HortScience 54(5):920–925. 2019. https://doi.org/10.21273/HORTSCI13880-19

Strategies for the Regeneration of *Paphiopedilum callosum* through Internode Tissue Cultures Using Dark–light Cycles

Nguyen Phuc Huy, Vu Quoc Luan, Le Kim Cuong, Nguyen Ba Nam, Hoang Thanh Tung, and Vu Thi Hien
Tay Nguyen Institute for Scientific Research, Vietnam Academy of Science and Technology, 116 Xo Viet Nghe Tinh, Dalat, Lam Dong, Vietnam

Dung Tien Le
Agricultural Genetics Institute, Vietnam Academy of Agriculture Sciences, Ha Noi 100000, Vietnam

Kee Yoeup Paek
Horticultural Science, Chungbuk National University, Cheongju 28644, Republic of Korea

Duong Tan Nhut[1]
Tay Nguyen Institute for Scientific Research, Vietnam Academy of Science and Technology, 116 Xo Viet Nghe Tinh, Dalat, Lam Dong, Vietnam

Additional index words. dark–light cycles, *Paphiopedilum callosum*, micropropagation, internode tissue

***Abstract. Paphiopedilum* spp. is one of the most commercially popular orchids because of its variety of shapes, sizes, and colors. However, it is at risk for extinction because of its exploitation. Regeneration of orchid plants using internode segments is extremely difficult. In this study, young *P. callosum* plants (1.5 cm) were exposed to eight dark–light cycles (14 days of dark and 1 day of light) for stem elongation to increase the number of nodes to obtain internode tissues. After 75 days of culture, the highest callogenesis (31.25%) was achieved when internode tissue was cultured on liquid Schenk and Hildebrandt (SH) medium containing 30 g·L^{-1} sucrose, 1.0 mg·L^{-1} Thidiazuron (TDZ), 1.0 mg·L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D), and cotton wool as the support matrix. The optimal media for induction of protocorm-like bodies (PLBs) were the same compositions as previously mentioned and were supplemented with 9 g·L^{-1} Bacto agar as the gelling agent. PLB clumps (5–6 PLBs/clump) produced the best shoots on medium containing 0.5 mg·L^{-1} α-Naphthaleneacetic acid (NAA) and 0.3 mg·L^{-1} TDZ. Among the organic substances tested, 200 g·L^{-1} potato homogenate (PH) added to Hyponex N016 medium supplemented with 1.0 mg·L^{-1} NAA, 30 g·L^{-1} sucrose, 170 mg·L^{-1} NaH_2PO_4, 1.0 g·L^{-1} peptone, and 9 g·L^{-1} Bacto agar resulted in the best rooting. The rooted plantlets with four to five leaves were acclimatized and had a 100% survival rate. The method presented in this research provides a strategy for the development of highly effective propagation of *Paphiopedilum* species using ex vitro explants for both conservation and horticultural purposes.**

Paphiopedilum callosum is a highly demanding ornamental plant. However, in its native habitat, *P. callosum* is rare and its distribution is restricted. There remain only severely fragmented subpopulations in Vietnam (central and south), Thailand, southern Laos People's Democratic Republic, Malaysia, and Cambodia. Naturally, this species often occurs in small groups, with very few individuals (Averyanov and Averyanova, 2003). Unfortunately, the abundance of the species has been significantly reduced in recent decades. Furthermore, the species is at risk because of ecological disturbances and degradation of its habitats through logging of forests for wood, deforestation, random cutting, soil erosion, fires, trampling, exploitation for horticultural purposes, and ruthless collection for regional and international trade. More generally, however, *P. callosum* is threatened by climate change, drought, tourism and leisure activities, urbanization, infrastructure development, and recreation activities with direct effects (e.g., destruction of plants) and indirect effects (e.g., alteration of habitat). In addition, the intrinsic factors of the population, such as its limited distribution and small number of mature individuals, threaten the existence of the species (Averyanov et al., 2003; Braem, 1988; Braem and Chiron, 2003; Cribb, 1987; Koopowitz, 2008). *P. callosum* has been assessed as endangered (EN), and a number of actions have been recommended to protect this species, such as the use of only cultivated specimens instead of wild plants and *ex situ* conservation (artificial propagation, re-introduction, and seed collection) (Averyanov and Averyanova, 2003; Averyanov et al., 2003).

Various methods, including asymbiotic germination in vitro, have been tested to overcome difficulties propagating *Paphiopedilum* spp. (Chen et al., 2004b; Ding et al., 2011; Pierik et al., 1988; Zeng et al., 2012, 2016). Furthermore, seed-derived shoots have been identified as efficient material for shoot multiplication of *Paphiopedilum* hybrids (Huang et al., 2001). Nhut et al. (2007) studied the in vitro stem elongation of shoot-derived plantlets of *P. delenatii* to obtain stem nodes for effective shoot regeneration and multiplication. Recently, micropropagation of *Paphiopedilum* spp. through callogenesis from seed-derived protocorm-like bodies (PLBs) has been reported (Hong et al., 2008; Lee and Lee, 2003; Lin et al., 2000; Long et al., 2010; Ng and Saleh, 2011). Nevertheless, seed setting and germination rates of many *Paphiopedilum* species/cultivars are extremely low, and these low rates are often affected by several unknown factors (Arditti, 2008; Pierik et al., 1988; Zeng et al., 2016).

