

## Histological Examination of Callogenesis in Bisected Protocorm Culture of Pigeon Orchid (*Dendrobium crumenatum* Swartz)

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### Abstract

Callogenesis is a prerequisite step for the initiation of callus-mediated plant regeneration. Three-month-old protocorms of *Dendrobium crumenatum* Swartz (pigeon orchid) were bisected transversely and cultured on modified Vacin and Went (VW) solid medium containing 20 g L<sup>-1</sup> sucrose, 2 g L<sup>-1</sup> Phytagel added with various concentrations of 1-naphthaleneacetic acid (NAA) (0, 0.1, 0.5 mg L<sup>-1</sup>) and 6-benzyladenine (BA) (0, 1, 2 mg L<sup>-1</sup>). The highest percentage of callus formation was obtained from the explant cultured on a modified VW supplemented with 0.5 mg L<sup>-1</sup> NAA and 1 mg L<sup>-1</sup> BA. The callus originated from the subepidermal layer of the bisected protocorm and subsequently formed nodular structure after culture for 8 weeks. The callus mass contained small isodiametric cells with prominent nuclei and nucleoli within dense cytoplasm.

**Keywords:** Bisected protocorm, *Dendrobium crumenatum*, nodular callus, subepidermal layer

### Introduction

*Dendrobium crumenatum* Swartz (pigeon orchid) is a sympodial orchid widespread in South and Southeast Asia. This orchid is valuable not only for its very unique floral features with attractive white fragrant flowers and synchronous flowering triggered by a sudden drop in temperature (about 10 °C) [1], but also for its medicinal properties exhibiting antimicrobial activity [2]. This orchid species is also used as an ideal plant for further research projects [3-5]. The effective way to enhance its commercial value can be achieved by studying the developmental biology, physiology and genetic transformation. Hence, tissue culture techniques, especially callus formation, is an appropriate method for rapid *in vitro* clonal propagation providing an excellent target material [6]. Particularly, successful plant regeneration via an intermediary callus phase is based on the amount of highly totipotent embryogenic callus which is a prerequisite for genetic transformation. In orchids, however, the well-established formation of callus is still very difficult and limited only to a few species, due to the slow growth and a tendency to become necrotic of the explants [7]. In previous studies, various concentrations and types of auxin and cytokinin so called plant growth regulators (PGRs) were used to study the callus formation in many *Dendrobium* species. Roy and Banerjee [8] revealed that callus formation from shoot-tip of *D. fimbriatum* Lindl. var. *oculatum* Hk. f. was achieved on a modified Knudson's C medium (KC) supplemented with 0.5 mg L<sup>-1</sup> 1-naphthaleneacetic acid (NAA) and 1 mg L<sup>-1</sup> 6-benzyladenine (BA). Likewise, Roy *et al.* [9] reported that a modified KC medium supplemented with 2 μM thidiazuron (TDZ) or 2 μM 6-benzylaminopurine (BAP) was an optimal medium for callus formation of *D. chrysotoxum* Lindl. In *Dendrobium* cv. Serdang Beauty, the highest fresh weight of callus was induced on a Murashige and Skoog (MS) medium containing 1.5 mg L<sup>-1</sup> indole-3-butyric acid (IBA) [10]. Moreover, the highest percentage of callus formation of *D. nanum* was obtained from the rhizome bud after culture on a MS basal medium added with 2.0 μM NAA and 1.2 μM kinetin [11]. Meesawat and Kanchanapoom [12] also reported that axillary bud-derived callus of *D. crumenatum* could be induced on a medium supplemented with a combination of

0.1 mg L<sup>-1</sup> NAA and 1 mg L<sup>-1</sup> BA. However, such study gave a low frequency and unidentified stimulus of callus formation. Thus, various concentrations of NAA and BA were added into the culture medium to optimize the concentration of PGRs for *in vitro* morphogenic response of transversely bisected protocorm and have focused only on callus formation to elucidate the ontogeny and alterations at cellular structure during callogenesis of *D. crumenatum*. Due to the effective system of micropropagation, it is essential to develop a reliable protocol for callus formation and to know the developmental pattern of callus from explants which can provide the advantage for orchid improvement. A clearer understanding of morphogenic response from the explants can provide an efficient plant tissue culture system for application in further research on plant regeneration and genetic transformation.

## Materials and methods

### Plant material

Six-week-old pods after hand cross-pollination of *D. crumenatum* orchid were collected and surface sterilized by dipping in 1.2 % sodium hypochlorite (NaOCl) solution (1.14 % active chlorine) with 2 drops of Tween 20 for 20 min. Then, they were rinsed with sterile distilled water (DW) 2 - 3 times. Sterilized capsules were dissected longitudinally and seeds were then scooped out into a 30 ml of liquid Vacin and Went (VW) medium [13] added with 2 % sucrose. The medium was adjusted to pH 5.3 with 1 N NaOH or 1 N HCl prior to autoclaving at 121 °C for 20 min. For protocorm formation, the cultures were incubated on an orbital shaker (120 rpm) at 25 ± 2 °C under a 16:8 h light/dark photoperiod and a light intensity of 23 μmol m<sup>-2</sup> s<sup>-1</sup> provided by cool daylight fluorescent lamps (36 Watts, Philips, Bangkok, Thailand) for 3 months.

