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IN VITRO INDUCTION OF POLYPLOIDY IN WHITE MULBERRY (*MORUS ALBA* VAR. S54) BY COLCHICINE TREATMENT

SARINEE CHAICHAROEN, ARIYA SATRABHANDHU AND MALEEYA KRUATRACHUE
Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.

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ABSTRACT

Young leaf explants of Morus alba var. S54 *were cultured on the modified Murashige and Skoog (MS) medium supplemented with 0.5 mg/l BA (N^6 - Benzyladenine) and 1.0 mg/l NAA (1-Naphthalene acetic acid) for callus initiation ; and 1.0 mg/l BA and 0.5 mg/l NAA for callus proliferation. Active callus tissues were soaked in sterilized aqueous colchicine solution at various concentrations (0.025 to 0.2%) for 3 to 7 days. Then the callus was placed on the medium containing 1.0 mg/l BA and 0.025 mg/l NAA for a shoot regeneration. For shoot growth, the regenerated shoots were subcultured on the medium containing 0.25 mg/l BA and 0.025 mg/l NAA. A root formation occurred when culturing an individual shoot on the half-strength modified MS medium containing 0.1 mg/l IBA (Indole-3-butyric acid). Determination of chromosome number was carried out by chromosome counts of squashed root-tip cells. The highest number of tetraploid plants could be obtained from 0.1% colchicine solution with 3 days soak period treatment (47.22%), whereas, other treatments produced lower number of tetraploid plants with the high percentage of mixoploid. The complete plants were successfully grown in pots containing soil. By comparison, the tetraploid plants exhibited slightly slower growth than diploid plants. Other characteristics of the plants were also observed to be different; the tetraploid plants have bigger stem diameter; greener and thicker leaves than those of diploid plants.*

INTRODUCTION

Many investigators had already reported on the superiority of polyploid mulberry over diploids[1,2]. Leaves obtained from polyploid plants had better quality for silkworm feeding than most of the diploid varieties[1]. It was also found that silkworms fed with leaves from these polyploid plants resulted in high egg production, heavy larval weight, good cocoon harvest and longer cocoon fiber for the raw silk production[2]. In addition, the polyploid *M. alba* cv. Kanva-2 exhibited an increase in plant height, size and weight. Their leaves showed an increase in water content and number of stomatal chloroplasts. Moreover, these plants had shorter internodes and petioles compared to those of the diploids.

Most mulberry breeders have succeeded in inducing polyploids through colchicine and promising hybrids have been obtained from the induced polyploids and diploids[3,4]. Autotetraploid mulberry of various species could be produced by the application of 0.4-0.5% colchicine or colchicine and X-ray irradiation to the vegetative buds or seedlings[5-9].

M. alba var. S54 is one of the mutants induced by a chemical mutagen and has become popularly cultivated for silkworm feeding in the Northeast of Thailand. The purpose of present study is to find the optimum ratio of auxin and cytokinin to promote a callus formation in young leaf explants of *M. alba* var. S54 and to induce polyploidy by colchicine solution of various concentrations.

MATERIALS AND METHODS

1. Callus induction

The expanding or recently expanded leaves, about 1.0 cm long, were excised from shoot cultures. Our explants were cultured in an upright position with only half of their lower portions dipped in the modified Murashige and Skoog (MS) medium[10] containing a vitamin mixture as proposed by Gamborg[11], 20 g/l saccharose, 8 g/l agar, 10% coconut water, and combination of 0, 0.5, 1, 2 mg/l BA and 0, 0.5, 1,2 mg/l NAA. About 10 to 15 of these explants were used per treatment and the experiments were done in 3 replicates.

The culture environment for all growth *in vitro* was maintained in a room at 25 ± 2° C, illuminated by cool-white fluorescent lamps at intensity between 3000-4000 lux with a 16-hour light period.

2. Induction of polyploidy

The explants for polyploid induction experiment were of those leaf derived callus. We needed a large number of these callus tissues, so callus proliferation was really essential.

