

## Plantlet Regeneration and Multiple Shoot Induction from Protocorm-Like Bodies (PLBs) of Medicinal Orchid Species, *Dendrobium crumenatum* Sw.

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Received: 20 November 2019, Revised: 20 May 2020, Accepted: 20 June 2020

### Abstract

To investigate the suitable medium for *in vitro* shoot regeneration and plantlet growth of *Dendrobium crumenatum* Sw., individual protocorm-like bodies (PLBs) (about 4 - 5 mm in diameter) of *Dendrobium crumenatum* Sw. derived from MS medium supplemented with 0.5 mg/L TDZ for 60 days of culture were cultured on 6 culture media; Murashige and Skoog (MS) medium, MS medium supplemented with 15 % (v/v) CW, MS medium supplemented with 15 % (v/v) CW and 0.2 % (w/v) AC, Vacin and Went (VW) medium, VW medium supplemented with 15 % (v/v) CW, VW medium supplemented with 15 % (v/v) CW and 0.2 % (w/v) AC. After 4 months of culture, MS medium containing 15 % coconut water (CW) gave the highest percentage of shooting and number of shoots per explant of 96.0 and 9.5, respectively with a significant difference from other media. The addition of 0.2 % (w/v) activated charcoal (AC) significantly increased the number of leaves and roots. PLBs developed into complete plantlets. MS medium supplemented with 15 % (v/v) CW and 0.2 % (w/v) AC and VW medium supplemented with 15 % (v/v) CW and 0.2 % (w/v) AC gave the highest number of roots per plantlet and root length at 5.3 roots and 34.9 mm, respectively. After the transfer of rooted shoots to the greenhouse, 95 % of the regenerated plantlets survived and grew vigorously. Plantlets grown *in vitro* were successfully acclimatized in the greenhouse and showed normal development.

**Keywords:** *Dendrobium crumenatum*, Germplasm conservation, *In vitro* conservation, Micropropagation, Thidiazuron

### Introduction

The Orchidaceae represents approximately 10 % of angiosperms. It is one of the largest, most diverse, and most important categories of botanically and commercially significant flowering plants with 20,000 - 30,000 species [1,2]. The floral characteristics of orchids cover an exceptionally wide range of different shapes, forms, sizes, and colorations, surpassing flowers of all the other angiosperms.

*Dendrobium crumenatum* Sw., commonly called pigeon orchid, is a member of the family Orchidaceae. It is native to India, Indochina, Taiwan, the Philippines, Malaysia, Indonesia, New Guinea, and Christmas Island. It is reportedly naturalized in Fiji, Hawaii, the West Indies, and the Seychelles. It grows in many localities from full sun to deep shade. *Dendrobium crumenatum* Sw. produces upright, sympodial, pseudobulbs that are swollen at the first 3 or 4 bottom nodes. The middle portion carries the leaves of 7 cm long and 2 cm wide that is very leathery. The top portion of the pseudobulbs carries flowers of about 2.5 cm

and pure white, with yellow markings on the labellum. The bloom cycle is triggered 9 days after a sudden drop in temperature, usually as a result of rain, although the same effect can be artificially created. *D. crumenatum* Sw. flowers are fragrant, but the scent lasts only for 1 day [3,4].

This orchid is valuable for its medicinal properties exhibiting antimicrobial activity [5]. Given the importance of *D. crumenatum* Sw., mass propagation for commercial cultivation requires a simple, economical, rapidly multiplying, and highly reproducible protocol. The present study was carried out to select the appropriate medium for shoot regeneration and plantlet development of *D. crumenatum* Sw. from calli-derived protocorm-like bodies (PLBs) under *in vitro* conditions and consequently on the *ex vitro* acclimatization process.

