Regeneration of Plantlets Through PLB (Protocorm-Like Body) Formation in *Phalaenopsis* 'Join Angle X Sogo Musadian'

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Abstract. Selection and regeneration of specific hybrids of *Phalaenopsis* typically takes a long time since this plant usually reproduces through seeds. This study was conducted to examine the best medium and explants for regeneration of *Phalaenopsis* 'Join Angle x Sogo Musadian'. *In vitro* and *in vivo* roots and leaves were used as explants, which were cultured in half-strength Murashige and Skoog medium supplemented with various combinations of plant growth regulators (TDZ, 2,4-D, NAA, BAP and IAA). The results showed that the *in vitro* roots produced the highest number of PLBs (49.3 PLBs) when they were cultured in medium containing 0.5 ppm NAA, 5 ppm BAP, and 0.5 ppm IAA. The *in vitro* leaf explants also regenerated PLBs, however, only two PLBs developed, i.e. when they were cultured in medium containing 1 ppm TDZ and 2 ppm 2,4-D. PLB germination into plantlets was performed by culturing each PLB on MS medium without plant growth regulators. Based on the results of the present study, it can be concluded that *in vitro* roots and half-strength MS medium supplemented with 0.5 ppm NAA, 5 ppm BAP, and 0.5 ppm IAA are the best explant and best medium respectively for plant regeneration through PLBs.

Keywords: *growth regulators;* in vitro *plant regeneration;* Phalaenopsis*; protocorm-like bodies; thidiazuron.*

1 Introduction

Phalaenopsis, also known as the moth orchid, is one of the most famous commercial orchids for potted plants and cut flowers. According to the American Orchid Society more than 75% of orchids in the market is from the genus *Phalaenopsis* [1]. *Phalaenopsis* has high aesthetic value and performs a good impression on consumers due to its attractive colors and shapes, as well as its long period of flowering time.

Efforts have been made to improve the quality of commercial orchids, especially through cross-pollination between selected plants. The hybrid will then regenerate through seed germination. This method has some disadvantages

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since the hybrid will show very diverse variations of phenotypes. Further selection of hybrid with desirable traits needs to be conducted, although a long period for selection is required. *In vitro* culture, i.e. through micropropagation, can be used as an alternative method for the regeneration of selected orchids in order to obtain uniform plants over a short time period. The propagule usually has the same character as its parent.

Attempts to regenerate *Phalaenopsis* orchids have been conducted by several researchers [2-4]. Propagation through PLBs has been obtained for the same orchid genus [4-6]. Different types of plant growth regulators (PGRs) and sources of explants have been used to induce PLBs. PLB induction on MS medium with addition of BAP and NAA [7] or TDZ [4] using leaf explants from plantlets have been reported. The use of 5.4 µM NAA and 4.5 µM TDZ has been applied to induce PLBs and plantlets from flower stalks of *Doritaenopsis* orchids [8].

The present study was carried out to investigate the possibility of inducing PLBs from *in vitro* and *in vivo* root and leaf explants of *Phalaenopsis* 'Join Angle x Sogo Musadian'. This orchid is a hybrid of *Phalaenopsis* 'Join Angle' and *Phalaenopsis* 'Sogo Musadian', which has large and thick leaves, a long spike (around 80 cm) and 6-10 white flowers. The diameter of each flower is around 8-10 cm.

2 Materials and Methods

2.1 Plant Materials

The explants used in this study were leaves and roots from *in vivo* and *in vitro* cultures. Young leaves and roots from a 2.5-year old *Phalaenopsis* 'Join Angle X Sogo Musadian' were used as the source for the *in vivo* explants. Meanwhile, the inflorescent nodes (Figure 1A) of this orchid were initially induced to produce plantlets (Figure 1B) in MS media supplemented with 3 ppm BAP. The leaves and roots from these plantlets were then used as the source for the *in vitro* explants.

Figure 1 Plantlets from the inflorescent stalk. (A) The first shoot developed in the inflorescent node. (B) Regenerated pantlets.

