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*Full Length Research Paper*

# Comparative micropropagation efficiency of diploid and triploid mulberry (*Morus alba* cv. $S_1$) from axillary bud explants

**S. Chattopadhyay[1,2]*, S. Gandhi Doss[1], S. Halder[1], A. K. Ali[1] and A. K. Bajpai[1]**

[1]Biotechnology Laboratory, Central Sericultural Research and Training Institute, Central Silk Board, Berhampore 742 101, West Bengal, India.
[2]Regional Muga Research Station, Central Silk Board, Boko 781 123, Assam, India.

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**Micropropagation ability of two cytotypes (2n = 2x = 28 and 2n = 3x = 42) of mulberry (variety $S_1$) was tested from axillary buds through organogenesis. Both shoot and rootlets development periods varied considerably with ploidy variations. The triploid shoots generally grew more vigorously in Murashige and Skoog (MS) medium fortified with 6-benzyl amino purine (6-BAP) than those of diploids. Maximum shoot initiation frequency (80%) was obtained with 4.4 µM of 6-BAP on 4.2 days of culture in triploid; whereas, the axillary buds sprouted within 3.5 days with 100% shoot initiation frequency in diploid cultures. Highest shoot length (4.8 and 5.6 cm per explant) was obtained at 8.8 and 4.4 µM 6-BAP supplementations in diploid and triploid cytotypes, respectively. Subsequently, regenerated shoots were transferred to auxin rich rooting media supplemented with either the different doses of indole acetic acid (IAA) or naphthalene acetic acid (NAA). Root initiation was more vigorous in diploid than triploids with both the tested growth regulators. Maximum number of rootlets per shoot (15) and root length (4.2 cm) were observed in MS basal medium supplemented with 4.0 µM NAA after 21 days of culture of diploid plants; while in triploid cytotype, maximum rootlets per shoot (8.3) were noticed with 4.4 µM NAA after 21 days of culture. Rooted plantlets were hardened in plastic cups containing sterile sand and soil mix (1:1; w/w) for 21 days and subsequently transferred to clay pots (l x b x h: 12 x 12 x 12 cm) in shade with a survival of 65 and 52% for diploid and triploid cytotypes, respectively. The time required for field establishment of micro-propagated triploid was about 60 days.**

**Key words:** *Morus alba*, diploid, axillary bud, organogenesis, shoot regeneration, triploid, hardening.

## INTRODUCTION

Mulberry (*Morus* spp.) is a multipurpose, predominantly dioecious, heterozygous and out breeding tree. The foliage of the plant is used mainly as a unique source of silk-worm (*Bombyx mori* L.) feed and cultivated in over 40 countries (Machii and Katagiri, 1991). Most of the natural species of *Morus* is diploid (2x = 28). But, triploids (2x = 42), developed by controlled crosses, are often superior with respect to foliage yield, quality and adaptability to environmental stimuli than diploids (Das and Prasad, 1974; Tojyo, 1985; Yang and Yang, 1989). A few triploid varieties, developed from controlled crosses like S-1635, Tr-10, RFS- 135, are presently under commercial exploitations. Still, field exploitation of many triploids is limited due to their relatively slow growth and multiplication rates (Hameda, 1963; Dzhafarov and Abbasov, 1967; Das, 1983). The diploid mulberry cultivar $S_1$ is widely used in eastern and north-eastern parts of India, but its triploid cytotypes, though qualitatively better with respects to certain foliage characters, is less popular due to poor

*Corresponding author. E-mail: soumenchatto@rediffmail.com. Fax: +91-3623-217384.

**Abbreviations: BAP,** 6-Benzyl amino purine; **IAA,** indole acetic acid; **IBA**, indole butyric acid; **MS,** Murashige and Skoog; **NAA,** naphthalene acetic acid.

growth and comparatively low multiplication efficiency. Moreover, the propagation methods of mulberry through hard wood stem cutting or grafting are besets with various problems (Ohyama and Oka, 1987). Production of saplings in nursery is time consuming (≥6 months) and irrigation intensive (Kapur et al., 2001). Besides, survival and vigor of stem cutting grown plants are inferior (Bapat et al., 1987; Zaman et al., 1997).

