J. Agric. Sci. Mansoura Univ., 32 (10): 8413 - 8434, 2007

HISTOLOGICAL, CHEMICAL AND EPIGENETIC STUDIES OF SNAPDRAGON (*Antirrhinum majus* l.) AND LARKSPUR *(Delphinium ajacis* l.) POLYPLOIDY LINES INDUCED BY COLCHICINE

Koriech, E.M.; Eman I. Moghazy; Y.M. Abd-El-Fattah and M. A. El-Sadek

Hort. Dept., Fac. of Agric., Suez Canal Univ., 41522 Ismailia, Egypt.

ABSTRACT

This study was carried out at Suez Canal University to report the production of tetraploid plants of snapdragon (*Antirrhinum majus* L.) and larkspur *(Delphinium ajacis* l.), with the ultimate aim of improving the ornamental qualities of these important floriculture plants. Chromosome doubling was achieved by the application of colchicine to either pre-soaked seed or to the apical meristems of young seedlings. Treatment of the ungerminated seed was the more efficient method in terms of numbers of tetraploid seedlings (up to 56.08% in *Antirrhinum majus* and 30.16% in *Delphinium ajacis*, as determined by chromosome counting of the root-tip nuclei). When colchicine was applied directly to the apical growing tip of cotyledon-stage seedlings, leaf and stem growth was temporarily affected but the plants eventually recovered. We conducted a preliminary screen for putative tetraploids based on the observation in other plant species of a correlation of stomatal size and count with ploidy. Stomatal studies revealed decrease in number with a concomitant increase in size of stomata across the lower leaf epidermis.

These techniques confirmed that the highest number of tetraploid plants in *Antirrhinum majus* was observed by soaking seeds in 1% colchicine for 24 hours operating time, while 0.50% for 48 hours gave the same results in *Delphinium ajacis*. In spite of the fact that polyploidization is a major driving force for plant genome evolution, results led to the conclusion that epigenetics evolved new morphological differences of the tetraploids included a more compact growth habit and shorter heights and darker leaves. These plants having a double chromosome number and nearly double DNA content. In addition, chlorophylls (a) and (b) were increased in ploidy incidence.

Keywords: polyploidy; colchicine; snapdragon (*Antirrhinum majus* l.); larkspur *(Delphinium ajacis* l.); tetraploid.

INTRODUCTION

Snapdragon and larkspur plants cultivated in Egypt for their cut flowers or as bedding plants. Many attempts to improve marketing were done through the agricultural practices. Generally, chromosome doubling or polyploidy (whole-genome duplication, WGD) resulting in good plants for exportation and local markets. Polyploidy could be artificially induced by some treatments, such as colchicine. Colchicine treatment is the classical method to induce doubling of chromosome number. The tetraploid plants were found to have large leaves with fewer large stomata than diploid plants, an indicator of increased ploidy level. Polyploids can establish a compatible relationship between alien cytoplasm and nuclei and between two divergent genomes, leading to rapid changes in genome structure, gene expression, and developmental traits such as fertility, inbreeding, apomixis, flowering

time, and hybrid vigor. Although the underlying mechanisms for these changes are poorly understood, some themes are emerging. There is compelling evidence that changes in DNA sequence, *trans*-acting effects, chromatin modifications, RNA-mediated pathways, and regulatory networks modulate differential expression of homoeologous genes and phenotypic variation that may facilitate adaptive evolution in polyploid plants and domestication in plants thus means the presence of epigenetic changes (Chen and Gao, 2007).

Chromosome counting which is time consuming and laborious is not suitable for detection mixoploids in tissues with lower proportion of dividing cells such as leaves (Jaroslav and Bartos, 2005). Stomata size, stomata frequency, pollen grain diameter and other changes in plant morphology were found to be useful indicators in the primary screening for new ploidy levels (Liu *et al.*, 2007). Stanys, *et al.,* (2006) stated that stomata length is a suitable parameter for identifying tetraploids. Stomata of tetraploid shoots of the same clone were approximately one third longer than in diploids.

Treatment of diploid plants to obtain tetraploids can be done through seed treatment, apical or adventitious meristem of seedlings or intact plants and *in-vitro* for cells, callus or portions grown on either solid or liquid medium (Kerr, 2001). Generally, polyploids seem, sometimes, to be more tolerant to stresses including drought and cold (Pašakinskienė, 2000) and poor soils (Buggs and Pannell, 2007) than their diploid progenitors.

The genus *Antirrhinum* (snapdragon) have 2n=16, is a member of the Family *Plantaginaceae* (Schwarz-Sommer *et al.*, 2003). *Delphinium* (larkspur) is a plant genus belongs to the Family *Ranunculaceae* and have 2n=16 (Dalgaard, 1986).

The purpose of this study is to induce polyploidization in snapdragon cultivar Rose Bicolor and larkspur cultivar Cliveden Beauty plants by colchicine treatment using plant attributes as primary indicators.

MATERIALS AND METHODS

The experiments were carried out at the Experimental Farm of the Faculty of Agriculture, Suez Canal University, during the two successive seasons of 2004/2005 and 2005/2006. The strain Rose Bicolor of *Antirrhinum majus* and the strain Cliveden Beauty of *Delphinium ajacis* were examined for their chromosome number and used in the present study.

Aqueous solution of colchicine was used to induce tetraploid plants of each *A. majus* cv. Rose Bicolor and *D. ajacis* cv. Cliveden Beauty. Two methods of treatments were used for seedlings and for seeds. In each treatment, five concentrations of aqueous solution of colchicine were used (0.00, 0.10, 0.25, 0.50 and 1.00%). Each concentration was used for different periods (soaking seeds for 1, 2, 3 or 4 days or immersing seedling apical meristem for 3, 5 or 7 days. In seedling treatment, the apices of fifty seedlings of four day's old were immersed using a piece of cotton saturated with the specific concentration of colchicine on its apical meristem. Similarly, in the treatment of soaking seeds, fifty seeds were used for different periods for

each concentration from both plants. Seeds of both plants were soaked in water for 24 hours and then transformed to the aqueous solution of colchicine and kept in the dark for the different periods.

For determining the effect of colchicine on the induction of polyploidy in snapdragon (*A. majus*) and larkspur (*D. ajacis*), data were recorded for histological studies including stomata characters (number of stomata, stomata length and stomata width), pollen characteristics (pollen grains viability, pollen grain diameter), chromosome number, and chemical component including (chlorophylls (a) and (b)) and Deoxyribonucleic acid content (DNA).

Statistical Analysis

Dates of the different variables were transformed according to their type when they need. The experimental designs were complete randomized block in a factorial layout according to Gomez and Gomez (1984). The differences were examined by t-paired test and Duncan's Multiple Range test to compare means of treatments.