2.1 Callus proliferation experiment

Four-week-old callus was first transferred from the induction medium (containing 0.5 mg/l BA and 1.0 mg/l NAA with 10% coconut water) to the modified MS medium containing different combinations of BA (0, 0.25, 0.5, 1.0 mg/l) and NAA (0, 0.25, 0.5, 1.0 mg/l) and subcultured on the same medium every 1,2,3 or 4 week. The callus culture was maintained on the modified MS medium containing various combinations of BA and NAA as listed above for 2, 4, 6, 8, 10 and 12 weeks (subculture every 2 weeks). Then, the callus was transferred to the shoot regeneration medium containing 1.0 mg/l BA and 0.025 mg/l NAA. The optimum callus age for shoot regeneration can therefore be evaluated.

2.2 Colchicine treatment

An active callus derived from the subcultured callus (6-8 weeks old) on the proliferation medium for 7 days was used for colchicine treatment. To test the effect of various concentrations of colchicine and soak period on polyploid induction in mulberry, colchicine at the concentrations of 0.025, 0.05, 0.1 and 0.2% was applied in combination with 3, 5, 7 and 10 days soak period.

Eighty pieces of callus were used for each treatment. The callus was transferred to a 125-ml Erlenmeyer flask containing 40 ml of filtered sterilized aqueous colchicine solution and incubated at 25° C on a rotary shaker at 80 rpm for different length of soak periods as listed above.

After soaking the callus in colchicine solution, they were rinsed with autoclaved distilled water twice and cultured on the proliferation medium for 2 weeks. The survival rate of the treated callus was recorded.

2.3 Plantlet regeneration from colchicine treated callus

To induce a shoot formation, the callus derived from each colchicine treatment were cultured on the modified MS medium supplemented with 1.0 mg/l BA and 0.025 mg/l NAA. Small shoots regenerated from each colchicine - treated callus were first counted before separating callus mass individually. Then, they were transferred to the modified MS medium containing the combination of 0.25 mg/l BA and 0 or 0.025 mg/l NAA. The root formation was obtained by transferring shoots of 2-3 cm long to half-strength modified MS medium supplemented with 0.1 mg/l IBA.

3. Cytological observation

A ploidy identification was made by chromosome count of root tips. These root tips of 1.0-1.5 cm long were collected from rooted plantlets and were pretreated in saturated aqueous 1-bromonaphthalene solution for 6-8 hours, fixed in 90% acetic acid for 20 minutes, rinsed with 95% ethanol and stored in 70% ethanol in a refrigerator. A hydrolytic maceration was carried out by using 1N HCl at 60° C for 10 minutes. The root tips were macerated, rinsed with distilled water and stained with Schiff's reagent for 1-2 hours at room temperature. Then, they were squashed in 2% aceto-orcein. Metaphase chromosomes were observed and counted under a light microscope.

4. Stomata observation

The observation of stomata was carried out on epidermal cells taken from the lower epidermis of two-month-old plants. These samples were placed in a drop of diluted Tween 20 (0.2%) on a glass microscope slide. The size and density of stomatal guard cells were observed.

5. Comparison of morphology between diploid and tetraploid mulberry (*M. alba* var. S54)

After the chromosome determination, these mulberry plants were washed with tap water to remove all adhering medium and grown in pots with autoclaved vermiculite for 2 months. Following morphological data were recorded from 20 diploid and 20 tetraploid mulberry plants : plant height, stem diameter, leaf number, width and lenght of leaf and leaf thickness. The method of t-test was used to evaluate the significant differences in morphological characters at the level of 0.01 probability between the diploid and tetraploid plants.

RESULTS

1. Callus proliferation experiment

Our results indicated that callus should be transferred to a fresh medium every 7 to 10 days or whenever browning substances appeared. In addition, we found that an induction medium was appropriate for the callus induction but not suitable for the callus multiplication. The effective medium for the callus proliferation was that of the modified MS medium containing 1.0 mg/l BA and 0.5 mg/l NAA. Moreover, the long term culture of mulberry callus should be avoided because the production of browning substances from callus inhibited its growth and decreased shoot regeneration. It was found that the callus culture should be maintained on the proliferation medium no longer than 10 weeks.

