In vitro flowering and *in vitro* pollination: methods that will benefit the orchid industry

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Summary

We have successfully developed a method to induce early *in vitro* flowering of plantlets in tropical *Dendrobium* hybrids (Hee et al. 2007; Sim et al. 2007, 2008). Using one of the hybrids, *Dendrobium* Chao Praya Smile, as a model system, methods for *in vitro* flowering and *in vitro* pollination are described. Flowers could be produced in culture about 5-6 months after seed sowing by appropriate treatment with benzyladenine (BA). This duration is much shorter than the 2-3 years required using conventional growing method in the green house. As a result, early evaluation of the characteristics of flowers is possible. Subsequent to *in vitro* flowering, pollination could be performed *in vitro* with formation of viable seeds. Hence the breeding cycles of orchid were reduced significantly. The usefulness of these methods and how they could benefit the orchid industry are discussed.

Introduction

Conventional orchid breeding is a lengthy process. The breeding process normally comprises of these essential stages: 1) pollination and seedpod maturation, 2) *in vitro* seed germination, protocorms development and growth of seedlings, 3) *ex vitro* establishment of seedlings and growing seedlings to maturity, and 4) assessment of flower characteristics and quality (Hee et al. 2007). Due to the long juvenile phase of orchids, the entire breeding cycle could be 3 to 5 years depending on the genotypes involved (Kamemoto et al. 1999; Hee et al. 2007). Orchid breeders usually have to grow the thousands of seedlings from each seedpod to maturity before flower quality can be evaluated (Sim et al. 2007). The effort, time, labor and capital investment in orchid breeding is tremendous and in most cases, only a few elite plants with desired characteristics are selected among the thousands of seedlings germinated from a seedpod.

In our laboratory, we have developed a method to induce *in vitro* flowering of some *Dendrobium* hybrids (Sim et al. 2007, 2008; Hee et al. 2007). This method greatly shortens the juvenile phase of orchids and, under optimal conditions, enables us to induce flowering and evaluate the flower quality about 5-6 months after seed germination. As a result, the duration required for breeding program could be shortened and tremendous cost could be saved. In addition, we demonstrated the success of *in vitro* pollination, seedpod formation, maturation and the production of future generations of seedlings *in vitro*. In this paper, we further report our experience with *in vitro* flowering of *Dendrobium* Chao Praya Smile as a model system and discuss the usefulness of *in vitro* flowering and *in vitro* pollination for the orchid industry.

Materials and Methods

Plant materials, culture media and culture conditions

Seeds from mature seedpods of *Dendrobium* Chao Praya Smile (*Dendrobium* Pinky × *Dendrobium* Kiyomi Beauty) were germinated aseptically in 90 mm Petri dishes with 25 ml of modified KC medium (Knudson 1946) supplemented with 2 % (w/v) sucrose, 15 % (v/v) coconut water and 0.3 % (w/v) Gelrite. The medium and subsequent media used were adjusted to pH 5.3 before autoclaving at 121 ˚C for 20 min.

Induction of in vitro flowering

The methods of Hee et al. (2007) and Sim et al. (2007, 2008) were followed. Briefly, eight-week-old protocorms were transferred to 50 ml of KH liquid culture medium (Hee et al. 2007) containing 2 % (w/v) sucrose, 15 % (v/v) coconut water and

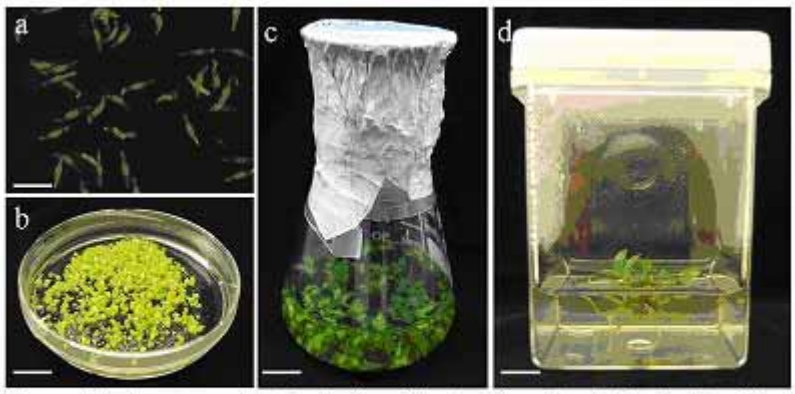

Figure 1 Various stages in the induction of *in vitro* flowering in *Dendrobium* Chao Praya smile. (a) Seeds after 4 days of germination. Bar = 0.5 mm. (b) Formation of protocorms after 8 weeks of germination. Bar = 1 cm. (c) Proliferation of protocorms in liquid medium. Bar = 1cm. (d) Transfer of morphologically normal seedlings to 2-layer medium. Bar = 1 cm.

