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## Micropropagation of *Eurycoma longifolia* Jack via Formation of Somatic Embryogenesis

<sup>1,2</sup>Sobri Hussein, <sup>2</sup>Rusli Ibrahim, <sup>1,3</sup>Anna Ling Pick Kiong, <sup>4</sup>Nor'aini Mohd Fadzillah and <sup>4</sup>Siti Khalijah Daud

<sup>1</sup>Department of Biochemistry, Faculty of Biotechnology and Biomolecular Science,  
Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Malaysia

<sup>2</sup>Malaysian Institute For Nuclear Technology Research, Bangi 43000 Kajang, Selangor Malaysia

<sup>3</sup>Department of Bioscience, Faculty of Engineering and Science,  
Universiti Tunku Abdul Rahman, 53300 Setapak, Kuala Lumpur, Malaysia

<sup>4</sup>Department of Biology, Faculty of Science, Universiti Putra Malaysia,  
43400 UPM Serdang, Selangor Malaysia

**Abstract:** The first successful micropropagation protocol for an important recalcitrant medicinal plant in Southeast Asia, *Eurycoma longifolia* Jack or Tongkat Ali as known in Malaysia was developed. In attempts to establish the somatic embryos of *E. longifolia*, the potential of cotyledon, zygotic embryo, leaf, petiole, stem and taproot in forming embryogenic callus were examined in the basal Murashige and Skoog (MS) medium supplemented with different auxins at various concentrations. Only cotyledon explants were able to form embryogenic callus in the presence of 1.0 mg L<sup>-1</sup> (w/v) of 2,4-dichlorophenoxyacetic (2,4-D). A higher yield (60%) of embryogenic callus was obtained when the Type 4 method dissected cotyledon explants were cultured in basal MS medium containing 0.5 mg L<sup>-1</sup> (w/v) of kinetin and 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D. The highest number of somatic embryos (45±2) was observed in the same medium formulation with the addition of 1.0 g L<sup>-1</sup> (w/v) activated charcoal. Subsequent transfer of these mature somatic embryos in basal MS media supplemented with 1.0 mg L<sup>-1</sup> (w/v) of kinetin produced a 90% of plantlet regeneration. The differences between the embryogenic and non-embryogenic callus were also determined based on the histological studies.

**Key words:** Somatic embryogenesis, plant regeneration, cotyledon, *Eurycoma longifolia*, tissue culture

### INTRODUCTION

*Eurycoma longifolia* Jack, a plant in the family Simaroubaceae is one of the most well known folk medicines in Southeast Asia. Locally known as Tongkat Ali, Bidara Pahit, Lempedu Pahit, Bidara Laut, Pasak Bumi in Indonesia, 'Tan-don' in Thailand and 'cay ba binh' in Vietnam<sup>[1,2]</sup>, 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<sup>[3]</sup>. Currently, there are three *Eurycoma* species that had been identified, namely, *Eurycoma longifolia*, *Eurycoma apiculata* and *Eurycoma harmandiana*, which was discovered recently in Thailand<sup>[4]</sup>. As a tree that cures hundred of diseases as called by Vietnamese<sup>[2]</sup>, many research had been carried out locally and internationally in determining the bioactive components that lead to its medicinal values especially its aphrodisiac properties<sup>[5,6]</sup>,

cytotoxic, antimalarial<sup>[7]</sup>, anti-tumor promoting and anti parasitic properties<sup>[8]</sup>.

The most common method of propagation for *E. longifolia* is through seeds. However, like other woody species, propagation of *E. longifolia* through seed is difficult due to the unreliable flowering habit and quick loss of viability as well as low seed germination rate and slow growth. At present, the commercial demand as well as the plant material used for extensive research on *E. longifolia* is met by collection from the wild. The plant is uprooted in the process of separating the root, which has lead to a serious shortage of the plants. Although *in vitro* propagation is a potential alternative for production of *E. longifolia* for commercial and conservation purposes, not many tissue culture studies have been conducted on this species. Thus, the development of rapid propagation systems for rare medicinal plants such as *E. longifolia* is a necessity in order to meet pharmaceutical needs and also to prevent

the plants from becoming endangered or extinct. Therefore, the main objective of this study was to develop efficient plantlet production using somatic embryogenesis system.

## MATERIALS AND METHODS

**Plant materials:** The source of *E. longifolia* plant used in this study was obtained from Natural Products Laboratory, Institute of Bioscience, UPM, Selangor, Malaysia. In order to reduce the problem of genotypic variation due to cross pollination, *E. longifolia* plant denoted as 11026-7 (Puchong) was used throughout the studies.

**Callus induction:** Surface sterilization of the explants was carried out in the initial stage in order to obtain the sterile explants for the callus induction studies. In this study, leaf, stem, taproot, cotyledon, petiole and embryo from *E. longifolia* plants were used as the explants. To obtain the sterile seed for *in vitro* germination purposes, soft seed coat (soft mesocarp) was removed and the cotyledon with hard mesocarp attached was dissected out and washed with tap water for 20 min prior to immersion in 20% (v/v) of commercial Clorox together with 2 to 3 drops of Tween 20 (Merck) as wetting agent for 15 min. The explants were then thoroughly rinsed in sterile distilled water for 5, 10 and 20 min, respectively. After two months of culture, the stem and taproot of the *in vitro* plantlets were cut aseptically at the length of 1.0 cm. The cut explants were then placed in the basal MS medium supplemented with 3% (w/v) of sucrose, 2.5 g L<sup>-1</sup> (w/v) of gelrite agar and various concentrations (0.0 to 5.0 mg L<sup>-1</sup> (w/v)) of auxins at pH 5.7. The cultures were incubated at 25±2°C and a 16 h photoperiod with cool white fluorescent light of 150 µmol m<sup>-2</sup> s<sup>-1</sup> (supplied by Philips TLD fluorescent light tubes). Auxins used in the callus induction studies were dicamba, picloram, 2,4-D, IAA, IBA and NAA all acquired from Sigma, USA. The experiments were conducted in ten replicates with three repetitions and were monitored daily.

Induction of callus from zygotic embryo explant was done by aseptically excised the zygotic embryos from the sterile seed. The cotyledons remained, which were lacked of zygotic embryo were dissected horizontally and separated into four equal parts. Both the zygotic embryos and cotyledon explants were cultured in the same media and culture conditions as mentioned in the stem and taproot explants were used.