2. Effects of colchicine concentration and soak period on callus

Table 1 shows the effects of colchicine concentration and soak periods on callus survival and percentage of survival callus that regenerated shoots. The untreated callus showed 95% of survival and 55.20 % of survived callus regenerated shoots. In the callus treated with colchicine, the highest percentage of callus survival (76.25%) (Fig.1A) and the highest percentage of callus survived that regenerated shoots (44.2%) (Fig.1B) were obtained from the callus soaked in 0.025% colchicine solution for 3 days. Shoots regenerated from untreated and colchicine-treated callus were rooted within 3 weeks on the half-strength modified MS medium containing 0.1 mg/l IBA (Fig.2).

TABLE 1. The effects of colchicine concentration and soak period on callus survival and percentage of survived callus that regenerated shoots.

Colchicine treatment		number of callus	Survival of callus (%)	Survived callus that regenerated shoot (%)
Concentration (%)	Soak period (days)			
Control (untreated callus)		200	95.00	55.20
0.025	3	200	76.25	44.20
0.025	5	200	68.75	32.70
0.025	7	200	62.50	32.00
0.05	3	200	67.50	42.60
0.05	5	200	60.00	35.40
0.05	7	200	52.50	32.50
0.1	3	200	61.25	26.50
0.1	5	200	50.00	22.50
0.1	7	200	8.75	0
0.2	3	200	0.00	0

