ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 8.4
IJAR 2021; 7(4): 100-106
www.allresearchjournal.com
Received: 19-02-2021
Accepted: 21-03-2021

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Polytene chromosome aberrations based genotoxicity appraisal by implementing insect genome

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Abstract
Present research work deals with evaluation of clastogenic and cytotoxic effects of dichlorvos using salivary gland polytene chromosomes. Exposure of pesticide was given *Drosophila melanogaster* larvae which were sacrificed for temporary squash preparation of polytene chromosome, along with controls. The structural aberration in treated stocks were incidences of ectopic pairing, inversion, breaks, chromosomal fusion, asynapsis and translocation with respective number 40, 23, 15, 12, 10 and 8 in treated stock while in control stock main aberrations are, inversion chromosomal fusion and ectopic pairing with respective value 32, 15 and 9 . Subsequently, statistical analysis was done by applying student "t" test and it was concluded that dichlorvos induced statistical significant genotoxicity in exposed larvae in comparison to control.

Keywords: polytene chromosome, genotoxicity appraisal, insect genome

Introduction
Genetic information is carried out by chromosome in the form of linear arrangement of genes. There are some special types of chromosomes including lamp brush chromosomes and polytene chromosomes, to meet physiological demand of body during specific developmental stages**.** Polytene Chromosomes are giant sized, multi-stranded chromosomes, formed by endoduplication of genetic material due to the repetitive rounds of DNA replication devoid of cell division, resulted in increased cell volume. Centromeric region of all chromosomes form chromocenter, which is usually a very fragile structure. Due to alignment of chromere in adjacent strand particular banding pattern of light and dark bands form, additionally presence of puff indicate the region of higher gene transcriptional activity. Due to multi-stranded structure of polytene chromosomes, it is easy to detect chromosomal aberration like deletions, inversions, translocations, asynapsis, fusion and break. Generally, regions of high transcriptional activity are lightly stained while dark bands represent highly condensed regions with low transcriptional activity. Bridges in 1935 first time prepared polytene photo map of *Drosophila melanogaster***.** Some region of polytene chromosomes form puff where high level of RNA transcription activity is there, such regions are called as Balbani ring. *Drosophila melanogaster* is widely as test model for various investigations related with genotoxicity evaluation of different mutagens through suitable methodologies. Additionally usage of non target organisms in environmental toxicology is needed to understand wide range of toxic consequences caused by pesticides or other pollutants on different organisms**.** The fruit fly *Drosophila Melanogaster* serves as a most noteworthy model organism because its adaptability to laboratory condition, short life span ,convenient handling and small diploid number (2n=8), therefore this dipteran insect is extensively studied and exploited organism for the investigation of many cellular and developmental processes which are widespread to higher organisms including humans. Drosophila *melanogaster* has been in use from last 100 years by Morgan, furthermore, elaborated and extensive study of concerned insect formulate significant assistance to the understanding of elementary biological processes.
For present genotoxicity evaluation dichlorvos was selected due to its broad spectrum insecticidal properties and its excessive applications in agricultural fields in Jalandhar distt. This chemical compound has been commercialized in 1961**.** Dichlorvos is a contact and stomach insecticides, widely used to control insects of agricultural field, buildings and

outdoor areas. Concerned chemical is used against aphids, spider, mites, caterpillars, thrips and whiteflies in greenhouse, outdoor fruit and vegetable crops. This insecticide acts on acetylcholinestrase enzyme associated with nervous system. Acute symptoms appear after exposure of chemical includes weakness, headache, and tightness in chest, blurred vision, salivation, sweating, nausea, vomiting, diarrhoea, and abdominal cramps.

In present research execution clastogenic and cytotoxic properties of dichlorvos is determined at sub lethal concentration which is usually much less than the exposure limit, actually applied in fields. Exposure of selected dose was given to first instar larvae, subsequently pesticide exposed larvae were reared unto third instar larvae which were used to prepare temporary squash preparation of polytene chromosomes. Good quality of chromosomes compliment with proper spreading and distinct banding pattern were selected for photography. Subsequently, obtained data was assessed for various type of chromosomal aberrations in pesticide exposed group and natural population. Observed number of chromosomal aberrations in treated stock was compared with that of control population. Subsequently, procured data from pesticide exposed group is compared with control stock and statistical analysis was done, which indicated statistically significant induced nontoxicity.

