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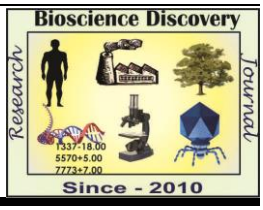

Cytotoxic effect of acridine orange and ethidium bromide on *Allium cepa* L. and *Oryza sativa* L.

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Abstract

Various studies on the effect of mutagenic chemicals on living systems have been conducted because of their adverse influence on genes that could be passed through generations after generations. Cytotoxic chemicals induce damages to the components of DNA and thus results into mutations. Acridine orange, a nucleic acid-selective fluorescence dye and ethidium bromide, another nucleic acid stain are used extensively in molecular biology laboratories that are known to exhibit mutagenic properties under chronic use. The present study envisaged to make convenient tests on two model biological systems (*Allium cepa* L. and *Oryza sativa* L.) for estimating the possible cytotoxic effect of both acridine orange and ethidium bromide respectively.The high mitotic index and lower percentage of abnormality was very much evident in roots treated with diluted mutagens. The mitotic index showed decreasing tendency with parallel increase in mutagenic concentrations. Binucleate, stickyanaphase, C-metaphase, polyploidy, unequal distribution of chromosomes, spiral nature of chromosomeand distortion of poles are some of the common abnormalities observed in all treatments. Both duration andconcentration of the treatment influenced the cell division.Further,growth behaviour of rice (*O.sativa)* showed decreasing tendency due to the effects of both the mutagens and revealed negative correlation like germination index, shoot length, root length, lowering of shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, relative water content and also the total chlorophyll content can be claimed to be a direct indication of the treated mutagens being harmful to all these essential aspects of plant life. The types and percentage of abnormalities in mitotic index of *A.cepa* and retarded growth behaviour of *O. sativa*confirmed the possible mutagenic effect of acridine orange and ethidium bromide on the crop and other biota in agro-ecosystem.

INTRODUCTION

Various studies on the effect of mutagenic chemicals on living systems have been conducted because of their adverse influence on genes that could be passed through generations after generations (Caritá *et.al.*, 2008). Cytotoxicity is the ability of chemicals to destroy the functioning of living cells. Cytotoxic chemicals induce damages to the components of DNA and thus results into mutations. Chronic exposure to low doses of these chemicals may affect biodiversity, while in humans it may increase the risk for cancer development. The genetic information can be duplicated, deleted, inserted or interact with DNA that regulates the fidelity of the genome, such as the spindle apparatus.Acridine orange (AO) (3-N, 3-N, 6-N, 6-N-Tetramethylacridine-3,6-diamine), a heterocyclic organic compound is a versatile nucleic acid-selective fluorescence dye used to stain acidic vacuoles, RNA and DNA in living cells.

The mutagenicity of these compounds is postulated as being due to the intercalation of the acridine molecule between adjacent nucleotide pairs of DNA (Drake, 1970). This results in the addition or removal of one or several base pairs, producing frame-shift mutations (Roth, 1974). Ethidium Bromide (EtBr) (**5-ethyl-6-phenylphenanthridin-5-ium-3, 8-diamine bromide**) another nucleic acid stain is used extensively in molecular biology laboratories. Even though EtBr is known as a strong mutagen, it is still being used in the laboratories as intercalators to visualize DNA (Hayashi, 2007).

Cytogenetic test analyses the frequency and type of chromosome aberrations in mitotic cells because it reflects early warning signs of adverse long term effect in the populations (Hose, 1994). The common onion, *Allium cepa*L. makes a convenient test system for estimating harmful effects of these chemicals on biological systems (Fiskesjo *et al.,* 1985, 1993; Rank *et al.,* 1998).

Another biological system to evaluate the seedling growth as well as other cytological characteristics for testing mutagenic sensitivity in plants is the rice plant, *Oryza sativa* L. (Amjad *et al.,*2002). Rice being a food crop, could be a model system for scientific research in laboratory conditions (Till *et al.,*2007). Hence, in the present work, an attempt has been made to assess the possible cytotoxic effect of both acridine orange and ethidium bromide on *Allium cepa* L. and *Oryza sativa* L. respectively.

