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

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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 
explotation. 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 calllogenesis 
(31.25%) was achieved when internode tissue was cultured on liquid Schenk and 
Hildebrandt (SH) medium containing 0.5 mg L−1 2,4-Dichlorophenoxyacetic acid (2,4-D), 
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 NaH2PO4, 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 

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by climate change, drought, tourism and leisure activities, urbanization, infrastructure 
development, and recreation activities with direct effects (e.g., destruction of plants) and 
direct 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 propaga-
tion, 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 hy-
brids (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 callo-
genesis 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/culti-
vars 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 ex-
plants (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. farrianneum, and P. insignis 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 disin-
fection, 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, re-
spectively.
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. 1b1) to induce stem elongation (Fig. 1b2 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. 1c1) and sterilized with 0.1% HgCl₂ treatments, elongated shoots were excised for callogenesis or callus induction; however, lateral buds extended from the nodes (data not shown).

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**Callogenesis.** After eight dark–light cycle treatments, elongated shoots were excised (Fig. 1c1) and sterilized with 0.1% HgCl₂ for 6 min and rinsed with sterilized distilled water five times. Then, the shoots were cut into five internode segments (Fig. 1c2) 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. 1e1). 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 mol⁻² s⁻¹ at a temperature of 25 ± 2 °C with 50% to 55% relative humidity (Fig. 1d1).

**Formation of shoots from PLBs.** PLB clumps (5–6 PLBs/clump) were transplanted to medium A supplemented 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. 1e1). 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 NaH₂PO₄, 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 transferred 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 histologico (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* ≤ 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.
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⁻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).

**Acclimatization of plantlets.** Results were obtained after 6 months of growth under greenhouse conditions with three types of substrates: 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

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

| PGRs (mg·L⁻¹) | Callogenesis ratio (%) | Induced position | Results |
|---------------|------------------------|------------------|---------|
| **TDZ 2,4-D** |                        |                  |         |
| 0             | 0 e                    | 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             | 0 e                    | Not induced      | No callogenesis |
| 0.5           | 0 e                    | Not induced      | No callogenesis |
| 0.6           | 0 e                    | Not induced      | No callogenesis |
| 1.0           | 15.0 e                 | Both sides of the internodes | Dark green, hard, and small callus cluster |
| 0.3           | 0 e                    | Not induced      | No callogenesis |
| 0.6           | 5.00 d                 | Both sides of the internodes | Very few callogenesis, small callus |
| 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 |

*Different letters in the column indicate significant differences in Duncan’s test (P < 0.05).*

PGR = plant growth regulator; TDZ = Thidiazuron; 2,4-D = 2,4-Dichlorophenoxyacetic acid.
of etiolation (Toyomasu et al., 1992). However, among reports of propagation of *Paphiopedilum* spp., there has been little discussion on 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 0.1 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 only 2,4-D. The 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 0.1 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 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 0.1 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 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 0.1 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 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 0.1 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 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 0.1 mg·L⁻¹ TDZ).
implemented with 2.0 mg L⁻¹ and NAA. The concentration of PH ranging from 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) in combination with 1.0 mg L⁻¹ NAA facilitated rooting. We found that supplementation 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, pheno-
litic 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 japo-
nicum, a rare epiphytic orchid.

The survival rate (100%) of this study is consistent with that of the study by Chyu-
am et al. (2010), who grew P. rothschildianum with four to five roots (survival rate of 90%). The high survival rates for Paphope-
dilum 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. Den-
sissimum plantlets with a root length of 3–6 cm and 4–5 leaves on peat and moss sub-
strate, 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 Paphiopedilum 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, plant-
lets 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 in-
volving other Paphiopedilum species and using this protocol should be performed to achieve toptipotent callus cultures, especially from tissues of elite varieties.

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