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CELL BIOLOGY AND MORPHOGENESIS

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An efficient direct induction of protocorm-like bodies from leaf subepidermal cells of *Doritaenopsis* hybrid using thin-section culture

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Abstract High-frequency protocorm-like body (PLB) formation directly from thin leaf sections of *Doritaenopsis* hybrid was achieved in order to develop a mass-scale propagation system. Concentrated efforts were made to study the effects of different cytokinins on in vitro PLB induction from thin leaf sections. Among the cytokinins tested, thidiazuron (TDZ) was found to be a more effective inducer of PLBs than benzyladenine and zeatin. A modified Murashige and Skoog medium supplemented with 9.0 μ*M* TDZ was found to be the optimum concentration for PLB development from thin leaf sections of *Doritaenopsis* hybrid. Of the two different explant types used in the present experiment, the highest percentage of PLB formation (72.3%) and highest number of PLBs (18) per explant were observed on thin leaf sections (1 mm thick), while only 20% (4.3 per explant) of comparatively large leaf segments (5 mm thick) were able to produce PLBs under the same culture conditions. Light microscopy observations indicated that the initial cell divisions for PLB formation occurred on the region near the cut surface and that an intact epidermal layer appeared to play an important role in PLB formation. Proembryo initiation occurred from several cells just beneath the intact epidermal cell, and globular PLBs were clearly visible after 3 weeks of culture and subsequently developed into mature PLBs.

Keywords *Doritaenopsis* · Protocorm-like body · Thidiazuron · Thin-section culture · Subepidermal cells

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Abbreviations *BA*: Benzyladenine · *CLB*: Callus-like body · *PLB*: Protocorm-like body · *TDZ*: Thidiazuron

Introduction

A wide range of attractive hybrids, varieties, or cultivars of monopodial orchids of the genus *Phalaenopsis*, which includes *Doritaenopsis* (*Doritis* × *Phalaenopsis*), have become economically important, mainly for use as cut flowers and in the potted plant industry. The need for mass propagation of selected elite genotypes has led to the development of several in vitro methods using shoot tips (Intuwong and Sagawa 1974), flower-stalk cuttings (Reisinger et al. 1976), root tips (Tanaka et al. 1976), and leaf segments (Kano 1971) as explants. Leaf segments derived from flower-stalk cuttings are preferred to those from adult plants because their use does not damage the mother plants (Tanaka 1992; Park et al. 1996, 2000). However, the number of PLBs produced is still low. Another problem encountered during the in vitro culture of *Phalaenopsis* is the long time period needed for the growth and multiplication of PLBs. Associated with the long culture period is somaclonal variation, which becomes a frequent and consistent event (Chen et al. 1998), often resulting in the development of undesirable characteristics. In order to propagate *Phaelaenopsis* efficiently, it is therefore necessary to develop an efficient and rapid method of PLB induction and also to reduce the time required for the growth and multiplication of PLBs.

Recently, a thin-cell layer method has been shown to be an efficient means for plant regeneration for orchids. Le et al. (1999) obtained high-frequency shoot regeneration from *Rhynchostylis gigantea* using thin cell layers of leaves. Similarly, a thin-section culture system for rapid regeneration of the monopodial orchid hybrid *Aranda* Deborah has been developed by Lakshmanan et al. (1995). However, this concept has not been exploited as a method for rapid plant production.

In this paper, we describe an efficient and rapid method of PLB regeneration for *Doritaenopsis* hybrid that

utilizes thin sections obtained from leaves as explants, and we report the efficiency of TDZ in stimulating PLB regeneration. The origin and development of PLBs from thin leaf sections is also detailed.

Materials and methods

Plant material

Leaves from 3-month-old in vitro plantlets were used as the source of explant material. These plantlets were derived from PLBs of *Doritaenopsis* New candy × *D.* (Mary Anes × Ever spring), the latter of which were obtained from in vitro culture of flower-stalk sections as described by Park et al. (2000).

