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High frequency early in vitro flowering of *Dendrobium* Madame Thong-In (Orchidaceae)

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Abstract We have successfully developed a method to induce early in vitro flowering of the self-pollinated seedlings of a tropical orchid hybrid, *Dendrobium* Madame Thong-In. Transition of vegetative shoot apical meristem to inflorescence meristem was observed when young protocorms were cultured in modified KC liquid medium. In contrast, protocorms cultured on Gelrite-solidified medium only produced axillary shoots and roots. CW was required to trigger the transitional shoot apical meristem and BA enhanced inflorescence stalk initiation and flower bud formation. However, normal flower development was deformed in liquid medium but developed fully upon transferring to two-layered (liquid over Gelrite-solidified) medium. Under optimal condition, in vitro flowering was observed about 5 months after seed sowing. Segregation of flower colours was observed in these seedlings and seedpods formed upon artificial pollination of the in vitro flowers.

Keywords *Dendrobium* Madame Thong-In · In vitro flowering · Seedlings · Tropical orchid

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Abbreviations AC: Activated charcoal · BA: 6-Benzyladenine · CW: Coconut water · KR: Gelrite-solidified medium · KRA: Gelrite-solidified medium with AC · K0: Basal liquid medium · K1: Basal liquid medium with 4.4 μM BA · K5: Basal liquid medium with 22.2 μM BA · K5RA: Gelrite-solidified medium with 22.2 μM BA and AC

Introduction

Orchids are normally grown for their exquisite flowers. In several tropical countries such as Thailand, Singapore and Malaysia, orchids (including *Dendrobium* hybrids) have become a major export cut flower crop. In many commercial *Dendrobium* hybrids, juvenile periods range between 2 and 4 years. Orchid breeders usually take a few years to grow the thousands of seedlings from each seedpod to maturity before flower quality can be evaluated. Therefore, the development of an early, in vitro flowering method will have significant impact on the orchid industry. Such a system allows earlier assessment of certain desired characteristics of the flowers such as size, shape, tones and variation of colours. Once the desired characteristics are selected, the clone could be mass propagated through tissue culture. In addition, such miniaturised orchid plantlets with flowers have potential commercial value as gifts or decoration. Furthermore, significant shortening of juvenile phase can provide a model system for studying flowering initiation and development.

Induction of precocious flowering of orchids in vitro had been shown in temperate orchids such as *Cymbidium ensifolium* (Wang et al. 1981, 1988; Wang 1988; Chang and Chang 2003), *Dendrobium candidum* (Wang et al. 1995, 1997) and a *Doriella* hybrid (Duan and Yazawa 1994). However, due to the nature of the materials (seeds and rhizome

for the species and floral stalk for the hybrid) used, the in vitro culture could not show segregation of the flower characteristics such as colour.

The present work aimed to shorten the juvenile period of a tropical orchid hybrid *Dendrobium* Madame Thong-In seedlings and induce early flowering in vitro. We have established a high frequency in vitro flowering system, using seeds from self-pollinated seedpods as starting materials. Effects of CW and BA on the induction of inflorescence meristems, and a two-layered (liquid over Gelrite-solidified) medium on flower development were also investigated for the hybrid under study. We demonstrated that segregation of the flower colour is expected to occur in the progeny. Hence, early assessment of the flower characteristics (colour, shape and size) is shown in our system.

Materials and methods

Plant material, culture media and culture conditions

Flowers of hybrid orchid *Dendrobium* Madame Thong-In were self-pollinated and the seedpods (3-month-old) obtained were surfaced sterilized by cleaning with 100% Clorox® (active ingredient 5.25% sodium hypochlorite), and then dipped into 95% (v/v) ethanol and burnt off. The seedpods were then dissected lengthwise and seeds were cultured in 9 cm petri dishes with 25 ml of modified KC medium (Knudson 1946) supplemented with 2% (w/v) sucrose, 15% (v/v) coconut water (CW, obtained from local green coconuts), with or without 0.03% (w/v) activated charcoal (AC, Sigma) and 0.3% (w/v) Gelrite (Kelco, San Diego, USA). Media were adjusted to pH 5.3 before autoclaving at 1 kg cm^{-2} (121°C) for 20 min. KC medium was used throughout the experiments as the basal medium.

Two-month-old green protocorms (2–3 mm height with two to three leaflets) were transferred to 40 ml basal liquid medium supplemented with 4.4 μM BA (K1) in 100 ml Erlenmeyer flasks on rotary shakers at 120 rpm for proliferation and multiplication. All cultures were incubated at 26 ± 2°C under a 16-h photoperiod of 35 μmol m^{-2} s^{-1} from daylight fluorescent lamps.

