4. DISCUSSION

4.1 Flowering pattern and pollen grain maturation

Although the inflorescence of the pigeon orchid, *D. crumenatum* Sw., consisted of two to three floral buds at different developmental stages, only the first (1st) floral bud at the responsive stage would blossom nine days after rainfall stimulation. The two other floral buds, the second (2nd) and the third (3rd) floral buds were initiated to develop sequentially. These two immature buds could further develop by sequential development, resulting from the same repeated stimulation. The reason why these floral buds stopped their growth at different development stages waiting for the appropriate stimulus should be further examined experimentally. It was interesting to further question that the 1st floral bud may produce some substance, which acts as an inhibitor, inhibiting the following the two floral buds (i.e. the 2nd and the 3rd buds) during the floral development. So, these developing floral buds were then stopped their own development waiting for the next stimulus. The stimulus required for process of both the flowering and the subsequent floral development is possibly the same. However, these floral buds must reach a state of maturity to be receptive to stimulus. The 1st floral bud at the responsive stage exhibited flower miniature completed with all floral structures and the microsporocyte or pollen mother cell was still at a pre-meiotic stage. The pollen grain of *D. crumenatum* resulted in bi-nucleate cell should be indicated to pollen maturity. These pollen grains were still in group called pollinia as in other orchids until anthesis (Galetto *et al.*, 1997).

4.2 Natural stimulus, physical and biological stimuli required for flowering

In nature, the synchronous flowering of *D. Crumenatum* Sw. orchid usually occurs nine days after a sudden drop in temperature, of about 8-10°C (7.66-9.69 °C) within 1-2 h, due to heavy rain. A gradually decreasing temperature could not stimulate flowering of this orchid. The number of opened flowers was positively correlated with the number of the flower miniatures in the responsive stage ready to flower at that time. An extra delay of flowering, 19 and 21 days after treatment, from both CR and HCR experiments might result from insufficient chilling and from rather short or prolonged cooling. In addition, the longer DTAs might also result from long photoperiod of the low light intensity of full moon. Photoperiod was important and critical when chilling (temperature below 10°C) requirement was not satisfied and the long photoperiods could be at least partly compensated for lacking of chilling (Battey, 2000). The insufficient cold-treated orchids placing under the greenhouse conditions might require a long photoperiod of full moon to induce flowering. The period of 19-21 days was enough for plants to pass through the day of full moon in tropical region. So, photoperiod should be a second trigger for flowering of this orchid. Because cold requirement of plants is a quantitative requirement, a duration time of 5 h was found to produce more blossom than a one-hour cooling. We did not succeed to get any flowers at all when the cooling lasted three hours. So, the minimal duration of low temperature for flowering could not be clearly identified. The situation could indeed be quite complicated. Should it be understood that a three-hour period was a kind of transition from insufficient to sufficient cooling? Recovery-time to becoming a full-grown plant is probably critical too, because the meristematic tissue could have been

damaged or injured by the cold treatment. It might be expected to find similar responses to those from the effect of long-lasting chilling with necrotic symptoms, as reported for certain tropical plants by Patterson and Reid (1990). Moreover, the appearance of the incomplete floral development and the flower abortion were commonly observed in many plant species grown in natural and experimental conditions (Kinet *et al.*, 1985). Some reports mentioned that exposure to cold enhanced subsequent GA biosynthesis in seeds potatoes and other tubers. During chilling of peach seeds, increases in several gibberellins took places, but these changes seem to be associated more with normal growth and seedling development than the release from dormancy (Powell, 1987; Evans, 1984). However, gibberellin production or export was severely inhibited by prolonged exposure to cold in corn root (Evans, 1984). In addition, chilling promoted the system of gibberellin synthesis in hazel nut but the formation of active gibberellins becomes operative only at higher temperature. Thus it was suggested that gibberellin content may be enhanced by chilling (Powell, 1987). Besides, there were many reports which suggested that there might be a relationship between chilling and the appearance of cytokinins including buds and seeds. While the others revealed little or no changes in cytokinins in response to chilling.

Endogenous gibberellins and cytokinins may be involved in the emergence of floral bud from dormancy. It was suggested by the fact that application of exogenous GA_3 and BA would induce floral bud-break. It seem possible, therefore, that the control of floral bud dormancy may involve an interaction between gibberellins and cytokinin. It means that one hormone may trigger changes in the endogenous level of the other and *vice versa*. Thus, BA and GA_3 might be a trigger to each other. It is

generally known that cytokinin (i.e. BA) can increase the content of endogenous GA_3 or can prevent the reduction of GA_3 content which is normally associated with a specific developmental stage (Evans, 1984). In addition, enhanced GA production seems to be involved in certain responses to cytokinin. Likewise in *D. crumenatum*, as a 'cold requiring' orchid, BA might cause an increase of GA_3 or prevent a reduction in GA, which then affects the flowering. It was possible that hormones interact at one or another phase in certain process such as when BA application is followed by applying GA or *vice versa.* It was mentioned that chilling mechanism which controlling dormancy operating through both cytokinin and gibberellin system in sequence. The result was also supported the floral stimulus was primarily a certain balance of known hormones and assimilates arriving at apex in a specific sequence (Bernier, 1981a; 1981b; Metzger, 1987). This phenomenon of floral stimulus would be consistent with the multi-factorial system of flowering, discovered in *Arabidopsis* (Berneir *et al*., 1993). This event has been found in some specific species. Moreover, pollen development might be disrupted by this effect. However, the continued development in microspore mother cells caused from enhancement of meiosis by cytokinin (Evans, 1984).

