# RESEARCH ARTICLES

## *IN VITRO* PLANT REGENERATION THROUGH YOUNG LEAF CULTURE IN MULBERRY (*MORUS ALBA* VAR. S54 X *MORUS ALBA* VAR. NOI)

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**ABSTRACT**

*Young leaf explants of mulberry (*Morus alba *var. S54 x* Morus alba *var. Noi), derived from aseptically grown shoots, were cultured on the modified Murashige and Skoog medium supplemented with various concentrations of $N^6$-Benzyladenine (BA) and 1-Naphthaleneacetic acid (NAA). The optimum concentrations of BA and NAA for callus initiation and proliferation were 0.5 mg/l BA plus 1.0 mg/l NAA and 1.0 mg/l BA plus 0.5 mg/l NAA respectively. The Callus was subcultured on the medium containing 1.0 mg/l BA and 0.025 mg/l NAA for shoot regeneration. To obtain shoot growth, the regenerated shoots were cultured on the medium supplemented with 0.25 mg/l BA. A root formation occurred when culturing an individual shoot on half-strength basal medium in the presence of 0.1 mg/l Indole-3-butyric acid (IBA). The addition of 0.1% activated charcoal was able to absorb browning substance during shoot growth and root formation stages. The plantlets were first transferred to sterilized vermiculite and then in the clay pots with soil. The complete plants 1-1.5 feet high have been grown in a trial field.*

## INTRODUCTION

Plant tissue culture techniques have become vitally important for clonal propagation and crop improvement. The plant regeneration *in vitro* has been studied extensively with herbaceous dicotyledon and monocotyledon[1,2], but relatively little work has been conducted on differentiation from callus cultures of woody species[3]. According to heterozygosity and long generation intervals, the propagation of woody plant by using conventional methods is difficult. Thus, the tissue culture technology is an entirely new approach for propagation and breeding of woody species. Several reports of successes of obtaining regenerated plants *in vitro* of woody species were listed[4]. These plant tissue culture techniques enhance high yield production of new plantlets in a short time.

Mulberry (*Morus* sp.) is an economically important plant in sericulture industry. Generally, the mulberry is propagated by stem cuttings, but some varieties with high nutrition value, such as Nakornrajsima 60, are difficult to root. The advantages of using tissue culture technique are high production of new plants in a short time and the applications in plant breeding programs to obtain spontaneous mutation by leaf culture. However, only a few of plant regeneration successes from mulberry callus have been reported[5, 6]. The present study demonstrates the possibility to induce shoot regeneration from leaf-derived callus of *Morus alba* var. S54 x *Morus alba* var. Noi. The complete plants were grown in clay pot containing soil and then in a trial field for further study.

## MATERIALS AND METHODS

The basal medium (BM) used in this experiment was the modified Murashige and Skoog (MS) medium which comprised the mineral salts of MS medium[7], vitamin mixture (B5),[8] 2% saccharose, and 0.8% agar. The pH of the medium was adjusted to 5.8 before autoclaving for 15 min. at 121°C. These ingredients of modified MS medium were shown in Table 1. Seeds of *M. alba* var. S54 x *M. alba* var. Noi were surface sterilized in 15% clorox plus 2 drops of Tween-20 for 15 minutes and rinsed 3 times with sterile distilled water. After the sterilization, seeds were germinated on the BM medium without any hormone. Their stem segments were excised from axenic seedlings and cultured on the BM medium with 1.0 mg/l $N^6$-Benzyladenine (BA) to get multiple shoot formations.

Young leaf explants (1.0 cm. long) were excised and cultured on the BM medium containing 16 combinations of BA (0, 0.5, 1, 2 mg/l) and 1-naphthalene acetic acid (NAA, 0, 0.5, 1, 2 mg/l) with 10% coconut water for callus initiation. The callus was transferred to the medium containing 16 combinations of BA (0, 0.25, 0.5, 1.0 mg/l) and NAA (0, 0.025, 0.5, 1.0 mg/l) for a callus proliferation.

