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Original Article

In vitro propagation through transverse thin cell layer (tTCL) culture system of lady's slipper orchid: *Paphiopedilum callosum* var. *sublaeve*

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Abstract

Paphiopedilum callosum var. *sublaeve*, a lady's slipper orchid native to southern Thailand, is protected under CITES (appendix I) due to habitat destruction and over-collection. The novel micropropagation technique for this endemic species was investigated. A 2-week preculture of seeds in distilled water before transfer to 1/2MS solid medium was the optimal conditions to promote seed germination in vitro. Shoot tip-derived transverse thin cell layer (tTCL) explants cultured on MVW solid medium containing 1.0 mg/L TDZ for 8 weeks provided the highest percentage of regenerated protocorm-like bodies (46.67±6.67), shoot formation (40.00±5.16), root formation (30.00±12.38) and survival rate (70.00±4.47). The acclimatized plantlets potted in sphagnum moss in the greenhouse grew well at 80% survival rate. No genetic variation was detected between the regenerated plantlets and their own mother plants based on RAPD marker.

Keywords: RAPD**,** somaclonal, transvers thin cell layer, *Paphiopedilum*

1. Introduction

Paphiopedilum, known as lady's slipper orchid, is a genus in the subfamily Cypripedioideae (Orchidaceae) (Cribb, 1998). Members of *Paphiopedilum*, listed in appendix I of CITES, are under extinction menace caused by over-collection and habitat destruction (Zeng, 2012). *Paphiopedilum callosum* var. *sublaeve*, a native species of southern Thailand, exhibits marvelous beautiful flowers, marbled and evergreen foliage (Figure 1A) and it is important to prevent the extinction. However, the conventional propagation including axillary bud division requires a period of time to produce a new shoot.

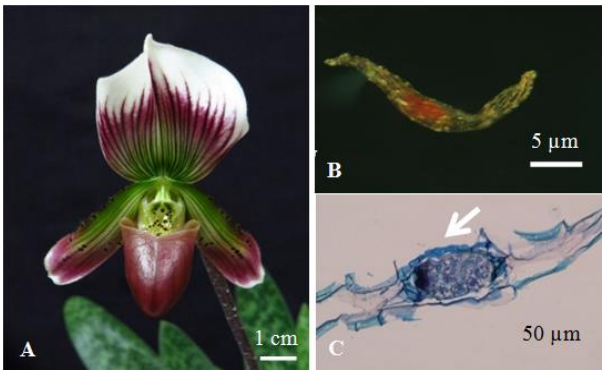

Figure 1. *Paphiopedilum callosum* var. *sublaeve*
A) Flower.
B) Viable seed with dark red-stained embryo (TTC test).
C) Seed coat with greenish-blue (arrow, TBO staining).

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Thin cell layer (TCL) technique, based on the use of very small explants derived from a limited cell number of uniform tissue, is useful for reducing the time period, producing a high frequency of shoot regeneration, and more competence than primary *in vitro* culture techniques (Zhao *et al.,* 2007). This TCL culture system could be used for the large scale production required for plant conservation. For instance, protocorm-like bodies (PLBs) of *Dendrobium malones* 'Victory' (Anjum *et al.,* 2006) and *Xenikophyton smeeanum* (Reichb.f.) (Mulgund *et al.,* 2011) were successfully induced from thin sections of leaf and shoot tips, respectively, in a short period of time. Vyas *et al*. (2010) also revealed that the secondary PLBs were induced from tTCL of primary PLBs of *Cymbidium* Sleeping Nymph. However, somaclonal variation caused by growth regulators during seed germination and development *in vitro* can arise and occur. Therefore, the effective molecular marker, such as RAPD marker has been introduced to investigate the genetic stability. Consequently, this study was conducted to investigate *in vitro* PLB production of *P. callosum* var. *sublaeve* via the tTCL technique and to determine genetic stability using RAPD marker.

2. Materials and Methods

2.1 Plant material

The capsules at 180 days after pollination (DAP) were collected from hand self-pollinated flowers of *P. callosum* var. *sublaeve* maintained in the greenhouse of Department of Biology, Faculty of Science, Prince of Songkla University, Thailand. Seed viability was examined using 1% 2, 3, 5-Triphenyltetrazolium chloride (TTC) assay (Vujanovic *et al.*, 2000).

