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Research Article

GENOTOXIC EFFECTS OF FURADAN AND ENODOSULPHAN ON (*ALLIUM CEPA*) ROOT TIPS

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ABSTRACT

Genotoxicity of pesticides for non-target organism and their influence on ecosystem are of worldwide concern. In the present study, the genotoxic effect of different concentrations of Furadan and Enodosulphan on (*Allium cepa*) root tips was evaluated through chromosome aberration assay (mitotic index and chromosome aberration rate). Both the pesticides showed genotoxic effects with variation with respect to dosage and exposure of time. The decline in mitotic index and increase in percentage of chromosomal aberration was observed as the concentration and duration of treatment was increased. The results showed that both of the tested pesticides are mito-depressive. Mitotic index of both Furadan and Enodosulphan treated root tip cells shows significant decrease in 50 and 100µg/ml concentration for 6 and 24 hrs. The silver staining technique is also done on root tip cells which are treated with Furadan and Enodosulphan 100µg/ml concentration for 24 hours duration. Silver staining results showed the presence of cell proliferation.

Keywords: Furadan, Enodosulphan, *Allium cepa,* Genotoxic, Silver staining.

INTRODUCTION

The use of pesticides in agricultural field is increasing rapidly. Extensive use of pesticides will lead damage to the ecosystem and also human health. There are more than 1000 chemicals classified as pesticides [1]. Most of the pesticides are chemicals that are used in agriculture for the control of pests, weeds or plant diseases. The first report of poisoning due to pesticide in India came from Kerala in 1958 where over 100 people dead after consuming wheat flour contaminated with parathion. Victims of pesticide poisoning accidents are often members of general population, for example in the recent catastrophe in Bhopal, India. There was an accidental release of toxic raw materials such as methyl isocyanate from pesticide manufacturing plant [2]. Cytogenetic damage associated with pesticide exposure has come from occupational exposure increase chromosomal damage among herbicide and insecticide sprayers during peak season was observed [3].

Pesticides are an important group of environmental pollutants and many of which are reported to be mutagenic [4]. Endosulfan is an organochloride pesticide. Chemical name is 6, 7, 8, 10, 10 - hexachloro -1, 5, 5, 6, 9, 9 alpha - hexahydro -6, 9-methano -2, 3, 4-benadioxathipein 3-oxide. Molecular formula is $C_9H_6C_{16}O_4$. The technical Enodosulphan is a brownish crystalline solid and it is a mixture of two isomers α and β. The isomer α is more insecticidal and is converted to more stable β at higher temperature. It is hydrolyzed slowly by water and more rapidly by acids and bases.

Carbofuran is an odorless white crystalline solid with a melting point range of 150-154° C. It is slightly soluble in water and highly soluble in N-methyl-2-pyrrolidone, dimethyl formaide, acetone and xylene. The chemical name of carbofuran is 2, 3 dihydro, 2, 2-dimethyl 7 benzo furanyl methyl carbamate ($C_{12}H_{15}No_3$) [5]. Carbofuran is a systemic acaricide, insecticide and nematicide used on much food crops mainly corn-alfalfa, sorgum, potatoes, sugar beets, canola, sunflower and mixed vegetables and on select non-food uses such as cotton, ornamentals and tobacco. Other names and registered trade names include Furadan, Curatera, Yaltox [6]. The present study is carried out to evaluate the genotoxic effects of Furadan and Enodosulphan on onion root tips by *Allium* test. Determination of mitotic index variations at different concentrations and exposure time was carried out. Evaluation and analysis of cell proliferation by AgNOR count.

Test Agents

Furadan and Enodosulphan samples were used for this study. Before each test was carried out, the effluent was equilibrated to room temperature (26 ± 2°C) and diluted with dechlorinated tap water to produce the dilutions investigated.

Assay Procedure

The assay was carried out using a plastic tube (Diameter, 2.3 cm; Length, 7.8 cm) in a rack. Dechlorinated tap water was used as control and for dilution of pesticides.