### Determination of seed viability

Seeds of 6-week-old capsule were soaked in 1 mL of 1 % solution of 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma-Aldrich Co., St. Louis, MO, USA) in the darkness at room temperature. They were then washed with DW for 2 - 3 times and centrifuged for 5 min at 3,000 rpm. The seeds were then examined for the color of the embryo using an Olympus SZH 10 stereomicroscope (Olympus Optical Co. Ltd., Tokyo, Japan) and the images were captured with an Olympus DP 72 digital camera (Olympus Optical Co. Ltd.). The seeds with completely dark red-stained embryos were classified as viable, whereas incompletely stained or unstained embryos were considered as non-viable. The data were collected and calculated as the percentage of viable seeds using the following formula [14,15].

$$\text{Percentage of viable seed} = \frac{\text{Red stained embryos} \times 100}{\text{Total seeds}} \quad (1)$$

### Callus formation

Three-month-old protocorms (approximately 2 - 3 mm in diameter) at the third stage of development [16] were bisected transversely and these segments were inoculated on a 10 ml of solid modified VW medium containing various concentrations of NAA at 0, 0.1 and 0.5 mg L<sup>-1</sup> (Fluka Chemie GmbH, Buchs, Switzerland) and BA at 0, 1 and 2 mg L<sup>-1</sup> (Sigma-Aldrich Co.). These PGRs were used either individually or in combination to examine their inductive effects on callus formation and other morphogenic responses. The medium was adjusted to pH 5.3 with 1 N NaOH or 1 N HCl and solidified with 0.2 % Phytigel (Sigma-Aldrich Co.) before autoclaving at 121 °C for 20 min. The inoculated protocorms were incubated in darkness for a week and then transferred to maintain under an illumination at 23 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux intensity provided by cool daylight fluorescent lamps (36 Watts, Philips) with a photoperiod of 16 h light and 8 h darkness at 25 ± 2 °C.

### Light microscopy observation

Samples were collected weekly and fixed in formalin-aceto-alcohol (FAA) II solution [1:1:18 v/v; formaldehyde (Ajax Finechem, Taren Point, Australia); glacial acetic acid (J.T. Baker, Phillipsburg, NJ, USA); 70 % ethyl alcohol (Merck, Billerica, MA, USA)] for 48 h. They were then dehydrated through a tertiary-butyl-alcohol series and embedded in Histoplast PE paraffin wax (Richard-Allan Scientific,

Kalamazoo, MI, USA). Sections were cut at 6  $\mu\text{m}$  thickness with a rotary microtome (Shandon Southern Product Ltd., Cheshire, UK) and stained with Delafiled's hematoxylin and safranin [17] for investigating the origin and general structures. Stained sections were viewed with light microscope (Olympus BX 51 TRF, Olympus Optical Co. Ltd.) with an in-built digital camera (Olympus DP 72, Olympus Optical Co. Ltd.).

### Statistical analysis

The experiment was performed based on a factorial experiment in completely randomized design (CRD) with 7 replications. Each replication included 4 pieces of the transversely bisected protocorm. The percentage of undeveloped and developed explants was recorded after a culture for 8 weeks. In particular, developed explants were focused to record the percentage of callus, protocorm-like bodies (PLBs) and shoot formation. The data were statistically analyzed with an analysis of various (ANOVA) and means were compared using the Duncan's multiple range test and least significant difference at a significance level of  $P \leq 0.05$  using SPSS version 19.

## Results and discussion

### Seed viability and protocorm formation

The viable seeds of 6-week-old capsule of *D. crumenatum* (Figure 1A) exhibited the percentage of  $97.15 \pm 0.88$  %. These seeds showed red-stained embryos after TTC test (Figures 1B and 1C) and they were then cultured in a VW liquid medium supplemented with  $20 \text{ g L}^{-1}$  sucrose for 3 months to induce the protocorms (Figure 1D) required for further experiment.



**Figure 1** Morphological characteristics of capsule, seed and protocorm of *Dendrobium crumenatum*. (A) Six-week-old capsule ( $bar = 0.7 \text{ cm}$ ); (B-C) Viable seeds showing red-stained embryos examined by TTC test; (D) Three-month-old protocorm exhibiting the appearance of shoot region (arrow) ( $bar = 1 \text{ mm}$ ).

