Improving Multiple Shoot Proliferation in Bamboo Mosaic Virus-free *Bambusa oldhamii* Munro Propagation by Liquid Culture

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Abstract. An in vitro method for obtaining bamboo mosaic virus (BaMV)-free plantlets of *Bambusa oldhamii* Munro was developed. BaMV-free meristems were incubated on MS basal medium supplemented with 0.45 μM thidiazuron (TDZ) to induce the development of multiple shoots. Multiple shoot proliferation was higher in stationary liquid culture than on semisolid medium. Cytokinin was the key component for inducing proliferation, and TDZ was the stable and effective cytokinin for proliferation in long-term subcultures. Multiple shoots rooted after 1 month in MS basal medium containing 10.74 to 26.85 μM α -naphthaleneacetic acid with a rooting efficiency of 83%. Healthy, well-developed plantlets were transferred to soil in pots and raised in a greenhouse. Those plants derived from tissue culture were more vigorous than the ones derived from the traditional in vivo vegetative propagation method, air layering. The tissue culture-derived plants could produce the culms after 15 months. Fifteen of 38 plants flowered 2 years after being transplanted to the field.

Bambusa oldhamii Munro, also known as “green timber bamboo,” is a well-known semitropical clumping bamboo species native to Taiwan and southern China (Hsu et al., 2000a). Because of its large size (up to 16.5 m high with culms as thick as 10 cm), it is grown commercially for timber and wood-related products. In addition, its very young green shoots are edible and considered to be a culinary delicacy in Taiwan. A serious problem in the commercial cultivation of *B. oldhamii* is the prevalence of viral disease, bamboo mosaic virus (BaMV). This virus can reduce quality and cause as much as a 50% decrease in yield (Hsu et al., 2000b). In vitro virus-free systems that promote the successful regeneration of plants have contributed significantly to crop improvement by overcoming many of the limitations of in vivo

propagation systems. In previous studies, the researchers used seeds, seedlings, or reproductive explants to establish somatic embryogenesis (Yeh and Chang, 1986) and multiple shoots proliferation (Kapoor and Rao, 2006; Nadgouda et al., 1990; Sood et al., 2002). However, it is very difficult to obtain bamboo reproductive tissues in the field. Lin and Chang (1998) used field-grown vegetative shoot meristems to induce multiple shoots in vitro, which were subsequently used as explants to establish somatic embryogenesis in *B. edulis* Munro (Lin et al., 2004). This method could provide the healthy plantlets without any seasonal limitations.

Unlike *B. edulis*, in our preliminary investigation, the multiple shoot proliferation of *B. oldhamii* is very low when these shoots were incubated on semisolid medium. The same problem also occurred in *Guadua angustifolia* (Jiménez et al., 2006). We report here an efficient protocol using in vitro shoot meristem culture to establish a BaMV-free micropropagation for *B. oldhamii*. We also report the in vivo flowering and the production of albino mutants in *B. oldhamii*.

Methods and Materials

Plant material and medium preparation. Shoots from field-grown *B. oldhamii* were used as the source of material for in vitro

culture. Total RNA was isolated from leaves, and dot-blot hybridization was carried out to detect the presence of BaMV as described by Hsu et al. (2000b). The meristems of shoots removed from BaMV-free plants were then used as explants to generate in vitro plants. Twenty nodes (≈ 5 cm long each) were excised and soaked in 2% sodium hypochlorite with sonication for 30 min. After three thorough washes in sterilized distilled water, the meristem explants were excised and cultured in 2-cm-diameter test tubes containing 10 mL semisolid MS basal medium (Murashige and Skoog, 1962) supplemented with 0.45 μM thidiazuron (TDZ; Sigma, St. Louis) as described by Lin and Chang (1998) under a 16/8-h (light/dark) photoperiod with artificial lighting provided by daylight fluorescent tubes (FL-30D/29, 40 W; China Electric Co., Taipei) at an intensity of 54 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. All tissue culture media contained MS salts (Murashige and Skoog, 1962), 3% (w/v) sucrose, and 2.2 g·L⁻¹ Gelrite. All plant growth regulators were added before the medium was adjusted to pH 5.7. The newly emerging multiple shoots were subcultured at 21-d intervals on the same medium.

