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Multiple Shoot Production In Vitro of the Tropical Timber Tree, Sentang (*Azadirachta excelsa* Linn.)

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Abstract. **Axillary buds from 5-month-old seedlings of *Azadirachta excelsa* Linn. were surface-sterilized twice with 1.35% (m/v) and 1.05% (m/v) of sodium hypochlorite for 25 and 15 minutes, respectively, before culturing on Murashige and Skoog (MS) medium containing combinations of BA and NAA. A combination of 4.4 μM BA + 0.5 μM NAA induced the most axillary buds to grow (eight per explant). Subsequent proliferation of the micropropagated shoots on this medium yielded abnormal shoots. The best medium for maximum proliferation of these micropropagated shoots contained 3.3 μM BA and 0.27 μM NAA. On this medium about four normal shoots were produced per explant. These findings indicate that two different media are needed for successful micropropagation of sentang. Chemical names used: N^6-benzylaminopurine (BA); 1-naphthaleneacetic acid (NAA).**

Azadirachta excelsa Linn., or sentang, is a timber species native to Borneo, and belongs to the family Meliaceae (Watson and Dallwitz, 1995). It is a fast-growing tree that can reach a height of 30 to 40 m with a diameter of 80 to 120 cm at 130-cm trunk height at 15 years of age. Sentang thrives on fertile and well-drained soil where the rainfall is 1.8 m or more per year. Mature trees flower once a year from February to April. Although sentang is routinely seed-propagated, asexual propagation methods are needed since sexual reproduction can result in unwanted genetic variation. According to Tan (personal communication), a sentang grower in Kedah, the viability of sentang seeds declines within 2 to 3 weeks of harvest, with germination rate dropping to between 50% and 75%. One of the difficulties encountered in establishing forest plantations of sentang is the lack of high-quality, uniform planting material.

In recent years, much progress has been made in the application of tissue culture methods to forestry improvement (Bonga and Durzan, 1987). For example, one of the nurseries in the United States has been actively producing plantlets of ≈180 azalea and rhododendron species and cultivars via tissue culture (Henke and Hughes, 1985). The immediate benefit that can be recognized via tissue culture technology is the ability to obtain a large number of propagules from a small number of elite trees. Trees can be produced more rapidly than with conventional propagation techniques. Moreover, tissue culture methods enable tailoring of production rates to the needs of the grower, since the propagules produced in vitro do not depend on in vivo vegetative cycles (Florino and Loreti, 1987).

The objective of this research was to propagate sentang by tissue culture and to determine the optimum medium and culture conditions for the induction of multiple shoots from axillary buds. A protocol to maintain sentang in vitro was also established.

Materials and Methods

Source and sterilization of explants. Tissues for the experiment were obtained from 5-month-old seedlings of sentang planted in polybags. Shoots ≈2 cm long were defoliated and surface disinfested by rinsing in running water, then washing in a solution of 2% (v/v) mild liquid detergent for 2 min. The shoots were then surface sterilized for 25 min under constant agitation in 1.35% (m/v) sodium hypochlorite solution to which a few drops of wetting agent (Tween 20) were added, and rinsed three times with sterile distilled water. Axillary buds were excised and resterilized with 1.05% (m/v) sodium hypochlorite for 15 min and rinsed three times with sterile distilled water. These axillary buds were used as explants for Expts. 1 and 2.

Expt 1. Effect of BA on multiple shoot formation. The explants were cultured in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing BA at 0.0, 8.8, 17.6, 26.4, 35.2, or 44.0 μM. The culture vessels used were glass tubes (15 × 2.5 cm) fitted with plastic caps. The experiment was conducted twice using a completely randomized design with 20 explants in each treatment. Cultures were placed under continuous lighting with cool-white fluorescent tubes at an intensity of 30 $\mu mol \cdot s^{-1} \cdot m^{-2}$ at 25 ± 2 °C. Explants were transferred to fresh media after 4 weeks, and the number of shoots per explant was recorded after 8 weeks of culture. Explants producing two or more shoots were classified as having multiple shoots.

