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ORIGINAL PAPER

Callus induction and shoot organogenesis from anther cultures of *Curcuma attenuata* Wall

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Abstract *Curcuma attenuata* is a highly valued ornamental. This study provides the first report on *C. attenuata* shoot organogenesis and plant regeneration. Immature anthers derived from 5 to 7 cm long inflorescences were isolated and cultured on different variations of Murashige and Skoog (MS) media to induce callus and then shoot organogenesis. When the 2-mm long anthers in which microspores were at the uninucleate developmental stage were cultured in the dark on MS medium containing 13.6 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.3 μM kinetin (KT) for 15 days and then transferred to 40 μmol m^{-2} s^{-1} fluorescent light for 30 days, the percentage callus induction reached 33.3 %. After callus was transferred to various differentiation media and cultured in the light, 33.1 % of all callus cultures could differentiate into adventitious shoots on MS medium supplemented with 22.0 μM 6-benzyladenine (BA), 0.53 μM α-naphthaleneacetic acid (NAA) and 1.4 μM thidiazuron (TDZ) after culturing for 60 days. Over 95 % of plantlets survived after transplanting plantlets into trays with a mixture of sand and perlite (2: 1) for 20 days. Chromosome number, determined from the root tips of young plantlets, indicated that all plantlets were diploid (2n = 84).

Keywords Callus differentiation · Plant regeneration · Shoot propagation · Root formation · Transplanting

Abbreviations

BA	6-Benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
NAA	α-Naphthaleneacetic acid
TDZ	Thidiazuron
KT	Kinetin
PGR	Plant growth regulator
MS	Murashige and Skoog

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Introduction

The genus *Curcuma* (Zingiberaceae) comprises more than 80 species of rhizomatous perennial herbs and is widespread in tropical Asia, extending to Africa and Australia (Purseglove et al. 1981). Within the genus *Curcuma*, *Curcuma attenuata* Wall is a highly valued ornamental, medical and edible plant which is indigenous to Burma and Thailand (Wallich 1828; Apavatjrut et al. 1996; Schaffer et al. 2011). It is considered as a novel ornamental plant in the floricultural market and grows well after having been introduced to Guangzhou, China. It is usually used in tropical and subtropical landscape design as a potted,

foliage and flower plant. It has a long flowering period (April to November) and vase cut flowers can last 20 days in Guangzhou. For these reasons it has seen an increasing demand in the world ornamental market.

Curcuma attenuata is a polyploid with 2n = 84 (chromosome number) and can be multiplied by seminal propagation (i.e., asexual division) of the rhizome (Apavatjrut et al. 1996; Jana et al. 2007). However, seeds obtained are limited, so usually traditional multiplication involves propagation by rhizome. Since it is difficult to keep pace with the demands created by market exploitation, it is thus essential to establish an efficient propagation and regeneration system, preferably in vitro, to ensure the clonal propagation of desired genotypes. Tissue culture and plant regeneration in vitro have not yet been reported for *C. attenuata*.

In this study, anthers were used as explants to induce callus, which was then used to form shoots and roots, i.e., plantlets. This study reports on an efficient plant regeneration and mass propagation system for *C. attenuata*.

Materials and methods

Plant material

Donor plants were grown in an open field in Guangzhou Agricultural Technology Promotion Center and South China Botanical Garden, China. The experiments were performed in summer (May to July). Immature inflorescences 5-7 cm long were used as explants (Fig. 1a).

Callus induction from immature anther culture

The surface of immature inflorescences was wiped clean with 70 % alcohol and cut into 2.5–3.5 cm long sections with a blade, disinfected with 0.2 % NaClO for 15 min then with 0.1 % $HgCl_2$ for 8–10 min, and then immersed in sterile distilled water 5–8 times and blotted dry on absorbent paper towels. The anthers (2 mm long at the uninucleate stage) were excised using tweezers and a sharp scalpel, and then inoculated onto 90 mm diameter Petri dishes containing Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) and different plant growth regulators (PGRs) (Table 1). Each dish was inoculated with 40 anthers and every treatment contained 200 anthers. All dishes contained 10 ml of agarified medium. All media contained 30 g l^{-1} sucrose and pH was adjusted to 5.8 with 1.0 N HCl or 1.0 N NaOH before adding 0.7 % agar (Sigma, USA) and autoclaving at 121 °C for 15 min. Dishes were placed in a culture room at 27 ± 2 °C in the dark for 15 days, and then exposed to 40 μmol m^{-2} s^{-1} fluorescent light in a 14-h photoperiod. Callus induction was investigated after culture for a total of 30 days.

