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Original Article

Optimization of efficient protocorm-like body (PLB) formation of *Phalaenopsis* and *Dendrobium* hybrids

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Abstract Optimization of the protocorm-like body (PLB) formation of *Phalaenopsis* and *Dendrobium* hybrids was performed by determining the effects of plant growth regulators (PGRs) and different parts and division sizes of the PLB. For both genera, the base part was the best for the proliferation of PLBs, yielding the highest number of PLBs on a PGR-free medium for *Phalaenopsis* and medium containing 0.1 μM NAA and 10.0μM BAP for *Dendrobium*. As regards the division size, four-division sections resulted in a higher PLB formation efficiency for *Phalaenopsis*, while two-division sections produced a higher PLB formation efficiency for *Dendrobium.* It is expected that these findings will be applicable to efficient PLB formation of other *Phalaenopsis* and *Dendrobium* orchids.

Keywords: Explant types, Orchids, Plant growth regulators (PGRs), Protocorm-like-body (PLB)

Introduction

With the global marketing of *Phalaenopsis* and *Dendrobium*, the orchid industry is now a substantial contributor to the economies of many ASEAN countries (Hew, 1994; Laws, 1995). Over 75% of all the orchids sold in the U.S. belong to the *Phalaenopsis* species, which replaced *Cattlyea* in the 1980s as the most popular orchid (Griesbach, 2002).

Phalaenopsis and *Dendrobium* are traditionally propagated by the cutting or division of offshoots, however, these methods slow the rate of multiplication and arrest the growth of the mother plant, making them ineffective for large scale production. Thus, *in vitro* propagation methods have been widely used for commercial production.

Many studies on the *in vitro* propagation of orchids have already been reported using various explants, such as stem nodes (Griesbach, 1983), leaf tissues/segments (Park et al., 2002), and root tip cultures (Park et al., 2003). Yet, *in vitro* multiplication using these methods is unfortunately very difficult and the rate of multiplication is also low. Thus, for the mass propagation of orchids, propagation using protocorms has been studied by Yam et al. (1991) and Chen et al. (2000). As a result, the production of PLBs from protocorms or PLBs and regeneration have been shown to be efficient (Chen and Chang, 2000; Chen and Chang, 2004; Naing et al., 2011). However, this requires precise cultural conditions, including the media composition and growth regulators (Chen et al., 2000; Chen and Chang, 2004). The horizontal sectioning of PLBs to produce next-cycle PLBs has also been demonstrated in the case of *Phalaenopsis* (Tanaka et al., 1984; Tanaka, 1987), along with high-frequency multiplication using similar techniques in the case of *Phalaenopsis gigantea* (Murdad et al., 2006).

Accordingly, this study attempted to optimize the precise media composition for the efficient *in vitro* propagation *of Phalaenopsis* and *Dendrobium* hybrids using different parts and division sizes of PLBs.

Materials and Methods

Plant materials

PLBs of *Phalaenopsis* and *Dendrobium* hybrids provided by Mingalardon Orchid Farm in Yangon, Myanmar, were used as the source of the explants.

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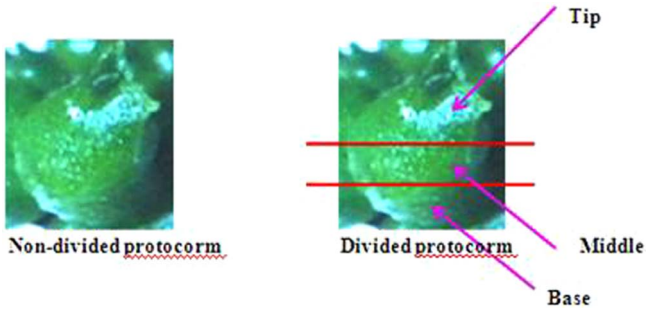

Figure 1A. Different initial materials: non-divided protocorm (intact) and divided protocorm.

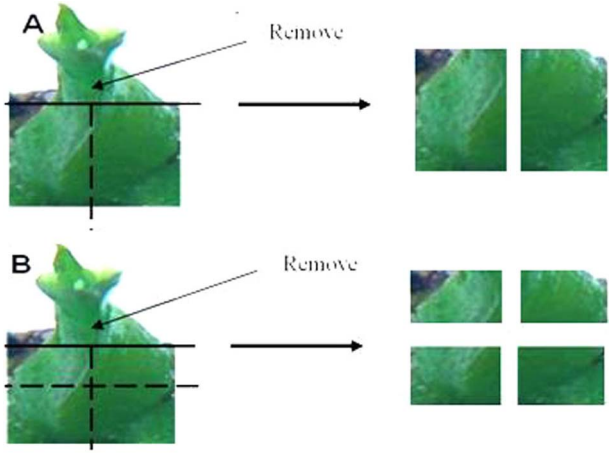

Figure 1B. Different sizes of divided protocorm: two-division sections (A) and four-division sections (B).

