# Micropropagation of an endangered Borneo Orchid, *Paphiopedilum rothschildianum* Callus using Temporary Immersion Bioreactor System

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## ABSTRACT

This research was aimed to propagate *Paphiopedilum rothschildianum* through protocorm like bodies (PLBs) induction from callus and shoot regeneration from PLBs using RITA® Temporary Immersion Systems. Callus were induced from seeds and protocorm explants on half-strength semi-solid MS medium supplemented with 0-22.6 M 2,4-dichlorophenoacetic acid (2,4-D) and 4.54 M 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ). Approximately 0.5 g callus were inoculated in a 1L RITA® vessel containing 150 ml liquid medium with immersion time of 5 min in every 125 min. Different concentrations (15 and 58 mM) of sucrose were evaluated for PLB formation from callus. The explants produced calli as early as 30 days. The percentages of explant forming callus after 90 days of culture were 77.04.5% and 94.411.0% respectively. Callus proliferation using RITA® system showed 3-fold increase in fresh weight (as compared to that cultured on semi-solid) and 135 PLBs per gram calli were regenerated. Regeneration capacity increased to 190 PLBs per gram calli when sucrose concentration in the medium was elevated from 15 mM to 58 mM.

**Key words:** Protocorm, Protocorm like bodies (PLB), RITA®, 2,4-dichlorophenoacetic acid (2,4-D), 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ)

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## INTRODUCTION

*Paphiopedilum rothschildianum*, endemic to Mount Kinabalu (Borneo, Malaysia) area is one of the rarest and most sought-after orchid species in the world (Van der Ent *et al*., 2015). It is classified as endangered and listed in Appendix I of the Convention on International Trade in Endangered Species (Mcgough *et al.* 2006). The slow growth rate of the species further accelerates its reduction in the wild. (Hennessy *et al*, 2000). Thus, a reliable propagation method is necessary to maintain the survival of the species. Micropropagation through liquid culture promotes higher growth rate of explant and requires less maintenance compared to semi-solid culture (Mehrotra *et al.,* 2010). This is mainly due to the efficient nutrient utilisation and gaseous exchange in liquid culture (Sandal *et al*., 2001; Sarasan *et al*., 2006). Successful liquid cultures have been reported for orchid species, such as Phalaenopsis (Park *et al*., 1996); *Epidendrum radicans* (Chen *et al*., 2002); *Aerides crispum* (Sheelavanthmath *et al*., 2005); Oncidium (Kalimuthu *et al.,* 2007); and *Satyrium nepalense* (Mahendran and Bai, 2009). Previous studies using seed and protocorm explants shown that totipotent callus were successfully induced on medium with 4.54 M TDZ in combination with different concentration of 2,4-D (Lin *et al*., 2000; Lee and Lee, 2003; Lu, 2004). However, explants in liquid culture are frequently associated with morphological disorder, most notably the hyperhydricity; the development of abnormal tissue with glassy and vitreous appearance (Mehrotra *et al.,* 2010), mainly due to the constant contact of explants with liquid medium. The culture condition in temporary immersion system has the potential to reduce hyperhydricity because the contact time between explants and liquid medium is reduced (Etienne dan Berthouly, 2002). The system has been proven to reduce hyperhydricity in *Musa sp., Hevea, Coffea, Ananas, Saccharum,* and *Manihot* spp. (Ziv, 2005). The concentration of sucrose in liquid medium also has a significant role in the culture condition. High concentrations of sucrose will affect the osmotic pressure in the culture and inhibits the formation of chlorophyll (Tokuhara and Mii, 2001). However, to date, there is no report on liquid culture for *P. rothschildianum.*

Hence, the objective of this work was to study the potential of temporary immersion system to enchance the micropropagation of *P. rothschildianum* by evaluating the effect of sucrose concentration in the medium.