MATERIALS AND METHODS

Selection of plant model: The present investigation was carried out in two model plants. They include *Allium cepa*L. (onion) for investigation on chromosomal aberrations and *Oryza sativa*L. (rice) for growth behaviour. Medium sized fresh onion bulbs were collected from the local market. Onion bulbs were grown on tap water for the initial root growth at room temperature. Young healthy roots were taken for the treatment of the chemical mutagen acridine orange.

Preparation of chemicals: Four different concentration of acridine orange (0.25µg/ml, 0.5µg/ml, 0.75µg/ml and 1.0µg/ml) were prepared and treated on healthy onion bulbs of uniform size for a period of 24, 48 and 72 hours each respectively. The onion bulbs grown in tap water were considered as control.

Cytological studies: Clean and healthy bulbs of onion were chosen for each treatment. The dry scales of onion bulbs were removed and then induced to rooting by placing them on glass of sandy soil with the base of the onion touching the surface of the soil at room temperature. When the roots grew upto 2.0 cm in length, they were cut and treated with, different prepared concentrations of acridine solution. Each concentration was kept for 24, 48 and 72hours respectively to study their cytotoxic effect. The root tips treated in distilled water was used as control. With an interval of 24 hours, 5-6 root tips were collected from treated onion bulbs for successive 3 days. The collection time was maintained at 8:00 am in the morning and the root tips were fixed in Carnoy's fluid (1 glacial acetic acid: 3 absolute alcohol) for 6 hours. After fixation, they were preserved in 70% alcohol for cytological investigations. Squashes were prepared using by acetocarmine stain and the slides were observed under light microscope.The fixed root tips were heated gently for 2-3 min on a spirit lamp containing 1.5% aceto-orcein solution and placed on a clean slide (Sharma and Sharma, 1980; Toijam *et al.,* 2013; Hore and Tanti, 2014; Sarma and Tanti, 2015). The dark portions of root tip were cut with a blade and put a cover slip on the tip. Microscopic studies were done by the gentle pressing on the cover slip and taping it to disperse the cell and observed under microscope (100X) and cells in mitosis were counted. Hereby, mitotic index (MI) was evaluated by analysing at least 500 cells per treatment (Tanti *et al.,* 2009). Chromosomal Abnormalities (CA) were calculated for each concentration and the values were expressed as mean ± SD. MI was calculated using following formula:

$$MI = \frac{\text{Total number of cells in division}}{\text{Total number of cells observed}}$$

Further, to investigate the effect of acridine orange and ethidium bromide on growth parameters, five different rice (*Oryza sativa*L.) varieties were selected*viz*., 'Mahsuri', 'Kola joha', 'IR-64', Salpona' and 'Huwagmani'.

Germinating Index (GI): Germinating Index (GI) is determined by the following formula (Li *et al.*, 2008).

$$GI = \frac{n}{d}$$

Where, 'n' is number of seedling emerging on day'd'
'd' is day after planting

After counting the GI, rice seedlings were transferred to small plastic glasses containing *i.e.* hydroponic culture containing Yoshida solution in such a way that their roots could sufficiently reached and be immersed in the nutrient solution. The different nutrient solutions were prepared by dissolving 0.25µg/ml, 0.5µg/ml and 1.0µg/ml of acridine orange and ethidium bromide solution into the required amount of Yoshida solution. The pH of the nutrient solutions were maintained at 5.8. Yoshida solution was used as control. After seven days, growth was measured in terms of root length, root fresh weight, root dry weight and shoot length, shoot fresh weight and shoot dry weight. Three plants were randomly selected from each roots and shoot samples were taken with the help of weighing balance. Immediately after taking them out of glasses and wiping out the moisture with tissue paper. The roots and shoots were oven dried at 80℃ for 72 hours in order to take their dry weight.

