Tetraploids Induction by Colchicine Treatment in *Hibiscus mutabilis*©

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Tetraploid plants of *Hibiscus mutabilis* L. with pink flower were obtained by colchicine treatment. Self-pollinated seeds were dipped into sulfuric acid for 360 minutes to break the impermeable seed coats. Seedlings with a root of 1–2 mm were treated by dipping into 0, 0.1, 0.3, or 1.0 mM colchicine solution for 12 or 24 h, respectively. Survival rate decreased according to colchicine concentration, and tetraploid induction increased according to colchicine concentration significantly. Many tetraploid plants were acquired with 0.3 mM colchicine regardless of treatment time. The width/length ratio in petal and sepal, the thickness of petal, the length of guard cell, and the pollen size in tetraploids were significantly larger than those of diploids. We decided that this tetraploid *H. mutabilis* is suitable as a garden plant.

INTRODUCTION

Cotton rose (*Hibiscus mutabilis* L.), which has white or pink flower, is a native woody plants in China and it has been planted widely in gardens as a summer flowering plant. However, the breeding of new cotton-rose selections is not progressing and there are few cultivars besides *H. mutabilis* var. *versicolor*. Although interspecific hybridization with other *Hibiscus* species has been tried, the breeding was not advanced because many are sterile. Tetraploid plants are able to produce fertile interspecific hybrids and tetraploid plants often show enlargement of various plant parts such as flowers and leaves. In this study, we tried tetraploid induction by colchicine treatment in cotton rose in order to breed new cotton-rose cultivars with high ornamental value.

MATERIALS AND METHODS

Plant Materials. Self-pollinated seeds of cotton rose with pink flower, which are planted in Gifu University, were collected in November 2009. Sixty seeds per treatment were dipped into sulfuric acid for 0, 5, 15, 30, 60, 120, 180, 240, 300, 360, 480, 600 min to scarify the impermeable seed coat. After sulfuric acid treatment the seeds were rinsed in running water for 1 h and placed on the moist filter paper in covered plastic Petri dishes. Two weeks later germinated seeds were counted.

Cholchicine Treatment. Germinated seeds with root length of 1–2 mm were dipped into colchicine solution (0, 0.1, 0.3, or 1.0 mM) containing 10% dimethyl sulfoxide (DMSO) for 12 or 24 h, respectively. Each treatment comprised 200 seedlings. After treatment the seedlings were rinsed in running water for 1 h, planted in plug trays which have 200 holes filled with peat moss and perlite (2 : 1, v/v), and grown in the greenhouse. Six weeks later the number of sprouting shoots was counted.

Tetraploid Analysis by Flow Cytometry. Ploidy analysis was carried out on the plantlets after five leaflets by flow cytometry **(**FCM) (Partec Ploidy Analyser, PAII). Nuclei were collected by chopping up the leaf segment of 0.5 cm around using sharp razor blade in 0.2 ml of Partec CyStain UV solution A. Subsequently nuclei were stained with 0.8 ml of Partec CyStain UV solution B containing 4'-6-diamidino-2-phenylindole (DAPI) and were filtered through a 30 μm mesh. *Hibiscus mutabilis* leaves from controls were used as the diploid standard.

Morphological Analysis. Fully expanded leaves and flowers of plants which were determined as diploid or tetraploid by FCM were used for morphological analysis. The leaf width/length ratio, the length of guard cells, the petal width/length, the sepal length/width, the petal weight/area and the pollen length were measured. The length of guard cells was measured by SUMP method (Suzuki's universal microprinting method) (Kawai, 1969).

RESULTS AND DISCUSSION

Germination Rate by Sulfuric Acid Treatment. In the relation between germination rate and sulfuric acid treatment (Fig. 1), no seeds germinated at 0 and 5-min sulfuric acid treatment. For sulfuric acid treatment times greater than 15 min, the germination percentage increased up to 360 min when a rate of 69.5% was reached. In treatment times longer than 360 min the germination percentage fell with increasing treatment time.

