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ORIGIN AND DEVELOPMENT OF CALLUS FORMATION FROM CULTURED BUD OF VANDA ORCHID

KAMNOON KANCHANAPOOM[a], THAVORN VAJRABHAYA[b] AND OBCHANT THAITONG[b]

[a]*Department of Biology, Faculty of Science, Prince of Songkla University, Hat-Yai, Songkla 90112, Thailand.*
[b]*Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok 10500, Thailand.*

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ABSTRACT

Lateral buds of Vanda x *Miss Joaquim were cultured on a modified agar medium containing 10% coconut water and 0.1 mg/l α-naphthalene acetic acid for one month before being transferred to a liquid medium. Histological analysis was carried out on paraffin sections stained with hematoxylin. The cultured buds on an agar medium resembled a typical angiosperm shoot apex. After leaf excision, some of these buds initiated meristematic cells on the abaxial position of young leaves. Serial sections revealed that callus formation originated from a single meristematic cell that had dense cytoplasm with a large nucleus. The calli were composed of parenchyma cells bounded by an epidermis-like layer and subsequently these cells differentiated into protocorm-like bodies when they were submerged in a liquid medium.*

INTRODUCTION

Success in clonal propagation of orchids through tissue culture has been reported by many researchers[1-6]. For commercial proposes, multiplication of a desired orchid plant by tissue culture technology is satisfactory. However, anatomical study and origin of callus tissue is inadequate. Morel[7] studied organogenesis of *Cymbidium* and *Cattleya* and reported that protocorm-like bodies (plb) usually appeared in epidermal and hypodermal layers. Intuwong and Sagawa[8] reported that young inflorescence of Sarcanthine orchid showed clusters of meristematic activities in the hypodermal layers of rachis, from which plb developed. Vajrabhaya and Vajrabhaya (personal communication) noted that new tissues of *Dendrobium* inflorescence derived from epidermis.

Since literature on the histology of orchid callus is still limited and most work was done on sympodial orchids, the purpose of this paper is to present the results on the origin of callus formation of monopodial orchids.

MATERIALS AND METHODS

Lateral buds of *Vanda* x Miss Joaquim, a monopodial orchid, which is the hybrid of *Vanda teres* (Roxb.) Lindl. x *Vanda hookerana* Rchb. f[9] were excised aseptically following the

method described by Kunisaki *et al*[10]. Briefly, stems were topped at the node of the seventh leaf and cut into 1.5-2 inch sections. These sections were surface-sterilized in the solution of 10% Clorox for 10 min. Leaf beyond the sheath was removed and sections were transferred to 5% Clorox for 5 min. After thoroughly rinsing in sterile distilled water, these buds were excised and placed on modified medium (Table 1) supplemented with 10% coconut water (CW) and 0.1 mg/l α-naphthalene acetic acid (NAA). The cultures were incubated at $27 \pm 2°C$ under fluorescent lamps adjusted to intensity ranging between 2,000-3,000 lux for 14 hours each day. A month after initial culture, young leaves covering the buds were cut. The defoliated explants were placed in a liquid medium without sucrose. Liquid cultures were continuously agitated at ca. 100 rpm on a reciprocating shaker for another 1-2 months. The serial sections (10 μm) were prepared from tissues fixed in FAA, dehydrated through a tertiary butyl alcohol series and subsequently stained in hematoxylin.

RESULTS

During the first month of culture on an agar medium, *Vanda* buds swelled considerably and became semi-spherical due to the development of leaf primordia (Fig. 1a). Observation under a light microscope revealed that the anatomy of the cultured buds was resembled natural buds (Fig. 1b). Removal of leaf primordium and apex was necessary before transferring to a liquid medium to induce proliferating bodies; if this is not done, the young leaves elongated and each bud grew into a single plantlet (Fig. 1c). Greater proliferation was achieved when buds were cultured in a liquid medium and the plb was green and healthy and there was no necessity for cutting these plb since they were separated easily by agitation (Fig. 1d).