Shoot organogenesis from callus culture

After anthers were cultured for a total of 80 days, callus that proliferated was transferred to jars (12 cm high and 8 cm in diameter) with different media (Table 2) for shoot organogenesis in the same light conditions as for callus induction. Shoot organogenesis was investigated in this process, which lasted 60 days. The experiments were repeated three times within 2 weeks.

Multiple shoot proliferation

The adventitious shoots pooled from shoot induction media were transferred equally to MS medium containing 6-benzyladenine (BA) (8.8 μM), α-naphthaleneacetic acid (NAA) (5.3 or 2.69 μM) and thidiazuron (TDZ) (1.4 μM) in some permutations and cultured in light, as described above (Table 3). Shoot proliferation was investigated after culturing for 45 days. The shoot proliferation coefficient (SPC) was calculated as the number of adventitious shoots after inoculation/number of adventitious shoots before inoculation. The experiments were repeated three times within 1 week.

Root formation and transplantation of plantlets

Adventitious shoot clumps were divided into single or very few shoots that were equally transferred to different MS media containing 2.69 μM NAA and/or 2.2 μM BA or no PGRs to induce roots (Table 4). After culturing for 30 days, root formation was investigated. In total, 300 well-developed, regenerated plantlets longer than 5 cm with 2–3 leaves were transplanted into a tray (50 × 40 × 10 cm) with a mixture of sand: perlite (2: 1). The tray was placed in a shed covered with a black net cutting 90 % of light and plantlets were irrigated with tap water once a day.

Data analysis

The callus induction culture experiments were conducted with a minimum of three replicates and all experiments were repeated three times. The data were reported as mean ± standard error. Means were analysed by Analysis of Variance and significant differences between means were compared by the Least Significant Difference (LSD) test using SPSS v. 17.0. For all comparisons, statistical significance was considered at $P \leq 0.05$.

Determination of chromosome number

Young root tips excised from 30 plantlets derived from all treatments between 10:30 and 11:30 am in spring, the period in which root tips are most actively growing. For

