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In vitro induction of tetraploidy in mulberry (*Morus alba* L.)

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Abstract A high frequency of tetraploidy was induced in mulberry (*Morus alba* L.) through apical bud treatment under *in vitro* conditions. Apical buds from in vitro-grown plants were treated with three different concentrations (0.05, 0.1 and 0.2%) of colchicine in MS medium for 24 h. Tetraploidy at a frequency of 39.4±4.8% was obtained using 0.1% colchicine, whereas the frequency of tetraploidy was significantly reduced to 16.7±2.3% when 0.2% colchicine was used. Morphological, histological and cytological evidence indicated a phenotypic and genomic similarity of in vitro- with ex vitro-induced tetraploids. Rooting of tetraploids was on basal medium containing 2.6 μM NAA. The recovery of tetraploids was 80.8% more efficient using the in vitro method instead of the ex vitro method. The use of the same colchicine medium for up to 4 weeks with additional explants was found to be equally effective for the induction of tetraploidy.

Key words Apical buds · Colchicine · In vitro induction · Tetraploids · Mulberry

Abbreviations *MS* Murashige and Skoog (1962) medium · *BA* 6-benzylamino purine · *NAA* α-naphthalene acetic acid · *PDB* para-dichloro-benzene

Introduction

Mulberry (*Morus* spp.), of the family Moraceae, is an economically important tree, and its leaf is the sole food for the silkworm (*Bombyx mori* L.). Triploid mulberries are superior to diploids with respect to leaf nutrition, genetic adaptability and resistance to environmental stress (Funabiki 1964). Triploids are developed through natural or controlled hybridization between diploid and tetraploid parents (Das et al. 1970). Since most of the natural triploids are unsuitable for commercial utilization due to their poor nutritional value and low propagation efficiency (Hameda 1963), desirable triploids are being developed using induced superior tetraploids for hybridization with diploids.Varieties with smooth, succulent leaves and high propagation efficiency are being utilized for the development of superior tetraploid parents.

There have been a number of reports on the induction of tetraploidy in mulberry through colchicine treatment of germinating seeds, seedlings and vegetative buds (Das et al. 1970; Dwivedi et al. 1986; Verma et al. 1986; Sikdar and Jolly 1994). However, in all cases the rate of induction of tetraploids was low (7–22.2%). Moreover, the treatment is lengthy, laborious and uneconomical due to a greater consumption of colchicine during the treatment. Varying environmental conditions are also suspected of inducing the appearance of mixoploidy (Sikdar and Jolly 1994).

The study described here was undertaken to induce mulberry tetraploids under controlled conditions through in vitro methods, so as to develop a suitable protocol for the maximum recovery of tetraploids and an economic use of colchicine.

Materials and methods

Apical buds (3–5 mm) excised from in vitro-grown plants of *Morus alba* L. var 'S1' were cultured on MS medium containing BA (8.8 μM) with four concentrations of colchicine (0.0, 0.05, 0.1 and 0.2%). The buds were kept on this medium for 24 h under a 16-h irradiance of 45 μmol m^{-2} s^{-1} in the culture room at 25°±1°C. After 24 h of treatment the buds were transferred to MS medium containing BA (8.8 μM) without colchicine. These were transferred on day 30 to fresh medium. For root induction, the growing microcuttings were transferred to MS medium containing 2.6 μM NAA on the 60th day. This protocol for the in vitro induction of tetraploidy was standardized and followed throughout the study. The experiment was repeated three times for confirmation of the efficacy of the protocol.

The percentage of survival of treated buds was recorded on the 60th day and compared with that of the control. Shoot length and number of leaves per shoot were recorded on regular intervals of 15 days. Similarly, root length and number of roots per plants were re-

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corded from growing plants after 30 days of growth on rooting medium.

For cytological confirmation of tetraploids, shoot tips of normal and tetraploid plants were pretreated separately with a saturated solution of para dichloro benzene (PDB) for 3 h. The pretreated shoot tips after washing were fixed in 1:3 proprionic-ethanol solution overnight. Staining was done with proprionic-orcein (2%) and squashed in 45% proprionic acid. Somatic chromosomes were studied using temporary slides.

Leaf size and thickness were measured from mature leaves. Stomatal frequency and size and number of chloroplasts per stoma of mature leaf were studied by fixation in 1:3 acetic-ethanol solution followed by staining in a 2% KI_3 solution for 1 min.