Experimental design and statistical analysis

There were two protocorms per flask and a total of 16–40 protocorms per treatment. Subculture was carried out every 3 weeks. For the studies on transitional shoot apical meristem, inflorescence meristem and flower bud development, protocorms were proliferated and maintained in K1. Based on their morphology, protocorms with inflorescence meristems were selected. They were classified into three stages: stage I, elongated protocorms (7–8 mm height); stage II, protocorms with visible inflorescence stalks; stage III, protocorms with flower buds. They were then cultured on a two-layered medium in Magenta GA7™ vessels (Magenta Corporation, Chicago). The two-layered medium consisted of 50 ml of 0.3% Gelrite-solidified basal medium (KR), or with addition of 0.03% activated charcoal (KRA = KR + 0.03% AC), overlaid with 20 ml of basal liquid medium supplemented with 22.2 μM BA (K5). Thus, the culture system was coded as K5/KR or K5/KRA accordingly (see below).

Media	Liquid layer		Gelrite-solidified layer		
	BA (μM)	CW (%)	BA (μM)	CW (%)	AC (%)
N0/NRA	0	0	0	0	0.03
N5/NRA	22.2	0	0	0	0.03
N5/N5RA	22.2	0	22.2	0	0.03
K0/KRA	0	15	0	15	0.03
K5/KRA	22.2	15	0	15	0.03
K5/K5RA	22.2	15	22.2	15	0.03

The protocorms were inserted into the Gelrite-solidified layer. There were two explants per GA7™ vessel. All the cultures were scored once every 3 weeks. The percentage of inflorescence with normal flowers was calculated as number of inflorescence stalks with at least one normal flower produced over the total number of inflorescence stalks with flowers (normal, near-normal and abnormal flowers) multiplied by 100. Abnormal flowers were defined as those with perianth that could not be differentiated into petals and sepals, and no reproductive organs were observed. Whereas near-normal flowers were those with extra or lesser number of floral parts and sometimes with multiple sets of pollinia.

Statistical analysis of the data was done by using the chi-square (χ^2) 2×2 contingency test (Zar 1984) to compare response to treatment with control, Yate's correction was done as degree of freedom is 1 (df = 1). The χ^2 test for k independent samples or groups was also used to assess the significance of differences among k independent groups (Siegel and Morgan 1996). To analyse the number of axillary shoots, root formation per explant or per protocorm and number of flower buds produced per inflorescence, one-way ANOVA and Tukey test were carried out (Zar 1984) using the MINITAB software.

Results

Effects of BA on the induction of inflorescence meristems

The seeds turned green within 2 weeks and grew into protocorms after 2 months of incubation on both KR and KRA Gelrite-solidified media (Fig. 1a). Young protocorms (2–3 mm height) were transferred to KRA (as control) and K5RA (KRA containing 22.2 μM BA) in GA7 vessels.

