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Chromosomal Aberration Assay in *Allium cepa* (Onion root tip):Atest system for evaluating Genotoxicity due to 2, 4-D

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Pesticides including herbicides, insecticides and fungicides are used extensively to improve crop yields. More than 2.5 million tons of pesticides are applied every year to agricultural crops worldwide (Vander werf, 1996). Some of the pesticides may run off to the aquatic environment which plays a vital role for functioning of ecosystem and is immediately related with human health. The increasing human population and industrial development has worsened the problem of disposal of anthropogenic chemicals and waste in aquatic environment. Large portions of these contaminants are potentially genotoxic and carcinogenic substances. Genotoxic in genetics is defined as a destructive effect on a cells genetic material (DNA,RNA). Genotoxic are mutagens and include both radiation and chemical genotoxins. There are three primary effects that genotoxins can have on organism by affecting their genetic information. Genotoxins can be carcinogens, mutagenes or teratogens. So these genotoxic chemicals are responsible for DNA damage in living system as well as a variety of aquatic organisms.
Cepa bulbs revealed different types of chromosomal aberrations which were apportioned in physiological aberrations (c- mitosis, delayed metaphase,stickiness, laggards,vagrants) and clastogenic aberration (chromosomal breaks, chromatin bridge and ring chromosome)
Keywords:- Chromosomal aberration , 2.4-D, onion root tip (*Allium cepa*)

Introduction :-

Genetic toxicology is a new division of Toxicology that identified and analysis the action agents with toxicity directed the hereditary component of living system Many contaminants present in surface water not only endanger survival and physiology of the organism but also induce genetic alteration in genetic materials and nonlethal, non–cytotoxic concentration is classified ''genotoxic''.

Genotoxicityis the important aspect of pharmacology that deals with the adverse effect of bioactive substance on living organisms. The genotoxic effect of 2,4-D be capability to act on genetic information and altering the structure of DNA thus interfering in prime cellular process like cell division. Genotoxic chemicals have similar in physical and chemical properties that enable here to interact with genetic materials.

The *Allium cepa* is a suitable indicator for determination of genotoxic agents in the sample taken from the environment. The genotoxic level of the agent under study is reflected by structural changes of the chromosomes and their changes number. Genotoxic effect of 2,4-D on DNA integrity as molecular bio marker, increase in percentage of hyper chromicity has been reported ie. Suggestive of structural introduced in DNA due to the binding of xenobiotics.

In present study efforts have beenmade to investigate the enotoxic effect of 2,4-D using chromosomal aberration in *Allium cepa*.

Genotoxic substance research is the great importance for the protection of the environment because it enables an insight into the influence of genotoxic substances on organisms. The goal of the research is to develop a valuable tool for the monitoring of the environment.

Genotoxic Effect of 2,4-D &Endosulfan on*Allium cepa*.

The first study on mitosis was carried out by levan (1938) by using colchcine colchicine on *Allium cepa* root meristem cells. In addition to this viciafaba, Tradescantiapaludosa, Pisum sativum, tlordeumvulgare, and crepis capillaries were also used for the same purpose (Amer and Ali 1983; Dryanosvka 1987; Kluge and podlesak 1985; Amer*et al.* 1999; Dimitrov 1994). *Allium* test is the most common one in order to determine the toxicity in the labs because of the storage and easy growing peculiarities of *Allium* (Fiskesjo 1985; Rank *et al.* 2002; Yazbasiogl*uet al.* 2003; Kava *et al.* 1994; Grant 1992). More over this system is well correlated with the data obtained from prokaryotic system (Fiskesjo 1985).

Material andmethod :-

Allium cepa (2n = 16) was used as test organism. The onions were kept cool and dry until cyto-toxicity testing just before use the outer scale of the bulbs were scraped away without destroying the root primordial the experiments were maintained in laboratory conditions and the roots were protected from direct sunlight in order to minimize fluctuation of the rate of division.

