DETECTION OF GENETIC DAMAGE INDUCED BY PLANT GROWTH HORMONE PUTRESCINE ON *Allium cepa* AND *Vicia faba*

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Plant hormones and other growth regulators are chemicals that play a vital role in different aspects of plant growth and development. Since their discovery; plant hormones remained the focus of interest in many botanical research areas. In nature plants are susceptible to different biotic and abiotic stresses. The response to these stresses occur using hormones and their signal transduction pathways. Even at the present time, when the role of genes in regulating all aspects of growth and development is considered of prime importance, it is still clear that the path of development is under the hormonal control, either via changes in hormone levels in response to change in gene transcription or with the hormones themselves as regulators of gene expression (Davies, 2004).

Polyamines such as putrescine, spermine and spermidine are one of the important growth regulating substances which are present in all living organisms. They are important for cell division as there is a positive correlation between the proliferative activity of cells and their contents (Heby and Persson, 1990; Marton and Pegg, 1995; Wallace *et al.,* 2003). The wide use of theses hormones imposes the importance to screen their mutagenic potentialities before their application to environment to avoid their negative impact on the quality of human life.

The present study aims to investigate the effect of putrescine on mitotic cell division in *Allium cepa* root tips and its ability to induce chromosomal aberrations. This work also focus on the use of image cytometry for the estimation of changes in cell cycle progression. In addition, the effect of putrescine on protein banding pattern in *Vicia faba* seeds was studied using sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique.

MATERIALS AND METHODS

Plant materials

Allium cepa (variety. Giza 6) and *Vicia faba* (var. Giza 2) were used as experimental plants for chromosome aberration assay and cell cycle analysis and characterization of protein profile, respectively.

Test chemical

Plant growth hormone putrescine, commonly used in the Egyptian agriculture, was tested in the present study. A series of gradual concentrations: 6.25, 3.12, 1.56 and 0.78 mM were used for different periods of time ranging from 3 to 24 hours. All treatments were carried out in the incubator at 20-25°C and continuous renewal of treatment solution was done.

1-Cytological procedures

A. Squash technique

Bulbs of *Allium cepa* were allowed to germinate in tap water. When the roots reached 2-3 cm long, they were transferred to the test solutions for the treatment time given in the text. The treated roots were fixed in Carnoy's solution [absolute alcohol: glacial acetic acid (3:1)] for 24 hours, then hydrolyzed in 1N HCL at 58°C for about 6-8 minutes, followed by staining with Leuobasic Fuchsin according to Darlington and La Cour (1976). Light green dye (0.3%) was used for staining the protoplasm. The root tips were squashed in 45% acetic acid and mounted in Canada balsam with a clean slide and placed in an oven at 25-35°C for 2-3 days to dry. The preparations were examined microscopically, 90 fields were completely analyzed for each concentration. The photomicrographs were taken from the prepared slides. The frequencies of different stages of mitosis as well as the frequencies of different mitotic abnormalities were determined. Mitotic indices and total abnormalities in each concentration were statistically analyzed using (t-test).

B. Cytophotometric measurements of nuclear DNA

The nuclear DNA was stained with Feulgen stain (Darlington and La-Cour, 1976) then, the content of the stained DNA can be estimated using Leica Qwin 500 image analyzer system (Bocking *et al.*, 1995). The nuclear-integrated optical density is the cytometric equivalent of its DNA content. The amount of DNA in the nuclei and the proportion of cells undergoing different phases of cell cycle were calculated from at least 100 cells for each treatments and control. These include cells with DNA amount less than 2C value, Cells with 2C DNA (G_1 Phase), cells with 3C-4C DNA (S-phase), cells with 4C DNA (G_2 Phase) and cells with DNA amount more than the 4C value (Danque *et al.*, 1993; Lee *et al.*, 1994; Huang *et al.*, 2008). t-test was used to compare the mean values of the factories studied.

2-Biochemical studies

Characterization of protein profiles was carried out using one dimensional sodium dodecyle sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide slab gel (12.5%) was prepared according to Laemmli (1970). Staining the gel with Coomassie blue R-250. The gels were first photographed using a camera and documented for further analysis by appropriate software.

