ORIGINAL ARTICLE

Somatic embryogenesis from young spikelets of the giant bamboo *Dendrocalamus asper* (Schult f.) Backer ex Heyne

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Abstract

Bamboos are an important worldwide non-timber forest product with rising interest due to their environmentally friendly applications. Besides its use as edible sweet shoots and culms for structural uses, the giant bamboo, *Dendrocalamus asper* is an imposing ornamental bamboo for horticulture. The present work aimed to establish in vitro callus culture and plant regeneration through somatic embryogenesis starting from young spikelets of *D. asper*. Pre-anthesis spikelets were collected, disinfected, and subjected to callus induction on MS basal medium supplemented by 2,4-Dichlorophenoxyacetic acid (2,4-D) at 0, 9, 18, 27, and 36 μM in combination with 9 μM of 2-iP or 9 μM Kinetin. The different calli types that developed were characterized and subcultured in 0, 4.5, 9, and 18 μM of 2,4-D in combination with 9 μM of 2-Isopentenyladenine (2-iP) or Kinetin for multiplication and somatic embryos regeneration. Additionally, the effect of explant cutting in half and its orientation on the culture medium were tested to improve callus induction The 2,4-D was essential for callus induction, and in combination with the two cytokinins resulted in embryogenic callus induction and somatic embryos regeneration. The subsequent reduction of this auxin to 4.5 μM resulted in somatic embryo maturation. Somatic embryos transferred to a plant growth regulator-free medium resulted in plantlet germination. The present work showed the feasibility of using bamboo spikelets as explants and the efficiency of using 2-iP in combination with 2,4-D to callus induction and in vitro plant regeneration through somatic embryogenesis.

Key message

Immature spikelets of the giant bamboo are feasible explants for in vitro callus induction and somatic embryogenesis.

Keywords Bamboo inflorescence · Callus induction · Micropropagation · Secondary somatic embryogenesis

Introduction

The subfamily Bambusoideae of family Poaceae comprises a wide diversity of bamboos with more than 1680 species (Soreng et al. 2017; Clark and Oliveira 2018), displaying a broad range of applications. The increasing worldwide interest in bamboo is due to their economic and environmental sustainability (Paudyal et al. 2019). Although practically every part of the bamboo is useful for food, medicine, handicrafts, construction, biomass, and primal matter for industry (Liese et al. 2015; Akinlabi et al. 2017), the most elegant application of bamboos is in the landscape ornamentation. Due to the spiritual or cultural values and the unique giant grass characteristics, the ornamental use of bamboos in gardens promotes benefits on physiological and psychological levels for the people (Wang et al. 2021; Zheng et al. 2021).

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Dendrocalamus asper is a tropical non-invasive bamboo with majestic culms that can reach up to 30 m of height and more than 15 cm of diameter. Due to their multipurpose (i.e. edible shoots and timber substitution) and ornamental features (i.e. the giant grass habit and the color dynamic of the culm development), *D. asper* have desired characteristics for horticulture (Li and Kobayashi 2004; Banik 2016).

D. asper can be propagated by seeds, culm cuttings, air-layering, offsets, macroproliferation, and micropropagation (Singh et al. 2012; Zang et al. 2019). Although seeds can be an easy way to obtain seedlings, the flowering cycle of *D. asper* is unpredictable and happens at intervals of 30–100 years. Seeds have short viability and an unknown genetic background (Arya et al. 1999; Banik 2016). Because of that, the vegetative propagation methods are the most practiced. However, they are not feasible for a large-scale plantlet production and large size of some propagules that needs high labor and transport costs (Arya et al. 1999). Further, rooting efficiency varies with seasons and between the genotypes (Ray and Ali 2017a).

For those reasons, micropropagation is desired for large-scale quality plantlets production, with additional advantages for breeding, ex situ conservation, and basic research purposes. Although several bamboo species already have some effort reported for somatic embryogenesis protocols development, most of the studies aiming for bamboo mass propagation are based on the organogenesis pathway (Singh et al. 2013). The microorganisms' manifestation during the in vitro cultures establishment and the wide variability of that cultures responses still being hindrances for the in vitro commercial plant production by both morphogenetic pathways (Ray and Ali 2017a, b).The bamboo in vitro culture

establishment can be reached from different explants, as isolated zygotic embryos, whole seeds, shoot apex, nodal segments, leaves, roots, and inflorescences (Ojha et al. 2009). Inflorescences can be the last somatic resource to in vitro regeneration of selected genotypes in the case of monocarpic flowering (Arya et al. 2008). Due to the scarcity of that kind of explant, few papers reported the flowering tissue employment for bamboos micropropagation. In Table S1 is presented a summary of relevant reports of inflorescence tissue culture approaches for Bambusa and Dendrocalamus species over the last decades. Those previous reports show the importance of the auxin 2,4-D for callus inductions in floral tissues and point to BAP and Kin as the most used cytokinins in the somatic embryogenesis of bamboos (Table S1). Due to the known callogenesis capacity of bamboo inflorecences, and the possibility to further regenerate somatic embryos from them, the present work aimed to establish a callus induction and plant regeneration through somatic embryogenesis from young spikelets of *Dendrocalamus asper*.