2.2 Effects of basal media and pretreatment duration on seed germination

The capsule was surface-sterilized with 1.2% sodium hypochlorite (NaOCl) for 20 min, rinsed with sterile distilled water (DW) 2-3 times and cut longitudinally. Seeds were scooped out and placed into sterile DW. They were suspended in approximately 125 seeds/mL in a 125-mL culture flask containing 20 mL of DW for various preculture periods (0, 1, 2 or 3 weeks) The cultures were maintained on a shaker (50 rpm) in the culture room (25±2°C) under dark conditions before transfer to 1/2 Murashige and Skoog (MS) basal media (Murashige & Skoog, 1962) or modified Vacin and Went (MVW) media (Vacin & Went, 1949). All media were supplemented with 10 g/L sucrose, 1 mg/L chitosan, 1.0 g/L activated charcoal (AC) and solidified with 6.8 g/L agar in which all concentrations were optimized in initial trials. The media were adjusted to pH 5.2 and all cultures were incubated under a 16-h photoperiod at irradiance of 23 µmol/m^2/s provided by cool white fluorescent tubes (Philips). Only the appearance of a swollen embryo with the ruptured testa would be considered to have successfully germinated. The experiment was performed with 3 replicates and repeated twice. Data were subjected to a two-way analysis of variance (ANOVA) followed by separating of mean using the Duncan's multiple range tests (DMRT) at P<0.05. The percentage of germinated seed was determined after culture for 30 days.

2.3 Effects of BAP and TDZ on *in vitro* morphogenesis

Aseptic shoot (1-1.5 cm height) of 4-month-old seedling (Figure 2A) was cut transversely into 2 tTCL explants each with a thickness of 0.5-0.6 mm. These tTCL explants (2 slices/bottle) were inoculated on 10 mL of modified Vacin and Went (MVW) medium supplemented with various concentrations of 6-benzylaminopurine (BAP) (0, 1.0, 5.0, and 10.0 mg/L) and thidiazuron (TDZ) (0, 0.1, 0.5, and 1.0 mg/L) alone or in combination (Table 1) with 15 replications (bottle). All media containing 0.2% AC were solidified with a combination of 5.5 g/L agar and 1 g/L Phytagel. After culture for 2 weeks, all explants were transferred to AC-free MVW medium to promote morphogenesis of several organs. The percentage of browning explant, shoot and root formation and PLB formation were recorded after culture for 8 weeks.

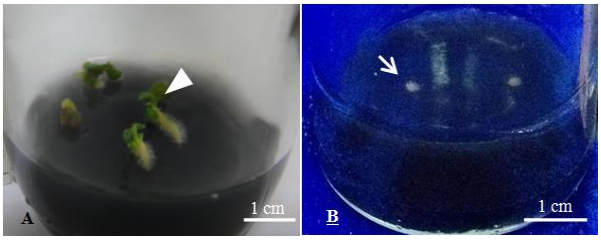

Figure 2. Explant sources for the tTCL culture system. A) Four-month-old seedling on MVW showing shoot (arrow head) explant of 0.5-1.0.cm tall which was then cut (3.0 mm below the apex) transversely into 2 pieces. B) The tTCL slice of 0.5 mm thickness (arrow) cultured on MVW medium added with 1.0 mg/L TDZ.

Table 1. Effects of pretreatment duration and basal medium type on seed germination of *P. callosum* var. *sublaeve*

Basal medium type	Duration time of pretreatment (week)	Percentage of seed germination (mean±SE)	Visual observation	
			Germination	Seedling*
1/2MS	0	16.86 ± 2.40^b	slow	pale green
	1	17.33 ± 0.86^b	slow	green and healthy
	2	37.47 ± 2.96^a	normal	green and vigorous
	3	19.67 ± 7.34^b	normal	green and vigorous
MVW	0	16.39 ± 1.68^b	slow	pale green
	1	17.33 ± 1.13^b	slow	green and healthy
	2	17.80 ± 1.72^b	normal	green and healthy
	3	22.48 ± 8.88^b	normal	green and healthy

Data were taken after a culture for 60 days and 120 days (*). The same letters in column are not significantly different at P≤0.05 as determined by DMRT.