Multiple shoot proliferation. To compare liquid culture [no shaking and shaking (orbital, 120 rpm)] and culture on semisolid medium for their ability to induce shoot proliferation, we cultured three clusters of shoots, each having three to five multiple shoots in 125-mL flasks, which either contained 25 mL semisolid medium or liquid medium supplemented with 0.45 μM of the TDZ counterpart and compared multiple shoot proliferation. The cultures were grown for 21 d under the same conditions as described, after which healthy regenerated plantlets were selected out and used as explants for the second and third subcultures. Multiple shoot proliferation was calculated as the number of multiple shoots after a 21-d incubation period divided by the initial number of multiple shoots. Data of multiple shoot proliferation rates were collected from nine clusters of multiple shoots ($n = 9$). For long-term observation, multiple shoots in one experiment were further subcultured for 21 d and then transferred to fresh medium with the same concentration. Each experiment was repeated three times.

For studying the effects of TDZ and cytokinin, one cluster of shoots having three to five shoots was incubated in 125-mL flasks with 25 mL liquid medium. In TDZ concentration experiments, 0, 0.05, 0.45, 2.27, and 4.54 μM of TDZ were tested. In cytokinin experiments, N⁶-[2-isopentenyl] adenosine (2-ip; Sigma), zeatin (Sigma), kinetin (Sigma), 6-benzylaminopurine (BA; Sigma), and TDZ were added to the basal medium and tested for their effects on multiple shoot proliferation. The cultures were grown and subcultured as described. Multiple shoot proliferation was calculated as the number of multiple shoots after a 21-d incubation period divided by the initial number of multiple shoots. For long-term observation,

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green multiple shoots in one experiment were further subcultured for 21 d and then transferred to fresh medium with the same concentration. Data on the multiple shoot proliferation rate were collected from three flasks ($n = 3$), and each experiment was repeated three times.

Rooting. In 2005 through 2006, to study the effect of auxins on rooting, one cluster of multiple shoots (three to five shoots) was cultured into a test tube containing 10 mL of MS basal medium supplemented with various components (Gelrite and auxin). In liquid experiments, 0 and 2.2 g·L⁻¹ Gelrite were added into the basal medium. In auxin experiments, α -naphthaleneacetic acid (NAA; Sigma), indole-3-acetic acid (IAA; Sigma), indole-3-butyric acid (IBA; Sigma), picloram (Sigma), 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma), and 3,6-dichloro-2-methoxybenzoic acid (dicamba; Sigma) were added to investigate the effect on rooting. The cultures were grown for 1 month under the same conditions as described. Data on rooting, root number, and root length were collected from 18 tubes ($n = 18$), and each experiment was repeated three times. Well-rooted plantlets were planted in plastic pots containing a soil mixture of peat, perlite, and vermiculite (1:1:1) and placed under greenhouse conditions that averaged 20 to 30 °C with a high relative humidity (90% to 100%).

Data collection. We observed the existence of root formation and visually counted the numbers of shoot and inflorescence. The shoot proliferation ratio and rooting are defined as follows:

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

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

Variance analysis was conducted using Costat (CoHort Software, Monterey, CA). Duncan's multiple range test was used for mean separation when significant treatment effects were present (Duncan, 1955).

Results and Discussion

Micropropagation. Multiple shoots developed from the meristems within 2 weeks of culture initiation in the semisolid culture systems containing 0.45 μM TDZ. However, in subsequent subcultures on semisolid medium supplemented with 0.45 μM TDZ, multiple shoot proliferation was slow (Table 1; proliferation = 1.20 times) and the lower leaves turned yellow and exhibited wilting symptoms. We attempted to increase the proliferation and growth of multiple shoots in *B. oldhamii* by using liquid culture supplemented with 0.45 μM TDZ and found that the multiple shoots grew well and with high rates of proliferation (Table 1,

Table 1. Effect of culture conditions on multiple shoot proliferation on MS basal medium supplemented with 0.45 μM thidiazuron.

	First subculture (21 d)	Second subculture (42 d)	Third subculture (63 d)
Semisolid	1.39 b	1.21 b	1.19 b
Liquid + shaking	2.70 a	1.53 b	1.42 b
Liquid	2.20 a	2.13 a	1.99 a

Green multiple shoot in one experiment were further subcultured for 21 d and then transferred to fresh medium with same concentration (first subculture, 21 d; second subculture, 42 d; third subculture, 63 d). Means followed by the same letter are not significantly different (least significant difference test, $P < 0.05$; Duncan, 1955).

Fig. 1A). With the exception of the first subculture, shaking did not increase the proliferation of multiple shoots in the long term (Table 1).