Review of the related literature: Following objectives were executed to achieve research targets

1. *Rearing of test organism*: To minimize the effect of environmental pollutants rearing of test organism was done in laboratory.
2. *Standardization of* LC_{20}: To determine the value of LC_{20}, done by exposing instar larvae to serial dilution for 24 hours .Subsequently, exact value of LC_{20} was calculated by probit analysis.
3. *Genotoxicity evaluation* : To study the effect of dichlorvos on the polytene chromosomes of *Drosophila melanogaster* and its comparison with natural population

Methodology: *Drosophila melanogaster* has been used as a test model in numerous investigations, to study correlation between chromatin structure and gene regulation due to presence of cytologicaly important polytene chromosomes. *Drosophila* is a holometabolous insect which complete life in four stages egg, larva, pupa and adult. Larval stage is concerned for maximum feeding, hence for digestion of food excessive production of saliva is required. There is only increase in volume of cells without increase in number of initial population of cells in salivary gland. Simultaneous with increase in volume, nuclei of cells also enlarge by forming specific type of giant sized, multi stranded polytene chromosomes at first instar stage. For formation of polytene chromosomes, homologous chromosomes undergo synapses, subsequently there is continuous endoduplication of genetic material without cell division. Chromosomes undergo many rounds of replication in the absence of cell division which resulted into approximately 1000 copies of DNA strands. In normal dividing diploid cells the DNA synthesis phase is followed by dividing phase but for polytene chromosome formation karyokinesis is not followed by Cytokinesis, hence repeated cycles of DNA synthesis results in polyploidy. Polytene chromosomes are present in ovary nurse cells, follicle cells surrounding oocytes, abdominal histoblasts, fat body cells, gut cells, and cells of the late pre pupal salivary gland. In case of *Drosophila*, approximately 1024 strands are present in one polygene chromosome.

Results and Discussion: For the present research work, genotoxicity of dichlorvos was evaluated by studying induced chromosomal aberrations (CA) in salivary polytene chromosomes. Complete compliment prepared from healthy and active third instar larva consists of six unequal and well banded elements comprising of a short X-chromosome and three much longer autosomes of unequal size. Generally all polytene chromosomes are connected by their centromere to form a fragile structure chromocenter including whole Y-chromosomes and short arm of X-chromosomes. From chromocenter six chromosomal elements can be seen radiating out from a common point (Fig.4). They are the right and left arms of chromosomes 2 and 3 (2R, 2L, 3R, 3L), a long arm of the X-chromosome and a short 4 chromosome (Fig.4). During squash preparation chromocenter gets disintegrated, all the three-chromosomes lie freely in the form of well stained and well spread elements of optimum stretching. For genotoxicity evaluation of dichlorvos insecticide, percentage frequency of structural aberrations like inversions, deletions, ectopic pairings, Asynapsis, fusions and breaks were considered to evaluated mutagenicity. Polytene chromosome analysis was carried out by dividing the experiment into three different sets for treated and control stocks. Accordingly, the first instar larvae were given treatment of LC_{20} of pesticide for 24 hours. For each individual set, a fixed number of 40third instar larvae were sacrificed for making the temporary squash preparations of the polytene chromosomes. The percentage frequency of various types of aberrations was calculated after which the mean and standard error and the level of significance of mutagenicity was calculated by applying student „t" test for each type of abnormality. The description of these different kinds of chromosomal rearrangements in the control and pesticides treated larvae of *Drosophila melanogaster* is as follows.

Inversion

Inversion is a structure chromosomal aberration, in which a segment of chromosome gets reversed from end to end. Generally with, by two simultaneous break, a segment of chromosome forms which can either be lost, leading to a deletion or may remain in its natural position due to immediate repair of breaks with the help of repair enzymes which are present in the nucleus. In case these two processes fail than this segment can rotate 180° degrees and get reinserted in the same place before the repair can take place. If such type of chromosomal aberration occur in both synapsed homologues then a reversion of bands become apparent in polytene chromosomes but if inversion is in heterozygous condition then formation of loop take place (Fig.7-10)

Ectopic pairing

In this chromosomal structural aberration, two separate polytene chromosome or different regions of same chromosome get connected to each other by thread like connection. Ectopic pairing can be interchromosomal and interchromosomal type (Fig.9-12).

Asynapsis

During formation of polytene chromosome, two homologues get synapsed but sometime due to effect of a chemical or by endogenous factor, synapsed chromosomes get separated which can occur rarely in nature but more frequently in experimental populations. The environmental factors in nature and treatment with a mutagen in the laboratory can cause these aberrations (Fig.11).