**Influence of NAA and BA on morphogenic responses**

Transversely bisected protocorms of *D. crumenatum* were cultured on a modified VW medium added with various concentrations of NAA (0, 0.1, 0.5 mg L<sup>-1</sup>) and BA (0, 1, 2 mg L<sup>-1</sup>). It was found that some explants became necrotic within 8 weeks, whereas more than 50 % of explants in all treatments showed a morphological response to the test medium. Such results clearly showed that the explants could be categorized as responsive and non-responsive explants, but there were no significant difference between the PGR treatments and control group (**Table 1**). After 8 weeks of culture, the responsive explants showed varied morphogenic responses namely callus, PLB and shoot as shown in **Figures 2 - 3**.

**Table 1** Effect of NAA and BA on the responses of protocorm segments of *Dendrobium crumenatum* after culture for 8 weeks on modified VW solid medium.

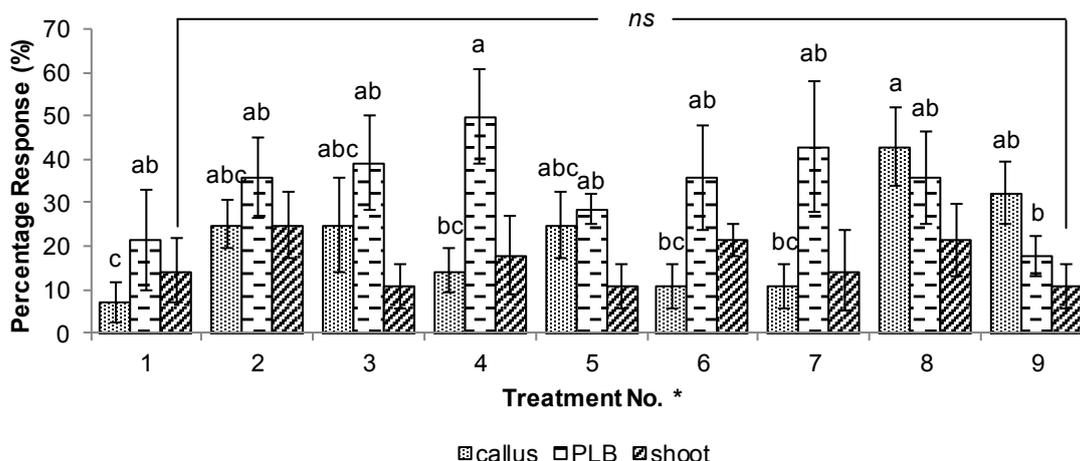
Plant growth regulators (mg L <sup>-1</sup> )		Non-responsive explants (%) <sup>ns</sup>	Responsive explants (%) <sup>ns</sup>
NAA	BA		
0	0	46.43 ± 15.84	53.57 ± 15.84
0	1	28.57 ± 10.10	71.43 ± 10.10
0	2	39.29 ± 12.02	60.71 ± 12.02
0.1	0	35.71 ± 13.20	64.29 ± 13.20
0.1	1	50.00 ± 12.20	50.00 ± 12.20
0.1	2	39.29 ± 9.22	60.71 ± 9.22
0.5	0	35.71 ± 12.02	64.29 ± 12.02
0.5	1	28.57 ± 10.10	71.43 ± 10.10
0.5	2	39.29 ± 13.20	60.71 ± 13.20

Data shown are the mean of 7 replicates ± standard error (S.E.). Comparison of the mean values within a column was analyzed using the Duncan's multiple range test at  $P \leq 0.05$ .  
<sup>ns</sup>: no significant difference

**Table 2** Analysis of variance for effect of plant growth regulators on formation of callus, PLB and shoot from transversely bisected protocorm of *Dendrobium crumenatum*.

Source of variation	Degrees of freedom	Mean square		
		Callus formation	PLB formation	Shoot formation
NAA	2	833.33 <sup>ns</sup>	158.73 <sup>ns</sup>	9.92 <sup>ns</sup>
BA	2	2172.62*	307.54 <sup>ns</sup>	128.97 <sup>ns</sup>
NAA × BA	4	416.67 <sup>ns</sup>	1289.68 <sup>ns</sup>	337.30 <sup>ns</sup>
Error	54	340.61	770.50	343.92

\* significant at  $P \leq 0.05$   
<sup>ns</sup>: no significant difference



**Figure 2** Effect of NAA and BA on callus, PLB and shoot formation from bisected segment of protocorm. Comparison of the mean value was analyzed using the Duncan's multiple range test or least significant difference at  $P \leq 0.05$ . The S.E. bars marked with different letters indicate the significant differences among treatments.