RESULTS AND DISCUSSION

A- Cytological studies

Plant hormones are naturally occurring compounds that are active at very low concentrations. Some of these hormones are used as growth promoters, while others as growth retardants. They represent essential link of many plant processes, control a diverse array of plant responses affecting growth and development, either directly or indirectly. There are many methods involved in determination of genotoxic action of chemicals (Uhi *et al*., 2003). Among them are chromosomal aberration and cytometry assay. Higher Plants represent excellent genetic assay systems for screening and monitoring the mutagenic effects of different chemicals (Grant and Owens, 2001, 2002 and 2006). *Allium cepa* is considered to be the master of model plants in genetic toxicity assessment (Rank and Nielsen, 1998). Genotoxicity of most chemicals is mainly concerned with two main items; determination of mitotic index (MI) and chromosome aberration assessment in treated plants. Mitotic index has been used as a parameter to assess the cytotoxicity of several agents (Kontek, *et al*., 2007; Pandey, 2008; Fisun and Rasgel, 2009). The effect of growth regulators on mitotic index and induction of chromosomal aberration have been studied by some authors (Ronchi *et al.,* 1976; Prasad and Das, 1977; Barakat, 2001; Howell *et al.,* 2007; Tabur and Demir, 2009).

One of the major effects of putrescine is its influence on the rate of mitotic division. In general, mitotic index (MI) values decreased gradually with the increase of putrescine concentration as compared with the control (Table 1). However, the lowest concentration (0.78 mM) and the moderate one (1.56 mM) showed a gradual increase in MI values with the increase of the period of treatment. The reduction in MI especially after treatments with high concentrations reflects the cytotoxic potential of putrescine. The reduction in MI may be due to blocking of cell cycle during interphase (Mohandes and Grant, 1972), inhibition of nuclear protein synthesis essential in the cell cycle (Kim and bendixen, 1987), blocking G_1 phase and preventing DNA synthesis (Glab *et al.,* 1994), suppressing DNA synthesis (Mohanty *et al.,* 2004) and interference with cell cycle preventing the progression of cells from G_2 phase to mitosis (keul and keul, 1994; Polit *et al.,* 2003).

Treatments with putrescine induced a significant percentage of abnormal mitosis. This percentage increased as the concentration of putrescine and the time of treatment increased (Table 1). The most frequent types of abnormalities were stickiness, micronuclei, disturbed phases, bridges, laggards, fragments, nuclear buds and polyploid cells (Plates 1 and 2). A characteristic type of cell death called apoptosis was recorded after 24 hours (Plate 1). Chromosome stickiness was common especially after treatment with high concentrations. Chromosome stickiness reflects highly toxic effects, usually of an irreversible type probably leading to

cell death (Rencüzogüllari *et al.,* 2001). Stickiness may be due to disturbances in cytochemically balanced reactions of nucleosomes formation (Jayabalan and Rao, 1987) and\or for defective functioning of one or two types of specific non-histone proteins involved in chromosome organization that are needed for chromatid separation and segregation (Gaulden, 1987).

Another common type of aberrations recorded at ana-telophase stages after putrescine treatments was chromosome and \or chromatin bridges. Their frequencies increased gradually with the increase of putrescine concentration. These bridges may be due to breakage and reunion with the formation of dicentric chromosome. Also bridges may be due to chromosome stickiness and subsequent failure of free anaphase separation (Gömürgen, 2005) and\or due to unequal translocation or inversion of chromosome segments (Kovalchuk *et al.,* 1998; Fisun and Goc Rasgele, 2009).

Most concentrations of putrescine caused complete inhibition of spindle fibers formation leading to the formation of C-metaphase either 2n or 4n. C-mitosis may be due to the poisons action of putrescine on the microtubules leading to block of mitosis at metaphase and depolymerization of spindle microtubules, thus producing colchicine-like effects (Morejohn and Fosket, 1986 and Aydemir *et al.,* 2008). If the effect on spindle is partial, it will lead to formation of disturbances and laggard abnormalities. These results are concomitant with those obtained by some investigators using different mutagenic agents (Haliem, 1990; Barakat and Hassan, 1997; Renata-Kontek *et al*., 2007; Singh, 2007).

