ROLE OF COLCHICINE AND PLANT GROWTH REGULATORS TO OVERCOME INTERSPECIFIC INCOMPATIBILITY

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Summary. *Gossypium arboreum* L. has desirable genes of biotic and abiotic resistance. It has been of interest to cotton breeders, however, introgression of these genes into tetraploid cultivated cotton was highly bottlenecked due to differences in ploidy level and morphology of chromosomes. Therefore, a study was conducted to find an efficient *in vivo* method of chromosome doubling by colchicine treatment in *Gossypium arboreum* L. and its subsequent introgression with *Gossypium hirsutum* L. The optimum colchicine treatment involved one drop of colchicine to the seedling tip applied in the course of three days. Stable autotetraploid plants were selected on the basis of chromosome counting at meiotic stages. Induced tetraploid plants were more robust with broader leaves, large bracts and higher optical density of genomic DNA than diploids. Mean stomatal lengths of diploid plants were smaller compared with the tetraploid. Induced tetraploid plants when kept as a female parent assisted interspecific hybridization (*Gossypium*). However, interspecific crosses failed when induced tetraploid plants were utilized as a male parent. This interspecies barrier was overcome by exogenous application of plant growth regulators, i.e. IAA and GA_3 to the stylar tissue within five minutes of pollination. The GA_3 concentration of 0.5 mg l^{-1} was found highly effective.

Key words**:** *Gossypium arboreum* L., colchicine, induced tetraploidy, plant growth regulators.

Abbreviations: IAA**-** indole-3-acteic acid, GA_3- gibberellic acid.

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INTRODUCTION

G. arboreum L. has desirable genes for abiotic tolerance and resistance to diseases (Innes et al. 1992; Stanton et al. 1992). On the other hand, upland cotton (*G. hirsutum*) varieties are high yielding and widely grown. However, they lack important characteristics present in diploid species of cotton and the cost of their production is therefore relatively high. It is worthwhile to introgress the genes for the above-referred desirable characters between arboreum and hirsutum cotton (Mehetre et al., 2003). However, by conventional breeding methods, the crosses between these two species of cotton are rarely successful due to abortion of embryo after fertilization because of differences in chromosome numbers of *G. arboreum* ($2n = 26$) and *G. hirsutum* ($2n = 52$). Attempts were also made to obtain the interspecific hybrid between cultivated *G. hirsutum* and *G. arboreum* through *in vitro* embryo rescue techniques. Limited success has been reported by this advent technique (Liu et al., 1992). Even if crosses are successful and hybrids are obtained, the resultant progeny will be triploid with sterility.

Colchicine ($C_{22}H_{25}O_6N$), a product extracted from the seeds and bulbs of *Colchicum autumnale* L., is probably the most widely used chemical for induction of polyploidy. In the late 1930s Blakeslee and Avery (1937) discovered that colchicine inhibited formation of spindle fibres, which resulted in polyploid cells. These cells were larger than diploid counterparts and greater cell volume frequently developed into thicker tissues, thus resulting in large size plant organs. It also affected stomata size, stomata frequency, pollen grain diameter and other plant morphological characteristics in new ploidy levels (Franzke and Ross, 1952; Dirks et al., 1956; Downes and Marshall, 1983).

Colchicine may be applied to induce autotetraploid plants in Asiatic cotton and direct crossing of these induced tetraploids of *G. arboreum* L. with cultivated tetraploids *G. hirsutum* would be a shortcut method for the transfer of desirable genes. The resulting interspecific hybrids would also be backcrossed to cultivated *G. hirsutum* to obtain plants with $2n = 52$ chromosomes having desired character combinations.

However, transmission of genes from induced autotetraploid proved very poor through pollen grains, primarily due to slower pollen tube growth (Coffin and Harney, 1978; Susiacuen and Álvarez, 1997). Plant hormones are known to control pollen tube growth (Kovaleva and Zakharova, 2003). Exogenous application of growth regulators has been used to facilitate interspecies crosses in many crops including cotton (Altman et al., 1988), wheat (Sitch and Snape, 1987) and tomato (Gordillo et al., 2003). In cotton, Altman (1988) compared exogenous hormone application with *in vitro* techniques i.e. ovule and embryo culture and found that exogenous hormones used with standard hybridization techniques were superior to *in vitro* methods.