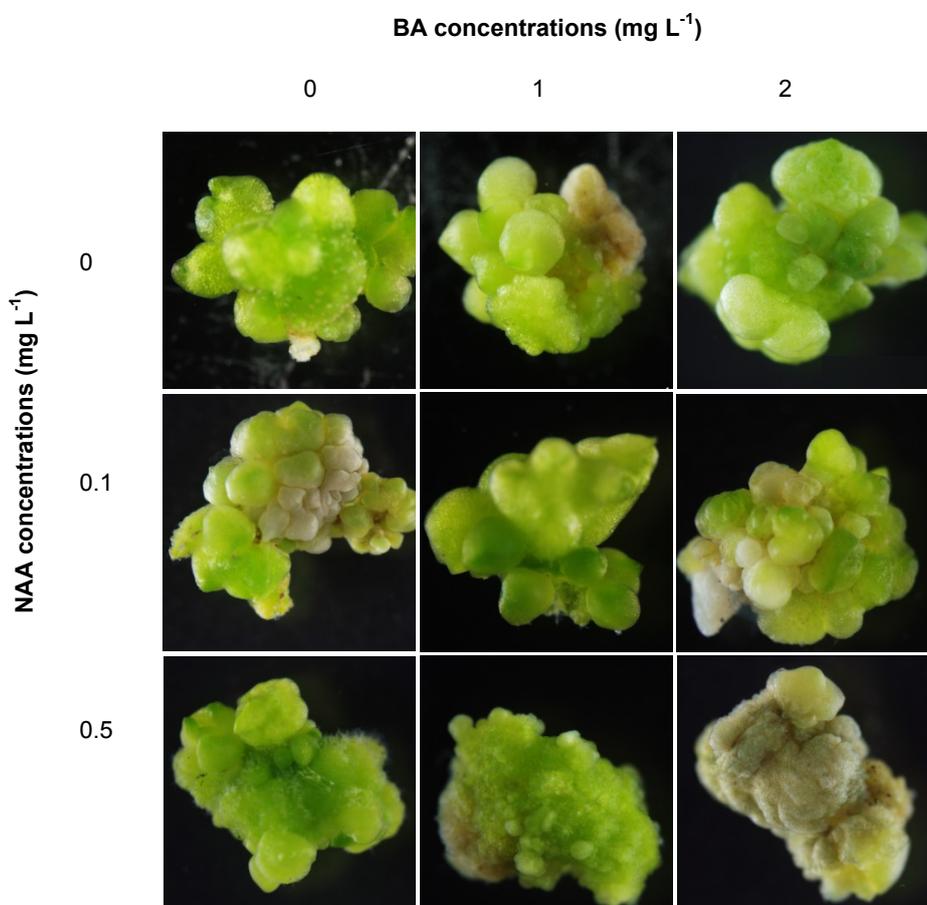
ns : no significant difference between treatment for shoot

\*Detail of various plant growth regulators in modified Vacin and Went medium (MVW)

- |  |  |
|--|--|
| 1 : MVW (control)  | 6 : MVW + 0.1 mg L <sup>-1</sup> NAA + 2 mg L <sup>-1</sup> BA |
| 2 : MVW + 1 mg L <sup>-1</sup> BA                              | 7 : MVW + 0.5 mg L <sup>-1</sup> NAA                           |
| 3 : MVW + 2 mg L <sup>-1</sup> BA                              | 8 : MVW + 0.5 mg L <sup>-1</sup> NAA + 1 mg L <sup>-1</sup> BA |
| 4 : MVW + 0.1 mg L <sup>-1</sup> NAA                           | 9 : MVW + 0.5 mg L <sup>-1</sup> NAA + 2 mg L <sup>-1</sup> BA |
| 5 : MVW + 0.1 mg L <sup>-1</sup> NAA + 1 mg L <sup>-1</sup> BA |  |

**Callus formation:** Callus formation was observed for all treatments (**Figure 2**). The control treatment gave the lowest percentage of callus formation ( $7.14 \pm 4.61$  %). From this result, callus formation can be induced on the medium without any exogenous PGRs. It indicated that the bisected segment of protocorm might contain an optimal balance endogenous PGR levels to induce callus formation as reported by Majumder [18]. However, this incidence of hormone-autonomous callus formation is an uncommon phenomenon in *D. fimbriatum* Lindl. var. *oculatum* Hk. f. [8], *D. chrysotoxum* Lindl. [9] and *Dendrobium* cv. Serdang Beauty [10]. Likewise, the percentage of callus formation on treatments with either NAA (at 0.1 mg L<sup>-1</sup> and 0.5 mg L<sup>-1</sup>) or BA (at 1 mg L<sup>-1</sup> and 2 mg L<sup>-1</sup>) alone were very low and not significantly different from the control (**Figure 2**). This result implies that the endogenous hormone within the explants might play a role in callus formation [19]. Khosravi *et al.* [10] also reported that the medium supplemented with NAA alone gave the PLBs-derived callus in *Dendrobium* cv. Serdang Beauty. Meanwhile, PGR combination treatments in particular 0.5 mg L<sup>-1</sup> NAA plus 1 mg L<sup>-1</sup> BA was found to be a potent treatment for callus formation which gave a significantly higher percentage of explants forming callus ( $42.86 \pm 8.99$  %) as compared with the control. This induced callus remained healthy and vigorous morphological appearance. Moreover, 0.5 mg L<sup>-1</sup> NAA combined with 2 mg L<sup>-1</sup> BA also provided the high percentage of callus formation ( $32.14 \pm 7.14$  %), but this callus was retarded and eventually became brown. Accordingly, the medium containing NAA in combination with BA at lower concentration appeared to significantly enhance the formation of callus (**Table 2**). However, the result revealed that the exogenously-applied auxin at both concentrations (0.1 and 0.5 mg L<sup>-1</sup>) in PGR combination treatments did not enhance the formation of callus. This result could be attributed to the fact that there was a sufficient level of endogenous auxin within the explants for a favorable ratio of