High concentrations of putrescine induced the formation of apoptosis that is a death process involved in the selective elimination of unwanted cells, comprises loss of cell to-cell contact, cytoplasmic shrinkage, membrane blebbing and fragmentation of DNA (Ellis, *et al.,* 1991; Martins and Earnshaw, 1997 and Green 1998). Cells undergoing cell death by necrosis or apoptosis are detected recently by cytokinesis-block micronucleus assay (Fenech *et al.,* 1999; Fenech, 2007). Micronuclei, nucleoplasmic bridge and nuclear buds which were observed after treatments with putrescine are biomarkers that are related to one another and reflect genetic instability (Fenech, 2007)

B- Cytophotometric measurements of nuclear DNA content in cells

The effect of the applied concentrations of putrescine on cell cycle phases after 3 and 24 hours treatments is documented in (Table 2 and Figs 19 and 20). After 3 hours treatment a progressive decreased in the proportion of cells with 2C value (G_1) was recorded as the concentrations of the hormone increased. On the other hand, the cell fractions with DNA content more than 4C were very high in all the concentrations applied and recorded 39.3 ± 0.779 in the roots treated with highest concentration as compared with the control which did not record any polyploid cell. The DNA index showed aneuploid condition demonstrating a

tetraploid case. All concentrations showed high percentage of cells at G_2 and S-phases that were recorded 45.2 ± 0.30 and 33.0 ± 0.32 after treatment with the concentration 0.78 Mm and 3.12 Mm as compared with control value of 23.8 ± 0.35 and 11.9 ± 0.34, respectively.

After 24 hours treatment roots treated with 6.25 mM putrescine showed absence of cells in G_1 phase, low percentage of S-phase (15.25 ± 0.22), high percentage of G_2 phase (26.27 ± 0.27) and very high polyploid cell fraction (58.48 ± 0.73). DNA index of this concentration was 2.18 ± 1.10 indicating sever aneuploid condition. The concentrations 3.12 and 1.56 mM showed decrease in the percentage of G_1 phase (10.29 ± 0.28 and 18.40 ± 0.20), increase in S-phase and G_2 phase than that of the control. Treatment with 3.12 mM putrescine produces very high polyploidy cells that reached (48.21 ± 0.64) and sever polyploidy was emphasized by DNA index that was 2.12 ± 0.79. On the other hand the concentration 0.78 mM showed high proportion of cells in S-phase (30 ± 0.26) than that of control. The DNA index was 1.36 ± 1.20 indicating mild aneuploidy.

Cells re-enter the cell cycle through G_1 phase are governed by a subset of cyclin and cyclin dependant kinases (CDKs) (Bird, 2003 and Moeller and Sheaff, 2005). Exposure the plants to DNA damaging agents can cause mutations and sometimes cell death. Protection from this damage is provided by two cell cycle checkpoints; DNA damage checkpoints and spindle checkpoint (Friedberg *et al*., 1995). DNA damage checkpoints are activated at G_1/S and G_2/M transition points that stop cell cycle progression to allow time for repair thereby preventing transmission of damaged or incompletely replicated chromosomes (O'Connor, 1997; Weinert and Hartwell, 1998). Many authors studied the effect of different stresses on the cell cycle progression (Reichheld *et al*., 1999, EL-Shazly, 2007; Ofer *et al*., 2008). The malfunctioning of cell cycle phases can be detected by deviation from normal cell progression through presence of aneuploidy (corresponding to changes in chromosome copy number and DNA breaks) and polyploidy (multiplication of normal DNA content). The pretreated *Allium cepa* root tips showed chromosomal abnormalities that are expected to be similar to aneuploid and polyploidy cases. It is widely accepted that chromosomal abnormalities occur predominantly in aneuploid malignant cells, leading to progressive development of cancer (Ghadimi, *et al*., 2000). Polyploidy shows reduction of mitosis through which cells undergoes successive DNA replications without any subsequent mitosis and cytokinesis leading to nuclear and cell enlargement (Melaragno *et al.*, 1993) and changes in cellular architecture that is known as endoreduplication (Sugimoto-Shirasu and Roberts, 2003). From the results of image cytometric analysis, it is clear that, there is obvious correlation between the higher ploidy percentage and chromosomal abnormalities, one of the causes for decrease in mitotic indices ob-

served in cytogenetic analysis for different exogenous treatment of plant hormone.

