3. RESULTS

3.1 Investigation of flowering pattern and pollen grain maturation

3.1.1 Flowering pattern and floral bud at the responsive stage

In this orchid, the short inflorescence consisted of two to three floral buds at different developmental stages (Figure 4). Each bud was covered by bracteal leaves. The pedicel of an opened flower (1) was clearly observed on the flowering day or the day of anthesis whereas the second (2) and the third (3) floral buds were forming the subsequent floral buds. The stage at which the floral development is halted was determined by the sequence of flowering events as shown in Figure 5. All buds in an inflorescence (Figure 6A) would be stimulated by a specific stimulus which triggered the subsequent floral development of this orchid. Only the first floral bud at the responsive stage, which showed the miniature version of flower completed with all floral parts (Figure 6B), was stimulated to anthesis nine days later (Figure 6C). The two other floral buds, namely, the second (Figure 6D, E) and the third (Figure 6D, F) developed sequentially after the same stimulus. That is the second floral bud (at the certain stage) developed the same as the first floral bud and the third floral bud (at the specific stage) developed the same as the second floral bud. Then, all buds stopped their development to wait for the next stimulus. Subsequent development occurred until there was no any floral bud in an inflorescence.

3.1.2 Time of pollen grain maturation

The pollen grain development of the first miniature flower was examined from the induction day (day0) to anthesis (day9). The developmental events during the period of time are shown in Figure 7 and Table 1 (Appendix E). It was found that the microsporocyte or pollen mother cell (2n) at day0 was still at a pre-meiotic stage (Pm). It was undergoing the first and the second meiotic division (Me I-Me II) to form dyad (D) and tetrad (T) on the fourth and the fifth day, respectively. The stage of uni-nucleate microspore was short and the microspore was immediately divided by mitosis to form the bi-nucleate pollen grain (Bp). This 2-celled pollen grain indicating maturation appeared six days after the natural stimulus. It showed a large vegetative nucleus (VN) and a small generative cell (GC). However, a group of pollen grains, known as pollinia, adhered together and remained together until anthesis. As a conclusion, one may say that the stage of two-celled pollen grains or mature pollen grains can be observed only three days before anthesis.

3.2 Determination of the natural stimulus and the effective stimuli required flor flowering

3.2.1 A sudden drop in temperature as a trigger

Data of temperature changes recorded by data logger were considered to fit with the flower blooming in nature. It was found that low temperature was critical and required for flowering in this orchid. In natural condition, the flower opening would be induced by the sudden drop of temperature resulting from heavy rain. This event coincided with the daily temperature changes

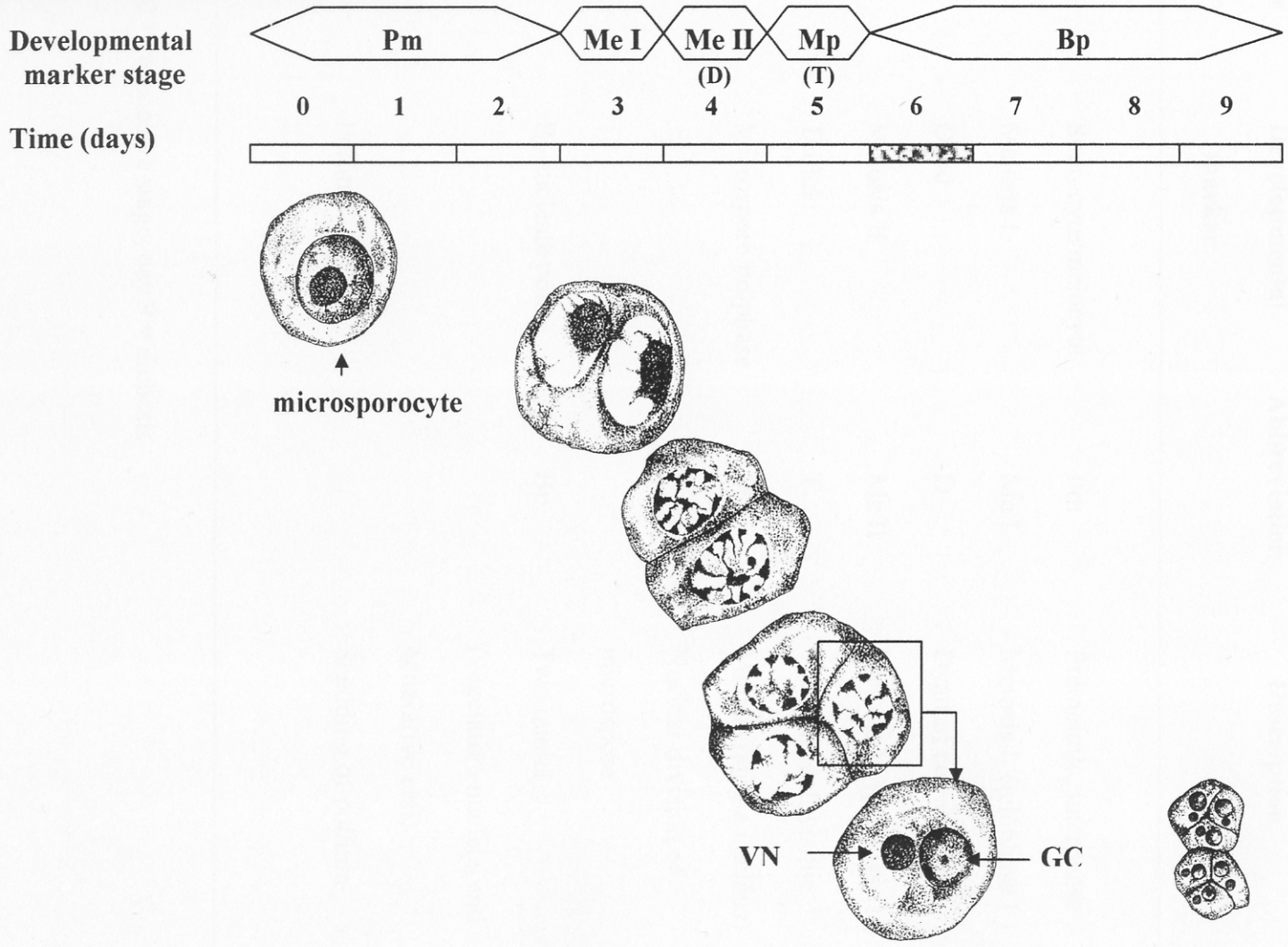

Figure 7 Pollen grain development of *D. crumenatum* from the induction day to the day of anthesis

