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AXILLARY SHOOT PROLIFERATION AND *IN VITRO* FLOWERING IN AN ADULT GIANT BAMBOO, *DENDROCALAMUS GIGANTEUS* WALL. EX MUNRO

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Summary

Continuous axillary shoot proliferation and *in vitro* flowering were achieved using single node explants from a mature (over 70-yr-old) field clump of *Dendrocalamus giganteus* (giant bamboo). The shoots proliferated in a basal Murashige and Skoog medium with 6 mg l^{-1} (26.6 μM) N^6-benzyladenine (BA) and 2% sucrose. The rate of shoot proliferation gradually increased to over three-fold before *in vitro* flowering took place. *In vitro* flowering was not the expression of a species-specific mechanism believed to occur during gregarious flowering, as the mother clump did not flower. The rate of shoot proliferation decreased at flowering, accompanied by reversion of flowering. The development of axillary meristems into vegetative or generative shoots depended on the level of BA. The possible role of BA, changes in the rate of shoot proliferation leading to build up, and release of stress in relation to flowering and its reversion are discussed.

Key words: Dendrocalamus giganteus; axillary shoot proliferation; *in vitro* flowering; floral reversion; N^6-benzyladenine; giant bamboo.

Introduction

Environmental cues signal commencement of flowering in many species. However, the signals that bring about flowering in bamboo in nature have remained a mystery. Most species of bamboo have long and unpredictable flowering cycles with some species flowering gregariously at species-specific intervals (Janzen, 1976). Such behavior is speculated to be genetically programmed. It is now known that genes control flowering in plants and that the expression of these genes is due to endogenous or exogenous signals. Some of these genes control the transition of the meristem from a vegetative to a reproductive state, while others control when to flower (Mandel et al., 1992; Hempel et al., 1998). By the generation of transgenic plants it has also been possible to demonstrate that these genes could be expressed in widely different plants (Weigel and Nilsson, 1995). This indicates the basic nature of flowering. Bamboo is reported to flower *in vitro* (Nadgauda et al., 1990); but whether the expression of flowering is due to the triggering of a genetically programmed mechanism or by exogenous factors has not been confirmed, as *in vitro* flowering has been reported only in seedling explants.

We have previously reported on flowering in a population of *Dendrocalamus giganteus* in nature (Ramanayake and Yakandawala, 1998). We now report the observation of *in vitro* flowering in continuously proliferating axillary shoots induced from a non-flowering adult clump over 70 yr old.

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Materials and Methods

Single node segments from healthy, vigorously growing field culms of *Dendrocalamus giganteus* were cultured as described by Ramanayake and Yakandawala (1997). The mother clump bore 58 well-developed culms and 83 stumps of harvested culms among those decaying in the dense undergrowth. The area occupied by the clump had a perimeter of 32 m, although the dead stumps extended well beyond. The clump was reported to be over 70 yr old.

In vitro shoots continuously proliferated for over 3 yr in a basal MS medium (Murashige and Skoog, 1962) with 6 mg l^{-1} (26.6 μM) N^6-benzyladenine (BA), 0.1 mg l^{-1} (0.5 μM) kinetin, 8% coconut water and 3% sucrose (MS1). The rate of shoot proliferation was determined at the onset, 12 mo., 18 mo., and when *in vitro* flowering was induced. A known number of shoots cultured in 6–10 replicate vessels containing 50 ml of MS1 medium were used initially. The number of shoots that developed at the end of subculture cycles in all vessels, originating from each replicate vessel, was used to calculate the mean shoot number per vessel and the total shoot number that developed at the end of five to six consecutive subculture cycles. The growth rate was calculated from the regression line obtained by plotting Ln (mean shoot number) versus number of subculture cycles.

The shoot proliferation medium, MS1, was modified with sucrose at 2%, 3%, and 4%, or by replacing BA with thidiazuron [1-phenyl-3-(1,2,3,-thiadiazol-5-yl)urea] (TDZ) at 1.5 or 3 mg l^{-1} (13.6 or 6.8 μM, respectively) or by eliminating coconut water. Six replicate vessels with a known number of shoots were used. The number of shoots per vessel was counted at the end of four to six successive subculture cycles. One-way analyses of variance or the Student's *t*-test was performed as required to analyze the effects of treatments.

After 6 mo., the proliferating shoots were maintained in MS2 medium, which was MS1 medium modified by eliminating coconut water and lowering sucrose from 3% to 2%.