RESULTS AND DISCUSSION

Allopolyploidy is a prominent mode of speciation in flowering plants. On allopolyploidy, genomic changes can take place, including chromosomal rearrangement and changes in gene expression, these processes is a major driving force for plant genome evolution (Adams, 2007). Genetic and epigenetic changes associated with newly synthesized allopolyploids have been well documented in a number of recent studies (Albertin *et al*., 2006 and Lukens *et al.,* 2006). According to Stupar *et al.,* (2007) the genetic and epigenetic instability associated with newly synthesized allopolyploids can be attributed to many potential factors, including dosage-regulated gene expression, nucleo-cytoplasmic interactions, homologous recombination, and other downstream factors associated with the merger of two subgenomes. They added that expression of the homologous genes in the new polyploids may be regulated by diverged regulatory elements associated with different subgenomes. Reconciling diverged regulatory pathways may play a significant role in driving the genetic and epigenetic changes observed in newly synthesized polyploids (Chen, 2007). According to these findings and the conclusions driven from observations of DNA content, stomata number per microscopic field, stomata length and stomata width would indicate that different studied lines or strains were found in this study. The following lines were recorded:

Antirrhinum majus			
Soaking seeds		**Immersion of apical meristem**	
Line number	**Treatment**	**Line number**	**Treatment**
Control	Diploid	Control.	diploid
A-1	0.25% colchicine for 48 hours	A-6	0.25% colchicine for 5 days
A-2	0.25% colchicine for 96 hours	A-7	0.50% colchicine for 7 days
A-3	0.50% colchicine for 48 hours	A-8	1.00% colchicine for 5 days
A-4	0.50% colchicine for 72 hours	A-9	1.00% colchicine for 7 days
A-5	1.00% colchicine for 48 hours		
8			
Soaking seeds		**Immersion of apical meristem**	
Line number	**Treatment**	**Line number**	**Treatment**
Control	diploid	Control.	diploid
D-1	0.10% colchicine for 48 hours	D-6	0.25% colchicine for 7 days
D-2	0.10% colchicine for 72 hours	D-7	0.50% colchicine for 5 days
D-3	0.25% colchicine for 72 hours	D-8	0.50% colchicine for 7 days
D-4	0.50% colchicine for 48 hours	D-9	1.00% colchicine for 5 days
D-5	1.00% colchicine for 48 hours		

1- Stomata characteristics (Number, length and width)

1-First experiment: (soaking seeds in different colchicine concentrations)

Data for average number of stomata per one mm^2, stomata length and width on the lower epidermis of fifth leaf for *A. majus* and *D. ajacis* are presented in Table (1) and illustrated in Photo (1)

The average number of stomata per microscopic field for the lower epidermis of fifth leaf of *A. majus* was 134.33±2.33 stomata in diploid plants, while it was ranged from 80.00±0.15 to 96.33±0.33 for tetraploid lines, in the first season, and from 83.33±0.88 to 94.00±0.00, in the second one. Meanwhile in *D. ajacis* there were 123.33±2.03 and 127.00±1.53 stomata in diploid plants, in the first and second seasons, respectively, while they ranged from 62.00±1.15 to 71.00±2.40 stomata for tetraploid lines, in the first season and from 62.67±0.33 to 72.00±1.00 in tetraploid lines, in the second season. There were more stomata in the leaves of diploid plants than tetraploids. A highly significant difference existed between diploid and tetraploid stomata density. The larger stomata size of the tetraploids was accompanied by a decrease in number of stomata per mm^2.

These data agrees with the finding of Gandhi and Patil (1997) on *Clitoria ternatea,* who stated that there was a significant decrease in stomata frequency in autotetraploid plants. In addition, similar results were reported by Gu *et al.,* (2005) on *Zizyphus jujube*, who showed that stomata characteristics differed markedly between diploid and tetraploid plants and the frequency of stomata were reduced significantly with polyploidy.

Data on the length of stomata are presented in Table (1) and illustrated in Photo (1). Concerning the length of stomata, results indicate the existence of highly significant differences between diploid and all tetraploid lines. The stomata of the former were shorter than those of the latter.

Daniel and Yao (1996) stated that the stomata length has been the most widely used because the measurement is simple, non-destructive, and does not require expensive instrument.

Data on the width of stomata are presented in Table (1) and illustrated in Photo (1). In *A. majus,* the average width was 18.30±0.87 µm for diploid plants, while it ranged from 26.58±0.65 to 30.29±1.13 µm and from 28.90±1.29 to 31.62±0.90 µm for tetraploid lines, in the first and second seasons, respectively. Meanwhile, in *D. ajacis* the average width of stomata of diploid leaf was 20.65±0.34 µm and 21.41±1.00 µm, in the first and the second seasons, respectively, whereas that of tetraploid lines, ranged from 31.40±00 to 33.28±0.18 µm, in the first season, and from 32.44±1.07 to 33.84±0.51 µm, in the second season. There were highly significant differences between diploid plants and tetraploids lines.

A similar conclusion was reached by several investigators as Sascha *et al.,* (2003) on *Acacia mearnsii*; Zaffar *et al.,* (2004) on saffron and Gu *et al.,* (2005) on *Zizyphus jujube*. They came to conclusions that stomata length, width, area and frequency enabled the greatest discrimination of diploid and tetraploid shoots and rapid indirect methods to identify ploidy level.

The results could be explained by the fact that colchicine increases the cell size and would consequently decrease the number of cells when counted in the same area Comai, (2000). These data agree with Gu *et al.,* (2005) on *Zizyphus jujube*, who showed that stomata characteristics differed markedly between diploid and tetraploid plant, while the frequency of stomata were reduced significantly.

II- Second experiment: Immersion of apical meristem.

Results pertaining to stomata characteristics for *A. majus* and *D. ajacis* as affected by colchicine application in both seasons are presented in Table (2) and Photo (1)

The number of stomata as recorded in Table (2) proved the existence of highly significant difference in this category between the diploid and the tetraploid plants for *A. majus* plant. The average number of stomata for diploids was 136.67±2.33, while it ranged from 82.00±1.15 to 90.00±1.15 in tetraploid plants, in the first season, and from 78.00±1.15 to 91.67±1.67, in the second season.

For *D. ajacis* plants, the average number of stomata per microscopic field was 126.33±0.88 and 124.67±0.88 in diploid plants, in the first and second seasons, respectively, and ranged from 61.33±1.45 to 69.00±0.58 and from 62.67±0.88 to 70.67±0.67 in tetraploid lines, in the first and the second seasons, respectively.

The statistical analysis with t-paired test for the average number of stomata per microscopic field indicated that highly significant differences were found between diploid and all tetraploid lines in both seasons of the study. The decrease in number of stomata was accompanied by large stomata size of the tetraploid.

This result could be explained by the fact that colchicine increases the cell size and would consequently decreases the number of cells when counted in the same area. (Comai, 2000). These results were in agreement

with the findings of Gandhi and Patil (1997) on *Clitoria ternatea,* who stated that there was a significant decrease in stomata frequency in tetraploid plants.

