

IMPROVED SOMATIC EMBRYOGENESIS FROM *COCOS NUCIFERA* (L.) PLUMULE EXPLANTS

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SUMMARY

Coconut is one of the most recalcitrant species to regenerate *in vitro*. Although previous research efforts using plumule explants have resulted in reproducible somatic embryogenesis, efficiency is only 4 or 10 somatic embryos per plumule without or with a brassinolide treatment, respectively. In order to increase the efficiency of somatic embryogenesis in coconut, two different approaches were evaluated and reported here: secondary somatic embryogenesis and multiplication of embryogenic callus. Primary somatic embryos obtained from plumule explants were used as explants and formed both embryogenic callus and secondary somatic embryos. The embryogenic calluses obtained after three multiplication cycles were capable of producing somatic embryos. The efficiency of the system was evaluated in a stepwise process beginning with an initial step for inducing primary somatic embryogenesis followed by three steps for inducing secondary somatic embryogenesis followed by three steps for embryogenic callus multiplication, and finally production of somatic embryos from callus. The total calculated yield from one plumule was 98 000 somatic embryos. Comparing this to the yield obtained from primary somatic embryogenesis results in about a 50 000-fold increase. When compared to the yield previously reported in the literature with the use of a brassinolide treatment, it is about a 10 000-fold increase in yield. The present protocol represents important progress in improvement in the efficiency of coconut somatic embryo production.

Key words: coconut; secondary somatic embryogenesis; callus multiplication.

INTRODUCTION

Coconut (*Cocos nucifera* L.) is one of the most recalcitrant species for *in vitro* regeneration. This is unfortunate, as there is a strong interest in coconut micropropagation due to a rapidly growing need for improved or selected coconut varieties, particularly those resistant to various diseases (Harnold and Randles, 1991; Arellano and Oropeza, 1995; Eden-Green, 1997). Research on coconut micropropagation has shown that somatic embryogenesis is a viable approach for *in vitro* plant regeneration (Hornung and Verdeil, 1999). The plumule explant, consisting of the shoot meristem and surrounded by leaf primordia excised from mature zygotic embryos, has proved to be the most responsive for embryogenic callus and somatic embryo formation (Hornung, 1995). The use of these explants has allowed the development of a reproducible micropropagation protocol (Chan et al., 1998; Sáenz et al., 1999) but with low efficiency, about four somatic embryos per plumule. The use of compounds such as brassinosteroids (Azpeitia et al., 2003) and anticytokinins (Azpeitia et al., 2003) has increased the number of somatic embryos per plumule by only 2.5-fold and 1.5-fold, respectively. Therefore, a higher efficiency of regeneration via somatic embryogenesis is required for mass micropropagation of elite coconut palms. However, it is important to consider that if we use plumules excised from zygotic embryos obtained by

cross-pollination, we would preclude cloning of individuals with known agronomic traits.

Somatic embryogenesis is defined as a process in which a bipolar structure, resembling a zygotic embryo, develops from a non-zygotic cell without vascular connection with the original tissue (Von Arnold et al., 2002). The resulting embryos can germinate and are converted into plantlets. Alternatively, somatic embryos can be used as explants to produce additional somatic embryos via secondary somatic embryogenesis (Vasic et al., 2001). Secondary somatic embryogenesis can be repeated several times, thus contributing significantly to increasing the yield of somatic embryo formation, especially in plants with low embryogenic capacity (Raemakers et al., 1995). For instance, somatic embryo production in *Theobroma cacao* increased by 30-fold after two cycles of secondary somatic embryogenesis (Maximova et al., 2002). Moreover, embryos obtained via secondary somatic embryogenesis are often better developed than those obtained via primary somatic embryogenesis. Secondary somatic embryogenesis has also been reported to help retain embryogenic competence during prolonged culture times, as long as 10 years in different species (Stamp and Henshaw, 1987; Baker and Wetzstein, 1995; Schavemaker and Jacobsen, 1995; Martinelli et al., 2001). On the other hand, a common practice to maintain and multiply callus is to subdivide it and repeatedly subculture these pieces. This results in the production of larger amounts of callus, as has been reported previously for various species (George, 1996). Neither secondary

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somatic embryogenesis nor callus multiplication has been reported for coconut. Therefore, based on the use of coconut plumule explants, the objectives of the present work were: (a) to determine if secondary somatic embryogenesis can be induced from somatic embryos; (b) to determine if embryogenic callus can be multiplied; and (c) to determine if these processes are useful to obtain greater yields of embryogenic calluses and ultimately somatic embryos.

