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UTILIZATION OF TISSUE CULTURE TECHNIQUE FOR PROPAGATION OF *MELIA AZEDARACH*

SARINEE CHAICHAROEN[a], SURIYA JANSAENGSRI[b], THATCHAI UMPRAI[c] AND MALEEYA KRUATRACHUE[a]

[a] *Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.*

[b] *Roi-ed Sericultural Experiment Station, Roi-ed, Thailand.*

[c] *Ministry of Forestry, Bangkok, Thailand.*

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ABSTRACT

The modified LS basal medium used in this study was composed of macronutrients, micronutrients from LS medium, vitamins of B5 proposed by Gamborg, and 10% coconut water. The explants of Melia azedarach *used in this experiment were young leaf, petiole, internode, terminal bud and axillary bud from young plants. The optimum concentration of BA for multiple shoot induction from axillary and terminal bud was 1 mg/l. Callus cultures could be readily initiated and proliferated from petiole and internode in the basal medium supplemented with 1 mg/l NAA plus 1 mg/l BA. The derived callus cultured on basal medium supplemented with 1 mg/l BA regenerated shoots which had a vigorous growth in basal medium with 0.25 mg/l BA. The individual shootlets regenerated vigorous roots within 7-10 days after immersion of the basal cut end into the solution of 1 mg/l IAA and 1 mg/l IBA for 1 day, then transferred to basal medium; 85% of the treated shoots rooted. One-month-old plantlets were then transplanted into pots filled with sterilized vermiculite under high humidity conditions. The survival percentage was about 70%. Complete plants were grown in the field.*

INTRODUCTION

Melia azedarach are fast growing trees, suitable for forestry orchard plantation because of their industrial economical value, short cycle of harvesting and tolerance to dry climatic conditions. The timber is useful and pretty. It can be beautifully varnished and sometimes can be sold as cedar-wood.[1] All parts of this tree including trunk, bark, leaf, fruit and seed are useful. It gives shade and is also a beautiful ornamental plant. Because of its value for many purposes, it has been classified as one of the multipurpose forest tree species[2] and might be one of the most important species in the future. It was in the first priority in forestry orchard plantation for producing raw material supply, veneer or plywood.

Some difficulties encountered in establishing forest plantation of *Melia azedarach* are unavailability of sufficient planting stocks, the low germination capacity of seeds and the very rare occurrence of promising trees. In general, *Melia azedarach* can be propagated through seedling but seeds selected from promising trees are very rare due to the serious destruction of natural forest and low percentage of seed germination which is less than 10%.[3] The multiplication of planting stock through vegetative propagation by using T-budding, root propagation or stem cutting is still a limit success and not suitable for large scale plantation. However, utilization of tissue culture in tropical forestry is still in its early developmental stage. Gupta *et al.*[4] had reported the propagation of 100 year-old teak trees by starting with the culturing of terminal buds and axillary buds on Murashige and Skoog (MS) media with 0.1 μg/ml kinetin and 0.1 μg/ml Benzyl adenine (BA). After shoot initiation and elongation,

rooting was accomplished by transferring to White's medium with indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and indole-3-propionic acid (IPA) each at 2 μg/ml for 48 hours and then to White's basal medium. Rooted plantlets were subcultured and additional shoots and plantlets were obtained from the axillary buds. Satrabhandhu and Chaicharoen[5] reported the possibility of mulberry mass propagation (Morus alba var. S54) through culture of young leaves. Tissue culture of *Melia azedarach* has had limitted success. Sanyal *et al.*[6] induced callus from young stem bark of *Melia azedarach* in MS plus 0.1-0.5 mg/l of IAA and BA in darkness. IAA at 0.5 mg/l induced rooting in 40% of tissue culture. Domecq[7] concluded that shoot tip explants of *M. azedarach* var. *gigantia* could be established on a modified MS basal medium with supplements of BA, IBA and gibberllins (GA3). Bud multiplication was favoured by transferring to MS medium without IBA. The best rooting response was obtained with a liquid, half strength basal medium containing IBA as the only growth regulator. After one week of high humidity in a greenhouse, the plantlets could survive and were transferred to the field.