## MATERIALS AND METHODS

### Plant materials

Seed explants were obtained from 6-month post pollination *P. rothschildianum* capsules collected from Poring and Kinabalu National Park Orchid Nurseries. The capsules were surface sterilised with 10% (v/v) Clorox solution containing 1 drop of Tween 20. Following surface sterilisation, the explants were rinsed 3 times with sterile distilled water. The sterilised capsules were then dissected longitudinally and some of the seeds were used as explants for callus induction. Protocorm explants were induced from seeds germinated on a half-strength MS medium (Murashige & Skooge, 1962).

### Callus induction and maintenance

Callus induction studies were conducted by using seed and protocorm explants cultured on the medium based on Hong *et al.* (2008). The medium comprised of half-strength MS basal medium supplemented with full-strength MS Vitamin, 2 g/l peptone, 170 mg/l $NaH_2PO_4$, 58 mM sucrose, 4.54 μM TDZ in combination with 2,4-D at various concentration (0, 4.52, 13.56, 22.6 μM) and 2.2 g/l Gelrite. The pH of medium was adjusted to 5.2 and autoclaved at 121 °C and 15 psi for 20 minutes. The medium was dispensed in 9 cm diameter Petri dishes and seed or protocorm explants were cultured on each Petri dish. Each treatment was replicated five times and repeated twice. All cultures were maintained at 25 ± 2 °C in darkness. Observations of the percentages of explant forming callus (total explants forming callus divided by total number of explants), and the size and quality of the callus formed were made every 10 days for 150 days which the explants were subcultured onto fresh medium at 30 days intervals.

### PLB induction and shoot regeneration

PLB induction and shoot regeneration studies were conducted by weighing 0.5 g calli in sterile condition and subsequently cultured in 1 L bioreactor RITA® vessel containing 150 ml half-strength

MS medium supplemented with 2.27 μM TDZ and 12.0 M BAP. Different treatments of sucrose (15 mM and 58 mM) were tested to evaluate the effect of sucrose concentration on hyperhydricity. The system was programmed with the immersion time of 5 minute every 125 minute. The inoculation density and immersion time were based on a previous study on Phalaenopsis by Young *et al.* (2000). The explants were harvested after 30 days of culture to obtain the fresh weight of callus and total number of PLB produced. Data were taken from 3 replicates. The calculations of regeneration capacity were based on total number of PLB produced divided by the initial weight of callus used.

**Data collection and statistical analysis**

All data were analyzed using SPSS (Statistical Package for Social Science) version 17.0 and subjected to analysis of variance (ANOVA). Duncan's multiple range tests were used to separate mean differences at 5% level. Qualitative data of the callus formed from seed or protocorm explants were collected using callus size and quality index as shown in Table 1.

## RESULTS AND DISCUSSION

**Callus induction**

Callus were successfully induced from seed and protocorm explants on the medium containing various concentration of 2,4-D (0-22.6 M) and 4.54 M TDZ after 30 days of culture. The growth of callus slowly increases in mass through the 90 days period (Plate 1A-D). All explants produced friable callus with creamy colour (Table 2). After 90 days of culture, the highest percentages of explants forming callus was obtained on MS medium containing 13.56 M and 4.54 M TDZ. Similar results were reported by Lin *et al.* (2000); Lee and Lee (2003); and Hong *et al.* (2008)

**Table 1** Callus size and quality index for qualitative data analysis

| Size | Index | Colour | Index | Type | Index |
|---|---|---|---|---|---|
| Small | + | browning | K | compact | c |
| Average | ++ | creamy | P | friable | f |
| Large | +++ | greenish | H | nodular | n |

where medium containing 22.6 μM 2,4-D and 4.54 μM TDZ gave the highest percentages of explants forming callus.