As for the leaf and petiole explants, the explants obtained *in vivo* were surfaced sterilized using 20% (v/v) of commercial Clorox for 15 min. After the leaf and petiole

explants were washed several times with sterile distilled water, leaf discs (5×5 mm) and petiole (5 mm) segments were excised and implanted in the same basal media as in other explants. All the experiments were conducted in ten replicates and repeated thrice.

After one week of incubation, the cultures were monitored daily to observe the callus initiation. The percentage of callus induction was observed after four weeks from the initial culture. To monitor the embryogenic capability and proliferation of the callus, all the callus initiated were maintained in the same fresh medium and subcultured at three weeks interval. An initial of 0.10 g fresh weight of three weeks old callus were cultured in the same medium. The growth of the callus expressed in dry weight was observed weekly while the day and percentage of embryogenic callus formation after three weeks, if any were also noted.

### Effects of different dissection methods using cotyledons:

The effects of six different dissection methods and culture positions on callus induction from cotyledon explants were further examined. In this study, the zygotic embryo was removed from each seed and only the cotyledons were used as the explants. Cotyledons of *E. longifolia* are lobed and each cotyledon was divided into two explants. The separated cotyledons were placed with either flat (Type 1) or lobed surface (Type 2) contacting the media as shown in Fig. 1a and b, respectively. As for the other four types of dissection method, the two cotyledons were further cut horizontally giving four cotyledon section explants per seed. For Type 3 (Fig. 1c) and Type 4 (Fig. 1d) dissection method, the cut cotyledon pieces were placed in the same manner as in the Type 1 and 2 dissection method whereas for Type 5 and 6 methods, the cut cotyledon pieces were placed either by the cut surface facing (Fig. 1e) or away (Fig. 1f) from the media.

All these differently dissected cotyledons were cultured in 100 mL Erlenmeyer flask containing the basal MS salts supplemented with MS vitamins, 3% (w/v) sucrose, 2.5 g L<sup>-1</sup> (w/v) gelrite agar and 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D at pH 5.7. The cultures were incubated at 25±2°C and a 16 h photoperiod with cool white fluorescent light of 150 µmol m<sup>-2</sup> s<sup>-1</sup> (supplied by Philips TLD fluorescent light tubes). After one week of incubation, the cultures were monitored daily to observe the callus initiation. The percentage of callus induction was observed after three weeks from the initial culture. This experiment was carried out in ten replicates for each dissection method with three repetitions.

**Embryogenic callus induction from potential explant:** For embryogenic callus induction, the most potential explant,

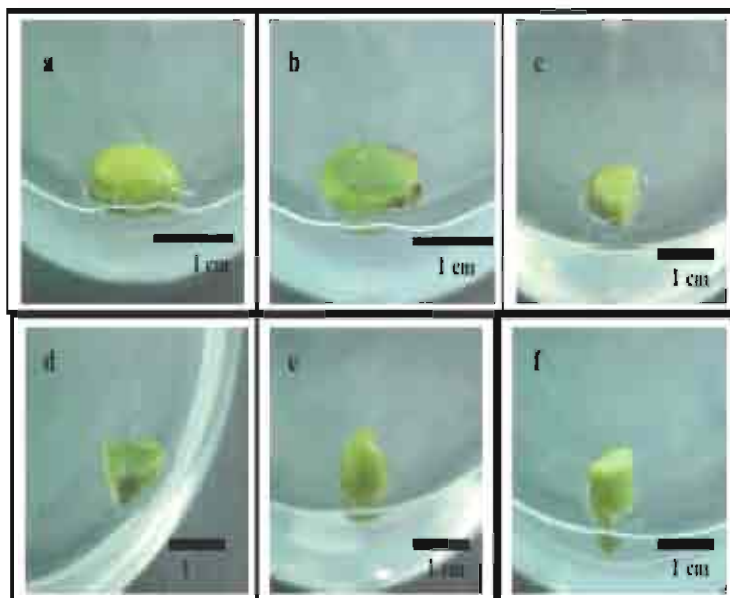


Fig. 1: Different dissection methods and culture positions applied for callus induction from cotyledon explants of *Eurycoma longifolia*. (a) Type 1 (b) Type 2 (c) Type 3 (d) Type 4 (e) Type 5 (f) Type 6

the cotyledons were dissected based on the best dissection method revealed in the previous studies. These explants were subsequently cultured into 100 mL Erlenmeyer flask containing basal MS medium supplemented either with 2,4-D alone or in combinations with cytokinins at the range of 0.5 to 3.0 mg L<sup>-1</sup> (w/v). The cytokinins studied were kinetin, BAP, zeatin and TDZ. All these cytokinins were purchased from Sigma, USA. The cultures were maintained at 25±2°C under 16 h photoperiod of 40 μmol m<sup>-2</sup> s<sup>-1</sup> light provided by cool white fluorescent lamps. Ten explants were used per treatment and the experiments were repeated thrice. The percentage of explants showing callus formation was calculated after three weeks of culture. All the callus initiated were maintained in the same fresh medium and subcultured at three weeks interval.

Careful selection and subculture of only the embryogenic sectors, which was characterized by the formation of green globular structure formed on the surface of callus was carried out to achieve efficient and long-term regeneration. At each subculture, the non-embryogenic and necrotic callus was carefully removed and only the immature somatic embryo (bright green color, globular shape) grown on the surface of the embryogenic callus were aseptically transferred to the fresh medium. The frequency of somatic embryogenesis, based on the appearance of visible green somatic embryo, was determined after three weeks of culture.

**Multiplication of somatic embryos:** For the multiplication studies, an initial number of 10±2 somatic embryos (approximately 0.10±0.01 g fresh weight) were cultured in the basal MS medium containing either 2,4-D alone or in combinations with kinetin at different concentrations and incubated at 25±2°C and a 16 h photoperiod with cool white fluorescent light of 150 μmol m<sup>-2</sup> s<sup>-1</sup> (supplied by Philips TLD fluorescent light tubes). Each treatment contains ten replicates and the experiments were repeated thrice. The numbers of embryos as well as the fresh weight of embryos were measured after sixth week of culture.

In order to reduce the browning effects and further increase the multiplication rate of the somatic embryos, activated charcoal was added at the concentration of 0.5, 1.0, 1.5 and 2.0 g L<sup>-1</sup> (w/v). The media without the activated charcoal was used as the control experiment. In this treatment, 10±2 somatic embryos (approximately 0.10±0.01 g fresh weight) were cultured in the basal MS medium supplemented with 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D and 0.5 mg L<sup>-1</sup> (w/v) of kinetin. The cultures were incubated at 25±2°C with 16 h of fluorescent light. The observation of the multiplication rate was carried out as mentioned above.