Reports on the use of tissue culture in forest trees are slowly increasing but further development and research need to be explored. Therefore, the present study was conducted towards the utilization of tissue culture for planting stock production of *Melia azedarach*.

METERIALS AND METHODS

The young leaves, petioles, internodes, axillary buds and terminal buds from promising seedlings (8 inches in height) of *Melia azedarach* selected from Roi-ed Provincial Forestry Office were washed with running tap water for 20 mins. The surface were sterilized by immersing in 70% ethanol for 20 secs and then were rinsed 3 times with distilled water. Next, the samples were shaken in 10% Clorox solution with 2 drops of Tween-20 for 15 mins. in Laminar air flow and then rinsed 3 times with sterilized distilled water. They were dissected into small pieces of 0.5-1 cm in length and transferred onto the modified Linsmaier and Skoog (mLS) medium which comprised of macronutrients and micronutrients of LS medium,[8] the vitamin mixture proposed by Gamborg *et al.*[9] in B5 medium, 6 g/l agar and 10% coconut water (Table 1).

The auxins used in this experiment were NAA, IAA, and IBA, the cytokinin used was 6-benzyladenopurine (BAP). The pH was adjusted to 5.7 before autoclaving for 15 mins. at 121° C under pressure of 15 lb/in.^{2}. The culture room for all growth in vitro was maintained at 25 ± 2° C. The light intensity was between 3000-4000 lux from cool-white fluorescent with a 16-hour light period.

The terminal buds and axillary buds were cultured on the modified LS basal medium supplemented with 0, 0.5, 1, 2 mg/l BA for multiple shoot production. The developed shoots were excised and transferred to a fresh medium of the same composition.

Callus cultures were initiated from the young leaves, internodes and petiole. The explants were cultured on the modified LS basal medium supplemented with the combinations of naphthalene acetic acid (NAA) (0. 0.5, 1, 2 mg/l) and BA (0, 0.5, 1, 2 mg/l). The derived callus was maintained by subculturing at 2 week intervals. After 3 passages, callus was transferred onto the modified LS basal medium supplemented with the combinations of NAA (0, 0.5, 1, 2 mg/l) and BA (0, 0.5, 1, 2 mg/l) to induce shoot regeneration. Callus with small shoots differentiated were transferred to the basal medium supplemented with the combinations of NAA (0, 0.25, 0.5, 1 mg/l) and BA (0, 0.25, 0.5, 1 mg/l) for shoot growth and shoot elongation.

The young shoots of 3-5 cm in height were excised at the base individually and transferred

to the following medium to induce rooting:

(a) Transferring young shoots to the modified LS basal medium or half strength of modified LS basal medium.

(b) Immersing the base cut end of young shoots in the individual combination of sterilized growth regulators IAA and IBA which were filtered through a millipore filter of 0.22 (m at various periods of time before being transferred to the hormone-free modified LS basal medium to investigate rooting.

(c) Transferring the young shoots to modified LS basal medium containing equal amount of NAA and IBA at levels of 0.1, 0.25 and 0.5 mg/l.

The plantlets with full root development (3-4 weeks rooting period) were uprooted and washed free of agar, then transplanted in clay pots which contained autoclaved vermiculite and covered with a plastic bag. They were watered every day and complete fertilizer solution was applied every 7 days. The pots were kept in the culture room for 4 weeks, before they were transferred to larger clay pots filled with soil and kept in the greenhouse.

RESULTS

Multiple shoots were developed when cultured on the modified LS medium supplemented with 0.5-2 mg/l BA (Table 2). The shoot numbers and shoot development from bud explants cultured on the medium containing 1 and 2 mg/l BA were greater than those on the medium containing 0.5 mg/l BA. Multiple shoots obtained from the buds cultured on the medium supplemented with 2 mg/l BA were small and unhealthy. The percentages of multiple shoot formation from axillary bud and terminal bud on the medium supplemented with 1 mg/l BA were 56.7% and 36.7%, respectively. These shoots were healthy and more satisfactory.