Allium cepa Root Growth Inhibition Assay

Onion bulbs (*Allium cepa* L., 2n=16) of the purple variety of average size (15-22 mm diameter) were purchased in the local market of Kottayam, Kerala. Bulbs were made germinated in common portable water (without any growth factors) during the course of 2-4 days. Then the bulbs were carefully removed without any damage to the roots. Infectious bulbs are discarded for the experimental purposes. The outer scales of onion bulbs and brownish bottom plates were removed without injuring the ring of root *Primordia*. Onion bulbs with good root growth were selected.

For root growth inhibition evaluation, freshly prepared stock extracts were diluted into 50µg/ml and 100µg/ml. Seven onion bulbs (with roots) were utilized for each concentration and the control (tap water). The base of each of the bulbs (roots) was suspended on the extracts inside 100 ml beakers in the dark for 6 to 24 h. At the end of the exposure period, root tips from these bulbs were cut and fixed in ethanol: glacial acetic acid (3:1, v/v). These were hydrolyzed in 1N HCl at 60° C for five minutes after which they were washed in distilled water. Two root tips were then squashed on each slide, stained with acetocarmine for 10 min and cover slips carefully lowered on to exclude air bubble. The cover slips were sealed on the slides with clear fingernail polish as suggested by [7].

This is to prevent drying out of the preparation by the heat of the microscope [8]. Six slides were prepared for each concentration and the control out of which five (at 1000 cells per slide) were analyzed at ×1000 magnification for induction of chromosomal aberrations. The mitotic index was calculated as the number of dividing cells per 1000 observed cells [9]. The frequency of aberrant cells (%) was calculated based on the number of aberrant cells per total cells scored at each concentration of each extract [10].

Microscopic Examination

All slides were coded and examined blind. The mitotic index (MI) was determined by the examination of 500 cells per concentration (100 cells per slide). Characterization of mitosis and chromosomal aberrations were scored in 100 cells per slide. Photomicrographs of some aberrant cells were taken. Mitotic Index was estimated as number of dividing cells over the total number of cells counted expressed in percentage. The phase indices were estimated as number of cells in each mitotic phase over the number of dividing cells expressed in percentage. Similarly, the percentage abnormal

cells were calculated as the number of aberrant cells over the number of dividing cells.

Argyrophilic Nuclear Organizer Region (AgNOR) Staining

Argyrophilic Nuclear Organizer Region (AgNOR) staining is also carried out to detect the number of AgNORs. Onion bulbs with roots (0.5-1.0 cm long) were exposed to 100 µg/ml concentrations of Furadan and Enodosulphan for 24 hours duration alone were analyzed. Bulbs kept in distilled water served as control. The treated root tips were washed with distilled water and fixed in freshly prepared 1:1 10% formalin and 1% hydroquinone for 24 hours. The fixed root tips are then stained with 2% silver nitrate solution. The root tips are washed in distilled water and refixed in 1:1 10% formalin and 1% hydroquinone in darkness for a minimum of two hours. Squash preparations are made with 45% acetic acid. The slides are then temporarily sealed and examined under a microscope and a number of AgNORs were counted and recorded.

RESULTS AND DISCUSSIONS

The cytotoxic and genotoxic effect of two pesticides Furadan and Enodosulphan on *Allium cepa* roots have been determined on the basis of chromosomal aberrations and mitotic indices. The root cells of *Allium cepa* treated with Furadan in all concentrations (50 and 100 µg/ml) for 6 and 24 hrs showed various types of chromosomal aberrations (Figure 1). The cytological aberrations observed in onion root tips were multi-polar anaphase, telomere puffing, disorted metaphase, metaphase clumping, laggards, strap nucleus, strap nucleus with micronucleus, anaphase bridge, telophase with one end puffing, C-metaphase, mature cell puffing and micronucleus (Plate 1).

