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A temporary immersion system improves *in vitro* regeneration of peach palm through secondary somatic embryogenesis

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• *Background and Aims* Secondary somatic embryogenesis has been postulated to occur during induction of peach palm somatic embryogenesis. In the present study this morphogenetic pathway is described and a protocol for the establishment of cycling cultures using a temporary immersion system (TIS) is presented.
• *Methods* Zygotic embryos were used as explants, and induction of somatic embryogenesis and plantlet growth were compared in TIS and solid culture medium. Light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to describe *in vitro* morphogenesis and accompany morpho-histological alterations during culture.
• *Key Results* The development of secondary somatic embryos occurs early during the induction of primary somatic embryos. Secondary somatic embryos were observed to develop continually in culture, resulting in non-synchronized development of these somatic embryos. Using these somatic embryos as explants allowed development of cycling cultures. Somatic embryos had high embryogenic potential ($65{\cdot}8 \pm 3{\cdot}0$ to $86{\cdot}2 \pm 5{\cdot}0$ %) over the period tested. The use of a TIS greatly improved the number of somatic embryos obtained, as well as subsequent plantlet growth. Histological analyses showed that starch accumulation precedes the development of somatic embryos, and that these cells presented high nucleus/cytoplasm ratios and high mitotic indices, as evidenced by DAPI staining. Morphological and SEM observations revealed clusters of somatic embryos on one part of the explants, while other parts grew further, resulting in callus tissue. A multicellular origin of the secondary somatic embryos is hypothesized. Cells in the vicinity of callus accumulated large amounts of phenolic substances in their vacuoles. TEM revealed that these cells are metabolically very active, with the presence of numerous mitochondria and Golgi apparatuses. Light microscopy and TEM of the embryogenic sector revealed cells with numerous amyloplasts, large nuclei and nucleoli, and numerous plasmodesmata. Plantlets were obtained and after 3 months in culture their growth was significantly better in TIS than on solid culture medium. However, during acclimatization the survival rate of TIS-grown plantlets was lower.
• *Conclusions* The present study confirms the occurrence of secondary somatic embryos in peach palm and describes a feasible protocol for regeneration of peach palm *in vitro*. Further optimizations include the use of explants obtained from adult palms and improvement of somatic embryo conversion rates.

Key words: *Bactris gasipaes*, tissue culture, somatic embryogenesis, clonal propagation, Picloram.

INTRODUCTION

Peach palm (*Bactris gasipaes*) is a caespitose, multipurpose palm tree that is widely distributed in the lowland humid Neotropics (Mora-Urpí *et al.*, 1997). Although it is listed as an underutilized crop, this species is one of the most useful palms in the Neotropics and the sole palm species that was domesticated for its fruits by Amerindians during the pre-Columbian period (Clement, 1988). Currently, the two major products from peach palm are the fruit, for local consumption, and the heart-of-palm, a gourmet vegetable extracted from the shoot apex and sold commercially worldwide (Clement, 2008). The heart-of-palm market is important in Latin America, and peach palm has numerous advantages for plantation production, such as perennial production from off-shoots, rapid growth rate and the possibility of fresh or minimally processed commercialization. Peach palm is also cultivated in Hawaii, Réunion Island, Indonesia and Malaysia.

Currently, major efforts towards the conservation of peach palm are based on the establishment of field germplasm banks, which are constantly threatened by biotic and abiotic factors, resulting in genetic erosion (Clement, 1997; Clement *et al.*, 2004). Seed banks are not an option because this species has recalcitrant seeds (Bovi *et al.*, 2004). Hence, *in vitro* conservation is suggested as the most promising technique for this purpose (Mora-Urpí *et al.*, 1997). At the Instituto Nacional de Pesquisas da Amazônia, Manaus, AM, Brazil, for instance, a core collection was created within the main collection and clonal propagation would permit its transfer to other institutions for further studies or use in breeding programmes. The formation of seed orchards with selected genotypes, as well as the large-scale clonal multiplication of

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selected palms, would also benefit from an *in vitro* regeneration protocol.

Both organogenesis and somatic embryogenesis pathways have been described for peach palm *in vitro* regeneration (Arias and Huete, 1983; Stein and Stephens, 1991), although the regeneration rate was limited. Hence, studies on somatic embryogenesis in peach palm were suggested to enhance the regeneration rate. The main factors affecting the induction of somatic embryogenesis from different types of explants have been identified (Steinmacher *et al.*, 2007*a*, *b*, *c*). It was suggested that during the induction of somatic embryos, the development of clusters of somatic embryos could be due to the development of secondary somatic embryos (Steinmacher *et al.*, 2007*a*), although little evidence was provided. The use of this morphogenetic pathway could be important in improving the regeneration rate of the whole procedure, providing cycling cultures. Cycling cultures can be obtained using cell suspensions or through the induction of secondary somatic embryos, and both have been described in palms (Teixeira *et al.*, 1995; Perez-Nunez *et al.*, 2006; Sané *et al.*, 2006); however, to the best of our knowledge no cycling cultures have been described in peach palm. Embryogenic friable callus, from which cell suspensions could be obtained (e.g. Teixeira *et al.*, 1995; Sané *et al.*, 2006), was observed to occur in peach palm using zygotic embryos as explants (Steinmacher *et al.*, 2007*a*), but some bottlenecks, such as their apparently random induction and the loss of embryogenic potential after several subcultures, makes secondary somatic embryogenesis the most attractive pathway for scaling-up *in vitro* regeneration. The use of zygotic embryos as explants in improvement and conservation programmes is inappropriate, although they may serve as an interesting model for peach palm somatic embryogenesis because a relatively high induction rate was observed (Steinmacher *et al.*, 2007*a*). In addition, the morpho-histological responses from zygotic embryos as explants were very similar to those observed from shoot meristem and leaf sheaths [i.e. first cell division on cells adjacent to the vascular tissue, callus growth through a meristematic zone and multicellular origin of the somatic embryos (Steinmacher *et al.*, 2007*a*, *c*)].

Somatic embryogenesis is an example of plant cell totipotency. It has been suggested that morphological and molecular mechanisms underlying plant cell totipotency are different from those controlling pluripotency, observed in the maintenance of root and shoot apices. However, pluripotent and totipotent cells have similar characteristics, including a high nucleus/cytoplasm ratio, a dense cytoplasm and a small fragmented vacuome (Verdeil *et al.*, 2007). Somatic embryogenesis involves the acquisition of embryogenic competence by somatic cells via their dedifferentiation and the reprogramming of their gene expression patterns (Harada, 1999; Feher *et al.*, 2003; Gaj *et al.*, 2005), possibly as a result of profound chromatin remodelling (Verdeil *et al.*, 2007). Secondary somatic embryogenesis is the process by which somatic embryos are formed from pre-existing somatic embryos or using somatic embryos as explants (Raemakers *et al.*, 1995). Usually, somatic embryos, which have higher embryogenic capacity than other explants, have been shown to increase the regeneration rate in several species (Raemakers *et al.*, 1995), and the resulting cultures maintain their morphogenic competence for several years (i.e. in sand grape (*Vitis rupestris*) (Martinelli *et al.*, 2001), thus increasing the potential regeneration rate several thousand-fold, as shown in coconut (*Cocos nucifera*) (Perez-Nunez *et al.*, 2006).

Temporary immersion systems (TIS), which involve flooding of plant tissue at regular time intervals, are often used for scaling-up or improving *in vitro* regeneration protocols and also offer the possibility of automating some culture stages. A semi-automated 'twin flask' TIS was initially developed for pineapple (Escalona *et al.*, 1999), and was later shown to improve the regeneration rate and plantlet quality for other plant species (Etienne and Berthouly, 2002). The number of regeneration protocols for other plant species using this system is increasing continually (Niemenak *et al.*, 2008; Sankar-Thomas *et al.*, 2008). Hence, an improved protocol for peach palm regeneration using TIS may be an alternative for the development of cycling cultures.