The average length of stomata of the diploid leaf was 22.33±1.06 µm whereas, that of tetraploid lines ranged from 39.44±0.97 to 43.08±1.18 µm, in the first season, and from 40.14±0.77 to 42.28±0.76 µm, in the second season. Generally, colchicine treatments increased average length of stomata for *A. majus*. This increase was found to be statistically highly significant, as shown in Table (2).

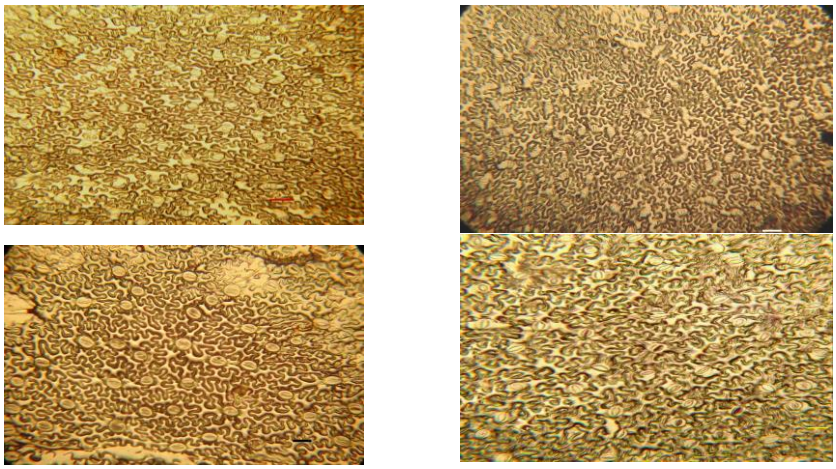
Results presented in Table (2) and Photo (1) illustrated the influence of colchicine application on stomata length for *D. ajacis* cv. Cliveden Beauty, in both seasons.

The average length of stomata of diploid leaves was 22.58±0.38 µm and 24.58±0.95 µm in the first and second seasons respectively, while it ranged from 46.83±0.32 to 48.64±0.69 µm and from 46.65±0.77 to 48.40±0.99 µm in tetraploid lines in the first and second seasons, respectively. There was a highly significant difference between diploid plants and all tetraploid lines.

Data in Table (2) show that the average stomata width of the diploid leaf of *A. majus* was 18.55 ± 0.55 µm and 18.78±0.15 in the first and second seasons, respectively, whereas, that of tetraploid lines ranged from 29.74±1.11 to 30.68±1.47 µm, in the first season and from 28.61±0.15 to 30.58±1.40 µm, in the second one. The larger stomata size of the tetraploids was accompanied by a decrease in number of stomata per mm^2. It can be noticed that average number of stomata/ mm^2 decreased, while stomata length and width increase due to polyploidy induction.

Data of stomata width for *D. ajacis* as affected by colchicine solution treatments are presented in Table (2). The average width of diploid leaf was 21.05±0.89 and 22.45±0.69 µm, in the first and second seasons, respectively, while it ranged from 33.88±0.49 to 35.91±0.85 µm and from 33.14±0.32 to 36.84±0.10 µm in tetraploid lines, in the first and the second seasons, respectively. From the above-mentioned results, it can be noticed that average number of stomata per microscopic field decreased, while stomata length and width increased due to polyploidy. Generally, colchicine treatments increased average width of stomata. This increase was found to be statistically highly significant with t-paired test.

Several investigators as Sascha *et al*., (2003) on *Acacia mearnsii* and Zaffar *et al*., (2004) on saffron reached similar results. They concluded that stomata length, width, area and frequency enabled the greatest discrimination of diploid and tetraploid shoots and are rapid indirect methods to identify ploidy level.



***Antirrhinum majus* cv. Rose Bicolor** ***Delphinium ajacis* cv. Cliveden Beauty**

Photo (1) Stomata characters in diploid (up) and tetraploid plants (down) of *Antirrhinum majus* cv. Rose Bicolor and *Delphinium ajacis* cv. Cliveden Beauty (bar 50 µm)

Table (1) Stomata number, length and width of diploid and tetraploid lines of *A. majus* cv. Rose Bicolor and *D. ajacis* cv. Cliveden Beauty, during the two successive of 2004/ 2005 & 2005/ 2006 seasons

	Lines		No. of stomata	Stomata length (µm)	Stomata width (µm)
Antirrhinum majus	First season, 2004/5	Control	134.33 ± 2.33	23.96±1.38	18.30±0.87
		A-1	90.00±0.00**	34.89±0.73**	29.17±1.48**
		A-2	95.67±2.33**	37.72±1.39**	26.58±0.65**
		A-3	80.00±1.15**	38.50±1.39**	27.26±0.26**
		A-4	92.00±1.53**	37.89±1.65**	30.29±1.13**
		A-5	96.33±0.33**	38.38±0.44**	28.65±0.92**
	Second season, 2005/6	Control	139.33±1.33	22.98±1.62	17.04±1.07
		A-1	94.00±0.00**	39.17±1.63**	31.62±0.90**
		A-2	89.67±0.58**	37.99±1.54**	29.69±0.24**
		A-3	83.33±0.88**	37.26±1.40**	29.12±1.30**
		A-4	86.33±0.88**	39.69±2.05**	30.58±1.86**
		A-5	90.67±0.67**	37.32±1.29**	28.90±1.29**
Delphinium ajacis	First season, 2004/5	Control	123.33±2.03	24.56±1.48	20.65±0.34
		D-1	65.00±1.73**	42.67±1.24**	33.28±0.18**
		D-2	62.00±1.15**	43.46±0.94**	32.19±0.27**
		D-3	66.67±1.67**	41.02±1.09**	31.40±0.56**
		D-4	71.00±2.40**	42.18±2.26**	31.71±0.94**
		D-5	66.33±1.76**	43.64±2.08**	31.80±0.78**
	Second season, 2005/6	Control	127.00 ± 1.53	23.09±0.74	21.41±1.00
		D-1	69.33±0.58**	40.89±2.06**	32.46±0.88**
		D-2	62.67±0.33**	44.66±1.70**	33.44±0.24**
		D-3	72.00±0.58**	42.31±1.39**	33.84±0.51**
		D-4	67.00±1.15**	40.69±0.97**	32.44±1.07**
		D-5	72.00±1.00**	42.76±0.92**	32.67±0.78**

Results are given as mean values ± standard error ** Significant differences at P < 0.01

Table (2):Stomata characteristics in the parental diploid and induced tetraploid lines of A*. majus* cv. Rose Bicolor and *D. ajacis* cv. Cliveden Beauty during both seasons of the experiment.