The DTA from exogenous hormone application (DTA= 9-11) in our tests was almost the same as in nature (DTA= 9) while the DTA from the experiment for cold requirement was 19-21. The flowering of *D. crumenatum* orchid could be made either by chilling them for 1 and 5 hr or by giving them the repeated application of GA_3 or BA. Because environmental factors i.e. low temperature had strong effects on all phase of plant growth and development, it was the fact that these parameters influence the content of endogenous hormones. When the microsporogenesis was considered,

the microspore mother cell began divide on the 2nd day after stimulation. It was mentioned that GA_3 firstly appeared to trigger the subsequent endogenous level of BA caused to meiotic cell division occurring clearly on the 2nd day. However, both cytokinin and auxin were reported that they were required for cell division in higher plants. This means that a drop in temperature might affect either cytokinin or GA_3 biosynthesis. From these results, the pathway of BA and GA_3 biosyntheses in sequence were the important pathways for the cold requiring plant to flowering (Powell, 1987). But it does not to absolutely conclude that either GA_3 or BA plays primary roles in the regulation of *D. crumentaum* flowering.

The more number of capsules resulting from fertilized flowers might depend on the visiting pollinators, for example, nocturnal insects which came when the flowers bloomed around full moon. Because the more pollinators meant the more successful fertilization, this might exert a secondary effect on the number of opened flowers. This orchid attracts in the late night and early morning, a lot of insects would possibly come from far due to its strong fragrance which is also considered very pleasant by humans. The white color of this flower probably increases their conspicuousness, but this only would function when there is some illumination. In fact, the coincidence between flowering and moonlight was found in the course of our one-year experiment, during which the pigeon orchid in nature was also monitored. In case of this orchid, it was possible that low light intensity of full moon might be the important factor for visiting pollinators. However, some interesting reports revealed that the low intensity of light might affect the number of opened flowers. Briefly, some short-day plants, which required a long night to induce flowering, received low light intensity (0.5 lux) during night, could promote a greater number of flowers than plants which

received the complete darkness. It was also reported that the light intensity from full moon on a cloudless night at latitude 50 to in tropical regions varied from 0.3 lux to 0.9 lux. It is of interest why low light intensity can produce a greater number of flowers than darkness. So, it is unknown whether the low light intensity from the moon light could be a second 'environmental' factor involved, related to pollination (personal observation) in this tropical orchid. This question should be explored in the near future.

4.3 *In vitro* plant regeneration through embryogenesis and organogenesis

The efficient procedure to produce the uniform plants showed that the HP medium was not suitable for the first phase of callus proliferation. This phase required a suitable concentration of the PGR and peptone to promote the calli to grow vigorously. It was noticed that peptone, one type of amino protein, was important for this phase. More importantly, there was significant difference between the two media with and without the peptone during the callus proliferation phase. Thus, the medium supplemented with combination of PGR and peptone could give the highest yield of healthy callus whereas the media supplemented with only PGR provided the lowest yield of callus. Vellupillai *et al*. (1997) 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. The proteins were, therefore, 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. Hence protein was important and required for this developmental stage as well. The calli could be also maintained by subculture monthly in the same medium like other orchids (Chang and Chang, 1998). The PLB formation stage needed the additive nutrient especially CW. The CW might be useful for promoting the callus to form PLBs. However, some proteins were necessary for this stage as well. These young PLBs were nearly round or oval and thereby similar in shape to the protocorms of *D. crumenatum* formed during seed development (Vellupillai *et al.*, 1997). The suitable media in previous developmental stage was the most effective and could make PLBs develop spontaneously to full plantlets. Thus, the HP medium would be selected for the last stage as well. The experiment also suggested that the peptone could be incorporated to the medium in all stages of development, but only as supplementary elements. The source of explants and origin of calli were important for regeneration pathway. In barley, the regenerative callus could be induced from the scutellum of the immature and leaf or coleoptile base of mature embryos. The regeneration pathway of callus derived from mature embryo could develop through either organogenesis or embryogenesis but the former was more common than the latter (Oka *et al*., 1995). In this study, the callus of *D. crumenatum* orchid induced from bud (meristem) could be regenerated through embryogenesis more than organogenesis. For the somatic embryo development, it should be mentioned that structures which first appeared like callus were actually embryogenic cells which converted into PLBs. The globular embryo-like structures were actually the infant PLBs induced via embryogenesis. It was also suggested that the process of somatic embryogenesis was involved in PLB formation. Thus, the

PLBs derived from some calli could be considered as somatic embryo and this type of callus was embryogenic. On the other hand, organogenetic formation of leaf primodia could be possibly noted. To sum up, the results revealed that healthy orchid plants could be obtained following regeneration via both somatic embryogenesis and organogenesis. In addition, this process of plant production was selected to produce more uniform plants for the subsequent experiment.