Thin-section culture

Five to seven segments, 1 mm thick, were cut transversely using a sharp surgical blade from the proximal (basal) portion of the youngest leaf of each plantlet. The sections were placed cut side down onto plastic petri dishes (10 cm in diameter) containing 25 ml of half-strength MS medium (Murashige and Skoog 1962) supplemented with different concentrations of TDZ (4.5–22.5 μ*M*), BA (2.2–22.2 μ*M*), or zeatin (2.3–22.8 μ*M*), 20% (v/v) coconut water, 10 mg l^{-1} adenine sulfate, and solidified with 2.3 g l^{-1} Gelrite (Duchefa, Haarlem). Since the usual thickness of leaf explants used for regeneration is 5 mm, leaf explants of this thickness were also cultured as controls. The thicker leaf explants were cultured using the same regeneration medium as above supplemented with 9.0 μ*M* TDZ, which had been determined to be the optimum concentration for PLB regeneration (Park, unpublished results). Approximately 40–42 leaf sections were cultured for thin-section culture and 15–16 leaf segments were cultured for thick leaf culture.

The pH of the medium was adjusted to 5.5, and all media were autoclaved for 20 min at 115°C (1.37×10^5 Pa). Cultures were incubated for 1 week in the dark at 27°C, after which they were transferred for further culture to a tissue culture room maintained at 25±1°C and a 16/8-h (light/dark) photoperiod with light supplied by cool-white fluorescent lamps (Kumho FL40D, Korea) at an intensity of 10 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF). After 6 weeks of culture, explants were evaluated in terms of percentage PLB formation and number of PLBs and CLBs per explant. Percentage of PLB formation was calculated based on the number of explants forming PLBs as a percentage of the total number of explants. For the number of PLBs per explant, only PLBs approximately 1 mm in size, with a round shape and shiny surface were counted. Irregular shaped bodies were counted as CLBs.

Plantlet regeneration and transplantation of plantlets

In order to obtain plantlets, we transferred PLBs to a modified Hyponex medium [Kano 1965; 6.5 N-4.5 P-19 K (g l^{-1}) + 20 N-20 P-20 K (g l^{-1})] supplemented with 30 g l^{-1} potato homogenate, 2 g l^{-1} peptone, 0.5 g l^{-1} activated charcoal (Duchefa), 20 g l^{-1} sucrose, and 5.5 g l^{-1} plant agar (Duchefa). The pH was adjusted to 5.5 before autoclaving. The cultures were maintained at 25°C under 16/8-h (light/dark) photoperiod (light intensity: 30 μmol m^{-2} s^{-1} PPF), and the plantlets were subcultured at 8-week intervals to fresh medium. The plantlets (5–6 cm in height) were transplanted to pots containing sphagnum moss and grown in the greenhouse under conditions of high humidity (60–70%) at a day/night temperature of 25/15°C and a 16/8-h (light/dark) photoperiod (light intensity: 500 μmol m^{-2} s^{-1} PPF.

Light microscopy

For histological observations, samples collected during different culture periods were fixed in a solution containing 2.5% glutaraldehyde and 1.6% paraformaldehyde buffered with 0.05 *M* phosphate buffer, pH 6.8, for 24 h at 4°C. Samples were dehydrated in an alcohol series and then embedded in Technovit 7100 (Kulzer, Germany) according to Yeung (1999). Serial 3-μm-thick sections were cut with glass knives on a Reichert-Jung 2040 Autocut rotary microtome. The sections were stained with Periodic acid Schiff's reaction for total carbohydrates, counterstained with amido black 10B for proteins or toluidine blue O for general histological organization (Yeung 1984), and then examined and recorded on Kodak color print film, ASA 200, using a Leica Aristoplan light microscope. At least 15 samples were processed and examined for each time point.

Ethylene measurement

Ethylene measurements were carried out using a gas chromatography (Hewlett Packard 6400, Germany) equipped with a porapack-Q column, a flame ionization detector, and an integrator. The operation temperatures for oven, injector and detector were 100°C, 150°C, and 200°C, respectively. N_2 at a flow rate of 30 ml min^{-1} was used as the carrier gas.

Experimental design and data analysis

Experiments were carried out in a randomized design and repeated twice with each treatment having five replicates. The data were subjected to Duncan's multiple range test using the SAS program (SAS Institute, Cary, N.C.).

