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Micropropagation of *Brasilidium forbesii* (Orchidaceae) through transverse and longitudinal thin cell layer culture

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ABSTRACT. An efficient *in vitro* propagation method was established for *Brasilidium forbesii* (Hook.) Campacci using transverse and longitudinal thin cell layer (tTCL and lTCL, respectively) culture systems. Six-month-old protocorms from *in vitro* germinated seeds were used for this study. TCLs (1.0-mm thick) from protocorms were grown on Woody Plant Medium (WPM) supplemented with benzyladenine (BA) (0.5–4.0 μM).The lTCL technique was more efficient for inducing protocorm-like bodies (PLBs) and regenerating shoots than the tTCL technique. The frequency of PLB formation was influenced by BA concentration, and the lTCL explant grown on a medium containing 2.0 μM BA produced the highest percentage of new protocorms (77%) with a total of 22.7 PLBs per explant, after the first subculture on the same medium. Plantlet development was optimal on WPM medium containing 3.0 g L^{-1} activated charcoal, and indole-3-butyric acid was not necessary for rooting. Regenerated plants were successfully acclimatized in a greenhouse after 16 weeks using vermiculite as the substrate (100% survival).

Keywords: epiphytic orchid, *in vitro* propagation, protocorms, protocorm-like body.

Micropropagação de *Brasilidium forbesii* (Orchidaceae) pela técnica 'thin cell layer' longitudinal e transversal

RESUMO. Foi estabelecido um método eficiente de propagação *in vitro* para *Brasilidium forbesii* (Hook.) Campacci utilizando a técnica 'thin cell layer' transversal (TCLt) e longitudinal (TCLl). Foram utilizados protocormos de seis meses obtidos da germinação *in vitro*. TCLs (1,0 mm de espessura) dos protocormos foram cultivados no meio 'Woody Plant Medium' (WPM), acrescido com benziladenina (BA) (0,5 a 4,0 μM). A técnica TCLl foi mais eficiente para indução de estruturas semelhantes a protocormos (ESPs) e regeneração de brotações do que a técnica TCLt. A frequência de formação de ESP foi influenciada pela concentração de BA e o explante TCLl, cultivado em um meio contendo 2,0 μM BA, produziu a mais alta percentagem de novos protocormos (77%), com um total de 22,7 PLBs por explante, após o primeiro subcultivo para o mesmo meio. O desenvolvimento das plântulas foi eficiente no meio WPM contendo 3,0 g L^{-1} de carvão ativado e o ácido indol-3-butírico (AIB) não foi necessário para o enraizamento. Plantas regeneradas foram estabelecidas com sucesso em casa de vegetação, utilizando vermiculita como substrato (100% de sobrevivência), após 16 semanas.

Palavras-chave: orquídea epífita, propagação *in vitro,* protocormos, estrutura semelhante a protocormo.

Introduction

Brasilidium forbesii (Hook) Campacci (synonym *Oncidium forbesii* Hook) is an endemic epiphytic orchid species found in the Atlantic Forest of Brazil. Orchids are grown primarily as ornamentals not only because of their exotic beauty but also because of their long shelf life. With the advance of agriculture and the constant destruction of their natural habitat, orchid species are collected indiscriminately, and this extractive activity threatens many species with extinction, drastically reducing their genetic variability in nature (VENDRAME et al., 2014). Orchids produce a large number of very fragile seeds in capsules that have no reserve material or endosperm; thus, they depend on mycorrhizal fungi for natural germination (MITRA, 1971). *In vitro* culture can be an effective technique for propagation and germplasm conservation of these species. However, successful *in vitro* orchid propagation is influenced by many factors, such as plant genotype and media composition (GNASEKARAN et al., 2012). In general, the type,

concentration and combination of growth regulators play an important role during *in vitro* propagation of many orchid species (ARDITTI; ERNST, 1993).