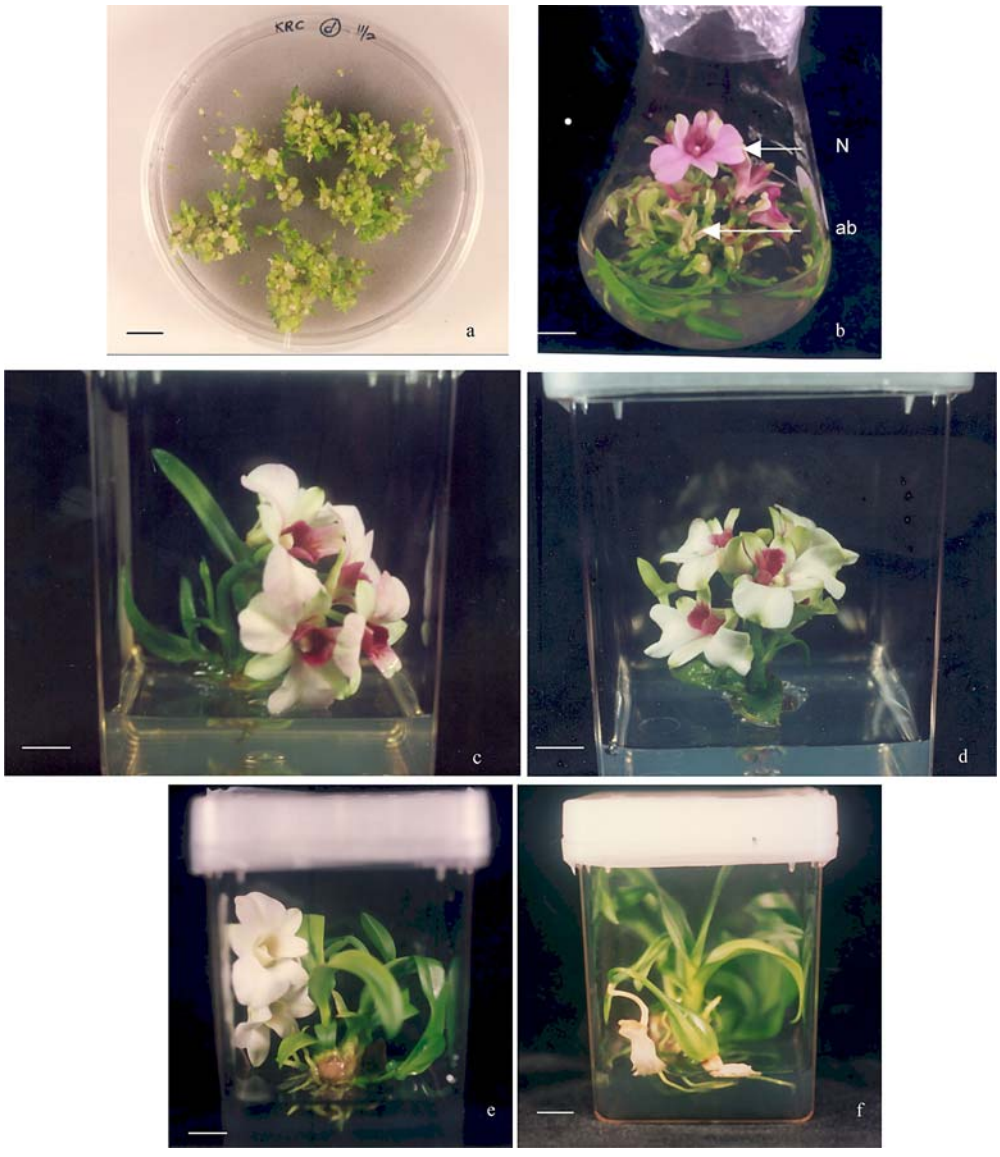

Fig. 1 In vitro flowering of *Dendrobium* Madame Thong-In. **a** Protocorms, **b** shoots with normal (N) and abnormal flowers (ab) in liquid medium, **c** flowers bloomed after 34 days, **d** shoot showing an inflorescence stalk with 6 flowers, **e** two white flowers before pollination, **f** one seedpod set after cross-pollinated. Bar: 1 cm

Protocorms rooted (90–100%) and produced axillary shoots (15–20%) within 3 weeks of culture. By the 18th week, all of them produced axillary shoots. These axillary shoots grew vigorously and some were taller than the primary shoots, but neither transitional shoot apical meristem nor inflorescence stalk was observed even after 5 months in culture.

Young protocorms from KRA were also cultured in liquid media, K0 (BA-free) and K1. Similarly, roots and axillary shoots were produced within 3 weeks. However, both root (78%) and shoot (100%) production were significantly higher for protocorms cultured in BA-enriched medium compared to that in hormone-free medium (41% for both root and shoot production) after 9 weeks of culture. However, on the 15th week, there were no significant differences between cultures in K1 and K0 with respect to shoot and root production, but the mean number of axillary shoots produced per protocorm was still significantly higher for cultures in K1, at $\alpha = 0.05$ (Table 1). Interestingly, transitional shoot apical meristems were induced in protocorms incubated in K1 and K0 media after 6 and 9 weeks, respectively. They eventually elongated and developed into inflorescence stalks. Only 18% of the protocorms produced inflorescence stalks in K0 compared to 83% in K1 after 12 weeks (Table 1). Inflorescence stalks were also observed in some of the induced axillary shoots. Flower buds were observed on the ninth week for cultures in K1, but none were induced in K0 even after 15 weeks of culture. Most of the flower buds in K1 were abnormal. Only those inflorescence stalks that grew out of the liquid surface produced normal flower buds, which eventually developed into normal flowers (Fig. 1b). Such flowers were purple, white or a combination of both colours. These flowering plantlets appeared to be complete miniature orchid plants with roots and leaves in flask. The size of the flowers

Table 1 Effects of BA on the young protocorms (germinated on KRA medium) of *D.* Madame Thong-In after 12 and 15 weeks

BA (μM)	No. of protocorms	% culture with inflorescence stalk[a]	% culture with flower bud[a]	% culture with axillary shoot[a]
12 weeks				
0	(i) 22	18(1.0) a	0	86(1.9 ± 0.2) a
4.4	(i) 18	83***(1.2 ± 0.1) a	60***(4.2 ± 0.8) a	100(3.5 ± 0.3) b
	(ii) 20	85***(1.8 ± 0.3) a	82***(6.3 ± 1.2) a	100(3.4 ± 0.2) b
15 weeks				
0	(i) 22	50(1.0) a	0	86(2.8 ± 0.2) a
4.4	(i) 18	94***(2.4 ± 0.3) b	82***(5.2 ± 1.1) a	100(5.6 ± 0.5) b
	(ii) 18	94***(2.4 ± 0.3) b	94***(8.3 ± 1.5) a	100(5.3 ± 0.5) b

(i) Rootless young protocorms. (ii) Young protocorms with tiny roots.

[a]Results of chi-square 2×2 contingency tests for comparison of the number of protocorms responding in medium with the control: $^{***}p < 0.001$. Mean number of inflorescence stalks and axillary shoots per protocorm or flower buds per inflorescence stalk ± SE in parentheses. Values followed by the same letter in the same column and same heading are not significantly different according to Tukey test at $\alpha = 0.05$ carried out on the number of inflorescence stalks or axillary shoots per protocorm, or flower buds per inflorescence as the variable.

was proportionally smaller compared to the mother plants grown in vivo.

