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Mitodepressive Activity of two Common Food Dyes on *Allium CEPAL*. Root Meristem

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Abstract: Food colours are used to increase the aesthetic qualities and to enhance the commercial viability of food products in the market. Many of the commercial food colours have been found to be potentially genotoxic in plant and animal models. This investigation was carried out to evaluate the mitodepressive effect of selected food dyes- Orange red and Apple green on *Allium cepa* L. root tip cells. Root tips of *A. cepa* L. were treated with four different concentrations (0.05, 0.1, 0.2 & 0.4 %) of the test dyes at an exposure time of 48 hours. Mitotic aberrations were evaluated by conventional aceto-orcein staining. The major mitotic abnormalities observed were single anaphase bridge, multiple anaphase bridges, sticky chromosomes, disoriented spindle apparatus and star anaphase. The results showed a concentration dependent variation in the abnormality index and mitotic index. The observations in the present investigation suggest the scope for further studies to establish the genotoxic potential of the food dyes assessing its safety for human consumption.

Keywords: Mitotic aberration, Orange red, Apple green, Mitotic index, cytotoxic.

I. INTRODUCTION

Ever since prehistoric times, man was fascinated to colour the objects of daily use. Earliest recorded use of food colour comes almost from India, China and Egypt in 1500 BC. Since then the trend of consumption of food coloured with food dyes has been increasing day by day. Colors play an important role in food identification, making the food more delightful. Before the mid 19th century food dyes used were of natural origin were extracted from natural sources, mainly from vegetables and fruits. But due to high cost, lack of availability of natural colouring material and difficulty in incorporating those in modern food processing, synthetic food colours has replaced the natural colouring substances.

Food coloring, or color additive, is any dye, pigment or substance that imparts color when it is added to food or drink. They come in many forms consisting of liquids, powders, gels, and pastes. Food coloring is used both in commercial food production and in domestic cooking. Colour additives are categorized as either dyes or lakes. Dyes dissolve in water and are manufactured as powders while lakes are the water-insoluble forms of the dyes. Lakes are more stable than dyes and are ideal for colouring products containing fats and oils, or items without enough moisture to dissolve the dyes. In the United States, the Food and Drug Administration divides food colour additives into two groups: certified, and exempt from certification. Certified colours are synthetically produced and used widely because they impart an intense, uniform color, are less expensive, and blend more easily to create a variety of hues. Colors that are exempt from certification include pigments derived from “natural” sources such as vegetables and animals as well as metals/minerals such as aluminum, silver, iron, and titanium dioxide. These are usually more expensive, and may add unwanted or unintended flavours to foods.

The control of the use of food dyes is based on the Acceptable Daily Intake (ADI), which, in turn, is based on the research findings and the recommendations of the Codex Committee on Food Additives and Contaminants (CCFAC) [1] Despite the required limits, the use of synthetic dyes in foods still raises a number of questions regarding their toxicity [2], [3]. These evaluations are extremely important in terms of establishing the potential of chemicals to cause cytotoxic, genotoxic and mutagenic conditions that can greatly contribute to the development of cancer [4]. The toxicity evaluations, in addition to warning about the permitted tolerance limits of dyes, have banned the use of some synthetic dyes worldwide, such as solid yellow, orange GGN, solid red, alizarin blue and scarlet GN because these additives revealed cytotoxicity, genotoxicity and mutagenicity in various test systems [5].

Bioassays with plants have been considered highly sensitive, rapid and simple for the monitoring of toxic effects of chemicals at the cellular level [6] and root meristem cells of *Allium cepa* L. have been shown as an efficient plant test organism for this type of evaluation [7] for their kinetic properties of proliferation, large chromosomes few in number ($2n = 16$), which facilitates their analysis [8], for enabling verification of alterations in the cell division rate (mitotic index) and cell aberrations [9], and for demonstrating satisfactory similarity to the results obtained with other bioassays, such as in animals and in cell cultures [10]. There

are reports which emphasize that even though the plant metabolism is different from that of animals, the results obtained by this system test are good toxicity analysis parameters at the cellular level, and have long been used to alert the population about the consumption of some foods and synthetic and natural medicines [10]-[12].

In this background, the present study was carried out by selecting the commonly used food colors- Orange red and Apple green to evaluate their mitodepressive effect by *Allium* assay. These dyes are commonly used in the preparation of ice creams, sweets and jellies in both urban and rural areas of the country.

II. MATERIALS AND METHODS

The test dyes Orange red and Apple green were procured from the local market. Bulbs of *A. cepa* L. weighing about 50 g were placed in coplin jars containing distilled water for growth of roots at room temperature. After two days the bulbs were transferred to coplin jars containing different concentrations of the test dyes and exposed for 48 hours. The test dye solutions were prepared by dissolving calculated amounts of the dyes in distilled water (0.05, 0.1, 0.2 & 0.4 %). Three replicates were maintained for each of the dye concentration and respective control was maintained by placing the bulb in distilled water.

After 48 hours of treatment, the roots were excised from the *Allium cepa* L. bulbs and the meristem tips were fixed in Methacarn fixative (3:1 Methanol : Acetic acid) for 24 hrs. The fixed roottips were hydrolyzed with 1 N HCl for 8 min and stained using 1% aceto-orcein stain for 30 min. Mitotic squash was prepared with 45% acetic acid and coverslips were sealed on the glass slides using clear nail polish.

The roottips were randomly selected from the triplicates and five slides for each concentration of the test dye were prepared and observed under 40X objective. The microscopic observations included number of cells under mitosis, cells in interphase and the presence of mitotic abnormalities. Mitotic index and abnormality index was calculated using the formulae:

$$\text{Mitotic index (MI)} = \frac{\text{Total number of dividing cells}}{\text{Total number of cells counted}} \times 100$$

$$\text{Abnormality index (AI)} = \frac{\text{Total number of abnormal cells}}{\text{Total number of dividing cells counted}} \times 100$$

Statistical Analysis

Statistical analysis was carried out by calculating the mean and standard errors of the mitotic index and abnormality index for all the concentrations of the test dyes. Data was expressed as Mean \pm Standard Error of Mean (SEM). Differences between the control and the different concentrations of the dyes were analyzed by means of Student's unpaired *t*-test. The level of significance for testing the statistical significance was 0.05.