Parallel to the development of a feasible protocol for peach palm somatic embryogenesis, morpho-histological studies enhance our comprehension of the process, allowing further optimizations and other uses for the protocol. Studies with coconut (Fernando *et al.*, 2003), African oil palm (*Elaeis guineensis*) (Schwendiman *et al.*, 1988), Juçara palm (*Euterpe edulis*) (Guerra and Handro, 1998), date palm *Phoenix dactylifera* (Sané *et al.*, 2006), macauba palm (*Acrocomia aculeata*) (Moura *et al.*, 2008) and peach palm (Steinmacher *et al.*, 2007*a*, *c*) showed that the first events of cell division were always observed in cells adjacent to the vascular tissue, resulting in primary calli or meristematic nodules from which somatic embryos developed.

In the present report, the occurrence of secondary somatic embryos during the induction of peach palm somatic embryogenesis was confirmed and the applicability of TIS for regeneration of peach palm *in vitro* was studied using a twin flask system.

MATERIAL AND METHODS

Plant material

Seeds from mature fruits of *Bactris gasipaes* Kunth, about 4 months after pollination from one selected open-pollinated tree (Mood1) of the Pampa Hermosa landrace grown in a commercial orchard in Ninole, Hawaii, USA, were used as sources of explants. The hard endocarp of the seeds was removed and the kernels (i.e. zygotic embryo enclosed in endosperm) were surface-sterilized by 1 min immersion in 70 % ethanol, followed by 40 min immersion in sodium hypochlorite solution, provided by a solution of 40 % commercial bleach (5 % active chloride), plus one drop of Tween 20 to each 100 mL of this solution (Steinmacher *et al.*, 2007*a*). Zygotic embryos were aseptically removed under a stereomicroscope.

Culture media and conditions

The zygotic embryos were transferred to Petri dishes containing 30 mL of somatic embryogenesis induction medium: MS (Murashige and Skoog, 1962) salts plus Morel and Wetmore (1951) vitamins, 3 % (w/v) sucrose, 500 mg L^{-1} glutamine (Duchefa, Haarlem, the Netherlands), 2·5 g L^{-1} Gelrite

(Duchefa), 1 µM $AgNO_3$ and 10 µM Picloram [4-amino-3, 5,6-trichloropicolinic acid (Duchefa)] (Steinmacher *et al.*, 2007*a*). Each Petri dish contained five zygotic embryos that were observed periodically to describe the development of the somatic embryos. After 12 weeks of culture, the somatic embryos were isolated and used as explants for the induction of secondary somatic embryos in two experimental systems: on solid medium and in a temporary immersion system. All the cultures were kept in darkness at 28 °C with a subculture interval of 6 weeks. For culture on solid medium, nine somatic embryos were cultured on 30 mL induction medium as previously described for induction of primary somatic embryogenesis, using 2·5 g L^{-1} Gelrite (Duchefa) as gelling agent. The TIS used in the present study was based on the 'twin flasks' system described by Niemenak *et al.* (2008). Briefly, the plant compartment consisted of 1-litre glass jars (Weck GmbH u. Co. KG, Wehr, Germany) within which 250–300 mg isolated somatic embryos were cultivated in small baskets made with 150-µm nylon sieves (Laborbedarf-Vertriebs GmbH, Berlin, Germany) attached to the bottom of a 250-mL Kautex polypropylene bottle (Rotilabo® Carl Roth, http://www.carlroth.com). The medium compartment was a 1-litre Schott Duran® (Mainz, Germany) bottle with 250 mL liquid induction culture medium as described above, but without the gelling agent. Every 6 h the medium was air-pumped into the plant compartment for 3 min of contact with the explants. The air was filter-sterilized through an autoclavable 0·2-µm filter (Midisart 2000, Sartorius, Goettingen, Germany). Callus was discarded in each subculture and isolated somatic embryos were further cultured. The induction rate was evaluated at the end of each subculture interval.

The influence of induction conditions on the maturation of somatic embryos was evaluated. After cycling cultures were established, embryogenic cultures obtained from cultures (1) cultivated continually on solid culture medium, (2) cultivated in TIS for 6 weeks and then on solid culture medium for 6 weeks, and (3) cultivated continually in TIS were transferred to maturation conditions. In all treatments, 300–400 mg embryogenic clusters were separated into small clusters of somatic embryos and transferred to Petri dishes containing 30 ml maturation culture medium [MS salts; Morel and Wetmore vitamins; 40 µM 2,4-D (2,4-dichlorophenoxyacetic acid); 10 µM 2-iP [2-isopentyladenine (6-dimethylaminopurine)]; 1·5 g L^{-1} activated charcoal; 1 g L^{-1} glutamine, 500 mg L^{-1} hydrolysed casein and 2·5 g L^{-1} Gelrite (Steinmacher *et al.*, 2007*a*)]. The cultures were kept in the dark at 28 °C for 4 weeks. Mature somatic embryos were than isolated and transferred to Petri dishes containing 30 mL conversion culture medium [MS salts; Morel and Wetmore vitamins; 20 µM 2-iP; 0·5 µM NAA (α-naphthaleneacetic acid); adapted from Steinmacher *et al.* (2007*a*)]. The cultures were transferred to light conditions (40–60 µmol m^{-2} s^{-1} provided by TLD 58 W/840 fluorescent lamps, Philips, Eindhoven, the Netherlands) at 25 °C for 4 weeks. Thereafter, somatic embryos were transferred to Petri dishes containing 30 mL MS salts plus Morel and Wetmore vitamins and 1·5 g L^{-1} activated charcoal for about 8 weeks until the plantlets reached 1–2 cm.

Plantlets obtained from the cultures induced in TIS and directly transferred to maturation conditions were selected, and their further growth was evaluated on solid culture medium and in TIS. The plantlets were weighed (200–250 mg each) and transferred to jars (Weck; 600 mL) containing 100 mL solid culture medium (eight plantlets per flask) and sealed with plastic film, or 24 plantlets for TIS treatment containing 300 mL of the same basal culture medium, except that the active charcoal was omitted. The same TIS described for induction of secondary somatic embryogenesis was used, but without the baskets. The cultures were kept under light conditions and subcultured at intervals of 4 weeks for 3 months. All the culture media were adjusted to pH 5·8 prior to adding 2·5 g L^{-1} Gelrite (Duchefa) as gelling agent and were autoclaved at 120 °C for 20 min.

The acclimatization procedure was adapted from that of Steinmacher *et al.* (2007*a*), keeping the plantlets in a high-humidity environment for the first 3 weeks. The entire acclimatization step was carried out in a growing room at 28 °C and with 16 h 80–100 µmol m^{-2} s^{-1} provided by HP-T Plus Lamps (Philips). To maintain the humidity the plantlets were transferred to plastic trays containing sand and watered with distilled water. The trays were placed inside of another plastic tray containing a transparent cover and this whole acclimatization apparatus was maintained in plastic bags for 3 weeks. After 3 weeks, the plastic bags were removed and the plantlets were watered every other day with distilled water and once a week with 5 mL per plantlet of modified Hoagland's solution. After 3 months survival and rooting of the plantlets were evaluated.

Histology

For light microscopy, samples were fixed in 2 % (w/v) paraformaldehyde/0·1 M phosphate buffer (pH 7·0), dehydrated in an ethanol series [30–100 % (v/v) in water] and embedded in LR White resin (London Resin Co Ltd, London, UK). Gelatine capsules filled with resin and samples were allowed to polymerize at 65 °C overnight. Specimens were cut into 1-µm-thick sections with a glass-knife in a semi-automatic microtome (Reichert Ultracut S, Leica), mounted onto glass slides with a drop of water and fixed over a hot plate (approx. 70 °C). For general histology, Toluidine blue O [0·5 % (w/v) in 0·1 M phosphate buffer] was used. For protein and polysaccharide localization the double-staining technique [with NBB (Naphtol Blue-Black) and PAS (Periodic Acid-Schiff)] or only PAS was used following Fisher (1968). For DAPI (4′-6-diamidino-2-phenylindole) staining, 100 µL of a stock solution (1 mg ml^{-1} in water) was diluted into 1 mL 0·1 M phosphate buffer (pH 7·0). The sections were incubated in one drop of the dilute solution for 5 min and washed once with 0·1 M phosphate buffer (pH 7·0). After removing the excess washing buffer, the samples were mounted with anti-fading Citifluor (Citifluor Ltd, London, UK) and visualized under UV excitation (excitation 355 nm; emission 450 nm) using an Olympus BH-2 microscope and photographed with a ColorView IIIu (Soft Imaging System, GmbH, Munster, Germany).

