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In vitro flowering of green and albino *Dendrocalamus latiflorus*

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Abstract To propagate *Dendrocalamus latiflorus*, we used in vivo inflorescences to produce calli on Murashige and Skoog basal (MS) medium supplemented with 3 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg/l kinetin, 250 mg/l polyvinyl pyrrolidone (PVP), and 1% coconut milk. Multiple shoots were generated on MS medium supplemented with 0.1 mg/l thidiazuron (TDZ). The green plantlets were successfully transferred to soil. Multiple albino shoots also regenerated and were able to proliferate on medium containing cytokinins, especially TDZ. Albino multiple shoots rooted in medium containing α-naphthaleneacetic acid (NAA), and callus formation was observed in the presence of 2,4-D and picloram. Green and albino regenerates flowered after 8 months of subculture. The flowering ratio increased to 44% after three treatments in medium containing 1 mg/l TDZ. Morphological observations revealed that the in vitro green and albino flower organs were normal. However, pollen derived from the in vitro flowers of both the green and albino plants were sterile.

Keywords Bamboo · Cytokinin · TDZ

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
BA	N^6-benzyladenine
IBA	indolebutyric acid
2-ip	2-isopentyl adenine

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MS	Murashige and Skoog medium
NAA	α-naphthaleneacetic acid
PVP	polyvinyl pyrrolidone
TDZ	thidiazuron

Introduction

Dendrocalamus latiflorus Munro. is a wildly distributed evergreen species locally known as ''Taiwan giant bamboo''. This giant bamboo, forests of which are abundant in Taiwan, is a valuable natural resource, with its mature culms used as building material and its young shoots being cultivated extensively for human consumption. However, *D. latiflorus* is susceptible to a potexvirus, Bamboo mosaic virus (BaMV), which poses a major obstacle to its commercial exploitation in Taiwan (Hsu et al. 2000). The development of a BaMV-resistant *D. latiflorus* line would provide a potential solution to this problem, but bamboo has a long juvenility period with extended breeding cycles and, consequently, it would be a difficult and protracted process to obtain virus-resistant seeds by means of hybrid breeding (Chang 1991, 1994; Chang and Ho 1997). Somatic embryogenesis in which seeds are used as the starting material has been reported to be a successful manner to propagate *Dendrocalamus* species (Yeh and Chang 1987; Rout and Das 1994); however, seed formation in *D. Latiflorus* is unreliable due to poor seed set during the sporadically occurring flowering periods (Chang 1991, 1994; Chang and Ho 1997). In vivo inflorescences are much easier to obtain and can also be used as starting material for a regeneration protocol.

Albino regenerates are frequently observed in bamboo tissue culture (Rout and Das 1994; Ho and Chang 1998; Lin and Chang 1998), and during our investigations on *D. latiflorus* we have isolated albino mutants among the multiple shoots. One of the mechanisms that has been proposed to explain the appearance of albino regenerates is the large-scale deletion and the accompanying loss of photosynthesis-related genes from the plastid DNA (Day and Ellis 1984; Dunford and Walden 1991; Harada et al. 1991; Harada et al. 1992; Kawata et al. 1997). As many chloroplast proteins are nuclear-encoded, the mechanism of regulating nuclear gene expression by the chloroplast is an important area of plant research. In vitro hybridization is a useful tool by which to investigate this area as it is difficult to obtain pollen from albino plants for hybridization purposes due to latter being unable to survive in the field. Consequently, in vitro flowering protocols which could provide the pollen and reproductive organs of albino plants would be very useful material for investigations on nuclear gene expression.

Various researchers have succeeded in inducing in vitro flowering in *Dendrocalamus* sp. within a few months (Nadgauda et al. 1990; Chambers et al. 1991; Rout and Das 1994; Gielis et al. 2001; Ramanayake et al. 2001) as well as the subsequent production of seeds (Nadgauda et al. 1990; Rout and Das 1994). Ho and Chang (1998) and Lin and Chang (1998) induced flowering in *Bambusa* albino regenerates and multiple shoots. These methodologies may provide the means to achieve the desired green-albino hybrids, but data are lacking on flowering in the albino plant and the effects of plant growth regulators on flowering in *Dendrocalamus*.

