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MICROPROPAGATION

Improved formation of embryogenic callus from coconut immature inflorescence explants

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Abstract Coconut is a crop with high economic potential; however, production is declining mainly because of palm senescence and diseases such as lethal yellowing. Therefore, there is great demand for palms selected for high productivity and resistance to diseases, and the best strategy for their production is micropropagation through somatic embryogenesis. It is currently possible to micropropagate coconut with highly efficient protocols using the plumule as the explant tissue, but these protocols are not useful for propagating an already fruit-bearing palm with known traits and measured performance. The objective of the present study was to define the conditions for the production and multiplication of embryogenic calluses from rachilla explants from immature inflorescences and to determine their capability to form somatic embryos that are able to convert into plantlets. Twenty-five media containing the plant-growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) combined in different concentrations were tested to induce somatic embryogenesis. A medium containing 0.65 mM 2,4-D and no BAP most efficiently induced the formation of callus with embryogenic structures, and through subculturing these structures as explants, embryogenic callus was produced from them and multiplied. Morphological and histological characterization showed that the callus obtained was indeed embryogenic, similar to that obtained from plumule. The results are encouraging and represent a strong basis for further studies aiming at the development of a protocol for massive propagation using rachilla explants from immature inflorescences, therefore enabling the cloning of fruit-bearing palms with known traits and measured performance.

Keywords Coconut · Somatic embryogenesis · Male inflorescence · Morphology · Histology

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Introduction

The coconut palm (*Cocos nucifera* L.) is a crop species with a rising economic value, cultivated in nearly 90 countries on 12 million ha (Solís-Ramos *et al.* 2012). Interest in coconut products has been rapidly increasing around the world over the past 10 years; in particular, the market for packaged coconut water has grown rapidly in Brazil, the USA, and Europe (Roolant 2014). This positive and sustained market growth needs to be matched with an increase in fruit production. However, losses caused by old age of palms, pests, and diseases, in particular lethal yellowing (LY) diseases in the Americas (Harrison and Oropeza 2008) and Africa (Eden-Green 1997) and cadang-cadang in the Philippines (Hanold and Randles 1991), are a significant challenge to increasing the scale of production in most coconut-producing countries.

In order to sustain the required production for the current and future demand of coconut products, producing countries need to renew their plantations and increase the cultivation area. To achieve this goal, a very large number of plants propagated from germplasm selected for disease resistance and high productivity is required. This increase in production capabilities is difficult to achieve while depending only on propagation through seed. For this reason, alternative means of propagation need to be considered, such as in vitro propagation or micropropagation by somatic embryogenesis (Solís-Ramos *et al.* 2012).

The development of micropropagation protocols for coconut has progressed slowly as this monocot species has been difficult to regenerate, but it has recently been achieved through somatic embryogenesis (Sáenz-Carbonell *et al.* 2013). Among the explants tested, plumules from zygotic embryos have been the most responsive tissue in terms of embryogenic callus formation, somatic embryo production, and somatic embryo conversion to plantlets (Chan *et al.* 1998; Azpeitia *et al.* 2003). A mass-propagation protocol has recently been developed, starting with plumule explants through the production of embryogenic callus and its multiplication, with estimated yields of 100,000 somatic embryos per explant (Pérez-Núñez *et al.* 2006).

This protocol, currently being scaled up in Mexico, can be used to propagate the progeny of selected plants from seeds obtained by controlled pollination. However, it is not useful to propagate hybrid palms using their seed because of segregation (Namboothiri *et al.* 2008). Furthermore, to propagate adult palms that are already bearing fruit and have known measured performance will require a different source of explant tissue such as rachillae from immature inflorescences, as early reports showed the formation of callus from rachillae (Brackpool *et al.* 1986; Sugimura and Salvaña 1989; Blake 1990). Further testing of these explants showed reproducible formation of embryogenic callus (Verdeil *et al.* 1994; Vidhana Arachchi and Weerakoon 1997), but the efficiency of formation of somatic embryos and plantlet conversion was not determined. More recent reports with other floral tissues, namely, anthers (Perera *et al.* 2008) and unfertilized ovaries (Perera *et al.* 2009), allowed the consistent formation of embryogenic callus, although with low frequencies. The abovementioned reports show that the use of floral tissue explants capable of forming embryogenic callus represents an opportunity to develop highly efficient protocols based on embryogenic callus multiplication, as reported for plumule explants (Pérez-Núñez *et al.* 2006).