MATERIALS AND METHODS

Plant material. Fruits were harvested 12–14 mo. after pollination from 15-year-old Green Malayan Dwarf coconut palms. Cylinders of endosperm containing embryos were excised in the field and placed in a 0.6% NaClO (w/v) solution during collection and then rinsed three times with distilled water. Once in the laboratory, under aseptic conditions, cylinders were washed in 70% ethanol for 3 min, rinsed three times with sterile distilled water for 5 min each, washed in 6% NaClO (w/v) solution for 20 min, and rinsed again three times with sterile distilled water. Embryos were excised from endosperm cylinders, washed in 0.6% NaClO solution (w/v) for 10 min, and rinsed with sterile distilled water three times. Plumules were excised from these embryos using a stereomicroscope and placed directly on a culture medium.

Primary somatic embryogenesis. Media preparation and culture conditions were carried out according to Azpeitia et al. (2003). Media I and II were each prepared using Y3 medium (Eeuwens, 1976), supplemented with 3 g l^{-1} gelrite and 2.5 g l^{-1} charcoal (acid-washed, plant cell culture tested), but medium I contained $600 \mu\text{M}$ 2,4-dichlorophenoxyacetic acid (2,4-D), while medium II contained $6 \mu\text{M}$ 2,4-D and $300 \mu\text{M}$ 6-benzyladenine (BA). All chemicals were supplied by Sigma (St Louis, USA). The medium pH was adjusted to 5.75 with NaOH before autoclaving for 20 min at 120°C . For callus induction, each explant was cultured for 3 mo. in a 35 ml glass vessel containing 10 ml of medium I. Cultures were kept under complete darkness at $27 \pm 2^\circ\text{C}$ without subculturing (condition I). For induction of primary somatic embryos, calluses were subcultured in a 100 ml glass vessel containing 25 ml of medium II. Cultures were kept under 16 h photoperiod ($45\text{--}60 \mu\text{mol m}^{-2} \text{ s}^{-1}$ PPF) at $27 \pm 2^\circ\text{C}$, and explants were subcultured once every 2 mo. (condition II).

Secondary somatic embryogenesis. Globular primary somatic embryos were excised and subcultured in medium I/condition I for induction of embryogenic callus. For induction of embryo development, calluses were then subcultured to medium II/condition II.

Callus multiplication. Embryogenic structures were isolated from embryogenic callus growing in medium I/condition I, and subcultured to the same medium and condition for 60–90 d to test if newly formed embryogenic callus could be obtained from them. This procedure was repeated twice. Embryogenic callus obtained was subcultured in medium II/condition II in order to test its embryogenic potential.

Histology. The histological procedures were carried out according to Buffard-Morel et al. (1992), but with slight modifications. Tissue samples were fixed in 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.2) for 24 h under negative pressure. Samples were dehydrated in a stepwise manner (1 h each step) using 30, 50, 70, 80, 90, 96, and 100% ethanol in water. This was followed by impregnation with JB-4 resin (Polyscience, PA, USA). Three micrometer sections were prepared from the resin-impregnated tissues with a microtome (HM 325, MICROM, Hellersbergstr, Neuss, Germany), equipped with a steel knife blade. Sections were double-stained with the periodic acid-Schiff reaction (PAS) combined with protein-specific naphthol blue-black. PAS will stain starch reserves and cell wall pink, and naphthol blue-black will specifically stain soluble or reserve proteins dark blue (Fisher, 1968).

Quantitative evaluation and statistics. Quantitative evaluation was carried out through a stepwise process as follows: the initial step was primary somatic embryogenesis (PSE), followed by three steps of secondary somatic embryogenesis (SSE_{1-3}), then three steps of embryogenic callus multiplication (ECM_{1-3}), and finally formation of somatic embryos from the embryogenic calluses obtained in ECM_2 and ECM_3 . In each step, a representative sample of the embryos or the embryogenic calluses obtained was used as explants for the following step. Then we determined the percentages of explants forming initial callus and embryogenic callus for each step and the amount of somatic embryos per embryogenic callus

(SE/EC) for PSE, SSE_{1-2} and ECM_{2-3} . Data presented correspond to means of six replicates, each with 100 explants for primary and secondary somatic embryogenesis, and three replicates each with 30 explants for embryogenic callus multiplication. Data were subjected to analysis of variance (ANOVA). Mean comparisons were determined by Student–Newman–Keuls test at $P < 0.05$.

