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Somatic Embryogenesis in Peach Palm Using the Thin Cell Layer Technique: Induction, Morpho-histological Aspects and AFLP Analysis of Somaclonal Variation

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• *Background and Aims* The thin cell layer (TCL) technique is based on the use of very small explants and has allowed enhanced *in vitro* morphogenesis in several plant species. The present study evaluated the TCL technique as a procedure for somatic embryo production and plantlet regeneration of peach palm.

• *Methods* TCL explants from different positions in the shoot apex and leaf sheath of peach palm were cultivated in MS culture medium supplemented with 0–600 μM Picloram in the presence of activated charcoal. The production of primary calli and embryogenic calli was evaluated in these different conditions. Histological and amplified fragment length polymorphism (AFLP) analyses were conducted to study *in vitro* morphogenetic responses and genetic stability, respectively, of the regenerated plantlets.

• *Key Results* Abundant primary callus induction was observed from TCLs of the shoot meristem in culture media supplemented with 150–600 μM Picloram (83–97 %, respectively). The production of embryogenic calli depends on Picloram concentration and explant position. The best response observed was 43 % embryogenic callus production from shoot meristem TCL on 300 μM Picloram. In maturation conditions, 34 ± 4 somatic embryos per embryogenic callus were obtained, and 45·0 ± 3·4 % of these fully developed somatic embryos were converted, resulting in plantlets ready for acclimatization, of which 80 % survived. Histological studies revealed that the first cellular division events occurred in cells adjacent to vascular tissue, resulting in primary calli, whose growth was ensured by a meristematic zone. A multicellular origin of the resulting somatic embryos arising from the meristematic zone is suggested. During maturation, histological analyses revealed bipolarization of the somatic embryos, as well as the development of new somatic embryos. AFLP analyses revealed that 92 % of the regenerated plantlets were true to type. The use of TCL explants considerably improves the number of calli and somatic embryos produced in comparison with previously described protocols for *in vitro* regeneration of peach palm.

• *Conclusions* The present study suggests that the TCL somatic embryogenesis protocol developed is feasible, although it still requires further optimization for *in vitro* multiplication of peach palm, especially the use of similar explants obtained from adult palm trees.

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

INTRODUCTION

The development of *in vitro* regenerative protocols in peach palm (*Bactris gasipaes*) has direct applications for clonal mass propagation of selected plants and may be integrated into breeding and conservation programmes (Mora-Urpí *et al.*, 1997). Today this species is important for its fruit, popular throughout its traditional distribution, and for its heart-of-palm, a gourmet vegetable extracted from the shoot apex. Various Latin American institutions have breeding programmes for one or both uses, where *in vitro* techniques for plantlet regeneration could be important tools. *In vitro* plantlet regeneration of peach palm was described for the first time by indirect organogenesis (Arias and Huete, 1983), later by somatic embryogenesis (Valverde

et al., 1987; Stein and Stephens, 1991) and by direct organogenesis (Almeida and Kerbauy, 1996). However, plantlet regeneration was often difficult, and to date there is no efficient protocol describing in detail the *in vitro* regeneration of peach palm from leaf sheaths or shoot meristems, which would be the perfect explant source for this caespitose palm. Valverde *et al.* (1987) described a protocol for *in vitro* regeneration of peach palm, although only ten embryogenic calli were obtained (out of 100 shoot apexes – where one explant could produce more than one callus) and each callus produced 2–8 somatic embryos. In the protocol described by Stein and Stephens (1991), only four calli cultures (out of 18 explants) producing >10 plantlets per culture were established. Both success rates are clearly insufficient for large-scale plantlet production. Thus, the *in vitro* regeneration of peach palm still requires the optimization of the protocols.

In the study of Valverde and co-workers, the production of somatic embryos was obtained with the use of Picloram. This auxin analogue has been successfully used for somatic

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embryogenesis induction in numerous plant species (Barro *et al.*, 1999; Groll *et al.*, 2001; Kaur and Kothari, 2004), and in peach palm our results confirm it as a reliable auxin source for inducing somatic embryogenesis from zygotic embryos and immature inflorescences (Steinmacher *et al.*, 2007*a*, *b*). During the development of a protocol for plantlet regeneration, morphological and histological aspects should be evaluated to elucidate the *in vitro* response, such as the origin and development of the calli and somatic embryos, as well as the characterization of the morphogenetic route. No histological observations of peach palm *in vitro* plantlet regeneration from leaf sheaths or shoot meristems have been reported. Sané *et al.* (2006) recently described a detailed protocol for *Phoenix dactylifera* regeneration through somatic embryogenesis with all the steps analysed histologically. They described the origin and development of the calli, as well as the histochemical alterations during *in vitro* culture, which may serve as markers for somatic embryogenesis competence.

Somatic embryogenesis is the preferred *in vitro* regenerative route for palms, as this morphogenetic pathway may increase the number of regenerated plantlets in comparison with organogenesis. Among other advantages, somatic embryogenesis permits creation of cycling cultures through the use of cell suspensions (Teixeira *et al.*, 1995; Sané *et al.*, 2006) or through secondary somatic embryogenesis (Perez-Nunez *et al.*, 2006). The production of somatic embryos capitalizes upon the totipotency of plant cells and involves the development of bipolar structures resembling zygotic embryos (Dodeman *et al.*, 1997). This morphogenetic route is influenced by several factors imposed by *in vitro* conditions (Feher *et al.*, 2003). Among these, the explant source and the developmental stage are considered key elements that alter cellular competence (Merkle *et al.*, 1995). In addition, the size of the explants greatly influences their morphogenetic capacity (Benkirane *et al.*, 2000; Delporte *et al.*, 2001), probably due to the establishment of a symplast domain (Bouget *et al.*, 1998; Haywood *et al.*, 2002) that maintains the coordinated development of the cells and tissues (Wu *et al.*, 2002; Roberts and Oparka, 2003). Roberts and Oparka (2003) define the symplast domain as a continuum between the cells of a specific tissue domain, allowing molecular movement through the plasmodesmata, and maintaining and coordinating morphogenetic activity in the tissue.