Such a protocol could be very useful to clone elite hybrid palms already known for high productivity and resistance to diseases. In a trial in Mexico, 12 coconut hybrids were evaluated for disease resistance and productivity, including hybrids locally produced and hybrids produced abroad and introduced into Mexico (Castillo *et al.* 2012). Two of the introduced hybrids (Malayan Red Dwarf × Vanuatu Tall and Malayan Red Dwarf × Tagnanan Tall) showed the highest copra production and lowest rates of loss due to LY disease (Castillo *et al.* 2012). Only a few plants of these two hybrids are present in Mexico.

Therefore, the objective of the present study was to define the conditions for the production and multiplication of embryogenic calluses and to determine their capability to form somatic embryos that are able to convert into plantlets. Rachilla explants obtained from the hybrids of interest were used.

Materials and Methods

Plant materials Rachilla explants were obtained from immature inflorescences of different developmental stages ranging from −4 to −10, with 0 corresponding to the youngest opened inflorescence (Perera *et al.* 2010). Three batches of immature inflorescences were collected. The first two batches were for experimental trials carried out to define conditions to induce embryogenic callus formation, and the third batch was for testing the defined conditions. Inflorescences used for the first batch (trial 1) were of stages −4, −5, and −6 from a 15-y-old Malayan Yellow Dwarf × Mexican Pacific Tall (MYD × MXPT) hybrid palm growing at San Crisanto, Yucatán, Mexico (21° 21′ 03.7″ N, 89° 11′ 07.9″ W). For the second batch (trial 2), the inflorescences used were from two 10-year-old Malayan Red Dwarf × Tagnanan Tall (MRD × TAGT) hybrid palms: the developmental stages were −6 to −9 for palm 1 and −7 to −10 for palm 2, both palms growing at Pailebot, Tabasco, Mexico (18° 16′ 04.4″ N, 93° 56′ 49.6″ W). The third batch of immature inflorescences, also from Pailebot, was obtained from five additional MRD × TAGT hybrid palms and five Malayan Red Dwarf × Vanuatu Tall (MRD × VTT) hybrid palms. For all the plants in this third batch, inflorescences were selected according to a size of approximately 10 cm in length.

Inflorescences obtained in the field were rinsed with distilled water and 70% (*v*/*v*) ethanol for 3 min during collection. Under aseptic laboratory conditions, the immature inflorescences were washed in 70% (*v*/*v*) ethanol for 3 min, rinsed three times with sterile distilled water, washed again in Cloralex 50% (*v*/*v*) (commercial bleach, 30 g L^{-1} NaOCl, Alen, Monterrey, Mexico) for 20 min, and finally rinsed three times with sterile water. The two spathes were excised, and the rachillae were separated and placed in a sterile solution of ascorbic acid (0.15 g L^{-1}), citric acid (0.1 g L^{-1}), and sucrose (30 g L^{-1}). All chemicals were of reagent grade (Sigma-Aldrich®, St. Louis, MO). Then, 0.3- to 0.5-mm rachilla slices were obtained and placed directly on culture medium.

Medium preparation and culture conditions Medium preparation and culture conditions were according to Pérez-Núñez *et al.* (2006). The preparation of the different media was based on a medium defined as medium I, prepared with Y3 medium formulation (Eeuwens 1976) supplemented with 3 g L^{-1} Gelrite™ and 2.5 g L^{-1} activated charcoal (Sigma-Aldrich®, C6289), without growth regulators. Medium pH was adjusted to 5.75 with KOH before autoclaving for 20 min at 120 °C. The media for both trial 1 and trial 2, designed to define the best conditions for induction of embryogenic callus, were prepared with the medium I formulation supplemented with growth regulators combined at different concentrations for testing explant response (details described in the "Experimental design" section below). Explants were

cultured for 90 d in a 35-ml glass vessel (Vitro, Tlalnepantla, Mexico) with polypropylene closure (Möller, Nezahuatcoyotl, Mexico) with 10 ml of medium, under complete darkness at 27 ± 2 °C and without subculturing.