Material e methods

Plant material

Branches with young synflorescences (clusters of spikelets) of *Dendrocalamus asper* (Fig. 1a) were collected in pre-anthesis stage. The material were accessed during an event of sporadic flowering of 20 clumps in a plantation of 600 plants, at Fazenda dos Bambus (− 23.110470°;
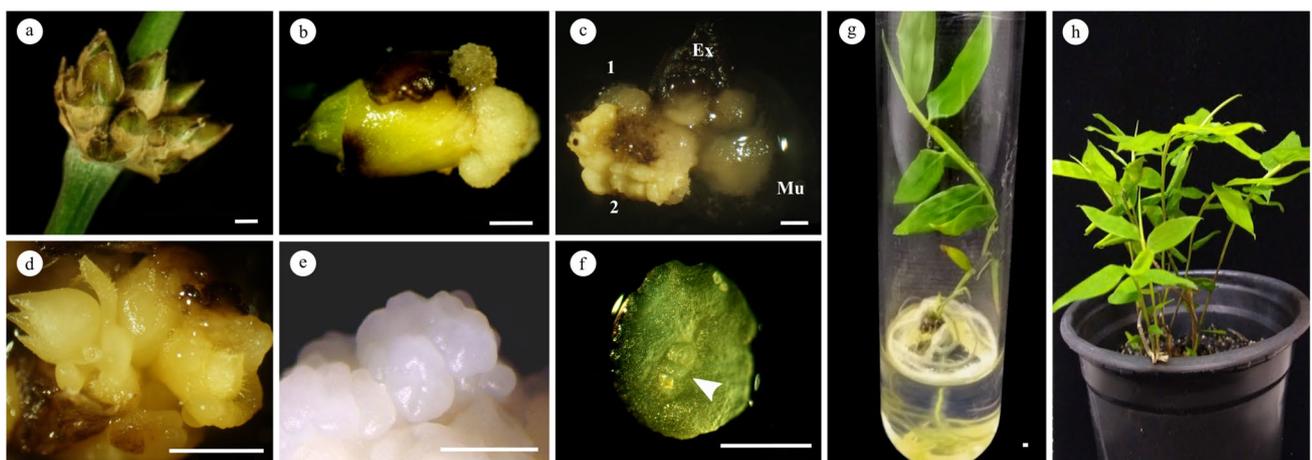
Fig. 1 Somatic embryogenesis from young spikelets of *Dendrocalamus asper*. **a** Synflorescence; **b** Callus induction on the basal region of spikelet after 30 days in vitro. **c** Calli of different morphological characteristics obtained from spikelet after 60 days [1- Type 1 callus, 2- Type 2 callus, Mu- Mucilaginous callus, Ex- Browned explant]; **d** Type 2 callus regenerating an spikelet-like structure. **e** Somatic embryos; **f** Somatic embryo with prominent embryonic axis (arrowhead) surrounded by scutellum tissue; **g** Plantlet germinated from somatic embryo; **h** Acclimatized plantlet. Bars = 1 mm

− 48.368193°), of the Jatobás Institute in the municipality of Pardinho, São Paulo, Brazil.

Explant preparation and disinfection

The explant disinfection method was carried out in three steps: (1) Branches with ± 10 cm containing about 10 developing synflorescences were selected. Prophylls and senescent glumes were removed before the branches were immersed in 70% (v/v) ethanol for two minutes and transferred to sodium hypochlorite solution (2% active chlorine) with Tween™ 20 (1 drop/100 ml) and constantly stirred for 20 min. The branches were then rinsed twice with sterile deionized water and exposed for 10 min to the chamber's airflow on sterile filter paper for drying; (2) Synflorescences were excised from the branches and immersed in 70% (v/v) ethanol for 30 s, followed by immersion in sodium hypochlorite solution (2% active chlorine) supplemented with Tween™ 20 for 5 min, and then rinsed with sterile deionized water; (3) The central spikelet of each synflorescence were selected (Fig. S1b) and their lowermostglume removed (Fig. S1c) before a new immersion in sodium hypochlorite solution (2% active chlorine) with Tween™ 20 for 15 min, followed by a triple wash in sterile deionized water. Before inoculation, another glume was removed and the spikelets were subjected to a further size reduction on their base to remove tissues damaged by chlorine and to wound the proximal florets of the spikelet (Fig. S1d).

Induction of callogenesis

The basal culture medium used in all the experiments was the MS saline formulation (Murashige and Skoog 1962), supplemented with 30 g L^{-1} of sucrose, 250 mg L^{-1} of polyvinylpyrrolidone, 2 ml L^{-1} of Morel's vitamins (Morel and Wetmore 1951), 1 g L^{-1} of glutamine, 1 g L^{-1} of Myo-inositol and 0.5 g L^{-1} of hydrolyzed casein. The plant growth regulators (PGR) were added before the pH of the medium was adjusted to 5.8. The media were sterilized by autoclaving for 15 min at 121 °C. Two experiments were carried out to determine: (1) the optimal 2,4-D concentration in combination with the cytokinins 2-Isopentenyladenine (2-iP) or Kinetin (Kin), and (2) the effect on callus induction in explants longitudinally halved cut and its orientation inoculation onto the culture media. For both experiments, the cultures were kept in the dark at 25 ± 1 °C, for 60 days, and the culture medium was refreshed on the 30th day.

Exp. 1: effects of 2,4-D combined with 2-iP or Kinetin on callus induction

After the disinfection procedure, the spikelet explants prepared as mentioned above were inoculated in culture media containing 0, 9, 18, 27, and 36 μM 2,4-D in combination with 9 μM 2-iP or 9 μM Kin. The cytokinin concentrations selected were that used by Yeh and Chang (1986a) for callus induction in *Bambusa oldhamii*. The treatments consisted of 8 replicate Petri dishes (60 mm × 15 mm), each containing 5 explants (subsamples) inoculated on 15 ml of culture medium. After 30 days, each subsample was evaluated, and binomial values (e.g. 0 for non-occurrence or 1 for occurrence) were recorded for microbial contamination, callus induction, browning, and swelling. The mean of the five subsample values in each Petri Dish was used for statistical analysis.

Exp. 2: effect of the explant halve cut and its orientation on the medium

After determining the optimal callus induction conditions, the disinfected spikelets were inoculated either non cut or longitudinally halved cut. These explants were placed onto the medium either as (1) cut surface in contact with the medium (downwards oriented) or (2) the cut surface upwards oriented. These treatments were carried out in 20 replicate Petri dishes each containing 4 explants (subsamples) inoculated on 15 ml of culture medium. After 30 days, each subsample was evaluated, and binomial values were recorded for callus induction and browning. The mean of the four subsample values in each Petri Dish was used for statistical analysis.