**Plantlet regeneration from mature somatic embryos:** Plant regeneration refers to the development of morphologically normal plantlets with a well-established

root and shoots with at least the first pair of leaves<sup>[9]</sup>. In order to obtain the optimal plant regeneration, mature cotyledonary embryos were aseptically transferred by forceps into six basal media, WPM<sup>[10]</sup>, MS<sup>[11]</sup>, NM<sup>[12]</sup>, DKW<sup>[13]</sup>, White<sup>[14]</sup> and B5<sup>[15]</sup> supplemented with zeatin, kinetin or BAP at 1.0 to 10.0 mg L<sup>-1</sup> (w/v). Ten replicates were used per treatment with three repetitions. After eight weeks of culture, the frequencies of plantlet regeneration were assessed. The time required for shoot formation, number of leaflets, rootlets and stem length of the plantlet produced were also recorded.

**Hardening and acclimatization:** The somatic embryo-derived plantlets with well-developed rootlets and shoots were thoroughly washed under tap water for 2 to 3 min to remove traces of agar-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 trays (59×30×12 cm) 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.

**Histological studies:** Two main samples, embryogenic and non-embryogenic callus as the control experiment were used in the histological studies. Histological studies, which involved the step of fixation, dehydration, embedding, sectioning, staining and mounting were carried out<sup>[16]</sup>. For fixation, the specimen was cut into the size of 2 to 5 mm with a sharp knife. The tissue was then fixed in vials containing FAA [70% ethanol: glacial acetic acid: formaldehyde at the ratio of 18:1:1 (v/v/v)]. The fixation was carried out at the room temperature within 24 h and the specimen was thoroughly washed for the dehydration process. After trimming, the block was ready for sectioning. The section was made with a thickness of 14 µm, whereby each trimmed cube contained a specimen. The slides were then left to dry in oven at 30 to 40°C for 2 days.

**Statistical analysis:** Variance analysis of the data was carried out and means were statistically compared using Tukey's test ( $\alpha=0.05$ ). Data were analyzed using the SPSS analysis (version 11.5) (SPSS Inc. USA).

## RESULTS AND DISCUSSION

**Effects of auxins on callus and embryogenic callus induction from different explants:** For most of the explants of *E. longifolia*, it was observed that calli started to develop at the cut regions after two weeks of culture. The calli were then further expanded to cover the whole explants surface.

In the treatment using cotyledon as explants, callus tissues formed were mostly friable or compact in texture and yellowish in colour when the medium was enriched with 2,4-D at the concentrations of 0.5 to 4.0 mg L<sup>-1</sup> (w/v). The greatest callus proliferation expressed in terms of dry weight was found on the medium containing full strength basal MS medium supplemented with MS vitamins, 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D followed by 2.0, 3.0, 4.0, 5.0 and 0.5 mg L<sup>-1</sup> (w/v) of 2,4-D with the yield for the respective treatment was 0.18, 0.14, 0.11, 0.10, 0.09 and 0.07 g DW/culture. After two subcultures, compact and nodular embryogenic calli was observed in cotyledon-derived callus cultured in 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D (Table 1). Bunches of globular and heart shaped embryoids were seen on the surface of the callus tissue. Even though the rate of embryogenic callus formation was quite low (30%), well developed and organized embryogenic callus was clearly distinguished from the non-embryogenic callus by the end of the fourth week. Embryogenic callus formation from cotyledon explants exposed to 2,4-D could have stimulated the accumulation of endogenous IAA, which had been considered to be important to the competence of carrot cells to undergo somatic embryogenesis<sup>[17]</sup>.

Apart from cotyledon, zygotic embryo explants also produced embryogenic callus at all the 2,4-D concentrations tested with the highest frequency (20%) was shown in 2.0 mg L<sup>-1</sup> (w/v) of 2,4-D. The zygotic embryo derived callus formed at various 2,4-D concentrations has different characteristics. They were either friable or compact callus with variable colorations, from yellowish to pale yellow. As for leaf explants, the data obtained revealed that the fastest callus formation was disclosed in 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D (14±2 days). Even though the highest callus induction rate was produced in this concentration (1.0 mg L<sup>-1</sup> (w/v) of 2,4-D), none of the callus developed was embryogenic. Leaf explants derived callus was soft and yellowish in colour in media supplemented with 2,4-D at 1.0 and 2.0 mg L<sup>-1</sup> (w/v).

The percentage response for callus induction and the nature of the response with various 2,4-D concentrations added to the basal MS medium in stem explants are also

Table 1: Effects of the basal MS medium supplemented with MS vitamins and different levels of 2,4-D on the callus and embryogenic callus induction from different explants of *E. longifolia*

