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BRIEF COMMUNICATION

Plant regeneration through direct somatic embryogenesis from leaf explants of *Dendrobium*

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Abstract

A protocol for induction of direct somatic embryogenesis, secondary embryogenesis and plant regeneration of *Dendrobium* cv. Chiengmai Pink was developed. Thidiazuron (TDZ) at 0.3, 1 and 3 mg dm^{-3} induced 5 - 25 % of leaf tip segments of *in vitro* grown plants to directly form embryos after 60 d of culture, and 1 mg dm^{-3} TDZ was the best treatment. Somatic embryos mostly formed from leaf surfaces near cut ends, and occasionally found on leaf tips. Higher frequency of embryogenesis was obtained in light than in darkness. During subculture, secondary embryos developed from outer cell layers of primary embryos. All combinations of NAA (0, 0.1, 1 mg dm^{-3}) and TDZ (0, 0.3, 1, 3 mg dm^{-3}) increased the multiplication rate of embryos. It takes about 8 months from embryo induction, plantlet formation to eventually acclimatization in greenhouse.

Additional key words: naphthaleneacetic acid, secondary somatic embryogenesis, thidiazuron

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Dendrobium orchids have lateral inflorescences with single or numerous flowers arising from a small group of bracts (Teob 1989). Methods for micropropagation of *Dendrobium* had been studied and were usually through protocorm-like-body or shoot formation using axillary buds, flower stalk cuttings, leaves, meristem tissues, pseudobulb segments, shoot tips and stem nodes as explants (Arditti and Ernst 1993, Manorama *et al.* 1986, Nayak *et al*. 1997, 2002). However, till present, there is no report described about somatic embryogenesis in this genus. In this communication, we establish a protocol for regeneration of a *Dendrobium* cultivar through direct somatic embryogenesis from leaf explants taken from *in vitro* grown plants.

Donor plants of *Dendrobium* cv. Chiengmai Pink were maintained on 1/2-strength Murashige and Skoog (1962) medium (1/2 MS medium) supplemented with [mg dm^{-3}]: myo-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), peptone (1000), NaH_2PO_4 (170), sucrose (20000), and Gelrite (2200). Leaf tip segments (1 cm long) taken from these donor plants were used as explants. Plant growth regulators were added prior to autoclaving. The pH of the media was adjusted to 5.2 with 1 M KOH or HCl prior to autoclaving for 15 min at 121 °C. Leaf explants were placed on the surfaces of 1/2 MS medium and were incubated in 55 × 15 mm Petri dishes (*Alpha Plus Scientific Corp.*, Taoyuan, Taiwan) under a 16-h photoperiod at irradiance of 28 - 36 μmol m^{-2} s^{-1} (daylight fluorescent tubes *FL-30D/29*, 40 W, *China Electric Co.*, Taipei, Taiwan) or in darkness. The temperature was 25 ± 2 °C. The subculture period was 2 months.

Leaf tip explants (about 1 cm in length) were cultured in light or darkness to test the effects of 2,4-D (0, 3, 10 mg dm^{-3}) and TDZ (0, 0.3, 1, 3 mg dm^{-3}) on induction of direct somatic embryogenesis. Five dishes each with

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; MS - Murashige and Skoog (1962) medium; NAA - naphthaleneacetic acid; TDZ - thidiazuron, 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea.
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four leaf explants were used for each treatment. Frequency of embryo-producing explants and number of embryos per responding explant were counted under a stereomicroscope (*SZH*, *Olympus*, Tokyo, Japan). Data were scored after 30 and 60 d of culture. Treatment means were compared following Duncan's Multiple Range Test (Duncan 1955).

One-month-old leaf-derived embryos were used to test the effects of combinations of NAA (0, 0.1, 1 mg dm^{-3}) and TDZ (0, 0.3, 1, 3 mg dm^{-3}) on multiplication rate of embryos and shooting embryos. Five replicates were performed in each treatment. Data were scored after 30 d of culture. Treatment means were compared following Duncan's Multiple Range Test.

Leaf-derived embryos were transferred to growth regulator-free 1/2 MS medium in light for conversion and further development. Plantlets were potted in *Sphagnum* moss for acclimatization in 50 % shaded greenhouse.

Tissues for histological observations were fixed in FAA (95 % ethyl alcohol : glacial acetic acid : formaldehyde : water, 10:1:2:7), dehydrated in a tertiary-butyl-alcohol series, embedded in paraffin wax, sectioned at 10 µm thickness and stained with 0.5 % safranin-O and 0.1 % fast green (Jensen 1962). Samples for scanning electron microscopy were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 4 h at 4 °C, dehydrated in ethanol (Dawns 1971), critical point dryer (*HCP-2*, *Hitachi*, Tokyo, Japan), and coated with gold in an ion coater (*IB-2*, *Giko Engineering Co*., Tokyo, Japan). A scanning electron microscope (*DSM-950*, *Carl Zeiss*, Jena, Germany) was used for examination and photography of the specimen.