The success of *Paphiopedilum* micropropagation from ex vitro–derived explants has been relatively limited. Its difficulty has been caused by contamination of ex vitro–derived explants and the poor development of explants (Huang, 1988; Stewart and Button, 1975). There have been only four reports of *Paphiopedilum* micropropagation from ex vitro–derived explants (Huang, 1988; Liao et al., 2011; Luan et al., 2015; Stewart and Button, 1975). Stewart and Button (1975) conducted a series of investigations of young and mature flower stems, tips of leaves, roots, stamens, ovaries, and terminal buds of *P. villosum*, *P. fairrieanum*, and *P. insigne* that were used to regenerate plants via callus and PLB induction. Huang (1988) demonstrated that 2- to 3-mm shoot tip meristems of a *Paphiopedilum* hybrid (*P. philippinense* × *P.* Susan Booth) could be used as explants to effectively improve the success rate of disinfection, although the explants grew slowly and most of them necrotized. Liao et al. (2011) reported that scape transverse slices of *Paphiopedilum* hybrids of *P.* Deperle and *P.* Armeni White could induce adventitious buds and regenerate as whole plants, respectively.

Received for publication 11 Jan. 2019. Accepted for publication 20 Feb. 2019.
This work was financially supported by the National Foundation for Science and Technology Development (NAFOSTED), Vietnam (No. 106-NN.01-2015.02). We are grateful to the Japan Society for the Promotion of Science (RONPAKU Program, VAST-11424) for a scholarship to the researcher. We also thank Prof. Takejiro Takamura (Kagawa University, Japan) for critical reading of and suggestions for our manuscript.
[1]Corresponding author. E-mail: duongtannhut@gmail.com.

No study has reported in vitro propagation of *P. callosum* using stem-elongated ex vitro explants as the source under dark–light cycles for plant regeneration through internode tissue cultures. The results of this study provide a new approach to micropropagation of *P. callosum* for commercial propagation.

Materials and Methods

Plant material. One-month-old ex vitro–grown young plants of *Paphiopedilum callosum* cultured on fern fiber in a greenhouse (Tay Nguyen Institute for Scientific Research, Dalat, Vietnam) that were ≈1.5 cm in height were harvested from donor plants and used as the initial explant source (Fig. 1a). These shoots were subjected to a total of eight dark–light cycles (i.e., 14 d in the dark and 1 d under light conditions; the shelf cultures were covered with black nylon during dark cycles) (Fig. $1b_1$) to induce stem elongation (Fig. $1b_2$ and 2a). Under dark conditions, orchid plants tended to elongate. However, if subjected to dark conditions for a long time, the plant will lose all pigment due to the lack of photosynthesis. Therefore, in this study, we used intermittent lighting conditions. Plants cultivated for 14 d in the dark were subjected to 1 d of light so that they could perform normal photosynthesis and retain pigment. This cycle was repeated until the plant had approximately five stem nodes (4-month-old plants). After these shoots were subjected to a total of eight dark–light cycles, the stem nodes were elongated. The internode tissues were used as explants near the axillary buds and were rejuvenated. Then, elongated shoots were cut at the younger internode stem for callogenesis.

Callogenesis. After eight dark–light cycle treatments, elongated shoots were excised (Fig. $1c_1$) and sterilized with 0.1% $HgCl_2$ for 6 min and rinsed with sterilized distilled water five times. Then, the shoots were cut into five internode segments (Fig. $1c_2$) and cultured on Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt, 1972) containing 30 g·L^{-1} sucrose (medium A) supplemented with 2,4-D (0.3–1.0 mg·L^{-1}) with or without TDZ at different concentrations (0.5–1.0 mg·L^{-1}). Cotton wool plugs were used as the substrates after being cut into pieces ≈5 × 5 cm and placed in vessels using a pincette. The pH of the medium was adjusted to 5.8 before it was autoclaved at 121 °C for 30 min. Explants were cultured under lighting condition for 75 d to induce callus (Fig. $1c_3$).

Induction of PLBs. To obtain PLBs, calli were divided into 0.1-g clusters and subcultured on medium A supplemented with 1.0 mg·L^{-1} 2,4-D in combination with various concentrations of TDZ (0.3–1.0 mg·L^{-1}) and 9 g·L^{-1} Bacto agar under fluorescent lamps with a light intensity of 15–20 µmol·m^{-2}·s^{-1} at a temperature of 25 ± 2 °C with 50% to 55% relative humidity (Fig. 1d).

Formation of shoots from PLBs. PLB clumps (5–6 PLBs/clump) were transplanted to medium A with 0.5 mg·L^{-1} NAA and different concentrations of BA (0.5–2.0 mg·L^{-1}) or TDZ (0.3–1.0 mg·L^{-1}) and 9 g·L^{-1} Bacto agar under light conditions described previously for shoot formation (Fig. 1e).

Root formation of in vitro–regenerated shoots. A single shoot with a height of 2 cm and 3 leaves were cultured on Hyponex N016 medium supplemented with 1.0 mg·L^{-1} NAA, 30 g·L^{-1} sucrose, 170 mg·L^{-1} NaH_2PO_4, 1.0 g·L^{-1} peptone (medium B), and 9 g·L^{-1} Bacto agar with different concentrations of coconut water (CW) (100–500 mL·L^{-1}), potato homogenate (PH), or banana homogenate (BH) (50–250 g·L^{-1}) under light conditions for rooting (Fig. 1f).

Acclimatization of plantlets. Plantlets with well-developed shoots and roots were taken out of the vessels, and the roots were washed in tap water to remove residual agar. Three hundred plantlets were then transplanted to plastic trays with three types of substrate (rice husk ash, coconut fiber, and fern fiber) and grown for 1 month before being transferred to 10-cm-diameter pots (with the same substrate) in the greenhouse (under natural light with <200 µmol·m^{-2}·s^{-1} photosynthetic photon flux density using sunshade nets) (Fig. 1g). The ambient temperature was ≈16–25 °C, and relative humidity was 60% to 90% in the greenhouse. Survival rates of the plantlets, new leaf formation, and soil plant analysis development (SPAD) values (the chlorophyll content index measured by SPAD 502; Konica Minolta, INC., Tokyo, Japan) after 6 months were recorded.