The medium containing 4.54 μM TDZ alone showed high percentages of explant forming callus for both seed (77.0%) and protocorm (89.3%). This indicates that *P. rothschildianum* seed and protocorm explants require very little or no 2,4-D for callus formation. The requirement for only cytokinin for induction of callus by *P. rothschildianum* was previously reported by Ng and Salleh (2010). In their study, callus formation by nodal stem explants of *P. rothschildianum* was observed on half-strength MS medium supplemented with kinetin (Ng and Salleh, 2010). The opposite results were observed by Hong *et al.* (2010) and Lin *et al.* (2000) on Paphiopedilum hybrid where the induction and proliferation of callus on medium without 2,4-D were unsuccessful. This shows that purebred species of Paphiopedilum produced

**Table 2** Formation of callus by protocorm and seed explant of *Paphiopedilum rothschildianum* explants after 90 days of culture on half-strength MS medium supplemented with 0-22.6 μM 2,4-D and 4.54 μM TDZ in darkness, 25 ± 2 ˚C

| Plant Regulators (μM) | | Explant formed callus (mean%±SD) | | Size and quality of callus | |
|---|---|---|---|---|---|
| **2,4-D** | **TDZ** | **Seed** | **Protocorm** | **Seed** | **Protocorm** |
| 0 | 0 | 33.6±7.6 f | 25.7 c | (K,P), c+,f+ | (K), c++ |
| 0 | 4.54 | 77.0±4.5 abc | 89.3 a | (P), f+++ | (K,P), c++, f+++ |
| 4.52 | 4.54 | 76.0±6.5 abc | 82.1 ab | (K), c++ | (P,K), c+, f+++ |
| 13.56 | 4.54 | 61.0±19.8 cd | 94.4 a | (K), c++ | (K,P),+, f+++ |
| 22.60 | 4.54 | 64.0±20.4 bcd | 89.3 a | (P), c++ | (K,P), c+, f+++ |

Note: Data were taken from 5 replicates with the same letters are not significantly different at $p<0.05$ using Duncan's Multiple Range Test. SD= Standard Deviation. Callus size and quality index; (K): browning, (P): creamy, (H): greenish, c: compact, f: friable, n: nodular, +: small, ++: average, +++: large.

adequate endogenous cytokinin for callus induction as compared to hybrid species (Palama *et al.,* 2010).

In the present study, the callus formation was considerably better on protocorm explants as compared to seed explants (Table 2). The highest percentages of explants forming callus (94.4%) was protocorm explants cultured on half-strength MS medium supplemented with 4.54 μM TDZ and 13.56 μM 2,4-D; while for seed explants, the highest (77.0%) was on medium with 4.54 μM TDZ. Furthermore, it was observed that the friability and size of callus formed on protocorm explants were qualitatively better than those on seed explants (Table 2, Figure 1). Hong *et al.* (2008) indicated that the regeneration capacity of seed-derived callus in their study on Paphiopedilum Alma Gavaert was better than the study by Lin *et al.* (2000) using protocorm-derived callus.

