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Protocorm-like body induction and subsequent plant regeneration from root tip cultures of *Doritaenopsis*

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Abstract

The effect of cytokinins thidiazuron (TDZ), benzyladenine or zeatin on protocorm-like body (PLB) induction from root tips of *Doritaenopsis* grown in vitro was studied. Among the cytokinins tested, TDZ was found to be more effective cytokinin in the induction of PLBs than benzyladenine (BA) or zeatin. On modified Murashige and Skoog medium (MS) supplemented with 2.3 μM TDZ the highest percentage of PLB formation (47.2%) occurred and each explant produced two to six PLBs. Histological observations indicated the existence of two kinds of pathways during PLB formation: direct PLB induction from the root meristem and callus-mediated PLB induction from cortical cells. During callus-mediated PLB regeneration, the initial cell divisions occurred in the cortex region of root apices followed by accumulation of callus like masses in the cortex region. These cell masses developed globular structures that developed into PLBs after 5 weeks of culture. Upon subculturing to a modified Hyponex medium, PLBs developed into plantlets that were successfully acclimatized in green house conditions.

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Keywords: *Doritaenopsis*; Protocorm-like bodies; Root tip cultures; Regeneration

1. Introduction

Doritaenopsis is a hybrid between *Doritis* and *Phalaenopsis*, and is a popular orchid species in the horticulture industry as cut flowers as well as potted plants. A wide range of hybrid varieties have been produced in this group and most of them are propagated by in vitro methods. Several micropropagation methods have been developed for *Phalaenopsis* using shoot tips, leaf segments, nodal and flower stalk cuttings (Arditti and Ernst, 1993 and references therein) [1]. Some of these procedures are applied to the propagation of *Doritaenopsis*, however, these methods will not produce large numbers of protocorm-like body (PLB).

Orchid roots have a well-developed metabolic capacity [2]; their ability to form buds is relatively limited under natural conditions. Some attempts to regenerate buds/PLBs from orchid root tips have been made, and multiplication was achieved in a limited number of species [3–7]. In this study, we attempted in vitro root tip culture in *Doritaenopsis* and report here a successful method of PLB induction and subsequent plantlet regeneration. The sequences of morphological and histological changes during PLB regeneration are verified.

2. Materials and methods

2.1. Plant materials

Roots of 3-month old in vitro plantlets were used as the source of explants. 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. [8,9].

Abbreviations: BA, benzyladenine; MS, Murashige and Skoog medium; PLB, protocorm-like body; TDZ, thidiazuron.

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2.2. Root tip culture

Root tips (< 0.5 cm) were dissected from plantlets and were cultured on half strength Murashige and Skoog [10] medium supplemented with different concentrations of thidiazuron (TDZ) (0.5–9.0 μM), benzyladenine (BA) (4.4–22.2 μM), or zeatin (24.6–22.8 μM), 20% coconut water, 10 mg/l adenine sulfate, and solidified with 2.3 g/l Gelrite (Duchefa). Coconut water was drained from ripe nuts (imported from The Philippines), filtered through 430 μm sieve and used immediately. Approximately 20 root tips were cultured in a plastic petri dish (10 cm diameter) containing 25 ml of medium. The pH of medium was adjusted to 5.5, and all media were autoclaved for 20 min at 115 °C (1.37×10^5 Pa). Zeatin was added by filter sterilization to the autoclaved medium. Cultures were incubated at 25 °C under cool-white florescent lamps (Kumho FL 40D, Korea) at an intensity of 30 μmol/m^2 per s photosynthetic photon flux (PPF) 16 h/day.

After 8 weeks of culture, explants were evaluated in terms of percentage PLB formation and number of PLBs per explant. The percentage of PLB formation was calculated based on the number of explants forming PLBs as a percentage of total number of explants. For the number of PLBs per explant, only PLBs approximately 1 mm in diameter, with a round shape were counted.

