

Embryogenic Callus Induction from Leaf Tip Explants and Protocorm-Like Body Formation and Shoot Proliferation of *Dimorphorchis lowii*: Borneon Endemic Orchid

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Received: April 17, 2016/ Accepted: September 13, 2016

ABSTRACT

Dimorphorchis lowii is a rare epiphytic orchid endemic to Borneo and very popular due to its ornamental value. For the purpose of mass propagation, procedures for *in vitro* propagation through callus were developed. The objectives of this study were to determine the effects of plant growth regulators (PGRs) on callus induction from leaf tip explant and the effects of complex additives on PLBs formation and shoot development. The best combination of PGR was 3.0 mg L⁻¹ Thidiazuron (TDZ) with 0.046 mg L⁻¹ NAA supplemented in half-strength medium (½ MS) (Murashige & Skoog, 1962). The percentage of survival and callus formation obtained were 96.0 % ± 19.8 and 52.0 % ± 16.5 respectively. Maximum shoot proliferation from PLBs was observed in Knudson C (KC) medium enriched with 15 % (v/v) coconut water. In this treatment, 10.2 ± 6.2 shoots were produced from one callus explant. Shoots were rooted on KC medium containing 15 % (v/v) coconut water and transferred in a medium mixture containing sphagnum moss, charcoal, brick and coco peat with the ratio of 1:1:1:1. After 2 months being acclimatized in the glasshouse, 78 % of plantlets survived. Histology observations showed that embryogenic callus derived from leaf tip explants might originate from epidermis and mesophyll cells and was capable of developing into complete plantlets.

Keywords: callus, *in vitro*, orchid, protocorm like-bodies (PLBs)

INTRODUCTION

Dimorphorchis lowii is an endemic orchid of Borneo and is one of the rarest epiphytic orchids, which commonly inhabiting the area with the elevation of 600-1800 meter above sea level (m asl) on Kinabalu, Tombuyukon and Trus Madi mountains in Sabah (Cribb & Bell, 2008). This

orchid possess unique and beautiful dimorphic feature, which means it has two distinct flowers on one inflorescent and has the prospect to be marketed as a valuable basket or potted flowers. The increasing popularity, high degree of rarity and its difficulty-to-locate have put this species among the most highly sought after species. Therefore, it is imperative to develop a method for multiplication to reproduce a great number of plantlets in order to fulfill the floriculture demand as well as for conservation purposes.

Dimorphorchis orchids, just like other monopodial orchids are asexually propagated using the top cuttings of the mother plants. Nevertheless, reproduction through this conventional technique can be detrimental to the stock plants as it poses threat due to the removal of meristematic tips (Gantait, Bustam, & Sinniah (2012). Conventional method through seed propagation is sometimes problematic as seed germination in natural environment is a slow process and it requires mycorrhizal or fungal partners' association for its germination (Pant & Thapa, 2012) and any disturbance in the habitat or physical environment during germination will destroy the whole population (Mathews & Rao, 1980). *In vitro* propagation of orchids by shoot tip culture is a well-developed method. However, by using shoot tip as explant means the most significant part that is actively growing has to be sacrificed. Therefore, a technique of reproduction using other insignificant parts such as leaves of the plant would be beneficial (Chugh, Guha, & Rao, 2009).

Successful culture of plantlet from leaf culture of many orchid species has been reported. Among them are *Aerides* (Devi, Devi, & Singh, 2013), *Aranda* (Gantait, Bustam, & Sinniah, 2012), *Phalaenopsis* (Kuo, Chen, & Chang, 2005; Khoddamzadeh et al., 2011), *Vanda* and *Dendrobium* (Martin & Madassery, 2006; Tee, Wong, Lam, & Maziah, 2010), and *Oncidium*

Cite this as: Jainol, J. E., & Gansau, J. A. (2017). Embryogenic callus induction from leaf tip explants and protocorm-like body formation and shoot proliferation of *Dimorphorchis lowii*: Borneon endemic orchid. *AGRIVITA Journal of Agricultural Science*, 39(1), 1-10. <http://doi.org/10.17503/agrivita.v39i1.895>

Accredited: SK No. 81/DIKTI/Kep/2011

Permalink/DOI: <http://dx.doi.org/10.17503/agrivita.v39i1.895>

(Mayer, Stancato, & Appezzato-Da-Glória, 2010). In leaf cultures, for example in *Renanthera* (Wu et al., 2012) and *Vanilla* (Janarthanam & Seshadri, 2008), wound surface of leaf explants had been reported to produce callus. Wu et al., (2012) reported that 99.67 % plantlet formation was obtained from callus originated from leaf segments of *Renanthera* orchid. Protocorm-like bodies (PLBs) generally have high potency to proliferate rapidly and regenerate into a whole plantlet (Bustam, Sinniah, Kadir, Zaman, & Subramaniam, 2013). Therefore, they are the most common target tissues used for mass propagation or producing a large number of uniform plantlets for commercial purposes.

