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ORIGINAL PAPER

Somatic embryogenesis from immature peach palm inflorescence explants: towards development of an efficient protocol

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Abstract Various factors affect the induction of somatic embryogenesis in peach palm (*Bactris gasipaes* Kunth). Among these, both the type and level of auxins had the greatest influence on in vitro responses, although the genotype and the developmental stage of the explants also influenced results. Younger inflorescences were more competent to respond to SE induction than more mature inflorescences and the use of a pre-treatment with 2,4-D (200 μM) in liquid MS culture medium also increased the embryogenic capacity, and diminished the development of flower buds. Higher oxidation rates were observed in explants maintained on 2,4-D-supplemented culture medium, while on 300 μM or 600 μM Picloram and Dicamba lower oxidation rates were observed. The progression from floral meristem to flower bud occurred at high frequency when low concentrations of auxins were used, independent of the type. Higher concentrations of Picloram or Dicamba reduced or even inhibited flower bud development. Picloram also enhanced the embryogenic induction rate more than 2,4-D and Dicamba, and among the concentrations evaluated 300 μM Picloram enhanced induction for both genotypes, with significant differences between genotypes. The best combination of variables used the least mature inflorescence (*Infl1*) from genotype I with the 2,4-D pre-treatment and 300 μM Picloram to generate 5 embryogenic calli from 18 explants; 26 embryos were obtained on average from each embryogenic callus. From these, eighteen embryos converted to plantlets and six of these survived transfer to the greenhouse.

Keywords Arecaceae · *Bactris gasipaes* Kunth · Tissue culture · Clonal propagation · Auxin

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2-iP	2-isopentyladenine (6-dimethylaminopurine)
Dicamba	3,6-dichloro-2-methoxybenzoic acid
MS	Murashige and Skoog's salts
NAA	Naphthalene acetic acid

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Picloram	4-amino-3,5,6-trichloropicolinic acid
SE	Somatic embryogenesis

Introduction

Peach palm (*Bactris gasipaes* Kunth—Arecaceae) is a neotropical palm that was probably domesticated in southwestern Amazonia. The palm is important today for both fruit and heart-of-palm production. The fruit is an excellent food, with starches, lipids, carotene and all essential amino acids (Yuyama et al. 2003). The heart-of-palm is composed of unexpanded juvenile leaves and subapical tissue, and has low concentrations of peroxidases and polyphenoloxidase, allowing in natura commercialization, as well as processing (Clement et al. 1999).

For this species, tissue culture techniques are considered to be the most efficient strategy for clonal plantlet regeneration, as well as for genetic conservation (Mora-Urpí et al. 1997), although a commercial protocol does not currently exist. Among various techniques, somatic embryogenesis (SE) offers the advantages of automated large-scale production and genetic stability of the regenerated plantlets (Guerra et al. 1999). However, for the clonal propagation of selected genotypes the development of protocols that allow regeneration from explants obtained from adult plants is necessary.

Success of SE in monocotyledonous species requires utilization of explants containing meristematic cells (Vasil 1987). Inflorescences from adult palm trees contain meristematic cells and are important explant sources because they can be obtained without damaging the donor tree (Verdeil et al. 1994).

Palm trees have been considered recalcitrant in tissue culture, although plantlet regeneration using inflorescence explants has been described for several species, including African oil palm (*Elaeis guineensis* Jacq.; Teixeira et al. 1994), coconut (*Cocos nucifera* L.; Verdeil et al. 1994), *Euterpe edulis* Mart. (Guerra and Handro 1998) and betel nut palm (*Areca catechu* L.; Karun et al. 2004). In peach palm, Arias (1985) was not able to induce a morphogenetic response from inflorescences cultured in vitro. However, the successful utilization of this explant source in peach palm was later described by Almeida and Kerbauy (1996). These authors observed that regeneration occurred through organogenesis at low frequency (about 11%), with a small number of plantlets being regenerated. Furthermore, regeneration was an apparently random response.

The present study aimed to determine the main factors involved in the acquisition of SE competence to permit plant regeneration from inflorescence explants of peach palm, with the goal of establishing a regenerative protocol through somatic embryogenesis for genetic conservation and improvement.