2.3. Plantlet regeneration and transplantation of plantlets

In order to obtain plantlets, PLBs were transferred to a modified Hyponex medium [11] [6.5N−4.5P−19K+20N−20P−20K] supplemented with 30 g/l potato homogenate, 2 g/l peptone, 0.5 g/l activated charcoal (Duchefa), 20 g/l sucrose, and 5.5 g/l plant agar (Duchefa). Potatoes homogenate, prepared by blending 30 g fresh potatoes with 200 ml of liquid medium for 1–2 min, was used immediately. The pH was adjusted to 5.5 before autoclaving. The cultures were maintained at 25 °C and a 16-h light:8-h dark photoperiod (light intensity: 30 μmol/m^2 per s PPF). The plantlets were subcultured every 8 weeks to fresh medium.

Plantlets (5–6 cm length) were transplanted to pots containing sphagnum moss and grown in the greenhouse under conditions of high humidity (60–70%) at a day/night temperature 25/15 °C and 16-h light:8-h dark photoperiod (light intensity: 500 μmol/m^2 per s PPF).

2.4. Light microscopy

Samples collected different culture periods were fixed in a solution containing 1.5% glutarldehyde and 1.6% paraformaldehyde buffered with 0.05 M phosphate buffer, pH 6.8, for 24–48 h at 4 °C. Samples were dehydrated in an ethanol series and then embedded in glycol methacrylate (Technovit 7100, Kulzer, Germany) according to the protocol of Yeung [12]. Serial 3-μm thick sections were cut using a autocut microtome (Leica RM 2165, Germany), mounted on glass slides, and stained with periodic acid-Schiff's reaction (PAS) for total carbohydrates, counterstained with 1% naphtol blue black or 0.05% toluidine blue O for 5 min [13], and then examined under a light microscope (Olympus Optical Co., LTD., Japan) at ×40 and ×200 magnification.

2.5. 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 multiple range test using the SAS program (SAS Institute, Cary, NC).

3. Results

The root tip explants cultured on a half Murashige and Skoog medium (MS) medium supplemented with different cytokinins become swollen after 4 weeks of culture. Subsequently PLBs were differentiated from the root tip explants after another 2–4 weeks (Fig. 1A, B, C). Fig. 2 shows the frequency of explant survival and frequency of explants that formed PLBs when cultured on various concentrations of BA, TDZ or zeatin. Explants devoid of BA, TDZ or zeatin did not show any response although low percentage of them survived more than 8 weeks. Both maximum percentage survival and morphogenic response of root tips was in a half MS medium supplemented with TDZ. On a half MS medium supplemented with 2.3 μM TDZ 71.1% of root tips survived and 47.2% of them developed two to six PLBs.

Observations made on serial longitudinal and cross sections of root tips revealed the occurrence of two differential developmental pathways during PLB regeneration. Root meristems were involved in the direct development of PLBs (Fig. 3F, G, H). Longitudinal sections of root tips showed elongation of the root meristem after 2 weeks of culture (Fig. 3F). Subsequently meristem differentiates into PLBs in 5 weeks of culture (Fig. 3G) and after 6–7 weeks multiple PLBs developed from the meristem (Fig. 3H). A different response was observed in the cortical region. Cross sections of root tips showed that the external part of root tip consists of a multi-layered velamen tissue (multiple epidermis on the aerial roots of epiphytic orchids, Fig. 3A). At the greater depth in the cortex, cells gain meristematic activity and divide continuously to form a meristematic zone (Fig. 3B). By the third week meristematic zones elaborated (Fig. 3C) and developed

