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Thidiazuron Induces High Frequency Direct Somatic Embryogenesis Growth from Cotyledon Culture of *Eurycoma longifolia*

(Tidiazuron Merangsang Pertumbuhan Somatik Embriogenesis daripada Kultur Kotiledon *Eurycoma longifolia*)

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ABSTRACT

Eurycoma longifolia *is one of the well-known herbal plants in Southeast Asian region due to its remarkable properties, especially the root part that can be used as aphrodisiac, anti-cancer, anti-malaria and anti-ulcer agent. Uncontrolled harvesting of this plant has reduced its population. Thus, an efficient protocol involving the induction of direct somatic embryogenesis from cotyledon culture of* E. longifolia *has been developed. 15% explants forming embryos were achieved on Modified Murashige and Skoog's (MMS) medium supplemented with 0.1 mg/L zeatin plus 0.2 mg/L indole-3-butyric acid (IBA). However, the addition of 0.12 mg/L thidiazuron (TDZ) has increased the percentage up to 22.0%. The maturation phase occurred in the same medium, thus decreased the frequency of subculture and the genetic instability of the embryos. Secondary somatic embryogenesis growth of 20%, which was the highest percentage of growth, was achieved from the primary embryos that were cultured in the MMS medium with the treatment of 2.0 mg/L IBA plus 0.075 mg/L TDZ. The results indicated that the direct somatic embryogenesis obtained can be further used to develop a protocol for plantlet regeneration of* E. longifolia.

Keywords: Cotyledon; Eurycoma longifolia*; secondary embryogenesis; thidiazuron; zeatin*

ABSTRAK

Eurycoma longifolia *adalah tumbuhan herba yang terkenal di kawasan Asia Tenggara memandangkan potensinya yang telah dikenal pasti terutamanya bahagian akar untuk mengubati malaria, ulser, kanser dan lemah tenaga batin. Penuaian tumbuhan ini yang dilakukan tanpa kawalan telah menyebabkan populasinya semakin berkurangan. Maka satu protokol cekap melibatkan aruhan secara terus somatik embriogenesis daripada pengkulturan kotiledon* E. longifolia *telah dijalankan. Pertumbuhan embrio sebanyak 15% telah berjaya dicapai menerusi media pertumbuhan Modified Murashige dan Skoog's (MMS) yang ditambah 0.1 mg/L zeatin dan 0.2 mg/L indole-3-butyric acid (IBA). Namun, penambahan 0.12 mg/L tidiazuron (TDZ) telah meningkatkan kadar pertumbuhan sehingga 22.0%. Pertumbuhan dan pertukaran embrio somatik ke fasa matang berlaku di dalam media pertumbuhan yang sama, oleh itu ia mengurangkan kekerapan subkultur dan ketidakstabilan genetik embrio tersebut. Pertumbuhan embrio sekunder sebanyak 20.0% iaitu pertumbuhan paling tinggi, telah dicapai daripada embroid primer yang dikulturkan di dalam media pertumbuhan MMS yang ditambah 2.0 mg/L IBA dan 0.075 mg/L TDZ. Keputusan ini menunjukkan pertumbuhan terus embrio somatik yang diperoleh berpotensi diaplikasikan untuk ke peringkat seterusnya dalam mikropropagasi* E. longifolia.

Kata kunci: Embrio sekunder; Eurycoma longifolia*; kotiledon; tidiazuron; zeatin*

INTRODUCTION

Eurycoma longifolia Jack is a slender, evergreen flowering tree belonging to the family Simaroubaceae that grows naturally in the jungles of Malaysia and Indonesia (Klin et al. 2009). Its root has been used traditionally to restore energy and vitality, enhance blood flow and function as herbal ingredient for women after child birth (Ismail et al. 1999). Due to its therapeutic benefits in traditional medicinal practices, *E. longifolia* has gained scientific interest among researchers, thus has increased its commercial value. The root extracts of *E. longifolia* have been proven to show anabolic properties (Hamzah & Yusof 2003), cytotoxic activity against tumour cells (Tee & Azimahtol et al. 2005), more potent antiplasmodial activity than chloroquine (Chan et al. 2004) and also anti-malaria properties (Chan et al. 1986).