Table 1 Markers for pollen grain development of *D. crumenatum* based on the cytological appearance

Time (day)	**Developmental marker**	**Abbreviation**	**Description**
0-2	Sporocyte/meiocyte	Pm	Premeiotic, interphase
3	Meiosis I	Me I	Meiosis I, (telophase I)
4	Dyad	D	Dyad of cell visible
4	Meiosis II	Me II	Meiosis II, (prophase II)
5	Tetrad	T	Tetrad of cell visible
5	Microspore prophase	Mp	Mitosis (single nucleus) Nuclear division of microspore
6-9	Bi-nucleate pollen	Bp	Two nuclei (vegetative nucleus and generative cell)
9	Pollinia		Shedding of pollinia

Day 0 = responsive stage, day 9 = anthesis

recorded by data logger. The marked decrease in temperature due to rainfall 9 days before anthesis would be a trigger for its floral development. However, only a sudden drop of at least 9-10 °C within 1-2 h would stimulate the breakage of dormant flower miniatures and flowering occurred nine days later (Figure 8 , Appendix F). The days to anthesis (DTA) for natural flowering after rainfall stimulation was exactly nine days, DTA=9. A gradual decrease of temperature on a cool day or cool night had no effect (Figure 9, Appendix F).

A lot of visiting insects were observed around the days of full moon and the more numbers of capsules resulting from successful fertilization appeared later. The insects would be as pollinator of this orchid. However, full moon might affect the more visiting of insects. Full moon was an indirect factor needed for successful pollination and fertilization of this orchid.

3.2.2 Low temperature as an effective physical stimulus

Due to the cold requirement of this orchid, duration of cold treatment is a principal parameter to induce flowering. Test results from cold–treated plants supported the importance of a speedy chilling (Table 2). Plants kept in dark, which endured for 1 and 5 h, at a temperature of 8 ± 2 °C, would break dormancy of flower miniature. Unfortunately, only few flowers would then normally open because the 19 days to anthesis (DTA=19) was more than in nature (DTA=9). Meanwhile, a warming-up in an incubator at 37 °C for 1 h before the cold treated experiment for 1 and 5 h would induce flowering too although after 21 days (DTA=21). The cold-treated plants for 3 h from both CR and HCR

Figure 8 A typical pattern of temperature variation throughout the day on induction days (d0).

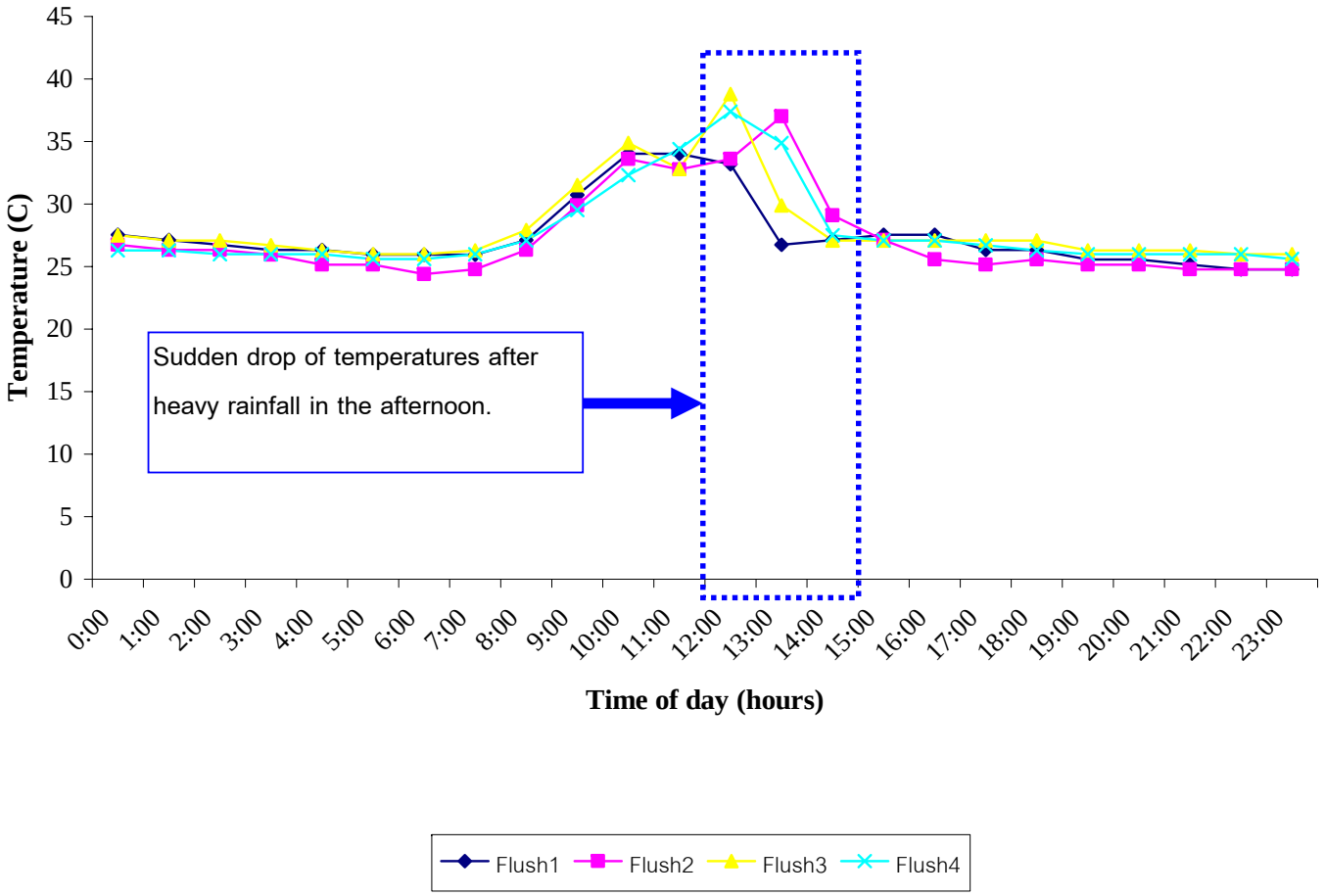

Figure 9 A typical pattern of temperature variation throughout the days on non-induction days

