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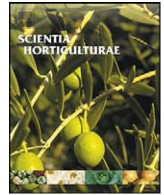

Short communication

High frequency early flowering from *in vitro* seedlings of *Dendrobium nobile*

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ABSTRACT

Dendrobium nobile Lindl. is a popular temperate Chinese orchid commonly marketed as a traditional medicinal plant. Seedlings of *Dendrobium nobile* Lindl. produced floral buds (33.3–34.8%) precociously on a defined basal medium (1/2 MS) containing paclobutrazol (PP_{333}) at 0.5 mg L^{-1} or thidiazuron (TDZ) at 0.1 mg L^{-1} within 4 months of culturing. The frequency of floral buds formation can be further increased to 95.6% by growing seedlings in a PN (PP_{333} 0.3 mg L^{-1} + NAA 0.5 mg L^{-1})-containing medium followed by transfer onto 1/2 MS medium with PP_{333} and TDZ (PP_{333} + TDZ). However, flower developed was deformed under 25 °C but it developed fully when grown in a lower temperature regime (23 °C/18 °C, light/dark) for 45 days. Under optimal condition, *in vitro* flowering was observed about 6 months after seed sowing.

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1. Introduction

Dendrobium is one of the largest genus in the family Orchidaceae (Bechtel et al., 1981). In China, there are 74 species (Tsi, 1999). The stems of some *Dendrobium* e.g. *D. nobile* species, are used as a tonic to improve digestion and for promoting the production of body fluid, nourishing 'yin' and eliminating 'evil-heat' (Yen, 1980; Anon., 1999). *D. nobile* is a rare and endangered perennial orchid endemic to China, mainly distributed in the mountain ranges of Southern China, such as Yun-nan, Gui-zhou, Si-chuan, Hai-nan, Guangxi, Hu-bei, Xi-zang provinces and Taiwan region. Flowering usually occurs between April and May (Chen and Ji, 1998). However, the juvenile period of this orchid is at least 3–5 years from seedlings to flower. This delay in flowering is a major problem in the propagation and breeding of herb *Dendrobium*.

Induction of precocious flowering of temperate *Dendrobium in vitro* had been reported for *Dendrobium candidum* (Wang et al., 1997) and *Dendrobium huoshanase* (Wen et al., 1999). Recently, there are reports of inducing early flowering of tropical *Dendrobium* seedlings (Sim et al., 2007; Hee et al., 2007). Sim et al. (2007) using a two-layered medium (liquid over Gelrite-solidified). This culture system also promoted *Dendrobium* Chao Praya Smile flowering and producing visible seeds within about 11 months (Hee et al., 2007). Unlike most tropical orchids, low temperature is an important factor for the reproductive transition in temperate *Dendrobium* orchids (Chen and Ji, 1998). When grown with only a single plant regulator, well-developed floral buds were not induced in *Dendrobium* and the flower bud either wither quickly or develop into abnormal flowers (Shao and Meng,1999).

The current work aims to shorten the juvenility phase and to induce normal flowering of a temperate orchid herb *D. nobile in vitro*. We have established a high frequency *in vitro* flowering protocol, using seeds from self-pollinated seed pods as starting materials. Growing seedling in medium with PP_{333} + TDZ followed by culture in lower temperature on flower development was also investigated. These findings are important for molecular and genetic studies on the mechanisms of flower induction and for advancing orchid breeding programs.

2. Materials and methods

2.1. Plant materials and growth conditions

Seed pods of *D. nobile* were surface disinfected by immersion in 75% ethanol for 30 s, followed by 0.1% $HgCl_2$ for 8 min and rinsed with sterile water. Seed pods were then dissected lengthwise and the seeds were spread on 1/2 MS medium (Murashige and Skoog, 1962) supplemented with 10% (v/v) ripe banana pulp and 0.4 mg L^{-1} α-naphthaleneacetic acid (NAA). Media were adjusted to pH 5.6 prior to autoclaving (121 °C for 20 min). 1/2 MS medium

Abbreviations: ABA, abscisic acid; BA, 6-benzyladenine; MS, Murashige and Skoog; NAA, α-naphthaleneacetic acid; PP_{333}, paclobutrazol; TDZ, thidiazuron; PA, PP_{333} 0.5 mg L^{-1} + ABA 0.5 mg L^{-1}; PN, PP_{333} 0.3 mg L^{-1} + NAA 0.5 mg L^{-1}; PGRs, plant growth regulators.