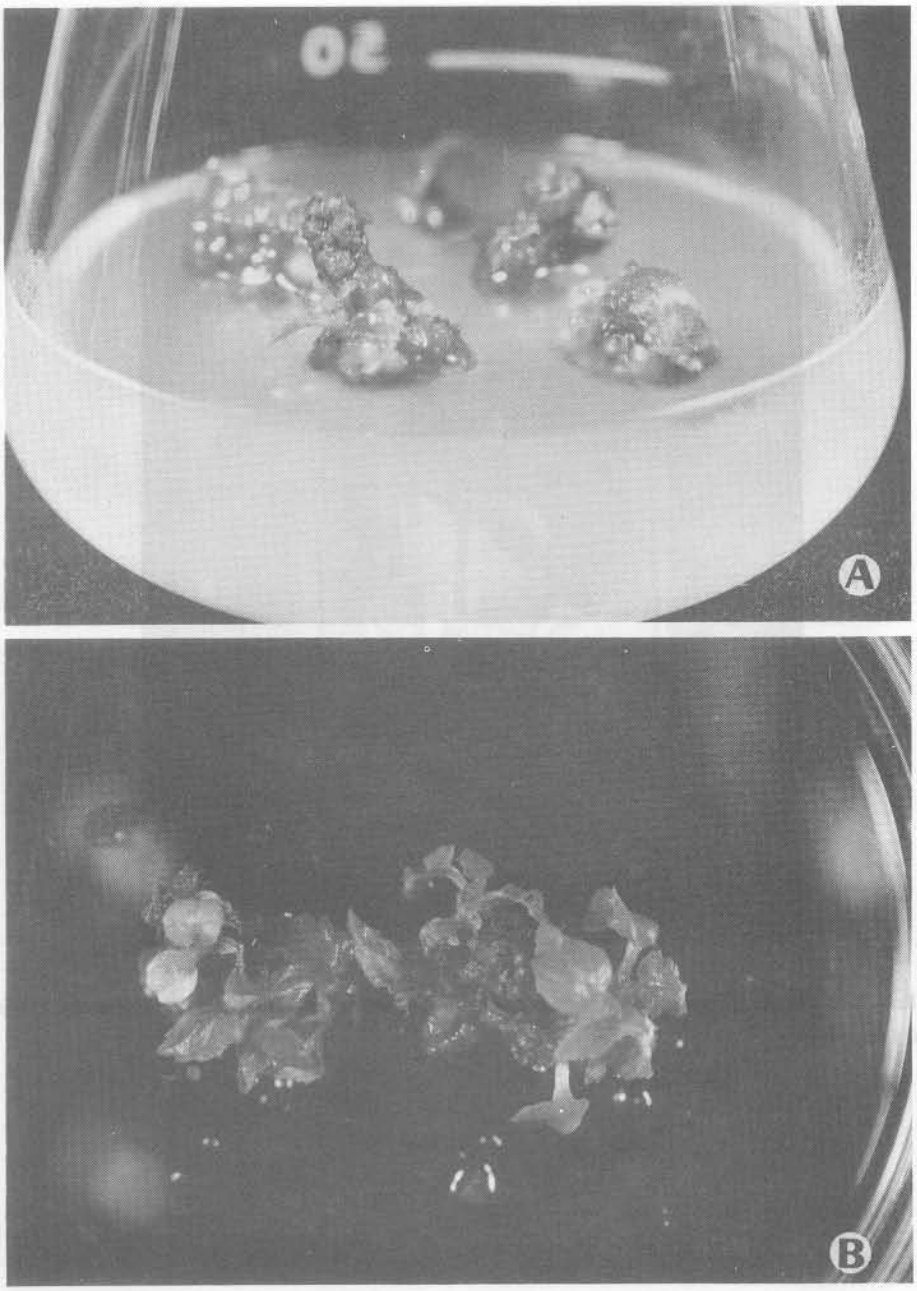

Fig. 1. A. Colchicine-treated callus of *M. alba* var. S54 cultured on the modified MS medium supplemented with 1.0 mg/l BA and 0.5 mg/l NAA after culture for 4 weeks.

B. Shoot regeneration derived from colchicine-treated callus cultured on the modified MS medium containing 10 mg/l BA and 0.025 mg/l NAA after culture for 4 weeks.

Fig. 2. Root formation of shoots regenerated from colchicine-treated callus on half-strength MS medium containing 0.1 mg/l IBA.

3. Determination of plants derived from untreated callus and colchicine-treated callus

Table 2 shows ploidy identification which was made by counting chromosome number of root tip cells. Among regenerated plants from untreated callus, 93.33% were diploids (2n=28,X=14) (Fig.3A), the other 1.67% were composed of tetraploids (2n=56,X=14) (Fig.3B) and 5% mixoploids. The use of colchicine treatment on callus increased the percentage of heteroploid production. Our results showed that with the increase of colchicine concentration and/or soak period, the percentage of tetraploid production was multiplied (Fig.4). The highest percentage (47.22%) of tetraploid production was obtained from 0.1% colchicine solution with 3 days soak period treatment (Fig.4). In this treatment, 11.11% became mixoploids while 41.67% remained diploids. Almost all of these mixoploids were the mixture of diploid and tetraploid cells.

Mixoploids that contained triploid (2n=42,X=14) (Fig.3C) and tetraploid cells were obtained from plants treated with 0.025% colchicine solution with 7 days soak period and those treated with 0.05% colchicine solution with 5 days soak period. Mixoploids containing diploid and near-tetraploid cells (2n=54,X=14) (Fig.3D) were obtained from plants treated with 0.05% colchicine solution with 7 days soak period. Moreover, several of the mixoploid plants had the mixture of diploid, triploid and tetraploid cells.

TABLE 2. Cytological observation of mulberry plant (*M. alba* var. S54) regenerated from untreated callus and colchicine-treated callus.