III. RESULTS

In the present study, the variations in mitotic indices, nature of mitotic aberrations and their frequency were analyzed to determine the cytotoxic potential of the test dyes. Table I describes the effect of Orange red dye on mitotic index of *A. cepa* L. root meristem cells treated with different concentrations of the dye for an exposure time of 48 hours. In comparison with the control with a value of 52.6 ± 0.36 , the mitotic index for Orange red dye at a concentration of 0.05% indicated a decrease in MI with a value of 49.6 ± 0.21 . The *t*-value obtained in the statistical analysis was 5.8094 and the *p* value was 0.0043 for 0.05% concentration. These values show that the results were significant at 0.05 level of probability. Similarly, it was observed that there is a statistically significant decrease in the mitotic indices at other concentrations of the dye used.

The mitotic indices obtained for the control and different concentrations of Apple green dye are tabulated in Table II. The results obtained were comparable to that of Orange red and also showed a statistical significant decrease in MI between control and treated samples.

The mitotic aberrations observed in case of both dyes used in the experiment are listed in Table 3. Anaphase single bridge was the most common aberration observed among all the concentrations while chromosomal break was the least observed aberration. The abnormality index calculated for each of the concentration is also listed in table III.

Figure 1 illustrates the normal phases of cell division and different mitotic aberrations observed. The percent frequency of mitotic aberrations as depicted by figure 2 shows that anaphase multiple bridge being the most common aberration with a percent value of 28.57 at a concentration of 0.1% Orange red. In case of Apple green dye, anaphase single bridge with a percent value of 37.03 at a concentration of 0.05% (Fig. 3) was the most common aberration. There was a concentration dependent decrease in the mitotic indices and a similar increase in the abnormality indices in case of both the dye treatments (Fig. 4 and Fig. 5).

Cytological observations of different cellular aberrations in all the concentrations of the test dyes on *A. cepa* L. clearly indicated the mito-depressive potential of the selected food dyes.

IV. DISCUSSION

Food products which are brightly coloured always attract the consumers and because of this many synthetic food colors have been used in the food industry. These food colors are believed to increase the commercial value of the food products. The food colors have been used even for domestic purposes which aim at simulating the color of the food product to be perceived as natural.

Food dyes are complex organic chemicals that were originally derived from coal tar, but now from petroleum. Since these food dyes are cheaper, more stable and brighter than natural colors, they are popularly used. The global production of food dyes is more than 8000 tons per year while India produces 2% of the total production [13]. There are reports on unscrupulous use of these food dyes in rural areas exceeding the permissible limits of the dyes [14].

In the present study, we have selected two most commonly used food dyes to assess their potential in inducing mitotic depression by *Allium* assay. The selected food dyes are Orange red and Apple green, which are blended colors and water-soluble.

Orange red is a combination of Carmoisine and sunset yellow while Apple green is a blend of tartrazine, brilliant blue and 16% sodium chloride. A blend of two or more dyes may produce an altogether different response than that of the individual components with respect to coloring abilities [15]. Carmoisine or azorubine is a synthetic red dye belonging to azo group. IUPAC name of carmoisine is disodium (E)-4- hydroxy-3[4-sulfonatophthalen-1-4) diazenyl naphthalene-1 sulfonate]. The use of this dye has been banned in Japan, Norway and US [16].

FD&C Yellow No. 6, the FDA-approved form of Sunset Yellow, is a water-soluble sulfonated azo dye that is used to color bakery foods, cereals, beverages, dessert powders, candies, gelatin desserts, sausage and many other foods, as well as cosmetics and drugs. The IUPAC name of sunset yellow is disodium 6-hydroxy-5-[(4-sulfophenyl)azo]-2-naphthalenesulfonate.

Apple green is composed of tartrazine, brilliant blue and 16% sodium chloride. FD&C Yellow No. 5, also known as Tartrazine, is used in bakery, beverages, pharmaceuticals, and cosmetics. The IUPAC name of tartrazine is Trisodium (4E)-5-oxo-1-(4-sulfonatophenyl)-4-[(4-sulfonatophenyl)hydrazono]-3-pyrazolecarboxylate. FD&C Blue No. 1 or Brilliant Blue 9 is a water-soluble coloring used in bakery products and drugs. The IUPAC name of brilliant blue is ethyl - [4 - [[4 - [ethyl -[(3 - sulfophenyl) methyl] amino] phenyl] - (2 - sulfophenyl) methylidene] - 1 - cyclohexa - 2, 5 - dienyldene] - [(3 - sulfophenyl) methyl] azanium [17].

These selected dyes were subjected to *A. cepa* assay to evaluate their mitodepressive activity. *Allium* test was first described by Levan and was subsequently considered widely as a practical and reliable system for the screening of environmental mutagens and carcinogens [18]-[20].

Allium assay enables the assessment of different genetic endpoints- mitotic index and chromosome aberrations. Mitotic index is used as an indicator of cell proliferation biomarkers which measures the proportion of cells in the mitotic phase of the cell cycle [21]. Chromosome aberrations analysis not only allowed estimation of genotoxic effects, but also enabled evaluation of their clastogenic and aneugenic actions [22].

The *Allium* test is suggested as a standard in environmental monitoring of pollutants and xenobiotics. Being a short-term test, it has many advantages: low cost, ease to handle, good chromosome conditions for the study of chromosome damage or disturbance of cell division including the evaluation of risks of aneuploidy [19].

The ability of the root cells to activate promutagens further widens the application areas of the *Allium* test. The root cells of *A. cepa* L. possess the mixed function oxidase system which is capable of activating promutagens or genotoxic chemicals. In this test, the appearance of stunted roots can be considered as an indicator of genotoxicity while wilting of roots can be a toxic response. The *Allium* test is a sensitive test and the positive results should be considered as a warning and also an indication that the tested chemical may be a risk to human health and to our environment [19], [23].