The investigation reported here had two objectives. First, we set out to develop an inflorescence-derived regeneration protocol for *D. latiflorus* that would be suitable for micropropagation and gene transformation applications. Second, we assessed the effects of various plant growth regulators on in vitro flowering in order to determine the key component(s) of flowering and juvenility of bamboo and, in addition to provide albino reproductive organs for further hybridization.

Materials and methods

Medium preparation

The basal medium contained MS salts (Murashige and Skoog 1962) and organic components (100 mg/l myo-inositol, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine-HCl, 1 mg/l thiamine-HCl, 2 mg/l glycine (all Sigma, St. Louis, Mo.). Sucrose was used as the source of carbon for all experiments. All growth regulators to be tested on in vitro flowering/shoot proliferation were added before the pH of the media was adjusted to 5.7. The media were then autoclaved at 121°C for 15 min.

Plant material and callus initiation

Two hundred inflorescences (1 ~ 2 cm long; Fig. 1A) were excised from one field-grown *D. latiflorus* plant in Taipei, Taiwan and soaked in 2% sodium hypochlorite with sonication for 10 min. After three thorough washes in sterilized distilled water, the young inflorescences were cut into small segments approximately 1–2 mm in length. Each segment was placed on 10 ml of semi-solid (0.22% Gelrite, Kelco, CA, USA) MS medium supplemented with 3 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma), 2 mg/l kinetin (Sigma), 250 mg/l polyvinylpyrrolidone (PVP; Sigma), and 1% coconut milk (Yeh and Chang 1987) in a test tube (20 x 150 mm) for the initiation of the callus to be used in regulation efforts. In total, 200 segments were cultured. Seventy percent of the segments contaminated and calli for this study were induced in the rest of segments. The calli that developed were subcultured on fresh media every 2 months for callus proliferation.

Multiple shoots were regenerated on semi-solid medium (0.22% Gelrite, Kelco, CA, USA) MS medium supplemented with 0.1 mg/l thidiazuron (TDZ; Sigma). This choice of regeneration medium was based on the results of an earlier investigation by Lin et al. (2004). The regenerates were maintained on MS medium supplemented with 0.1 mg/l TDZ with subculture every 21 days.

Effects of plant growth regulators on albino shoot proliferation

Because an individual shoot of bamboo is very difficult to proliferate (Lin and Chang 1998), we used shoot clusters as the starting material for the albino shoot proliferation experiments. Three clusters of multiple shoots, each containing three to five shoots, were placed in a 125 ml flask (diameter: 10 cm) containing 25 ml of semi-solid medium (0.22% Gelrite) . Five concentrations of TDZ (0, 0.01, 0.05, 0.1, 1 mg/l) were added to the MS basal medium to test the effects of TDZ on albino shoot proliferation and in vitro flowering. The effects of various cytokinins were investigated by adding the following cytokinins to the semi-solid basal medium at the concentrations given: 0.1 mg/l TDZ; 1 and 5 mg/l kinetin; 1 and 5 mg/l 2-isopentyl adenine (2-ip); 1 and 5 mg/l 6-benzyladenine (BA); 1 and 5 mg/l zeatin (Sigma). Data were collected from three flasks each containing three multiple shoot clusters ($n = 9$).