Expt 2. Effect of BA and NAA combinations on multiple shoot formation. Procedures were identical with those used in Expt. 1 except for the concentrations of BA and NAA used.

Expt. 2a. In a preliminary study, the explants were cultured on MS media supplemented with BA at 0.0, 8.8, 17.6, or 26.4 μM and NAA at 0, 1, 2, 3, or 4 μM arranged in a factorial design for a total of 20 combinations. Twenty explants were used in each treatment and the experiment was conducted twice.

Expt. 2b. In a more detailed study, explants were cultured on MS media containing BA at 0.0, 2.2, 4.4, 6.6, 8.8, 11.0, or 13.2 μM and NAA at 0.0, 0.5, or 1.0 μM arranged in a factorial design, for a total of 21 treatments. Again, 20 explants were used per treatment and the experiment was repeated twice. Choice of these BA and NAA concentrations was based on the best results of the preliminary study.

Expt 3. Effects of BA and NAA on axillary shoot formation. Media containing combinations of BA at 0.0, 1.1, 2.2, or 3.3 μM and NAA at 0.0, 0.27, or 0.54 μM arranged in a factorial design were tested for their effects on micropropagated shoots obtained from the previous experiment. Culture vessels used were 100-mL conical flasks. Twenty explants were cultured per treatment and the experiment was carried out twice. Micropropagated shoots were also grown and transferred as in Expt. 1. The number of shoots produced was recorded after 8 weeks.

Statistical analysis. Data obtained from Expts. 1, 2, and 3 were analyzed using analysis of variance and the means compared using Duncan's multiple range test at $P = 0.05$. All the experiments that were repeated twice were treated as blocks, giving two replications (blocks) for each treatment. All percentage values were arcsin transformed for normal distribution prior to analysis (Zar, 1996).

Results and Discussion

Expt. 1. Effect of BA on multiple shoot formation. The number of shoots produced was significantly affected by BA concentration. In the absence of BA, few shoots were formed, with 66.7% of the explants forming callus while only 6.7% formed single shoots

Table 1. Effects of BA concentration on percentage of sentang explants forming shoots in vitro. (Expt. 1).

No. shoots	BA (μM)					
per culture	0.0	8.8	17.6	26.4	35.2	44.0
1	6.7	20.0	53.3	20.0	6.7	0.0
>1	0.0	6.7	13.3	0.0	0.0	0.0
Total	6.7	26.7	66.6	20.0	6.7	0.0

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(Table 1). Of the six initiation media tested, only two media containing BA at 8.8 and 17.6 μM induced multiple shoots. Twenty percent of the explants cultured on 8.8 μM BA produced single shoots and 6.7% produced multiple shoots. Values for the medium containing 17.6 μM were 53.3% and 13.3%, respectively. However, the shoots formed were abnormal or hyperhydric. Shoots were translucent and the leaves thickened, turgid, and brittle. Explants in medium containing 35.2 μM BA only formed 6.7% single shoots. At 44 μM, the presence of BA was inhibitory; shoots failed to form and explants only swelled with callus forming around the edges of the cut end. Therefore, the optimum concentration of BA to induce multiple shoot formation is 17.6 μM. These results suggest that lower BA concentrations are better for shoot initiation.

Expt. 2. Effect of BA and NAA combinations on multiple shoot formation. Expt. 2a. Culture medium with NAA alone had no significant effect on the percentage of explants forming multiple shoots (Fig. 1). No shoots were produced in medium devoid of BA. The optimum concentration for BA alone was at 8.8 μM; higher concentrations inhibited multiple shoot formation. The addition of NAA at 1 μM to BA at 8.8 μM gave a better effect on shoot proliferation. According to Lakshmi (1981), shoot apices of *Eucalyptus citriodora* cultured on medium supplemented with NAA and BA produced more vigorous and sturdy shoots. However, when a higher concentration of NAA was combined with BA at 8.8 μM, the production of multiple shoots declined. Out of the 20 media that were tested, only one (8.8 μM BA and 1 μM NAA) induced >50% of the explants to form multiple shoots; some of these explants also formed callus. Based on these results, the balance between auxin and cytokinin content in the medium needed further refinement for multiple shoot formation. A narrower range of BA and NAA concentrations was therefore tested.