When inflorescences (spikelets) were induced *in vitro*, the number of culture vessels bearing spikelets and the number of spikelets per vessel were counted every time shoots were subcultured. When the spikelets showed signs of no further development, 50 were removed and the spikelet length, number of florets per spikelet, number of stamens, anther and pistil length, and color were observed; and also acetocarmine (0.5% in 45% acetic acid)

TABLE 1

EFFECT OF SUCROSE ON SHOOT PROLIFERATION IN *DENDROCALAMUS GIGANTEUS*

Subculture cycle	Mean shoot number per vessel		
	2% sucrose	3% sucrose	4% sucrose
At 5 mo.			
0[a]	7.0 ± 0.4	7.2 ± 0.76	6.5 ± 0.6
1	10.8 ± 1.0	12.6 ± 1.2	10.2 ± 1.1
2	16.4 ± 2.0	19.6 ± 1.7	12.7 ± 1.9
3	15.4 ± 2.0	14.8 ± 0.8	10.5 ± 1.8
4	17.8 ± 1.8	21.2 ± 1.7	11.6 ± 3.7
5	34.3^{z} ± 6.2	39.2^{z} ± 2.2	13.3^{y} ± 3.1
At 12 mo.			
0[a]	7.2 ± 0.2	6.6 ± 0.6	5.8 ± 0.4
1	19.1 ± 3.8	16.2 ± 1.6	12.3 ± 1.8
2	19.1 ± 2.4	30.2 ± 3.9	26.2 ± 2.2
3	21.4 ± 2.0	26.8 ± 1.4	27.3 ± 1.2
4	24.2^{y} ± 1.3	45.6^{y} ± 5.1	111.3^{z} ± 16.6

Treatment means with the same letter along a row are not significantly different.

[a] Beginning of subculture 1.

TABLE 2

EFFECT OF BA ON SHOOT PROLIFERATION IN *DENDROCALAMUS GIGANTEUS* (29 MO. AFTER CULTURE INITIATION)

Subculture cycle	Mean shoot number per vessel in BA levels[a]			
	0 mg l^{-1}	3 mg l^{-1}	6 mg l^{-1}	12 mg l^{-1}
0[b]	16.8 ± 1.1	17.8 ± 1.6	18.8 ± 1.7	18.0 ± 1.2
1	14.0^{x} ± 1.0	29.8^{y} ± 3.5	30.3^{z} ± 3.1	38.0^{z} ± 4.5
2	10.6^{y} ± 1.3	18.4^{y} ± 4.9	38.0^{y} ± 3.4	45.9^{z} ± 7.7
3	0.0^{x}	17.5^{x} ± 2.8	35.2^{y} ± 1.1	61.6^{z} ± 9.5
4	0.0^{x}	25.5^{y} ± 3.0	49.1^{z} ± 1.0	50.2^{z} ± 3.5

Mean shoot numbers with the same letter along a row are not significantly different.

[a] Mean shoot number per vessel at the end of a subculture cycle.

[b] Beginning of subculture 1.

squashes of microspores were made. For comparison, spikelets bearing florets at anthesis were examined from a field clump that flowered in the locality of the mother.

The non-flowering shoot clusters were separated and cultured in MS2 medium with different levels of BA (0, 3, 6, and 12 mg l^{-1}; 0, 13.3, 26.6, and 53.2 μM, respectively), in six replicate vessels to determine the effect of BA on flowering. The shoots and spikelets that developed were counted when they were subcultured. The mean shoot number per vessel and the total number of shoots that developed during six subculture cycles were used to analyze the effect of BA.

Development of isolated *in vitro* spikelets cultured on semisolid medium containing MS2 with agar and BA at 3 or 6 mg l^{-1} (13.3 or 26.6 μM) was observed.

Results and Discussion

In vitro *shoot proliferation*. A 70-yr-old clump of *Dendrocalamus giganteus* responded by continuous axillary shoot proliferation. Previously our group obtained proliferation only from seedling (Rajapakse, 1992) and juvenile buds explanted during the appropriate rainfall seasons (Ramanayake and Yakandawala, 1997).

The rate of shoot proliferation increased from an initial 1.6-fold per 9–14-day subculture cycle to 3.25-fold just before *in vitro* flowering, after which it dropped to 1.73-fold. During flowering after 29 mo. in culture the shoots turned brittle and were often damaged.