For a shoot induction, a callus was cultured on the medium containing 25 combinations of BA (0, 0.25, 0.5, 1.0, 2.0 mg/l and NAA (0, 0.025, 0.05, 0.1, 0.2 mg/l). Small shoots (1-2 cm. high) regenerated from the callus were excised and transferred to the medium supplemented with 0.25 mg/l for shoot growth. To obtain a root formation, shoots were cultured on half-strength BM medium containing either indole-3-butyric acid (IBA) or NAA at the concentration of 0.1 and 0.2 mg/l.

The mulberry tissue culture is always faced with browning substances which is lead to growth decline and finally tissues death. To solve this problem, some activated charcoal was added into the culture medium at the concentrations of 0, 0.05, 0.1, 0.2 and 0.4% (w/v). The activated charcoal was added in different culture media provided for various stages of the mulberry tissue culture; the medium with 0.5 mg/l BA and 1.0 mg/l NAA for callus induction, the medium with 1.0 mg/l BA and 0.5 mg/l NAA for callus proliferation, the medium with 1.0 mg/l BA and 0.025 mg/l NAA for shoot regeneration, the medium with 0.25 mg/l BA and 0.025 mg/l NAA for shoot growth, and 0.5 modified MS medium with 0.1 mg/l IBA for root formation.

The culture environment for all growth *in vitro* was maintained at temperature 25±2°C, illuminated by cool-white fluorescent lamps at the light and intensity between 3000-4000 lux with a 16 hour-light and 8 hour-dark periods alternately.

TABLE 1 Basic ingredients of the modified Murashige and Skoog (MS) medium.

| Ingredients | mg/l |
|---|---|
| $(NH4)_2\ NO_3$ | 1,650 |
| $KNO_3$ | 1,900 |
| $CaCl_2.2H_2O$ | 440 |
| $MgSO_4.7H_20$ | 370 |
| $KH_2PO_4$ | 170 |
| $FeSO_4.7H_2O$ | 27.8 |
| $Na_2EDTA$ | 33.6 |
| $MnSO_4.4H_2O$ | 22.3 |
| $ZnSO_4.7H_2O$ | 8.6 |
| $H_3BO_3$ | 6.2 |
| KI | 0.83 |
| $Na_2MoO_4.2H_2O$ | 0.25 |
| $CuSO_4.5H_2O$ | 0.025 |
| $CoCl_2.6H_2O$ | 0.025 |
| myo-inositol | 100 |
| nicotinic acid | 1.0 |
| pyridoxine.HCl | 1.0 |
| thiamine.HCl | 10.0 |
| saccharose | 20,000 |

TABLE 2 The effects of 16 combinations of BA and NAA on callus formation of young leaf explants of *M. alba* var. S54 x *M. alba* var. Noi after culture for 4 weeks.

| NAA (mg/l) | Callus formation (%) | | | |
|---|---|---|---|---|
| BA (mg/l) | 0 | 0.5 | 1 | 2 |
| 0 | 0 | 40 | 60 | 40 |
| 0.5 | 20 | 40 | 80 | 40 |
| 1 | 20 | 40 | 80 | 40 |
| 2 | 0 | 20 | 40 | 20 |

When roots were fully developed (3 to 4 weeks rooting period), the plantlets were uprooted. The agar was removed and the plantlets were transferred to clay pots containing autoclaved vermiculite and covered with plastic bags. They were irrigated with complete fertilizer every week and sufficient amount of water every day. The pots were kept in the culture room under the same condition as the culture vessels for 4 weeks and during this period the plastic covers were punched. Then they were transferred to clay pots with soil in the green house.

## RESULTS AND DISCUSSION

When the stem segments were placed on BM medium containing 1.0 mg/l BA, multiple shoot formations occurred within 4 weeks (Fig. 1). Shoots derived from this stage were multiplied on the same medium which produced numerous shoots in a few weeks. Young leaves were used as a source of explant to establish callus culture. The advantages of using only leaf explants were 1) many explants can be derived from a single shoot and 2) cytological and morphological studies of regenerants and the mother plants are available. Culturing the explants on the medium in the presence of 0.5-1 mg/l BA and 1.0 mg/l NAA callus formation, whereas the other combinations of BA and NAA produced the lower callus formation (Table 2). The optimum callus proliferation was obtained when transferring callus to the medium with 0.5 mg/l BA plus 1 mg/l NAA (Fig. 2). Moreover, the addition of coconut water in the culture medium during the callus initiation stage enhanced fast growing callus. However, coconut water was omitted during the callus proliferation stage due to the high production of browning substances.