Measurement of relative water content (RWC): Relative water content (RWC) was determined by weighing shoot and floating it on deionised water for four hours at constant temperature in diffused light. When shoot became fully turgid, it was reweighed and dried for 72 hours and weight was determined. The shoot RWC was calculated by the following formula (Barrs and Weatherly, 1962).

$$RWC\ (\%) = X\ 100\ \frac{(FW-DW)}{(TW-DW)}$$

Where, FW=Fresh weight, DW=Dry weight and TW=Turgid weight

Estimation of total chlorophyll content: Chlorophyll a, chlorophyll b and total chlorophyll were determined by using the method postulated by Arnon (1949). Fresh leaves of 300mg were crushed in mortar and pestle with 5ml of 80% acetone. The absorbance was taken at 663nm and 645nm using spectrometer against 80% acetone as blank.

$$\text{Total chlorophyll} = \frac{(20.2\times A645) + (8.02\times A663)}{W} \times 1000$$

RESULTS AND DISCUSION

Effect of acridine orange on chromosome behaviour: The chemically untreated onion root tips revealed normal behaviour of chromosomes comprising all the stages with normal metaphase 2n = 16. However, the root tips collected from the growing media containing 0.1µg/ml, 0.5µg/ml, 0.75µg/ml and 1.0µg/ml concentrations for 24, 48, 72 hours of exposure time in acridine solution, various abnormalities such as cell vacuolation, larger chromosome at prophase stage, chromatid bridges at early anaphase, broken metaphase, swollen chromosome, disintegrated nucleus, clumped chromosomes in metaphase were observed. A distinct dose dependent increase in chromosomal anomalies was observed in all treatments (Table-1 & Fig-3). Thequantitative results found to be statistically highly significant when compared to control ($P<0.001$). The frequency of nuclear disintegration was found to be higher during 24h exposure while, the frequency was reduced in 48 and 72h exposures respectively. The cytotoxicity levels of an agent can be determined by the increase or decrease in the mitotic index (Leme *et al.*, 2009).The mitotic index (MI) reduced greatly with the increase in concentration and duration.

Lowering of MI in treated root meristem could be due to inhibition of DNA synthesis (Sudhakar *et al*., 2001). The high frequency of mitotic abnormalities such as chromosome divergence, lagging chromosome, nuclear disintegration, chromosomefragmentation, distortion of pole in anaphase and C-metaphase induced by acridine orange affects on mitotic spindles and altering the orientation of chromosomes at various stages of the cell cycle. Impairment of mitoic spindle function is probably due to the interaction of acridine orange with tubulin-SH group (Kuriyama and Sakai, 1974).Observation of chromosome stickiness is another type of abnormality induced by acridine orange. The stickiness presumably is due the intermingling of chromatin fibres,which leads to sub chromatid connections between chromosomes (Klasterska *et al*.,1976).

Table-1: Mitotic indices and chromosomal aberrations observed in *A. cepa* root meristem treated with acridine orange.

Conc. (µg/ml)	Duration (hours)	Total Analysed Cells	No. of Cells Showing Division	Total aberrant Cells	Mitotic Index (%) [Mean+SE]	Chromosomal Aberration (%) [Mean+SE]
Control	-	1017	847	50	8.31±0.16	4.89±078
0.25	24	1068	844	79	7.8±0.22	9.18±0.86
0.5		1033	574	98	5.54±0.12	17.11±0.91
0.75		1049	492	110	4.69±0.1	22.45±0.74
1.0		1071	483	119	4.46±0.9	17.5±0.29
0.25	48	1048	680	119	6.49±0.13	17.5±0.29
0.5		1060	462	124	4.26±0.1	26.92±0.65
0.75		1029	390	116	3.79±0.05	29.98±171
1.0		1088	732	160	6.75±0.17	21.89±1.76
0.25	72	1065	606	251	5.7±0.1	41.6±2.11
0.5		1029	537	166	5.2±0.04	47.55±0.77
0.75		1050	184	104	1.74±0.03	56.27±1.1
1.0		1055	634	280	6.0±6.01	44.31±0.45

Effects of AO and EtBr on growth pattern of rice: Determination of mutagens efficiency is necessary for its use in mutation breeding (Makeen*et al*., 2010). In this present study, both acridine orange and ethidium bromide showed influence on seed germination and seedling growth in five varieties of rice.