The effect of media. As shown in Table 1, a high level of sucrose (4%) adversely affected shoot growth initially (only ca. 13.3 shoots compared to 34.3 and 39.2 shoots in 2 and 3% sucrose, respectively, at 5 mo.). However, 12 mo. later when the rate of shoot proliferation increased, the shoot number doubled with 2, 3, and 4% sucrose to *c.* 24.2, 45.6, and 111.3 shoots, respectively.

Surprisingly, the shoot number significantly increased from 24 ± 1.3 to 65.6 ± 12.7 with the elimination of coconut water after 6 mo. of culture. The replacement of BA (6 mg l^{-1}) with TDZ did not improve shoot development, although TDZ was reported to be beneficial for *D. strictus* (Singh et al., 2000). After three subculture cycles the 30.5 ± 2 shoots in 1.5 mg l^{-1} TDZ were significantly the highest compared to 21.4 ± 2 shoots formed in BA. But those in 3 mg l^{-1} TDZ developed into compact masses of a large number of buds, which remained small and turned brown during long subcultures.

The level of BA affected shoot development. Shoots did not survive in the absence of BA, as also observed in *Bambusa tulda* (Saxena, 1990) and *D. asper* (Arya et al., 1999). The lowest shoot number per vessel (25.5) developed in 3 mg l^{-1} BA after four subculture cycles (Table 2), and the shoot number per vessel in 6 mg l^{-1} (49.1) and 12 mg l^{-1} (50.2) BA did not differ significantly. However, the total number of 633 shoots that formed in 6 mg l^{-1} BA was higher than the 231 shoots in 12 mg l^{-1} BA after six subcultures (data not shown). This was because the compact clusters of small shoots that were produced in the higher BA level made it difficult to separate during subculture, and they developed at a slower rate than those in the lower level. Thus, 6 mg l^{-1} BA was the optimum level for shoot proliferation of both adult (this study) and juvenile shoots reported earlier (Ramanayake and Yakandawala, 1997). This level is higher than that used in other bamboo species, e.g. 22 μM (0.5 mg l^{-1}) for *B. arundinacea*, *D. brandisi*, and *D. strictus* (Nadgauda et al., 1990, 1997), 8×10^{-6} *M* (1.8 mg l^{-1}) for *B. tulda* (Saxena, 1990), 2 mg l^{-1} for *D. strictus* (Ravikumar et al., 1998), and 3 mg l^{-1} for *D. asper* (Arya et al., 1999).

In vitro *flowering*. A most significant event that occurred after a long time, 29 mo., in culture was flowering. Inflorescences (spikelets) were observed among the vegetative shoots. Although only two or three spikelets per cluster appeared at first, larger clusters of over 10 spikelets with only a few vegetative shoots developed later (Fig. 1C). *In vitro* flowering in bamboo was first reported by Nadgauda et al. (1990) in *B. arundinacea*, *D. brandisii*, and *D. strictus*. It was first reported in *D. giganteus* by Rajapakse (1992). Other species of bamboo have also flowered *in vitro* (Chambers et al., 1991; Rout and Das, 1994; Gielis et al., 1997). Except for Gielis et al. (1997), all others reported precocious flowering in juvenile shoots derived from seedlings. This is the first report of continuous shoot proliferation and *in vitro* flowering in a tropical adult bamboo (70 yr old).

In vitro spikelets and florets were basically similar to specimens off an unrelated field clump examined at the same time and to those observed earlier (Ramanayake and Yakanadawala, 1998), except for some differences and abnormalities (Fig. 1A, B). The lemma of *in vitro* florets tapered to a point and the margins opened out, unlike