Callus multiplication and embryos regeneration

Calli cultures induced in both cytokinins combined with 18 μM 2,4-D for sixty days were transferred to media with 0, 4.5; 9, and 18 μM 2,4-D in combination with their respectives cytokinins (9 μM) to induce further development.These treatments were carried out in 8 replicate Petri dishes each containing 5 calli (subsamples) inoculated on 15 ml of culture medium. At 30 days of transfer to the new treatments, each subsample was evaluated, and binomial values were recorded callusbrowning, embryos regeneration, and the frequency of each type of callus obtained.

Statistical analysis

Percentage data were obtained by averaging the binomial values of occurrence in each Petri dish. The analysis of variance assumptions was verified by analyzing the residues distribution of the model and the application of Bartlett's homoscedasticity test. When necessary, the percentage values were submitted to sine arc transformation. Statistical analyzes were performed in the R Studio environment (R 3.6.2). Variance analysis was performed using the "base",

"stats" (R Core Team 2019), and "agricolae" (Mendiburu 2019) packages.

Somatic embryo germination and acclimatization

Somatic embryos were transferred to the basal culture medium without PGR for maturation and plantlet germination. The cultures were maintained at 25 ± 1 °C for 15 days in the dark and subsequently exposed to the 16 h photoperiod under white LED lamps (80 µmol cm^{-2} s^{-1}) for another 30 days. The plantlets that developed were transferred to basal medium in test tubes for 30 days for axillary branching, elongation, and root development before the acclimatization. The plantlets were next transferred to 1 L pots containing a commercial substrate (Tropstrato FT—Vida Verde). Plants were acclimatized in a 65% shading greenhouse under intermittent nebulization.

Histochemical analysis

Samples of spikelet showing initial callogenesis on the 7th day after callus induction, type 1 to type 2 callus development with initial protoderm development, and isolated somatic embryos were fixed in glutaraldehyde for 48 h. The material was dehydrated in an increasing ethanol series up to anhydrous conditions (Ruzin 1999). Therefore, the samples were infiltrated in hydroxyethyl methacrylate (Leica Historesin, Germany). Cross-sectional and longitudinal sections of 5 µm thickness were obtained in a rotating microtome RM 2125 (Leica, Germany) and serial sections stretched onto slides. The sections were stained with toluidine blue in pH 6.8 phosphate buffer according to O'Brien et al. (1964) for the observation and identification of anatomical structures. The images were captured in a DP71 camera attached to the BX-40 microscope (Olympus, Japan).

Results and discussion

Explant disinfection effectiveness

The three-step disinfection method and the compact arrangement of florets in spikelets resulted in a high percentage of of viable cultures. In the first experiment, microbial contamination of only 4.2% was observed after 30 days of in vitro introduction. In experiment 2, only 3.7% of the explants were contaminated. In both experiments these few contaminated explants manifested a late and slow-growing microorganism with a few showing mycelial growth. Bamboo in vitro cultures are commonly considered as hard-to-establish due to their microorganisms association and morphological characteristics, which usually can make disinfection difficult (Tsay et al. 1990; Ray and Ali 2017b). Endogenous contamination

is also an important constraint during establishing bamboo in vitro cultures, mainly in explants obtained from mature and field-grown plants (Oprins et al. 2004). Bamboo inflorescences have many layers of bracts surrounding the innermost tissues, which are younger and morphogenetically more responsive. These bracts provide a protective layer but at the same time make the disinfection procedure difficult, since they create a physical barrier that prevents the contact of the ethanol and chlorine solution with the contaminated tissues. Thus, performing asepsis in three-steps, combined with the interspersed removal of these outermost bracts allows both the removal of more differentiated and less responsive tissues, as well as efficient tissue asepsis. Only one-step 2% sodium hypochlorite soaking with sonication of *Dendrocalamus latiflorus* inflorescence segments resulted in the loss of 70% of the explants due to contamination (Lin et al. 2007). Arya et al. (2008) reported the loss of 30–40% of the explants due to contamination during induction of shoots from *D. asper* inflorescences. Other studies, in which bamboo floral explants were used, did not mention the contamination manifestation after disinfection (Yeh and Chang 1986a, b; Lin et al. 2003a, b; Lin et al. 2005; Qiao et al. 2013).

Callus induction and browning

In the first few days after in vitro culture establishment, a swelling of the explants was observed, most notably in the treatments containing only cytokinins (2-iP or Kin). Percentage of 52% and 25% of swollen explants were observed respectively in the presence of 9 µM 2-iP or 9 µM Kin. Yeh and Chang (1986a, b) reported similar results of swollen explants during callus induction from two *Bambusa* species when inflorescence explants were used. In the 2,4-D free treatments (cytokinin-alone), 77% of the explants showed tissue browning from the second subculture, 62% in KIN, and 90% in 2-iP. Several bamboos show spikelets with basal buds, which can develop new inflorescences known as pseudospikelets (McClure 1966). In a medium containing the cytokinin Thidiazuron, *Bambusa edulis* inflorescences could be maintained in the adult phase and proliferate in vitro (Lin et al. 2003a, b). On the other hand, vegetative shoots were obtained from *D. giganteus* and *D. asper* spikelets by the in vitro flowering reversion in a medium containing the cytokinin Benzilaminopurine (Ramanayake and Yakandawala 1998; Arya et al. 2008). In the present work, no regeneration was obtained from spikelets in the only-cytokinins treatments, probably due to the removal of indeterminate buds during the disinfection procedure (Fig. S1c).

