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Tissue Culture Note

Multiple shoots formation of an important tropical medicinal plant, *Eurycoma longifolia* Jack

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Abstract *Eurycoma longifolia* Jack is well known among the communities in Southeast Asia because of its aphrodisiac properties and its effectiveness as the cytotoxic, anti-malarial, anti-ulcer, anti-tumor promoting and anti-parasitic agent. Micropropagation through direct plant regeneration from *in vivo* shoot tip explants was carried out. The highest regeneration percentage (90%) and multiple shoots formation were obtained with the basal Murashige and Skoog (MS) medium supplemented with 5.0 mg l^{-1} kinetin. Roots were induced after 14 days of culture in the basal MS medium supplemented with 0.5 mg l^{-1} of indole-3-butyric acid. Plantlets regenerated from shoot tip explants survived well with no morphological differences from parent plants after two months of transplantation to soil.

Key words: *Eurycoma longifolia*, micropropagation, multiple shoots, shoot tip.

Eurycoma longifolia Jack or locally known as Tongkat Ali, Bidara Pahit, Lempedu Pahit, Bidara Laut, Pasak Bumi in Indonesia, 'Ian-don' in Thailand and 'cay ba binh' in Vietnam (Choo and Chan 2002) is a plant in the family Simaroubaceae. It is a tall slender shrub tree commonly found as an understorey in the lowland forests at up to 500 m above the sea level in Burma, Indochina, Thailand, Malaysia, Sumatra, Borneo and the Philippines (Ang et al. 2002). *E. longifolia* becomes the targeted medicinal plants in the local community in Southeast Asia mainly owing to its aphrodisiac property. The most common method of propagating *E. longifolia* is through seeds. However, being a recalcitrant plant, the seeds have a low percentage of germination and it takes a long time to germinate due to extremely immature state of zygotic embryo at the time of dispersal. The gradual disappearance of this plant is due to the indiscriminate collection of the taproot as the raw material for the drug preparations. Therefore, it needs to be rapidly mass-multiplied on a commercial scale to comply with the need of the herbal and pharmaceutical industry. The existence of tissue culture technology can play an important role in this regard, with the added advantage of maintaining disease-free plants (Aly et al. 2002). In view of *E. longifolia*'s potential commercial value as a plantation crop as well as to conserve its germplasm, formation of the multiple shoots and subsequent rooting of the *in vitro* shoots were carried out as this *in vitro* micropropagation protocols has not been reported elsewhere.

The source of *E. longifolia* plant used in this study was obtained from Institute of BioScience, Universiti Putra Malaysia, Selangor, Malaysia. Surface sterilization of the *in vivo* explants was initiated by washing under running tap water for 30 min and further surface-sterilized using 15% (v/v) of commercially available Clorox for 15 min. The sterile explants were then rinsed for three times with sterile distilled water for 5, 10 and 15 min, respectively. The *in vivo* shoot tips were aseptically trimmed to about 1.5 cm in length and inoculated into 100 ml Erlenmeyer flask containing the basal MS medium supplemented with cytokinins (BAP, kinetin and zeatin) at the concentrations of 1.0 to 10.0 mg l^{-1}, 3% (w/v) of sucrose and 2.5 gl^{-1} of Gelrite at the pH value of 5.7. The cultures were kept at $25\pm2°C$ and a 16 hours photoperiod with cool white fluorescent light of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ (supplied by Philips TLD fluorescent light tubes). For each treatment, ten replicates

Abbreviations: BAP, Benzylaminopurine; IAA, Indole-3-acetic acid; IBA, Indole-3-butyric acid; Kinetin, 6-furfurylaminopurine; MS, Murashige and Skoog medium; NAA, naphthaleneacetic acid.

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Table 1. Effects of different concentrations of kinetin on the shoot formation from *in vivo* shoot tip explants of *Eurycoma longifolia* after two months of culture in the basal MS medium.

Concentrations of kinetin ($mg\,l^{-1}$)	Regeneration[1] (%)	Number of shoots[1]	Stem length[1] (cm)	Number of leaftelts[1]	Number of rachis[1]	Number of leaflets per rachis[1]
0.0	30 a	1.0±0.2 a	1.5±0.2 a	3.0±0.5 a	1.0±0.2 a	3.0±0.4 a
1.0	40 a	1.0±0.2 a	1.7±0.1 a	4.0±0.6 a	2.0±0.3 a	2.0±0.3 a
2.0	50 b	1.0±0.2 a	1.7±0.2 ab	24.0±3.6 b	3.0±0.5 b	8.0±1.2 d
3.0	70 c	2.0±0.3 b	1.7±0.2 ab	27.0±2.0 b	4.0±0.6 b	7.0±1.1 c
4.0	80 d	3.0±0.4 c	2.0±0.4 b	32.0±2.0 c	5.0±0.7 c	6.0±0.9 b
5.0	90 d	4.0±0.2 d	2.0±0.6 b	34.0±3.0 c	4.0±0.6 b	8.0±1.2 d
6.0	50 b	2.0±0.3 b	2.0±0.3 b	30.0±5.0 c	5.0±0.7 c	6.0±1.0 b