Callus multiplication was carried out by using embryogenic structures isolated from the calluses as explants. They were cultured for 90 d in a 35-ml glass vessel with 10 ml of medium II (prepared with medium I formulation supplemented with 0.65 mM 2,4-dichlorophenoxyacetic acid [2,4-D]), under complete darkness at 27 ± 2 °C and without subculturing. For induction of somatic embryo formation, embryogenic calluses were cultured for 30 d in a 100-ml glass vessel (Vitro, Tlalnepantla, Mexico) with polypropylene closure (Möller, Nezahuatcoyotl, Mexico) with 25 ml of medium III (prepared with medium I formulation supplemented with 0.325 mM 2,4-D) under complete darkness at 27 ± 2 °C and without subculturing.

For germination of somatic embryos and shoot formation, embryogenic calluses were cultured in a 100-ml glass vessel with 25 ml of medium IV (prepared with medium I formulation supplemented with 0.006 mM 2,4-D, 0.3 mM 6-benzylaminopurine [BAP], and 0.0046 mM gibberellic acid [GA_3]) under a 16-h photoperiod (45–60 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by Tri-Phosphor (F32T8, 6500 K, 32 W) daylight tubes (MAGG^{MR}, Tlatilco, Mexico) at 27 ± 2 °C and subculturing every 2 mo for a total of three subcultures.

For plantlet formation, shoots were cultured in a 500-ml glass vessel (Vitro) with polypropylene closure (Möller) with 50-ml medium V (prepared with medium I formulation supplemented with 0.006 mM 2,4-D and 300 mM 6 BAP), under a 16-h photoperiod (45–60 μmol m^{-2} s^{-1} PPFD) at 27 ± 2 °C, and subculturing every 2 mo for a total of three subcultures.

The 2,4-D concentration of 0.65 mM used in medium II was defined experimentally from trials I and II (see the "Results" section). The growth regulator concentrations in media III, IV, and V were according to Pérez-Núñez *et al.* (2006).

Experimental design As described above, two trials were performed to define conditions for the induction of embryogenic callus from rachilla explants. For the first one, 25 media were prepared by combining 5 concentrations of 2,4-D with 5 concentrations of BAP and were referred to as treatments T1 to T25 (Table 1). These media were evaluated to determine from which one the best embryogenic callus induction response could be obtained from rachilla explants of immature inflorescences of stages −4, −5, and −6 of an MYD × MXPT hybrid palm.

The three best treatments resulting from the first trial were evaluated in a second trial to determine what conditions produced the best embryogenic callus induction response from rachilla explants of immature inflorescences of stages −6 to −9 of palm 1 and stages −7 to −10 of palm 2 of the MRD × TAGT hybrid.

Table 1. Media with different combinations of 2,4-D and BAP concentrations used for somatic embryogenesis induction

BAP (mM)	2,4-D (mM)				
	0.01	0.05	0.2	0.4	0.65
0	T1	T6	T11	T16	T21
0.01	T2	T7	T12	T17	T22
0.02	T3	T8	T13	T18	T23
0.04	T4	T9	T14	T19	T24
0.05	T5	T10	T15	T20	T25

2,4-D 2,4-dichlorophenoxyacetic acid, *BAP* 6-benzylaminopurine

Histology Histological procedures followed Buffard-Morel *et al.* (1992) with slight modifications as outlined by Sáenz *et al.* (2006) and Montero-Cortés *et al.* (2010). The samples of tissues to be studied were placed for 24 h in a pH 7.2 phosphate buffer solution containing 4% (*w*/*v*) paraformaldehyde. They were then subjected to dehydration in an aqueous solution of ethanol, increasing concentration stepwise from 30 to 100% (*v*/*v*) within a 7-h period. The samples were impregnated with JB-4R resin (Polyscience, Warrington, PA), and 5-μm sections were obtained with an HM 325 Microtome (Thermo Fisher Scientific, Waltham, MA) and stained with periodic acid-Schiff (PAS) reagent combined with protein-specific naphthol blue-black according to Fisher (1968).