The thin cell layer (TCL) technique utilizes very small explants and was first described in *Nicotiana tabacum* (Tran Thanh Van *et al.*, 1974). It could be an alternative method to disrupt symplast domains, as well as to modulate *in vitro* response. This procedure gave enhanced results for numerous *in vitro* culture systems (Tran Thanh Van and Bui, 2000), including *Digitaria sanguinalis* (Van Le *et al.*, 1997), *Oryza sativa* (Nhut *et al.*, 2000), *Elaeis guineensis* (Teixeira *et al.*, 1994) and *Cocos nucifera* (Samosir *et al.*, 1998).

In vitro culture may cause disturbances to the genome organization of regenerated plantlets, resulting in somaclonal variation (Larkin and Scowcroft, 1981). To evaluate possible somaclonal variation in regenerated plants, DNA should be randomly and evenly analysed (Polanco and Ruiz, 2002). Molecular markers, such as amplified fragment length polymorphisms (AFLPs) (Vos *et al.*, 1995), allow the analysis of genomic DNA using a multilocus approach with high sensitivity. The AFLP method combines the reproducibility of restriction fragment analysis and the power of the polymerase chain reaction (PCR) in a straightforward technique (Mueller and Wolfenbarger, 1999). AFLP markers have been used to detect somaclonal variation in plants (e.g. Carolan *et al.*, 2002; Polanco and Ruiz, 2002; Pontaroli and Camadro, 2005). The sensitivity of the restriction analysis and the use of stringently controlled PCRs often allow the detection of point mutations (insertions/deletions), and are proving useful in detecting somaclonal variation during somatic embryogenesis (e.g. Cervera *et al.*, 2002). In *P. dactylifera*, AFLPs were more sensitive than random amplification of polymorphic DNA (RAPD) markers to detect genomic alterations in regenerated plantlets (Saker *et al.*, 2006); in *E. guineensis*, for example, the RAPD technique did not detect differences between groups of somaclonal variants (Rival *et al.*, 1998). Also in *E. guineensis* the AFLP technique was used successfully with methylation-sensitive restriction enzymes to detect variants from *in vitro* culture (Matthes *et al.*, 2001). The AFLP technique has already been used for distinguishing peach palm landraces (Clement *et al.*, 2002).

The TCL technique associated with different Picloram concentrations was evaluated to develop an efficient procedure for somatic embryogenesis and plantlet regeneration of peach palm. In addition, histological and AFLP analyses were conducted in order to verify the *in vitro* morphogenetic responses, as well as the genetic stability of the regenerated plantlets.

MATERIALS AND METHODS

Plant material

Nearly mature fruits, about 12 weeks after pollination, were collected from one selected open-pollinated plant of peach palm of the Yurimaguas population of the Pampa Hermosa landrace kept at the Instituto Nacional de Pesquisas da Amazônia (INPA) germplasm collection, Manaus, Amazonas, Brazil. The hard endocarps were removed and the kernels (i.e. zygotic embryo enclosed by endosperm) were surface-sterilized during 1 min immersion in 70 % ethanol, followed by a 40 min immersion in sodium hypochlorite solution, provided by a solution of 60 % commercial bleach (2·0–2·5 % active chlorine), plus one drop of Tween-20® in each 100 mL. Thereafter, in aseptic conditions, the kernels were rinsed three times in sterile distilled water. Zygotic embryos were aseptically removed from the kernels under a stereoscopic microscope and transferred to test tubes containing 10 mL of basal culture medium [MS salts (Murashige and Skoog, 1962) plus Morel's vitamins (Morel and Wetmore, 1951), 3 % sucrose, 1·5 g L^{-1} activated charcoal and gelled with 7·0 g L^{-1} Agar (Merse)]. The culture medium was adjusted to pH 5·8 prior to adding the gelling agent and was autoclaved for 15 min at 1 kgf cm^{-2}. The cultures were kept at 26 ± 1 °C in a 16 h
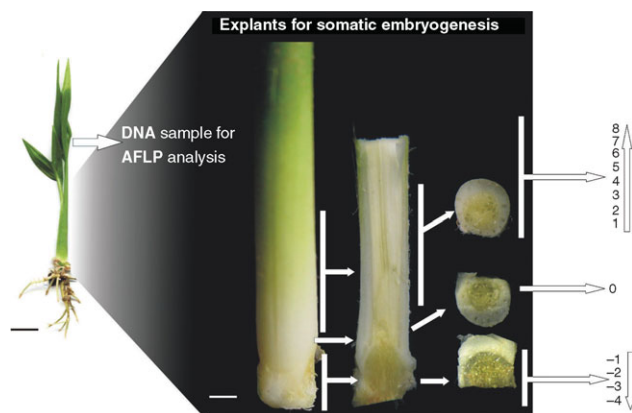
F I G. 1. Schematic diagram showing the origin of the explants utilized in the present study (dark scale bar = 1·75 cm, white scale bar = 3 mm).

light period, with 50–60 µmol m^{-2} s^{-1} intensity provided by cool-white fluorescent lamps (Sylvania), until the plantlets reached 5–8 cm in height.

The leaves, roots, haustorial tissue and the most external green leaf sheath of the plantlets were removed. The leaves were individually identified and frozen for subsequent AFLP analysis. The remaining tissue was transversely sectioned in 0·7–1 mm slices to obtain different histogenic layers. Some of these explants were composed only of sub-apical tissues or of leaf sheath and apical meristem, as well as different leaf developmental stages. A similar procedure was described for *D. sanguinalis* (Van Le *et al.*, 1997), although in the present study the explants were individually evaluated. The sub-apical tissues were numbered −4 to −1, the apical meristem 0, and the upper sections 1–8 (Fig. 1) to evaluate the effect of the explant's position on the production of embryogenic calli and somatic embryos. Thus, 13 transverse sections were obtained from one plantlet and inoculated in one Petri dish. The original orientation of the explant was maintained.

