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Stomatal Size, Stomatal Frequency and Pollen Grain Diameter as Indirect Method for Identification of Ploidy Levels in Cotton

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ABSTRACT

Seeds of diploid local variety cotton (*Gossypium arboreum*), PM2, were treated with 0, 0.1 and 0.5% colchicine solutions at duration of 24 and 36 hours for polyploidy induction. Seed germination, seedling survival, stomatal size, stomatal frequency, percent pollen grain sterility and pollen grain diameter were recorded. The results showed seed germination, seedling survial and stomatal frequency to be negatively correlated with colchicine concentrations and durations of seed soaking while numbers of plant having large stomatal size and % pollen grain sterility have positive correlation. Stomatal size increased whereas stomatal frequency decreased. No correlation between pollen grain diameter and colchicine concentrations as well as durations of seed soaking was found.

Key words: cotton, *Gossypium arboreum*, colchicine, polyploidy, stomatal size

INTRODUCTION

Native cotton, *Gossypium arboreum*, originally cultivated in Thailand consists of both white and brown cottons which have short and coarsely staples making them much harder to spin and weave. It is diploid with "A" genome and posses 13 pairs of chromosome (2n = 2x = 26). American upland cotton, *G. hirsutum*, was introduced to replace the native one in the early 1950's. (Na Pompeth, 1994). It is allotetraploid or amphidiploid of "AD" genomes with 26 pairs of chromosome (2n = 4x = 52). This *G. hirsutum* was derived from hybridization between *G. thuberi* and *G. arboreum* having both white and brown long staples. The commercial cultivated cotton in Thailand nowaday is mainly white cotton, whose yields are low due to serious pest problems. Among the reported pests, cotton jassid (*Amrasca biguttula biguttula* (Ishida)), cotton bollworm (*Helicoverpa armigera* H?bner) and plant bug (*Megacoelum biseratense*) are some of the key pests. (Hormchan *et al*, 1997; Hormchan and Wongpiyasatid, 1999; Khaing *et al*, 2002). It was noticed that *G. arboreum*, the Old World species, seemed to be tolerated to the mentioned insects compared to *G. hirsutum*, the New World species.

Interspecific hybridization between *G. hirsutum* and *G. arboreum* will increase the genetic diversity as well as transferring of insect resistance from *G. arboreum* to *G. hirsutum*, the commercial cultivated species. However, interspecific hybridization may not be successful due to different chromosome numbers and sizes or even different genomes. This problem may be solved by doubling of chromosome numbers. Doubling of chromosome

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could be artificially induced by chemical treatments such as nitrous oxide (N_2O), oryzalin and colchicine (Subrahmanyan and Kasha, 1975; van Tuyl *et al.*, 1992; Bouvier *et al.*, 1994; Wongpiyasatid *et al.*, 2003). Treatment of various plant parts with colchicines, an alkaloid derived from *Colchicum autumnale*, has become the most applied technique.

Colchicine has been used successfully to induce the formation of polyploidy in several plant species, such as orchids (Hick, 2003), roses (Anonymous, 2005), barley (Subrahmanyam and Kasha, 1975), cotton (Wongpiyasatid *et al.*, 2003). Stadler *et al.* (1989) pointed out that different plant species might require very different effective concentrations of colchicine.

Stomatal size and density, number of chloroplasts in the guard cells of the stomata, length of these cells, ratio between the dimensions of certain organ and size of pollen grains are possible means of estimating the level of ploidy but these parameters are not applicable to all species (Dore, and Essad, 1996)

Evan (1955) and Speckman *et al.* (1965) reported that length of stomata was the accurate indicator of polyploidy levels in many plants. Write (1976) also showed that stomatal measurement was a quick method to determine whether or not most leaves on a branch were polyploidy. Preliminary test of polyploidy induction in *G. arboreum* using colchicine treatment was reported (Wongpiyasatid *et al*, 2003). Colchicine solutions derived from powder and drug tablets were compared for the effectiveness of chromosome doubling. It was found that colchicine solution derived from powder gave better results in polyploidy induction than solution from drug tablets. The purpose of this study was to induce chromosome doubling in *G. arboreum* by colchicine treatment and to study whether stomatal size, stomatal frequency and pollen grain diameter could be used as indirect method for preliminary identification of ploidy levels in cotton.