When 1-cm-long leaf tip segments of *Dendrobium* cv. Chiengmai Pink cultured on TDZ-containing 1/2-MS medium, clusters of somatic embryos directly formed from leaf tip regions (Fig. 1*A*) and surfaces near cut ends (Fig. 1*B*) within 30 d in light. Generally, leaf cells of two specific regions could directly form embryos, including

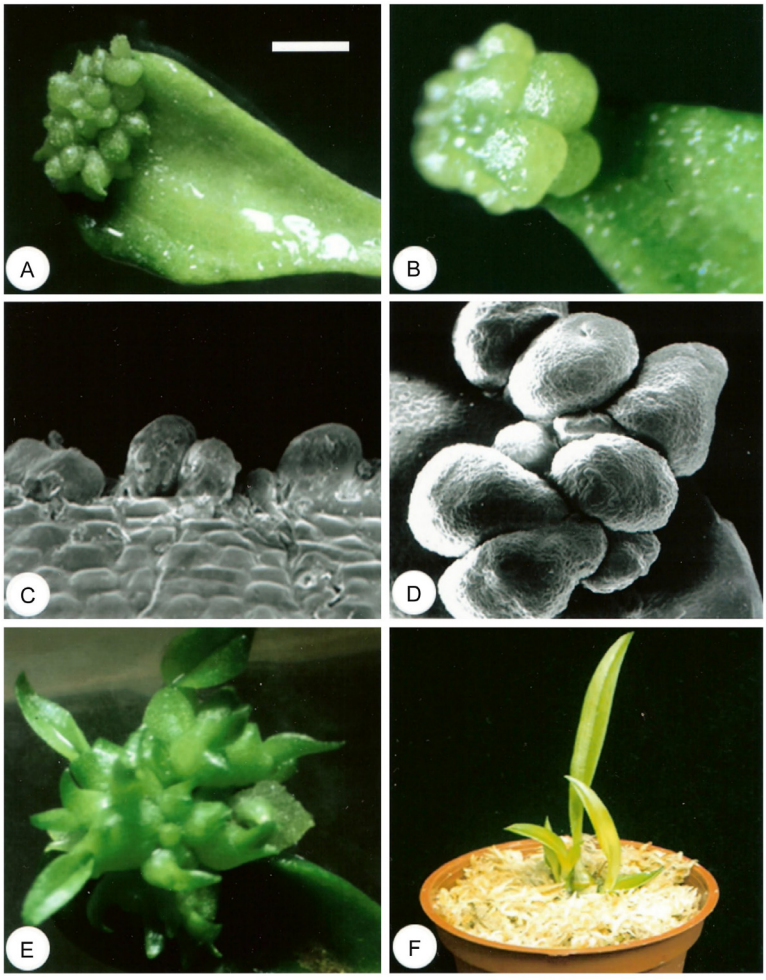

Fig. 1. Direct embryo formation, secondary somatic embryogenesis and plant regeneration of *Dendrobium* cv. Chiengmai Pink (*bar in upper left* refers to all panels): *A* - somatic embryos directly formed from the adaxial surface near the cut end after 30 d of culture in light (*bar* 2 mm); *B* - a cluster of embryos directly formed from the leaf tip region after 30 d of culture in light (*bar* 4 mm); *C* - a scanning EM photo for the early stage of direct embryo formation from leaf epidermal cells (*bar* 200 µm); *D* - a cluster of leaf-derived embryos (*bar* 900 µm); *E* - leaf-derived embryos formed shoots (*bar* 3 mm); *F* - regenerated plantlets (*bar* 1.5 cm).