Culture media and conditions

The explants were inoculated in Petri dishes containing the basal culture medium described above, supplemented with 500 mg L^{-1} glutamine, and the gelling agent was substituted by 2·5 g L^{-1} Phytagel®. Picloram was added at different concentrations (0, 150, 300 or 600 µM) to evaluate its effect on the production of embryogenic callus. No sub-culture was applied until embryogenic calli were obtained. Further analyses were carried out only with calli produced on 300 or 600 µM Picloram-enriched media. The embryogenic calli obtained were maintained on a maturation medium composed of basal medium enriched with 40 µM 2,4-D (2,4-dichlorophenoxyacetic acid), 10 µM 2-iP [2-isopentyladenine (6-dimethylaminopurine)], 1 g L^{-1} glutamine and 0·5 g L^{-1} hydrolysed casein, with sub-cultures at 4-week intervals. The somatic embryos were selected on the basis of their shape (well formed, globular to oblong) and colour (white to yellowish). Fully developed somatic embryos were transferred to conversion medium, constituted by basal medium without activated

charcoal and containing 24·6 µM 2-iP and 0·44 µM NAA (naphthalene acetic acid), and cultured for 30 d. The somatic embryos were then transferred to basal culture medium until conversion, and plantlets >6 cm tall were then acclimatized. The culture medium was adjusted to pH 5·8 before the addition of the gelling agent; it was then autoclaved for 15 min at 1 kgf cm^{-2}, and 30 mL were transferred to Petri dishes or flasks. During the induction and maturation of somatic embryos, the cultures were kept in the dark at 26 ± 1 °C. For somatic embryo conversion and plantlet growth, the cultures were kept in a growth chamber at 26 ± 1 °C under a 16 h light period with 50–60 µmol m^{-2} s^{-1} intensity provided by cool-white fluorescent lamps (Sylvania).

Histological procedure

Samples were collected at critical periods of *in vitro* culture and fixed for 48 h at 4 °C in 0·2 M phosphate buffer (pH 7·3) containing 2·5 % paraformaldehyde. Thereafter, the samples were washed with the same buffer without fixative and dehydrated in a graded alcohol series (30–100 %), twice for 15 min at each step. The samples were embedded in Leica Historesin® and alcohol (1 : 1, v/v) overnight, followed with pure resin for 24 h. Gelatin capsules with the samples were filled with resin and allowed to polymerize following the manufacturer's instructions. Afterwards, 5–7 µm sections were obtained with a manual microtome, mounted on a slide with a drop of water and stained with 0·5 % toluidine blue O in 0·1 M phosphate buffer (pH 6·8).

AFLP analyses

Eight 'mother-plants' (F1–8; the seedlings used to extract explants) and three regenerated plantlets (a–c) from each mother-plant were randomly sampled; four sets (mother-plant and their regenerated plantlets) were regenerated using 300 µM Picloram (F1–4) and four using 600 µM Picloram (F5–8). DNA was extracted according to Doyle and Doyle (1990) with slight modifications. Leaves were collected and about 200 mg of plant material were ground with a mortar and pestle in liquid nitrogen, transferred to a 1·5 mL microtube containing 1·0 mL of DNA extraction solution [2 % cetyltrimethylammonium bromide (CTAB); 0·7 M NaCl; 50 mM Tris–HCl; 10 mM EDTA pH 8·0; and 0·1 % β-mercaptoethanol] and incubated at 60 °C for 1 h with occasional swirling. An organic extraction was carried out by adding 0·6 mL of chloroform–isoamyl alcohol (24 : 1) to the mixture. The tubes were centrifuged at 8000 *g* for 5 min, the supernatant phase transferred to a new microtube and 10 µL of a 10 % CTAB solution (10 % CTAB; 1·2 M NaCl) was added to each sample, followed by 1·0 mL of cold 100 % ethanol. The samples were then incubated at −4 °C overnight. After centrifugation, the ethanol was discarded and the DNA pellet was washed with 1·0 mL of 70 % ethanol during 1 min. After discarding the ethanol, the DNA was dried at room temperature, diluted in 70 µL of TE buffer with RNase A (10 mM Tris–HCl pH 8·0; 1 mM EDTA; 10 µg mL^{-1} RNase A), quantified in a 0·8 % agarose gel and stored at −20 °C until use.

The AFLP reactions were performed as described by Vos *et al.* (1995), with slight modifications as described by Samuel Hazen of Michigan State University (available at http://www.msu.edu/user/hazensam/aflp/AFLPprotocolMSU.html). Six selective primer combinations (M-CTG/E-AAC, M-CTG/E-AAG, M-CAG/E-AGC, M-CAG/E-ACT, M-CAG/E-AAG and M-CAC/E-ACA) were used, based on the screening by Clement *et al.* (2002). All PCRs were carried out in a Peltier Thermal Cycler PTC-100 (MJ Research). The fragments were separated in a 6 % denaturing polyacrylamide gel at 75 W for 3 h. Silver nitrate was used for staining (Caetano-Anollés and Gresshoff, 1994) and the bands were visualized under white light. AFLP products were manually scored as present or absent, and transformed into a binary matrix for data analysis. Only reliable bands between 50 and 800 bp were scored.

Statistical procedure

Four Picloram concentrations were evaluated (0, 150, 300 or 600 mM), as was the effect of initial explant position, on primary callus induction and embryogenic callus production. The experiment was a factorial in a completely randomized design, with five repetitions. Each repetition was composed of at least four Petri dishes and each Petri dish had all 13 histogenic layers from one plant. After 3 and 5 months of culture, the primary callus and somatic embryogenesis induction rates, respectively, were evaluated. The data were transformed by $\sqrt{(x + 5)}$ and submitted to analysis of variance (ANOVA); means were compared with Duncan's multiple range test at 95 % significance.