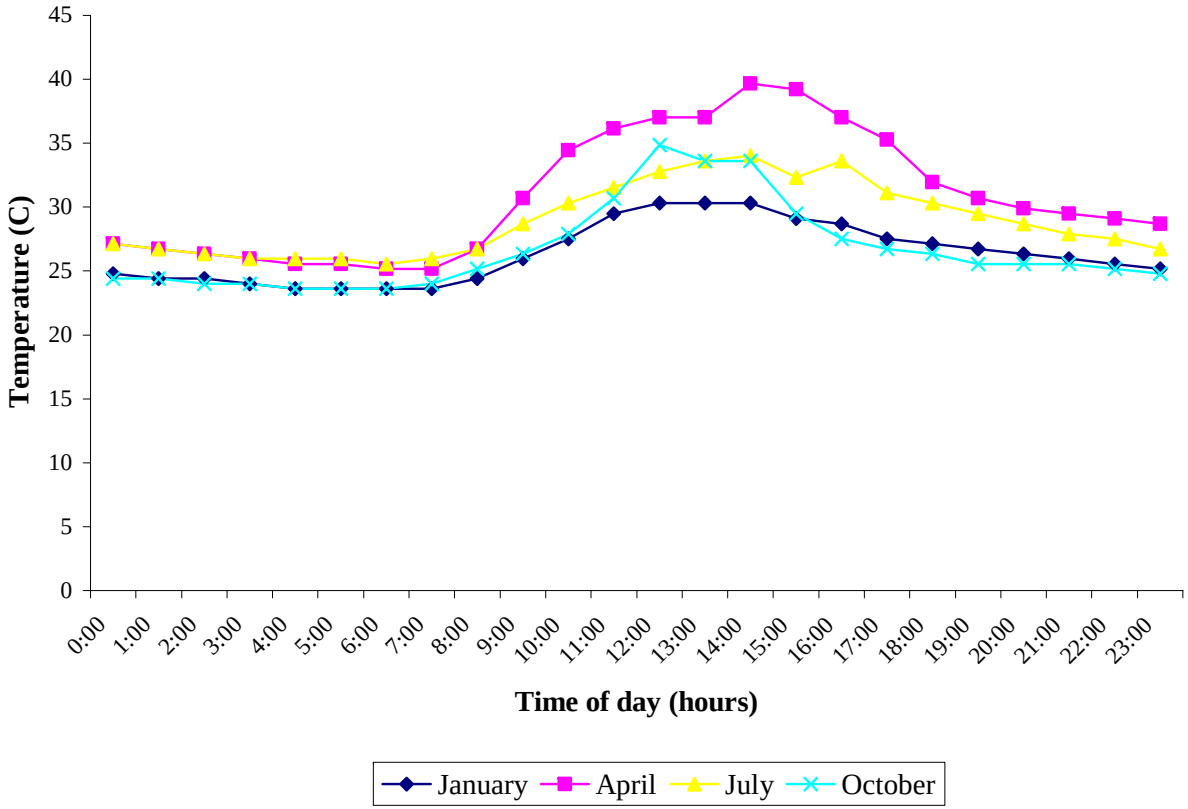

Table 2 Effect of various periods of cold treatment on the flowering of *D. crumenatum*. *

Duration of cold treatment (h)	DTA	No. of plant flowered	No. of opened flower / No. of plant	**Percentage of flowering on the anthesis day	Remark
Control (0)	-	-	-	-	-
CR experiment					
1	19	1	1/1	16.67	some AB
3	-	-	-	-	most AB
5	19	1	3/1	16.67	some AB
7	-	-	-	-	most SE
8	-	-	-	-	most SE
HCR experiment					
1	21	1	1/1	16.67	some AB
3	-	-	-	-	most AB
5	21	2	2/2	33.33	some AB
7	-	-	-	-	most SE
8	-	-	-	-	most SE

* Plant after given treatment was held in natural greenhouse. There were 6 individual plant / treatment.

** Number of flowered plant/ treatment

CR Cold treatment at 8 ± 2 °C in refrigerator, in darkness

HCR Plants were held in incubator (37 °C) for 1 h prior to cold treatment

DTA Number of days to anthesis (after the beginning of treatment)

\- Plants did not flower

AB Failure of flower development, flower miniature aborted

SE Senescence of leaves

experiments failed to flower. Most developing floral buds aborted since the third day after treatment. While the duration time of 7 and 8 h resulted in some floral buds aborted in inflorescence. They failed to continued floral development. In addition, damage due to chilling injury was generally observed in longer cooling treatment, i.e. 7 or 8 h, both in CR and HCR experiments. The morphological signs of this injury manifest themselves in gradual yellowish leaves, dry leaves and wilted shoots, and these symptoms would be more serious the longer the duration of cold treatment. Some aborted flower miniatures would then also be seen.

3.2.3 A separate application of BA and GA_3 hormone as an effective biological stimulus

The application of BA at 10^{-1} and 10^{-2} M were the most effective for flowering. They showed the DTAs of flower 9 and 11, especially DTA of flower 9 which was similar to those under natural conditions (DTA=9) (Table 3). The developing floral buds (58.33%) could be obtained from all six individual plants of 10^{-1} M BA application. It was also found that these developing flower miniatures treated with 10^{-1} M BA had gradually died since the fifth day but the others (14.29%) could further develop to anthesis on the supposed day, nine and 11 days after given treatment (Figure 10). The application of BA at 10^{-1} and 10^{-2} M showed the percentage of flowering response of 8.33% and 9.09%, respectively. Applying GA_3 was also effective at 10^{-2} M because it resulted in DTA of flower 10 and the percentage of flowering response was 12.50%. However, a combination GA_3 and BA

Table 3 Effect of plant growth regulators (PGRs) application on the flowering of *D. crumenatum*.

Concentration of PGRs (Molar)	Percentage of Response on the 4th day			Percentage of Response on the 9th day		DTA[1]
	Death	No response	Developing Flowers	Developing Flowers to anthesis[2]	Flowers to anthesis[3]	
untreated	-	-	-	-	-	-
BA						
10^{-1}	-	41.67	58.33*	14.29**	8.33***	9,11
10^{-2}	-	90.91	9.09	100.00	9.09	11
10^{-3}	68.75	31.25	-	-	-	-
10^{-4}	50.00	50.00	-	-	-	-
GA_3						
10^{-1}	81.20	18.18	-	-	-	-
10^{-2}	75.00	12.50	12.50	100.00	12.50	10
10^{-3}	40.00	60.00	-	-	-	-
10^{-4}	11.76	88.24	-	-	-	-
BA+ GA_3						
10^{-1}	58.52	11.76	29.41	-	-	-
10^{-2}	58.33	25.00	16.67	-	-	-
10^{-3}	35.71	64.29	-	-	-	-
10^{-4}	45.45	54.55	-	-	-	-

(1) DTA is the time from the beginning of hormone application to anthesis, (2) From developing flowers on the 4th day to anthesis

(3) From developing flowers on the 0th day (beginning of treatment) to anthesis

(*) = 14 developing flowers/24 treated flowers, (**) = 2 opened flowers/14 developing flowers, (***) = 2 opened flowers/24 treated flowers

At 10^{-1} BA application, developing flowers were induced from all six individual plants in the treatment.