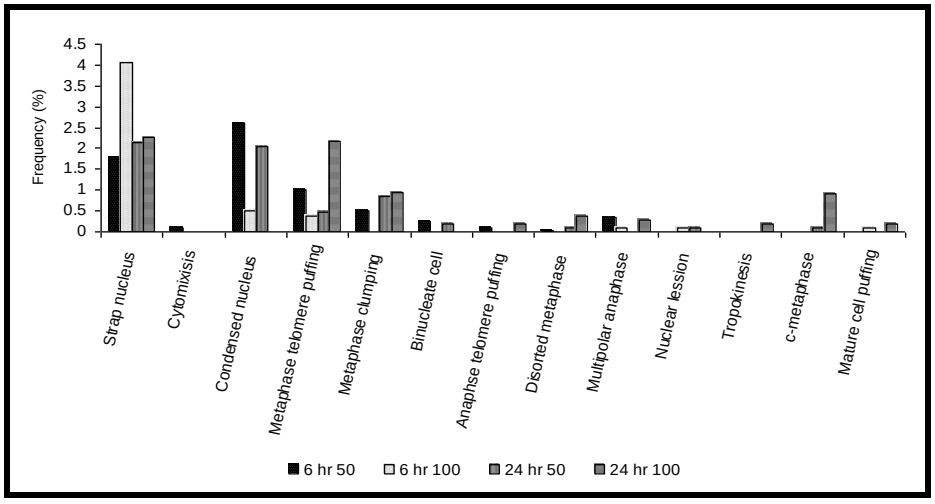
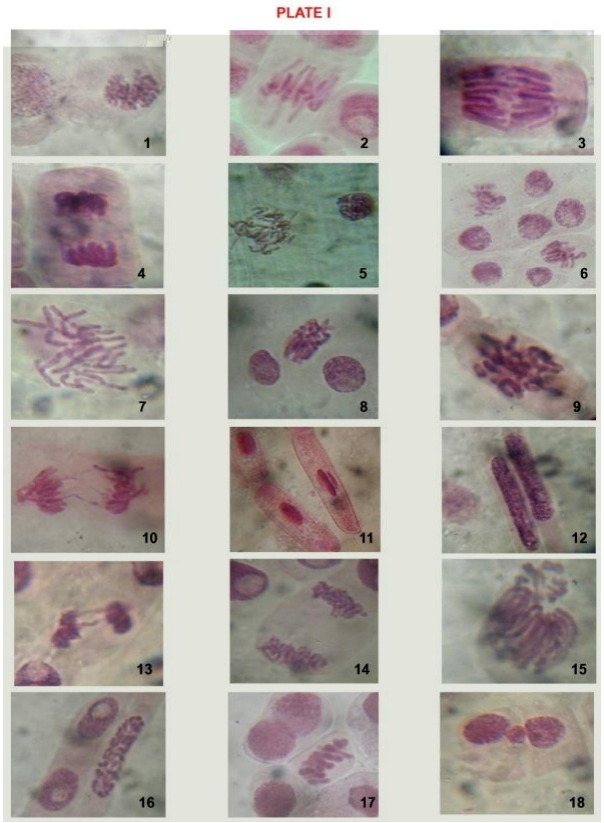


Figure 1: Percentage of aberration in Furadan treated root tip cells of *Allium cepa.*



Stages of cell division: 1.Prophase 2.Metaphase 3.Anaphase 4. Telophase.
Types of Aberrations: 5.Multipolar anaphase 6.Telomere Puffing 7.Disoriented metaphase 8.Late prophase showing puffing and stickness 9.Metaphase sticking 10.Laggards 11.Strap nucleous with micronucleus 12.Strap nucleous 13.Anaphase bridge 14.Telophase with one end puffing 15 C-metaphase 16.Mature cell division showing puffing 17.High degree of clumping 18. Micronucleus.

Control cell examined under microscope showed normal four stages of mitosis. Multipolar or distorted anaphase was observed in 100µg/ml concentration of cell treated with Furadan for 6 and 24 hours duration. Telomere puffing was observed in both 50 and 100 µg/ml concentration of cells treated with Furadan for 6 and 24 hours duration.

Disoriented metaphase was observed in all concentrations for 6 to 24 hours treatment of Furadan (Plate 1). Late prophase showing puffiness and stickiness were observed in Furadan treated cells with 100µg/ml concentration for 24 hours duration. Chromosome laggards were observed in cells treated with Furadan. Strap nucleus and strap nucleus with micronucleus were observed more frequently in all concentration for 6 and 24 hrs treatment of Furadan.