For the AFLP analysis, the plantlets regenerated on 300 or 600 μM Picloram were compared with their mother-plant. The relationship among individuals was assessed by computing Dice's similarity coefficient (Dice, 1945). The similarity matrix was used as input for a principal coordinate analysis (PCO), implemented with NTSYS-pc 2·0 (Rohlf, 1998). The differences between the genetic similarities and the number of fragments lost or gained in the

two Picloram concentrations were tested. The significance of the difference was measured using an exact probability test (Raymond and Rousset, 1995), based on Fisher's test for RxC contingency tables. The analysis was performed with RxC (Miller, 1997), using 1000 batches of 10 000 replicates and 1000 de-memorization steps for the Markov chain method.

RESULTS

Callus and somatic embryo production and plantlet regeneration

The induction of primary calli was strongly influenced by the interaction between the Picloram concentration and the explant position ($P < 0.01$). On culture medium devoid of Picloram, some explants presented the development of a meristem and root growth from sub-apical tissue, but no callus was observed and most of the explants showed intense oxidation after 4 weeks of culture. The highest primary callus induction was observed in explants from the apical meristem region on culture media supplemented with Picloram, ranging from 83 to 97 % induction with 150–600 μM Picloram, without significant differences among these treatments, but differing from the results in the absence of Picloram (Fig. 2). Explants from sub-apical tissues (-1 and -2) showed significantly higher induction of primary calli on 300 and 600 μM Picloram culture media, with 74 and 71 %, respectively, not differing from the explants from the apical meristem (Fig. 2). Explants from the upper layers (1–8) showed induction of primary calli with 600 μM Picloram, ranging from 50 to 10 %. For those explants composed of leaf sheath (1–8), a horizontal response gradient was also observed, as the outer leaf sheath showed low response and eventual oxidation, while the inner leaf sheaths or leaf primordia showed the development of primary callus. The most responsive leaf sheath explants were 1–3 (Fig. 2; see also figures available online at
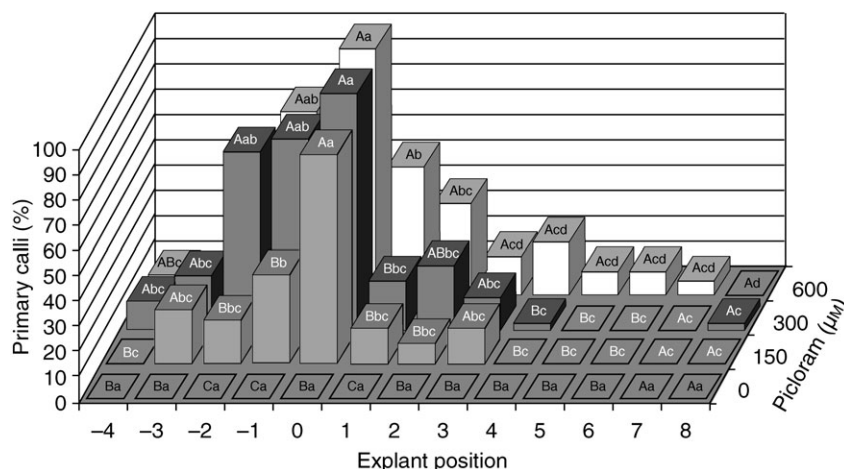

FIG. 2. Influence of explant position and Picloram concentration (0, 150, 300 or 600 μM) on primary callus induction of peach palm. Upper case letters represent statistical differences among the Picloram concentrations (at each explant position) and lower case letters represent differences among the explant positions (at each Picloram concentration) according to Duncan's test.

FIG. 3. Influence of explant position and Picloram concentration (0, 150, 300 or 600 µM) on embryogenic callus production of peach palm. Upper case letters represent statistical differences among the Picloram concentrations (at each explant position) and lower case letters represent differences among the explant positions (at each Picloram concentration) according to Duncan's test.
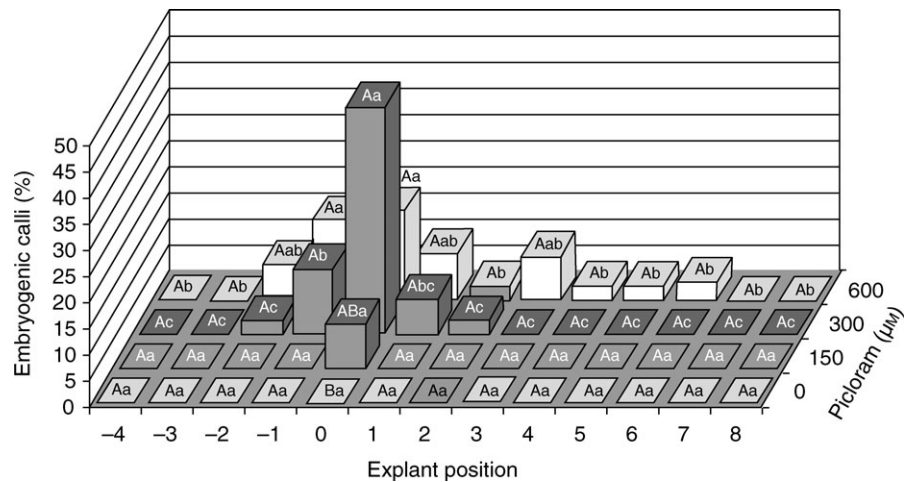
http://www.cca.ufsc.br/lfdgv/AoBs), which coincide with the region containing leaf primordia.

