Effects of Organic Additives and Plant Growth Regulators on Protocorm Development of *Dendrobium lowii*

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ABSTRACT: A simple and efficient growth protocol was develop for *Dendrobium lowii,* an endangered and Borneo's endemic epiphyte orchid, using four month old protocorms as explant sources produced by asymbiotic seeds germination. Protocorms of *Dendrobium lowii* were cultured on Knudson C (KC) media supplemented with organic additives (coconut water, tomato juice and banana pulp) or plant growth regulators (NAA, Zeatin and BAP) at different concentrations and observed for protocorm development. Among all organic additives tested, medium containing banana pulp at 25g/L induced the highest growth index value of 593.3 after 240 days of culture. This treatment also promoted 100% production of shoot and 93.3% of root formation compared to other treatments. Addition of 2g/L peptone or 15% (v/v) coconut water had significantly induced 16.7% protocorms proliferation. The supplementation of 6 µM NAA promotes similar responses for growth index of 563.3. The treatment induced up to 86.7% and 83.3% of protocorms forming shoots and roots, respectively. The study also revealed that the addition of 2 or 4 µM of NAA and 4 or 6 µM BAP is suitable for shoot induction, however with poor rooting formation. This finding is important for conservation and horticultural manipulation of the species.

KEYWORDS: Growth index; Orchidaceae, proliferation; Knudson C

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INTRODUCTION

There are estimated about 250,000 species of flowering plant with *Orchidaceae* as the biggest family in plant kingdom which estimated to be around 20,000 to 30,000 species (Heywood, 1978). *Dendrobium* is one of the largest genera in the Orchidaceae family, with about 1,100 species, of which at least 300 have been cultivated (Wood, 2006). *Dendrobium* is well known in the *Orchidaceae* family for their complex fabricated and long lasting colourful flowers (Talukder *et al.*, 2003). *Dendrobium lowii* is an endemic orchid and can only be found in Borneo (Sabah, Sarawak and Kalimantan) in the altitude about 900m (3,000 ft.) (Wood, 2006). This orchid has high commercial value because of its attraction such as sweet odour and beautiful flowers besides having many usages in various fields (Bose *et al.*, 1999). This species is rare in the wild, also due to the deforestation, and uncontrolled cultivation from natural habitat, the species is currently reported in the Appendix II of the CITES (subjecting international trade in specimens of selected species to certain controls.). In order to conserve the species from extinction and to increase the population size, plant tissue culture can play a significant role (Roy *et al.*, 2011). Concept of plant tissue culture was firstly thought by Haberland in 1902 which then was rapidly developed by White, Steward, Gautheret, Skoog, Street, Nitsch, Morel and many more (Ernst & Arditti, 1993). *In vitro* propagation of orchid offer an opportunity for the selection of various desirable traits and, produced high quality and uniform plantlets throughout a year under disease-free conditions

regardless of the seasons and weathers (Pola *et al.*, 2009). The addition of organic additives and plant growth regulators in culture medium has been reported recently to enhance protocorm proliferation and regeneration in some Borneo orchids such as *Phalaenopsis gigantea, Dimorphorchis rossii, Dimorphorchis lowii, Vanda dearei* and *Vanda helvola* (Murdad *et al.*,2006; David *et al.*, 2010; Jualang *et al.*, 2014; Jualang *et al.*, 2015; David *et al.*, 2015, Jainol & Jualang, 2016). Therefore, the purpose of this research was to study the effect of organic additives and plant growth regulators on regeneration and proliferation of *D. lowii* protocorm.

MATERIALS AND METHODS

Source of Explants

Four months old protocorms of *Dendrobium lowii* obtained from *in vitro* seeds germination were used as explants.