Explants	Concentration (mg L <sup>-1</sup> )	Days callus start to form*	Callus morphology	% of explants forming callus*	Days embryo start to form*	% of calli forming embryogenic culture*	Growth after 8 weeks (g DW/culture)*
Cotyledon	0.0	0a	NR	0a	0a	0a	0.01a
	0.5	17b	Friable, yellowish	50b	0a	0a	0.07b
	1.0	14b	Compact, yellowish	90c	20b	30b	0.18d
	2.0	16b	Friable, yellowish	80c	0a	0a	0.14cd
	3.0	18b	Friable, yellowish	70bc	0a	0a	0.11bc
	4.0	18b	Friable, brown	50b	0a	0a	0.10bc
Zygotic embryo	5.0	19b	Hard, brown	50b	0a	0a	0.09bc
	0.0	0a	NR	0a	0a	0a	0.01a
	0.5	18b	Friable, yellowish	40bc	13b	10b	0.04ab
	1.0	16b	Compact, yellowish	80de	14b	10b	0.09d
	2.0	15b	Compact, yellowish	90e	14b	20b	0.10d
	3.0	17b	Friable, yellowish	60cd	15b	10b	0.08cd
Leaf	4.0	18b	Friable, yellowish	40bc	15b	10b	0.07bcd
	5.0	18b	Friable, pale yellow	30b	15b	10b	0.05bc
	0.0	0a	NR	0a	0a	0a	0.01a
	0.5	17b	Friable, yellowish	80d	0a	0a	0.04b
	1.0	14b	Soft, yellowish	90d	0a	0a	0.07c
	2.0	16b	Soft, yellowish	90d	0a	0a	0.04b
Stem	3.0	16b	Friable, pale yellow	70cd	0a	0a	0.03ab
	4.0	18b	Friable, yellow	50bc	0a	0a	0.03ab
	5.0	18b	Friable, yellow	30b	0a	0a	0.02b
	0.0	0a	NR	0a	0a	0a	0.01a
	0.5	19b	Soft, yellowish	40bc	0a	0a	0.06b
	1.0	16b	Hard, yellowish	80e	0a	0a	0.10b
Taproot	2.0	17b	Hard, yellowish	60cde	0a	0a	0.18c
	3.0	17b	Hard, yellowish	70de	0a	0a	0.07b
	4.0	18b	Hard, yellow	50bed	0a	0a	0.08b
	5.0	18b	Hard, brown	30b	0a	0a	0.06b
	0.0	0a	NR	0a	0a	0a	0.01a
	0.5	18b	Soft, yellow	40bc	0a	0a	0.04ab
Petiole	1.0	16b	Friable, yellow	70d	0a	0a	0.11c
	2.0	16b	Friable, yellow	60cd	0a	0a	0.07bc
	3.0	17b	Friable, yellow	60cd	0a	0a	0.08bc
	4.0	18b	Friable, brownish	30d	0a	0a	0.06b
	5.0	19b	Friable, brownish	30d	0a	0a	0.04ab
	0.0	0a	NR	0a	0a	0a	0.01a
Petiole	0.5	15b	Friable, pale yellow	60b	0a	0a	0.08a
	1.0	14b	Friable, pale yellow	70b	0a	0a	0.09b
	2.0	14b	Friable, pale yellow	80b	0a	0a	0.10b
	3.0	16b	Friable, pale yellow	70b	0a	0a	0.11b
	4.0	17b	Friable, pale yellow	60b	0a	0a	0.08b
5.0	17b	Friable, pale yellow	60b	0a	0a	0.03b	

NR indicates that no callus formation from the explants after four weeks of culture (NR= No Respond)

\* Means followed by the same letter(s) are not significantly different ( $p < 0.05$ ) using Tukey's test. (N=3)

shown in Table 1. Similar to the leaf explants, no somatic embryos formation was observed although 80% of the stem explants examined induced callus in 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D. In terms of texture, most of the callus produced was hard in texture and yellowish in colour. Only soft yellowish callus was developed in 0.5 mg L<sup>-1</sup> (w/v) of 2,4-D. As for the taproot explants, 70% of the explants formed callus after 16±2 days of culture in 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D. All the calli formed did not show any sign of somatic embryo formation. In the meantime, observation on petiole explants showed that the maximum rate of callus formation could be achieved in 2.0 mg L<sup>-1</sup> (w/v) of 2,4-D after 14±2 days of culture. Alike the leaf and stem explants of *E. longifolia*, the callus formed in the entire 2,4-D concentrations tested was not embryogenic

callus as formation of green embryoid was not observed on the surface of the callus after eight weeks of culture.

In general, this study proved that auxin is usually required for the induction of somatic embryogenesis with 2,4-D being used alone or in combination with other auxins<sup>[18]</sup>. A lower percentage (20%) of embryogenic callus formation in zygotic embryo explants compared to the cotyledon explants (30%) indicating that the cotyledon was more competent for embryogenic callus formation in *E. longifolia*. Besides, the embryogenesis from zygotic embryo explant has been described as coming from 'pre-embryogenic determined cells' and it is rather an 'embryo cloning' whereby a zygotic embryo is induced to replicate itself indefinitely<sup>[19]</sup>. Therefore, cotyledon explants were chosen as the best explants for further experiments.

Table 2 Effects of the basal MS medium supplemented with different levels of 2,4-D in combination with kinetin on the callus and embryogenic callus induction from cotyledon explants of *E. longifolia*

2,4-D (mg L <sup>-1</sup> )	Kinetin (mg L <sup>-1</sup> )	Days callus start to form*	Callus morphology	% of explants forming callus*	Days embryo start to form*	% of calli forming embryogenic culture*
0.0	0.0	0a	NR	0a	0a	0a
	0.5	0a	ES	0a	0a	0a
	1.0	0a	ES	0a	0a	0a
	2.0	0a	ES	0a	0a	0a
	3.0	0a	ES	0a	0a	0a
0.5	0.0	21cd	Fnable, yellowish	50c	0a	0a
	0.5	19bcd	Soft, yellow	40bc	0a	0a
	1.0	20bcd	Soft, yellow	40bc	0a	0a
	2.0	21cd	Soft, yellow	30b	0a	0a
	3.0	22 d	Soft, yellow	30b	0a	0a
1.0	0.0	14b	Compact, yellowish	90d	20bc	30a
	0.5	15bc	Compact, yellowish	80d	18b	60b
	1.0	17bcd	Compact, yellowish	80d	21c	50ab
	2.0	17bcd	Fnable, yellowish	50c	22c	40 ab
	3.0	18bcd	Fnable, yellowish	40bc	30d	40 ab
2.0	0.0	16bcd	Fnable, yellowish	70d	0a	0a
	0.5	18bcd	Fnable, yellow	70d	0a	0a
	1.0	18bcd	Fnable, yellow	50c	0a	0a
	2.0	19bcd	Fnable, yellow	40bc	0a	0a
	3.0	19bcd	Hard, yellow	40bc	0a	0a
3.0	0.0	18bcd	Fnable yellowish	70 d	0a	0a
	0.5	18bcd	Fnable yellowish	50c	0a	0a
	1.0	20bcd	Fnable yellowish	50c	0a	0a
	2.0	21bcd	Fnable yellowish	40bc	0a	0a
	3.0	22bcd	Fnable yellowish	40bc	0a	0a

NR indicates that no callus formation from the explants after four weeks of culture

ES represents the explants enlarge in size

No embryogenic callus was formed in the basal MS medium supplemented with 2,4-D in combination with other cytokinins such as BAP, zeatin and TDZ

\*Means followed by the same letters are not significantly different ( $p < 0.05$ ) using Tukey's test (N=3)

#### Embryogenic callus induction from potential explant: In

this study, six weeks of observation revealed that the ratio of 2,4-D and kinetin in the induction medium clearly affected the frequency of somatic embryogenesis in *E. longifolia*. As in *Foeniculum vulgare*<sup>[20]</sup> that required the combination of cytokinin and auxin for embryogenic callus induction, the combination of 2,4-D and kinetin also resulted in the highest percentage of embryogenic callus induction from the cotyledon explants of *E. longifolia*. High percentage of embryogenic callus induction could be due to the fact that auxin and cytokinin are the main regulators in plants involved in the regulation of cell division and differentiation<sup>[21]</sup>. Besides, the inclusion of kinetin in the medium could enhance the efficiency of calli to produce somatic embryo that could mature normally<sup>[22]</sup>.