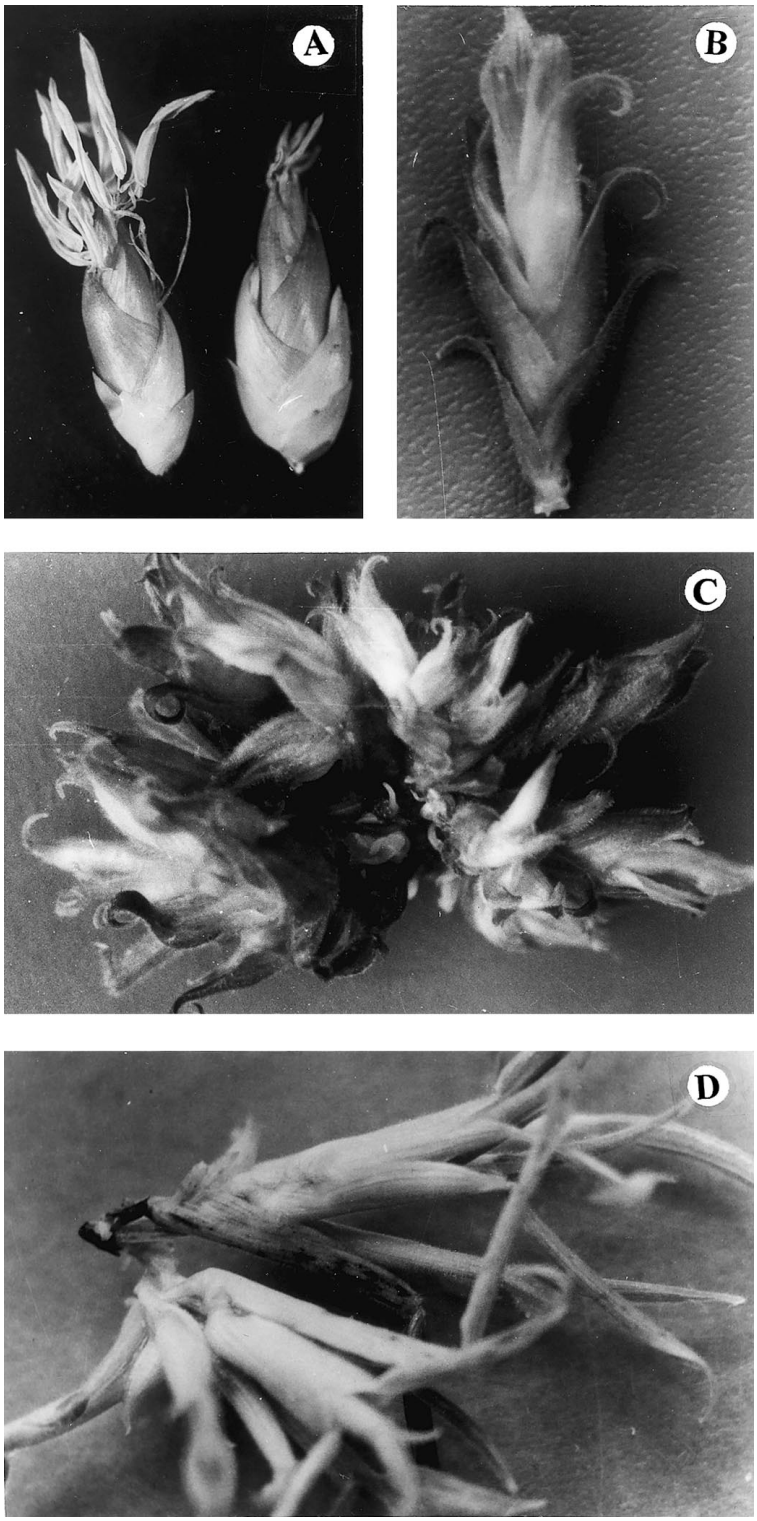

FIG. 1. Flowering in *Dendrocalamus giganteus*. *A*, Fully developed *in vivo* spikelets with anthers exserted (×2.5); *B*, fully developed *in vitro* spikelet (there is no anthesis) (×3); *C*, a cluster of *in vitro* spikelets (×3); *D*, vegetative shoots formed during floral reversion (×3).

those in field clumps where the lemma was concave. Spikelets were narrower and longer due to a loose arrangement of florets, and varied more in length (17.0 ± 2.6 mm) than field specimens (15.3 ± 0.6 mm). The mean floret number per spikelet was higher *in vitro* (6.1 ± 1.0) than *in vivo* (5.1 ± 0.2). In *B. arundinacea* too, *in vitro* spikelets had a wider variation in length, and had more florets that were smaller than those *in vivo* (Nadgauda et al., 1997).

Florets in the field had a constant number of six stamens, whereas

it ranged from 0 to 12 *in vitro*, with only 34% bearing six stamens. Anthers did not dehisce *in vitro* as they did in the field. *D. strictus* anthers also did not dehisce when it flowered *in vitro* (Singh et al., 2000). In *B. arundinacea*, anther dehiscence *in vitro* was limited to a few florets and pollen viability was low (Nadgauda et al., 1997). Acetocarmine squashes of microspores in field specimens of *D. giganteus* stained pink and had thick sculptured walls. Those *in vitro* did not stain, were empty, and were lens-shaped due to collapsed thin walls. Nadgauda et al. (1997) and Singh et al. (2000) also made similar observations. Although most *in vitro* florets were normal with a single pistil, a relatively high proportion (24%) had double pistils and a few (4%) were staminate. Such abnormalities were rare in the field. Neither the style nor the stamens of *in vitro* florets elongated as seen in field clumps during anthesis (Fig. 1A, B). There was no seed set, but seed set is rare in this species in nature (Ramanayake and Yakandawala, 1998). Singh et al. (2000) also observed that florets did not open fully, the stigma remained within and there was no seed set *in vitro*. In *B. arundinacea*, stamens and pistils were shorter *in vitro* and anthesis was not complete in many florets (Nadgauda et al., 1997). However, seed set occurred to a low extent.