All these curative properties are provided by the 65 compounds that were detected in the *E. longifolia* root in the group of quassinoids, squalene derivatives and triterpenes (Kuo et al. 2003). Other compounds such as 13, 21-dihydroeurycomanone have shown antiplasmodial activity (Klin et al. 2009) and eurycomanone has a potential cytotoxic effect against cancerous liver cell (Zakaria et al. 2009). The species also contain alkaloid compound,

such as 9-methoxycanthin-6-one that exhibits cytotoxic activity (Kardono et al. 1991), anti-cancer properties along with anti-microbial agent (Choo et al. 2000) and also anti-malaria properties (Chan et al. 2004). A study was conducted to improve 9-methoxycanthin-6-one productivity from callus cultures of *E. longifolia* (Rosli et al. 2009).

With an increasing demand for the root part of *E. longifolia* and the shortage of its supply, there is a need to develop an efficient mass propagation of this species. *In vitro* propagation from callus explants was reported via indirect somatic embryogenesis from cotyledon culture of *E. longifolia* (Hussein et al. 2005). However, to the best of our knowledge, *in vitro* mass propagation from direct somatic embryogenesis has not been reported. In addition, the embryogenic pathway is a suitable methods to learn about the morphophysiological and molecular aspects involved in cellular differentiation (Rocha et al. 2012), the biochemical pathways during the development of embryogenesis (Balzon et al. 2013) and the propagation of non-seed producing plants or plants that do not respond to conventional propagation techniques such as cuttings (Anthony et al. 2004). Generally, the application of auxin and cytokinin is a key factor in the determination of embryogenic response. Other growth regulators such as abscisic acid (ABA), zeatin and thidiazuron (TDZ) that belongs to the phenylureas, have become the alternative for high-frequency direct regeneration of somatic embryo (Jiménez 2005). As such, cotyledon explants *E. longifolia* were cultured on Modified Murashige and Skoog (1962) basal medium with zeatin, IBA, ABA, TDZ and silver nitrate ($AgNO_3$) to determine the optimum growth conditions for the induction, development and germination of somatic embryos.

Materials and Methods

PLANT MATERIALS

Young seeds of *E. longifolia* were collected from the herbal garden in the Museum of Terengganu. The seeds were sterilized with 70% ethanol for 1 min and later with 20% sodium hypochlorite (NaOCl) together with 3 drops of Tween 20© for 20 min. Thereafter, the seeds were rinsed with sterilized distilled water. Each seed coat was then cut open. The two lobed cotyledons in the seed were dissected horizontally into three equal parts with the cut surface of explants facing the media.

EMBRYO INDUCTION MEDIUM

For direct induction of somatic embryos, Modified Murashige and Skoog (MMS) containing a combination of IBA (0.2, 0.4, 0.8 mg/L) and zeatin (0.05, 0.1 and 0.2 mg/L) was used as the basal medium. Each Petri dish consisted of ten explants and cotyledons cultured in a growth regulators-free medium that served as the control.

EMBRYOGENESIS GROWTH

A series of ABA (0.05, 0.1, 0.2 and 0.4 mg/L), $AgNO_3$ (0.5, 1.0, 2.0 and 4.0 mg/L) and TDZ (0.015, 0.03, 0.06 and 0.12 mg/L) were added in MMS that contained combination of 0.2 mg/L IBA and 0.1 mg/L zeatin. Although treatment with 0.4 mg/L IBA plus 0.1 mg/L zeatin showed the highest percentage of embryo growth (Table 1), treatment 0.2 mg/L IBA plus 0.1 mg/L zeatin was selected as a control because there was no significant difference between the two treatments. Each Petri dish consisted of ten explants.

