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In Vitro Callus Induction from Seed, Leaf and Protocorm Explants of *Paphiopedilum rothschildianum*

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Abstract: This research was conducted to investigate the effects of different types of explants on the formation of *Paphiopedilum rothschildianum* callus. Seed, Leaf Segments (LS), Seed-Derived Protocorms (SDP) and Secondary Protocorms (SP) were cultured on half-strength (MS) Media Supplemented with 5 mg/L 2, 4-Dichlorophenoacetic acid (2,4-D) and 1 mg/L 1-phenyl-3-(1-23-Thiadiazol-5-yl)-urea (TDZ). Lower percentage of seed 73.5±14.3 and SP 65.0±22.4% formed callus as compared to SDP 87.5±16.9%. However, the mass of callus induced was highest on SP as compared to SDP and seed explants. Leaf explants failed to form callus and died after 40 days of culture. Callus started to form on seed, SDP and SP after 30 days of culture. Further, study on SDP shows that 3 months old SDP cultured on medium with 1 mg/LTDZ and 4 mg/L 2,4-D was the best explant for callus induction with 82.5%±16.9 explant forming callus recorded as compared to 81.3±12.5 and 72.0±11.0 for 1 and 6 months old SDP, respectively. Calli produced from the present study slowly regenerates into Protocorm Like Bodies (PLBs) and develop shoots and leaflets that further grow into plantlets after 120 days of culture on development medium.

Key words: Seed-derived protocorms, secondary protocorms, Protocorm Like Bodies (PLBs), 2, 4-dichlorophenoacetic acid, 1 mg/L 1-phenyl-3-(1-,23-Thiadiazol-5-yl)-urea (TDZ), callus

INTRODUCTION

Paphiopedilum rothschildianum is a rare slipper orchid endemic to the area around Mount Kinabalu, Sabah, Malaysia (Cribb, 1998). Being rare, the species is classified as endangered, the Convention on International Trade in Endangered Species (CITES) is discussed in this study. As with other Paphiopedilum, the species has a very slow growth rate, consequently propagating the orchid through the natural ways takes several years. Hence, multiplying the orchid through tissue culture techniques is seen as the best alternative. Micropropagation has been applied to propagate many orchid species in large scales, the techinuqe produced high mass of plants in a considerably short time and with a lower cost. Micropropagation through callus culture is now widely used to mass propagate many orchids, these inculde *Cymbidium ensifolium* var. misericors (Chang and Chang, 1998); Phaelonopsis (Ishii *et al*., 1998; Chen *et al*., 2000; Tokuhara and Mii, 2001); *Pleione formosana* Hayata (Lu, 2004); *Dendrobium fimbriatum* (Roy and Banerjee, 2003). Only a few research on callus culture of slipper orchids had been reported; Lin *et al*. (2000) and Hong *et al*. (2008) successfully induced callus of Paphiopedilum hybrids using seed-derived protocorm and seed respectively as explants while Lee and Lee (2003) established callus culture of *Cypripedium formosanum* using seed-derived protocorm. To date, there is no reports on callus culture for *P. rothschildianum*. Hence, the objective of this research was to evaluate the potential of different explants to produce callus.

MATERIALS AND METHODS

Plant material: *Paphiopedilum rothschildianum* capsules were collected from Poring and Kinabalu National Park Orchid Nursery. The capsules were surface sterilised by immersing in 10% (v/v) chlorox followed by rinsing three time with sterile distilled water. Sterilesed capsules were then disected logitudinally and some of the seeds were used as explants. To obtain protocorms, a portion of the seeds were germinated on half strength by

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Murashige and Skooge, medium (MS) and the resulting protocorms were used as explants designated as Seed-Derived Protocorms (SDP). Subsequently, some of the protocorms formed from these seeds were cultured on a multiplication medium (modified RE medium) and new protocorms or 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 1×1 cm and used as explants designated as Leaf Segments (LS). For SDP explants, 1, 3 and 6 months old explants were also tested on MS Medium Supplemented with 1 mg/L TDZ and 4 or 5 mg/L 2, 4-D.

Callus induction: To induce callus, medium based on Hong *et al.* (2008) was employed. The medium was comprised of half strength MS basal medium supplemented with full strength MS Vitamin, 2 g/L peptone, 170 mg/L NaH_2PO_4, 20 g/L sucrose, 5 mg/L 2, 4-D with 1 mg/L TDZ and 2.2 g/L gelrite. The study on the effect of age of SDP explants were conducted using two different combinations of PGRs; 1 mg/L TDZ with 4 and 1 mg/L TDZ with 5 mg/L 2, 4-D. The pH of medium was adjusted to 5.2 and autoclaved at 121°C and 15 psi for 20 min. The medium was dispersed in 9 cm diameter petri dishes and four explants of seed, SDP, SP and LS were cultured on each petri dish. Each treatment was replicated 5 times. All cultures were maintained at 25± 2°C in darkness. Observations were made every 10 days for 150 days and explants were subcultured onto fresh medium at 4 weeks intervals.