Seed germination: Seed germination is an important parameter used to gauge the response of plants to mutagenic treatments germination percentage was observed with an increase in the concentration of AO. The reduction in the germination percentage induced by AO treatment was less as compared to that in the EtBr. In ethidium bromide treatment, the maximum was 5.48 (0.25µg/ml EtBr) and minimum was 1.01(1.0µg/ml EtBr). Mutagenic treatments revealed a gradual decreasing trend in germination from lower to higher doses (Sunil *et al*., 2011). The treatment with acridine orange and ethidium bromide inhibits the germination of percentage of seed. The overall seed germination index is more than control, acridine orange and ethidium bromide both produce toxic effect on germination. In the mixture treatment of AO and EtBr on rice varieties, it was observed gradually response of plants to mixture chemicals.

Shoot and root length under mutagenic stress: The seedling shoot height in control plants was 9.67cm (Table- 3). It was maximum (9.16 cm) at 0.25µg/ml AO and minimum (6.6cm) at 1µg/ml AO. The gradual decrease in seedling shoot height was recorded with an increase in the concentration of AO (Table- 3).

Table- 2: Effects of AO and EtBr on seed germination of rice.

Chemical conc.(µg/ml)	Mahsuri	Kolajoha	Huwagmoni	IR-64	Salpona
Control	5.07±0.50	2.91±1.25	1.32±0.30	3.81±0.86	2.83±0.26
0.25AO	5.91±0.61	3.78±0.14	1.48±0.28	4.70±0.89	3.37±0.86
0.5AO	4.95±0.21	3.28±0.89	1.45±0.24	3.80±0.17	3.16±0.22
1.0 AO	3.40±0.64	3.01±0.94	1.12±0.14	3.51±0.41	3.54±0.70
0.25EtBr	5.48±0.96	3.92±2.95	1.37±0.01	4.68±0.16	3.28±0.38
0.5EtBr	5.42±0.53	3.77±0.10	1.07±0.56	3.65±0.21	2.74±0.06
1.0EtBr	4.11±0.54	3.21±0.28	1.01±0.66	3.41±0.01	3.31±0.89
0.25(AO+EtBr)	4.26±1.24	3.11±0.80	1.28±0.08	4.96±2.39	3.37±0.04
0.5(AO+EtBr)	4.17±0.67	3.11±0.80	1.28±0.8	3.24±0.02	3.29±0.04
1.0(AO+EtBr)	4.09±0.51	3.22±0.20	0.89±0.45	3.99±0.77	2.88±0.16

In EtBr treatment, it was reduced with the increase in the doses of ethidium bromide but at 0.5µg/ml, it was increased more than control (Table- 4). The highest shoot height (9.76cm) was observed in 0.5µg/ml EtBr while the lowest (7.97 cm) was noted in 0.25µg/ml EtBr concentration (Table- 4). The seedling height reduction induced by acridine orange was more as compared to that of ethidium bromide. This indicates that 1.0µg/ml conc. of AO and 0.25µg/ml EtBr of treatments have an inhibitory effect on seedling shoot height (length of root and shoot). The reduction in length of root and shoot was attributed to the effects of mutagens on the physiological system (Gaul, 1977).

Table- 3: Effects of AO conc. on seedling pattern of rice.

Chemical conc. (µg/ml)	Seedling shoot length (cm)	Seedling root length (cm)	Fresh weight of shoot (mg)	Dry weight of shoot (mg)	Fresh weight of root (mg)	Dry weight of root (mg)
Control	9.67±1.7	9.87±1.95	0.3±0.006	0.03±0.02	0.107±0.018	0.012±0.02
0.25AO	9.16±1.6	10.46±1.79	0.023±0.008	0.003±0.01	0.013±0.013	0.004±0.002
0.5AO	7±0.43	6.5±0.85	0.022±0.002	0.001±0.005	0.011±0.008	0.004±0.002
1.0AO	6.6±1.03	7.13±3.78	0.017±0.003	0.008±0.002	0.009±0.007	0.004±0.003