2.4 Plantlet acclimatization

Regenerated shoots were transferred to modified Murashige and Skoog (MMS) medium for plantlet growth (Kaewubon *et al.,* 2010). The plantlets with 3-4 roots were then transplanted into a 3-inch pot containing sphagnum moss in a greenhouse and the survival rate was recorded at one month after transplanting.

2.5 Histological analysis

The seeds and *in vitro* morphogenesis responses were histologically confirmed. After culture for 8 weeks, the PLBs, shoots, and roots derived from thin sections were collected and fixed in FAAII (formaldehyde: glacial acetic acid: 70% ethyl alcohol; 1:1:18 v/v/v) for 48 h. Fixed tissues were dehydrated in a tertiary-butyl-alcohol series, embedded in Histoplast PE and thin sectioned (6 µm thick) by a rotary microtome. Sections were stained with Delafield's hematoxylin and Safranin to examine the general structure (Ruzin, 1999). Seed samples were stained with toluidine blue O (TBO) to detect lignin and some phenols (Feder & O'Brian, 1968).

2.6 Analysis of genetic variation

2.6.1 Plant materials and DNA isolation

The mother (M) seedlings and their regenerated (R) plants were randomly collected to determine the genetic stability. Four-month-old seedlings which were cultured on 1/2MS medium containing 1 mg/L chitosan were used as mother plants. The R plants were induced from tTCL explants which were cultured on MVW medium containing TDZ (0, 0.1, 0.5, and 1.0 mg/L) in combination with BAP (0, 1.0, 5.0, and 10.0 mg/L) for 12 weeks (Table 2). The total genomic DNA was extracted from the young leaf samples (100 mg/sample) of M and R plants following the protocol described by Chung *et al.* (2006) with some adaptation. DNA concentration and purity were determined by spectrophotometer and the samples were diluted to a concentration of 20 ng/l.

2.6.2 PCR-based RAPD

The amplification reaction was done in 24.5 µL which consisted of 2.5 µL 10x buffer (100 mM Tris-HCl, 500 mM KCl, 0.01% gelatin), 1 µL of template DNA, 2 µL dNTP mix (100 mM), 0.25 µL of 5 u/µL *Taq* DNA polymerase, 1.5 µL of 10 µM primer (Table 3), and 17.25 µL deionized water. DNA amplification was carried out in a thermal cycler for an initial denaturation step of 3 min at 94ºC before beginning the cycling protocol. An amplification cycle consisted of 40 sec at 94ºC, 1 min at 37ºC and 1 min at 72ºC. A total of 40 cycles were performed. The cycling was terminated with a final extension at 72ºC for 10 min. After amplification, DNA fragments were separated by 1.5% agarose (molecular biology grade) gel electrophoresis at 100V in 0.5x Tris-acetate-EDTA (TAE) buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA; pH8.0), stained with ethidium bromide (0.1µL/mL). The DNA bands were then photographed under ultraviolet light using a photo documentation system. A 100-base pair ladder was used to estimate the size of RAPD bands to nearest 50 base pairs. In this study, 90 primers were taken for initial screening, as only 11 primers producing repeatable bands were analyzed.

Amplification product profiles were scored for the presence (1) or absence (0) of bands. Genetic similarity between pairs was calculated according to Jaccard's similarity coefficient followed by cluster analysis by the SIMQUAL module (Anderson *et al.,* 1993).

Table 2. Effects of BAP and TDZ on morphogenic responses of tTCL explants of *P. callosum* var. *sublaeve* cultured on MVW medium for 8 weeks.