## Materials and methods

### Plant materials and surface sterilization

Main shoots of *D. crumenatum* Sw. (15 - 25 cm long) were harvested from plants grown in the greenhouse at the Faculty of Science and Technology, Nakhon Si Thammarat Rajabhat University. The stalks were cut into the nodal segments, each holding 1 axillary bud. Surface sterilization of nodal segments (about 3 - 4 cm in length) was performed according to Meesawat and Kanchanapoom [6]. The sterilized *D. crumenatum* Sw. buds were cultured on MS medium supplemented with 3 % (w/v) sucrose, a combination of 1.0 mg/L BA and 0.1 mg/L NAA, 0.2 % (w/v) peptone, and 0.2 % (w/v) AC at pH 5.7 to initiate callus from the modified method as described by Meesawat and Kanchanapoom [6]. The callus proliferation was observed after 1 month of culture. These calli were then transferred to the same medium. The subculture monthly was recommended to produce more totipotent calli than the subsequent experiment. For protocorm-like bodies (PLBs) formation, calli at 1 month of culture were transferred to MS medium supplemented with 0.5 mg/L Thidiazuron (TDZ) for 60 days of culture from the method as described by Rittirat *et al.* [7].

### Influence of different basal media on *in vitro* shoot regeneration and plantlet growth of *Dendrobium crumenatum* Sw. after culture for 4 months

To investigate the suitable medium for *in vitro* shoot regeneration and plantlet growth of *Dendrobium crumenatum* Sw., individual protocorm-like bodies (PLBs) (about 4 - 5 mm in diameter) of *Dendrobium crumenatum* Sw. derived from MS medium supplemented with 0.5 mg/L TDZ for 60 days of culture (Figure 2) [7] were cultured on 6 culture media; Murashige and Skoog [8] (MS) medium, MS medium supplemented with 15 % (v/v) CW, MS medium supplemented with 15 % (v/v) CW and 0.2 % (w/v) AC, Vacin and Went (VW) [9] medium, VW medium supplemented with 15 % (v/v) CW, VW medium supplemented with 15 % (v/v) CW and 0.2 % (w/v) AC.

The culture media were solidified with 0.75 % (w/v) agar-agar (commercial grade). The pH of MS [8] and VW medium [9] was adjusted to 5.7 and 5.0, respectively with 1 N KOH or 1 N HCl prior to autoclaving for 15 min at 121 °C. The coconut water was taken out from the green coconut and added to the basal medium. All cultures were maintained at 25 ± 2 °C under a 16 h photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux density (PPFD). The percentage of shooting, number of shoots per explant, number of leaves per explant, leaf length, leaf width, percentage of rooting, number of roots per explant, and root length were recorded and statistically compared after 4 months of culture

### Transplantation of plantlets/greenhouse acclimatization

The complete plantlets derived from MS medium supplemented with 15 % (v/v) CW and 0.2 % (w/v) AC for 4 months of culture were taken out from culture bottles and rinsed thoroughly with tap water to remove residual nutrients and agar from the plantlets without causing damage to roots. These plantlets were treated with 0.1 % fungicide (bavistin) solution for 5 min and again washed with sterile water. All plantlets were blot dried and then transplanted to pots containing sterilized coconut husks. All plantlets were grown in the greenhouse with 70 - 80 % relative humidity and about 12 h photoperiod, 300 - 400 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux density (PPFD) (shaded sunlight) and 33 ± 2 °C to 30 ± 2

°C day/night temperature. The young plants were sprayed with water twice a day for 2 months. The percentage of plantlet survival was recorded.

#### Experimental design and statistical analysis

All the experiments were conducted in a completely randomized design (CRD) with 5 replicates per treatment and the experiments were repeated 3 times. The results are expressed as mean  $\pm$  SE of 3 experiments. The data were analyzed by ANOVA using SPSS version 20 and the mean values were separated using Duncan's multiple range test (DMRT) at a 5 % probability level.