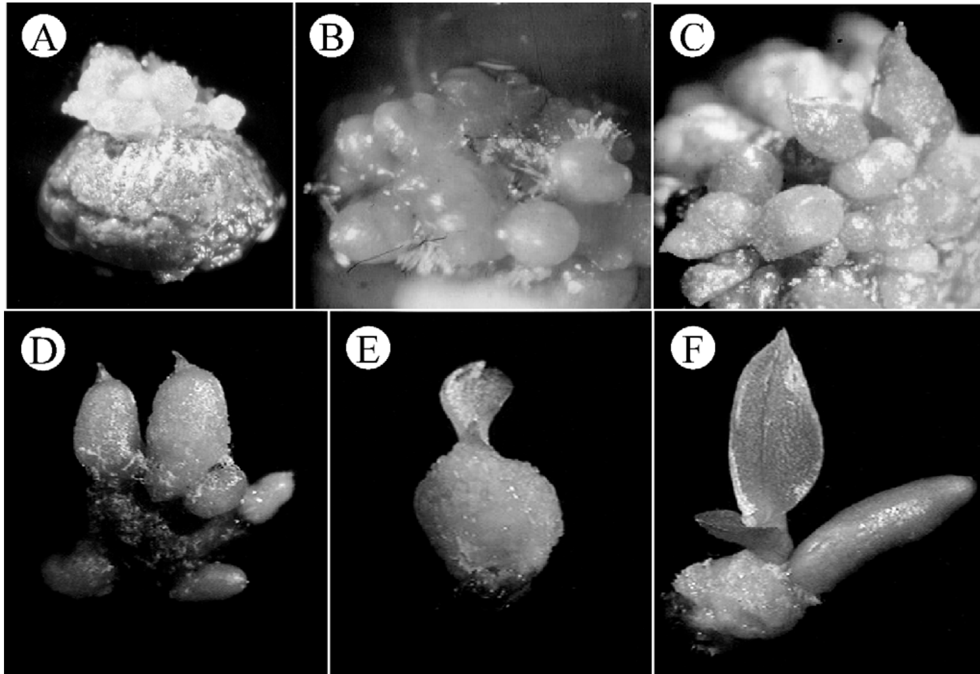

Fig. 1. Induction of PLBs and plant regeneration from root tips of *Doritaenopsis.* (A) PLB development on root tip after 4 weeks of culture on 1/2 MS medium with 2.3 μM TDZ. PLBs developed after 5 (B) and 6 (C) weeks of culture. (D) Developing PLBs on modified Hyponex medium. (E) PLBs with expanding sheath leaves. (F) A rooted plantlet.

into callus like tissue (Fig. 3D). Subsequently from these cell masses globular structures emerge out after 5 weeks of culture (Fig. 3E). A number of mature PLBs were clearly visible on the surface of the explant after 6 weeks of culture.

Upon sub-culturing to modified Hyponex medium PLBs enlarged in their size after 2 weeks (Fig. 1D). Subsequently PLBs showed protrusion of leaves on the apical region (Fig. 1E). The two sheath leaves and PLBs enlarged and elongated sequentially upon sub-culturing to fresh medium, PLBs developed into normal plantlets (Fig. 1F). Plantlets grew further after another two subcultures (Fig. 4A). The regenerated plantlets about 3–4 cm in height with a pair of leaves and three to four roots were then potted to sphagnum moss and acclimatized in greenhouse. These plants grew well and developed into normal plants after 12 weeks of transplantation (Fig. 4B).

4. Discussion

The study of orchid root morphogenesis has not received as much attention as the development of aerial plant organs. Root tips of orchids species have been considered generally recalcitrant to form PLB or callus in vitro, such is the case, for example, with *Epidendrum*, *Oncidium*, and *Cattleya* plants [14,4,6]. Among the three

