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Tissue Culture of Some Dipterocarps and *Agathis* in Brunei

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Summary : Tissue culture of nodal and shoot-tip sections from Dipterocarpaceae (*Cotylelobium burckii, Dryobalanops aromatica, Shorea albida, Shorea curtisii)* and *Agathis borneensis* was carried out. Mature embryos and/or cotyledonal nodes of seeds from *Dryobalanops aromatica, Shorea parvifolia, Upuna borneensis* were cultured on the defined media. Multiple shoots were obtained from shoot-tip culture of *Agathis borneensis* on the 0.5 Gamborg's (G) medium containing 1mg/l of zeatin. Bud elongation and callus formation from the nodal segments of the stem of *Dryobalanops aromatica* on the 0.25 G medium containing 1mg/l IBA were also observed. *In vitro* germination of *D. aromatica* and *Upuna borneensis* was successful. Plantlets formed from embryos were acclimated easily. It was observed that the embryo material of these two species could be stored for at least 10 months *in vitro.*

1 Introduction

Reforestation of Dipterocarps requires good methods of propagation from limited material, but conventional methods, such as cutting or grafting, are usually difficult for these species. Utilization of tissue culture for micropropagation or germplasm conservation seems useful to solve these problems. Results of several cases of tissue culture from tropical forest trees have been reported. Among them, the most practically applied species may be teak (*Tectona grandis)* in Thailand.

Micropropagation by tissue culture of teak plus trees more than 100 years old was successful (GUPUTA, 1980). Now, 10 000 plantlets of micropropagated teak are planted out annually (SASAMOTO, 1989). Nevertheless, in other tropical tree species, practical usage of tissue culture for micropropagation is rare except for Eucalyptus or Acacia. Especially in the case of Dipterocarpaceae, valuable timber trees of South East Asia, only preliminary works have been done. In 1983, SMITS *et al.* first cultured leaf ribs of *Shorea curtisii, Shorea obtusa, Dipterocarpus grandiflorus,* obtaining callus or rooting from them (SMITS, 1983). Axillary shoot formation from a nodal stem segment of *Anisoptera costata* and *in vitro* germination of an embryo of *Dryobalanops lanceolata* were observed (ISHII, 1989). Recently, *in vitro* regeneration of *Shorea robusta* was reported (JAIN, 1991). Shoots with well-developed green leaves were proliferated from axillary buds of nodal segments of aseptically grown seedlings. These were rooted in IAA and Biotin-treatment, but few details of the study have been provided. Two orthodox species of *Dipterocarps (D. alatus* and *D. intricatus)* were cultured *in vitro, D. intricatus* only was *in vitro* regenerated but habitution was unsuccessful (LININGTON, 1991). These reports show the possibility of tissue culture in the propagation of Dipterocarps, the seed storage and mass propagation of which present problems. In this report, the tissue culture of Dipterocarps and Agathis of Brunei is described.

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2 Materials and methods

2.1 Nodal and shoot-tip section of seedlings

Up to 6-year-old seedlings of the following Dipterocarps and Agathis were used as a source of explants :

#1 : *Agathis borneensis* (Tulong)
#2 : *Cotylelobium burckii* (Resak durian)
#3 : *Dryobalanops aromatica* (Kapur peringgi)
#4 : *Shorea albida (Alan)*
#5 : *Shorea curtisii* (Meranti seraya)

The nodal and shoot-tip segment of each species were surface sterilized with 70% ethyl alcohol, 10% clorox, 0.3% mercuric chloride and then washed with sterilized distilled water. Ten to twenty mm length explants which include the nodal or shoot-tip segments were cut and cultured in the defined basic media listed on Table 1 in 24mm×150mm test tubes or 100 to 300ml flasks.

The pH of the media was adjusted to 5.7 to 5.9 before autoclaving at 120℃ for 15minutes. The tubes and flasks with explants were placed in an air-conditioned room at a temperature of 23 to 28℃. The light condition was 16hours photoperiod per day with a fluorescent lamp of approximately 3 000 lx.

Some of the containers were placed in an incubator at a constant temperature (28℃) under the same light conditions.

2.2 Seeds

Seeds of *Dryobalanops aromatica, Shorea parvifolia,* and *Upuna borneensis* were collected at Bukit Basong in the Andulau Forest Reserve on 7th August, 1991, while seeds of *Dryobalanops aromatica* were collected on 12th August, 1991 at Bukit Beruang in the Tutong District.