Statistical analysis A completely randomized design was used in both trials. The data presented correspond to means. In every trial, one explant was considered an experimental unit and a replicate. The number of replicates used per treatment varied between 30 and 105; the number of replicates in each case is indicated in corresponding table legends. Data were subjected to analysis of variance (ANOVA), and the procedure for mean comparisons (Tukey test at $P < 0.05$ or $P < 0.01$) was performed with SAS version 9.0 software.

Results

Induction of callus from rachilla explants By the end of the 90-d culture period, some of the rachilla explants from batch one, stage −6 (outer spathe length of 12.3 cm) inflorescence had formed calluses that had necrotic tissue associated with pearly white tissue (Fig. 1*A* parts *a* and *b*). The formation of calluses with embryogenic structures (described in detail below) was associated with certain treatments. Four of them were combinations of 0.4 mM 2,4-D with or without BAP, five contained 0.65 mM 2,4-D with or without BAP, and the

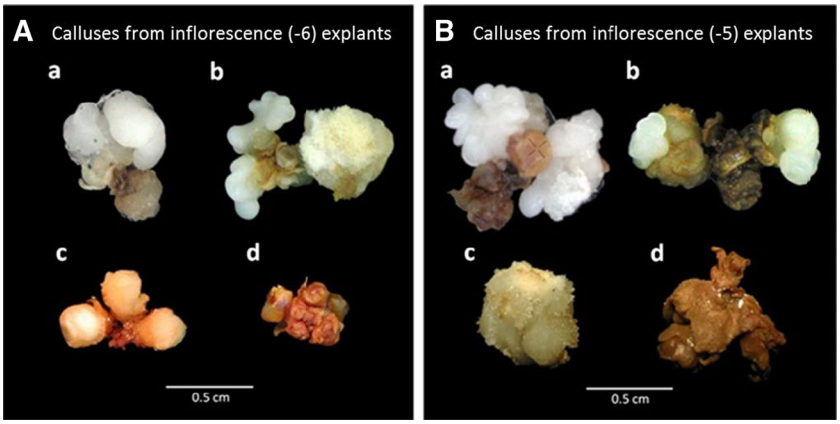

Fig. 1. Formation of callus from rachilla explants of a Malayan Yellow Dwarf × Mexican Pacific Tall (MYD × MXPT) hybrid palm. *A* Rachilla explants (inflorescence development stage −6) after 90 d of in vitro culture on medium I. *a*, *b* Explants that formed calluses with embryogenic structures. *c*, *d* Explants that formed spongy callus (*c*) or without callus formation, showing necrotic tissue (*d*). *B* Rachilla explants (inflorescence development stage −5) after 90 d of in vitro culture on medium II. *a*, *b* Explants that formed calluses with embryogenic structures. *c*, *d* Explants that formed spongy callus (*c*) or without callus formation, showing necrotic tissue (*d*).

last one contained 0.01 mM 2,4-D and 0.04 mM BAP (Table 2).

Similar results were obtained with rachilla explants from stage −5 (22.3 cm) inflorescences (Fig. 1*B* parts *a* and *b*). Pearly white structures formed only on media with 0.65 mM 2,4-D, with or without BAP (Table 3). In the rest of the treatments with explants from stage −6 or −5 inflorescences, growth was observed, but it was spongy callus (part *c* of Figs. 1*A* and *B*) or necrotic tissue (part *d* of Figs. 1*A* and *B*).