PGR leading to callus formation in agreement with previous reports by Khosravi *et al.* [10]. Thus, the application of exogenous auxin at a low level did not impact the total amount of auxin and subsequently did not influence the specific ratio of auxin to cytokinin. Meanwhile, a high concentration of exogenous cytokinin not only increases the structural rigidity of the cell wall leading to a decreasing amount of water uptake and subsequently decelerating cell division [20], but also induces a programmed cell death process in the plant cell [21]. Thus, cytokinin at high concentration has an inhibitory effect on callus formation even combined with exogenous auxin as shown in this present result. Accordingly, the enhancement of callus formation of *D. crumenatum* might still require a high level of exogenous auxin to balance auxin-cytokinin ratio. This finding is consistent with the previous studies exhibiting the addition of auxin combined with cytokinin play a key regulator to regulate cell division, an essential process for callus formation in many orchids such as *D. fimbriatum* Lindl. var. *oculatum* Hk. f. [8], *Phalaenopsis* [22] and *Vanilla planifolia* [23]. Because auxin and cytokinin act synergistically to regulate cell cycle in which auxin effects DNA replication, whereas cytokinin activates mitosis [24]. In addition, histological analysis showed that the callus mass originated near the cut surface of the explant and was easily distinguishable from the explants (Figures 4A and 4B).

