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Full Length Research Paper

Exploring the effects of colchicine treatments on polyploidization in two Diploid Cotton Varieties (*Gossypium herbaceum L. and G. arboreum L.*)

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Anti-mitotic agents such as colchicine have been used to induce polyploidy in various plants. Here we examined the effects of different doses of colchicine on polyploidy induction in two cotton species (*Gossypium herbaceum* and *Gossypium arboreum*). The data reveal that the dose of colchicine, treatment duration, genotype and their combined effects on the induction of polyploidy and plant growth rate were statistically significant. Increase of either the concentration of colchicine or treatment duration showed adverse affects on seed viability in both species examined. The colchicine treatment is more effective when the length of hypocotyls is between 4-7 mm. The buds of *G. arboreum* treated with 0.9% colchicine exhibited more tetraploid cells sixteen hours post treatment. Among three methods employed, *in vitro* treatment of embryos can be conducted in a little space and needs a little amount of colchicines. However, high risk of contamination and high sensitivity of embryos to colchicine is a draw back of this method. Treatment of intact meristems with colchicine requires more amounts of the material and increases the risk of adverse environmental effects. Furthermore, plants treated with this method are susceptible to environmental stimuli including high temperature and should be kept away from direct sunlight. Together, our data reveal that treatment of the cotton seeds with colchicine was more efficient and reliable compared to other methods examined. The optimum dose of colchicine and the incubation time should be adjusted for each variety under various environmental conditions.

Key words: Polyploidization, *Gossypium*, Colchicin, embryo.

INTRODUCTION

Colchicine ($C_{22}H_{25}O_6N$), a product extracted from the seeds and bulbs of the plant *Colchicum autumnale* L., as well as other anti-mitotic agents such as amiprophos-methyl oryzalin and trifluralin have long been used to induce polyploids (Blakeslee and Avery, 1937; Stanys et al., 2004). These types of chemicals act by binding to the tubulin dimmers, preventing the formation of micro-tubules, and consequently, spindle fibers during cell division (Petersen et al., 2003). Thus, colchicine can effectively arrest cell division at the early anaphase stage. At this phase, the chromosomes have been duplicated but mitosis has not yet taken place and restriction of cell wall formation at this stage results in the polyploid cells. They are generally larger than their diploid counterparts and frequently develop into thicker tissues, resulting in large-sized plant organs (Vainola, 2000). In addition, pollen grain diameter as well as the size and number of stomata are generally increased in the polyploids (Blakeslee and Aery, 1937; De Jesus-Gonzales and Weathers, 2003; Chauvin et al., 2003).

The ploidy manipulation is considered as a valuable tool in genetic improvement of many plants including the *Solanum* spp. (Chauvin et al., 2003), citrus (Wu and Mooney, 2002), pomegranate (Shao et al., 2003), *Allium* spp. (Jakse et al., 2003) and azaleas (De Schepper et al., 2004). An attempt to increase the ploidy level has been

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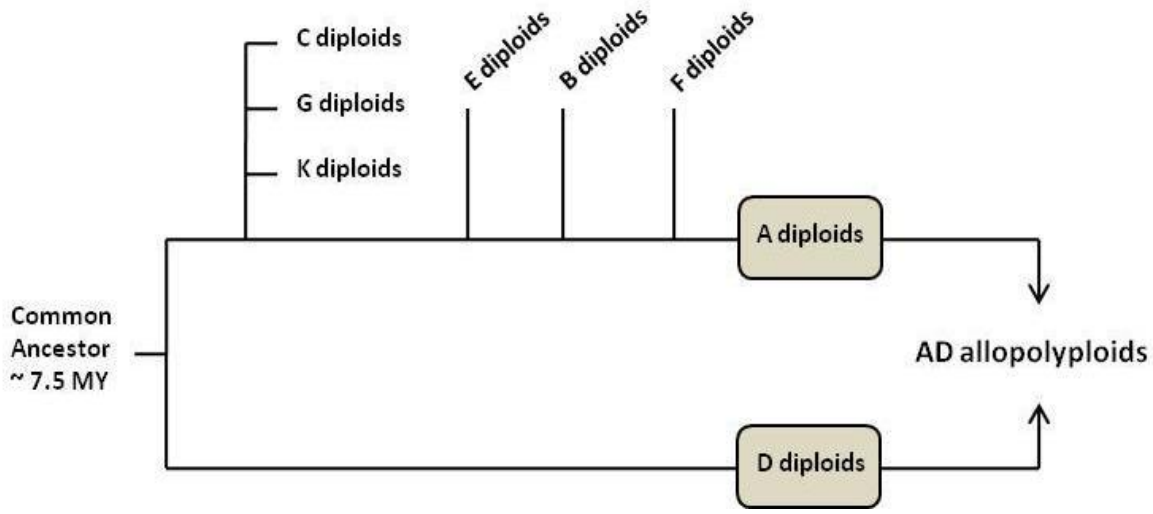

