Journal of Tropical Biology and Conservation 15: 1–14, 2018 ISSN 1823-3902
E-ISSN 2550-1909

Research Article

PLB Regeneration of *Paphiopedilum rothschildianum* using Callus and Liquid Culture System

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Abstract

This research was conducted to rapidly propagate *Paphiopedilum rothschildianum* using semi-solid and liquid culture systems. Calli were induced from seed, leaf segments (LS), seed-derived protocorms (SDP) and secondary protocorms (SP) cultured on half-strength semi-solid MS media supplemented with 0-22.6 µM 2,4-dichlorophenoacetic acid (2,4-D) and 4.54 µM 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ). Regeneration of PLB from callus was optimized on semisolid medium and were evaluated in various concentrations and types of PGRs. PLB regeneration was further optimized using callus originating from a different induction medium, and cultured on different concentrations (0, 15 and 58 mM) of sucrose. For PLB regeneration using liquid culture system, 0.5 g callus were inoculated in a temporary immersion bioreactor system (RITA®) containing 150 ml liquid medium with immersion time of 5 minutes in every 125 minutes. SE, SDP and SP explants produced calli as early as 30 days with the percentages of explant forming callus at 77.0% ± 4.5, 94.4% ± 11.0, and 66.7% ± 14.4 respectively after 90 days of culture. The calli regenerated on medium supplimented with 2.27 µM TDZ and 12.0 µM BAP, but with very low percentage (15.0% ± 13.7 callus produced the average of 3 PLBs). PLB regeneration capacity increased to 37.5% ± 13.7 with the average of 5.9 PLBs for callus originating from an induction medium containing 4.54 µM TDZ, when a lower sucrose concentration (15 mM) was used in the regeneration medium. Callus proliferation using RITA® system showed an almost 2-fold increased in fresh weight and 168 PLBs per gram calli were regenerated. In contrast to semi-solid culture, the regeneration capacity in liquid culture system increased to 190 PLBs per gram calli when sucrose concentration in the medium was elevated from 15 mM to 58 mM.

Keywords: Seed-derived protocorms, secondary protocorms, temporary immersion system, Temporary Immersion System (TIS), RITA® bioreactor system.

Received 07 June 2016
Reviewed 08 November 2017
Accepted 21 December 2017
Published 15 October 2018

Introduction

Paphiopedilum rothschildianum is a rare slipper orchid species which is endemic to the area around Mount Kinabalu, Sabah, Malaysia (Cribb, 1998). Being rare, this species has been classified as endangered and is listed in *Appendix I* of the Convention on International Trade in Endangered Species (CITES; CITES Appendix 2008). According to Arditti & Ernst (1993), the propagation of this orchid species in the natural way takes several years due to its slow growth rate. Hence, multiplying the orchid through tissue culture techniques is seen as the best alternative. Micropropagation has been applied to propagate many orchid species to produced high mass of plants in a considerably short time and with a lower cost (Zeng et al., 2016). Micropropagation through callus culture is now widely used in the mass propagation of orchids, including *Cymbidium ensifolium var. misericors* (Chang & Chang, 1998); *Phaelonopsis* (Ishii et al., 1998; Chen et al., 2004; Tokuhara & Mii, 2001); *Drynaria Quercifolia* (Hegde et al., 2006); *Pleione formosana Hayata* (Lu, 2004); and *Dendrobium fimbriatum* (Roy & Banarjee, 2003). Only a few researches on callus cultures of slipper orchids have been reported. Callus of *Paphiopedilum hybrids* was induced from seed-derived protocorm (Lin et al.,2000) and seed (Hong et al.,2008) as explants. Another slipper orchid, *Cypripedium formosanum* callus culture was also established using seed- derived protocorm (Lee & Lee, 2003). The propagation of *P. rothschildianum* through *in vitro* formation of secondary protocorm-like bodies (PLBs) from primary PLB developed from stem-derived callus was also recently conducted by Ng & Salleh (2011). Liquid culture system is another alternative means to propagate orchids, it provides higher growth rate of explant and less maintenance as compared to semi-solid culture (Mehrotra et al., 2007). Liquid culture system has been widely used for orchid propagation, this includes *Phalaenopsis* (Park et al., 1996); *Cymbidium* (Da Silva et al., 2006); *Epidendrum radicans* (Chen et al., 2002); *Satyrium nepalense* (Mahendran & Narmatha Bai, 2008); *Oncidium* (Kalimuthu et al., 2007); and *Aerides crispum* (Sheelavanthmath et al., 2005). To date, no report on liquid culture for *P. Rothschildianum* has been documented. The culture system has potential for large-scale propagation of *P. rothschildianum*. Therefore, the objective of this work was to develop protocols for propagation of *P. rothschildianum* using callus on semi-solid and liquid culture systems.