For scanning electron microscopy (SEM), the samples were fixed in 3 % (v/v) glutaraldehyde in 0·1 M phosphate buffer (pH 7·0) and dehydrated in an ethanol series [30–100 % (v/v) in water], followed by an acetone series [30, 50 and

100 % (v/v) acetone in ethanol]. The samples were dried to the critical point with liquid CO_2 in a CPD 030 critical point dryer (Bal-TEC, Leica, http://www.leica-microsystems.com), affixed to aluminium stubs and coated with gold palladium in a SCD 050 sputter coater (Bal-TEC, Leica). The mounted specimens were examined with a Philips XL 20 scanning electron microscope.

For transmission electron microscopy (TEM), samples were fixed in 4 % (v/v) glutaraldehyde in 0·1 M cacodylate buffer (pH 7·0) for 2 h at room temperature. Thereafter the samples were transferred to fresh fixative solution and maintained overnight at 4 °C. The samples were then rinsed in the same buffer without glutaraldehyde three times (15 min each) and post-fixed in 1 % (v/v) OsO_4 in 0·1 M cacodylate buffer at 4 °C for 2 h. After rinsing in cacodylate buffer, the samples were dehydrated in a graded acetone series and embedded in Spurr's resin. Ultrathin sections (80–100 nm) were cut with an Ultracut E ultramicrotome (Reichert-Jung, Vienna, Austria), collected over a Formvar-coated copper grid, and stained with 1 % uranyl acetate and 0·1 % lead citrate for 10 min each.

Statistical analysis

A completely randomized design was used for all experiments. For the experiment of induction of secondary somatic embryogenesis, three repetitions comprising three Petri dishes each were evaluated for solid culture medium or three repetitions comprising two flasks for TIS. The data were subjected to ANOVA and when necessary the SNK test was used to compare means using STATISTICA v.6 (StatSoft, Inc.). The variables evaluated were the percentage of callus, spongy tissue development and induction rate of secondary somatic embryos. The percentage induction was additionally separated into three embryogenic-capacity categories [low (<5 somatic embryos), medium (5–15) and high (>15)] because during SEM it was difficult to count the exact number of somatic embryos.

For plantlet growth, the experiment was conducted in a completely randomized design containing five replications, with each replication represented by one flask with solid or liquid culture medium. The variables evaluated were plantlet fresh weight, total number of plantlets and plantlet height (with three classes: <3·5 cm, 3·5–6·5 cm, >6·5 cm). For plantlet acclimatization, the final survival rate (%), average height (cm), rooting rate (%) and average number of roots per plantlet were evaluated. The results were subjected to ANOVA as above.

RESULTS

Induction of primary somatic embryogenesis

Zygotic embryos (Fig. 1A) cultivated on Picloram-enriched culture medium showed swelling and callus growth during the first week of culture. Globular structures arising from callus were observed within 4–6 weeks of culture (Fig. 1B). These globular structures were whitish and usually only a few isolated globular structures were observed on each explant at this time. Development of additional somatic embryos occurred exactly in the sectors where the globular structures first appeared (Fig. 1C), resulting in clusters of somatic embryos at the end

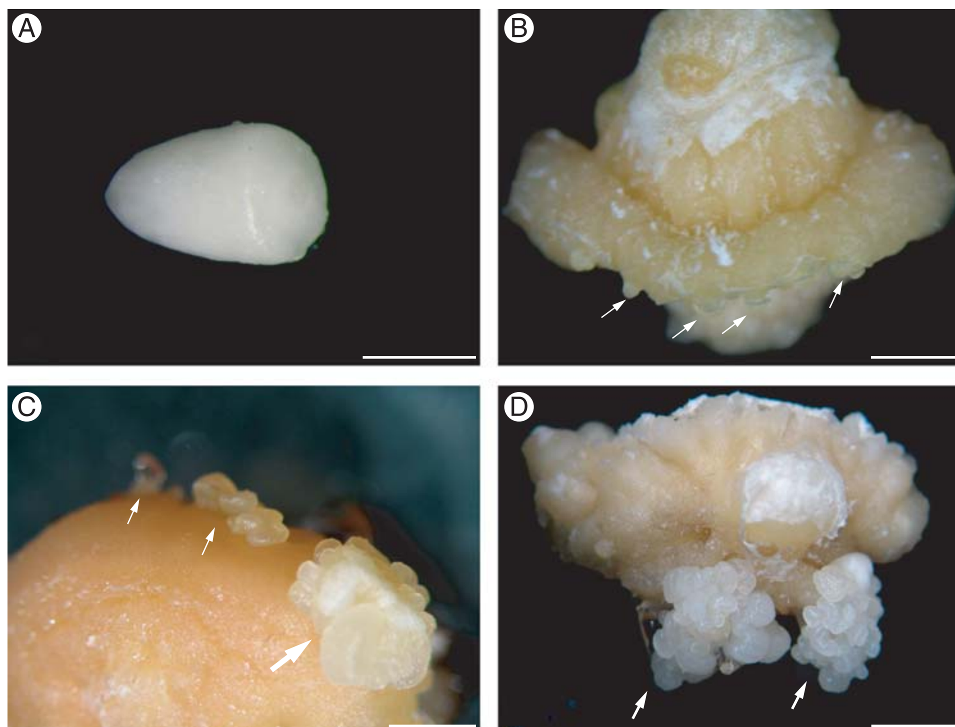

FIG. 1. Induction of somatic embryogenesis from peach palm zygotic embryos. (A) Mature zygotic embryo of peach palm used as explant. (B) Initial development of globular structures resembling somatic embryos on the callus (arrows) after 4–6 weeks of culture. (C) Further development of globular somatic embryos (arrows) where somatic embryos had previously developed. (D) Development of a cluster of somatic embryos (arrows) on the callus after 6 weeks of culture. Scale bars: (A) = 1 mm, (B) = 3 mm, (C, D) = 2 mm.

of 12 weeks of culture. These clusters of somatic embryos were observed on all parts of the callus, but usually clusters in contact with the culture medium developed more somatic embryos (Fig. 1D). Approximately 40 % of the explants developed somatic embryos.

Histological techniques were used to describe the sequence of development of individually selected somatic embryos. Globular structures at the onset of polarization (i.e. elongated cells) and the presence of a well-delimited protoderm were the first clearly distinguishable stages of somatic embryo development in culture (Fig. 2A). Further development included their elongation, development of procambium and differentiation of the shoot meristem pole (Fig. 2B). Somatic embryos transferred to maturation conditions had well-developed procambium and a developing sheath base around the shoot meristem (Fig. 2C). After the somatic embryos were transferred to conversion conditions, their development included a well-differentiated shoot meristem completely enclosed by the sheath base (Fig. 2D).

SEM revealed the initial development of the isolated globular structures (Fig. 3A), which resulted in the development of small clusters of somatic embryos (Fig. 3B). Alterations in the morphology of the somatic embryos were observed in particular, but not exclusively, in the sector where the sheath base resulted in a mushroom-like structure (Fig. 3B). From these somatic embryos, secondary somatic embryos developed, resulting in clusters of somatic embryos (Fig. 3C). This confirms that the development of clusters of somatic embryos in peach palm is due to the development of secondary somatic embryos. In the clusters of somatic embryos, several developmental stages could be observed, revealing non-synchronized development. This was due to the fact that secondary somatic embryos are continually produced in these conditions, where secondary somatic embryos arose also from globular somatic embryos (Fig. 3D), and that primary somatic embryos also developed at different points on a callus at different times.