In the case of explants from stage −4 (34.3 cm) inflorescence (not shown), no callus with pearly white structures was formed; the calluses formed showed only the appearance of spongy tissue as observed in some calluses from inflorescences of stages −6 and −5 (part *c* of Figs. 1*A* and *B*, respectively).

In the case of inflorescence stage −5, calluses with pearly white structures were formed only on media with 0.65 mM 2,4-D, with or without BAP (Table 3). In the rest of the treatments, with explants from either stage −6 or −5 inflorescences, growth was observed, but it was spongy callus (part *c* of Figs. 1*A* and *B*) or necrotic tissue (part *d* of Figs 1*A* and *B*). With explants from stage −4 inflorescence, no callus with

Table 2. Formation of callus with embryogenic structures from coconut rachilla explants (inflorescence stage −6) after 90 d of *in vitro* culture in 25 different treatments

mM	Percentage of explants producing embryogenic callus				
2,4-D; BAP	0.01	0.05	0.2	0.4	0.65
0	0a	0a	0a	3.3a	16.6a
0.01	0a	0a	0a	6.6a	16.6a
0.02	0a	0a	0a	13.3a	16.6a
0.04	3.3a	0a	0a	10.0a	10.0a
0.05	0a	0a	0a	0.0a	6.6a
C.V.	127.18				

Each treatment was prepared with medium I formulation supplemented with different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) concentrations, as shown. The rachilla explants were obtained from an inflorescence of development stage −6 from a Malayan Yellow Dwarf × Mexican Pacific Tall (MYD × MXPT) hybrid coconut palm. The data shown are averages of 30 repetitions. Values followed by the same letter within the same column are not significantly different according to Tukey's test ($P \leq 0.01$). Significance and coefficient of variation (C.V.) were determined using a $(\sqrt{x}+1)$ transformation

Table 3. Formation of callus with embryogenic structures from coconut rachilla explants (inflorescence stage −5) after 90 d of in vitro culture in 25 different treatments

mM	Percentage of explants producing embryogenic callus				
2,4-D BAP	0.01	0.05	0.2	0.4	0.65
0	0c	0c	0c	0c	18.4a
0.01	0c	0c	0c	0c	0.0c
0.02	0c	0c	0c	0c	10.5ab
0.04	0c	0c	0c	0c	0.0c
0.05	0c	0c	0c	0c	2.6bc
C.V.	85.54				

Each treatment was prepared with medium I formulation supplemented with different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) concentrations, as shown. The rachilla explants were obtained from an inflorescence of development stage −5 from a Malayan Yellow Dwarf × Mexican Pacific Tall (MYD × MXPT) hybrid coconut palm. Data shown are averages of 38 repetitions. Values followed by the same *letter* within the same *column* are not significantly different according to Tukey's test ($P \leq 0.01$). Significance and coefficient of variation (C.V.) were determined using a $(\sqrt{x}+1)$ transformation

pearly white structures was formed with any of the 25 treatments used (not shown).

From a quantitative point of view, the percentages of rachilla explants from inflorescence stage −6 forming embryogenic callus by day 90 varied from 3.3 to 16.6% among those treatments that showed a response (Table 2). The highest yields were obtained using 0.65 mM 2,4-D combined with different concentrations of BAP, but the differences among BAP levels were not statistically significant. In the case of rachilla explants from inflorescence stage −5, the percentage of embryogenic callus formation varied from 2.6 to 18.4% among those treatments that showed a response (Table 3). These responses were obtained when 0.65 mM 2,4-D was used, regardless of the presence of BAP. The highest percentage was obtained without BAP, which was significantly better than most other treatments.

Histological analysis of callus induced from rachillae At day 30 of culture, transverse sections showed that the body of the rachilla was still present and that callus tissue (Fig. 2*A*) was formed from the floral meristem (Fig. 2*B*). The center of the rachilla showed groups of procambium and xylem cells (Fig. 2*C*). At day 60, transverse sections showed a compact structure (Fig. 2*D*) formed by stained small meristematic cells at the periphery, and the inner body of the callus was formed by parenchyma and xylem cells (Fig. 2*E*, *F*). The peripheral cells had a visible nucleus of irregular shape, without a clearly defined nucleolus. The cells also revealed densely stained cytoplasm, indicating high metabolic activity (Fig. 2*G*).