Results

The survival of leaf thin sections and the production of PLBs were dependent on the types and concentrations of cytokinins in the medium. Among the different cytokinins tested, both maximum percentage survival and maximum morphogenic response of thin sections were recorded in medium containing TDZ (Table 1). A modified

Table 1 Effects[a] of different cytokinins on the stimulation of in vitro PLB and CLB induction in thin leaf sections of *Doritaenopsis* hybrid

Cytokinin concentration (μ*M*)	Survival rate (%)	PLB formation (%)	CLB formation (%)
0.0	8.67f	0.34e	0.57g
TDZ			
4.5	64.79c	31.62b	4.94e,f
9.0	88.47a	72.30a	2.08f,g
13.5	89.61a	28.71b	9.20c,d
22.5	87.86a	17.81c	11.56c
BA			
2.2	73.25b	4.75d,e	17.25b
4.4	65.00c	0.00e	32.13a
11.1	58.33c,d	8.33d	30.23a
22.2	51.67d	22.33c	31.63a
Zeatin			
2.3	36.75e	0.00e	3.13e,f,g
4.6	42.25e	1.38e	6.05d,e
11.4	34.67e	0.00e	5.33e,f
22.8	37.50e	0.00e	0.00g

[a] Each value represents the mean of five replications and in each column, the means followed by the same letter are not significantly different as indicated by Duncan's multiple range test (P=0.05)

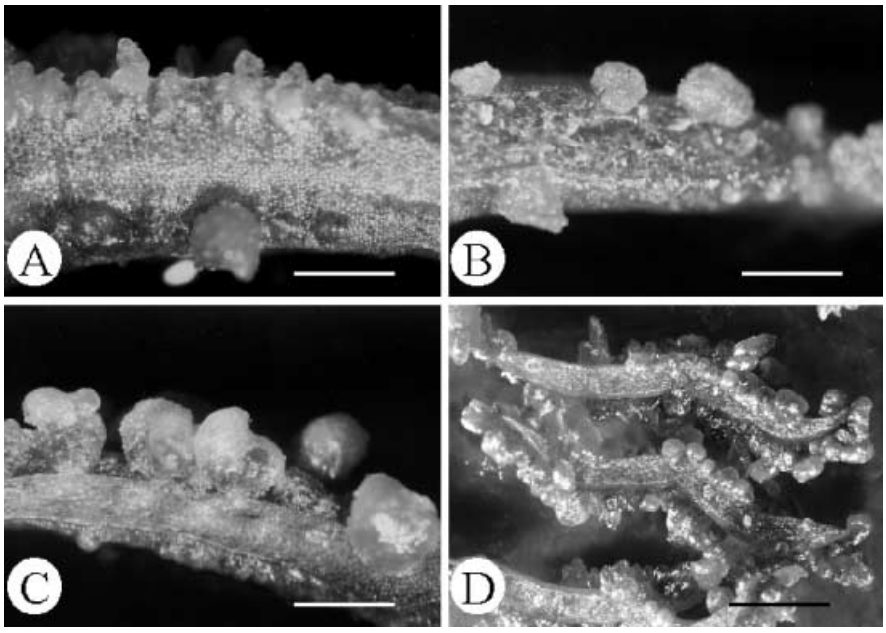

Fig. 1A–D PLB regeneration from thin leaf sections of the *Doritaenopsis* hybrid during varying lengths of culture periods. **A** Cut edge of thin leaf sections showing initiation of PLB. *Bar*: 1.5 mm. **B** PLB primordia formation after 3 weeks of culture. *Bar* 2 mm. **C**, **D** Formation of mature multiple PLBs along the cut surface after 4 weeks and 6 weeks of culture, respectively. *Bar* 2 mm (**C**), 3 mm (**D**)

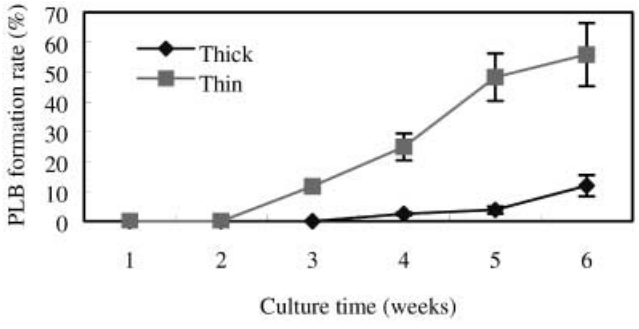

Fig. 2 Rate of PLB formation during different culture periods from thin leaf sections and thick leaf segments of *Doritaenopsis* hybrid (mean ± standard error)