This study investigated (I) the effects of PGR on callus induction from leaf tip explants and (II) the effect of complex additives on the PLBs formation and shoot development of *D. lowii*. Histology and scanning electron microscope observations were also conducted to study the callus induction from leaf tip explants.

MATERIALS AND METHODS

The mother plant was collected in early 2009 from a local nursery in Sabah, Malaysia. This plant was brought to the Orchid Unit nursery at University Malaysia Sabah, grown and maintained in the glasshouse conditions. In December 2009, seeds were obtained and cultured in Vacin & Went (VW) (Vacin & Went, 1949) medium added with 10 % (v/v) potato homogenate. Protocorms developed from these seed cultures were then transferred to half-strength MS ($\frac{1}{2}$ MS) (Murashige & Skoog, 1962) medium containing 2.0 % (w/v) sucrose and 0.2 % (w/v) yeast extract. This served as the staging medium for explants source. During series of subcultures, protocorms grew into multiple shoots. Multiple shoots bearing 3-4 leaves each were chosen as source for leaf explants. The first two leaves from the top with the diameter of 2-5 mm were chosen and dissected with a sterile sharp scalpel and the tips were cut approximately 5-7 mm in length.

Medium Preparation and Culture Condition

In this study, three media formulations were used as basal media: Vacin and Went (VW), modified MS containing half-strength macro and micro nutrients ($\frac{1}{2}$ MS), and Knudson C (KC) (Yam & Arditti, 2009). VW enriched with 10 % (v/v) potato homogenate was used for seed

germination, $\frac{1}{2}$ MS was used as basal medium for protocorm development into seedlings in which the leaves were used as the source of explants in experiment I. $\frac{1}{2}$ MS was also used for basal medium in experiment I, and KC was used as basal medium in experiment II. All media were supplemented with 2 % sucrose and solidified with 0.8 % agar powder (Sigma, USA). $\frac{1}{2}$ MS was also enriched with 100 mg L⁻¹ myo-inositol, 0.5 mg L⁻¹ pyridoxine-HCl, 0.1 mg L⁻¹ thiamine-HCl, 0.5 mg L⁻¹ nicotinic acid and 2 mg L⁻¹ glycine. Different PGRs (TDZ or NAA) and concentrations were added to $\frac{1}{2}$ MS medium in as treatments experiment I, and different complex additives and concentrations (15 % (v/v) coconut water, 0.2 % (w/v) peptone or 0.2 % (w/v) yeast extracts) were added to KC medium as treat-ments in experiment II.

The pH of the media was adjusted to 5.2-5.3 and agar powder was weighed and added prior to autoclaving for 20 minutes at 121 °C and 15 kPa. The autoclaved medium was poured aseptically into sterile plastic petri dishes (60 mm x 15 mm, Sterilin, UK) at 20 ml for each petri dish. These petri dishes were allowed to cool down, covered and then sealed with parafilm M[®] (American National Cam[™], Menasha, WI) before used. For callus induction experiment, cultures were maintained at 25 ± 2 °C under continuous darkness. For PLB formation and shoot development experiment, cultures were maintained at 25 ± 2 °C under continuous cool-white fluorescent light with photon flux density (PFD) of 15-25 µmol m⁻² s⁻¹.

Effect of PGRs on Embryogenic Callus Induction from Leaf Tip Explants

PGR tested in this investigation included TDZ at 0.22, 0.33, 1.0, 3.0 and 4.0 mg L⁻¹ and NAA at 0.046, 0.5 and 1.0 mg L⁻¹. Both TDZ and NAA were applied individually or in combination. $\frac{1}{2}$ MS medium supplemented with 2.0 % (w/v) sucrose was used as basal medium.

Effects of Complex Additives on PLBs Development and Shoot Proliferation from Embryogenic Callus Derived from Leaf Tip Explants

Green embryogenic calli was cut aseptically into clumps of approximately 3-5 mm in diameter and the clumps were used as explants. In this study, three types of complex additives namely coconut water at 15 % (v/v), peptone at 0.2 %

Juddy E. Jainol and Jualang Azlan Gansau: *Embryogenic Callus Induction from Leaf Tip Explants*.....