2-Biochemical studies

Table (3) and Plate (3) demonstrate the effect of the plant hormone putrescine on protein banding patterns of *Vicia faba* seed treated for 24 hours. Treatment seeds with different concentrations of this hormone induced considerable variations in protein banding patterns. The major variations are expressed as changes in band intensity and appearance or disappearance of some bands. These results are in accordance with the investigations carried by (Gottschalk and wolf 1983; Abou-Deif and Mohamed, 2007). The total number of the protein bands was 20 bands; ranged from the molecular weight of 250 to 10 KDa. The highest numbers of bands 17 were recorded after treatments with 0.78 mM putrescine while the least number of bands 11 was recorded after treating *Vicia faba* seeds with 6.25 mM putrescine. Five of these bands are common bands while 15 bands are polymorphic bands. The most visible changes in the protein patterns were the appearance of 6 new bands which have the molecular weight of 250, 215, 170, 160, 75 and 20 KDa. On the other hand, 9 bands were disappeared. The disappearance of polypeptides during growth hormone treatments compensates the increased synthesis of others. Changes in protein synthesis under growth hormone treatments may be due to changes in the efficiency on mRNA translation and/ or the regulation of RNA transcription, transport and stability. Changes in band intensity could be explained on the basis of cytological abnormalities produced by putrescine. The presence of laggards and bridges support this conclusion.

SUMMARY

Much attention was paid for putrescine as important plant hormone involved in stress signalling and response in plant. The effect of putrescine on mitotic activity, induction of mitotic abnormalities and changes in the different parameters of the cell cycle has been investigated. In addition, the effect of putrescine on protein banding pattern in *Vicia faba* seeds was studied using sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique. In general, detection of genotoxicity either by chromosome aberration and cytometric analysis or by SDS-PAGE represents acceptable methods to indicate the mutagenic potentialities of different chemicals. Higher concentrations of the plant growth hormone putrescine caused harmful effects at both cytogenetic and biochemical levels. At the cytological level putrescine caused reduction in mitotic index and induced a number of chromosomal abnormalities. The cell cycle analysis showed drastic alternation in different cell cycle phase percentages and the DNA indices as compared with control. The protein patterns showed qualitative and quantitative variations among the investigated samples. It is worthy to mention that gene mutation and changes in the gene expression have to be considered as the reasonable interpretation for the observed banding pattern changes.

This study emphasizes that putrescine has high mutagenic potentialities expressed in its ability for induction of mitotic abnormalities, alternation of cell cycle phase and induction of considerable variations in protein banding patterns. The programmes of plant growth hormone putrescine application should take into account the concentration of the hormone and the nature of plant. So, it will be necessary to test the mutagenic potential of putrescine on more intensive basis especially on non target systems before it is recommended for wider use in agricultural field.

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Table (1): Frequency of different types of prophase, metaphase and ana-telophase abnormalities, mitotic index and mean percentage of abnormal mitosis after treating *Allium cepa* root tips with different concentrations of putrescine for 3, 6 and 24 hours.