	Lines		No. of stomata	Stomata length (µm)	Stomata width (µm)
Antirrhinum majus	First season, 2004/5	Control	136.67±2.33	22.33±1.06	18.55±0.55
		A-6	90.00±1.15**	41.40±0.79**	30.44±1.27**
		A-7	88.00±1.53**	39.44±0.97**	30.68±1.47**
		A-8	85.33±1.33**	43.08±1.18**	29.74±1.11**
		A-9	82.00±1.15**	40.12±0.45**	30.07±0.66**
	Second season, 2005/6	Control	132.67±2.67	21.90±1.27	18.78±0.15
		A-6	82.67±1.33**	42.28±0.76**	29.60±0.57**
		A-7	91.67±1.67**	41.85±1.09**	30.58±1.40**
		A-8	78.00±1.15**	41.27±1.84**	29.49±0.67**
		A-9	87.00±1.53**	40.14±0.77**	28.61±0.15**
Delphinium ajacis	First season, 2004/5	Control	126.33±0.88	22.58±0.38	21.05±0.89
		D-6	69.00±0.58**	47.97±0.71**	33.88±0.49**
		D-7	65.67±0.88**	46.83±0.32**	34.07±0.95**
		D-8	61.33±1.45**	48.64±0.69**	35.34±0.75**
		D-9	63.00±1.15**	47.42±0.45**	35.91±0.85**
	Second season, 2005/6	Control	124.67±0.88	24.58±0.95	22.45±0.69
		D-6	70.67±0.67**	46.77±0.49**	35.21±1.12**
		D-7	66.33±0.67**	46.89±0.46**	33.14±0.32**
		D-8	62.67±0.88**	46.65±0.77**	34.50±0.77**
		D-9	65.67±1.86**	48.40±0.99**	36.84±0.10**

Results are given as mean values ± standard error
**** Significant differences at P < 0.01**

2- Pollen grain characteristics

First experiment: (Soaking seeds in different colchicine concentrations)

The average diameter and percentage of stainable pollen grains for *A. majus* and *D. ajacis* as indicator of polyploidy are presented in Table (3) and illustrated in Photo (2)

As for *A. majus* pollen grain viability was significantly decreased with polyploidy, in both seasons. Data for the first season showed that average percentage of stainable pollen grains was 94.67±0.33% in diploid plants, meanwhile it ranged from 79.33±0.33 to 84.20±0.59% in tetraploid lines (Table 3) and Photo (2). Also, for *D. ajacis* data presented in Table (3) and Photo (2) indicated that average percentage of pollen grain viability was 86.11±0.59 and 85.56±0.87% in diploid plants, in the first and second seasons, respectively, whereas it ranged from 72.00±1.92 to 75.11±0.56 % in tetraploid lines, in the first season, and from 73.67±0.33 to 76.33±0.51 %, in the second season.

It is clearly shown that incidence of polyploidy decreased pollen fertility. In conclusion, the fertility of tetraploid and diploid plants was decreased as ploidy level increased.

These results were in accordance with those reported by Barufaldi *et al*., (2003) on *Lotus glaber* and Eeckhaut *et al.*, (2004) on *Spathiphyllum*

wallisii. They stated that a highly significant difference was detected between diploid and tetraploid plants in the pollen grains viability.

To determine the effect of polyploidy on the diameter of pollen grains, 100 pollen grains from each inflorescence for *A. majus* and *D. ajacis* were measured. The data taken from Table (3) showed that there were highly significant differences between diploid and tetraploid plants in pollen grain diameter. Average diameter of pollen grain was significantly increased, using t- paired test between lines and control plants due to polyploidy. Data shown in Table (3) and Photo (2) indicated that the diameter of pollen grain of tetraploid lines was larger than that of the diploids. However, Tsvetova and Ishin (1995) on Sorgam stated that the size of pollen grains is seen as a possible method for identifying polyploidy plants.

These results agreed with those reported by Barufaldi *et al*., (2003) on *Lotus glaber;* Oliveira *et al*., (2004) on *Stevia rebaudiana;* Richa and Srivastava (2004) on *Helianthus annus* and Eeckhaut *et al*., 2004 on *Spathiphyllum wallisii*. They reported that a positive correlation was found between pollen size and number of chromosomes or the ploidy level.

Second experiment: Immersion of apical meristem

Data of pollen characteristics for *A. majus* and *D. ajacis* as affected by colchicine solution treatments are presented in Tables (3) and Photo (2)

Data for Pollen grains viability (%) obtained in the second experiment were similar to those of the first experiment. The average percentage of stainable pollen grain of *A. majus* was 94.00±0.58% in diploid plants, and it ranged from 78.67±0.67% to 83.00±0.58% in tetraploid lines in the first season. The same behavior was also observed in the second season. In addition, in *D. ajacis,* the average percentage of pollen grains fertility was 85.22±0.22% and 85.56±0.87% in diploid plants, in the first and second seasons, respectively, while it ranged from 74.67±0.88 to 76.67±0.33 and from 72.67±0.67 to 75.33±0.88% in tetraploid lines, in the first and the second seasons, respectively.

The statistical analysis of the average of pollen grains viability indicated that pollen grains viability was highly significant decreased with polyploidy induction. Finally, from the above-mentioned results it could be concluded that induction of polyploidy decreased pollen grains viability. These results are similar to those reported by Jamwal and Kaul (1997) on *Apium graveolens* and Gandhi and Patil (1997) on *Clitoria ternatea*.

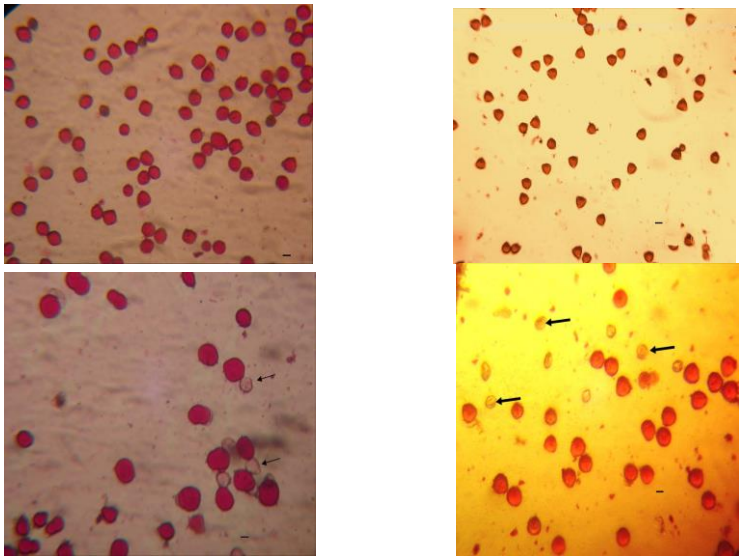
Results presented in Tables (3) and Photo (2) illustrated the influence of colchicine application on pollen grain diameter for *A. majus* and *D. ajacis,* in both seasons. The statistical analysis using t-paired test showed a highly significant increase in the diameter of pollen grains indicating a favorable response towards polyploidy.

The large size of pollen grains is one of the main characteristics of polyploidy as reported by Oliveira *et al.,* (2004) on *Stevia rebaudiana* and Rauf *et al.,* (2006) on *Gossypium arboreu*m. They stated that the pollen grains of tetraploid lines were larger than those of diploid plants.

Table (3) Pollen grains diameter and pollen fertility of parental diploid and different tetraploid lines of *Antirrhinum majus* and *Delphinium ajacis* cv. Cliveden Beauty during both seasons of the experiment.