Albino mutants were also observed in the liquid medium supplemented with 0.45 μM TDZ (Fig. 1B). These mutants were able to grow and proliferate in the same medium as their normal counterparts (Fig. 1C). Albino mutants also occurred in another system (Lin and Chang, 1998). Unlike the high percentage and early occurrence (second subculture) of albino mutants in *B. edulis* culture (Lin and Chang, 1998), there were only three mutants generated in subsequent cultures. Compared with *B. edulis*, the mechanism of the mutagenesis is also different in *B. oldhamii*. The albino mutants of *B. edulis* were generated because of the deletion in the chloroplast genome. In *B. oldhamii*, by using the genomic polymerase chain reaction method, there was no deletion in these mutants (data not shown). This phenomenon indicates that the mechanisms of albino generation are different in *B. edulis* and *B. oldhamii* (Liu et al., 2007).

The presence of 0.45 to 4.54 μM TDZ promoted shoot proliferation in each of the 21-d subcultures (Table 2). The highest shoot proliferation occurred in 2.27 μM TDZ. In subsequent experiments, multiple shoots were incubated in liquid MS basal medium supplemented with 0.45 μM TDZ.

Cytokinins other than TDZ also enhanced shoot proliferation. Although the highest shoot proliferation was found in the first subculture in medium supplemented with 22.2 μM BA, there was no significant difference among TDZ, zeatin, and kinetin tested (Table 3). The cytokinin 2-ip could not enhance shoot proliferation in the second and third subcultures (Table 3).

Fig. 1. Liquid culture of *Bambusa oldhamii*. (A) Normal shoot growth after 21 d of culture in liquid medium supplemented with 0.45 μM thidiazuron (TDZ) (bar = 2 cm). (B) Albino shoots regenerated in liquid medium with 0.45 μM TDZ after 21 d (bar = 2 cm). (C) Albino shoots proliferating in liquid medium with 0.45 μM TDZ after 21 d (bar = 2 cm).

Table 2. Effect of thidiazuron (TDZ) concentrations on shoot proliferation.

TDZ (μM)	First subculture (21d)	Second subculture (42 d)	Third subculture (63 d)
0	1.18 c	1.04 c	1.34 c
0.05	1.68 c	1.54 c	1.55 c
0.45	2.64 b	2.63 b	2.49 ab
2.27	4.90 a	5.80 a	2.95 a
4.54	2.71 b	3.50 b	1.90 bc

Cultures were grown in light condition on MS basal medium containing 30 g·L⁻¹ sucrose. Green multiple shoot in one experiment were further subcultured for 21 d and then transferred to fresh medium with same concentration (first subculture, 21 d; second subculture, 42 d; third subculture, 63 d). Means followed by the same letter are not significantly different (least significant difference test, $P < 0.05$; Duncan, 1955).

The addition of the auxin NAA to the liquid culture medium increased the percentage of rooting. Only 22% of the multiple shoots rooted on hormone-free semisolid medium; this percentage increased to 78% after the explants were transferred to liquid medium containing 26.85 μM NAA (Fig. 2A) with the roots developing from the base of the young shoots (Fig. 2B). All of the auxins tested enhanced rooting, with the exception of 2,4-D, picloram and dicamba at the higher concentration (Table 4). NAA was the most effective auxin for root induction, and there was no difference between 10.74 and 26.85 μM . The auxin treatments affected the morphology and length of the roots. NAA treatment resulted in more and longer roots relative to the other auxins (Table 4). Although the lower concentration (9.05 μM) of 2,4-D, picloram, and dicamba increased rooting and root number, it inhibited root length (Table 4, Fig. 2C).

The results showed that multiple shoots proliferated in the liquid MS basal medium supplemented with 0.45 μM TDZ than rooting in the liquid MS medium supplemented with 26.85 μM NAA. This liquid axillary shoot proliferation protocol was repeated three times. The multiple shoots were subcultured for 1, 3, or 8 years before the rooted plantlets were transferred to a greenhouse and eventually a field. There was no significant difference between the plantlets derived from 3-year and 8-year subcultures. According to these data, this protocol could be used in the commercial propagation for long-term propagation. In *B. edulis*, after 8 years of subculture, many somaclonal varieties were produced (Lin et al., 2005). This phenomenon is valuable for physiological investigation; however, the mutant plantlets have no commercial value. In the future, we will apply this protocol to *B. edulis* to control the somaclonal variation.