4.4 *In vitro* floral bud induction system and flowering

The Orchidaceae exhibits diversity in both vegetative and floral forms. Studying flower formation *in vitro* can provide a model system for studying induction and development (Jumin and Ahmad, 1999). A simultaneous effect of BA in the production of floral buds was reported in other plants such as soybean (Julian and Wyndale, 1992), bamboo (John and Nadgauda, 1999) and Chinaberry (Handro and Floh, 2001). Chambers *et al*. (1991) reported that flowering of the bamboo *Dendrocalamus hamiltonii* Munro only occurred in treatments containing BA in the tested range, and not in growth regulator-free controls. However, the greatest frequency of cultured explants giving rise to flowers occurred when the explants were transferred to a growth regulator free culture medium. The cytokinin BA may merely facilitate the growth of pre-existing floral meristems. These reports suggest the important role of BA in activating the switch-on mechanism for flower induction. BA promotes the transition to reproductive stage in many orchid species (Kostenyuk *et al*., 1999; Paek *et al*., 1989; Wang *et al*., 1993). In our experiment BA at 25 μM was effective to initiate floral buds and the role of BA was probably due to the activation

of endogenous cytokinin in ascending xylem sap (Srinivasan and Mullin, 1978). Using scanning electron micrographs and sectioned materials, morphologically distinct stages of vegetative shoot apical meristem development to transition shoot apical meristem were defined. In the beginning all meristems were vegetative and flat and eventually became domed. The narrow flattened vegetative meristem (stage 0) at the apex was identified as in vegetative stage while the broad flattened vegetative meristem (stage 1) was characterized as the first morphological sign committed to floral development (Foster *et al*., 2003). The domed apical meistem initiated three to four bracts before converting to a floral meristem. Besides these, the event of bract primordium initiation, the flattening of shoot apical meristem (SAM) and formation of flower primordium were revealed and analyzed during the transitional phase of *Dendrobium* Madame Thong-Ing cultured *in vitro* (Yu and Goh, 2000). The early floral development pattern of this orchid was consistent with the results of the appendicular epigynous development (Appendix G), the common type of epigynous organogenesis type with inferior ovary position, described by Kuzoff *et al.* (2001) and with general floral organogenesis (Evans and Dickinsons, 1996). This type showed a convex floral apex followed by the flattened apex during perianth initiation, whereas the hypogynous type generally exhibited a convex floral apex throughout floral organ organogenesis. Morphological changes of the shoot apex prior to floral development are widely recognized as signs of the transition from vegetative to floral development in a wide range of species (Steeves and Sussex, 1989). Results from *in vitro* floral induction system, it was possible that the flower bud developed abnormally after median sepal (S1) initiation because no subsequent development of anther (A1) initiation was detected

Many representatives of the Orchidaceae usually have long juvenile periods-up to 13 years and delayed transition to reproductive development (Arrditti, 1992). Thus the shortening of juvenility in the pigeon orchid is of biotechnological interest. In orchid there are several reports on *in vitro* induction of the early flowering (Kerbauy, 1984; Duan and Yazawa, 1994; 1995; Paek *et al*., 1989; Arditti and Ernst, 1992). Although flowering in many plant species is considered to be a complex process regulated by a combination of environmental and genetic factors, this developmental step is also associated to maturity. In this regard, we did not observe flowering *in vitro*. Sugatha and Chandra (1997) have shown that *Melia azedarach* clones produced from seedling tissue cultures bore flowers in the first year of establishment. Furthermore, failure of *in vitro* flowering may be due to the absence of the stimulus mentioned earlier.

The culture medium composition and the environmental conditions accelerated the growth rate and thus shorten the vegetative growth period. The anthesis of this orchid from the natural and the induced sources was synchronized. This is in contrast to the report of bamboo flowering since the natural anthesis was highly synchronized and took place in the morning hours whereas the anthesis under *in vitro* conditions was not synchronized and occurred at any time during day or night (Nadguada *et al*., 1997).

In conclusion this research has shown that flower buds could be induced on mini-shoots of the pigeon orchid during *in vitro* culture but flowering was stimulated in the greenhouse under natural conditions. Furthermore accurate experimentation will be necessary to verify if floral buds in this orchid could be induced *in vitro*. The

data reported here are novel and bring out the importance of this plant as well as a better understanding of *in vitro* orchid flower induction.