The production of embryogenic calli was evaluated 5 months after inoculation and followed the same response pattern as primary callus induction (compare Figs 2 and 3), with interaction between Picloram concentration and explant position ($P < 0.05$). On culture medium with 300 µM Picloram, explants from the apical meristem region showed 43 % embryogenic callus production, while on 150 or 600 µM Picloram this was 8 and 17 %, respectively (Fig. 3), without significant differences among these values. The production of calli with embryogenic characteristics in explants from the upper layers (3–8) was observed only on 600 µM Picloram-supplemented culture medium, ranging from 3 to 9 % (Fig. 3).

On the maturation culture medium, an average of 34 ± 4 somatic embryos was obtained per embryogenic callus, without differences between the 300 and 600 µM Picloram treatments (36 ± 5 and 32 ± 5 somatic embryos per embryogenic callus, respectively). Fully developed somatic embryos were obtained, and those selected had a 45.0 ± 3.4 % conversion rate, again without differences between the two Picloram concentrations. Plantlets were obtained (Fig. 4F) and were transferred to culture flasks and later acclimatized (Fig. 4G). During the acclimatization step, approximately 80 % of the plantlets survived and were transferred to the greenhouse, where all survived after 2 months.

Morpho-histological aspects

After 1 week of culture, swelling and growth of the explant were observed, resulting in the development of primary callus. The primary calli were yellow in colour, compact, with radial but non-organized growth (Fig. 4A) from which somatic embryos arose (Fig. 4D). Interestingly, while the meristem region (explant 0) was the most responsive, morphological analyses revealed that embryonic calli also arose from tissues adjacent to the apical meristem (i.e. leaf primordia), while the apical meristem itself showed only an initial growth and occasionally primary callus development (Fig. 4B, C). The embryogenic calli were transferred to maturation culture medium, where growth of somatic embryos as well as signs of polarization were observed (Fig. 4E). In addition, in maturation conditions, several developmental stages could be observed, revealing non-synchronized somatic embryo production.

Histological analyses of fresh green tissue showed that peach palm has mesophyll composed of parenchymatic isodiametric cells with collateral vascular bundles distributed along the tissue and intercalated with fibre bundles (Fig. 5A). About 4–5 weeks after inoculation on culture medium supplemented with Picloram, the first cellular division events were observed to occur simultaneously in several cells adjacent to the vascular tissue, while the remaining parenchymatic cells degenerated (Fig. 5B); these actively dividing cells progressed to burst through the epidermis of the explants, resulting in primary callus (Fig. 5C). Additionally, primary callus induction did not occur simultaneously, as some calli were observed to develop later on the explant, without an apparent relationship to their proximity to vascular tissue or meristems. The radial growth of the primary calli was ensured by the presence of a meristematic zone composed of small meristematic isodiametric cells (Fig. 5D).

Subsequently, development of globular structures arising from the primary callus was observed. Histological analyses revealed that these structures arose from the meristematic zone, and presented a well-delimited protoderm and signs of polarization (Fig. 5E), characteristics of somatic embryos at the globular stage. After transfer of these embryogenic calli to the maturation culture medium, some cells of the peripheral zone of the primary callus also exhibited embryogenic competence, resulting in the development of additional globular somatic embryos (Fig. 5F). Most of the well-formed somatic embryos developed and showed complete polarization, illustrated by the presence of procambium (Fig. 5G), and could be converted into plantlets (Fig. 4F).
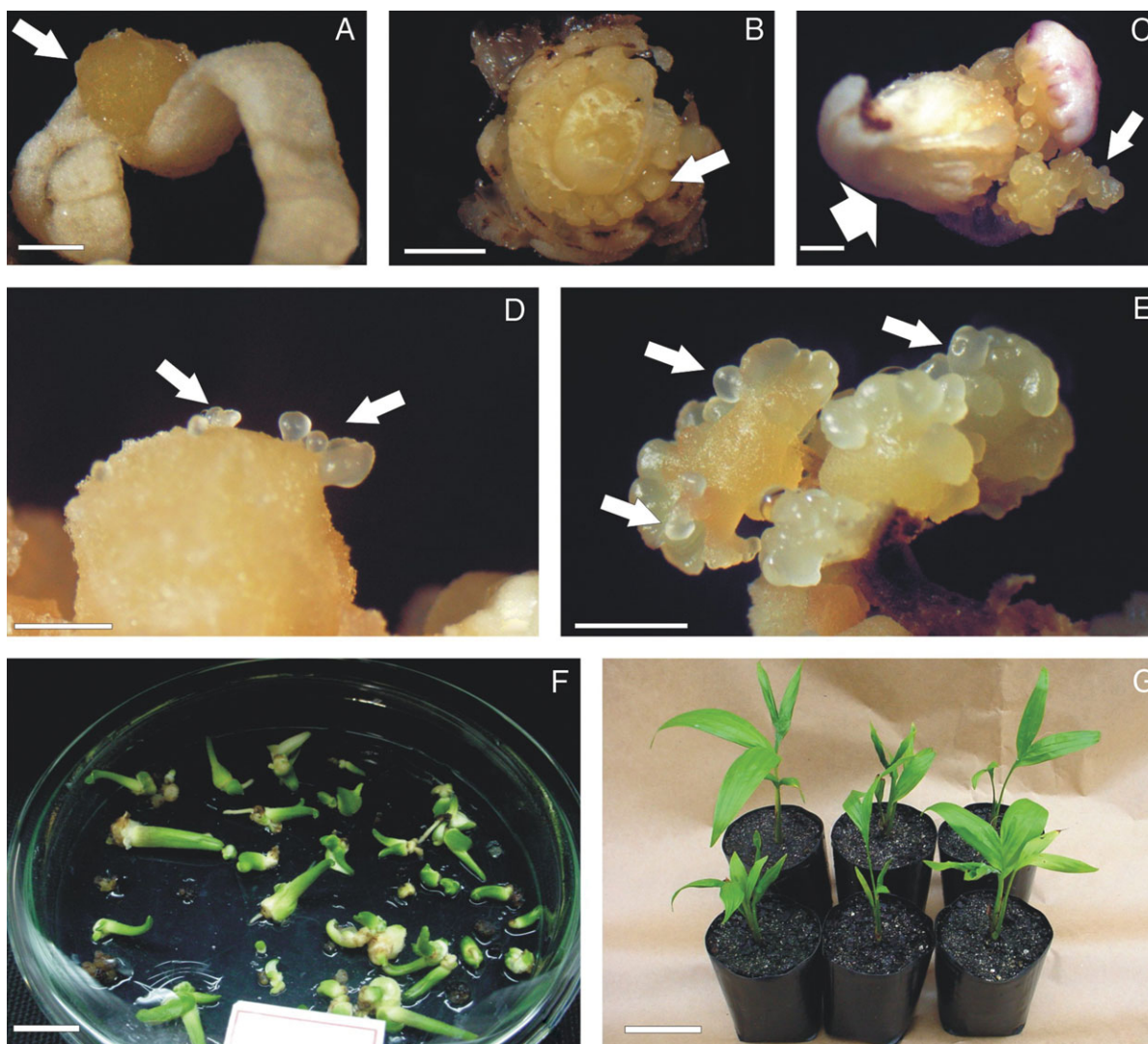
F<small>IG</small>. 4. Somatic embryogenesis and plantlet regeneration from thin cell layers of peach palm. (A) Primary callus (arrow). (B) Primary callus induction from shoot meristem TCL: note the development of primary callus (arrow) in tissue surrounding the shoot meristem. (C) Embryogenic callus from shoot meristem TCL: note the development of the shoot meristem (thick arrow) and embryogenic callus (thin arrow). (D) Somatic embryo development (arrow) from primary callus. (E) Embryogenic callus on maturation culture medium. (F) Plantlets obtained from converted somatic embryos. (G) Acclimatized plantlets. Scale bars: A = 1 mm; B–D = 2·5 mm; E = 1 cm; F, G = 2·5 cm.