The seedling root height in control plants was 9.87cm (Table- 3). In the acridine orange treatments, it was maximum (10.46cm) at 0.25µg/ml and minimum (6.5cm) at 0.5µg/ml. Although gradual decrease was recorded with an increase in concentration but at 1µg/ml AO root height was increased (7.13cm) than control (Table-3& Fig-1). An inhibitory reduction was obtained in EtBr treatment where maximum (6.04cm) was at 0.25µg/ml EtBr and minimum (3.5cm) at 1µg/ml concentration where inhibitory effects were the most (Table- 4, Fig-2).

According to Datta *et al*., (1992), the reduction in root length may be the result of marked suspension of mitotic division, affected nuclear condensation causing irregular distribution of chromosomes bridges and fragmentation.

Table 4: Effects of EtBr on seedling pattern of rice.

Chemical conc. (µg/ml)	Shoot length (cm)	Root length (cm)	Shoot fresh weight (mg)	Shoot dry weight (mg)	Root fresh weight (mg)	Root dry weight (mg)
Control	9.67±1.7	9.87±1.95	0.30±0.007	0.04±0.10	0.1±0.18	0.001±0.08
0.25EtBr	7.97±0.057	6.04±0.94	0.026±0.004	0.004±0.002	0.01±0.001	0.002±0.002
0.5 EtBr	9.76±1.62	5.34±0.68	0.028±0.004	0.005±0.003	0.008±0.001	0.004±0.004
1.0 EtBr	8±0.5	3.5±0	0.0203±0.005	0.009±0.007	0.001±0.002	0.005±0.002

Shoot and root fresh weight and dry weight: The results (Table- 3 & 4) indicated that the mutagens treatments on rice had significant effects on fresh and dry weight of shoots and roots. In every parameter, all were inhibited by the mutagens as decreasing order. The fresh weight of shoot and root was highest at 0.25µg/ml and lowest at 1.0µg/ml of acridine orange concentration (Table- 3). In this present study, there was a reduction in dry weight of root and shoot arising out of mutagenic treatment of AO. Again, in the EtBr treatments, the fresh and dry weights of shoots and roots were decreased like acridine orange. Both the mutagens showed the same effects on these parameters.

Relative water content: The relative water content of the shoot in control plants was 0.036mg. In this present investigation, the results of water content of rice in different concentrations of acridine orange were decreased in an increase of doses of treatment. It was maximum at 0.25µg/ml and minimum at 1.0µg/ml of AO. At ethidium bromide also decreased with an increase of conc. of chemicals. It was maximum at 0.25µg/ml and minimum at 1.0µg/ml of AO (Table- 5).

Table- 5: Effects of acridine orange and ethidium bromide on relative water content.

Chemical conc. (µg/ml)	Relative water content of shoot in acridine orange (%)	Relative water content of shoot in ethidium bromide (%)
Control	18.46	18.46
0.25	57.56	49.96
0.5	47.57	85.45
1.0	3.83	60.02

Total chlorophyll content: The chlorophyll content in rice seedling enhanced more than control (Table-6). Fluctuation of chlorophyll content in rice may be due to inducing nature of mutagens. Similar results on seed germination bioassays to assess toxicity of molasses fermentation based bulk drug industry effluent were reported (Nagda *et al*.,2006; Srivastava and Sanhai 1987). Higher concentration of wastewater are inhibitory to synthesis of chlorophyll molecules particularly chlorophyll *a* (khan *et al*., 2011). In both the mutagens, significant difference result is obtained in between the 645nm and 663nm wavelength.

Table-6: Effects of AO and EtBr on total chlorophyll content.