To assess the effectiveness of the colchicine medium for repeated use, we cultured additional explants on the same colchicine medium 15 and 30 days after the initial culture.

The data on ex vitro induction of tetraploids from a separate experiment, where swollen axillary buds were treated with 0.2%, 0.4% and 0.6% aqueous colchicine solution using the cotton swab technique (Das et al. 1970), were utilized here for comparison.

Results and discussion

Survival of treated buds

After 60 days of treatment the survival of buds was recorded on the basis of the greenness of the buds. Nongrowing, brown buds were considered to be dead, while green, growing buds were considered to have survived. The survival rates were 86.7±5.4%, 66.7±5.4% and 40.0±5.4%, respectively, in 0.05%, 0.1% and 0.2% colchicine, as compared to the 100% survival in the control (Table 1). There was an inverse relation between colchicine concentration and survival of buds. This is in agreement with findings using ex vitro conditions (Das et al. 1970; Dwivedi et al. 1986; Sikdar et al. 1994).

Bud emergence and shoot growth

The first visible effect of colchicine treatment was delayed sprouting and growth of the treated buds. In untreated explants, bud emergence and growth initiation were observed within 3 days of inoculation, whereas treated explants started growth 5–8 days after their transfer to the colchicine-free medium. The growth of treated buds was slower than that of the controls (Figs. 4, 5). The concentration of colchicine was observed to have a marked influence on the growth of shoots (Fig. 1). The lengths of the shoots varied from 2.5±0.14 cm to 1.76±0.02 cm after 60 days of growth using the three concentrations of colchicine, with the longest (2.5±0.14 cm) at 0.05% and the shortest (1.76±0.02 cm) at 0.2% colchicine. Untreated shoots were 8.8±0.28 cm long after 60 days of culture (Fig. 1). The retarded growth rate may be due to the reduced rate of cell division that results from the physiological disturbance caused by colchicine (Swanson 1957). Similar observations of initial retardation of growth were also reported from *ex vitro* studies by earlier workers (Sikder and Jolly 1994). However, under in vitro conditions the presence of

Table 1 Effect of colchicine on induction of mulberry tetraploids under in vitro and ex vitro conditions

Treatment[a]	Concentration of colchicine (%)	Duration (h)	Survival of buds	Tetraploids recovered
In vitro	0.00	24	100.0±0.0	00.0±0.0
	0.05	24	86.7±5.4	30.9±1.9
	0.10	24	66.7±5.4	39.4±4.0
	0.20	24	40.0±5.4	17.0±2.3
Ex vitro	0.20	6	100.0±0.0	00.0±0.0
		9	100.0±0.0	00.0±0.0
		12	100.0±0.0	00.0±0.0
	0.40	6	100.0±0.0	00.0±0.0
		9	100.0±0.0	22.2±9.1
		12	100.0±0.0	11.1±9.6
	0.60	6	88.9±5.2	00.0±0.0
		9	77.8±9.1	11.1±5.6
		12	44.4±5.2	00.0±0.0

[a] Number of buds treated in each replication varied from 9 buds in ex vitro to 15 buds in in vitro conditions; experiments were repeated three times

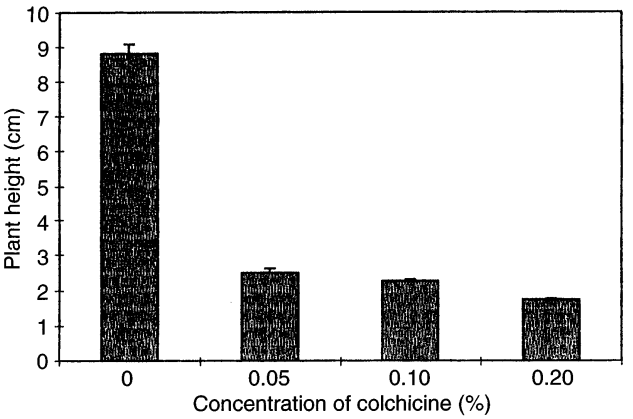


Fig. 1 Effects of concentration of colchicine on shoot growth of mulberry tetraploids after 60 days of growth on colchicine-free medium