Figure 1. Schematically evolutionary history of the cotton (*Gossypium* spp.).

conducted seeking different goals in various plants. In citrus, tetraploid (4n) parents were produced to create seedless triploids by crossing (4n) and (2n) parents (Wu and Mooney, 2002). In the medicinal plants, *Scutellaria* spp. (Gao et al., 2002) and *Artemisia* spp. (De Jesus-Gonzalez and Weathers, 2003), tetraploidy increases the amounts of the secondary metabolites, baicalin and artemisinin. In azalea, chromosome doubling has been used to obtain new ornamental characteristics (De Schepper et al., 2004). In addition, the polyploids also provide a wider germplasm base for breeding studies (Thao et al., 2003).

Cotton (*Gossypium* sp.) has been developed as a particularly useful group for studies of the polyploidy (Wendel and Cronn, 2003; Adams and Wendel, 2004). The diploid cottons are classified into eight genome groups (A-K) based initially on cytology and refined by molecular systematic studies (Wendel and Cronn, 2003). There are two major branches of the diploid *Gossypium* species; one comprising the New World (D- genome group) and the other containing all other genome groups, variously distributed in the Old World (A-genome group) (Figure 1). The polyploidization between an A-genome diploid and a D-genome diploid approximately 1.5 million years ago created the AD allotetraploid group of cotton (Senchina et al., 2003) . Induction of polyploidy in inter-specific or intergeneric hybrids results in duplication of the two genomes present in the hybrid and formation of an allopolyploid. This allows for continued introgression of desired genes into the cultivated gene pools (Olsen et al., 2006).

To better understand the fate of polyploidization in two diploid cotton species, we analyzed the phenotypic and cytologic effects of various doses of colchicine on plants treated in various developmental stages and for different periods.

MATERIALS AND METHODS

Plant material and colchicine treatment

Gossypium herbaceum and *Gossypium arboreum* were obtained from the Iranian Cotton Research Institute, Gene Bank in 2003-2004.

Seed treatment

The cotton seeds were arranged among the wet paper towels incubated at 25 - 27˚C for germination. After 24 - 30 h, seeds were immersed for 4, 8, 12 and 16 h in distilled water supplemented with 0, 0.2, 0.4, 0.6 or 0.9 percent colchicine. Upon completion of their incubation, the seeds were rinsed thoroughly with distilled water and planted carefully inside the wet paper towels and incubated in 25 - 27˚C. After 36 h, ploidy level of the root tip meristemic tissues was determined by squash method as described by Singh (1993).

Isolated embryo treatment

The cotton seeds were surface sterilized in 70% ethanol for 1 min, washed and soaked in distilled water for 8 h for softness of woody cotton seed coat. The whole embryos were excised from seeds, and they were placed as downward or upward on ½ × Murashige and Skoog (MS) nutrient medium containing 0, 100, 500, 1000 or 2000 ppm colchicine (Stanys et al., 2004). After 24 h, embryos were rinsed three times with liquid medium and distilled water and grown on MS nutrient medium without colchicine (Stanys, 1997; Stanys et al., 2004). The root tips were collected after 24-36 h and used for preparation of microscopic slides. The ploidy level of the rooted regenerated was evaluated by counting the chromosome numbers in the cells of root meristems (Singh, 1993).