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Table 1
PGRs combination in pre-culture media for the *D. nobile* plantlets.

PGR combination	PP_{333} (mg L^{-1})	NAA (mg L^{-1})	ABA (mg L^{-1})
PGRs-free	–	–	–
PN	0.3	0.5	
PA	0.5	–	0.5

Table 2
Culture conditions for the *D. nobile* plantlets.

	PP_{333} + TDZ (mg L^{-1})	Culturing temperature (°C)
CK1	0 + 0	25
CK2	0.5 + 0.05	25
CK3	0.8 + 0.08	25
CK4	1.0 + 0.10	25
T1	0 + 0	23/18 (light/dark)
T2	0.5 + 0.05	23/18 (light/dark)
T3	0.8 + 0.08	23/18 (light/dark)
T4	1.0 + 0.10	23/18 (light/dark)

Table 3
Effects of BA, TDZ and PP_{333} on the floral buds formation of *D. nobile* plantlets.

	% floral buds formation (±S.E.)
0	0 ± 0 g
BA 0.05	0 ± 0 g
0.1	5.9 ± 1.3 f
0.5	13.3 ± 2.3 d
1.0	20.0 ± 2.2 c
TDZ 0.05	31.9 ± 3.4 ab
0.1	34.8 ± 3.4 a
0.5	15.6 ± 2.3 d
1.0	12.6 ± 2.6 de
PP_{333} 0.05	8.9 ± 2.2 ef
0.1	17.0 ± 4.6 cd
0.5	33.3 ± 3.9 a
1.0	28.9 ± 2.2 b

Data were recorded after 120 days of culture. In all column, means ± S.E. followed by the same letters are not significantly different at the $p < 0.05$ level of significance.

was used as the basal medium for all experiments in this study. The cultures were maintained in a culture room kept at 25 °C with a 12 h photoperiod provided by white fluorescent light at 60 μmol m^{-2} s^{-1}.

2.2. Experimental series

In the first experimental series, 2–3 months old seedlings were used. Induction of floral buds *in vitro* was by treatment with BA, TDZ or PP_{333} at different concentrations (0, 0.05, 0.1, 0.5 and 1.0 mg L^{-1} respectively). In the second experimental series, the 2–3 months old seedlings that were pre-cultured on 1/2 MS medium with PA (PP_{333} 0.5 mg L^{-1} + ABA 0.5 mg L^{-1}) or PN were transferred to an inductive media supplemented with PP_{333} + TDZ or PGRs (plant growth regulators)-free media (as control). (1) The seedlings were pre-cultured on 1/2 MS medium supplemented with PA for 35 days (Table 1), then transferred to inductive media (I, II, III, IV) or PGRs-free media (CK); (2) the seedlings were pre-cultured on 1/2 MS medium supplemented with PN for 90 days, then transferred onto inductive media (CK2, 3, 4; T2, 3, 4) or PGRs-free media (CK1, T1)at different temperatures for 45 days (Table 2).

2.3. Data handling

Each treatment involved nine flasks (five plantlets/flask), and each experiment was replicated three times. The percentage of floral buds, normal flowers and abnormal flowers were counted within 120 days. Data were analyzed using the analysis of variance and Duncans multiple range test at $p < 0.05$ level of significance.

3. Results

3.1. Effects of BA, TDZ and PP_{333} on the induction of floral buds

We did not observe any flowering in the control plants cultured on 1/2 MS medium during the 120-day period of observation (Table 3). Table 3 shows that, given appropriate concentrations of BA, TDZ or PP_{333}, 20.0–34.8% of *D. nobile* shoots produced floral buds (Fig. 1A). BA at high dose (1.0 mg L^{-1}) was more effective for floral buds induction (20.0%) in cultures than that of lower doses (ranging from 0 to 0.5 mg L^{-1}). TDZ promoted floral buds formation (31.9–34.8%) better than BA at low concentrations (0.05–0.1 mg L^{-1}), but floral buds (12.6–15.6%) induction were reduced at high concentrations (0.5–1.0 mg L^{-1}). PP_{333} at high