Data collection and statistical analysis: All data were analyzed using SPSS (Statistical Package for Social Science) Version 22.0 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

Combination of 2, 4-D (5 mg/L) and 1 mg/L TDZ did successfully induce and stimulate the growth of callus on seeds, SP, SDP explants. Callus became visible after 30 days of culture and slowly increases in mass through the 150 days period (Plate 1 A-H). All explants produced friable callus with creamy colour. Similar results were reported by Lin *et al.* (2000), Lee and Lee (2003) and Hong *et al.* (2008) with 5, 2, 4-D and 1 mg/L TDZ gave the highest percentanges of explants forming callus. Unfortunately, LS failed to produce callus and died after 40 days of culture (Plate 1I-J). Stems, root tips and leaves

Table 1: Formation of callus by different types of *Paphiopedilum rothschildianum* explants on ½ (MS) Medium Supplemented with 1 mg/L TDZ+5 mg/L 2, 4-D in darkness, 25±2°C

	Percentage of explant forming callus at different days (mean%±SD)		
Explants	30	90	150
Seed	7.5±4.3[b]	NA	73.5±14.3[a]
SDP	56.7±24.5[a]	85.4±22.5[a]	87.5±16.9[a]
SP	41.7±21.2[a]	50.0±35.4[a]	65.0±22.4[a]
Leaf	0	0	0

[a, b]Data were taken from 5 replicates with the same latters are not significantly different at $p<0.05$ using Duncan's multiple range test, SD = Standard Deviation

Table 2: The sizes of callus induced on *Paphiopedilum rothschildianum* explants on ½ MS Medium Supplemented with 1 mg/L TDZ+5 mg/L 2, 4-D in darkness, 25±2°C based on the cultured days

	Callus size at different days		
Explants	30	60	90
Seed	+	++	++
SDP	+	++	++
SP	++	+++	+++

Callus mass were cataloged as large (+++); moderate (++); small (+)

of Paphiopedilum hybrids failed to produce callus when cultured on media containing combination of TDZ and either 2, 4-D and PBOA as well as medium containing 2, 4-D in combination of TDZ, BA or 2ip as reported by Lin *et al.* (2000) (Fig. 1).

The highest percentages of explants forming callus after 150 days of culture was SDP (87.5%±16.9) followed by seeds (73.5%±14.3) and SP (65.0%±22.4) (Table 1). These results are in contrast with the report made by Lin *et al.* (2000) which showed seed explants formed better callus than SDP. Although, SDP gave the highest percentage of explant forming callus, it was SP explants that showed higher mass of callus formed on explants (Table 2). Better formation of callus by SP explants might due to the carry over effect as SP explants originated from multiplication medium which contained PGRs.

Makara *et al.* (2010) conclude that PGR like TDZ has a carry-over effect from his study on banana. They found that the proliferation rates of shoots originating from basal cycle medium with various TDZ concentrations were significantly higher than those from 5 mg/L BAP, thus, suggested that TDZ had a high carry over effect, enabling the shoots to continue proliferating on the hormone free medium (Makara *et al.*, 2010).

Further study on the age of SDP explants showed that the percentage of callus formation on 3 months old SDP was significantly higher (70.0%±19.7, 65.0%±22.4) as compared to 1 month old SDP (43.8%±12.5, 40.0%±22.5) for every treatments with 4 mg/L and 5 mgL 1 2, 4-D, respectively, after 30 days of culture (Table 3 and 4; Fig. 2). After 90 days of culture, the percentage of explants forming callus on 3 months old SDP increased to