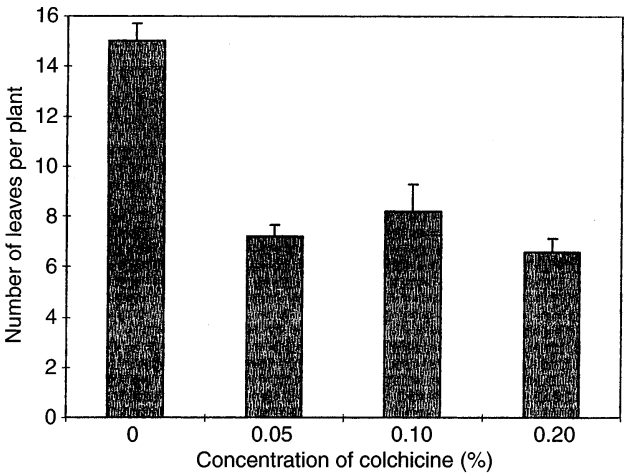


Fig. 2 Variation in number of leaves in mulberry tetraploids using different concentrations of colchicine after 60 days growth on colchicine-free medium

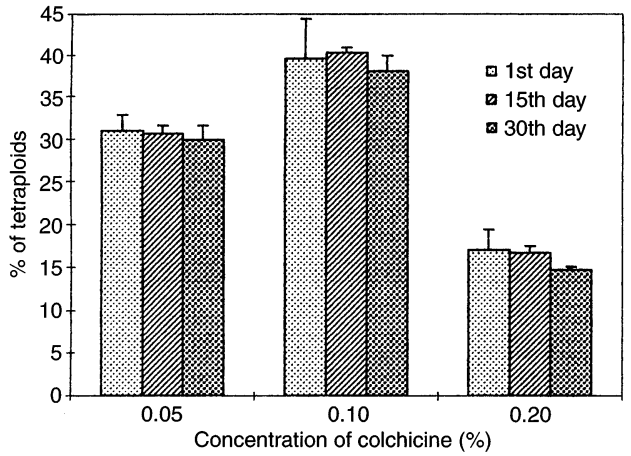


Fig. 3 Efficacy of the same colchicine medium for induction of tetraploidy in additional explants of mulberry

Table 2 Morphological and anatomical characteristics of in vitro induced mulberry tetraploids

Characteristics	Diploid (2 n=28)	Tetraploid (2 n=56)
Length of leaf (cm)	1.5±0.1	2.2±0.2
Breadth of leaf (cm)	1.1±0.7	1.7±2.3
Thickness of leaf (μm)	185.0±0.0	342.6±2.3
Stomatal frequency/mm^2	471.7±18.9	452.5±39.8
Length of stomata (μm)	20.8±0.7	41.4±2.7
Breadth of stomata (μm)	18.9±0.7	30.3±1.2
Number of chloroplast/stomata	12.1±0.8	23.7±0.7
Number of roots/plant	15.0±0.3	6.6±0.2

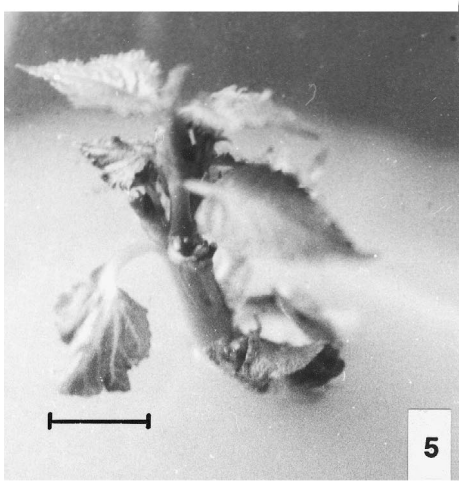
BA may have enhanced the division of polyploidized cells and resumption of growth.

Leaf characteristics

The basal leaves of shoots that developed from colchicine-treated buds were distorted while the upper newly formed leaves were large, thick and dark green, with stronger venation and deeper serration (Fig. 7A,B). Such an increase in thickness could be due to an increase in cell size (Dwivedi et al. 1986). The length and breadth of the leaves were also greater in colchicine-induced plants (2.2±0.22 cm and 1.7±0.10 cm) compared to that of untreated plants (1.48±0.12 cm and 1.12±0.07 cm) (Table 2). The number of leaves that emerged after 60 days of growth also varied using the three different concentrations of colchicine (Fig. 2).