Orchids were once considered to be particularly difficult-to-propagate plants *in vitro*, but thin cell layer (TCL) technology has helped develop methods for their tissue culture, making mass clonal propagation easier and more reproducible (TEIXEIRA DA SILVA, 2013). The TCL system employs various small-sized explants from different plant organs excised either longitudinally [longitudinal thin cell layer (lTCL)] or transversally [transverse thin cell layer (tTCL)] (TRAN THANH VAN, 2003). TCL culture systems are promising and efficient with regard to the total output of orchid plantlets compared to other conventional *in vitro* methods for rapid regeneration of orchids. However, these culture systems have not yet been completely exploited for propagating commercially important orchids (HOSSAIN et al., 2013). In orchids, tTCl has been successfully employed for protocorm-like bodies (PLBs) of *Cymbidium aloifolium* and *Dendrobium nobile* (NAYAK et al., 2002) and *Cymbidium* Sleeping Nymph (VYAS et al., 2010), and lTCL has been used to produce PLBs of the hybrid *Cymbidium* Twilight Moon 'Day Light' (TEIXEIRA DA SILVA; TANAKA, 2006). Thus, to obtain rapid plant regeneration with a high frequency, TCL culture methods were exploited for mass propagation of *B. forbesii*. The effects of benzyladenine (BA) on PLB induction, of indole-3-butyric acid (IBA) and activated charcoal on plantlet development, and substrates on acclimatization of TCL explants were therefore evaluated.

Material and methods

Plant material, seed sterilization, and *in vitro* germination

Seeds of *B. forbesii* stored at –80°C for one year were surface sterilized with 0.75% (v v^{-1}) sodium hypochlorite plus 0.1 Tween® 20 for 5 min., followed by five rinses with distilled, autoclaved water. Seeds were germinated on Woody Plant medium (WPM) (LLOYD; McCOWN, 1980) and the protocorms from 6-month-old *in vitro* cultured plants were used as the explant source.

tTCL and lTCL culture

The protocorms (approximately 2.0 mm in thickness) were cross sectional and longitudinally sliced into two pieces, approximately 1.0 mm in thickness, using a sharp surgical blade under sterile conditions. The tTCL and lTCL explants were inoculated on WPM without a growth regulator or with different concentrations of BA (0.5, 1.0, 2.0, and 4.0 μM). The apical protocorm sections were placed cut side down, and the basal and lateral sections were placed cut side up on the culture medium. The tTCL and lTCL sections were cultured in petri dishes; 10 basal and 10 apical explants and 20 lateral sections (designated as section 1 and 2) were inoculated in each dish, and five dishes were used for each treatment.

The cultures were incubated for four weeks in the dark and then transferred to a 16-h 8-h^{-1} (light/dark) photoperiod for four additional weeks. Visual observations were conducted weekly. TCL explants with small clumps were subcultured using the same treatment after eight weeks. The percentage of explants forming PLBs and the average number of PLBs regenerated per responsive explant were recorded after eight weeks of initial culture and after eight additional weeks of the first subculture.

Shoot development and rooting

Multiple shoot clusters from the TCLs were transferred to a growth regulator-free medium and cultured for eight weeks. For shoot development and rooting, individual shoots (1.0 ± 0.2 cm in length) were inoculated on WPM without a growth regulator or supplemented with 1.0, 2.0, or 4.0 μM IBA or 1.0, 2.0, or 3.0 g L^{-1} activated charcoal. Ten shoots were cultured in each flask with six repetitions per treatment for the IBA experiment and nine repetitions for the activated charcoal experiment. After eight weeks, shoot and root length, average number of roots, and weight of the fresh mass were recorded.

Acclimatization of regenerated plantlets

The regenerated young plants (2.0 ± 0.5 cm in length) with three to four expanded leaves per shoot and well developed roots were removed from the culture medium, washed gently with water to remove traces of agar, and transferred to polystyrene trays (3.5 cm^2) containing Tecnomax®, fine-textured vermiculite, a mixture of Tecnomax® and vermiculite (1:1), of Tecnomax® and coconut powder (1:1) or vermiculite and coconut powder (1:1). Ten plants were transplanted per substrate with five replicates each. The plants were maintained in a greenhouse at room temperature (25 ± 3°C), and manually irrigated every three days. After 16 weeks, the shoot and root length, the average number of roots, and fresh mass were recorded.