Data obtained also disclosed that only low concentration of 2,4-D and kinetin concentrations, which is 0.5 to 3.0 mg L<sup>-1</sup> (w/v) of kinetin in combination with 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D was capable of inducing the somatic embryo formation as exhibited in Table 2. Application of 2,4-D at a higher concentration than cytokinin was found to affect negatively proembryo induction and proliferation, possible due to a toxic effect of the excessive 2,4-D, which is a known herbicide. Among all the combinations of 2,4-D and kinetin tested, the best treatment for the highest embryogenic callus formation (60%) was 0.5 mg L<sup>-1</sup> (w/v) of kinetin and 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D (Fig. 2a). The percentage of embryogenic callus formation which represents the

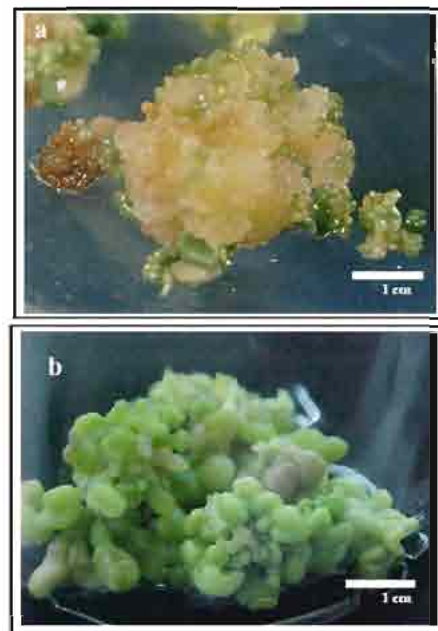


Fig. 2: Induction and multiplication of somatic embryos of *E. longifolia*. (a) Induction of embryogenic callus in 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D + 0.5 mg L<sup>-1</sup> (w/v) of kinetin (b) Multiplication of somatic embryo in 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D + 0.5 mg L<sup>-1</sup> (w/v) of kinetin and 1.0 g L<sup>-1</sup> (w/v) of activated charcoal

response capacity of the cotyledon explant to the medium achieved in this combination was 60%. Somatic embryos of *E. longifolia* induced in this combination were either formed individually or in groups on the surface of the embryogenic callus with many green globular structures. The influences of exogenously applied auxins preferentially 2,4-D on the induction of the somatic embryogenesis are well documented. It has been suggested that the presence of auxins in the culture medium influences the formation of non-polar proembryos affecting the further development of somatic embryos in the maturation phase<sup>[23]</sup>. On the other hand, cytokinin act directly on the cell cycle, where there are probably requested in the regulation of the synthesis of proteins involved in the formation and operation of the mitotic spindle<sup>[24]</sup>.

Meanwhile, significant variation was also noted in this study with the changes in 2,4-D concentration, particularly in combination with higher levels of kinetin. The percentage of somatic embryogenesis was significantly reduced by increasing the kinetin level from 1.0 mg L<sup>-1</sup> (w/v) to 3.0 mg L<sup>-1</sup> (w/v). For instance, in the combination of 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D and 3.0 mg L<sup>-1</sup> (w/v) of kinetin, only 40% of embryogenic callus was formed. This might be due to the addition of both an auxin and a cytokinin at high concentrations was associated with increased embryo proliferation and embryo development to torpedo-shaped stage rather than triggering the embryogenic callus induction. Thus, this study further agreed that a particular ratio of auxin and cytokinin in the medium might be necessary for the induction of somatic embryogenesis. The current study on *E. longifolia* also suggested that the balance between the auxin and cytokinin is probably more essential than their absolute concentrations for the initiation of embryogenic cultures although the absolute concentration of plant growth regulators is also important. Apart from that, the study also found that the substitution of kinetin with other cytokinins such as zeatin, TDZ and BAP in combination with 2,4-D in the induction medium failed to stimulate somatic embryogenesis in *E. longifolia*.

**Multiplication of somatic embryos:** Table 3 demonstrates the data on the multiplication of somatic embryos in MS medium supplemented with different levels of kinetin and 2,4-D. High frequency of embryos multiplication was significantly observed in the combination of 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D and 0.5 mg L<sup>-1</sup> (w/v) of kinetin, with the number of embryos produced were 35±2 after six weeks of culture. Great occurrence of embryogenesis in this combination could possibly be caused by the reduced

Table 3: Effects of the basal MS medium supplemented with 2,4-D in combination with kinetin on the multiplication of somatic embryos derived from cotyledon explants of *E. longifolia*.

2,4-D (mg L <sup>-1</sup> )	Kinetin (mg L <sup>-1</sup> )	Number of embryos*	Fresh weight at the 6th weeks (g FW/culture)*
0.0	0.0	10a	0.10a
	0.5	11ab	0.10a
	1.0	19cde	0.28def
	2.0	15abcd	0.21cd
	3.0	13abc	0.12ab
1.0	0.0	24efg	0.29a
	0.5	35h	0.40i
	1.0	30gh	0.37hi
	2.0	29gh	0.31efgh
	3.0	27fg	0.29defg
2.0	0.0	20cdef	0.25cdef
	0.5	27fg	0.31efgh
	1.0	26efg	0.28defg
	2.0	24efg	0.22cd
	3.0	19cde	0.19bc
3.0	0.0	18bcde	0.22cd
	0.5	25efg	0.36ghi
	1.0	26efg	0.33fgh
	2.0	23defg	0.29defgh
	3.0	22defg	0.25cde

\* Means followed by the same letter(s) are not significantly different ( $p < 0.05$ ) using Tukey's test. (N=3)

level of the growth regulators adsorbed by the embryos or due to the genetic difference<sup>[25]</sup>. Many species required supplementation of cytokinin along with auxin for optimum response and in some cases to prevent necrosis of callus. This requirement for exogenous cytokinin could be related to the maintenance of a proper balance between auxin and cytokinin, which act synergistically to regulate cell division. Moreover, cytokinin supplied to plant cell not only stimulate cell division through regulation of specific genes, but also act as membrane stabilizer and anti-ageing substance by reducing free radicals that could destroy membrane integrity<sup>[26]</sup>.