Colchicine treatment		number of	Ploidy identification (%)		
Concentration (%)	Soak period (days)	plant	Diploid	Tetraploid	Mixoploid
Untreated callus		50	93.33	1.67	5.00
0.025	3	50	88.89	4.94	6.77
0.025	5	50	79.60	6.12	14.28
0.025	7	50	70.00	3.33	26.67
0.05	3	50	78.87	18.31	2.82
0.05	5	45	58.14	13.95	27.91
0.05	7	40	56.41	10.26	33.33
0.1	3	40	41.67	47.22	11.11
0.1	5	38	39.74	8.69	52.17

4. Comparison of morphology between diploid and tetraploid mulberry (*M. alba* var. S54)

About 82% of regenerated plants survived from the acclimation process and were grown in pots containing soil. Comparison of morphology between diploid and tetraploid mulberry at the same age revealed that tetraploid plants exhibited slightly slower growth than diploid plants. The tetraploid plants have bigger stem diameter; greener, larger and thicker leaves than diploid plants (Table 3). The differences of the stem diameter, the width and length of leaf and the leaf thickness in diploid and tetraploid mulberry were found to be statistically significant ($p<0.01$).

It was also found that chromosome doubling was accompanied by enlargement of guard cell pairs. The averages of the width and length of guard cell pairs of tetraploids are statistically larger than those of diploids ($p<0.01$). Similar results were obtained when the density/mm^2 of guard cell pairs of diploid and tetraploid leaves was compared (Table 3). The tetraploids have larger stomata but lower density than diploids. The diploid and tetraploid mulberry plants, which are successfully grown in soil are shown in Fig.5.

TABLE 3. Morphological characteristics, the size and density of stomata of diploid and tetraploid mulberry (*M. alba* var. S54).

Characteristics	Size (mm)		Significance of difference
	Diploid	Tetraploid	
Plant height	195	170	ns
Stem diameter	2.08	4.49	s
Leaf number	12	10	ns
Width & length of leaf	38.5, 49.1	48.8, 59.2	s
Thickness of leaf	1.90	2.45	s
Width & length of stomata	0.16, 0.23	0.22, 0.34	s
Density of stomata (number/mm.^{2})	230	129	s