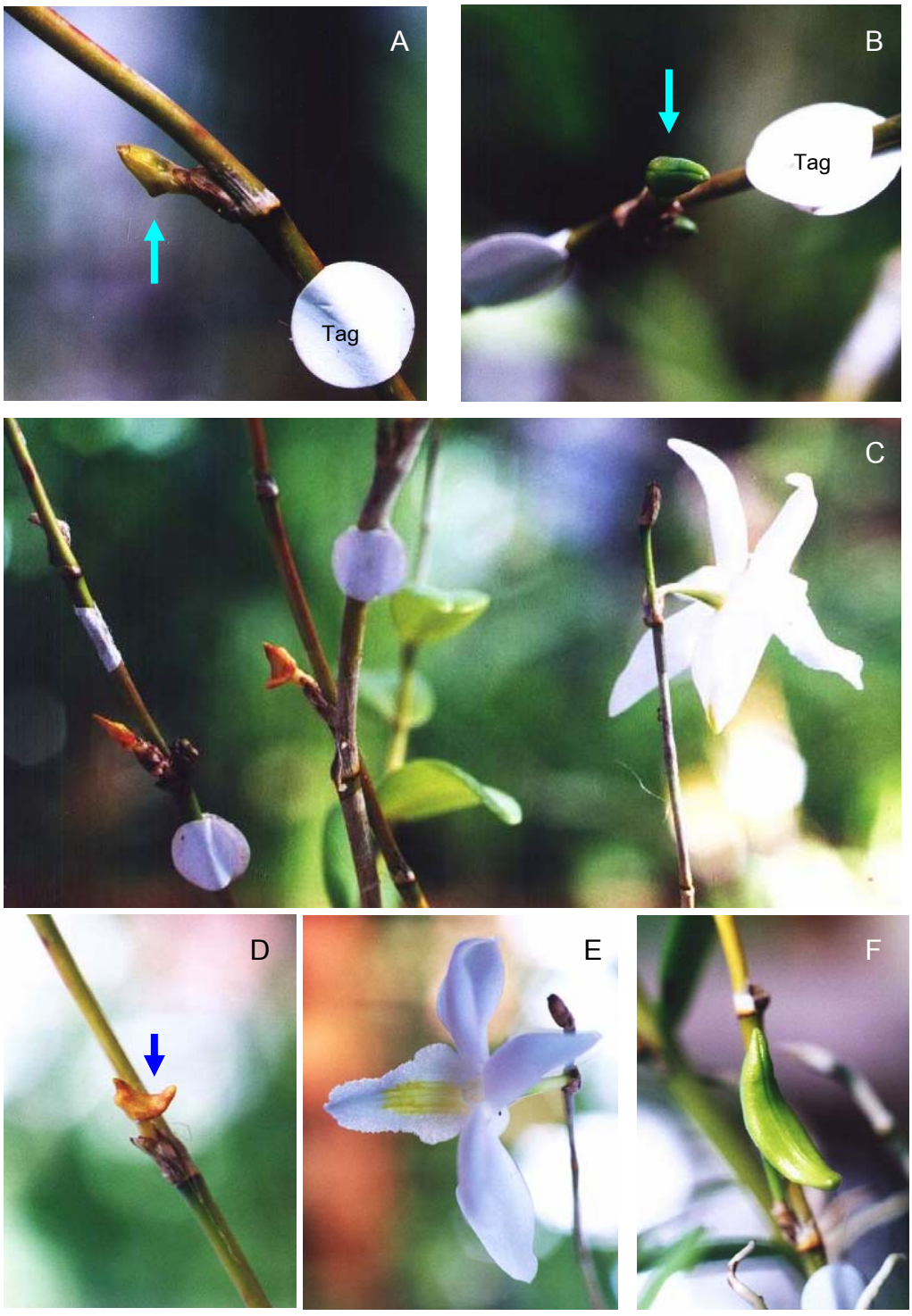

Figure 10 Photographs show flowering response of plants treated with 10^{-2} M BA. They were taken 5 (A, B) and 9 (C-F) days after hormone treatment.

treatment did not cause any anthesis (Table 3). The characteristics of no response were also noticed from several given treatments.

3.3 *In vitro* plant regeneration through embryogenesis and organogenesis

3.3.1 Developmental phases of plant regeneration

The stages of orchid development are described as follows. The one-month-old bud (Figure 11A) was transferred to the CI medium. The callus turned green or yellowish and formed nodular compact structures (NC) within four weeks (Figure 11B). These structures further proliferated and developed into PLBs by the 10^{th} week, enlarged and differentiated to regenerated shoots (Figure 11C) 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 two to three roots before *in vivo* transfer (Figure 11D).

3.3.2 Suitable media for the three main developmental phases

The callus proliferation of *D. crumenatum* was promoted significantly on VW medium supplemented with PGR and peptone when compared with VW medium without any addenda or supplemented with only PGR (Table 4). Thus, VW medium with PGR (1 mg l^{-1} BA and 0.1 mg l^{-1} NAA) and 2 g l^{-1} peptone was the most effective for callus proliferation phase. This medium was selected as the standard maintenance medium for this phase and for subculturing the totipotent calli. The VW media containing only either CW or peptone were more effective to promote callus