The presence of tiny roots in the young protocorms did not affect their response towards BA-enriched medium when compared to rootless young protocorms in terms of axillary shoot formation, root production, percentage of cultures with inflorescence stalks and flower buds (Table 1).

Effects of CW

Young protocorms (about 2 mm height) with two tiny leaflets from KR medium were transferred to 40 ml of basal medium containing 0, 5, 10, 15, 20 and 30% of CW to investigate the role of CW in the transitional shoot apical meristems.

During the first 3 weeks of culture, the increase in height and number of leaves were rather slow. Protocorms in CW-free medium hardly grew and turned brown easily compared to those in media supplemented with CW (5–30%). Roots and axillary shoots were produced within 4 weeks of culture. On the 15th week, 38% of the protocorms were with roots and axillary shoots in the control, i.e. medium without CW. More than 83% of the protocorms in CW-enriched media produced roots and axillary shoots.

Inflorescence stalk was induced after 9 weeks in CW-enriched medium. Although 5% CW was enough to trigger this initiation, inflorescence stalks induced in low concentration of CW (5 and 10%) did not grow very well and disintegrated after some time. Percentage of protocorms with inflorescence stalks increased as CW concentrations increased, with the optimum (65%) at 20% CW. However, no flower buds were observed even after 15 weeks of culture in these inflorescence stalks.

The presence of CW was effective in inducing inflorescence stalk, roots and axillary shoots compared to the control. In general, CW promoted vegetative growth and to a lesser degree induced inflorescence meristem, but did not support the formation of flower buds. However, when these protocorms with inflorescence stalks were transferred to the two-layered flowering medium (K5/KR), normal flower buds were initiated within 3 weeks, and they developed into normal flowers within 6 weeks of culture.

Response of induced protocorm towards the two-layered culture medium

Different stages of the protocorms (shoots) were transferred from K1 liquid medium to two-layered culture media, such as N0/NRA, N5/NRA, N5/N5RA, K0/KRA, K5/KRA and K5/K5RA. Protocorms with visible inflorescence induced in K0 liquid medium were also transferred to media K0/KRA, K5/KRA, K5/KR and K0/K5R to compare with those protocorms maintained in K1.

Induction of inflorescence stalks from stage I protocorms

Inflorescence stalks became visible after 3 weeks of culture upon transferred to two-layered medium. By the ninth week, about 40–55% of the protocorms produced inflorescence stalks. Flower buds were observed in cultures on K5/KRA and K5/K5RA. The flowers bloomed after 6 weeks of incubation and they were normal. Usually, there were only 1 or 2 flowers per inflorescence.

Roots were also observed in 3-week-old cultures. In the absence of CW, BA (N5/NRA), significantly inhibited the mean number of roots produced in the cultures. The presence of CW in the two-layered medium (K0/KRA) increased the mean markedly to 11.3 ± 0.9 roots per protocorm. Addition of BA and CW to the two-layered culture media further increased the mean number of roots to 15.6, for protocorms in K5/KRA.

Axillary shoots were observed within 2 weeks. On average there were 2–3 shoots per protocorm in the media supplemented with BA and/or CW compared to the control (N0/NRA), which had 1.4 ± 0.2 shoots each. The presence of BA in both layers (K5/K5RA) significantly increased the mean number of axillary shoot production to 2.9 ± 0.4.

Induction of flower buds from stage II protocorms

The inflorescence stalks produced flower buds at high frequency when protocorms with visible inflorescence stalks induced in K1 liquid medium were transferred to the two-layered medium. It was possible that flower bud meristems were initiated in K1 before transferring or treatments. As high as 75% of the inflorescence stalks formed flower buds in N0/NRA (CW-free) after 3 weeks, but declined to 40% in the ninth week as the abnormal flower buds aborted. When cultured on media supplemented with BA (N5/NRA and N5/N5RA), 75–80% of the shoots produced an average of two flower buds per inflorescence. On K0/KRA (CW-enriched medium), 65% of the inflorescence stalks produced flower buds. About 70% of the flower buds bloomed by the sixth week and 90% by the ninth week of culture. Normal, near-normal and abnormal flowers could be borne on the same inflorescence.

The presence of CW and BA in the two-layered media increased flower bud formation significantly. Shoots in media K5/KRA and K5/K5RA also produced higher number of normal flowers, i.e. 75% and 68% respectively. As many as five normal flowers were obtained in K5/KRA. The highest mean number of flower buds (3.4 ± 0.4) was obtained in K5/K5RA. The majority of these plants were complete plantlets as 85–95% of the cultures produced roots, with slightly lower of 70% for cultures in N5/NRA. Also, 88–100% of the protocorms produced axillary shoots, with an average of three each after 9 weeks.