### Results and discussion

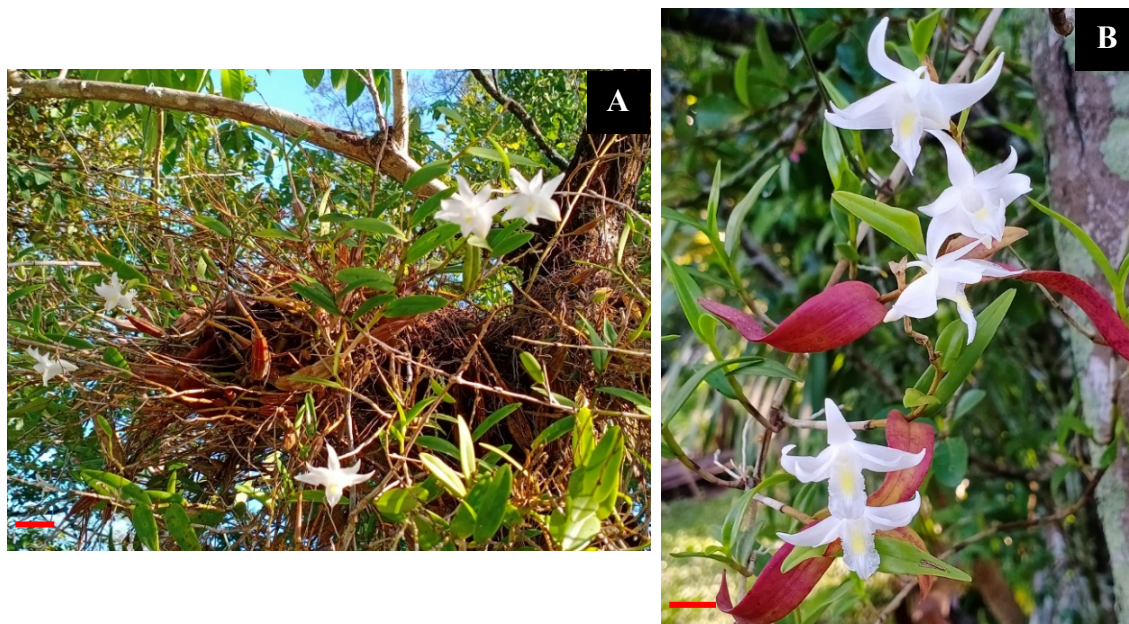
#### Influence of different basal media on *in vitro* shoot regeneration and plantlet growth of *Dendrobium crumenatum* Sw. after 4 months of culture

To investigate the suitable medium for *in vitro* shoot regeneration and plantlet growth of *Dendrobium crumenatum* Sw., individual protocorm-like bodies (PLBs) (about 4 - 5 mm in diameter) of *Dendrobium crumenatum* Sw. derived from MS medium supplemented with 0.5 mg/L TDZ for 60 days of culture were cultured on 6 culture media.

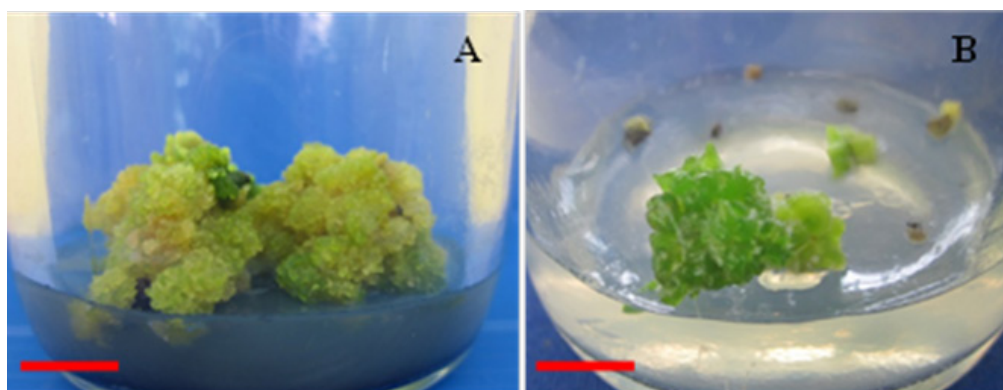
In this present study, PLBs produced green friable calli when cultured on MS or VW medium supplemented with 15 % (v/v) CW and 0.2 % (w/v) AC after 2 months of culture. Among the 6 media tested, the MS medium supplemented with 15 % (v/v) CW had the highest shooting percentage (96.0 %) when compared to the MS medium supplemented with 15 % (v/v) CW and 0.2 % (w/v) AC (56.0 %), the VW medium supplemented with 15 % (v/v) CW (41.0 %), the VW medium supplemented with 15 % (v/v) CW and 0.2 % (w/v) AC (11.0 %), the MS medium (0.0 %) and the VW medium (0.0 %). The PLBs were pale green to yellow when they were cultured on MS medium without 15 % (v/v) CW and 0.2 % (w/v) AC. Similar results were found in VW medium without 15 % (v/v) CW and 0.2 % (w/v) AC. However, these PLBs died after 4 months of culture (**Table 1**). MS medium supplemented with 15 % (v/v) CW gave the highest percentage of shooting and number of shoots per explant of 96.0 and 9.5, respectively (**Table 1**). A statistically significant difference was observed among treatments. The percentage of responding shooting was significantly lower ( $p \leq 0.05$ ) on MS or VW medium (**Table 1**).