Formation of fully developed embryogenic callus from embryogenic structures The white pearly structures (referred to as embryogenic structures from here on) from the calluses obtained with explants from inflorescence stages −6 and −5 were isolated and subcultured on medium II. This operation was repeated two times, starting each time from the embryogenic structures obtained from the calluses formed in the previous subculture (Table 4 part *A*). In each of the three subcultures, new calluses with embryogenic structures were formed without necrotic or spongy tissues. Figure 3 shows the development of embryogenic callus from embryogenic structure explants during the last subculture. Within the first 45 d of culture, the explants grew and developed translucent structures with an ear-like shape that became more abundant with time. By day 75, new structures resembling embryogenic structures grew on the translucent structures. The first embryogenic structures observed were globular embryogenic structures followed by the appearance of elongated embryogenic structures, both of which became more abundant with time. By day 90, fully developed embryogenic calluses were formed.

The proportion of embryogenic structure explants forming fully developed embryogenic callus increased with each subculture from 9.3 to 57.1% (explants originated from stage −5 inflorescence) and from 11.1 to 44.9% (explants originated from stage −6 inflorescence) (Table 4 part *A*).

Histological analysis of embryogenic callus formation from embryogenic structures Histological analysis of isolated embryogenic structures (Fig. 4*A*) placed on medium II to induce the formation of embryogenic callus showed that by day 30 of culture, translucent structures with an ear-like shape had developed on compact callus. These translucent structures consisted of meristematic cells with a densely stained cytoplasm (Fig. 4*B*). Within the next 30 d, the translucent structures became more abundant and larger, and by day 60, histological sections showed arrays of small meristematic cells in their peripheral tissues (Fig. 4*C*). Above these tissues, globular embryogenic structures formed by day 75 (Fig. 4*D*), followed by the appearance of elongated embryogenic structures, both of which increased in size and number by day 90

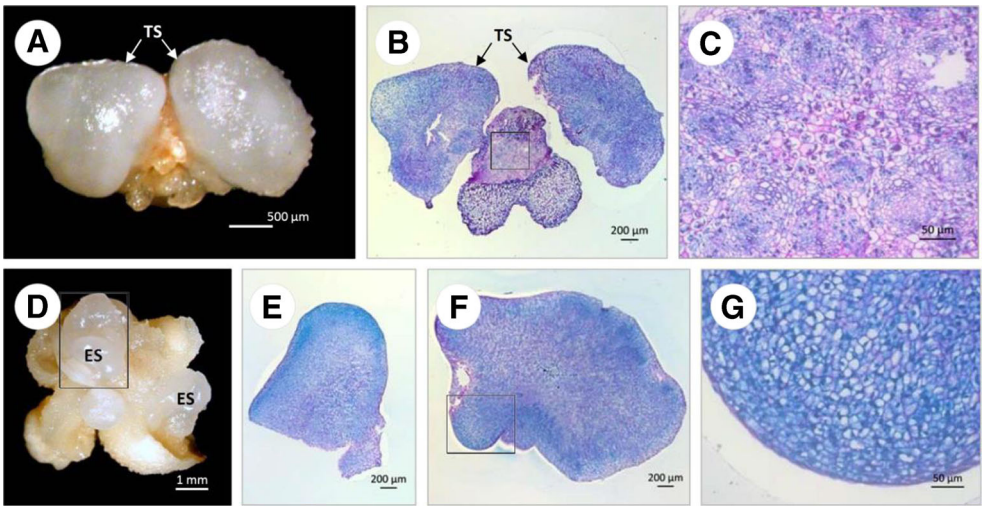

Fig. 2. *A* Callus with pearly white structures formed after 30 d of culture on rachilla explant of a Malayan Yellow Dwarf × Mexican Pacific Tall (MYD × MXPT) hybrid palm, and *B* corresponding histological section. *C* Magnification (×40) of section designated in (*B*), showing vascular cambium and xylem tissues. *D* Callus after 60 d with formations resembling embryogenic structures, and *E*, *F* corresponding histological sections of these formations. *G* Magnification (×40) of part of section shown in (*F*). *ES* embryogenic structure, *TS* translucent structure.