The tested explants, young leaves, petioles and internodes cultured on the modilied LS medium supplemented with 0.5-1 mg/l NAA and 0.5-1 mg/l BA, all became swollen and showed some callus initiation within 7-10 days. Later, some showed further growth, but others exhibited only slow growth or ceased growing. After 4 weeks of culturing, most of the callus from young leaf explants turned yellow and brown. Some leaf explants on the medium with 1 mg/l NAA plus 1 mg/l BA showed root formation. Callus mass was obtained from petiole and internode explants. The growth of callus formed on the modified LS medium supplemented with 1 mg/l NAA plus 1 mg/l BA (Fig. 1A) was better than the callus on the medium containing equal concentrations of NAA and BA at the level of 0.5 mg/l and 2 mg/l, respectively. No callus formation was observed from explants cultured on the medium without any growth regulator or with 2 mg/l BA (Table 3).

The investigation of the optimal ratio of NAA and BA on adventitious shoot induction revealed that the basal medium with 2 mg/l NAA plus 2 mg/l BA inhibited shoot regeneration absolutely. Moreover, no shoot formation occurred on the medium contained only NAA. The most effective medium for shoot regeneration was modified LS basal medium supplemented with 1-2 mg/l BA, while the higher the concentration of BA, the greater the number of shoots regenerated, but the more difficult to enhance growth of the shoots induced as well. The suitable medium for shoot regeneration with healthy growth was modified LS basal medium supplemented with only 1 mg/l BA, which gave an average of nine regenerated shoots (Fig. 2).

The shoots regenerated from callus cultured on the modified LS basal medium containing 0.25 mg/l BA showed good growth and elongation when compared with the other combinations. Shoots of 3-5 cm in height could be obtained within 6-8 weeks of culture (Fig. 1B). Some