The seeds were washed thoroughly and surface sterilized with 70% ethyl alcohol for 3 min. 10% - 50% clorox then 0.3% mercuric chloride for 5 to 10 min. and rinsed with sterilized water. Seed coats were then removed and embryo and/or cotyledonal node cultured.

2.3 Subculture

Initially cultured explants collected from Alan and Kapur, and seedlings *in vitro* grown from embryos of Kapur and Upun Batu were subcultured and/or acclimated. Explants were subcultured for multiple bud induction; segments of *in vitro* grown seedlings were also subcultured. Acclimation of *in vitro* grown seedlings was carried out by covering them with a polythene bag for 1 week, then perforating the bags to reduce humidity. Pots were set in a green house with a 1 minute per hour water spray.

Table 1. Composition of basic culture media (mg/l)

	Gamborg	0.5MS	WPM
Macro-nutrients			
NH_4NO_3	—	825	400
$(NH_4)_2SO_4$	134	—	—
KNO_3	3 000	950	—
KH_2PO_4	—	85	170
K_2SO_4	—	—	990
$NaH_2PO_4 \cdot 2H_2O$	169.6	—	—
$MgSO_4 \cdot 7H_2O$	250	185	370
$CaCl_2 \cdot 2H_2O$	150	220	96
$Ca(NO_3)_2 \cdot 4H_2O$	—	—	556
Micro-nutrients			
FeNaEDTA	40	18.35	36.7
$MnSO_4 \cdot 7H_2O$	13.2	11.15	22.3
$ZnSO_4 \cdot 7H_2O$	2	4.3	8.6
H_3BO_3	3	3.1	6.2
$CoCl_2 \cdot 6H_2O$	0.025	0.0125	0.025
$CuSO_4 \cdot 5H_2O$	0.025	0.0125	0.025
KI	0.75	0.415	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.125	0.25
Organic compound			
myo-inositol	100	50	100
Nicotinic acid	1	0.25	0.5
Thiamine HCl	10	0.05	1
Pyridoxine HCl	1	0.25	0.5
Glycine	—	1	2
Sucrose	20 000	20 000	20 000
Agar	8 000	8 000	8 000

3 Results and discussion

3.1 Initial culture of nodal and shoot-tip section of seedlings

Sterilization procedures, media compositions and results are shown in Table 2. Mild surface sterilization was difficult and imperfect. Strong sterilization using mercuric chloride solution was effective, but chemical damage to the explant sometimes occurred.

When shoot-tips of *Agathis borneensis* were cultured on the half-strength Gamborg's medium (0.5 G) containing 1mg/l of zeatin, multiple shoots were obtained (Fig. 1).

Table 2. Dipterocarps and Agathis tested for tissue culture initiation from nodal and shoot-tip segments of potted or naturally regenerated seedlings.

Species	Sterilization	Media	Results
Agathis borneensis (Tulong)	*70%EtOH 1min 10% clorox 15min +1 drop Bentowett	*0.25G + IBA 1mg/l	*shoot elongation
	*70% EtOH 1min 10% clorox 10min +1 drop Bentowett	*G + Zea 1mg/l	*no response
	*ditto	*0.5G + Zea 1mg/l	*multiple shoot
	*ditto	*0.25G + Zea 1mg/l	*shoot elongation
	*ditto	*0.25G + IBA 1mg/l	*shoot elongation
	*70% EtOH 3min 10% clorox 10min +1 drop Bentowett	*0.25G + IAA 1mg/l	*no response
Cotylelobium burckii	*70% EtOH 3min 10% clorox 10min 0.3% $HgCl_2$ 5min	*2G + BAP 1mg/l	*browning
	*ditto	*G + BAP 1mg/l	*browning
	*ditto	*0.5G + BAP 1mg/l	*browning
	*ditto	*0.25G + BAP 1mg/l	*browning
	*ditto	*0.25G + IBA 1mg/l	*browning
Dryobalanops aromatica (Kapur peringgi)	*70% EtOH 3min 10% clorox 10min	*0.25G + IBA 1mg/l	*callusing and bud elongation
	*70% EtOH 30min 10% clorox 10min	*0.25G + IBA 1mg/l	*callusing
	*70% EtOH 3s 10% clorox 10min 0.3% $HgCl_2$ 5min	*G + Kin 1mg/l	*swelling callus
		*0.5G + Kin 1mg/l	*callus
		*0.25G + Kin 1mg/l	*callus
		*0.25G + IAA 1mg/l	*callus
	70% EtOH 3min 10% clorox 10min 0.3% $HgCl_2$ 5min	*0.5G + BAP 1mg/l +NAA 0.01mg/l	*callus
		*0.5G + BAP 1mg/l +NAA 0.1mg/l	*callus swelling
		*0.5G + BAP 1mg/l +NAA 1mg/l	*callus
		*0.5G + BAP 1mg/l +NAA 10mg/l	*callus
Shorea albida (Alan)	*70% EtOH 1min 10% clorox 15min	*0.25G + IBA 1mg/l	*callus