Callus induction occurred in all treatments containing 2,4-D (Table 1), and most of them originated from the basal cut end of the spikelet (Fig. 1b). An increase in the 2,4-D resulted in higher callus induction, and did not depend on the

Table 1 Percentage of callus induction and browning in *Dendrocalamus asper* spikelet explants after 30 days in treatments with 2,4-D combined to Kin and 2-iP for callogenesis

CK (9 μM)	2,4-D (μM)	Callus Induction (%)	Browning (%)
2-iP	0	00.0 ± 00.0 c	87.5 ± 05.3 a
	9	40.0 ± 10.7 b	65.0 ± 07.3 ab
	18	72.5 ± 11.9 a	32.5 ± 11.9 cd
	27	65.0 ± 08.2 a	67.5 ± 07.5 ab
	36	77.5 ± 08.0 a	20.0 ± 08.4 d
Kin	0	02.5 ± 02.5 c	52.5 ± 10.0 bc
	9	30.0 ± 11.9 b	70.0 ± 11.9 ab
	18	37.5 ± 04.5 b	72.5 ± 05.2 ab
	27	45.0 ± 10.5 b	60.0 ± 10.7 ab
	36	40.0 ± 08.4 b	57.5 ± 08.0 bc

Mean ± Standard error. Values followed by the same letter in the column are not significantly different according to the Duncan test ($p < 0.05$). Means obtained from 8 replicates (n = 40)

CK Citokynin, *2,4-D* Dichlorophenoxyacetic acid, *2-iP* 2-isopentenyl adenine, *Kin* Kinetin

type of cytokinin combined with it (Table 1). Even though callogenesis was also induced when 2,4-D was combined with Kin, the auxin increment did not significantly increase the callus induction. The combination 18 μM 2,4-D + 9 μM 2-iP was considered as optimal due to high callus induction (72.5%) associated with a browning of only 32.5% in explants. Although the 36 μM 2,4-D combined with 9 μM 2-iP showed higher callus induction (77.5%) and lower browning of explants (20%), an high 2,4-D concentration can increase the likelihood of somaclonal variation. It has been reported that 2,4-D induced callogenesis and somatic embryogenesis in several bamboo species (Obsuwan et al. 2019). In young shoots of *D. hamiltonii* (Godbole et al. 2002) and zygotic embryos of *P. edulis* (Yuan et al. 2013), callus induction occurred only in the presence of 2,4-D in the culture medium and the frequency of callus induction

was affected by its concentration. For callus induction in leaves, roots, and nodal segments of *D. asper* 30 μM 2,4-D was necessary (Ojha et al. 2009). In inflorescence cultures of *B. oldhamii*, *D. latiflorus*, and *D. asper*, callus was induced using 13.5–27 μM of 2,4-D in the culture medium (Prutpongse and Gavinlertvatana 1992).

Histological sections of explants revealed cell proliferation on floret subtending tissues (i.e., palea and lemma) after 7 days of induction in 18 μM 2,4-D + 9 μM 2-iP (Fig. 3a). Those thin vascularized tissues were responsive to callus induction probably due to their high surface-to-mass ratio associated with vascular disruption during desinfection procedure. Considering that the palea and lemma are two bract-like structures, i.e., a modified leaf (Lombardo and Yoshida 2015), it is worthwhile investigating if our protocol may be successfully applied to other young vegetative explants as well.

Calli morphology

Most of the cultures showed more than one type of calli, in the first 30 days of induction (Table 2; Fig. 1). In general, three distinct types of calli were obtained: Initially a less common mucilaginous translucent callus with whitish cell clusters appeared (Fig. 2a). The most common was the type 1 callus which was transluscent to yellowish in colour with a friable texture and small-nodular growth (Fig. 2b, c). The type 2 callus was yellowish to white, with a compact appearance and initial protoderm development, showing a smoother surface from where somatic embryos developed (Fig. 2e). A gradual and successive regeneration occurred from muscillaginous to type 1 callus to type 2 callus. Type 1 callus could be obtained from the mucilaginous, and the type 2 embryogenic calli were usually derived from type 1 callus (Fig. 2b, d). Histological analysis of type 2 callus revealed a protoderm organization around a meristematic center (Fig. 3b). Qiao et al. (2013) reported the development

Table 2 Frenquency of type 1 and type 2 calli, somatic embryos regeneration, and browning percentages of *Dendrocalamus asper* calli cultures derived from young spikelets, during the somatic embryogenesis multiplication step

CK (9 μM)	2.4-D (μM)	Callus occurrence Type 1 (%)	Type 2 (%)	Embryo regeneration (%)	Browning (%)
2-iP	0.0	44.4 ± 06.1 bc	22.2 ± 06.1 ab	16.7 ± 09.9 ab	55.6 ± 06.1 a
	4.5	72.2 ± 13.8 ab	44.4 ± 12.2 ab	22.2 ± 09.6 ab	16.7 ± 09.9 ab
	9.0	94.4 ± 4.81 a	27.8 ± 08.9 ab	22.2 ± 06.1 ab	5.56 ± 04.8 b
	18.0	100.0 ± 00.0 a	16.7 ± 11.8 b	16.7 ± 11.8 ab	00.0 ± 00.0 b
Kin	0.0	25.9 ± 08.8 c	58.3 ± 11.8 a	43.7 ± 08.3 a	27.1 ± 12.6 ab
	4.5	90.5 ± 08.9 a	33.3 ± 09.6 ab	00.0 ± 00.0 b	23.8 ± 13.1 ab
	9.0	71.4 ± 10.6 ab	09.5 ± 08.9 b	09.5 ± 05.7 b	33.3 ± 13.6 ab
	18.0	25.0 ± 17.7 c	25.0 ± 11.3 ab	00.0 ± 00.0 b	58.3 ± 17.7 a

Mean ± Standard error. Values followed by the same letter in the column are not significantly different according to the Duncan test ($p < 0.05$). Means obtained from 8 replicates (n = 40)