The data obtained also showed that when high levels of cytokinin were included in the media with 2,4-D, the frequency and the number of somatic embryos generated were reduced significantly. This can be clearly observed in the treatments using 1.0 mg L<sup>-1</sup> (w/v) of 2,4-D in combination with either 1.0, 2.0 or 3.0 mg L<sup>-1</sup> (w/v) of kinetin whereby only 30±3, 29±4 and 27±2 embryo were observed, respectively. Meanwhile, in the absence of plant growth regulators, no significant increment in terms of embryo multiplication was observed after sixth weeks of culture. The development of somatic embryos depends on the concentration of auxin and relatively low auxin concentration is helpful for the differentiation of somatic embryos as well as bipolar growth establishment<sup>[27,28]</sup>.

Normally, a somatic embryo passes through typical globular, heart shape and torpedo shape stage and further develops into mature cotyledonary embryo<sup>[29]</sup>. These stages were also clearly observed in the somatic embryos of woody species such as *Azadirachta excelsa* (Jack) Jacobs. Similar to *Azadirachta excelsa* (Jack) Jacobs, one



of the woody species<sup>[30]</sup>, developmental stages involved in the somatic embryogenesis of *E. longifolia* are green globular to heart embryos, torpedo and cotyledonary embryos. Somatic embryogenesis from callus culture is generally an efficient method giving a high frequency of plant regeneration<sup>[31]</sup>. However, retaining cells at their juvenile stages of differentiation and development is important to maintain their regenerable capability. In other word, if the cells in a culture have fully differentiated, this culture may have lost its totipotency or its regenerable capacity<sup>[32]</sup>.

**Effects of activated charcoal on the multiplication of somatic embryos:** In multiplying the somatic embryos of *E. longifolia*, a browning effect of the cultures was observed after three weeks of culture in medium deficient of activated charcoal. Browning effect caused poor somatic embryogenesis efficiency as previously observed in *Vitis vinifera*<sup>[33]</sup>. Supplementation of the multiplication medium with 1.0 g L<sup>-1</sup> (w/v) of activated charcoal reduced the browning effect in *E. longifolia* and caused an increase in the number of embryos produced to as high as 45±2 after six weeks of culture (Fig. 2b) compared to only 36±3 of embryos formed in the control medium, which lacked activated charcoal. The function of activated charcoal in absorbing potentially harmful substances including phenolics secreted into culture medium by

plant cells might explain the effective multiplication of *E. longifolia* in the activated charcoal forfeited medium.

The sign of germination, including the appearance of haustorial, plumular and radicular nodules occurred in *Hyophorbe lagenicaulis* embryo after culture in the medium containing 2.0 g L<sup>-1</sup> (w/v) activated charcoal<sup>[34]</sup>. Nevertheless, the data gained in this study revealed that activated charcoal with the concentrations higher than 1.0 g L<sup>-1</sup> (w/v) caused a decrease in somatic embryo multiplication in *E. longifolia* whereby only 0.54±0.06 g FW/culture and 0.35±0.05g FW/culture of somatic embryos were observed in 1.5 and 2.0 g L<sup>-1</sup> (w/v) activated charcoal, respectively.

Most of the somatic embryos of *E. longifolia* formed in this study had a normal structure and showed a low frequency of callusing, which indicates that somatic embryogenesis of *E. longifolia* might be a promising system of *in vitro* plant propagation. In general, embryogenic callus of *E. longifolia* underwent division and resulted in the formation of pro-embryos that could be observed more often and clearly in the presence of 1.0 g L<sup>-1</sup> (w/v) activated charcoal compared to the control treatment. The pro-embryos divided and developed into globular embryos (Fig. 3a) within one week. The globular embryos were then further developed into heart-shape embryos (Fig. 3b) showing a bipolar nature within one week. Differentiation of heart-shaped embryos into the

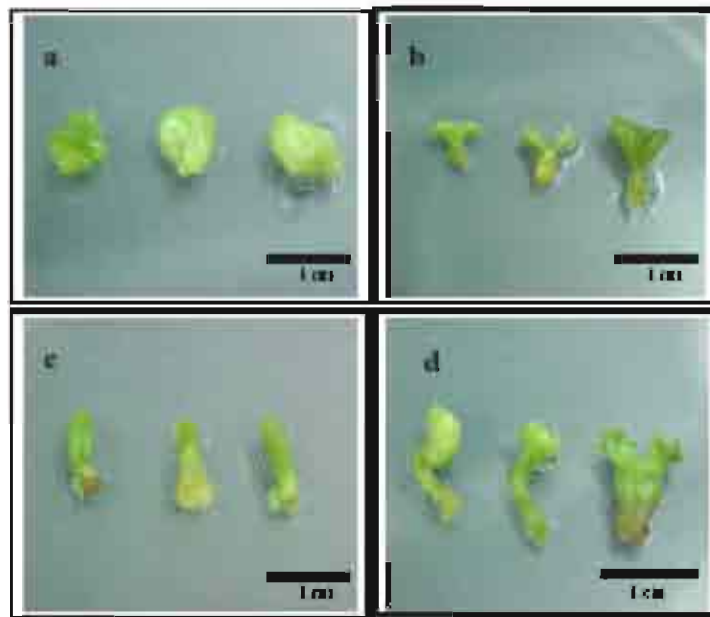


Fig. 3: Developmental stages in the somatic embryos of *E. longifolia*. (a) Early globular stage and late globular stage (b) Early heart stage and late heart stage (c) Early torpedo stage and late torpedo stage (d) Early cotyledonary stage and late cotyledonary stage

Table 4: Effects of different basal media (MS, NM, WPM, DKW, B5 and White) supplemented with cytokinins (Kinetin, BAP and zeatin) at various concentrations (1.0 to 10.0 mg L<sup>-1</sup>) on the plantlet regeneration from the somatic embryos of *E. longifolia*