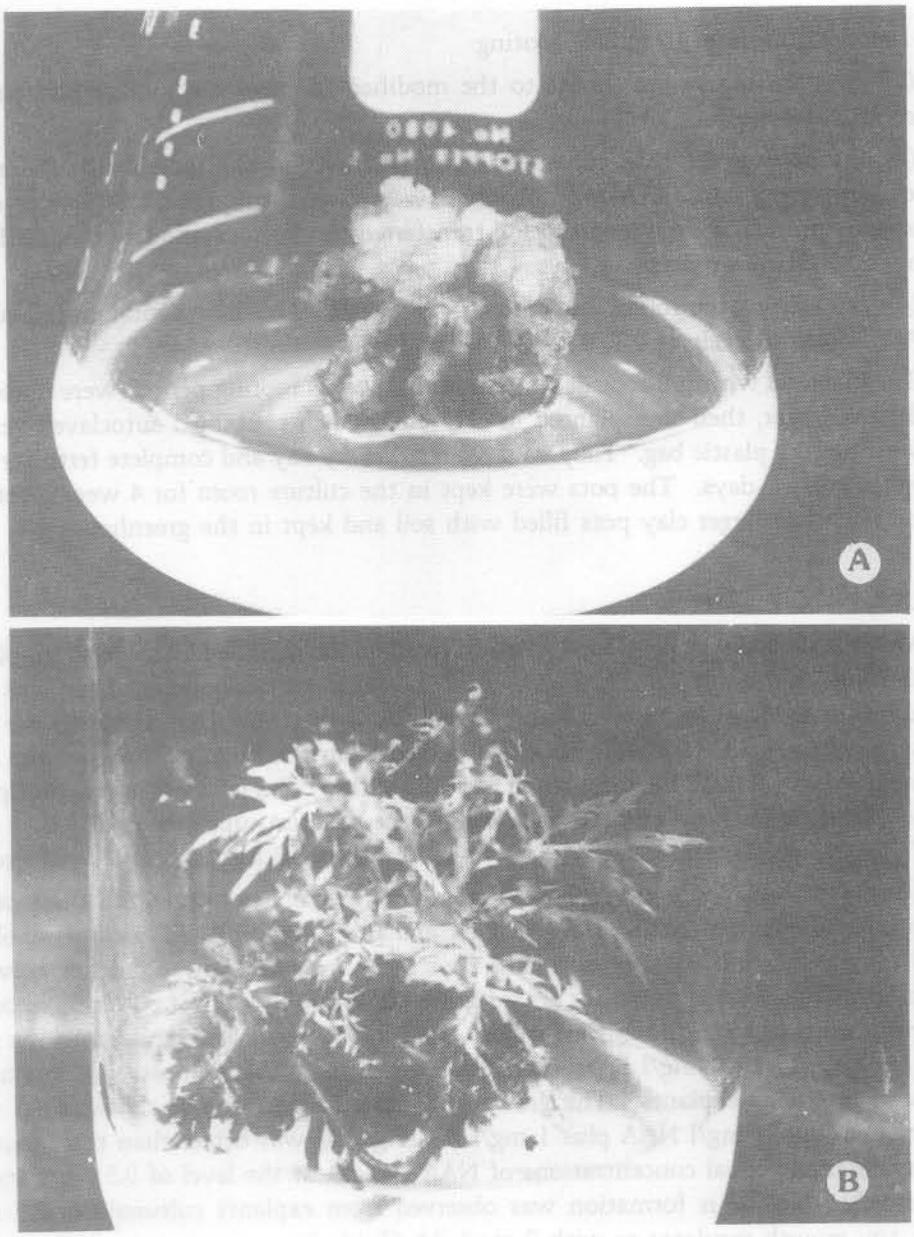

Fig. 1. A. Callus of 2 months old from internode explant cultured on mLS basal medium.

B. Shoot regeneration from callus on mLS medium with 1mg/l BA.

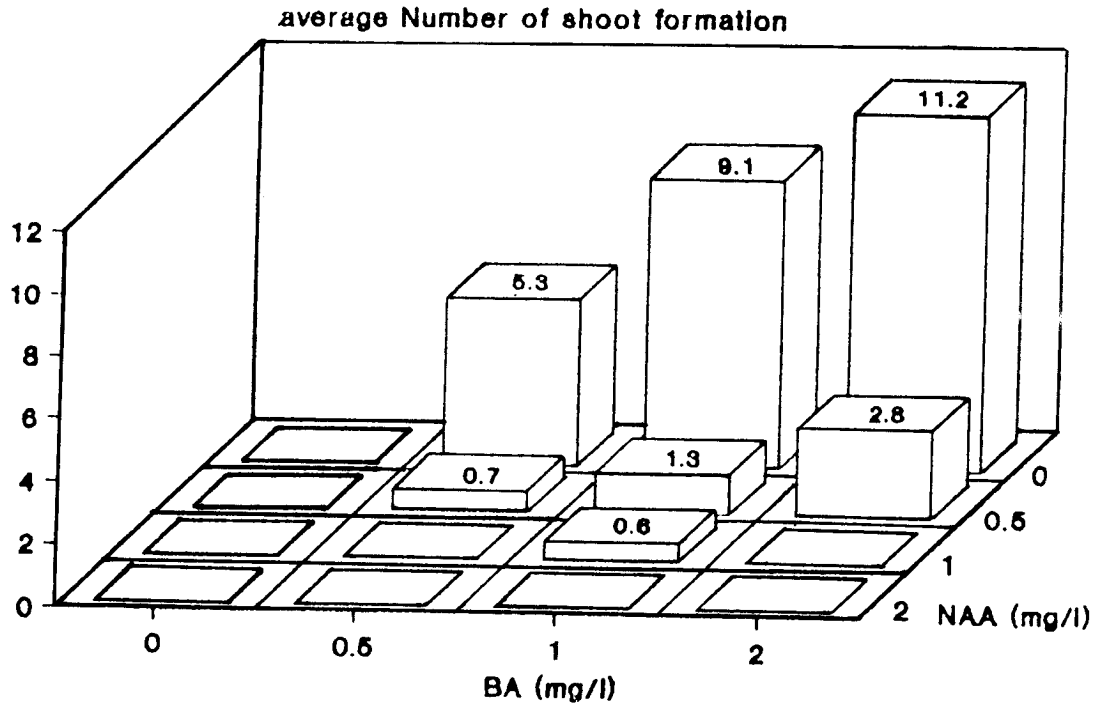

Fig. 2. The effect of NAA and BA on shoot formation from callus of *Melia azedarach* after culture for 4 weeks.