Anaphase bridge were observed in 100µg/ml concentration for 6 and 24 hrs hour treatment with Furadan (Figure 2 and 3). Telophase with one end puffing observed in cells treated with Furadan of concentration 100µg/ml for 24 hours. C-metaphase another type of abnormality found in 50µg/ml concentration of Furadan treated cell for 24-hour duration. Mature cell puffing was observed in 100µg/ml concentration for 6 and 24 hrs treatment of Furadan. A high degree of clumping was also observed in Furadan treated cells.

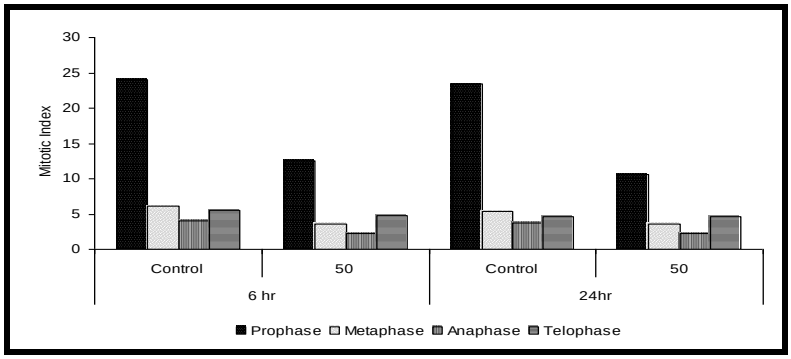


Figure 2: Mitotic index of *Allium cepa* root tip cells treated with Furadan (50µg/ml).

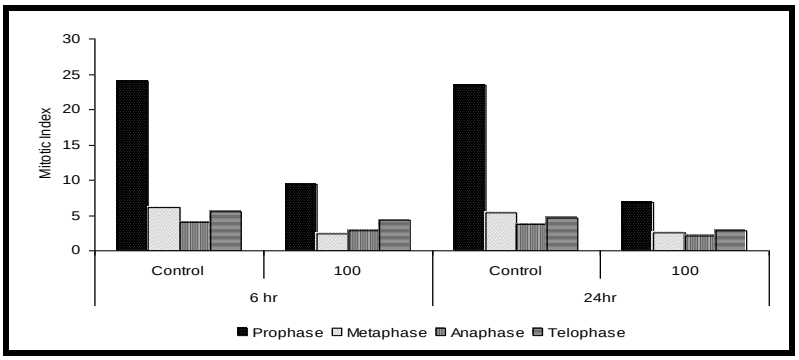


Figure 3: Mitotic index of *Allium cepa* root tip cells treated with Furadan (100µg/ml).

Micronucleus was found in 100µg/ml concentration of Furadan treated cell with duration of 24 hours. The mitotic index of Furadan treated root tip cells shows significant decrease in 50 and 100µg/ml concentrations for 6 to 24 hours. The root tip cells of *Allium cepa* treated with Enodosulphan concentrations for 6 to 24 hours showed various types of chromosomal aberrations. The cytological aberrations observed in onion root tip cells are strap nucleus, telomere puffing, scattered metaphase or disoriented metaphase, metaphase clumping, multipolar anaphase, laggards, star anaphase, anaphase bridge, nuclear lesions, bi-nucleate cell, nucleoids, tropokinesis, cell showing one end anaphase stage and another end uncoiled telophase puffing. Strap nucleus one of the major abnormality was observed in all concentrations for 6 and 24 hrs hour treatment of Enodosulphan (Figure 4, 5 and 6). Metaphase clumping was observed in both 50 and 100 µg/ml concentration of cells treated with Enodosulphan for 24 hours duration.