Therefore, a study was conducted to induce: (i) polyploidy in *G. arboreum* by colchicine treatment and identifying tetraploid plants by karyotypic analysis (ii) in-

trogression from *Gossypium hirsutum* L. (2n=52); *Gossypium arboreum* L (2n=52). The effect of exogenous application of hormones on *G. hirsutum* L. to facilitate the introgression from *Gossypium arboreum* L (2n=52) was also studied.

MATERIALS AND METHODS

Two cotton genotypes belonging to *G. arboretum* (2n= 26) viz., FH-228 and HK-113 were used throughout the experiments. Three different methods of colchicine treatment: soaking of seeds in colchicine solution (1%), application of colchicine solution (1%) to growing tip of plants and dipping of whole seedlings except the radical in colchicine solution (1%) were tested. Three replications and twenty pots of each genotype per replication (each pot containing three seeds) were used for colchicine application whereas ten pots per replication per genotype were treated with distilled water as control.

Microscopic studies

The meiotic studies were carried out at 1500x magnification, by staining immature pollens taken from each colchicines-treated plant with acetocarmine. Stomatal and pollen diameters were measured at 40x and 20x magnification, respectively. The values obtained were computed into micrometer.

Flower and leaf area measurements

The first borne flowers on both the tetraploid and the control were used for measurements of reproductive organs. On the 8th node of all plants leaves were tagged as they appeared on the main stem and after 30 days the leaves were outlined on a graph paper and their area was measured in cm^2.

Genome DNA extraction and quantification

Genomic DNA of tetraploid and control plants was extracted according to the CTAB method (Doyle and Doyle, 1987) and its optical density was measured using a spectrophotometer.

Self- and interspecific crosses

The flowers of (i) *Gossypium arboreum L.* 2n=26; (ii) *Gossypium arboreum* L. 2n=52 and (iii) *Gossypium hirsutum L.* 2n=52 were emasculated one day before opening as apparent from the candle shaped floral bud. Pollens were gently removed with the help of forceps. The stigma was then covered with soda straw tube to avoid any foreign pollen contamination. Emasculated flowers were tagged with the date of

emasculation. The emasculated flowers were pollinated in the next morning. Some floral buds were tied with a thread in order to obtain self-pollinated seeds.

Exogenous hormone application

Stock solutions of gibberellic acid ^{3} (GA^3), and indole-3-acetic acid (IAA) (Sigma-Aldrich Chemical; St Louis, MO) were prepared by dissolving the appropriate amount in ethanol for GA3 or NaOH for IAA to yield final concentrations of 0.00,0.25,0.50,0.75,1.0,2.0 mg l^{-1}. Three drops of each hormone solution or water were applied using a medicinal dropper to the *Gossypium hirsutum* L. cv. HR-VO within five minutes after pollination with pollens from autotetraploid arboreum cv. FDH-228 and HK-113. Three replications were made each including ten plants.

Seed germination (percentage) was measured only in method one. Plant height measurements and karyotype analysis were done for all plants obtained from the three colchicine application methods. Stomata size, pollen diameter, leaf area, DNA optical density and floral characteristics were measured only in the confirmed tetraploid and control plants of both cultivars.

RESULTS

Germination percentage (method1) and plant height (method 1, 2, 3) of colchicines-treated plants were very low when compared to control (Table 1). Growth abnormalities and inhibition after colchicine treatment continued for more than one month after which surviving plants of both cultivars in all experiments resumed normal growth and development.