The investigation of the optimum ratio of BA and NAA on adventitious shoots induction revealed that 2.0 mg/l NAA inhibited shoot regeneration absolutely. Similarly, no shoot formation occurred on the medium containing only NAA. The most effective combination of BA and NAA for shoot regeneration was 1.0 mg/l BA plus 0.025 mg/l NAA (Table 3 and Fig. 3). The result of this study was different from the experiment of Oka and Ohyama[5] as well as Mhatre *et al.*[6] Oka and Ohyama reported the formation of shoot buds directly from leaves without petioles of *Morus alba* cultured on MS medium supplemented with 1.0 mg/l BA. Whereas Mhatre *et al.* derived shoot buds regeneration directly and via callus from leaves of *Morus indica* which were presoaked in MS medium containing 2.0 mg/l BA before culturing on the medium of the same composition.

For shoot growth, it was necessary to excise the small shoots from the callus mass and transferred them to the medium containing 0.25 mg/l BA or combined with 0.025 mg/l NAA. The medium containing only 0.1 mg/l NAA or combining with 0.125, 0.25 and 0.5 mg/l BA caused the formation of soft yellowish-brown callus at the cut ends of the shoot. This callus was removed before transferring to root induction medium.

The formation of root was achieved by subculturing shoots on the root induction medium for 3-4 weeks. The best rooting was found on the half-strength modified MS medium supplemented with 0.1 mg/l IBA (Table 4 and Fig. 4); roots were formed at the basal parts of the shoots. The auxins IBA and NAA stimulated root numbers, but they did not exert any significant effect on root elongation. The half-strength modified MS medium

TABLE 3 The effects of 20 combinations of BA and NAA on shoot formation from callus of *M. alba* var. S54 x *M. alba* var. Noi after culture for 4 weeks.

| NAA (mg/l) / BA (mg/l) | Shoot formation (%) | | | | |
|---|---|---|---|---|---|
| | **0** | **0.025** | **0.05** | **0.1** | **0.2** |
| 0 | 0 | 0 | 0 | 0 | 0 |
| 0.25 | 10 | 10 | 10 | 0 | 0 |
| 0.5 | 40 | 60 | 40 | 40 | 0 |
| 1 | 60 | 80 | 60 | 40 | 0 |
| 2 | 40 | 40 | 20 | 10 | 0 |

TABLE 4 The effects of culture media and auxins on root formation in *M. alba* var. S54 x *M. alba* var. Noi after culture for 4 weeks.

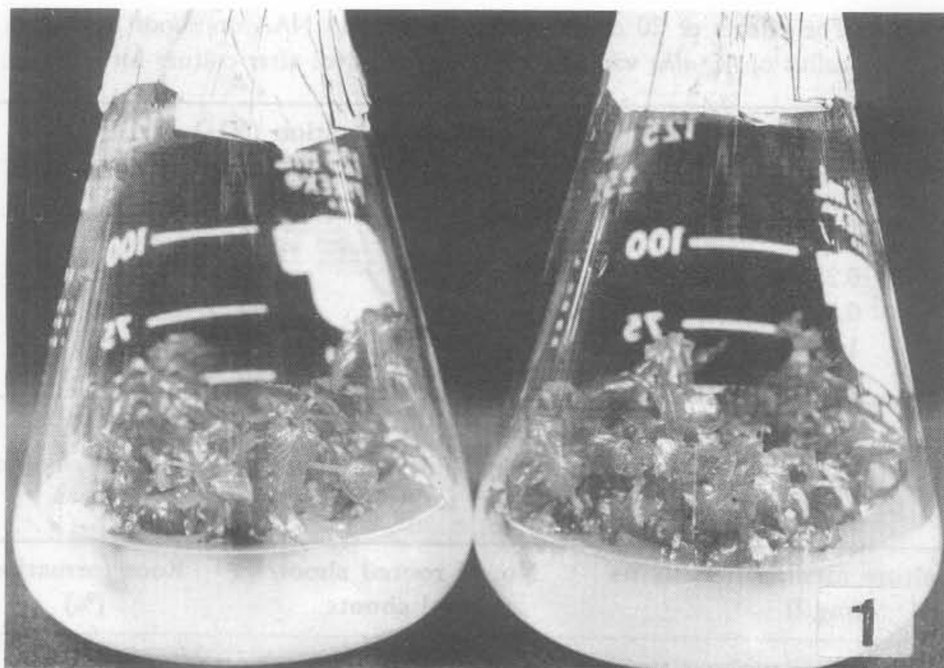
| Culture media plus auxins (mg/l) | No. of rooted shoot/ total shoots | Root formation (%) |
|---|---|---|
| mMS medium without auxin | 9/20 | 45 |
| 1/2 mMS | 14/20 | 70 |
| 1/2 mMS + 0.1 mg/l IBA | 18/20 | 90 |
| 1/2 mMS + 0.2 mg/l IBA | 16/20 | 80* |
| 1/2 mMS + 0.1 mg/l NAA | 15/20 | 75* |
| 1/2 mMS + 0.2 mg/l NAA | 11/20 | 55* |