Fusion

A chromosomal fusion can take place between broken ends of the same or different polytene chromosomes.

Break

Chromosomal break is the commonest manifestation of a mutagen in which, depending upon the extent of its effect there can be the fragmentation of chromosomes.

Photographic Section Control Stocks

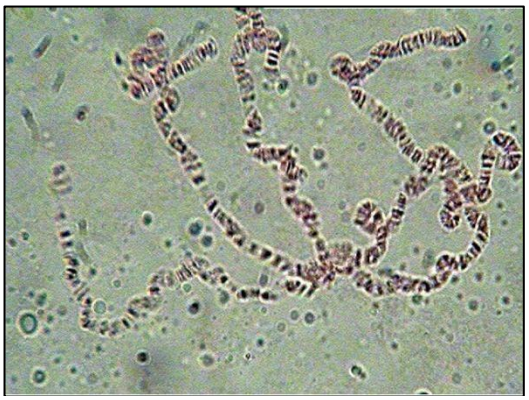

Fig 1: Normal compliment

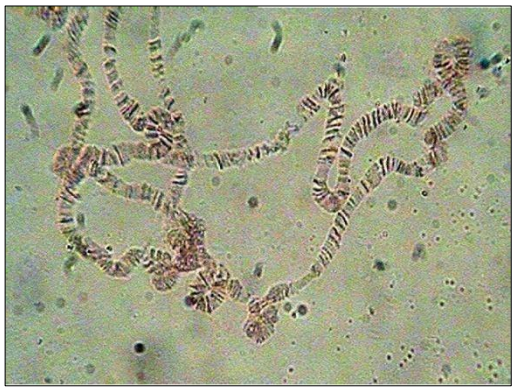

Fig 2: Inversions

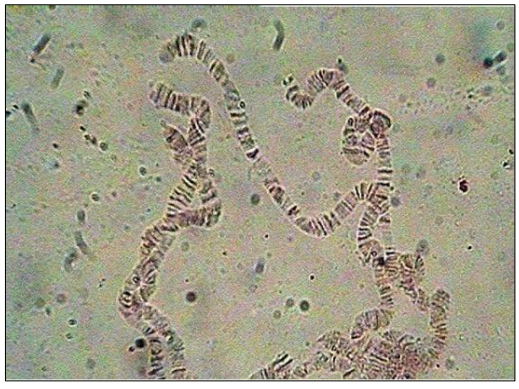

Fig 3: Chromosomal fusion

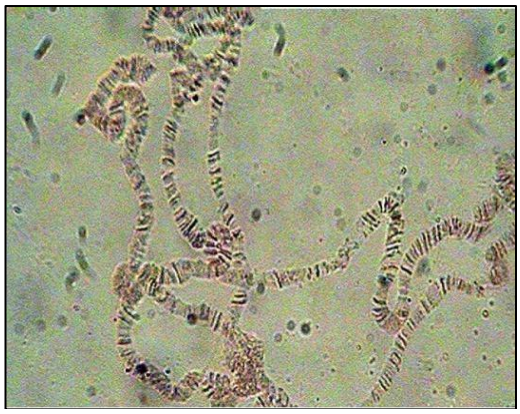

Fig 4: Chromosomal fusion

Treated Stocks

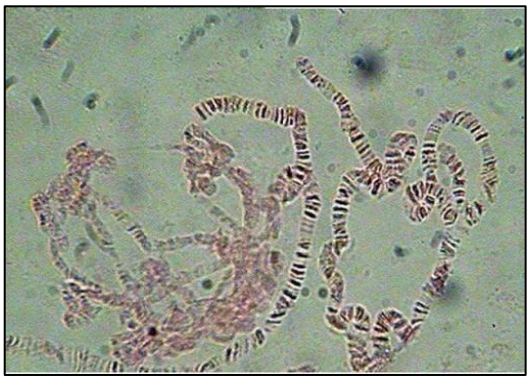

Fig 5: Inversion

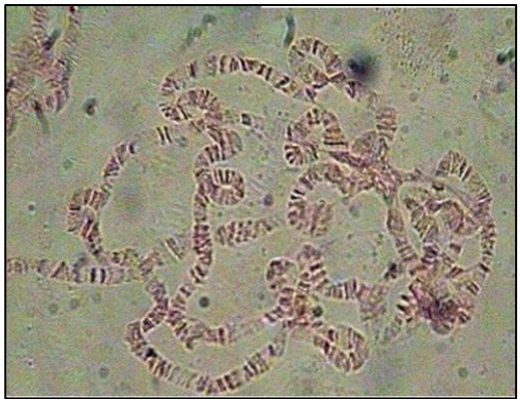

Fig 6: Inversion

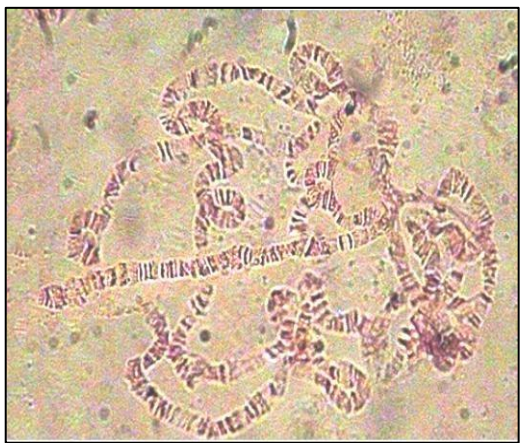

Fig 7: Inversion and Ectopic pairing pairing

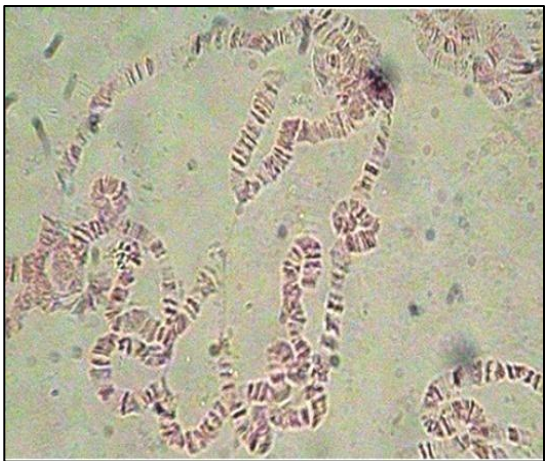

Fig 8: Inversion and Ectopic

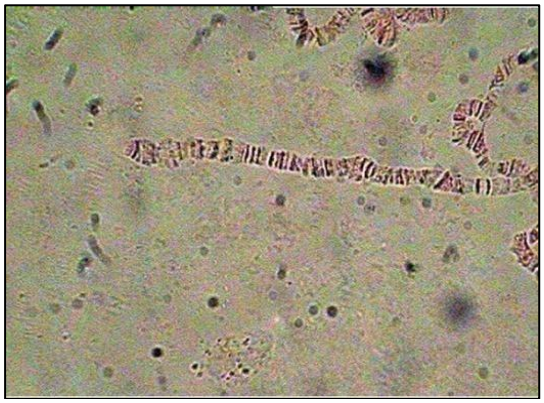

Fig 9: Ectopic pairing

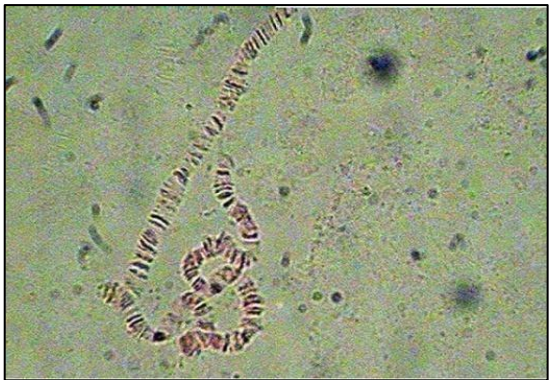

Fig 10: Ectopic Pairing

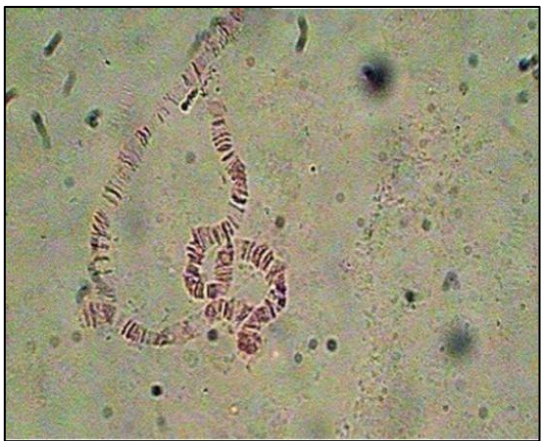

Fig 11: Ectopic pairing

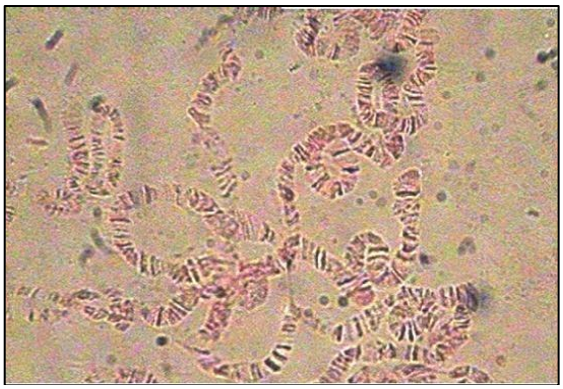

Fig 12: Asynapsis