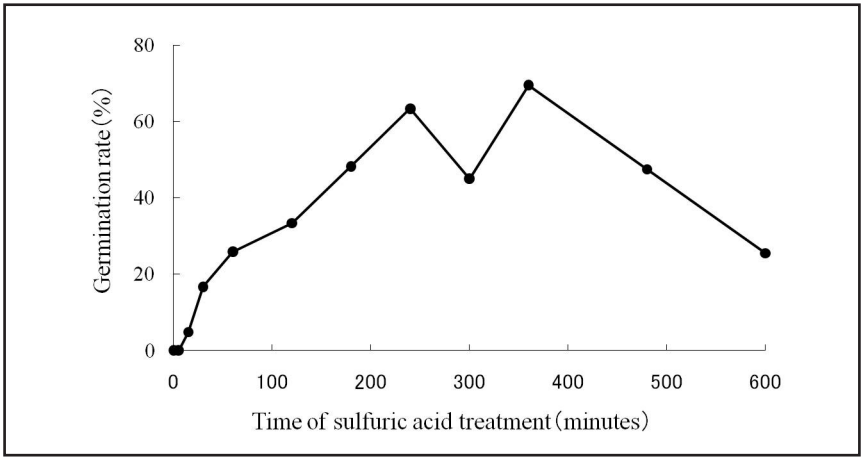

Figure 1. Relation between germination rate and time of sulfuric acid treatment.

Hibiscus genera have a hard seed coat and germination is inhibited by impermeability. Sakhanokho (2009) indicated that *H. dasycalyx* and *H. acetosella* seed germination was enhanced by sulfuric acid treatment for 10 to 20 min. In this study, however, only 16.7% of *H. mutabilis* seeds germinated by a 30 min sulfuric acid treatment, and the optimal time of breaking the impermeable seed coats was the 360 min treatment. Therefore, we decided that the seed coat of *H. mutabilis* was

Table 1. Survivals and polyploids induction by colchicine treatment.

Treatment (h)	Concentration of colchicines (mM)	Sprouting (%)	Number for FCM analysis	2x (%)	4x (%)		Others (%)	Remarks*
12h	0	142 / 200 (71.0[x]) d[z]	129	129 (100.0) d[z]	0 (0.0[y]) a	(0.0[y]) a[z]	0 (0.0[y]) a[z]	
	0.1	118 / 200 (59.0) c	118	102 (86.4) c	7 (5.9) b	(3.5) b	9 (7.6) b	(2x+4x : 9)
	0.3	79 / 200 (39.5) b	91	24 (26.4) b	56 (61.5) c	(28.0) d	11 (12.1) b	(2x+4x : 11)
	1.0	46 / 200 (23.0) a	44	3 (6.8) a	33 (75.0) c	(16.5) c	8 (18.2) b	(2x+4x : 3, 4x+8x : 2, 8x : 3)
24h	0	125 / 200 (62.5) BC	125	125 (100.0) C	0 (0.0) A	(0.0) A	0 (0.0) A	
	0.1	129 / 200 (64.5) C	111	82 (73.9) B	25 (22.5) B	(12.5) B	4 (3.6) A	(2x+4x : 4)
	0.3	104 / 200 (52.0) B	106	12 (11.3) A	81 (76.4) C	(40.5) C	13 (12.3) B	(2x+4x : 5, 4x+8x : 4, 8x : 4)
	1.0	23 / 200 (11.5) A	38	5 (13.2) A	25 (65.8) C	(12.5) B	8 (21.1) B	(4x+8x : 3, 8x : 5)
12h	-	385 / 800 (48.1)	382	258 (67.5)	96 (25.1)	(12.0)	28 (7.3)	
24h	-	381 / 800 (47.6)	380	224 (58.9)	131 (34.5)	(16.4)	25 (6.6)	
-	0	267 / 400 (66.8)	254	254 (100.0)	0 (0)	(0.0)	0 (0)	
-	0.1	247 / 400 (61.8)	229	184 (80.3)	32 (14)	(8.0)	13 (5.7)	
-	0.3	183 / 400 (45.8)	197	36 (18.3)	137 (69.5)	(34.3)	24 (12.2)	
-	1.0	69 / 400 (17.3)	82	8 (9.8)	58 (70.7)	(14.5)	16 (19.5)	
Total number		766 / 1600 (47.9)	762	482 (63.3)	227 (29.8)	(14.2)	53 (7.0)	

[z]Different letters indicate a significant difference according to Tukey's multiple range test ($P<0.05$).