When the stage II protocorms were subjected to different concentrations of BA in the upper liquid layer (K1 = 4.4 μM, K2 = 8.9 μM, K5 = 22.2 μM BA) overlaid on KR Gelrite-solidified layer, BA increased flower bud production to 87% in K1/KR after 9 weeks compared to 38% in K0/KR (control, without BA). With higher concentration of 22.2 μM BA in the liquid layer (K5/KR), 90% produced flower buds with an average of 5.7 flower buds per inflorescence. When AC was added to the Gelrite-solidified layer, 56–67% of the cultures produced flower buds in K0/KRA, K1/KRA and K2/KRA. With high BA of 22.2 μM in the liquid layer, all the inflorescence stalks produced flower buds with an average of 3 each.

Shoots with visible inflorescence stalks induced in K0 liquid medium were also treated with two-layered medium such as, K0/KRA (as control), K5/KRA, K5/KR and K0/K5R. After 9 weeks, 69–75% of the cultures in BA-enriched media produced an average of two flower buds each but none was observed in the control (Table 2).

No flower buds were observed in the control (K0/KRA) where BA was not incorporated into the two-layered medium even after 9 weeks of culture (Table 2). More than 68% of the cultures in BA-enriched media produced flower buds within 3 weeks of culture. On average there were two flower buds per inflorescence by the ninth week (Table 2). About half of the flowers bloomed by the sixth week, but, the majority of flowers were only near-normal or abnormal.

Flower development in stage III protocorms

Flowers bloomed within 3 weeks, upon transferring these stage III protocorms to two-layered media. On H_2O/KRA, cultures with flower buds were reduced to 39% after 9 weeks, as flower buds aborted. However, when cultured on the same medium without AC (H_2O/KR), all of the inflores-

Table 2 Effects of BA on the flower development of *D.* Madame Thong-In stage II protocorms (induced in K0 medium) after 9 weeks of culture

Media	No. of protocorms	% culture with flower bud[a,b]	% inflorescence with		
			Normal flowers[a,c]	Near-normal flowers[a,c]	Abnormal flowers[a,c]
K0/KRA	18	0	0	0	0
K5/KRA	14	71***(2.0 ± 0.3) a	20	30	70***
K5/KR	16	69***(2.2 ± 0.3) a	0	45**	45**
K0/K5R	24	75***(2.3 ± 0.3) a	6	39*	67***

[a]Results of chi-square 2 × 2 contingency tests for comparison of the number of protocorms responding in medium with the control: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

[b]Mean number of flower bud per inflorescence ± SE in parenthesis. Values followed by the same letter in the same column are not significantly different according to Tukey test at $\alpha = 0.05$ carried out on number of flower buds per inflorescence as the variable.

[c]Percentage of inflorescence with normal flowers was taken as number of inflorescence stalks with at least one normal flower produced over the total number of inflorescence stalks with flowers (normal, near-normal and abnormal flowers) × 100. The same formula is also applied to % inflorescence with near-normal and abnormal flowers.

Table 3 Development of flowers from *D*. Madame Thong-In stage III protocorms after 9 weeks of culture

Media	No. of protocorms	% culture with flower bud[a,b]	% inflorescence with		
			Normal flowers[a,c]	Near-normal flowers[a,c]	Abnormal flowers[a,c]
H_2O/KRA	13	39(2.2 ± 0.5) a	40	80	80
H_2O/KR	10	100**(5.2 ± 1.0) a	50	40	80
K5/KRA	20	100***(4.5 ± 0.5) a	80	45	25
+K5/KRA	20	100***(6.8 ± 1.2) a	35	50	90

[a]Results of chi-square 2 × 2 contingency tests for comparison of the number of protocorms responding in medium with the control, H_2O/KRA: $^{**}p < 0.01$; $^{***}p < 0.001$.

[b]Mean number of flower buds per inflorescence ± SE in parenthesis. Values followed by the same letter in the same column are not significantly different according to Tukey test at $\alpha = 0.05$ carried out on number of flower buds per inflorescence as the variable.

[c]Percentage of inflorescence with normal flowers was taken as number of inflorescence stalks with at least one normal flower produced over the total number of inflorescence stalks with flowers (normal, near-normal and abnormal flowers) × 100. The same formula is also applied to % inflorescence with near-normal and abnormal flowers.

+ Initial protocorms with more than three flower buds.

cence stalks still bore the flower buds, with an average of 5.2 buds (Table 3). Half of these developed into normal flowers. When cultured on K5/KRA, more new flower buds developed, a mean number of 4.5 buds each were produced and 80% of the inflorescences developed normal flowers (Fig. 1c and d). As many as 11 white flowers were recorded with nine normal and two near-normal flowers in one inflorescence.

Volume of liquid medium in two-layered medium affecting flower bud formation and flower development in stage II protocorms

Since the liquid medium was important in two-layered method for flowering, the volume of the liquid medium could be crucial for flower development. Protocorms with visible inflorescence stalks in K1 were selected and cultured in K5/KRA for further development.

Flower buds were observed as early as 3 weeks of culture in all the treatments. However, abortion of flower buds occurred, especially in cultures with 0–15 ml of K5 in the liquid layer. Shoots or plantlets with flower buds increased as the volume of the liquid medium increased and reached its maximum at 20 ml (Table 4). High frequency (55–100%) of flower buds was developed in shoots cultured in two-layered media compared to 28% in the Gelrite-solidified medium only.