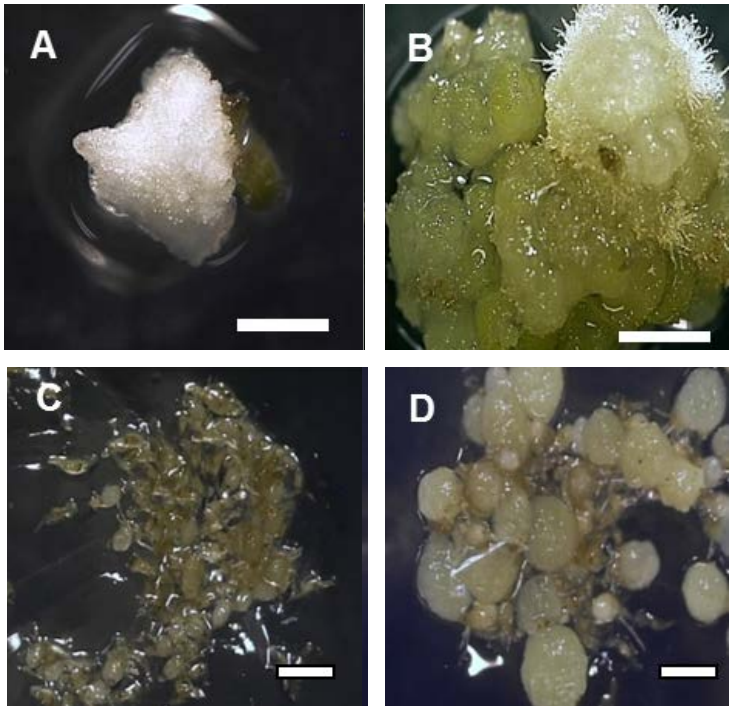


**Figure 1** Callus induction of *Paphiopedilum rothschildianum* on half-strength MS (Murashige & Skoog, 1962) medium in dark, 25 ± 2 °C. A) Callus formed on protocorm after 30 days of culture (bar = 0.18cm); B) Callus proliferation on protocorm after 90 days of culture (bar = 0.19 cm); C) Seed explants after 30 days of culture (bar = 0.05 cm); D) Callus formation on seed explants after 90 days of culture (bar = 0.21 cm)

**PLB induction and shoot regeneration**

Callus explant in temporary immersion system shows a 3-fold increase in fresh weight with regeneration capacity of 168 PLBs per gram calli. PLB regeneration capacity increases to 190 PLBs per gram calli when the sucrose concentration was elevated from 15 mM to 58 mM (Table 3). This may due to efficient distribution and utilisation of nutrient in liquid medium (Sandal *et al.,* 2001). This was supported by Ziv (2005) who stated that elevating sucrose concentration led to higher biomass of Boston fern (*Nephrolepis exaltata*).

The regeneration capacity of 190 PLBs per gram calli within 30 days of culture achieved in this study is better than previous reports on propagation of *Paphiopedilum* species using semi-solid culture system; Lin *et al.* (2000) with regeneration capacity of 7 shoot buds/ plantlet per 0.1 g calli, Hong *et al.* (2008) obtained 4.7 PLB/shoots per 5 mg calli, and Ng and Salleh (2010) achieved 4.1 PLBs per explant. It was observed that callus cultured in 1 L RITA® system vessel containing 150 ml half-strength MS showed no sign of vitrification or hyperhydricity (Figure 2). PLBs regeneration and development

**Table 3** Formation of PLB from *Paphiopedilum rothschildianum* callus after 30 days of culture in 1 L RITA® system vessel containing 150 ml half-strength MS supplemented with 2.27 M TDZ and 12.0 M BAP with different concentrations of sucrose.

| Carbon source (mM) of sucrose | Fresh weight Increased* (g) | Average number of PLB | Regeneration capacity** (PLB per gram callus) | Size and quality of callus*** |
|---|---|---|---|---|
| 15 | 0.06 ±0.01 | 3.6 | 168 | (H), f++ |
| 58 | 0.302 ±0.17 | 4.0 | 190 | (H), f+++ |

Average of 3 replications.

* Fresh weight of callus obtained after 30 days of culture. (Mean ± Standard Deviation)

** The calculations of regeneration capacity were based on total number of PLB produced divided by the initial weight of callus used.

*** Callus size and quality index; (K): browning, (P): creamy, (H): greenish, c: compact, f: friable, n: nodular, +: small, ++: average, +++: large.