s = significant; ns = not significant

Significant difference at $p < 0.01$

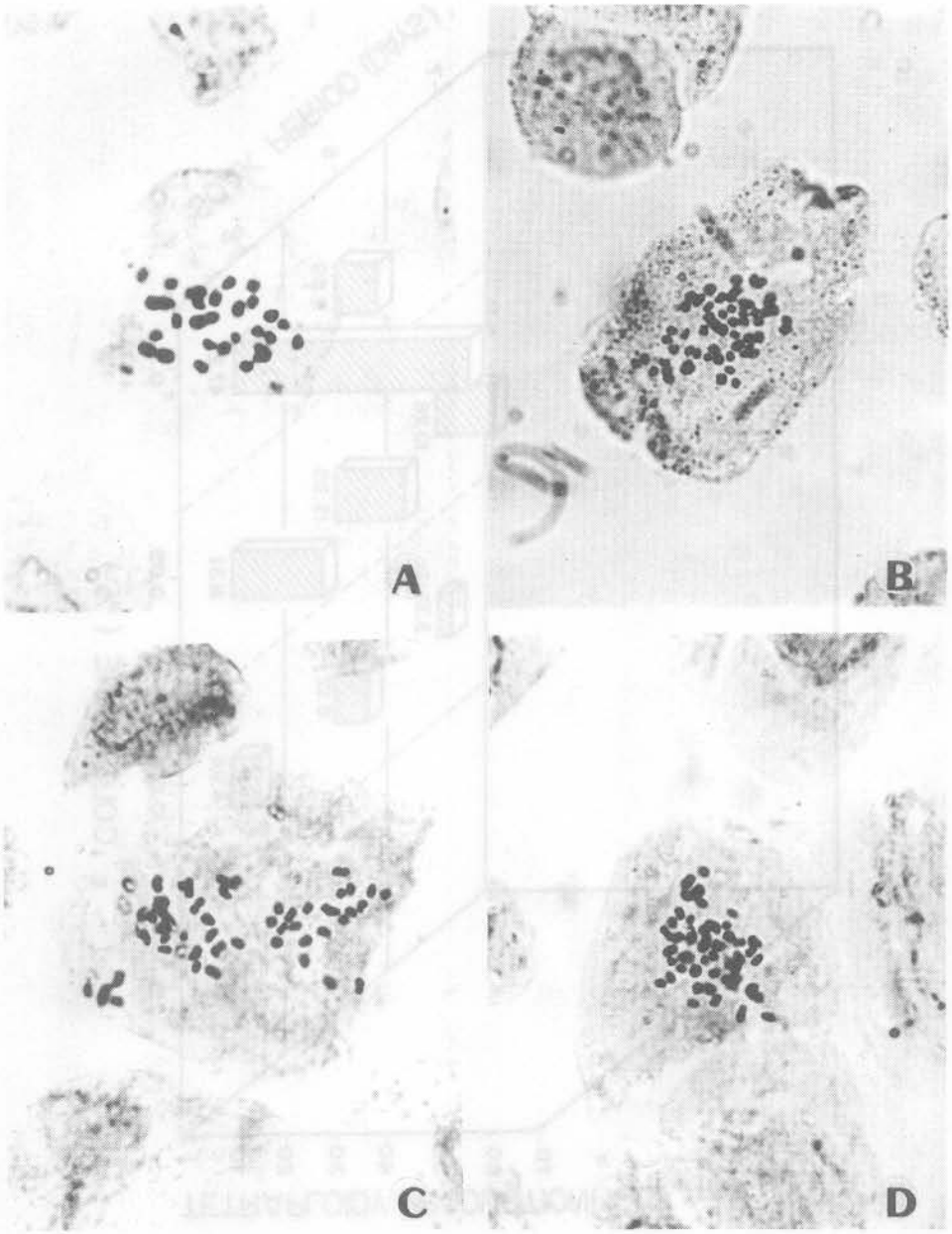

Fig. 3. Chromosome numbers of *M. alba* var. S54 A. Diploid cell (2n=28,X=14). B. Tetraploid cell (2n=56,X=14). C. Triploid cell (2n=42,X=14). D. Near-tetraploid cell (2n=54,X-14). X2680