2.2 Induction of PLBs

The *in vivo* leaf and root explants were surface sterilized with 10% NaClO for 8 min and rinsed with sterilized distilled water prior to culture in the *in vitro* condition. All explants (from both *in vitro* and *in vivo* source) were then cultured on half-strength MS [9] solid medium supplemented with various combinations of plant growth regulators, namely TDZ (1 or 3 ppm), 2,4-D (1 or 2 ppm), NAA (0.5 ppm), IAA (0.5 ppm), and BAP (1, 5 or 10 ppm) (Table 1). The pH of the media was adjusted to 5.6-5.8. The cultures were placed in the dark for 1 month and then transferred to an incubation room for incubation under a 16-hour photoperiod. Subcultures were conducted every 4 weeks.

Table 1 Composition of medium for PLB induction.

No.	Concentration of Plant Growth Regulator (ppm)				
	TDZ	2,4-D	NAA	IAA	BAP
1	1	1	-	-	-
2	1	2	-	-	-
3	3	1	-	-	-
4	3	2	-	-	-
5	-	-	0.5	0.5	1
6	-	-	0.5	0.5	5
7	-	-	0.5	0.5	10

2.3 Regeneration of Plantlets from PLBs

Developed PLBs were inoculated individually; they were then transferred into half-strength MS medium without plant growth regulator. The pH of the medium was adjusted to 5.6-5.8. These cultures were then incubated under a 16-hour photoperiod and subcultured every 4 weeks.

3 Results and Discussion

3.1 The Effect of Plant Growth Regulator in PLB Induction

In this research, PLBs were formed in *in vitro* and *in vivo* root explants as well as *in vitro* leaf explants. Fresh hand section of the PLBs showed that the structure originated from epidermal and subepidermal cells of the *in vitro* leaf explants was divided actively to form protuberances (Figure 2). The *in vivo* leaf explants did not regenerate PLBs. Instead, these explants formed embryogenic cells (Figure 3A and 3B) when they were cultured in media containing TDZ and 2,4-D or NAA, IAA and BAP (Table 2). These protuberance(s) continued to multiply (Figure 3C, 3D and 3E) and developed to form PLBs after 16-24 weeks of culture (Figure 3F). The formed PLBs shot primordia after 18-24 weeks of culture (Figure 3G), and shoots and roots regenerated after 32 weeks

of culture (Figure 3H). Similar results have been observed in *Oncidium* orchids [10], where the PLBs were directly differentiated from the leaf epidermal tissue. In *Doritaenopsis* orchids, PLBs have been developed from subepidermal tissue [11]. In some other orchids, regeneration of PLBs occurred indirectly through embryogenic callus culture [12,13]. PLB pattern formation, therefore, varies among Orchidaceae depending on the explant tissue used and the culture condition.

Table 2 Morphogenic response in various kinds of explants cultured in media supplemented with various combinations of growth regulators.

Medium	Explant types			
	In Vitro		*In Vivo*	
	Root	Leaf	Root	Leaf
1 ppm TDZ + 1ppm 2,4-D	-	Embryogenic cells	-	Embryogenic cells
1 ppm TDZ + 2ppm 2,4-D	-	Embryogenic cells	-	PLBs
3 ppm TDZ + 1ppm 2,4-D	-	Embryogenic cells	-	Embryogenic cells
3 ppm TDZ + 2ppm 2,4-D	-	Embryogenic cells	-	Embryogenic cells
0.5 ppm NAA + 1 ppm BAP + 0.5 ppm IAA	-	-	PLBs	Embryogenic cells
0.5 ppm NAA + 5 ppm BAP + 0.5 ppm IAA	PLBs	-	PLBs	Embryogenic cells
0.5 ppm NAA + 10 ppm BAP + 0.5 ppm IAA	-	-		Embryogenic cells

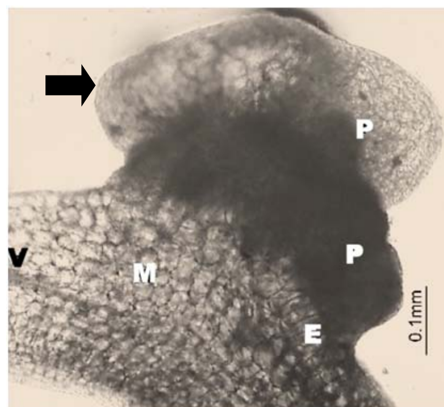

Figure 2 Fresh hand section of *in vitro* leaf explant with PLB (arrow) that is formed on its surface. Note: E – epidermis; M – mesophyll; P – protuberances; V – vascular tissue.