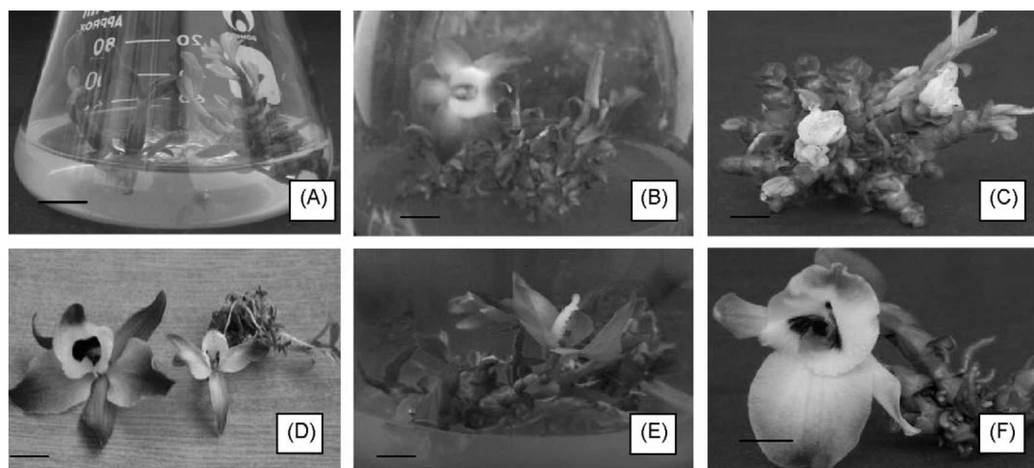

Fig. 1. *In vitro* flowering of *D. nobile* Lindl. induced by PGRs. (A) Inflorescences formed on TDZ-containing medium, (B) normal flowers formed PA-containing medium, (C) 11 florets per shoot, (D) comparison of the *in vitro* flowering seedlings with the parental plants *in vivo*, (E) the floral bract blooming, (F) the abnormal flower without complete organs. Bar: 1 cm.

Table 4
Effects of PA pre-treatment on flowering induced by PP_{333} + TDZ *in vitro*.

Treatment	PP_{333} + TDZ (mg L^{-1})	% floral buds formation (±S.E.)	% normal flowers (±S.E.)	% abnormal flowers (±S.E.)
CK	0 + 0	21.5 ± 3.4 c	10.5 ± 1.8 b	27.4 ± 2.5 b
I	0.5 + 0.05	37.0 ± 3.4 b	12.1 ± 1.1 b	29.9 ± 4.7 ab
II	0.5 + 0.10	33.3 ± 2.3 b	0 c	31.3 ± 5.6 ab
III	1.0 + 0.05	34.1 ± 3.4 b	0 c	37.2 ± 5.1 a
IV	1.0 + 0.10	62.2 ± 3.9 a	15.4 ± 1.1 a	38.1 ± 2.2 a

Data were recorded after 120 days of culture. In each column, means ± S.E. followed by the same letters are not significantly different at the $p < 0.05$ level of significance.

Table 5
Effects of PN pre-treatment and lower temperature on flowering induced by PP_{333} + TDZ *in vitro*.

	% floral buds formation (±S.E.)	% normal flowers (±S.E.)	% abnormal flowers (±S.E.)
CK1	23.0 ± 3.4 e	33.2 ± 1.9 e	11.2 ± 3.6 b
CK2	54.8 ± 3.4 d	42.0 ± 5.9 d	6.7 ± 2.1 cd
CK3	95.6 ± 5.9 a	27.8 ± 3.1 ef	23.3 ± 2.4 a
CK4	62.2 ± 5.9 cd	22.7 ± 1.7 f	19.1 ± 2.1 a
T1	25.9 ± 2.6 e	74.1 ± 2.4 b	0 e
T2	64.5 ± 5.9 c	81.6 ± 2.1 a	0 e
T3	83.7 ± 5.6 b	68.8 ± 6.1 bc	4.4 ± 1.3 d
T4	69.6 ± 4.6 c	62.8 ± 5.6 c	8.6 ± 2.1 bc

Data were recorded after 120 days of culture. In each column, means ± S.E. followed by the same letters are not significantly different at the $p < 0.05$ level of significance.

concentrations (0.5–1.0 mg L^{-1}) promoted floral buds formation (28.9–33.3%) but dramatically reduced the percentage of floral buds (8.9–17.0%) at low concentrations (0.05–0.1 mg L^{-1}). TDZ (0.05–0.1 mg L^{-1}) and PP_{333} (0.5–1.0 mg L^{-1}) were more effective for inducing floral buds of *D. nobile* plantlets than BA.