Usually pesticides exposure is associated with acute and chronic consequences on animal and man. Excessive application of synthetic chemicals in field disturb the biotic components by eliminate food sources needed by the animals, forcing them to relocate, change their diet or starve, additionally concerned chemicals also affect the biodiversity rich in pollinator insects, birds, wild life, domestic animals, fish and livestock. The use of un-prescribed pesticides in inappropriate doses is not only disturbing the soil conditions but also destroying the healthy pool of bio-control agents that normally co-exist with other flora and fauna. Acute consequences in human beings include mild head ache, flu, skin rashes, nausea, dizziness and diarrhoea while chronic poisoning can cause cancer, genetic defects and fertility problems (Decock *et al*., 1994; Tielmans *et al*., 1999 ; Rojas *et al*., 2000) [13, 55, 46], birth defects (Restrepo *et al*., 1990; Petrelli *et al*., 2000), miscarriages, leukaemia, non-Hodgkin's lymphoma, hormonal changes, liver damage, kidney disease, suppression of the immune system, asthma, allergic dermatitis, respiratory complications (Zuskin *et al*., 1993) [64], autoimmune, neurological and behavioural disorders. Children are highly vulnerable to the toxic effects of pesticides as they remain in increased contact with floors, lawns and playgrounds (Bell, 2001; Grisolia, 2002; Kiesecker, 2002; Schreinemachers, 2003; Richard *et al*., 2005) [2, 19, 25, 50, 44]. In the rural areas the pesticide use had been posing risks of short and long term health problems in farm workers and their families. Epidemiological studies on cancer in farmers have yield conflicting results. For example, meta-analysis showed that farmers were at risk of developing specific tumors, including leukaemia (Daniels *et al*., 1997; Zahm *et al*., 1997; Zahm and Ward, 1998) [12, 62, 61] and multiple myeloma (Khuder and Mutgi, 1997) [24]. Workers who mix, load or apply pesticides were found to be at greater risk due to spills, splashes, careless handling and inadequate protective measures. Today a number of organizations are working in collaboration to reduce the harmful consequences of pesticide use. These organizations are U .S. Environmental Protection Agency (USEPA), World Health Organization(WHO), Food and Agriculture Organization (FAO), United Nations Environment Programme (UNEP), United Nations Commission on Trade and Development (UNCTAD) and United Nations Institute for Training and Research (UNITAR) which recommend guidelines to reduce risks from pesticides and the necessary information about them such as, its residual hazards, environmental fate, acute, sub chronic and chronic toxicity, spray drift and effects on non target organisms. In India, acts such as Prevention of Food Adulteration Act (PFAA) 1954,