Optimization of Culture Medium Compositions for Protocorm Development

Protocorm proliferation and regeneration were investigated on KC medium (Knudson C, 1946) supplemented with 2% (w/v) sucrose, and treated with organic additives or plant growth regulators. Four types of organic additives tested are coconut water, tomato juice (10%, 15% and 20% v/v), banana pulp (25, 75 and 125 g/L) and peptone (2 g/L). Plant growth regulators tested in this study are Naphthalene acetic acid (NAA), Zeatin and 6-Benzylaminopurine (BAP) at concentrations of 2, 4, 6 μM, respectively. Basal medium devoid of any organic additive or plant growth regulator served as control. The medium pH was adjusted to 5.3±0.02 and solidified with 0.8% (w/v) agar (Sigma) prior to autoclaving for 20 min at 15 psi, 121°C. The cultures were maintained at 24±2 ^{0}C under a 24 h d^{-1} photoperiod with a PPF of 20–50 μmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes (Philips, Malaysia).

Experimental Design and Statistical Analysis

All experiments were carried out in a completely randomized design (CRD) with five replicates per treatment and each replicate contains five protocorms. Observations were recorded every 30 days for 240 days for protocorm development. Sub culture was performed every 60 days. Growth index (GI) was measured according Arditti (1967). Data were subjected to analysis of variance (ANOVA) and means were compared by the Duncan's multiple range test at $p<0.05$ using the SPSS ver. 20 (SPSS Inc., USA).

RESULTS AND DISCUSSION

Effect of Organic Additives on Protocorm Proliferation and Development

After 240 days of culture, protocorms treated with all types of organic additives produced one to two leaves, with roots formation and the growth index (GI) value ranged from 483.3 to 593.3 (Table 1, Figure 1). Protocorm treated with 25 g/L of banana pulp showed the highest GI values of 593.3 with all (100%) protocorms were successfully developing shoots and 93.3% of protocorms producing root. The current finding was supported by Vyas *et al.* (2009) where maximum number

of shoots and root was observed in *Dendrobium lituiflorum* when cultured on KC media supplemented with 12.5% and 25.0% (v/v) of banana extract, respectively.

Table 1. Effect of organic additives on protocorm development of *D. lowii*

Treatment	Percentage of proliferation (%)	Percentage of protocorm forming shoot (%)	Percentage of protocorm forming root (%)	Growth index (GI)
Control	0^{d}	73.3^{d}	26.7^{f}	483.3
Peptone (2 g/L)	16.7^{a}	100.0^{a}	76.7^{c}	576.7
BP (25 g/L)	0^{d}	100.0^{a}	93.3^{a}	593.3
BP (75 g/L)	0^{d}	96.7^{b}	83.3^{b}	580.0
BP (125 g/L)	0^{d}	90.0^{b}	66.7^{d}	550.0
TJ (10%, v/v)	0^{d}	93.3^{b}	86.7^{b}	580.0
TJ (15%, v/v)	13.3^{b}	93.3^{b}	86.7^{b}	573.3
TJ (20%, v/v)	0^{d}	75.0^{d}	40.0^{e}	485.0
CW (10%, v/v)	6.7^{c}	93.3^{b}	93.3^{a}	583.3
CW (15%. v/v)	16.7^{a}	93.3^{b}	83.3^{b}	576.7
CW (20%, v/v)	13.33^{b}	86.7^{c}	63.3^{d}	543.3

Note: Means followed by the same letter (s) within each column are not significantly different at $p < 0.05$, according to Duncan's multiple range tests. BP – banana pulp; TJ – Tomato juice; CW – coconut water.