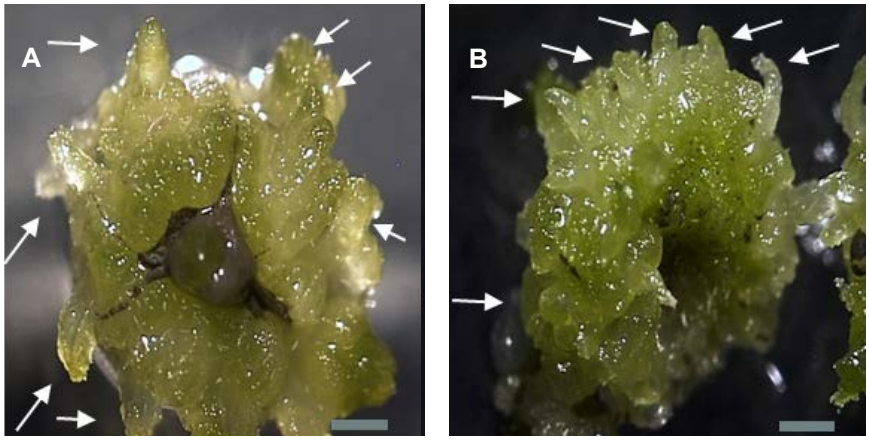


**Figure 2** Formation of PLBs on *Paphiopedilum rothschildianum* callus cultured in 1 L RITA® system vessel containing 150 ml half-strength MS (Murashige & Skoog, 1962) supplemented with 2.27 μM TDZ and 12.0 μM BAP (bar = 1 mm). A) PLB/shoots formed in medium with 15 mM sucrose after 30 days of culture. B) PLB/shoots formed in medium with 58 mM sucrose after 30 days of culture

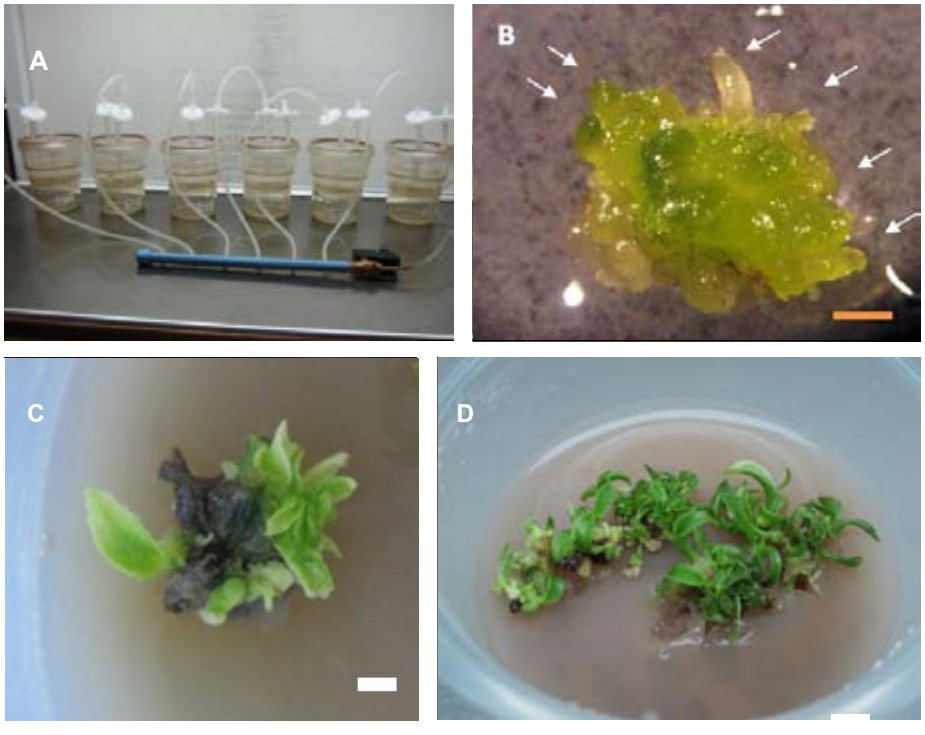


**Figure 3** PLBs regeneration and development of *Paphiopedilum rothschildianum* callus cultured in 1 L RITA® system vessel containing 150 ml half-strength MS (Murashige & Skoog, 1962) supplemented with 2.27 μM TDZ and 12.0 μM BAP (bar = 1 mm). A) RITA® system programmed at 5 min immersion time once every 125 min. B) Multiplied PLBs transferred to development medium. C) PLBs developed into shoot after 14 days of culture on development medium. D) Formation of plantlet after 150 days of culture

of *Paphiopdilum rothschildianum* callus was shown in Figure 3. This work proved that temporary immersion system is able to minimise the hyperhydricity problem associated with explant cultured in liquid culture.

## CONCLUSION

Based on the present study conducted, a protocol for propagation of *P. rothschildianum* using temporary immersion system has been established. This study proved that seed and protocorm explants able to formed callus after 30 days of culture. The subsequent PLB induction and shoot regeneration using temporary immersion system produced up to 190 PLBs per gram calli. The method described in this study shows the potential for further optimisation of temporary immersion system in the enhancement of the species propagation.

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