In vitro Seed Germination of *Coelogyne asperata* Lindl. (Orchidaceae)

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ABSTRACT A protocol for *in vitro* seed germination of *Coelogyne asperata* Lindl. has been established successfully. Immature seeds from 182 days old capsule were cultured on three different basal media; Murashige and Skoog (MS), Knudson C (KC), and Vacin and Went (VW) and maintained under continuous light at 25 ± 2 ˚C. After 30 days of culture (DAC), more than 90% of seeds were germinated on KC and VW media and about 84% of seeds germinated on MS medium. The incorporation of organic additives, including coconut water, potato homogenate and tomato juice each at 10% (v/v) in KC medium was tested to determine their effect on seed germination of *C. asperata*. The result revealed that KC basal medium alone without addition of organic additives promoted over 90% of seed germination at 30 DAC. Therefore, the protocol of using standard KC basal medium for *C. asperata* seeds germination could be suggested for mass propagation and conservation of this wild scented orchid.

KEYWORDS: Orchidaceae, Asymbiotic seed germination, Knudson C, Organic additives
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INTRODUCTION

Orchids belong to the Orchidaceae family are well-known for their aesthetic and good commercial values. They are practically found in all-natural ecosystems with large number of species, and some orchid species are locally abundant. However, due to life cycle's distinctiveness, several orchids have low geographic and altitudinal distribution ranges, so they can be seriously threatened (Seaton *et al*., 2010). Additionally, increases in demand coupled with attractive price for wild orchids have encouraged orchid poaching, consequently resulted in many orchids species lose their natural habitat, thus endangered the plant (Hinsley *et* al., 2018). In fact, the entire Orchidaceae family is currently included in Appendix-II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

Coelogyne asperata is an indigenous orchid species under *Coelogyne* genus, distributed in many parts of Southeast Asia and some parts of Oceanic archipelagos such as in Maluku Islands, Solomon Islands and Papua New Guinea (Wood & Cribb, 1995). Naturally, the orchid can be found on tree branches and humid rock faces especially near to river. This plant typically flowers from May until June with raceme pendulous about 1 ft long. The overhanging inflorescence reaches a length of up to 12 in/30 cm and consists of 10-15 flowers (Gravendeel, 2000). Like many other species of orchids, *C. asperata* is also threaten for being endangered not only due to its intriguingly attractive and commercially profitable, but also due to their scarcity and rareness, especially if it is cultivated from the wild (Coleman, 2001). Therefore, a prompt action for mass propagation of *C. asperata* is crucial to conserve this native species.

Plant tissue culture has been applied for years in conserving plant germplasm. The *in vitro* germination comprises a set of techniques widely utilized for *in vitro* propagation, which results in higher germination rates compared to natural germination process (Araújo, 2004). However, major disadvantage of adapting *in vitro* germination is that the high cost of components used for the

culture medium, particularly the plant growth hormone such as naphthalene acetic acid (NAA), indole acetic acid (IAA) and benzyl amino purine (BAP) (Daud *et al*., 2011). Some organic sources such as coconut water and fruit juice contained significant amount of vitamins, amino acids and organic constituents which can act as growth regulators, making these organic sources as an excellent organic additive for *in vitro* cultivation (Daud *et al*., 2011). Additionally, these organic additives also can be acquired at significantly lower cost compared to commercially available growth regulators. The beneficial of organic additives and NAA in promoting the multiplication shoot and roots multiplication of a Black Orchid hybrid (*C. pandurata x C. rumphii*) has been reported previously by Hartati *et al.* (2017). Therefore, the current study was conducted to establish a protocol for *in vitro* seed germination of this native orchid *C. asperata* by assessing the effects of basal media and supplementation of organic additives in culture medium.

METHODOLOGY

Sample preparation and surface sterilization

Pods of *Coelogyne asperata* were harvested after 182 days of hand pollination and brought back to the laboratory of Unit for Orchid Studies, Universiti Malaysia Sabah. Dry petals attached to the pod were removed, and pod was cleaned thoroughly under running tap water for 30 mins. The pod was surface sterilized for 15 mins in a 30% (v/v) of Clorox ® solution containing two drops of Tween-20 and rinsed with sterile distilled water for five times. The pod was then dipped in 95% (v/v) of ethanol and passed briefly through the flame.

Effect of basal media on in vitro seed germination

Three different basal media including Murashige and Skoog (MS) (Murashige & Skoog, 1962), Knudson C (KC) (Knudson, 1946) and Vacin and Went (VW) (Vacin & Went, 1949) were tested in this study. The pH of every medium was adjusted accordingly and added with 0.8% (w/v) of agar as solidifying agent prior autoclaving at 120 ˚C, 15 psi for 20 min. About 25ml of medium was dispensed into petri dish. Under a laminar flow hood, the sterilized pod was then cut longitudinally with blade, and the powdery mass of seeds were sown onto culture medium.