CK Citokynin; *2,4-D* Dichlorophenoxyacetic acid; *2-iP* 2-isopentenyl adenine; *Kin* Kinetin
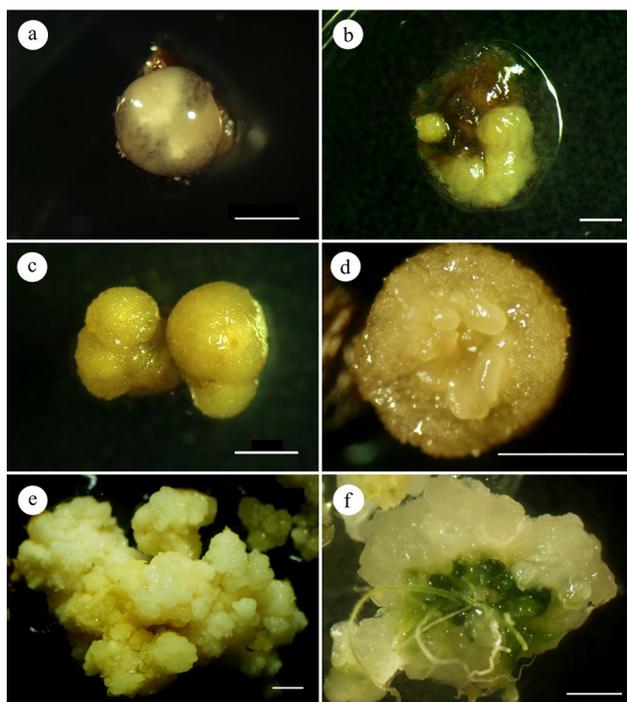
Fig. 2 *Dendrocalamus asper* spikelet-derived types of calli. **a** Mucilaginous callus; **b** Type 1 callus differentiating from a mucilaginous callus; **c** Type-1 callus; **d** Type-2 callus differentiating from type-1 callus; **e** Type-2 callus regenerating somatic embryo; **f** Secondary somatic embryogenesis on a scutellum tissues of a fused somatic embryos cluster. Bars = 2 mm

Fig. 3 Histological analysis in *Dendrocalamus asper* calli and somatic embryos obtained from young spikelets. **a** Longitudinal section showing initial callogenesis in lemma tissue after 7 days in callus induction medium; **b** Longitudinal section of type 2 callus developing from a type 1 callus, showing an organized protoderm (arrowhead); **c** Cross-section of the scutellar somatic embryo showing meristematic centers (arrowhead) around the embryo axis; **d** Longitudinal section of a somatic embryo, *Sc* Scutellum, *Pl* Plumule, *SM* Shoot Meristem, *Eb* Epiblast, *Cl* Coleorhiza. Bars = 100 μm

of embryogenic calli derived from primary calli generated from *D. latiflorus* anthers. The simultaneous occurrence of different types of calli was also reported for *B. glaucescens* (Jullien and Van 1994). In general, type 1 callus was fast-growing and more prone to browning as compared to type 2.

A few of type-1 calli regenerated root clusters, probably originating from somatic embryos that were not identified as so during the selective subculture or even the direct root regeneration from type-1 callus. As soon as the roots touched the culture medium surface, a mucilaginous loose callus developed, similar to that reported by Yeh and Chang (1986a, b) and Chang and Lan (1995). This observation also highlights the potential of bamboo roots as a vegetative starting material for in vitro callus induction.

The combination of 2,4-D with the two different cytokinins, Kin and 2-iP, affected a differential responses of the explants to browning and the type of calli that developed (Table 2). 2,4-D in combination with 2-iP promoted the induction of type 1 callus (72.2–100%) compared to its combination with Kin (25.0–90.5%). Kin brought about a higher percentage of type 2 callus in the absence of 2,4-D (58.3%) and further regeneration of somatic embryos. When in combination with 2iP, the 2,4-D total removal or its reduction to 4.5 μM in the culture media

promoted development of type 2 calli (22.2–44.4%). The type of cytokinin influenced the browning in the cultures. In 2-iP alone-medium or in combination with reduced levels of 2,4-D, there was an increase in calli browning (16.7–55.6%), especially on the type 1 calli. In the Kin-containing media, 2,4-D did not influence browning (23.8–58.3%). The browning incidence is usually observed during the multiplication step of bamboo calli cultures. Qiao et al. (2013) reported the common occurrence of *D. latiflorus* anther-derived calli during the multiplication step.

After sixtydays on the induction medium, type 2 callus, spikelet-like structures (Fig. 1d), and somatic embryos were regenerated (Fig. 1e–f). In induced callus from *D. latiflorus* anthers, shoots were obtained during the induction step (Qiao et al. 2013). Yeh and Chang (1986b) reported the somatic embryos' regeneration during the 16 months long culture on 2,4-D and Kin-containing medium. In the present work, an increase in the cytokinin:auxin ratio promoted type 2 callus and subsequent somatic embryos regeneration. Similar observations occurred for *D. hamiltonii* with the gradual reduction of 2,4-D and NAA concomitant with BAP increases in the culture medium (Godbole et al. 2002). As in many other plants, a higher cytokinin to auxin ratio s can enhance shoot and embryo regeneration from bamboo callus too. In bamboos calli cultures, a concentration-ratio

of 2:1 (cytokinin: auxin) was feasible to regenerate shoots (Prutpongse and Gavinlertvatan 1992).