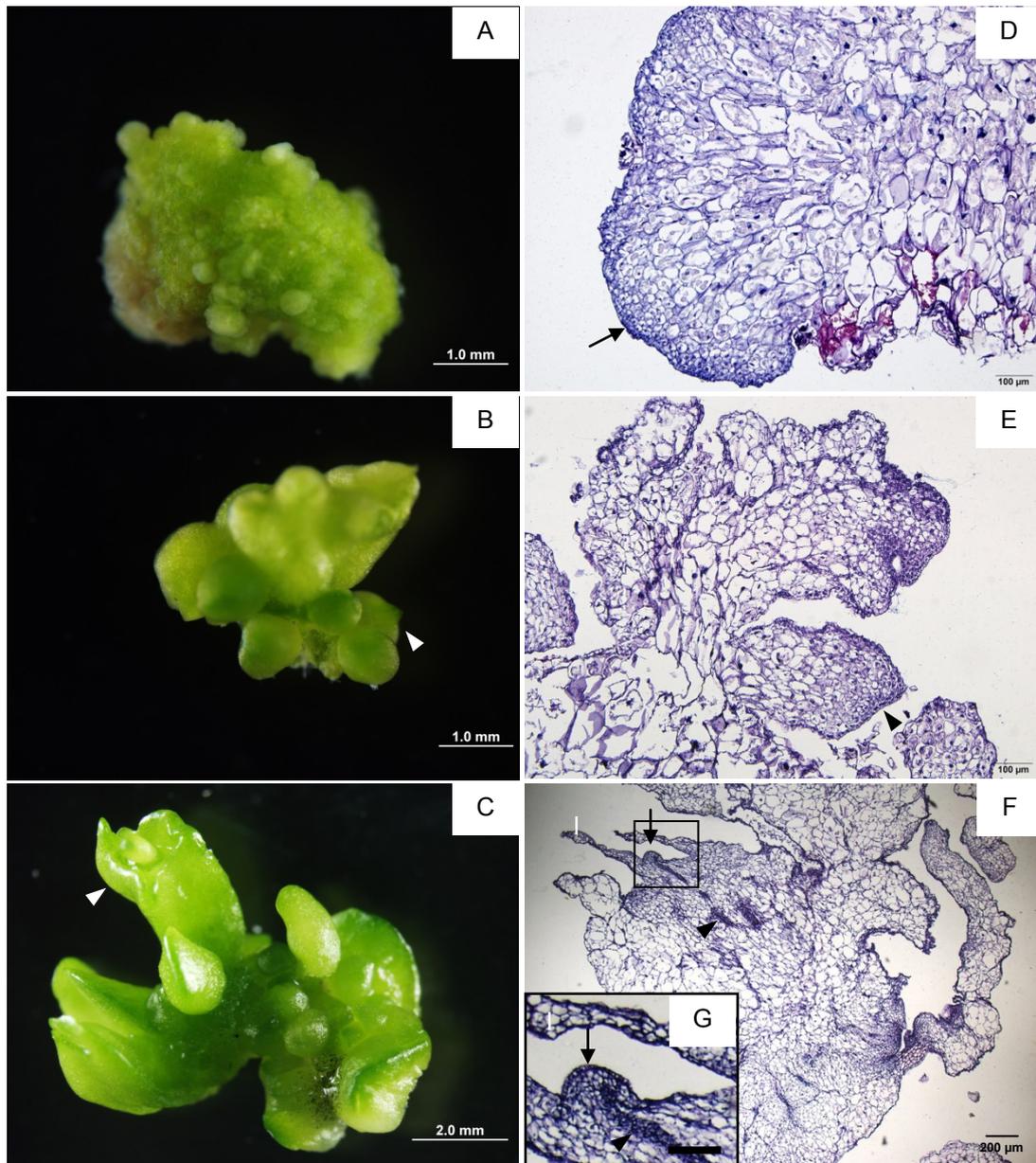


**Figure 3** Effect of different ratios of NAA to BA on the developmental pattern of protocorm segment after culturing for 8 weeks on modified VW medium.

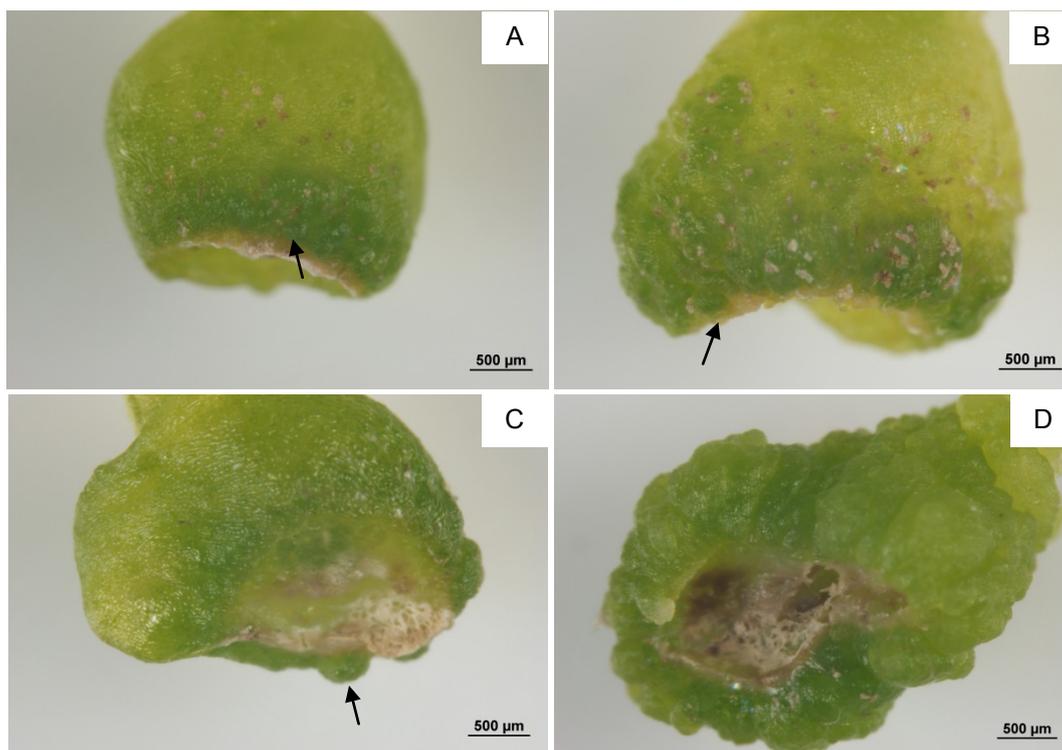
**Protocorm-like bodies (PLBs) formation:** PLB was formed in all PGR treatments including the control. However, NAA alone (at  $0.1 \text{ mg L}^{-1}$ ) produced the highest percentage of PLB formation ( $50.00 \pm 10.91 \%$ ) (**Figure 2**). The PGR combination treatments also gave PLB formation, but lower than individual PGR treatments which all exhibited no significant difference from the control ( $21.43 \pm 11.48 \%$ ). This present result was similar to that of the previous studies reported on *D. chrysotoxum* Lindl. [9] and *Dendrobium* Bobby Mesina Red. [25] with the medium containing only  $1 \mu\text{M}$  NAA and  $3 \text{ mg L}^{-1}$  NAA, respectively. Dipti *et al.* [26] mentioned that NAA was noted to be the crucial factor on the initiation of early and globular stages of PLB which is a prerequisite for the formation of PLB. Furthermore, the PLB formation of *D. crumenatum* on BA-alone treatment was in agreement with the observation of Kaewjampa *et al.* [27] who reported that the highest rate of PLB formation of *Cymbidium* Waltz 'Idol' was achieved on a medium with BA alone at  $1$  or  $10 \text{ mg L}^{-1}$ . Similarly, the increase in average number of PLBs and the percentage of PLB formation of *D. kingianum* was also obtained on a medium with BA alone particularly at low concentration ( $0.1 \text{ g L}^{-1}$ ) [28]. Moreover, histological studies showed that PLB occurred directly from transversely bisected protocorm segment without any intervening callus phase (**Figures 4C and 4D**). These PLBs showed an elongated shape with densely packed cells and a depression at the apical region (**Figure 4D, arrow**). Accordingly, this PLB formation was referred to as one type of morphogenesis called direct somatic embryogenesis as mentioned by Quiroz-Figueroa *et al.* [29].