(w/v) and yeast extract at 0.2 % (w/v) were used to investigate the PLBs development and subsequent shoot formation from embryogenic callus of *D. lowii*. KC medium supplemented with 2.0 % (w/v) sucrose was used as basal medium. Once shoots were established, they were transferred into 100 ml Erlenmeyer flask containing 20 ml of KC medium supplemented with 15 % (v/v) coconut water for shoot elongation and root formation.

Data Analysis

All experiments were performed in a Complete Randomized Design (CRD) with five replicates. For embryogenic callus induction experiment, one petri dish represented one replicate and in each replicate contained 20 leaf tip explants. Data on percentage of survival, percentage of callus formation, callus density and color were recorded after 60 days of culture. For PLBs shoot proliferation from embryogenic callus derived from leaf tip explants, one petri dish represented one replicate and in each replicate contained 20 callus clumps. Data on percentage of survival rate, percentage of PLB formation, number of shoots, and number of leaves were recorded after 60 days and 120 days of culture. Significance of treatment effects on these data were analyzed using ANOVA, $p < 0.05$ and comparison between mean values of treatments were made by Duncan Multiple Range Test using SPSS version 20 (IBM Corporation, New York, USA)

Histology and Scanning Electron Microscope Observation on Embryogenic Callus Derived from Leaf Tip Explants

Light Microscopy

Tissues for histological observation were secured in PAA solution (15 Picric acid: 1 Acetic acid), dehydrated in an ethanol series, and lodged in paraffin wax. The materials embedded in paraffin were sectioned at 5 μm and stained with hematoxylin-eosin. The observation and photography were done using light microscope (Leica ICC50 HD, UK).

Scanning Electron Microscopy (SEM)

The samples were fixed in 2 % (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) and dehydrated in an ethanol series. The samples were dried to the critical point with liquid CO_2 in a critical point dryer (CPD) (Leica EM300,

Austria), affixed to aluminium stubs and coated with gold palladium in a sputter coater (EMITECH, K550X, England). The mounted specimens were examined with scanning electron microscope (Zeiss EVO MA10, U.K).

RESULTS AND DISCUSSION

Effect of PGRs on Embryogenic Callus Induction from Leaf Tip Explants

The effects of PGRs is shown in Table 1. Incorporation of TDZ and NAA at a concentration ranges from 1.0 to 3.0 mg L^{-1} and 0.046 to 1.0 mg L^{-1} , respectively in $\frac{1}{2}$ MS medium was crucial in promoting the percentages of survival of explants and embryogenic callus formation. The highest percentage of explant survival was observed in medium containing 3.0 mg L^{-1} TDZ and 0.046 mg L^{-1} NAA at 96.0 % \pm 19.8 and 1.0 mg L^{-1} TDZ and 0.046 mg L^{-1} NAA at 92.0 % \pm 15.3. There were no significant differences between these two treatments. However, there were significant differences observed when compared to the other concentrations of TDZ and NAA as shown in Table 1. For embryogenic callus induction, it was observed that medium containing 3.0 mg L^{-1} TDZ and 0.046 mg L^{-1} NAA produced the highest percentage of callus formation (52.0 % \pm 16.5), while other PGR treatments produced only 18-34 % of the explants forming callus.

The control treatment produced only 2 % \pm 1.4 of explants forming callus. These results indicated that the presence of PGR was not essential for callus induction; however, addition of either TDZ or NAA or both PGRs in the medium was beneficial, and the best medium for callus induction was MS + 3.0 mg L^{-1} TDZ and 0.046 mg L^{-1} NAA. White embryogenic calli was formed and covered almost all the surface of the leaf tip explants (Figure 1A and 1B). Similar study was done by Chen & Chang (2006) with leaf tip explants of *Phalaenopsis amabilis*. Chen & Chang (2006) reported that when leaf explants of *Phalaenopsis amabilis* were cultured on hormone-free medium or medium added with NAA alone at 0.1 or 1.0 mg L^{-1} , the explants turned necrotic and no embryo was formed. However, in the presence of TDZ at 0.1, 1.0 and 3.0 mg L^{-1} either alone or in combination with NAA at 0.1 or 1.0 mg L^{-1} , somatic embryos (SE) directly formed at the surface of explants, without any interfering callus formation.