with/without 11.1 µM BA in 100 ml Erlenmeyer flasks. The flasks were placed on rotary shakers at 120 rpm for proliferation. After 3 passages of sub-culturing in KH liquid medium at 3 weeks intervals, the seedlings were transferred to two-layer (Sim et al. 2007) KH medium in Magenta GA7^{TM} containers. The two-layer culture media consisted of 50 ml of Gelrite-solidified medium topped with a layer of liquid medium of same volume and composition (Sim et al. 2007). All cultures were incubated at 25 ± 2 ˚C and a 16-h photoperiod of 40 µmol m^{-2} s^{-1} from daylight florescent lamps.

In vitro pollination

To perform pollination, flowers produced in culture were self-pollinated in a laminar flow hood using a pair of forceps. Upon pollination, the plantlets were observed for seedpods formation. These seedpods were harvested upon maturation and cut open. Embryos from these *in vitro* seedpods were germinated on modified KC medium (Knudson 1946).

Results and discussion

Induction of in vitro flowering

As reported earlier (Hee et al. 2007), after culturing for a few days, the embryos enlarged and turned green in color (Fig. 1a) and subsequently developed into protocorms (Fig. 1b) . Eight-week-old protocorms proliferated in liquid medium. In this medium, protocorms developed into seedlings with tiny leaves (Fig. 1c). Morphologically normal seedlings were selected and were then transferred to the 2-layer medium containing 11.1 µM BA (Fig. 1d). About 4-8 weeks after transferring, single floral bud or inflorescence stalk could be observed developed from the shoot apex of the plantlets (Fig. 2a). About 70% of the selected protocorms were successfully induced to produce flowers *in vitro* (Fig. 2b). Thus, the approximate duration required from seed sowing to flowering was about 6 months.

In this method, BA is required for effective induction of *in vitro* flowering. No flowering was observed from plantlets cultured in medium without BA. In *Dendrobium* Madame Thong-In, as high as 94 % of seedlings cultured in 2-layered medium supplemented with 4.4 uM BA were observed to have inflorescent stalks after 15 weeks of culture (Sim et al. 2007). Cytokinins are important signals in flowering (Bernier et al. 1993, Bonhomme et al. 2000, Lindsay et al. 2006) and are commonly incorporated in the culture media for *in vitro* flowering (Bernier 1988; Peeters et al. 1991). Addition of the cytokinin iPAdos (isopentenyl adenosine) was found to be effective in inducing early bolting and flower bud formation *in vitro* in *Arabidopsis* (He and Loh 2002). Exactly how BA caused early flowering of *Dendrobium* orchids remains to be elucidated. However, using

Table 1 Characteristic of green-house grown and *in vitro Dendrobium* Chao Praya Smile at flowering.

Characteristics	Green-house grown plant	*in vitro* plantlets
Average height at bolting of one shoot (cm)	12.1 ± 0.8	2.9 ± 0.3
Average fresh weight of one shoot (g)	28.8 ± 1.3	0.67 ± 0.03
Duration of flowering period (day)	28 - 30	20 - 23
Size of flowers (diameter, cm)	4	2 - 2.5
Number of leaves at bolting	5 - 6	4 - 5
Range of leaf length (shortest, longest)	(7.8 ± 0.3, 12.5 ± 0.3)	(0.61 ± 0.02, 1.66 ± 0.06)
Average number of flower buds per plant	12	4