Flowers could bloom in 3 weeks of culture but these were usually the abnormal flowers with undifferentiated perianths. More than 60% of the flowers bloomed by the sixth week and 90% by the ninth week. The abnormal flowers usually wilted within 1–3 weeks, whereas the normal and near-normal flowers could last 3–4 weeks. In the control (KRA), only 10% of the inflorescences produced normal flowers. The highest percentage of normal flowers produced was with 20 ml of K5 (75%), followed by 43% in 30 ml of K5 (Table 4). However, if cultured on just K5RA (Gelrite-solidified only), 46% of the inflorescence stalks produced flower buds and only 36% of these inflorescence produced normal flowers.

Table 4 Volume of K5 on KRA in two-layered medium affecting the flower bud induction and flower development of *D*. Madame Thong-In after 9 weeks of culture

K5 (ml)	No. of protocorms	% culture with flower bud[a,b]	% culture with normal flower[a]	% culture with root[a,b]	% culture with axillary shoot[a,b]
0	36	28(1.8 ± 0.3) a	10	100(10.7 ± 1.0) ab	97(2.5 ± 0.2) a
5	44	55*(2.5 ± 0.4) a	21	95(13.8 ± 1.1) a	91(2.7 ± 0.2) a
10	40	58*(1.7 ± 0.2) a	26	93(11.3 ± 0.8) ab	90(2.53 ± 0.1) a
15	40	58*(2.0 ± 0.2) a	17	93(11.6 ± 1.0) ab	93(2.9 ± 0.2) a
20	20	100***(2.9 ± 0.2) a	75**	90(6.8 ± 0.9) b	90(2.9 ± 0.3) ab
30	40	88***(2.7 ± 0.3) a	43	90(9.8 ± 1.2) ab	98(3.9 ± 0.3) b

[a]Results of chi-square 2 × 2 contingency tests for comparison of the number of protocorms responding in medium with the control: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

[b]Mean number of roots or axillary shoots per protocorm, or flower buds per inflorescence ± SE in parentheses. Values followed by the same letter in the same column are not significantly different according to Tukey test at $\alpha = 0.05$ carried out on number of roots or axillary shoots per protocorm, or flower buds per inflorescence as the variable.

Table 5 Effects of BA and AC on the flower development of *D*. Madame Thong-In protocorms after 9 weeks of culture

Abbreviation of media	No. of protocorms	% culture with flower bud[a,b]	% inflorescence with normal flowers[a,c]	% inflorescence with near-normal flowers[a,c]	% inflorescence with abnormal flowers[a,c]
K0/KR	24	38(2.1 ± 0.4) a	11	22	89
K1/KR	38	87***(4.5 ± 0.6) ab	42	39	70
K5/KR	40	90***(5.7 ± 0.5) b	50	58	42*
K0/KRA	17	65(1.9 ± 0.3) a	27	9	64
K1/KRA	34	56(3.3 ± 0.5) a	53	32	74
K2/KRA	21	67(2.8 ± 0.5) a	21	14	79
K5/KRA	20	100***(2.9 ± 0.2) a	75***	45	30***

[a]Results of chi-square 2 × 2 contingency tests for comparison of the number of protocorms responding in medium with the control, K0/KR: $^{*}p < 0.05$; $^{***}p < 0.001$.

[b]Mean number of flower bud per inflorescence ± SE in parenthesis. Values followed by the same letter in the same column are not significantly different according to Tukey test at $\alpha = 0.05$ carried out on number of flower buds per inflorescence as the variable.

[c]Percentage of inflorescence with normal flowers was taken as number of inflorescence stalks with at least one normal flower produced over the total number of inflorescence stalks with flowers (normal, near-normal and abnormal flowers) × 100.

Effects of BA in the liquid layer and AC in the Gelrite-solidified layer on flower bud production

Shoots with visible inflorescence stalks were subcultured with different concentrations of BA (K1 = 4.4 μM, K2 = 8.9 μM, K5 = 22.2 μM BA) over KR layer (Table 5). The presence of BA increased the percentage of inflorescence stalks with flower buds from 38% (K0/KR) to 87% in K1/KR (Table 5). With higher concentration of BA in the liquid layer, K5/KR, 90% produced flower buds and on average there were 5.7 ± 0.5 flower buds per inflorescence. Only 11% of the flowers produced were normal in the case of K0/KR, but the frequency of normal flowers increased as BA concentration increased. Abnormal flowers were significantly lesser in K5/KR.

When AC was added to the Gelrite-solidified layer, the frequency of flower buds was 65% in the K0/KRA (Table 5). The frequency did not increase when BA concentration was lower than 22.2 μM (K5). With 22.2 μM BA in the liquid layer, 100% of the inflorescence stalks produced flower buds. When activated charcoal was added to the Gelrite-solidified layer the mean number of flower buds was lower than that in K5/KR. But the percentage of normal flowers per inflorescence was higher, i.e. 75%, and only 30% produced abnormal flowers (Table 5).