		Lines	Pollen grains viability (%)	Pollen grain diameter (µm)	Lines	Pollen grains viability (%)	Pollen grain diameter (µm)
Antirrhinum majus	First season, 2004	Control	94.67±0.33	18.42±0.47	Control	94.00±0.58	17.83±0.66
		A-1	79.33±0.33**	26.97±0.79**	A-6	80.67±1.20**	26.97±0.46**
		A-2	81.89±1.42**	27.21±1.41**	A-7	82.33±0.33**	28.64±0.74**
		A-3	84.20±0.59**	27.84±1.36**	A-8	78.67±0.67**	28.73±1.16**
		A-4	80.10±0.10**	29.07±1.49**	A-9	83.00±0.58**	27.55±1.17**
		A-5	80.53±1.89**	26.88±0.71**			
	Second season,	Control	95.33±0.51	17.09±1.04	Control	94.33±0.33	18.72±0.79
		A-1	83.67±0.38**	28.54±1.30**	A-6	78.67±0.88**	29.12±1.07**
		A-2	82.56±0.68**	29.84±0.56**	A-7	81.67±0.33**	27.55±0.25**
		A-3	83.44±0.59**	29.08±0.80**	A-8	79.33±0.88**	29.00±0.50**
		A-4	81.56±0.87**	30.22±0.46**	A-9	79.00±0.58**	27.86±0.19**
		A-5	81.11±0.40**	28.12±0.94**			
Delphinium ajacis	First season,	Control	86.11±0.59	29.33±1.67	Control	85.22±0.22	27.89±1.13
		D-1	72.00±1.92**	43.38±1.86**	D-6	76.00±0.58**	47.16±0.58**
		D-2	75.11±0.56**	47.06±1.04**	D-7	74.67±0.88**	45.83±0.22**
		D-3	74.67±0.84**	46.67±0.83**	D-8	75.11±0.49**	48.58±0.58**
		D-4	74.11±0.22**	45.58±1.34**	D-9	76.67±0.33**	49.08±1.17**
		D-5	74.33±1.35**	45.00±0.72**			
	Second season,	Control	85.56±0.87	28.56±0.84	Control	85.56±0.87	28.56±0.48
		D-1	74.67±0.88**	46.67±1.10**	D-6	74.00±0.58**	44.61±0.69**
		D-2	73.67±0.33**	46.00±0.52**	D-7	75.33±0.88**	48.66±0.23**
		D-3	76.00±0.58**	44.71±0.96**	D-8	72.67±0.67**	49.12±0.68**
		D-4	75.33±0.88**	47.33±0.96**	D-9	73.67±0.67**	48.00±0.43**
		D-5	76.33±0.51**	47.06±0.81**			

Results are given as mean values ± standard error
**** Significant differences at P < 0.01**



***Antirrhinum majus* cv. Rose Bicolor** **_Delphinium ajacis_ cv. Cliveden Beauty**

Photo (2) Pollen grains of *Antirrhinum majus* cv. Rose Bicolor and *Delphinium ajacis* cv. Cliveden Beauty showing increase in size and decrease in viability of pollens in tetraploid plants (down) compared with diploid plants (up) (bar 10 µm)

3- Chemical components: Pigments content

First experiment: (Soaking seeds in different colchicine concentrations)

The effect of colchicine treatments on chlorophylls (a) and (b) in plant leaves are presented in Table (4).The statistical analysis indicated that tetraploid lines in the two seasons had a significant effect on the content of both chlorophylls (a) and (b) more than the diploid plants.

Data in Table (4) showed that the average content of chlorophylls (a) and (b), respectively, in the diploid plant was 12.50±0.35 mg/g and 5.79±0.29 mg/g fresh weight, in the first season, and 11.97±0.26 mg/g fresh weight and 5.46±0.11 mg/g, in the second season. Meanwhile, it ranged from 16.33±0.38 to 17.09±0.02 mg/g fresh weight and from 12.13±0.73 to 13.25±0.16 mg/g fresh weight, in the first season, and it was ranged from 16.63±0.44 to 17.04±0.25 mg/g fresh weight and from 12.08±0.39 to 13.13±0.43 mg/g fresh weight, in the second season, in the tetraploid lines.

It is clear that chlorophyll a and b increased due to polyploidy. Similar results were reported by Derake (1994) on *Gerbera jamesonii*. Besides, the finding of Kermani *et al.,* (2003) on *Rosa*; Zaffar *et al.,* (2004) on saffron and Mathura *et al*., (2006) on *Acacia mearnsii* were in harmony with the obtained results, whereas leaves of tetraploid plants were darker green in color than that of the diploid plants interestingly.

As for *D. ajacis*, data for the content of chlorophylls (a) and (b) in plant leaves are presented in Table (4). The statistical analysis using the t- paired test indicated that tetraploid plants in the two seasons had a significant increase in the content of both chlorophyll a and b. The average content of chlorophyll a and b in the diploid plant was 8.03±1.34 and 3.84±0.49 mg/g fresh weight,

in the first season, and 7.66±0.18 and 3.21±0.10 mg/g fresh weight, in the second season respectively. Meanwhile, it was ranged from 15.90±0.29 to 16.88±0.20 mg/g fresh weight and from 9.40±0.69 to 12.05±0.74mg/g fresh weight in the first season, and it ranged from 16.36±0. 41 to 16.80±0.22 mg/g fresh weight and from 10.69±0.03 to 12.45±0.87 mg/g fresh weight, in the second season, in tetraploid lines.

From the above-mentioned results, it is clear that chlorophyll (a) and (b) increased due to polyploidy. Obtained results were in agreement with the results reported by Silva *et al.,* (2000) on *Cattleya intermedia;* Kermani *et al.,* (2003) on *Rosa* and Zaffar *et al.,* (2004) on saffron that leaves of tetraploid plants were darker green in color than those of the diploid interestingly. In addition, Mathura *et al*., (2006), stated similar results on *Acacia mearnsii.* They found that chlorophyll a and b increased due to polyploidy.

Second experiment: Immersion of apical meristem.

The results in Table (4) show the effect of colchicine solution application on chlorophyll a and b contents, in both seasons of experiment. The trend of results, in both seasons was obtained in tetraploid lines in the second experiment. The average content of chlorophyll a and b respectively in the diploid plant was 12.26±0.40 and 5.82±0.28 mg/g fresh weight and ranged from 16.33±0.38 to 17.09±0.02 mg/g fresh weight and from 10.76±1.19 to 13.22±0.63 mg/g fresh weight in tetraploid lines, in the first season. The same trend was obtained in the second season.

Finally, the above-mentioned results show clearly that chlorophylls (a) and (b) increased due to polyploidy induction. Similar results were reported by Kermani *et al.,* (2003) on *Rosa*; *Zaffar et al.,* (2004) on saffron and Mathura *et al*., (2006) on *Acacia mearnsii*. These findings were in harmony with the obtained results in the present investigation that leaves of tetraploid plants were darker green in color than those of the diploid ones interestingly.