Cytokinins (mg/L)		Percentage PLB induction (mean±SE)	Number of PLBs/ explant (mean±SE)	Percentage root formation (mean±SE)	Number of roots/ explant (mean±SE)	Percentage shoot formation (mean±SE)	Number of shoots/ explant (mean±SE)	Browning percentage (mean±SE)	Survival Percentage (mean±SE)
TDZ	BAP								
0	0	3.33 ± 3.33^{b}	1.00 ± 0.57^{b}	3.33 ± 3.33^{b}	2.00 ± 0.58^{a}	13.33 ± 4.21^{b}	1.67 ± 0.33^{ab}	100.00 ± 0.00^{a}	20.00 ± 7.30^{b}
0	1.0	23.33 ±12.02^{b}	1.33 ± 0.33^{b}	6.67 ± 4.21^{b}	1.00 ± 0.58^{ab}	6.67 ± 6.67^{b}	2.00 ± 0.58^{ab}	96.67 ± 3.33^{a}	33.33 ±12.29^{b}
0	5.0	23.33 ±10.85^{b}	1.00 ± 0.57^{b}	13.33 ± 6.67^{ab}	0.67 ± 0.33^{ab}	13.33 ±13.33^{b}	1.00 ± 0.00^{b}	100.00 ± 0.00^{a}	40.00 ±13.66^{b}
0	10.0	6.67 ± 4.21^{b}	1.00 ± 0.57^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	3.33 ± 3.33^{b}	2.00 ± 0.58^{ab}	100.00 ± 0.00^{a}	10.00 ± 6.83^{b}
0.1	0	16.67 ± 9.54^{b}	1.00 ± 0.57^{b}	3.33 ± 3.33^{b}	1.00 ± 0.58^{ab}	3.33 ± 3.33^{b}	1.00 ± 0.58^{b}	100.00 ± 0.00^{a}	23.33 ± 9.54^{b}
0.5	0	16.67 ± 6.14^{b}	1.00 ± 0.58^{b}	13.33 ± 4.21^{ab}	0.67 ± 0.67^{ab}	16.67 ± 8.03^{b}	3.00 ± 1.00^{ab}	93.33 ± 4.21^{a}	40.00 ±10.33^{b}
1.0	0	46.67 ± 6.67^{a}	3.33 ± 0.33^{a}	30.00 ±12.38^{a}	1.00 ± 0.58^{ab}	40.00 ± 5.16^{a}	1.33 ± 0.33^{b}	76.67 ±12.02^{b}	70.00 ± 4.47^{a}
0.1	1.0	20.00 ± 7.30^{b}	0.33 ± 0.33^{b}	13.33 ± 6.67^{ab}	1.00 ± 0.58^{ab}	6.67 ± 6.67^{b}	1.00 ± 0.00^{b}	100.00 ± 0.00^{a}	30.00 ±11.25^{b}
0.1	5.0	6.67 ± 4.21^{b}	1.67 ± 0.88^{b}	10.00 ± 4.47^{ab}	1.33 ± 0.33^{ab}	13.33 ± 6.67^{b}	3.67 ± 1.45^{a}	100.00 ± 0.00^{a}	23.33 ± 9.54^{b}
0.1	10.0	20.00 ± 8.94^{b}	0.33 ± 0.33^{b}	10.00 ± 6.83^{ab}	1.00 ± 0.00^{ab}	3.33 ± 3.33^{b}	1.00 ± 0.58^{b}	93.33 ± 4.21^{a}	26.67 ± 8.43^{b}
0.5	1.0	10.00 ± 6.83^{b}	1.33 ± 0.67^{b}	10.00 ± 6.83^{ab}	0.67 ± 0.33^{ab}	16.67 ± 9.54^{b}	1.00 ± 0.58^{b}	96.67 ± 3.33^{a}	33.33 ± 9.89^{b}
0.5	5.0	6.67 ± 4.21^{b}	1.00 ± 0.58^{b}	10.00 ±10.00^{ab}	1.00 ± 0.58^{ab}	13.33 ± 9.89^{b}	1.00 ± 0.58^{b}	96.67 ± 3.33^{a}	26.67 ±11.15^{b}
0.5	10.0	16.67 ± 6.14^{b}	1.00 ± 0.58^{b}	10.00 ± 6.83^{ab}	0.33 ± 0.33^{b}	6.67 ± 4.21^{b}	1.00 ± 0.58^{b}	93.33 ± 4.21^{a}	30.00 ± 6.83^{b}
1.0	1.0	16.67 ± 6.14^{b}	1.00 ± 0.58^{b}	10.00 ± 4.47^{ab}	1.00 ± 0.58^{ab}	6.67 ± 6.67^{b}	1.00 ± 0.58^{b}	100.00 ± 0.00^{a}	23.33 ± 9.54^{b}
1.0	5.0	10.00 ± 6.83^{b}	0.33 ± 0.33^{b}	16.67 ± 6.14^{ab}	1.00 ± 0.58^{ab}	16.67 ± 9.54^{b}	1.00 ± 0.58^{b}	100.00 ± 0.00^{a}	36.67 ± 8.03^{b}
1.0	10.0	16.67 ± 6.14^{b}	1.33 ± 0.88^{b}	10.00 ± 4.47^{ab}	0.67 ± 0.33^{ab}	16.67 ± 8.03^{b}	1.33 ± 0.88^{b}	100.00 ± 0.00^{a}	36.67 ±14.06^{b}