Chemical conc. (µg/ml)	Chlorophyll content
Control	0.00117
0.25AO	0.0113
0.5AO	0.0113
1.0AO	0.0126
0.25EtBr	0.0100
0.5EtBr	0.0150
1.0EtBr	0.0091

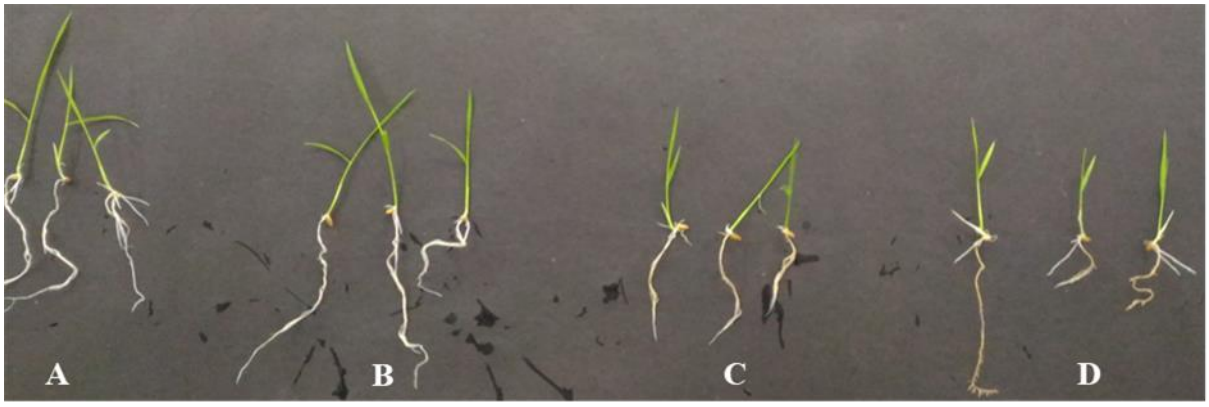

Fig-1: Effect of acridine orange on rice, A-Control, B- 0.25µg/ml, B-0.5µg/ml, D -1.0µg/ml

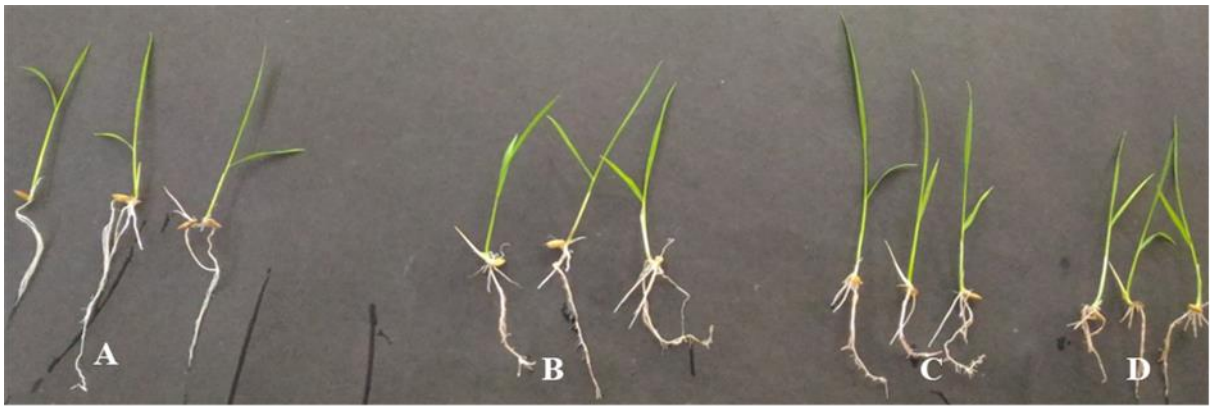

Fig-2: Effect of ethidium bromide on rice, A-Control, B- 0.25µg/ml, B-0.5µg/ml, D -1.0µg/ml