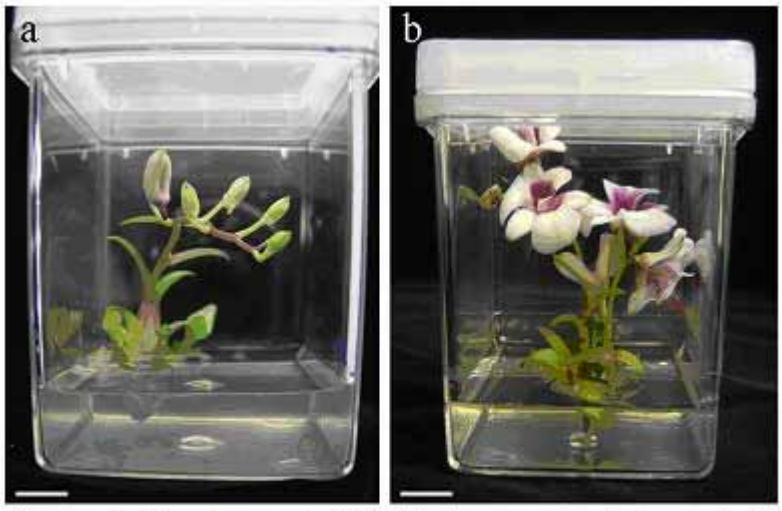

Figure 2 Development of (a) inflorescence stalk, and (b) flowers in *Dendrobium* Chao Praya Smile in culture. Bar = 1 cm and 1.2 cm, respectively, in (a) and (b).

D. Madame Thong-In as experimental materials, we found that as a result of BA application, endogenous IP and IPA increased to unsually high levels (Sim et al. 2008). Thus it was suggested that the increase of endogenous IP and IPA is associated with early *in vitro* flowering in *Dendrobium* (Sim et al. 2008). Experiments to investigate whether similar surge of IP and IPA occurs in *in vitro* flowering *Dendrobium* Chao Praya Simile cultures are in progress.

The 2-layered (liquid/gelrite solidified) medium apparently is important for successful induction of *in vitro* flowering. No flowers were observed from plantlets of *D.* Chao Praya Smile (Hee et al. 2007) and *D*. Madame Thong-in (Sim et al. 2007, 2008) cultured in medium solidified with gelrite alone. However, in *D.* Second Love, shoots could be induced to produce flowers *in vitro* after culturing in Phytagel solidified medium with 1.8 µM TDZ (Ferreira et al. 2006). The exact reason remains to be elucidated.

Figure 3 Comparison of *in vitro* flowering and flowering in green-house grown plant of *Dendrobium* Chao Praya Smile. Bar = 4 cm.

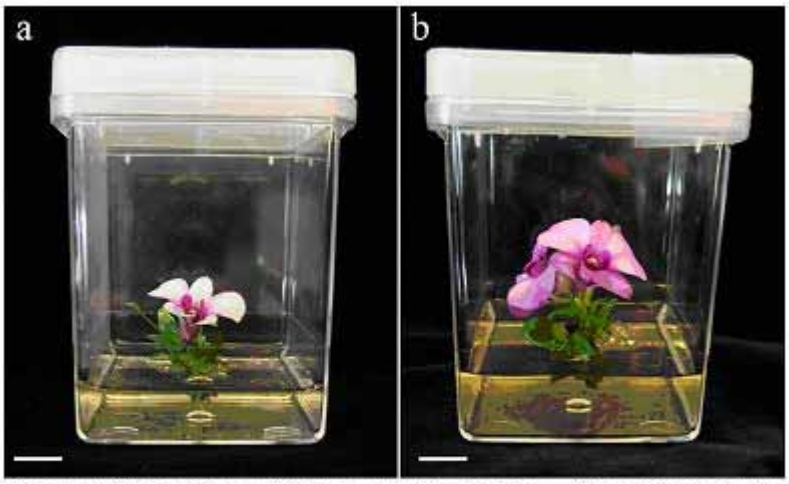

Figure 4 Production of flowers in *Dendrobium* Chao Praya Smile in culture. (a) and (b) are plantlets with one and two flowers, respectively. Bar = 1 cm.

Plantlets flowering in vitro

The *in vitro* flowering plantlets were about 2-3 cm height excluding the inflorescence whereas a normal flowering green-house grown plant measured about 11-13 cm height (Fig. 3, Table 1). Table 1 summarized some characteistics between *in vitro* flowering plantlets and green-house grown plants.