Table 2. (Continued)

Species	Sterlization	Media	Results
Shorea albida (Alan)	*70% EtOH 10s 10% clorox 10min +1 drop Bentowett	*G + BAP 1mg/l	*browning
		*0.5G + BAP 1mg/l	*browning
		*0.25G + BAP 1mg/l	*browning
		*0.25G + IBA 1mg/l	*browning
	*70% EtOH 3min 10% clorox 10min +1 drop Bentowett 0.3% $HgCl_2$ 5min	*2G + BAP 1mg/l	*browning
		*G + BAP 1mg/l	*browning
		*0.5G + BAP 1mg/l	*browning
		*0.25G + BAP 1mg/l	*browning
		*0.25G + IBA 1mg/l	*no response
	*10% clorox 10min +1 drop Bentowett 0.3% $HgCl_2$ 5min	*G + Kin 1mg/l	*browning
		*0.5G + Kin 1mg/l	*browning
		*0.25G + Kin 1mg/l	*browning
		*0.25G + IAA 1mg/l	*no response
	*70% EtOH 10min 20% clorox 10min +1 drop Bentowett 0.3% $HgCl_2$ 5min	*0.5G + BAP 1mg/l +NAA 0.01mg/l	*no response
		*0.5G + BAP 1mg/l +NAA 0.1mg/l	*axillary bud
		*0.5G + BAP 1mg/l +NAA 1mg/l	*swelling
		*0.5G + BAP 1mg/l +NAA 10mg/l	*swelling
Shorea curtisii (Meranti seraya)	*70% EtOH 10min +1 drop Bentowett 0.3% $HgCl_2$ 5min	*0.5MS +NAA 0.2mg/l	*no response
		*0.5MS +BAP 0.2mg/l +NAA 0.2mg/l	*no response
		*0.5MS +BAP 2mg/l +NAA 0.2mg/l	*no response
		*0.5MS +BAP 10mg/l +NAA 0.2mg/l	*no response
		*WPM + NAA 0.2mg/l	*contaminated
		*WPM + BAP 0.2mg/l +NAA 0.2mg/l	*contaminated
		*WPM + BAP 2mg/l +NAA 0.2mg/l	*contaminated
		*WPM + BAP 10mg/l +NAA 0.2mg/l	*contaminated
		*0.5WPM + BAP 1mg/l	*contaminated

Note : EtOH : ethyl alcohol IAA : Indole-3-acetic acid Zea : Zeatin
$HgCl_2$: mercuric chloride IBA : Indole-3-butyric acid NAA : Naphthalen acetic acid
BAP : benzylaminopurine Kin : Kinetin G : Gamborg

Fig. 1. Multiple shoot formation in the shoot-tip culture of *Agathis borneensis* on the 0.5 G medium containing 1mg/l of Zeatin.

Shoot elongation was observed from the shoot-tip sections cultured on the quarter strength Gamborg's medium (0.25G) containing 1mg/l of zeatin or 1mg/l IBA(Fig. 2). Longer shoot-tips were better sources for tissue culture than the round shorter type.

Single bud elongation and callus formation were observed from the nodal segments of the stem of *Dryobalanops aromatica* cultured on 0.25G containing 1mg/l of IBA(Fig. 3).

In the case of *Shorea albida*, swelling and callusing of shoot-tip segments were observed on the 0.25G medium containing 1mg/l of IBA. Axillary bud initiation from nodal segment was also observed on the 0.5G medium containing BAP 1mg/l and NAA 0.1mg/l (Fig. 4).