Cotyledinary meristem treatment

Seeds were planted and grown in a green house under normal conditions (16 h light period, temperature 27^{o}C and humidity 65%). To induce polyploidy, various concentrations of colchicine aqueous solution (0.2, 0.4, 0.6 and 0.9 percent) were applied for 24 h with

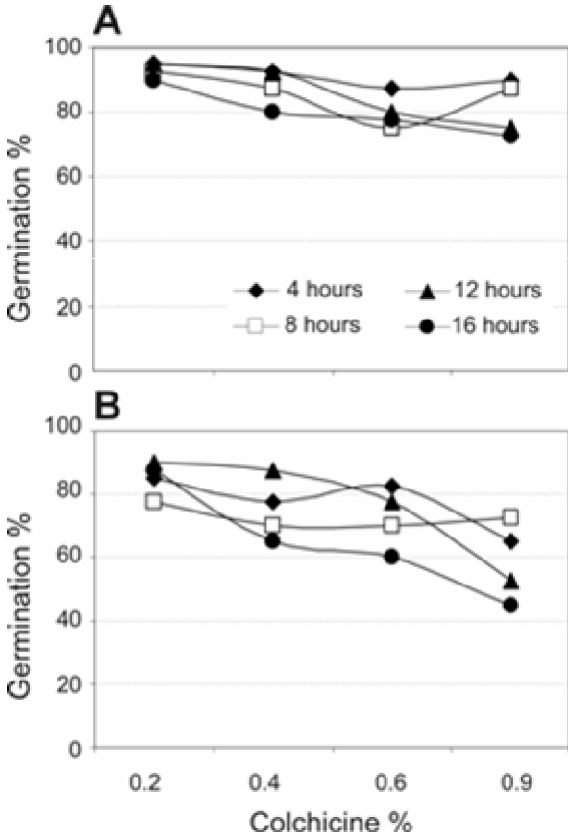

Figure 2. Effects of different concentrations of colchicine on the germination rate of *G. herbaceum* (A) and *G. arboreum* (B).

cotton wool on the terminal meristem of the young plants just after cotyledon expansion, or adult plants. During this treatment, cotton plantlets were kept at room temperature in a water -saturated atmosphere to avoid water evaporation from the cotton wool; this procedure was necessary to prevent concentrating the colchicine, which would burn the terminal meristem (Liu et al., 2001). After 3 days, plants were screened and the percentage of healthy plants as well as probable morphological abnormalities was scored in plantlets. For adult plants the height of the plants was measured at the time of treatment and one or two weeks post treatment.

Chromosome counting

The root tips of the controls and the polyploids were treated in 2 mM 8-hydroxyquinoline (Alishah et al., 2007) for 5 - 6 h at 18˚C, washed three times with distilled water and were subsequently fixed in freshly prepared absolute ethanol-glacial acetic acid (3:1) for 24 h at 4˚C and finally, stored in 70% ethanol at 4˚C. Metaphase spreads were obtained by a squash, hydrolyzed in 1 N hydrochloric acid for 10 - 15 min at 60˚C (45 - 60 min at room temperature) and stained with aceto orcein (Sheidai et al., 1996; Singh, 1993). At least 50 cells from each sample were screened and the number of cells with polyploidy was determined by squash method and microscopic visualization.

Statistical analysis

Experimental design used in each trial was a randomized complete block design (RCBD) with two factors in four replications. The first factor was dose of colchicine (D) (0, 0.2, 0.4, 0.6, 0.9 percent for seed and meristem treatment, or 0, 100, 500, 1000, 2000 ppm for embryo treatment) and second one was colchicine treatment periods (T) (4, 8, 12 and 16 h). The analysis of variance (ANOVA) for the assessed traits was carried out based on the RCBD description. The mean grouping analysis was done with Duncan's multiple range test (DMRT). All data analysis was conducted using SAS and SPSS software systems, SAS (1990).