In this study, we observed a direct relationship between macroscopic and microscopic parameters for all the concentrations of the test dyes. Concentration-dependent decrease in root growth was observed by root length measurements (Fig.6).

Mitotic aberrations are the changes in mitotic phases of the cell cycle which also include chromosomal aberrations. It can be classified as clastogenic aberrations that are chromatin bridge, chromosomal breaks and ring chromosomes and physiological aberrations which are c-mitosis, vagrants, stickiness, delayed anaphase and laggards [24].

In all the concentrations of the test dyes used, we could observe a concentration-dependent decrease in mitotic indices and an increase in abnormality indices. Such a decrease in the mitotic index indicates that the components of the dye interfere in the normal process of mitosis. Our results showed anaphase single bridge as the most common mitotic aberrations among the ten different aberrations observed. Chromosomal bridges may be due to the chromosomal stickiness and subsequent failure of free anaphase

separation or may be a result of unequal translocation or inversion of chromosome segments [25]. In case of 0.05% Apple green treatment, a highest of 37.03% anaphase single bridges was observed. This was followed by multiple anaphase bridges which accounted for a highest of 28.5% in case of 0.1% Orange red. Stickiness of chromosomes was another common aberration observed in all the treatments. Apple green at a concentration of 0.05% showed 22.22% of sticky chromosomes among all the aberrations followed by 17.07% in case of 0.05% Orange red. This aberration would have resulted from an increased chromosomal contraction and condensation or depolymerization of DNA and dissolution of nucleoproteins. Chromosomal stickiness reflects an irreversible toxic effects leading to cell death [26]. The presence of sticky chromosomes or condensation of chromosomes below their normal size at metaphase might also be due to delay in chromosomal movement by the treatment of dyes [27].

In case of metaphase abnormalities, the major ones observed were distorted metaphase, and disoriented. These can be collectively called as colchicine type mitosis (c-mitosis). C-mitosis refers to the effect of colchicines which prevents the assembly of spindle fibres and results in scattering of the chromosomes over the cells [28]. C-mitotic abnormalities were observed only in the different concentrations of Orange red dye treatment. The occurrence of disoriented chromosomes might be due to action of Orange red on the microtubules of the spindle fibres. The dye could have affected the failure of chromosomal alignment at the equatorial plate. This may be due to dysfunction of spindle and energy deficiency causing a delay in the division of centromeric region [16].

Diagonal anaphase was observed indicating abnormal orientation of mitotic apparatus or distorted mitotic spindles causing disoriented mitosis [16]. Star anaphase was observed significantly in all the concentrations of Apple green. Earlier reports have shown the appearance of star anaphase chromosomes with the treatment of terbutol. The researchers have proposed that this aberration might be explained by an effect on microtubule organizing centers [29].

In case of higher concentrations of both the dyes, chromosomal breaks(4.08%) were observed. Our results are in accordance with the earlier studies which report that high doses of metanil yellow and fast green FCF induce chromosomal breaks and micronucleus [30]. Nuclear lesions were observed in some of the cells under Apple green treatment. Such types of lesions have been observed earlier in *Allium cepa* L. treated with adriamycin [31].

It was observed that the frequency of clastogenic aberrations were much higher than the physiological aberrations in the present investigation. The results of mitodepressive effect of synthetic dyes obtained in the study are in agreement with the earlier studies [32-36].

V. CONCLUSIONS

Food industry is an evergreen industry with fast growth in terms of economics and innovation. To meet the market demands, there is always a constant effort in increasing the attractiveness of food commodities by using food colors and additives. However, some of these intentionally added food dyes of synthetic origin are known to be potentially mutagenic. The results obtained in the present investigation clearly indicates cytotoxic activity of the dyes, Orange red and Apple green on root meristem of *A. cepa* L. The observations in case of Orange red are in agreement with the earlier reports. To the best of our literature search, we report the mitodepressive effects of the dye Apple green on *A. cepa* L. root meristem for the first time. However, further studies are essential to accurately assess the potential risks of mutagenicity and carcinogenicity of these food colors.

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TABLE I

EFFECT OF DIFFERENT CONCENTRATIONS OF ORANGE RED ON ROOT MERISTEM OF *A. cepa* AT EXPOSURE TIME OF 48 HOURS

Sl. No	Concentration of the dye	Average root length (cm)	Total cells examined	Total No. of dividing cells	Mitotic index	SD +/-	SE
1	Control	3.1	676	356	52.6	0.63	±0.36
2	0.05	2.8	612	304	49.6	0.36	±0.21
3	0.1	2.5	605	284	46.9	0.35	±0.20
4	0.2	2.2	580	256	44.1	0.17	±0.10
5	0.4	1.8	576	244	42.3	0.16	±0.09

SD – Standard Deviation

SE – Standard Error

TABLE II

EFFECT OF DIFFERENT CONCENTRATIONS OF APPLE GREEN ON ROOT MERISTEM OF *A. cepa* AT EXPOSURE TIME OF 48 HOURS

Sl. No	Concentration of the dye	Average root length (cms)	Total cells examined	Total number of dividing cells	Mitotic index	SD +/-	SE
1	Control	3.2	572	247	43.1	0.37	±0.21
2	0.05	2.95	563	233	41.3	0.36	±0.20
3	0.1	2.7	560	227	40.5	0.35	±0.20
4	0.2	2.65	542	203	37.4	0.16	±0.09
5	0.4	2.2	531	188	35.4	0.21	±0.12