3.2. Effects of PA pre-treatment on flowering induced by PP_{333} + TDZ in vitro

It was observed that 21.5% of the plantlets produced floral buds, in which 10.5% of them developed into normal flowers (Fig. 1B) cultured on PGRs-free medium (CK) during the 120-day period of observation (Table 4). With PGRs added, (treatment, I–IV) more floral buds (33.3–62.2%) were induced. Moreover, the number of flowers initiated from each shoot also increased, reaching the highest value of 11 florets (Fig. 1C). Among the four treatments tested, treatment IV was the most effective for floral buds formation (62.2%) and flower induction (53.5%), about 15.4% of them ended up with complete organs. However, such flowers were undersized compared to that of normal flower but there was no difference in their coloration (Fig. 1D).

In general, normal floral buds were initiated within 6 weeks, and they developed into normal flowers within 5 weeks of culture (Fig. 1E). No significant differences were observed in the development of flower bud into flower among the other treatments ($p < 0.05$). Less than 40% of them, even if bloomed, were smaller and mostly abnormal, having perianths that could not be differentiated into petals and sepals, or columns or the reproductive organs were absent (Fig. 1F).

3.3. Effects of PN pre-treatment and lower temperature on flowering induced by PP_{333} + TDZ in vitro

We observed 23.0% of the plantlets produced floral buds, in which 33.2% of them developed into normal flowers (Fig. 1B) cultured on PGRs-free medium (CK1) subjected to 25 °C during 120-day period of observation (Table 5). More floral buds formation were observed (54.8–95.6%) in the other controls (CK2–4).

About 22.7–42.0% of such floral buds developed into normal flowers within 5 weeks of culture. Usually, there were only one or two flowers per inflorescence (Fig. 2B). Among the controls (CKs),

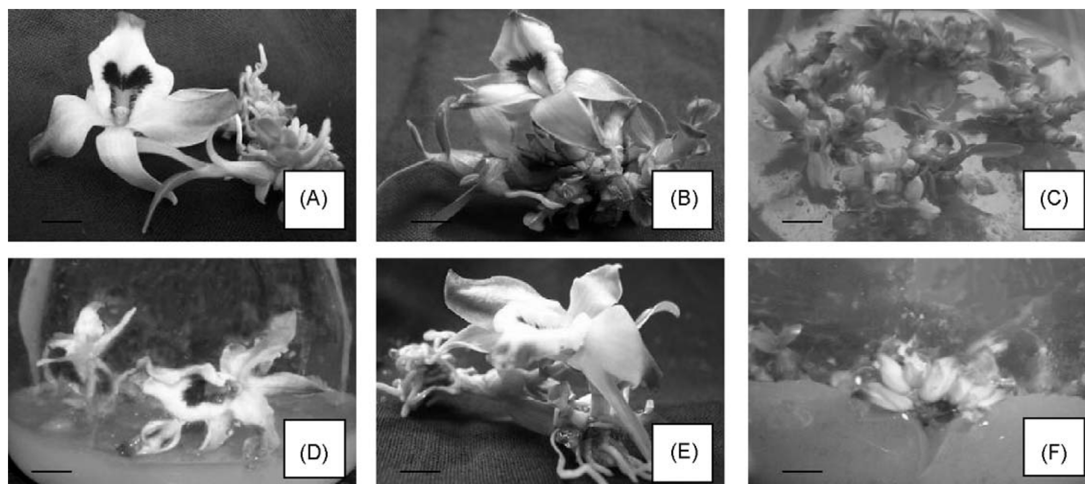

Fig. 2. *In vitro* flowering of *D. nobile* induced by PGRs and lower temperature. (A) Normal flowers formed PN-containing medium, (B) one flower per inflorescence, (C) high rate of floral buds induction, (D) high frequency of normal flowers, (E) the normal flower bloomed 3–4 weeks, (F) 10 floral buds per inflorescence. Bar: 1 cm.