3.2 Initial culture of embryos and/or cotyledonal nodes

Embryos and/or cotyledonal nodes of three Dipterocarps species were cultured as shown in Table 3.

For embryo germination, larger containers were better than smaller ones. After a few days, roots appeared first, then the cotyledon opened (Figs. 5,6). After 10 months, embryos of Kapur and Upun Batu still survived and the leaves opened. Figs. 7 and 8 show the Dipterocarps cultured *in vitro*. *In vitro* germinatated materials provide a good source for subculture.

3.3 Subculture

After one-and-a-half months initial culture, segments from *in vitro* grown Kapur were subcultured to 0.5G media (NAA 0.2mg/l, BAP 2-10mg/l). The results eight months later are shown in Table 4. A relatively high concentration of BAP was good for multiple bud formation. Subcultured explants of Kapur to the 0.5G media containing 0.5mg/l of BAP and 0-500mg/l of glutamine showed no response except the formation of one bud in the medium containing 10mg/l of glutamine.

Protuberance mass obtained in the initial culture of Alan on WPM containing 1mg/l of BAP and NAA was subcultured to the 0.5G media containing 0.5mg/l of BAP and 0-500mg/l of glutamine. Since no clear response was obtained, these were subcultured again to the 0.5G medium containing 2.5g/l of activated charcoal. They were still fresh after 2 months.

The explants from Upun Batu subcultured on the 0.5G media containing 0.5mg/l of BAP and 0.1-1mg/l of 5,6-dichloro-3-indoleacetic acid (di-Cl-IAA) produced callus at the base of the hypocotyl. Multiple buds were observed in subcultured explants on the WPM medium containing BAP 0.5 mg/l

Fig. 2. Shoot elongation in shoot-tip culture of *Agathis borneensis* on the 0.25G medium containing 1mg/l of IBA.

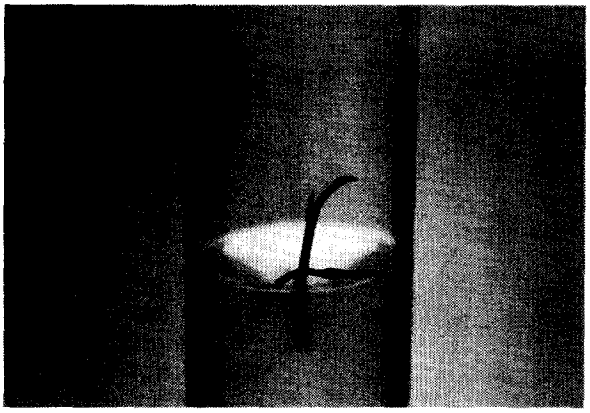

Fig. 3. Bud elongation and callus formation from the nodal segments of the stem of *Dryobalanops aromatica* on the 0.25G medium containing 1mg/l of IBA.

Fig. 4. Swelling and axillary bud initiation from the nodal segment of *Shorea albida*.

Table 3. Dipterocarps tested for tissue culture initiation from embryos and cotyledonal nodes