SD – Standard Deviation

SE – Standard Error

TABLE III

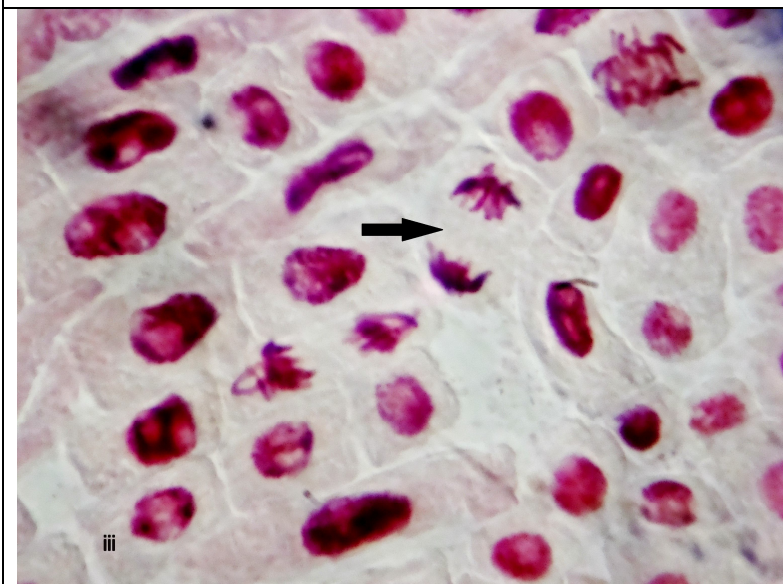
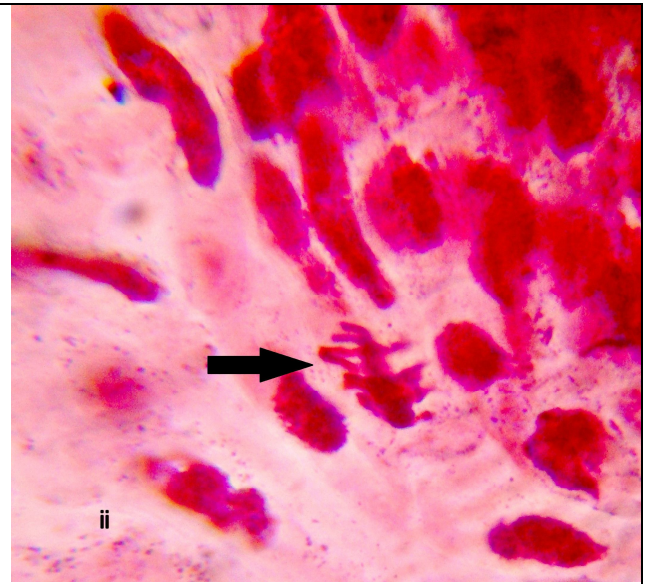
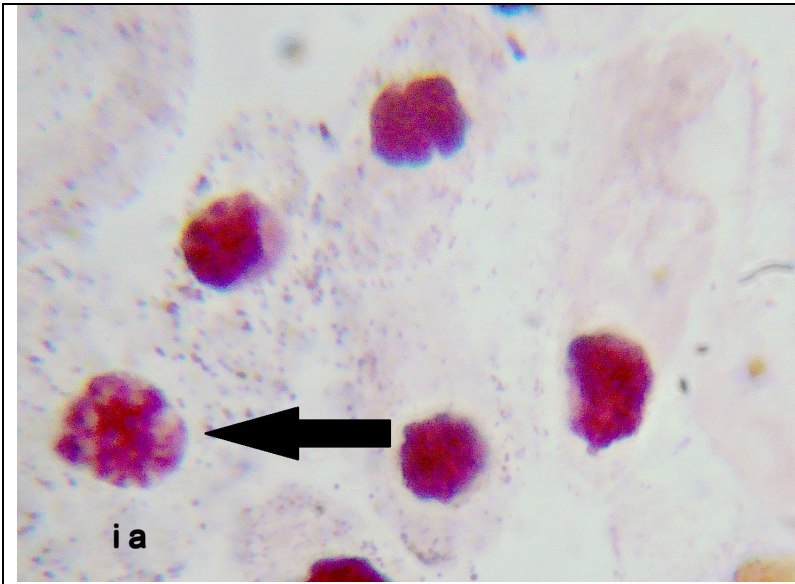
NATURE AND NUMBER OF MITOTIC ABERRATIONS OBSERVED IN ROOT MERISTEM OF *A. cepa* TREATED WITH THE FOOD DYES