The *in vitro* leaf explants produced PLBs following culture in medium containing TDZ and 2,4-D. In medium containing NAA, BAP and IAA, these explants formed embryogenic cells (Figure 3A). A similar result is mentioned by Chen & Chang [4], where the combination of TDZ and 2,4-D could induce embryogenic callus in *Phalaenopsis* leaf explants. Cui, *et al*. [14] stated that TDZ is commonly used to induce PLB in orchids. Kuo, *et al*. [3], Chen & Chang [4] and Niknejad, *et al.* [6] obtained similar results when TDZ was used for PLB induction in leaf explants of *Phalaenopsis* orchids. When the existence of TDZ inside the cell is relatively stable compared to other growth regulators, it will not be degraded easily by cytokinin oxidase [14]. TDZ plays a role in regulating the endogenous hormone effect, providing nitrogen in the tissue and also stimulating the ROS compound that plays a role in somatic embryo formation [15,16]. Vasser, *et al.* [17] showed that TDZ can modulate endogenous auxins, which in synergism with cytokinins constitute the inductive signal for embryogenic expression to induce somatic embryogenesis in geraniums. In orchids, PLBs are considered somatic embryos because cytologically they produce a similar hydroxyproline-rich protein (HGRP), a cell wall marker protein, as zygotic embryo [18].

Table 3 Total average number of PLBs that arose from various explants.

Medium	Number of PLBs			
	In Vitro		***In Vivo***	
	Leaf	**Root**	**Leaf**	**Root**
1 ppm TDZ + 1ppm 2,4-D	-	-	-	-
1 ppm TDZ + 2ppm 2,4-D	2	-	-	-
3 ppm TDZ + 1ppm 2,4-D	-	-	-	-
3 ppm TDZ + 2ppm 2,4-D	-	-	-	-
0.5 ppm NAA + 1 ppm BAP + 0.5 ppm IAA	-	27.33	-	-
0.5 ppm NAA + 5 ppm BAP + 0.5 ppm IAA	-	49.33	-	2
0.5 ppm NAA + 10 ppm BAP + 0.5 ppm IAA	-	-	-	-

In the present study, numerous PLBs were developed from *in vitro* and *in vivo* root explants following culture in medium containing 0.5 ppm NAA, 0.5 ppm IAA, and 1 ppm or 5 ppm BAP (Table 3). The ability of root explants to produce PLBs has been observed in *Cyrtopodium paranaense* [19]. The growth regulators used in those experiments, however, were different from the growth regulators used in our experiment. PLBs were developed after culture in medium containing 10.2 μM IAA and 9 μM TDZ. Meanwhile, research conducted by Tokuhara & Mii [7] showed that PLBs could be induced using 0.5 μM NAA and 4.4 μM BAP in *Phalaenopsis* shoot explants. Cytokinin (BAP) and auxin (IAA and NAA) are frequently used to produce somatic embryos as well as PLBs. Exogenous auxins (NAA and IAA) and cytokinin (BAP) can stimulate proliferation and cell differentiation to form somatic embryos or PLBs [19]. Pasternak, *et al.* [20] have stated that exogenous auxin and cytokinin can

activate kinase proteins that are related with cdc2 protein activity. It plays a role in activating and stopping the process of cell division. Utami, *et al*. [21] explained that more cdc2 proteins were found in the leaf explants of the *Phalaenopsis* orchid after culturing it in media containing 2 ppm NAA.