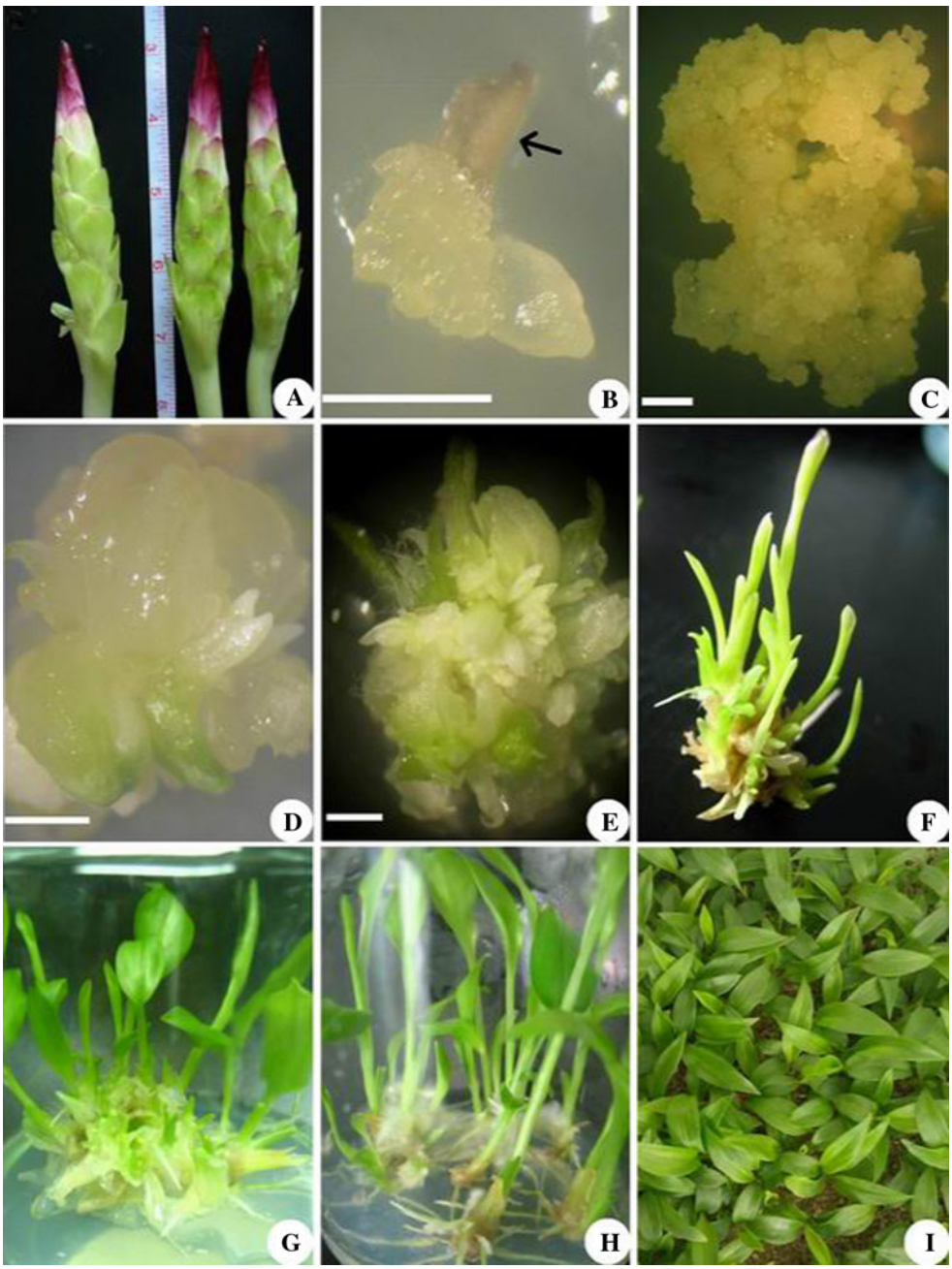

Fig. 1 Callus induction and shoot organogenesis from anther culture of *Curcuma attenuata* Wall. **a** Immature inflorescence used for culture explants or as the edible part; **b** Callus induction from anther culture on MS medium supplemented with 13.6 μM 2,4-D and 2.3 μM KT after cultured for 20 days (*black arrow* indicates that the anther did not open showing that the callus originated from the anther's surface and not from inner tissue); **c** Callus proliferation on the medium supplemented with 22.2 μM BA and 0.53 μM NAA; **d** Differentiation of callus on MS medium supplemented with 22.2 μM BA, 0.53 μM and 1.4 μM TDZ after culture for 60 days; **e** Adventitious shoot formation from callus after culturing for 80 days on MS medium supplemented with 22.2 μM BA, 0.53 μM NAA and 1.4 μM TDZ; **f** Shoot proliferation on MS medium supplemented with 4.4 μM BA and 1.06 μM NAA after culturing for 30 days. **g** Proliferation of adventitious shoots on MS medium supplemented with 8.8 μM BA, 2.69 μM NAA and 1.4 μM TDZ after culture for 45 days; **h** Root formation on MS medium supplemented with 2.2 μM BA and 2.69 μM NAA; **i** Transplanted in vitro-derived plantlets in a tray (*bars* = 2 mm)

squashes, the metaphase chromosomes were evaluated using root tips (Zhang et al. 2010). The material was pre-treated with 0.002 M 8-hydroxyquinoline for 2–3 h, a saturated aqueous solution of paradichlorobenzene for 4–5 h, or an ice water mixture solution for 24 h at 4 °C. Root tips were fixed with freshly prepared Carnoy fluid

Table 1 Effects of different PGRs on callus induction from anther culture on MS media after culturing for 15 days in darkness and then 30 days in light

PGRs in induction media (μM)					Callus induction (%)*	Observed results
2,4-D	KT	BA	NAA	TDZ		
4.5					56.3a	Callus, friable, slow growing
	4.5				0f	Swollen, necrosis
			5.4		18.7c	Callus, roots
		4.4			0f	Swollen, necrosis
				4.5	0f	Swollen, necrosis
4.5				0.14	9.7d	Granular callus, white
4.5		4.4		0.14	3.3e	Yellowish soft callus, fast growing
4.5		13.2			3.7e	Swollen and little callus
		26.4	0.1		10.2d	Swollen, white soft callus
13.6	0.9				15.6c	Soft callus, yellowish
13.6	2.3				33.3b	More soft callus, yellowish