Methodology

Plant materials

Paphiopedilum rothschildianum capsules 6-month post pollination were collected from Poring and Kinabalu National Park orchid nurseries. The capsules were surface sterilised with 10% (v/v) chlorox solution containing 1 drop of tween 20 followed by rinsing with sterile distilled water three times. The sterilised capsules were then disected longitudinally and some of the seeds were used as explants for callus induction and designated as Seed Explant (SE). To obtain protocorms, a portion of the seeds were germinated on a half strength MS medium (Murashige & Skooge, 1962) and the resulting protocorms were used as explants designated as Seed-derived Protocorms (SDP). Subsequently, some of the protocorms were cultured on a multiplication medium and secondary protocorms formed on the protocorm explants were designated as Secondary Protocorms (SP). Leaves from *in vitro* plantlets of *P. rothschildianum* were cut into pieces of 1 cm × 1 cm and used as explants designated as Leaf Segments (LS).

Callus induction

To induce callus, the medium based on Hong et al. (2008), was used. The medium was comprised of half strength MS basal medium supplemented with full strength MS Vitamin, 2g/l peptone, 170 mg/l NaH_2PO_4, 58 mM sucrose, various concentration of 2,4-D (0-22.6 µM) with a constant concentration of TDZ (4.54 µM) and 2.2 g/l Gelrite. The pH of medium was adjusted to 5.2 and autoclaved at 121ºC and 15 psi for 20 minutes. The medium was dispensed in 9 cm diameter Petri dishes and four explants of SE, SDP, SP and LS were cultured on each Petri dish. Each treatment was replicated five times and all cultures were maintained at 25 ± 2˚C in darkness. Observations were made every 10 days for 90 days and explants were subcultured onto fresh media at four week intervals.

PLB Regeneration

PLB regeneration from callus was first optimized on semi-solid media, and the best medium was subsequently applied on RITA®. To induce PLB, semi-solid media based on Hong et al. (2008) were supplemented with NAA (0-26.85 µM), 2,4-D (0-9.04 µM), TDZ (0-9.08 µM) and BAP (0-22.20 µM) at various concentrations and combinations. To evaluate the effect of origin of callus (induction medium) on PLB regeneration, callus induced from seed explant (SE) cultured on semi-solid half strength MS media containing 4.54 µM TDZ alone or a in combination with 13.6 µM 2,4-D were used. To evaluate the effect of carbon source, medium with sucrose concentration of 0, 15 and 58 mM were

tested. To evaluate PLB regeneration in liquid culture system, 0.5 g callus were inoculated in 1 L RITA® system vessel containing 150 ml liquid medium. The immersion time was set to 5 minutes in every 125 minutes. All cultures were maintained at 25 ± 2°C in a 16 hour photo period. Observations were made every 10 days for 150 days and explants were sub-cultured onto a fresh medium at four week intervals.

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 conducted for mean comparisons of data collected using $P < 0.05$.

Results and Discussion

Callus Induction

Callus was induced on medium containing a combination of 22.6 µM 2,4-D and 4.54 µM TDZ for SE, SP, and SDP explants. Figure 1 (Plate 1A-H) showed that the callus became visible after 30 days of culture and slowly increased in mass through the 150 days period. The morphology of the callus formed on average was friable and creamy coloured. The auxin to cykonin ratio was considered standard for callus induction experiments for slipper orchids as reported by Lin et al. (2000); Hong et al. (2008); and Lee & Lee (2003) where 22.6 µM 2,4-D and 4.54 µM TDZ gave the highest percentages of explants forming callus. The results indicate that the combination of auxin and cytokinin in the culture medium play important roles in dedifferentiation of plant cell (Pierik, 1997). LS failed to produce callus and died after 40 days of culture (Plate 1I-J). Lin et al. (2000) had reported the failure of stems, root tips and leaves of *Paphiopedilum* hybrids to produce callus when cultured on media containing TDZ, 2,4-D, PBOA, BA, and 2ip. Different types of explants has been used for *in vitro* clonal propagation of *Paphiopedilum* (Chugh et al., 2009). The success rate of the culture depends on the source, types, maturity and treatment of the explants (Trigano & Gray, 1999).