Treatment mM	% of Prophase abnormalities			% of metaphase abnormalities							% of anaphase-telophase abnormalities						Mean MI % ±SE	Mean % of abnormalmitosis ±SE
	Stick	Split	Irrg.	CM(2N)	CM(4N)	Star	Break	Stick	Dist.	Lag.	Lag.	Brid.	Multi.	Break	Stick	Dist.		
3 hours																		
Control									2.15	2.15						3.88	4.20±0.08	2.69±0.90
0.78125	4.76		3.57		1.12		1.12	7.87	8.99	3.37	1.02	8.16	1.02		5.11	4.08	3.37±0.15	16.97±1.41
1.5625	13.04	10.15	15.94	8.00		2.00		2.00	30.00	8.00	4.29	14.29		4.29	12.85	8.57	2.49±0.01	43.92±0.26
3.125	15.69	3.92	27.45	1.85	3.70	1.86	3.70	27.78	24.07	5.56	5.95	13.11	3.57	4.76	28.57	9.52	2.35±0.05	61.38±0.26**
6.25	19.38	16.00	27.12			7.41		70.37	11.11	0.00		26.37	2.50	7.50	16.25	12.5	1.92±0.09*	68.32±1.99**
6 hours																		
Control		0.82	0.82	0.99					2.97		1.19					2.38	4.64±0.07	3.08± 0.81
0.78125	1.89	0.94	5.50	2.33			2.33	1.16	15.12	3.48	0.83	3.30	1.65	0.83	0.83	4.13	3.95±0.10	13.97± 0.39
1.5625	4.48	2.99	17.90	5.56	1.39	1.39	1.39	6.94	25.00	6.94	1.35	9.46		1.35	8.12	14.86	2.60±0.05	36.62± 2.02*
3.125	21.31	9.84	34.42	3.85	1.92		7.69	42.31	15.38	3.85	8.43	21.66		5.00	23.33	10.00	2.15±0.03*	69.36± 1.08**
6.25	10.53	12.50	39.47	1.59		3.17	6.35	46.03	11.11	6.35	4.17	30.56	8.33	5.56	19.44	11.11	2.04±0.03**	74.25± 1.01**
24 hours																		
Control		1.56							1.00			1.32					4.46±0.03	1.32± 0.54
0.78125	3.33	6.67	12.22	5.95	2.38	1.19	2.38	9.53	15.48	3.57	0.86	4.31	0.86	0.86	6.91	7.75	3.99±0.05	27.24± 0.25
1.5625	5.88	2.35	14.12	5.80	1.45		2.90	8.70	20.28	4.35	7.07	11.11		3.03	11.11	15.15	3.00±0.05*	37.94± 1.77*
3.125	20.00	4.62	36.92	1.43	1.43	4.29	2.86	37.14	21.43	12.85	6.85	24.66	1.75	1.75	23.29	12.38	2.54±0.05*	71.43± 0.18**
6.25	16.46	5.71	65.71	4.35		1.45		36.23	27.54	10.14	5.08	35.59		1.69	30.53	8.47	2.00±0.07**	81.99± 0.66**

* Significant from control at 0.05 level (t-test). ** Significant from control at 0.01 level (t-test).

Table (2): Effect of putrescine on the cell cycle parameters in root meristematic cells of *Allium cepa* treated for 3 and 24 hours.

3 hours						
Conc. Mm	DNA < 2c ± SD	G1 phase ± SD	S-phase ± SD	G2 phase ± SD	DNA > 4C ± SD	DNA index± SD
6.25	0	2.6± 0.281	29.9±0.263	28.2±0.294	39.3± 0.779	1.93 ± 1.18
3.125	0	3.0± 0.082	27.0±0.3	33.0±0.317	37.0±0.865	1.93± 1.14
1.5625	0	9.3± 0.187	33.3±0.281	25.0±0.237	32.4±1.45	1.92± 1.70
0.78125	1.9± 0.042	19.2± 0.266	45.2±0.302	15.4±0.303	18.3±1.03	1.6 ± 1.28
Control	26.7±0.19	47.6±0.281	23.8±0.354	11.9±0.344	0.0	1 ± 0.70
24 hours						
6.25	0	0	15.25 ± 0.22	26.27 ± 0.27	58.48 ± 0.73	2.18 ± 1.10
3.125	1.79 ± 0.01	10.29 ± 0.28	25.0 ± 0.31	14.1 ± 0.37	48.21 ± 0.64	2.12 ± 0.79
1.5625	1.60 ± 1.03	18.40 ± 0.20	36.0 ± 0.28	29.60 ± 0.27	14.40 ± 1.05	1.6 ± 1.18
0.78125	0	44.62 ± 0.25	30.0 ± 0.26	16.15 ± 0.27	9.23 ± 0.77	1.36 ± 1.20
Control	16.67 ± 0.19	47.62 ± 0.28	23.81 ± 0.35	11.91 ± 0.34	0	1 ± 0.70

Table (3): Effect of putrescine on protein banding patterns of *Vica faba* seeds separated by SDS-PAGE for 24 hours.

Band No.	Mol. WT.	putrescine				
		Lane 1 (Control)	Lane 2 6.25 mM	Lane 3 3.12 mM	Lane 4 1.56 mM	Lane 5 0.78 mM
1	250	-	-	-	++	++
2	215	-	-	++	++	++
3	200	++	+	-	-	-
4	170	-	-	++	++	++
5	160	-	+	+	-	-
6	150	++	-	+	+	+
7	100	+	+	-	-	+
8	90	+	-	-	-	+
9	75	-	-	+	++	+
10	73	+	+	+	+	+
11	50	+	+	+	+	+
12	40	+	+	+	++	+
13	37	+	+	-	+++	+
14	32	+	-	-	+	+
15	27	+++	+++	+++	+++	+++
16	25	+	+	-	+	+
17	20	-	-	-	+	+
18	13	++	++	++	++	++
19	12	++	-	+	++	++
20	10	++	++	++	-	-
Total	20	14	11	12	14	17

Types of mitotic abnormalities induced by putrescine in *Allium cepa* root tips.