SECONDARY EMBRYO INDUCTION

To stimulate secondary somatic embryogenesis for *E. longifolia*, only primary somatic embryo clumps were detached and individually inoculated on MMS media without growth regulators (control) or with IBA (0.5, 1.0 and 2.0 mg/L) and TDZ (0.05, 0.075, 0.1 and 0.2 mg/L). The numbers of primary embryos that produced secondary embryogenesis were recorded after six weeks of culture by counting under on a fluorescent microscope (DM5000B, Leica, Germany) equipped with a digital camera in a bright-field contrast.

CULTURE MEDIA AND *IN VITRO* GROWTH CONDITIONS

The Modified MS medium contained the following macronutrients: 1320 mg/L NH_4NO_3, 1520 mg/L KNO_3, 264 mg/L $CaCl_2 \cdot 2H_2O$, 444 mg/L $MgSO_4 \cdot 7H_2O$ and 340 mg/L KH_2PO_4. The content of micronutrients was 33.8 mg/L $MnSO_4 \cdot 4H_2O$, 0.5 mg/L CuSO4·5H_2O, 17.2 mg/L $ZnSO_4 \cdot H_2O$, 12.4 mg/L H_3BO_3, 1.7 mg/L KI, 0.5 mg/L $Na_2MoO_4 \cdot 2H_2O$ and 0.05 mg/L $CoCl_2 \cdot 6H_2O$. The content of iron-EDTA and supplements was according to MS medium. For all the experiments, the media which contained 3% sucrose and 1 g/L of activated charcoal were adjusted to pH5.7 and solidified with 0.25% phytagel. Cultures were maintained in a controlled environment growth room at 25± 2°C in the darkness.

HISTOLOGY PREPARATION OF EMBRYOS

The anatomical examination was carried out in order to determine the origin of the secondary somatic embryos. Primary and secondary embryos for histological studies were taken from the six weeks old incubation. The tissues were fixed with 50.0% formalin acetic acid (FAA) for 48 h at room temperature. After being dehydrated in a series of ethanol (30 and 50%) followed by ascending tertiary butyl alcohol (TBA) (60, 70, 85, 95 and 100%) and lastly by parraffin oil, the embryos were embedded in paraffin wax at 60°C and cut into 10.0 mm sections by rotary microtome (RM 2245, Leica, Germany). The sections were then transferred and affixed to the glass slides. After staining with safranin and fast green, the specimens were mounted with cover slips by using Canada balsam mounting media. Permanent slides were then examined under a fluorescent microscope (DM5000B, Leica, Germany) equipped with a digital camera in a bright-field contrast.

Statistical Analysis

All the experiments were conducted according to a complete randomized design by taking 60 explants per treatment (20 explants in three replicates). The stages of embryo developments which are globular, torpedo and cotyledonary were observed. For embryo induction medium and enhancing the embryogenesis growth experiments, after 6 weeks of culture, the percentage of explants forming embryos was counted as number of embryos growth from the explants per total explants times hundred.

The statistical analysis was done through the analysis of variance between groups (ANOVA) and Duncan's Multiple Ranges Test (DMRT) using the Statistical Package for Social Science (SPSS) program. Data were expressed as mean of three determinations±Standard Error (SE).

Results

Direct Embryogenesis from Cotyledon Culture on Embryo Induction Medium

Three weeks after being cultured, the first somatic embryos began to appear at the cut edge of the cotyledon (Figure 1(a) and 1(b)) but the rate of growth was very low. Observation from week 3 till 8 found that the highest percentage of explants forming embryos was in week 6 and the rate of growth in week 7 till 8 was constant (data not shown). All the treatments showed direct formation of somatic embryogenesis (Table 1). However callus was observed in several explants.

The growth of callus was unexpected since the aim of this present study was to induce direct somatic embryogenesis from the cotyledon. The treatments might be suitable for callus induction but after being sub-cultured into the same media, there was no response from the callus neither it was proliferated nor became embryos (data not shown). This might be due to either the callus might not be embryogenic or the treatments were not suitable for callus subculture.