RESULTS

Primary somatic embryogenesis. Plumule explants cultured in medium I/condition I formed callus along the outer leaf primordia surrounding the shoot meristem within 15–30 d following culture. This callus will hence be referred to as initial callus. This callus was hard and white or beige in color (Fig. 1a), as previously reported by Azpeitia et al. (2003). Histological analysis showed the presence of meristematic cells in the periphery of the callus (Fig. 2a).

Initial callus eventually evolved into embryogenic callus. The developmental changes included the formation of ear-shaped translucent structures that appeared after 45 d (Fig. 1b). Histological analysis of these structures showed the presence of densely stained small meristematic cells located in their periphery (not shown).

At about 60 d, globular and then elongated structures began to develop along the surface of the translucent structures (Fig. 1c); these were compact, smooth and easily distinguishable. Since somatic embryos eventually developed from these structures (see below), these will be referred to henceforth as embryogenic structures. Histological analysis of these structures showed the presence of densely stained small meristematic cells (Fig. 2b). Meristematic cells eventually developed into meristematic nodules, located along the periphery of the embryogenic structures, but below a layer of unstained cells forming the protoderm (PD) (Fig. 2c). Most calluses reaching this stage of development did so within 90 d and will henceforth be referred to as embryogenic calluses.

When embryogenic calluses were transferred to medium II/condition II, somatic embryos were observed on the embryogenic structures within 30–40 d following transfer (Fig. 1d). Early somatic embryos were 1–2 mm in diameter, globular and white. Histological analysis of these embryos did not reveal a well-defined meristem but did reveal a few layers of meristematic cells (Fig. 2d). By day 40, somatic embryos developed into elongated shapes, and some fused embryos were observed. Embryos were capable of germination and postgerminative development in medium II/condition II (Fig. 1e).

Evaluation of yields showed that the percentage of explants forming initial callus was 83.33 and 38.16 for embryogenic callus, and the production of somatic embryos was 5.16 SE/EC (Table 1).

Secondary somatic embryogenesis. Embryos obtained through primary somatic embryogenesis were cultured in medium I/condition I to determine if they were capable of forming initial and embryogenic calluses. By day 15, initial callus formation surrounding the embryo was observed; this callus was hard and colored white or beige (Fig. 1f). Histological analysis showed that the proliferating tissue forming a ring around the embryo contained meristematic cells (Fig. 2e, f). Similar to primary somatic embryogenesis, most of these cells were observed in the periphery of the callus. These cells also revealed a more densely stained cytoplasm.