Histological analyses revealed that the sub-epidermal cell layers had a more intense reaction to Toluidine blue (Fig. 4A). No suspensor-like tissue was observed and the somatic embryos had a broad basal area fused to the maternal tissue, but no vascular connections with the explant were observed by light microscopy. As somatic embryo development progressed, intense staining was observed in the sheath base region (Fig. 4B) from where new somatic embryos arose (Fig. 4C). As with SEM analysis, light histology analysis revealed the initial development of secondary somatic embryos from globular somatic embryos (Fig. 4D).

Induction of secondary somatic embryogenesis and plantlet regeneration

Peach palm somatic embryos were used as explants and different morphological responses were observed, including spongy tissue, callus and somatic embryo development. Picloram-enriched culture medium was effective in inducing secondary somatic embryogenesis and on average peach palm somatic embryos had elevated embryogenic capacity, ranging from

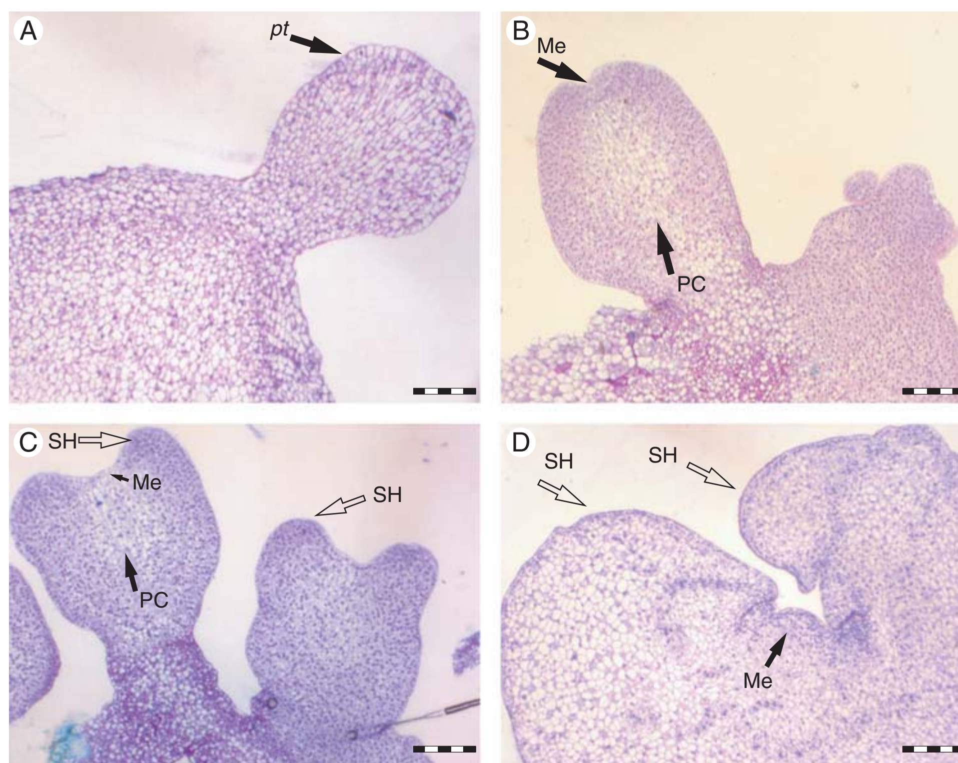

FIG. 2. Histological analyses of the development of peach palm somatic embryogenesis. (A) Globular somatic embryo with well-developed protodermis (*pt*). (B) Elongated somatic embryos showing the initial differentiation of the procambium (PC) and shoot meristem (Me). (C) Mature somatic embryo revealing complete development of the procambium as well as the sheath base (SH). (D) Somatic embryo in conversion conditions revealing a well-formed shoot meristem enclosed by the sheath base. Scale bars = 200 μm.

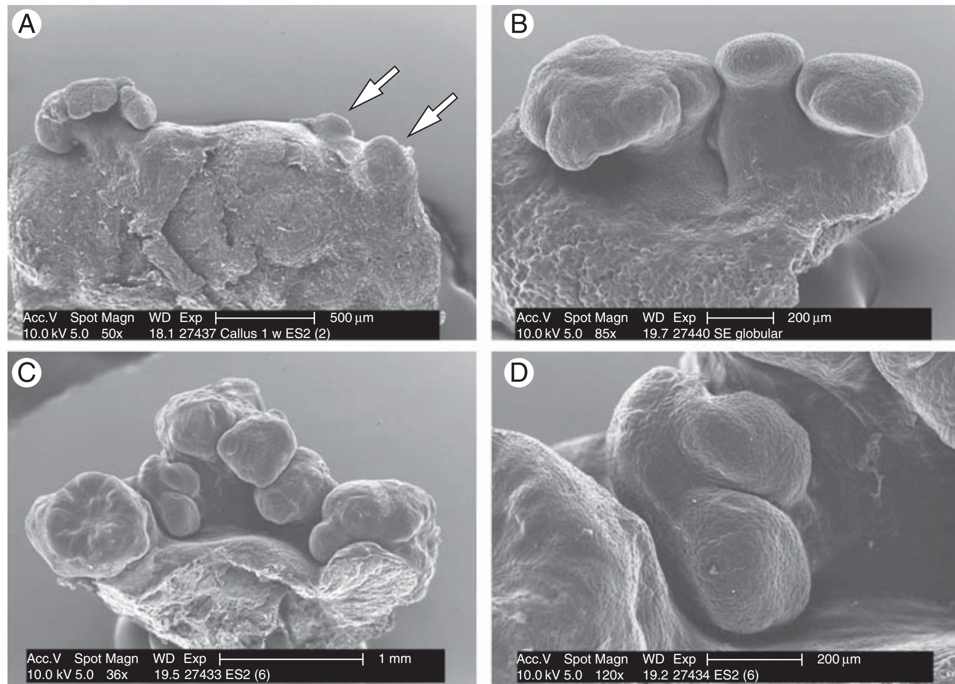

FIG. 3. Scanning electron microscopy analyses during the induction of peach palm somatic embryos. (A) Initial development of isolated globular structures (arrows). (B) Small clusters of primary somatic embryos. (C) Development of secondary somatic embryos, resulting in a cluster of somatic embryos. (D) Globular somatic embryo revealing the development of secondary somatic embryos. Scale bars: (A) = 500 µm, (C) = 1 mm, (B, D) = 200 µm.