Note: mMS medium = modified MS medium
1/2 mMS = half-strength modified MS medium
* The soft brownish callus formed at the base of shoots.

TABLE 5 The effects of activated charcoal on tissues culture growth of *M. alba* var. S54 x *M. alba* var. Noi after 4 weeks of culture.

| Activated charcoal % (w/v) | Mulberry tissues growth | | | | |
|---|---|---|---|---|---|
| | Leaf callus induction period | Callus proliferation period | Shoot induction period | Shoot growth period | Root formation period |
| 0 | ++++ | ++++ | ++++ | ++ | ++ |
| 0.05 | ++ | +++ | ++ | ++ | ++ |
| 0.1 | + | ++ | + | ++++ | ++++ |
| 0.2 | – | + | – | + | ++ |
| 0.4 | – | + | – | – | + |

\+ = explant growth
– = no explant growth



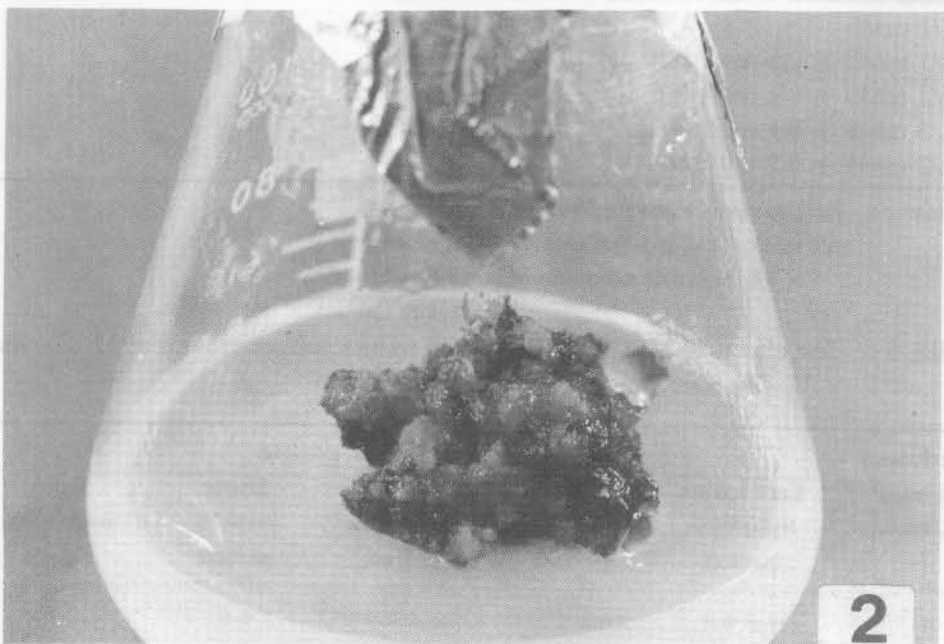




**Fig. 1.** Multiple shoot formation derived from stem segments grown on BM medium containing 1.0 mg/l BA.

**Fig. 2.** Callus tissues obtained from young leaf explant grown on the medium containing 0.5 mg/l BA and 1.0 mg/l NAA.

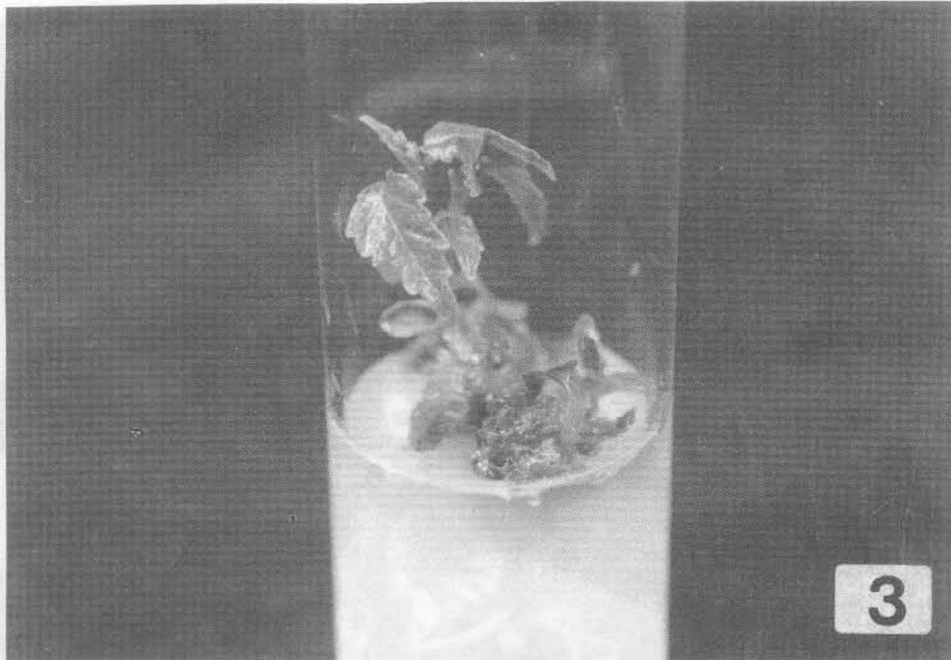