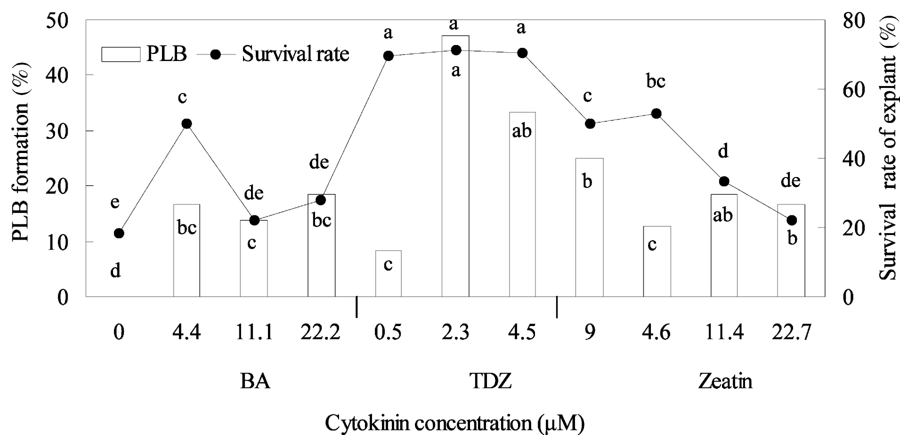

Fig. 2. Survival rate and frequency of PLB developing root tips of *Doritaenopsis* on MS medium supplemented with different concentrations of BA, TDZ, or zeatin.

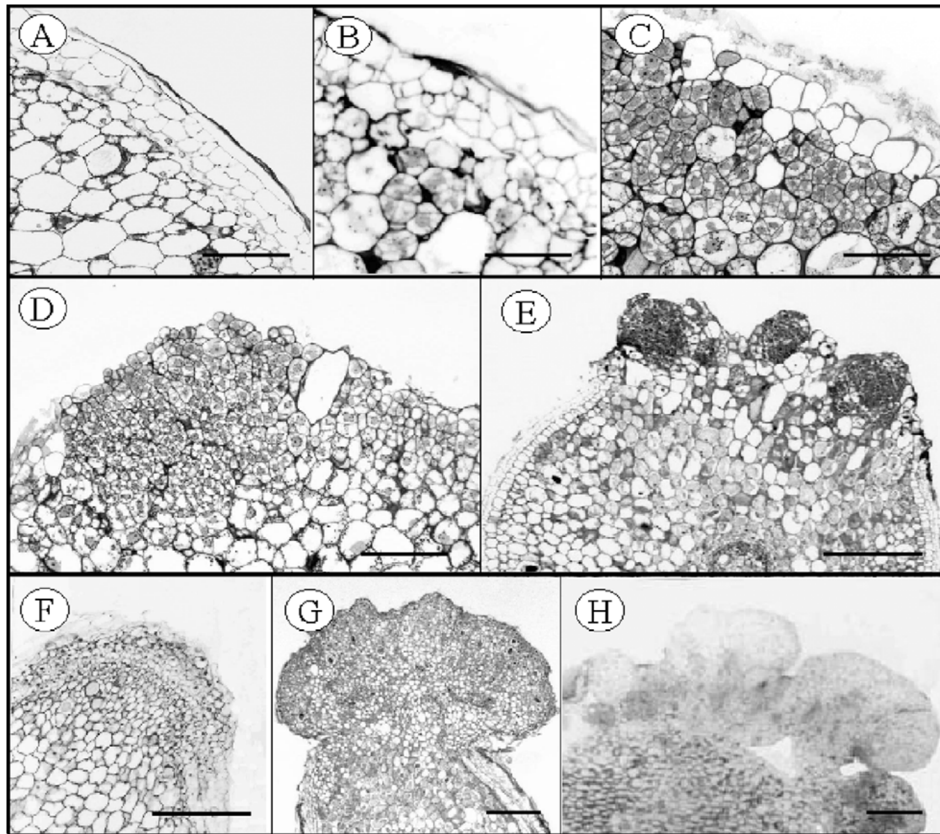

Fig. 3. Histological observations during PLB regeneration from root tips of *Doritaenopsis*. (A) Cross section of the root after 3 days in culture showing multilayered velamen and cortex tissue (bar = 200 μm). Cross section of root tips showing active cell divisions in the cortex region after 2 (B) and 4 (C) weeks of culture (bar = 200 μm). (D) Development of callus after 4 weeks of culture (bar = 400 μm). (E) Protrusion of globular structures from the callus (bar = 600 μm). (F) Longitudinal section of root tip after 2 weeks of culture (bar = 600 μm). (G) Longitudinal section of root tip after 6 weeks in culture showing development of protocorm (bar = 1 mm). (H) Longitudinal section of root tip after 8 weeks in culture showing development of multiple PLBs (bar = 1 mm).