Mean in each column followed by the same letters are not significantly different at P≤0.05 as determined by DMRT.

Table 3. Number and size ranges of the amplified RAPD bands of *P. callosum* var. *sublaeve* which were cultured on MVW containing different concentrations of TDZ and BAP for 8 weeks.

Primer	Sequence (5'-3')	Number of amplified band	Size ranges (bp)	GC %
OPA-04	AATCGGGCTG	17	180-2000	60
OPA-09	GGGTAACGCC	13	210-1500	70
OPU-12	TCACCAGCCA	18	100-1500	60
OPU-13	GGCTGGTTCC	11	210-1200	70
OPZ-3	CAGCACCGCA	15	200-1400	70
OPZ-11	CTCAGTCGCA	12	320-1800	60
OPA-A-11	ACCCGACCTG	14	200-1150	70
OPA-B-4	GGCACGCGTT	17	200-1800	70
OPA-B-8	GTTACGGACC	12	190-1100	60
OPA-D-15	TTTGCCCCGT	14	180-1300	60
UBC-719	GGTGGTTGGG	16	380-1600	70
Total		159		-
$\bar{x}$		14.45		-

3. Results and Discussion

3.1 Effects of pretreatment duration and basal media on seed germination

The dark red-stained viable seeds (Figure 1B) were 28.0%. The exhibited seeds on the 1/2MS medium gave a higher percentage of seed germination than on the VW medium. This was in contrast to the Long *et al.* (2010) study that reported the VW medium provided a higher percentage of seed germination in four *Paphiopedilum* species than the 1/2MS. Thus, the composition of macro- and micronutrients was one of the factors required for growth and development. However, no significant difference were observed between the 1/2MS and VW media in seed germination of *Coelogyne nervosa* A. Rich (Abraham *et al.,* 2012) and *P. wardii* Sumerh (Zeng *et al.,* 2012).

There were interaction effects between medium type and water-pretreatment period on seed germination (Table 4). This study was similar to Godo *et al.* (2010) who reported that the water presoaking could enhance seed germination of *Calanthe tricarinata* Lindl. Waes and Debergh (1986) also reported that the pre-soaking of *Dactylorhiza maculata* seeds for 15 min in 5% $Ca(OCl)_2$ and 1% Tween-80 followed by 24 h in sterile DW was the optimal condition for its germination and the pretreatment period was species-dependent. Many plant species needed the pretreatment step because a water soluble inhibitor could be leached and the seed coat became permeable to water and other nutrients. In this species, the seed coats were stained greenish blue with TBO (Figure 1C) indicating the presence of lignin and polyphenol in the cell wall. The hydrophobic characteristic of these substances was reported to be a crucial barrier to the uptake of water and nutrients (Lee *et al.,* 2006). Consequently, the demolition of the barrier substances allowed more water and oxygen absorption to embryo. Therefore, changing the physical characteristics of the testa by shaking (Kuath *et al.,* 2008) or soaking seeds in water (Linden, 1992) might improve seed germination due to making the testa tissue more permeable.