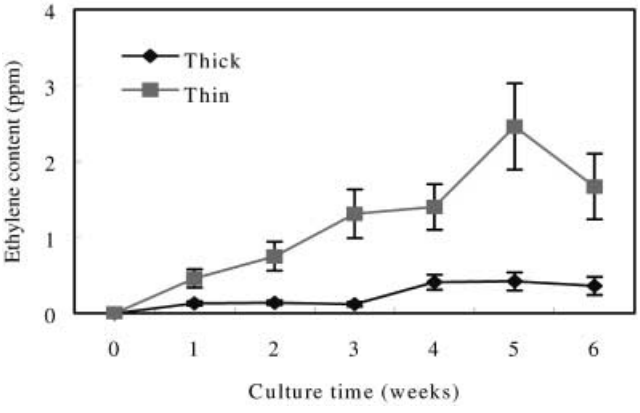

Fig. 3 Comparison of the number of PLBs formed per explant of *Doritaenopsis* hybrid from thin leaf sections and thick leaf segments after 6 weeks of culture (mean ± standard error)

MS medium supplemented with 9.0 µ*M* TDZ was the optimum concentration for PLB development from thin leaf sections of *Doritaenopsis* hybrid. Globular structures were seen after 2 weeks of culture (Fig. 1A); these were distinctly visible along the edges of the cut surface of the thin sections after 3 weeks of culture (Fig. 1B). These globular proembryos further differentiated into mature PLBs during subsequent culturing (Fig. 1C, D). On all of the media, PLBs always regenerated directly near the epidermis of the thin sections and never from callus. Explants that did not respond to culture eventually became necrotic. All of the BA concentrations induced more CLBs than the TDZ and zeatin treatments (Table 1); these CLBs were not able to develop into PLB or callus.

A comparative analysis of PLB production by thick leaf segments (5 mm) and thin leaf sections (1 mm) showed that the latter produced 4.5-times more PLBS than thick leaf segments (Fig. 2) in the PLB induction medium supplemented with 9.0 µ*M* TDZ after 6 weeks of culture. Changes in ethylene content during the culture period were also investigated (Fig. 3). In thin-section culture, the ethylene contents were remarkably higher than in thick leaf culture for all culture periods, especially after 2 weeks (onset of regeneration) and 5 weeks (vigorous regeneration stage).

Once transferred to the Hyponix medium, PLBs germinated and developed into normal plantlets (Fig. 4A). These plantlets readily acclimatized to greenhouse conditions after being transferred to the *Sphagnum* moss medium (Fig. 4B) and continued to develop into normal plants. No phenotypic variations were observed among them during their vegetative growth.

Cross sections of thin leaf sections that had been cultured for only 3 days on PLB induction medium revealed that mitotic divisions had already began by this time near the adaxial epidermis. The cytoplasm-rich subepidermal