Microscopic examination of the serial sections showed that eight buds out of twenty five initiated meristematic cells on the leaf epidermis of young leaves (Fig. 2a). The newly formed tissue originated from this single meristematic cell that had a dense cytoplasm with a large, well-stained nucleus (Fig. 2b). Rich cytoplasm of the cell was quite conspicuous in contrast to the original surrounding cells. Fig. 2a also showed scattered single meristematic cells on the abaxial position of young leaves.

The first division of the single cell was anticlinal, giving rise to the two-celled stage (Fig. 2c). The second division of the two-celled stage took place in both cells simultaneously, forming the four-celled stage (Fig. 2d). Shortly afterwards in four-celled stage, daughter cells underwent periclinal division (Fig. 2e) resulting in the formation of a globular structure (Fig. 2f). From this stage, rows of meristematic cells increased in extent by anticlinal and periclinal division so that callus mass developed in large numbers over this area (Fig. 3a). The sections also showed that the calli were composed of small prominent and isodiametric parenchyma cells formed on the surface of this callus (Fig. 3b) resulting in shoot apex-like structure or meristemoid (Fig. 3c). Many meristemoids in different stages were observed over a broad area of the leaf and showed no continuity of vascular traces to parental tissue (Fig. 3d).

DISCUSSION

It is considered that *Vanda* callus was first initiated while cultured on an agar medium owing to the removal of leaf primordium. Effect of wounding has been clearly shown on a

TABLE 1. Composition of medium used in culturing *Vanda* buds

Constituents	Concentrations (mg/l)
Major elements	
KNO_3	2,500
$MgSo_4.7H_2O$	400
$NH_4H_2PO_4$	300
$CaCl_2.2H_2O$	200
Iron compound	
$F_eSO_4.7H_2O$	15
Na_2EDTA	20
Minor elements	
$MnCl_2.4H_2O$	2
H_3PO_3	1
$ZnSO_4.7H_2o$	0.1
$CoCl_2.6H_2O$	0.02
$CuCl_2.2H_2O$	0.01
Organic addenda	
Myo-inositol	100
Nicotinic acid	5
Glycine	2
Pyridoxin HCl	0.5
Thiamine HCl	0.5
Folic acid	0.5
Biotin	0.05
Sugar	
Sucrose	20,000
Auxin	
NAA	0.1

