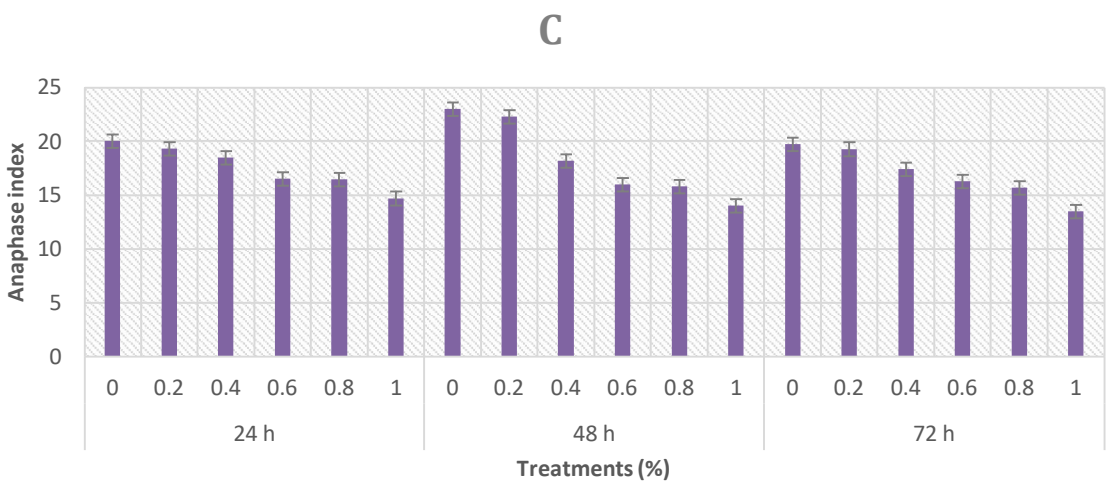
Table 2: Percentage of various Chromosomal (CA) Aberration and Relative Abnormality Rate (RAR) in root meristem of *A. cepa* exposed to different concentration of Chlorpyrifos after 24, 48 and 72 h.

Concentration of test Pesticide	Duration of Treatments	Total no. of cell observed.	Types of Chromosomal Aberration (CA)							Percentage Aberrant Cell (PAC)	Relative Abnormality Rate (RAR)
			Stickiness	C-Metaphase	Disturbed Anaphase	Clumping Anaphase	Laggard	Bridge	Micronuclei		
Control	24 h	1031		100	-	-	-	-	-	0.09 ± 0.05	0.62 ± 0.04
	48 h	1031	100	-	-	-	-	-	-	0.19 ± 0.05	0.62 ± 0.40
	72 h	1048	-	100	-	-	-	-	-	0.47 ± 0.16	0.61 ± 0.85
0.2%	24 h	1048	50	-	50	-	-	-	-	1.04 ± 0.19	1.33 ± 0.38
	48 h	1000	25	50	25	-	-	-	-	1.8 ± 0.21	2.7 ± 1.18
	72 h	1011	20	40	40	-	-	-	-	2.27 ± 0.13	3.57 ± 1.06
0.4%	24 h	1028	60	-	20	20	-	-	-	0.09 ± 0.05	3.84 ± 1.31
	48 h	1058	28.57	14.28	28.57	28.57	-	-	-	0.37 ± 0.16	5.78 ± 1.19
	72 h	1081	22.22	22.22	33.33	11.11	11.11	-	-	0.64 ± 0.13	7.82 ± 0.07
0.6%	24 h	988	9.09	27.27	18.18	18.18	18.18	9.09	-	1.21 ± 0.26	10.09 ± 1.69
	48 h	1021	16.66	25	8.33	16.66	16.66	8.33	8.33	1.95 ± 0.25	12 ± 2.84
	72 h	998	25	25	18.75	12.5	12.5	-	6.25	2.4 ± 0.42	17.39 ± 0.65
0.8%	24 h	1032	27.77	27.77	22.22	16.66	-	5.55	-	0.09 ± 0.10	21.17 ± 1.47
	48 h	1050	20	15	10	20	15	10	10	0.47 ± 0.14	26.31 ± 3.18
	72 h	1030	19.04	9.52	14.28	19.04	14.28	14.28	9.52	0.87 ± 0.1	30 ± 7.16
1.0%	24 h	1110	21.73	13.04	8.69	21.73	21.73	8.69	4.34	1.44 ± 0.03	33.82 ± 0.28
	48 h	1042	20.83	8.33	12.5	16.66	16.66	12.5	12.5	2.01 ± 0.51	42.1 ± 6.83
	72 h	1012	20.68	17.24	10.34	13.79	17.24	10.34	10.34	2.86 ± 0.64	55.76 ± 8.39