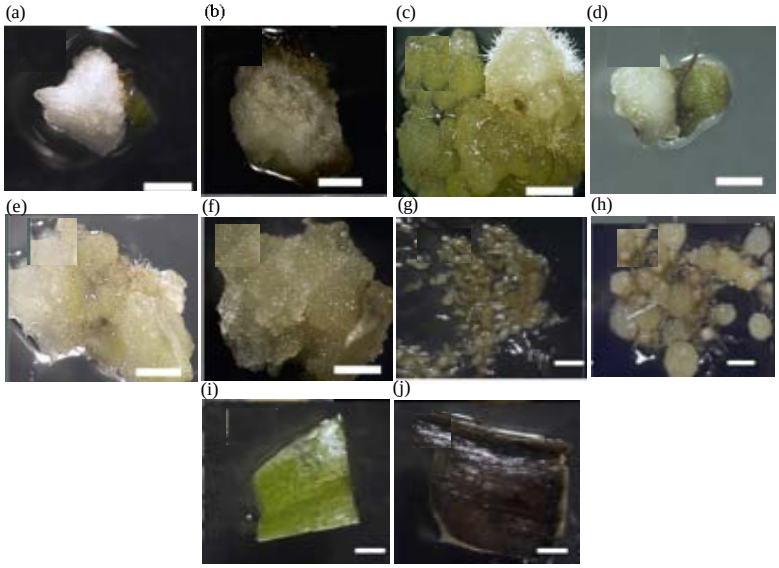

Fig. 1: Callus induction of *Paphiopedilum rothschildianum* on ½ MS medium with 1 mg/L TDZ and 5 mg/L 2, 4-D in darkness, 25±2°C; a) SDP after 30 days of culture (bar = 0.18 cm); b) SDP after 60 days of culture (bar = 0.19 cm); c) SDP after 90 days of culture (bar = 0.19 cm); d) SP after 30 days of culture (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) and j) LS after 40 days of culture (bar = 0.21 cm)

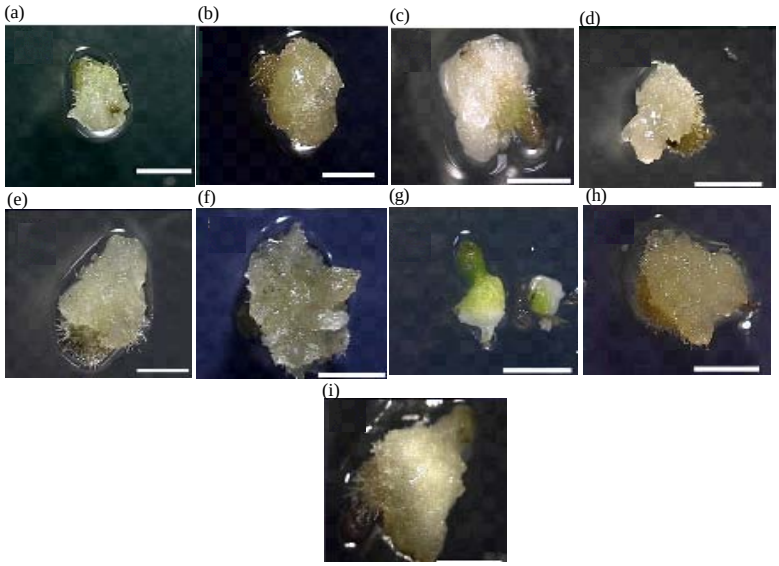

Fig. 2: Callus induction of *Paphiopedilum rothschildianum* on ½ MS medium with 1 mg/L TDZ+4 mg/L 2, 4-D in darkness, 25±2°C (bar = 2.5 mm) Callus formed on 1-month-old SDP explants; a) After 30 days of culture, b) Slowly increase in size after 60 days of culture; c) Proliferate further after 90 days of culture; callus formation was faster on 3 months old SDP; d) After 30 days of culture, e) Proliferated well after 60 days of culture; f) The mass of callus further increase after 90 days of culture. Lower mass of callus was observed on 6 months old SDP; g) After 30 days of culture; h) The mass of callus slowly increase after 60 days of culture and i) and the size of callus further increase after 90 days of culture

Table 3: The effect of age of Seed-Derived Protocorm (SDP) on callus induction of *Paphiopedilum rothschildianum* on ½ (MS) Medium Supplemented with 1 mg/L TDZ and different concentration of 2, 4-D in darkness, 25±2°C

Age of explants (months)	2, 4-D (mg/L)	Percentage of explant forming callus at different days (mean%±SD)		
		30	60	90
1	4	43.8±12.5^{ab}	68.8±12.5^{ab}	81.3±12.5^{a}
	5	40.0±22.5^{b}	65.0±22.4^{ab}	70.0±32.6^{ab}
3	4	70.0±19.7^{a}	82.5±16.9^{a}	82.5±16.9^{a}
	5	65.0±22.4^{ab}	80.0±20.9^{a}	80.0±20.9^{a}
6	4	16.0±8.9^{c}	68.0±11.0^{ab}	72.0±11.0^{ab}
	5	16.0±16.7^{c}	48.0±11.0^{c}	48.0±11.0^{b}

$^{a, b}$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

Table 4: The sizes of callus induced on *Paphiopedilum rothschildianum* 1 and 3 months old explants on ½ (MS) Medium Supplemented with 1 mg/L TDZ and different concentration of 2, 4-D in darkness, 25±2°C