Material and methods

Plant material

Eight immature inflorescences from two open-pollinated plants from the Yurimaguas population of the Pampa Hermosa landrace, kept at the Instituto Nacional de Pesquisas da Amazônia (INPA) germplasm collection, Manaus, Amazonas, Brazil, were shipped to Florianópolis, Santa Catarina. The external spathes were removed and the inflorescences, still enclosed by the internal spathes, were surface-sterilized by immersion in 70% ethanol for 5 min., followed by air-drying in aseptic conditions. Thereafter, the internal spathes were removed (Fig. 1A), and the rachillae were separated from the inflorescences and used as explants.

In order to evaluate the influence of the inflorescences' developmental stage on SE, these were classified as *Infl1*, *Infl2* and *Infl3*, according to the external spathes' size—from 5–8 cm, 8–12 cm and 12–16 cm, respectively. According to Clement (1987), these inflorescences are formed in the axils of leaves 2 to 5, 6 to 9, 10 to 15, respectively, where leaf 1 is the newest expanded leaf in the crown.

Culture media and conditions

The effect of a pre-treatment before explant extraction and induction of SE was tested, as

described for *Euterpe edulis* Mart. in vitro culture (Guerra and Handro 1998). The basal culture medium [containing MS salts (Murashige and Skoog 1962), Morel vitamins (Morel and Wetmore 1951), 3% (w/v) sucrose, 500 mg L^{-1} glutamine, 1.5 g L^{-1} activated charcoal] was enriched with 200 μM 2,4-D and the inflorescences were kept in test tubes containing 25 mL of liquid culture medium during four weeks with occasional agitation.

After the pre-treatment, the rachillae were sectioned into slices 1 to 2 mm thick and inoculated into Petri dishes containing 25 mL of the basal culture medium supplemented with different auxins (2,4-D, Picloram or Dicamba) at different concentrations (150, 300 or 600 μM). The cultures were maintained at a temperature of 25 ± 2°C in the dark for 32 weeks without subculture. The embryogenic calli were then transferred to a maturation culture medium composed of the basal culture medium plus 40 μM 2,4-D, 10 μM 2-iP, 500 mg L^{-1} hydrolyzed casein and with the glutamine concentration increased to 1 g L^{-1}, and were sub-cultured at four week intervals. Mature and well formed somatic embryos were selected and transferred to a regeneration medium composed by the basal culture medium plus 24.5 μM 2-iP and 0.44 μM NAA, gelled with 2.5 g L^{-1} Phytagel®, and 25 mL was spread evenly in Petri dishes and these were incubated at 25 ± 2°C under light with an intensity of 50–60 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps (Sylvania) during four weeks. Thereafter, the somatic embryos were transferred to Petri dishes containing 25 mL of the basal culture medium gelled with Agar 0.7% (w/v) until conversion. Converted somatic embryos were transferred to 300 mL flasks containing 30 mL of the same culture medium until the plantlets were 6 cm tall. In all culture media the pH was adjusted to 5.8 before addition of the gelling agent, and all the components being autoclaved at 121°C and 1 kgf cm^{-2} for 15 min.

The plantlets had their root systems pruned to approximately 2 cm and acclimatization was carried out in trays containing 3 × 3 cm cells with commercial substrate (PlantMax® Fi). The trays were placed inside a plastic box covered with glass to allow light entry and to reduce water exchange (Fig. 1G). These plantlets were kept under 16 h light periods with 100–130 μmol m^{-2} s^{-1} light intensity provided by cool-white fluorescent (Sylvania) and high pressure sodium vapor lamps (Empalux—VST).

Parameters measured and statistical procedure

The present study evaluated the effect of genotype, inflorescence maturity, pre-treatment, auxin type and auxin concentration, with replications composed of three Petri dishes, containing one rachillae per dish. The inflorescences *Infl1*, *Infl2* and *Infl3* yielded an average of 6, 13 and 18 explants per Petri dish, respectively. The variables evaluated were oxidation, flower bud development and SE induction. The different responses were evaluated once at the end of the culture period and calculations were based on the initial number of explants. Explants that showed intense browning were considered to be oxidized; usually these explants do not progress toward other in vitro responses. Flower bud development was characterized by continued normal development (Fig. 1B) and did not progress to any other response. Embryogenic calli were usually composed of both globular and nodular structures, allowing the characterization of this morphogenetic response.