This study was undertaken to evaluate the growth of *D. crumenatum* Sw. PLBs as influenced by 6 commonly used culture media in orchid. Our results showed that MS medium was superior to VW medium and both media containing 15 % (v/v) CW gave better results than those without CW. CW is known to act like a cytokinin substance, hence promote cell division and growth of the protocorms [10].

Organic supplements have been shown to stimulate seed germination and seedling growth of many orchids [11-14]. However, their effects are complex and may vary depending on species, the types of explants or the developmental stages. In *Cypripedium macranthos*, as compared to coconut water, the potato and banana homogenates appeared to have a significant influence on the seedlings growth [15]. The presence of coconut water improved seed germination of *Cypripedium* species [16,17], but its nutrient compositions might be not sufficient for the subsequent growth of *Cypripedium macranthos* seedlings.



**Figure 1** *Dendrobium crumenatum* Sw. plants growing in natural habitat. (A); Mature plants of *D. crumenatum* Sw. and (B); flowers of *D. crumenatum* Sw. showing pure glittering white and a bright yellow disc on the lip. (Scale bar, A = 3 cm and B = 1 cm).



**Figure 2** *In vitro* propagation of *Dendrobium crumenatum* Sw. (A); callus derived from explant on MS medium containing 3 % (w/v) sucrose and 0.76 % (w/v) agar (commercial grade), combination of 1.0 mg/L BA and 0.1 mg/L NAA, 0.2 % (w/v) peptone and 0.2 % (w/v) AC and (B); initiation of PLBs on calli cultured on MS medium supplemented with 0.5 mg/L TDZ for 60 days. (Scale bar = 1 cm).

**Table 1** Influence of different basal media on *in vitro* shoot regeneration of *Dendrobium crumenatum* Sw. after 4 months of culture.

Basal media	Shooting (%)	No. of shoots per explant (mean ± SE) <sup>a</sup>	No. of leaves (mean ± SE) <sup>a</sup>	Leaf length (mm) (mean ± SE) <sup>a</sup>	Leaf width (mm) (mean ± SE) <sup>a</sup>
MS	0.0 <sup>c</sup>	0.0 ± 0.0 <sup>c</sup>	0.0 ± 0.0 <sup>c</sup>	0.0 ± 0.0 <sup>c</sup>	0.0 ± 0.0 <sup>c</sup>
MS + 15 % CW	96.0 <sup>a</sup>	9.5 ± 0.4 <sup>a</sup>	3.8 ± 0.2 <sup>b</sup>	28.2 ± 0.9 <sup>a</sup>	8.9 ± 0.4 <sup>a</sup>
MS + 15 % CW + 0.2 % AC	56.0 <sup>b</sup>	3.8 ± 0.2 <sup>b</sup>	5.0 ± 0.3 <sup>a</sup>	26.8 ± 0.9 <sup>a</sup>	5.3 ± 0.2 <sup>b</sup>
VW	0.0 <sup>c</sup>	0.0 ± 0.0 <sup>c</sup>	0.0 ± 0.0 <sup>c</sup>	0.0 ± 0.0 <sup>c</sup>	0.0 ± 0.0 <sup>c</sup>
VW + 15 % CW	41.0 <sup>b</sup>	4.0 ± 0.3 <sup>b</sup>	3.3 ± 0.3 <sup>b</sup>	17.7 ± 0.3 <sup>b</sup>	5.3 ± 0.2 <sup>b</sup>
VW + 15 % CW + 0.2 % AC	11.0 <sup>c</sup>	4.0 ± 0.5 <sup>b</sup>	3.2 ± 0.2 <sup>b</sup>	20.6 ± 0.6 <sup>b</sup>	5.6 ± 1.7 <sup>b</sup>