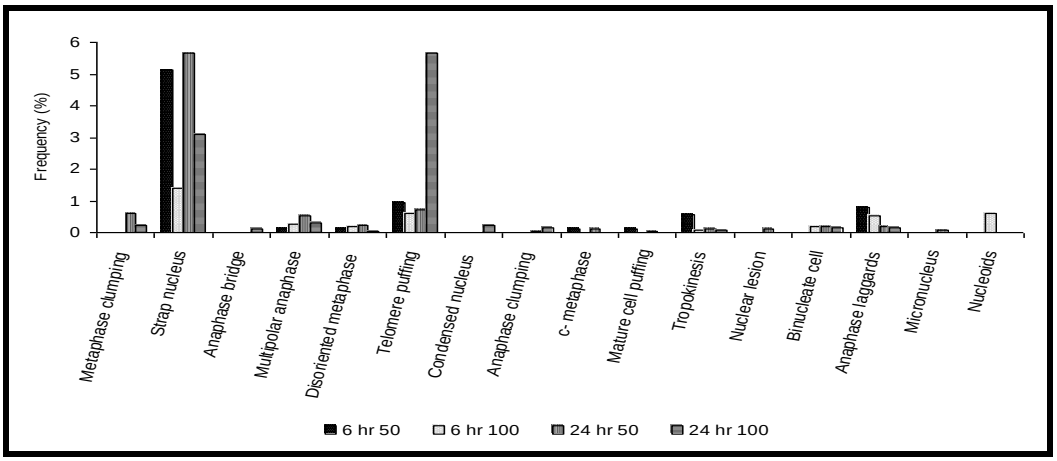


Figure 4: Percentage of aberration in Endosulphan treated root tip cells of *Allium cepa.*

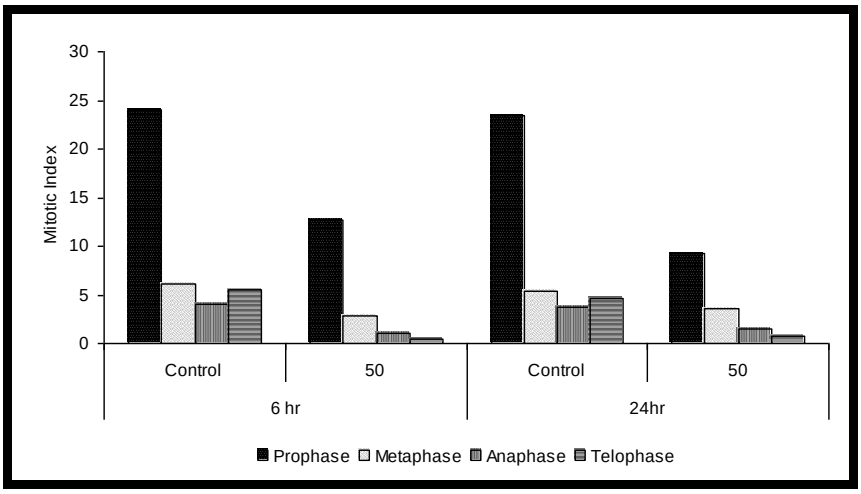


Figure 5: Mitotic index of Allium cepa root tip cells treated with Endosulphan (50µg/ml)

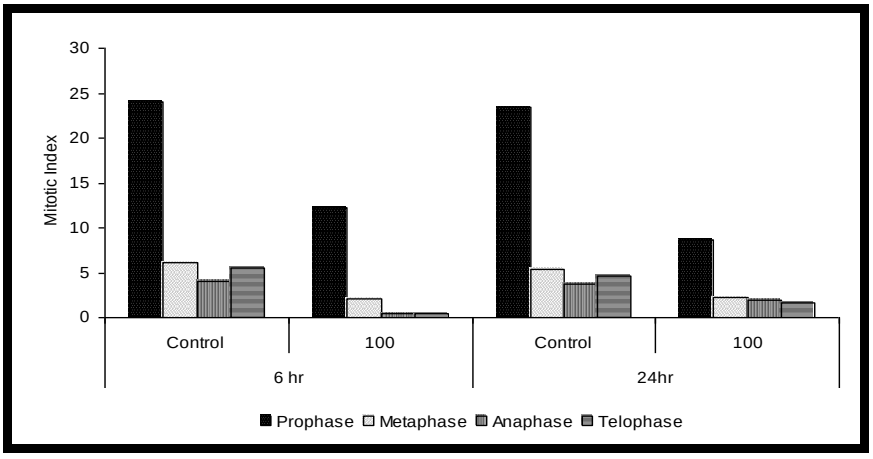


Figure 6: Mitotic index of *Allium cepa* root tip cells treated with Endosulphan (100µg/ml).