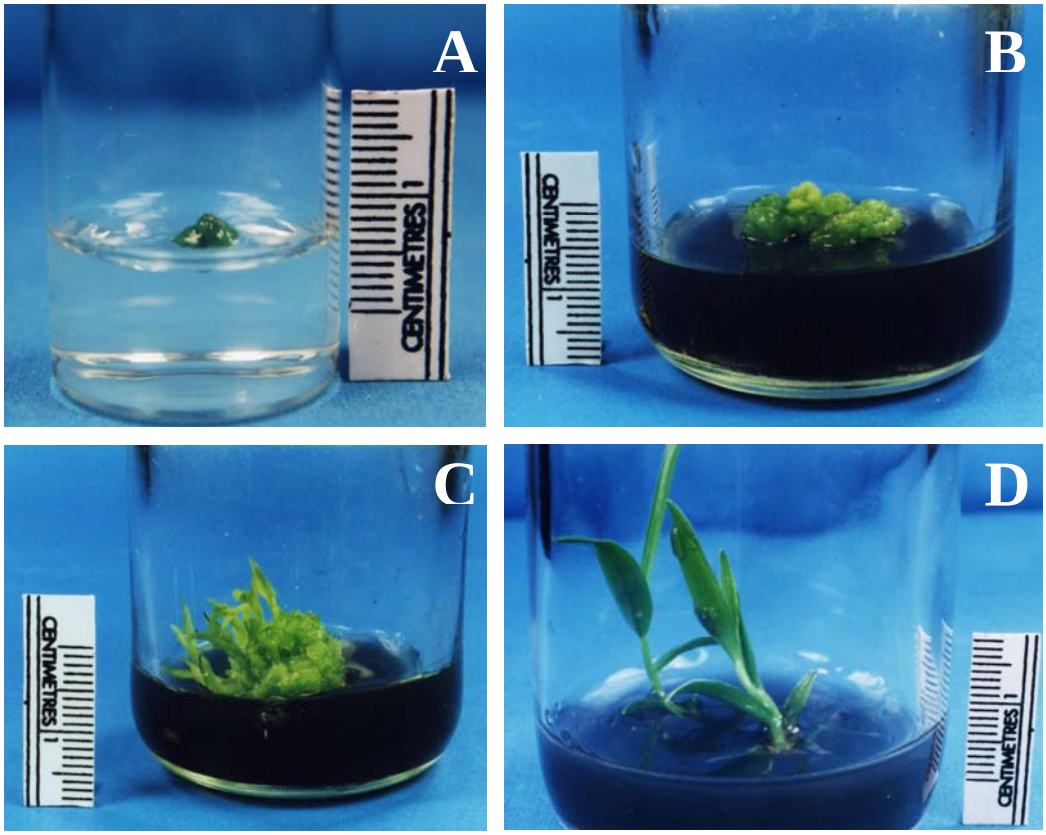

Figure 11 Developmental phases of plant regeneration. The 1-month-old bud (A) was induced to form callus which was then proliferated (B). The regenerated shoots were formed (C) and rooting shoots containing two to three expanded leaves were transplanted to greenhouse conditions.

Table 4 Effects of basal media supplemented with various substances on various developmental phases of *D. crumenatum*

(Week of cultures) Growth phase	Treatment No *	VW	HP
(The 4th week)		Means of increase 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 increase in fresh weight of callus or PLBs formation per 100 mg of callus (mg ±SE)[1]	
2) PLBs formation	1	45.76 ± 15.21ab	-
and proliferation	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	-

(continued)

(The 16th week)		Means number of regenerated shoots per g 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 were 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

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 addition of peptone (Table 4). 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 CW was more effective than peptone. From visual observation, both 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 largest numbers of PLBs were formed on VW medium with PGR and CW. At this stage, the calli continued to proliferate and to produce PLBs. There was no significant differenct between the medium with only CW and the medium with a combination of PGR and CW. However, as PGR might 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 only peptone or only PGR were ineffective in this stage.

In the plantlet regeneration stage, there was no significant difference among regenerated shoots obtained from any medium tested. However, the hormone-free medium with peptone gave more vigorous shoots than the others. Besides, PLBs themselves seemed to have the potential for further development.

3.3.3 Plant development via either somatic embryogenesis and organogenesis

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

Meristematic tissues consisting of the densely stained cells arose in the proliferating epidermal or the subepidermal tissues (Figure 12A) 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 (Figure 12B) and embryo-like structure (Figure 12C, D) could be observed from these regions. Moreover, the independent organogenesis of leaf primordia was established. It was noticed that some shoot meristem had a few leaf primodia but it did not have any bipolar structure (Figure 13A, B), so it was regarded as a meristem organogenetically formed .However, their vascular tissues formed a complex inside the callus later. The embryo–like structures in NC could be found while the organogenetic formation of leaf primordium was observable but the embryogenesis occurred more than organogenesis (data not shown). Furthermore, the regeneration pathway of these calli would be both through embryogenesis and organogenesis.