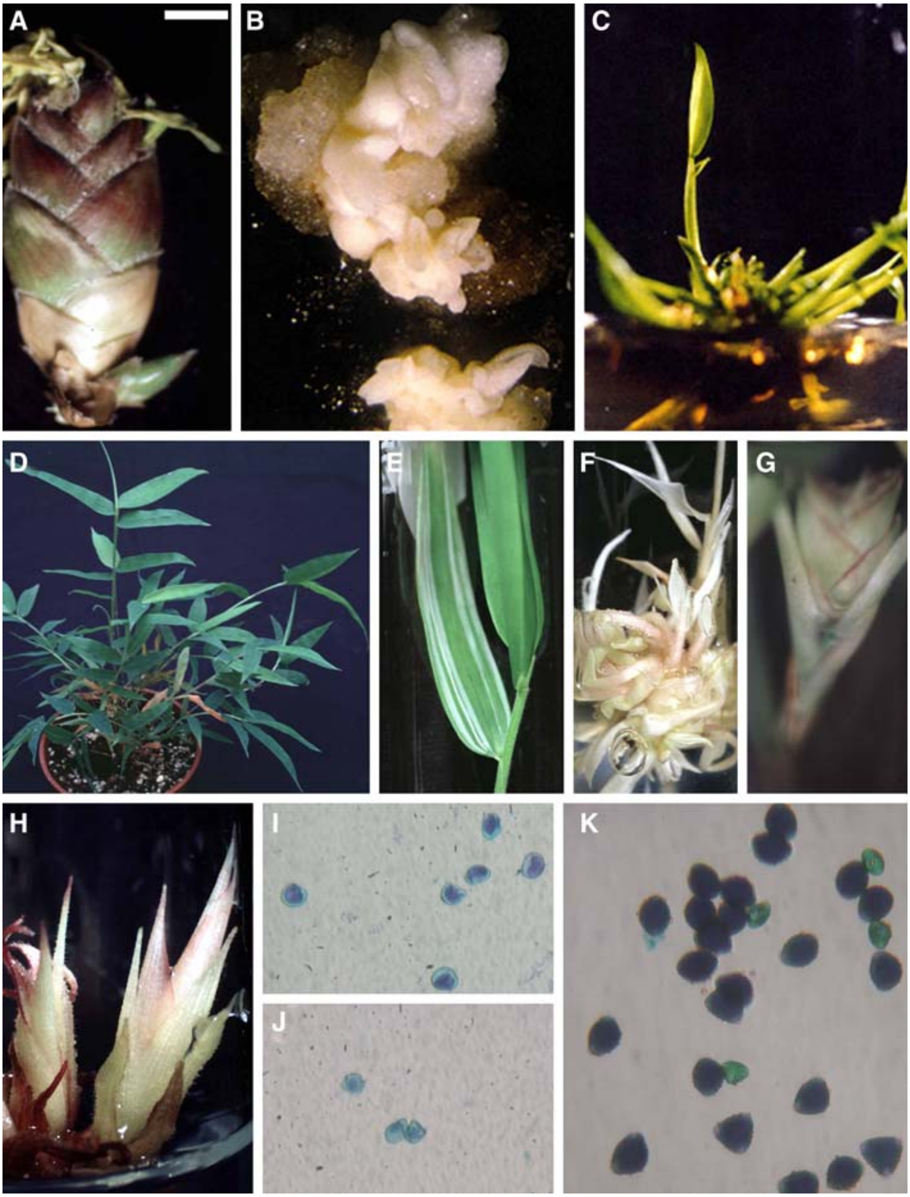

Fig. 1 (**A**–**K**) In vitro morphogenesis of field-grown inflorescence of *D. latiflorus* and their derived regenerates. (**A**) In vivo inflorescence (bar = 1 mm). (**B**) The calli derived from inflorescence (bar = 2 mm). (**C**) Multiple shoots proliferated in subculture on basal medium plus 0.1 mg/l TDZ (bar = 1 cm). (**D**) Three-month-old healthy potted plants derived from callus regeneration (bar = 2 cm). (**E**) Green-white chimera shoots in subculture on basal medium plus 0.1 mg/l TDZ (bar = 1 cm). (**F**) Albino regenerates occurring in subcultured multiple shoot proliferation (bar = 1 cm). (**G**) Green regenerate flowering in subculture on basal medium plus 0.1 mg/l TDZ (bar = 5 mm). (**H**) Albino flower occurring among the population of subcultured albino shoots cultured on basal medium plus 0.1 mg/l TDZ (bar = 5 mm). (**I**, **J**) Lack of staining with Alexander's stain indicates that these pollen grains are male sterile in yellow anther (K) and brown anther (L) (200x). (**K**) Pollen of *Tropaeolummajus* L. served as the positive control in the staining method (200x)

Effects of auxin on rooting and flowering of albino shoots

Five clusters of multiple shoots, each containing three to five shoots, were placed in a 125 ml flask (diameter: 10 cm) containing 25 ml semi-solid MS basal medium supplemented with one of the following auxins (Sigma) at one of the concentrations to be tested: 0, 2, and 5 mg/l α-naphthaleneacetic acid (NAA); 2 and 5 mg/l 2,4-D; 2 and 5 mg/l picloram). Data were collected from three flasks of five multiple shoots ($n = 15$).

Growth conditions

The calli were cultured on medium at 26°C in the dark and regeneration studies and multiple shoots for proliferation were cultured under a 16/8-h (light/dark) photoperiod with artificial light (daylight fluorescent tubes, 40 W) provided at an intensity of 54 μmol m^{-2} s^{-1}. Rooted green plantlets were then transferred to soil in pots and raised in a greenhouse without hardening.