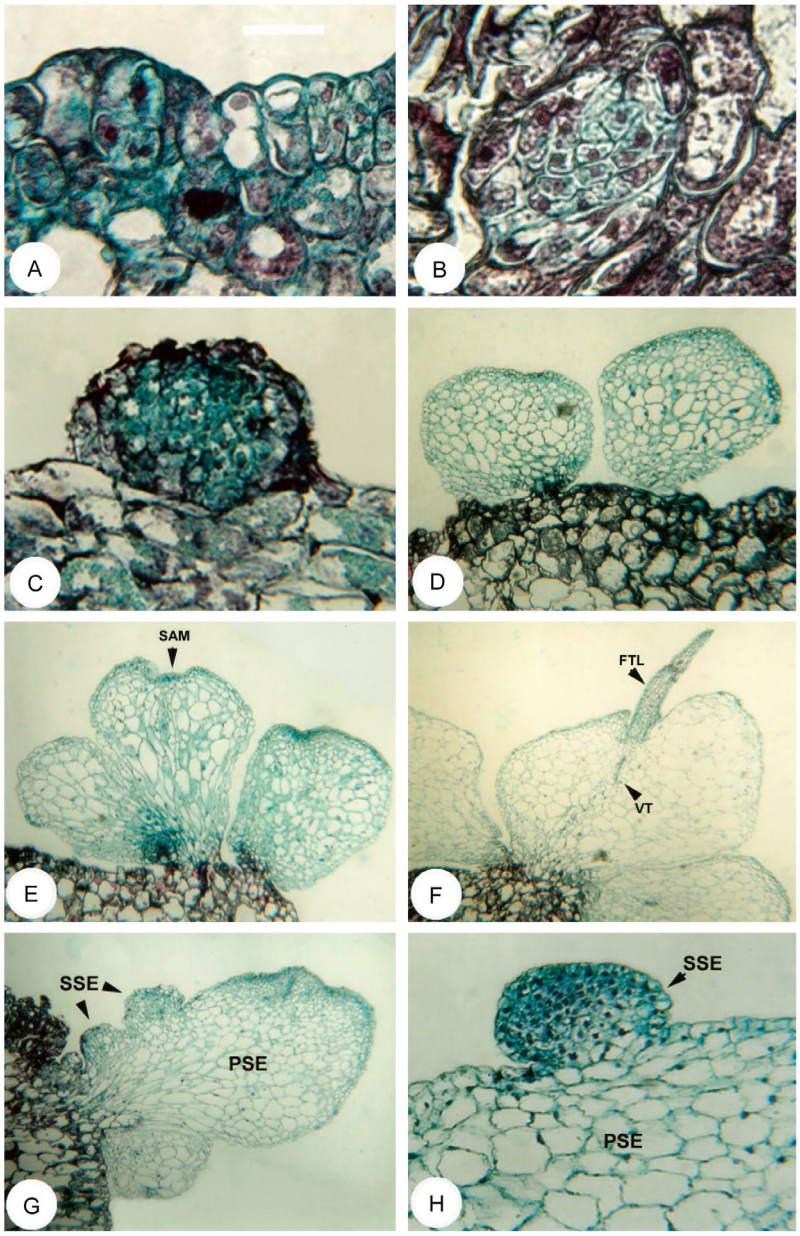

Fig. 2. Histological observation on direct somatic embryogenesis and secondary embryogenesis from leaf explants of *Dendrobium* cv. Chiengmai Pink (*bar in upper left* refers to all panels): *A* - meristematic cells originated from leaf epidermal cell layers (*bar* 0.15 mm); *B* - meristematic cells originated from mesophyll cells (*bar* 40 µm); *C* - a globular embryo with densely stained embryonic cells (*bar* 300 µm); *D* - globular embryos (*bar* 1.5 mm); *E* - developing embryos with shoot apical meristem (SAM) on a leaf explant (*bar* 1.2 mm); *F* - a mature embryo with first true leaf (FTL) and vesicular tissue (VT) (*bar* 600 µm); *G* - secondary embryos (SSE) formed from the posterior region of a primary embryo (PSE) (*bar* 600 µm); *H* - a secondary embryo (SSE) formed from the interior region of a primary embryo (PSE) (*bar* 1 mm).

leaf surfaces near cut ends and leaf tips. By contrast, both sides of leaf surfaces between tips and cut ends had no embryogenic response. In another orchid, *Oncidium*, generally, leaf tips had the highest ability to form embryos (Chen *et al*. 1999, Chen and Chang 2001). However, in *Dendrobium*, leaf surfaces near cut ends had the highest ability to directly form embryos, and occasionally, embryogenesis could be found on leaf tips.