All values are the mean of triplicates. ± S.D. (n=3); different from the control ($P \leq 0.05$)


A
Prophase index
50
45
40
35
30
25
20
15
10
5
0
0 0.2 0.4 0.6 0.8 1 0 0.2 0.4 0.6 0.8 1 0 0.2 0.4 0.6 0.8 1
24 h
48 h
72 h
Treatments (%)


B
Metaphase index
30
25
20
15
10
5
0
0 0.2 0.4 0.6 0.8 1 0 0.2 0.4 0.6 0.8 1 0 0.2 0.4 0.6 0.8 1
24 h
48 h
72 h
Treatments (%)


C
Anaphase index
25
20
15
10
5
0
0 0.2 0.4 0.6 0.8 1 0 0.2 0.4 0.6 0.8 1 0 0.2 0.4 0.6 0.8 1
24 h
48 h
72 h
Treatments (%)

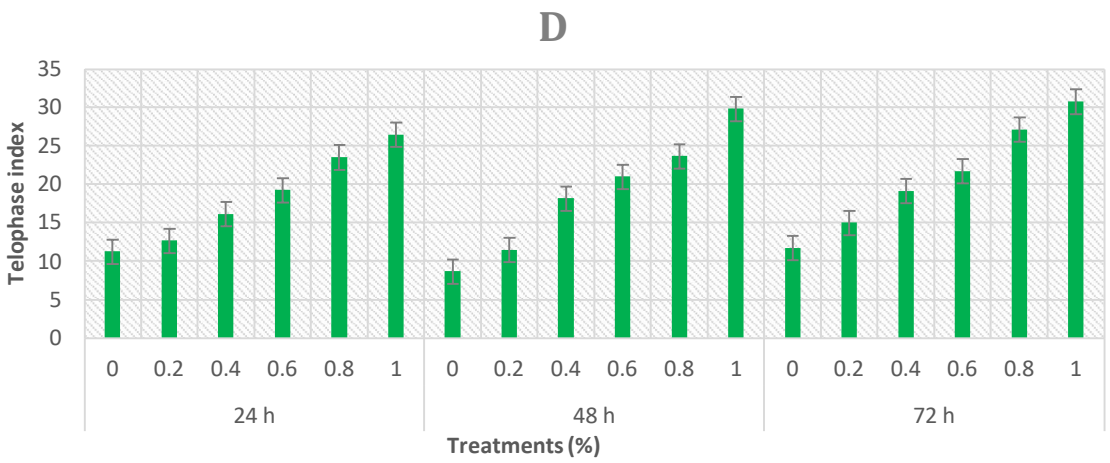
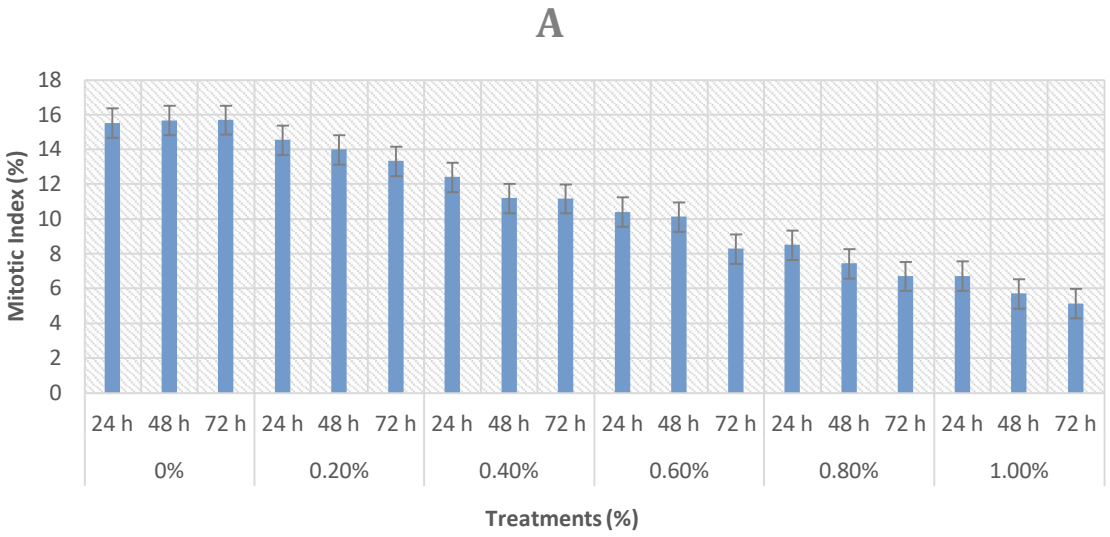
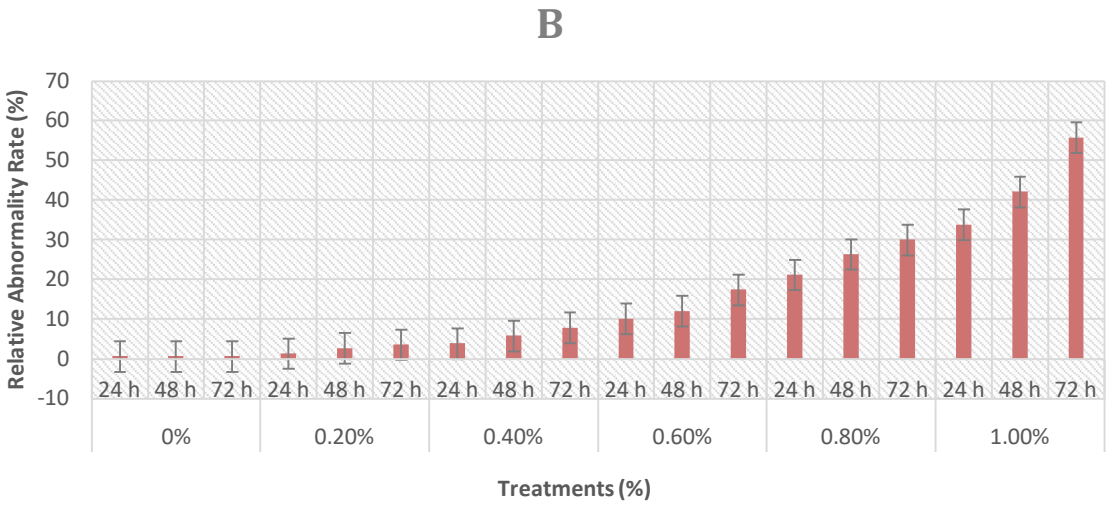


Figure 2: Effect of different concentration (%) and period of treatment of Chlorpyrifos on phase index (Prophase, Metaphase, Anaphase, Telophase) in *Allium cepa* L. at 24, 48 and 72 h.