The meiotic studies were carried out for all plants obtained by all methods of colchicines application to confirm the auto-tetraploid plants. Among all the methods, shoot apex treatment at the seedling stage (Method 2) was found most effective followed by seedling dipping in colchicine solution (Method 3) (Table 2). Method 2 was not only effective in producing tetraploid plants but also resulted in a high sur-

Table 1. Average germination percentage and plant height of *G. arboreum* (FDH-228 and HK-113) after colchicine treatment compared to the respective controls

Experiment	Treatment	Germination (%)		Plant height (cm)	
		FDH-228	HK-113	FDH-228 ±SE	HK± SE
1	Control	-	-	51.5±2.23	39± 3.67
	Treated	-	-	27.66± 2.89	27.66±10.89
2	Control	78	86	50.11±1.12	43.7±4.22
	Treated	11	14	16.37±0.67	21.74±0.78
3	Control	-	-	52.25±1.87	41.5±3.31
	Treated	-	-	43.34±2.23	36.33±3.5

Table 2. Efficiency of three different methods of colchicine application to induce ploidy in two *G. arboretum* cultivars

Cultivar	**Method 1**		**Method 2**				**Method 3**			
	Treated*	Tetraploid	Treated**	A.P[1]	C.P[2]	S.T[3]	Treated**	A.P	C.P	S.T
FDH-228	180	0	180	43	61	16	180	71	9	8
HK-113	180	0	180	74	31	05	180	26	28	4

[1] Aneuploid; [2] Chimeral Plants; [3] Stable tetraploid
Method 1 (Seed treatment); Method 2 (Shoot apex treatment); Method 3 (Seedling dipping)
* number of treated seeds
** Seedling treated

vival rate. However, both methods (2 and 3) were also involved in the production of chimeras and aneuploids.

The confirmed stable tetraploid plants were further used to study the different plant attributes. Compared to the control plants, the autotetraploids plants of both cotton varieties had epidermis with larger stomata size, but lower stomatal frequency (Table 3). It was found that leaves of autotetraploid plants of FDH-228 and HK-113 had 74.55% and 82.02% lower stomata than untreated plants. Polyploid leaves were much larger than 2n plant leaves ranging from 100.3% to 125.18%. Similarly, comparison of genomic DNA extracted from leaves of two-ploidy levels showed higher DNA optical density for autotetraploids from both cultivars (Table 3). Pollens appear to be round in structure in both autotetraploid and control plants under stereomicroscope and some increase in pollen diameter of tetraploids was also observed (Table 4). However, the number of anthers per flower was greatly reduced in autotetraploid plants as compared to diploid arboreum (Table 4). Some other floral parts also showed increased length in autotetraploid plants as compared with diploids. Thus, autotetraploid plants showed an increase in petal length by 51.5% and 21.6% in cultivars FDH-228 and HK-113, respectively (Table 4) while the increase observed in bract length was by 145.56 % and 60% in FDH-228 and HK-113, respectively (Table 4).

Doubling of the chromosome number of the diploid cotton plants (*G. arboretum*) improved the cross ability of polyploids. Some seeds were obtained as a result of interspecific crosses. However, crosses between G. *arboreum* (2n=26) and *G. hirsutum* or G. *arboreum* (2n=52) ended in complete failure (Table 5). High success rate was obtained when tetraploid plants of *G. arboreum* var. FDH-228 were treated with *Gossypium hirsutum* cv. HR-VO pollens (Table 5). The bolls had slower growth and produced lower seeds (Table 5).

Direct crosses between *G. arboreum* (2n=52) and *G. hirsutum* (2n=52) proved to be successful, but reciprocal crosses (*G.hirsutum* 2n=52 x *G. arboreum* 2n=52) ended in complete failure. Flowers dropped one or two days after pollination. Therefore, different concentrations of two plant hormones were applied to the stylar tissues

Table 3. Anatomical, chemical and morphological differences in leaves of control and tetraploid plants of two cotton genotypes FDH-228 and HK-113