Species	Sterilization	Media	Results
Dryobalanops aromatica (Kapur peringgi)	*70%EtOH 3min 10% clorox 10min	*G + Zea 1mg/l	*rooting
		*0.5G + Zea 1mg/l	*greening
		*0.25G + Zea 1mg/l	*rooting
		*G + BAP 1mg/l	*rooting
		*0.25G + BAP 1mg/l	*rooting
		*G + Kin 1mg/l	*rooting
		*0.5G + Kin 1mg/l	*rooting
		*0.25G + Kin 1mg/l	*rooting
		*0.25G + IAA 1mg/l	*rooting
	*70% EtOH 3min 10% clorox 10min 0.3% $HgCl_2$ 7min	*0.5G + BAP 1mg/l +NAA 0.01mg/l	*germination
		*0.5G + BAP 1mg/l +NAA 0.1mg/l	*germination
		*0.5G + BAP 1mg/l +NAA 1mg/l	*germination
		*0.5G + BAP 1mg/l +NAA 10mg/l	*germination
		*WPM + NAA 0.2mg/l	*germination
		*WPM + BAP 0.2mg/l +NAA 0.2mg/l	*germination
		*WPM + BAP 2mg/l +NAA 0.2mg/l	*germination nodular structure
		*WPM + BAP 10mg/l +NAA 0.2mg/l	*germination swelling
Shorea parvifolia (Meranti serang punai)	*70% EtOH 3min 10% clorox 10min	*G + Zea 1mg/l	*small bud
		*0.5G + Zea 1mg/l	*bud
		*0.25G + Zea 1mg/l	*browning
		*0.25G + IBA 1mg/l	*browning bud
Upuna borneensis (Upun batu)	*70% EtOH 3min 10% clorox 10min	*G + BAP 1mg/l	*germination
		*0.5G + BAP 1mg/l	*germination
		*0.25G + BAP 1mg/l	*germination
		*0.25G + IBA 1mg/l	*germination
		*G + Zea 1mg/l	*germination
		*0.5G + Zea 1mg/l	*greening
		*0.25G + Zea 1mg/l	*greening
		*0.25G + IAA 1mg/l	*no response
		G + Kin 1mg/l	*germination
		0.5G + Kin 1mg/l	*germination
		0.25G + Kin 1mg/l	*germination
		0.25G + IAA 1mg/l	*greening

Note : Abbreviations are the same as in Table 2.

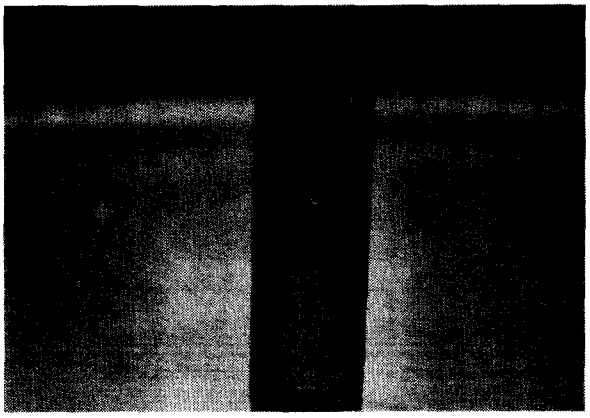

Fig. 5. *In vitro* germination of *Dryobalanops aromatica.*

Fig. 6. *In vitro* germination of *Upuna borneensis.*

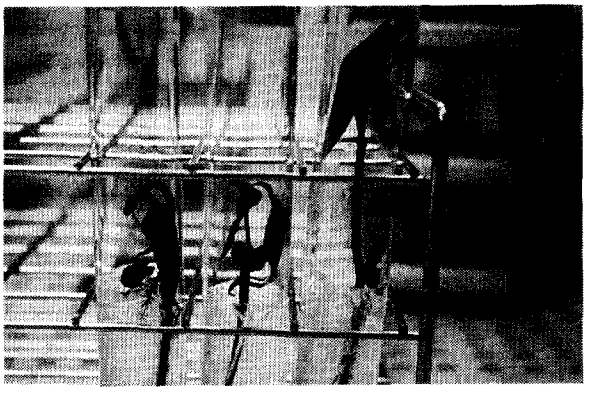

Fig. 7. *In vitro* plantlet formation of *Dryobalanops aromatica.*

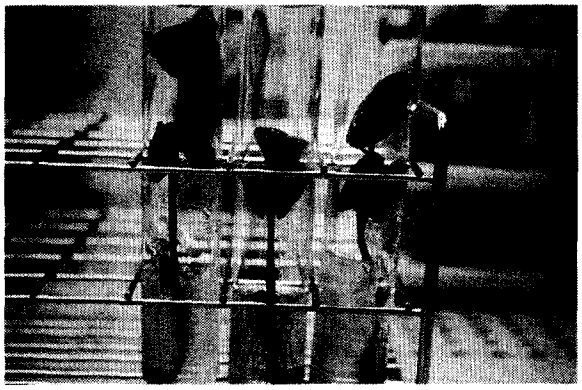

Fig. 8. *In vitro* plantlet formation of *Upuna borneensis*.