Culture medium and conditions

The WPM medium was supplemented with 3% (w/v) sucrose and gelled using 0.6 % (w/v) agar Vetec®. The pH was adjusted to 5.8 with 0.1 N NaOH or HCl before the addition of agar. The media were autoclaved at 121°C for 20 min. Petri dishes (150 mm in diameter and 20 mm in height) containing 40 mL of semi-solid WPM were used for the TCL experiments. Flasks (6.2 cm in diameter and 12.5 cm in length) containing 40 mL of WPM were used for the shoot development and rooting experiments. All cultures were incubated at 25 ± 1°C/19 ± 1°C (day/night) under a 16-h/8-h (day/night) photoperiod provided by white fluorescent tubes at an intensity of 40 μmol m^{-2} s^{-1}.

Experimental design and statistical analysis

Experiments were performed using a completely randomized design and repeated at least once. All data were statistically analyzed by analysis of variance (ANOVA), and means were compared by Tukey's test at $p < 0.05$ using Assistat 7.6 beta software.

Results and discussion

Induction and regeneration of PLBs from lTCL and tTCL

The PLB development pattern of *B. forbesii* was similar in all treatments including the control. Small white protuberances gradually emerged on the explants within two (Figure 1A) to four weeks of culture under dark conditions (Figures 1B and 1C). Globular PLBs and greenish-yellow protuberances were observed on the surface of the TCL explants after four weeks in the dark, followed by two weeks of culture in a 16-h photoperiod (Figure 1D), and these protuberances gradually increased in size and developed into shoots by the end of eight- to 12 weeks under a 16-h photoperiod condition (Figure 1E).

Of the two techniques tested, lTCL was more effective than tTCL for PLB formation, and subculturing using the same BA concentration increased the formation frequency and total number of PLBs. The type of lTCL section, designated as 1 and 2, showed no effect on the PLB regeneration process after culture initiation and the first subculture using the same treatment (Table 1). BA concentration only influenced PLBs regeneration frequency, however caused no significant difference in the average number of PLBs per explants for lTCl (Table 1). Explants cultured in medium supplemented with 2.0 μM BA were most effective for regenerating PLBs from lTCL protocorms after eight weeks (69%) and after the first subculture (77%) than those inoculated on medium with 4.0 μM BA (46 and 50% for culture initiation and the first subculture, respectively) (Table 1). The average number of PLBs was affected neither by the type of lTCL section nor by the BA concentration. The subculture in the same medium gave an increase in the regeneration frequency and the average number of PLBs in all treatments (Table 1).

Approximately 30% of the tTCL explants and 60% of the lTCL sections produced 14.9 – 19.5 PLBs per responsive explant within eight to 16 weeks of culturing on growth regulator-free WPM (Tables 1 and 2). Paudel and Pant (2012) also observed that protocorms of *Esmeralda clarkei* responded readily on Murashige and Skoog (MS) medium supplemented with or without growth regulators. In contrast to the present study, for TCLs of some orchid species, death occurred after two to three weeks on a medium without growth regulators (ZHAO et al., 2007; RANGSAYATORN, 2009).

Table 1. Effect of BA on PLB induction in lateral sections (lTCL) of *Brasilidium forbesii* protocorms cultured on WPM culture medium eight weeks after culture initiation and at the end of the first subculture.