PLB formation from intact or different parts of PLB

Intact or transversally divided parts of PLBs (tip, middle, and basal portions) shown in Figure 1A were cultured on an MS medium supplemented with 15% (v/v) CW and different concentrations of α-naphthaleneacetic acid (NAA , 0, or 0.1 μM) in combination with 6-benzylaminopurine (BAP, 0, 0.5 or 10.0 μM).

PLB formation from different division sizes of intact PLB

For this experiment, the tips of the protocorms were trimmed off and the rest of the PLBs evenly sectioned into two-division sections (2-3 mm in length, 1-1.5 mm in width) or four-division sections (1-1.5 mm in length and width) using a surgical blade under aseptic conditions, as shown in Figure 1B. These explants were then cultured in the same media composition as described above.

Culture conditions

The pH of the medium was adjusted to 5.8 before gelling with a 7.0 g/L agar. All the media were autoclaved at 121°C and 105 kPa pressure for 15 minutes. The culture vessels were 100ml bottles that included 20 ml of the medium and were closed using cotton plugs. After culturing, the culture vessels were incubated in a culture room at 25±2°C under a 16-hour photoperiod using cool white fluorescent lamps with approximately 25 $\mu mol^{-1}m^{-2}s^{-1}$.

Experimental design and statistical analysis

The experiments were arranged using a Randomized Complete Block Design (RCB) with two factors. Five explants were used per treatment with seven replicates. The PLB formation frequency and mean number of PLBs per explant were recorded after 8 weeks of culture. The collected data were statistically analyzed using DMRT

Results and Discussion

PLB formation from intact or different parts of PLBs

When intact or different parts of the PLBs (tip, middle, and basal parts) were cultured on the media supplemented with different combinations of NAA and BAP, protuberances were observed for both orchid genera after 2 weeks of culturing, and the formation

Table 1. Effects of Different parts of PLBs and PGRs on frequency of PLB formation and mean number of PLBs per explants of *Phalaenopsis* hybrid and *Dendrobium* hybrid

PGRs (μM)		Esplant part	*Phalaenopsis* hybrid		*Dendrobium* hybrid	
NAA	BA		PLBs formation %	Mean no of PLBs per explant	PLLBs formation %	Mean no of PLBs per explant
0	0	NDP	0.0	0.0	6.7	2.5
		Tip	20.0	2.8	10.0	1.2
		Middle	40.0	11.7	8.0	12.7
		Base	44.0	13.3	51.4	11.0
0.1	5.0	NDP	0.0	0.0	8.0	12.1
		Tip	8.0	0.7	42.8	15.5
		Middle	20.0	3.0	45.0	9.0
		Base	32.0	11.0	53.3	17.3
0.1	10.0	NDP	0.0	0.0	20.0	3.2
		Tip	0.0	0.0	14.3	3.5
		Middle	0.0	0.0	55.0	13.3
		Base	0.0	0.0	57.1	18.7

of PLBs was subsequently observed after 6 weeks of culturing.

In both genera, the basal parts resulted in a higher percentage of PLB formation than the other parts (Table 1). However, the *in vitro* response to PLB formation by the two genera varied distinctly according to the different PLB parts and PGR concentrations used. No PLB formation was recorded from the intact PLBs of *Phalaenopsis*, whereas the intact PLBs of *Dendrobium* did respond. Moreover, for *Phalaenopsis*, although the basal part was found to be the best for PLB formation with most treatments, no response was observed when cultured on the medium containing 0.1 μM NAA and 10.0 μM BAP. In contrast, for *Dendrobium,* the basal part resulted in similar percentages with all treatments. In addition, for *Phalaenopsis,* PLB formation favored the PGR-free medium over the media containing PGRs, whereas, for *Dendrobium,* the media containing PGRs resulted in higher PLB formation.

Similar to PLB formation, for both genera, the basal part was found to have a more inductive effect on the number of PLBs than the other parts, although a higher number of PLBs per explant was generally obtained from *Dendrobium*. Notwithstanding, the highest number of PLBs per explant (11.6) was recorded from the basal part of *Phalaenopsis* cultured on a PGR-free medium (Figure 2A). Thus, among the tested media, the PGR-free medium showed a better response than the media with added PGRs. For *Phalaenopsis,* the middle part was found to have a more inductive effect on the number of PLBs per explant than the tip part, while the intact PLBs did not respond at all. In contrast, for *Debderobium,* the inductive effect of the protocorm parts on the number of PLBs varied distinctly according to the PGR concentration, although the number of PLBs induced by the basal part showed superiority with most treatments. Furthermore, for the basal part, the medium containing 0.1 μM NAA and 10.0 μM BAP was found to encourage an increase in the number of PLBs per explant (18.7) (Figure 1D).