*Different letters within the column (Callus induction %) indicate significant differences according to the LSD test ($P \leq 0.05$) following ANOVA (% values not arc-sine transformed)

Table 2 Effects of different PGRs on shoot organogenesis from the anther induced callus on MS media after culturing for 60 days in light

PGRs in induction media (μM)			Shoot organogenesis (%)*	Observed results
BA	NAA	TDZ		
0	5.3	0	0e	Little callus and slim roots
4.4	0	0	0e	Callus turned brown and died finally
0	0	4.6	0e	Swollen callus, granular
8.8	0	1.4	0e	Callus proliferation, granular
13.2	2.69	0	8.3d	Callus turned green; roots and shoots
22.2	0.53	0	11.6c	Shoots
13.2	2.69	1.4	25.0b	Shoots
22.2	0.53	1.4	33.1a	Shoots
8.8	2.69	1.4	20.0b	Shoots and roots
0	0.53	1.4	0e	Granular callus proliferation, roots

*Different letters within the column (Shoot organogenesis %) indicate significant differences according to the LSD test ($P \leq 0.05$) following ANOVA (% values not arc-sine transformed)

Table 3 Effects of different PGRs on shoot proliferation of *C. attenuata* on MS media after culturing for 45 days in light

PGRs in induction media (μM)			Mean shoot number/explant*	Observed results
BA	NAA	TDZ		
17.8	2.69	0	4.9c	Shoots and roots
8.8	2.69	0	5.0c	Stronger shoots and roots
8.8	0	1.4	6.8b	Stronger shoots
8.8	2.69	1.4	10.2a	Shoots and shorter roots

*Different letters within the column indicate significant differences according to the LSD test ($P \leq 0.05$) following ANOVA

Table 4 Root formation in *C. attenuata* on different rooting media containing different PGRs

PGRs in induction media (μM)		Rooting time (days)	Root number*	Rooting observation
NAA	BA			
0	0	20	4.9c	Short roots
2.69	0	10	10.0b	Long and slim roots
2.69	2.2	7	12.4a	Long and strong roots

*Different letters within the column indicate significant differences according to the LSD test ($P \leq 0.05$) following ANOVA

(ethanol alcohol: glacial acetic acid = 3:1). Another method was to fix root tips with freshly prepared Carnoy fluid immediately after collection for 20–24 h at room temperature. Direct fixing of root tips without any pre-treatment was optimum for detecting metaphase chromosomes. Incidentally, chromosome condensation was also better and centromeres were easily discerned. The fixed samples were hydrolyzed in 1.0 N HCl for 8 min at room temperature after washing with distilled water three times, then stained in 1 % aceto-orcein for 15 min and squashed on a glass slide after being heated slightly. The squashed sections were observed under a microscope (Zeiss Axioplan 2), and photographs were taken with an automatic camera. Ploidy analysis was repeated three times.

Results

Induction of callus and shoot organogenesis from anther culture

Anthers cultured on different media for 15–30 days showed different responses (Table 1): single use of 4.5 μM 2,4-D in the induction medium could induce a high percentage of callus (56.2 %). This callus was compact and friable. Initially, 5.4 μM NAA could induce callus from which some roots emerged within a month. However, 2, 4-D and NAA applied singly could not result in shoot organogenesis from callus in the same time period. The use of 4.5 μM kinetin (KT), 4.4 μM BA or 4.5 μM TDZ alone in induction medium could not induce callus and explants generally necrosed. 2,4-D (4.5 μM), when combined with a low concentration (0.9–2.3 μM) of KT or 0.14 μM TDZ, could induce a wide range of callus (9.7–33.3 %). Alternatively, a low concentration of NAA combined with a high concentration of BA (26.4 μM) could also induce callus (Fig. 1b).