Histological study. Samples were fixed in Formalin acetic acid alcohol (FAA; formaline, acetic acid, and 70% ethanol as 5:5:90), dehydrated with Deshidratante histológico (Biopur SRL, Rosario, Argentina), embedded in paraffin wax (Paraplast Plus®; Sigma-Aldrich, Germany), and sectioned into 8- to 10-µm-thick serial sections with a rotary microtome. Sections were mounted on glass slides, stained with safranin-Astra blue (Luque et al., 1996), and observed under an optical microscope (×40).

Statistical analyses. All treatments were performed in triplicate, and each replicate included 20 cultures of 250-mL vessels (each vessel contained 40 mL of medium and 3 explants). The means were compared using Duncan's multiple range test using SPSS (version 16.0; IBM, Armonk, NY) with $P \leq 0.05$ (Duncan, 1995).

Results

Callogenesis. Table 1 and Fig. 2a show the callogenesis capacity results after 75 d; nodal cultures were excised from ex vitro–elongated shoots. Few calli (callogenesis formed at both internodes) and a small, light green callus were induced from stem nodes (1 cm) of *P. callosum* on media with only 2,4-D added (maximum callogenesis rate of 6.25% on medium containing 1.0 mg·L^{-1} 2,4-D).

In the current study, there were significant differences in the callogenesis capacity of the *P. callosum* explants cultured on media with combinations of 2,4-D and TDZ and on media with only 2,4-D. The results (Table 1) indicated that the highest callogenesis rate (31.25%) was recorded on medium combined with 1.0 mg·L^{-1} of 2,4-D and 1.0 mg·L^{-1} TDZ. The callus emerged on the cut surface of internodes (Fig. 2b, c) excised from the elongated stem nodes.

The ability of callogenesis differs depending on the location of the cut surface of the internodes. This rate decreased from the first internode (from the shoot tip) to the fourth internode. The fifth internode did not form callogenesis or callus induction; however, lateral buds extended from the nodes (data not shown).

Induction of PLBs. Table 2 shows the effects of 2,4-D and TDZ on the induction of PLBs after 75 d of culture. On medium with 1.0 mg·L^{-1} 2,4-D alone, a few yellow–green PLBs were observed. These PLBs turned brown and necrotic after 75 d of culture. There were significant increases in PLB induction when different concentrations of TDZ (0.3–1.0 mg·L^{-1}) were added to culture

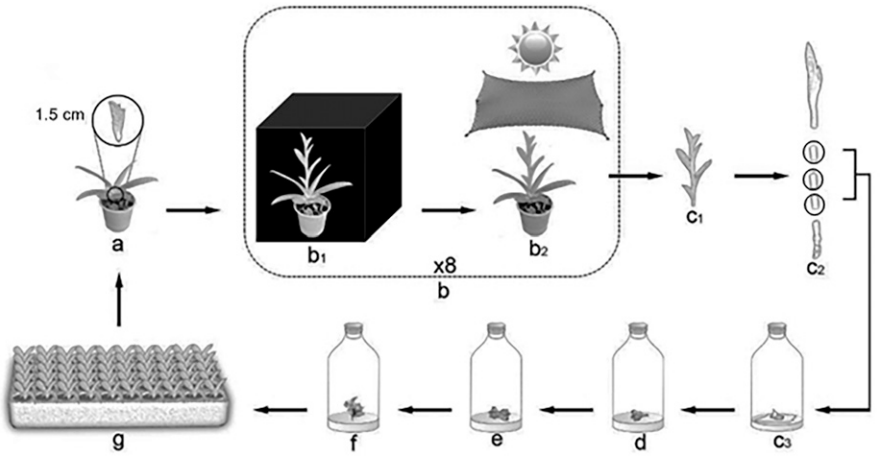

Fig. 1. Plant regeneration through internode tissue cultures of *Paphiopedilum callosum*. **(a)** Ex vitro plant. **(b)** Plant exposed to dark **(b_1)**-light **(b_2)** cycles of 14 d/1 d for stem elongation of the eighth replicate. **(c_1)** Elongated shoot excision. **(c_2)** Internode segments. **(c_3)** Callus induction. **(d)** PLB induction. **(e)** Shoot formation. **(f)** Plant regeneration. **(g)** Acclimatization in the greenhouse.

Table 1. Callus formation from ex vitro *P. callosum* stem nodes after 75 d of culture.

PGRs (mg·L^{-1})				
TDZ	2,4-D	Callogenesis ratio (%)	Induced position	Results
0	0	0 e[z]	Not induced	No callogenesis, browning young internode
	0.3	0 e	Not induced	No callogenesis
	0.6	0 e	Not induced	No callogenesis
	1.0	6.25 d	Both sides of the internodes	Very few callogenesis, small and light green callus
0.5	0	0 e	Not induced	No callogenesis
	0.3	0 e	Not induced	No callogenesis
	0.6	5.00 d	Both sides of the internodes	Very few callogenesis, small callus
	1.0	15.00 c	Both sides of the internodes	Dark green, hard, and small callus cluster
1.0	0	0 e	Not induced	No callogenesis
	0.3	7.50 d	A few locations on young internodes	Very few callogenesis, small and light green callus
	0.6	20.00 b	Young internodes excised from the elongated stem nodes	Light yellow, spongy callus cluster
	1.0	31.25 a	Young internodes excised from the elongated stem nodes	Light yellow, spongy callus cluster

[z]Different letters in the column indicate significant differences in Duncan's test ($P \leq 0.05$).
PGR = plant growth regulator; TDZ = Thidiazuron; 2,4-D = 2,4-Dichlorophenoxyacetic acid.

media in combination with 2,4-D, and the highest number of PLBs per explant (15.33 PLBs) was recorded when 1.0 mg·L^{-1} 2,4-D was used in combination with 1.0 mg·L^{-1} TDZ. These PLBs were bright green (Fig. 2d). Histological observations of PLBs were performed after 75 d of culture (Fig. 2e).