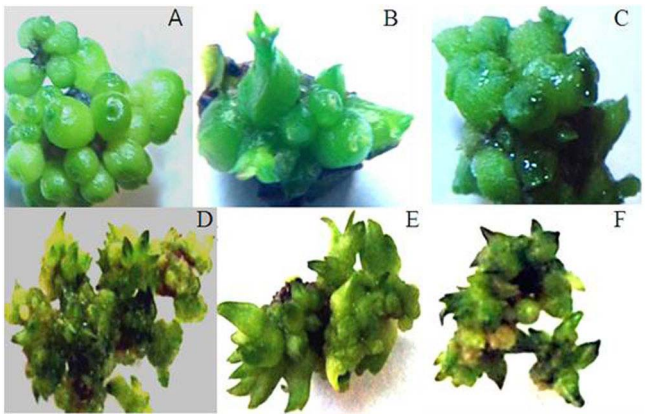

Figure 2. Proliferation of PLBs from protocorm of *Phalaenopsis* and *Dendrobium* hybrids: proliferated PLBs on basal part of protocorm (A), 2-division size (B), and 4-division size (C) of divided protocorms cultured on PGR-free medium in *Phalaenopsis* and cultured on 0.1 μM NAA + 10.0 μM BAP supplemented medium in *Dendrobium* (D, E, and F).

Therefore, the findings of this experiment provide a valuable message for PLB propagation from different parts of orchid PLBs. Dividing the PLBs resulted in more PLB formation than using intact PLBs. This was related to the wounds and the results of activating quiescent cells near the cut surface (Imaseki, 1985; Murdad et al., 2006). Murdad et al. (2006) also reported that this activation includes the initiation of cell proliferation and biochemical changes within the quiescent unwounded cells near the wound. Increased endogenous hormone production in certain plant organs after wounding may also have been one of the factors involved in PLB formation (Rosenstock et al., 1978). Among the protocorm parts used, the tip parts developed fewer PLBs than the basal or middle parts, and the site of PLB formation was not seemingly confined to the location of the apical meristem (Begum et al., 1994). Histological observation has also revealed that regenerated PLBs are generally formed from the epidermal layer of the posterior region of the protocorm (Chen and Chang, 2004). Thus, since the basal part consists of large parenchymatous cells and functions as a storage organ (Batygina et al., 2003), the basal part can be assumed as the most suitable for use as an explant.

In the case of *Phalaenopsis,* the maximum PLB formation was observed on a PGR-free medium. Chen and Chang (2004) also reported that NAA inhibited direct embryo formation from protocorms of *Phalaenopsis amabilis* var. *formosa* Shinmadzu. Therefore, it can be assumed that an NAA-added medium can inhibit PLB formation in some species of *Phalaenopsis*. Moreover, the type of explant may also be a factor, since the amount of PGR added to the culture medium can differ depending on the explant type, i.e., shoot, leaf, stem, or root. For *Phalaenopsis* and *Doritaenopsis*, Tokuhara and Mii (1993) reported that the highest frequency of PLB formation was obtained from shoot tips on a medium supplemented with 0.1 mg/L NAA and 20 mg/L BA. Meanwhile, in the case of root explants, the highest frequency of PLB formation was reported to occur on a medium supplemented with 2.3 μM TDZ (Park et al., 2003). Leaf and stem explants have also been reported to require a PGR-supplemented medium to induce PLBs (Murthy and Pyati, 2001; Tee et al., 2010; Luo et al., 2008; Tanaka, 1992). In contrast, protocorm explants have been shown to produce the maximum PLB formation on a culture medium without PGR (Murdad et al., 2006). Thus, the PLB propagation results of *Phalaenopsis* in the current study were similar to the previous findings of Murdad et al. (2006).

Conversely, for *Dendrobium*, the 0.1 μM NAA+10.0 μM BAP added medium resulted in the maximum PLB formation. Tee et al. (2010) previously reported that BAP was the most efficient for PLB formation from shoot tip cultures of *Dendrobium*, plus the production of PLBs was very high in the presence of NAA. The *in vitro* response to PGRs has also been found to be different between *Phalaenopsis* and *Dendrobium* hybrids, seemingly due to the content of endogenous substances depending on the genotype.