Table 4. Assessment of pretreatment duration (P), basal medium type (B) and their interactions on seed germination of *P. callosum* var. *sublaeve*

Main factors	Percentage of seed germination			
	df	Mean square	F	*p*
Pretreatment duration (P)	3	304.546	4.834	*
Basal medium type (B)	1	200.335	3.180	ns
Interaction PxB	3	301.604	4.788	*
Error	36			
Total	44			

* = significant level at $\alpha = 0.05$, ns = not significant

Soaking the seeds in water for 2 weeks followed by a culture on 1/2MS gave vigorous, green seedlings with normal germination and the highest germination percentage (37.47±2.96%) which was significantly different from that on MVW (17.80±1.72%) (Table 1). In contrast, water-pretreated seeds for 0 and 1 week followed by culture on both 1/2MS and MVW exhibited slow germination. The pale green seedlings were obtained from no water-pretreatment. Therefore, seed pretreatment with water for 2 weeks could enhance seed germination of this orchid.

3.2 Effects of BAP and TDZ on morphogenesis of tTCL explants

3.2.1 PLB response

The highest percentage of PLB induction (46.67 ±6.67) and number of PLBs per explant (3.33±0.33) were obtained on MVW medium supplemented with 1.0 mg/L TDZ alone (Table 2). This result conformed to Niknejad *et al.* (2011) who reported that the highest percentage of PLB induction and number of PLB per explant of *Phalaenopsis gigantean* were gained from using 1.0 mg/L TDZ alone. In addition, basal segment explants of *Dendrobium* Sonia 'Earsakul' cultured on 1/2MS medium supplemented with 1.0 mg/L TDZ alone could be induced to form somatic embryos at 10.71% and at 0.5 PLB/explant (Juntada *et al.,* 2015). The application of TDZ alone at higher concentration (3 mg/L) gave the highest number of PLBs per leaf explant in *Phaleanopsis bellina* (Rchb.f.) Christenson (Khoddamzadeh *et al.,* 2011) and *Phaleanopsis* cv. 'Surabaya' (Balilashaki *et al.,* 2015).

3.2.2 Shoot response

The highest percentage of shoot induction (40.00±5.16) and high number of shoots/explant (3.00±1.00) were observed in MVW medium containing 1.0 mg/L TDZ alone and 0.5 mg/L TDZ alone, respectively. Meanwhile, the same medium consisting of 5 mg/L BAP alone gave a lower percentage of shoot induction (13.33±13.33) than those of 0.5-1.0 mg/L TDZ alone (Table 2). Srivastava *et al.* (2015) reported that application of 9.1 µM TDZ alone to Knudson C (KC) medium gave the highest number of shoot buds/shoot segment of *Aerides ringens* (Lindl.) Fischer. Besides, 1/2MS medium containing 1.0 mg/L TDZ alone was also suggested

to provide a higher number of shoot/explant than the BA in *Dendrobium aqueum* Lindley (Parthibhan *et al.,* 2015). Therefore, TDZ had a stronger effect than BAP on shoot induction of *P. callosum* var. *sublaeve*. This is possibly due to the ability of TDZ to resist cytokinin oxidase (Huetteman & Preece, 1993) providing an internal suitable balance between cytokinin and auxin (Saxena *et al.,* 1992) and enhancing the synthesis of adenine type cytokinins (Baghel & Bansal, 2014). However, application of BAP alone was reported to provide the highest number of shoots/explant of *Paphiopedilum callosum* (Wattanawikkit *et al.,* 2011), *Aerides odorata* Lour (Devi *et al.,* 2013), and *Vanda coerulea* (Jitsopakul *et al.,* 2013).