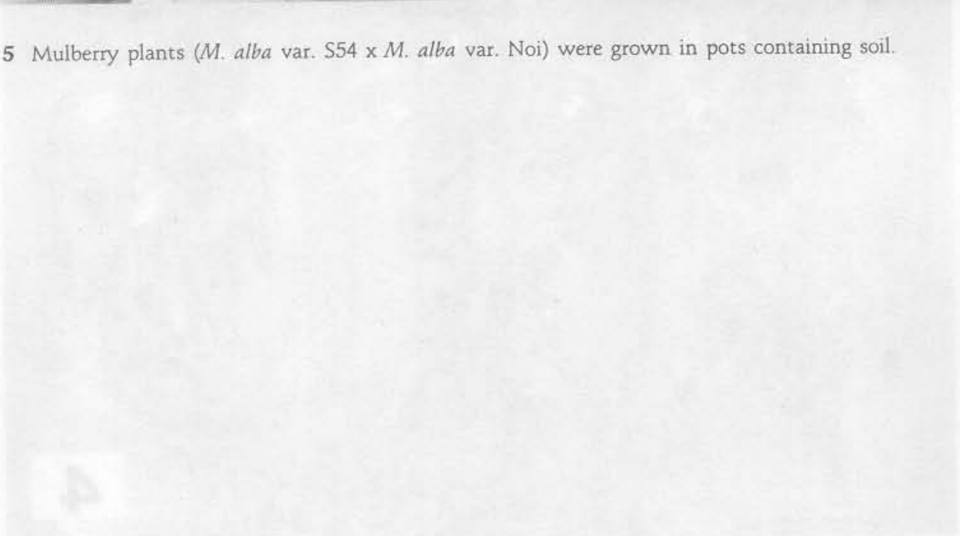


**Fig. 3.** Shoot regeneration from callus on the medium containing 1.0 mg/l BA and 0.025 mg/l NAA.

**Fig. 4.** Root formation occurred when shoot was cultured on half-strength BM medium supplemented with 0.1 mg/l IBA.

Fig. 5 Mulberry plants (*M. alba* var. S54 x *M. alba* var. Noi) were grown in pots containing soil.



without hormone enhanced lower rooting percentage than a medium containing IBA but stimulated root .elongation. The culture media with 0.2 mg/l IBA, 0.1 and 0.2 mg/l NAA caused a root formation with soft brownish callus formation at the cut ends of shoots. The formation of callus led to root breaking when plantlets were transferred from agar medium. The results also showed that IBA was more effective for root formation than NAA in all concentrations tested.