**Shoot formation:** Shoot formation was also observed in all treatments at different frequency (**Figure 2**). Particularly, the presence of  $1 \text{ mg L}^{-1}$  BA gave the highest percentage of shoot formation ( $25.00 \pm 7.72 \%$ ), but were not statistically difference from the other treatments. The successful shoot formation in the present study could imply that the low concentration of exogenous BA might be the most effective concentration for the shoot formation of transversely bisected protocorm. Similar results were reported by Habiba *et al.* [30] who mentioned that BA could enhance the direct shoot formation from the explants of *Epidendrum* 'Rouge Star No. 8' as shown by the high percentage of shoot formation and the high average number of shoots per explant. Habiba *et al.* [30] also mentioned that BA is one of cytokinin which involves in cell division, shoot differentiation and modification of apical dominance. Moreover, histological features confirmed that shoot (**Figure 4E**) originated directly from the explants via direct organogenesis. The obtained shoot had shoot apical meristem, leaf primordia and vascular strand (**Figures 4F and 4G**).

Hence, this study proposed that the specific morphogenic response of bisected protocorm was controlled by the correct combination of PGRs, cytokinin and auxin, as confirmed by the histological aspect.



**Figure 4** *In vitro* morphogenesis of *Dendrobium crumenatum* culturing on modified VW medium containing NAA and BA for 8 weeks. The explants were cultured on a medium supplemented with 0.5 mg L<sup>-1</sup> NAA in combination with 1 mg L<sup>-1</sup> BA, 0.1 mg L<sup>-1</sup> NAA and 1 mg L<sup>-1</sup> BA showing (A) callus mass (B) PLB (arrow head) and (C) shoot (arrow head), respectively. Longitudinal section of explants exhibiting (D) callus mass (arrow) at the cut surface; (E) PLB with a small depression at shoot apex (arrow head); (F) shoot with leaf primordium (l), shoot apical meristem (arrow) and vascular strand (arrow head); (G) magnified view of boxed region of (F) (*bar* = 100 μm).