Screening of tetraploids

The initial screening for tetraploids was based on stomatal morphology and stomatal chloroplast counts (Mochizuki and Sueoka 1955). Further confirmation was by chromosome counts. Stomatal frequency was 471.7±18.9 per square millimeter in diploids and 452.5±39.9/mm^2 in tetra-



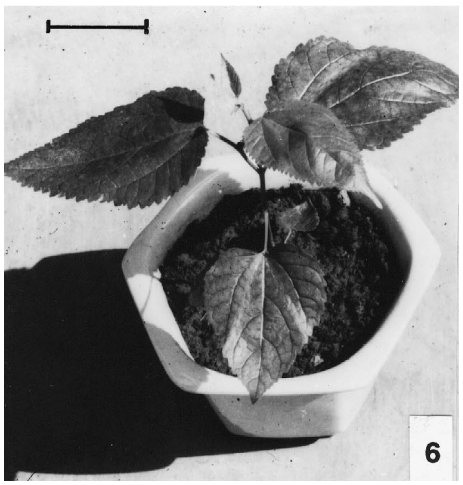


Fig. 4 Diploid mulberry plant from untreated apical bud after 60 days of growth on MS medium. *Bar*: 1.6 cm

Fig. 5 Tetraploid mulberry from treated apical bud after 30 days of growth on colchicine in MS medium. *Bar*: 1.3 cm

Fig. 6 Tetraploid mulberry developed from in vitro-treated apical bud, growing in soil. *Bar*: 6.3 cm)