cytokinins used in this study, TDZ was efficient in induction of PLBs (Fig. 2). TDZ was first reported to have cytokinin activity by Mok et al. [15] and has been reported to induce shoot regeneration in many orchid species [16–18]. In this study TDZ induced both direct and callus-mediated PLB regeneration. PLB regeneration is comparable to somatic embryogenesis pathway in orchids [19], and hence TDZ is more potent cytokinin for induction of both organogenesis as well as embryogenesis. TDZ alone or in combination with auxins has

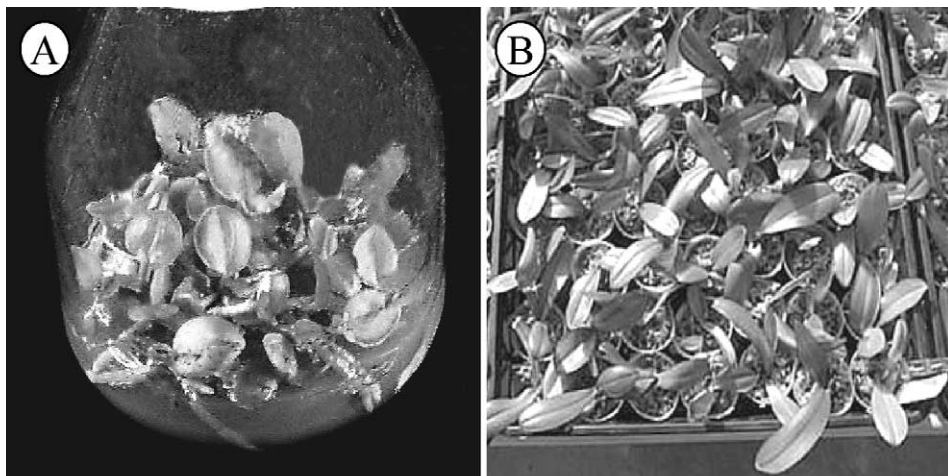

Fig. 4. (A) Actively growing plantlets on Hyponex medium 8 weeks after culture. (B) Acclimated plants growing in greenhouse.

been used to induce embryogenesis in *Cymbidium ensifolium* [20], *Oncidium* [21,22].

Although extensive research has been carried out on PLB induction from in vitro cultures in orchids, there are few structural details known on the development of PLBs or their cellular origin. Histological observations made in the present studies revealed that root apical meristem involved in direct PLB formation (Fig. 3F, G, H). Kerbauy [23,5] and Colli and Kerbauy [3] have also shown direct PLB regeneration from root tips of *Catesetum*. In contrast to this in other orchid species a callus stage has proved to be a necessary condition for in vitro regeneration [14,4,6]. Histological observations made in the cortical regions revealed that the initial division occurred in several cells led to the formation of callus (Fig. 3B, C, D). Subsequently callus differentiated globular bodies (Fig. 3E) that developed into PLBs.

The regenerated PLBs easily developed into plantlets on Hyponex medium. These plantlets were subsequently acclimatized in the greenhouse.

Using root tip culture plants can be regenerated on a large scale, and the mother plant can also be conserved since using root tips as the explant does not destroy mother plants. The number of roots available through the year, and ease of using them as explants makes roots an ideal explant for in vitro propagation of *Doritaenopsis*.