Effect of organic additives on in vitro seed germination

Three types of organic additives such as coconut water (CW), tomato juice (TJ) and potato homogenate (PH) at concentration of 10% (v/v) respectively, were added into KC basal medium to determine their effect in promoting seed germination of *C. asperata*. KC medium without organic additives served as a control.

Data collection

All treatments consisted of five independent replicates and were maintained under a 24h light condition at 25 ± 2 °C. Observation was performed every 3 days interval (denominated as days after cultured – DAC) and germination responses were recorded following the seed germination and embryo development classification by Semiarti *et al*., 2010.

Statistical analysis

A completely randomized design was used in all experiments and subjected to the analysis of variance (ANOVA) and the means values were separated by Duncan's multiple range test with 95% confidence level (at P=0.05), using IBM SPSS 27 statistics software.

RESULTS AND DISCUSSION

Seed germination and protocorm development

Orchid seeds are unique due to the presence of unorganized embryo cells and devoid of functional endosperm, therefore exogenous water and nutrients is obligatory for germination (Rasmussen *et al*., 2015). The germination stages of *Coelogyne asperata* from seed to seedling were described in Figure 1. Stage 1 (Figure 1A) is considered as no growth of embryo (first day of culture). After five DAC, the undifferentiated tissues of the seeds swelled up by imbibing water and nutrients, increasing cell number through repeated cell divisions to form an embryo (Stage 2) (Figure 1B) The yellowish swollen embryo turned to green and ready to discharge from seed coat after 10 DAC. A completely discharged embryo was considered germinated and protocorm was developed (Stage 3) (Figure 1C). After 40 DAC, a pointed shoot apex on the protocorm started to develop leaf primordium (Stage 4) (Fid. 1D). A second leaves appeared afterward within 50 DAC (Stage 5) (Figure 1E) and third leaves were developed after 60 DAC (Stage 6) (Figure 1F).

Figure 1. Developmental stages of *Coelogyne asperata* seed. (A) Stage 1; (B) Stage 2; (C) Stage 3; (D) Stage 4; (E) Stage 5; (F) Stage 6. Bar (A)-(C): 0.2 mm; (D)-(F): 1 mm.

Effect of basal media on seed germination

Three types of basal media were tested for *in vitro* seed germination of *C. asperata*. Within 10 DAC, the highest germination percentage was recorded on KC medium with 20.59±9.97% of seeds germinated up to stage 3 (Table 1), meanwhile no significant difference was observed in MS and VW media. After 30 DAC, seeds cultured in both KC and VW basal media were observed with more than 90% of germination percentage, compared to MS with only 84.50±5.51% of seeds germination.

Table 1. Effect of basal media on seed germination of *Coelogyne asperata*

Basal medium	Germination percentage (%) (Mean ± S.D)		
	10 DAC	20 DAC	30 DAC
MS	7.98±4.08^{b}	53.8±7.15^{b}	84.50±5.51^{b}
VW	5.72±3.83^{b}	78.8±7.46^{a}	93.69±4.73^{a}
KC	20.59±9.97^{a}	79.4±8.14^{a}	92.34±4.33^{a}

Means ± SD (standard deviation) of 5 replicates. Means in each column followed by the same letters are not significantly different at $p< 0.05$ as determined by DMRT. DAC: Days after culture; MS: Murashige and Skoog; VW: Vacin and Went; KC: Knudson C.

The three basal media (MS, KC, and VW) that were utilized in this study contained similar components and concentration of carbon source (sucrose) and gelling agent, but varying in concentration of mineral salts of macro, micro- nutrients as well as vitamins. Germination rate and percentage of *C. asperata* seeds were improved on KC and VW media, despite of having comparatively low concentration of macro and micro-nutrients as well as vitamins compared to MS medium. Surprisingly, the finding in this study is contradicting with the previous works that prefer MS medium in seed germination of several *Coelogyne* spp. such as *C. nervosa* (Abraham *et al*., 2012),

C. fuscescens (Koirala *et al*., 2013) and *C. flaccida* (Kaur & Bhutani, 2014). According to Yam *et al.* (2002), the nutritional requirements of germinating orchid seeds are varied from one species to another. Compared to the other species of *Coelogyne* orchids, *C. asperata* might require lower amount of minerals and additives to strive optimally, as it germinates and grow better in less rich media such as KC and VW. The suitability of KC medium in asymbiotic seed germination was also reported in other epiphytic orchids, including *Vanda dearei* (Jualang *et al*., 2014), *Aerides ringens* (Srivastava *et* al., 2015) and *Cymbidium aloifolium* (Suriya *et al*., 2017). Other factor that might contributes to the successful germination of orchid was the age of capsule (Bakar *et al*., 2014). In the present study, *C. asperata* capsules have been harvested after 182 days of pollination corroborating a study done by Lestari (2015), which indicated that *C. asperata* capsules with age of more than 120 days exert higher explants germination compared to capsules with aged of less than 100 days. This factor might as well contribute to the high germination percentage as observed in all tested media.