Each flowering plantlets produced one inflorescence stalk with an average of 3-4 flower buds. More than 70 % of the flowering plantlets could produce 2 flowers, while the remaining produced single flower (Fig. 4). The flowers produced by *in vitro* flowering of *D.* Chao Praya Smile (Fig. 5) and *D.* Madame Thong-In are smaller than the flowers produced by greenhouse-grown plants (Sim et al. 2007, Hee et al. 2007). Nevertheless, the shapes are normal and could be used for preliminary evaluation. In addition, experience with *D.* Madame Thong-In revealed segregation of colors in *in vitro*-produced flowers in the progeny of an orchid hybrid. There were at least 4 distinctive types of flowers obtained with respect to flower color (Sim et al. 2007). Thus selections of floral shapes and colors are achievable using such *in vitro* cultures.

In vitro pollination and seed production

Seedpods were formed upon self-pollination of flowers produced in culture. Fig. 6a shows the development of seedpod in culture. The whole duration for seedpod to reach maturation in culture ranged from 12 weeks to 14 weeks. Fig. 6b shows the seeds formed from these seedpods. These seeds were viable, germinated (Fig. 6c) and able to grow into seedlings. The success of *in vitro* pollination and viable seeds formation in *D.* Chao Praya Smile (Hee et al. 2007) and *D.* Madame Thong-In (Sim et al. 2007) demonstrate that the gametes produced from *in vitro* flowers were functional. Thus,

such a system could also be used for early determination of hybrid fertility.

Conclusion

Orchids are grown mainly for their exquisite flowers (Sim et al. 2007). Thus in any orchid breeding program, evaluation and selection of the flower quality such as flower shape and colour are important. In this paper, we have demonstrated the possibility of inducing *in vitro* flowering of *Dendrobium* must earlier than time required for conventional breeding method. Not only flowers could be induced early in culture when compared to conventional orchid growing methods, but seedpods with viable seeds could also be produced (Fig. 4, Hee et al. 2007; Sim et al. 2007). The whole breeding cycle, therefore, could be shortened. A comparison of the stages in both methods is explained in the diagrams below (Fig. 7).

Using these methods, the estimated duration from seed germination to *in vitro* embryos production has been shortened from 35 months to about 10 months (Hee et al. 2007). The method of *in vitro* embryo production would have produced 6 generations of embryos with the time that required for 2 generations in conventional orchid breeding (Hee et al. 2007).

Figure 5 Flowers of *Dendrobium* Chao Praya Smile developed *in vitro* and in green-house grown plant. Flowers a and b were developed *in vitro*. Flower c was obtained from green-house grown plant. Bar = 1cm.

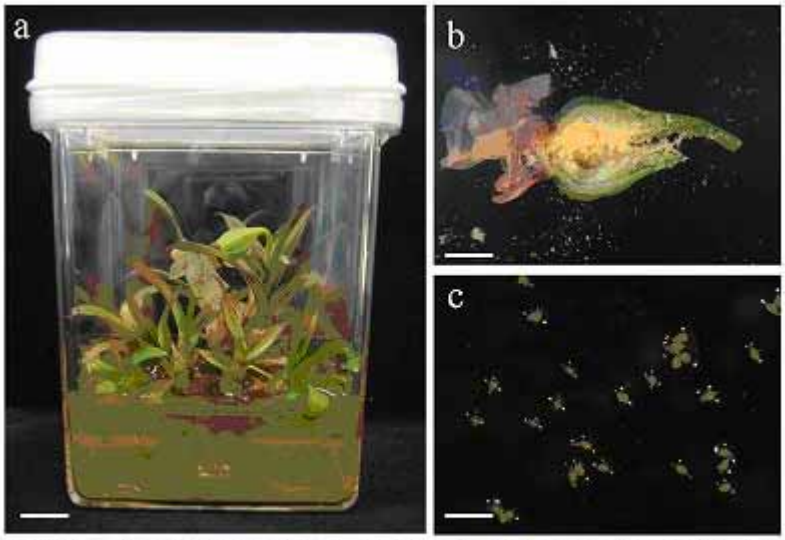

Figure 6 *In vitro* pollination and seed production in culture. (a) Development of seedpod in culture. Bar = 1cm. (b) Seeds from the seedpod developed in culture. Bar = 5mm. (c) Seeds after 1 week of germination. Bar = 1 mm.