Data of the average chlorophyll a and b content in plant leaves of *D. ajacis* are presented in Table (4). The statistical analysis using the t- paired test indicated that tetraploid plants, in the two seasons, had a significant effect on the content of both chlorophylls (a) and (b). The average content of chlorophylls (a) and (b) in the diploid plant was 7.99±0.35 and 3.57±0.02 mg/g fresh weight, in the first season, and 8.32±0.17 and 3.91±0.35 mg/g fresh weight in the second season. Meanwhile it ranged from 15.79±0.33 to 16.87±0.20 mg/g fresh weight and 10.69±0.2 to 12.66±1.12 mg/g fresh weight, in the first season while ranged from16.08±0.05 to17.27±0.33 mg/g fresh weight and from 10.36±0.19 to 12.86±1.43 mg/g fresh weight, in the second season, in tetraploid lines.

Table (4) Chlorophyll a and b contents in plant leaves of diploid and different tetraploid lines of *A. majus* cv. Rose Bicolor and *D. ajacis* cv. Cliveden Beauty during both seasons of the experiment.

		Lines	Chlorophyll a (mg/g f.w)	Chlorophyll b (mg/g g f.w)	Lines	Chlorophyll a (mg/g f. w.)	Chlorophyll b (mg/g f. w.)
Antirrhinum majus	First season, 2004/5	Control	12.50±0.35	5.79±0.29	Control	12.26±0.40	5.82±0.28
		A-1	16.33±0.38*	12.36±0.40**	A-6	16.33±0.38**	10.76±1.19*
		A-2	16.69±0.22**	12.56±0.30**	A-7	16.69±0.22**	11.52±0.86**
		A-3	16.89±0.13**	12.46±0.49**	A-8	16.89±0.13**	13.22±0.63**
		A-4	17.09±0.02**	13.25±0.16**	A-9	17.09±0.02**	12.21±0.35**
		A-5	16.89±0.13**	12.13±0.73**			
	Second season, 2005/6	Control	11.97±0.26	5.46±0.11	Control	11.79±0.74	5.43±0.58
		A-1	16.63±0.44**	12.45±0.63**	A-6	16.75±0.35**	11.67±0.69**
		A-2	16.94±0.74**	12.63±0.65**	A-7	16.59±0.48**	11.30±0.48**
		A-3	16.95±0.46**	12.08±0.39**	A-8	16.40±0.51**	11.86±0.92**
		A-4	16.73±0.37**	13.13±0.43**	A-9	16.62±0.25**	11.92±0.60**
		A-5	17.04±0.25**	12.22±0.56**			
Delphinium ajacis	First season, 2004/5	Control	8.03±1.34	3.84±0.49	Control	7.99±0.35	3.57±0.02
		D-1	16.85±0.19**	11.66±0.44**	D-6	16.87±0.20**	12.13±1.19**
		D-2	16.12±0.51**	10.25±1.54**	D-7	16.71±0.16**	10.96±0.56**
		D-3	15.90±0.29**	9.40±0.69**	D-8	16.20±0.30**	12.66±1.12**
		D-4	16.84±0.14**	11.64±0.39**	D-9	15.79±0.33**	10.69±0.20**
		D-5	16.88±0.20**	12.05±0.74**			
	Second season, 2005/6	Control	7.66±0.18	3.21±0.10	Control	8.32±0.17	3.91±0.35
		D-1	16.36±0.41**	10.72±1.16**	D-6	16.20±0.56**	11.13±0.61**
		D-2	16.70±0.55**	12.41±0.16**	D-7	16.38±0.18**	10.63±0.25**
		D-3	16.57±0.05**	10.69±0.03**	D-8	17.27±0.33**	12.86±1.43**
		D-4	16.50±0.26**	12.06±1.71**	D-9	16.08±0.05**	10.36±0.19**
		D-5	16.80±0.22**	12.45±0.87**			

Results are given as mean values ± standard error
**** Significant differences at P < 0.01**

4- Ploidy incidence percentage

1- First experiment (Soaking seeds in different colchicine concentrations)

Data in Table (5) show the effect of colchicine treatments on induction of polyploidy in *A. majus*. It can be concluded that either colchicine treatments or time of application had no effect on the mixoploidy produced during both seasons. Obviously, the higher colchicine concentration as 1% for either 24 or 48 hours produced more tetraploids during both seasons.

In soaked seeds treatments, the more effective concentration for inducing tetraploid in *A. majus* cv. Rose Bicolor was 1.00 % for 48 hours, in the first season, and the same treatment in the second season (Table 5). The percentage of tetraploid plants in the treatments of 1.00% for 48 hours; 1.00% for 24 hours; 0.25% for 96 hours; 0.50% for 72 hours; 0.25 for 48 hours; 0.50% for 24 hours and 0.50% for 48 hours was 43.33, 56.08, 30.00, 17.25, 8.62, 7.82 and 7.02, respectively.

Second experiment: Immersion of apical meristem.

Data presented in Table (5) are for plants of which size of pollen grains and other characteristics were examined for detection of polyploidy. Results on plants raised from treated seedlings indicated that the effective concentrations for inducing tetraploid plants in *A. majus* cv. Rose Bicolor were 1.00% for 7 days, 0.50 % for 7days, 1.00%for 5 days, 0.5% for 5 days, 0.25% for 5 days and 1.00% for 3 days. The highest percentage of tetraploid plants, as 49.64 and 53.88% was produced from the treatment that received 1.00% colchicine solution for 7days, in the first and second seasons, respectively. On the other hand, mixoploid plants were observed in this experiment as show in Table (5).

As for *D. ajacis* data presented in Table (6). Results on plants raised from treated seedling indicate that the highest numbers of tetraploid plants were obtained from treating seedlings with 0.25% colchicine for 7 days. The percentage of tetraploid plants was 38.97 and 41.29% was produced from the treatment which received 0.25% colchicine solution for 7 days, in the first and second seasons, respectively. On the other hand, mixoploid plants were observed in this experiment as show in Table (6).

5- Deoxyribonucleic acid content (DNA) (ng/uL)

Data given in Table (7) show the DNA content obtained from both diploid and tetraploid lines during both seasons of study, measured by the method described by Mingai (2002) (Nano Drop® ND-1000 spectrophotometer). Analysis of the selected lines clearly revealed the existence of four groups of DNA ploidy levels:

- A group of individuals presented relative DNA content very similar to control (polyploidization did not occur) (Figures 1 and 2).
- A group of individuals that had two or more ploidy levels in the same tissue, which means that polyploidization, did not occurred in all cells of the treated tissues. These plants were classified as mixoploid (Figures 1 and 2).
- A group of individuals characterized by relative DNA content superior by about nearly 115% to control. Individuals of this group were considered aneuploids (Figures 1 and 2).

- 5

- A group of individuals with relative DNA content superior by about 100% as compared to control. This group was classified as having doubled chromosome numbers (Figures 1 and 2).