Shoot formation. The results of shoot formation are presented in Table 3. Medium supplemented with NAA alone did not result in shoot regeneration from PLBs, whereas PLBs cultured on media containing NAA in combination with BA or TDZ successfully induced shoot (3.25–8.00 shoots/explant, 4.75% to 60.00%) after 120 d of culture. A high number of shoots regenerated (4.75 shoots/explant) when PLBs were cultured on medium supplemented with 2.0 mg·L^{-1} BA or 0.6 mg·L^{-1} TDZ and 0.5 mg·L^{-1} NAA. Nevertheless, the results of this study indicated that the highest shoot formation (60.00%, 8.00 shoots/explant) was obtained when using 0.3 mg·L^{-1} TDZ in combination with 0.5 mg·L^{-1} NAA (Fig. 2f, g).

Root formation of in vitro–regenerated shoots. The effects of organic nutrients on root formation of *P. callosum* are presented in Table 4 and Fig. 2h. The addition of CW, PH, and BH on medium B with different concentrations showed positive effects on root formation of *P. callosum* after 90 d of culture. The presence of organic amendments significantly increased not only the number of roots and root length but also the shoot development, including the number of leaves, leaf length, and total fresh weight (Table 4). The results showed that low concentrations of CW (100 mL·L^{-1}) and BH (50–100 g·L^{-1}) facilitated rooting, with 4.13, 4.18, and 4.20 roots/shoot, respectively. However, high concentrations of these organic nutrients inhibited root formation and shoot development (Table 4). In the present study, PH was suitable for rooting; nevertheless, PH at high concentrations was not effective for rooting (Table 4). The optimal concentration of PH for root formation and shoot growth was 200 g·L^{-1}, resulting in the highest number of root formations (4.33 roots/shoot), root length (4.6 cm), number of leaves (5.5 leaves/shoot), leaf length (5.43 cm), and total fresh weight (1.65 g/plantlet) (Fig. 2h, Table 4).

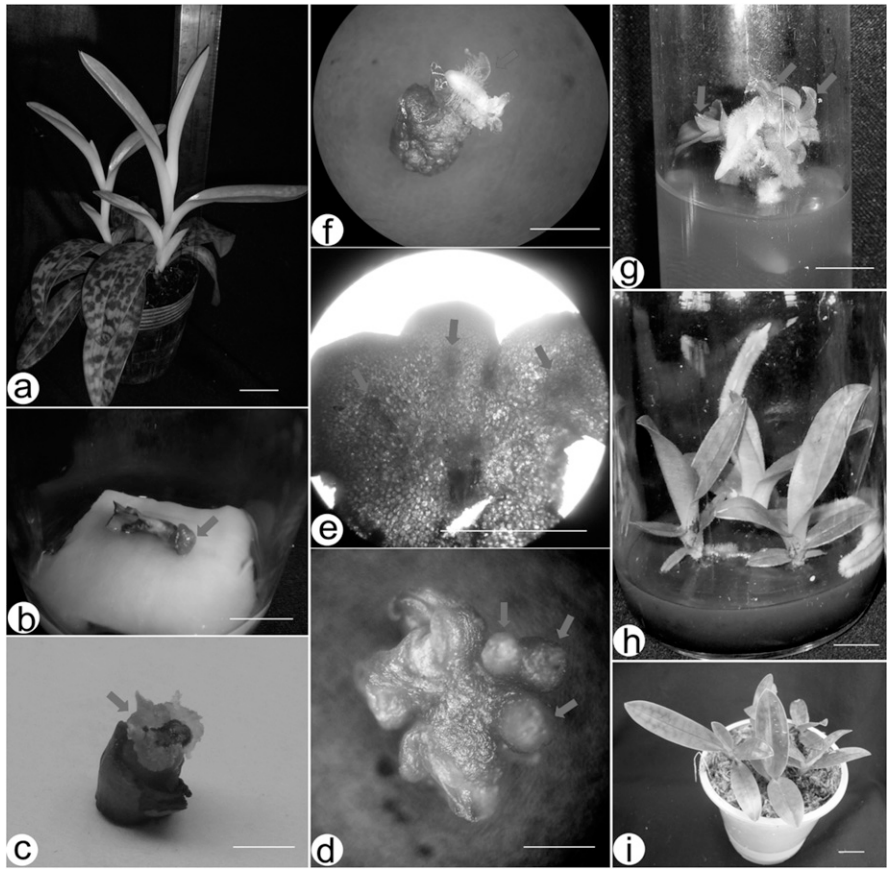

Fig. 2. Plant regeneration of *Paphiopedilum callosum*. Arrow indicates the critical positions. Bar: 1 cm. **(a)** *P. callosum* shoots elongated under dark–light cycles of 14 d/1 d for stem elongation of the eighth replicate. **(b, c)** Callus emergence (arrows) at the cut surfaces of internodes after 75 d of culture. **(d)** Protocorm-like body (PLB) induction (arrows). **(e)** Histology of PLB (arrow) under optical microscope (×40). **(f, g)** Shoot formation (arrow). **(h)** Plant regeneration. **(i)** The 6-month-old plants in the greenhouse.

Acclimatization of plantlets. Results were obtained after 6 months of growth under greenhouse conditions with three types of substrate: rice husk ash, coconut fiber, and fern fiber. Plantlets had a survival rate of 100% and 2.00–2.33 newly formed leaves; these results were not significant (Table 5). However, the length and width of leaves were significantly different. Plantlets grown on rice husk ash and coconut fiber had short, light green leaves that grew slowly (data not shown). Plantlets grown on fern fiber (Fig. 2i) had long, dark green leaves that grew well. SPAD values were different between substrates (Table 5). Plants grown on fern fiber had the highest SPAD value (38.17); this indicated that fern fiber is optimal for the growth and development of plants.