Effect of organic additives on seed germination of C. asperata

The effect of organic additives added into KC basal medium for the germination of *C. asperata* seed was determined. Although the germination rate was marginally slower compared to the control, all treatments exert similar effect on germination percentage at 70 DAC (Table 2) with the germination percentages are more than 95%, regardless of being added with organic additives or not. During this time, most of the protocorms with leaf primordia (stage 3) were developed into stage 4 and stage 5 with formation of one to two leaves (Figure 2D and 2E). The growth development of *C. asperata* from early days of culture to 70 DAC on KC medium alone is summarized in Figure 2.

Table 2. Effect of organic additives supplemented in KC medium on seed germination of *Coelogyne asperata*

Treatment	Germination percentage (%) (Mean ± S.D)			
	10 DAC	30 DAC	50 DAC	70 DAC
Control	22.33±2.38^{a}	92.44±4.33^{a}	97.63±2.09^{a}	99.93±0.15^{a}
CW 10% (v/v)	19.97±2.31^{a}	77.61±9.26^{b}	91.11±3.66^{b}	98.55±1.37^{a}
PH 10% (w/v)	20.34±4.55^{a}	87.98±5.40^{a}	94.55±2.49^{ab}	99.11±1.41^{a}
TJ 10% (w/v)	17.18±10.78^{a}	48.03±7.89^{c}	75.33±7.27^{c}	97.35±3.14^{a}

Means ± SD (standard deviation) of 5 replicates. Means in each column followed by the same letters are not significantly different at $p< 0.05$ as determined by DMRT. DAC: Days after culture; MS: Murashige and Skoog; VW: Vacin and Went; KC: Knudson C.

Supplementation of organic additives to orchid culture medium could have stimulatory or inhibitory effect on orchids' seed germination, which caused by the fluctuation in carbohydrates, protein, fat, vitamins, phenols, amino acids, fibre, hormones, sterols and organic acids contents (Arditti *et al*., 1982). The present study revealed that supplementation of organic additives in culture medium does not improve the germination rate of *C. asperata*. Surprisingly, *C. asperata* was able to germinate at better rate while achieving high germination percentage in un-supplemented KC basal media within 30 DAC. Similar with the findings by Lo *et al.* (2004), seeds of *Dendrobium tosaense* were germinated successfully on a medium without complex additives. In contrast, Abraham *et al*. (2012) and Hartati *et al*. (2017) have demonstrated the beneficial effects of coconut water on seed germination of *Coelogyne nervosa* and Black Orchid hybrid (*C. pandurata x C. rumphii*), respectively. The benefits of other organic additives such as tomato juice, potato homogenate, and banana homogenate in promoting seed germination and seedling growth have been demonstrated in many orchid species including *Vanda helvola* (David *et al*., 2015); *Dendrobium lowii* (Gansau *et al*., 2016) and *Cypripedium macranthos* (Huh *et al*., 2016).

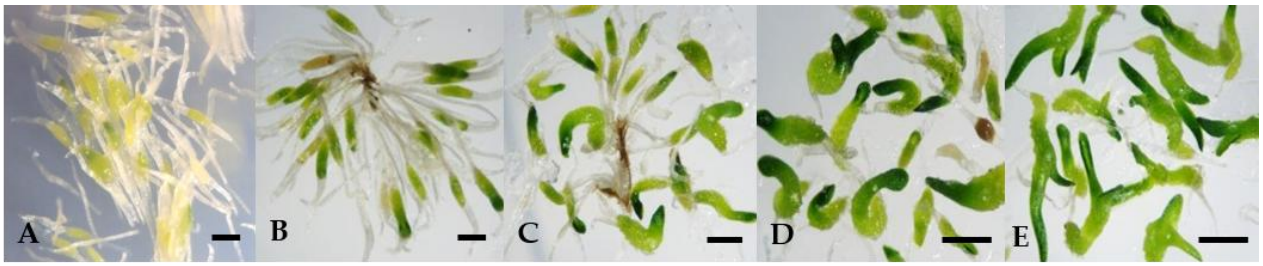

Figure 2. Seeds germination of *Coelogyne asperata* on KC medium alone. (A) 5 DAC; (B) 10 DAC; (C) 30 DAC; (D) 50 DAC; (E) 70 DAC. Bar (A-B): 0.2 mm; (C-E): 1 mm. DAC = Days after culture.

CONCLUSION

A protocol for *in vitro* seed germination of *Coelogyne asperata* was established in the present study. *C. asperata* seeds were successfully germinated and developed into seedling on KC basal medium alone. The finding suggests a simple and standard use of a medium for *C. asperata in vitro* seeds germination could potentially adapted for a large-scale propagation as well as for a conservation mean of *C. asperata*.

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