Scattered metaphase one type of abnormality observed in all concentration for 6 and 24 hours treatment of Enodosulphan. Telomere puffing was observed in all concentrations for 6 and 24 hrs hour treatment of Enodosulphan. It is one of the most common and also huge percentage abnormality noticed in the genotoxic assay. Multipolar or disorted anaphase was observed in all concentrations for 6 and 24 hrs treatment of Enodosulphan. Lagging chromosomes another type of aberration found in both concentrations (50 and 100 µg/ml) for all duration of treatment.

Star anaphase at one end with tropokinesis was observed only at cells treated with Enodosulphan (Plate 2). Tropokinesis were observed in all concentration for 6 and 24 hrs treatment of Enodosulphan. Dead cells were observed more frequently in cells treated with Enodosulphan with a concentration of 100µg/ml. Anaphase bridge were observed only in 100ug/ml concentrations of 24 hours treated cells with Enodosulphan.

Bi-nucleate cells were observed in all concentrations of Enodosulphan treated cells for 6 and 24 hours duration. Nucleoids, one of the rarely seen abnormality obtained in cells treated with 100µg/ml concentration of Enodosulphan for 6-hour duration. Cell showing one end at anaphase stage and another end uncoiled telophase puffing was observed in Enodosulphan treated cells. Nuclear lesions were observed in cells treated with Enodosulphan with a concentration 50µg/ml for 24 hours sample. The mitotic index of Enodosulphan treated root tip cells shows significant decrease in 50 and 100µg/ml concentrations for 6 and 24 hrs. All cells show a significant decrease in mitotic index when compared with control cells.

The AgNOR count was done on root tip cells treated with 100µg/ml concentration of Furadan and Enodosulphan for 24 hours and 100µg/ml concentration of Enodosulphan treated cells with 6-hour duration. Furadan treated cells showed more dots (Table 1). In Enodosulphan treated, root tip cells the percentage of dots were more than two and four (Table 2). In this silver staining technique ring nucleolus, dense AgNOR silver stained root cap cells and extrusion of nuclear materials from nucleus all these are observed (Plate 2). In control the percentage of cells with more than two AgNOR dots was zero.

Table 1: AgNOR count on root tip cells of *Allium cepa* treated with 100µg/ml concentration of Furadan for 24 hours.

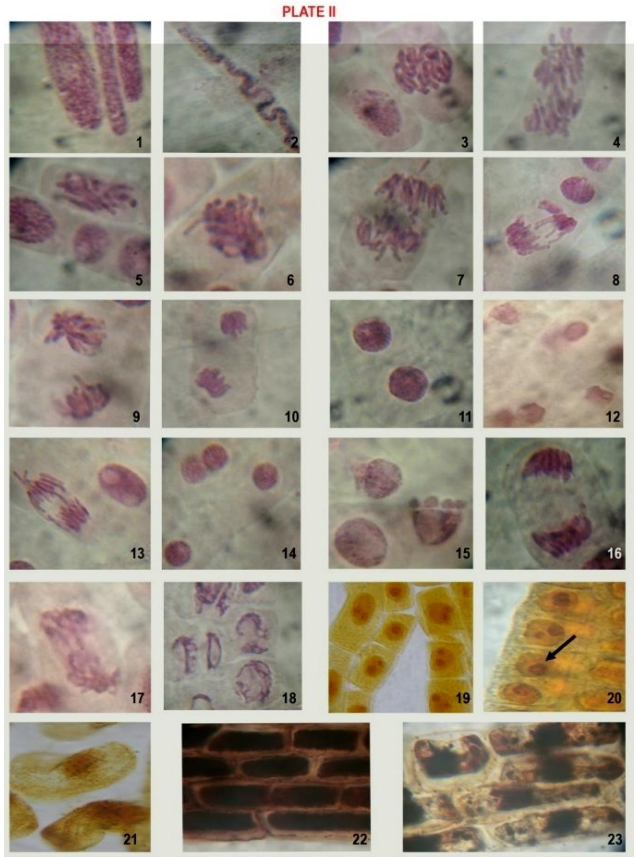
Control				**100 µg/ml Furadan treated cells**					
Cells with 1 or 2 AgNOR dots*		Cells with more than 2 AgNOR dots		Cells with 1 or 2 AgNOR dots*		Cells with more than 2 AgNOR dots*		Cells with more than 4 AgNOR dots*	
6	24	6	24	6	24	6	24	6	24
198	400	Nil	Nil	69	77	22	114	51	159