RESULTS AND DISCUSSION

In order to determine the optimum concentration of colchicine and the duration of the treatment, cotton seeds were treated with various concentrations of the colchicine for different periods, as indicated in the materials and methods and the ratio of viable seedlings after treatment were scored. Figure 2 shows that for both species examined, an increase in the concentration of colchicine or in the treatment period had adverse effects on seed viability. In both species, increasing the incubation time from 4 - 16 h decreased the percentage of viable seedlings from 87 - 95% to 72 - 90%. The results also show that *G*. *arboreum* was more sensitive to the applied doses of colchicine than the *G*. *herbaceum* (Table 1).

The results of one way analysis of variance (ANOVA) for this experiment are shown in Table 1 and indicate that the effects of colchicine dose (D) (P 0.01), genotype (G), D × treatment duration (T) as well as D ×T × G were all significant (P 0.05). However, the effects of replica-tion, D×G and D×T, were not significant. In addition, the effects of colchicine dose, the combining effects of G×D, D×T (P 0.01), and the effects of G×D×T (P 0.05) on tetraploid cells were all significant but the effects of genotype and G×T were not statistically significant (Table 2).

During the colchicine treatment of the germinating seeds, some buds were seriously damaged so that their further growth was abolished. Our data suggest that when the length of hypocotyls is between 4-7 mm the colchicine treatment is more effective. To investigate the polyploidy fate in healthy growing buds post colchicine treatment, we counted the number of chromosomes in these buds. Treatment of the seedlings with 0.2% colchicine did not have any effects on ploidy level of the buds (data not shown). By increasing the dose of colchicine to 0.4%, tetraploidy was observed only in 6.6% of the *G. arboreum* buds (Table 1). Figures 3 also shows that the response of species examined to colchicine doses above 0.4% was not the same. Sixteen hours post treatment, the buds of *G. arboreum* treated with 0.6% and that of *G. herbaceum* treated with 0.9% colchicine exhibited more tetraploid cells (Figures 3 and 5). Similar results indicating various responses of different species or varieties of wheat, primrose, pomegranata and sweet beet to the colchicine have also been reported (Peterson et al., 2003; Shao et al., 2003; Thao et al., 2003).

Further, we analyzed the effects of colchicine on cotton embryos grown *in vitro*. The response of complete embryos of examined cotton species to colchicine as pre-

Table 1. Different colchicine doses effect at the various ploidy stages.

Genotype	Time (h)	Colchicine dose (%)											
		0.2			0.4			0.6			0.9		
		TCS	STC	VS	TCS	STC	VS	TCS	STC	VS	TCS	STC	VS
G. herbaceum	4	0.50	0.0	95.1	0.21	0.0	92.2	1.53	0.0	87.1	23.53	20.0	90.1
	8	0.00	0.0	92.4	0.00	0.0	98.0	3.25	5.5	75.0	52.38	22.1	87.6
	12	0.23	0.0	95.3	0.00	0.0	92.0	8.72	11.0	80.2	86.68	94.1	75.1
	16	0.27	0.0	90.6	1.02	0.0	80.3	26.27	22.2	77.1	94.40	95.4	72.2
G. arboreum	4	0.00	0.0	85.2	0.81	0.0	77.0	10.83	0.0	82.3	83.47	25.2	65.4
	8	0.00	0.0	77.1	0.00	0.0	70.5	20.78	6.2	70.0	91.70	75.1	72.2
	12	0.25	0.0	90.0	1.46	0.0	87.2	88.94	37.2	77.0	95.02	92.8	52.0
	16	0.66	1.0	87.1	7.00	6.6	65.4	92.14	92.5	60.8	68.06	68.0	45.3

VS, Viable seeds after colchicine treatment (%); STC: seeds with tetraploid cells (%); TCS, Percent of tetraploid cells in methaphase preparations (%).

