In Vitro Plant Regeneration through Embryogenesis and Organogenesis from Callus Culture of Pigeon Orchid (*Dendrobium crumenatum* Sw.)

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Abstract

In vitro clonal propagation of pigeon orchid (*Dendrobium crumenatum* Sw.) through callus from bud culture was carried out. The suitable conditions for three main stages of plant regeneration, callus proliferation, PLBs formation and proliferation as well as regeneration of shoots were examined. The callus proliferation required the combination of 0 1 mg l^{-1} NAA, 1 mg l^{-1} BA, 2 g l^{-1} AC and 2 g l^{-1} peptone to promote growth. PLBs formation stage needed additive 10% CW while the stage of shoot regeneration had occasion to the condition from previous steps of experiment. Peptone could be added in the media for all three developmental stages but it must be used as a minor substance only. Histological observation suggested that plant regeneration was developed through both embryogenesis and organogenesis.

Keywords: protocorm-like bodies, *Dendrobium crumenatum*, callus, orchid, histology, embryogenesis, organogensis

Abbreviations : AC- activated charcoal; BA- 6-Benzyladenine; CW- Coconut water; HP- Hyponex basal medium; NAA- α- Naphthaleneacetic acid; NC- Nodular compact structure; PGR- Plant growth regulator; PLBs- protocorm- like bodies; VW- Vacin and Went basal medium

1. Introduction

The pigeon orchid (*Dendrobium crumenatum* Sw.) is a tropical epiphytic orchid and grows on almost every old tree in the open country. Its life history is typical of many other Malayan orchids, and has many uncommon features, such as, its fragrant white flowers [1]. All plants in the area can flower simultaneously through the year and the flowers open in a short time (1-2 days). So the pigeon orchid is interesting for many physiologists, developmental biologists, molecular geneticists as well as plant breeders.

The tissue culture technique has advanced rapidly in recent years. Somatic embryogenesis and organogenesis have become acceptable techniques for clonal propagation. Thus, the establishment of an efficient *in vitro* plant regeneration is the basis for various further studies, for instance, *in vitro* flowering system [2] and genetic modification studies using genetic manipulation [3]. Besides, the *in vitro* flowering system, in particular based on somatic embryogenesis and organogenesis is still useful to genetic analysis of expression of specific gene products during floral development. In addition, there are fewer variations in clonal propagation between plantlets obtained from tissue culture and seed- derived plantlets. In the latter case, most of their characteristics are not uniform [4]. Hence a method for high number explants with few unvarying characters, suitable for subsequent experiment was desirable. Furthermore, the use of single genotype-derived cultures especially plants developed via embryogenesis, will not only solve the problems of genotype effects but also provide the high

regeneration, fast growing and uniform explants [5].

The present study was attempted to develop an efficient plant regeneration through intermediate callus proliferation. Additionally, the study investigated whether the media had any impact on growth or not. Thus, the various addenda were also incorporated into the media to study their effects on stages of development. We chose to investigate the suitable media for each phase of development to produce the high number explants used for subsequent experiments. The histological observation during morphogenesis was made to investigate if almost all of calli- derived plantlets will develop through whether embryogenesis or organogenesis.

2. Materials and methods

2.1 Plant materials and surface sterilization

The axillary buds of *D. crumenatum* were used to obtain protocorm-like bodies (PLBs) and plantlets. Main shoots (15-25 cm. long) were harvested from plants grown in greenhouse at Department of Biology, Faculty of Science, Prince of Songkla University. The stalks were cut into the nodal segments each holding one axillary bud. These nodal segments (about 3-4 cm in length) were first washed with tap water and a few drop of detergent (Teepol) , then rinsed with water 2-3 times. After removing of their sheaths they were surface sterilized with 20% clorox containing 1-2 drops of Tween 20 for 20 min. The series of clorox percentage were used as 10%, 5% for 10 and 5 minutes respectively. Finally the excised buds were washed with sterile distilled water 2-3 times and cultured on the Vacin and Went (VW) medium [6] supplemented with 20 g l^{-1} sucrose to promote bud growth. The medium was adjusted pH at 5.3 with NaOH or HCl. The medium was solidified using 2 g l^{-1} Gelrite and autoclaved at 121 °C and 1.05 kg cm^{-2} for 20 min. The cultures were kept at 25 ± 2 °C at 16 hr photoperiod under an illumination of 20 µmol m^{-2} s^{-1} photosynthetic photon lux density provided by Grolux lamps. These buds were maintained for 4 weeks before being transferred to the callus intiation (CI) medium.

