2. MATERIALS AND METHODS

For *D. crumenatum* Sw., there has been limited available information, and that needs to be confirmed by experimental studies. In order to understand the flowering behavior and the developmental events, the flowering aspects were carried on. Therefore, the research consisted of four parts as the followings:

Firstly, this part consisted of the investigation of flowering behavior including the characteristics of the stage at which the floral buds responded to stimulus and the time when most pollen grains were mature. The integrated results of natural floral development served as a control in experiment of the *in vitro* floral induction system as well.

Secondly, this part consisted of the determination of factors affecting the breakage of dormant floral bud, for instance, lowering the temperature and applying hormone.

Thirdly, this part was a prerequisite of the last part. This part was an attempt to artificially induce flowering. Therefore, a method for producing high number explants with a few unvarying characters, required for the experiment of *in vitro* flowering, was desirable. Based on somatic embryogenesis and organogenesis, a method for efficient plant regeneration through callus culture was attempted.

Finally, the *In vitro* floral bud induction and flowering were performed.

2.1 Investigation of flowering pattern and pollen grain maturation

2.1.1 Plant materials and conditions

The wild orchid, *Dendrobium crumenatum* Sw. collected from the local forest were potted and maintained under shade house conditions in the greenhouse of the Department of Biology, Faculty of Science, Prince of Songkla University and used in the experiments.

2.1.2 Sample collection

The floral buds obtained from inflorescences of naturally growing plants were randomly chosen. These inflorescences were collected daily at 0800-0900 hr for ten days from the induction day (day0) to anthesis (day9). Other samples taken 20, 25 and 30 days after blooming were also inspected. Three to five inflorescences of each individual shoot (at least 6 individual shoot) were sampled on each day. The light microscopy (LM) and the scanning electron microscopy (SEM) provide ample opportunity for detailed observation of anatomical and morphological changes during the course of floral development in nature. These samples were then prepared for LM and SEM. The samples, collected from day0 to day9, were also prepared using the paraffin method and observed under LM.

2.1.3 Microscopy observations

The paraffin method was used for LM preparation (Johansen, 1940; Ruzin, 1999). The samples were fixed in FAA II containing 5 % (v/v) formaldehyde, 5%

(v/v) acetic acid and 90% (v/v) of 70% ethanol. They were dehydrated through a series of graded ethanol treatments, after which the samples were infiltrated and embedded in paraplast plus (melting point 56 °C) using Histo-embedder (model Jung, Leica, Germany). Serial sections of 10-12 μm thickness were cut on a rotary microtome. Sections were stained with either safranin and fast green or hemotoxylin and safranin (Ruzin, 1999).

For SEM imaging, the samples were dissected under a stereo microscope, fixed at 4 °C for at least 3 hrs in 10% (v/v) formaldehyde, 5% (v/v) acetic acid, 45% (v/v) ethanol and 1% (v/v) Triton X-100. After being rinsed three times in 0.1 M phosphate buffer, pH 7.2, these tissues were dehydrated in a graded ethanol series, critical point dried using CO_2, affixed to stubs with double stick tape, and coated with gold. These samples were imaged, using a scanning electron microscope JEOL 5800LV operated at 10-15 kV and the hard copies were then visually examined.

2.2 Determination of the natural stimulus and the effective physical and biological stimuli required for flowering

2.2.1 Data collection and the natural flowering

Temperature and relative humidity in the greenhouse were continuously recorded using a data logger (HOBO pro series). The dates of natural flowerings outdoors throughout the whole year were recorded. When any natural flowering appeared, the typical temperature throughout the day on induction day (d0) was

checked using previously recorded data from the data logger. Temperature variations on non induction days were also considered. The day of full-moon was simultaneously recorded for use as an additional observation.

2.2.2 Experiment to test the effective physical stimulus

The cold treatment (CR) and pre-heat treatment before cold treatment (HCR) experiments were set to determine the minimum period (h) of low temperature to break dormancy. Mature orchids with inflorescence stems were divided into two groups called CR and HCR. Each group had six treatments and each treatment consisted of six individual plants surrounded by moist coconut peat filled in pots. For CR group, plants were incubated for cold treatment in refrigerator (8±2 °C) in darkness for 0 (for control), 1, 3, 5, 7 and 8 h For HCR group, plants were incubated in an incubator (37 °C) for 1 hr. before cold treatment (8±2 °C) for various periods of time as described previously. After that, plants from both CR and HCR groups were subsequently transferred to the warmer condition of the greenhouse. The plants were watered every morning and inspected for signs of morphological changes and expected development of inflorescence.

2.2.3 Experiment to test the effective biological stimulus

The exogenous hormone was applied to determine which of the plant growth regulators (PGRs) probably acted as a biological stimulus. Three sets of exogenous tests were carried out with GA_3, BA, and a combination of GA_3 and BA at various concentrations: 0 (for control), 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} M containing 0.05% Tween-20 (13 treatments). In the same manner as in the cold-treated experiment, each

treatment contained six individual plants (six replications). Three to four inflorescences per plant were chosen. The total of 18-24 inflorescences (samples) per treatment were tagged. The control and treated pots were maintained in the greenhouse. The plants were watered daily in the morning before hormone treatment. The Tween-20 was first applied using cotton wool on a tagged inflorescence, followed by hormone application. A 1 μL amount of hormone solution was applied on one tagged inflorescence using a micropipette. The PGRs were applied twice a day at 0800 and 1600 h for two successive days.