BA concentration (μM)	PLBs regeneration (%)			Average number of PLBs per responsive explants			
Culture initiation	Section 1	Section 2	Mean[a]	Section 1	Section 2	Mean[a]	Total
0.0	60.0	62.0	61.0 ab	7.3	7.6	7.4 a	14.9
0.5	46.0	56.0	51.0 ab	7.0	6.7	6.9 a	13.7
1.0	54.0	58.0	56.0 ab	8.7	8.4	8.6 a	17.1
2.0	74.0	64.0	69.0 a	7.1	7.6	7.4 a	14.7
4.0	42.0	50.0	46.0 b	8.1	9.1	8.6 a	17.2
Mean[b]	55.2 A	58.0 A		7.7 A	7.9 A		
1st Subculture							
0.0	70.0	60.0	65.0 ab	11.3	8.1	9.7 a	19.4
0.5	58.0	62.0	60.0 ab	10.7	11.5	11.1 a	22.2
1.0	74.0	70.0	72.0 a	12.3	12.3	12.3 a	24.6
2.0	82.0	72.0	77.0 a	10.5	12.2	11.4 a	22.7
4.0	52.0	48.0	50.0 b	11.1	10.2	10.7 a	21.4
Mean[b]	67.2 A	62.4 A		11.2 A	10.9 A		

[a,b]Means within a column followed by the same lower case letter and means within a line followed by the same upper case letter do not differ significantly according to Tukey's test ($p \leq 0.05$).

Incorporation of a cytokinin into the medium (0.5 – 2.0 μM) was essential for enhancing the frequency of PLB formation from tTCL; however, a BA concentration above the optimum level (2 μM) inhibited PLB development during culture initiation and after the first subculture, as it was also observed with the lTCL method (Table 2). The most appropriate BA concentration for a high frequency of PLB regeneration was 2.0 μM, and it was independent of the type of protocorm section (apical or basal) used (Table 2). The higher average number of PLBs (9.3) per tTCL was therefore attained on the media with 1.0 μM BA, whereas the concentration of 4 μM produced fewer PLBs (5.8) after eight weeks (Table 2). Subculturing PLBs on medium containing the same BA concentration resulted in slow proliferation (Table 2). However, use of basal sections resulted in a higher average number of PLBs per explant (10.2) than those of the apical (8.3) sections after the first subculture at the same BA concentration (Table 2).

Rapid and direct PLB regeneration from lTCL and tTCL protocorms without mediation of a callus is an efficient method for mass propagation in *B. forbesii*. This factor is of most importance because plants produced by direct regeneration exhibit greater genetic stability than those produced from a callus, as reported by Sheelavanthmath et al. (2005) for *Aerides crispum*. Furthermore, this novel tissue culture method is more efficient than others *in vitro* culture methods making mass clonal propagation easier and more reproducible (TEIXEIRA DA SILVA, 2013), as reported for *C. aloifolium* and *D. nobile* (NAYAK et al., 2002) as well as for *D. candidum* (ZHAO et al., 2007).

The analysis of PLB production from protocorms using tTCL and lTCL technique showed that the latter produced more PLBs regardless of BA addition. In this study, the type of section and the explant used are therefore very important for regeneration using the TCL technique. Protocorms are also excellent explants for PLB induction and subsequent plant regeneration of *A. crispum* (SHEELAVANTHMATH et al., 2005), *Phalaenopsis gigantea* (MURDAD et al., 2006), and *Cymbidium* Sleeping Nymph (VYAS et al., 2010).

The type of lTCL segment showed no effect on PLB regeneration of *B. forbesii*, whereas segments from basal tTCL protocorms produced a greater number of PLBs (10.2) than segments from the apical part (8.3), only after the first subculture. Similar results were obtained for segments from the basal parts of PLBs in *Doritaenopsis* that responded better in proliferation of new PLBs (AMAKI; HIGUCHI, 1989). Murdad et al. (2006) also reported that using trimmed base protocorm culture method is an efficient *in vitro* technique for the rapid propagation of *P. gigantea* and the injuries caused by the cutting process may play an important role in the formation of new protocorms through proliferation. According to Ferreira et al. (2015), protocorm consist of meristematic cells which are rich in auxins and have high metabolic activity; so the removal of the leaf apex overcome the apical dominance, which combined with the addition of a cytokinin in the culture medium promoted the regeneration of PLBs, as reported for *Epidendrum secundum*.