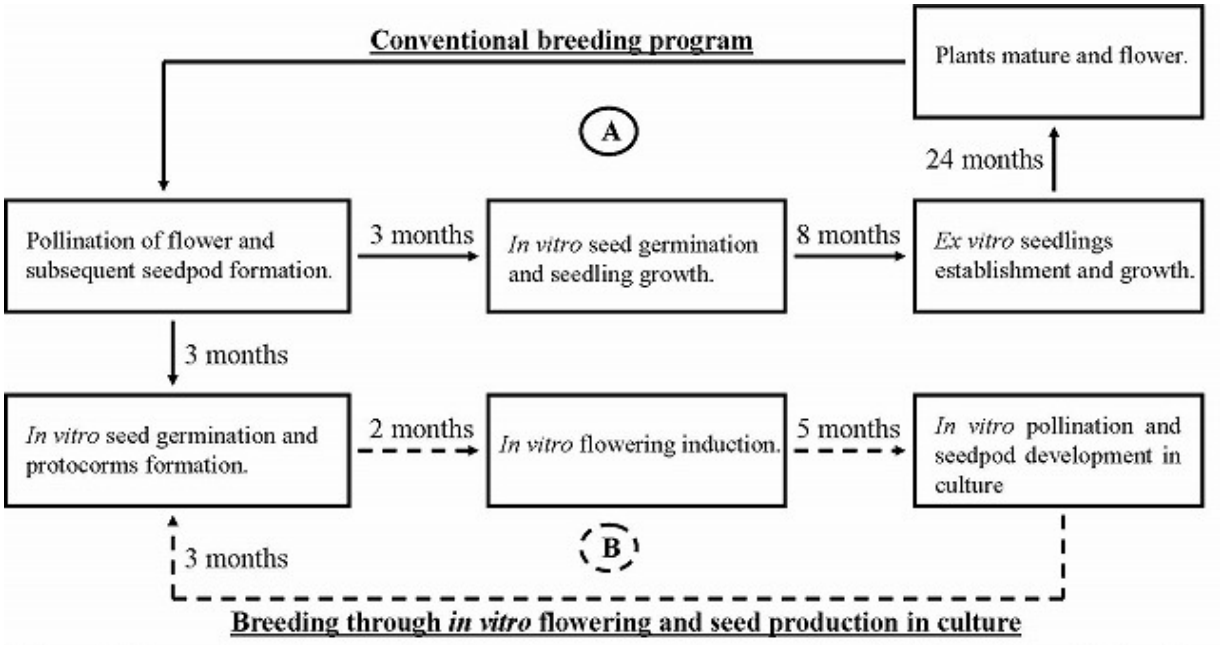

Figure 7 Comparison of conventional breeding program (A, approximately 35 months) with breeding through *in vitro* flowering and seed production in culture (B, approximately 10 months).

To conclude, the advantages of using *in vitro* flowering and *in vitro* pollination in orchid breeding could be summarized as:

1) Flower shape and color could be evaluated 5-6 months after seed germination instead of 2-3 years using conventional breeding methods.

2) The segregation of flower colors in hybrid plantlets *in vitro* can facilitate selection.

3) *In vitro* pollination allows early assessment of hybrid fertility.

Most important of all, there will be tremendous saving of time, effort, space, manpower and costs in orchid breeding by adopting such *in vitro* flowering and pollination methods in orchid breeding programs. These methods will undoubtedly contribute to and benefit the orchid industry as a whole.

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試験管内での開花と受粉：ラン産業に利益もたらす方法

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摘要

我々はデンドロビュウムの交配種で早期に試験管内で開花させる方法を開発した (Hee et al. 2007; Sim et al. 2007, 2008) 。 *Dendrobium* Chao Praya Smile を試験管内開花と受粉のモデル系として使った方法を紹介する。適当な BA 処理によって播種後５～６ヶ月で開花させることができる。これは通常の開花が２～３ヶ年必要なことと比べる、大変短期間である。したがって、花の特性検定が早期に可能である。続いて試験管内の受粉と発芽可能な種子形成が可能であった。その結果、育種年限が著しく短縮した。この方法のラン産業における有用性について考察する。