As an alternative, sapling utilization state of mulberry could be achieved in relatively short duration with better quality through micropropagation (Oka and Ohyama, 1986). Indeed, mulberry regeneration protocols from the apical/axillary shoot buds and nodal explants have been reported from number of genotypes (Mhatre et al., 1985; Jain et al., 1990; Sharma and Thorpe, 1990; Chattopadhyay et al., 1990; Bhau and Wakhlu, 2001). But, report on the comparative micropropagation efficiency of diploid and triploid varieties or different ploidy levels of same mulberry variety are scanty. Secondly, in vitro shoot initiation in *Morus* spp. is greatly influenced by the age of explants, season and nature of growth regulators (Khurana et al., 2003). Most of the protocols is genotype specific and may not be applicable to all valuable cultivars (Bapat et al., 1987; Sahoo et al., 1997). As a result, very little information is available on the micropropagation potential of commercial mulberry variety $S_1$. Therefore, the objective of the study was to establish and compare direct regeneration protocols of diploid and triploid cytotypes of this important mulberry variety.

## MATERIALS AND METHODS

### Explants preparation

The axillary buds from two cytotypes (2n = 2x =28 and 2n =3x = 42) of mulberry (*Morus alba* cv. $S_1$) were collected from 60 days old plants maintained at the experimental field of our Institute. The explants were washed under running tap water, followed by immersion in 0.1% (w/v) carbendazim for 20 min and rinsed thoroughly with distilled water. Then, the explants were treated with 0.2% (w/v) cetavlon for 10 min, 5 min in 0.1% (w/v) aqueous mercuric chloride solution in a sterile bottle under laminar airflow, and finally washed 4 to 5 times with sterile distilled water.

### Induction and development of shoots

The disinfected segments were cultured in conical flask containing 100 ml of sterile MS basal medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, 0.7% (w/v) agar (Himedia, India) and various doses of 6-BAP (range: 2.2 to 22.2 µM) for shoot induction. The pH of the media was adjusted to 5.7 prior to autoclaving at 121°C for 15 min. All cultures were incubated at 25 ± 2°C under 16 h photoperiod (light intensity 300µmol $m^{-2}$ $s^{-1}$) provided by cool white fluorescent light source. The cultures were maintained by regular sub-culturing at 28 days intervals on fresh MS medium.

### Rooting and transplantation

Regenerated shoot (2 to 3cm in length) obtained from 28 days culture were transferred aseptically to the culture tubes (250 x 15 mm) containing MS medium supplemented with 2% (w/v) sucrose, 0.7% (w/v) agar with various doses of auxins like IAA (1.4 to 7.3 µM and NAA ( 1.3 to 6.7 µM) for root induction. After 21 days, profusely rooted plantlets were taken out of the tubes and agar was gently removed by washing in sterile water. The plantlets were transferred to plastic cups (7 x 5 cm) containing mixture of sand and soil (1:1 w/v) moisten with half strength MS basal liquid medium and grown at 25 ± 2°C with 68 to 78% RH under 16 h photoperiod for 21 days. The plantlets covered with polythene bag were adequate to achieve such humidity. Subsequently, the hardened plants (5 ± 2 cm) were transferred to clay pots (l x b x h: 12 x 12 x 12 cm) containing sand, soil and farmyard manure (1:1:1 w/w/w) and kept at glass house under shade for 60 days.

### Analyses

Each experiment was repeated thrice with 10 replication per treatment. Data was recorded at 7 days interval for specific period of treatment. Analysis of variance was conducted using CRBD. The mean values were partitioned with least significant difference ($p > 0.05$) in appropriate cases (Gomez and Gomez, 1984).

## RESULTS AND DISCUSSION

The field grown triploid cytotype of mulberry variety $S_1$ had significantly higher foliage morphological characters than diploid (Table 1). Besides, length, breadth and size of stomata were also greater in triploids. However, primary shoot length (after 60 days of ground level pruning) and survival of triploid plants, measured as a function of growth and multiplication efficiencies, were 23 and 49% lesser in triploid than diploid plants.