The *Allium cepa* is commonly used as a test organism because it is cheap, easily available and has advantages over other short-term tests. Among the endpoints of *Allium cepa* root chromosomal aberrations, detection of chromosomalaberration have been the most used one to detect genotoxicity/antigenotoxicity along the years. The mitotic index and chromosomal abnormalities are used to evaluate genotoxicity and micronucleus analysis is used to verify mutagenicity of different chemicals. The *Allium cepa* root chromosomal aberration assay is widely used to determine genotoxic and antigenotoxic effects of different plant extracts.(Namita Khanna*and Sonia Sharma, 2000).

Mitotic Index: The percentage of cells undergoing mitosis or it is defined as the ratio of no. of cells in the dividing phase to the total number of cells observed. This will help to identify the region of most mitotic activities. Mitotic index helps us to quantify the cell division. The meristematic region in the root tip is the actively growing region and thus the mitotic index is high.

Mitotic index = n/N × 100

n: Total number of dividing cells observed

N: Total number of cells in the field of microscope

Mitotic index is used to quantify the differences in cell division when environmental parameters are changed.

Procedure

1. Cut roots from onion plant using a scalpel.
2. Trim the tip of each root to 1 cm; use only the tapered end of the root tip.
3. Use forceps to place 2–3 root tips (use only the 1-cm tips) on a glass microscope slide.
4. Place them in a solution containing Hydrochloric acid (1N) and ethanol in 1:1 concentration.
5. Allow the root tips to soak in the mixture for 1 minutes.
6. After one minute, Put the material in water and wash off the solution.
7. Place the root tip on a slide. Use a paper towel to blot away excess water.

10. Using a clean, graduated pipette, add 2–3 drops of Aceto carmine stain to the root tip.
11. Allow the root tips to soak in the stain for 3 minutes.
12. Use a paper towel to blot away excess stain.
15. Place a cover slip on the root tissue. Gently apply pressure on the cover slip to squash the root tissue.
16. Using low magnification on the microscope, focus on the root cells. Switch to medium power or high power as necessary to easily visualize the inside of the onion root cells.
17. Study all of the squashed tissue to locate cells in each stage of the cell cycle. Repeat same procedure for all concentrations (Flinn Scientific, Inc. 2016)

Allium root growth test and determination of $EC_{50.}$

Clean and healthy onion bulbs were allowed to produce roots in distilled water. After 2-3 days the onion bulbs with freshly emerged roots were placed on coupling jars filled with different concentration of 2,4-D&endosulfan , for four days (96hrs). The toxicity assay is performed as semi-static exposure test after every 24hrs the test solutions wee replaced by fresh solutions sets were measured (length of 10 roots from each bulb) at the end of exposure time. The relative reduction of root length was calculated as the percentage of the deviation from the control. The effective concentration (EC_{50}) value was determined as the effective concentration for 50% growth inhibition. Experiments were carried out in triplicate.

Cytogenetic parameter:

Different concentration (0.1, 0.5, 0.75 and 1ppm) of 2,4-D&endosulfan were used in the treatment of *Allium cepa*. Onion bulbs were rooted in distilled water for 24hrs. The five bulbs which have approximately same root length were transferred to each test solutions. After the completion of 3hrs. treatment the root tips 1-2cm long of each bulb were cut carefully and fixed in fixative (ethanol : glacial acetic acid in 3:1 ratio) for 24hrs in a refrigerator at 4-b0C. The root tips were washed twice in cold water for 10 minutes each and allowed to dry. A solution of INHCL at 600C (Matsumoto *et al*; 2006) was added to the root tips for 10 min. The root tips were again washed with distilled water. The HCl treatment was repeated.

Statistical analysis

The mean value with standard deviation (SD) for each root length was calculated from value obtained from individual bulbs and it was compared to the corresponding control value. The ‘t’ test was conducted manually to ascertain if the difference was statistically significant or not for root growth.

Result :- The present investigation were carried out on root tip of *Allium cepa* .

Effect of 2,4-D (Chromosomal aberration) in *Allium cepa*.