The data were subjected to the General Linear Model (GLM) and means were compared using least significant difference (LSD) procedures from STATISTICA v7 (StatSoft, Inc., 2004).

Results and discussion

Somatic embryogenesis can be considered as the ultimate expression of the totipotentiality of plant cells and it is well established that the expression of this feature is under genetic control (Ezhova 2003). In the present study, a significant influence ($P < 0.01$) of the genotype on all the morphological responses was observed, with genotype II showing the best SE induction (Tables 1–3). Considering that the two genotypes were from the same landrace where little among-accession genetic divergence has been detected

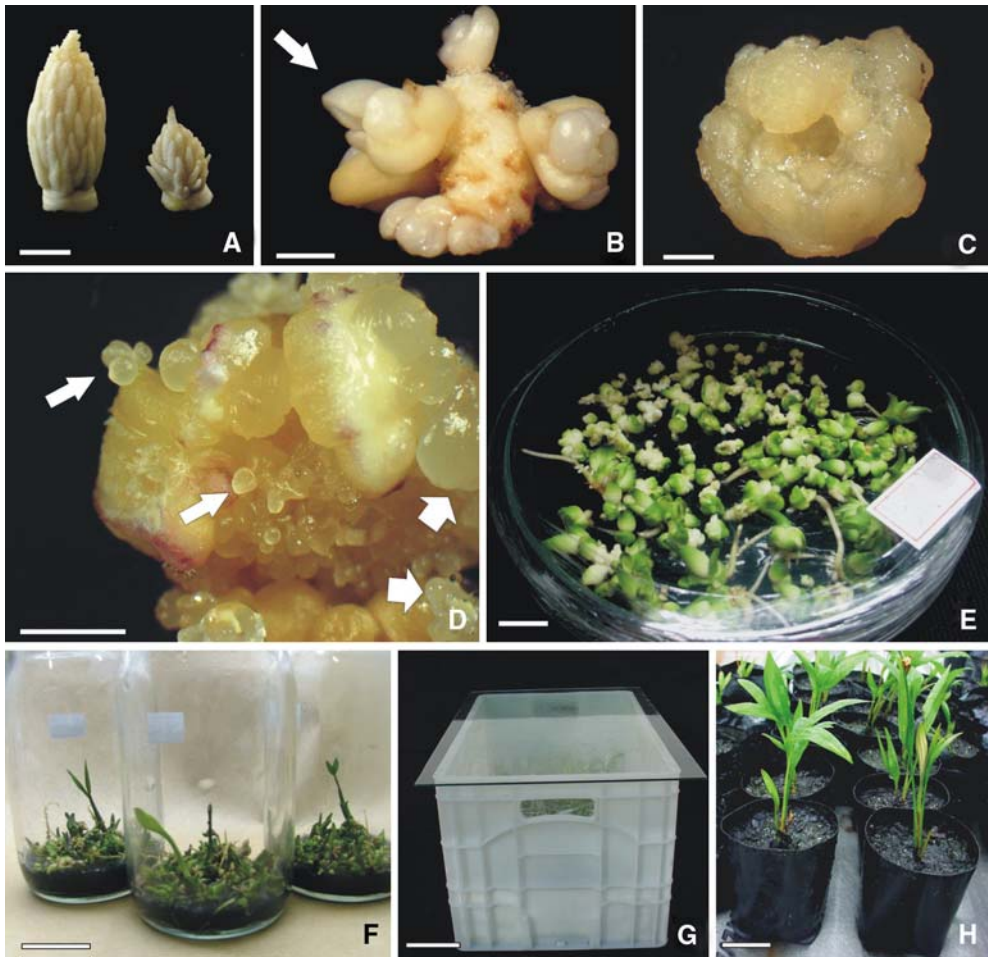

Fig. 1 Somatic embryogenesis and plantlet regeneration from immature inflorescences of peach palm (*Bactris gasipaes* Kunth, Arecaceae). (**A**) Immature inflorescences utilized as explant source (bar = 1 cm). (**B**) In vitro development of flower bud (arrow) (bar = 1 mm). (**C**) Non-organized cellular proliferation of explants (bar = 2.5 mm). (**D**) Somatic embryogenic induction—note the development of globular somatic embryos (thin arrow) and nodular tissue (thick arrow) (bar = 2.5 mm). (**E**) Converted somatic embryos (bar = 1 cm). (**F**) Plantlets showing the development of shoot and root (bar = 2.5 cm). (**G**) Acclimatization apparatus (bar = 12.5 cm). (**H**) Acclimatized plantlet (bar = 2.5 cm)