Expt. 2b. No shoots formed in medium without BA or NAA (Table 2). NAA alone at 0.5 μM initiated shoot formation, but only a small percentage of the explants produced multiple shoots; at 1.0 μM NAA alone no shoots were formed (Fig. 2). When BA was used alone, the response increased as the concentration of BA increased to 11 μM. Adding NAA at 0.5 or 1.0 μM increased response to BA and the best response was at 4.4 to 8.8 μM BA. NAA at 0.5 μM combined with BA at 4.4 μM induced the highest number of explants to produce multiple shoots (eight shoots per explant). This medium seems to be the most suitable for producing multiple shoots using axillary buds as explants. The significant interaction (F values = 5.29*) of NAA and BA concentrations illustrated that a combination of both growth regulators was necessary to support maximum shoot performance and multiplication in sentang.

Expt. 3. Effects of BA and NAA on axillary shoot formation. When media supplemented with 4.4 μM BA and 0.5 μM NAA were used for subsequent subculture of the micropropagated shoots, abnormal shoots with curled and brittle

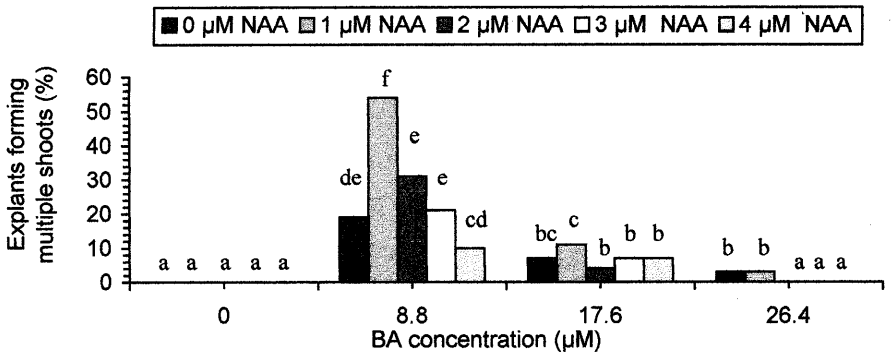

Fig. 1. Effects of BA and NAA on initiation of shoots from axillary buds of sentang explants grown for 8 weeks on Murashige and Skoog medium. F values for NAA, BA, and their interaction were 2.99^{ns}, 41.77**, and 9.52*. (^{ns,*,**}Nonsignificant or significant at $P \leq 0.05$ or 0.01. Bars followed by the same letter are not significantly different by Duncan's multiple range test, $P = 0.05$. Untransformed data shown.)

Table 2. Effects of combinations of BA and NAA on the number of shoots formed per axillary bud explant of sentang in vitro. (Expt. 2b).

NAA	BA (μM)						
(μM)	0.0	2.2	4.4	6.6	8.8	11.0	13.2
0.0	0.0 ± 0.0[z]	2.3 ± 0.5	2.7 ± 0.6	3.3 ± 0.8	2.7 ± 0.8	3.0 ± 0.8	2.7 ± 0.6
0.5	2.0 ± 0.0	3.1 ± 0.7	7.7 ± 1.5	3.9 ± 1.9	3.9 ± 1.1	3.8 ± 1.1	3.7 ± 1.1
1.0	0.0 ± 0.0	3.2 ± 1.4	4.4 ± 1.5	4.7 ± 1.2	3.7 ± 1.1	3.6 ± 0.9	3.0 ± 0.7

[z]Mean ± SD.