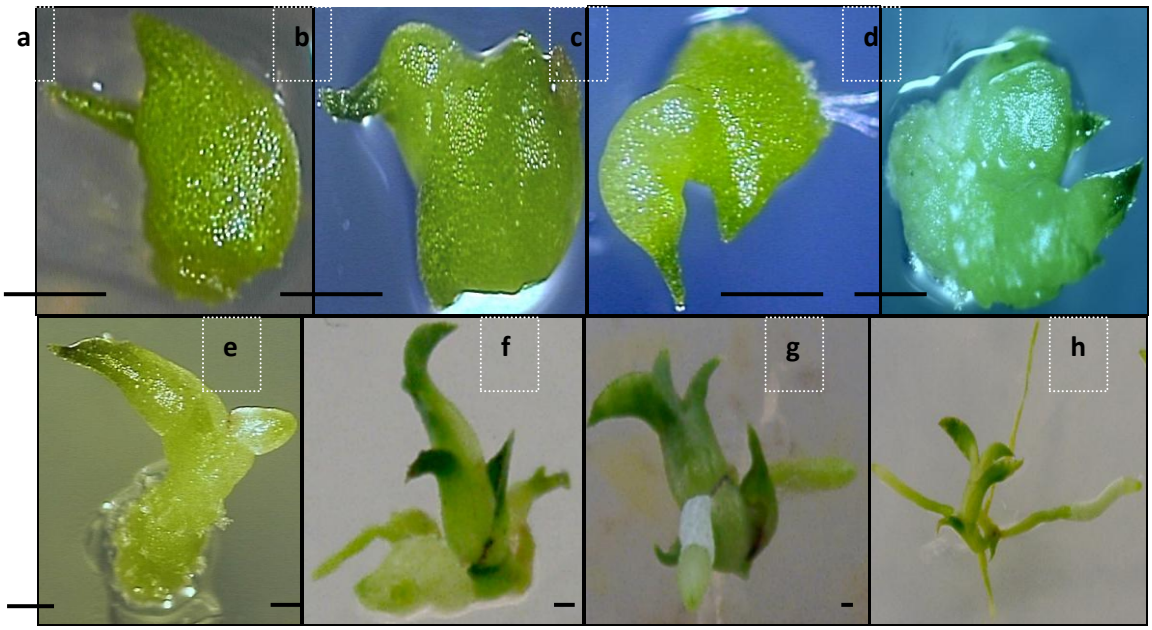

Figure 1. Growth development of *D. Lowii* protocorm. (a) Stage three of matured protocorm; (b-c) Early indication of shoot developement at the apical protocorm; (d) Protocorm proliferation; (e-f) Shoot with developed leaves; (g) Root initiation on seedling; (h) *D. lowii* seedling (Bar is 2 mm).

Kaur and Bhutani (2012) also found that the highest regeneration frequency of 28-week old protocorms *Cymbidium pendulum* with healthy shoots and root formation was recorded in banana homogenate (50 g/l). Previously, Huang *et al.* (2001) also stated that the addition of 20 g/l banana extract showed high development of shoot and root for cloning of *in vitro* orchid *Paphiopedilum*. According to Arditti (1967) and Van Staden and Stewart (1975), banana extract contains high natural cytokinin which provide the differentiation and shoot development. Ernst and Arditti (1993) also added that the cell division-inducing compound that present in banana fruit may also be responsible for the enhancing effect on orchid embryo development and differentiation.

Apart from banana treatment, the addition of 2 g/L of peptone was also suitable for shoot induction. In this treatment, all protocorms responded 100% in shoot formation (Figure 1e) and 76.7% of protocorms forming roots (Table 1, Figure 1g). Previous study by David *et al.* (2015) also stated the beneficial effect of 0.1% (w/v) peptone in promoting highest number of leaves in *Vanda helvola.* Peptone being water soluble protein hydrolysate with very high amino acid content promotes growth of cultures (Chugh *et al.*,2009). Some reports have shown a positive effect on the growth of explants, including embryo production in *Oncidium* (Chen & Chang, 2002). The addition of 10% (v/v) coconut water was also beneficial in promoting shoot and root of *D. lowii*. This treatment had promoted up to 93.3% of shoot and root formation (Table 1). Coconut water is a complex additive which contains amino acids, organic acids, nucleic acids, vitamins, carbohydrates, plant regulators with zeatin and minerals (Ge *et al.*, 2005). The promotory effect on morphogenesis, is related to its growth regulator content such as cytokinins (Chugh *et al.*, 2009). The effectiveness of coconut water in promoting shoot and root formation of protocorms was also demonstrated in *Vanda dearei* and *Dimorphorchis rossii* endemics orchid in Sabah (Jualang *et al.*, 2014; Jualang *et al.*, 2015).