The highest percentage of cotyledon produced embryos was 15.0% in the treatment of 0.1 mg/L Zeatin plus 0.4 mg/L IBA. Between 4 and 8 weeks of culture, most of the embryos were globular or heart-shape. From week 7 till 8, the somatic embryos developed into the torpedo and cotyledon stage (Figure 1(d) and 1(e)).

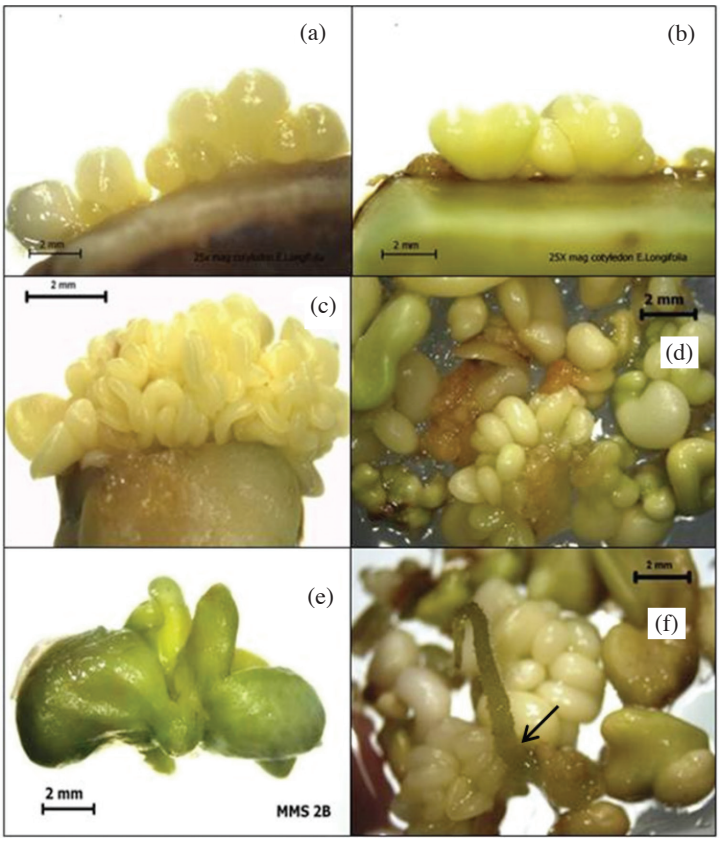

FIGURE 1. Macroscopic observation of somatic embryogenesis from cotyledon explants of *E. longifolia* cultivated on MMS medium (a) Early globular embryogenesis at cut edge of the cotyledon after 4 weeks of culture with the treatment of IBA (0.2 mg/L), zeatin (0.1 mg/L), ABA (0.1 mg/L), (b) Heart shape stage of the embryos, (c) Multiplication of secondary embryos from a primary embryos, (d) Somatic embryos in globular and heart-shaped in secondary embryos induction medium with TDZ (0.075 mg/L) and IBA (0.5 mg/L), (e) Cotyledon stage of the embryos and (f) Somatic embryos with long hypocotyls on MMS medium supplemented with TDZ (0.05 mg/L) and IBA (0.5 mg/L)

EFFECTS OF $AGNO_3$, ABA AND TDZ IN ENHANCING THE EMBRYOGENESIS GROWTH

To improve the rate of embryo growth, the cotyledons were cultured in the media containing zeatin and IBA with the addition of $AgNO_3$, ABA or TDZ. The percentage of embryo induction decreased compared with control when cotyledons were cultured in the treatment containing 1.0, 2.0 or 4.0 (mg/L) $AgNO_3$, 0.4 mg/L ABA and 0.03 mg/L TDZ (Table 2). The highest percentage of direct embryogenesis was from the addition of 0.12 mg/L TDZ (22.0%). Notably, the growth rate of control (15.0%) was slightly lower than in the previous culture (Tables 1 and 2). This may be due to the difference in terms of the time the seeds were taken as explants that might change the endogenous level of growth regulator in the cotyledon of *E. longifolia*.