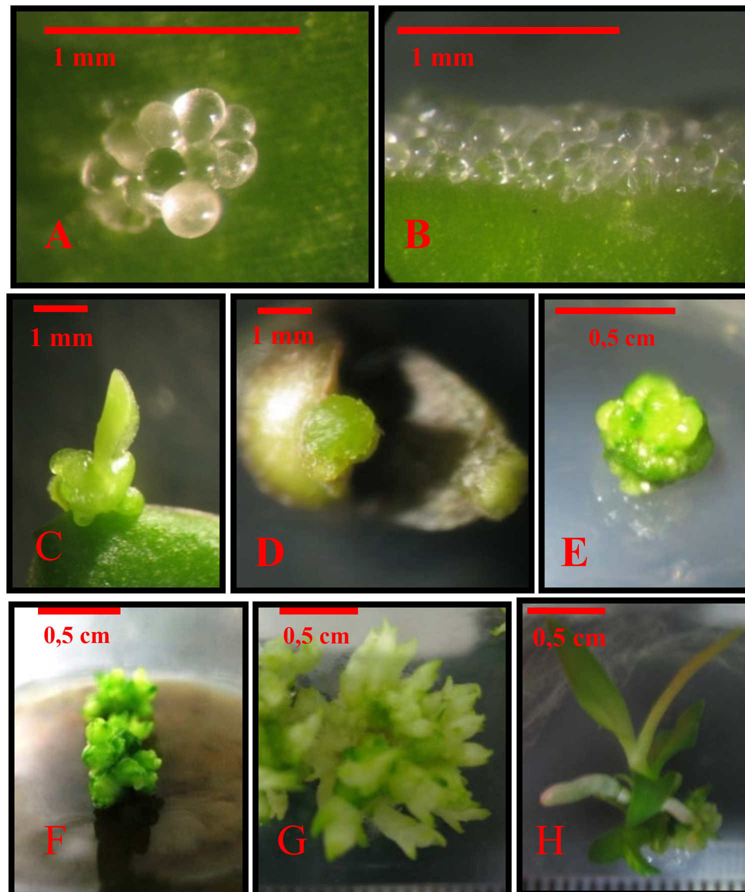

Figure 3 (A) Embryogenic cells on *in vitro* leaf explants; (B) embryogenic cells on *in vivo* leaf explants; (C) PLBs on *in vitro* leaf explants; (D) beginning of PLBs forming on *in vivo* root explants; (E) beginning of PLBs forming on *in vitro* root explants; (F) PLBs; (G) multiplicated PLBs; (H) plantlet.

3.2 Plantlet Regeneration from PLBs

In this study, all PLBs germinated into plantlets 8 weeks after being transferred into germination medium. Each PLB still developed additional shoots; about 3.5 ± 1.93 plantlets were formed in each PLB. A protocorm-like body (PLB) is a structure that possesses leaf and root primordia, so this structure still keeps its

ability to form new leaves, roots or even shoots when it is cultured in basal medium without growth regulators. The studies of Kuo, *et al.* [3], and Chen & Chang [4] and Young, *et al*. [5] also showed that PLBs can germinate to become plantlets when they are cultured in media without plant growth regulators.

4 Conclusions

In this study, PLBs could be induced from *in vitro* leaves and roots as well as *in vivo* roots. The highest number of PLBs was regenerated from *in vitro* root explants during culture in medium containing 0.5 NAA, 5 ppm BAP and 0.5 ppm IAA. All of the induced PLBs succeeded to germinate into plantlets in half-strength MS media without plant growth regulator.

References

[1] Griesbach, R.J., *Development of Phalaenopsis Orchids for the Mass-Market*, Trends in New Crops and New Uses, Janick, J. & Whipkey, A. Eds, ASHS Press, Alexandria, pp. 458-465, 2002.

[2] Košir, P., Škof, S. & Luthar, Z., *Direct Shoot Regeneration from Nodes of Phalaenopsis Orchids*, Acta Agriculturae Slovenica, **83**, pp. 233-242, 2004.

[3] Kuo, H.L., Chen, J.T. & Chang, W.C., *Efficient Plant Regeneration Through Direct Somatic Embryogenesis from Leaf Explants of Phalaenopsis 'Little Steve'*, *In Vitro* Cell Development Biology-Plant, **41**, pp. 453-456, 2004..

[4] Chen, J.T. & Chang, W.C., *Direct Somatic Embryogenesis and Plant Regeneration From Leaf Explants of Phalaenopsis amabilis*, Biologia Plantarum, **50**(2), pp. 169-173, 2006.

[5] Young, P.S., Murthy, H.N. & Yoeup, P.K., *Mass Multiplication of Protocorm Like Bodies Using Bioreactor System and Subsequent Plant Regeneration in Phalaenopsis*, Plant Cell, Tissue and Organ Culture, **63**, pp. 67-72, 2000.

[6] Niknejad, A., Kadir, M.A. & Kadzimin, S.B., *In Vitro Plant Regeneration from Protocorms-Like Bodies (PLBs) and Callus of Phalaenopsis gigantea (Epidendroideae: Orchidaceae)*, African Journal of Biotechnology, **10**(56), pp. 11808-11816, 2011.