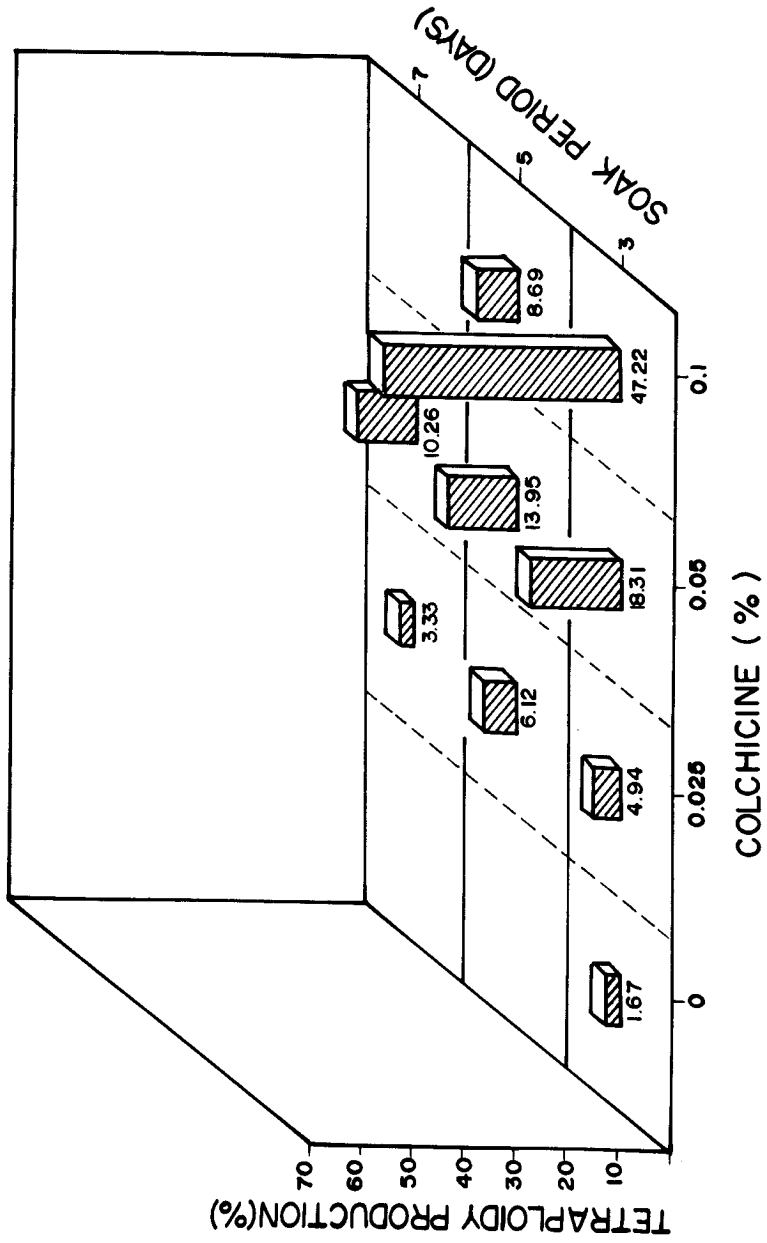

Fig. 4. Histogram showing percentage of tetraploid plants derived from untreated and colchicine -treated callus.

Fig. 5. Mulberry plants (*M. alba* var. S54) of two months old. Left : diploid plant (2n=28,X=14). Right : tetraploid plant (2n=56,X=14).

DISCUSSION

The study of long term culture of mulberry callus showed that the callus older than 10 weeks failed to regenerate shoots. Six- to eight-week-old callus was optimum for a shoot induction and it was used in polyploid induction experiment. In comparison with the untreated callus, those soaked in colchicine solution showed the reduction in survival and the percentage of callus that regenerated shoots. It was reported that colchicine not only prevented the function of the division mechanism, but also killed cells and tissues[12]. To achieve the shoot regeneration, either from the untreated callus or the callus soaked in colchicine solution, it was necessary to culture the callus on the modified MS medium supplemented with 1.0 mg/l BA and 0.025 mg/l NAA.

From our results, the number of shoots regenerated from the colchicine-treated callus was more than those derived from the untreated callus. Infact, effect of colchicine on shoot production improvement in *Prunus* was already reported[13]. Colchicine is known to arrest mitosis at metaphase by the disruption of spindle fibers, however, the mode of action of colchicine in organogenesis is still unknown. The shoots were rooted on half-strength modified MS medium containing 0.1 mg/l.IBA. The disadvantage of the auxin treatment was that it caused a callus formation and inhibited a root elongation. The presence of the callus between the root and shoot resulted in a poor vascular connection which decreased the survival of mulberry plantlets[7].

The percentage of polyploid plants obtained from the untreated callus was very low and unpredictable as compared to the percentage of those derived from the colchicine-treated callus. The higher colchicine concentration and longer soak period, the higher percentage of polyploid production was. The present study demonstrated the possibility of polyploid induction by soaking callus in aqueous colchicine solution followed by plant regeneration through the tissue culture technique. The colchicine solution of 0.1% with 3 days treatment appeared to give the best result in the production of tetraploid plantlets (47.22%). There were several studies that reported the use of colchicine solution in vitro induction of polyploidy in monocotyledons and dicotyledons[14,15]. Vajrabhaya and Chaicharoen[14] reported the effect of 0.05% and 0.02% colchicine solution with 3, 5 and 10 days soak period on polyploid induction in *Dendrobium* hybrid callus. Among 130 plants regenerated from the diploid stocks, 63.07% became tetraploids, while 11.54% remained diploids[14]. Espino and Vazques[15] reported that the leaf explants of *Saintpaulia ionantha* cultured on the MS medium containing 150 mg/l colchicine for 4 days enhanced 46% polyploid plants which were composed of 26% tetraploids, 2% octoploids and 18% mixoploids[15].