Browning substances were the major problem of *M. alba* var. S54 x *M. alba* var. Noi tissue culture which always occurred along with some kinds of cultured explant. The occurrence of these substances caused tissue death. A chemical analysis of the browning substances which occurred in the plant tissue culture showed that they were of phenolic compounds[9]. The cause of browning substances production may be due to damage of the tissue by dissection and the culture medium containing high sugar content and/or coconut water.

To solve this problem, various levels of activated charcoal were tested. The results showed that all of the explants that were cultured on the medium without activated charcoal required frequent subculture every 7 to 10 days to avoid the release of browning substances. In the addition of 0.1% (w/v) activated charcoal, the subculture period should be proceeded for every 2 to 3 weeks. However, lower or higher levels of activated charcoal did not improve mulberry tissues growth. The reduction of the concentration of activated charcoal to 0.05%, the subculture interval was not different from the medium without this substance, while 0.2% and 0.4% activated charcoal caused poor mulberry tissue growth. The use of activated charcoal at the proper stage of culture was also important. From the experiment, it appeared that during callus induction, callus proliferation and shoot induction processes, activated charcoal was not necessary to be supplemented in the culture medium. Addition of activated charcoal contributed to the decline of plant tissue growth and vice versa in shoot growth and root formation (Table 5). Although 0.1% activated charcoal was available, however, transferring of the explants to a fresh medium when browning appeared to be better in preventing tissue death.

Approximately 80% of the plants survived when the roots were induced on half-strength modified MS medium without hormone or in the presence of 0.1 mg/l IBA. Plants cultured on the medium containing 0.2 mg/l IBA and 0.2 mg/l NAA, frequently formed friable callus at the cut ends of shoots and about 20-30% of plants survived from acclimation process. The success of transplanting and survival of potted plants depends on the quality and number of roots (Fig. 5). All of the mulberry plants in the clay pots from the green house have been grown in the trial field for further study.

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## บทคัดย่อ

ใบอ่อนของหม่อนพันธุ์ผสม *Morus alba* var. S54 x *Morus alba* var. Noi ได้จากยอดอ่อนของต้นหม่อนที่เพาะเลี้ยงไว้ในขวดทดลอง นำใบอ่อนมาเลี้ยงบนวุ้นอาหารสูตรดัดแปลง MS ที่ใส่ฮอร์โมน BA และ NAA ความเข้มข้นต่าง ๆ กัน ความเข้มข้นที่เหมาะสมของ BA 0.5 มก./ลิตร และ NAA 1.0 มก./ลิตร ชักนำให้เกิดเนื้อเยื่อแคลลัส และความเข้มข้น BA 1.0 มก./ลิตร และ NAA 0.5 มก./ลิตร ทำให้เนื้อเยื่อแคลลัสเจริญเติบโตดี เนื้อเยื่อแคลลัสที่เลี้ยงบนวุ้นอาหารที่มี BA 1.0 มก./ลิตร และ NAA 0.025 มก./ลิตร ชักนำให้เกิดต้นอ่อน นำต้นอ่อนเหล่านี้มาเลี้ยงบนวุ้นอาหารที่มี BA 0.25 มก./ลิตร ทำให้ต้นอ่อนเจริญเติบโตดี การชักนำให้ต้นอ่อนเกิดรากโดยนำต้นอ่อนมาเลี้ยงบนวุ้นอาหารที่มีสารอาหารเพียงครึ่งเดียว โดยใส่ฮอร์โมน IBA 0.1 มก./ลิตร การใส่ถ่าน 0.1% สามารถดูดสารสีน้ำตาลที่เกิดขึ้นในระยะที่ต้นหม่อนมีการเจริญเติบโตและเกิดรากได้ นำต้นอ่อนของหม่อนลงปลูกในเวอร์มิคิวไลท์ที่อบฆ่าเชื้อแล้ว และปลูกลงในกระถางดิน เมื่อต้นหม่อนสูงประมาณ 1-1.5 ฟุต จึงนำไปปลูกในแปลงทดลอง