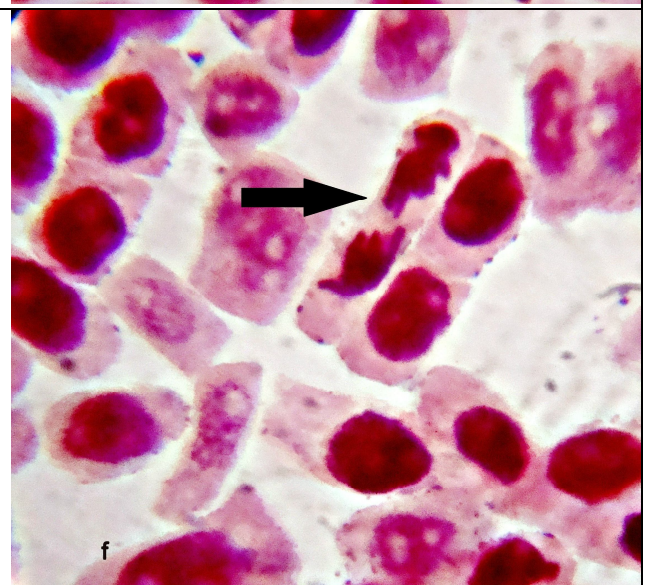
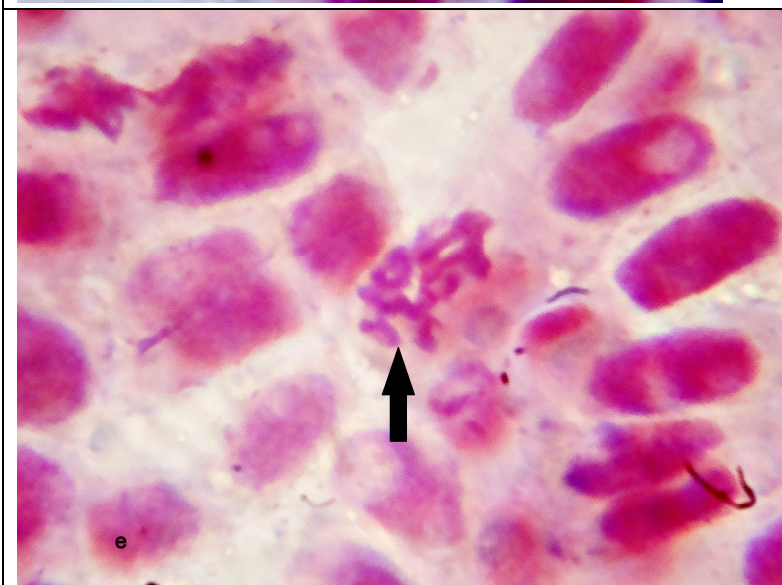
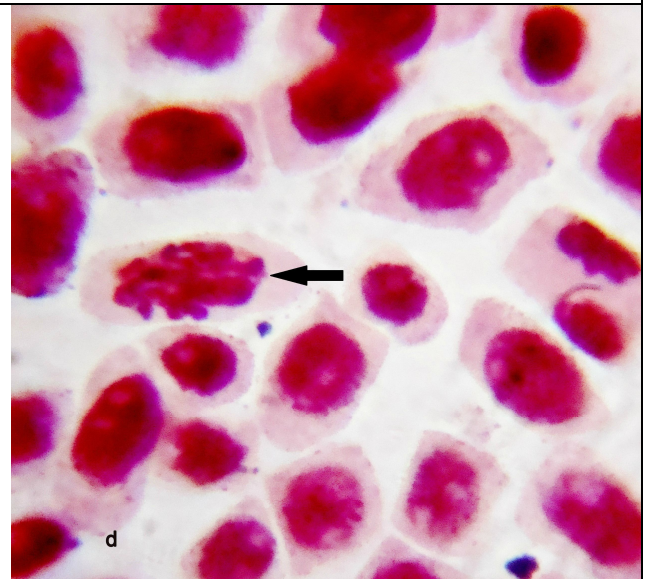
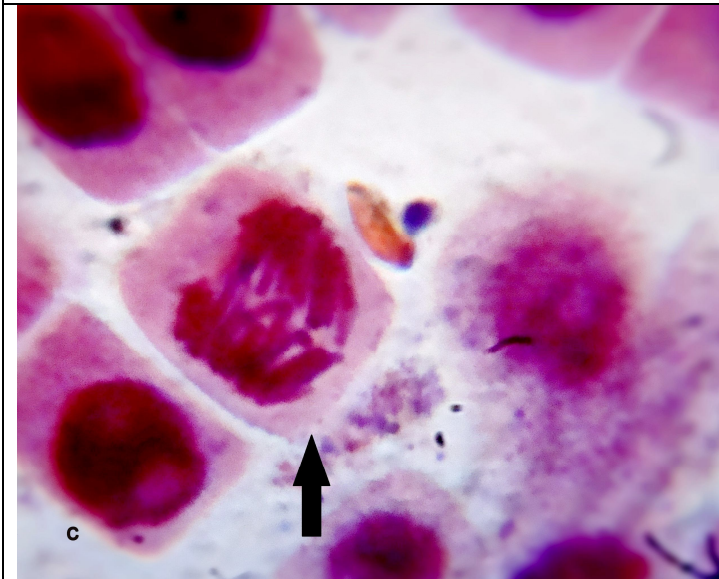
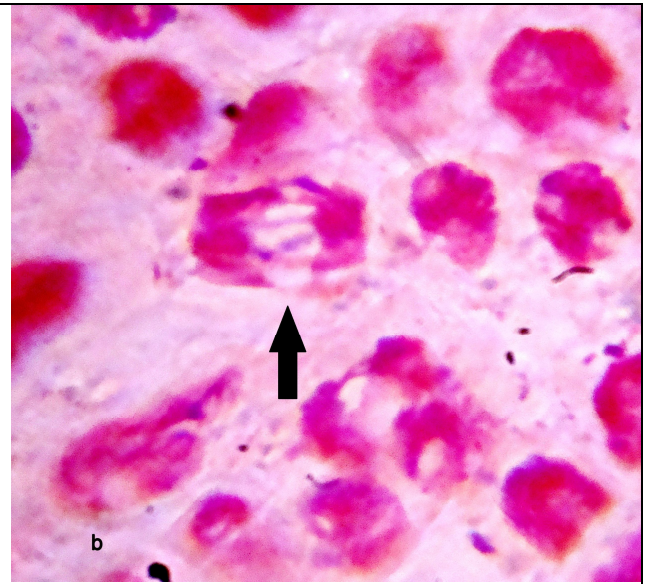
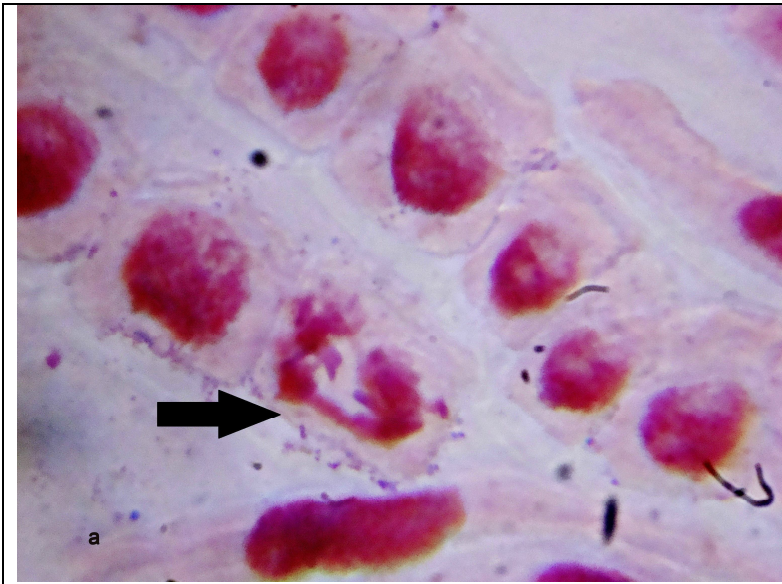
Food Dye	Orange red	Apple green