2.2 Initial callus production

The 4- week- old buds growing on VW agar medium were transferred to the CI medium. The underwater dissection technique was used. The CI medium contained the basal salts of VW supplemented with 20 g l^{-1} sugar, combination of 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA, 2 g l^{-1} peptone, 2 g l^{-1} Gelrite and 2 g l^{-1} Activated Charcoal (AC) at pH 5.3 (The medium was selected on the basis of preliminary experiment). The callus proliferation was observed after one month of cultures. These calli were then transferred to the same medium. The subculture monthly was recommended to produce more totipotent calli than the subsequent experiment.

2.3 Sequence of plant regenration obtained from PLBs

Cultured media and conditions

The basic salts of VW and Hyponex (HP; N: P: K = 5-10-5) media supplemented with or without various addenda were examined to be the effective and suitable one on various stages of growth : callus proliferation, PLBs formation and shoot differentiation respectively. Apart from complex addenda, 1mg l^{-1} BA and 0.1mg l^{-1} NAA, were also incorporated into medium. Five modified media were tested while using the basal media without supplement as the control : (1) added 10% coconut water (CW), (2) added 2 g l^{-1} peptone, (3) added the combination of 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA (4) added 10% CW besides the combination of 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA (5) added 2 g l^{-1} peptone besides the combination of 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA. All media were adjusted to pH 5.3 using either HCl or NaOH prior to autoclaving. The 20 g l^{-1} sucrose and 2 g l^{-1} AC were added to all media. The VW and HP formulae were solidified with 2 g l^{-1} Gelrite and 7.2 g l^{-1} agar respectively. All the cultures were maintained in the same conditions described previously.

Callus proliferation

To investigate the suitable medium for callus proliferation, the cultured media supplemented with different addenda described before were used. The 5ml of each medium was dispensed into each 20 ml screw-cap vial. The amount in fresh weight of 100mg of established callus was inoculated into each vial. The increase in fresh weight of callus was taken after four weeks of culture.

PLBs formation and proliferation as well as regenerated shoots

In order to multiply the amount of PLBs, the fresh weight of callus and PLBs was recorded at the end of the 10^{th} week. These calli and PLBs were next displaced to each bottle with 10 ml of the same medium. After that, the mean number of the regenerated shoots was recorded at the end of the 16^{th} week of culture.

Plantlet production

In order to create plantlets, the regenerated shoots with 2-3 leaves were detached from shoot clumps and transferred to VW medium (pH 5.3) supplemented with 2 g l^{-1} sucrose, 10% CW and 7.2 g l^{-1} agar. Afterwards, these healthy plantlets were surrounded by moist coconutpeat before being filled in the minipots.

2.4 Data analysis

The experiment was designed in completely randomized design (CRD) and every treatment had 6 replications. It was repeated twice. Both fresh weights of callus proliferation and PLBs formation were recorded at the end of the 4^{th} week and the 10^{th} week.The growth of cultures was measured in terms of increase in fresh weight (mg). The differentiated shoots were recorded as the number of shoots at the 16^{th} week of the experiments. The number of regenerated shoots from PLBs in each bottle after the 16^{th} week of cultures were counted and presented as the number of regenerated shoots produced per gram of PLBs. The data were analyzed using ANOVA and subjected to Duncan's multiple range test (DMRT) at a significance level of P= 0.05, using SPSS v.10 software. These data were separately analyzed for each set of experiments. The square root transformation was used for PLBs formation and proliferation experiment before analysis of variance [7]. The Kruskal-Wallis test was used to analyze the mean difference of regenerated shoots in the last growth phase

2.5 Histology analysis

The cultures were collected as representative samples. They were then fixed in FAA II (formalin : acetic acid : 70% ethanol ; 5 : 5 : 90), dehydrated through the ethyl-butyl alcohol series and embedded in paraplast plus (m.p 60-62 °C). Specimens were cut into 10 μm thick sections on rotary microtome and stained with hematoxylin as well as safranin and fast green for microscopic observation [8].