The DNA content represented the increasing of DNA in the same cell that means that ploidy status can be found. The trend of results, in both seasons, was parallel. The data obtained showed that there was an increase in DNA content for samples taken after 150 days from transplanting the tetraploid lines over the diploid or control plants. DNA content is a good indicator of ploidy. This method also reduces the time to determine ploidy level in plants. The optical density is then used to evaluate ploidy level.

Results are also supported by studies regarding *in vitro* induction of polyploidy in watermelon and estimation based on DNA content carried by Raza *et al*., (2003). In harmony with these results, Galitski *et al*., (1999) found that the increase for DNA per cell presumably results in an increase in nuclear size and a reduction in the nuclear surface area/volume ratio.

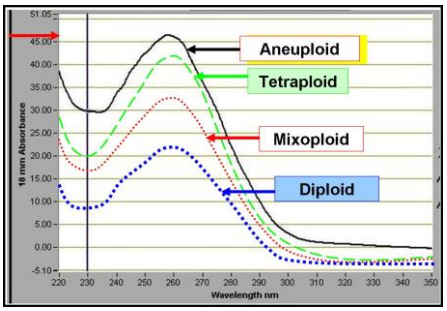


Figure (1) DNA content of aneuploid, tetraploid, mixoploid and diploid plants of *Antirrhinum majus* cv. Rose Bicolor

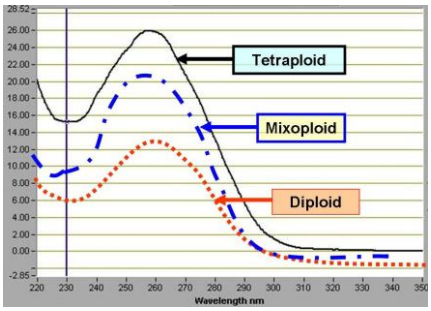


Figure (2) DNA content of tetraploid, mixoploid and diploid plants of *Delphinium ajacis* cv. Cliveden Beauty

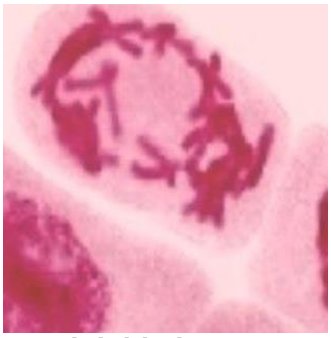
Table (7) DNA content of diploid and different polyploidy lines of *Antirrhinum majus* cv. Rose Bicolor as measured by Nano Drop® ND-1000 spectrophotometer in both soaked seeds and seedlings treatments with colchicine ,in two seasons for experiment.

DNA Content (ng/uL)							
First season, (2004/2005)							
Antirrhinum majus				**Delphinium ajacis**			
Lines	**Soaked seeds**	**Lines**	**Seedlings treatment**	**Lines**	**Soaked seeds**	**Lines**	**Seedlings treatment**
Control	1173.63	Control	1074.90	Control	669.13	Control	673.33
A-1	2216.08**	A-6	2148.08**	D-1	1289.11**	D-6	1260.44**
A-2	2199.13**	A-7	2091.38**	D-2	1208.52**	D-7	1287.46**
A-3	2228.52**	A-8	2048.53**	D-3	1264.38**	D-8	1271.18**
A-4	2203.19**	A-9	2030.20**	D-4	1277.86**	D-9	1255.59**
A-5	2249.66**			D-5	1287.96**		
Second season, (2005/2006)							
Lines	**Soaked seeds**	**Lines**	**Seedlings treatment**	**Lines**	**Soaked seeds**	**Lines**	**Seedlings treatment**
Control	1092.97	Control	1078.63	Control	676.67	Control	686.67
A-1	2073.08**	A-6	2065.00**	D-1	1276.78**	D-6	1283.25**
A-2	2160.20**	A-7	2073.21**	D-2	1274.13**	D-7	1250.02**
A-3	2080.50**	A-8	2104.56**	D-3	1251.38	D-8	1267.17**
A-4	2131.76**	A-9	2023.32**	D-4	1258.81**	D-9	1257.37**
A-5	2040.27**			D-5	1270.67**		

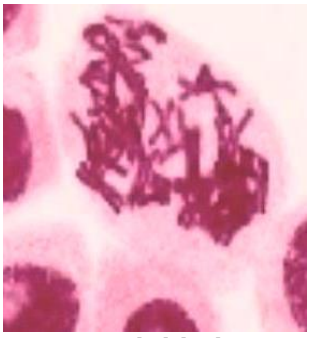
**** Significant differences P < 0.01**

6-Chromosomes number

Smears with acetocarmine were made to count the chromosome number in root tip of the tetraploid and diploid plants. In the diploid plants of *A. majus* cv. Rose Bicolor, 16 pairs of chromosome were observed at metaphase; on the other hand, 32 pairs were observed at the same stage in tetraploid plants, (Photo 3). As for *D. ajacis* cv. Cliveden Beauty in diploid plants, 8 pairs of chromosomes were observed at metaphase, on the other hand 16 pairs were observed at the same stage in tetraploid plants (Photo 4). Kellogg (2003) reviewed that polyploidy raises a problem for gene regulation. Recent studies suggest that this is caused by epigenetic mechanisms that somehow modify chromatin. The results from the present study as well as several previous investigations (Chen, 2007) indicated that newly synthesized polyploids lines of both snapdragon and larkspur were undergo.

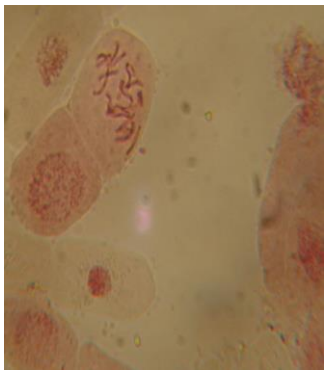


Diploid plants

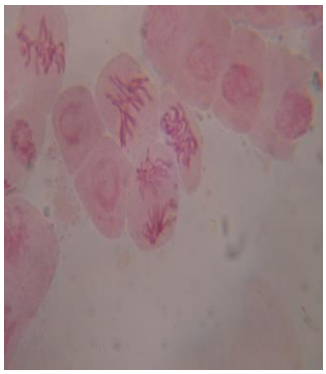


Tetraploid plants

Photo (3) Chromosome number in the root tips of diploid and tetraploid plants of *Antirrhinum majus*, at metaphase stage. Eight pairs of chromosome are observed in diploid and sixteen pairs in tetraploid plants (x = 1500)



Diploid plants



Tetraploid plants

Photo (4) Chromosome number in the root tips of diploid and tetraploid plants of *Delphinium ajacis* cv. Cliveden Beauty, at metaphase stage. Eight pairs of chromosome are observed in diploid and sixteen pairs in tetraploid plants (x = 400)

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دراسات سيتولوجية وكيميائية والوراثة الفوقية على نباتى حنك السبع والعايق لإحداث سلالات متضاعفة باستخدام الكولشيسين