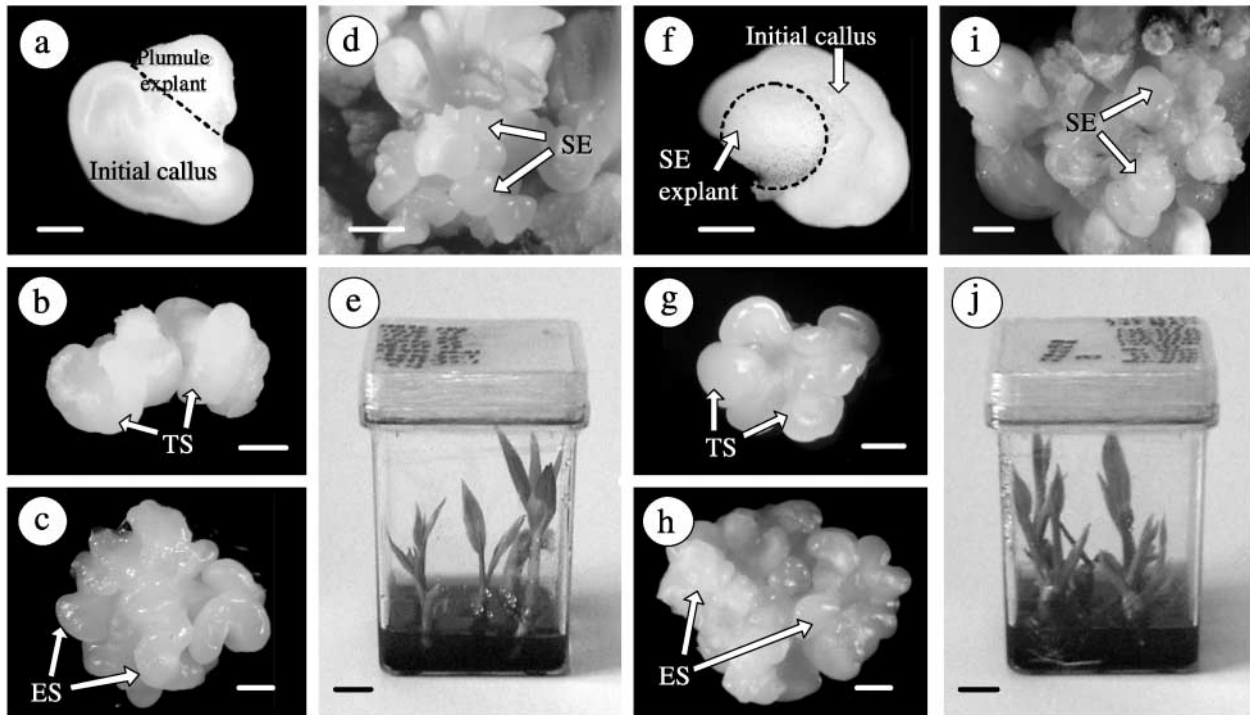


FIG. 1. Morphological development of coconut plumule cultures during primary (*a–e*) and secondary (*f–j*) somatic embryogenesis. Explants (plumules or somatic embryos) cultured in medium I/condition I forming initial callus (*a, f*). These eventually develop ear-shaped translucent structures (TS) (*b, g*) prior to becoming embryogenic callus (*c, h*) with characteristic embryogenic structures (ES). Embryogenic calluses are then transferred to medium II/condition II, where they form somatic embryos (SEs) (*d, i*). Some of these embryos germinate and convert into plantlets (*e, j*). Scale bars for *a–d* and *f–i* = 1 mm; and for *e* and *j* = 1 cm.

The development of initial callus into embryogenic callus occurred within 60 d, as opposed to the 90 d period for primary somatic embryogenesis, but morphological (Fig. 1*g, h*) and histological (Fig. 2*g*) development were identical.

Embryogenic calluses were transferred to medium II/condition II and further development into somatic embryos was observed from embryogenic structures within 20–25 d of culture. These new somatic embryos were identical to those formed during primary somatic embryogenesis (Fig. 1*i*). Although fused embryos were also observed, these were less abundant than those observed during primary somatic embryogenesis. Somatic embryos obtained through secondary somatic embryogenesis were used as explants for further development of initial callus, embryogenic callus, and somatic embryos. Secondary somatic embryos were capable of germination and postgerminative development in medium II/condition II (Fig. 1*j*).

During primary and secondary somatic embryogenesis (and multiplication of embryogenic callus, see below), some of the initial callus produced a sponge-like callus that did not develop into an embryogenic callus; this will henceforth be referred as non-embryogenic callus. When this type of callus (30 d of culture in medium I/condition I) was analyzed histologically, no meristematic cells were observed, as cells were large in size, loose, having a small nucleus, and lacking protoderm (Fig. 2*h*).

The results for yield evaluation showed that the percentage of explants forming initial callus was 81.66 for SSE₁, 76.0 for SSE₂, and 81.8 for SSE₃ (Table 1). The percentage of explants forming embryogenic callus was 46.16 for SSE₁, 42.33 for SSE₂, and 56.0

for SSE₃ (Table 1). The production of somatic embryos was 4.66 SE/EC for SSE₁, and 6.33 for SSE₂; this was not evaluated for SSE₃ (Table 1).

Multiplication of embryogenic callus. Isolated embryogenic structures from embryogenic callus (Fig. 3*a, b*) were subcultured in medium I/condition I in order to determine whether these were capable of forming initial and embryogenic calluses. Formation of initial callus in isolated embryogenic structures was observed after 15 d and was noticeable for only a few days. Histological sections showed presence of meristematic cells with densely stained cytoplasm in peripheral tissues (Fig. 3*c, d*). This was followed immediately by formation of translucent structures that became noticeable after 30 d (similar to those in Fig. 1*b, g*). Histological observations of these structures showed meristematic nodules (Fig. 3*e*). By day 45–60, embryogenic structures formed on the surface of translucent structures. The process of formation of initial and embryogenic calluses during multiplication of embryogenic callus showed no differences from those reported for primary or secondary somatic embryogenesis.