Whether in light or darkness, explants became necrotic and no embryos were obtained on growth regulator-free or 2,4-D-containg medium after 60 d of culture (Table 1). In our previous reports in *Oncidium* and *Phalaenopsis*, 2,4-D is a negative factor in direct embryo induction (Chen and Chang 2001, 2006). The present data indicate that 2,4-D also retarded embryogenesis from leaf explants of *Dendrobium*. Moreover, in

Oncidium and *Phalaenopsis*, light is not an essential factor during direct embryo induction (Chen *et al*. 1999, Chen and Chang 2006). However, working with *Dendrobium*, explants cultured in light have higher embryogenic response than in darkness (Table 1). Despite of TDZ concentration, light enhanced both frequency of embryogenesis and embryo numbers per explant (Table 1). In light, 5 - 10 % of explants formed an average of 2 - 2.5 embryos per responding explant after 30 d of culture on TDZ-containing media (Table 1). After 60 d of culture, higher embryogenic frequencies and embryo numbers were obtained than 30 d (Table 1). The best treatment was at 1 mg dm^{-3} TDZ, and 25 % explants were induced to form an average of 10.4 embryos per responding explant (Table 1). In the presence of TDZ, we obtained lower frequency of embryogenesis in *Dendrobium* leaf cultures when compared with *Oncidium* and *Phalaenopsis* (Chen and Chang 2001, 2006).

Table 1. Effects of light and TDZ on direct somatic embryogenesis from leaf explants of *Dendrobium*. Data were scored after 30 and 60 d of culture. Percentage of direct somatic embryogenesis was determined as embryo-producing explants divided by number of explants (20) in each treatment. Mean number of embryos per responding explant was measured as total number of embryos divided by embryo-producing explants in each treatment. Means followed by same letters in a column are not significantly different from each other according to Duncan's multiple range test ($P < 0.05$).

TDZ [mg dm^{-3}]		Embryogenesis [%] 30 d	60 d	No. embryos [$explant^{-1}$] 30 d	60 d
0	light	0 a	0 b	0	0
0.3		5 a	10 ab	2.0	2.7
1.0		10 a	25 a	2.0	10.4
3.0		10 a	15 ab	2.5	2.7
0	dark	0 a	0 b	0	0
0.3		0 a	5 b	0	1.0
1.0		0 a	10 ab	0	1.5
3.0		0 a	5 b	0	2.0

From SEM observation, somatic embryos originated from leaf epidermal cells (Fig. 1*C*). Subsequently, embryos developed into globular shape and usually aggregate in clusters (Fig. 1*D*). At maturity, embryos could be easily dislodged from each other and parent explants. After 60 d of culture, these embryos enlarged and formed shoots (Fig. 1*E*). Roots developed 45 - 60 d after transferring these embryos to growth regulator-free 1/2 MS medium (Fig. 1*F*). Plantlets (about 6 cm in height) with 5 - 6 leaves and 2 - 3 roots were obtained from 60-d-old embryos after 3 passages of subculture with two month intervals (Fig. 1*G*).

Table 2. Effects of NAA and TDZ on multiplication rate of embryos of *Dendrobium*. Data were scored after one month of culture. Five replicates were performed in each treatment. Multiplication rate was determined as final numbers of embryos divided by initial numbers of embryos. Means followed by same letters in a column are not significantly different from each other according to Duncan's multiple range test ($P < 0.05$).

NAA [mg dm^{-3}]	TDZ [mg dm^{-3}]	Multiplication rate
0	0	1.79 b
0	0.3	2.47 ab
0	1	3.50 ab
0	3	2.53 ab
0.1	0	3.67 ab
0.1	0.3	3.27 ab
0.1	1	3.94 ab
0.1	3	2.56 ab
1	0	2.95 ab
1	0.3	3.20 ab
1	1	3.39 ab
1	3	5.17 ab

Small clusters of primary embryos were used to test the effects of NAA and TDZ on embryo development and secondary embryogenesis. When maintained on growth regulator-free medium, embryos gradually formed shoots and some were found to be bearing secondary embryos on outer surfaces of sheath leaves and basal parts. All combinations of NAA and TDZ enhanced the multiplication rate of embryos (Table 2). The best treatment was at 1 mg dm^{-3} NAA plus 3 mg dm^{-3} TDZ with 5.17 multiplication rate of embryos after 30 d of culture (Table 2).

In this present communication, we developed a simple and efficient protocol for induction of direct somatic embryogenesis of *Dendrobium* cv. Chiengmai Pink, and successfully obtained plantlets from regenerated embryos. Regeneration system through direct somatic embryogenesis is suitable for further studying on physiology and morphology status of embryo development, mass propagation and genetic transformation in *Dendrobium*.

References

Arditti, J., Ernst, R. (ed.): Micropropagation of Orchids. Pp. 467-520. John Wiley & Sons, New York 1993.

Chen, J.T., Chang, W.C.: Effects of auxins and cytokinins on direct somatic embryogenesis from leaf explants of *Oncidium* 'Gower Ramsey'. - Plant Growth Regul. **34**: 229-232, 2001.

Chen, J.T., Chang, W.C.: Direct somatic embryogenesis and plant regeneration from leaf explants of *Phalaenopsis amabilis*. - Biol. Plant. **50**: 169-173, 2006.