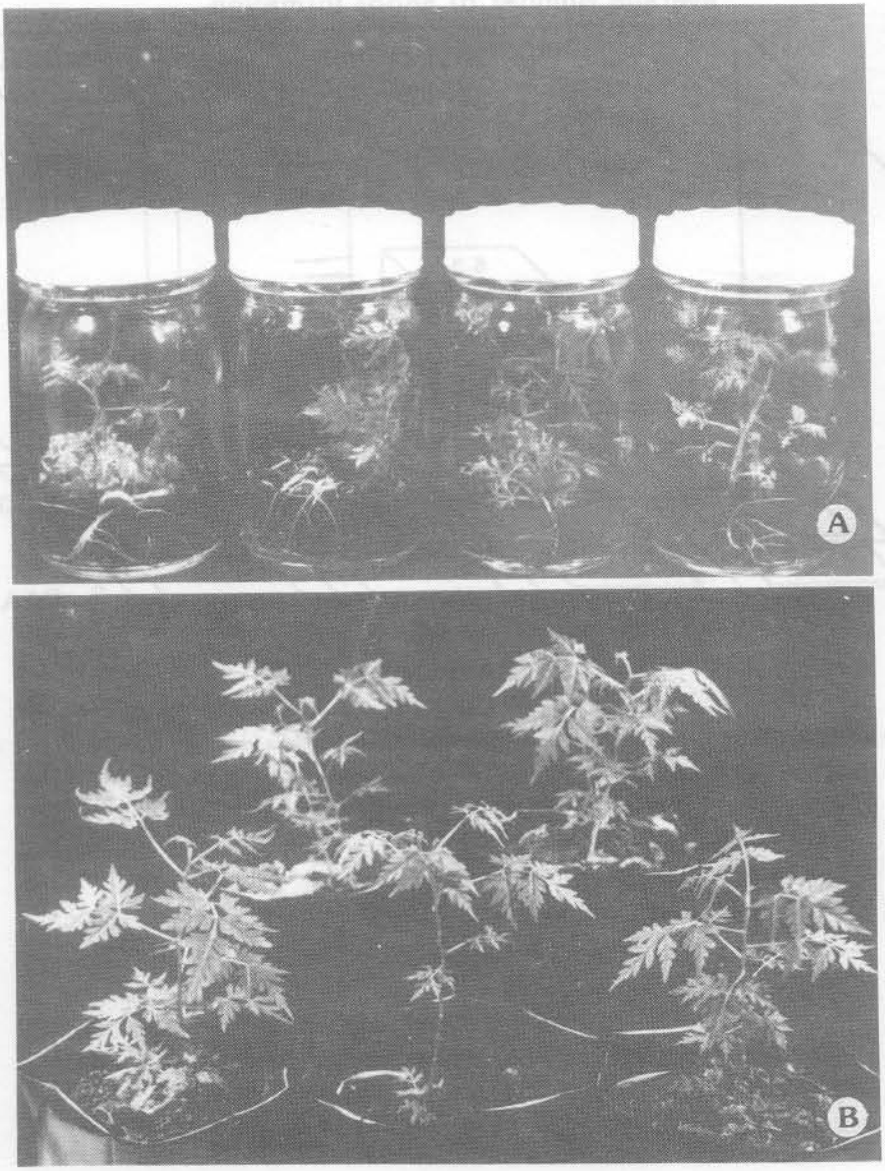

Fig. 3. A. Plantlets of 4 weeks old cultured on mLS basal medium with healthy roots in the medium.

B. *Melia* plantlets grown in the pots filled with soil and kept in the greenhouse before transplanting to the field.

multiple shoots were formed at the basal part of shoots cultured in the medium containing 1 mg/l BA and 0.25 mg/l NAA plus 1 mg/l BA. After 4 weeks of the experiments, no root formation occurred from the shoots cultured on either full-strength modified LS basal medium or half-strength modified LS basal medium even with the addition of activated charcoal 0.5-1 g/l.