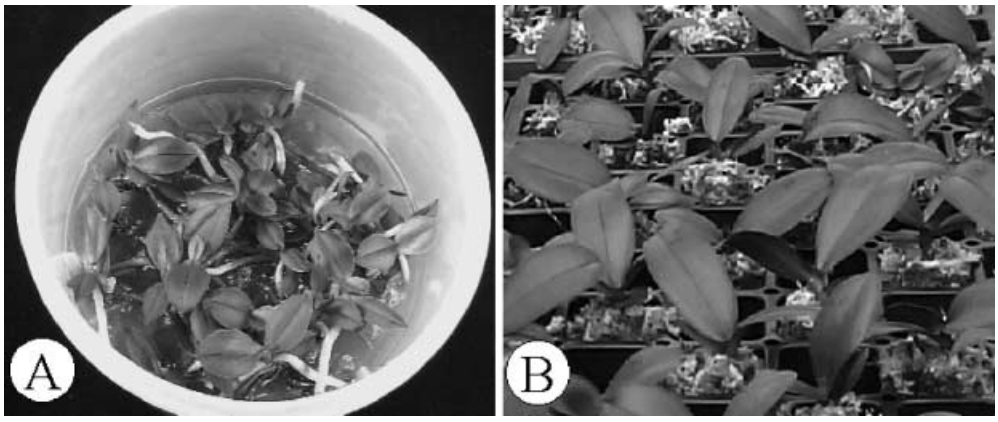

Fig. 4A, B Plantlets derived from PLBs. **A** Plantlets regenerating from PLBs on Hyponex medium after 3 months, **B** 2-month-old acclimatized plantlets grown in the greenhouse

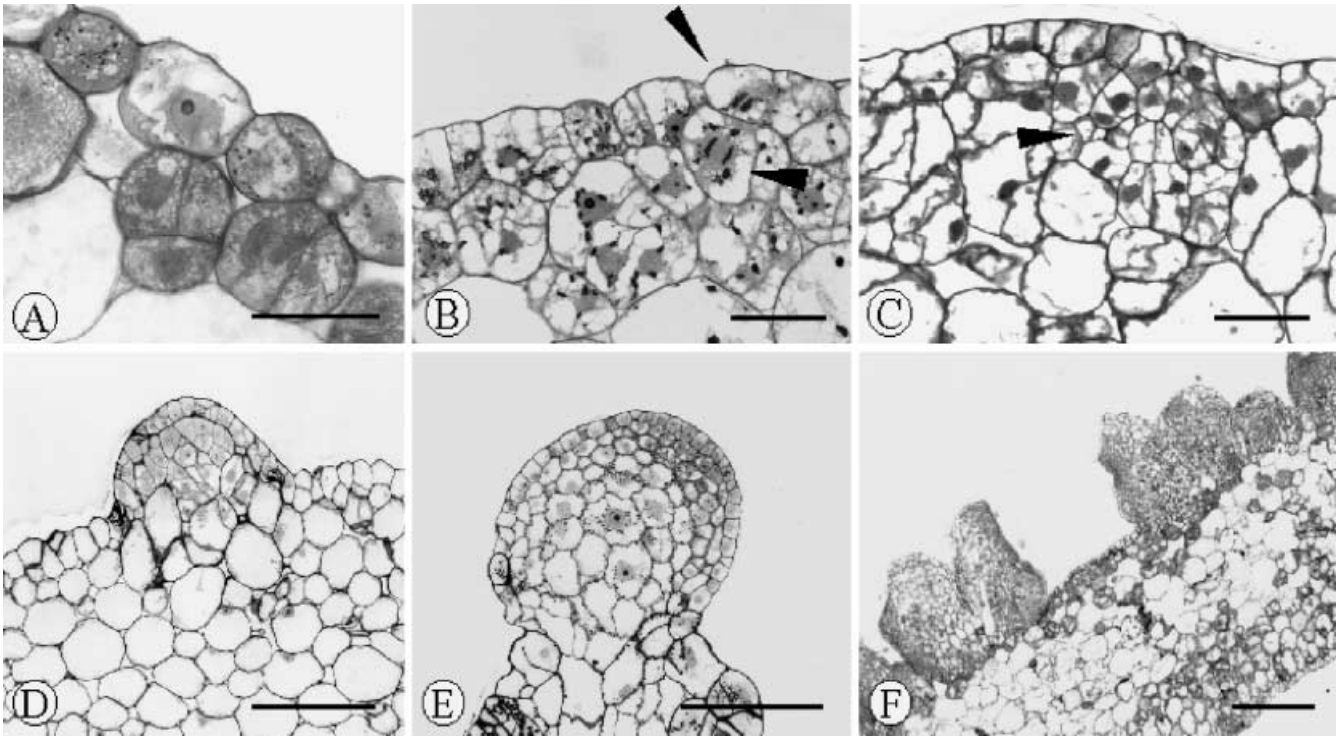

Fig. 5A–F Cross sections of thin leaf sections of *Doritaenopsis* hybrid showing PLB formation. **A** Mitotic division occurring in subepidermal cells with a dense cytoplasm after 3 days of culture. *Bar* 50 µm. **B** Concomitant with the mitotic activities of the subepidermal cells, the epidermal cells also begin to divide. *Arrowheads* cells undergoing active divisions. *Bar* 100 µm. **C** Formation of cell division centers just beneath the epidermis after 7 days of culture. *Bar* 100 µm. **D** Continual mitotic activity within the growth center results in the formation of a small protuberance 3 weeks after culture. *Bar* 200 µm. **E** A developing globular-shaped PLB after 4 weeks of culture. *Bar* 200 µm. **F** Mature PLBs after 6 weeks of culture. *Bar* 1.5 mm