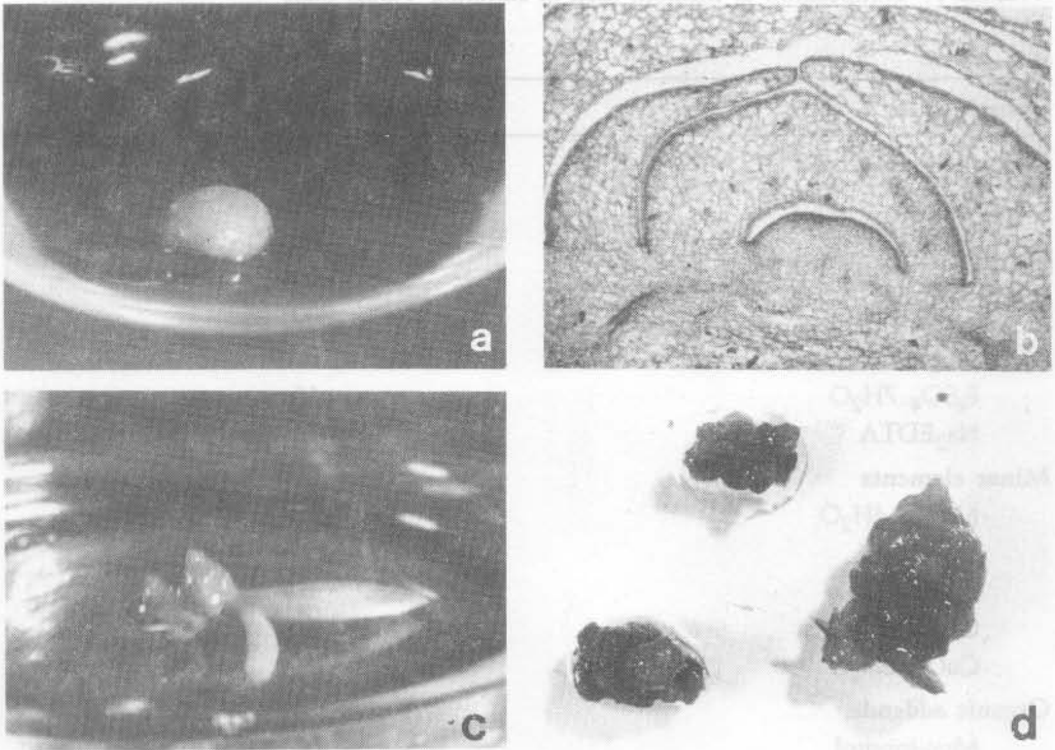

Fig. 1. Lateral bud of *Vanda* cultured on an agar medium (a) semi-spherical shape (b) cross section of bud (c) single plantlet from an uninjured bud cultured in a liquid medium (d) protocorm-like bodies forming on the surface of defoliated bud.