Discussion

Callogenesis. It is well known that in the absence of light, shoot elongation could be promoted in plants with the general attributes

Table 2. Effects of 2,4-D in combination with Thidiazuron (TDZ) on protocorm-like body (PLB) induction for *P. callosum* after 75 d of culture.

PGRs (mg·L^{-1})		PLB induction			
2,4-D	TDZ	ratio (%)	PLBs/explant (no.)	Fresh wt (g)	Results
1.0	0	100 a[z]	3.00 d	0.14 c	Few yellowish green PLBs that became brown and necrotic after 75 d of culture
	0.3	100 a	6.67 c	0.19 b	Yellow–green PLBs
	0.6	100 a	9.33 b	0.22 a	Green PLBs
	1.0	100 a	15.33 a	0.24 a	Bright green PLBs, good growth

[z]Different letters in the same column indicate significant differences in Duncan's test ($P \leq 0.05$).
PGR = plant growth regulator.

Table 3. Effects of α-Naphthaleneacetic acid (NAA) in combination with BA or Thidiazuron (TDZ) on shoot formation for *P. callosum* after 120 d of culture.

PGRs (mg·L^{-1})							Results	
NAA	BA	TDZ	Shoot formation (%)	Shoots/explant (no.)	Shoot ht (cm)	Leaves (no.)	Shoot	Root
0.5	0	0	0.00 g [z]	0.00 d	0.00 e	0.00 c	No regeneration of shoots from PLBs	No root formation
	0.5	0	11.25 e	3.25 c	2.15 a	4.00 ab	Small, long, and weak shoots	Many large roots
	1.0	0	43.75 c	3.25 c	2.08 ab	3.67 ab	High, dark green shoots	Many long roots
	2.0	0	51.25 b	4.75 b	1.87 abc	3.67 ab	Good growth, dark green shoots	Short roots
	0	0.3	60.00 a	8.00 a	2.00 ab	3.33 b	Good growth, dark green shoots	Short roots
	0	0.6	30.00 d	4.75 b	1.77 bc	3.33 b	Good growth, dark green shoots	Few roots
	0	1.0	4.75 f	3.25 c	1.23 d	4.00 ab	Small, short, and weak shoots	Few roots

[z]Different letters in the same column indicate significant differences in Duncan's test ($P \leq 0.05$).
PGR = plant growth regulator.

Table 4. Effects of coconut water (CW), potato homogenates (PH), and banana homogenates (BH) on the growth of *P. callosum* shoots after 90 d of culture.

Organic amendment		Roots/shoot (no.)	Root length (cm)	Leaves/shoot (no.)	Leaf length (cm)[z]	Total fresh wt (g)	Results
Control		3.93 cd [y]	3.23 fg	3.73 h	3.40 e	1.08 e	Small green shoots
CW (mL·L^{-1})	100	4.13 b	3.33 f	4.48 d	4.38 c	1.43 abc	Vigorous dark green shoots
	200	3.90 cde	3.75 e	4.40 de	5.13 b	1.65 a	Vigorous dark green shoots
	300	3.80 de	3.70 e	4.48 d	4.43 c	1.48 ab	Vigorous light green shoots
	400	2.93 f	3.10 g	3.23 j	3.55 ef	1.30 bcde	Vigorous light green shoots
	500	1.85 i	1.55 i	3.25 j	2.55 g	0.78 f	Vigorous light green shoots
PH (g·L^{-1})	50	4.18 b	4.03 cd	4.75 c	4.40 c	1.30 bcde	Vigorous light green shoots
	100	4.20 ab	4.10 cd	4.78 c	5.08 b	1.45 abc	Vigorous light green shoots
	150	4.13 b	4.00 d	5.00 b	5.05 b	1.53 ab	Light green shoots, 1–2 new shoots/vessel
	200	4.33 a	4.60 a	5.50 a	5.43 a	1.65 a	Light green shoots, 1–2 new shoots/vessel
	250	3.78 e	3.75 e	4.25 ef	4.03 d	1.28 bcde	Light green shoots, slow growth, 1–2 new shoots/vessel
BH (g·L^{-1})	50	4.20 ab	4.15 c	4.50 d	3.63 e	1.35 bcd	Vigorous dark green shoots
	100	4.13 b	4.38 b	4.23 f	4.60 c	1.50 ab	Vigorous dark green shoots
	150	2.70 g	3.10 g	4.00 g	3.25 f	1.23 cde	Vigorous light green shoots
	200	2.48 h	2.53 h	3.50 i	3.23 f	1.18 de	Vigorous light green shoots
	250	1.73 i	1.60 i	3.25 j	2.35 g	0.75 f	Vigorous light green shoots

[z]Data were measured as the average of the first, second, and third leaves from the shoot tip.
[y]Different letters in the same column indicate significant differences in Duncan's test ($P \leq 0.05$).

Table 5. Effects of substrates on plantlet growth in the greenhouse after 6 months of cultivation.

Substrate	Survival rate (%)	New leaf formation	SPAD value	Results
Rice husk ash	100 a[z]	2.00 a	23.45 c	Leaves are short, light green; plants grew slowly
Coconut fiber	100 a	2.33 a	27.32 b	Leaves are short, light green; plants grew slowly
Fern fiber	100 a	2.33 a	38.17 a	Leaves are long, dark green; plants grew well

[z]Different letters in the same column indicate significant differences in Duncan's test ($P \leq 0.05$).

of etiolation (Toyomasu et al., 1992). However, among reports of propagation of *Paphiopedilum* spp., there has been little discussion of the application of dark conditions to obtain elongated stem nodes as a highly efficient method of generating explants. For *Paphiopedilum* hybrids of *P. Deperle* and *P. Armeni* White, the scape transverse slices could induce adventitious buds and regenerate into whole plants (Liao et al., 2011). It was found that 1.5- to 3.0-cm sections of flower buds of *P.* Deperle were able to produce shoots, but only sections of flower buds longer than 2.5 cm on *P.* Armeni White were regenerated. Recently, Luan et al. (2015) reported that the best stem elongation of *P. delenatii* in vitro shoots was obtained in the dark after 4 months of culture. These shoots were then maintained under fluorescent light for 60 d before being excised into single nodes and transferred to ex vitro conditions. However, plants had extreme difficulty regenerating internodal segments because of the lack of nodes. In this study, we efficiently regenerated *P. callosum* from internodal segments devoid of nodes.