Plate (1)

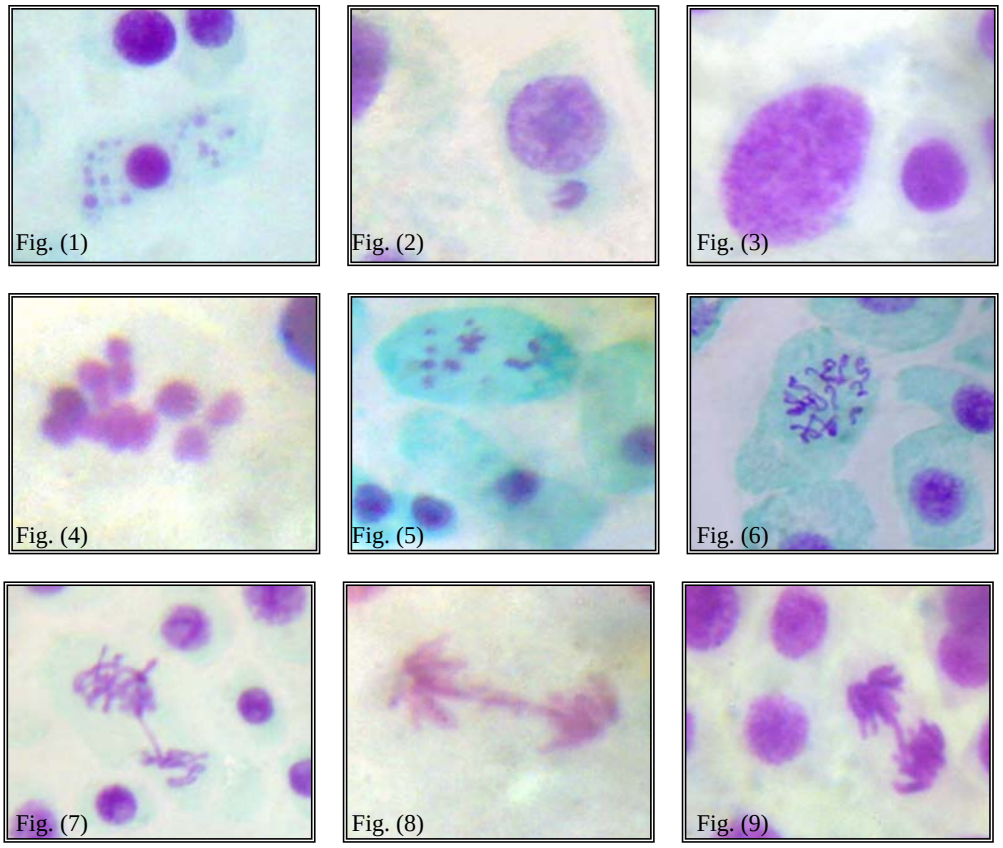


Fig. (1): Multi-micronucleate cell after treatment of root tips with 6.25 mM putrescine for 24 hours.
Fig. (2): Interphase with one micronucleus after treatment with 6.25 mM putrescine for 24 hours.
Fig. (3): Endoreduplication (restitution cell) after treatment cells with 6.25 mM putrescine for 6 hours.
Figs (4 and 5): Apoptotic cells observed at after treating root tips for 6 and 24 hours with 6.25mM putrescine.
Figs (6 and 7): Split prophase after treatment with 1.56 mM putrescine for 24 hour.
Figs (8 and 9): Anaphase Bridge after treatment with 3.12 mM putrescine and 1.56 mM putrescine for 24 hours.