Morphological abnormalities such as stiffness and discolouration of roots were observed in higher concentrations (i.e., 1.0ppm) along with decrease in root growth (Fig:- 25, Table-12). As is evident the root growth decreased with increasing the concentration (0.1,0.5,0.75 & 1.0 ppm) of 2,4-D. It was observed that above 1.0ppm there was no root growth during 96hrs treatment. After fourth day treatment of growth in the control , the average length of root 17.12cm. Dose-response curve obtained between the concentration of 2,4-D and *Allium* root growth determined the effective concentration (EC_{50}) value with retards 50% root growth as 0.5ppm.

Table.1:- The root length after treatment in EC_{50} was 8.70cm.

Dose (ppm)	Average length (mm)	Growth (%)	Decrease in growth (%)
Conltol	17.12	100.00	0.00
0.1	14.50	84.69	-15.31
0.5	8.70	50.56	-49.44
0.75	5.34	31.19	-68.81
1.00	5.10	9.34	-90.66

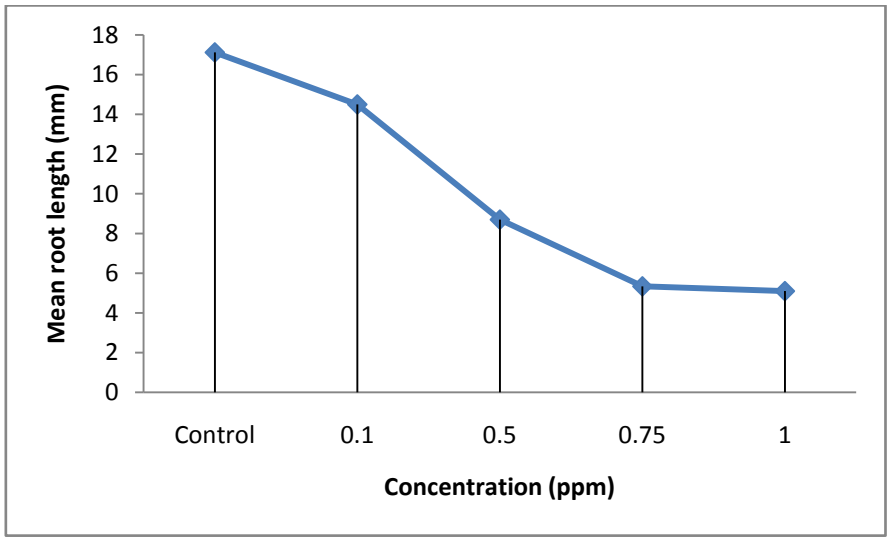

Fig. 1 :- Graphical representation of growth of root Tip

Exposure to different concentration (0.01 to 1.0ppm) of 2,4-D significantly and dose dependently inhibited the mitotic index in the root tip cells of *Allium cepa*. The table indicates that there was an exponential relationship between the percentage of aberrations and concentrations of herbicides. There were significantly differences in the herbicide concentrations and the control (P>0.05). Mitotic index significantly decreased in the herbicide concentration as compared to control at each treatment. The percentage of mitotic index was significantly low at 1 ppm compared to other concentrations.

Table.2 :- Effect of 2,4-D on mitotic cell division of*Allium cepa*.

Treatment conc. (ppm)	No. of cells observed	No. of dividing cells	Mitotic index (% ± SE)
Control	2639	718	27.21± 0.017
0.1	2817	618	21.94 ± 0.025
0.5	2618	541	20.66 ± 0.065
0.75	2914	468	16.06 ± 0.009
1.0	2516	324	12.88 ± 0.044

Effect on chromosomal aberrations

Results of Geno toxicity tests with *Allium cepa* were evaluated (Table – 1&2). The result shows that the highest concentration (1.0 ppm) showed the highest toxicity followed by 0.75, 0.5 and 0.1 ppm in the same order. The plate 1 to 7 shows the different types of changes in the organization and morphology of the chromosome in the root tips exposed to 2,4-D. As is evident from Figures and tables, seven types of chromosomal aberrations were recorded in anaphase-telophase cells.