3. Results and discussion

3.1 Growth phase of plant regeneration

The stages of orchid development were described as follows. The one month old bud (Fig 1A) was transferred to the CI medium. The callus turned green or yellowish and formed nodular compact structures (NC) within four weeks (Fig 1B). These structures further proliferated and developed into PLBs by the 10^{th} week, enlarged and differentiated to regenerated shoots (Fig 1C) by the 16^{th} week. The regenerated shoots could be separated and transferred to fresh media. They grew to small plantlets having two expanded leaves and 2-3 roots before *in vivo* transfer.

3.2 Effect of various supplements on each growth phase

The callus proliferation of *D. crumenatum* was promoted significantly on VW medium supplemented with PGR and peptone when compared with VW medium without no any addenda or added with only PGR (Table1). So, VW medium with PGR (1 mg l^{-1} BA and 0.1 mg l^{-1} NAA) and peptone (1gl^{-1}) was the most effective for the callus proliferation phase. This medium was selected as the standard maintenance medium for this phase and for subculturing the totipotent calli. The VW medium containing only either CW or peptone were more effective to promote callus growth than the VW medium supplemented with only PGR. The callus proliferation phase required not only low concentration of PGR but also additive nutrients such as peptone to promote their growth because the quantity of callus was enhanced significantly by the addition of peptone (Table 1). However, for HP medium test, the medium without any additive substances showed the minimum of increased fresh weight of callus compared with HP medium containing other addenda, for instance, CW or peptone. The results are similar to that of VW medium testing. But the CW was more effective than peptone. From visual observation, both of VW and HP medium tests showed that the calli obtained from any media supplemented with peptone were greener. In addition, growth of callus from VW medium was more vigorous

than that from HP medium. Unfortunately, after subculture, the calli from the HP experiment had no further development and eventually died. The result may suggest that HP medium was not suitable for this important stage. It was noticed that peptone, one type of amino proteins, was important for this phase. Vellupillai *et al* [9] reported that at least two proteins (Mr. 14 and 33 kDa) appeared to be synthesized and stored during seed development and rapidly metabolized during protocorm development. So, the proteins were important for nutrition during the period of embryo development into seedling of orchid *D. crumenatum*. These proteins could be the major seed-storage proteins as observed in other plants. Likewise in this experiment, when peptone was incorporated to the medium it could make callus grow vigorously. In addition, peptone was the most effective to promote callus growth and proliferation. So. protein was important and required for this developmental stage as well. It was possible that some proteins were necessary for PLB development of *D. crumenatum* as well. Besides, the calli could be also maintained by subculturing monthly in the same medium like other orchid [10].

The largest number of PLBs were formed on VW medium with PGR and CW. In this stage the calli could continue to proliferate as well as to produce PLBs. There was no significant difference between the medium with only CW and the medium with combination of PGR and CW. However, as PGR would be useful to cause the proliferation, increase of the fresh weight of PLBs and callus obtained from medium supplemented with PGR and CW was higher than that from the medium containing only CW. The media with with only peptone or only PGR were ineffective in this stage. Therefore, CW would be useful to promote the callus to form PLBs.

In the plantlet regeneration stage, there was no significant difference among regenerated shoots obtained from any media tested. However, the hormone-free medium with peptone gave more vigorous shoots than the others. Besides, PLBs themselves seemed to have the efficiency for further development. It was possible that the suitable media in previous developmental stage was the most effective and could make PLBs develop spontaneously to full plantlets. Thus, the HP medium could be selected for this stage as well.

3.3 Callus-derived shoots develop via embryogenesis and organogenesis in vitro

The calli could be further developed along two morphogenic routes : (1) somatic embryo development and (2) production of shoot by organogenesis.

Meristematic tissues composed of densely stained cells arose in the proliferating epidermal or subepidermal tissues (Fig 2A) and showed an appearance of nodular compact structures (NC). These cells of the dermal system began to divide, giving rise to meristematic tissues which further developed into NC. In addition, globular embryo (Fig 2B) and embryo-like structure (Fig 2C, 2D) could be observed from these regions. The globular embryo- like structures were actually the infant protocorm–like bodies induced via embryogenesis. These young PLBs were nearly round or oval and thereby similar in shape to the protocorms of *D. crumenatum* formed during seed development [9]. Thus, the PLBs derived from some calli could be considered as somatic embryo and such type of callus was embryogenic.