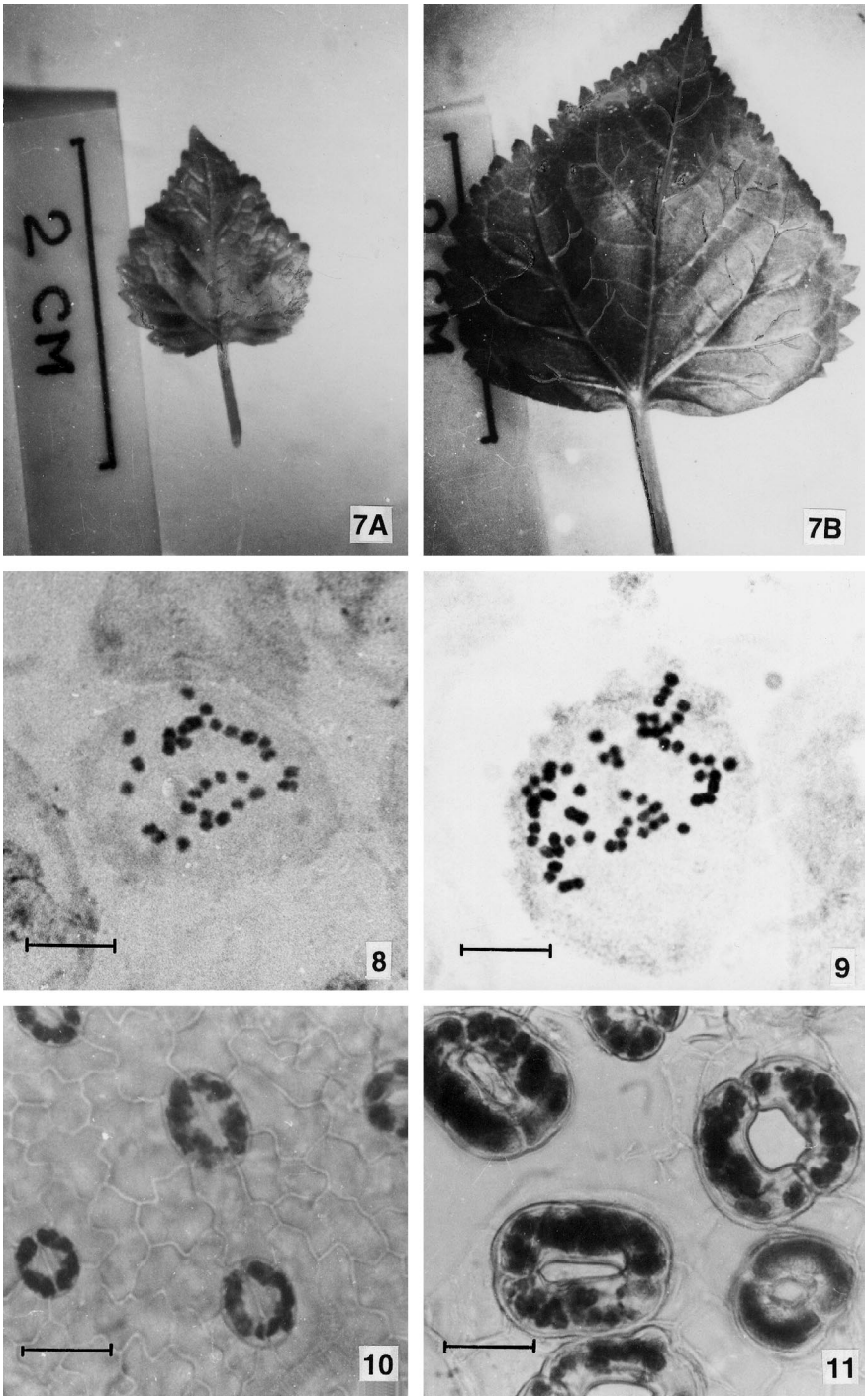


Fig. 7 **A** Leaf of in vitro-grown diploid mulberry plant. **B** Leaf of in vitro-grown tetraploid mulberry plant

Fig. 8 Somatic chromosomes of diploid mulberry plant, 2n= 28. *Bar*: 8 μm

Fig. 9 Somatic chromosomes of tetraploid mulberry plant, 2n=4x=56. *Bar*: 8 μm

Fig. 10 Stomata of in vitro-grown diploid mulberry plant. *Bar*: 20 μm

Fig. 11 Stomata of in vitro-grown tetraploid mulberry plant. *Bar*: 20 μm

ploids. The average length and breadth of the stomata were 20.8±0.7 μm and 18.9±0.7 μm respectively in diploids and 41.4±2.7 μm and 30.3±1.2 μm in tetraploids. Similarly, the chloroplast number per stoma was 12.1±0.8 in diploids and 23.7±0.7 in tetraploids (Table 2). In tetraploids the size of stomata and number of chloroplasts were almost double those observed in diploids,while the frequency of stomata was lower (Figs. 10,11). Sikder et al. (1986) used this technique to screen tetraploids after colchicine treatment of the apical bud of *Morus alba* L. under *ex vitro* conditions. They found the average number of stomatal chloroplasts to be 9.4 in diploids and 18.6 in tetraploids. In the present study tetraploids also had a higher number of stomatal chloroplasts (almost double) than diploids. Cytological studies further confirmed the tetraploid status of tetraploids. The mitotic chromosome number of diploid plants was 28 and that of tetraploid plants was 56 (Figs. 8, 9). Regular distribution of 56 chromosomes in the metaphase plate of each plant derived from all colchicine concentration treatments revealed no mixoploidy among the tetraploids.

Mixoploidy is a major problem in the recovery of tetraploids using ex vitro conditions. However, in in vitro methods occurrence of mixoploids may have been minimized by growing the treated plants under more favourable conditions. The controlled environment, temperature and photoperiod may have favoured the synchronous division of meristematic cells and eliminated the chances of mixoploidy among the tetraploids induced under in vitro conditions, thereby contributing to the exceptionally high recovery of tetraploids. Similarly, BA in the medium may have promoted a more rapid growth of the treated buds, resulting in a higher recovery of tetraploids.

Recovery of tetraploids

The highest percentage (39.4±4.8%) of tetraploids was recovered using 0.1% colchicine and the lowest (17.0±2.3%) using 0.2% colchicine (Table 1). A moderate percentage of recovery (30.9±1.9%) was noted using 0.05% colchicine. Use of the same colchicine medium for additional treatment of buds after 15 and 30 days of initial culture showed negligible variation in the recovery of tetraploids (Fig. 3).Thus, this method provides an efficient technique by which colchicine can be utilized economically for the mass induction of tetraploids (Fig. 6).

Root formation and growth

Root initiation was observed 10 days after transfer to rooting medium in both diploid and tetraploid plants obtained using different concentrations of colchicine. However, tetraploids obtained using 0.2% colchicine required 12 days for rooting. A higher number of roots was found in diploid plants (15.0±0.28) than in tetraploid ones (6.6±0.22).

The present investigation revealed that in vitro treatment of the apical bud with 0.1% colchicine for 24 h was more effective than the other concentrations tested for the induction of tetraploidy in mulberry. Similarly, the same colchicine medium can be used repeatedly for the induction of tetraploidy for up to 30 days without any loss in induction capacity. The present study, therefore, represents a novel method for induction for the large-scale induction of tetraploids in mulberry.

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