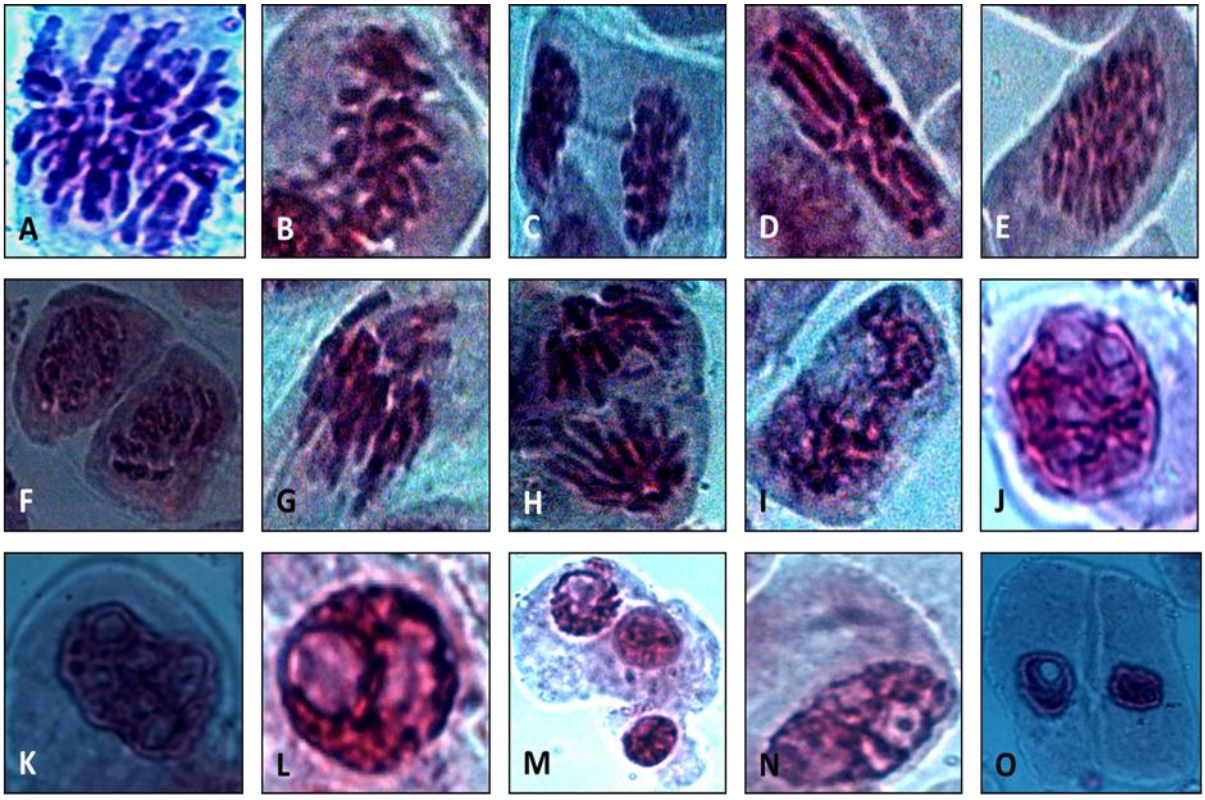

Fig-3: Chromosome abnormalities in the various phases of mitotic cell division of acridine solution treated onion root tips. A-B. C-Metaphase, C. Anaphase Bridge, D. C-Anaphase, E. Polyploidy, F. Spiral nature of chromosome, G. Sticky anaphase, H. Syncytium form in anaphase, I. Nuclear disintegration, J-L. Resident chromosome in metaphase, M. Binucleate cell, N. Micronuclei, O. Nuclear lesion and fragmented prophase.

CONCLUSION

Effects of different chemical mutagens in plant have immense potential for the genetic variation leading to the crop improvement and agricultural sustainability. The present study provides an evidence for the possible genotoxic effect of acridine orange on plant system. Various types of chromosomal aberrations occurred in onion (*Allium cepa*L.) root meristem. While growth parameters of rice (*Oryza sativa* L.) like the decreasing germination index, decreasing shoot length, decreasing root length, lowering of shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, relative water content and also the total chlorophyll content can be claimed to be a direct indication of the treated mutagens being harmful to all these essential aspects of a plant life. Therefore,the results of the present work indicated that genetic variation could be created by chemical mutagens like acridine orange and ethidium bromide. Moreover, it is necessary that in addition to routine physico-chemical and biological analyses, the industrial effluents should also be evaluated for the cytological damages caused by acridine orange and ethidium bromide to plant cells.

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