Incorporation of a cytokinin into the culture medium is essential for enhancing the frequency of PLB formation in *B. forbesii*, regardless of TCL technique used.

Table 2. Effect of BA on PLBs induction in tTCL explants of *Brasilidium forbesii* cultured on WPM, eight weeks after culture initiation and after the first subculture.

BA concentration (μM)	PLBs regeneration (%)			Average number of PLBs per responsive explants			
Culture initiation	Basal	Apical	Mean[a]	Basal	Apical	Mean[a]	Total
0.0	36.0	30.0	33.0 bc	9.0	7.5	8.2 ab	16.5
0.5	46.0	32.0	39.0 ab	9.9	7.1	8.5 ab	17.0
1.0	42.0	36.0	39.0 ab	9.9	8.8	9.3 a	18.7
2.0	50.0	60.0	55.0 a	7.8	8.4	8.1 ab	16.2
4.0	14.0	18.0	16.0 c	6.6	5.1	5.8 b	11.7
Mean[b]	37.6 A	35.2 A		8.6 A	7.4 A		
1st Subculture							
0.0	34.0	32.0	33.0 ab	9.8	9.7	9.8 a	19.6
0.5	52.0	40.0	46.0 ab	9.9	7.2	8.6 a	17.1
1.0	50.0	44.0	47.0 a	11.3	10.0	10.6 a	21.3
2.0	52.0	66.0	59.0 a	9.6	9.1	9.4 a	18.7
4.0	14.0	28.0	21.0 b	10.2	5.5	7.9 a	13.4
Mean[b]	40.0 A	42.0 A		10.2 A	8.3 B		

[a,b]Means within a column followed by the same lower case letter and means within a line followed by the same upper case letter do not differ significantly according to Tukey's test ($p \leq 0.05$).

The analysis of PLB production using lTCL and tTCL explants showed that 2.0 μM of BA was more responsive for *B. forbesii*. Similarly, BA is also effective for regenerating other orchid species, such as *Dendrobium nobile* (NAYAK et al., 2002) and *Coleogyne cristata* (NAING et al., 2011).

Sheelavanthmath et al. (2005) also obtained the optimal results for *A. crispum* when protocorm sections were used as explants on a medium supplemented with 1.0 μM BA, and all the explants responded and developed an optimum of 49.1 PLBs per explant. They also demonstrated the importance of using juvenile explants such as protocorms (4-week-old) for *A. crispum*, and the responses observed by these authors may have been better than ours because we used older protocorms (24-week-old).

Shoot development and rooting

Shoot growth and root formation were observed in all the tested media after three weeks of culture, indicating that auxins are not necessary for these processes. The root and shoot length (2.7 cm), the average number of roots (3.4), and the fresh mass (0.15 g) were not show significantly different among all IBA concentrations tested. Similar results, in which shoots developed into well-rooted plants on growth regulator-free culture medium, were obtained for *A. crispum* (SHEELAVANTHMATH et al., 2005), *D. draconis* (RANGSAYATORN, 2009) and *O. flexuosum* (MAYER et al., 2010).

The addition of activated charcoal favored the development of more vigorous shoots, and the charcoal treatments were better than control with regard to the average number of roots and length of shoots. The addition of 3.0 g L^{-1} activated charcoal was beneficial for explant development (average number of roots, 4.2; longest root length, 2.1 cm; shoot length, 3.6 cm) (Table 3, Figure 1F).

Activated charcoal has been frequently used in tissue culture to improve cell growth and development and induce rooting of micropropagated shoots in several plants (THOMAS, 2008) Activated charcoal also improved the *in vitro* plant quality of *Miltonia flavescens* and *Laelia flava* (MORAES et al., 2005) and was efficient for *in vitro* rooting of *Dendrobium* hybrids (MARTIN; MADASSERY, 2006) in half-strength MS medium supplemented with 2.0 g L^{-1} charcoal and for *O. tigrinum* in MS medium with 1.0 g L^{-1} charcoal (MATA-ROSAS et al., 2011).