cells divided first (Fig. 5A). Concomitant with the mitotic activities of the subepidermal cells, the epidermal cells also began to divide (Fig. 5B). Cell divisions were restricted mainly to regions of the adaxial epidermal and subepidermal cell layers near the cut surface of the explant. By day 7 of PLB induction, some dividing subepidermal cells had organized into growth centers (Fig. 5C). After 3 weeks of PLB induction, most of the growth centers had gradually protruded above the surface of the epidermis, resulting in the formation of a small protuberance that was easily observed on the explants (Fig. 5D). Continual divisions within these protuberances resulted in PLB formation. After 4 weeks of culture, PLB structures began to take shape. A gradient of cell size was noted, with smaller cells occupying the future shoot pole (Fig. 5E). A number of mature PLBs were clearly visible on the surface of the explant after 6 weeks of culture (Fig. 5F). For those growth centers formed just beneath the injured epidermis, only small globular structures appeared, and these structures failed to develop into PLBs (Fig. 6A, B).

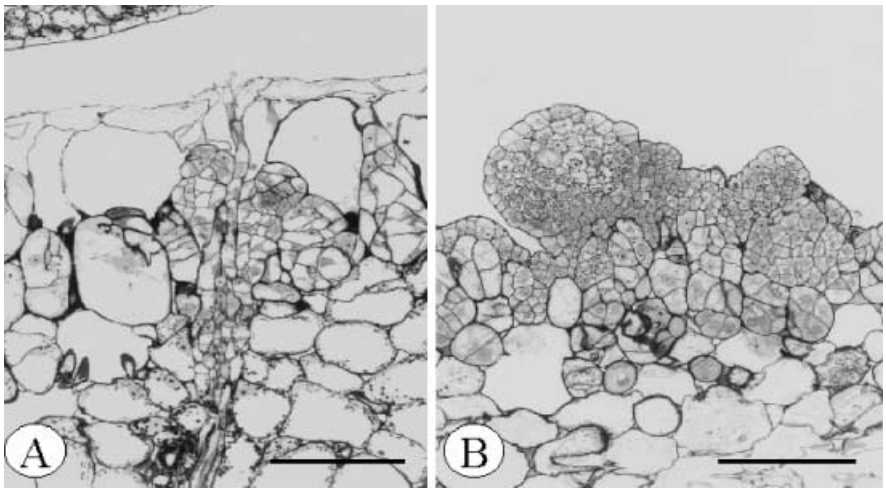

Fig. 6A, B Sections of a thin leaf explant of *Doritaenopsis* hybrid showing the influence of the epidermis on PLB formation after 3 weeks of culture. **A** Wounded epidermis and subtending mesophyll cells. Although mitotic divisions were found, these cells failed to organize into growth centers leading to PLB formation. *Bar* 200 µm. **B** The formation of a PLB developing in areas having an intact epidermal layer. *Bar* 200 µm

Discussion

The thin-leaf section culture method described here is an efficient in vitro technique for the rapid propagation of one hybrid of *Doritaenopsis.* Significant differences in the PLB induction rate were observed between the TDZ-treatment and the zeatin or BAP treatment. It is generally believed that TDZ, a phenylurea derivative, is more active in stimulating adventitious shoot formation than somatic embryogenesis (Huetteman and Preece 1993). However, our findings illustrate that TDZ strongly stimulated PLB (a unique type of somatic embryo in orchid) formation from thin leaf sections. TDZ has shown similar promotive effects with respect to regeneration for a number of species (Chen and Chang 2001; Prakash et al. 2001), including *Phalaenopsis* and *Doritaenopsis* (Ernst 1994; Chen and Piluek 1995). Huetteman and Preece (1993) have suggested that hyperhydricity and shoot fasciation are associated with TDZ treatment. However, these were not observed in our study.