Effect of the explant cutting

The longitudinal halve-cutting of explants increased the callus induction percentage compared to those non cut (Table 3). The orientation of these half-spikelets onto the culture medium also influenced the callus induction. The explant cut-surface in contact with the culture medium reduced the callus induction. The inoculation of half-spikelet with the cut-surface upward oriented resulted in a significant increment in the callus induction in relation to the non cut explant. This step led to a callus induction in almost 100% of the explants. The explant cutting and its orientation on the medium did not affect the contamination. In all treatments, most calli first appeared on the basal cut end region of the spikelets. Similar observations were reported (Godbole et al. 2002), in which initial swelling and cellular proliferation began from the cut ends segmented shoots of *D. hamiltonii*. Explants inoculated with cut-surface oriented upward developed calli from the borders tissues. It was also observed callus initiation from the anthers. After 30 days, a subsequent subculture on the same induction medium of those anther-derived calli resulted in browning, and embryonic callus (type 2) was not observed. Although anther-derived callus could result in haploid plants, interesting for breeding purposes (Tsay et al. 1990), the low callus induction compared to the enclosing tissues of spikelet showed the need for improvement on the callus induction from anthers. The spikelet longitudinal cutting enhanced the callus induction by relieving the physical blocking of the enclosing tissues. Additionally, mechanical wounding is recognized and commonly adopted for cellular differentiation induction by overexpression of stress-related genes (Fehér et al. 2003; Wójcik et al. 2020). Furthermore, the explant size-reduction increases the surface-area-to-volume ratio, promoting the stress triggered by the in vitro environment. Large explants or intact organs can show, even that explanted, a well-organized symplastic pathway and stable cellular metabolism, which can lead to recalcitrance to in vitro responses. The wounding of tissues often results

in symplastic domain rupture and can stimulate the establishment of new domains and plasmodesmata cell-to-cell communication, promoting differentiation and pluripotentiality expression (Bonga 2017). Even though the small size of the spikelets used as explants for those experiments, the halve cuttingshowed a feasible and straightforward technique to increase the explant number, callus induction area, and the callus induction rate. This result suggests that using the thin cell layer technique may be beneficial to increase callus induction from bamboo spikelets.

Secondary somatic embryogenesis

A change in the scutellum was observed when somatic embryos were transferred to a medium containing the higher 2,4-D levels (9–18 µM). This occurred mainly on somatic embryos in late developmental stages. The scutellar tissue of zygotic and somatic embryos is reported to be 2,4-D-responsive inother *Dendrocalamus* species (Sumathi et al. 2003; Zhang et al. 2010). In the present study, during the selective multiplication, when easily detachable and round-shaped somatic embryos were isolated and transferred to 18 µM 2,4-D + 9 µM 2-iP (induction medium) only a minor growth on the root region of the embryo axis was observed. At the same time, a cellular proliferation occurred around the somatic embryo axis, especially on the scutellum tissue, where it was possible to observe meristematic centers (Fig. 3c). This observation led to an understanding of the development of secondary embryo clusters fused to the scutellar tissues and the effect of 2,4-D in inducing secondary embryogenesis in the scutellum tissues. Thus maintenance of embryogenic calli in media with 2,4-D led to secondary embryogenesis from the regenerated embryos. Plant regeneration from these fused embryo clusters (Fig. 2f) was inefficient because of their non-synchronic maturation. Long-term culture in the presence of 2,4-D in the medium led to clusters of reduced-size fused somatic embryos, which did not undergo maturation and did not germinate into plantlets. Therefore, to develop a reliable protocol for scale-up plant production in *D. asper*, further studies focusing on the control of secondary somatic embryogenesis is

Table 3 Callus induction and explant contamination percentages of *Dendrocalamus asper* young spikelets in the function of longitudinal halve cutting and the explant orientation on the culture medium

Longitudinalcutting	Explant orientation on medium	Induction (%)	Contamination (%)
–	Non-cut	80.4 ± 04.5 b	05.0 ± 02.3 a
+	Cut surface downward oriented	63.3 ± 06.7 c	03.7 ± 02.0 a
+	Cut surface upward oriented	98.7 ± 01.2 a	02.5 ± 01.7 a

Mean ± Standard error. Values followed by the same letter in the column are not significantly different according to the Duncan test ($p < 0.05$). Means obtained from 20 replicates (n = 80)

recommended to improve somatic embryo development and plant regeneration.

Plantlet's regeneration

Somatic embryos regenerated in all the tested media during the multiplication. Normal somatic embryos were translucent to white in color showing a regular rounded to spherical shape with the embryonal axis on their central region. Most of them were easily detachable from the callus surface. Plantlets developed (germinated) from round somatic embryos with a smooth epidermis faster than in those with the rough epidermis or fused scutellum (Fig. 2f). Greening of the embryonic axis was observed after 7–10 days of 16 h photoperiod exposition, and shoots and roots developed after 10–14 days. Histochemical analysis showed a considerable starch accumulation in the scutellum tissue (Fig. 3d), which probably caused the yellowish to the white color of the somatic embryos. Similar features were observed in *Phyllostachys edulis* somatic embryos (Yuan et al. 2013). That increased starch content on scutellum tissue was probably due to the sucrose enriched medium and the lack of endosperm and aleurone layer on somatic embryos. Both structures are primarily responsible for the starch accumulation and starch related-enzymes in seeds. In somatic embryos of *D. hamiltonii*, the scutellum substituted those structures in the amylase accumulation and starch deposition (Godbole et al. 2004). In the present work, the successfully germinated in plantlets were transferred to test tubes with basal medium, where synchronous rooting and shoot elongation was observed. The typical leaves development of embryonic seedlings was also observed during the plantlet elongation (Fig. 1g). After 45 days, converted plants started tillering and were acclimatized (Fig. 1h).