Means followed by different letters within the column are significantly different from others at 5 % level by DMRT.

<sup>a</sup>Values represent means ± standard error.

For the rooting of shoots, MS or VW medium fortified with 15 % (v/v) CW or 15 % (v/v) CW and 0.2 % (w/v) AC were used (**Table 2**). Significant rooting response was observed in MS or VW medium containing 15 % (v/v) CW or 15 % (v/v) CW and 0.2 % (w/v) AC (**Table 2**). Among different treatments, the highest number of roots was observed in MS medium containing 15 % CW and 0.2 % AC with the average value of 5.3 per explant. The highest root length was observed in the VW medium containing 15 % CW and 0.2 % AC (34.9 mm) (**Table 2**). However, in support of the present finding, superiority of AC in root induction was also reported by [18]. Thus, among the different rooting media, AC was found to be most effective for the rooting of shoots of *D. crumenatum* Sw. This result was contrary to the previous findings of rooting in *Aerides odorata* [19] and *Micropera pallida* [20] showing a positive response in medium with IAA. Similarly, Pradhan [21] and Asghar *et al.* [22] observed a maximum number of roots on medium fortified with IBA than in NAA in orchid species.

**Table 2** Mean response of plantlet growth to basal media of *Dendrobium crumenatum* Sw. after 4 months of culture.

Basal media	Rooting (%)	No. of roots per explant (mean ± SE) <sup>a</sup>	Root length (mm) (mean ± SE) <sup>a</sup>
MS	0.00 <sup>c</sup>	0.0 ± 0.0 <sup>d</sup>	0.0 ± 0.0 <sup>c</sup>
MS + 15 % CW	70.00 <sup>b</sup>	3.0 ± 1.3 <sup>c</sup>	24.0 ± 0.2 <sup>d</sup>
MS + 15 % CW + 0.2 % AC	90.00 <sup>a</sup>	5.3 ± 0.2 <sup>a</sup>	30.9 ± 0.3 <sup>b</sup>
VW	0.00 <sup>c</sup>	0.0 ± 0.0 <sup>d</sup>	0.0 ± 0.0 <sup>c</sup>
VW + 15 % CW	70.00 <sup>b</sup>	3.7 ± 0.2 <sup>b</sup>	28.5 ± 0.2 <sup>c</sup>
VW + 15 % CW + 0.2 % AC	90.00 <sup>a</sup>	3.9 ± 0.2 <sup>b</sup>	34.9 ± 0.3 <sup>a</sup>

Means followed by different letters within the column are significantly different from others at 5 % level by DMRT.

<sup>a</sup>Values represent means ± standard error.



AC often promotes orchid shoot proliferation. AC has been reported to promote multiple shoot formation in nodal segments of *A. formosanus* [23]. AC has beneficial and harmful effects in the culture medium, depending upon the medium, explant, and plant-growth regulators used. The beneficial effects of AC on tissue responses *in vitro* could be attributed to:

1. providing a dark environment by darkening the medium [24];
2. adsorption of harmful substances produced by either the media or explant [25,26];
3. adsorption of plant-growth regulators and other organic compounds [27-29]; or
4. release of substances naturally present in or adsorbed by AC [30-32].