AFLP analyses of the regenerated plantlets

The AFLP primer combinations used in this study generated a total of 252 fragments, ranging from 30 to 59, with a mean of 41·7 fragments per primer combination (Table 1). The PCO analysis based on Dice's similarity index explained 36 % of the total variation in the first two axes. Six out of eight plant sets grouped in clusters formed by the mother-plant and its respective clones (Fig. 6). The two clones with lowest genetic similarity diverged significantly from their plant sets: clone 4c displayed 81 % identity to its mother-plant and clone 8b displayed 69 % identity. However, clone 8b also had a relatively high level of missing data, so the results on this plantlet may be inconclusive. A fragment was considered to be missing data when it was present but weakly stained compared with an adjacent strongly stained band.

The two Picloram concentrations (300 or 600 µM) had no effect on the mean similarities among clones and their respective mother-plant: 96 and 95 %, respectively. Ten of 12 plantlets with genetic similarities to their respective mother-plant of ≥97 % were produced with 300 µM Picloram, while the 600 µM Picloram yielded nine of 12 plantlets with genetic similarities in this range (Table 2). Considering all AFLP primer combinations, a higher frequency of fragment loss (90 fragments for 300 µM and 124 fragments for 600 µM) than of fragment gain (44 fragments for 300 µM and 27 fragments for 600 µM) was observed. The comparison between Picloram treatments revealed significant differences between treatments regarding loss ($P = 0·017$) or gain ($P = 0·0002$) of AFLP fragments.
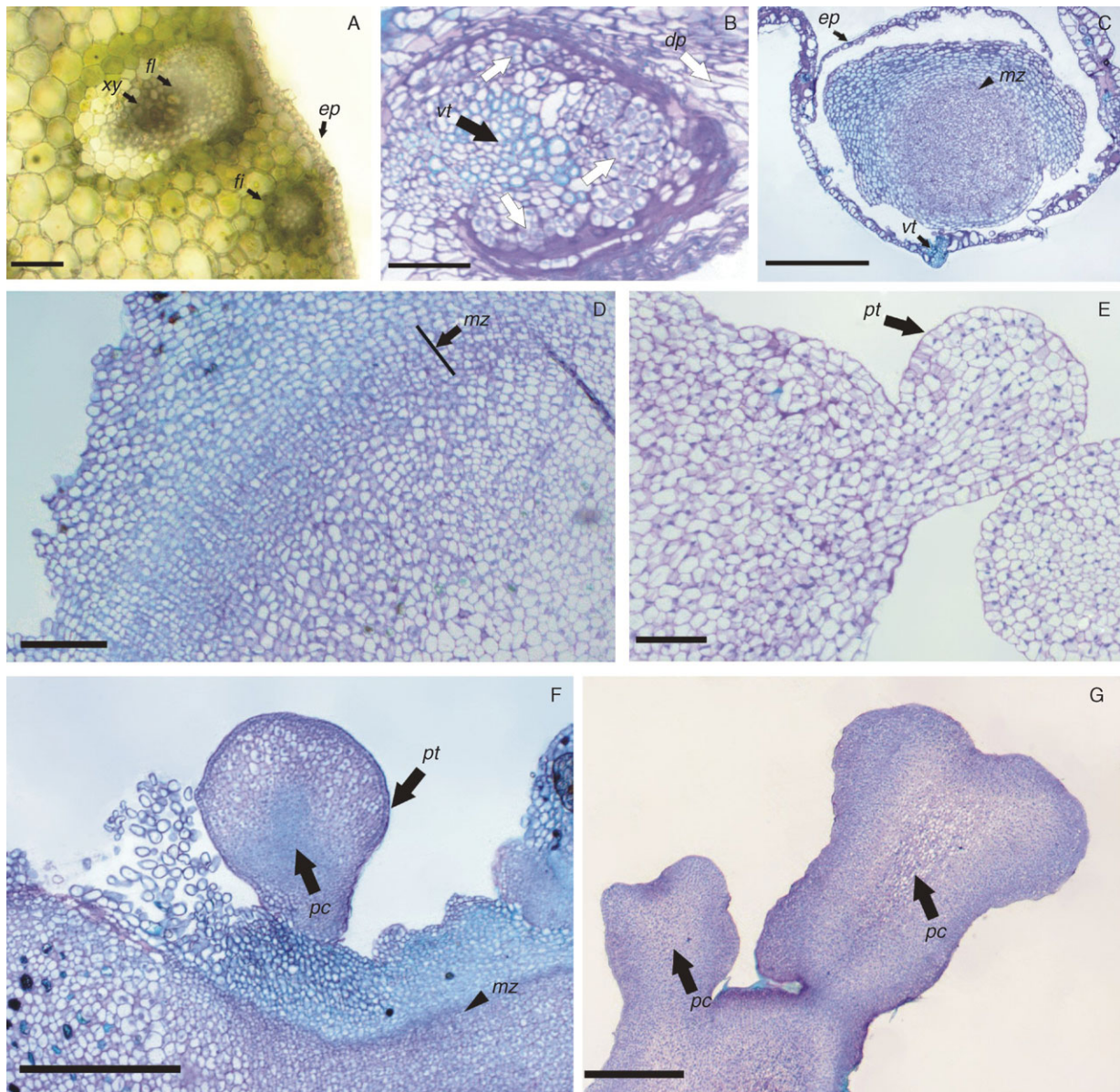
FIG. 5. Histological analyses of peach palm somatic embryogenesis. (A) Histological section of fresh tissue to illustrate the organization of a mature tissue evidencing a collateral vascular bundle distributed along the tissue and intercalated with a fibre bundle. (B) First cell division events (arrow) observed in cells adjacent to the vascular tissue and the degenerating parenchyma (dp, arrow). (C) Primary callus induction: note the formation of a meristematic zone (arrowhead). (D) Details from the meristematic zone (arrow). (E) Globular somatic embryo, showing polarization signals and a well-defined protodermis. (F) Globular somatic embryos observed in maturation culture conditions. (G) Mature somatic embryos showing a well-developed procambium and without vascular connection with the matrix tissue. Abbreviations: *xy*, xylem; *fl*, phloem; *fi*, fibre bundle; *ep*, epidermis; *dp*, degenerating parenchyma; *vt*, vascular tissue; *pt*, protodermis; *pc*, procambium; *mz*, meristematic zone. Scale bars: A, B, D, E = 200 μm; C, F, G = 500 μm.