Insecticide Act1968, Insecticide Rules 1971 and Environment Protection Act 1986 were implemented for their safer use. Awareness about the deleterious effects of pesticides on genetic material and reproductive system of living organisms led to the development of important subjects of Ecotoxicology and Genetic Toxicology which deal with the assessment of the mutagenic effects of chemicals and physical agents on the biology of living systems with special emphasis on their genetic material (Muller, 1927; Sax, 1938; Russell, 1951; Ghosh *et al.*, 1990; Ni *et al*., 1994; Nath *et al*., 1996; Grisolia, 2002) [35, 19, 47, 18, 40, 39, 19]. Today, a number of protocols are available for genotoxicity assessment of pesticides by using different test organisms, as a result of which genotoxic and reprotoxic properties of several commonly used pesticides are now well documented (Wyrobek *et al.*, 1981; Onfelt and Klasterska, 1983; Schrage and Dixon, 1985; Rotenberg and Weinstein, 1991; Davis *et al.*, 1992; Baranski, 1993; Guillelte *et al.*, 1994; Jadaramkunti and Kaliwal, 1999, 2001, 2002; Grisolia, 2002; Maclellan *et al*., 2004) [60, 19]. Motivated by the significance of genotoxicity studies, the present investigations were undertaken, as a result of which, the present text deals with the genotoxicity assessment studies of dichlorvos on the genetic material of *Drosophila melanogaster* taken as experimental models. Dichlorvos (2, 2 dichlorovinyl dimethyl phosphate), is an organophosphates insecticide that interfere the normal activity of acetyl cholinesterase, essential for proper functioning of the nervous system. This insecticide is used, worldly as a contact and stomach insecticide (Meister, 1992) [30]. In an investigation, Murthy, 1986 observed that the concerted insecticide is toxic to aquatic organisms. As this chemical is highly toxic, can get inside the body through inhalation, ingestion and dermal absorption (OHSI, 1991) [42], furthermore the toxicity of this formulation increases with UV light (TOXNET, 1985) [56]. EPA, classified this chemical as highly toxic (toxicity class-I) (Howard, 1989) [21]. Genotoxic properties of this pesticide are documented by few studies (Cabrera, 2000; Sasaki *et al*., 2000; Wang *et al*., 2003) [9, 49, 57]. Ames test indicated it as possible mutagen (Brusick *et al*., 1980; Leifer *et al*., 1981) [8, 26], whereas Wang *et al*., (2003) [57] reported that Dichlorvos induced sister chromatid exchange and chromosomal aberrations in Chinese hamster ovary (CHO) cell line. In a study it was concluded that vitamin E which proved quite effective in reducing genotoxicity of other pesticide, has failed in lowering deleterious consequences of dichlorvos. Gupta, *et al*, 2007 [54] observed that dichlorvos reduce reproductive potential of Drosophila melanogaster. Shukla, *et al*., 2010 [53] observed that dichlorvos caused gradual increase in DNA damage with increase in concentration and duration of exposure in *Mystus vittatus.*

Conclusion

Genotoxicity assessment has become crucial before their actual application in the fields. For this, the subjects like Ecotoxicology and genetic toxicology occupy major positions as they deal with the assessment of the effects of chemicals and physical agents of daily use on the hereditary material and environment of living systems. Some of these harmful effects can be assessed directly by measuring their interaction with the DNA through the mechanism of DNA repair, production of gene mutations and chromosome alterations. Although the effects of pesticides are generally target-specific yet, in actual practice, they are not always selective for intended target species as they also tend to damage the non target sources, including man. Inspired by the work done so far in the subject area of genetic toxicology, the present topic of research entitled "Polytene chromosome aberrations based genotoxicity appraisal by implementing insect genome" was carried out. *Drosophila melanogaster* was selected as test model, to analyse consequences of selected insecticides on polytene chromosomes of larval stage. Exposure of selected dose was given for 24 hours duration and rearing of larvae was done under controlled conditions. Clastogenic and cytotoxic consequence of dichlorvos was determined by analysing induced chromosomal aberrations in treated stocks with control population. It was concluded that, insecticide dichlorvos has maximum tendency to induce ectopic pairing, followed by inversion, chromosomal fusion, breaks, Asynapsis and translocation whereas in control population comparatively low number of structural aberrations were observed. Concerned chemical was found to make chromosomes sticky which could be possible reason, disorganized structural integrity of chromosomes. The selected dose is much lower than the concentration which is exposure limit in field. Review of literature of this chemical suggest that it possess drastic consequences on a biotic and biotic components prevailing in ecosystem, therefore intense research work is required to be carried out on concerned chemical so that suitable exposure limits could be designated. Additionally efforts should be made by scientific community to find out alternatives of pesticides by implementing botanical extracts and simultaneously integrated pest management programmes should be implemented to reduce the consequences of these mutagens

Future Scope of Study: Scientific literature suggests that dichlorvos is a potential toxic chemical for flora and fauna of an ecosystem. At sub lethal , LC_{20} concerned chemical has induced significant genotoxicity by causing elevated structural chromosomal aberrations such as ectopic pairing, inversion, breaks, chromosomal fusion, asynapsis and translocations in comparison to control stocks. Therefore, it is essential fact that alternatives of chemical pesticides should be formulated by using comparatively less genotoxic chemicals.

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