Table 4. The effect of BAP concentration on the subculture of *Dryobalanops aromatica*

BAP concentration (mg/l)	Subcultured no. of segments	Results (no. of segments)
2	6	no response(6), browning(6)
4	5	small protuberances(3), browning(5)
6	6	green hypocotyl(3), small protuberances(1), shoot(1), browning(2)
8	6	shoots(1), green cotyledon(1), browning(5)
10	4	multiple buds(1), green hypocotyl(1), browning(2)

0.5G medium containing NAA 0.2mg/l

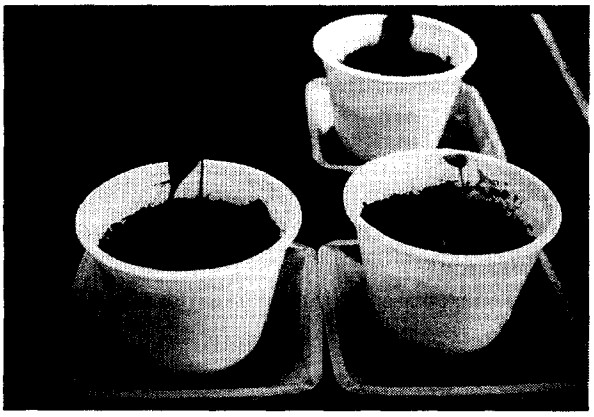

Fig. 9. Acclimatized plantlets grown *in vitro* from *Dryobalanops aromatica* and *Upuna borneensis*.

and di-Cl-IAA 0.5mg/l solidified with gellan gum.

3.4 Acclimation of the *in vitro* grown plantlets

In vitro grown plantlets from embryos of Kapur and Upun Batu were successfully acclimated. They were potted out to the green house (Fig.9) and will be planted in the field.

From this screening test, the shoot-tip of *Agathis* was found to be the best explant for the initiation of tissue culture. In other tropical species, such as cedro or teak, shoot-tip explant was also used and a good result obtained. However, collection of a large number of shoot tips from a limited number of plant material was difficult. One solution may be the use of stem nodal segments. As shown in this study, the nodal section of the *Dryobalanops aromatica* seedling has the ability to produce new buds under certain conditions. If sterilization of explants was more reliable and a high rate of budding possible, this could be a good source for micropropagation by tissue culture. This is also one option for *Shorea albida* (Alan) propagation. At present, however, it is difficult to obtain good juvenile material for tissue culture from this species. We used 5-year-old seedlings which were naturally regenerated in the 1986 fruiting year. Even though they were still small in size (ca 1m height), physiologically they did not appear juvenile. Spray pretreatment of cytokinins to the mother material, for example, must be tried in the future for rejuvenation.

For tropical forests where genetic diversity is very important, micropropagation from many seed sources may have an advantage over simple clonal propagation from limited material. Seeds are also good material for tissue culture because they are more juvenile than saplings or adult trees. In this study, rooting and cotyledon development from the embryos of some Dipterocarps were observed and *in vitro* germinated seedlings were obtained. This juvenile material may be useful for further subculture and propagation.

Obtaining *in vitro* shoots from Alan is still difficult, however, meristematic protuberances from nodal segments will be good sources for tissue culture study,such as somatic embryo formation which is ideal for micropropagation. If multiple buds obtained in the subculture of Upun Batu and Kapur grow well, repetitive subculture will produce many shoots.

The tissue culture technique is used, not only for micropropagation but also for the storage of germplasm. It appeared that among dipterocarps in Brunei, embryos of Kapur and Upun Batu easily

can be stored *in vitro* for at least 10 months. Those material can be planted out with an acclimation technique providing planting stock at any time.

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ブルネイ産フタバガキ科数種と*Agathis*の組織培養

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摘　要

ブルネイ産フタバガキ科の*Cotylelobium burckii, Dryobalanops aromatica, Shorea albida, Shorea curtisii*と針葉樹*Agathis borneensis*の茎節と茎頂培養を行った。同じく，*Dryobalanops aromatica, Shorea parvifolia, Upuna borneensis*について，胚や子葉節の培養を行った。*Dryobalanops aromatica*と*Upuna borneensis*ではそれぞれ，茎節と子葉節からの多芽体の形成がみられた。*Shorea albida*では茎節からの発芽と膨潤が観察された。*Dryobalanops aromatica*と*Upuna borneensis*では，胚の培養によって，個体が再生され，その順化に成功した。それらの胚は少なくとも10か月間試験管の中で生存し，フタバガキ科の試験管内保存の可能性が示された。

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