Possible causes of flowering. The gregarious flowering of bamboo has been attributed to a species-specific internal clock (Janzen, 1976). Thus, the *in vitro* flowering reported in seedling-derived shoots was held to be the expression of precocious cohorts (Banik, 1994). If this were so, the mother clump of our study should have flowered *in vivo* concomitantly with its descendent shoots *in vitro*. But the mother clump did not flower. Axillary shoot cultures of a 6-yr-old juvenile clump of the same species also flowered, while the mother culms in the field were not flowering (unpublished). Gielis et al. (1997) made similar observations in temperate species of bamboo. Therefore it is likely that physiological factors have indeed caused flowering.

A cytokinin, mainly BA, has been a medium constituent in many reports of *in vitro* flowering, e.g. ginseng (Chang and Hsing, 1980), *Phaleanopsis* (Duan and Yazawa, 1995), rattan (Ramanayake, 1999), and *Murraya paniculata* (Jumin and Ahmad, 1999). Coconut water, also known to contain cytokinins, was once thought to be the cause of *in vitro* flowering when it was first reported in bamboo (Nadgauda et al., 1990). Adenine sulfate and TDZ too have been present during *in vitro* flowering in bamboo (Rout and Das, 1994; Singh et al., 2000). Another cytokinin, isopentyladenine, and polyamines induced *in vitro* flowering in *Cichorium intybus* (Bais et al., 2000). Tanaka et al. (1995) showed that kinetin and IAA, which induce *in vitro* flowering in thin cell layers of tobacco, can also induce the expression of the *S*-adenosyl-L-homocysteine hydrolase gene which encodes a cytokinin-binding protein formed during early flower bud formation. Zeatin riboside and isopentyladenine were shown to increase in the shoot apex shortly after floral induction in *Sinapsis alba* (Bernier et al., 1993). Therefore it is evident that cytokinins are implicated in the floral transition.

The level of BA affected the morphogenetic pathway of axillary meristems during flowering. With the optimum level of BA ($6\,mg\,l^{-1}$) for shoot proliferation, the ratio of spikelets to vegetative shoots (65/633) showed that development was mainly vegetative. At non-optimal levels of 3 or $12\,mg\,l^{-1}$ vegetative development was suppressed when 66 spikelets/71 vegetative shoots and 63 spikelets/231 vegetative shoots formed, respectively.

BA induced only vegetative shoots for a long period of $2\frac{1}{2}$ yr, indicating that specific flowering signals developed later. Gielis et al. (1997) observed that bamboo under stress conditions of high light intensities and drought flowered, while those in more favorable conditions grew vegetatively. They also found that superoxide dismutase, ascorbate peroxidase, and catalase, which generally form during oxidative stress, were higher in reproductive tissues than in vegetative tissues. Shoot cultures are under stress when overcrowded. The rise in shoot proliferation observed with progressive subculture could lead to the development of too many shoots in vessels. Seedling tissues of bamboo are also known to proliferate rapidly, but the changes in their rate of shoot proliferation with *in vitro* flowering have not been reported. Once flowering was induced there was a drop in shoot proliferation. At this time floral reversion took place. Floral reversion does not normally happen in nature, unless growth has been disturbed by unusual environmental conditions or when flowering occurs out of season (Battey and Lynden, 1990). Reversion of flowering and incomplete development of florets with the absence of viable pollen observed in *D. giganteus* shoot cultures indicate that floral induction took place under physiological conditions that were not stable or fully conducive for flowering.

Recent studies in *Arabidopsis thaliana* have identified a large number of genes that control flowering. A model comprising four pathways of flowering has been put forward (Levy and Dean, 1998; Pineiro and Coupland, 1998). Bamboo could also have several pathways of flowering. Although flowering in nature in bamboo is unpredictable it is more frequent *in vitro*. Therefore, the study of factors involved in the pathway that lead to *in vitro* flowering could lead to the development of a predictable system of flowering in this group of plants.

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