*** Approximately**

Table 2: AgNOR count on root tip cells of *Allium cepa* treated with 100µg/ml of Endosulphan for 24 hours.

Control				100 µg/ml Endosulphan treated cells					
Cells with 1 or 2 AgNOR dots*		Cells with more than 2 AgNOR dots		Cells with 1 or 2 AgNOR dots*		Cells with more than 2 AgNOR dots*		Cells with more than 4 AgNOR dots*	
6	24	6	24	6	24	6	24	6	24
196	386	Nil	Nil	73	82	28	121	62	163

*** Approximately**



Types of Aberration: 1 & 2.Strap nucleous 3.Metaphase clumping 4.Scattered metaphase 5&6. Telomere puffing 7.Multipolar anaphase 8.Laggards 9.Star anaphase at one end with tropokinesis 10.Tropokinesis 11&12.Dead cells 13.Anaphase bridge 14.Binucleate cell 15.Nucleoids 16.Mature cell showing tropokinesis 17.Cell showing one end anaphase stage another end telophase puffing 18. Nuclear lesion 19.AgNOR Staining (normal cell) 20.Ring nucleoli 21.Cells with more than two AgNOR dots 22 & 23. Dence AgNOR silver staining in root cap cells.

Clumping of chromosome at metaphase was more frequent, that might be due to the action of disulphide and sulfhydryl linkages which enter in to spindle formation as suggested by [11,12] revealed that the thickening and swelling of chromosome induced chromosomes to stick together which formed a compact clump in *Euphorbia geniculate* by treatment of herbicides 2, 4-D and sodium arsenate. [13] suggested that toxic substance might cause membrane structure alterations resulting in permeability changes by interference with chromosomal aberrations. Malignant tissues frequently exhibit abnormal nuclear morphology including variability in nuclear shape [14,15] have reported the suppression of cell plate formation was an essential step for the formation of bi-nucleate cell.

According to [16] anaphase bridges might be due to the formation of dicentric chromosomes as a result of breakage and reunion of broken chromosomes. [17] is of opinion that anaphase bridges may be due to unequal exchange of dicentric chromosomes. Disturbed or disoriented metaphase is found in both Furadan and Endosulphan treated cells. Disturbed metaphase has been induced due to the disturbance of spindle apparatus as revealed by[18].. When investigating the effect of pesticides on *Vicia faba plant*. Disoriented metaphase may be to spindle dissolution. The reason for spindle dissolution could be due to alteration of the gene controlling the spindle mechanism.

Lagging chromosomes are observed in anaphase in both 6 and 24 hour Endosulphan treated root tip cell with concentration 50 and 100µg/ml respectively. Chromosome laggards also observed in 100µg/ml Furadan treated root tip cells of 24 hours treatment. [19] opined that lagging chromosome at anaphase might be due to the fact that spindle fibers were inactivated. In *Allium cepa*, laggards and bridges were noticed due to the treatment of herbicide avenoxan [20]. The above finding suggests that lagging chromosomes or fragments originate from alteration of the spindle. Condensed nucleus is found frequently in Furadan treated samples. Comparatively less rate of condensed nucleus is found in 24 hour treated 100µg/ml concentration of Endosulphan. Condensed nucleus irregular profiles are being hallmarks of apoptosis [21]. The nuclear is common feature of apoptosis like cell death or programed cell death [22].