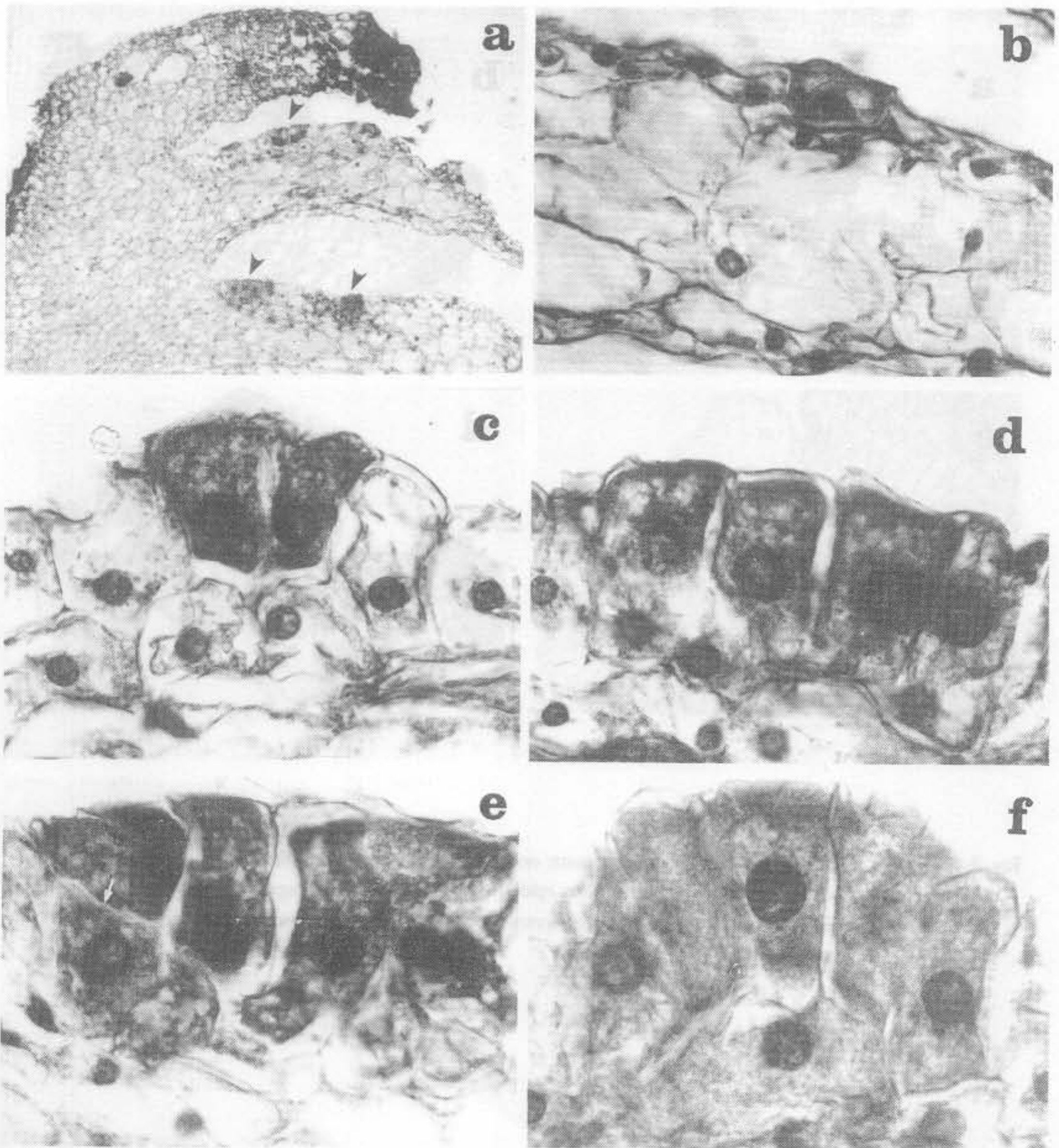

Fig. 2. Histology of *Vanda* bud cultured on an agar medium after leaf excision showing (a) scattered meristematic cells on young leaves (arrows) (b) a single meristematic cell with densely stained cytoplasm and nucleus (c) the two-celled stage derived from anticlinal division of the single cell (d) the four-celled stage (e) appearance of periclinal division (arrow) at the four-celled stage (f) the rising of daughter cells resulted from periclinal division.