Media	Hormone	Concentration (mg L <sup>-1</sup> )	Percent (%) of Regeneration*	Days shoot start to form*	No. of leaflets*	Rootlets length(cm)*	Stem length (cm)*	
MS	Kinetin	0	0a	0a	0a	0.0a	0.0a	
		1	90c	31b	14d	4.8e	4.5e	
		2	50b	35bc	10c	3.1d	3.7d	
		3	40b	37bc	8c	2.5c	2.9c	
		4	30b	40c	4b	1.3b	2.5b	
	BAP	5	30b	42c	2b	0.5b	1.3b	
		0	0a	0a	0a	0.0a	0.0a	
		1	40b	30b	8c	4.5e	3.5de	
		2	40b	37c	7c	2.3c	2.1b	
		3	30b	40c	4b	2.4c	3.2d	
	Nitsch	Zeatin	4	30b	40c	5b	3.3d	3.8e
			5	30b	42c	6bc	1.5b	2.7c
			0	0	0a	0a	0.0a	0.0a
			1	30	29b	6bc	3.8d	2.5d
			2	30	43c	5b	1.4b	1.2b
		3	30	42c	8cd	2.5c	1.9c	
		4	40	40c	10d	1.6b	3.0e	
		5	30	41c	6bc	3.5d	2.1cd	

No plantlet regeneration was observed from the somatic embryos of *E. longifolia* cultured in the basal:

MS medium supplemented with zeatin,

Nitsch medium supplemented with BAP and zeatin,

WPM, DKW, B5 and White medium supplemented either with kinetin, BAP or zeatin.

No plantlet regeneration was observed at cytokinins concentrations higher than 5.0 mg L<sup>-1</sup>.

\* Means followed by the same letter(s) are not significantly different ( $p < 0.05$ ) using Tukey's test. (N=3)

torpedo stage as shown in Fig. 3c also took place within one week. Finally, the torpedo shape embryos were also differentiated into the cotyledonary stage (Fig. 3d) within one week. It was observed that all the four stages of the somatic embryos developed in the basal MS medium fortified with the same concentration of auxin, cytokinin and 1.0 g L<sup>-1</sup> (w/v) activated charcoal. Rapid development of cotyledonary stage embryos with the presence of activated charcoal was also recorded in *Aesculus hippocastanum* L.<sup>[35]</sup>

**Plantlet regeneration from mature somatic embryos:** In this studies, the efficiency of six different basal media; MS, NM, WPM, DKW, B5 and White medium supplemented with different cytokinins, namely BAP, kinetin and zeatin at various concentrations on plantlet regeneration was examined. However, from all the combinations of media and cytokinins tested, only three combinations; the basal MS medium supplemented with kinetin, BAP as well as NM enriched with kinetin at various concentrations managed to show significant plantlet regeneration after two months of culture. Thus, only the results obtained from these three combinations were further discussed.

The study revealed that kinetin at lower concentration (1.0 to 4.0 mg L<sup>-1</sup> (w/v)) is an ideal cytokinin in stimulating regeneration from somatic embryos of *E. longifolia*. This could be due to the genes that trigger cellular differentiation in culture can be selectively influenced by growth regulators<sup>[36]</sup>. Kinetin

generally gave better results in plantlet regeneration than other cytokinins tested. The result obtained revealed that increased concentrations of kinetin brought about a decrease in the regeneration rate whereby plantlet formation was only 50, 40 and 30% in 2.0, 3.0 and 4.0 mg L<sup>-1</sup> (w/v) of kinetin, respectively. At higher concentration of kinetin (5 mg L<sup>-1</sup> (w/v) in this study), only 30% of the mature embryos produced shoots. On the contrary, a 90% of regeneration rate was observed in embryos treated with 1.0 mg L<sup>-1</sup> (w/v) of kinetin. Similarly, in *Medicago arborea*, which is also a woody species, its somatic embryos were successfully germinated in the regeneration medium that containing 1.0 mg L<sup>-1</sup> (w/v) of kinetin<sup>[37]</sup>.

As shown in Table 4, it was found that the mature embryos of *E. longifolia* required approximately 31±2 days of culture to regenerate into plantlet in the basal MS medium supplemented with 1.0 mg L<sup>-1</sup> (w/v) of kinetin. This period is significantly shorter compared to the three and five months period needed in forming shoot from somatic embryos of *Buchloe datyloides*<sup>[38]</sup> and the woody species, *Calliandra tweedii*, accordingly<sup>[39]</sup>. Observation on leaflets formation as shown in Table 4 disclosed that the highest number of leaflets (14.0±0.7) was formed in 1.0 mg L<sup>-1</sup> (w/v) of kinetin whilst 10.0±0.5, 8.0±0.4, 4.0±0.2 and 2.0±0.1 of leaflets were obtained in 2.0, 3.0, 4.0 and 5.0 mg L<sup>-1</sup> (w/v) of kinetin, respectively. Likewise in the rootlets formation, the longest rootlets, 4.8±0.2 cm was also attained in plantlet regenerated from embryos treated with 1.0 mg L<sup>-1</sup> (w/v) of kinetin. This was then followed



Fig. 4: Plantlet regenerated from somatic embryos of *E. longifolia* after two months of culture in  $1.0 \text{ mg L}^{-1}$  (w/v) of kinetin



Fig. 5: Somatic embryo derived plant of *Eurycoma longifolia* after two months of transplantation into the soil with the composition of nitrogen, phosphate, potassium at the ratio of 1: 3: 2

by  $3.1 \pm 0.2$ ,  $2.5 \pm 0.1$ ,  $1.3 \pm 0.1$ ,  $0.5 \pm 0.0$  cm rootlets which was achieved in 2.0, 3.0, 4.0 and  $5.0 \text{ mg L}^{-1}$  (w/v) of kinetin. Similarly, the same profile as in rootlets formation was detected in stem whereby shorter stem was observed in increased concentrations of kinetin. The longest stem obtained was  $4.5 \pm 0.2$  cm in  $1.0 \text{ mg L}^{-1}$  (w/v) of kinetin. Rooting of regenerated plantlets could occur without the presence of rooting plant growth regulators such as IBA and IAA because they were developed from somatic embryos, which typically form rootlets by themselves<sup>[40]</sup>.

By taking the three main parameters, number of leaflets, rootlets and stem length into consideration, it was found that among all the concentrations tested,  $1.0 \text{ mg L}^{-1}$

(w/v) of kinetin (Fig. 4) managed to produce healthy and balance plantlets in terms of leaflets ( $14 \pm 0.7$ ), stem ( $4.5 \pm 0.2$  cm) and rootlets ( $4.8 \pm 0.2$  cm) formation. Even though shoots were also produced in 4.0 and  $5.0 \text{ mg L}^{-1}$  (w/v) of kinetin, the plantlet produced was not as healthy as in  $1.0 \text{ mg L}^{-1}$  (w/v) of kinetin and the regeneration rate in these concentrations was low. Many embryogenic systems, including woody plant species like conifers appear to produce somatic embryos that are capable of germination and plant establishment but do not fully mature, resulting in slow germination and initial growth<sup>[41]</sup>.