In vitro pollination

Twenty in vitro flowers were self- or cross-pollinated artificially using pollinia from the in vitro flowers, but in vitro pollination was not successful. Although some of the ovaries became swollen, the flowers wilted and dropped. When dissected, no seeds were observed in the swollen ovary.

Since some of the ovaries became swollen after pollination, it was speculated that the pollinia obtained in the in vitro flowers were either non-fertile or not mature. Anthers from in vivo grown mother plants were harvested, surface-sterilized and the pollinia from such flowers were used to pollinate the in vitro flowers (Fig. 1e). The ovaries became swollen and grew into seedpods (Fig. 1f). This showed that the ovaries of the in vitro flowers were functioning but the anthers might not be so. After 3 months, the seedpods were dissected and seeds that appeared whitish in colour, were germinated on KRA medium. The density of the seed was low compared to the seedpods pollinated in vivo.

Most of the seeds appeared to be non-fertile (about 0.1% of the seeds were fertile) and only a few seeds germinated. Green protocorms were observed within 3 weeks and they eventually grew into plantlets in KR or KRA medium. Young protocorms (2-month-old) transferred to K1 liquid medium for 6 weeks flowered upon transfer to two-layered medium.

Comparison of the characteristics of parental plants (in vivo) and in vitro seedlings at flowering

The average vegetative growth period of *D*. Madame Thong-In under normal cultivation was about 30 months (Table 6). The pseudobulbs were about 20–30 cm in height and inflorescence length could be as long as 32 cm with an average of 13 flowers per inflorescence stalk. As many as 18 flowers were recorded per inflorescence in some cases. Flowers are of light purple petals and light green sepals with diameter of 3–3.5 cm. Flowers on inflorescence could last for a month.

In the present study, the process of seed germination to in vitro flowering took about 5–6 months depending on the stage of protocorms used. The juvenile period was shortened to 1/5 of the normal in vivo vegetative growth period. The diameter of the flowers was 2–2.5 cm (Fig. 2a). Since these seedlings were from self-pollinated seedpod, segregation of the colours was observed. There were at least four distinctive types of flowers obtained, in terms of colour

Table 6 Comparison of the characteristics of parental plants in vivo and in vitro seedlings at flowering

Characteristics	Parental plants (in vivo)	Seedlings at flowering (in vitro)
Duration of vegetative growth before flowering (months)	30	5
Height of pseudobulbs (cm)	20–30	1–1.5
Number of leaves per shoot	11–13	5–9
Leaf length (cm)	5–13	0.5–1.5
Inflorescence length (cm)	25–32	1.5–3.0
Average number of flowers per inflorescence	13	4
Flower diameter (cm)	3.0–3.5	2.0–2.5
Longevity of open flower (days)	30	21–28

Fig. 2 **a** Comparing the in vitro flowering seedlings with the mother plant. **b** Seedlings from the same seedpod with flowers. Bar: 2 cm

(Fig. 2b). Therefore, early assessment of flower colours is possible within 6 months in vitro.

Discussion

Flowering is a unique developmental event in plants which involves the transition of vegetative shoot apex to form either an inflorescence or a floral meristem, and followed by initiation and subsequent maturation of the floral organs. In *Dendrobium* Madame Thong-In, CW was necessary for the growth of protocorms and transition of vegetative shoot apical meristem to inflorescence meristem. This was demonstrated in the CW-enriched liquid medium (K0, Table 1) and 5% CW was enough to trigger this transition mechanism. CW contains sugars, vitamins, amino acids and plant growth regulators (Tulecke et al. 1961; Raghavan 1966). This study revealed that CW is important for inflorescence stalk induction in *D*. Madame Thong-In. However, similar to *Doriella* Tiny (Duan and Yazawa 1994), CW did not induce the formation of flower buds in *D*. Madame Thong-In (Table 1). This showed the complexity of flowering process as CW alone is not enough for successful flowering.

Cytokinins are important signals in flowering (Bernier et al. 1993; Lejeunne et al. 1994; Bonhomme et al. 2000). Cytokinin is also a common requirement for in vitro flowering (Nitsch and Nitsch 1967; 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). When early flowering was induced (by triacontanol, lanthanum and cerium) in cytokinin-free medium, a significant increase in endogenous concentration of the IP subfamily in the root and leaf tissues was observed (He and Loh 2002). However, incorporation of cytokinins in the medium was found to depress flowering in vitro in rapid-cycling *Brassica napus* (Koh and Loh 2000). In this study, BA alone in the medium was not effective for floral induction. However, in the presence of CW, BA enhanced earlier formation of inflorescence stalks and promoted flower bud induction but normal flower development did not occur in the liquid medium, as the flower buds obtained were abnormal. A similar effect was observed in *Lemna gibba* G3, where BA complemented the effect of ethylenediamine-di-*o*-hydroxyphenylacetic acid (or salicylic acid) on flowering (Pieterse and Müller 1977). Nadgauda et al. (1990) suggested that cytokinins might be involved in flowering perhaps with CW supplying the inositol and cytokinin oxidase inhibitors, which promote cytokinin responses. The role of cytokinins in floral evocation might be in controlling early mitotic activity, precocious initiation of axillary meristems, and increased rate of appendage production by the meristems (Scorza and Janick 1980). Also, cytokinin was cited as a probable component of a multi-factored flowering stimulus (Bernier 1988).