Table 3. Effect of activated charcoal on elongation and rooting of shoots from protocorm explants of *Brasilidium forbesii* cultured on WPM medium for eight weeks.

Activated charcoal (g L^{-1})	Average number of roots	Mean length of the longest root (cm)	Shoot length (cm)
0.0	2.2 ± 0.707 b	1.3 ± 0.845 a	1.6 ± 0.707 b
1.0	3.6 ± 2.121 a	1.9 ± 0.707 a	2.8 ± 2.121 a
2.0	3.5 ± 2.121 a	1.7 ± 1.060 a	3.0 ± 1.414 a
3.0	4.2 ± 0.707 a	2.1 ± 0.707 a	3.6 ± 1.414 a

Means within a column followed by the same letter do not differ significantly according to Tukey's test ($p \leq 0.05$).

Acclimatization of plantlets

Plantlets were readily acclimatized and established in all substrates showing the emergence of new leaves after four weeks, with a survival rate of 100% in greenhouse conditions. Comparisons between each of the variables assessed are shown in Table 4. Vermiculite alone or mixed in equal part with powder coconut (1:1) was the most efficient substrate only for root length (Table 4, Figure 1G). Similar results were obtained for *Dendrobium candidum* with 95% survival rate in the greenhouse using vermiculite as a substrate (ZHAO et al. 2007).

Based on our results, it can be concluded that thin longitudinal and transversal protocorm sections of *B. forbesii* produced PLBs in large numbers at a low cost. Adding BA (2.0 μM) was necessary to increase the regeneration frequency of PLBs, and rooting (100%) was not dependent on auxins. Plantlets were successfully acclimatized (100%) using vermiculite as a substrate. An advantage of this protocol is that it requires addition of growth regulator at only one step and in low concentration (2.0 μM BA). Moreover, if we germinate seeds from one capsule *in vitro*, at least 1,000 good quality protocorms can be produced after six months. The TCLs from protocorms grown on WPM supplemented with 2.0 μM BA can produce 17,479 plantlets from lTCL and 11,033 from tTCL after 14 months. Thus, TCL culture is a highly efficient culture system for regeneration of *B. forbesii* plants from protocorms.

Table 4. Effect of substrates used for acclimatization on *Brasilidium forbesii* plantlet development after 16 weeks.

Substrate	Average number of roots	Length of longest root(cm)	Shoot length (cm)	Fresh mass (g)
Tecnomax®	3.6 ± 2.828 a	1.4 ± 0.707 b	4.8 ± 0.707 a	0.1 ± 0.074 a
Vermiculite	4.0 ± 1.414 a	3.2 ± 2.121 a	6.1 ± 1.414 a	0.2 ± 0.148 a
Tecnomax® and vermiculite (1:1)	3.8 ± 0.707 a	2.0 ± 0.707 b	6.0 ± 1.060 a	0.2 ± 0.251 a
Tecnomax® and coconut powder (1:1)	3.0 ± 2.828 a	1.7 ± 0.707 b	4.6 ± 0.353 a	0.1 ± 0.074 a
vermiculite and coconut powder (1:1)	3.3 ± 0.707a	3.3 ± 1.060 a	5.6 ± 1.767 a	0.2 ± 0.022 a

Means within a column followed by the same letter do not differ significantly according to Tukey's test ($p \leq 0.05$).