The process of subdividing embryogenic callus and subculturing the isolated pieces in medium I/condition I was carried out for two additional cycles. Again, initial and embryogenic callus developed in each of these two cycles, showing no morphological or histological differences in relation to the observations for the first embryogenic callus multiplication cycle. When explants obtained from the center of an embryogenic callus were subcultured, they formed non-embryogenic callus, and therefore were not used.

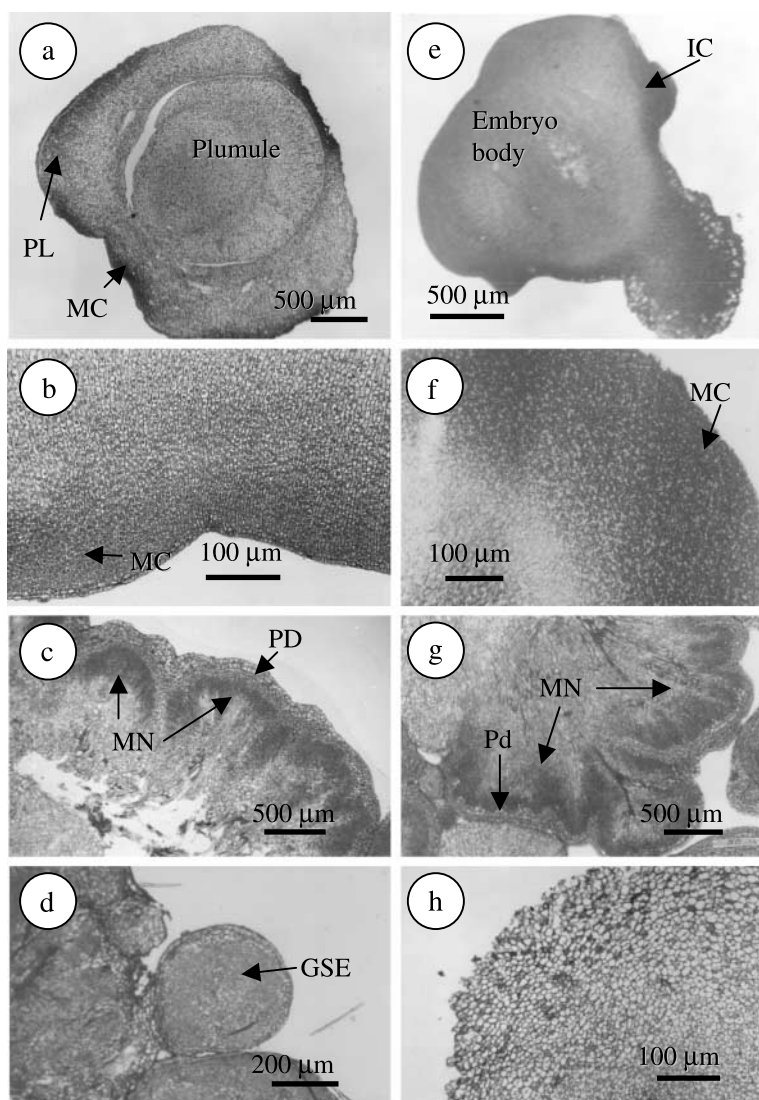


FIG. 2. Histological sections from coconut cultures during primary (*a–d*) and secondary somatic embryogenesis (*e–g*). (*a*) Section of a plumule explant showing growth of plumular leaves (PL) and presence of meristematic cells (MC). (*b*) Section of an embryogenic callus showing the presence of meristematic cells in the periphery. (*c*) Section of an embryogenic callus showing meristematic nodules (MN) and protoderm (PD) formation. (*d*) Section of an embryogenic callus showing a globular somatic embryo (GSE). (*e*) Section of a primary somatic embryo showing formation of initial callus (IC) on the middle part of the embryo body. (*f*) Section of callus showing the presence of meristematic cells (MC). (*g*) Section of an embryogenic callus showing meristematic nodules (MN) and protoderm (Pd) formation. (*h*) Section of a non-embryogenic callus obtained during secondary somatic embryogenesis, showing the absence of protoderm and meristematic nodules.

In order to evaluate if the embryogenic calluses obtained during embryogenic callus multiplication were able to form somatic embryos, they were subcultured into medium II/condition II. Somatic embryos formed from 20 to 25 d of culture.