Treatment	Range of stomata length (μm)		Stomata length(μm)		Stomata frequency		Leaf area (cm^2)		DNA Optical density	
	FDH-228	HK-113	FDH-228	HK-113	FDH-228	HK	FDH-228	HK	FDH-228	Hk-113
Auto-tetraploids	32.2-41.4	27.6-38.7	37.11±1.25	34.23±3.01	157±19.30	178±15.47	236.22±21.89	187.96±18.96	0.060±0.005	0.056±0.007
Control	20.7-23	18.1-19.4	21.4±057	18.25± 1.0	274±33.01	32433.02	104.90±10.92	92.45±31.02	0.038±0.002	0.028±0.004
Difference			73.41% increase	87.56% increase	74.55% decrease	82.02% decrease	125.18% increase	100.3% increase	58% increase	100% increase

Table 4. Anatomical and morphological differences in flowers of control and tetraploid plants of two cotton genotypes FDH-228 and HK-113

Treatment	Pollen diameter (μm)		Petal length (cm)		Bract length (cm)		Anther No.	
	FDH-228 ±SE	HK-113±SE	FDH-228±SE	HK-113±SE	FDH-228±SE	HK-113±SE	FDH-228±SE	HK-113±SE
Autotetraploids	120±13.42	112.66±7.55	2.98 ±0.25	3.94 ±0.017	4.57± 0.25	2.03±0.3	51.7 ±2.44	46.22±3.55
Control	98.2±28.5	101.77±32.44	1.96 ±0.01	3.24 ±0.13	1.78± 0.34	1.27±0.55	101.3±5.67	92.33±4.25
Difference	22.20 % increase	10.70% increase	50.5% increase	21.60% increase	145.56% increase	60% increase	96% decrease	100% decrease

Table 5. Number of bolls and seeds per boll obtained as a result of various crosses

Variety	Ploidy level	No. of boll set			
		PIT /TFP**	Seeds/ Boll	PGH/ TFP**	Seeds/ Boll
FDH-228	**Diploid**	0/96	0.00±0.00	0/350	0±0.00
	Tetraploid	18/81	21.18±3.13	7/253	9.33±1.15
HK-113	**Diploid**	0/76	0.00±0.00	0/266	0±0.00
	Tetraploid	4/39	22.23±2.73	2/117	8.50±0.5

* Pollinated with induced tetraploid (PIT)/Total flowers pollinated (TFP)
** Pollinated with *G. hirsutum* L (PGH)/ Total flower pollinated (TFP)

Table 6. Effect of exogenous application of plant hormones after treatment with induced tetraploid pollens cv. FDH-228 and HK-113 to *Gossypium hirsutum* L. cv. HR-VO

Hormone	IAA (Indole-3-acetic acid)				GA3 (Giberellic acid ^{3})			
(mg l^{-1})	Pollinated with FDH-228		Pollinated with HK-113		Pollinated with FDH-228		Pollinated with HK-113	
	No. boll set	Seeds/ boll	No. boll set	Seeds/ boll	No. boll set	Seeds/ boll	No. boll set	Seeds/ boll
0.00	0	0.00±0.00	0	0.00± 0.00	0	0.00 0.00	0	0.00± 0.00
0.25	2	7.50±0.50	2	9.00±0.00	1	7.00± 0.00	0	0.00 0.00
0.50	2	11.50± 0.50	2	12.00±1.42	9	11.33± 2.00	8	11.62± 1.92
0.75	4	11.25±1.32	6	11.67±1.63	2	8.00± 0.00	2	9.50± 0.50
1.00	6	10.33±2.23	8	9.88±1.46	1	7.00± 0.00	1	9.00 0.00
2.00	1	6.00± 0.00	1	6.00± 0.00	1	6.00± 0.00	1	8.00± 0.00

within five minutes after pollination to enhance introgression in to G. *hirsutum* (Table 6). Our results showed that both cultivars were more sensitive to giberellic acid than to IAA (Table 6). The highest numbers of interspecific bolls or seeds per boll were obtained when 0.5 mg l^{-1} of GA_3 was applied to the style tissue. The comparison between the two cultivars showed that HK-113 was more sensitive to IAA whereas FDH-228 was found more sensitive to GA_3 (Table 6). As far as IAA is concerned, the highest number of interspecific bolls was obtained when 1.0 mg l^{-1} of IAA was applied to the style (Table 6). However, an increase in boll number did not necessarily lead to increased number of seeds per boll. The highest number of seeds per boll was obtained when 0.5 mg l^{-1} of IAA was applied to the stylar tissues in both varieties (Table 6).