Moreover, the independent organogenesis of leaf primordia could be established. It was noticed that some shoot meristem had a few leaf primodia but it did not have any bipolar structure (Fig 3A, 3B), so it was regarded as a meristem organogenetically formed. However, their vascular tissues would form a complex inside the callus later. The embryo – like structures in NC could be found while the organogenetic formation of a leaf primordia were observable but the embryogenesis occurred more than organogenesis (Data not shown). Thus, the embryo-like structures in NC could be found while the organogenetic formation of a leaf primordia were observable. Furthermore, the regeneration pathway of these calli could be both through embryogenesis and organogenesis. However, Oka *et al* [11] reported that the regeneration pathway depended on the source of calli. The origin of the regenerative callus induced from immature and mature embryos in barley were the scutellum and leaf or coleoptile base respectively. The regeneration pathway of callus derived from mature embryo could be either through organogenesis and embryogenesis but the former was more common than the latter. Thus, the source of explant was important for regeneration pathway. In this study *D.*

crumenatum orchid callus induced from bud (meristem) could be regenerated through embryogenesis more than by organogenesis. In addition, this process of plant production was selected to produce more uniform plants for the subsequent experiment.

4. Conclusion

The callus proliferation phase required a suitable concentration of PGR and peptone to promote the calli to grow vigorously. The PLBs formation stage needed additive nutrients especially that of CW. The experiment also suggested that the peptone could be incorporated to the medium in all stages of development, but only as a supplementary element. More importantly, it was necessary for the callus proliferation stage because there was significant difference between the two media with and without the peptone : the medium added with both PGR and peptone could give the highest yield of healthy callus whereas the media supplemented with only PGR provided the lowest yield of callus.

For the somatic embryo development, it should be mentioned that structures which first appeared like callus were actually embryogenic cells which got converted into PLBs. It was also suggested that the process of somatic embryogenesis was involved in PLB formation. On the other hand, organogenetic formation of leaf primodia could be possibly be noted. To sum up, the results revealed that the healthy orchid plants could be obtained following regeneration via both somatic embryogenesis and organogenesis.

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6. References

[1] Holttum, R.E., The Story of Pigeon Orchid, In: Plantlife in Malaya, Longman, UK, pp. 121-141, 1964.

[2] Chang, W.C., and Hsing.Y.I., *In vitro* Flowering of Embryoids Derived from Mature Root Callus of Ginseng (*Panax ginseng*), Nature, Vol. 28., pp. 341-342, 1980.

[3] Woodson, W.R., Biotechnology of Floricultural Crops, HortScience, Vol. 26, No. 8, pp. 1029-1033, 1991.

[4] Ishii, Y., Takamura, T., Goi, M. and Tanaka, M., Callus Induction and Somatic Embryogenesis of *Phalaenopsis,* Plant Cell Reports, Vol. 17, pp. 446-450, 1998.

[5] Wang, Z., Lehmann, D., Bell, J. and Hopkins, A., Development of an Efficient Plant Regeneration System for Russian Wildrye (*Psathyroatachys juncea*), Plant Cell Reports, Vol. 20, pp. 797-801, 2002. (Published Online 14 Dec 2001; DOI : 10.1007/s00299-001-0410-3)

[6] Vacin, E.F., and Went, F.W., Some pH Changes in Nutrient Solutions, Bot. Gaz, Vol. 110, pp. 605-613, 1949.

[7] Zar, J.H., Biostatistical Analysis, Third Edition, Prentice-Hall International, Inc., USA, 1996.

[8] Johansen, D.A., Plant Microtechnique, McGraw-Hill Co, Ltd., New York,USA, 1940.

[9] Vellupillai, M., Swarup, S. and Goh, C.J., Histological and Protein Changes During Early Stages of Seed Germination in the Orchid, *Dendrobium crumenatum*, Journal of Horticultural Science, Vol. 72, No. 6, pp. 941-948, 1997.

[10] Chang, C. and Chang, W.C., Plant Regeneration from Callus Culture of *Cymbidium ensifolium* Var. Misericors, Plant Cell Reports, Vol.17, pp. 251-255,1998.

[11] Oka, S., Saito. N. and Kawaguchi, H., Histological Observation on Initiation and Morphogenesis in Immature and Mature Embryo Derived Callus of Barley (*Hordeum vulgare* L.), Annals of Botany, Vol. 76, pp. 487-492,1995.