The work described in this report provides further evidence to enhance our knowledge of the dark–light cycle developmental pathway, known as etiolation, for ex vitro shoot elongation during micropropagation of *P. callosum*. The callogenesis rate, however, was low when explants were cultured on media supplemented with 2,4-D only. This result is consistent with that of the study by Sherif et al. (2016), who demonstrated that low callogenesis rates of 10.7% and 12.7% for the node and internode, respectively, of *Anoectochilus elatus* were obtained on medium with only 2,4-D. In this study, the callogenesis capacity was significantly higher when *P. callosum* stem nodes were cultured on media with combinations of 2,4-D and TDZ (31.25% on medium with 1.0 mg·L^{-1} of 2,4-D and 1.0 mg·L^{-1} TDZ). Lin et al. (2000) found higher callogenesis rates for a 1-year-old stem of a *Paphiopedilum* hybrid on a medium with 1.0 mg·L^{-1} of 2,4-D and 1.0 mg·L^{-1} TDZ and on a medium with 10.0 mg·L^{-1} of 2,4-D and 0.1 mg·L^{-1} TDZ (45%

and 65%, respectively). The culture medium containing TDZ along with 2,4-D induced callus formation of different orchids, including *Cymbidium, Phalaenopsis*, *Paphiopedilum*, and *Oncidium* (Chang and Chang, 1998; Chen and Chang, 2000; Chen et al., 2000; Hong et al., 2008; Jheng et al., 2006; Lin et al., 2000).

The decreased callogenesis of callus induction from the first to fifth internodes may be explained by the age of the explants. The first and second internodes (near the shoot tip) will be younger than those on the base internodes. Therefore, internodes as far away as the shoot tip do not easily induce the callus. A possible explanation for these results may be that callogenesis efficiency could depend on species/cultivars, explant sources, as well as culture media. The key advantage of this study was that ex vitro stem nodes were used as the initial explants; in previous studies, callogenesis from in vitro asymbiotic seed germination was reported.

Induction of PLBs. Lin et al. (2000) reported that the combination of TDZ (0.5–3.0 mg·L^{-1}) and NAA was produced via PLB formation of hybrid *P. callosum* 'Oakhil' × *P. lawrenceanum* 'Tradition'. In contrast, studies by Hong et al. (2008) and Ng and Saleh (2011) of *Paphiopedium* Alma Gavaert and PLB formation from the callus showed that the best responses were found with 5 mg·L^{-1} NAA (4.7 PLBs/explant) and 0.9 mg·L^{-1} Kinetin (4.1 PLBs/explant), respectively. The results of this study using *P. callosum* showed higher PLB induction capacities (19–24 PLBs/explant) on media containing TDZ (0.3–1.0 mg·L^{-1}) in combination with 1.0 mg·L^{-1} 2,4-D compare to the results of previous reports. This proved that different plant growth regulators (PGRs) are required for suitable induction of PLBs in different species of *Paphiopedilum* (Masnoddin et al., 2018).

Shoot formation. Research of *P. villosum* var. *densissimum*, *P. insigne* (Lindl.) Stein, *P. bellatulum* (Rchb. f.) Stein, and *P. armeniacum* identified that combinations of BA and NAA resulted in effective shoot organogenesis after 3 months of culture (Long et al., 2010). In this study, the high shoot formation (4.75 shoots/explant) was recorded 120 d after PLBs were transferred to medium supplemented with 2.0 mg·L^{-1} BA and 0.5 mg·L^{-1} NAA. However, the combination of 0.3 mg·L^{-1} TDZ and 0.5 mg·L^{-1} NAA resulted in a maximum shoot formation from PLBs of *P. callosum*, with eight shoots per explant (Table 3, Fig. 2f, g). Combinations, concentrations, and the ratio of PGRs are important for shoot formations in orchids (Dohling et al., 2012). It was possible that shoot induction of *P. callosum* was affected by both NAA and TDZ. This finding agrees with the results of Lin et al. (2000), who showed suitable combinations of NAA and TDZ for shoot bud formation and plant regeneration in hybrid *P. callosum* 'Oakhil' × *P. lawrenceanum* 'Tradition'. Studies by Kishor and Devi (2009) and Jitsopakul et al. (2013) involving *Aerides vandarum* Reichb.f × *Vanda stangeana* Reichb.f, and *Vanda coerulea*, respectively, also showed that TDZ combined with NAA provided a high number of shoots per explant.

Root formation of in vitro–regenerated shoots and acclimatization of plantlets. Zeng et al. (2013) investigated the effects of BH on the rooting capacity of *P. hangianum* and found that 100 g·L^{-1} BH added to rooting medium containing 1.0 or 2.0 mg·L^{-1} NAA was determined to be most suitable for the highest rooting percentage (85% to 91%) and tallest shoots (5.3–5.6 cm). The results of this study also indicated that BH at low concentrations (50–100 g·L^{-1}) with 1.0 mg·L^{-1} NAA facilitated rooting. We found that supplements of PH at different concentrations (100–200 g·L^{-1}) resulted in the highest rooting capacities of *P. callosum* when compared with other organic matter (CW and BH) and the control (organic matter–free). The highest root formation occurred on medium containing PH because potato is a rich source of carbohydrates, protein, fat, vitamins, phenolic compounds, amino acids, and fatty acids (Islam et al., 2003). The benefits of PH were also reported by Seon et al. (2018), who investigated rooting of *Thrixspermum japonicum*, a rare epiphytic orchid.