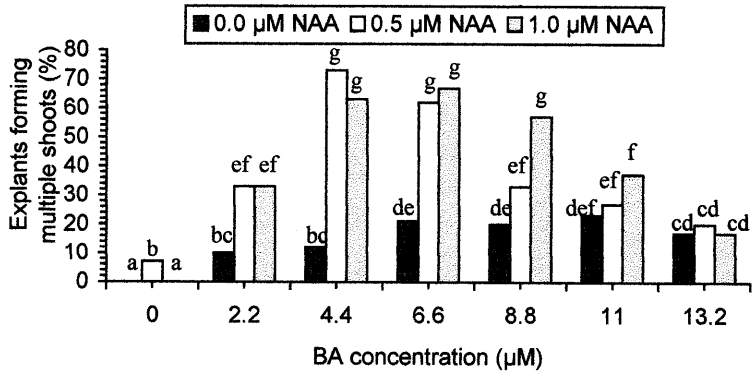

Fig. 2. Multiple shoot formation initiated from axillary bud explants of sentang cultured for 8 weeks on Murashige and Skoog medium with BA and NAA. F values for NAA, BA, and their interaction were 10.77**, 10.61**, and 5.29*. (^{ns,*,**}Nonsignificant or significant at $P \leq 0.05$ or 0.01. Bars followed by the same letter are not significantly different by Duncan's multiple range test, $P = 0.05$. Untransformed data shown.)

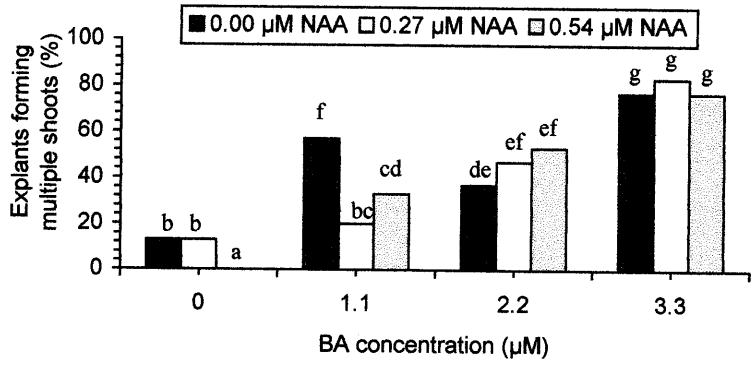

Fig. 3. The effect of BA and NAA combinations on sustained shoot multiplication of sentang grown for 8 weeks on Murashige and Skoog medium. F values were NAA 0.55^{ns}, BA 15.78**, and their interaction 6.921*. (^{ns,*,**}Nonsignificant or significant at $P \leq 0.05$ or 0.01. Bars followed by the same letter are not significantly different by Duncan's multiple range test, $P = 0.05$. Untransformed data shown.)