EFFECTS OF IBA AND TDZ IN SECONDARY EMBRYOS INDUCTION

The growth rate for secondary somatic embryogenesis in various concentrations of IBA and TDZ varied from 10.0% to 68.0% (Table 3). Overall, the secondary embryo inductions were favored in medium supplemented with

TABLE 1. The effects on percentage of embryo induction from cotyledon culture of *E. longifolia* in the embryo induction medium containing zeatin and IBA

Zeatin	IBA	Percentage of explants forming embryos	Percentage of callus induction	Percentage of explants forming callus and somatic embryos
0.0	0.0	1.00± 0.69^{g}	13.33±0.21^{f}	0.0±0.00^{f}
0.0	0.2	2.00±0.92^{g}	11.67±0.17^{f}	3.33±0.21^{e,f}
0.0	0.4	3.50± 1.09^{f,g}	5.00±0.22^{f}	3.33±0.21^{e,f}
0.0	0.8	2.50± 0.99^{g}	23.33±0.21^{e}	8.33±0.17^{d,e}
0.05	0.0	4.50± 1.14^{e,f,g}	45.00±0.21^{c,d}	15.00±0.22^{b,c}
0.05	0.2	5.00±1.15^{d,e,f,g}	46.67±0.33^{b,c,d}	18.33±0.17^{e,f}
0.05	0.4	7.50±0.99^{c,d,e,f}	48.33±0.31^{b,c,d}	20.00±0.00^{a,b}
0.05	0.8	8.00± 0.92^{c,d,e}	41.67±0.31^{d}	6.67±0.21^{a,b}
0.1	0.0	9.50±0.50^{b,c}	31.67±0.31^{e}	6.67±0.21^{d,e}
0.1	0.2	14.00±1.12^{a}	48.33±0.43^{c,d}	20.00±0.00^{d,e}
0.1	0.4	15.00± 1.15^{a}	53.33±0.42^{a,b,c}	21.67±0.17^{a}
0.1	0.8	11.00±0.69^{a,b,c}	60.00±0.37^{a}	11.67±0.17^{c,d}
0.2	0.0	11.00± 0.69^{a,b,c}	51.67±0.31^{a,b,c,d}	8.33±0.17^{d,e}
0.2	0.2	12.50± 0.99^{a,b}	45.00±0.42^{c,d}	18.33±0.17^{a,b}
0.2	0.4	9.50± 0.50^{b,c}	51.67±0.5^{a,b,c}	15.00±0.22^{b,c}
0.2	0.8	9.00± 0.69^{b,c,d}	56.67±0.33^{a,b}	11.67±0.17^{c,d}

Data were recorded after 6 weeks of culture incubation. Values that represent the mean±SE following the same letter within columns are significantly different, according to Duncan's Multiple Range Test (p<0.05)

TABLE 2. Effects of $AgNO_3$, ABA and TDZ in enhancing the embryogenesis growth

Zeatin (mg/L)	IBA (mg/L)		Percentage of explants forming embryos
0.1	0.2	Control	15.33±1.92^{a,b,c}
		$AgNO_3$ (mg/L)	
0.1	0.2	0.5	17.33±1.18^{a,b}
0.1	0.2	1.0	8.00±2.00^{c,d,e}
0.1	0.2	2.0	2.67±1.18^{e}
0.1	0.2	4.0	2.67±1.18^{e}
		ABA (mg/L)	
0.1	0.2	0.05	18.67±1.33^{a,b}
0.1	0.2	0.1	19.33±1.82^{a}
0.1	0.2	0.2	11.33±2.15^{b,c,d}
0.1	0.2	0.4	7.33±1.18^{d,e}
		TDZ (mg/L)	
0.1	0.2	0.015	16.67±1.26^{a,b}
0.1	0.2	0.03	16.67±1.59^{a,b}
0.1	0.2	0.06	22.00±1.45^{a}
0.1	0.2	0.12	22.00±2.00^{a}