The survival rate (100%) of this study is consistent with that of the study by Chyuam et al. (2010), who grew *P. rothschildianum* with four to five roots (survival rate of 90%). The high survival rates for *Paphopedilum* sp. may also be due to the genetic characteristics of each species (Chen et al., 2004a; Liao et al., 2011; Zeng et al., 2016). Our results obtained for *P. callosum* were higher than those obtained by Long et al. (2010); after planting *P. villosum* var. *Densissimum* plantlets with a root length of 3–6 cm and 4–5 leaves on peat and moss substrate, the plantlets grew slowly and the survival rate was low (≈60%) at 2 months. In this study, the optimal growth of plantlets was cultivated on fern fiber, which provided better physiological conditions and endured under moist, humid conditions for plantlet acclimatization of *Paphopedilum* sp.

Conclusion

The results of this study showed that internode tissue obtained from ex vitro shoots elongated during dark–light cycles are suitable explants for callus induction of *P. callosum*. Medium containing 1.0 mg·L^{-1} TDZ and 1.0 mg·L^{-1} 2,4-D was found to be most suitable for PLB induction, and highly effective shoot formation was recorded when PLBs were sub-cultured on SH medium containing 0.3 mg·L^{-1} TDZ and 0.5 mg·L^{-1} NAA. The concentration of PH ranging from 100 to 200 g·L^{-1} was determined to be effective for the rooting stage. Finally, plantlets were successfully acclimatized and had a survival rate of 100% after being transferred to ex vitro conditions. Although the genetic stability of regenerants was not investigated, plants derived from callus-derived PLBs have successfully grown in the greenhouse and displayed no abnormalities. These results contribute to the existing knowledge of using ex vitro–derived explants (internode tissue) for effective micropropagation via callus and PLB induction of *Paphiopedilum* species, especially *P. callosum*. Further research involving other *Paphiopedilum* species and using this protocol should be performed to achieve totipotent callus cultures, especially from tissues of elite varieties.

Literature Cited

Arditti, J. 2008. Micropropagation of orchids. 2nd ed. Blackwell Publishing Ltd., Malden, MA.

Averyanov, L., P. Cribb, P.K. Loc, and N.T. Hiep. 2003. Slipper orchids of Vietnam. Compass Press Limited, The Royal Botanic Gardens, Kew.

Averyanov, L.V. and A.L. Averyanova. 2003. Updated checklist of the orchids of Vietnam. Vietnam National University Publishing House, Hanoi.

Braem, G.J. 1988. *Paphiopedilum*. A monograph of all tropical and subtropical Asiatic slipper-orchids. Brucke-Verl. Schmersow, Hildesheim.

Braem, G.J. and G.R. Chiron. 2003. *Paphiopedilum*-Tropicalia. Voreppe, France.

Chang, C. and W.C. Chang. 1998. Plant regeneration from callus culture of *Cymbidium ensifolium* var. misericors. Plant Cell Rep. 17(4):251–255.

Changchun, D., Y. Hong, and L. Fangyuna. 2004. Factors affecting the germination of *Paphiopedilum armeniacum*. Acta Bot. Yunnanica 26(6):673–677.

Chen, J.T. and W.C. Chang. 2000. Plant regeneration via embryo and shoot bud formation from flower-stalk explants of *Oncidium* Sweet Sugar. Plant Cell Tissue Organ Cult. 62(2):95–100.

Chen, Y.C., C. Chang, and W.C. Chang. 2000. A reliable protocol for plant regeneration from callus culture of *Phalaenopsis*. In Vitro Cell. Dev. Biol. Plant 36(5):420–423.

Chen, T.Y., J.T. Chen, and W.C. Chang. 2004a. Plant regeneration through direct shoot bud formation from leaf cultures of Paphiopedilum orchids. Plant Cell Tissue Organ Cult. 76(1):11–15.

Chen, Z.L., X.L. Ye, C.Y. Liang, and J. Duan. 2004b. Seed germination in vitro of *Paphiopedilum armeniacum* and *P. micranthum*. Acta Hortic. Sinica. 31(4):540–542.

Chyuam, Y.N., N.M. Saleh, and F.Q. Zaman. 2010. In vitro multiplication of the rare and endangered slipper orchid, *Paphiopedilum rothschildianum* (Orchidaceae). Afr. J. Biotechnol. 9(14):2062–2068.

Cribb, P. 1987. The genus *Paphiopedilum*. Royal Botanic Gardens, Kew in association with Collingridge, Kew, London.

Ding, C.Q., L. Li, and N.H. Xia. 2011. Aseptic sowing and in vitro seedling culture of *Paphiopedilum micranthum* T. Tang and F.T. Wang. North Hort. 5:115–117.

Dohling, S., S. Kumaria, and P. Tandon. 2012. Multiple shoot induction from axillary bud cultures of the medicinal orchid, *Dendrobium longicornu*. AoB Plants pls032:1–7.

Duncan, D.B. 1995. Multiple range and multiple F test. Biometrics 11:1–42.

Hong, P.I., J.T. Chen, and W.C. Chang. 2008. Plant regeneration via protocorm-like body formation and shoot multiplication from seed-derived callus of maudiae type slipper orchid. Acta Physiol. Plant. 30:755–759.

Huang, L.C. 1988. A procedure for asexual multiplication of *Paphiopedilum* in vitro. Amer. Orchid Soc. Bull. 57:274–278.

Huang, L.C., C.J. Lin, C.I. Kou, B.L. Huang, and T. Murashige. 2001. *Paphiopedilum* cloning in vitro. Scientia Hort. 91:111–121.