From the results, mixoploid plants obtained from both the untreated callus and the colchicine - treated callus contained 3 different chromosome numbers (diploid, triploid and tetraploid) and were sectorial chimera. The occurrence of mixoploid plants with sectorial chimera was also reported in *S. ionantha* cultured on the MS medium containing colchicine[15]. Espino and Vazques[15] explained that the formation of chimeric plants could occur only on spindle fiber of certain stages of nuclear division. Other explanations for the occurrence of mixoploid were the high stability of *M. alba* var. S54 and the predomination of diploid cells above tetraploid cells.

A comparison of morphological characteristics between the diploid and tetraploid mulberry plants of the same age showed that the tetraploid plants exhibited gigantism in many characters such as stems, leaves and size of stomatal guard cells.

It is realized that the application of tissue culture techniques incorporated with the proper colchicine concentration and soak period could produce mulberry plants with doubling in chromosome numbers which are valuable in mulberry breeding programs.

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บทคัดย่อ

นำใบอ่อนของหม่อน *Morus alba* var S54 มาเพาะเลี้ยงบนวุ้นอาหารสูตรดัดแปลง Murashige และ Skoog (MS) ซึ่งใส่ฮอร์โมน BA ความเข้มข้น 0.5 มก/ลิตร และ NAA ความเข้มข้น 1.0 มก/ลิตร เพื่อชักนำให้เกิดเนื้อเยื่อแคลลัสและบนวุ้นอาหารที่ใส่ BA 1.0 มล/ลิตร และ NAA 0.5 มก/ลิตร เพื่อให้เนื้อเยื่อแคลลัสเจริญเติบโตดี เนื้อเยื่อแคลลัสเมื่อแช่ในสารละลายโคลชิซีนที่มีความเข้มข้นต่างๆ กัน (0.025-2%) เป็นเวลา 3-7 วัน แล้ว นำเนื้อเยื่อแคลลัสมาเลี้ยงบนวุ้นอาหารที่ใส่ BA ความเข้มข้น 1.0 มก/ลิตร และ NAA ความเข้มข้น 0.025 มก/ลิตร เพื่อให้เนื้อเยื่อแคลลัสเจริญเป็นต้นอ่อน แล้วนำต้นอ่อนไปเลี้ยงบนวุ้นอาหารที่ใส่ ฮอร์โมน BA ความเข้มข้น 0.25 มก/ลิตร และ NAA ความเข้มข้น 0.025 มก/ลิตร เพื่อ ให้ต้นอ่อนเจริญเติบโต นำต้นอ่อนมาเลี้ยงบนวุ้นอาหารสูตรดัดแปลง MS ที่มีสารอาหารเพียงครึ่งเดียว โดยใส่ IBA ความเข้มข้น 0.1 มก/ลิตร เพื่อให้ต้นอ่อนออกราก การตรวจสอบโครโมโซมหม่อนโดยการนับจำนวนโครโมโซมของเซลล์จากปลายราก ผลการทดลองพบว่า ได้ต้นหม่อนเตตราพลอยด์จำนวนมากที่สุด (47.22%) จากการแช่เนื้อเยื่อแคลลัสในสารละลายโคลชิซีน 0.1% เป็นเวลา 3 วัน แต่การใช้สารละลายโคลชิซีนความเข้มข้นอื่นๆ ได้ต้นหม่อนเตตราพลอยด์จำนวนน้อยกว่าและได้ต้นมิกโซพลอยด์เป็นจำนวนมาก นำต้นหม่อนดิพลอยด์และเตตราพลอยด์ที่สมบูรณ์มาปลูกลงในกระถางที่มีดิน จากการเปรียบเทียบพบว่าต้นเตตราพลอยด์มีการเจริญเติบโตช้ากว่าต้นดิพลอยด์เล็กน้อย แต่ต้นหม่อนเตตราพลอยด์มีลำต้นที่ใหญ่กว่า มีใบสีเขียวเข้มและหนากว่าดิพลอยด์