Table 1. Effects of plant growth regulator types and concentrations on embryogenic callus induction from leaf tip explants of *D. lowii* cultured in ½ MS medium supplemented with 2.0 % (w/v) sucrose under continuous darkness at temperature of 25 ± 2 °C after 60 days of culture

Cytokinin/Auxin (mg L ⁻¹)		Percentage of Survival (%)	Percentage of callus formation (%)	Density of callus	Color of callus
TDZ	NAA				
Control		24.0±8.0 ^g	2.0±1.47 ^{d*}	+	Greenish-white
0.22	0.00	64.0±28.5 ^{cd}	18.0±10.4 ^{bc}	+	Greenish-white
0.33	0.00	60.0±24.5 ^{cde}	22.0±11.5 ^{bc}	+	Yellowish-white
1.0	0.00	48.0±20.5 ^{def}	24.0±13.1 ^{bc}	+	Greenish-white
3.0	0.00	90.0±30.3 ^{ab}	28.0±17.4 ^{bc}	++	Greenish-white
0.0	0.046	75.0±43.9 ^{bc}	28.0±17.4 ^{bc}	++	Yellowish-white
0.0	0.5	65.0±48.3 ^{cd}	25.0±13.9 ^{bc}	+	Greenish-white
0.0	1.0	42.0±29.8 ^{efg}	0.0±0.0 ^e	-	-
1.0	0.046	92.0±15.3 ^a	34.1±25.8 ^{bc}	++	White
3.0	0.046	96.0±19.8 ^a	52.0±16.5 ^a	+++	White
3.0	0.5	34.0±8.9 ^{fg}	12.0±8.8 ^{bc}	+	Yellowish-white
3.0	1.0	86.0±21.1 ^{ab}	28.0±17.9 ^{bc}	++	White
4.0	0.5	62.0±25.0 ^{cd}	18.0±10.4 ^{bc}	+	Greenish-white
4.0	1.0	50.0±26.0 ^{def}	14.0±9.1 ^{bc}	+	Greenish-white

Remarks: *Mean values within a column followed by the same letters are not significantly different at p < 0.05 according to Duncan's Multiple Range Test. Note. +: callus at tip, ++: callus at tip and midsection, +++: callus covered all surfaces, -: no callus

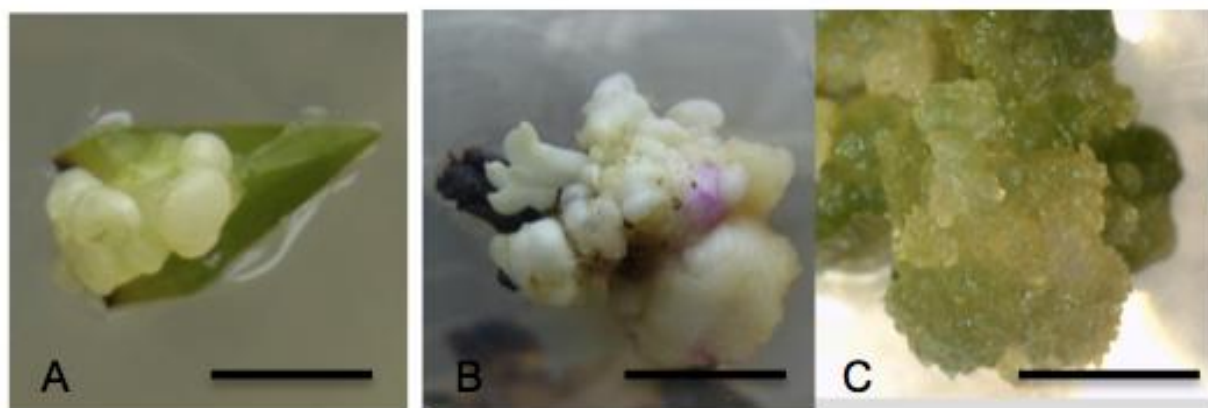


Figure 1. Embryogenic callus induction from leaf explant. **A.** Callus induction from leaf tip explant cultured on ½ MS medium supplemented with 3.0 mg L⁻¹ TDZ and 0.046 mg L⁻¹ NAA after 30 days of culture (DOC). **B.** Callus induction from leaf tip explant cultured on ½ MS medium supplemented with 3.0 mg L⁻¹ TDZ and 0.046 mg L⁻¹ NAA after 60 DOC and **C.** after 90-100 DOC when transferred under 24hr light. (Scale bars = 5 mm).