Plate (2)

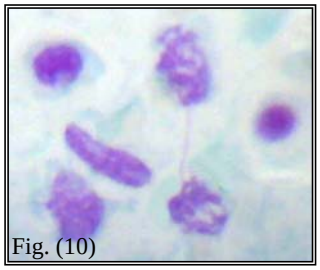


Fig. (10)

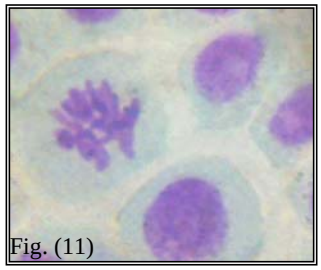


Fig. (11)

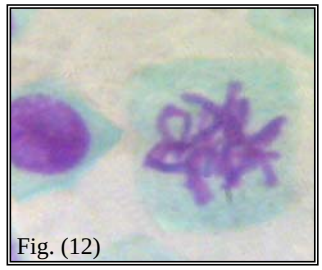


Fig. (12)

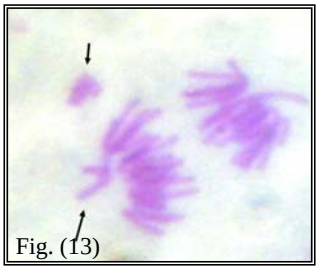
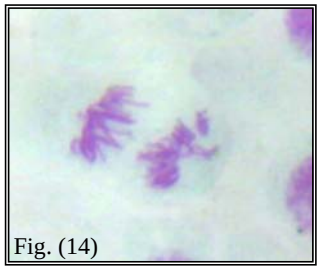


Fig. (13)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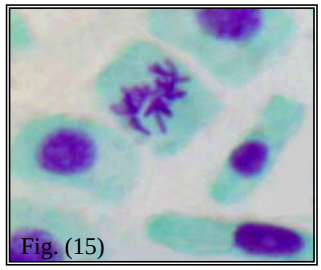
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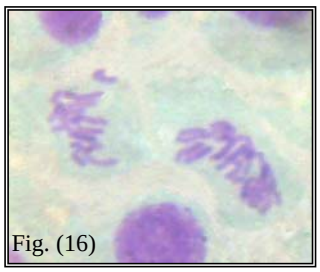
Fig. (16)


Fig. (17)

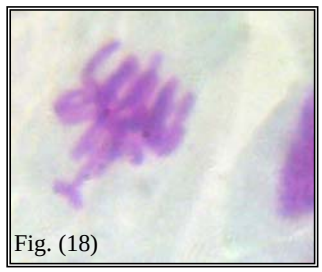
Fig. (18)

Fig. (10): Telophase with chromatid bridge after treatment with 3.12 mM putrescine for 3 hours.

Fig. (11): Sticky metaphase after treating root tips with putrescine 1.56 mM putrescine for 24 hours.

Fig. (12): Disturbed metaphase after treating root tips with 1.56 mM putrescine for 3 hours.

Fig. (13 and 14): Anaphase with free chromosomes after treatment with 1.56 mM putrescine and 3.12 mM putrescine for 3 and 24 hours.

Fig. (15): Disturbed metaphase after treatment with 3.12 mM putrescine for 6 hours.

Fig. (16): Laggard chromosome after treatment with 1.56 mM putrescine for 6 hours.

Fig. (17): Metaphase with a number of laggard chromosomes after treatment with 0.78 mM putrescine for 24 hours.

Fig. (18): Metaphase with a laggard chromosome after treatment with 1.56 mM putrescine for 3 hours.