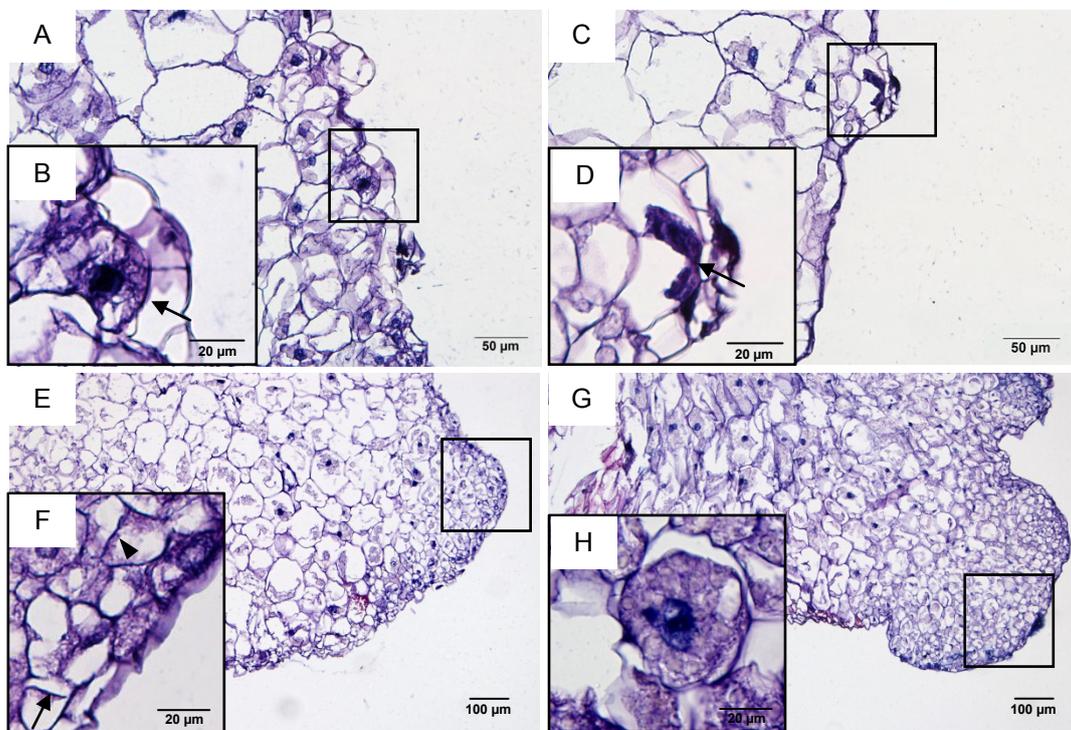


**Figure 5** Callogenesis of *Dendrobium crumenatum* on the modified VW medium supplemented with 0.5 mg L<sup>-1</sup> NAA and 1 mg L<sup>-1</sup> BA. Three-month-old protocorms were transversely bisected showing (A) swollen protocorm and early callus formation (arrow), (B) small clusters of callus (arrow), (C) callus mass (arrow) and (D) large amount of compact greenish callus mass after culture for 1, 2, 4 and 8 weeks, respectively.

#### **Histological observation of callogenesis**

The origin of the callus and its ontogeny were investigated during bisected protocorm culture on the modified VW medium added with 0.5 mg L<sup>-1</sup> NAA plus 1 mg L<sup>-1</sup> BA. After culturing for a week, the explants began to swell (**Figure 5A**) and showed the onset of cell division near the wound site which was possible to observe from a densely stained subepidermal cell having a dense cytoplasm and large nucleus (**Figures 6A** and **6B**). Then, the single originated callus cell continued to divide in an anticlinal orientation for producing more cells leading to an increase in the size of the callus mass (**Figures 5B, 6C** and **6D**). The callus mass became more obvious (**Figures 5C** and **6E**) and increased their cells by periclinal and anticlinal divisions (**Figures 6F** and **6G**) after culturing for 4 weeks. The obtained callus cells were clearly distinguished from original cells of the explants. The callus mass continued to grow and eventually form nodular compact green structure within 8 weeks of culture (**Figures 5D** and **6H**). This structure was comprised of small and isodiametric parenchyma cells having a prominent nucleus and a conspicuous nucleolus within densely stained cytoplasm (**Figure 6I**). This result indicates that wounding may be considered as a trigger of cell division giving rise to the onset of callus forming, similar to that reported by Ikeuchi *et al.* [31]. Moreover, a single subepidermal cell containing a dense cytoplasm and large nucleus was proposed as an origin of callus mass of *D. crumenatum*. The characteristics of this originated cell showed an actively dividing cell which may be associated with the formation of callus. Similarly, Lombardi *et al.* [32] reported that the callus of *Passiflora cincinnata* Mast. initiated from the subepidermal cell of leaf discs. This present result also showed the callus cells continued to divide and

produce more callus cells leading to an increase in the size of the callus mass. This induced compact callus comprised of small isodiametric cells with a prominent nucleus and nucleolus within densely stained cytoplasm which is in agreement with Creemers-Molenaar *et al.* [33] who reported that histological features of callus cell could indicate a high regenerative potential of their cells due to the appearance of their meristematic characteristics. Moreover, the histological observation confirmed that PGRs may play a key role related to the callogenesis pathway as mentioned by Hamidvand *et al.* [34].



**Figure 6** Histological analysis of callogenesis of *Dendrobium crumenatum*. (A-B) Callus originated from the subepidermal cell (arrow) of protocorm segment after a week of culture; (C-D) Subepidermal cell divided in an anticlinal plane (arrow) after 2 weeks of culture; (E-F) Obvious callus mass (arrow) containing meristematic cells undergo (F) periclinal (arrow head) and anticlinal divisions (arrow) after 4 weeks of culture; (G-H) Nodular compact callus comprising small isodiametric cells with a conspicuous nucleus and nucleolus within densely cytoplasm after 8 weeks of culture. (B, D, F, H magnified of box region of A, C, E, G)

## Conclusions

The present study revealed that a medium containing  $0.5 \text{ mg L}^{-1}$  NAA in combination with  $1 \text{ mg L}^{-1}$  BA gave a high percentage of bisected protocorm segment forming a callus with a significant difference from the PGR-free medium (control) and confirmed by histological observations. Calluses originated from the subepidermal cell of transversely bisected protocorm segment that divided in anticlinal plane. Then, the callus cells underwent peri- and anticlinal division within 4 weeks of culture. Induced calluses continued to grow and gave rise to the nodular compact callus after 8 weeks of culture. The obtained callus is useful and can then be used for further experiments in orchid improvement.

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