The highest frequency of SE formations were found in media containing 3 mg L⁻¹ TDZ without NAA, or in combination with 0.1 or 1.0 mg L⁻¹ NAA, i.e., 93.8 %, 87.5 % and 81.3 %, respectively. In line with these results, Tariq, Ali, & Abbasi (2014) also reported that 2.0 mg L⁻¹ TDZ in combination with 1.0 mg L⁻¹ NAA produced optimum result in callus formation from leaf explant of *Artemisia absinthium*. In this study, greenish-white callus development was seen on

PGRs-free medium but the rate was very low at only 2.0 % ± 1.4. Gantait, Bustam, & Sinniah (2012) found that in control medium, 32.9 % of leaf explants were able to induce embryogenic calli even without any PGRs support. The best concentration of TDZ in this study was similar to the result reported by Khoddamzadeh et al., (2011), where leaf explants produced the highest embryogenic calli or PLBs at 71.87 % when

Juddy E. Jainol and Jualang Azlan Gansau: *Embryogenic Callus Induction from Leaf Tip Explants*.....

cultured in ½ MS medium supplemented with 3.0 mg L⁻¹ TDZ alone.

PGR(s) proportions have been known to have species-specific effect on callus and PLB induction from leaf explants (Shimura & Koda, 2004; Gantait, Bustam, & Sinniah, 2012). TDZ is a substituted phenyl urea with cytokinin-like activity (van Staden, Zařímalová, & George, 2008; Jitsopakul, Thammasiri, & Ishikawa, 2013). The ascendancy of TDZ in inducing somatic embryos (infant PLBs) from leaf explants was recounted in numerous orchid species including *Oncidium* (Mayer, Stancato, & Appezzato-Da-Glória, 2010), *Doritaenopsis* (Park, Yeung, Chakrabarty, & Paek, 2002), *Dendrobium* and *Phalaenopsis* (Kuo, Chen, & Chang, 2005). TDZ is also useful for rapid plant regeneration through organogenesis (Kishor & Devi, 2009; Jitsopakul, Thammasiri, & Ishikawa, 2013), somatic em-bryogenesis (Jheng, Do, Liauh, Chung, & Huang, 2006; Gow, Chen, & Chang, 2009), or PLB formation of orchids (Mayer, Stancato, & Appezzato-Da-Glória, 2010; Khoddamzadeh et al., 2011; Roy, Sajeev, Pattanayak, & Deka, 2012). NAA is a synthetic auxin plant growth regulator that is routinely used in *in vitro* propagation of orchid to promote callus induction such as in *Dendrobium* (Mei, Danial, Mahmood, & Subramaniam, 2012; Salam, Salam, & Mohanty, 2013), *Vanilla* (Tan, Chin, & Alderson, 2011), *Oncidium* (Mayer, Stancato, & Appezzato-Da-Glória, 2010), *Phalaenopsis* (Khoddamzadeh et al., 2011), and *Renanthera* (Wu et al., 2012).

Callus from leaf tip explant cultured on ½ strength MS medium turned green when transferred under 24hr light after 90-100 DOC (Figure 1C).

Effect of complex additive on PLB development and shoot proliferation from embryogenic callus derived from leaf tip explants

Table 2 shows the result obtained after 120 days of cultures. 15 % (v/v) coconut water presented the highest percentage of survival and PLBs formation both at 72.0 % ± 11.9 followed by 0.2 % (w/v) yeast extract at 62.0 % ± 13.5 in both percentages of survival and PLBs formation.

There were no significant differences between the effects of these two complex additives. However, significant differences were observed between these two complex additives in terms of number of PLBs per explant with 15 % (v/v) coconut water produced the highest 16.9±6.5 and 0.2 % (w/v) yeast extract produced 9.9±2.9. In both media, the emergence of nodular structures from the callus was seen after 20-30 days of culture (Figure 2A). These nodular tissues later progressed further and developed into aggregates of PLBs after 60 days of culture (Figure 2B). After 120 days of culture, 15 % (v/v) coconut water produced a significantly higher percentage of PLBs forming shoot at 70.0 % ± 42.0 with 8.9±2.9 shoots and 5.5±2.6 leaves (Figure 2C) followed by 0.2 % (w/v) peptone at 40.0 % ± 20.2 with 4.5±1.2 shoots and 2.7±1.1 leaves.