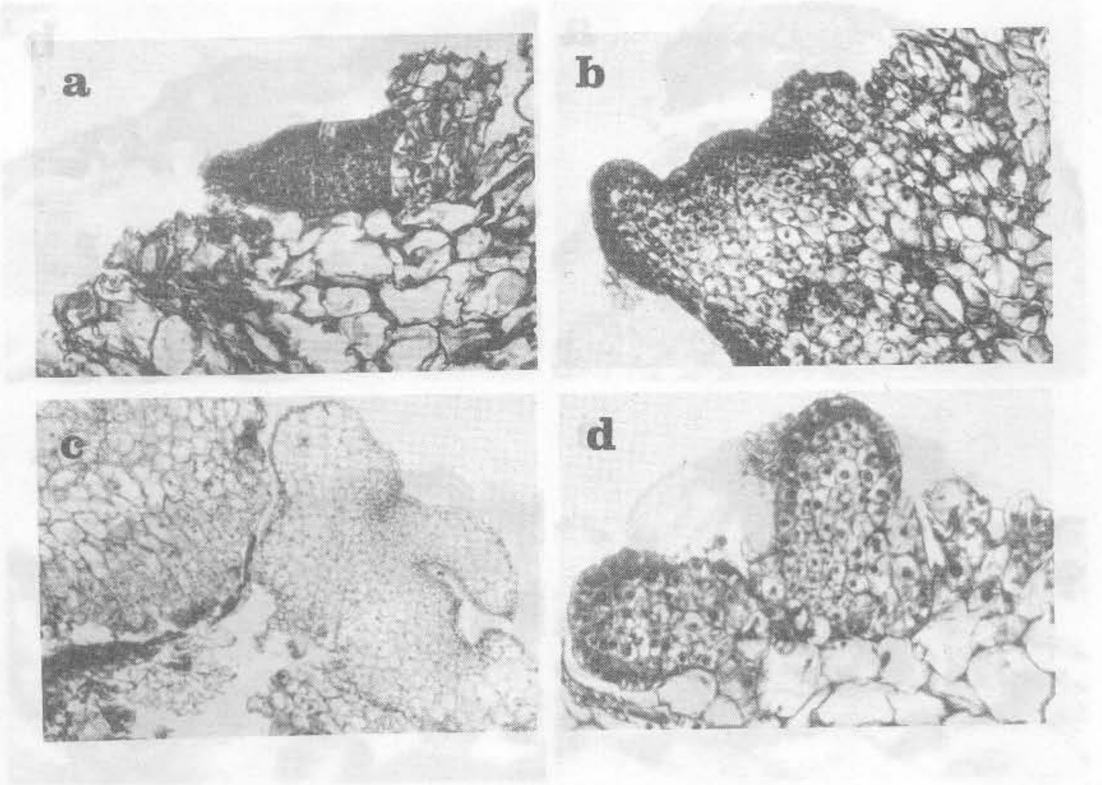

Fig. 3. (a) Callus originating from a single meristematic on the surface of young leaf (b) compact callus composed of small dense parenchyma cells bounded by an epidermis-like layer (c) later stage of (b) show growing point or meristemoid (d) formation of several meristemoids without direct connection to the leaf cells below.

variety of explants[8, 10, 11, 12]. Besides wounding, the balance of concentrations of auxins and cytokinins is changed and the new balance promotes callusing. Additions of CW to the medium invariably enhance callus proliferation since CW contains several growth regulators.

Multiplication of plb is usually carried out in a liquid medium. The advantage of a liquid medium is that released waste substances are diluted and not accumulated around the explants. There is better O_2 supply in a liquid medium and the transport of nutrients is more effective, hence increase in growth rate of plb. Growth and proliferation of plb depend on the type of tissue and the composition of the nutrient medium used[13]. In our experiment the addition of sucrose, the source of carbon and energy in all synthetic media, affected growth of *Vanda* buds. This is in accord with other vandaceous orchids[10, 12, 14]. The reason for this is not quite understood; however, electron microscopic studies have shown that cells of tissue in a medium containing sucrose have lost their integrity, and parts of the cell ultrastructure were damaged. Elimination of sucrose from the medium produced healthy tissues[14]. Toxic substances produced by the interaction of sucrose, CW and other nutrients during autoclaving is probably another factor causing buds to turn yellow and become necrotic[15].

In orchid tissue culture from meristem, inflorescence and leaf[7, 8], the calli formed at first on explants differentiated into plantlets. In the culture of buds reported here, the single epidermal cells which retain meristematic potential develop indirectly somatic embryogenesis with an intervening callus stage. Such indirect embryogenesis has been described in *Ranunculus sceleratus*[16] and *Daucus carota*[17]. In early stages of the regeneration of these plants, the mode of epidermal cell division is quite similar to that of embryogenesis from *Vanda* leaf epidermis.

REFERENCES

1. Morel, G. M. (1960). Producing virus-free *Cymbidium*. *Amer. Orch. Soc. Bull.* **29**, 495-497.
2. Sagawa, Y., Shoji, T. and Shoji, T. (1966). Clonal propagation of *Cymbidium* through shoot meristem culture. *Amer. Orch. Soc. Bull.* **35**, 118-122.
3. Scully, R. M. (1966). Stem propagation of *Phalaenopsis*. *Amer. Orch. Soc. Bull.* **35**, 40-42.
4. Churchill, M. E., Arditti, J. and Ball, A. E. (1977). Clonal propagation of orchids from leaf tips. *Amer. Orch. Soc. Bull.* **40**, 109-113.
5. Kerbauy, G. B. (1984). Plant regeneration of *Oncidium varicosum* (Orchidaceae) by means of root tip culture. *Plant Cell Reports.* **3**, 27-29.
6. Shimasaki, K. and Uemoto, S. (1990). Micropropagation of a terrestrial *Cymbidium* species using rhizomes developed from seeds and pseudobulbs. *Plant Cell, Tissue and Organ Culture* **22**, 237-244.
7. Morel. G. M. (1971). Morphogenesis of stem apical meristem cultivated *in vitro* : Application to clonal propagation. In *Morphogenesis in Plant Cell, Tissue and Organ Culture-International Symposium*.(Johri, B.M. ed.) Delhi UGC Center of Advanced Study in Botany, University of Delhi, pp. 5-12.
8. Intuwong, O. and Sagawa, Y. (1973). Clonal propagation of Sarcanthine orchids by aseptic culture of inflorescence. *Amer. Orch. Soc. Bull.* **42**, 209-215.
9. Holttum, R. E. (1953). Flora of Malaya. Vol 1. Orchid of Malaya. pp. 16-18. Government Printing Office, Singapore.
10. Kunisaki, J. T., Kim. K. K. and Sagawa, Y. (1972). Shoot-tip culture of *Vanda*. *Amer. Orch. Soc. Bull.* **41**, 435-439.
11. Tse, A. T. Y., Smith, R. and Hackett, W. P. (1971). Adventitious shoot formation on *Phalaenopsis* nodes. *Amer. Orch. Soc. Bull.* **40**, 807-810.