عيد محمد قريش – ايمان اسماعيل مغازى – يحيى محمد عبد الفتاح – محمد أحمد الصادق

قسم البساتين - كلية الزراعة جامعة قناة السويس

أجريت هذه الدراسة بمزرعة كلية الزراعة جامعة قناة السويس خلال الموسمين 2005/2004 و 2006/2005 لاحداث التضاعف الوراثى فى نباتى حنك السبع والعايق باستخدام الكولشيسن سواء بنقع البذور او معاملة القمة النامية للبادرات.باستخدام تركيزات وفترات زمنية مختلفة . تمت الدراسة على كل نبات على حدة وشملت الدراسة الآتى :

- نقع البذور فى محلول الكولشيسين بتركيزات: صفر، 0.1 ، 0.25 ، 0.50 ، 1.0 % ولفترات زمنية 24، 48،72 ،96ساعة.
- معاملة القمة النامية للبادرات بالكولشيسن بنفس التركيزات السابقة ولفترات زمنية 3 ، 5 ، 7 أيام.

تمت دراسة الصفات التالية : صفات الثغور (عدد الثغور فى وحدة المليمتر المربع – طول الثغر – عرض الثغر) صفات حبوب اللقاح (حيوية حبوب اللقاح - حجم حبوب اللقاح) وعدد الكروموسومات والمركبات الكيميائية (كلوروفيل أ ، ب) و كمية الحامض النووى الدى اوكسى ريبوزى).

كانت أهم النتائج المتحصل عليها هى :

- وجد ان أعلى عدد من النباتات المتضاعفة لحنك السبع تم الحصول عليها من نقع البذور فى 1% كولشيسن لمدة 24 ساعة ، بينما فى نبات العايق تم الحصول على نفس النتيجة عند نقع البذور فى 0.50 % كولشيسين لمدة 48 ساعة .
- كان عدد الثغور فى النباتات الرباعية أقل منه فى النباتات الثنائية بينما زاد طول وعرض الثغر فى النباتات الرباعية.
- اتضح من النتائج أن إحداث التضاعف يؤدى الى إنخفاض حيوية حبوب اللقاح بينما زاد حجمها فى النباتات الرباعية.
- لوحظ أن أوراق النباتات المتضاعفة كانت أغمق لونا من أوراق النباتات الثنائية ولذا وجدت زيادة معنوية فى تركيز كل من الكلوروفيل أ ، ب فى أوراق النباتات الرباعية فى كلتا التجربتين .
- وجد تضاعف فى كمية الحامض النووى الدى اوكسى ريبوزى فى النباتات الرباعية عن النباتات الثنائية نتيجة لحدوث التضاعف.

Table (5) Effect of different colchicine concentrations and soaking time on induction of polyploidy plants of *Antirrhinum majus* cv. Rose Bicolor in two seasons for the experiment.

Colchicine concentration (%)	Soaking time (h)	1st season 2004/5		2nd season 2005/6		Duration (day's)	1st season 2004/5		2nd season 2005/6	
		Mixoploidy %	(4x) %	Mixoploidy %	(4x) %		Mixoploid %	(4x) %	Mixoploid %	4x %
0.00	24	0.00	0.00	0.00	0.00	3	0.00	0.00	0.00	0.00
	48	0.00	0.00	0.00	0.00	5	0.00	0.00	0.00	0.00
	72	0.00	0.00	0.00	0.00	7	0.00	0.00	0.00	0.00
	96	0.00	0.00	0.00	0.00					
0.10	24	0.00	0.00	0.00	0.00	3	0.00	0.00	0.00	0.00
	48	0.00	0.00	0.00	0.00	5	0.00	0.00	0.00	0.00
	72	0.00	0.00	0.00	0.00	7	6.14	0.00	5.25	0.00
	96	0.00	0.00	0.00	0.00					
0.25	24	0.00	0.00	0.00	0.00	3	0.00	0.00	0.00	0.00
	48	0.00	8.62	0.00	9.68	5	14.32	13.24	13.40	9.02
	72	0.00	0.00	0.00	0.00	7	0.00	28.80	0.00	25.02
	96	0.00	30.00	0.00	27.18					
0.50	24	0.00	7.82	0.00	7.57	3	9.30	0.00	12.37	0.00
	48	0.00	7.02	0.00	6.73	5	6.21	10.86	5.51	13.79
	72	0.00	17.25	0.00	15.45	7	0.00	40.42	0.00	37.45
	96	0.00	0.00	0.00	0.00					
1.00	24	0.00	56.08	0.00	54.85	3	0.00	4.44	0.00	5.46
	48	0.00	43.33	0.00	41.11	5	0.00	22.42	0.00	20.59
	72	0.00	0.00	0.00	0.00	7	0.00	49.64	0.00	53.88
	96	0.00	0.00	0.00	0.00					

Table (6) Effect of different concentrations and treatment duration with colchicine on induction of polyploidy in *Delphinium ajacis* cv. Cliveden Beauty in two seasons for the experiment.

Colchicine concentration %	Soaking time (h)	1st season 2004/5		2nd season 2005/6		Duration (days)	1st season 2004/5		2nd season 2005/6	
		Mixoploidy %	(4x) %	Mixoploidy %	(4x) %		Mixoploidy %	(4x) %	Mixoploidy %	(4x) %
0.00	24	0.00	0.00	0.00	0.00	3	0.00	0.00	0.00	0.00
	48	0.00	0.00	0.00	0.00	5	0.00	0.00	0.00	0.00
	72	0.00	0.00	0.00	0.00	7	0.00	0.00	0.00	0.00
	96	0.00	0.00	0.00	0.00					
0.10	24	0.00	0.00	0.00	0.00	3	0.00	0.00	0.00	0.00
	48	3.00	4.36	6.00	0.00	5	0.00	0.00	0.00	0.00
	72	0.00	8.45	0.00	0.00	7	6.14	0.00	0.00	0.00
	96	0.00	6.84	0.00	0.00					
0.25	24	9.00	0.00	11.00	0.00	3	0.00	0.00	0.00	0.00
	48	8.00	0.00	8.00	9.68	5	14.32	0.00	0.00	0.00
	72	0.00	9.55	0.00	0.00	7	0.00	38.97	0.00	41.29
	96	28.00	0.00	24.00	27.18					
0.50	24	27.00	10.18	29.00	7.57	3	9.30	19.67	10.00	22.76
	48	25.00	30.16	22.00	6.73	5	6.21	8.04	0.00	8.84
	72	0.00	16.20	0.00	15.45	7	0.00	25.76	0.00	24.04
	96	0.00	0.00	0.00	0.00					
1.00	24	0.00	11.79	0.00	54.85	3	0.00	15.44	0.00	17.71
	48	0.00	25.40	0.00	41.11	5	0.00	8.11	0.00	5.26
	72	0.00	0.00	0.00	0.00	7	0.00	0.00	0.00	0.00
	96	0.00	0.00	0.00	0.00					