3.4 *In vitro* floral bud induction system and flowering

3.4.1 Some stages of the natural floral development served as control

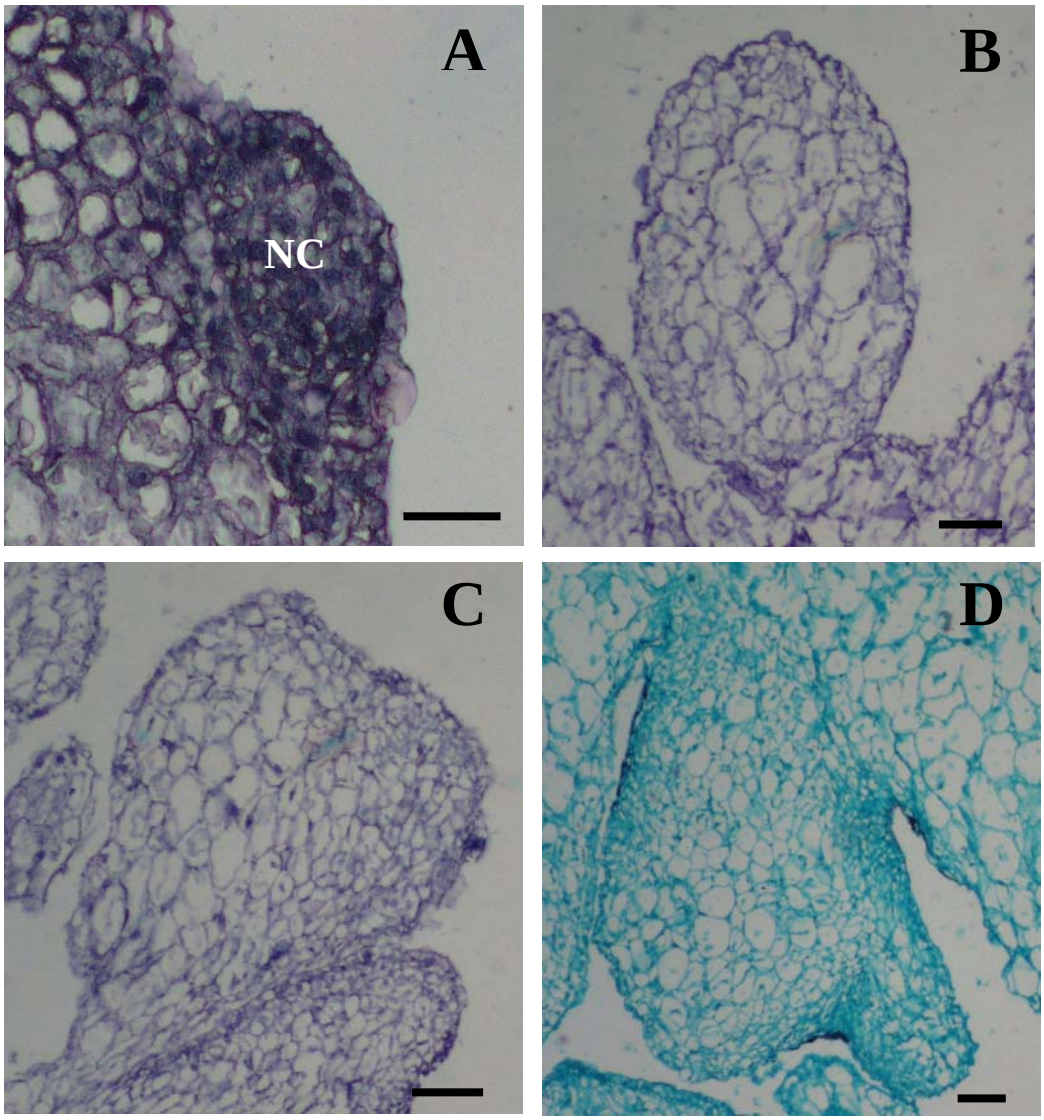

Figure 12 Plant developed through somatic embryogenesis. Meristematic tissues showing an appearance of nodular compact callus (NC), that corresponded compact callus macroscopically observed (A).Various types of embryonic structures (B-D) show a globular embryo-like structure (B), forming small PLB (C), and somatic embryo with asymmetric structure (D).

PLB- protocorm-like body. (A; Bar= 50 μm, B-D; Bar= 100 μm)

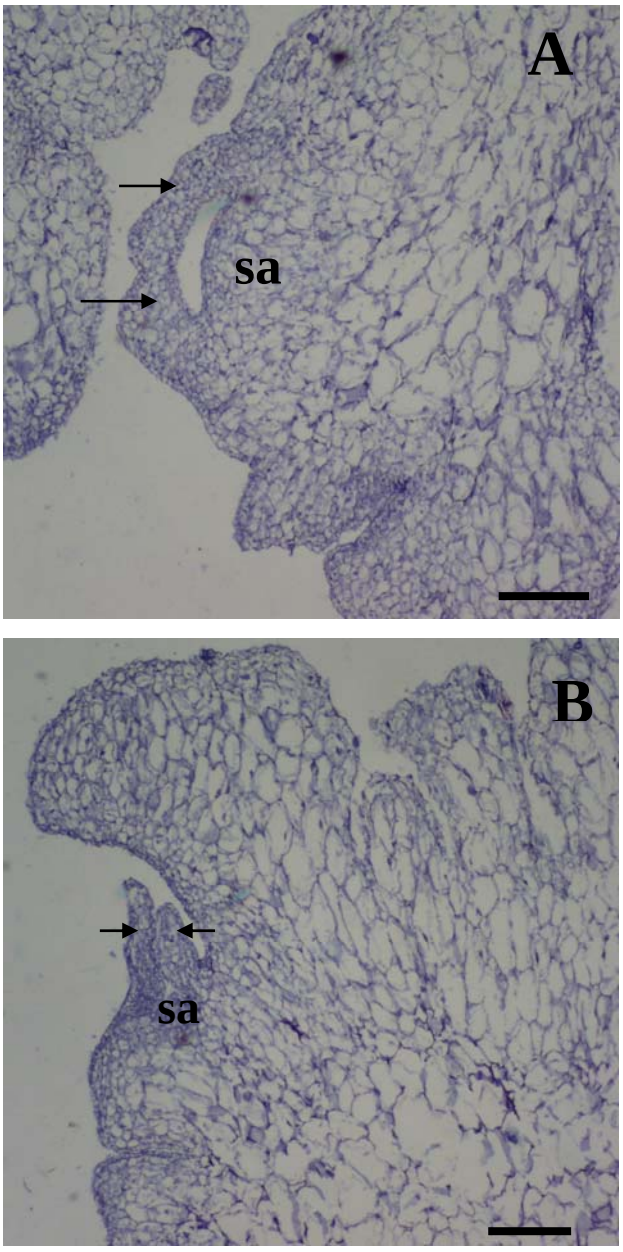

Figure 13 Shoots developed through organogenesis (A-B). Shoot apical meristem (sa) with leafy organ structures (arrows) independently growing apart from the root. Bar= 200 μm.

It was found that the floral development of *D. crumenatum* was in accordance with those of Epidendroideae and Vandoideae described by Kurzweil (1987). Based on analysis of SEM, scanning electron micrographs of some stages of floral buds in a non-resuspinate position were defined (Figure 14A, C). The sequence of perianth initiation became visible as the lateral sepal primordia (S2 and S3) followed by the lip (L or median petal) primordium. The abaxial side divided and elevated into the lateral petal primordia (P1 and P2) followed by the median sepal primordium (S1) resulting in the last perianth member initiation. The anther primordium (A1) was initiated from the abaxial ridge above S1 and the pronounced doming of the anther primordium was observed when all sepals (S1-S3) were positioned higher to cover the young floral bud (Figure 14C). Images of sectioned floral bud at the same stage of development were shown on Figure 14B and 14D. At the end of the normal floral development, young floral bud was covered by all sepals (S1-S3).

3.4.2 *In vitro* floral organ induction

The initiation of floral parts was recognized as a commitment to flowering. The mini-shoots derived from PLBs were induced to undergo floral organogenesis *in vitro*. Floral buds were not formed on SIM with the low concentration of BA. In contrast, floral buds were formed on FIM at higher concentration of BA, indicating that formation of floral buds was induced by or required BA. However, these mini-shoots in FIM produced one bud whereas plants grown in the greenhouse produced two to three flower buds on each inflorescence.