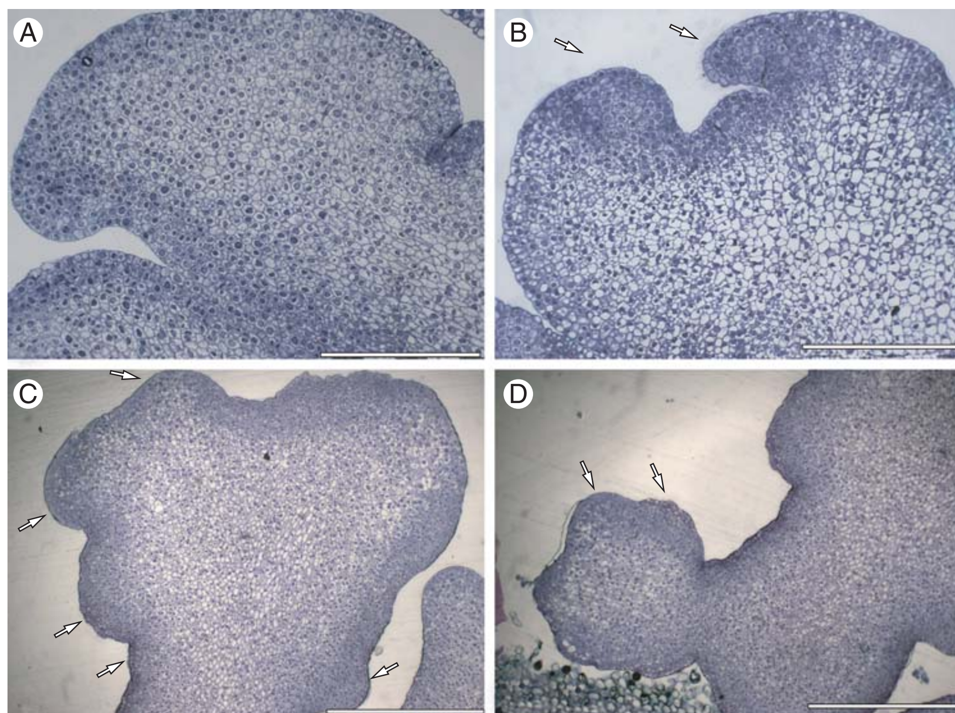

FIG. 4. Histological analyses of the development of peach palm somatic embryos stained with Touludine blue. (A) Histological analysis of peach palm primary somatic embryos revealing intense cell division on sub-epidermal cells. (B) Developed somatic embryo showing intense staining in the sheath base region (arrows). (C) Development of secondary embryos (arrows) from elongated somatic embryo. (D) Initial development of secondary somatic embryos from globular somatic embryos (arrows). Scale bars: (A, B) = 200 µm, (C, D) = 500 µm.

TABLE 1. *Percentage of induction of secondary somatic embryogenesis of peach palm in different culture conditions and during different cycles of 6 weeks each*

	Callus	Spongy tissue	Development of secondary somatic embryos			
			Total	Low	Medium	High
1st solid	7·7 ± 2·1[bc]	26·2 ± 2·9[b]	66·2 ± 3·1[b]	14·4 ± 1·7[b]	35·5 ± 2·0[a]	16·3 ± 2·7[b]
2nd solid	7·6 ± 2·8[bc]	26·2 ± 2·4[b]	65·8 ± 3·0[b]	21·8 ± 2·4[b]	37·8 ± 4·3[a]	6·2 ± 2·0[bc]
3rd solid	1·3 ± 0·5[c]	11·1 ± 4·2[a]	86·6 ± 4·1[a]	35·5 ± 6·0[a]	41·3 ± 8·6 [a]	9·7 ± 4·5[bc]
4th solid	0·0 ± 0·0[c]	31·1 ± 1·8[b]	70·2 ± 2·3[ab]	40·8 ± 2·5[a]	26·6 ± 2·5[ab]	2·6 ± 0·9[c]
2nd TIS	21·4 ± 2·7[a]	0·0 ± 0·0[c]	78·6 ± 2·7[ab]	16·5 ± 2·2[b]	13·3 ± 2·9[b]	48·8 ± 4·5[a]
3rd TIS	16·0 ± 3·1[ab]	0·0 ± 0·0[c]	83·9 ± 3·0[a]	13·4 ± 2·3[b]	19·5 ± 4·2[ab]	50·9 ± 3·0[a]
4th TIS	15·3 ± 3·5[ab]	0·0 ± 0·0[c]	84·6 ± 3·5[a]	9·7 ± 1·0[b]	10·7 ± 3·7[b]	64·2 ± 5·8[a]
TIS/Solid	9·3 ± 2·4[bc]	0·4 ± 0·4[c]	86·2 ± 5·0[a]	4·9 ± 2·7[b]	13·7 ± 2·6[b]	63·5 ± 6·7[a]

Data are means ± s.e. Means followed by different lower-case letters in the column represent statistical differences identified by SNK analysis ($P < 0{\cdot}05$).

TABLE 2. *Maturation of peach palm secondary somatic embryos on maturation culture medium*

		30 days culture on maturation medium	
Treatment	Initial weight (mg f. wt)	Increment (g)	Somatic embryos (100-mg explant)
Solid	343 ± 9·3	6·89 ± 0·74[b]	0·0 ± 0·0[b]
TIS	349 ± 13·7	3·92 ± 1·27[a]	62·1 ± 16·2[a]
TIS/Solid	370 ± 12·2	3·96 ± 1·03[a]	44·9 ± 7·8[a]

Data are means ± s.e. Means followed by different lower-case letters in the column represent statistical differences identified by SNK analysis ($P < 0{\cdot}05$).

65·8 to 86·2 % of secondary somatic embryo development in all conditions (Table 1). Statistical differences ($P < 0{\cdot}05$) were observed for the total rate of induction only in the first cycle (Table 1). A decreasing rate of embryogenic capacity was observed on solid culture medium for this group: 16·3 ± 2·7 % of the explants showed more than 15 somatic embryos per explant in the first cycle, whereas after four cycles (6 weeks each) only 2·6 ± 0·9 % of the explants could be classified as high capacity. On TIS, 48·8–64·2 % of the explants showed high embryogenic capacity (Table 1), without statistical differences between these values, but with significant differences ($P < 0{\cdot}05$) compared with solid culture medium. Transfer of somatic embryos from TIS to solid culture medium showed high embryogenic capacity (86·2 %), with most explants showing more than 15 somatic embryos (63·5 %), without differences with the TIS treatment. No development of spongy tissue was observed in TIS, while callus development was significantly ($P < 0{\cdot}05$) higher in TIS than on solid culture medium, varying from 15·3 to 21·4 % (Table 1).

Upon transferring somatic embryo clusters to maturation conditions for 4 weeks, differences among culture conditions were observed. The highest increase in fresh weight was observed for clusters of somatic embryos cultivated exclusively on solid culture medium (Table 2). This increase was due mainly to the development of a spongy haustorial-like tissue. Embryogenic clusters from TIS or TIS/solid medium had high numbers of mature somatic embryos after 4 weeks in maturation conditions, without statistical differences between these treatments (Table 2). The conversion capacity of somatic embryos was around 30 % (data not shown) and plantlets were obtained.

Small plantlets (200–250 mg each) were cultivated on solid culture medium or transferred to TIS. The final fresh weight of the plantlets was higher in TIS than on solid culture medium (Table 3). Plantlet height was also influenced by culture conditions. After 3 months of culture on solid culture medium, no plantlets taller than 6·5 cm were observed, while in TIS 51·1 ± 11·4 % of the plantlets were taller than 6·5 cm (Table 3). Additionally, newly formed shoots were observed in TIS as well as on solid culture medium; it is not clear if these shoots developed from fused somatic embryos or from the development of off-shoots, as peach palm is caespitose. Root development was observed only occasionally during the culture period.

Upon transferring the regenerated plantlets to acclimatization conditions, 65 % of TIS-grown plantlets and 97 % of solid culture medium plantlets survived after 3 months. On the other hand, plantlet height, rooting rate and number of roots per plantlet were significantly higher in plantlets from TIS than from solid culture medium (Table 4). Successful rooting of TIS-grown plantlets (75·1 %) was much higher than those grown on solid culture medium (12·5 %) and all plantlets were allowed to grow further.

Morpho-histological aspects of secondary somatic embryo development

Using somatic embryos as explants resulted in secondary somatic embryos developed directly on the explants. As observed during the induction of primary somatic embryogenesis, secondary somatic embryos developed frequently, but not exclusively, from the sheath base and somatic embryos appeared with a broad basal area fused to maternal tissue without vascular connections to the explant tissue. Once somatic embryogenesis was triggered, continual development of somatic embryos was again observed in all tested conditions, resulting in clusters of somatic embryos.

Histochemical analyses revealed that starch accumulation generally precedes the development of somatic embryos (Fig. 5A). A multicellular origin, involving niches of subepidermal and epidermal cells (Fig. 5B), is hypothesized for

TABLE 3. *Comparison of TIS and solid culture medium on subsequent peach palm plantlet growth*

Treatment	Initial weight (mg f. wt)	Final weight (g f. wt)	Total no.	Class of plantlet height (%)		
				<3·5 cm	3·5–6·5 cm	>6·5 cm
TIS	213 ± 2	1·34 ± 0·2	177	16·9 ± 5·2	32·0 ± 7·8	51·1 ± 11·4
Solid	215 ± 1	0·97 ± 0·1	54	41·6 ± 8·1	58·4 ± 8·1	0·0 ± 0·0

Data are means ± s.e.