An important finding of this investigation was that thin leaf sections of *Doritaenopsis* hybrid produced PLBs in greater numbers than thick leaf segments, the most commonly used explants in *Doritaenopsis* tissue culture (Tanaka 1992; Arditti and Ernst 1993). The thin-cell layer culture has been shown to be a valuable system for studying problems related to plant morphogenesis in vitro (Tran Thanh Van 1981; Tran Thanh Van Et al. 1990). The removal of thin cell layers from plants most likely removes influences of maternal origin and allows cells to develop alternate developmental pathways under the influence of components and culture conditions (Lakshmanan et al. 1995). In our culture system, wounding caused by the excision process may have played an important role in PLB induction. In several species, wound culture methods, like transverse thin-cell layer culture (Carimi et al. 1999; Le et al. 1999) and thin-section culture (Lakshmanan et al. 1995), have been successfully used for high-frequency regeneration from explants. As a result of the wounding-response phenomena, quiescent unwounded cells near the cut surface become active and initiate cell proliferation (Imaseki 1985). Several biochemical changes (degradation of starch, increase in peroxidase and polyphenol oxidase activities) are associated within the otherwise quiescent unwounded cells near the wound (Imaseki 1985). However, the relationship between these metabolic changes and PLB formation is still ambiguous and remains to be examined further. The gaseous growth regulator, ethylene, has a profound effect on the metabolic changes induced by the wounding of plant tissue (Imaseki 1985), and it has been reported that ethylene plays an important role in the induction of shoot organogenesis and/or somatic embryogenesis in a number of species (Reynolds 1987; Cho and Kasha 1989). In thin-section culture, higher levels of ethylene were detected than with thick-leaf segment culture. There is a good correlation between changes in ethylene content and the time of PLB regeneration, which indicates that ethylene gives a positive signal for PLB induction during thin-section culture.

PLB induction revealed that PLBs developed directly from the subepidermal layers near the wounded region. Although extensive research has been carried out on in vitro PLB induction in cultivated orchids, there are few structural details known on the development of PLBs or their cellular origin, especially from thin leaf sections. The histological observations reported here provide evidence that PLB induction from thin leaf sections is direct – i.e., without an intermediate callus phase. Morphological changes were visible after 3 weeks of culture, while histological sections displayed cellular modifications as early as 3 days post-culture. The pattern of PLB development reported here was produced by simultaneous divisions of several cells and thus was multicellular in origin. An interesting observation of this investigation was that the growth centers formed just beneath the injured epidermis did not form PLBs. Although cells continued to divide, the cell mass failed to organize into a PLB as a defined protoderm layer was absent. This suggests that subepidermal cells do not have the ability to differentiate

into protoderm cells; hence, organized development cannot take place. During zygotic embryo development, the formation of the protoderm is the first histo-differentiation event of a proembryo. The formation of a surface layer may provide a defined physical boundary so that other organized developmental events can take place.

On the basis of the study reported here, it can be concluded that thin leaf sections of *Doritaenopsis* hybrid produced PLBs in greater numbers than the 5-mm-thick leaf segments, the most commonly used explants in *Doritaenopsis* tissue culture, and that this concept can be exploited as a method of rapid plant propagation. Furthermore, the histological study on the developmental pattern of PLB induction has lead to a better understanding of the development of PLBs and their cellular origin and will consequently result in higher regeneration rates that should benefit clonal propagation. This system may also prove useful for future studies elucidating the genes involved in subsequent differentiation processes of PLB induction.