MATERIALS AND METHODS

Seeds of *Gossypium arboreum*, a diploid variety PM2 (light brown cotton) with "A" genome (2n = 2x = 26) were polyploidy induced in 3 treatments of 0, 0.1 and 0.5% concentrations of colchicine solution derived from powder sold by Sigma Chemical Co. Thirty seeds per treatment, with 3 replicates, were employed. In each treatment, 30 seeds were put into the nylon bag after which was soaked in a beaker with colchicine solution. The beaker was then placed on the shaker under fume hood in order to obtain the well blended solution. At the end of 24 and 36 hours soaking period, seeds were washed in running tap water for 6 hours, air-dried and later 3 seeds were planted in each pot. Fifteen days after planting (DAP), the numbers of seedling were counted and % germination was calculated. The numbers of surviving plant were also counted at 90 DAP and were used in $LC_{50(90)}$ calculation. Stomatal size and frequency of each plant in each treatment were recorded. Pollen grains diameters were measured and % pollen sterility of plants with large stomata were determined.

Stomata measurement

Length of stomata

Completely opened mature leaves from 3 months old plants were sampled (avoiding very young or very old leaves), 5 leaves/plant. A peel of epidermis from the lower surface of the lamina was removed with a fine scalpel. The peel was then mounted on a drop of water between slide and coverslip. Stomata lengths were measured under 400X light microscope equipped with and ocular micrometer, 10 measurements/leaf (5 from left and 5 from right sides of the mid vein). The value obtained were computed into micrometer (μm) using stage micrometer. Averaged stomatal length and standard deviation were determined.

Stomata density

To obtain stomata density, countings were undertaken under 40X light microscope. Surface cells of 5 leaves/plant, treated and the control were examined, 10 measurements/plant. The countings were undertaken from both left and right sides of each leaf.

Measurement of pollen grain diameter

This was conducted with plants having large stomata. The new blooms were brought in the lab and pollen grains were then shaken onto slides. Acetocarmine drops were applied on pollen grains after which were covered with cover slips allowing observation under light microscope and measurement with an ocular micrometer. Ten pollens per bloom, 3 blooms per plant were measured.

Determination of % pollen sterility

This was also executed with plants having large stomata. Three blooms per plant were used. Fertile and sterile pollen grains per each bloom were counted. The numbers were used in % pollen sterility determination.

RESULTS AND DISCUSSION

In the experiment on % germination of cotton seeds soaked in colchicine solution at different levels of concentration, it was found that % seed germination decreased with the increasing doses of colchicine. Table 1 shows the greater % germination reduction when the soaking duration was higher. The results agreed with Raphiphan (2000) who reported the duration of soaking seeds and colchicine concentrations had significant effects on the numbers of day required for total germination of *Ipomoea quamolic* Linn. The highest colchicine concentration showed the least germination percentage. In addition, among the surviving seedlings, some were noticed to gradually die especially seedlings in the treatments employing high colchicine doses along with long soaking period.

The relationship between colchicine concentrations and % survival of 90 days old seedlings in various durations treatment found $LC_{50(90)}$ at 24 and 36 hours after soaking to be 0.35 and 0.22% respectively (Figure 1). The results were similar to those reported by Parakarn *et al* (2002) which indicated the rate of survival to be inversely related with colchicine concentration and soaking duration. At 0.5% concentration, % survival of cotton plant were 27 and 3 at 24 and 36 hours after soaking respectively. In most cases, the mortality appeared to be due to poor seedling vigor resulting in an ability to overcome the toxic effect of colchicine (Jensen; 1974). Addink (2002) stated that too high concentration of colchicine could inhibit the development of living part resulting in mortality of organism. Table 2 and 4 present the

Table 1 Averaged percent germination and plant survival (90 days after germination) of *G. arboreum*, PM2, after colchicine treatments at different concentrations and durations of seed soaking.

Duration of seed soaking (h)	Concentration of colchicine (% wt/v)	Avg. germination (% of control)	Plant survival (% of control)
24	0	100	100
	0.1	92	87
	0.5	77	27
36	0	100	100
	0.1	91	57
	0.5	58	3

average stomatal sizes of PM2 seed soaked in colchicine solution at different concentrations and times. The average stomatal sizes of the control group were found to be 23.97±0.90 and 23.77±0.70 μm 24 and 36 hours after soaking in water, respectively. Treatments soaked in colchicine solution were separated into 2 groups, one with stomatal size less than or equal to 27 μm and the other with those greater than 27 μm comparing to the greatest stomatal size of the control. When statistically analyzed, it was found that the average of large stomatal size was significantly different from that of the control. Increasing concentration of colchicine, the treatment was also noticed to increase the numbers of plant with large stomatal size compared with that of equal concentration. Thirty-six hours of soaking, the treatments gave more percentages of plant with large stomatal sizes which indicated that both concentrations and soaking durations tended to increase efficacy in chromosomal increase of cotton.

As for stomatal frequency, Table 3 shows that the greater the stomatal size, the lesser, the stomatal frequency.