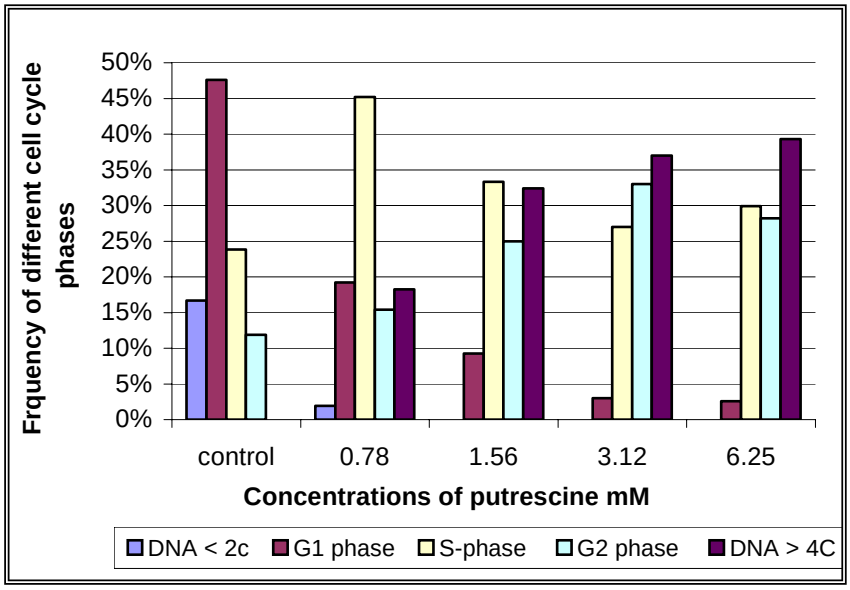

Fig. (19): Effect of putrescine on cell cycle parameters after treatment of *Allium cepa* root tips for 3 hour treatment.

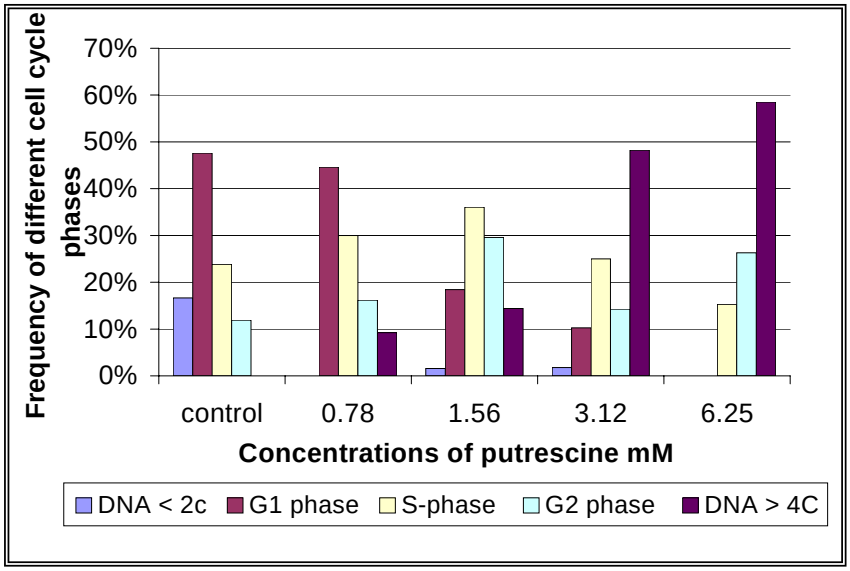

Fig. (20): Effect of putrescine on cell cycle parameters after treatment of *Allium cepa* root tips for 24 hour treatment.

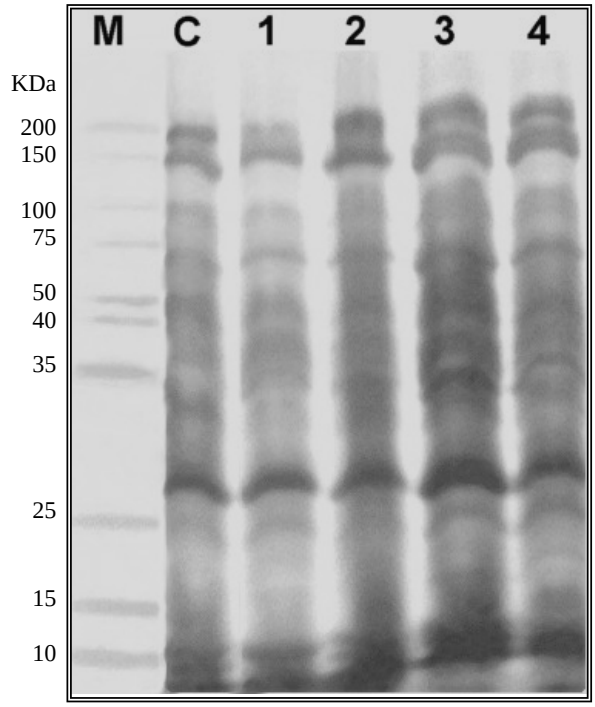

Plate (3): Electrophotograph of protein banding patterns of *Vica faba* seeds separated by SDS-PAGE after treatment with different concentrations of putrescine for 24 hours.
M= Marker Lane 1 (C) = control
Lane 2 (1) = 6.25 mM
Lane 3 (2) = 3.12 mM
Lane 4 (3) = 1.56 mM
Lane 5 (4) = 0.78 Mm