[1] All values are means±S.D. Means followed by the same letters are not significantly different (p<0.05) using Tukey's test. Thirty explants were cultured at each concentration.

were taken and repeated three times. Results were statistically compared using Tukey's test (α=0.05).

It is evident from Table 1 that MS medium supplemented with different cytokinins at different concentrations showed variation in the regeneration percentage and number of shoots formed. Among the three cytokinins tested, kinetin-treated explants achieved the highest regeneration percentage compared to BAP and zeatin. The data obtained revealed that the maximum regeneration percentage (90%) could be successfully obtained in 5.0 $mg\,l^{-1}$ of kinetin. However, there was no sign of shoot formation at concentrations higher than 6.0 $mg\,l^{-1}$ of kinetin (data not shown). Multiple shoots were formed in this study. Based on the number of shoots formed per explant, it was found that the supplementation of 5.0 $mg\,l^{-1}$ of kinetin produced the maximum number of shoots (4.0±0.2 per explant) (Figure 1a). Addition of 4.0 $mg\,l^{-1}$ of kinetin into the basal MS medium also influenced the number of shoot produced. A total of 3.0±0.4 shoots per explant was observed in this treatment. At 3.0 and 6.0 $mg\,l^{-1}$ of kinetin, 2.0±0.3 shoots were produced, whilst other concentrations tested within the range did not show any sign of multiple shoots formation. Besides the kinetin treatment, the formation of 2.0±0.1 shoots per explant was also observed in 3.0, 4.0 and 5.0 $mg\,l^{-1}$ of BAP. Study also revealed that 5.0 $mg\,l^{-1}$ of zeatin was the only concentration that managed to produce 2.0±0.1 shoots per explant, while other treatments within the range only formed one shoot in each explant.

Analysis on the leaflets formation disclosed that there was no significant difference in the treatments using 4.0 to 6.0 $mg\,l^{-1}$ kinetin, whereby all these treatments produced more than 30 leaflets. The number of leaflets per rachis was maximum (8.0±1.2) in the media containing either 2.0 or 5.0 $mg\,l^{-1}$ of kinetin, which was then followed by the treatment with 3.0 (7.0±1.1), 4.0 (6.0±1.0), 6.0 (6.0±1.0), 0.0 (3.0±0.4) and 1.0 mgl^{-1} of kinetin (2.0±0.3). Meanwhile, the highest number of rachis (5.0±0.7) was obtained in explants treated with 4.0 and 6.0 mgl^{-1} of kinetin. In the meantime, the concentrations of 4.0, 5.0 and 6.0 $mg\,l^{-1}$ of kinetin

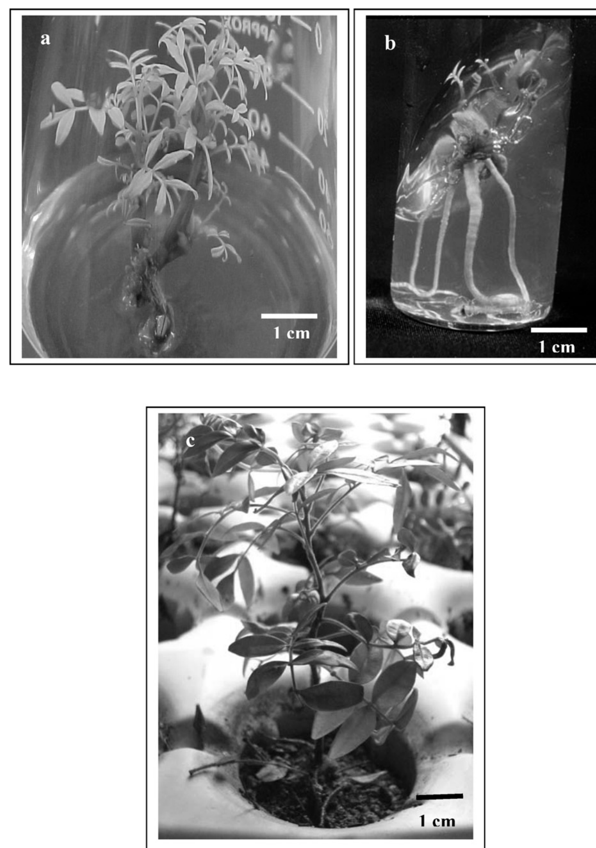

Figure 1. Plantlet regeneration in *Eurycoma longifolia*. (a) Multiple shoots formed in MS medium supplemented with 5.0 $mg\,l^{-1}$ kinetin. (b) Roots formed in MS medium supplemented with 0.5 $mg\,l^{-1}$ IBA. (c) *In vitro* rooted plantlets after two months of transplantation into the soil with the composition of nitrogen, phosphate, potassium at the ratio of 1 : 3 : 2.