Table 2. ANOVA results for colchicine effects on polyploidy in seed and embryo

Source of Variation	Degree of freedom (df)	Means of Squares			Degree of freedom (df)	Means of Squares	
		Embryo treatment				Seed treatment	
		ETC	EWN	VE		STC (log x)	VS
Replication (R)	3	0.798 **	0.525 *	242.12 **	3	6.78 ^{ns}	12.45 ^{ns}
Genotype (G)	1	0.532 *	2.45 **	187.80 *	1	14.18 ^{ns}	33.80 *
Dose (D)	3	1.194 **	0.977 **	176.38 **	3	126.51 **	48.92 **
Time (T)	1	0.621 *	1.112 *	192.72 *	3	51.43 *	39.35 *
G*D	3	0.360 *	0.743 *	139.91 *	3	70.22 **	22.45 ^{ns}
G*T	1	0.412 ^{ns}	0.541 ^{ns}	168.40 ^{ns}	3	29.45 ^{ns}	19.58 ^{ns}
D*T	3	0.368 *	0.415 ^{ns}	128.25 *	9	41.45 **	36.19 *
G*D*T	3	0.595 **	1.355 **	201.11 **	9	31.48 *	29.45 *
Error	45	0.121	0.182	46.60	93	15.08	12.15

Ns, Non significance.
* and **, Significant at 5% and 1% levels; respectively.
Vs, Number of viable seedling post treatment; STC, Number of seeds with tetraploid cells.
VE, Number of viable embryos with normal growth.
EWN, Number of embryos without normal growth.
ETC, Number of embryos with tetraploid cells.

sented in Table 3 indicates that cotton embryos are very sensitive to increasing doses of colchicine. In many embryos, chromosomes were shorter and more condensed, and many cells exhibited abnormal morphology and increased rate of necrosis when the concentration of colchicine is increased from 1000 - 2000 ppm (data not shown). The data indicate that germination ability of the embryos isolated from *G. herbaceum* was better than that in *G*. *arboreum*. The highest rate of appearance of tetraploid cells was observed in plants treated with 1000 ppm colchicine for 24 h. Under these conditions, the percentage of tetraploid cells in the embryos of *G. arboreum* that survived the treatment was almost twice as that in *G. herbaceum* (Table 3). The results of analysis of variance for this experiment is shown in Table 2 with the effects of colchicine dose and the effects of G × D × T on the number of embryos with normal growth. The number of embryos with abnormal growth as well as the number of tetraploid cells is significant (P 0.1). In addition, the effects of genotype on the number of embryos with abnormal growth (P 0.1) or with normal growth and on the number of tetraploid cells, as well as the effect of treatment period on all three above mentioned traits (P 0.5) are significant. Together, all three examined factors independently, or their combined effects on various aspects of colchicine treatment, were

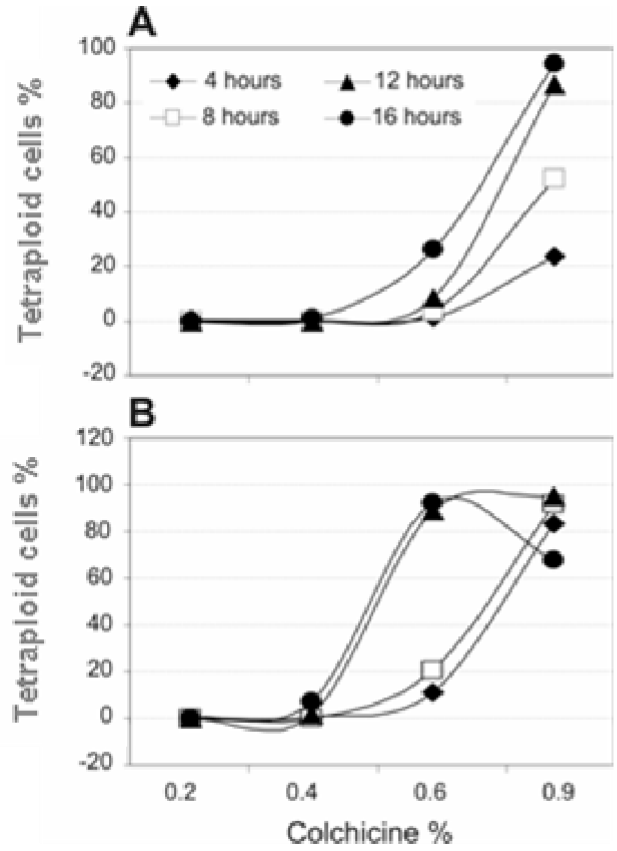

Figure 3. Effects of different concentrations of colchicine on the induction of tetraploid cells of *G. herbaceum* (A) and *G. arboreum* (B).