Shoot organogenesis from callus culture

On differentiation media supplemented with only NAA, BA or TDZ (Table 2), callus could not proliferate or differentiate into shoots. When the medium contained 22.2 μM BA, 0.53 μM NAA and 1.4 μM TDZ, most callus proliferated easily (33.1 %) (Fig. 1c) while on medium containing 2.69 μM NAA and 13.2 or 22.2 μM BA, callus could differentiate easily into both shoot buds and adventitious roots. As NAA concentration decreased and BA concentration increased, more adventitious shoots (buds) could differentiate (Fig. 1d, e). When TDZ was added to the medium, callus became granular then developed into adventitious shoots (Table 2). As culture time was extended, one clump of adventitious shoots eventually developed from one callus clump (Fig. 1f).

Multiple shoot proliferation

On propagation medium supplemented with 8.8 μM BA, 2.69 μM NAA and 1.4 μM TDZ, the SPC reached 10.2 after culture for 30 days (Fig. 1g). On MS medium supplemented with 8.8 μM BA and 2.69 μM NAA, the SPC reached 5.0 within 30 days. However, many adventitious roots formed from the base of shoots. As the concentration of BA increased to 17.8 μM, the leaves of adventitious shoots became deformed and shoot growth slowed. When 8.8 μM BA was combined with 1.4 μM TDZ in the propagation medium, SPC reached 6.8, but almost no roots were visible. BA (8.8 μM), TDZ (1.4 μM) or NAA (2.69 or 5.3 μM), when used singly in the shoot propagation media, did not result in adventitious shoot proliferation. However, on medium containing 5.3 μM NAA, adventitious shoots developed a few thin roots and some callus (Table 2).

Root formation and transplantation of plantlets

On medium containing 2.2 μM BA and 2.69 μM NAA, roots formed within 7 days. After culturing for a total of 30 days, each shoot could develop a mean of 12.4 roots (Fig. 1h). When the medium contained only 2.69 μM NAA, some thin roots developed by the 10th day; after culturing for 30 days, each shoot could develop a mean of 10.0 roots. Medium free of PGRs could also develop roots. However, root development was slow and means root number/shoot decreased to 4.9 (Table 4). More than 95 % of a total of 300 plantlets that were acclimatized survived and grew normally within 20 days (Fig. 1i). The entire process from callus induction to fully acclimatized plantlets took approximately 1 year to achieve.

Determination of chromosome number and somaclonal variation

All acclimatized plantlets tested were diploid (2n = 84) (Fig. 2) and no variation among regenerated plants was obvious.

Discussion

A mass propagation and regeneration system has been established for many species of the genus *Curcuma*. Axillary buds have been used as explants for establishing a tissue culture system in *C. longa* (Balachandran et al. 1990; Sit and Tiwari 1997; Prathanturarug et al. 2003a, b, 2005) and *C. alismatifolia* (Udomdee et al. 2003). Rhizome buds

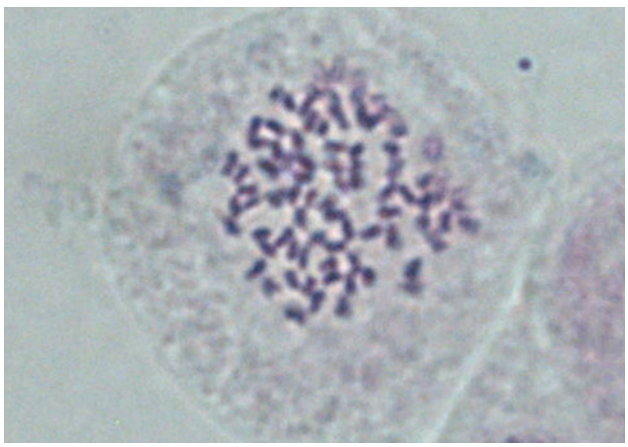

Fig. 2 Chromosome number (2n = 84) of acclimatized *Curcuma attenuata* plantlets (n = 42) indicating it is a diploid

of *C. domestica*, *C. aeruginosa* and *C. caesia* inoculated aseptically on MS medium with varying levels of BA and KT could produce multiple shoots (Balachandran et al. 1990). Freshly sprouted shoots of *C. aromatica* could form new shoots on MS medium supplemented with BA alone (4.4–30.8 μM) or a combination of BA (4.4–22.0 μM) and KT (2.3–4.7 μM) (Nayak 2000). Rhizomes and leaf sheaths of *C. amada* could be used as explants to induce adventitious shoots and in mass propagation (Prakash et al. 2004). In vitro protocols for plantlet regeneration and medium-term genotype conservation of eight wild species of *Curcuma* (*C. aeruginosa*, *C. aromatica*, *C. brog*, *C. caesia*, *C. malabarica*, *C. raktakanta*, *C. soloensis* and *Curcuma* sp.) were optimized, although they were genotype-dependent and were significantly influenced by the type and concentration of cytokinins used (Tyagi et al. 2004). Our study represents the first regeneration system for *C. attenuata*.