Micronucleus frequency observed in cells with 50µg/ml concentration of Endosulphan for 24 hours duration. Micronuclei often results from the acentric fragments or lagging chromosomes that fail to incorporate in to the daughter nuclei during telophase of the mitotic cell and can cause cell death due to deletion of primary genes [23]. The induction of micronuclei in root meristems of *Allium cepa* or any cell of any other organism is the manifestation of chromosome breakage and disturbance of the mitotic process due to spindle abnormalities. Micronuclei considered as indication of true mutation effect [24]. The induction of micronuclei suggests that the tested mixtures contained constituents that are clastogenic or are spindle inhibitor.

Disorganized or multipolar anaphase is observed in both 50 and 100µg/ml concentration of cells treated with Endosulphan for 6 and 24 hours. In the case of Furadan only 100µg/ml concentration of cells shows this abnormality. These abnormalities may due to numerous centrosomes, multipolar and disorganized spindle assembly. Furadan might have induced centrosome amplification in mitotic cells. Nucleoids are observed in Endosulphan treated cells with concentration of 100µg/ml for 6-hour duration. Only 0.61 percentage aberration is noticed. The nucleoids are conspicuous in the dyad cytoplasm from anaphase 1 through the interphase and again in the tetrad from anaphase 11 until well after completion of the cleavage. The nucleoids are nevertheless obliviously chemically similar to normal nuclei in respect to RNA and protein. Cytomixisis is observed in treated cells with Furadan 50µg/ml solution for 24 hours. Cytomixisis consists in the migration of chromatin material between meiocytes through cytoplasmic connections; cytomixisis creates a variation in the chromosome number.

Tropokinesis is a spindle abnormality found in cells 6 and 24 hours treatment with Furadan and Endosulphan. Tropokinesis occur due to the abnormal orientation or misbehavior of spindle fibers. During cell division the daughter chromosomes move towards the center of the cell, instead to the Centre, daughter nuclei were formed at the corners in tropokinesis partial inactivation of the spindle may lead to irregular anaphase [25]. The mitotic index of Furadan treated root tip cells shows a significant decrease in 50 and 100µg/ml treated cells for 6 and 24 hours duration. The mitotic index of 50µg/ml treated cells for 6 hours is 23.3 and 24 hour is 21.2. Mitotic index for 100µg/ml treated cell is 19.0 for 6-hour treatment and 14.4 for 24 hours. The result shows a considerable decrease occur in prophase

and in metaphase stages. The reduction in prophase and metaphase may be due to arrest of cells in G1 phase and retardation in pace of events during S or G2 phase.

The mitotic index of Endosulphan treated root tip cells shows 17.1 and 15.2 percentage on 50µg/ml concentration for 6 and 24 hours respectively 100µg/ml concentration shows 15.1 and 14.6 percentage of division for 6 and 24 hours duration of treatment. In all concentration and duration of time the mitotic index is significantly less than from control. The study shows the eduction in mitotic index may due to arrest of cells in G1 phase and retardation in pace of events during S or G2 phase. The decrease in anaphase and telophase may be due to spindle abnormalities. Nuclear Organizer region (NORs) is the tool used by cytogeneticist for the study of chromosomal disorders. Compared to normal cell the frequency of NORs is significantly higher with in molecules of malignant cells. It was decided that a NOR study using silver staining can be used for characterizing malignancy AgNORs appear as brown or black dots with in a yellowish background of nucleus. The number of AgNORs, their size and heterogeneity represents proliferative cellular activity, thus it can be used for detecting malignancy and grading tumor [26].

CONCLUSION

Treatment with Furadan and Endosulphan on *Allium cepa* root tip produced chromosomal aberrations such as strap nucleus, metaphase clumping, telomere puffing, scattered metaphase, multipolar anaphase, laggards, anaphase bridge, star anaphase, micronucleus, bi-nucleate cell, C-metaphase, mature cell puffing, tropokinesis, nuclear lesion, nucleoids, cell death. These aberrations may lead to cancer, cell death mitotic delay and genetic changes. It has been found out that Furadan has high deleterious effect in long-term duration of treatment when compared with Endosulphan.

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