TABLE 4. *Peach palm plantlet growth and survival rate after 3 months of acclimatization*

Treatment	No. of plantlets	Survival (%)	Height (cm)	Rooting (%)	Roots per plantlet
TIS	133	65^{b}	$11{\cdot}2^{a}$	$75{\cdot}1^{a}$	$2{\cdot}3^{a}$
Solid	36	97^{a}	$6{\cdot}6^{b}$	$12{\cdot}5^{b}$	$1{\cdot}3^{b}$

Means followed by different lower-case letters in the column represent statistical differences identified by ANOVA ($P < 0{\cdot}05$).

these somatic embryos. However, in cultures on solid culture medium only a few cells showed the presence of amyloplasts (Fig. 5C), whereas in cultures from TIS most subepidermal cells showed starch accumulation. As development of somatic embryos progressed, starch accumulation was observed only in the basal area of the somatic embryos or later on those sectors where other somatic embryos would develop (Fig. 5D). However, starch accumulation could not be systematically correlated with cell embryogenic capacity, as in culture sub-epidermal cells also accumulated large amounts of starch without developing into somatic embryos, especially in TIS conditions.

Ultrastructural analyses of the embryogenic sector revealed small starch granules in the cells of the protodermis, while sub-epidermal cells had larger amyloplasts that were also more abundant and well distributed in the cells (Fig. 6A). The cells contained numerous small vacuoles and a large central nucleus with prominent nucleolus (Fig. 6B), no cell-wall thickening, and plasmodesma was often observed connecting the cells (Fig. 6C). These results together suggest the multicellular origin of the somatic embryos and epidermal and sub-epidermal cells for the origin of the secondary somatic embryos. Also in this embryogenic sector, mitotic events could be observed by DAPI staining and a higher nucleus/cytoplasm ratio was observed in epidermal and sub-epidermal cells compared with those that would result in callus growth (Fig. 7A). More numerous mitotic events were observed in the sub-epidermal cell layer and these cells also had centrally located nuclei with one or two nucleoli (Fig. 7B).

Histological analyses of callus revealed the presence of a specific zone of small cells (Fig. 8A), as well as the presence of an epidermis-like layer (Fig. 8B). Only a few amyloplasts were observed in some areas of the callus (Fig. 8C). In the area where the embryogenic sector was in contact with the callus sector, an intense metachromatic reaction with Toluidine blue O was observed in the vacuoles (Fig. 8D) of a specific layer of cells usually associated with the development of somatic embryos. This metachromatic reaction indicates the accumulation of large amounts of phenolic substances in the vacuoles of the cells. Ultrastructural analysis of these phenol-storing cells revealed the presence of a large vacuole containing electron-dense substances, numerous mitochondria, small amyloplasts, plastids also containing electron-dense substances and Golgi complexes (Fig. 8E), all suggesting metabolically active cells.

DISCUSSION

The occurrence of secondary somatic embryogenesis during the induction of peach palm somatic embryogenesis, as previously hypothesized (Steinmacher *et al.*, 2007*a*), was confirmed on auxin-enriched culture medium with light microscopy and SEM in the present study. The occurrence of secondary somatic embryos in primary culture conditions was observed in Bermuda grass (Li and Qu, 2002) and the constant requirement of plant growth regulators for the development of somatic embryos has been described in several monocot species [i.e. banana (Khalil *et al.*, 2002); see also the review by Raemakers *et al.* (1995)]. In the present study, the use of this morphogenetic pathway also allowed the establishment of an effective protocol for the induction of secondary somatic embryogenesis and permitted the development of a cycling culture.

In peach palm induction of both primary and secondary somatic embryos was achieved with Picloram-enriched culture medium, confirming that this as an effective auxin analogue for peach palm somatic embryogenesis, as previously demonstrated (Valverde *et al.*, 1987; Steinmacher *et al.*, 2007*a*, *b*, *c*). This auxin analogue also induced embryogenic competence in African oil palm (Teixeira *et al.*, 1995) and was the most suitable auxin source for somatic embryogenesis in arecanut palm (*Areca catechu*, Karun *et al.*, 2004) and macauba palm (Moura *et al.*, 2008). Auxins have been shown to act like a molecular glue binding to its TIR1 receptor and promoting ubiquitin-dependent degradation of Aux/IAA repressor proteins, activating the auxin response elements (Guilfoyle, 2007; Tan *et al.*, 2007). Naturally occurring auxin (indol acetic acid – IAA) and synthesized auxin analogues (i.e. NAA and 2,4-D) showed the same activity (Guilfoyle, 2007; Tan *et al.*, 2007). It is thought that auxin analogues also have a dual role during the induction of somatic embryogenesis, one related to auxin signalling and the other to a stress component (Feher *et al.*, 2003) that also changes the endogenous content of auxins (Jiménez, 2005). Picloram is thought to have a stronger auxin effect compared with other auxin analogues (i.e. 2,4-D) and this observation is supported by the fact that germinating *Arabidopsis* seeds

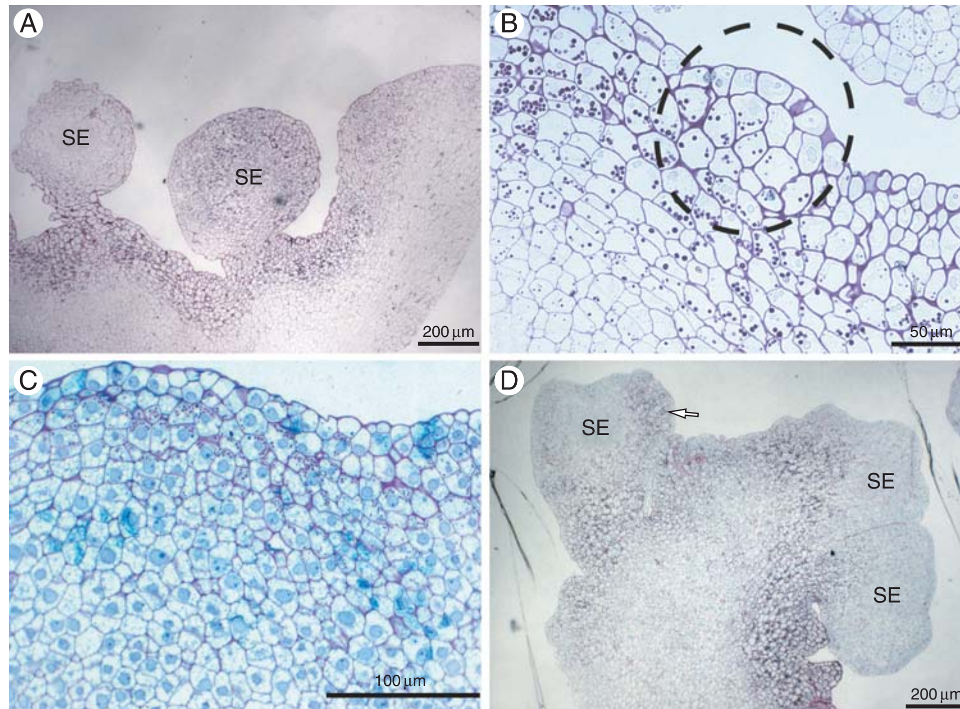

FIG. 5. Histochemical analyses during the development of peach palm secondary somatic embryos (SE). (A) Cluster of somatic embryos from TIS showing high starch accumulation. (B) Possible origin of somatic embryos involving sub-epidermal and epidermal cells (circle). (C) Samples cultured only on solid culture medium. (D) Further development of somatic embryos showing specific starch accumulation in those sectors where other somatic embryos would develop (arrow). Scale bars: (A) = 200 μm, (B) =50 μm, (C) = 100 μm, (D) = 200 μm.