The highest number of shoots/explant was obtained on MVW medium that contained 0.1 mg/L TDZ in combination with 5.0 mg/L BAP but it was not significantly different with 0.5 mg/L TDZ alone. Whereas, the application of TDZ combined with BAP was reported to give the maximum number of shoots/explant from *Rhynchostylis gigantean* (Le *et al.,* 1999) and *Dendrobium chrysanthum* Wall. ex Lindl (Hajong *et al.,* 2013). According to a model of cytokinin action in plant cell, the cytokinin-binding protein (CBP) has two different binding sites. One site binds adenine-type natural cytokinins and the other side binds phenylurea-type cytokinins (Guo *et al.,* 2011; Nielsen *et al.,* 1995). It was possible that shoot induction of *P. callosum* var. *sublaeve* was affected by both BA (binding to adenine-type CBC) and TDZ (binding to the phenylurea-type CBC). Moreover, TDZ (0.5 mg/L) combined with NAA (1.0 mg/L) could provide a high number of micro-shoots/seedling of hybrid orchid (*Aerides vandarum* Reichb.f x *Vanda stangeana* Reichb.f) (Kishor & Devi, 2009). Jitsopakul *et al.* (2013) also reported that MVW medium containing 2.0 mg/L TDZ in combination with 0.5 mg/L NAA provided a high mean number of shoots/explant of *Vanda coerulea*.

3.3.3 Root response

Root formation exhibited in almost all treatments, except the one containing 10.0 mg/L BAP alone could not induce root formation. This result was similar to Wattanawikkit *et al.* (2011) who revealed that 1/2MS medium supplemented with a high concentration of BAP at 100 µM could not induce root formation of *Paphiopedilum callosum*.

The highest percentage of root formation (30.00 ±12.38) and number of roots/explant (1.00±0.58) were obtained on MVW medium with 1.0 mg/L TDZ added (Table 2). Thus, TDZ might provide the mobility of endogenous auxins (such as indol-3-acetic acid, IAA) or auxin-like bioregulators. The synergistic effects of auxin and cytokinin combination may establish the inductive signal for root induction (Ahmadian *et al.,* 2013) Therefore, TDZ at 1.0 mg/L in this study was able to enhance the percentage of root formation.

However, PGR-free medium gave the highest number of roots/explant (2.00±0.58). Majumder *et al.* (2010) reported that PGR-free KC medium could enhance the rooting ability of *Dendrobium farmeri* Paxt. within 3 weeks. This was because of the balance between endogenous auxin and cytokinin and they are the key regulators of *in vitro* organogenesis (Nordstrom *et al.,* 2004). Therefore, a high endogenous auxin/cytokinin ratio in explants cultured on PGR-free MVW medium could induce root formation.

3.3.4 Browning response and survival rate

Most explants gradually became brown after a culture for 3 weeks. Explants on MVW medium containing high BAP (10 mg/L) exhibited high browning (100%), low survival rate (10.00±6.83%), low PLB induction (6.67± 4.21%), and low shoot formation (3.33±3.33%). This study conformed to Mondal *et al.* (2013) who revealed that BAP at a high concentration inhibited PLB induction of *Doritis pulcherrima*. However, Jitsopakul *et al.* (2013) reported that shoot tips of *Vanda coerulea* cultured on MVW medium supplemented with 5.0 mg/L BAP provided a high survival rate but gave rise to a low number of shoots and roots per explant. Further, the application of BAP (10.0 mg/L) in combination with TDZ (0.1- 1.0 mg/L) gave a higher survival rate than the 10 mg/L BAP alone.

Explants on MVW medium supplemented with TDZ at a high level (1.0 mg/L) exhibited green, the lowest browning (76.67±12.02%), and the highest survival (70.00± 4.47%). This result disagreed with Mulgund *et al.* (2011) who reported that high TDZ level led to browning, necrosis, and eventually death of the *Xenikophyton smeeanum* (Reichb.f). Therefore, the types with varying ratios of growth regulators may need adjustment depending on the plant species.

3.4 Histological assessment

The cytokinins affecting morphogenesis responses were histologically examined. The tTCL explants cultured on MVW medium containing 1 mg/L TDZ provided PLBs (arrow, Figure 3A), shoots (a dashed line with an arrow, Figure 3A) and root (arrow head, Figure 3A). Histological observation displayed the shoot with shoot apical meristem (SAM) and leaf primodium (LP) presenting cells with densely stained cytoplasm (Figure 3B). This zone had high meristematic activity involving formation of new meristematic cells. PLBs also exhibited the masses of small embryogenic cells containing dense cytoplasm and large nuclei (Figure 3C).