Table 2. Effect of complex additives types and concentrations on PLBs development and shoot proliferation from callus culture of *D. lowii* cultured on KC medium supplemented with 2.0 % (w/v) sucrose under 24-hour light photoperiod with PFD of 15-25 μmol m⁻² s⁻¹ at 25 ± 2 °C after 60 and 120 days of culture

Complex additive Type and concentration (w/v, v/v)	Percentage of survival (%) [†]	Percentage of proliferated explant (%) [*]	Number of new PLBs formed [*]	Percentage of PLBs formed shoot (%) ^{**}	Number of shoots ^{**}	Number of leaves ^{**}
Control	40.0 ± 20.5 ^b	40.0 ± 20.5 ^b	5.8 ± 1.9 ^c	2.0 ± 1.6 ^b	1.8 ± 0.1 ^b	1.1 ± 0.5 ^c
0.2 % peptone	48.0 ± 20.2 ^b	48.0 ± 20.2 ^b	6.5 ± 4.0 ^c	40.0 ± 20.2 ^a	4.5 ± 1.2 ^b	2.7 ± 1.1 ^b
0.2 % YE	62.0 ± 13.5 ^a	62.0 ± 13.5 ^a	9.9 ± 2.9 ^b	46.0 ± 20.1 ^a	3.1 ± 1.9 ^b	1.7 ± 0.4 ^b
15 % CW	72.0 ± 11.9 ^a	72.0 ± 11.9 ^a	16.9 ± 6.5 ^a	70.0 ± 42.0 ^a	8.9 ± 2.9 ^a	5.5 ± 2.6 ^a

Remarks: Mean values within a column followed by the same letters are not significantly different at p<0.05 according to Duncan's Multiple Range Test; ^{*} Data collected after 60 days of culture; ^{**} Data collected after 120 days of culture

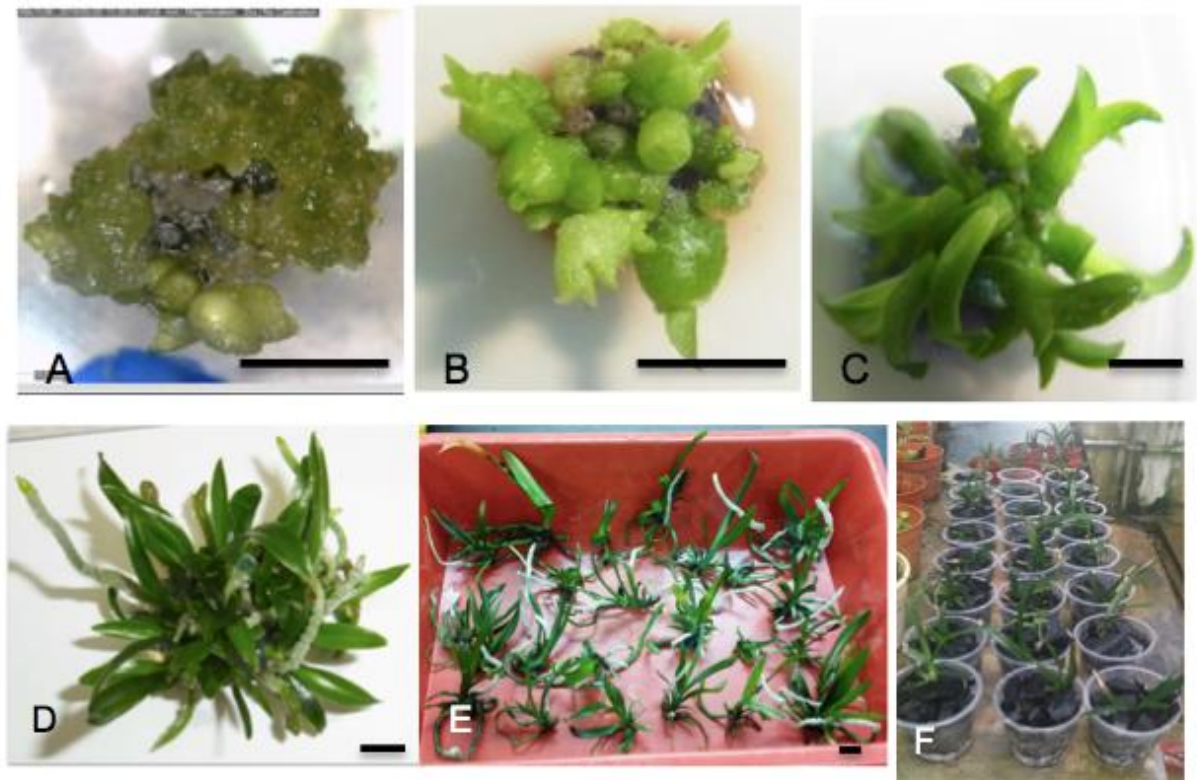


Figure 2. Plantlet development from callus: **A.** Callus cultured on KC medium added with 15 % (v/v) coconut water. **B.** PLBs with shoots after 50-60 DOC and **C.** Shootlets after 120 DOC. (Scale bars = 5 mm). **D.** Plantlets formed when cultured on KC medium supplemented with 15 % (v/v) coconut water after 150-180 DOC. **E.** Plantlets were separated from clumps and prepared for acclimatization. **F.** Plantlets acclimatization after 2 months. (Scale bars = 10 mm)