Table 1 Effects of basal media supplemented with various substances on different growth phase of *D. crumenatum* development

(Week of cultures) Growth phase	Treatment No *	VW	HP
(The 4th week)		Means of increased in fresh weight of callus per 100 mg of initial callus (mg ± SE)[1]	
1) Callus proliferation	1	65.54 ± 15.31a	131.67 ± 25.52a
	2	99.08 ± 18.32ab	380.00 ± 69.08c
	3	115.29 ± 17.75ab	291.67± 55.02bc
	4	63.94 ± 12.30a	150.83 ± 16.67a
	5	87.36 ± 13.30ab	366.67 ± 57.77c
	6	129.04 ± 24.19b	202.50 ± 27.83ab
(The 10th week)		Means of increased in fresh weight of callus or PLBs formation per 100 mg of callus (mg ±SE)[1]	
2) PLBs formation and proliferation	1	45.76 ± 15.21ab	-
	2	124.25 ± 48.35d	-
	3	51.69 ± 20.01ab	-
	4	46.89 ± 12.07a	-
	5	215.87 ± 51.77cd	-
	6	122.05 ± 43.60bc	-
(The 16th week)		Means number of regenerated shoots per gm PLBs (No. of regenerated shoots ± SE)[2]	
3) Regenerated shoots	1	6.70 ± 3.49ns	-
	2	2.18 ± 1.09ns	-
	3	15.34 ± 7.09ns	-
	4	6.13 ± 6.13ns	-
	5	11.89 ± 4.64ns	-
	6	0.00[3]	-

Each value represents mean ± SE (SE: standard error)

[1] Means followed by the same letter do not differ significantly within each column as indicated by one-way ANOVA followed by DMRT at P=0.05. Tests were replicated at least two times.

[2] The Kruskal-Wallis test was used to examine the mean difference.

[3] Data is constant. It has been omitted from testing

ns no significant difference

- Little or no callus development to subsequent growth phase. The calli stopped their development.