Data were recorded after 6 weeks of culture incubation. Values that represent the mean±SE following the same letter within columns are significantly different, according to Duncan's Multiple Range Test (p<0.05)

TABLE 3. The effects of IBA and TDZ in secondary embryos induction with every treatment were added 1 g/L of activated charcoal

IBA	TDZ	Percentage of primary embryos with secondary embryos	Percentage of secondary embryos growth
0.0	0.0	16.0 ± 0.24 ^{a,b,c}	66.0 ±1.35^{a,b}
0.0	0.05	10.0 ± 0.31 ^{c,d,e}	28.0 ± 1.06 ^{a,b}
0.0	0.075	10.0 ±0.01 ^{c,d,e}	24.0 ±0.60 ^{a,b}
0.0	0.1	6.0 ± 0.24^{e,d}	18.0 ± 1.00 ^{a,b}
0.0	0.2	14.00 ± 0.24 ^{a,b,c,d}	36.0 ±0.78 ^{a,b}
0.5	0.05	12.00 ± 0.20 ^{c,d,e}	30.0 ±0.43 ^{a,b}
0.5	0.075	18.00 ± 0.20 ^{a,b}	32.0 ±0.43 ^{a,b}
0.5	0.1	6.00 ± 0.24^{e,d}	12.0 ±0.57 ^{a,b}
0.5	0.2	16.0 ±0.24 ^{a,b,c}	42.0 ±0.5 ^{a,b}
1.0	0.05	14.0 ±0.24 ^{a,b,c,d}	42.0 ±0.72 ^{a,b}
1.0	0.075	8.0 ±0.20^{c,d,e}	10.0 ±0.25^{b}
1.0	0.1	6.0 ±0.24 ^{d,e}	10.0 ±0.33^{b}
1.0	0.2	8.0 ±0.20 ^{c,d,e}	12.0 ±0.29^{b}
2.0	0.05	8.0 ±0.20 ^{c,d,e}	28.0 ±1.50 ^{a,b}
2.0	0.075	20.0 ±0.32 ^{a}	68.0 ±0.43 ^{a,b}
2.0	0.1	10.0 ±0.31 ^{c,d,e}	44.0 ±1.56 ^{a,b}
2.0	0.2	4.0 ±0.24^{e}	20.0 ±0.01^{b}

Data were recorded after 6 weeks of culture incubation. Values that represent the mean±SE following the same letter within columns are significantly different, according to Duncan's Multiple Range Test (p<0.05)

IBA plus TDZ and fewer embryos were grown in the media only containing TDZ. The highest percentage of secondary somatic embryo growth was from the treatment of 2.0 mg/L IBA plus 0.075 mg/L TDZ (20.0%). The secondary embryos had formed over the entire surface of cotyledons of the embryos (Figure 1(d)) and developed into the cotyledonary stage within 8 weeks (Figure 1(e)).

ANATOMICAL OBSERVATION

A multicellular origin of the secondary somatic embryos was emphasized. Light microscopy of the embryogenic sector showed densely stained cells (Figure 2(a)) which were meristematic. Histological observations showed early evidence of embryo primordia (Figure 2(a)) as densely-stained groups of five or more meristematic cells, located within the tissues of the primary embryo and these groups of cells probably developed into secondary embryos. The secondary embryo cells derived from the primary one (Figure 2(b)). These primordia evolved into globular embryos that grew to the cotyledonary-stage through the heart-shape (Figure 2(c)) and they were observed to have bipolar embryonic axis. Figure 2(d) shows meristematic region of a shoot apex.