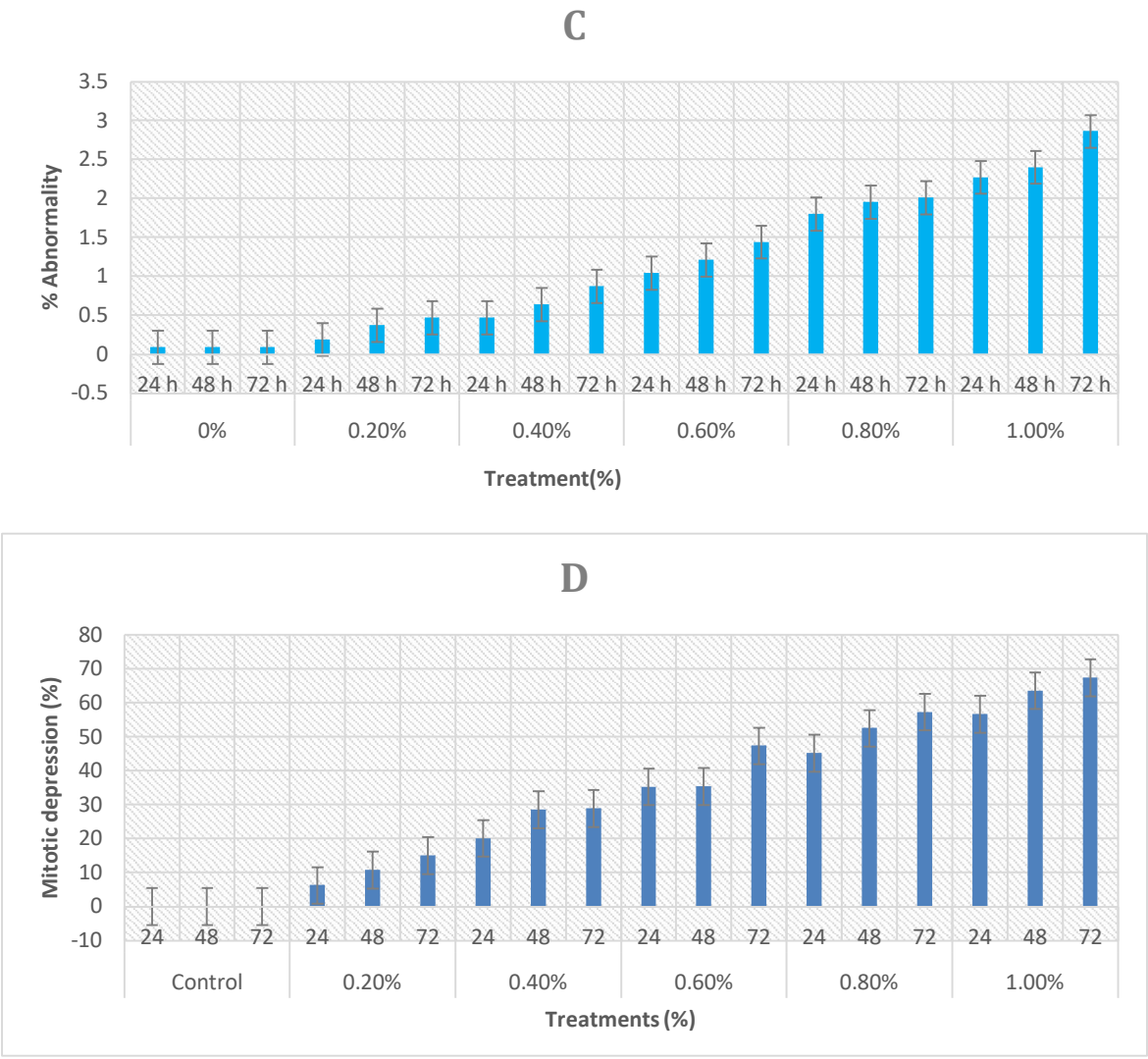


Figure 3: Effects of different dose and duration (hr) of Chlorpyrifos on [A] Mitotic index [B] Relative abnormality (%); [C] and Abnormality (%); [D] Mitotic depression in *Allium cepa* L. at 24, 48 and 72 h. All values are mean of triplicates. ±S.D.

CONCLUSION

On the basis of observation made on different parameter it came to know that in most of the slides prepared from the treated root tips have various cytological abnormalities due to mutagenic effects of pesticide. The pesticide Chlorpyrifos 20% EC has a mito-depressive effect on the mitotic index, and also cause severe cytological and chromosomal aberration on the cells in concentration dependent manner. It has capability to produce variety of mutants and chromosomal aberration even below in the recommended dose. For this reason, it is important to examine the cytogenotoxicity influence of insecticide before considering their application in agricultural goal.

Authors contribution statement

The first author Pravin Kumar, Ph. D. Research Scholar is responsible for sampling, testing, observation and data analysis of the work done and wrote the paper with input from Dr. Vinod Prasad, Ph. D. supervisor. Finally, both authors have discussed the results and contributed to the manuscript.

Conflict of interest

Conflict of interest declared none by authors.

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