* Details of various addenda in VW and HP media ; Treatments No

1 ; Basal medium**

2 ; Basal medium + 10% coconut water

3 ; Basal medium + 2 g l^{-1} peptone

4 ; Basal medium + 0.1 mg l^{-1} NAA + 1 mg l^{-1} BA

5 ; Basal medium + 0.1 mg l^{-1} NAA + 1 mg l^{-1} BA + 10% coconut water

6 ; Basal medium + 0.1 mg l^{-1} NAA + 1 mg l^{-1} BA + 2 g l^{-1} peptone

** Basal medium contained 20 g l^{-1} sucrose and 2 g l^{-1} AC at pH 5.3

VW; Basal medium of Vacin and Went (1949) , HP; Basal medium of Hyponex

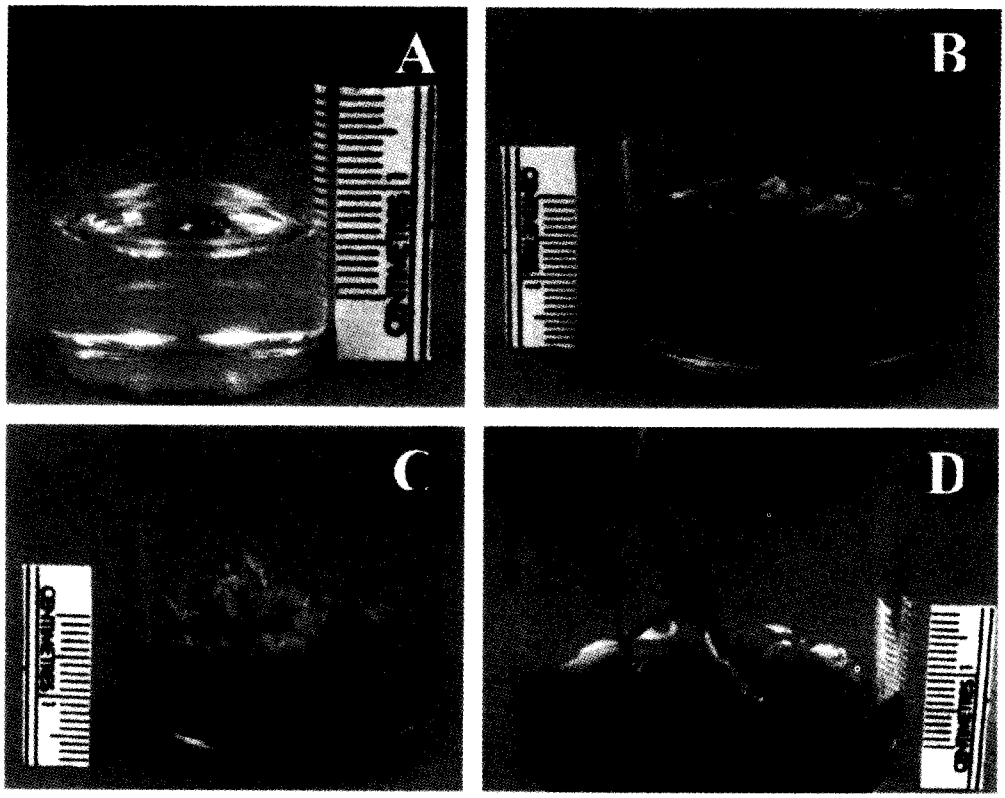

Fig I. Developmental stages of plant production. (A) The 1- month- old bud shows sign of growth on VW medium containing 20 9 1-1 and 2 9 1-1 Gelrite. (B) Callus proliferation is induced on vw medium supplemented with combination of 0.1 mg 1-1 NAA and 1.0 mg 1-1 HA, 20 9 1-1 sucrose, 2 9 1-1 peptone, 2 9 1-1 Gelrite as well as 2 9 1-1 AC. (C) The regenerated shoots are formed. (D) Plantlets containing 2-3 expanded leaves and 1-3 root(s) after transferred to VW basal medium.