Conclusions

The present study described a somatic embryogenesis protocol from young spikelets of the giant bamboo, *Dendrocalamus asper* (Fig. S2). A 3-step disinfection procedure using ethanol and sodium hypochlorite in spikelet explants gave rise to more than 95% of explants to be successfully in vitro introduced. That procedure, combined with the embryogenic competence of the young enclosing tissues of the florets, resulted in an efficient in vitro callus culture establishment. 2,4-D was essential for callus induction, and the cytokinins (2-iP and Kin) tested showed an effect on the type of callus obtained from spikelet and their embryogenic capacity. The longitudinal halve-cutting of spikelet before inoculation further increased the callus induction and the number of explants per spikelet, improving the efficiency of in vitro introduction. When combined with 2-iP

or Kin, reduction of 2,4-D concentration resulted in a rapid somatic embryo regeneration from the calli. These results corroborated the already known benefits of using Kin for bamboo micropropagation and showed that 2-iP as a feasible alternative for bamboo somatic embryogenesis from floral explants. The subculture of the somatic embryos to the 18 μM 2,4-D + 9 μM 2-iP (induction medium) led to secondary embryogenesis from scutellum tissues, resulting in fused and asynchronous embryos maturation. The somatic embryos obtained were converted into plantlets in a PGR-free medium. Embryogenic cultures induced from spikelets allow the large-scale propagation of selected genotypes. They are also an important method for rescue and ex situ conservation of elite genotypes in a monocarpic flowering event. In terms of future research would be useful to extend current findings by improving the steps of somatic embryo regeneration and plantlets germination. One of the most important future demands is how many embryos could be obtained from a single explant and the further analysis of their genetic fidelity to the mother plants.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

Akinlabi ET, Anane-Fenin K, Akwada DR (2017) Applications of bamboo. In: Akinlabi ET, Anane-Fenin K, Akwada DR (eds) Bamboo: the multipurpose plant. Springer, Cham, pp 179–219

Arya S, Sharma S, Kaur R, Dev Arya I (1999) Micropropagation of *Dendrocalamus asper* by shoot proliferation using seeds. Plant Cell Rep 18:879–882. https://doi.org/10.1007/s002990050678

Arya S, Satsangi R, Arya ID (2008) Direct regeneration of shoots from immature inflorescences in *Dendrocalamus asper* (edible bamboo) leading to mass propagation. Bamboo Science & Culture 21:14–20

Banik RL (2016) Dendrocalamus nees. In: Banik RL (ed) Silviculture of South Asian priority bamboos. Tropical forestry. Springer, Singapore, pp 177–192

Bonga JM (2017) Can explant choice help resolve recalcitrance problems in in vitro propagation a problem still acute especially for adult conifers? Trees 31:781–789. https://doi.org/10.1007/s00468-016-1509-z

Chang WC, Lan TH (1995) Somatic embryogenesis and plant regeneration from roots of bamboo (*Bambusa beecheyana* Munro var. beecheyana). J Plant Physiol 145:535–538. https://doi.org/10.1016/S0176-1617(11)81784-0

Clark LG., Oliveira RP (2018) Diversity and evolution of the new world bamboos (Poaceae: Bambusoideae: Bambuseae, Olyreae). In: Proceedings of the 11th World bamboo congress, Xalapa, Mexico. The World Bamboo Organization, Plymouth, pp 35–47.

Fehér A, Pasternak TP, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tiss Organ Cult 74:201–228. https://doi.org/10.1023/A:1024033216561

Godbole S, Sood A, Thakur R, Sharma M, Ahuja PS (2002) Somatic embryogenesis and its conversion into plantlets in a multipurpose bamboo, *Dendrocalamus hamiltonii* Nees et Arn. Ex Munro. Curr Sci 83:885–889

Godbole S, Sood A, Sharma M, Nagar PK, Ahuja PS (2004) Starch deposition and amylase accumulation during somatic embryogenesis in bamboo (*Dendrocalamus hamiltonii*). J Plant Physiol 161:245–248. https://doi.org/10.1078/0176-1617-01219

Jullien F, Van KTT (1994) Micropropagation and embryoid formation from young leaves of *Bambusa glaucescens*' Golden Goddess'. Plant Sci 98:199–207. https://doi.org/10.1016/0168-9452(94)90010-8

Li Z, Kobayashi M (2004) Plantation future of bamboo in China. J for Res 15:233–242. https://doi.org/10.1007/BF02911032

Liese W, Welling J, Tang TKH (2015) Utilization of bamboo. In: Liese W, Köhl M (eds) Bamboo: the plant and its uses. Springer, Switzerland, p 299

Lin CC, Lin CS, Chang WC (2003a) In vitro flowering of *Bambusa edulis* and subsequent plantlet survival. Plant Cell, Tissue Organ Cult 72:71–78. https://doi.org/10.1023/A:1021281217589

Lin CS, Chen CT, Lin CC, Chang WC (2003b) A method for inflorescence proliferation. Plant Cell Rep 21:838–843. https://doi.org/10.1007/s00299-003-0571-3

Lin CS, Lin CC, Chang WC (2005) Shoot regeneration, re-flowering and post flowering survival in bamboo inflorescence culture. Plant Cell Tiss Organ Cult 82:243–249. https://doi.org/10.1007/s11240-005-0883-9

Lin CS, Liang CJ, Hsaio HW, Lin MJ, Chang WC (2007) In vitro flowering of green and albino *Dendrocalamus latiflorus*. New for 34:177–186

Lombardo F, Yoshida H (2015) Interpreting lemma and palea homologies: a point of view from rice floral mutants. Front Plant Sci 6:61. https://doi.org/10.3389/fpls.2015.00061

McClure FA (1966) A glossary of the bamboos. Taxon 15:220–235. https://doi.org/10.2307/1215968

Mendiburu F (2019) Agricolae: Statistical Procedures for Agricultural Research. R package version 1.3–1. https://cran.r-project.org/package=agricolae

Morel G, Wetmore RH (1951) Fern callus tissue culture. Am J Bot 38:141–143. https://doi.org/10.2307/2437837

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

O'Brien T, Feder N, McCully ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59:368–373. https://doi.org/10.1007/BF01248568

Obsuwan K, Duangmanee A, Thepsithar C (2019) In vitro propagation of a useful tropical bamboo, *Thyrsostachys siamensis* Gamble, through shoot-derived callus. Hortic Environ Biotechnol 60:261–267. https://doi.org/10.1007/s13580-018-00119-z

Ojha A, Verma N, Kumar A (2009) In vitro micropropagation of economically important edible bamboo (*Dendrocalamus asper*) through somatic embryos from root, leaves and nodal segments explants. Res Crops 10:430–436

Oprins J, Grunewald W, Gillis K, Delaere P, Peeters H, Gielis J (2004) Micropropagation: a general method for commercial bamboo production. In: Proceedings of the 7th world bamboo congress, New Delhi, India. The World Bamboo Organization, Plymouth, pp 1–11.