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References

[1] J. Arditti, R. Ernst, Micropropagation of Orchids, Wiley, New York, 1993.
[2] M.E. Churchill, G.A. Ball, J. Arditti, Tissue culture of orchids-II. Methods for root tips, Am. Orchid Soc. Bull. 41 (1972) 726–730.
[3] S. Colli, G.B. Kerbauy, Direct root tip conversion of *Catesetum* into protocorm like bodies, effect of auxin and cytokinin, Plant Cell Tiss. Org. Cult. 33 (1993) 39–44.
[4] G.B. Kerbauy, Plant regeneration of *Oncidium varicosum* (Orchidaceae) by means of root tip culture, Plant Cell Rep. 3 (1984) 27–29.
[5] G.B. Kerbauy, Regeneration of protocorm like bodies through in vitro culture of root tips of *Catasetum* (Orchidaceae), Z. Plfanzenphysil. 113 (1984) 287–291.
[6] J. Stewart, J. Button, Development of callus and plantlets from *Epidendrum* root tips cultured in vitro, Am. Orchid Soc. Bull. 47 (1978) 607–612.
[7] M. Tanaka, Y. Senda, A. Hasegawa, Plantlet formation by root-tip culture in *Phalaenopsis*, Am. Orchid Soc. Bull. 45 (1976) 1022–1024.
[8] S.Y. Park, H.N. Murthy, K.Y. Paek, Rapid propagation of *Phalaenopsis* from floral stalk derived leaves, In Vitro Cell. Dev. Biol. Plant 38 (2002) 168–172.
[9] S.Y. Park, H.N. Murthy, K.Y. Paek, Mass multiplication of protocorm like bodies using bioreactor system and subsequent plant regeneration in *Phalaenopsis*, Plant Cell Tiss. Org. Cult. 63 (2000) 67–72.
[10] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant. 15 (1962) 473–497.
[11] K. Kano, Studies on the media for rapid orchid seed germination, Mem. Fac. Agril Kagawa Univ. 20 (1965) 1–68.
[12] E.C. Yeung, The use of histology in the study of plant tissue culture systems-some practical comments, In Vitro Cell. Dev. Biol. Plant 35 (1999) 137–143.
[13] E.C. Yeung, Histological and histochemical staining procedures, in: I.K. Vasil (Ed.), Cell culture and Somatic Cell Genetics of Plants, Laboratory Procedures and Their Applications, vol. 1, Academic press, Orlando, 1984, pp. 689–697.
[14] G.B. Kerbauy, In vitro conversion of *Cattleya* root tips cells into protocorm-like bodies, J. Plant Physiol. 138 (1991) 248–251.
[15] M.C. Mok, W.S. Mok, D.J. Armostong, K. Shudo, Y.I. Sogai, T. Okamoto, Cytokinin activity of *N*-phenyl-*N′*-1,2,3-thiadiazol-5ylurea (thiadiazuron), Phytochemistry 21 (1982) 1509–1511.
[16] J.T. Chen, W.C. Chang, Effect of auxins and cytokinins on direct somatic embryogenesis of leaf explants of *Oncidium* 'Grower Ramsey', Plant Growth Regul. 34 (2001) 229–232.
[17] R. Ernst, Effect of thidiazuron on in vitro propagation of *Phalaenopsis* and *Doritaenopsis* (Orchidaceae), Plant Cell Tiss. Org. Cult. 39 (1994) 273–275.
[18] N.R. Nayak, S. Patnaik, R.P. Rath, Direct shoot regeneration form foliar explants of epiphytic orchid *Acampae praemorsa* (Roxb.) Blatter and McCann, Plant Cell Rep. 16 (1997) 583–586.
[19] G. Morel, Clonal multiplication of orchid, in: C.L. Withner (Ed.), The Orchids: Scientific Studies, Wiley, New York, 1974, pp. 169–222.
[20] C. Chang, W.C. Chang, Plant regeneration from callus of *Cymbidium ensifolium var. misericors*, Plant Cell Rep. 17 (1998) 251–255.
[21] J.T. Chen, W.C. Chang, Effect of auxins and cytokinins on direct somatic embryogenesis of leaf explants of *Oncidium* 'Grower Ramsey', Plant Growth Regul. 34 (2001) 229–232.
[22] J.T. Chen, W.C. Chang, Efficient plant regeneration through somatic embryogenesis from callus cultures of *Oncidium* (Orchidaceae), Plant Sci. 160 (2000) 87–93.
[23] G.B. Kerbauy, S. Colli, Increased conversion of root tip meristems of *Catasetum fimbriatum* into protocorm-like bodies mediated by ethylene, Lindleyana 12 (1997) 59–63.