- Sticky chromosomes – Chromosomes that fail to condense completely at metaphase chromatin masses which are undistinguishable as chromosomes are seen as clumps in extreme cases cells having sticky chromosomes lack spindle fibers.
- Multipolar anaphases and telophases – these are cells in anaphases and telophase stages that have more than two spindle poles instead of the normal two.
- Anaphase or telophase bridge – Dicentric chromosomes that form a bridge between both poles at anaphase or telophase caused mainly by the breakage & fusion of chromosomes.
- C- Mitosis – Mitotic cells that lack spindle fibers with unattached whole lying scattered throughout the cells. The effect is commonly produced in cells treated with the spindle poisious.
- Laggard - These are whole chromosomes that fail to migrate to either pole at anaphase because of possible damage to kinetochore.

The total mean percentage of aberrations (C-mitosis, multipolar delayed anaphases, Vagarant or laggard, Sticky-metaphase, chromosomal bridge at telophase, sticky telophase bridges & multipolar anaphase bridge) according to total cells were 12.50%. The c-mitosis & laggard were found to the most common followed by chromosomal bridge & telophase multipolar anaphase, multipolar delayed anaphase, sticky telophase bridge & sticky c-mitosis, multipolar delayed anaphase & sticky metaphase were not found in control. It is also clear that the total chromosomal aberrations increases with the increase in the concentration of herbicide.

Table :- 3 . Different types of chromosomal aberration induced by 2,4-D.

Conc (ppm)	No. of cells	cm	MDA	Lg	sm	cbt	stb	mpa	Total % ± SE
Control	718	0	0	2	0	3	3	2	101.39 ± 0.012
0.1	618	4	5	4	6	5	3	6	335.34 ± 0.026
0.5	541	7	7	6	7	7	6	8	488.87 ± 0.031
0.75	468	9	10	12	7	10	9	10	6714.32 ± 0.26
1.0	324	18	13	16	9	15	14	12	9729.94 ± .029
Total	-	38	35	38	29	37	35	38	-

Cm → C-mitosis
MDA → Multipolar delayed anaphase
Lg → Laggard
Sm → Sticky mitosis
Cbt → Chromosomal bridge at telophase
Stb → Sticky telophase bridge
Mpa → Multipolar anaphase

The mitotic division showed the presence of 16 chromosome in fig.2

Fig-2 :- Mitosis in onion root tip at control.