The results for yield evaluation showed the percentage of explants forming initial callus was 91.6 for ECM₁, 97.5 for ECM₂, and 98.5 for ECM₃ (Table 1). The percentage of explants forming embryogenic callus was 72.7 for ECM₁, 90.0 for ECM₂, and 82.5 for ECM₃ (Table 1). The production of somatic embryos was 7.20 SE/EC for ECM₂, and 7.5 for ECM₃ (Table 1); this was not evaluated for ECM₁.

Cumulative yields. Based on the data in Table 1, we calculated the cumulative figures for total yields of explants, embryogenic callus, and somatic embryos produced throughout the process.

Explant numbers could increase from 100 plumules, the starting amount in primary somatic embryogenesis, to 1129 somatic embryo explants in SSE₃ and to 1597284 embryogenic structure explants in ECM₃ (Table 2). Embryogenic callus production increased from 38 in primary somatic embryogenesis to 632 in SSE₃ and to 1309773 in ECM₃ (Table 2). Finally, the amount of somatic embryos increased from 196 in primary somatic embryogenesis to 1129 in SSE₂ and to 9823297 in ECM₃ (Table 2). Although conversion of embryos to plantlets was out of the scope of the present study, it was calculated for primary somatic embryogenesis that about 12% of embryos formed plantlets. We have no data for the conversion of secondary somatic embryos or those obtained after the embryogenic callus multiplication steps.

TABLE 1

FORMATION OF INITIAL CALLUS (IC), EMBRYOGENIC CALLUS (EC) AND SOMATIC EMBRYOS PER EC (SE/EC) AFTER PRIMARY SOMATIC EMBRYOGENESIS (PSE) AND SEVERAL STEPS OF SECONDARY SOMATIC EMBRYOGENESIS (SSE) AND EC MULTIPLICATION (ECM)

| Step | Type of explant | <i>n</i> explants ^z | % IC ^y | % EC ^y | SE/EC ^y |
|------------------|-----------------|--------------------------------|-------------------|-------------------|--------------------|
| PSE | Plumule | 100 | 83.33 ± 8.66 a | 38.16 ± 10.24 b | 5.16 ± 1.6 a |
| SSE ₁ | SE | 100 | 81.66 ± 15.2 a | 46.16 ± 5.77 b | 4.66 ± 0.51 a |
| SSE ₂ | SE | 100 | 76.0 ± 14.17 a | 42.33 ± 8.16 b | 6.33 ± 1.36 a |
| SSE ₃ | SE | 100 | 81.8 ± 8.2 a | 56.0 ± 17.0 ab | – |
| ECM ₁ | Piece of EC | 30 | 91.6 ± 11.72 a | 72.7 ± 23.4 a | – |
| ECM ₂ | Piece of EC | 30 | 97.5 ± 2.5 a | 90.0 ± 10.0 a | 7.20 ± 0.28 a |
| ECM ₃ | Piece of EC | 30 | 98.5 ± 1.5 a | 82.5 ± 6.5 a | 7.5 ± 1.1 a |

^zFrom the total yields of SE or EC obtained at the end of PSE, SSE₍₁₋₃₎ or ECM₍₁₋₃₎, respectively, only a part was used, 100 (six replicates) for PSE and SSE₍₁₋₃₎, and 30 (three replicates) for ECM₍₁₋₃₎.

^yFigures presented are means with standard deviation and different letters within columns denote significant differences ($P < 0.05$).

DISCUSSION

Reproducible coconut somatic embryogenesis from plumule explants has been previously achieved with low efficiency (Chan et al., 1998; Sáenz et al., 1999; Azpeitia et al., 2003). In order to improve the system, secondary somatic embryogenesis and embryogenic callus multiplication were developed. These approaches have been useful in other species to obtain greater

yields of embryogenic callus and somatic embryos (Raemakers et al., 1995; George, 1996; Maximova et al., 2002). Neither secondary somatic embryogenesis nor embryogenic callus multiplication has previously been reported for coconut.

To determine whether secondary somatic embryogenesis could be initiated, primary somatic embryos were evaluated for formation of initial callus, embryogenic callus, and secondary somatic embryos, and all these stages of development were indeed obtained.