Cotton with large stomatal size and reduced stomatal frequency had potential to be polyploidy or had increased chromosome numbers. Evan (1955) and Speckman *et al* (1965) also reported that length of stomata was the accurate indicator of polyploidy levels in many plant species. As a result, the stomatal size and stomatal frequency could be used as the indicators of polyploidy level for cotton. However, the counting of chromosome number of cotton with large stomatal size which will be further conducted, will accurately indicate whether such speculation is accordingly.

The optimum concentration of colchicine for polyploidy induction varied widely among different species, methods of application, plant parts, etc. The experiment found that 24 hours of seed soaking in 0.1 and 0.5% colchicine solution yielded plants with 55.56 and 78.57% large stomata (Table 2) and 87 and 27% seedling survival of the control, respectively (Table 1). While seed soaking for 36 hours gave quite increasing stomotal size, yet, % survival was very low. The optimum concentration of colchicine for *G. arboreum* should, therefore, be 0.35% which was $LC_{50(90)}$ value of 24 soaking hours (Figure 1).

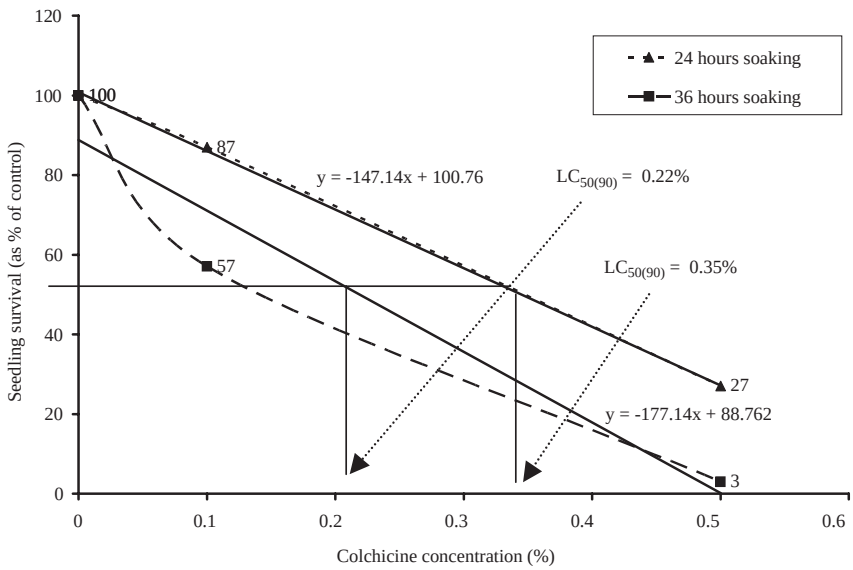

Figure1 Relationship between colchicine concentration and survival of 90 days old seedling (as % of control) at 24 and 36 hours soaking durations.

Table 2 Ranges, means and standard deviations of stomatal size of cotton variety, PM2 (*G. arboreum*) seeds soaked in colchicine solution of different concentrations and soaking durations.

Duration of seed soaking (h)	Concentration of colchicine (%)	Total no. of plants	No. of plants with large stomatal size	Plants with large stomatal size (%)	Stomatal size (μm)1/			
					Normal		Large	
					Range	Mean ± S.D.	Range	Mean ± S.D.
24	0	67	0	0.00	22.10 – 26.90	23.97±0.90	–	–
	0.1	54	30	55.56	22.30 – 25.70	23.93±0.98	27.25 – 35.10	31.47±1.99
	0.5	14	11	78.57	24.95 – 25.20	25.07±0.13	32.20 – 34.15	33.30±0.56
36	0	64	0	0.00	22.00 – 25.95	23.77±0.70	–	–
	0.1	33	22	66.67	23.35 – 26.10	24.32±0.75	32.60 – 38.75	33.68±1.28
	0.5	1	11	100	–	–	35.70	35.70±1.21

1/ ≤ 27.00 mm classifed as normal; > 27.00 mm classified as large

Table 3 Ranges, means and standard deviations of stomatal frequency of cotton variety, PM2 (*G. arboreum*) seeds soaked in colchicine solution of different concentrations and soaking durations.

Duration of seed soaking (h)	Concentration of colchicine (%)	Total no. of plants	No. of plants with large stomatal size	Plants with large stomatal size (%)	No. of stomata per 1 mm			
					Normal Stomatal size		Large Stomatal size	
					Range	Mean ± S.D.	Range	Mean ± S.D.
24	0	67	0	0.00	163.49 – 274.94	236.48±16.52	–	–
	0.1	54	30	55.56	133.13 – 277.83	222.52±37.55	91.57 – 155.42	119.51±23.79
	0.5	14	11	78.57	200.00 – 224.82	213.61±12.58	90.60 – 133.25	110.97±11.93
36	0	64	0	0.00	210.60 – 289.15	241.83±17.57	–	–
	0.1	33	22	66.67	209.88 – 246.51	230.29±10.31	56.51 – 132.65	109.51±17.64
	0.5	1	1	100	–	–	96.27	96.27±5.42

Table 4 Means of normal and large stomatal size and stomatal frequency of cotton plants grown from seed soaking in different colchicine solutions and durations.