Figure 1. (Plate A-J): Callus induction of *Paphiopedilum rothschildianum* on ½ MS (Murashige & Skoog, 1962) medium in darkness, 25 ± 2°C. **A)** SDP after 30 days of culture (bar = 0.18 cm); **B)** SDP after 60 days of culture (bar = 0.19 cm); **C)** SDP after 90 days of culture (bar = 0.19 cm); **D)** SP after 30 days of culture (bar = 0.19 cm); **E)** SP after 60 days of culture (bar = 0.19 cm); **F)** SP after 90 days of culture (bar = 0.19 cm); **G)** Seed after 30 days of culture (bar = 0.05 cm); **H)** Seeds after 80 days of culture (bar = 0.21 cm); **I)** LS after 10 days of culture (bar = 0.22 cm); **J)** LS after 40 days of culture (bar = 0.21 cm).

Table 1. Formation of callus by different types of *Paphiopedilum rothschildianum* explants after 90 days of culture on ½ MS (Murashige & Skoog, 1962) medium supplemented with 0-22.6 µM 2,4-D and 4.54 µM TDZ in darkness, 25 ± 2°C.

Plant Growth Regulators (µM)		Explant formed callus (mean%±SD)				Size and quality of callus			
TDZ	2,4-D	SE	SDP	SP	LS	SE	SDP	SP	LS
0	0	33.6 ± 7.6^{f}	25.7 ± 9.8^{f}	40.0 ± 13.7^{ef}	0^{g}	(K,P), c+,f+	(K), c++	(K,P), c++	-
4.54	0	77.0 ± 4.5^{abc}	89.3 ± 13.4^{a}	41.7 ± 14.4^{ef}	0^{g}	(P), f+++	(K,P), c++, f+++	(H, K), n+++, c+++	-
4.54	4.52	76.0 ± 6.5^{abc}	82.1 ± 18.9^{a}	66.7 ± 14.4^{bcd}	0^{g}	(K), c++	(P,K), c+, f+++	(H,K), c+++, n+++	-
4.54	9.04	-	87.5 ± 13.7^{a}	58.3 ± 14.4^{cde}	-	-	(K,P), +, f+++	(H,K), c+++, n+++	-
4.54	13.56	61.0 ± 19.8^{cd}	94.4 ± 11.0^{a}	62.5 ± 14.4^{cd}	0^{g}	(K), c++	(K,P),+, f+++	(H,P), n+++, c+++	-
4.54	18.08	-	82.1 ± 18.9^{a}	55.6 ± 9.6^{de}	-	-	(K,P), c+, f+++	(K,P), c+++	-
4.54	22.6	64.0 ± 20.4^{bcd}	89.3 ± 13.4^{a}	56.3 ± 12.5^{de}	0^{g}	(P), c++	(K,P), c+, f+++	(P,H), c+++, n+++	-

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.

The highest percentages of explants forming callus after 150 days of culture was SDP (94.4 ± 11.0) followed by SE (77.0 ± 4.5) and SP (66.7 ± 14.4) (Table 1). These results are in contrast with that reported by Lin et al. (2000) which showed seed explants formed better callus than SDP. This may due to the fact that SDP was grown on fresh medium as compared to the dormant nature of the seed explants. These findings suggest that the responsiveness of explant was affected by the maturity and responsiveness of the cell and tissue (Murthy & Pyati, 2001). Table 1 also showed that, on average although SDP showed the highest percentage of explant forming callus, it was SP explants that showed higher mass of callus formed on explants. Better formation of callus by SP explants might be due to the carry over effect of PGR as the SP explants originated from a multiplication medium containing TDZ. This is supported by the finding made by Makara et al. (2010) from their study on banana where higher concentration of endogenous PGRs may increase plant growth and proliferation. They found out that the proliferation rates of shoots originating from basal cycle medium with various TDZ concentrations were

significantly higher than those from 22.2 mM BAP, which suggested that TDZ had a high carry over effect enabling the shoots to continue proliferating on the hormone free medium (Makara et al. 2010).