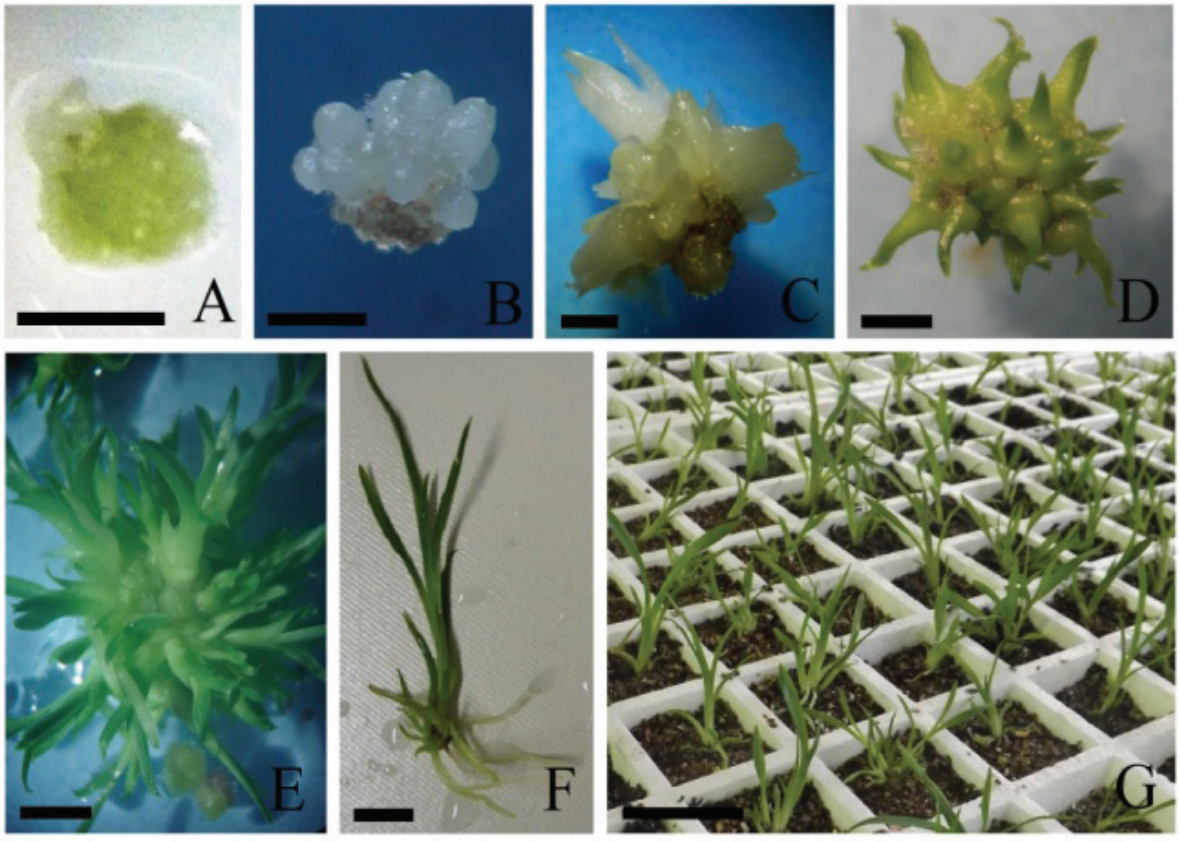

Figure 1. Plant regeneration and developing PLBs from protocorm through lTCL section of *Brasilidium forbesii* grown on WPM medium with 1.0 μM BA. (A) Initiation of PLB after two weeks in dark (bar = 1 mm); (B) New PLBs forming within three weeks in dark (bar = 1 mm); (C) Developing PLBs four weeks after culture in dark. (bar = 1 mm). (D) Initiation of shoots after four weeks in dark and two weeks of culture in a 16h/8h (light/dark) photoperiod (bar = 1 mm); E) Shoots cultured four weeks in dark and 12 weeks in a 16h/8h (light/dark) photoperiod (bar = 5 mm). (F) Plantlet developed on WPM medium supplemented with 3 g L^{-1} of activated charcoal (bar = 10 mm); (G) Transplanted plantlets after 16 weeks of acclimatization in the greenhouse (bar = 35 mm).

Conclusion

This is the first paper describing direct PLB development through culture of thin longitudinal and transversal section of *B. forbesii* from protocorms. The efficient initiation of PLBs and subsequent conversion into shoots using a cytokinin alone (BA) at a low concentration (2.0 μM) from protocorms provides a simple, inexpensive, and effective protocol for mass propagation and conservation of this ornamental orchid in a short period of time.

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