Overall, coconut water at 15 % (v/v) showed the superiority compared to the other two complex additives (peptone & yeast extract). Coconut water is extensively used in the plant micropropagation due to its growth enhancer substance and cytokinin-type action that assist cell division and stimulate fast growth (Arditti, 2008; Yong, Ge, Ng, & Tan, 2009; Prando, Chiavazza, Faggio, & Contessa, 2014). Coconut water contains high levels of Z-type cytokinin (trans-zeatin riboside, trans-zeatin O-glucoside, dihydrozeatin O-glucoside, trans-zeatin, dihydrozeatin, trans-zeatin riboside-5'-monophosphate), iP-type cytokinin (N⁶-isopentenyladenine), kinetin, kinetin riboside and ortho-topolin in addition to sugars, vitamins, minerals and amino acids (Yong, Ge, Ng, & Tan, 2009; Tan, Cheng, Bhat, Rusul, & Easa, 2014). Murdad et al., (2006) reported that the production

of PLBs in *Phalaenopsis gigantean* is improved with the addition of coconut water in the culture medium. The PLB and callus induction in *Cymbidium* hybrids is also promoted by the coconut water in the culture media (Teixeira da Silva & Tanaka, 2006). Coconut water has also been reported to enhance shoot multiplication in *Vanilla planifolia* (Kalimuthu, Senthilkumar, & Murugalatha, 2006).

Rooted shoots (Figure 2D & Figure 2F) were obtained after culturing clusters of shoots on KC medium containing 15 % (v/v) coconut water for 4 to 5 months and these plantlets were transferred in a medium mixture containing sphagnum moss, charcoal, bricks and coco peat with the ratio of 1:1:1:1. After 2 months, 78 % of plantlets aged between 1-1½ year survived after being transferred to the glasshouse (Figure 2F).

Juddy E. Jainol and Jualang Azlan Gansau: *Embryogenic Callus Induction from Leaf Tip Explants*.....