Statistical analysis

The number of shoots was recorded by visual counting at the beginning of the experiments. We observed root formation and visually counted the numbers of shoots and inflorescences. The shoot proliferation ratio, rooting, callus formation, and flowering were defined as follows.

$$\text{shoot proliferation ratio} = \frac{\text{No. of shoots after incubation}}{\text{No. of original shoots}}$$

$$\text{rooting (\%)} = \frac{\text{No. of multiple shoots with root} \times 100}{\text{No. of total multiple shoots}}$$

$$\text{callus formation (\%)} = \frac{\text{No. of multiple shoots with callus} \times 100}{\text{No. of total multiple shoots}}$$

$$\text{flowering (\%)} = \frac{\text{No. of multiple shoots with flower} \times 100}{\text{No. of total multiple shoots}}$$

Variance analysis was conducted using Costat (CoHort software, USA). Duncan's multiple range test was used for mean separation when significance treatment effects were present (Duncan 1955).

Pollen staining

Green and albino anthers were collected from in vitro flowers at noon, and pollen viability was examined using a mixture of malachite green, acid fuchsin, and orange G (Alexander 1969).

Results

Plant regeneration

The medium developed by Yeh and Chang (1987) for obtaining callus from seeds of *D. latiflora* as explants was used in the present experiment for obtaining callus from the inflorescences of *D. latiflorus* (Fig. 1A). This was a deliberate choice based on the limited availability of *D. latiflorus* inflorescences. Following the protocol of Yeh and Chang (1987), we obtained white and pale-yellow callus from the surfaces of the explants after 1 month of culture. These calli were subcultured on fresh media every 2 months in the dark (Fig. 1B). These *D. latiflorus* calli turned brown and were unable to regenerate on MS medium supplemented with 2 mg/l NAA and 1 mg/l BA as reported in the Yeh and Chang (1987) protocol for *D. latiflora*. However, they were able to regenerate and multiple shoots were able to proliferate on MS medium supplemented 0.1 mg/l TDZ (Fig. 1C), which has been reported to be the optimal medium for *B. edulis* somatic embryo germination (Lin et al. 2004). The plantlets that subsequently developed from the multiple shoots of *D. latiflorus* had well-developed roots and were transplanted into soil (peat:vermiculite:perlite = 1:1:1; Fig. 1D).

Effects of TDZ concentration and cytokinins on albino shoot proliferation and flowering

After four subcultures, green-white chimera (Fig. 1E) and albino (Fig. 1F) regenerates appeared among the multiple shoots. We then manipulated the concentrations and types of growth regulators to determine the best combination for optimal albino shoot proliferation and flowering. The inclusion of TDZ in the culture medium increased albino shoot proliferation (Table 1), and the effect was concentration-dependent: there was no significant difference in albino shoot proliferation on medium containing between 0 and 0.01 mg/l TDZ, while higher concentrations (0.05 ~ 1 mg/l) significantly increased albino shoot proliferation. Although multiple shoots could be proliferated on 0.1 mg/l TDZ, the efficiency was not stable and the proliferation ratio was variable (1.58–3.41). Although not significantly greater than proliferation with 1 mg/l, multiple shoots did show the highest and most stable proliferation on medium containing 1 mg/l TDZ (Table 1).

Compared to the no cytokinin controls, cytokinins other than TDZ were also able to promote albino shoot proliferation, although at different efficiencies (Table 2). The highest overall ratio of proliferation was obtained with TDZ but this was not always significantly better than other cytokinins (Table 2). BA and kinetin had a promoting effect but were relatively less efficient in the second 21-day subculture (second treatment). Albino shoots

Table 1 The effect of TDZ concentration of *D. latiflorus* albino multiple shoot proliferation and flowering

TDZ (mg/l)	1st treatment (21 days)		2nd treatment (42 days)		3rd treatment (63 days)	
	Shoot proliferation	Flowering (%)	Shoot proliferation	Flowering (%)	Shoot proliferation	Flowering (%)
0	1.04 b*	0.0 a	1.00 c	0.0 a	1.00 b	0.0 b
0.01	1.36 b	11.1 a	1.06 c	0.0 a	1.22 b	11.1 b
0.05	1.94 a	0.0 a	1.21 bc	0.0 a	2.48 a	0.0 b
0.1	2.72 a	0.0 a	1.58 ab	11.1 a	3.41 a	0.0 b
1	3.42 a	11.1 a	5.65 a	22.2 a	3.58 a	44.4 a
P value	0.0025	0.5801	0.0000	0.1705	0.0003	0.046