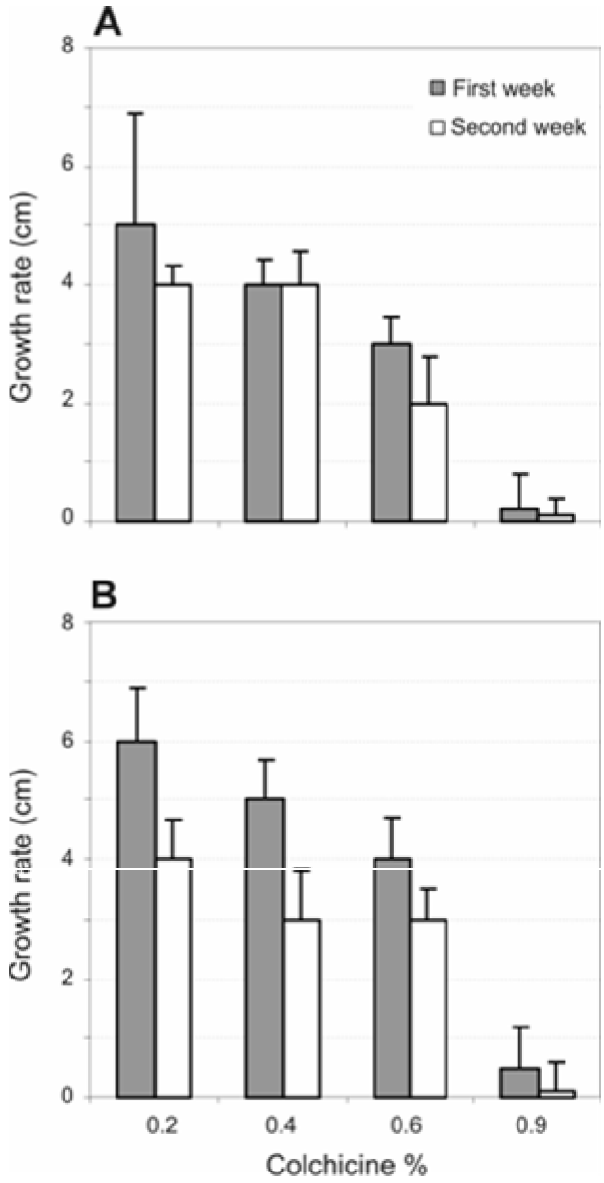

Figure 4. Effects of different concentrations of colchicine on growth rate (%) of *G. herbaceum* (A) and *G. arboreum* (B) with time (weeks).

effective. Comparable results have been reported for *Miscantus sinensis* (Peterson et al., 2003), pomegranate (Shao et al., 2003) and *alocacia* (Thao et al., 2003). Treatment of embryos with colchicine *in vitro* is an effective method which needs considerably less space and a little amount of colchicine. On the other hand, the high risk of contamination, higher sensitivity of embryos to colchicine as well as to the environmental stimuli might be considered as draw backs of this method.

To further investigate the effects of colchicine on induction of polyploid cells in cotton, apical meristems of seedlings or mature plants were treated with various doses of the colchicine and the results are presented in Figure 4. The data shows that the growth rate of seedlings decreased with increasing concentrations of colchicine, suggesting that the colchicine may halt the cell division in apical meristems leading to the growth arrest. Hansen et al. (1995) reported that defining the optimum concentration of colchicine in such experiments is very difficult. To increase the reproducibility of experiments, we kept a careful eye on environmental conditions such as elimination of plants from being exposed to direct sunlight and tight control of humidity. Our results show that treatment of plants with 0.9% colchicine for 12 h completely abolished the growth (data not shown). The results indicate that increasing the dose of colchicine would lead to a decline in the growth rate of the plants (Figure 4). In both species examined, increasing the dose of colchicine from 0.2 - 0.9% decreased the growth rate of the plants, suggesting that the apical meristem of the plants is very sensitive to this anti-mitotic compound.

Conclusions

The colchicine is an anti-mitotic agent that can bind to microtubules and inhibit formation of spindle fibers during

Table 3. Different colchicines dose effects in the embryo cells.