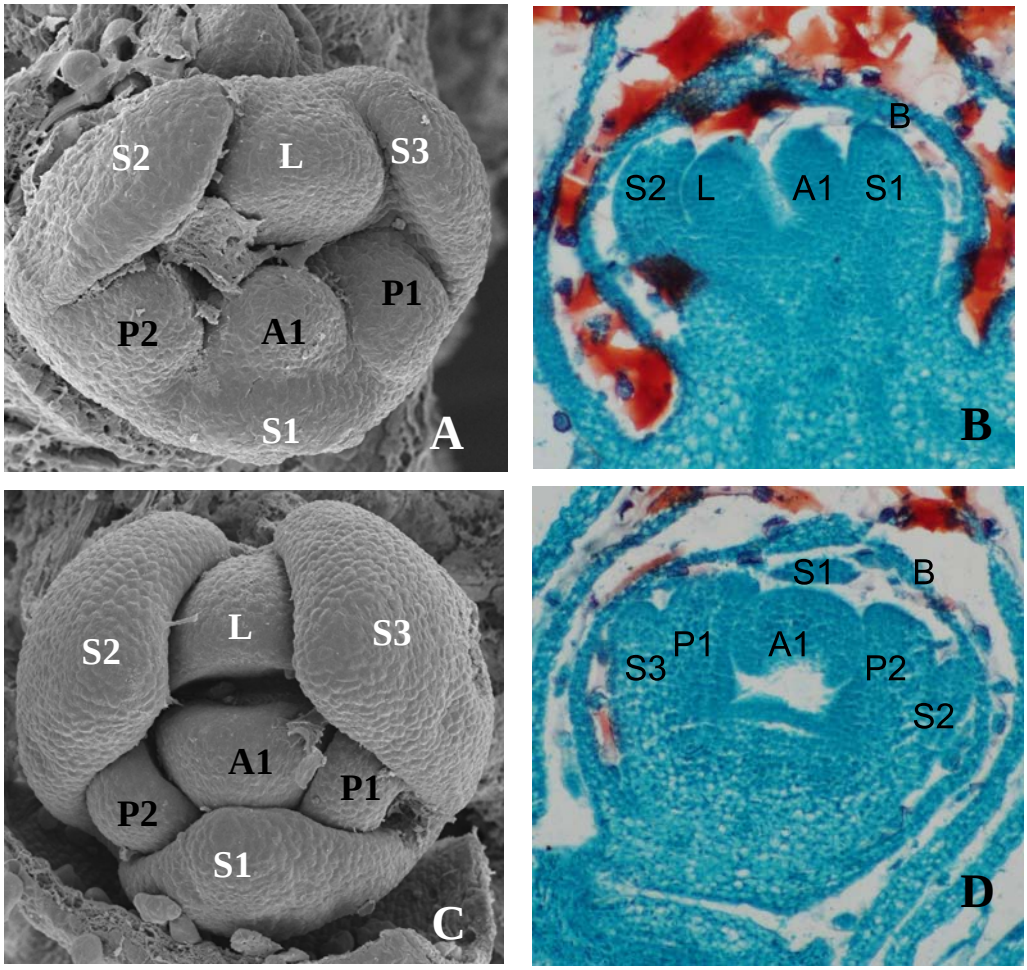

Figure 14 SEM (left) and LM (right) micrographs show the floral buds of *D. crumenatum* at some stages of the natural floral development. Longitudinal sections of floral buds correspond to A (B) and C (D). A1: anther primodium, L: lip primodium, P1-P2: petal primordia, S1-S3: sepal primordia. (A, C: top view and non-resuspinate position, B: lateral view, D: front view)

LM and SEM evaluation were used to explore the apical morphological changes and to find when floral organ initiation began. It was found that the early floral development *in vitro* was formed from the terminal buds of some explants. Under these culture conditions, the mini-shoots in SIM developed typical vegetative shoot apical meristem (SAM) with two leaf primordia (lp) within one month in culture (Figure 15A). After the exposure to BA in FIM, treated plants produced transition shoot apical meristem at week 8 of the culture, but the apices remained flat with the initiation of the first bract primordial (bp) (Figure 15B). At week 10 of the culture, the second bract primordium and the flower primordium (fp) were initiated (Figure 15C). By the week 15, the apex had assumed a convex, domed shape by an increase in the number of cells across the whole apex (Figure 15D). Domed apical meristem initiated three to four bracts; each had a wide lamina similar to vegetative leaves. Sepal (S) and petal (P) primordia were initiated at week 25 and their developments were observed at week 30; however, SEM and section micrographs showed a malformed floral bud at week 32 with S1 covering all of S2 and S3 (Figure 15E, F). Unfortunately, no sign of column initiation was detected from our experiment. The sequence of development events of the *in vitro* mini-shoots is diagrammed in Figure 16.