* There were three flasks for each treatment, and three clusters of multiple shoots in each flask ($n = 9$). Each experiment was run in triplicate within the column. Means followed by the same letter are not significantly different (Least significant difference test, $P < 0.05$ Duncan 1955)

Table 2 Influence of cytokinin type and concentration on *D. latiflorus* albino multiple shoot proliferation and flowering

Cytokinin	(mg/l)	1st treatment (21 days)		2nd treatment (42 days)	
		Shoot proliferation	Flowering (%)	Shoot proliferation	Flowering (%)
–	–	1.19 b*	0.0 a	1.00 c	0.0 a
TDZ	0.1	3.09 a	11.1 a	6.97 a	22.2 a
BA	1	2.60 ab	0.0 a	4.08 b	11.1 a
	5	3.34 a	0.0 a	3.83 b	0.0 a
Zeatin	1	1.15 b	22.2 a	1.09 c	11.1 a
	5	1.29 b	0.0 a	1.56 c	0.0 a
2ip	1	1.32 b	0.0 a	1.00 c	0.0 a
	5	1.19 b	0.0 a	1.56 c	0.0 a
Kinetin	1	1.35 b	0.0 a	1.31 c	0.0 a
	5	2.25 ab	0.0 a	3.79 b	0.0 a
P value		0.0000	0.0600	0.0000	0.0975

*There were three flasks of each treatment and three clusters in each flask ($n = 9$). Each experiment was run in triplicate within a column. Means followed by the same letter are not significantly different (Least significant difference test, $P < 0.05$; Duncan 1955)

did not show significant proliferation when either zeatin or 2-ip was added to the proliferation medium. This result was consistent after two treatments.

After eight subcultures in the 8 months following regeneration, both in vitro green (Fig. 1G) and albino (Fig. 1H) regenerated plantlets flowered. Continuous subculturing of the green multiple shoots increased the flowering ratio. After 11 subcultures in 11 months, the flowering ratio was 26.7% in the green regenerates.

In TDZ treated clusters, the ratio of multiple shoot flowering in the 21-day subculture (first treatment) was only 0–11% (Table 1). After three subcultures in medium supplemented with 1 mg/l TDZ, the flowering ratio increased, reaching 44% in the third treatment cycle.

In the cytokinin experiments, flowering occurred on MS medium supplemented with 0.1 mg/l TDZ, 1 mg/l zeatin, and 1 mg/l BA, respectively, but it did not occur in the cytokinin-free control treatments. However, there was no statistically significant difference among these cytokinin treatments or the control with respect to flowering between the two subcultures (Table 2).

Effects of auxins on rooting, flowering and callus formation of albino shoots

Multiple shoots could only root on medium supplemented with NAA, with the highest rooting ratio obtained when 5 mg/l was added to the medium, reaching 46.7% after 2 months (Table 3). In the 2,4-D and picloram treatments, calli formed on the bottom of the explants. Multiple shoots also flowered in the medium supplemented with auxin and, after 2 months, 8.9–22.2% of the plants had flowered (Table 3).

Morphological examination of the albino and green inflorescences revealed that the in vitro flowers remained intact. The anthers were initially yellow, and then turned brown. Both the yellow and brown anthers had sterile pollen (Fig. 1I, J) which could not germinate in vitro. One of the green regenerates was maintained in the greenhouse for 2 months at

Table 3 The effect of different auxins on rooting, callus formation, and flowering of *D. latiflorus* albino multiple shoots, after 2 months of treatment

Auxin	(mg/l)	Rooting (%)	Callus formation (%)	Flowering (%)
NAA	0	0.0 b*	0.0 D	0.0 b
	2	13.3 b	0.0 d	17.8 ab
	5	46.7 a	0.0 d	0.0 b
IAA	2	0.0 b	0.0 d	0.0 b
	5	0.0 b	0.0 d	22.2 a
IBA	2	0.0 b	0.0 d	8.9 ab
	5	0.0 b	0.0 d	17.8 ab
2,4-D	2	0.0 b	33.3 b	0.0 b
	5	0.0 b	20.0 c	0.0 b
Picloram	2	0.0 b	100.0 a	17.8 ab
	5	0.0 b	100.0 a	0.0 b
P value		0.0014	0.0000	0.1270

*There were three flasks of each treatment and five clusters of multiple shoots in each replicate ($n = 15$). Each experiment was run in triplicate within a column. Means followed by the same letter are not significantly different (Least significant difference test, $P < 0.05$; Duncan 1955)

which time it flowered. However, the plantlet died after in vivo flowering. The in vivo florets were able to open but there was no seed formation.