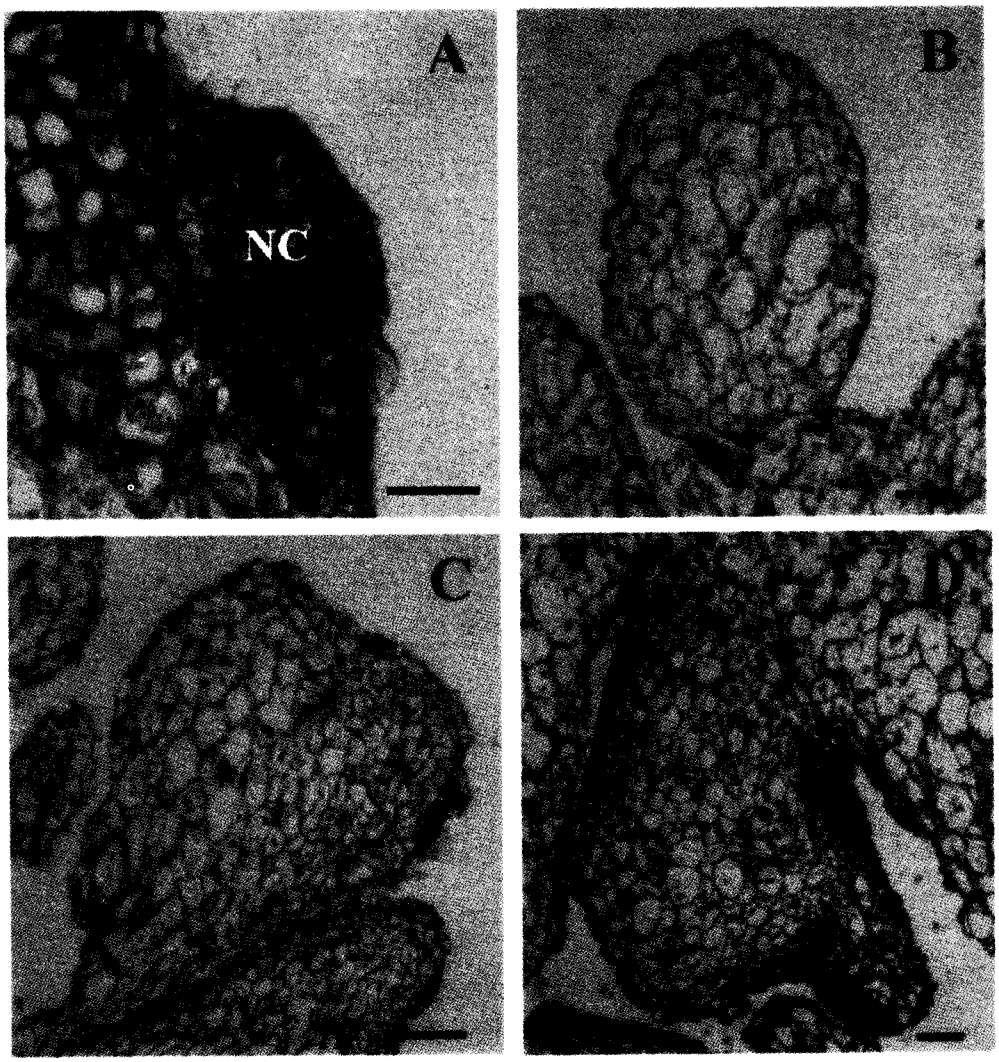

Fig 2. Somatic embryogenesis in Dendrobium crumenatum Sw. (A) Meristematic tissues composes of densely stained small cells arise in the peripheral region of dennal tissues and shows an appearance of nodular compact callus; NC, that correspond to compact callus ınacroscopically observed. Bar= 50 1.lIn. (B-D) The various morphological types of embryonic structures. (B) A globular embryo-like structure and (C) forming small PLB (D) somatic embryo showing asymmetric structure. NC- nodular compact structure; PLB- protocorm like body.
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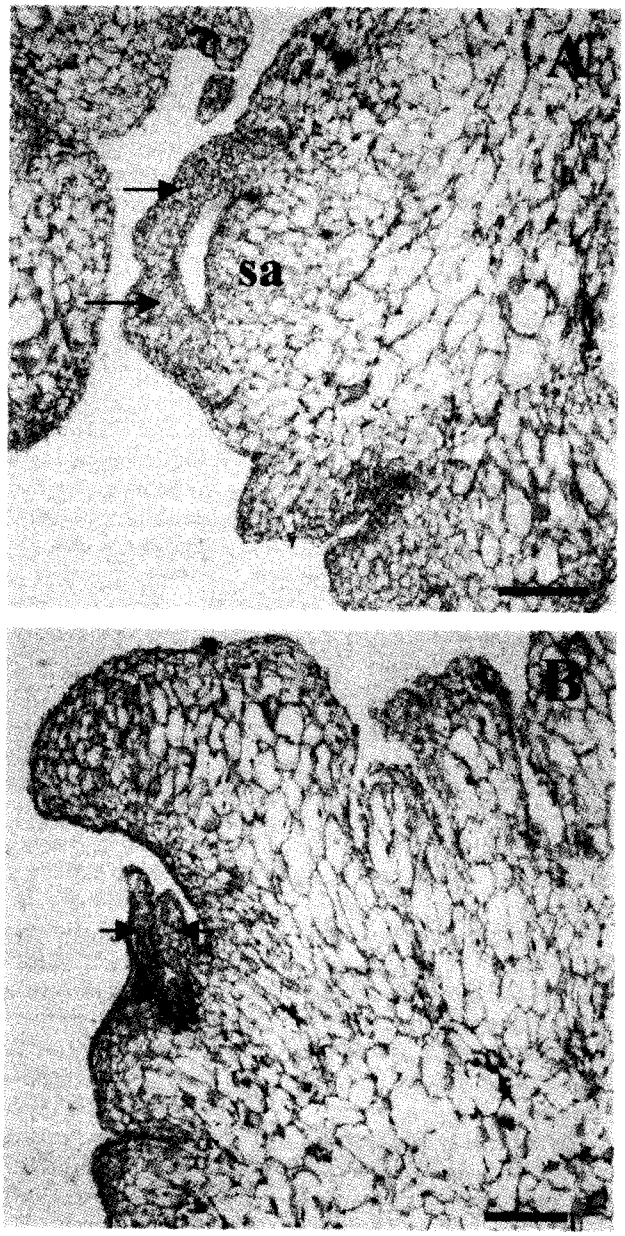

Fig 3. Regeneration through organogenesis of Dendrobium crumenatum Sw. (A-B) Showong shoot apical meristems (sa) with leafy organ structures (arrows) independently grow apart from root. Bar= 200 ~.