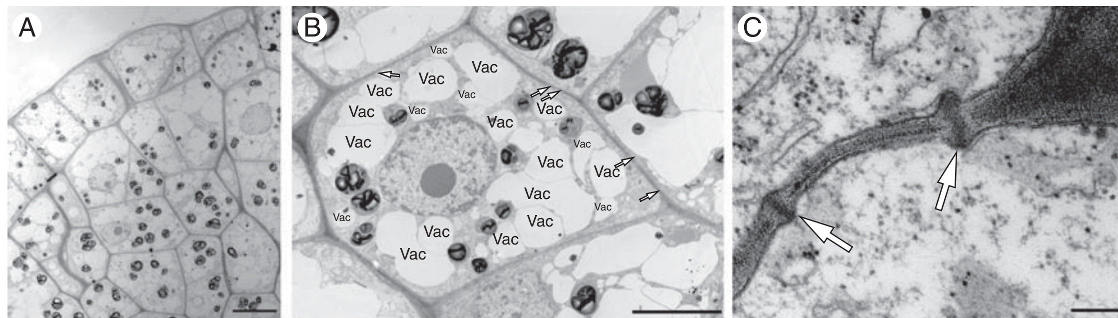

FIG. 6. Ultrastructural analyses of peach palm embryogenic cells. (A) Embryogenic sector including epidermal and sub-epidermal cells. (B) Example of a cell of the embryogenic sector contained numerous small vacuoles (Vac) and numerous plasmodesma (arrows). (C) Aspect of the plasmodesma. Scale bars: (A) = 10 μm, (B) = 5 μm, (C) = 200 nm.

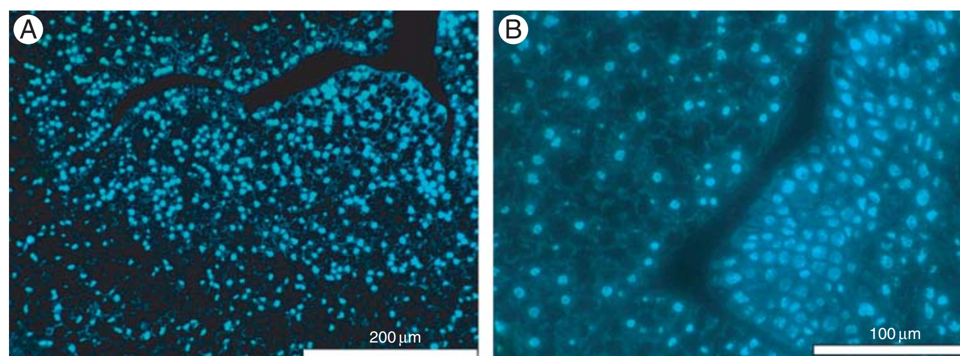

FIG. 7. Mitotic events in the embryogenic sector revealed by DAPI staining. (A) Higher nucleus/cytoplasm ratio observed in sub-epidermal cells. (B) Mitotic events during the initial development of peach palm secondary somatic embryos. Scale bars: (A) = 200 μm, (B) = 100 μm.

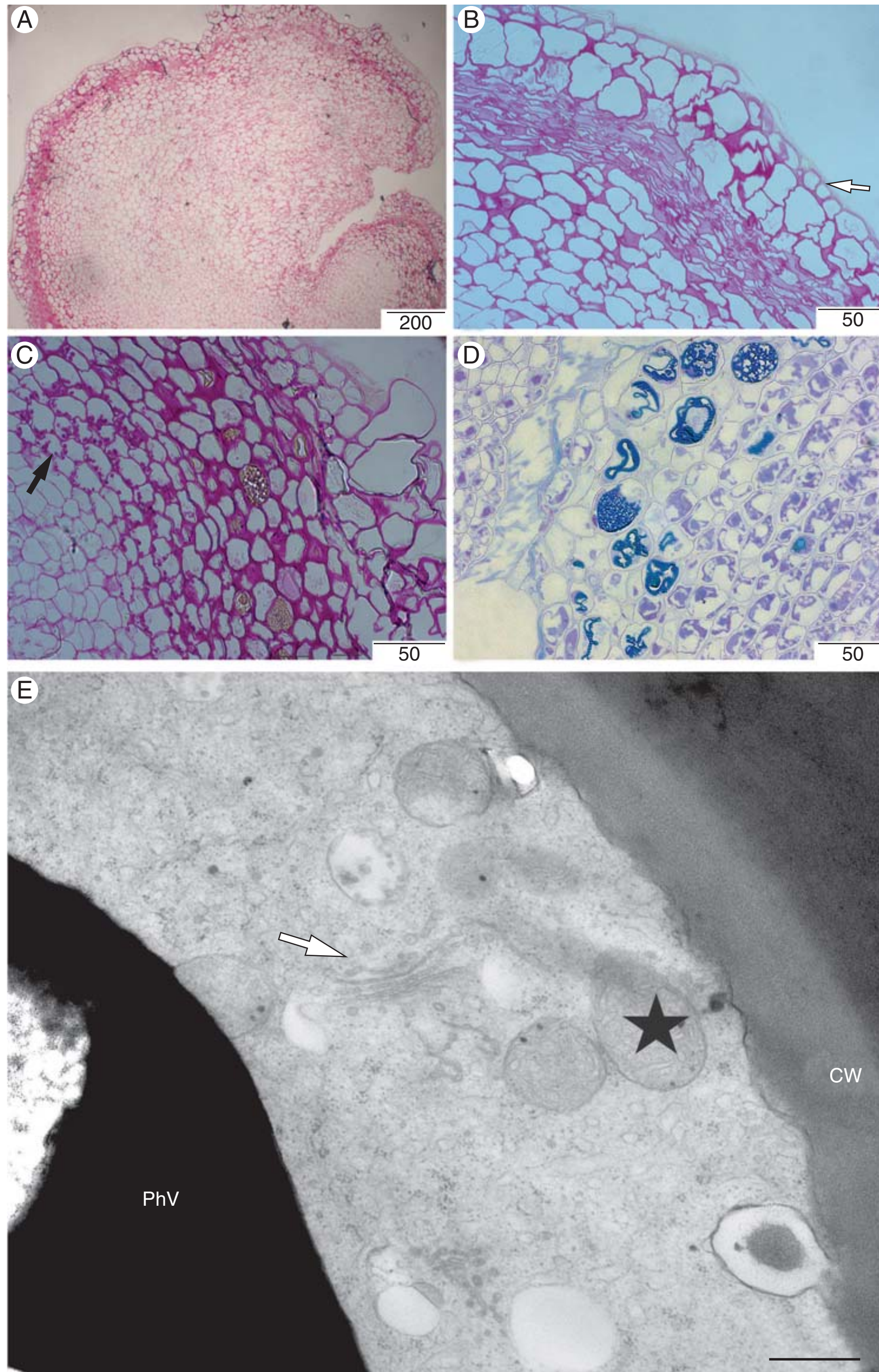

FIG. 8. Histological and ultrastructural aspects of the callus sector. (A) General view of callus stained with PAS reagent. (B) Detailed view of A showing an epidermis-like cell layer (arrow). (C) Presence of amyloplast in the callus sector (black arrow). (D) Histological section of the callus sector in contact with the embryogenic sector revealing the accumulation of phenolic substances in the cells. (E) Detailed view of the phenol-storing cells showing numerous mitochondria (stars), and Golgi complex (arrow). Scale bars: (A) = 200 μm, (B, C) = 50 μm, (D) = 500 μm, (E) = 500 nm.

in Picloram-enriched culture medium mimicked an auxin over-producing mutant (*sur2*) (Delarue *et al.*, 1998). However, other differences between 2,4-D and Picloram in signalling cascades may also exist, as a mutation in one receptor altered the response to Picloram but not to 2,4-D and IAA (Walsh *et al.*, 2006).