Table 1 Influence of genotype and inflorescence maturity (*Infl1*—5–8 cm, *Infl2*—8–12 cm and *Infl3*—16–12 cm) on oxidation rate, flower bud development and SE induction from peach palm inflorescences

Inflorescence maturity	Oxidation Rate (%)			Flower Bud Development (%)			SE induction (%)		
	Genotype I	*Genotype II*	Mean	*Genotype I*	*Genotye II*	Mean	*Genotype I*	*Genotype II*	Mean
Infl1	11.7^{abB}	32.9^{aA}	22.3^{a}	30.4^{bA}	20.2^{bB}	25.3^{b}	1.5^{aB}	7.1^{aA}	4.3^{a}
Infl2	13.5^{aB}	22.5^{bA}	18.0^{a}	27.1^{bA}	30.1^{aA}	28.6^{b}	0.0^{aA}	0.6^{bA}	0.3^{b}
Infl3	5.1^{bA}	7.7^{cA}	6.4^{b}	45.1^{aA}	36.6^{aB}	40.9^{a}	0.5^{aA}	1.5^{bA}	1.0^{b}
Mean	10.1^{B}	21.0^{A}		34.2^{A}	28.9^{B}		0.6^{B}	3.0^{A}	

A two-way interaction between inflorescence maturity and genotype ($P < 0.05$) was observed for all variables. The values with different capital letters show significant genotype differences and with different small letters in the columns show significant differences for the inflorescence maturity, according to the LSD test

with microsatellite markers (Doriane Rodrigues, Univ. Amazonas, pers. com., 2006), this strong genotypic influence suggests that protocols need to be ample enough to capture these genetic differences. The influence of the genotype on SE induction in peach palm was also observed using leaf sheaths as explants (Stein and Stephens 1991). Similar effects of genotype on SE induction were observed in date palm (*Phoenix dactylifera* L.; Al-Khayri and Al-Bahrany 2004) and on the development of friable embryogenic callus in African oil palm (Teixeira et al. 1995). However, SE induction from coconut inflorescences did not show a genotype effect (Verdeil et al. 1994).

The inflorescences' developmental stages also had a significant ($P < 0.01$) influence on in vitro responses, as well as a significant interaction with genotype ($P < 0.05$). The oxidation rate was higher in younger inflorescences (*Infl1–Infl2*), where genotype II presented the highest rate (Table 1). Development of flower buds (Fig. 1B) was not influenced by the developmental stage of the inflorescence explant, although older inflorescences tended to grow faster. The maturity of the inflorescences influenced SE induction in both genotypes, with *Infl1* showing a 3–4-fold increase compared with *Infl3*, suggesting that the youngest inflorescences were more competent to respond to SE induction than more developed inflorescences (Table 1). Similar response gradients have been described for other species. Callus induction in wheat was inversely correlated with inflorescence size (Benkirane et al. 2000). In *Euterpe edulis*, SE induction was observed only when inflorescences at the first developmental stage were used, while older inflorescences showed oxidation and flower bud development (Guerra and Handro 1998). The same pattern was described for betel nut palm (Karun et al. 2004) and coconut (Verdeil et al. 1994).

The utilization of a pre-treatment before transferring the explant, in order to reduce oxidation and increase the induction rate, was a strategy employed in other palm in vitro systems, such as *Euterpe edulis* (Guerra and Handro 1998). In the present study, a significant interaction between pre-treatment and genotype was found for oxidation rate ($P < 0.01$), with genotype II responding to the pre-treatment while genotype I did not (Table 2). One important effect of the pre-treatment was the reduction in flower bud development, by approximately 2.5-fold for both genotypes. The pre-treatment also significantly effected SE induction ($P < 0.01$), increasing its rate at least 2-fold for both genotypes, without interactions. A pre-treatment step also enhanced SE in coffee (*Coffea arabica* L.; Quiroz-Figueroa et al. 2002), orchardgrass (*Dactylis glomerata* L.; Alexandrova and Conger 2002) and kodo millet (Vikrant and Rashid 2003). Nhut et al. (2000) observed that the pre-treatment increased SE induction in rice (*Oryza sativa* L.) and suggested that this step alters the endogenous hormonal balance. In addition, the pre-treatment step could stress the cells, triggering cell division (Feher et al. 2003).