Discussion

Effects of TDZ concentration and other cytokinins on albino shoot proliferation and flowering

Cytokinins are a class of plant growth substances that are active in promoting cell division as well as being active in numerous physiological processes, including plant cell growth and differentiation. As such, they are a common requirement for *in vitro* flowering (Scorza 1982) and have been shown to be a key factor for obtaining in vitro flowering of bamboo (Nadgauda et al. 1990; Chambers et al. 1991; Rout and Das 1994; Lin and Chang 1998). More specifically, TDZ has been found to be capable of inducing flowering in *B. edulis* (Lin and Chang 1998; Lin et al. 2003) and *D. strictus* (Singh et al. 2000) although, the optimal concentration varied between the two species: 1 mg/l in *Dendrocalamus* (Singh et al. 2000) and only 0.1 mg/l in *Bambusa* (Lin and Chang 1998).

TDZ is known to be a potent cytokinin in woody plant tissue culture (Huetteman and Preece 1993). Lin et al. (2003) did not observe any difference among the cytokinins in inducing reproductive growth of multiple shoots of *B. edulis*. In the present assessment on the effect of different cytokinins on in vitro flowering for the long-term subculture of *D. latiflorus*, we observed that flowering occurred in the TDZ, zeatin, and BA treatments (Table 2). However, there was no significant difference between these treatments and cytokinin-free control.

In the present investigation when the influence of TDZ concentration on flowering was observed, there was no significant difference in flowering between the various TDZ concentrations in the first subculture (first treatment; Table 1). However, the high-TDZ treatment (1 mg/l) significantly increased the flowering ratio only after the third subculture (third treatment). Based on this result, we conclude that long-term, high concentration TDZ is a common requirement for in vitro flowering of albino *D. latiflorus*.

Effects of auxins on rooting, flowering and callus formation of albino shoots

Flowering and rooting were our two major aims in studying the effect of auxins on *D. latiflorus* multiple shoots. Rout and Das (1994) found that bamboo could flower in indole-3-butyric acid (IBA)-supplemented medium, while Lin et al. (2003) observed that *B. edulis* somatic embryo-derived multiple shoots could also achieve 2–29% flowering when cultured in medium containing NAA, 2,4-D, and IBA (Lin et al. 2003). Bamboo multiple shoots are notably difficult to root in tissue culture (Nadgir et al. 1984; Prutpongse and Gavinlertvatana 1992; Chaturvedi et al. 1993). Although numerous studies have indicated that NAA is the most effective auxin for the induction of root regeneration in many plant species (review by Hu and Wang 1983), Lin and Chang (1998) used 2,4-D to induce rooting by adult bamboo (*Bambusa edulis*)-derived multiple shoots. When all of the most important auxins (NAA, IBA, 2,4-D and picloram) were tested in the present investigation for their effect on rooting, callus formation, and flowering, we found that *D. latiflorus* could only root in NAA medium and that the rooting percentage (46.7%) was lower than that of *B. edulis* (5 mg/l NAA: 80%; Lin et al. 2003). There was no significant difference in flowering ratio between the auxin treatments. These results are in agreement with those obtained with *B. edulis* (Lin et al. 2003).

Pollen development

In previous *Dendrocalamus* studies, flowering led to the formation of normal seeds when BA and IBA were supplemented to the medium (Nadgauda et al. 1990; Rout and Das 1994). Singh et al. (2000) also demonstrated that 20% of the pollen derived from the anther induced by TDZ was normal. However, in this study, the experimental results showed that *D. latiflorus* did not form seeds when the multiple shoots were cultured in BA-, IBA-, and TDZ- supplemented media. *D. latiflorus* can produce seed in the field. Future investigations will aim at determining the effects of medium components on *D. latiflorus* pollen development. In future investigations, we will elucidate pollen development, seed formation and hybridization in *D. latiflorus*.

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