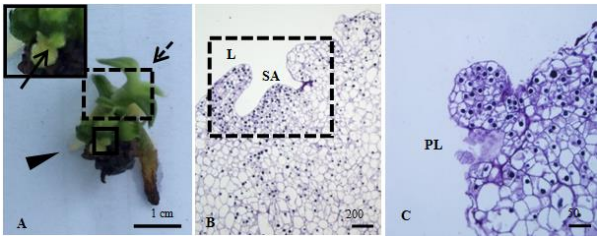

Figure 3. The transverse thin cell layer (tTCL) of shoot tips of *P. callosum* var. *sublaeve* cultured on MVW medium supplemented with 1.0 mg/L TDZ exhibiting A) shoot (a dashed line with an arrow), PLBs (arrow) and roots (arrow head). Longitudinal section of explant presenting B) shoot and C) PLB regenerated from tTCL. SAM, shoot apical meristem; LP, leaf primodium.

3.5 Plantlet acclimatization

Sixteen-week-old regenerated shoots on MVW medium supplemented with 1.0 mg/L TDZ (Figure 4A) were transferred to MMS medium. These plantlets displayed 3-4

Figure 4. Growth promotion of *P. callosum* var. *sublaeve*. A) Clump of developing new shoots (arrows) originated from tTCL shoot piece (arrow head) cultured on MVW medium supplemented with 1 mg/L TDZ for 16 weeks. B) Regenerated plantlets after transfer to growth-promoting medium for 10 weeks. C) Greenhouse-grown plants in pot containing sphagnum moss.

roots (Figure 4B) after culture for 10 weeks. They were then transplanted into sphagnum moss with 80% survival rate in a greenhouse (Figure 4C).

3.6 Analysis of genetic stability

Eight pairs of the regenerated plants and the mother plants were randomly chosen to examine the genetic stability. The eleven primers having 60-70% guanine-cytosine (GC) content and clear polymorphic DNA bands were successfully amplified (Table 3). These primers generated fragments that varied from 11 (OPU-13) to 18 (OPU-12) and the sizes ranged from 100 bp (OPU-12) to 2,000 bp (OPA-04). A total of 159 bands were obtained with an average of 14.45 bands per primer and 95% were polymorphic. Results indicated the percentage of GC affected the amplification which conformed to the report of Gnat *et al.* (2015) who revealed that using a primer having a high GC% in the RAPD technique supported the generation of an adequately high number of amplicons in amplification of *Astragalus glycyphyllos*. However, Padmalatha and Prasad (2008) claimed that percentage of GC did not affect the amplification of *Centella asiatica*. The RAPD profiles of 16 samples (8 pairs of M and their R plants) using OPU 12 are shown in Figure 5. Results revealed that the regenerated plants and the mother plants presenting identical RAPD profiles indicated the mother-regenerated plant pairs had genetic stability.

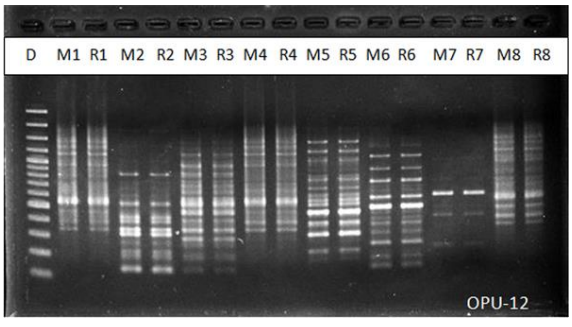

Figure 5. RAPD profiles of the mother seedlings (M) and their regenerated plants (R) generated by OPU-12. D: 100bp DNA ladder.

4. Conclusions

The recent protocol for *in vitro* propagation of *P. callosum* var. *sublaeve* was found to cause no genetic variation. Water-pretreated seeds in the dark for 2 weeks before transfer were required for seed germination. Morphogenesis responses that included PLBs, shoots, and roots were successfully induced from tTCL explants of shoot tips cultured on MVW containing 1.0 mg/L TDZ. No genetic variations could be detected among the mother plants and their regenerants as revealed by RAPD markers.

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