The individual shoots were immersed 1-3 days in the combined solution of IAA and IBA at various concentrations of 0, 0.25, 0.5 and 1 mg/l before transferring to modified LS medium. The combination of 1 mg/l IAA and 1 mg/l IBA for 1 day immersion induced rooting in 7-10 days. This combination gave the highest percentage of root induction which later produced many long and healthy roots (Fig. 3A) in modified LS basal medium. The results also showed that IBA was more effective than IAA in root induction. Decreasing the immersion period from 24 hours to 1, 3, 5, 7, 10 or 18 hours and treating with higher concentrations of IAA and IBA (1, 1.5 and 2 mg/l each) proved less effective in causing root induction than treatment in solutions of 1 mg/l IAA plus 1 mg/l IBA for 24 hours.

The semi-solid cultured media that contained NAA and IBA caused callus formation at the base cut end of the shoots. Media containing higher doses of auxin induced higher percentages of rooting (Table 4) but all the induced roots were short, big and stunted with no growth appearing even if transferred to fresh basal medium immediately when root primordia appeared in 7-10 days. Successful rooting of the shoots was achieved in about 85% of the cases. The plantles were grown in sterilized vermiculite in the culture room at temperature 25±2° C. About 70% of the plants survived (Fig. 3B) and were grown in Northeast Thailand.

DISCUSSION

From the present study, the modified LS basal medium containing 1 mg/l BA was fround to be optimum for multiple shoots production of *Melia azedarach* from vegetative bud explants. The result corresponded to that of Demecq[7] cultured shoot tips of *M. azedarach* on the MS medium with BA and GA3. An average of 4 shoots per bud explant can be produced, showing high potential to reproduce numerous shoots directly within 3 months. The advantage of this technique is evident that the axillary shoot system is genetically the most stable one and only root induction is required for plantlet formation.[10] The use of petiole and internode as explants to induce multiple shoots through callus culture showed that modified LS basal medium containing 1 mg/l NAA was optimum for callus induction and proliferation. Young leaf explants cultured in this medium showed some callus initiation and some regenerated roots at the cut end of the midrib, but no distinct callus mass was observed because leaf explants had tiny parenchymatous cells.

From the present study, the long term culture of all explants presented some problems in the occurrence of browning substance released into the medium which inhibited tissue growth especially for leaf explants. The more mature the explants used, the more browning substance was released. These substances are classified into phenolic compounds. In this experiment, adding activated charcoal into the medium and frequently subculture to fresh medium could inhibit browning substance formation.

The study on the long term culture of *Melia azedarach* callus showed that callus older than 3 months was difficult to induce shoot but callus of 2 months old was optimum for shoot induction. The length of time in culture prior to shoot differentiation leads to increases in genetic aberrations of one sort or another leading to subsequent differences in growth response and/or expression of morphological traits.

TABLE 1. The components of modified LS basal medium for tissue culture of *Melia azedarach*.

	mg/l		mg/l
NH_4NO_3	1,650	Na_2EDTA	37.3
KNO_3	1,900	$FeSO_4.7H_2O$	27.8
$CaCl_2$ $2H_2O$	440	myoinositol	100.0
$MgSO_4.7H_2O$	370	nicotinic acid	1.0
KH_2PO_4	170	pyridoxine-HCl	1.0
H_3BO_3	6.2	thiamine-HCl	10.0
$MnSO_4$ $4H_2O$	22.3	sucrose	20 g/l
$ZnSO_4.4H_2O$	8.6	coconut water	10%
KI	0.83	pH	5.7
$Na_2MoO_4.2H_2O$	0.25		
$CuSO_4.5H_2O$	0.025		
$CoCl_2.6H_2O$	0.025		

TABLE 2. The effect of BA on multiple shoot formation from axillary buds and terminal buds after 4 weeks of culture.

BA (mg/l)	Multiple shoot formation (%)	
	axillary bud	terminal bud
0	0	0
0.5	33.3	20.0
1	56.7	36.7
2	46.7	30.0

Table 3. The effect of NAA and BA on callus formation of internode and petiole after 4 weeks of culture.