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References

Arditti J, Ernst R (1993) *Phalaenopsis*. Micropropagation of orchids. Wiley, New York

Carimi F, De Pasquale F, Crescimanno FG (1999) Somatic embryogenesis and plant regeneration from pistil thin cell layers of Citrus. Plant Cell Rep 18:935–940

Chen JT, Chang WC (2001) Effects of auxins and cytokinins on direct somatic embryogenesis on leaf explants of Oncidium 'Grower Ramsey'. Plant Growth Regul 34:229–232

Chen WH, Chen TM, Fu YM, Hsieh RM, Chen WS (1998) Studies on somaclonal variation in *Phalaenopsis*. Plant Cell Rep 18:7–13

Chen YQ, Piluek C (1995) Effects of thidiazuron and N6-benzylaminopurine on shoot regeneration of *Phalaenopsis*. Plant Growth Regul 16:99–101

Cho UH, Kasha KJ (1989) Ethylene production and embryogenesis from anther cultures of barley (*Hordeum vulgare*). Plant Cell Rep 8:415–419

Ernst R (1994) Effects of thidiazuron on in vitro propagation of *Phalaenopsis* and *Doritaenopsis*. Plant Cell Tissue Organ Cult 39:273–275

Huetteman CA, Preece JE (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tissue Organ Cult 33:105–119

Imaseki H (1985) Hormonal control of wound-induced responses, In: Pharis RP, Reid DM (eds) Hormonal regulation of development. Springer, Berlin Heidelberg New York, pp 485–512

Intuwong O, Sagawa Y (1974) Clonal propagation of *Phalaenopsis* by shoot tip culture. Am Orchid Soc Bull 43:893–895

Kano K (1965) Studies on the media for orchid seed germination. Mem Fac Agric Kagawa Univ 20:1–68

Kano K (1971) The possibility of vegetative propagation by leaf segment culture in *Phalaenopsis* and *Vanda*. Abstr Jpn Soc Hortic Sci Autumn Meet, pp 270–271

Lakshmanan P, Loh CS, Goh CJ (1995) An in vitro method for rapid regeneration of a monopodial orchid hybrid *Aranda* Deborah using thin section culture. Plant Cell Rep 14:510–514

Le BV, Phuong NTH, Hong LTA, Tran Thanh Van K (1999) High frequency shoot regeneration from *Rhynchostylis gigantea* (Orchidaceae) using thin cell layers. Plant Growth Regul 28: 178–185

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497

Park SY, Kakuta S, Kano A, Okabe M (1996) Efficient propagation of protocorm-like bodies of *Phalaenopsis* in liquid medium. Plant Cell Tissue Organ Cult 45:79–85

Park SY, Murthy HN, Paek KY (2000) Mass multiplication of protocorm-like bodies (PLBs) using bioreactor system and subsequent plant regeneration in *Phalaenopsis*. Plant Cell Tissue Organ Cult 63:67–72

Prakash E, Khan SV, Meru E, Rao KR (2001) Somatic embryogenesis in *Pimpinella tirupatiensis* Bal. And Subr. an endangered medicinal plant of Tirumala hills. Curr Sci 81: 1239–1241

Reisinger DM, Ball EA, Arditti J (1976) Clonal propagation of *Phalaenopsis* by means of flower-stalk node cultures. Orchid Rev 84:45–52

Reynolds TH (1987) A possible role for ethylene during IAA induced pollen embryogenesis in anther cultures of *Solanum carolinense* L. Am J Bot 74:967–969

Tanaka M (1992) Micropropagation of *Phalaenopsis* spp. In: Bajaj YPS (ed) High-tech and micropropagation. Biotechnology in agriculture and forestry, vol 20. Springer, Berlin Heidelberg New York, pp 246–268

Tanaka M, Senda Y, Hasegawa A (1976) Plantlet formation by root-tip culture in *Phalaenopsis*. Am Orchid Soc Bull 45: 1022–1024

Tran Thanh Van K (1981) Control of morphogenesis in in vitro cultures. Annu Rev Plant Physiol 32:291–311

Tran Thanh Van K, Richard L, Gendy CA (1990) An experimental model for the analysis of plant/cell differentiation: thin cell layer concept, strategy, methods, records and potential. In: Rodriguez R, Tames RS, Durzan DJ (eds) Plant aging: basic and applied approaches. Plenum, New York, pp 215–224

Yeung EC (1984) Histological and histochemical staining procedures. In: Vasil IK (ed) Cell culture and somatic cell genetics of plants, vol 1. Laboratory procedures and their applications. Academic, Orlando, pp 689–697

Yeung EC (1999) The use of histology in the study of plant tissue culture systems-some practical comments. In Vitro Cell Dev Biol Plant 35:137–143