Secondary somatic embryogenesis has already been proved to be an excellent morphogenetic process for several species,

such as coconut (Perez-Nunez *et al.*, 2006), cassava (*Manihot esculenta*) (Groll *et al.*, 2001) and cacao (*Theobroma cacao*) (Maximova *et al.*, 2002). It has been shown that somatic embryos, especially during the first developmental stages, have higher embryogenic capacity than other explants (Raemakers *et al.*, 1995). This could be attributed to the presence of higher levels of somatic embryogenesis-related transcripts, such as the somatic embryogenesis-related kinase (SERK) and leafy cotyledon (LEC), which are still being expressed at the first developmental stages of somatic embryos (Schmidt *et al.*, 1997; Kwaaitaal and de Vries, 2007; Alemanno *et al.*, 2008; Sharma *et al.*, 2008).

The combination of a secondary somatic embryogenesis pathway with TIS also increases propagation efficiency in several species, for example rubber (*Hevea brasiliensis*) (Etienne *et al.*, 1997), coffee (Albarran *et al.*, 2005) and cacao (Niemenak *et al.*, 2008). To the best of our knowledge, however, there are no conclusive reports on the use of TIS and secondary somatic embryogenesis in palms. In our study, efficient cycling cultures could be established only on TIS, while on solid culture medium a decreasing rate of highly embryogenically capable cultures and an increasing rate of spongy tissue development were observed. Other advantages of TIS could be related to the absence of nutrient gradients (as in solid culture medium), nutrient uptake without permanent hypoxia and the frequent renewal of the *in vitro* atmosphere, where ethylene accumulates (Etienne and Berthouly, 2002). The presence of silver nitrate, a known blocker of ethylene perception, was shown to greatly increase somatic embryogenesis of peach palm (Steinmacher *et al.*, 2007*a*).

Stress is an important factor related to the acquisition of embryogenic competence (Pasternak *et al.*, 2002) and the temporary flooding in TIS could create different forms of stress, such as temporary hypoxia, which is sensed by the cells as a sort of stress triggering short-term metabolic adaptations. During hypoxia plant cells adopt a carbohydrate-conserving response to reserve starch and hexose sugars for use in aerobic metabolism upon recovery from the stress (Fukao and Bailey-Serres, 2004). This strategy also decreases oxygen consumption and improves plant re-growth after reoxygenation (Geigenberger, 2003). Therefore, it is tempting to suggest that peach palm explants adopted such an adaptation strategy, because, according to our histological analysis, greater starch accumulation was observed in TIS-cultivated explants. Additionally, in the present study, greater starch accumulation was observed in the embryogenic sector in particular, while in the callus sector starch was rarely observed. Starch accumulation is considered a marker for embryogenic capacity in several systems, including oil palm and coconut (Schwendiman *et al.*, 1988; Verdeil *et al.*, 2001). However, in peach palm starch accumulation could not be systematically correlated to somatic embryogenesis, as some cells accumulated starch without developing into somatic embryos, as also observed in rattan somatic embryogenesis (Goh *et al.*, 2001), while in pineapple-guava (*Feijoa sellowiana*) non-embryogenic tissue accumulated much more starch (Canhoto *et al.*, 1996). Therefore, it remains to be determined if starch accumulation is more related to hypoxia or other metabolic pathways than to the specific development of somatic embryos. Even in well-oxygenated surroundings, plant tissues that have high metabolic activity can become hypoxic, especially those that lack large intercellular air spaces or contain cells that are poorly vacuolated (Geigenberger, 2003), characteristics similar to those observed in the present study.

Histological analyses revealed cells with embryogenic characteristics, such as small cells with small vacuoles, large nuclei and dense cytoplasm. Such cells were usually found as niches of sub-epidermal and epidermal tissue. In coconut somatic embryogenesis, sub-epidermal embryogenic cells also had a dense cytoplasm (Saenz *et al.*, 2006). At the onset of somatic embryo development, DAPI staining also revealed that these cells were at different stages of division or with centrally located nuclei with one or two nucleoli, while in the callus sector much less signal was observed and the nuclei were usually in the periphery of the cells. This agrees with the observation that cells able to produce somatic embryos are mitotically more active than non-embryogenic cells (Pasternak *et al.*, 2002).

Somatic embryos may originate from either unicellular or multicellular pathways and, in our study, niches of epidermal and sub-epidermal cells participated in the formation of peach palm secondary somatic embryos. Ultrastructural analyses revealed that peach palm somatic embryogenesis follows a pathway similar to that described for pineapple-guava (Canhoto *et al.*, 1996), in which embryogenic cells were connected by plasmodesmata through their cell walls. Additionally, peach palm somatic embryos also appeared with a broad basal area in contact to maternal tissue, but without vascular connection with the maternal tissue, and histological analyses showed no co-ordinated cell division during the initial developmental steps. All these characteristics suggest the multicellular origin of the somatic embryos (Quiroz-Figueroa *et al.*, 2006). In coconut, both multicellular and unicellular pathways have been observed (Verdeil *et al.*, 2001; Fernando *et al.*, 2003), although plantlets have been regenerated mostly from somatic embryos with a multicellular origin (Perera *et al.*, 2007).

Histological analyses of the callus sector revealed a sector of phenol-rich cells exactly on the border of the callus, especially in the sectors where somatic embryos developed. Similar results were also observed in pineapple-guava, and these phenol-storing cells were hypothesized to form a barrier between the somatic embryos and the mother tissue during induction and development of somatic embryos (Reis *et al.*, 2008). Blockage of symplastic transport is also thought to be a major driver of morphological alterations (Pfluger and Zambryski, 2001) and it is possible that for the development of somatic embryos symplastic isolation is required also at the multicellular level. Such isolation can occur physically through the thickening of the cell wall and closing of the plasmodesmata by deposition of callose (Dubois *et al.*, 1990; Verdeil *et al.*, 2001), as well as by deposition of phenolic and lipophilic substances on cell walls (Pedroso *et al.*, 1995), or through the development of barrier cells (i.e. phenol-storing cells) between somatic embryos and mother tissue (Reis *et al.*, 2008), as observed in the present study. As these phenol-storing cells are metabolically very active, with the presence of numerous mitochondria and

Golgi apparatuses, their specific roles during the development of somatic embryos remain to be elucidated.

In the present study, somatic embryos were transferred to maturation conditions prior to conversion. These steps can still be considered as a bottleneck to a successful protocol, as some mature somatic embryos were fused or no mature somatic embryos were observed from those cultures induced only on solid culture medium. Additionally, a relatively low (around 30 %) conversion rate was observed and the regenerated plantlets had deficient root development *in vitro*. Previous studies of acclimatization of peach palm plantlets suggested that the *in vitro*-grown roots were not functional (Arias, 1985) and they were removed, allowing new roots to develop during the acclimatization step (Steinmacher *et al.*, 2007*a*). Plantlets produced in TIS had lower survival rates than those from solid culture medium; however, TIS-grown plantlets showed enhanced growth and higher rooting rate, suggesting physiologically better plantlets. Among the other vantages of TIS, the ventilation of the culture containers may also result in plantlets that are more capable of growing in *ex vitro* conditions. In coconut, increased capacity of *in vitro*-grown plants to control water loss was related to the ventilation of the flasks (Talavera *et al.*, 2001). This suggests that TIS could be an alternative technique for the growth of other palm species.

In conclusion, the occurrence of secondary somatic embryos during the induction of somatic embryogenesis in peach palm was confirmed. This morphogenetic pathway allowed the development of a protocol suitable for *in vitro* multiplication of peach palm using a TIS and cycling cultures. Although zygotic embryos were used as explants, somatic embryogenesis was already obtained from inflorescences and shoot meristems (Steinmacher *et al.*, 2007*b*, *c*), making this protocol useable for selected genotypes. In fact, a pilot project is underway for the mass propagation of selected peach palm genotypes from Hawaii. The maturation and conversion conditions in this protocol must also be improved, possibly using storage proteins as a quality marker. The use of a TIS may also be an interesting strategy for the scaling-up of other palm tree *in vitro* protocols.

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