3.4.3 Flowering of some induced plants after placing under natural greenhouse conditions.

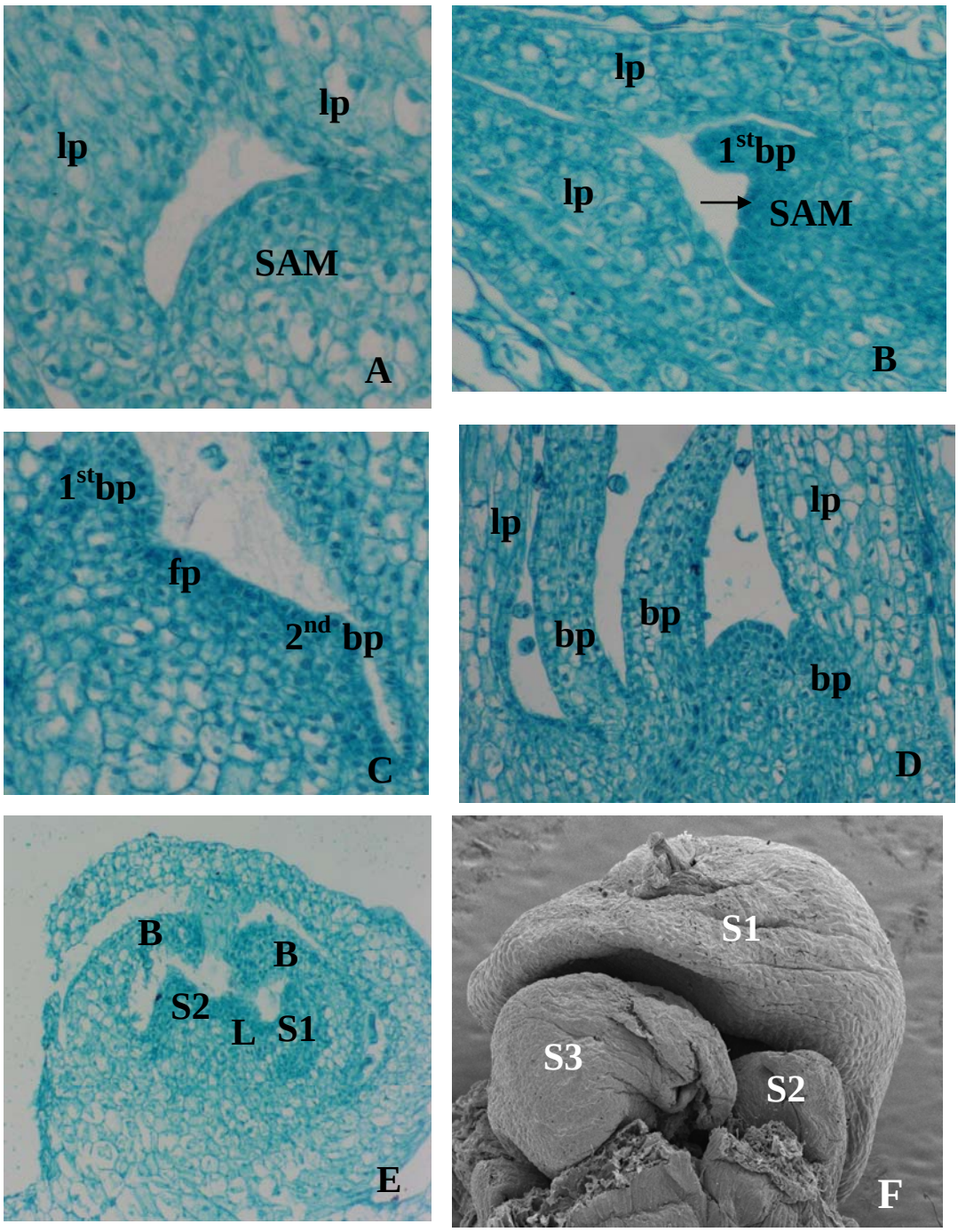

Figure 15 Median longitudinal sections of the sequence of development events in cultured mini-shoots of *D. crumenatum*. Shoot apical meristem with two leaf primordia (A). Flat vegetative meristem (B). Flower primordium (C) and domed meristem initiation (D). A 30-week old of malformed floral bud (E) and SEM of a 32-week old floral bud showing abnormality (F).

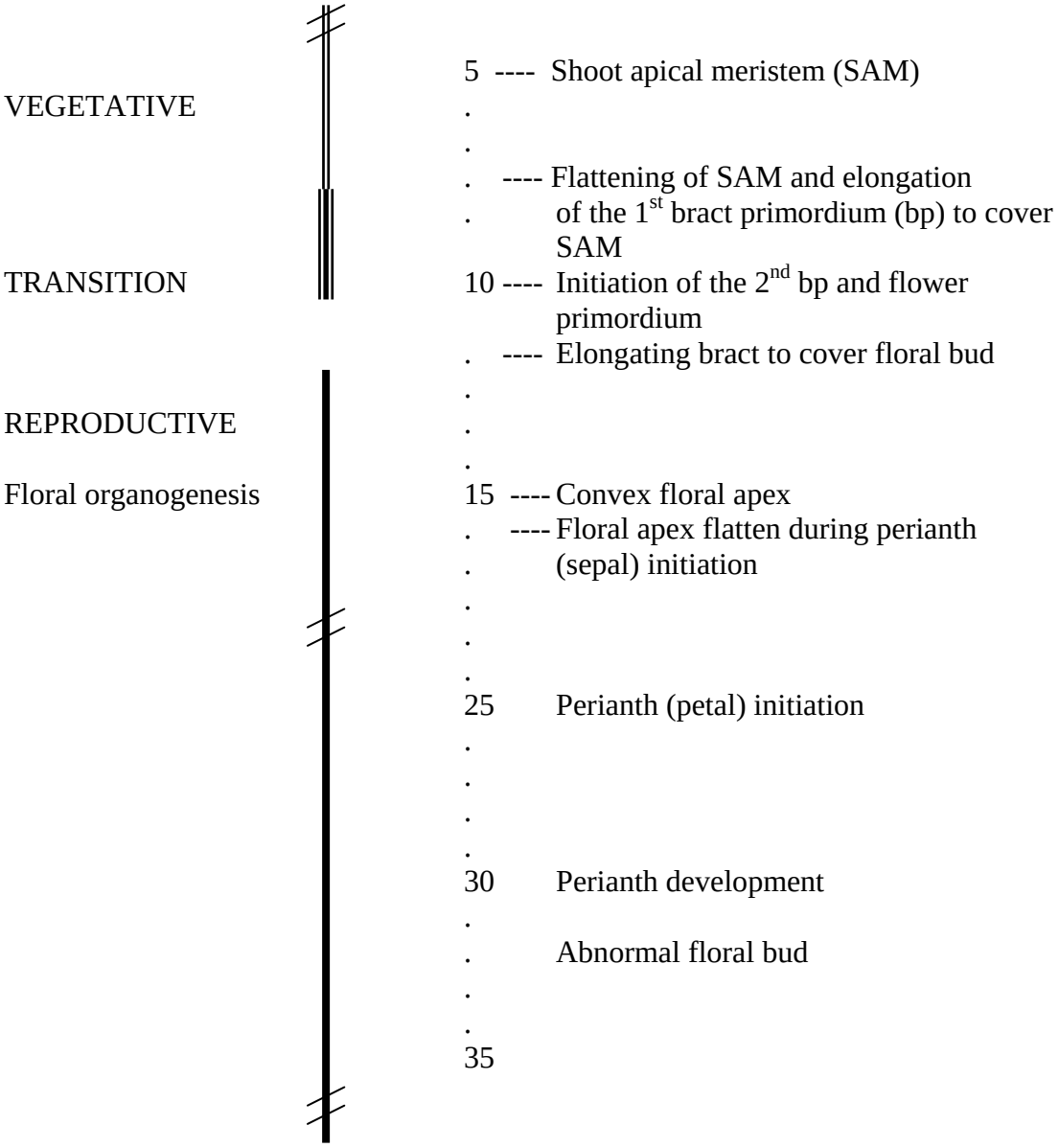

Figure 16 Schematic illustrating the developmental events of *D. crumenatum* cultured *in vitro*

Although there was no success in getting *D. crumenatum* to flower *in vitro*, common flowering could be found from some plants after placing in natural conditions.

In our experiment, the induced floral bud was unable to develop further into a flower *in vitro* if they remained in the same medium. In contrast, flowering from these floral buds occurred when transplanting to the natural greenhouse conditions. In total, it took about 8-12 months to obtain flowers. The structure of the flowers was morphologically resembled to those produced on the mother plant (Figure 17). It was interesting that flowering of non-induced and induced plants which were maintained under the same natural conditions in a greenhouse was achieved simultaneously nine days after the rainfall stimulation. Thus, at this stimulating period, the anthesis in both non-induced and induced plants was also synchronized.

Figure 17 Flowering of the induced plant after placing under natural greenhouse conditions. The yellowish leaves (arrow) had appeared since the beginning of stimulation.