DISCUSSION

The use of the TCL technique offers promise for peach palm *in vitro* plant regeneration. Explant size has an important role in peach palm *in vitro* response, as in our preliminary experiments low callus induction was observed from explants that were 1 cm thick (data not shown). Small explants also presented higher morphogenetic capacity in *Triticum durum* (Benkirane *et al.*, 2000) and in *T. aestivum* (Delporte *et al.*, 2001). Benkirane *et al.* (2000) postulated that larger explants maintain normal tissue interactions, and such interactions may inhibit cell division through maintenance of symplast domains. Additionally, explants with reduced size showed synthesis of new cell wall components, such as oligosaccharides, that can act as signals to the cell to re-enter the mitotic cycle (Tran Thanh Van and Bui, 2000). Small explants also present higher surface contact with the culture medium, and can be considered to be more stressed, increasing the cell's metabolism (Feher *et al.*, 2003).

The present study confirms the use of Picloram as a reliable auxin analogue to trigger somatic embryogenesis

TABLE 1. *Mean number of fragments scored and number of fragments lost and gained for each AFLP primer pair combination from peach palm clones regenerated through somatic embryogenesis in two concentrations of Picloram*

Primer combination	No. of fragments	300 μM Picloram		600 μM Picloram	
		Fragment loss	Fragment gain	Fragment loss	Fragment gain
AAC/CTG	30	13	9	10	0
AAG/CTG	44	12	12	7	9
AGC/CAG	40	16	10	25	4
ACT/CAG	49	25	6	23	3
AAG/CAG	30	4	0	13	0
ACA/CAC	59	20	7	46	11
Overall	252	90	44	124	27

The exact test (Raymond and Rousset, 1995) revealed a significant difference between Picloram treatments for fragment loss ($P = 0.017$) and fragment gain ($P = 0.0002$).
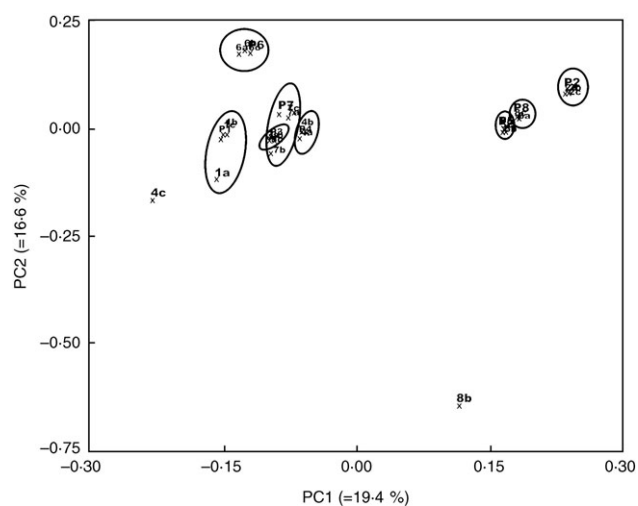

FIG. 6. Principal coordinate analysis (PCO) based on Dice's similarities, revealing the genetic relatedness of eight seedling–clone sets of peach palm analysed using 262 AFLP markers.

in peach palm, as observed by Valverde *et al.* (1987) and Steinmacher *et al.* (2007*a*, *b*). In interaction with the Picloram concentration, explant position influenced *in vitro* response, revealing a response gradient for primary callus production from layers 1 to 8 cultivated on 600 μM Picloram. In addition, a horizontal gradient was observed as outer leaf sheaths presented a low morphogenetic response and greater oxidation compared with inner leaf sheaths. The original explant position also had a fundamental role in the acquisition of somatic embryogenesis competence in *Coffea arabica* (Quiroz-Figueroa *et al.*, 2002), *Dactylis glomerata* (Somleva *et al.*, 2000; Alexandrova and Conger, 2002) and *Avena sativa* (Nuutila *et al.*, 2002), and both vertical and horizontal gradients were also observed in *D. sanguinalis* (Van Le *et al.*, 1997).