On the MS basal medium alone, the axillary buds showed no sign of differentiation. When explants were cultured on medium supplemented with different doses of 6-BAP, morphogenic responses at various degrees were apparent in both cytotypes. Many workers proved the superiority of 6-BAP than other growth regulators for mulberry shoot induction from various types of explants (Narayan et al., 1989; Chitra and Padmaja, 1999; Kavyashree et al., 2006), and we have restricted our shoot induction experiment exclusively with this synthetic cytokinin. The dose of 6-BAP was observed to have a marked influence on sprouting and growth (Table 2). Earliest sprouting of buds was observed on 3.5 days with 8.8 µM and 4.2 days with 4.4 µM 6-BAP treated diploid and triploid plants, respectively (Table 2). Corol-lary to the obtained field results (Table 1), in vitro growth of triploid shoots was better than diploid at lower concentrations of 6-BAP (Figure 1). But there was no difference in the shoot number per explants in both cytotypes. The length of shoots varied maximally (5.5 fold in diploid and 7.7 fold in triploid plants) across the tested doses (2.2 to 22.2 µM) of growth regulator. The highest shoot length was achieved with 8.8 and 4.4 µM of 6-BAP treated diploid (6.6 cm) and triploid (7.0 cm) explants, respectively after 28 days of culture. Average shoot length was 14% more in triploid than diploid cytotypes. Our results

**Table 1.** Morphological and anatomical characteristics of field grown diploid and triploid cytotypes of mulberry (*M. alba* L. cv. $S_1$).

| Character | Diploid (2n = 28) | Triploid (2n = 42) |
|---|---|---|
| Length of leaf (cm) | 14.0 ± 1.1 | 16.4 ± 1.5 |
| Breadth of leaf (cm) | 9.1 ± 0.7 | 11.2 ± 0.5 |
| Petiole l (cm) | 2.8 ± 0.4 | 3.4 ± 0.5 |
| Petiole b (mm) | 1.4 ± 0.2 | 1.8 ± 0.3 |
| Single leaf area ($cm^2$) | 82.4 ± 12.2 | 124.1 ± 1.9 |
| Leaf thickness (µm) | 121.9 ± 5.9 | 138.2 ± 10.6 |
| Stomatal frequency (no $mm^{-2}$) | 877.9 ± 82.7 | 570.4 ± 24.5 |
| Stomatal length (µm) | 16.6 ± 1.6 | 20.4 ± 1.2 |
| Stomatal breadth (µm) | 10.1 ± 7.7 | 11.6 ± 1.1 |
| Stomatal size ($\mu m^2$) | 166.8 ± 32.5 | 238.1 ± 31.5 |
| Survival (%) | 72.6 ± 8.7 | 36.4 ± 4.6 |
| Shoot length (cm) | 121.2 ± 9.1 | 93.6 ± 7.9 |

Data are mean ± SE (n = 20); Parameters were measured from 60 days after ground level pruning of six years old plant.

**Table 2.** Comparative effect of different concentrations of 6-BAP on shoot induction from axially bud explants of diploid and triploid cytotypes of mulberry (*M. alba* L. cv. $S_1$) after 28 days of culture.

| 6-BAP (µM) | Day to sprout of axillary bud | | Explant producing shoot (%) | | Maximum shoot length (cm) | | Average shoot length (cm) | |
|---|---|---|---|---|---|---|---|---|
| | 2n | 3n | 2n | 3n | 2n | 3n | 2n | 3n |
| 2.2 | 9.0 ± 0.8 | 7.0 ± 0.4 | 60 | 60 | 1.2 ± 0.4 | 1.7 ± 0.2 | 0.8 ± 0.4 | 1.3 ± 0.4 |
| 4.4 | 8.0 ± 0.4 | 4.2 ± 0.3 | 90 | 80 | 1.9 ± 0.3 | 7.0 ± 0.6 | 1.2 ± 0.7 | 5.6 ± 1.4 |
| 8.8 | 3.5 ± 0.2 | 6.3 ± 0.2 | 100 | 70 | 6.6 ± 0.8 | 3.4 ± 0.2 | 4.8 ± 1.8 | 2.6 ± 0.8 |
| 17.8 | 4.3 ± 0.3 | 8.1± 0.5 | 80 | 60 | 4.0 ± 0.4 | 1.2 ± 0.4 | 3.2 ± 0.8 | 0.8 ± 0.4 |
| 22.2 | 6.1± 0.4 | 10.1±0.7 | 70 | 70 | 2.9 ± 0.2 | 0.7 ± 0.1 | 2.6 ± 0.3 | 0.5 ± 0.2 |