Paudyal K, Adhikari S, Sharma S, Samsudin YB, Paudyal BR, Bhandari A, Birhane E; Darcha G; Trinh TL, Baral H (2019) Framework for assessing ecosystem services from bamboo forests: lessons from Asia and Africa 255 CIFOR. https://doi.org/10.17528/cifor/007433

Prutpongse P, Gavinlertvatana P (1992) In vitro micropropagation of 54 species from 15 genera of bamboo. HortScience 27:453–454. https://doi.org/10.21273/HORTSCI.27.5.453

Qiao G, Li H, Liu M, Jiang J, Yin Y, Zhang L, Zhuo R (2013) Callus induction and plant regeneration from anthers of *Dendrocalamus latiflorus* Munro. In Vitro Cell Dev Biol Plant 49:375–382. https://doi.org/10.1007/s11627-013-9498-8

R Core Team (2019). R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Ramanayake SMSD, Yakandawala K (1998) Incidence of flowering, death and phenology of development in the giant bamboo (*Dendrocalamus giganteus* Wall. ex Munro). Ann Bot 82:779–785. https://doi.org/10.1006/anbo.1998.0754

Ray SS, Ali MN (2017a) Factors affecting macropropagation of bamboo with special reference to culm cuttings: a review update. N Z J for Sci 47:17. https://doi.org/10.1186/s40490-017-0097-z

Ray SS, Ali N (2017b) Biotic contamination and possible ways of sterilization: a review with reference to bamboo micropropagation. Braz Arch Biol Technol 60:1–11. https://doi.org/10.1590/1678-4324-2016160485

Ruzin SE (1999) Plant microtechnique and microscopy. Oxford University Press

Singh SR, Dalal S, Singh R, Dhawan AK, Kalia RK (2012) Micropropagation of *Dendrocalamus asper* {Schult. & Schult. F}. Backer ex k. Heyne): an exotic edible bamboo. J Plant Biochem Biotechnol 21:220–228. https://doi.org/10.1007/s13562-011-0095-9

Singh SR, Singh R, Kalia S, Dalal S, Dhawan AK, Kalia RK (2013) Limitations, progress and prospects of application of biotechnological tools in improvement of bamboo—a plant with extraordinary qualities. Physiol Mol Biol Plants 19:21–41. https://doi.org/10.1007/s12298-012-0147-1

Soreng RJ, Peterson PM, Romaschenko K, Davidse G, Teisher JK, Clark LG, Barberá P, Gillespie LJ, Zuloaga FO (2017) A world-wide phylogenetic classification of the Poaceae (Gramineae) II: an update and a comparison of two 2015 classifications. J Syst Evol 55:259–290. https://doi.org/10.1111/jse.12262

Sumathi R, Malliga P, Yashodha R, Gurumurthy K (2003) Enhanced somatic embryogenesis and plant regeneration in bamboo (*Dendrocalamus strictus*). Plant Cell Biotechnol Mol Biol 4:9–16

Tsay HS, Yeh CC, Hsu JY (1990) Embryogenesis and plant regeneration from anther culture of bamboo (*Sinocalamus latiflora* (Munro) McClure). Plant Cell Rep 9:349–351. https://doi.org/10.1007/BF00232396

Wang Y, Qu H, Bai T, Chen Q, Li X, Luo Z, Lv B, Jiang M (2021) Effects of variations in color and organ of color expression in urban ornamental bamboo landscapes on the physiological and psychological responses of college students. Int J Environ Res Public Health 18:1151. https://doi.org/10.3390/ijerph18031151

Wójcik AM, Wójcikowska B, Gaj MD (2020) Current perspectives on the auxin-mediated genetic network that controls the induction of somatic embryogenesis in plants. Int J Mol Sci 21:1333. https://doi.org/10.3390/ijms21041333

Yeh ML, Chang WC (1986a) Plant regeneration through somatic embryogenesis in callus culture of green bamboo (*Bambusa oldhamii* Munro). Theoret Appl Genetics 73:161–163. https://doi.org/10.1007/BF00289269

Yeh ML, Chang WC (1986b) Somatic embryogenesis and subsequent plant regeneration from inflorescence callus of *Bambusa beecheyana* Munro var. beecheyana. Plant Cell Rep 5:409–411

Yuan JL, Yue JJ, Wu XL, Gu XP (2013) Protocol for callus induction and somatic embryogenesis in Moso bamboo. PLoS ONE 8(12):e81954. https://doi.org/10.1371/journal.pone.0081954

Zang Q, Liu Q, Zhuge F, Wang X, Lin X (2019) In vitro regenerations via callus induction in *Dendrocalamus asper* (Schult.) Backer. Propag Ornam Plants 19:66–71

Zhang N, Fang W, Shi Y, Liu Q, Yang H, Gui R, Lin X (2010) Somatic embryogenesis and organogenesis in *Dendrocalamus hamiltonii*. Plant Cell Tiss Organ Cult 103:325–332. https://doi.org/10.1007/s11240-010-9783-8

Zheng J, Tarin MWK, Jiang D, Li M, Ye J, Chen L, He T, Zheng Y (2021) Which ornamental features of bamboo plants will attract the people most? Urban for Urban Green 61:127101. https://doi.org/10.1016/j.ufug.2021.127101