During in vitro culture, some explants (20% general mean, data not shown) were able to dedifferentiate, resulting in undifferentiated actively growing tissue (Fig. 1C), which later differentiated into somatic embryos. The SE induction was characterized by the development

Table 2 Influence of genotype and pre-treatment in liquid culture medium with 2,4-D (200 μM) during four weeks on oxidation rate, flower bud development and SE induction from peach palm inflorescences

Pre-treatment	Oxidation rate (%)			Flower bud development (%)			SE induction (%)		
	Genotype I	*Genotype II*	Mean	*Genotype I*	*Genotype II*	Mean	*Genotype I*	*Genotype II*	Mean
Absence	9.2^{aA}	8.9^{bA}	9.0^{b}	45.2	43.1	44.1^{a}	0.4	1.8	1.1^{b}
Presence	11.0^{aB}	33.1^{aA}	22.0^{a}	23.1	14.9	19.0^{b}	1.0	4.3	2.6^{a}
Mean	10.1^{B}	21.0^{A}		34.1^{A}	29.0^{B}		0.7^{B}	3.0^{A}	

Significant influence for pre-treatment ($P < 0.01$) and for genotype ($P < 0.01$) was observed for all variables. A two-way interaction between pre-treatment and genotype ($P < 0.05$) was observed only for oxidation rate. The values with different capital letters show genotype significant differences and with different small letters in the columns show significant differences for the pre-treatment, according to the LSD test

Table 3 Influence of genotype and different auxin types and concentrations on oxidation rate, flower bud development and SE induction from peach palm inflorescences

Auxin type/ concentration		Oxidation rate (%)			Flower bud development (%)			SE induction (%)		
		Genotype I	*Genotype II*	Mean	*Genotype I*	*Genotype II*	Mean	*Genotype I*	*Genotype II*	Mean
2,4-D	150	29.5^{aB}	45.8^{aA}	37.6^{a}	56.3^{bA}	51.8^{abA}	54.0^{b}	0.0^{bA}	0.0^{bA}	0.0^{c}
	300	15.1^{bB}	31.4^{bA}	23.2^{b}	72.4^{aA}	63.9^{aA}	68.1^{a}	0.0^{bA}	0.0^{bA}	0.0^{c}
	600	20.5^{abA}	12.9^{cA}	33.4^{b}	22.0^{cA}	30.3^{cA}	26.1^{c}	0.0^{bA}	1.4^{bA}	0.7^{c}
Dicamba	150	2.7^{cB}	30.7^{bA}	16.7^{b}	74.1^{aA}	45.9^{bB}	60.0^{ab}	0.0^{bA}	0.0^{bA}	0.0^{c}
	300	3.4^{cA}	13.4^{cA}	8.4^{c}	14.5^{cdA}	11.2^{dA}	12.8^{d}	0.0^{bB}	10.5^{aA}	5.2^{b}
	600	1.1^{cB}	24.1^{bA}	12.6^{c}	0.0^{eA}	1.8^{dA}	0.9^{e}	0.0^{bA}	1.7^{bA}	0.8^{c}
Picloram	150	11.8^{bcA}	8.8^{cA}	10.3^{c}	53.9^{bA}	52.8^{abA}	53.3^{b}	1.3^{abA}	0.9^{bA}	1.1^{c}
	300	3.9^{cA}	12.4^{cA}	8.1^{c}	12.7^{cA}	3.1^{dA}	7.9^{de}	4.7^{aB}	11.4^{aA}	8.0^{a}
	600	2.9^{cA}	10.0^{cA}	6.4^{c}	2.8^{deA}	0.0^{dA}	1.4^{e}	0.0^{bA}	1.2^{bA}	0.6^{c}
Mean		10.1^{B}	21.0^{A}		34.3^{A}	28.9^{B}		0.6^{B}	3.0^{A}	

A three-way interaction between auxin type and its concentrations and genotype was observed for all variables ($P < 0.05$). The values with different capital letters show genotype significant differences and with different small letters in the columns show significant differences for the auxin type and concentration, according to the LSD test

of white to yellowish globular or nodular structures (Fig. 1D).