Explants (5 cm) with axillary bud were collected from 60 days old plant and placed on solidified MS basal medium supplemented with various doses of 6-BAP. Data are mean ± SE of the three independent experiments with 10 replications in each occasion.

are in agreement with the previous findings of 6-BAP mediated shoot induction and proliferation (Kim et al., 1985; Jain and Dutta, 1992; Bhau and Wakhlu, 2001) and also supports the notion that 6-BAP had a less response to shootlets generation at relatively higher doses in mulberry (Hossain et al., 1992; Pattanaik et al., 1996). Additionally, results indicated that effect of in vitro organogenesis from axillary buds of two different ploidy levels of a commercial variety of mulberry were significantly different. The obtained result of better shoot regeneration potential of triploid over diploid mulberry supports the observations in pinto peanut (Rey and Mroginski, 2006), asparagus (Kunitake et al., 1998) and cucumber (Sarreb et al., 2002).

Regenerated 28 days old *in vitro* shoots (length 2 to 3 cm) were excised and transferred to half strength MS medium fortified with different doses of auxins like IAA and NAA. Shoots were unable to produce any root primordia in the absence of auxin (data not shown). Contrary to the obtained results of shoot regeneration, diploid plants were more vigorous (in both auxins) in root formation than triploids (Figure 2, Table 3). NAA appeared to be more effective for root induction and growth, than IAA in both cytotypes. Maximum root induction was observed with 4.0 and 5.4 µM of NAA supplemented culture of diploid (15 rootlets per shoot) and triploid (8.3 rootlets per shoot) shoots, respectively. Subsequently, moderate to high rooting frequency was observed in both the cytotypes with two tested growth regulators. The average root length was found to be highest in 2.9 µM IAA (4.0 cm) and 4.0 µM (3.8 cm) NAA supplemented cultures of diploids; while in triploid cultures, the highest average root length was observed at 4.4 µM IAA (6.0 cm) and 5.4 µM NAA (2.3 cm) supplemented cultures after 21 days (Table 3). Our results confirm the observations that *in vitro* rooting was maximally influenced by NAA (Jain et al., 1990; Vijay-Chitra and Padmaja, 1999) and contradicts the IAA dominated maximal root proliferation views of others (Yadav et al., 1990; Karthiravan et al., 1995). Additionally, it was also revealed that *in vitro* cultured triploid plants require more auxin for root development than diploid, and NAA is the most suitable auxin in both cytotypes.

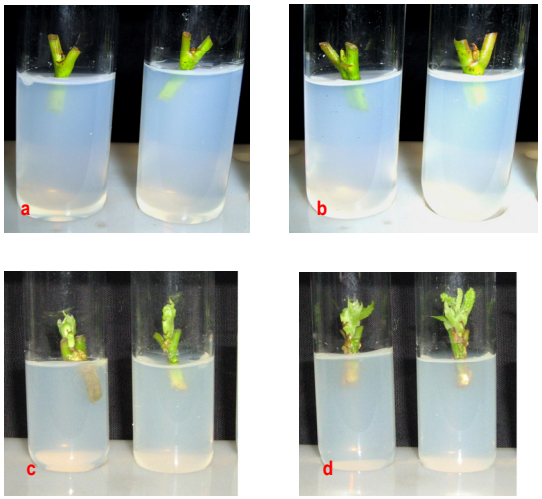


**Figure 1.** *In vitro* sprouting and growth of axillary buds of diploid (a, c) and triploid (b, d) cytotypes of mulberry (*Morus alba* L. cv. $S_1$) with 2 and 1.0 mg L-1 6-BAP containing MS medium, respectively.