Liquid medium was superior for initiation inflorescence stalks in *D*. Madame Thong-In as compared to agar media used for flower initiation in tissue cultures of other monocot plants such as date palm (*Phoenix dactylifera*) seedling (Ammar et al. 1987) and *Dendrocalamus hamiltonii* (Chambers et al. 1991). In *D*. Madame Thong-In, protocorms incubated in KC Gelrite-solidified medium supplemented with either CW (KRA) or both CW and BA of 22.2 μM (K5RA) did not produce inflorescence stalk even after 18 weeks in culture. However, with the same nutrient composition, i.e. CW (15%) in the KC liquid medium (K0), inflorescence stalks were observed within 9 weeks, though no flower buds were initiated after 15 weeks of culture (Table 1). These data showed that besides the composition of nutrients and culture conditions, physical state of the medium also played an important role for inflorescence stalk induction or transition of vegetative meristems to inflorescence meristems. A similar situation was demonstrated in pepper where flowers were only initiated in liquid medium and not in agar medium (Tisserat and Galletta 1995). Tisserat and Galletta (1995) attributed the factors responsible for flowering in pepper to the physical culture environment and not the nutrient medium because shoot tips were grown on the same MS medium without growth regulators for the culture systems used. Kerbauy (1984) also indicated that in *Oncidium varicosum*, medium composition and environmental conditions seemed to accelerate the growth rate of the pseudobulbs developed and thus shortened the vegetative period. Effect of subculture time and age of the original culture also affected in vitro flower induction in roses (Wang et al. 2002). In *Dendrobium* hybrids, effectiveness of nutrient uptake by plants in Gelrite-solidified media and impurities from gelling agent may affect flower initiation in Gelrite-solidified media compared to liquid medium (Table 4). However, liquid layer alone did not provide the necessary support and did not encourage normal flower development as orchid is not an aquatic plant. Apparently, the two-layered medium strategy adopted here provides the better alternative for its growth and particularly, flower development.

The two-layered medium strategy allowed normal development of flower buds to maturity. Addition of BA in the upper liquid layer enhanced flower bud formation (Table 2) and normal development of flowers. Addition of AC in the Gelrite-solidified layer reduced the effective BA concentration for flowering as AC is known to adsorb many types of molecules, including growth substances added to the culture medium (Weatherhead et al. 1978). In general, stage I protocorms were less responsive to two-layered medium in terms of flower bud formation as compared to stage II protocorms. So far, there was no report on in vitro flowering study using two-layered medium. But, the two-layered

method had been used with success in protoplast cultures of *Petunia* (Power et al. 1976), *Linum usitatissimum* (Barakat and Cocking 1983), *Lithospermum erythrorhizon* (Maeda et al. 1983) and *Citrus mitis* (Sim et al. 1988). In *Citrus mitis*, it was found that two-layered method enhanced somatic embryo formation as compared to Gelrite-solidified and liquid media (Sim et al. 1988). Flower morphogenesis is known to be regulated by hormonal, nutritional and environmental factors. Apparently, in *D*. Madame Thong-In, besides the nutrients and plant growth regulator(s) in the culture medium, physical state of the medium i.e. liquid or Gelrite-solidified medium, and probably air exchanges (liquid medium) also played an important role in in vitro flowering. Experiments on volume of the upper liquid in the two-layered medium (Table 4) further demonstrated the importance of this strategy for successful flower bud induction and flower development. In the two-layered medium, 20 ml liquid K5 medium was optimal for flower bud formation. This implied that the level of liquid also affects the response of the shoot.

In *D*. Madame Thong-In, seeds were used as the starting material to initiate cultures. The process of seed germination to flowering took about 5–6 months depending on the stage of protocorms used. In the field, seed germination to flowering takes about 30 months for this hybrid (Table 6), thus the juvenile period was shortened to 1/5 or less after the treatment in tissue culture system.

This study is the first report on segregation of flower colours in in vitro flowering of orchids (Fig. 2b) in the progeny of an orchid hybrid. Some plants produced only white flowers (Fig. 1e) or pink flowers (Fig. 1b), while others produced white flowers with red tint in the lower half of the labellum or a combination of pale green and different shades of pink on petals and sepals (Fig. 1c and d). There were at least four distinctive types of flowers obtained with respect to flower colour. Therefore, early assessment of flower colours is possible within 6 months using our in vitro flower induction method. Hence, this shortens the time required for normal evaluation (at least 2 years), reduces the labour costs and optimizes the space required for normal orchid breeding. This system will be highly beneficial for orchid breeders and helpful for the multi-million dollar orchid cut flower industry in South East Asia.

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