[7] Tokuhara, K. & Mii, M., *Induction of Embryogenic Callus and Cell Suspension Culture from Shoot Tips Excised from Flower Stalk Buds of Phalaenopsis (Orchidaceae), In Vitro* Cell Development Biology-Plant, **37,** pp. 457-461, 2001.

[8] Vendrame, W.A. & Maguire, I., *In Vitro Propagation and Plantlet Regeneration from Doritaenopsis Purple Gem 'Ching Hua' Flower Explant*, Hortscience, **42**(5), 1256-1258, 2007.
[9] Murashige, T. & Skoog, F. *A Revised Medium for Rapid Growth and Bioassay with Tobacco Tissue Culture*, Physiologia Plantarum, **15**, pp. 473-495, 1962.
[10] Chen, J.T., Chang, C. & Chang, W.C., *Direct Somatic Embryogenesis on leaf explants of Oncidium 'Gower Ramsey' and Subsequent Plant Regeneration,* Plant Cell Reports, **19**, pp. 143-149. 1999
[11] Park, S.Y., Yeung, E.C., Chakrabarty, D. & Paek, K.Y., *An Efficient Direct Induction of Protocorm-like Bodies from Leaf Subepidermal Cells of Doritaenopsis Hybrid using Thin-section Culture,* Plant Cell Reports, **21**, pp. 46-51, 2002.
[12] Ishii, Y., Takamura, T., Goi, M. & Tanaka, M., *Callus Induction and Somatic Embryogenesis of Phalaenopsis*, Plant Cell Reports, **17**, pp. 446-450, 1998.
[13] Tokuhara, K. & Mii, M., *Highly-efficient Somatic Embryogenesis from Cell Suspension Cultures of Phalaenopsis Orchids by Adjusting Carbohydrate Sources*, *In Vitro* Cellular & Developmental Biology. Plant, **39**, pp. 635-639, 2003
[14] Cui, J., Liu, J., Deng, M., Chen, J. & Henny, R.J., *Plant Regeneration through Protocorm-like Bodies Induced from Nodal Explants of Syngonium podophyllum 'White Butterfly'*, HortScience, **43**, pp. 2129-2133, 2008.
[15] Schulze, J., *Improvements in Cereal Tissue Culture by Thidiazuron: A Review*, Fruit, Vegetable and Cereal Science and Biotechnology,**1**, pp. 64-79, 2001.
[16] Murthy, B.N.S., Murch, S.J. & Saxena, P.K., *Thidiazuron: A Potent Regulator of In Vitro Plant Morphogenesis, In Vitro* Cell Development Biology-Plant, **34**, pp. 267-275, 1998.
[17] Vasser, C., Qureshi, J.A., Gill, R. & Saxena, P.K., *Morphoregulatory Role of Thidiazuron: Substitution of Auxin and Cytokinin Requirement for the Induction of Somatic Embryogenesis in Geranium Hypocotyl Cultures,* Plant Physiology, **99**, pp. 1704-1707.1992
[18] Lee, Y-I., Hsu, S-T. & Yeung, E.C., *Orchid Protocorm-like Bodies are Somatic Embryos,* American Journal of Botany, **100**(11), pp. 2121-2131, 2013.
[19] Guo, W.L.G., Chang, Y.C.A. & Kao, C.Y.K., *Protocorm-like Bodies Initiation from Root Tips of Cyrtopodium paranaense (Orchidaceae),* Hortscience, **45**, pp. 1365-1368, 2010.
[20] Pasternak, T., Miskolczi, P., Ayaydin, F., Meszaros, T., Dudits, D. & Feher, A., *Exogenous Auxin and Cytokinin Dependent Activation of*

CDKs and Cell Division in Leaf Protoplast-derived Cells of Alfalfa, Plant Growth Regulation, **32**, pp. 129-141, 2000.

[21] Utami, E.S.W., Sumardi, I., Taryono & Semiarti, E., *Effect of α-Naphtaleneacetic Acid (NAA) on Somatic Embryogenesis of Moth Orchid Phalaenopsis amabilis (L.)*, Biodiversitas, **8**(4), pp. 295-299, 2007. (Text in Indonesian)