Genotype	Time (h)	Doses (ppm)											
		100			500			1000			2000		
		EG	TE (%)	TCS	EG	TE (%)	TCS	EG	TE (%)	TCS	EG	TE (%)	TCS
G. herbaceum	12	100	0.0	0.83	80.0	1.8 (9.0)	11.76	84.1	2.5 (11.3)	47.96	96.0	2.9 (10.7)	54.44
	24	90.1	0.0	0.0	88.1	1.9 (8.6)	13.77	91.3	3.8 (17.3)	80.92	92.5	3.7 (16.1)	28.93
G. arboreum	12	85.2	0.0	0.0	96.0	4.2 (19.1)	7.04	92.4	5.6 (24.3)	75.1	89.1	2.6 (10.4)	27.62
	24	96.3	0.0	1.63	92.2	5.0 (20.0)	22.03	95.3	6.1 (32.1)	83.89	96.3	4.3 (15.0)	22.90

EG, Percent of germinated embryos; TE, average of embryos with induced tetraploid cells (percent in parenthesis)
TCS, percent of tetraploid cells in metaphase preparations.

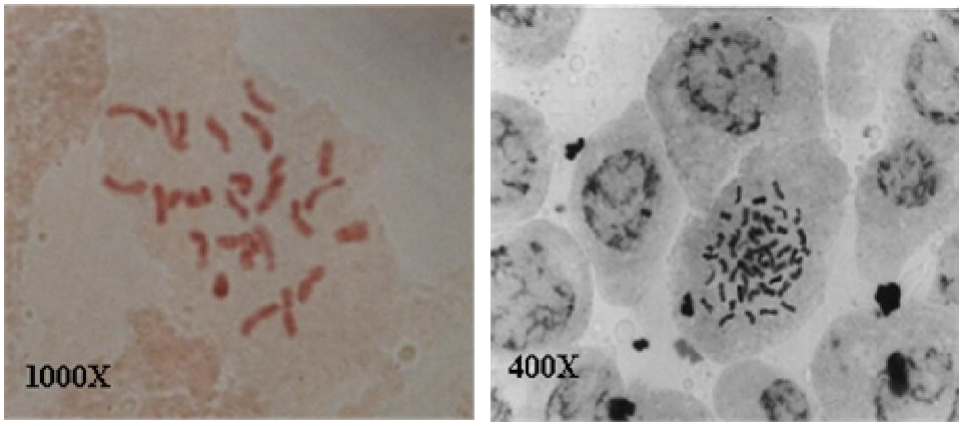

Figure 5. Chromosome number in diploid (A; 2n = 2x = 26) (*G. herbaceum* L.) and polyploid (B) cotton induced by colchicine treatment.

the cell division resulting in formation of the polyploid cells. In addition, optimum effect of the colchicine treatment can be achieved when the cells are actively dividing. The rate of the polyploidization in a cell depends on both the dose of colchicine and the duration of treatment. The optimum treatment conditions lead to reasonable induction of tetraploid cells while most of the treated cells are still alive and can be rescued. Here the responses of the seeds, embryos and apical meristems of two diploid species of cotton to the colchicine were monitored. Our data reveals that the dose of colchicine, treatment duration, genotype and their combined effects on the generation of polyploidy and plant growth rate are statistically significant. Among three methods employed to induce polyploidy in cotton, *in vitro* treatment of embryos can be conducted in a little space and needs little colchicines. However, high risk of contamination and high sensitivity of embryos to the colchicine is a draw back of this method. Treatment of intact meristems with the colchicine requires more amounts of the material and increases the risk of environmental adverse effects. Furthermore, plants treated in this method are susceptible to environmental stimuli including high temperature and should be kept away from direct sunlight. On the other hand, treatment of the cotton seeds with the colchicine was more efficient and reliable compared to the other methods examined. Our data suggest that for polyploidy induction, 16 h incubation of the *G. arboreum* and *G. herbaceum* seeds with 0.6 and the 0.9% colchicine, respectively, gives the best results. The optimum dose of colchicine and the incubation time depends on the species and environmental conditions.

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