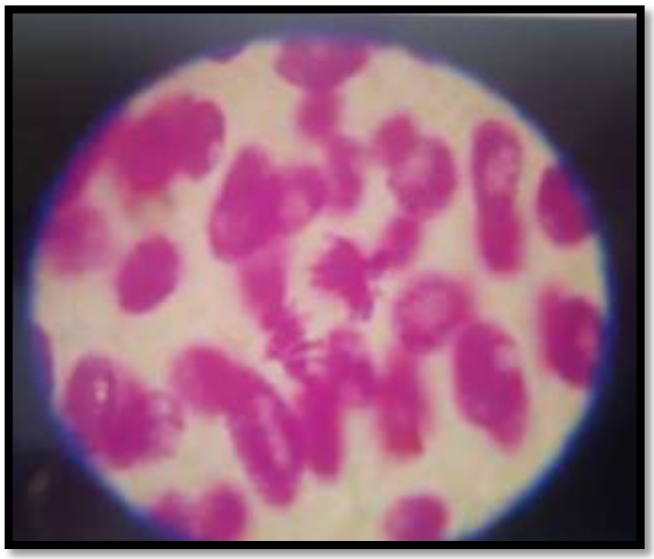

Fig -3 :- Mitosis in Onion root tip after treatment of 2,4-D

Fig-4:- Leggard treated with 2,4-D

Fig- 5:- Multipolar delayed anaphase treated with 2,4-D

Fig-6:- Showing sticky mitosis metaphase treated with 2,4-D

Fig- 7:- Showing sticky telophafe bridge treated with 2,4-D

Discussion

The *Allium* metaphase test is also suitable for the detection of radiation, since radiation causes similar CAs as genotoxic substances (Al-Sabti 1989). So these two tests, for radioactive emissions and genotoxic substances, can be combined in a single testing session. A triradial three-arm chromosome configuration arises by the interaction of two chromosomes each having an isochromatide breakage. Many types of triradial chromosomes can be found in mammalian cells. The main reason for this type of damage is ionizing radiation that induces the formation of dicentric and ring chromosome (IAEA 1986; Hatayoglu and Orta 2007). Genotoxic substance research is of great importance for the protection of the environment because it enables an insight into the influence of genotoxic substances on organisms. The goal of the research is to develop a valuable tool for the monitoring of the environment.

In the present study though there is significant decrease in the mitotic index at all the concentrations. But the decrease below 50% with respect to the control was observed only at the highest concentration. The EC_{50} value determined for the test compound was 0.5 ppm. According to Rank and Nielsen (1977) the mitotic index will never be below half of the control. Thus, the failure of rest of the concentrations to reduce the MI below 50% of this control is in agreement with the results of Rank and Nielsen (1977).

However, investigations of recent years appears to be more consistent. Holland *et al.* (2002); showed that 2,4-D increased the number of nucleus at the highest non-toxic level.

The mutagenic effects observed in cells treated with the 2,4-D &Endosulfan included C-mitosis, stickiness chromosomal bridges, multipolar anaphase and leggards. These results are in agreement with Aellium*cepa* root with different herbicides, such as sencorer and Gasegard (Haliem 1990; Monsa 1982), Cyanazine (Papes*et al.* 1989) carbetamex and Paradoneplus (Badr 1983). Cytotoxic effects of the herbicides used in the present case were in conformity with saxena*et al.*2004; Gul *et al.* 2006; Mustafa and Arikan 2008.

C-mitosis one of the most mommon anomaly observed mayu occur due to disturbed microtubules by 2,4-D and this results in C-mitosis. Stickiness is induced either by the effect of herbicides on chromosomal protein attributed to the improper folding of chromosome fibers or may be due to the action of herbicides on the polymerization process, resulting in the fragmentation of chromosomes and bridges forms sticky chromosomes ((EI- Ghamery*et al.* 2000). Stickiness is accepted as an indicator of toxicity which results in cell death (Fiskerjo, 1985; EI- Ghamery*et al.* 2000).

Multipolar spindles are generally characteristic of cancer cells. Cells with multipolar spindles are characterized by more than two centrosomes, usually four, sometimes have a second metaphase plate. The multiple chromosomes segregate to opposite ends and the spindles attach to the chromosomes haphazardly, when anaphase occurs in these cells, the chromosomes are separated abnormally and results is aneuploidy of both daughter cell (Kryukov , F, 2011). This can lead to loss of cell viability (Sato, Norihito, 2004) and chromosome instability (Neil *et al.* 2009). A possible cause of multipolar spindle formation involve regulation of protein kinase family known as Aurora kinase. Deregulation of these proteins can lead to multipole centrosome formation & aneuploidy (Jingyan Fu, 2011).

Levan (1938) described C-metaphase or C-anaphase as an inactivation of the spindle followed by a random scattering of the condensed chromosomes in the cell. It has also been suggested by Yildiz and Arikan (2008) that large number of laggard chromosomes and C-anaphases indicated that a test compound acted as a strong spindle inhibitor. Badr*et al.* (1992) attributed the induction of anaphase / telophase bridges to chromosome breaks,

Stickiness and breakage and remain of the broken ends of chromosomes. Therefore the anaphase/ telophase brides in the present study suggested a clastogenic effect of 2,4-D. The 2,4-D thus caused abnormal DNA condensation, abnormal chromosome coiling and inactivation of spindle. The 2,4-D therefore have the potential to cause aneuploidy in exposed organisms and adverse human health & environmental effects.

Conclussion:-

In summary , genetic toxicology investigates the interaction between chemical and physical agents with genetic material which shows subsequent adverse effect , such as cancer or genetic disease in further generation . These changes in genetic material of organism can be detected by cytogenetic assay.

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