Chen, J.T., Chang, C., Chang, W.C.: Direct somatic embryogenesis from leaf explants of *Oncidium* 'Gower Ramsey' and subsequent plant regeneration. - Plant Cell

Rep. **19**: 143-149, 1999.
Dawns, C.J.: Biological Techniques in Electron Microscopy. - Barnes and Noble, New York 1971.
Duncan, D.B.: Multiple range and multiple F test. - Biometrics **11**: 1-42, 1955.
Jensen, W.A.: Botanical Histochemistry. - Freeman, San Francisco 1962.
Manorama, P., Rao, A.N., Goh, C.J., Loh, C.S.: Leaf callus development in *Aranda* and *Dendrobium*. - In: Fifth ASEAN Orchid Congress. Pp. 102-109. National University of Singapore, Singapore 1984.
Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant **15**: 495-497, 1962.
Nayak, N.R., Rath, S.P., Patnaik, S.: *In vitro* propagation of three epiphytic orchids, *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch and *Dendrobium moschatum* (Buch-Ham) Sw. through thidiazuron-induced high frequency shoot proliferation. - Sci. Hort. **71**: 243-250, 1997.
Nayak, N.R., Sahoo, S., Patnaik, S., Rath, S.P.: Establishment of thin cross section (TCS) culture method for rapid micropropagation of *Cymbidium aloifolium* (L.) Sw. and *Dendrobium nobile* Lindl. var. *oculatum* Hk. f. - Sci. Hort. **94**: 107-116, 2002.
Teob, E.S. (ed.): Orchids of Asia. - Times Books International, Singapore 1989.

Price, W.C., Rana, N., Sample, V.A. (ed.): **Plantations and Protected Areas in Sustainable Forestry.** - Food Products Press, an Imprint of the Haworth Press, Inc., Binghampton 2006. 156 pp. USD 19.95. ISBN 13: 978-1-56022-139-9. Co-published simultaneously as Journal of Sustainable Forestry 21 (4), 2005.

Sustainability seems to become a very popular not only among environmentalists, but also scientists, economists and politicians. This term is often misused in order to add some importance and insistency to the topic that is only vaguely related to the survival of human societies on this planet. Fortunately, this is not the case of this reviewed book. As stated in the Introduction by V. Alaric Sample, President of the Pinchot Institute for Conservation, *"What is new, is the broader recognition of the urgent need to protect the remaining biological diversity in high conservation value forests – and, at the same time, meet more of humanity's material needs from renewable natural resources such as wood*".

The contributions of this book really deal with precise assessment of both forests values and managed tree plantations. It could be of general interest, that the world's annual wood harvest (about 1.6 billion cubic meters) requires only one fifth of the world forest area. Although the wood consumption is expected to further rise, considerable supply could be gained from tree plantations. The benefits as well as problems of the intensively managed industrial plantations are discussed in much detail in the individual contributions. As the volume contains only 8 papers, their titles could be listed in this review in order to illustrate the potential impact of plantations and protected areas on sustainable forestry.

This book starts with some basic information about the editors. The Introduction explains the main reasons for paying so much attention to changes in forest management. The very content deals with "The Environmental Benefits of Tree Plantations", "Integrating Protected Areas, Plantations, and Certification", "Industrial Plantation Forestry: Do Local Communities Benefit?", "Environmental Aspects of the Intensive Plantations/Reserve Debate", "Mitigating Environmental and Social Impacts of Intensive Plantation Forestry", "Investing in Forestry: Opportunities and Pitfalls of Intensive Plantations and Other Alternatives", "A vision for World Forests: Results from the Council on Foreign Relations Study", and "Sustainable Forestry and Biodiversity Conservation: Toward a New Consensus". The book terminates with a detailed Index.

I very much appreciate that the contributions are not overwhelmed with vague sentences on sustainability and its importance. Any reader would find here a lot of valuable data as well as qualified analyses of the role of forests in the world economy and local communities. The book clearly indicates, that sustainability need not be an irrational idea but a noble ambition for researchers and managers, economists and politicians. As I started with a quotation, I would like to terminate with another one from the last contribution written again by V.A. Sample: *"We have an obligation to protect our remaining "hot spots" of biological diversity – and bear our share of the local, short-term economic effects of doing so – and, at the same time, meet our share of the demand for renewable wood and fiber that we ourselves generate, without shifting un undue burden on biologically rich forests in other regions of the world."*

It is a pleasure for me to recommend this book by far not only to specialists in forests and tree plantations, but to anybody who is deeply concerned about the future fate of the world ecosystems.

L. NÁTR (*Praha*)