PLB Regeneration

PLB formation from callus proved to be very slow and low in number. Only 15.0% ± 13.7 callus formed PLB (average of 3 PLBs per callus) on the semi-solid medium supplimented with 2.27 µM TDZ and 13.32 µM BAP after 120 days of culture (Table 2, Figure 2). The other PGRs failed to produce PLB. Lin et al. (2000) suggest that the combination of TDZ with auxin is crucial for the regeneration of PLB for *Paphiopedilum*. However, the results were in contrast with the study conducted by Hong et al. (2008) and Ng & Salleh (2011). Hong et al. (2008) were able to regenerate PLB for *Paphiopedilum* Alma Gavaert using 26.85 µM NAA while Ng & Salleh (2011) used 4.0 µM kinetin to regenerate PLB for *P. rothschildianum*.

Table 2. Formation of PLB on half-strength MS (Murashige & Skoog, 1962) with different types and concentration of PGRs as explants after 150 days of culture.

Plant Growth Regulators (µM)				Callus formed PLB (mean% ± SD)	PLB per explant
NAA	2,4-D	BAP	TDZ		
0	0	0	0	0	0
0	0	4.44	2.27	0	0
0	0	13.32	2.27	15.0±13.7	3
0	0	22.20	2.27	0	0
0.27	0	0	2.27	0	0
2.69	0	0	2.27	0	0
5.37	0	0	2.27	0	0
26.85	0	0	2.27	0	0
0	2.26	0	2.27	0	0
0	4.52	0	2.27	0	0
0	6.79	0	2.27	0	0
0	9.08	0	2.27	0	0
0	2.26	0	4.54	0	0
0	4.52	0	4.54	0	0
0	6.79	0	4.54	0	0
0	9.08	0	4.54	0	0

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.

This proved that different PGR were required to succesfully regenerate PLB for different species of *Paphiopedilum*. In addition, PLB regeneration was proven to be difficult for *Paphiopedilum* species as demonstrated by Lin et al. (2000) and Hong et al. (2008). They reported that PLB formation on callus was achieved only after 120 days and 150 days of culture respectively.

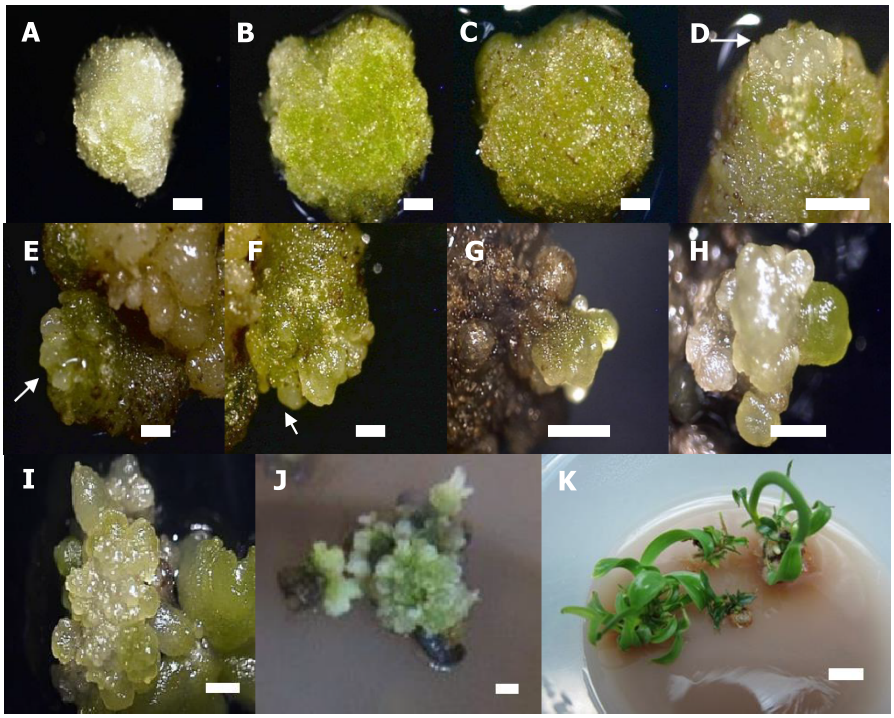

Figure 2. The formation of PLBs from callus cultured on half-strengh MS medium supplemented with 2.27 µM TDZ and 12.0 µM BAP (bar = 1 mm). **(A)** Callus proliferated after 30 days of culture. **(B)** Callus become greenish in colour after 60 days of culture. **(C)** **(C)** Callus become more greenish after 90 days of culture. **(D)** PLB formed on callus after 120 days of culture. **(E)** Formation of PLB become more apparent after 150 days of culture. **(F)** Magnification of the PLB formed. **(G)** Callus turned brown but PLB continue to grow and multiply. **(H)** PLB further multiply after 240 days of culture. **(I)** Multiplied PLBs transferred to development medium. **(J)** PLBs developed into shoot after 14 days of culture on development medium. **(K)** Formation of plantlet after 150 days of culture.