produced a similar pattern in terms of stem elongation. The length of the stem for the respective treatment was 2.0±0.4, 2.0±0.6 and 2.0±0.3 cm.

Healthy shoots (2.0±0.2 cm in length) induced from shoot tip explants were transferred into the basal MS medium containing different auxins (IBA, IAA and NAA) at the range of 0.1 to 0.6 $mg\,l^{-1}$ for rooting. The effects of these auxins on root induction as well as the length of the roots were examined after two months of culture. The results revealed that among the three auxins

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Table 2. Effects of IBA on root induction in the shoots regenerated from shoot tip explant of *E. longifolia*.

Concentration of IBA ($mg\,l^{-1}$)	Days root start to form[1]	Percentage of root formation (%)	Root length (cm)
0.0	NR a	0 a	0.0 a
0.1	NR a	0 a	0.0 a
0.2	NR a	0 a	0.0 a
0.3	NR a	0 a	0.0 a
0.4	20 c	50 b	4.0 b
0.5	14 b	90 c	8.0 c
0.6	NR a	0 a	0.0 a

[1] NR indicates that no root was induced from the shoots after two months of culture. Means followed by the same letters are not significantly different ($p<0.05$) using Tukey's test. (N=3).

tested, only treatments using IBA at the concentrations of 0.4 and 0.5 mgl^{-1} showed a sign of root formation from the new shoots. About 90% of the root formation occurred in the treatment using 0.5 $mg\,l^{-1}$ of IBA, while only 50% was observed in 0.4 $mg\,l^{-1}$ of IBA (Table 2). The formation of roots started to be visible after 14 days of culture in the treatment using 0.5 $mg\,l^{-1}$ of IBA, while a significant longer period, 20 days, was required for the root formation in 0.4 $mg\,l^{-1}$ of IBA. Observation after two months showed that the roots formed in the treatment using 0.5 $mg\,l^{-1}$ of IBA could be elongated until 8.0±1.0 cm in length (Figure 1b). New shoots treated with NAA or IAA in any of the concentrations tested tended to form callus at the base of the shoots. The possible reason could be that IBA is more resistant than IAA to chemical degradation in tissue culture media, both during autoclaving and at room temperature (Cuenca et al. 1999). Although shoots grown in rooting media containing 0.6 $mg\,l^{-1}$ of IBA also formed callus at their cut ends, the callus formed was less than that observed in NAA or IAA. According to Juliani et al. (1999), shoots contain high levels of endogenous auxins and the addition of exogenous auxin caused the inhibition of root development, thus resulted in callusing at the base of the shoots.

The plantlets with well-developed roots and shoots were thoroughly washed under tap water for 2 to 3 min to remove traces of Gelrite-gelled medium sticking to them. *In vitro* hardening was done in culture bottles containing water and covered with polypropylene caps. After two weeks, the caps were opened and plantlets were transferred to plastic pots containing mixture of soil, that have the composition of nitrogen, phosphate, potassium at the ratio of 1 : 3 : 2. Plants were maintained at the environment with 25 to 28°C. The plants were initially covered with glass to maintain high humidity. After three weeks, the glasses were removed. The percentage of plant survival was calculated after two months. The *in vitro* rooted plantlets were successfully acclimatized with 70% survival rate. Acclimatized plantlets were healthy and well developed when transferred to the soil. The plants grew as high as 9.0±1.5 cm and no morphological difference from the parent plants was shown after two months of transplantation (Figure 1c). Many authors showed that leaves formed *in vitro* do not develop their photosynthetic capability, but they can function as storage organs to be a source of carbohydrates for newly developing leaves (Borkowska, 2001). The poor photosynthetic capability could have caused the dying of some micropropagated *E. longifolia* plantlets during the acclimatization process.

The present study is the first report on successful *in vitro* propagation of *E. longifolia* by shoot tip culture and subsequent rooting. The established *in vitro* micropropagation protocols offer a potential system for improvement, conserving and mass propagation of *E. longifolia* for outplantings to nursery plots.

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