**Table 3.** Comparative effect of different concentrations of auxin based growth regulators on root induction from the diploid and triploid shoots of mulberry (*M. alba* L. cv. $S_1$) after 21 days of culture.

| Growth regulator (µM) | Explant producing root (%) | | Root (number explant$^{-1}$) | | Maximum root length (cm) | | Average root length (cm) | |
|---|---|---|---|---|---|---|---|---|
| | 2n | 3n | 2n | 3n | 2n | 3n | 2n | 3n |
| **IAA** | | | | | | | | |
| 1.5 | 80 | 80 | 11± 0.4 | 3.0 ± 0.5 | 3.9 ± 0.4 | 4.1 ± 0.2 | 3.4 ± 0.5 | 3.1 ± 1.0 |
| 2.9 | 100 | 100 | 13 ± 0.5 | 4.0 ± 0.6 | 4.5 ± 0.7 | 5.0 ± 0.4 | 4.0 ± 0.5 | 3.5 ± 1.5 |
| 4.4 | 90 | 90 | 7 ±0.5 | 6.6 ± 1.0 | 3.1± 0.6 | 7.1 ± 0.7 | 2.8 ± 0.2 | 6.0 ± 1.0 |
| 5.9 | 90 | 80 | 8 ± 0.6 | 4.5 ± 0.6 | 2.8 ± 0.5 | 6.4 ± 0.5 | 2.6 ± 0.2 | 5.7 ± 0.7 |
| 7.3 | 80 | 80 | 8 ± 0.5 | 4.8 ± 0.8 | 2.6 ± 0.2 | 4.7 ± 0.6 | 2.3 ± 0.3 | 4.3 ± 0.4 |
| **NAA** | | | | | | | | |
| 1.3 | 70 | 70 | 10 ± 0.4 | 2.3 ± 0.7 | 2.8 ± 0.3 | 1.9 ± 0.8 | 2.4 ± 0.5 | 1.6 ± 0.3 |
| 2.7 | 90 | 90 | 14 ± 0.4 | 4.0 ± 0.4 | 3.6 ± 0.5 | 2.8 ± 0.9 | 3.4 ± 0.2 | 1.8 ± 1.0 |
| 4.0 | 90 | 90 | 15 ± 0.5 | 7.0 ± 1.0 | 4.2 ± 0.9 | 2.2 ± 0.3 | 3.8 ± 0.3 | 2.1 ± 0.1 |
| 5.4 | 100 | 100 | 9 ± 0.3 | 8.3 ± 1.1 | 2.1±0.4 | 2.5 ± 0.2 | 1.8 ± 0.4 | 2.3 ± 0.2 |
| 6.7 | 90 | 90 | 7 ± 0.2 | 5.5 ± 0.5 | 1.7±0.2 | 2.1± 0.4 | 1.4 ± 0.4 | 1.8 ± 0.3 |

Regenerated shoots (2 to 3 cm) were collected from the 28 days old culture and placed on solidified MS basal medium supplemented with various doses of auxin based growth regulators. Data are mean ± SE of the three independent experiments with 10 replications in each occasion.