Table 3. Formation of PLB on half-strength MS (Murashige & Skoog, 1962) with different concentration of sucrose using *Paphiopedilum rothschildianum* callus developed from medium supplemented with different PGRs as explants.

Callus source	Sucrose concentration (mM)	Callus regenerate (mean% ± SP)	Average number of PLB	Time taken for PLB formation (Days)
Developed from ½ MS medium supplemented with 4.54 µM TDZ	0	0	-	-
	15	37.5 ± 13.7[a]	5.9	42
	58	33.3 ± 14.4[bc]	2.7	45
Developed from ½ MS medium supplemented with 4.54 µM TDZ and 13.6 µM 2,4-D	0	0	-	-
	15	16.7 ± 14.4[b]	2	66
	58	0	-	-

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.

The regeneration capacity increased to 37.5% ± 13.7 callus forming PLB (average of 5.9 PLBs per callus) after only 42 days of culture when lower concentration of sucrose was used in the regeneration medium (Table 3). This showed that lower concentration of sucrose are more suitable for the formation of PLB for *P. rothschildianum* callus. This was supported by Chen & Chang (2002), who reported that higher embryogenic response of *Oncidium* 'Grower Ramsey' leaf explant when they were cultured on the medium containing lower concentration of sucrose. Plant regeneration for cell suspension culture of *Phalaenopsis* species was also more successful using the medium with lower concentration of sucrose (Tokuhara & Mii, 2001). Faria et al. (2004) indicated that high concentration of sucrose resulted in carbohydrate accumulation in the medium and subsequently causing retardation of photosynthesis.

The results in Table 3 also showed that the PGRs used to induce callus affect the callus regeneration capacity. It is observed that lower 2,4-D concentration in the callus induction medium was able to increase the callus regeneration capacity. This was supported by Lu (2004), who indicated that the capability of *Pleione formosana* Hayata callus to regenerate PLB was affected by the concentration of 2,4-D and TDZ in the callus induction medium. Lin et al. (2000) indicated that callus originating from medium supplemented with single treatment of 2,4-D or 2,4-D results in little growth and eventually becomes necrotic. The present study suggests that high concentration of auxin in callus induction medium inhibit PLB regeneration for *P. rothschildianum* callus. This may due to the carry over effect of 2,4-D from induction medium onto the

regeneration medium, similar to the study conducted by Makara et al. (2010). This also proves that high concentration of auxin may inhibit the morphogenesis of plant tissue cultured *in vitro* (Smith, 2013).

For liquid culture experiment, the average PLB formation was higher (4.0 PLBs) on medium with higher sucrose concentration as compared to 3.6 PLBs on medium with lower sucrose concentration (Table 4). The PLBs was then transferred onto a development medium and successfully developed into plantlet after 150 days of culture (Figure 3C-E). The result was different from semi-solid culture, where lower concentration of sucrose was better for PLB formation. This indicates that the optimal sucrose concentration for PLB regeneration of *P. rothschildianum* callus in liquid medium was different compared to a semi-solid medium. This maybe due to efficient distribution and utilisation of nutrients in liquid medium (Sandal et al., 2001). This was supported by Ziv (2005) who stated higher sucrose concentration produced higher biomass of Boston Fern *(Nephrolepis exaltata)*. This finding could also be due to the osmotic pressure in liquid medium not being affected by high concentration of carbon as opposed to semi-solid medium (Pierik, 1997).

Table 4. Formation of PLB from *PaphiopedilPLB um rothschildianum* callus after 30 days of culture in 1 L RITA® system vessel containing 150 ml half-strength MS (Murashige & Skoog, 1962) with different types of carbon source.

Carbon source	Fresh weight increased (gram)	Average of PLB	Total PLB per final weight
58 mM of sucrose	0.30 ± 0.17	4.0	95/0.82 g
15 mM of sucrose	0.06 ± 0.01	3.6	84/0.65 g

Note: Data were taken from 3 replicates. Fresh weight of callus obtained after 30 days of culture. SD= Standard Deviation.