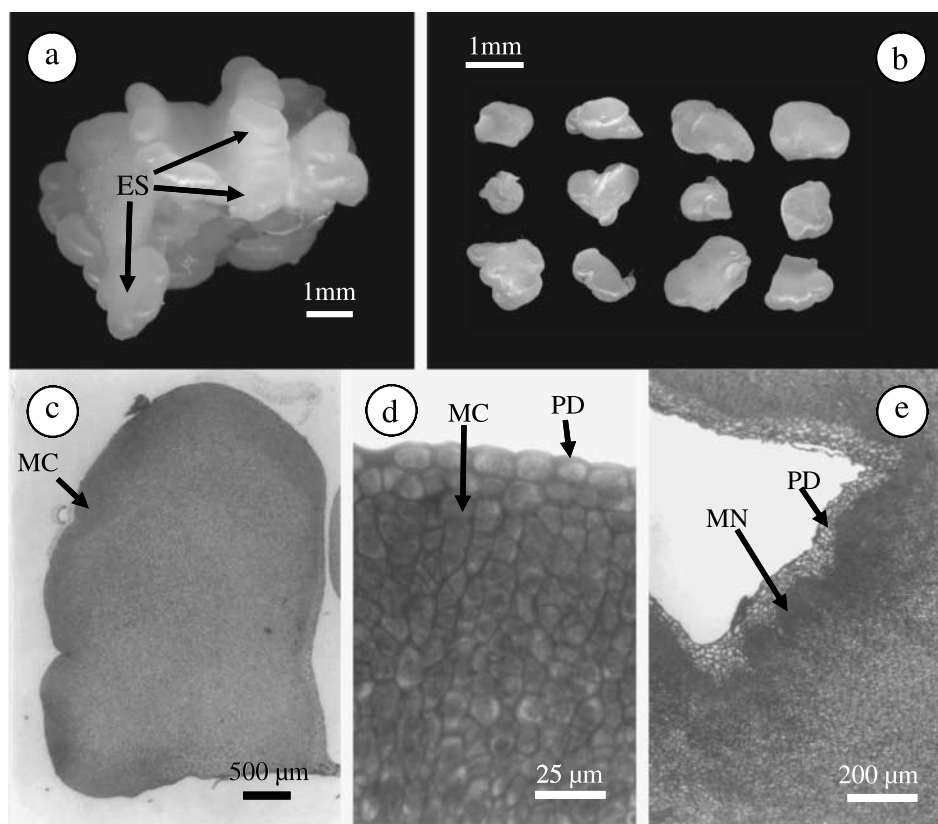


FIG. 3. (a) Morphological and histological observations during the process of embryogenic callus multiplication. An embryogenic callus showing embryogenic structures (ES). (b) Isolated ES used as explants to start the process. (c) Section of an isolated ES showing the presence of meristematic cells (MC) in the periphery and (d) amplified view of peripheral tissue showing densely stained MC and the presence of a protoderm (PD). (e) A section of an embryogenic callus showing the presence of meristematic nodules (MN) underneath cell layers of PD.

TABLE 2

CALCULATED CUMULATIVE YIELDS OF EMBRYOGENIC CALLUS (EC) AND SOMATIC EMBRYOS (SE) AFTER PRIMARY SOMATIC EMBRYOGENESIS (PSE) AND SEVERAL STEPS OF SECONDARY SOMATIC EMBRYOGENESIS (SSE) AND EC MULTIPLICATION (ECM)

| Step | Slicing | Explants ^a | EC ^a | SE ^a |
|------------------|---------|-----------------------|---------------------|-----------------------|
| PSE | – | 100 | 38 ± 10.2 | 196 ± 60.87 |
| SSE ₁ | – | 196 | 90 ± 11.32 | 421 ± 46.72 |
| SSE ₂ | – | 421 | 176 ± 34.3 | 1129 ± 240 |
| SSE ₃ | – | 1129 | 632 ± 271.4 | – |
| ECM ₁ | × 15 | 9480 | 6826 ± 2708.5 | – |
| ECM ₂ | × 20 | 136 520 | 122 868 ± 19 306.8 | – |
| ECM ₃ | × 13 | 1 597 284 | 1 309 773 ± 146 828 | 9 823 297 ± 2 307 528 |

^aFigures calculated with percentages reported in Table 1 extrapolating to total yields for each step.