[y]Number of plants/number of sprouting.

[x]Number of plants/number of treated plants.

*Remarks indicate the kind of ploidy and that number.

thicker than those of *H. dasycalyx* and *H. acetosella*. The growth of germinated seedlings was not increased by sulfuric acid treatments over 360 min. This may indicate that sulfuric acid treatments over 360 min injured the embryo and germination was inhibited.

Effect of Colchicine Treatment. Germination rate was high for control (12 h: 71%, 24h: 62.5%) and decreased with increasing concentration of colchicine (Table 1). The treatment of 1 mM colchicine inhibited sprouting of seedlings significantly. The seedlings which did not elongate had normal opened cotyledons but no elongated root. The high concentration of colchicine strongly inhibited cell division of the shoot and root apical meristems. It therefore appears that the seeds treated with a high concentration of colchicine could not elongate root and differentiate leaves after treatment.

Tetraploid Analysis by Flow Cytometry. Flow cytometry analysis showed that a lot of polyploid plants were produced (Table 1 and Fig. 2). All plants in 0 mM colchicine treatment were diploid and the percentage of diploids decreased with increasing colchicine concentration. The tetraploid percentages within seedlings in 0.3 and 1.0 mM colchicine treatments were over 60% regardless of treatment time, but in 0.1 mM colchicine treatment were significantly lower (12 h: 5.9%, 24 h: 22.5%). The tetraploid percentage within treated plants in 1.0 mM colchicine treatment were lower than that in 0.3 mM, and the octoploid plants and chimeras with 8x were observed in 1.0 mM. From these results, we decided that 0.3 mM colchicine was the optimum concentration for tetraploid induction regardless of treatment time.

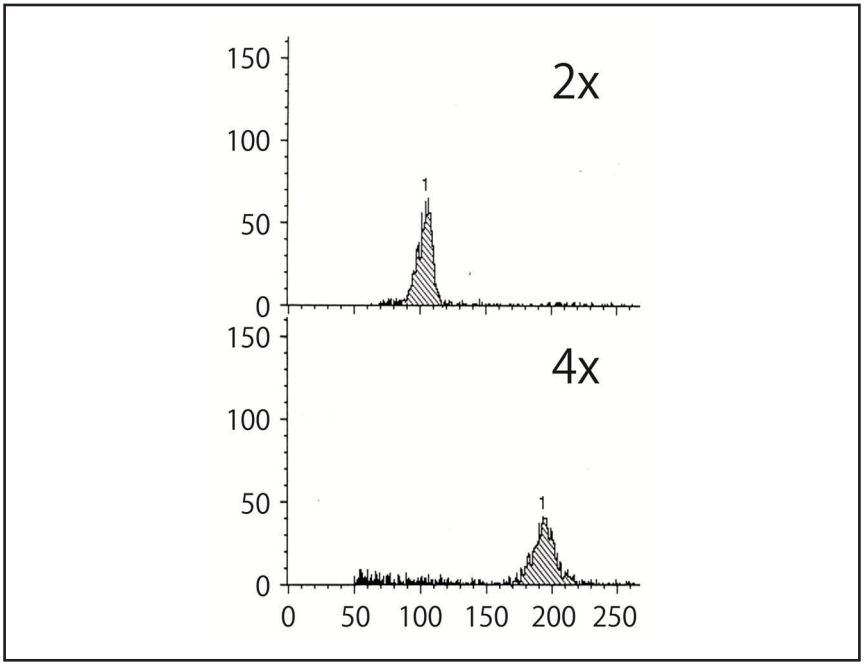

Figure 2. Flow cytometric histograms of 2x and 4x plants.