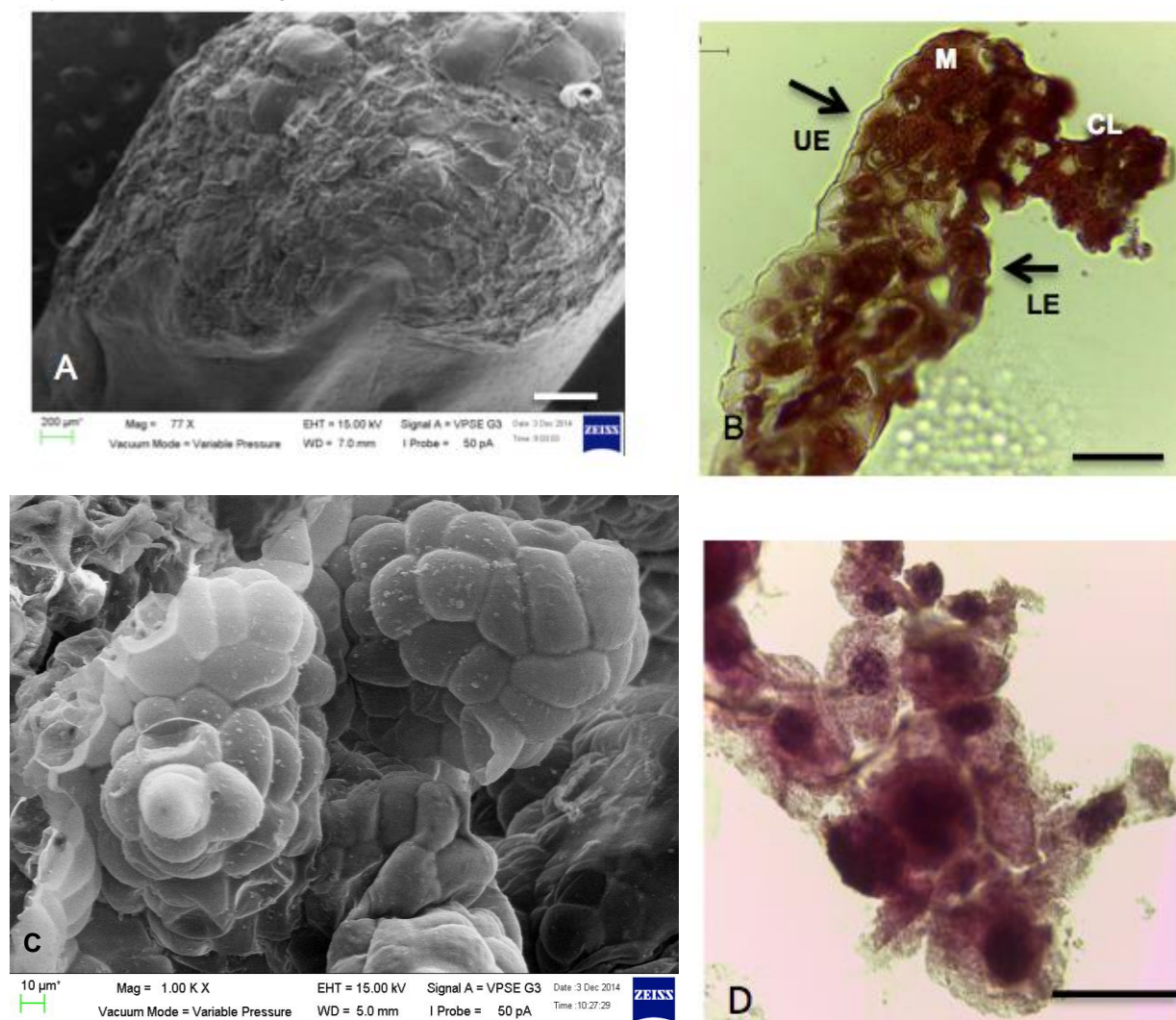


Figure 3. Scanning electron microscope (SEM) and histology view on **A**. SEM view on embryogenic callus formed on the abaxial part of leaf tip explant, scale bar = 400 μm , 77 x. **B**. Light microscopic views of leaf tip showed longitudinal section of explant showing cell dedifferentiation in callus that produced unorganized cell mass, upper & lower epidermis (UE) & (LE), mesophyll cell (M), callus (CL), scale bar = 50 μm , 40x. **C**. Single cells divide and developed into PLBs of different sizes, scale bars = 40 μm , 1.00K x. **D**. Cross section of calli with multi-globular compact callus cells, scale bar = 50 μm , 40x (Jainol, 2016).

Histology and Scanning Electron Microscope Observations on Embryogenic Callus Derived from Leaf Tip Explants

Embryogenic callus derived from leaf tip explants shows cells dedifferentiation (Figure 3A-B). Begum, Tamaki, Tahara, & Kako (1994) reported that callus formation from orchids used explants that contained meristematic cells or epidermal cells, suggesting that callus cells arise from these types of cells. Figure 3B shows the cross-section through the leaf tip explants which

showed densely lower epidermis and mesophyll cells underwent dedifferentiation. Most of the time, callus developed at the cut ends of leaf tip explants, suggesting that the wounding might have triggered dedifferentiation of cells to form callus. Kuo, Chen, & Chang (2005) reported that the highest ability of *Phalaenopsis* leaf explants to form embryos was found at adaxial surfaces near cut ends on TDZ-containing medium. Figure 3C-D show that the callus masses composed of spherical, compact and loose aggregates of cells.

Spherical single cells can be seen embedded within the friable callus. These cells started to divide and gave rise to somatic embryos or PLBs of different sizes. Globular-stage somatic embryo or PLBs might originate either from the cells within the callus or from the callus surface. These PLBs developed into shoots that would form complete plantlets when cultured on KC medium supplemented with 15 % (v/v) coconut water.

CONCLUSION

The present study demonstrated that the success of embryogenic callus induction from leaf tip explants of *Dimorphorchis lowii* is greatly influenced by plant growth regulators. Leaf tip explants were able to produce embryogenic callus in medium free of PGR but the frequency was very low (2 %). In this study, the best result in embryogenic callus formation on leaf tip explants was obtained at combination of 3.0 mg L⁻¹ TDZ and 0.046 mg L⁻¹ NAA. Furthermore, 15 % (v/v) coconut water was found to be the best complex additive to promote PLBs development and subsequent shoot formation from embryogenic callus of *D. lowii*. Histology observations revealed that, embryogenic callus might have originated from epidermis and mesophyll cells and these calli have the capability to multiply and form complete plantlets. However, further study needs to be conducted to study cell differentiation, and the structural and biological functions of cells involved in the initiation and formation of embryogenic callus.

ACKNOWLEDGMENT

We thank Tissue culture laboratory, University Malaysia Sabah for providing the facilities and equipment for this research.

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