Similarly, secondary somatic embryos were also capable of developing initial callus, embryogenic callus, and secondary somatic embryos. When primary and secondary somatic embryogenesis were compared morphologically and histologically, they were found to be very similar with only minor differences. With both types of explants, plumule or somatic embryos, initial callus developed from the peripheral tissues as has been reported for other species such as *Vitis rupestris* S. (Martinelli et al., 2001) and *Quercus suber* L. (Puigderrajols et al., 2001). During primary and secondary somatic embryogenesis, it was common to find fused embryos. However, these were more abundant in primary somatic embryogenesis (data not shown).

The formation of initial callus during secondary somatic embryogenesis took no longer than the time needed for primary somatic embryogenesis, but the formation of embryogenic callus and somatic embryos was more rapid during secondary somatic embryogenesis. Therefore, secondary somatic embryogenesis was more efficient than primary somatic embryogenesis. A similar difference has been reported for cassava cultures (Stamp and Henshaw, 1987).

To investigate whether coconut embryogenic callus could be multiplied, the embryogenic calluses were subdivided and isolated pieces of embryogenic structures were used as explants. Our findings demonstrated that embryogenic structures produced new embryogenic callus that was capable of forming somatic embryos; hence, the strategy of embryogenic callus multiplication is possible in coconut *in vitro* culture. Developmentally, the process is very similar to that observed for primary and secondary somatic embryogenesis, except that initial callus formation is barely noticeable, with the formation of translucent structures taking place within a few days of culture; and very importantly, newly formed embryogenic callus was able to form somatic embryos. Formation of embryogenic callus and somatic embryos during embryogenic callus multiplication was more rapid than callus formation from primary somatic embryogenesis using plumule explants.

Quantitative performance of both secondary somatic embryogenesis and embryogenic callus multiplication was evaluated to determine if either process could be useful to improve the embryogenic callus and somatic embryo yields. The percentage of explants forming initial callus during primary somatic embryogenesis was 83.3, for secondary somatic embryogenesis it was 76–81.8, and for embryogenic callus multiplication it was 91.6–98.5. In all cases, the differences were not statistically significant.

The percentage of explants forming embryogenic callus during primary somatic embryogenesis was 38.1, for secondary somatic embryogenesis it was 42.3–56.0, and for embryogenic callus multiplication it was 72.7–90.0, the last being significantly larger than the first two. In the case of somatic embryo formation, the figures varied from 4.6 to 7.5 SE/EC without significant differences. The results show that each of the morphological responses studied for secondary somatic embryogenesis and embryogenic callus multiplication were not quantitatively reduced in relation to primary somatic embryogenesis and were reproducible. For overall yields of the process, 100 plumules resulted in a total of 1.3 million embryogenic calluses and 9.8 million somatic embryos, or 13 000 embryogenic calluses and 98 000 somatic embryos per single plumule. When comparing these yields with the present yields for primary somatic embryogenesis, the increases were about 35 000-fold for embryogenic callus formation and about 50 000-fold for somatic embryo formation. Previous figures in the literature for primary somatic embryogenesis (Azpeitia et al., 2003) were 360 somatic embryos per 100 plumules or about 1000 embryos if explants were treated with 22(S);23(S)-homobrassinolide. Therefore, the present embryo yield is about 27 000-fold greater if compared with the result by Azpeitia et al. (2003) obtained without brassinolide treatment or still 10 000-fold greater if compared with that obtained with brassinolide treatment. In our experiments, brassinolide treatment was not tested.

It is important to stress that after three steps of embryogenic callus multiplication, the calluses were still able to produce embryos. These embryos were tested as explants, and they produced embryogenic callus and somatic embryos (data not shown). Since the repetitive use of callus multiplication has the risk of losing embryogenic competence (George, 1996; Das Neves et al., 1999), perhaps an alternate use of secondary somatic embryogenesis and embryogenic callus multiplication can be carried out several times to exploit their respective advantages, which include conservation of embryogenic competence and a large multiplication capacity. This way, if the protocol is extended with further steps of embryogenesis and embryogenic callus multiplication, we should expect to obtain a larger yield than the one reported here.

The protocol reported here represents important progress towards practical application by showing a way to improve the efficiency of coconut somatic embryo production. As mentioned previously, the use of plumule explants from zygotic embryos obtained by cross-pollination will preclude the application of this protocol for cloning of palm individuals with known agronomic traits. However, this could be

overcome if embryos are obtained by self-pollination of isogenic palms, or alternatively, by replacing plumules with a type of explant obtained from a mature palm with known traits. However, attempts to achieve the last have not been successful so far in obtaining reproducible production of embryogenic callus and somatic embryos.

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