Age of explants (months)	Concentration of 2, 4-D (mg/L)	Sizes of callus at different days		
		30	60	90
1	4	+	++	+++
	5	+	++	+++
3	4	++	++	+++
	5	++	++	+++
6	4	+	++	+++
	5	+	++	+++

Callus mass were cataloged as large (+++); moderate (++); small (+)

82.5%±16.9 for medium 4 mg/L 2, 4-D and 80.0±20.9 for medium with 5 mg/L 2, 4-D. In addition, after 30 days of culture, the size of callus formed on 3 months old SDP (Table 4, Fig.2d) was bigger as compared to 1 month old SDP (Fig. 2a) and 6 months old SDP (Fig. 2g). These results indicates that the formation of callus was faster on 3 months old SDP. This is supported by Chen *et al.* (2000) in their study that shows 2 months old protocorm explants of Phalaenopsis was better for callus induction than 1 month old protocorm. The maturity of explants known to have cirtical impact on their gowth and development in in vitro culture. The results on Table 3, also shows that 6 months old SDP give the lowest percentage of callus formation on both treatment of 4 mg/L 2, 4-D (72.0±11.0%) and 5 mg/L 2, 4-D (48.0±11.0%). This shows that callus induction also affected by the age of SDP explants. Younger explants consists if more responsive and actively dividing cells. Feng and Chen (2014) suggest that 2 months old seedlings of *Phalaenopsis aphrodite* were more suitable for inducing embryogenesis than 4 months old seedlings. The present study shows that, although, younger explants give better response to callus formation on SDP explants, the maturity of explants also plays an important role in their growth and development. Explant aged 1 months old was young but

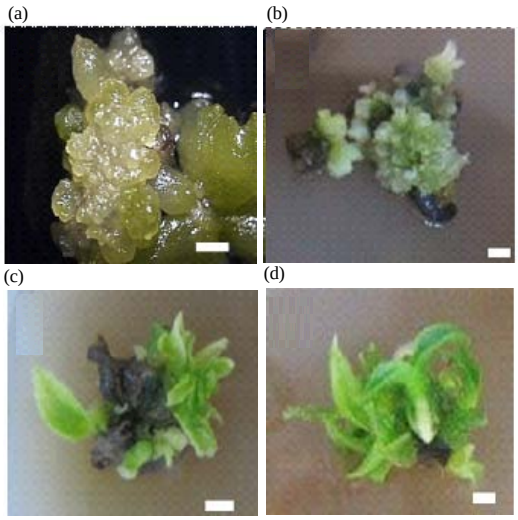

Fig. 3: Growth and development of Protocorm Like Bodies (PLBs) regenerated from callus induced on 3 months old SDP (Bar = 1 mm); a) Callus induced was transferred to regeneration medium and slowly regenerated into PLBs; b) PLBs then multiplied and transferred to development medium where it undergoes growth and development after 14 days of culture; c) The formation of shoots and leaflets was observable after 50 days of culture; d) The shoots and leaflets slowly developed into plantlets after 120 days of culture

not matured while 6 months old SDP was too matured. Therefore, the study conclude that 3 months old SDP was the best explant for callus induction because of its young and matured cells.

The results also indicate that lower concentration of 2, 4-D was better for callus induction on all SDP explants with different age (Table 3). The highest percentage of explant forming callus (82.5%±16.9) was on the medium with 1 mg/L TDZ+4 mg/L 2, 4-D. This indicates that SDP explants contain sufficient endogenous auxin for callus induction. The addition of 2, 4-D may limit the capacity of the explant to produce callus. The effect of endogenous auxin in combination with cytokinin were demonstrated by Khosravi *et al.* (2008) on Dendrobium. Their study showed that the combination of exogenous cytokinin and endogenous auxin significantly increased the regeneration response of *Dendobrium calli*. However, increasing the concentration of auxin may limit the explant response. This was shown in the study by Chen and Chang (2001) in which the presence of endogenous auxin reduced the rate of direct embryogenesis from leaf explants of Oncidium "Grower Ramsey".

Calli originated from 3 months old SDP was transferred to regeneration medium and slowly regenerates into Protocorm Like Bodies (PLBs) formation (Fig. 3a). The PLBs then transferred

to development medium and slowly develop shoots and leaflets that further grow into plantlets after 120 days of culture (Fig. 3b-d).

CONCLUSION

This research proved that callus can be induced on *P. rothschildianum* explants within 90 days and although, SDP explants formed the highest percentage of callus it was SP explant that produced the largest biomass.

RECOMMENDATIONS

Further study on SDP explants showed that 3 months old SDP was the best explant source for callus induction. This is mainly due to the level of maturity of the explant. The calli obtained from the present work was able to regenerate into plantlets after 120 days of culture on the development medium.

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