Different explants have been used to induce callus and then shoot organogenesis in the genus *Curcuma* (Xu et al. 2006). Different *C. longa* explants (leaf base, root tips and axillary buds from rhizomes) were stimulated by exogenous polyamines, combined with NAA or with BA, to produce callus and its subsequent differentiation (Viu et al. 2009). Callus could be induced from the leaf base of turmeric (*C. longa*) by 2.2 μM BA and 26.5 μM NAA (Salvi et al. 2001) and by NAA (5.4 μM) and BA (8.8 μM) from rhizome buds and shoot tips of *C. longa* (Sunitibala et al. 2001). 2,4-D (9.1 μM), when used singly, could also induce callus from *C. amada* leaves and rhizomes (Prakash et al. 2004). Plant regeneration from callus cultures of *C. aromatica* was possible with 2,4-D (9.1 μM) and 2.3 μM KT (Mohanty et al. 2008). Callus could be induced from the base of adventitious shoots of *C. kwangsiensis* on MS medium containing 1.4 μM TDZ, 4.4 μM BA and 2.3 μM 2,4-D (Zhang et al. 2011). In *C. alismatifolia*, regeneration has been reported from young inflorescences (Wannakrairoj, 1997). Plant regeneration from the culture of immature of *C. longa* inflorescences was possible by direct shoot development on MS basal medium supplemented with BA (22.0 or 440 μM) in combination with 0.9 μM 2,4-D or 0.54 μM NAA and 4.5 or 9.0 μM TDZ in combination with 0.57 μM IAA (Salvi et al. 2000). *C. alismatifolia* inflorescences were used as explants and, when inoculated on MS basal medium containing 44.0 μM BA and 0.57 μM NAA for 1 month, they developed and reverted to vegetative shoots directly from flower organs (or floral buds) and not via callus (Toppoonyanont et al. 2005); moreover, they located at the same positions and were arranged spirally within the bracteole, similarly to *ex vitro* shoots (Udomdee et al. 2003). All of these reports showed that callus were induced from different explants by using of 2,4-D single or NAA in combination with BA, KT or TDZ. However, our study on *C. attenuata* is the first study in the genus *Curcuma* in which regeneration has been shown to be possible from anther culture. There is always the risk, however, that 2,4-D can induce callus that results in regenerants with high levels of somaclonal variation, although this was not the case in this study.

In this study, 2,4-D could induced callus when applied singly or in combination with KT. However, the callus could not differentiate into shoots at later culture stages when 2,4-D and NAA were used alone for callus induction. Furthermore, when NAA was used alone for callus induction, some root-like organization took place. All the cytokinins tested (KT, TDZ or BA) could not induce callus from anther culture (Table 1), although the anthers become swollen. When these were combined with 2,4-D in callus induction media, they decreased callus induction percentage indicating that auxin (NAA) was a key factor to induce callus from anther culture. After callus was induced, both auxins and cytokinins could enhance callus proliferation except for BA (4.4 μM) when used singly. Shoot organogenesis took place under different PGR combinations (Table 2). TDZ (1.4 μM) could enhance differentiation into shoots and shoots, but not when used alone. BA could be used for callus induction, callus differentiation and plantlet proliferation. However, a high concentration of BA (26.2 μM) was needed for callus induction and a low concentration of BA (8.8, 13.2 or 22.2 μM) was necessary for shoot differentiation and plantlet proliferation.

We succeeded in establishing an efficient shoot organogenesis, mass proliferation and plant regeneration system for *C. attenuata* using immature anthers as explants. This study indicates that anther culture was efficient in terms of callus induction and shoot organogenesis without ploidy differences in regenerated plantlets. The use of anthers as explants has never been reported for the genus *Curcuma* and has only reported on another genus *Zingiber officinale* (ginger) in the Zingiberaceae (Samsudeen et al. 2000). This will provide a foundation for mass propagation and future transgenic biotechnological applications and also for haploid breeding.

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