In the present study, the production of embryogenic calli from explants of the apical meristem (explant 0) was highest on culture medium with 300 μM Picloram. The induction of embryogenic callus followed the same tendency as primary callus development, suggesting that the morphogenetic competence of the explants was governed by the capacity of the explant cells to re-enter the mitotic cycle, as suggested by Tran Thanh Van and Bui (2000). Although somatic embryogenesis induction might not be simply related to the cell cycle or to a certain level of mitotic activity of the cells in the explants (Doleželová *et al.*, 1992), it has been shown that the formation of callus is correlated with cell cycling and that the subsequent origin of somatic embryos initially involves cycling of cells at the margins of the callus of Arabidopsis (Raghavan, 2004). In the present study, most initiation of somatic embryos was also associated with cycling of cells, but from a meristematic zone formed within the callus. Also, cells able to produce somatic embryos were mitotically more active than non-embryogenic cells (Pasternak *et al.*, 2002). In contrast, the *in vitro* recalcitrance of *C. nucifera* was linked to a block in the cell cycle (Sandoval *et al.*, 2003). This suggests that the TCL technique may be an interesting strategy for the induction and control of *in vitro* morphogenesis in other palm species, as is the case with peach palm.

Histological analyses showed that the first cell divisions occurred in cells adjacent to vascular tissue, resulting in primary calli. In palms, cells adjacent to the vascular tissue apparently have higher morphogenetic capacity. Studies with *C. nucifera* (Fernando *et al.*, 2003), *E. guineensis* (Schwendiman *et al.*, 1988), *Euterpe edulis* (Guerra and Handro, 1998) and *P. dactylifera* (Sané *et al.*, 2006) 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 similar to those observed in the present study.

Somatic embryos were observed arising from the meristematic zone of primary calli 5 months after inoculation, characterizing the indirect pattern of somatic embryogenesis with possible multiple cellular origins (Williams and Maheswaran, 1986). These somatic embryos were observed at the first developmental stage, and presented a well-delimited protoderm and polarization signals. In *C. nucifera*, the same pattern was observed, although in this species a fragmentation of the meristematic zone was observed prior to the development of the somatic embryos, which could therefore have uni- or multicellular origins (Verdeil *et al.*, 1994; Dussert *et al.*, 1995; Fernando *et al.*, 2003).

TABLE 2. *Summary of the AFLP analysis of somaclonal variation in regenerated peach palm, based on Dice's coefficient of similarity between the mother-plant and its clone*

300 μM Picloram			600 μM Picloram		
Mother-plant	Clone	Coefficient of similarity	Mother-plant	Clone	Coefficient of similarity
F1	1a	0·89	F5	5a	0·99
	1b	0·99		5b	0·99
	1c	0·99		5c	0·99
F2	2a	0·99	F6	6a	0·97
	2b	0·98		6b	0·94
	2c	0·99		6c	0·98
F3	3a	0·99	F7	7a	0·99
	3b	0·99		7b	0·92
	3c	0·97		7c	0·99
F4	4a	0·98	F8	8a	0·99
	4b	0·98		8b	0·69
	4c	0·81		8c	0·99
Mean		0·96	Mean		0·95

The exact test (Raymond and Rousset, 1995) revealed no significant difference ($P = 0.75$) between Picloram treatments for Dice's coefficient of similarity.

Considering that in the present study relatively high concentrations of Picloram were used to stimulate somatic embryo production and that *in vitro* culture conditions affect the organization of plant genomes (Larkin and Scowcroft, 1981), AFLP analyses were conducted to determine to what extent the present conditions could generate divergent plantlets. Although most of the clones revealed a similarity greater than 97 % to the mother-plant (see Table 2 and Fig. 6), some degree of somaclonal variation appears to exist in regenerated peach palm plantlets as some minor changes of the banding patterns were observed (see Supplementary figures available online at http://www.cca.ufsc.br/lfdgv/AoB) and two plantlets were off type (Fig. 6). The two clones with similarities lower than 81 % to the mother-plant showed normal morphology. In *Codonopsis lanceolata*, normal phenotypes often had genotypic alterations to some degree (Guo *et al.*, 2006), and the authors postulated that such differences imply that most genomic changes occurred in non-coding regions with little effect on gene expression, and/or that the change could be a recessive mutation, without changes in the phenotype. In the present study, independently of the Picloram concentration, the most common mutation observed in the more divergent plantlets was the loss of fragments, implying that most mutations were in the restriction sites of the enzymes.

Summarizing, the present study suggests that the TCL somatic embryogenesis protocol developed is feasible, although it still requires further optimization for *in vitro* multiplication of peach palm. Additionally, in the present study, the explants were obtained from *in vitro* grown plantlets; however, similar explants can be obtained easily from adult palms since peach palm produces off-shoots that will allow the cloning of selected adult plants. More detailed studies are being conducted to evaluate the genotype effect of the donor plant on *in vitro* responses, as well as the incidence of somaclonal variation. Further studies will also elucidate to what extent the genomic alterations detected by AFLP might affect the phenotype of adult plants. Additionally, an embryogenic cell suspension was recently obtained in *P. dactylifera* (Sané *et al.*, 2006) from calli similar to those described in the present study, and the adaptation of the present protocol to conform with that from *P. dactylifera* may create an embryogenic cell suspension of peach palm.

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