NAA (mg/l) / BA (mg/l)	Callus formation (%)			
	0	**0.5**	**1**	**2**
0	0	20	20	10
0.5	10	60	20	20
1	10	10	80	20
2	0	10	10	60

Table 4. The effect of culture media and auxins on root formation in *Melia azedarach* after culture for 4 weeks.

Culture media plus auxins (mg/l)	No. of rooted shoot / Total shoot	Rooting (%)
mLS+0.1 mg/l NAA +0.1 mg/l IBA	1/20	5
mLS+0.25 mg/l NAA +0.25 mg/l IBA	5/20	25
mLS+0.5 mg/l NAA +0.5 mg/l IBA	10/20	50

The differentiated shoot grew vigorously in shoot growth medium containing 0.25 mg/l BA and reached the optimum height of 4-5 cm within 6 weeks. The individual shoot immersed in the combination of 1 mg/l IAA and 1 mg/l IBA sterile solution for 1 day before transferring to modified LS basal medium produced vigorous and active roots within 10 days, while the others were less effective. From the combinations tested, IBA showed more effectiveness than IAA and NAA in root induction.

Propagation of *Melia azedarach* by conventional methods of breeding is time-consuming and often inefficient owing to low-seed set, poor germination and great variability. The advantage of using tissue culture technique to propagate this plant could be applied in order to overcome these barriers and may prove to be a short cut in reducing the normal life cycle which may take several months. In this experiment we obtained high yield production of plantlets of *Melia azedarach*.

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บทคัดย่อ

วุ้นอาหารสูตรดัดแปลงที่ใช้ในการทดลองนี้ประกอบด้วยธาตุอาหารตามสูตรอาหารของ Linsmaier และ Skoog โดยใช้วิตามินตามสูตรอาหาร B5 ของ Gamborg และเติมน้ำมะพร้าว 10% จากการทดลองใช้ชิ้นส่วนของใบอ่อน ก้านใบ ปล้อง ตายอดและตาข้างของต้นอ่อนเลี่ยน พบว่าสูตรอาหารที่มี BA 1 มิลลิกรัม/ลิตร ชักนำให้เกิด multiple shoot ในตายอดและตาข้าง สูตรอาหารที่เหมาะสมในการชักนำให้ก้านใบ ปล้อง เกิดแคลลัสและเพิ่มปริมาณแคลลัสคือสูตรอาหารที่เติม NAA 1 มิลลิกรัม/ลิตร ร่วมกับ BA 1 มิลลิกรัม/ลิตร เมื่อย้ายแคลลัสมาเลี้ยงบนวุ้นอาหารที่มี BA 1 มิลลิกรัม แคลลัสสามารถเจริญเป็นต้นอ่อน ซึ่งเจริญเติบโตดีในสูตรอาหารที่ลด BA ลงเหลือ 0.25 มิลลิกรัม/ลิตร ต้นอ่อนขนาด 4-5 ซม. เมื่อนำมาชักนำให้เกิดรากด้วยวิธีต่างๆ พบว่าสามารถให้เปอร์เซนต์การออกรากสูงสุด 85% เมื่อแช่โคนต้นอ่อนในสารละลายที่มี IAA 1 มิลลิกรัม/ลิตร ร่วมกับ IBA 1 มิลลิกรัม/ลิตร นาน 1 วัน ก่อนย้ายมาเลี้ยงบนอาหารที่ไม่เติมสารควบคุมการเจริญเติบโต และสามารถออกรากได้ภายใน 7-10 วัน โดยมีรากยาวและแข็งแรง เมื่อต้นอ่อนอายุ 1 เดือน ย้ายลงปลูกในกระถางที่ใส่เวอมิคิวไลท์ รดน้ำทุกวันและรดปุ๋ยสัปดาห์ละ 1 ครั้ง คลุมด้วยถุงพลาสติกเพื่อให้มีความชื้นพอเหมาะ พบว่าต้นอ่อนที่ปลูกมีเปอร์เซนต์รอดประมาณ 70% ต้นเลี่ยนที่สมบูรณ์ได้นำไปปลูกในแปลงทดลอง

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