12. Vajrabahya, M. and Vajrabhaya, T. (1970). Tissue culture of *Rhynchostylis gigantea,* a monopodial orchid. *Amer. Orch. Soc. Bull.* **39,** 907-910.
13. Teo, C. K. H. (1980). Studies on some aspects of orchid tissue culture. In *Proceedings of the 9th World Orchid Conference.*(Sukshom, K. ed.) Amarin Press, Bangkok, Thailand, pp. 211-214.
14. Teo, C. K. H. and Wong, C. H. (1978). Effect of sucrose on the growth of protocorms of *Holttumara* Loke Tuck Yip. *The Orchid Review.* **86,** 285-289.
15. Puhan, Z. and Martin, S. M. (1970). The industrial potential of plant cell culture. *Prog. Ind. Microbiol.* **9,** 13-39.
16. Konar, R. N., Thomas, E. and Street, H. E. (1972). Origin and structure of embryoids arising from epidermal cell of stem of *Ranunculus sceleratus* L. *J. Cell Sci.* **11,** 77-93.
17. Kato, H. and Takeuchi, M. (1966). Embryogenesis from the epidermal cells of carrot hypocotyl. *Scientific Papers of the College of General Education University of Tokyo* **16,** 245-254.

บทคัดย่อ

เลี้ยงตาช้างแวนด้ามิสโจอะคิมบนอาหารวุ้นสูตรดัดแปลงที่มีน้ำมะพร้าว 10% และ NAA 0.1 มิลลิกรัมต่อลิตร เป็นเวลา 1 เดือน ก่อนย้ายไปเลี้ยงในอาหารเหลวสูตรเดียวกัน ศึกษาลักษณะกายวิภาคตาแวนด้าเมื่อเลี้ยงในหลอดทดลอง โดยการตัด section ต่อเนื่องด้วยวิธีการฝังพาราฟิน ย้อม section ด้วยสีฮีมาทอกไซลิน พบว่าตาข้างที่เลี้ยงในหลอดทดลอง มีลักษณะทั่ว ๆ ไปตามแบบฉบับปลายยอดของพืชพวกแองจิโอสเปิร์ม เมื่อตัดเอาใบอ่อนออก พบว่าตาข้างบางอันมีการสร้าง เซลล์เมอริสเตมตรงตำแหน่ง abaxial ของใบอ่อน จากการศึกษากายวิภาคของแคลลัสที่เกิดพบว่า มีจุดกำเนิดมาจากหนึ่งเซลล์ ซึ่งมีไซโทพลาซึมหนาแน่นย้อมติดสีเข้ม นิวเคลียสใหญ่ กลุ่มแคลลัสประกอบด้วยเซลล์พาเรนไคมา ต่อมามีชั้นคล้ายเอพิเดอมิส ล้อมรอบ เมื่อนำแคลลัสนี้ไปเลี้ยงในอาหารเหลวก็จะได้ก้อนโปรโตคอร์มขึ้นมา