**Hardening and acclimatization:** Rooted plantlets from the plantlet regeneration were successfully acclimatized and had an 80% survival rate. Acclimatized plantlets were healthy, green and well developed when transferred to soil. The regenerated plants attained a height of  $9.0 \pm 1.0$  cm two months after transplantation. All of them are identical to the parent plant in the nature environment. No morphological difference was observed after two months in the soil (Fig. 5). Although a 100% of survival rate could not be achieved, these results on *E. longifolia* is better than that reported in *Corydalis yanhusuo* whereby none of the 30 converted somatic embryos of *Corydalis yanhusuo* survived when transplanted for acclimatization<sup>[42]</sup>.

**Histological studies:** The embryogenic callus of *E. longifolia* formed was confirmed through histological analysis as the formation of embryogenic cells can be correlated with the characteristic morphological changes<sup>[21]</sup>. Figure 6a and b represent the differences between embryogenic callus and non-embryogenic callus under 400X and 100X magnifications. The formation of vascular strand (vs) and shoot meristem (sm) could be clearly visible in embryogenic callus of *E. longifolia* as shown in Fig. 6c. However, in non-embryogenic callus (Fig. 6d), bigger and highly vacuolated cells were observed. No nucleus was seen in this callus that indicated the structure of non-embryogenic callus. Development of bi-polar embryos at heart and torpedo stages was also noticed in embryogenic callus of *E. longifolia* as displayed in Fig. 6e and f under 40X magnifications. Meanwhile, Fig. 7a exhibits the formation of secondary somatic embryo developed from the primary somatic embryo. Secondary somatic embryo formation is a common process involved in the somatic embryogenesis in many woody species. Several secondary globular structures were also observed (Fig. 7b). Normal somatic embryos were also observed loosely attached to the surface of calluses and matured asynchronously. The mature embryo had two well-developed cotyledons, shoot

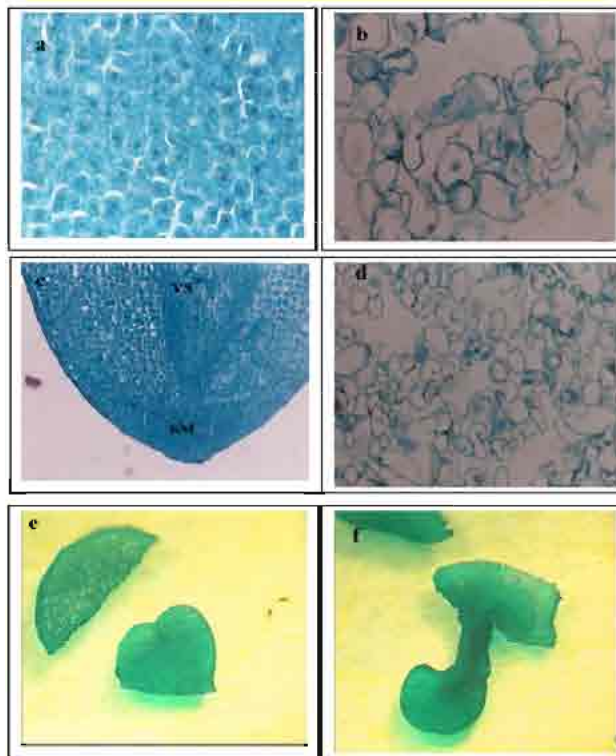


Fig. 6: Structure and development of somatic embryo in *Eurycoma longifolia*. (a) High magnification of somatic embryo section (magnification 400X) (b) Non-embryogenic callus (magnification 200X) (c) Longitudinal section of the embryogenic callus, (VS) representing a vascular strand and (SM) show the shoot meristem (magnification 100X) (d) Non-embryogenic callus (magnification 100X) (e) Showing the heart shape (magnification 40X) (f) Cross section of torpedo shape (magnification 40X)

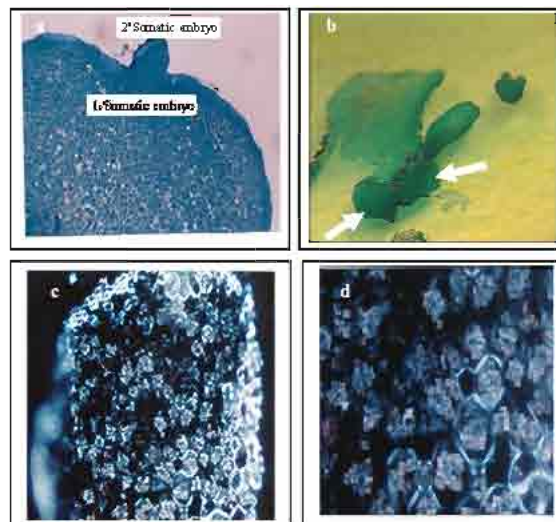


Fig. 7: Structure and development of somatic embryo in *E. longifolia*. (a) Direct somatic embryogenesis showing primary and secondary embryos (magnification 100X) (b) Longitudinal section through an embryogenic callus showing several secondary globular structures (magnification 40X) (c) Starch structure observed in the embryogenic callus (magnification 200X) (d) Starch structure observed in the embryogenic callus (magnification 400X)

and root primordia and provascular traces that were already present before their complete maturation.

Numerous starch granules (Fig. 7c and d) were also detected in the embryogenic callus of *E. longifolia*. A characteristic feature of *in vitro* callus cells is the rapid appearance of starch in callus cells, marked the differences existing between embryogenic and non-embryogenic cells<sup>[43]</sup>. The presence of high levels of hydrocarbon reserves such as starch has been reported at the beginning of several *in vitro* development processes<sup>[44]</sup>. Numerous starch grains were usually found in cells near the meristematic tissues derived from immature and mature embryos<sup>[45]</sup>. The possible role of this polysaccharide in plant cells remains unclear, although it has been suggested that it could act as an energy source or as an essential osmotic agent for development<sup>[43,44]</sup>. The degradation of starch give rise to the formation of glycolytic intermediates that, when catalyzed oxidatively, supply high levels of ATP that can be used by cellular metabolism<sup>[43]</sup>.

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