In this study, it was observed that supplementation of organic additives had contribute to protocorm proliferation (Figure 1d). The addition of 2 g/L of peptone and 15 % (v/v) of coconut water promoted 16.7% of protocorms to proliferate, respectively. Previous study done by Murdad *et al.* (2006) reported that coconut water at 10% (v/v) was the most effective in proliferation (5.68 ±10.14%) of trimmed protocorm of *Phalaenopsis gigantea*. Kaur and Bhutani (2012) also found that the addition of coconut water (10%) and peptone (2 g/l) into medium demonstrated optimum for abundant multiplication of protocorm-like bodies (PLBs) from 28-week old protocorms *Cymbidium pendulum*.

Effect of plant growth regulators on protocorm development

On average, all protocorms responded well when treated with plant growth regulators with GI value ranged from 410.0 to 563.3 after 240 days of culture (Table 2, Figure 1). Among plant growth regulators, treatment of 6 µM NAA promoted the highest GI value of 563.3 with 86.7% of protocorms producing shoots and 83.3% of protocorms forming root (Table 2). It was also observed that protocorms treated in all treatments containing NAA and 4-6 µM BAP showing a good response for shoot induction. Several studies have supported the current finding, i.e., the application of NAA was favorable in shoot production on protocorms of some orchids such as *Phaphiopedilum, Dendrobium candidum* and *Vanda coerulea* (Huang *et al.*, 2001; Zhao *et al.*, 2008; Roy *et al.*, 2011). The beneficial effect of BAP was also reported by Roy *et al.* (2011), that higher concentration of BAP (8.88 µM) is the best concentration for inducing shoot from protocorm of *Vanda coerulea.* In contrast, Zeng *et al.* (2013) found that half-strength MS supplemented 1.0 or 2.0 mg/L N6-benzyladenine (BAP) was most suitable for the induction and proliferation of protocorm-like bodies (PLBs) from protocorms 60 days after germination. The beneficial effect of BAP not only can be found on orchids but also in *Zingiber officinale* Rosc. 'Tambunan' (David *et al.*, 2016). Cytokinins namely BAP, Kinetin and Zeatin were needed in the culture to improve the cell differentiation, proliferation of shoot and morphogenesis (Smith, 1992). Besides that, cytokinins also play important role in induction and regeneration of shoot for most of the plants (Gamborg & Philips, 1995). However, in this study, the application of plant growth regulators did not influence the protocorm proliferation of *D. lowii.*

Table 2. Effect of plant growth regulators on protocorm development of *D. lowii*

Treatment	Percentage of proliferation (%)	Percentage of protocorm forming shoot (%)	Percentage of protocorm forming root (%)	Growth index (GI)
Control	0	73.3[b]	26.7[c]	483.3
NAA (2 μM)	0	80.0[a]	16.7[d]	493.3
NAA (4 μM)	0	86.7[a]	40.0[b]	526.7
NAA (6 μM)	0	86.7[a]	83.3[a]	563.3
Zeatin (2 μM)	0	73.3[b]	26.7[c]	493.3
Zeatin (4 μM)	0	76.7[b]	26.7[c]	486.7
Zeatin (6 μM)	0	43.3[c]	16.7[d]	410.0
BAP (2 μM)	0	76.7[b]	26.7[c]	496.7
BAP (4 μM)	0	80.0[a]	33.3[c]	503.3
BAP (6 μM)	0	86.7[a]	16.7[d]	500.0

Note: Means followed by the same letter (s) within each column are not significantly different at $p < 0.05$, according to Duncan's multiple range tests

CONCLUSION

In conclusion, the growth and development of *Dendrobium lowii* protocorms were significantly enhanced by the addition of organic additives and 2-4 μM NAA and 4-6 μM BAP in KC medium. The use of organic additives in the present study showed favorable result in formation of shoot and root and subsequently regeneration of a complete plantlet and hence may contribute to a simpler and economical plant culture media because additives such as banana and coconut water are easily available throughout the year as compared to plant growth regulators. Thus, this protocol can be used as simple and efficient *in vitro* mass propagation of *Dendrobium lowii* orchid and to reestablish this species back to the wild.

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