Table 2. Effects of different sizes of explants and PGRs on frequency of PLB formation and mean number of PLBs per explants of *Phalaenopsis* hybrid and *Dendrobium* hybrid

PGRs (μM)		Esplant size	*Phalaenopsis* hybrid		*Dendrobium* hybrid	
NAA	BA		PLBs formation %	Mean no of PLBs per explant	PLLBs formation %	Mean no of PLBs per explant
0	0	Two-Division	20.0	3.7	26.7	8.0
		Four-division	36.0	8.0	0.0	0.0
0.1	5.0	Two-Division	6.7	2.0	30.0	12.3
		Four-Division	0.0	0.0	24.0	6.7
0.1	10.0	Two-Division	0.0	0.0	36.7	15.3
		Four-Division	0.0	0.0	20.0	11.7

Thus, the type and level of PGR necessary in an *in vitro* culture apparently depend on the species, genotype and explant size (Kane, 1996).

For the efficient regeneration of *Phalaenopsis* protocorms, it was very encouraging that the MS medium without PGR worked well in this study. However, in the case of *Dendrobium*, the medium containing (0.1 μM NAA+5.0 μM BAP) and (0.1 μM NAA+10.0 μM BAP) was required to obtain the maximum PLB formation. Therefore, each genotype needs a specific PGR regime even when using the same kind of explant.

In brief, based on the findings in this study, further experimentation is needed to identify suitable culturing methods, such as liquid cultures, shaking cultures, and explant orientation, for more efficient regeneration protocols.

PLB formation from different division sizes of PLB

The effects of the different sizes of the explants and PGRs on the frequency of PLB formation by the *Phalaenopsis* and *Dendrobium* hybrids are shown in Table 2. The highest PLB formation frequency (36%) resulted from the four-division sections of *Phalaenopsis* on the PGR-free medium, while the two-division sections of *Dendrobium* showed the highest PLB formation (36.7%) when cultured on the medium with 0.1 μM NAA and 10.0 μM BAP. Although the number of PLBs per explant did not differ significantly between the two genera for the different sizes of the divided protocorms, the maximum number of PLBs induced per explant did vary. For *Phalaenopsis* the number of PLBs (8.0) per explant was higher from the four-division sections than from the two-division sections on the PGR-free medium (Figure 2B and C), whereas, for *Dendrobium,* the number of PLBs (15.3) per explant was higher from the two-division sections than from the four-division sections on the 0.1 μM NAA and 10.0 μM BAP-added medium (Figure 2E and F).

Thus, based on the results of this experiment, dividing the protocorms into four-division sections produces the maximum PLB formation in the case of *Phalaenopsis*, whereas two-division sections produce the maximum PLB formation in the case of *Dendrobium*. These differences in the regeneration capability of the two orchid genera would seem to be influenced by the genotype. Tanaka (1992) also reported that the regeneration capability in terms of PLB formation could even differ within one genus. It is a routine pathway that protocorms or PLBs are divided into small sections and inoculated on a medium. One small section of a protocorm or a PLB can then induce many PLBs after 6-8 weeks of culture. In this way, PLB propagation can be expanded to a commercial level. Therefore, sectioning a protocorm is an important factor in propagation, as each section plays a vital role in the multiplication of PLBs.

In this study, for *Phalaenopsis*, the medium without PGR obtained the maximum survival percent and PLB formation for both sizes of explant. However, for *Dendrobium*, 0.1 μM NAA and 10.0 μM BAP needed to be added to obtain the highest PLB formation frequency and number of PLBs per explant. Plus, these findings confirmed the results of the first experiment, i.e., that PLB proliferation is related to the genotype and PGR requirements.

In conclusion, for maximum regeneration capability, this study found that a divided protocorm was more efficient than a non-divided protocorm for both *Phalaenopsis* and *Dendrobium* hybrids. More specifically, the basal part of the protocorm was most efficient as an explant for PLB formation. However, it is recommended that both the basal and middle parts are used to obtain satisfactory results in PLB propagation. When dividing the protocorm, in the case of *Phalaenopsis,* a higher percentage of PLB formation and the maximum number of PLBs per explant were observed from four-division sections, whereas, in the case of *Dendrobium*, the same results were obtained from two-division sections. For both orchid genera, a PGR-free medium produced satisfactory results for PLB formation, although for *Dendrobium*, the addition of 0.1 μM NAA and 10.0 μM BAP showed the best results. Yet, while the PGR level can differ depending on the genotype or species, protocorm sectioning can be used for any orchid. Therefore, this finding will be applicable to other *Phalaenopsis* and *Dendrobium* orchids.

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