DISCUSSION

2,4-Di-chlorophenoxy-acetic acid (2,4-D) was commonly used as an auxin to induce the somatic embryogenesis. However, in this present study, IBA and Zeatin can also be applied as a somatic embryogenesis inducer (Table 1). In fact, the results showed that high competency of *E. longifolia* cotyledon explants had consistently formed somatic embryos directly without an intervening callus phase. Direct somatic embryogenesis will reduce the genetic instability that often associated with somatic embryos obtained indirectly from callus (Rai & McComb 2002).

According to Dhandapani et al. (2007), the frequency of somatic embryogenesis development in cells and tissues does not only depend on the explant types, but also on the combinations and concentrations of plant growth regulators. The present results showed that the combinations of TDZ with IBA and zeatin in MMS has increased the percentage of embryo growth. There were also some reports of TDZ, $AgNO_3$ (Vasic et al. 2001), ABA (Bell et al. 1993; Nishiwaki et al. 2000) and zeatin (Fujimura & Komamine 1975) that has promoted the somatic embryogenesis process.

The results (Table 2) showed the role of TDZ is a potent inducer of direct somatic embryogenesis with addition of IBA and zeatin. The effectiveness of TDZ was possibly due to its capacity to stimulate endogenous cytokinin biosynthesis that would alter endogenous cytokinin metabolism and the TDZ itself is highly resistant to degradation by cytokinin oxidase. Thus, it possesses a strong cytokinin-like activity that exceeds the other commonly used adenine-type cytokinins including benzylaminopurine or kinetin (Li et al. 1998). Study from Rocha et al. (2012) also showed that the greatest embryogenic response of *Leucopogon verticillatus* was from the treatment of TDZ and zeatin combined with IAA with the first appearance of somatic embryo growth after 3 weeks of inoculation.

The direct formation of somatic embryos was suppressed by the addition of absicsic acid (ABA) (Table 2). This demonstrates that the addition of ABA might inhibit the biochemical reaction of IBA and zeatin in certain concentrations. Inhibition or suppression of embryo induction by the addition of growth regulators is rare,

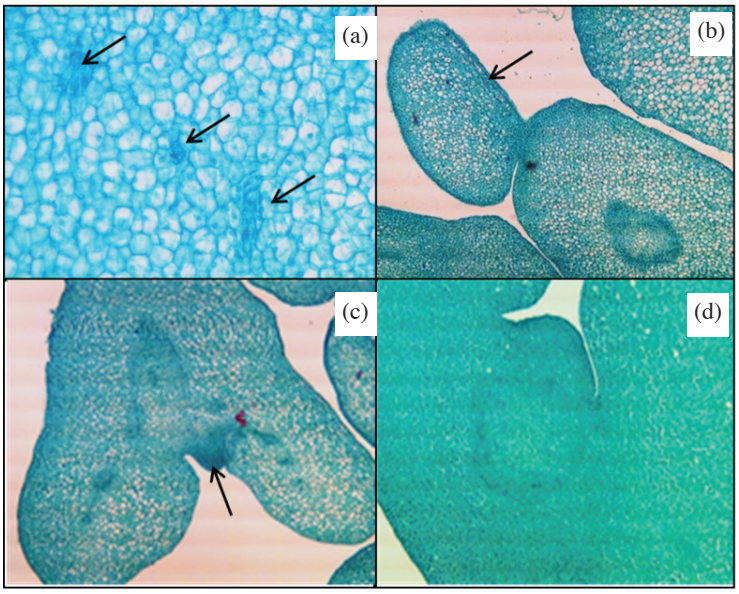

FIGURE 2. Direct somatic embryogenesis and secondary embryogenesis growth in cotyledon cultures of *E. longifolia* (a) Intensely-dividing meristematic cells (arrows), (b) Secondary embryo cells derived from the primary one (arrow), (c) Heart shaped embryo with embryonic axis (arrow) and (d) Meristematic region of a shoot apex

since most studies have reported the use of auxin for the induction of somatic embryo (Wu et al. 2007). ABA controls the embryo formation and maturation, the initiation and progressing of tissue desiccation and the induction of seed dormancy (Santa-Catarina et al. 2006). So, it was suggested that the addition of ABA will induce the maturation phase of somatic embryogenesis, but not the somatic embryogenesis from the explant. Besides, the response of the explant tissue to ABA is different between the species. For instance, an exposure to the abscisic acid during the induction as well as the differentiation stage reduced the number of somatic embryos obtained in *Medicago sativa* (Ruduś et al. 2001). Meanwhile, ABA is commonly utilized to promote somatic embryo development in many coniferous genera, including *Picea*, *Larix* and *Pinus* (Stasolla & Yeung 2003).