CK3 was the most effective in inducing floral buds (95.6%) in *D. nobile* (Fig. 2C), but resulted in 23.3% abnormal flowers.

When cultured at lower temperature (23 °C/18 °C, light/dark), more than 60% of the floral buds developed into normal flowers, and less than 10% produced abnormal flowers (Table 5). It was observed that flower developed normally and fully in T1 and T2 treatments (Fig. 2D). Among the four treatments (Ts), T2 is more effective in inducing normal flowers (81.6%). Moreover, such flowers could last around 3–4 weeks (Fig. 2E), although the number of floral buds (64.5%)were slightly reduced. With increasing of PP_{333} + TDZ concentration, the number of floral buds per inflorescence dramatically increased (T3 and T4), as many as 10 floral buds were obtained (Fig. 2F), but the number of abnormal flowers (4–9%) also increased. In general, the emergence of floral buds was delayed by about 3 weeks in comparison to that of the control (CKs).

4. Discussion

The inductive effect of BA on flowering have been shown *in vitro* and described in reports with orchids (Wang et al., 1995; Duan and Yazawa, 1994; Kostenyuk et al., 1999; Wen et al., 1999; Sim et al., 2007; Hee et al., 2007).

However, our results suggest otherwise, BA was not effective for floral induction in *D. nobile*. In contrast, treatment with TDZ at low doses (0.05–0.1 mg L^{-1}) was effective in promoting floral buds formation. These results agreed with the findings of Chang and Chang (2003) who reported that TDZ has a stronger inductive effect than BA on the flowering of *Cymbidium ensifolium in vitro*.

It was reported that PP_{333}, when applied as a collar drench, foliar spray, or trunk injection could induce flower bud initiation in *E. globulus* Labill. and *E. nitens* (Griffin et al.,1993; Hasan and Reid, 1995). In some fruit crops, e.g. apple tree (cv. Red Delicious), sweet cherry (*Prunus avium* L.) and sour cherry (*Prunus cerasus*) the inhibition of vegetative growth was apparent which were applied by PP_{333} (Meilan,1997). In our present study, PP_{333} at high doses (0.5–1.0 mg L^{-1}) promoted floral buds formation. This result disagrees with the findings that PP_{333} totally blocked the inductive effects of cytokinin (Kostenyuk et al., 1999) and significantly delayed flower bud formation and anthesis (Guo et al., 2004).

Wang et al. (1997) have earlier reported that the flowering frequency was further increased to more than 80% by pre-treatment of protocorms in an ABA-containing medium followed by transfer onto MS medium with BA. In our experiments, PP_{333} + TDZ induced over half of shoots that were pre-cultured by PA to flower (Table 4). A similar result was demonstrated in our previous study on *Dendrobium moniliforme* (L.) SW. (Wang et al., 2006). In addition, nearly 100% of shoots was induced to floral buds after pre-cultured in PN-containing medium followed by transfer onto 1/2 MS medium with PP_{333} + TDZ (Table 5).

D. nobile Lindl. was distributed at an elevation of 500–1700 m above sea level (Chen et al., 1998), with an annual average temperature of 18–21 °C and January average temperature of over 8 °C (Ran, 2002). Hence, low temperature is responsible for flowering induction of *D. nobile* Lindl. In the case of T2 (Table 5), when combined with lower temperature, PP_{333} + TDZ promoted significantly more floral buds to flowering (81.6%) and prolonged the flower longevity.

In conclusion, PP_{333} or TDZ promoted plant maturity and the formation of floral buds. Precocious flowers can be developed in an inductive media with PP_{333} + TDZ added and subjected to lower temperature (23 °C (light)/18 °C (dark)). Our protocol shortens the time required for normal flower development evaluation (normally it takes at least 3 years) and reduces the labor costs. These findings will be highly beneficial to orchid breeders and breeding programs. It is necessary, however, to further study how the endogenous hormones function in flowering induction in others herb *Dendrobium*.

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