Conc. (%)		CO	0.05	0.1	0.2	0.4	CO	0.05	0.1	0.2	0.3
Mitotic aberrations (Number of aberrant cells)	Abnormal prophase	-	-	-	-	-	-	01	01	02	02
	Distorted metaphase	-	04	04	05	05	-	-	-	-	-
	Disoriented metaphase	-	02	03	03	03	-	-	-	-	-
	Disoriented spindle apparatus	-	06	06	07	10	-	04	06	07	09
	Anaphase multiple bridge	-	08	14	15	14	-	-	11	10	09
	Anaphase single bridge	-	10	11	11	12	-	10	12	12	11
	Sticky chromosome	-	07	08	10	10	-	06	06	06	07
	Star anaphase	-	-	-	06	06	-	04	05	05	05
	Abnormal telophase	-	04	03	03	04	-	02	02	04	04
	Chromosomal break	-	-	-	02	02	-	-	-	01	02
	Total no. Of aberrant cells	-	41	49	62	66	-	27	43	47	49
	Total no. of dividing cells	356	304	284	256	244	247	233	227	203	188
	Abnormality index	-	13.48	17.25	24.21	27.04	-	11.58	18.94	23.15	26.06
	SD +/-		0.14	0.26	0.45	0.25		0.245	0.205	0.254	0.251
SE		±0.08	±0.15	±0.26	±0.14		±0.14	±0.11	±0.14	±0.15	

SD - Standard Deviation

SE - Standard Error





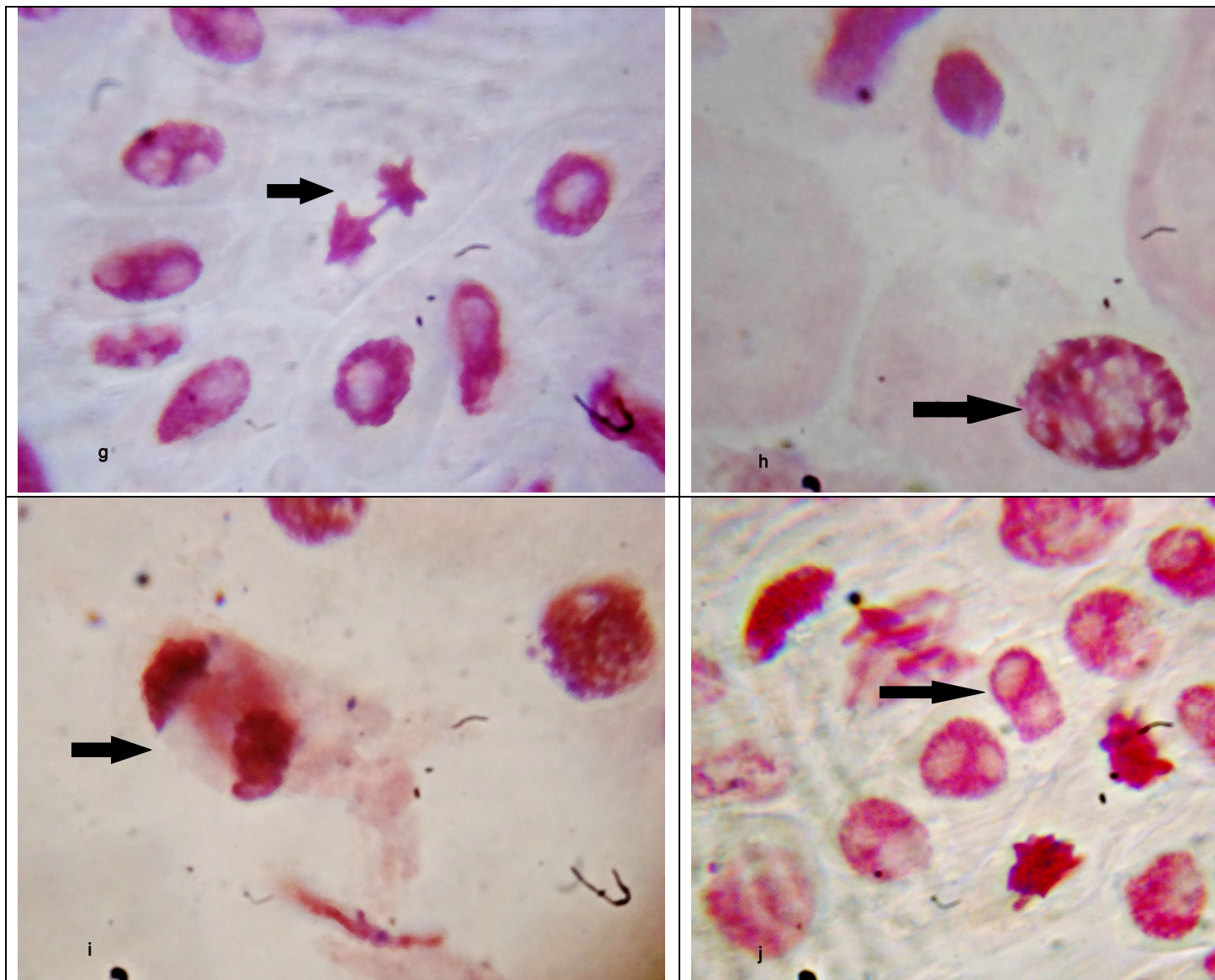


Fig. 1 Mitotic aberrations observed in root meristem of *Allium cepa* L.:

i. Normal prophase; ii. Normal metaphase; iii. Normal anaphase; iv. Normal telophase; a. Anaphase single bridge; b. Anaphase multiple bridge; c. Sticky chromosome; d. Distorted metaphase; e. disoriented metaphase; f. Disoriented spindle apparatus; g. Star anaphase; h. Abnormal prophase i. Abnormal telophase; j. Nuclear lesions

Magnification- 400 X

Fig. 3: Nature of mitotic aberrations in *A. cepa* L meristem cells treated with different concentrations of Apple green dye