Hardening of the plants in the greenhouse under high humidity conditions was found to be highly beneficial for successful acclimatization. Well-developed *in vitro* derived shoots along with roots were transferred into sterilized coconut husks for *ex vitro* establishment of *D. crumenatum* Sw. Ninety-five percent of the regenerated plantlets survived and grew vigorously. Plantlets grown *in vitro* were successfully acclimatized in the greenhouse and showed normal development (**Figure 3**).

The ultimate success of micropropagation depends on acclimatization. During this period, a higher percentage of plant losses occur due to several reasons [33]. Therefore, it is necessary to obtain quality plantlets under *in vitro* conditions to ensure a high survival percentage and appropriate growth under greenhouse conditions. In order to survive *ex vitro*, a plantlet must achieve a growth stage with an appropriate sprout number, foliage area, and radicular system, considering roots number and length [34]. The successful rooting of the shoots and the number of roots per shoot are the key factors for acclimatization of *Dendrobium crumenatum* Sw. plantlets. Improvement of *in vitro* rooting techniques and conditions will facilitate the production of a greater number of roots and overall healthy plantlets which helps in better survival of plantlets upon transferring them to *ex vitro* conditions [35].

The transplantation stage continues to be a major bottleneck in the micropropagation of orchid. A substantial number of micro propagated plantlets fail to survive when transferred from *in vitro* conditions to a greenhouse or field environment. The greenhouse or field environment have substantially lower relative humidity, higher and intense light levels that are stressful to micro propagated plants compared to *in vitro* conditions [36].



**Figure 3** *Dendrobium crumenatum* Sw.; the complete plantlets derived from MS medium supplemented with 15 % (v/v) coconut water (CW) and 0.2 % (w/v) activated charcoal (AC) for 4 months of culture with well-expanded leaves in pots containing sterilized coconut husks after 2 months of transplanting. (Scale bar = 1 cm).

A simple and easy technique for the medium-term *in vitro* conservation of *D. crumenatum* Sw. has been developed. *Ex situ* conservation methods played an important role in the conservation of plants. The main methods used in *ex situ* conservation are maintenance of living plants through cultivation, *in vitro* conservation, and encapsulation. An *in vitro* plant regeneration protocol was successfully established for medicinal species, *Dendrobium crumenatum* Sw. by culturing PLBs. Using this protocol, it is possible to produce viable uniform and healthy plants with a maximum survival rate that can be used for large scale cultivation. Furthermore, the protocol may facilitate the conservation of this fragrant, medicinal orchid in a natural population.

### Conclusions

The data presented herein indicate that for rapid multiplication of *D. crumenatum* Sw. using different media could be effectively employed which otherwise exhibits extremely slow growth. MS medium containing 15 % CW gave the highest percentage of shooting and number of shoots per explant of 96.0 and 9.5, respectively after 4 months of culture with a significant difference from other media. The addition of 0.2 % (w/v) AC significantly increased the number of leaves and roots. After 4 months of culture, PLBs developed into complete plantlets. MS medium supplemented with 15 % CW and 0.2 % (w/v) AC and Vacin and Went (VW) medium supplemented with 15 % CW and 0.2 % (w/v) AC gave the highest number of roots per plantlet and root length at 5.3 roots and 34.9 mm, respectively. After the transfer of rooted shoots to the greenhouse, 95 % of the regenerated plantlets survived and grew vigorously. Plantlets grown *in vitro* were successfully acclimatized in the greenhouse and showed normal development.

### Acknowledgements

This research was financially supported by Prince of Songkla University, Pattani Campus, Pattani, Thailand (Grant No. 59005). The authors would like to thank the Department of Technology and Industries, Faculty of Science and Technology, Prince of Songkla University, Pattani Campus, Pattani, Thailand, for providing laboratory facilities for this investigation.

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