The plants derived from tissue culture were more vigorous than those derived from the traditionally vegetative propagated plants (Table 5). After 1 year of growth, the plants derived from tissue culture were taller and had more shoots than their traditionally propagated counterparts.

In vivo flowering. The original plants derived from tissue culture flowered in Jan. 2006. In 2006 and 2007, 20% of the traditionally propagated (air layering and cutting) plants of *B. oldhamii* flowered in the field of southern Taiwan (A.S. Chen, personal communication). Interestingly, 15 plants derived from this protocol flowered after 2 years of growth in the field (40% [15 in 38]). The flowers were normal in appearance and consisted of a lemma and palea containing six stamens and an ovary but no seed formation.

Conclusion

A protocol for the liquid multiple shoot proliferation of bamboo was established using

Table 3. Effect of cytokinin on shoot proliferation of *Bambusa oldhamii*.

Cytokinin (μM)	First subculture (21d)	Second subculture (42 d)	Third subculture (63 d)
—	1.28 b	1.11 c	1.11 c
2-ip (24.1)	1.28 b	1.08 c	1.23 bc
BA (22.2)	3.11 a	2.20 a	1.85 ab
Kinetin (23.2)	2.25 ab	1.92 ab	1.78 ab
TDZ (0.45)	2.24 ab	2.17 ab	2.34 a
Zeatin (22.8)	1.64 b	1.85 ab	1.44 bc

Cultures were grown in light on MS basal medium containing 30 g·L⁻¹ sucrose. Green multiple shoot in one experiment were further subcultured for 21 d and then transferred to fresh medium with same concentration (first subculture, 21 d; second subculture, 42 d; third subculture, 63 d). Means followed by the same letter are not significantly different (least significant difference test, $P < 0.05$; Duncan, 1955).

2-ip = N⁶-[2-isopentenyl] adenosine; BA = 6-benzylaminopurine; TDZ = thidiazuron.

Fig. 2. Effect of auxins on the rooting of *B. oldhamii* shoots cultured in liquid medium. (A) Root formation after a 21-d culture in medium supplemented with 26.85 μM α -naphthaleneacetic acid (NAA) (bar = 0.5 cm). (B) Root formation after a 14-d culture in the medium supplemented with 28.54 μM indole-3-acetic acid (IAA). *Root. (C) Root formation in medium supplemented with 9.05 μM 2,4-dichlorophenoxyacetic acid (2,4-D).

TDZ and vegetative tissues from BaMV-free selected elite field-grown plants. Multiple shoot proliferation was higher in stationary

liquid culture than on semisolid medium. These multiple shoots could provide the material for callus initiation. The tissue

Table 4. Effect of different auxins on rooting.

Auxin ^z	Concn (μM)	Rooting (%)	No. of roots	Root length (cm)
—		25 cd	0.35 bc	1.50 de
NAA	10.74	83 a	3.39 a	7.19 a
	26.85	83 a	4.28 a	7.75 a
IAA	11.42	61 ab	1.50 b	3.85 bc
	28.54	61 ab	1.44 b	4.69 b
IBA	9.80	42 bc	1.31 b	2.49 cd
	24.60	45 bc	1.05 bc	2.18 cde
Picloram	8.28	52 abc	1.03 bc	0.35 de
	20.70	0 d	0.00 c	0.00 e
2,4-D	9.05	56 abc	1.89 b	0.57 de
	22.62	29 cd	1.18 b	0.07 e
Dicamba	9.05	44 bc	1.28 b	0.39 de
	22.62	33 bc	1.07 bc	0.11 e

Means followed by the same letter are not significantly different (least significant difference test, $P < 0.05$; Duncan, 1955).

^zNAA = α -naphthaleneacetic acid; IAA = indole-3-acetic acid; IBA = indole-3-butyric acid; picloram = 4-amino-3,5,6-trichloropicolinic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; dicamba = 3,6-dichloro-2-methoxybenzoic acid.

Table 5. Effect of propagation methods on the growth of *B. oldhamii* plantlets in the field.

	Ht (cm) ^z	No. of branches
Tissue culture-derived	289 a	5.56 a
Air layering method-derived	161 b	3.78 b

^zData are from the experiment with nine replicates after 1 year of growth in the field ($n = 9$). Means followed by the same letter are not significantly different (least significant difference test, $P < 0.05$; Duncan, 1955).

culture-derived plantlets were more vigorous than the plantlets derived from the traditional method.

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