2.2.4 Flowering response and the data recording

For a qualitative measurement of flower development, flower initiation is the proportion of plants that have initiated at least one flower after a given treatment (Bernier *et al*., 1981). With the clear-cut responses obtained (flower opened), it is not unnecessary to carry out a statistical analysis of the data (Heide, 1997). The flowering response of treated plants was observed and recorded in percentage of flowering response along with the morphological changes of developing floral buds. The time from the end of cold treatment and the time from the first day of hormone application to anthesis were counted in terms of the number of days to anthesis (DTA) for cold and PGRs application experiments, respectively.

2.3 *In vitro* plant regeneration through embryogenesis and organogenesis

2.3.1 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 a greenhouse at the Department of Biology, Faculty of Science, Prince of Songkla University. The stalks were cut into 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 drops 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 Tween20 for 20 min. The series of Clorox percentage used was 10%, 5% for 10 and 5 minutes respectively. Finally, the excised buds were washed with sterile distilled water 2-3 times and cultured on Vacin and Went (VW) medium (Vacin and Went, 1949 and Appendix C) supplemented with 20 g l^{-1} sucrose to promote bud growth. The pH of this medium was adjusted to 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 under a 16 h photoperiod using 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 initiation (CI) medium.

2.3.2 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, a 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). Callus proliferation was observed after one month of culture. These calli were then transferred to the same medium. The subculture monthly was done to produce enough totipotent calli for the subsequent experiment.

2.3.3 Investigation of suitable media for the three main developmental phases

2.3.3.1 Culture 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 find the effective and suitable one for various stages of growth: callus proliferation, PLBs formation and shoot differentiation. Apart from complex addenda, 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA, were also incorporated into the media. Five modified media were tested while used the basal media without supplement as the control: (1) supplemented with 10% coconut water (CW), (2) supplemented with 2 gl^{-1} peptone, (3) supplemented with a combination of 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA (4) supplemented with 10% CW besides the combination of 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA (5) supplemented with 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. Sucrose 20 g l^{-1} and AC 2 g l^{-1} 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 under the same conditions as described previously.

2.3.3.2 Callus proliferation

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

2.3.3.3 PLBs formation and proliferation phase

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 transferred to bottles each containing with 10 ml of the same medium.

2.3.3.4 Regenerated shoot phase and plantlet production

Mean number of the regenerated shoots was recorded at the end of the 16^{th} week of culture. In order to produce plantlets, the regenerated shoots with 2-3 leaves were detached from shoot clumps and transferred to VW medium at 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 coconut peat before being filled in the mini-pots.

2.3.3.5 Data analysis

The experiment was a completely randomized design (CRD) and every treatment has 6 replications. It was repeated twice. Fresh weight of both callus proliferation and PLBs formation were recorded at the end of the 4^{th} and the 10^{th} week. The growth of cultures was measured in term of increase in fresh weight (mg). The differentiated shoots were recorded as 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} weeks of culture 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 (Zar, 1996). The Kruskal-Wallis test was used to analyze the mean difference of regenerated shoots in the last growth phase.

2.3.4 Histological observation

These representative samples were investigated whether plants regenerated through embryogenesis and organogenesis. The samples were randomly collected from various developmental phases and prepared using the paraffin method for LM observation. The sample preparation for LM was described previously in 2.1.3

2.4 *In vitro* floral bud induction system and flowering

The initiation of some floral parts, for example, sepal or petal, was recognized as a initial sign of flowering. The callus-derived protocorm- like bodies (PLB) obtained from the previous experiment (2.3) were used as materials.

2.4.1 Vegetative growth: mini-shoot induction

These PLB were cultured for vegetative growth in modified liquid Knudson (KC) medium (Knudson, 1946 and Appendix D) supplemented with 5 μM BA, 20 gl^{-1} sucrose, 15% (v/v) coconut water (designated shoot induction medium; SIM). The pH of the medium was adjusted to 5.3 with KOH before autoclaving at 121 °C, 1.1 kg/cm^2 for 20 minutes. They were placed on a rotary shaker with an agitation speed of 120 rpm under a 16 hr photoperiod under an illumination of 20 μmol $m^{-2}s^{-1}$ photosynthetic photon flux density provided by Gro-lux lamps. These cultures were maintained at 24 ± 2 °C. The young mini-shoots were obtained after four weeks of culture and they were subsequently subcultured once a month.

2.4.2 Reproductive growth: floral bud induction

The young mini-shoots were obtained after four weeks of culture and they were transferred to liquid KC medium containing 25 μM BA, 20 gl^{-1} sucrose and 15% (v/v) coconut water (designated flower induction medium; FIM) for floral bud induction. The cultures were maintained for four weeks prior to the first sampling was collected. Subsequently, the samples were harvested once a week. The monthly subculture was done until explants with emerging shoot apices (1-2 cm long) were

evidenced and then transferred to the solid SIM overlaid with liquid FIM. The plantlets of 5-8 cm length (approximately 8-12 weeks after culture on solid medium) were transplanted to a coconut bed placed in the greenhouse conditions. The flowering time was observed and recorded.

2.4.3 Histological observation

The morphological changes of natural floral buds and shoot apices of *in vitro* grown mini-shoots were observed using LM and SEM. Some stages from the natural development were used as a control to compare with some stages obtained from the in vitro floral induction system. LM and SEM preparation were described previously in 2.1.3.

2.4.4 Outdoor flowering

The plantlets of 5-8 cm length (approximately 8-12 weeks after culture on solid medium) were transplanted to coconut bed placed under greenhouse conditions. The flowering time was observed and recorded.