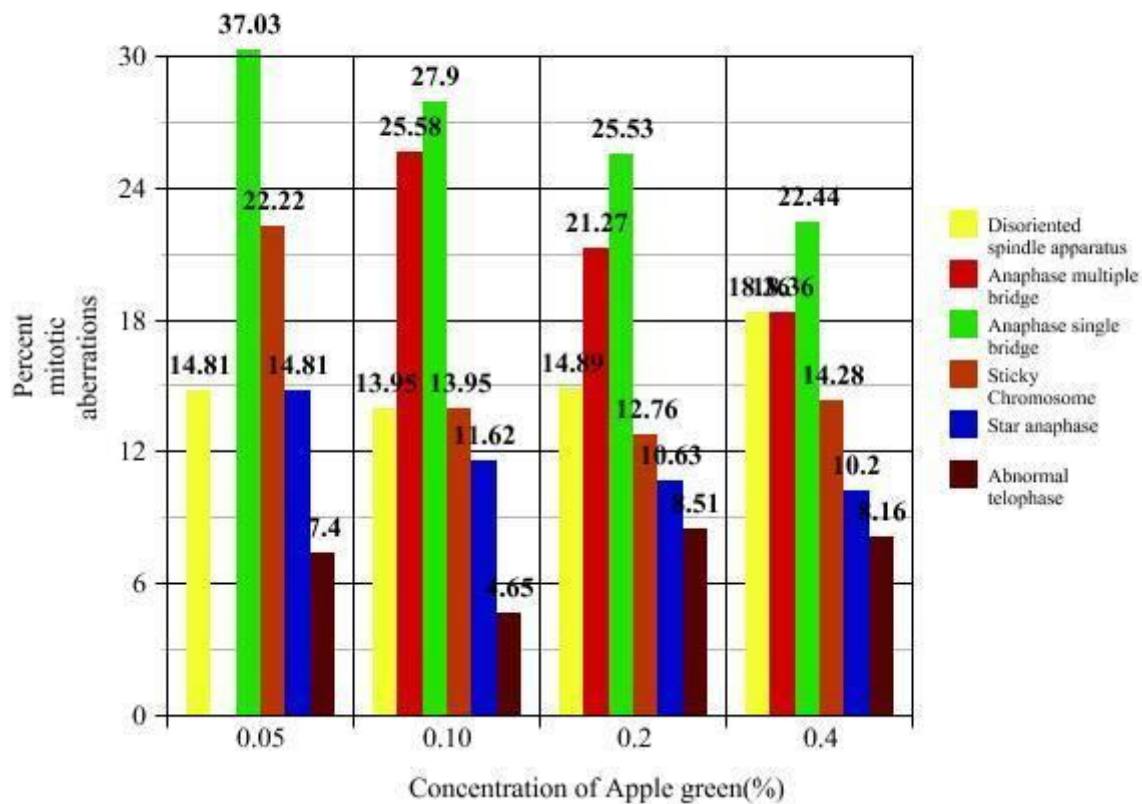


Fig. 2: Nature of mitotic aberrations in *A. cepa* L meristem cells treated with different concentrations of Orange red dye

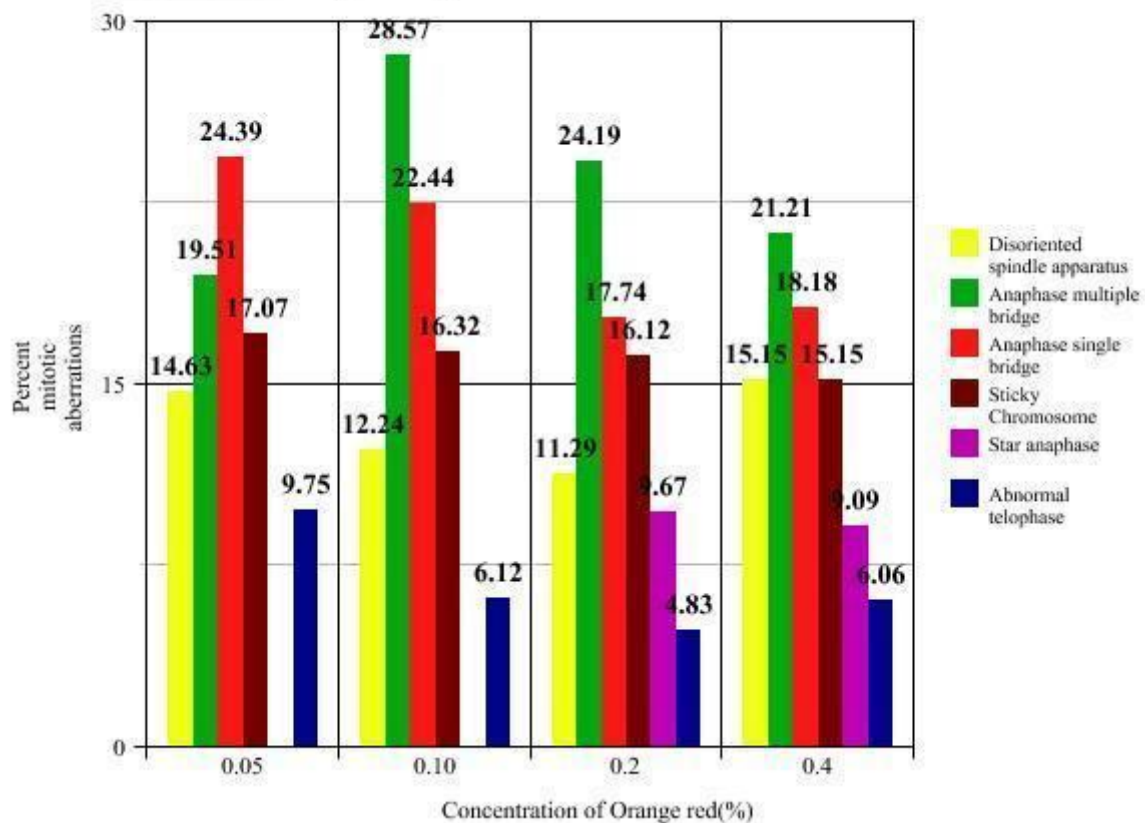


Fig.5: Effect of different concentrations of Apple green on MI and AI of onion root meristem