Islam, M.O., A.R. Rahman, S. Matsui, and A.K.M. Prodhan. 2003. Effects of complex organic extracts on callus growth and plb regeneration through embryogenesis in the *Doritaenopsis* orchid. Jpn. Agr. Res. Q. 37:229–235.

Jheng, F.Y., Y.Y. Do, Y.W. Liauh, J.P. Chung, and P.L. Huang. 2006. Enhancement of growth and regeneration efficiency from embryogenic callus cultures of *Oncidium* 'Gower Ramsey' by adjusting carbohydrate sources. Plant Sci. 170(6):1133–1140.

Jitsopakul, N., K. Thammasiri, K. Ishikawa, M. Wannajuk, P. Sangthong, S. Natapintu, and K. Won-In. 2013. Efficient adventitious shoot regeneration from shoot tip culture of *Vanda coerulea*, a Thai orchid. Sci. Asia 39(5):449–455.

Kishor, R. and H.S. Devi. 2009. Induction of multiple shoots in a monopodial orchid hybrid (*Aerides vandarum* Reichb. f×*Vanda stangeana* Reichb. f) using thidiazuron and analysis of their genetic stability. Plant Cell Tissue Organ Cult. 97(2):121–129.

Koopowitz, H. 2008. Tropical Slipper Orchids: *Paphiopedilum* and *Phragmipedium* species and hybrids. Timber Press, Portland, OR.

Lee, Y.I. and N. Lee. 2003. Plant regeneration from protocorm-derived callus of *Cypripedium formosanum*. In Vitro Cell. Dev. Biol. Plant 39:475–479.

Liao, Y.J., Y.C. Tsai, Y.W. Sun, R.S. Lin, and F.S. Wu. 2011. In vitro shoot induction and plant regeneration from flower buds in *Paphiopedilum* orchids. In Vitro Cell. Dev. Biol. Plant 47(2):702–709.

Lin, Y.H., C. Chen, and W.C. Chang. 2000. Plant regeneration from callus culture of a *Paphiopedilum* hybrid. Plant Cell Tissue Organ Cult. 62:21–25.

Long, B., A.X. Niemiera, Z.Y. Cheng, and C.L. Long. 2010. In vitro propagation of four threatened *Paphiopedilum* species (Orchidaceae). Plant Cell Tissue Organ Cult. 101:151–162.

Luan, V.Q., N.P. Huy, N.B. Nam, T.T. Huong, V.T. Hien, N.T.T. Hien, N.T. Hai, D.K. Thinh, and D.T. Nhut. 2015. Ex vitro and in vitro *Paphiopedilum delenatii* Guillaumin stem elongation under light-emitting diodes and shoot regeneration via stem node culture. Acta Physiol. Plant. 37:136.

Luque, R., H.C. Sousa, and J.E. Kraus. 1996. Métodos de coloracao de Roeser (1972) e Kropp (1972) visando a subtituicao do azul do astra por azul de alciao 8GS ou 8GX. Acta Bot. Bras. 10:199–212.

Masnoddin, M., R. Repin, and Z.A. Aziz. 2018. PLB regeneration of *Paphiopedilum rothschildianum* using callus and liquid culture system. J. Trop. Biol. Conserv. 15:1–14.

Ng, C.Y. and N.M. Saleh. 2011. In vitro propagation of *Paphiopedilum* orchid through formation of protocorm-like bodies. Plant Cell Tissue Organ Cult. 105:193–202.

Nhut, D.T., D.T.T. Thuy, N.T. Don, V.Q. Luan, N.T. Hai, K.T.T. Van, and C.C. Chinnappa. 2007. Stem elongation of *Paphiopedilum delenatii* Guillaumin and shoot regeneration via stem node culture. Propag. Ornam. Plants 7(1):29–36.

Pierik, R.L.M., P.A. Sprenkels, D.H. Van, and D.M.Q.G. Van. 1988. Seed germination and further development of plantlets of *Paphiopedilum ciliolare* Pfitz in vitro. Scientia Hort. 34:139–153.

Schenk, R.U. and A.C. Hildebrandt. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50:199–204.

Seon, K.M., D.H. Kim, K.W. Kang, and I. Sivanesan. 2018. Highly competent in vitro propagation of *Thrixspermum japonicum* (Miq.) Rchb. f., a rare epiphytic orchid. In Vitro Cell. Dev. Biol. Plant 54:302–308.

Sherif, N.A., T.S. Kumar, and M.V. Rao. 2016. In vitro regeneration by callus culture of *Anoectochilus elatus* Lindley, an endangered terrestrial jewel orchid. In Vitro Cell. Dev. Biol. Plant 52(1):72–80.

Stewart, J. and J. Button. 1975. Tissue culture studies in *Paphiopedilum*. Amer. Orchid Soc. Bull. 44:591–599.

Toyomasu, T., H. Yamane, I. Yamaguchi, N. Murofushi, N. Takahashi, and Y. Inoue. 1992. Control by light of hypocotyl elongation and levels of endogenous gibberellins in plantlets of *Lactuca sativa* L. Plant Cell Physiol. 33:695–701.

Zeng, S.J., K.L. Wu, J.A. Teixeira da Silva, J.X. Zhang, Z.L. Chen, N.H. Xia, and J. Duan. 2012. Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh., an endangered terrestrial orchid. Scientia Hort. 138:198–209.

Zeng, S.J., W.C. Huang, K.L. Wu, J.X. Zhang, J.A. Teixeira da Silva, and J. Duan. 2016. In vitro propagation of *Paphiopedilum* orchids. Crit. Rev. Biotechnol. 36(3):521–534.

Zeng, S.J., J. Wanga, J. Wua, K. Wua, J.A. Teixeira da Silva, J.X. Zhang, and J. Duan. 2013. In vitro propagation of *Paphiopedilum hangianum* Perner & Gruss. Scientia Hort. 151:147–156.