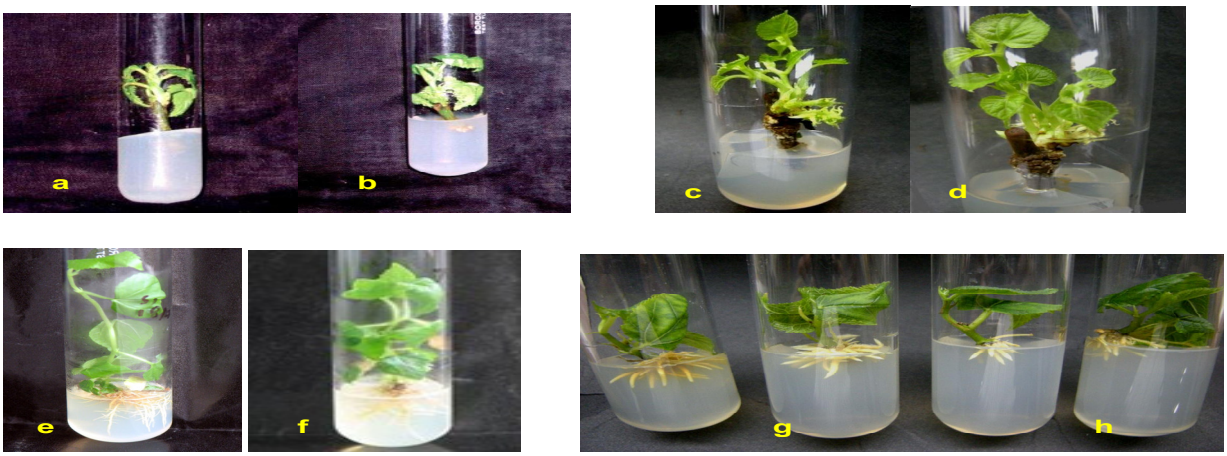


**Figure 2.** Different stages of organogenesis of diploid and triploid cytotypes of mulberry (*M. alba* L. cv. $S_1$): Induction of diploid (a and c) and triploid (b and d) shoots from axillary buds on MS medium containing 8.8 and 4.4 µM of 6-BAP, after 14 and 28 days of culture, respectively. Rooting of diploid (e and g) and triploid (f and h) from the shoots on MS medium supplemented with 4.0 and 4.4 µM of NAA, respectively after 15 and 21 days of culture, respectively.


**Figure 3.** Different stages of hardening of diploid and triploid cytotypes of mulberry (*M. alba* L. cv. $S_1$): Hardened (a and c) and transplanted (b and d) 30 and 60 days old diploid and triploid plants, respectively, maintained at shade condition.

However, higher concentrations of all tested auxins (≥5.9 µM) were inhibitory to root induction and supported the observation of Vijayan et al. (1998).

Regenerated plants (4 to 5 cm) were hardened with 93 and 82% success rate after 28 days of plastic cup maintenance (Figure 3) for diploid and triploid cytotypes,

**Table 4.** Morphological and anatomical characteristics of 2n and 3n cytotypes of mulberry (*M. alba* L. cv. $S_1$) after 60 days hardening under shade condition.

| Character | Diploid (2n = 28) | Triploid (2n = 42) |
|---|---|---|
| Survival (%) | 65.4 ± 6.2 | 52.3 ± 5.3 |
| Shoot length (cm) | 114.3 ± 7.4 | 107.4 ± 10.2 |
| Branch (no plant$^{-1}$) | 3.4 ± 0.5 | 5.6 ± 0.8 |
| Length of leaf (cm) | 13.8 ± 0.8 | 16.1 ± 1 |
| Breadth of leaf (cm) | 9.5 ± 0.6 | 10.8 ± 0.8 |
| Single leaf area (cm$^2$) | 75.3 ± 8.6 | 116.3 ± 11.4 |
| Stomatal frequency (no mm$^{-2}$) | 799.4 ± 70.7 | 512.6 ± 50.2 |
| Stomatal size (µm$^2$) | 178.3 ± 41.1 | 220.5 ± 37.6 |

Data are mean ± SE (n = 20).

respectively. Subsequently, transferred clay potted plants in shade showed about 65 and 52% survival in diploid and triploid plants, respectively after 60 days of growth at glasshouse (Table 4). The survival of triploid improved significantly than field grown triploid of identical age. Otherwise, the regenerated plants after hardening did not show any appreciable variation in morphological and stomatal features when compared with the respective field grown cytotypes. Most significantly, sapling utilization state of micropropagated plants was attained with about 55% less time (140 days) than the reported period of nursery raised saplings from stem cutting clones of 6 to 8 months (Dandin et al., 2001).

In conclusion, our findings describe for the first time, comparative protocols for micropropagation of diploid and triploid cytotypes of an elite mulberry variety, and extended the possibility of rapid regeneration of a slow growing and less multiplication efficient triploid for field exploitation.

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