The auxin type and its concentrations are thought to have the greatest influences on in vitro culture. In the present study, a significant three-way interaction ($P < 0.05$) was observed between the genotype, auxin type and its concentration (Table 3). When the oxidation rate was evaluated in terms of the auxin type and its concentration, 2,4-D presented the highest rate in both genotypes. For the development of flower buds, the presence of 150 μM of auxin, independent of type, stimulated the development of such structures, as did the use of 300 μM 2,4-D. On the other hand, 600 μM Picloram and Dicamba reduced the rate of flower bud development or even completely inhibited it in both genotypes. Although in vitro development of flower buds has been described for peach palm (Almeida and Kerbauy 1996), the present study showed that when low Picloram or Dicamba concentrations, or when 2,4-D at any concentration were used, higher flower bud development rates were observed. Similar results were obtained in African oil palm, where 2,4-D stimulated flower bud development, while the absence of growth regulators allowed oxidation and explant death (Teixeira et al. 1994). Other studies also reported that flower bud development from immature inflorescences under in vitro conditions was influenced by both culture medium composition (mainly related to auxin) and inflorescence maturity (Guerra and Handro 1998; Karun et al. 2004; Teixeira et al. 1994; Verdeil et al. 1994).

The auxin type and its concentration played important roles in SE induction. Picloram at 300 μM induced highest SE in both genotypes, although with a statistical difference between the genotypes (Table 3). Additionally, Dicamba (300 μM) induced a similar response in genotype II only; 2,4-D did not induce a noticeable SE response. The reasonably good results in the presence of Picloram follows the trend observed by Valverde et al. (1987) with apical meristems, suggesting that Picloram is the most suitable auxin type for peach palm SE induction, as it is for wheat (Barro et al. 1999) and betel nut palm (Karun et al. 2004) inflorescences.

Although SE induction rate was relatively low, the number of somatic embryos and their conversion capacity were high, with an average of 25.8 ± 1.6 somatic embryos being formed from each embryogenic callus after transfer to the maturation culture medium. The higher organic nitrogen and lower auxin concentration of the maturation medium and the use of cytokinin in the conversion medium allowed 70.3 ± 2.9% conversion of the mature somatic embryos to plantlets. In African oil palm, the use of organic nitrogen increased storage protein accumulation (Morcillo et al. 1999). In coconut the use of a step with low auxin concentration resulted in higher plantlet regeneration (Fernando and Gamage 2000) and the inclusion of a cytokinin was important for

conversion of oil palm (Aberlenc-Bertossi et al. 1999) and *Euterpe edulis* (Guerra and Handro 1998) somatic embryos. Hence, our results are in agreement with results obtained in other palms. However, in the present study, as well as for African oil palm (Teixeira et al. 1993), the nodular structures formed were not always capable of conversion into plantlets. These non-convertible nodular structures may be somatic embryos with arrested development due to deficient polarization (Yeung 1995), but this will require further study to confirm.

Plantlets with well-balanced shoot and root development (Fig. 1E) were obtained. The regenerated plantlets (Fig. 1F) were successfully acclimatized (78%—51 out of 65) in the acclimatization apparatus (Fig. 1G). However only 45% (29 out of 65) survived 4 months after transfer to the greenhouse (Fig. 1H). With regard to peach palm, it was suggested that an underdeveloped or poorly formed root system could result in low survival during the acclimatization step (Arias 1985). In the present study, the in vitro-grown root systems were pruned and new roots grew (data not shown), thus indicating that Arias' observation may not always be relevant. A yellowing of the leaves was often observed, culminating in the death of some plantlets after transfer to the greenhouse. Such behaviour has been described in other species and could be related to the photosynthetic apparatus of the plantlets (Rival et al. 1997) and/or inadequate plant nutrition under these culture conditions.

In addition to further investigations on the effect of genotype, the ontogenetic developmental sequence of the somatic embryos requires more work. The further improvement of this research protocol will benefit breeding and conservation programs with this species.

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