DISCUSSION

Our results on plant height were in agreement with Wright (1976) who reported that induced tetraploid plants seemed to grow more slowly and growth abnormalities were the first indication of successful colchicine treatment. Growth inhibition after

colchicine treatment was also confirmed by Stebbin (1984) who showed that the decreased growth rate of polyploids was caused by the reduced rate of cell division. The supply of the cells with auxins was interrupted, the respiratory ratio was reduced and activities of many enzymes were diminished.

The results of stomatal length and frequency agreed with those of Evan (1955) and Speckman et al. (1965) who reported that stomata length was the accurate indicator of the polyploid level in many plants. Wright (1976) also showed that stomatal measurement was a quick way to determine whether most of the leaves on the branch were polyploid. Similarly, Uhlik (1981) reported that the polyploid plants had gigantic characteristics, such as thicker and wider leaves with a greater stomata size and larger flowers. Stomata size and frequency, as well as pollen grain diameter were preliminary indicators of ploidy level in plants of *Bromos inermis*. A positive correlation between stomatal length and pollen diameter was found. This means that pollen diameter increase with increasing ploidy level. Induced polyploids also showed high DNA content.

The increase in dimensions and area was probably due to the fact that cells with a larger complement of chromosomes grow larger to maintain a constant ratio of cytoplasmic to nuclear volume, and express more proteins.

From the results obtained, it appeared that chromosome doubling in Asiatic diploid species can be used as a tool to overcome the incompatibility experienced in *G. arboreum* x *G. hirsutum* species which was reported difficult in many cases (Mehetre et al., 2003). One or two self-pollinated bolls in tetraploid plants were also found confirmatory for successful induction of polyploidy (Mehetre et al., 2003).

Exogenous application of plant hormones was found successful to overcome interspecies barriers. Application of plant hormones to the stylar tissues may result in enhanced pollen tube growth. A positive physiological role of GA_3 and IAA in the pollen tube growth has been previously reported (Singh et al., 2002; Koveleva et al., 2005). Koveleva et al. (2005) reported that exogenous application of IAA and GA_3 promoted pollen tube growth while cytokinin hindered its growth. Similarly, Kovaleva and Zakharova (2003) found significant changes in hormonal status of stylar tissues following pollination in self-compatible and incompatible *Petunia hybrida* L. The pollen tube growth after self-incompatible pollination was accompanied by a 5-fold increase in cytokinin content in the stylar tissues while self-compatible pollination brought an increase in the IAA contents in style. The auxin/cytokinin status of the style may be involved in controlling pollen tube growth.

In addition to the above report, there are very few successful examples of successful introgression from Asiatic cotton. A disease of localized occurrence was cotton rust caused by *Puccinia cacabata*. It was of importance in the Southwestern U.S. and Northwestern Mexico, where its incidence is variable but its economic impact can be significant. Fungicidal control of the disease is costly and not fully satisfactory. Few cultivars of *G. arboreum* were rust-resistant and all the other species were

found susceptible. Through artificial polyploids and interspecific hybrids plus a back-crossing scheme accompanied by continued screening for resistance, success was achieved in transferring this resistance into agronomically acceptable germplasm of *G. hirsutum*. Now rust resistant cultivars are available for planting in those areas where rust was a significant problem and the costs of disease control and disease loss are thereby avoided (Kohel and Lewis, 1984).

Such type of strategy will be used to introgress resistant genes for the abiotic and biotic resistance in upland cotton to overcome some localized problems.

The true tetraploid plants of *Gossypium arboreum* identified in this study will be permanently maintained in the department and F_1s obtained will be back-crossed to agronomically acceptable varieties of G. *hirsutum* L.

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