The addition of $AgNO_3$ along with 2,4-D has been shown to promote the frequency of embryogenesis in *Paspalum scrobiculatum* (Rashid 2002) and in fertile plant recovery from shoot apex explants of *Allium cepa* (Ramakrishnan & Ceasar 2013). However, the addition of $AgNO_3$ in the treatment was expected to increase the somatic embryogenesis growth rate since it is a potent inhibitor to reduce the biological action of ethylene, which influences cell division and cytodifferentiation (Ramakrishnan & Ceasar 2013). A higher concentration of silver nitrate (1.0-4.0 mg/L) strongly inhibited the formation of embryos, indicating an inhibitory effect of silver nitrate but at low concentrations (0.5 mg/L). Silver nitrate slightly increased the production of somatic embryogenesis compared with the control (Table 2). The same results were observed in the somatic embryogenesis of *Coffea* where at low concentrations, $AgNO_3$ improved embryo yield, while higher doses negatively affected the regenerative capacity (Fuentes et al. 2000). Silver nitrate had also decreased the phenolic accumulation in culture medium for somatic embryogenesis induction in *Eucalyptus globulus,* but the somatic embryogenesis potential was severely repressed (Pinto et al. 2008).

Primary embryos of *E. longifolia* at the cotyledonary stage appeared to be capable of producing secondary embryos without an intervening callus phase. This phenomenon of embryo-to-embryo proliferation has been described as direct secondary or accessory embryogenesis (Śliwińska et al. 2008). Secondary embryos of *E. longifolia* were able to develop on solid media without growth regulators but higher somatic embryogenesis growth was observed on the treatment of 2.0 mg/L plus 0.075 mg /L TDZ (Table 3). Our findings differed from Hussein et al. (2005) who reported that *E. longifolia* with the addition of 2,4-D was found to be a prerequisite for an effective induction of secondary embryogenesis.

TDZ alone seemed to be less responsive for secondary somatic embryogenesis induction. However, combination with IBA exhibited a reasonable number of somatic embryos (Table 3). Naing et al. (2012) also reported that no formation of embryo from leaf explants of *Chrysanthemum* cv. Euro when cultured on the medium containing TDZ alone, but combination with 2,4-D produced secondary embryos growth.

The anatomical examinations (Figure 2) illustrate the origin of the secondary somatic embryos derived from the peripheral region and also from inside the tissues of the primary embryo. Shi et al. (2010) also reported that secondary somatic embryos in camphor tree (*Cinnamomum camphora*) were produced on the surfaces of the cotyledons and radicular ends of maternal somatic embryos. According to Raemakers et al. (1995), cells that were capable of direct somatic embryogenesis were physiologically similar to

those in zygotic embryo. These cells were frequently found either in tissue before the onset of embryogenesis or in the developing zygotic embryo.

Although it is more common to find studies in which the combination of auxins and cytokinins allows a major production of somatic embryos, our results showed that when auxins and cytokinins were added to the medium with the addition of other growth regulators, the yield of somatic embryos was superior compared with the treatment of the combination of auxin and cytokinin alone. This study also showed that cotyledon explants possess a high competency to form direct somatic embryos, which the maturation of the embryos occurs in the same medium. The relatively short period of time required to obtain direct embryos indicates the great potential of the cotyledon to be used as the explant in the mass production of *E. longifolia*. Further studies to germinate somatic embryos up to *ex vitro* conditions are needed.

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