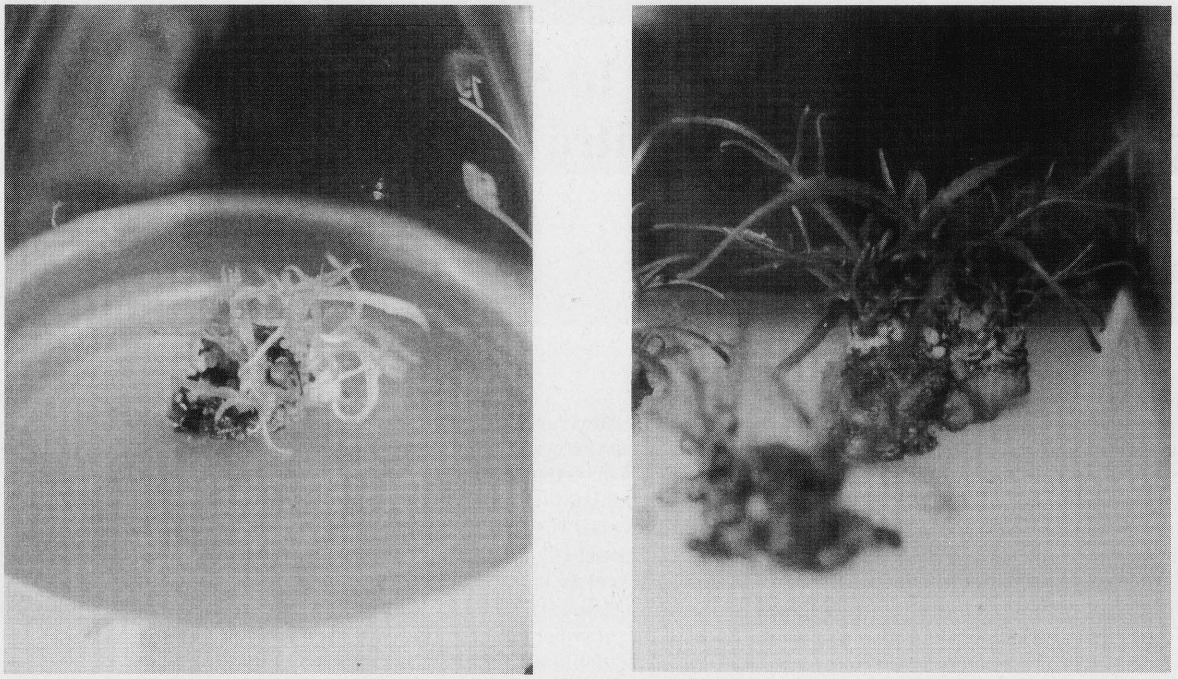

Fig. 4. Formation of abnormal shoots when Murashige and Skoog (MS) medium with BA at 4.4 μM and NAA at 0.5 μM was used for subsequent subculture of the micropropagated shoots (**left**) and production of normal shoots from micropropagated shoots on MS medium containing BA at 3.3 μM and NAA at 0.27 μM (**right**).

leaves were formed (Fig. 3). Therefore, a narrower range of BA and NAA were combined in a factorial design to select a suitable multiplication medium for the micropropagated shoots. Use of BA alone promoted multiple shoot formation at 1.1 to 3.3 μM (Fig. 4), but NAA alone at 0.27 μM had no effect on the percentage of explants producing multiple shoots. A concentration of 0.54 μM NAA alone was inhibitory, and NAA had no consistent effect upon response to BA. A medium with 3.3 μM BA and 0.27 μM NAA induced the highest percentage (83%) of explants to produce normal multiple shoots. Even though the mean number of shoots obtained from explants cultured in medium with 2.2 or 3.3 μM BA alone was similar to that cultured in 3.3 μM BA and 0.27 μM NAA, the shoots formed were abnormal and hyperhydric (Table 3). Therefore, medium with 3.3 μM BA and 0.27 μM NAA was chosen as the proliferation medium.

Table 3. The effects of combinations of BA and NAA on sustained shoot multiplication of sentang in vitro. (Expt. 3).

NAA	BA (μM)			
(μM)	0.0	1.1	2.2	3.3
0.00	2.0 ± 0.0[z]	2.6 ± 1.0	4.1 ± 1.7	4.1 ± 1.4
0.27	3.0 ± 1.2	3.0 ± 0.9	3.8 ± 1.4	4.3 ± 2.1
0.54	0.0 ± 0.0	3.7 ± 1.7	2.8 ± 1.0	2.3 ± 0.5

[z]Mean ± SD.

From this study, we observed that for subsequent subculture of micropropagated shoots the concentration of BA required decreased from 4.4 μM to 3.3 μM, and these shoots still required a small amount of NAA (0.27 μM) to obtain a maximum rate of shoot multiplication and an acceptable shoot quality. Micropropagated shoots that initially required higher concentrations of growth regulators required lower concentrations after the initial culture period. This result is similar to those seen with the Bromeliaceae, where shoot-tip explants required much lower concentrations of growth regulators when subcultured several times (Pierik, 1987). Our experiments clearly indicate that a change in medium between the initial establishment of explants (from the field) and the later proliferation and maintenance of shoot cultures is required to obtain normal, healthy, micropropagated shoots. Overall growth from axillary bud explants is best initiated using BA at 4.4 μM and NAA at 0.5 μM. Further multiplication on BA at 3.3 μM and NAA at 0.27 μM induces good multiplication rates and excellent shoot morphology. This study demonstrates that micropropagation of sentang is possible with axillary bud explants and the technique can provide high quality and uniform planting material for reforestation. The techniques can be readily adapted for a commercial production system. However, further work is needed on the shoot elongation and root development of the microshoots.

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