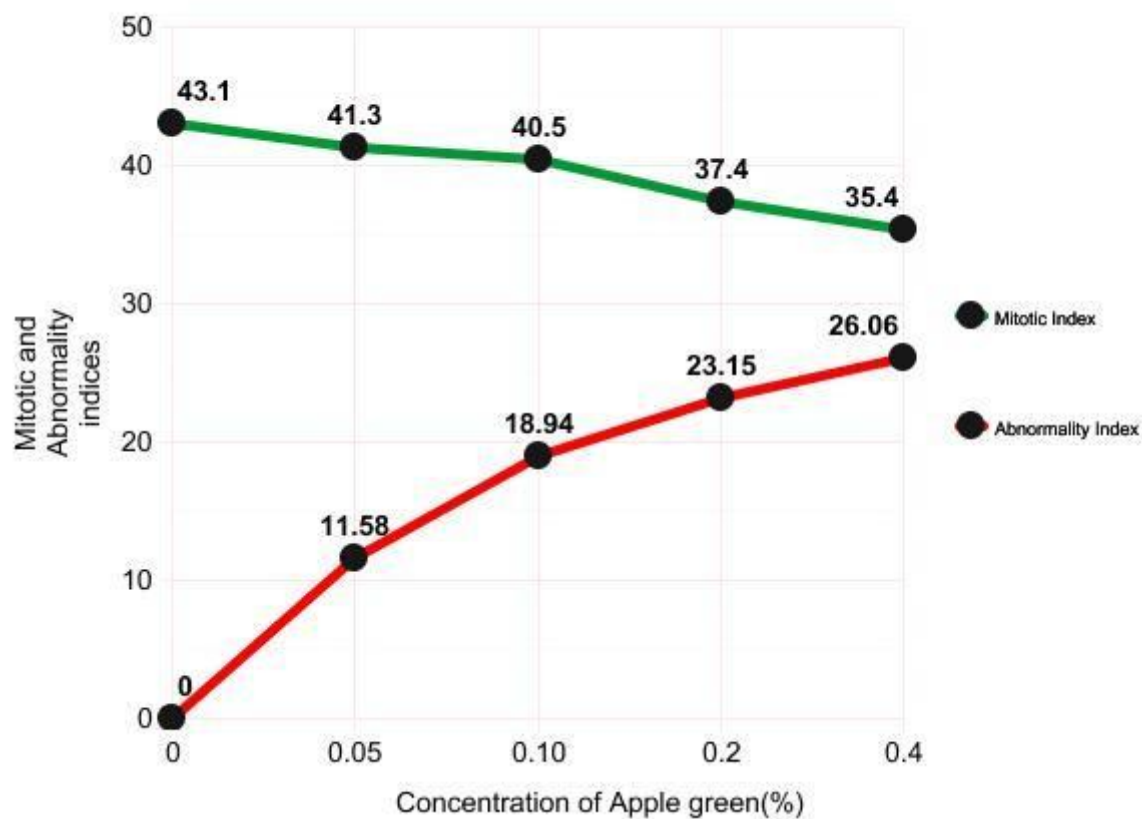


Fig. 4: Effect of different concentrations of Orange red on MI and AI of onion root meristem

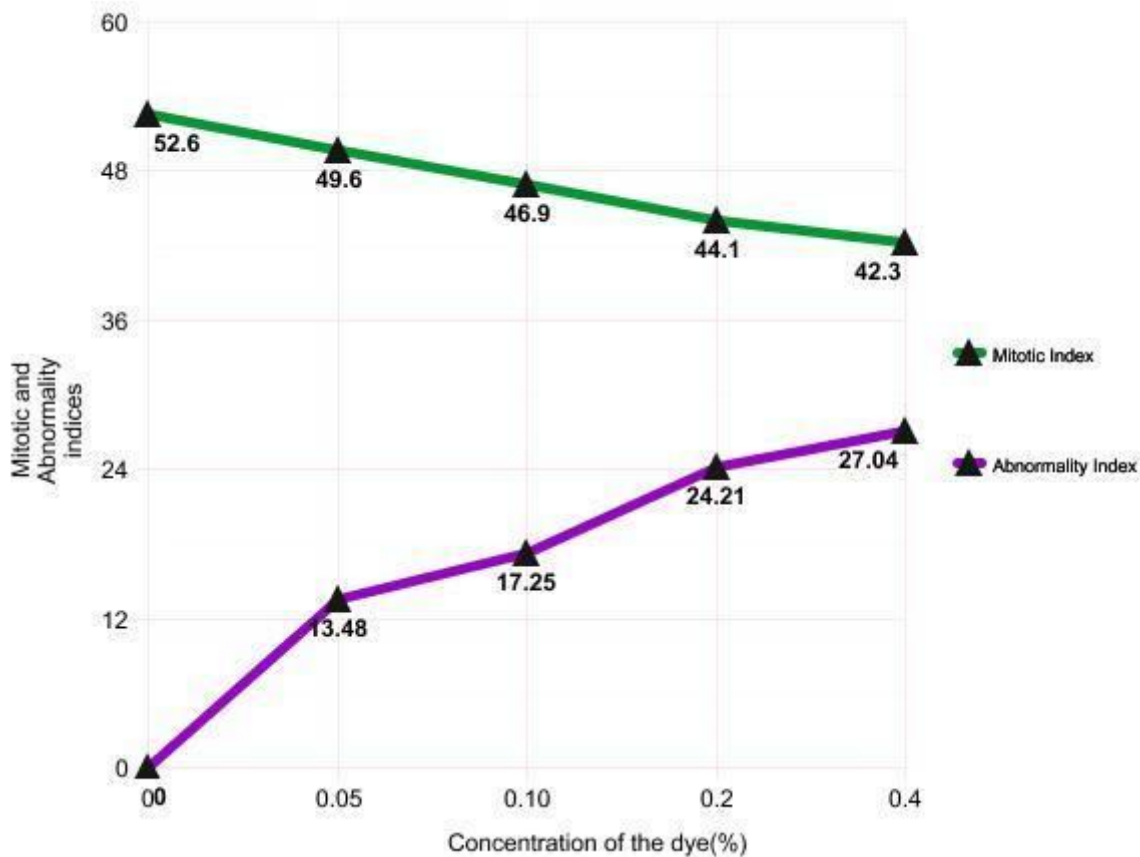


Fig. 6: Effect of Orange red on root growth in *Allium cepa* L.



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