Duration of seed soaking (h)	Concentration of colchicine (%)	Stomatal size[1/]	Mean stomatal size (μm)[2/]	Mean stomatal no. per 1 mm^2 [2/]
	0	Normal	23.97e	236.48a
24	0.1	Normal	23.93de	222.52a
		Large	31.47c	119.51b
	0.5	Normal	25.07d	213.61a
		Large	33.30b	110.97b
	0	Normal	23.77e	241.83a
36	0.1	Normal	24.32de	230.29a
		Large	33.68b	109.51b
	0.5	Large	35.70a	96.27b

[1/] ≤ 27.00 mm classified as normal; > 27.00 mm classified as large

[2/] means within each column followed by a common letter are not significantly different as determined by DMRT (p = 0.05)

Compared with the plant with normal stomatal size, the one with large stomata would greatly vary in growth. The high concentration of colchicine solution plus long soaking duration were noticed to cause the treated seeds to give low-height plants. Aside from variation in height, the plants with large stomata also bloomed later than the control, and produced some morphology different from the control such as, thick and course leaves, a lot of branching.

Such characteristics were in accordance with Balkanjieva (2003) who reported that colchicine treatments usually produce negative side effects such as irregularities in mitotic division, growth retardation and chromosomal deficiencies. These effects might lead to the inhibition of plant growth and development particularly in the first days after planting.

Chlorophyll mutation was also noticed in this study which was due to the fact that colchicine induced mutation other than doubling of chromosomes. Jensen (1974) mentioned that in addition to the negative side effects of colchicine, like mitotic irregularities, growth retardation, etc., other mutagenic effects including quantitative changes have been reported for various crops in literature.

When cotton bloomed, pollen grain diameter of every plant with large stomata were measured and percent pollen sterilities of these plants compared to those of the control were checked (Table 5). With increasing concentration of colchicine solution and soaking duration, % sterility of pollen grain was found to increase. Mean differences of pollen grain diameter among the treatments were small. There was an overlap in the ranges of pollen diameter among the treatments as well. Thus, pollen grain diameter is not recommended to be used as an indirect method for identification of ploidy level of cotton. The result did not agree with Evans (1955) and Tan and Dunn (1973) who stated that pollen diameters of red clover, white clover, lucerne and *Bromus inermis* are positively correlated with ploidy level.

CONCLUSION

According to the experiment, the following conclusions were made :

1. With increasing concentration of colchicine solution and soaking duration, % germination and survival of seedling decreased

Table 5 Ranges, means and standard deviations of fertiled and steriled pollen grain (%) and pollen grain diameter of large stomatal size and low stomatal frequency of colchicine treated cotton plants.

Duration of seed soaking (h)	Concentration of colchicine (%)	Fertiled pollen (%)		Steriled pollen (%)		Pollen grain diameter (μm)	
		Range	Mean	Range	Mean ± S.D.	Range	Mean ± S.D.
24	0	88.27 – 95.32	92.18±3.59[1/]	4.68 – 11.73	7.82±3.59	11.40 – 11.90	11.68±0.26
	0.1	59.40 – 97.69	78.76±12.33	2.31 – 37.21	21.24±12.33	11.10 – 12.10	11.70±0.28
	0.5	8.45 – 94.70	65.60±20.38	5.3 – 91.55	34.40±20.38	11.50 – 14.55	11.93±0.61
36	0	88.27 – 95.32	92.18±3.59	4.68 – 11.73	7.82±3.59	11.40 – 11.90	11.68±0.26
	0.1	38.86 – 95.48	72.99±17.99	4.52 – 61.14	27.01±17.99	9.05 – 14.20	11.65±1.15
	0.5	52.80 – 54.07	53.44±0.89	45.93 – 47.20	46.56±0.89	13.50 – 14.20	13.85±0.50

while % plants with large stomata increased.

2. Stomatal size was negatively correlated with stomatal frequency.

3. Percent sterility of pollen grain increased with the increasing concentration of colchicine solution and soaking duration whereas pollen grain diameter had no correlation with either concentration or duration.

4. Stomatal size and stomatal frequency can be used as indirect methods for identification of ploidy level of cotton whereas pollen grain diameter is not recommended.

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