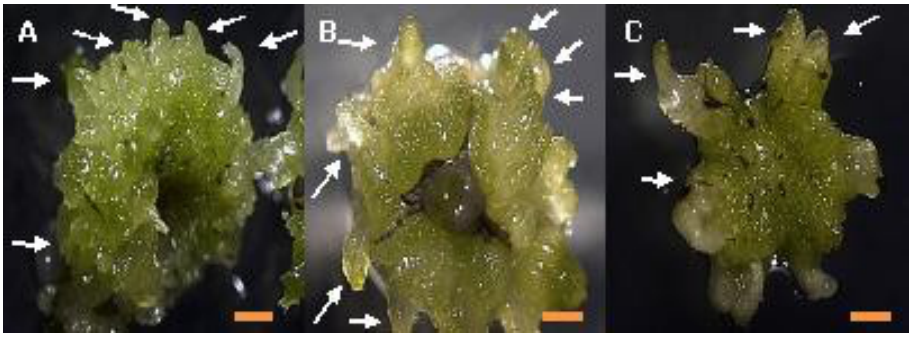

Figure 3. Formation of PLB/shoots on *Paphiopedilum rothschildianum* callus after 30 days of culture in 1 L RITA® system vessel containing 150 ml half-strength MS (Murashige & Skoog, 1962) with different types of carbon source (bar = 1 mm). **A)** PLB/shoots formed in medium with 58 mM sucrose. **B)** PLB/shoots formed in medium with 15 mM sucrose. **C)** PLB/shoots formed in medium with 15 mM glucose.

Table 5. Comparison between regeneration capacity of *Paphiopedilum rothschildianum* callus in liquid culture system and semisolid culture.

Culture system	Carbon source	Regeneration capacity (PLB per gram callus)
Liquid medium in 1 L	15 mM sucrose	168
RITA® vessel	58 mM sucrose	190
Semisolid medium in 9mm	15 mM sucrose	113
Petri plate	58 mM sucrose	45

Note: The calculation of regeneration capacity were based on total number of PLB produced divided by the initial weight of callus used.

For the comparison of the regeneration capacity between liquid culture and semi-solid culture, it showed more than a 2-fold increase in PLB formation on callus cultured in liquid culture as compared to semi-solid culture (Table 5). This is similar to the study conducted by Nayak et al. (2002) on PLB regeneration of *Cymbidium aloifolium* (L.) Sw. and *Dendrobium nobile* Lindl PLB in semi-solid and liquid medium. Their results showed that PLB regeneration of both orchid species was significantly enhanced compared to semi-solid medium. Furthermore, in the liquid culture system, more explants can be cultured in a larger container, thus increasing productivity. This is mainly due to the efficiency of their culturing conditions, media transfer and sterilization procedures. In contrast, the semi-solid culture system is limited by size of the culture container, intensive labour work and poor aseptic condition (Etienne & Berthouly, 2002). The RITA® culture vessel used in the present study was also able to avoid the hyperhydricity problems normally associated with explants in liquid culture, thus producing healthy PLBs (Figure 3A-B). The system used a temporary contact between the explants and liquid medium, which can greatly reduced hyperhyricity (Etienne & Berthouly, 2002). Teherefore, the current work proved that the temporary immersion system provides many advantages that further enhance the PLB regeneration of *P. rothschildianum* callus compared to the conventional system.

Conclusion

Based on the present study, a protocol for propagation of *P. rothschildianum* using callus on semi-solid and liquid culture systems has been established. This work proved that callus can be induced on *P. rothschildianum* explants within 30 days, where SDP explants formed the highest percentage of callus. PLB formation from callus was achieved using 2.27 µM TDZ and 12.0 µM BAP. The regeneration capacity increased by using different concentration of sucrose. Morever, liquid culture system using RITA® further increased the regeneration

capacity; producing up to 190 PLBs per gram calli. Future studies will be directed to further optimise the liquid culture system including volume of media, inoculum density and immersion time.

Acknowledgements

The authors wish to thank Sabah Park Orchid Nursery for supplying *P. rothschildianum* seeds and Universiti Malaysia Sabah for funding the research under the Fundamental Research Grant Scheme (GPS0009-SG-1/2009).

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