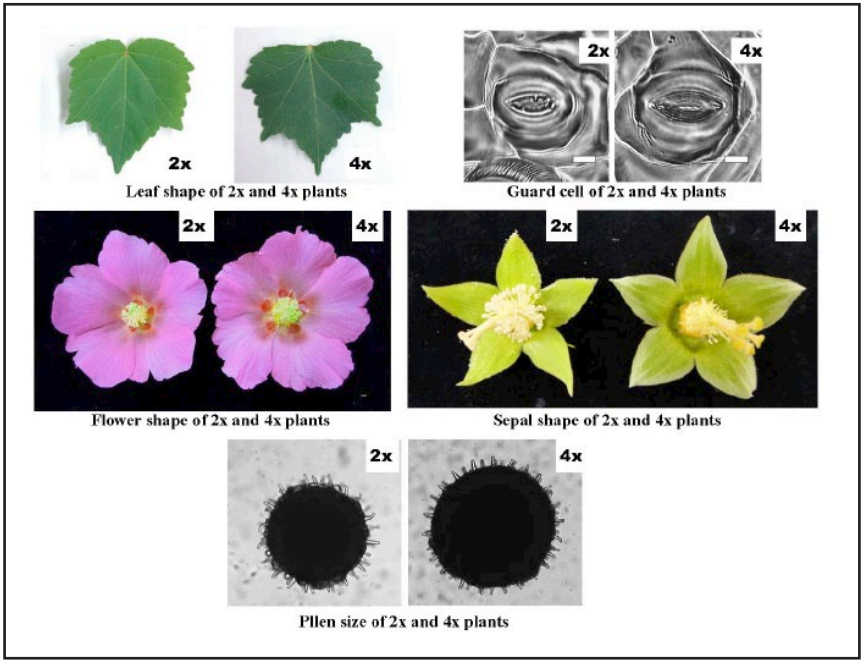

Figure 3. Shape of leaf and flower in 2x and 4x plants.

Table 2. Comparison of diploid and tetraploid plants.

	2x		4x		Test of significance
	Average	Standard deviation	Average	Standard deviation	
Length of guard cell (μm)	4.58	±0.58	5.49	±0.66	**
Length/width ratio in petal	0.90	±0.08	0.98	±0.10	*
Petal weight (g/cm^2)	16.14	±0.75	30.19	±1.55	**
Length/width ratio in sepal	0.69	±0.06	0.80	±0.09	**
Pollen size (μm)	140.23	±12.29	173.61	±7.48	**

Significant differences (** : p = 0.01, * : p = 0.05, NS : No significant)

Although the gains of tetraploid in *Rosa* species were 0.2% to 0.3% in our past reports (Fukui and Yokota, 2007; Sugimoto et al., 2010), the tetraploid percentage within treated plants at dipping treatment of colchicine in 1.0 mM for 24 h was high with 40.5% in this result. Therefore, we considered that the seed of *H. mutabilis* had higher activity of cell division in shoot apical meristem than those of *Rosa* species.

Morphological Analysis. The results of morphological analysis are shown in Figure 3 and Table 2. The tetraploid leaf looked thicker and darker than the diploid leaf, but there were no differences in size and shape. The lengths of guard cells of tetraploids were significantly larger than those of diploids (Fig. 3 and Table 2). Plant height for tetraploids was about 1m and was shorter than diploids.

Tetraploids bloomed in September 2010. The petal of tetraploids was wider in comparison with diploids, and the flower shape was roundish because parts of the petals overlapped (Fig. 3 and Table 2). The petals of tetraploids were thicker than that of diploids. The sepals of tetraploids were wider in comparison to diploids, and pollen grains were larger than that of diploid pollen.

From these results, the characteristics of *Hibiscus* tetraploid plants were a more compact shape, deeper green leaves, and overlapping and thicker petals. We, therefore, decided that this tetraploid *H. mutabilis* is suitable as a garden plant. Additionally the tetraploid *H. mutabilis* has ability to make triploid plants by cross-pollination with diploid plants and interspecific hybrids by cross-pollination with other *Hibiscus* species.

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