Table 4. Embryogenic callus and somatic embryo formation from different starting tissues originally obtained from rachilla explants from inflorescences at developmental stages −5 and −6 of a MYD × MXPT hybrid coconut palm

Action induced	Starting tissue for culture[z]	Subculture	Inflorescence stage	
			−5	−6
			% explants responding	
A. Embryogenic callus formation	▪ Embryogenic structure explants	1	9.3	11.1
		2	30.0	30.2
		3	57.1	44.9
			% calluses responding	
B. Somatic embryo formation on embryogenic callus	▪ Fully embryogenic callus	4	NA	21.4
			% explants responding	
C. Embryogenic callus formation	▪ Somatic embryos	5	NA	18.1
	▪ Embryogenic structure explants	6	NA	15.6
		7	NA	36.6

MYD × *MXPT* Malayan Yellow Dwarf × Mexican Pacific Tall, *NA* not available

[z] Embryogenic structure explants and somatic embryos were cultured for 90 d on medium II; fully embryogenic callus was cultured for 30 d on medium III

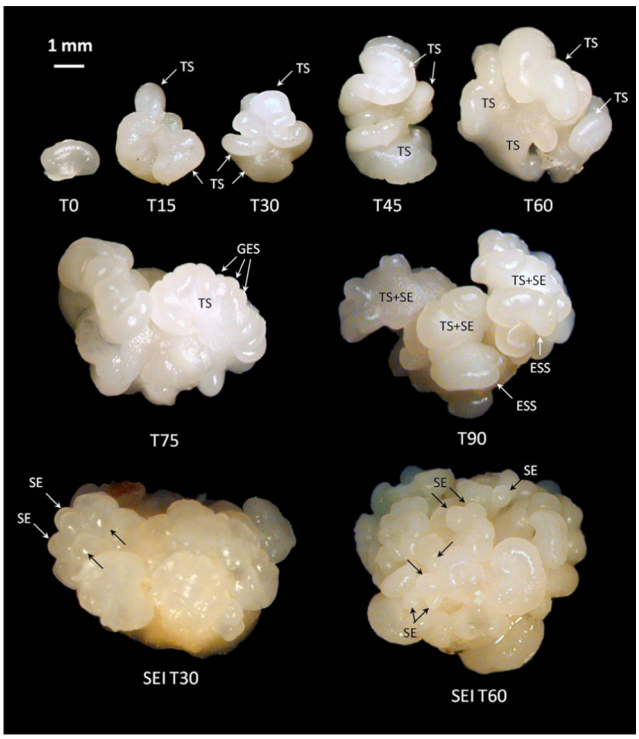

Fig. 3. Morphological changes occurring during the development of embryogenic callus induced from embryogenic structures used as explants. These embryogenic structures were isolated from embryogenic calluses originally obtained from coconut rachilla explants of a Malayan Yellow Dwarf × Mexican Pacific Tall (MYD × MXPT) hybrid palm. Explants were cultured in vitro on medium II for the formation of embryogenic callus (T0–T90 culture times from 0 to 90 d). Embryogenic calluses were subcultured on medium III for somatic embryo induction (*SEI T30-T60* culture times 30 and 60 d). *TS* translucent structure, *TS+ES* translucent structure with embryogenic structure, *GES* globular embryogenic structure, *EES* elongated embryogenic structures, *SE* Somatic embryo, *SEI* somatic embryo induction.

(Fig. 4*E*). Histological sections also showed that embryogenic structures apparently formed from meristematic cells surrounded by a protoderm (Fig. 4*E*). The inner part of the callus was formed of xylem and parenchyma cells (Fig. 4*F*, *G*). The cells of the embryogenic structures were densely stained, the nuclei were not round, and the nucleoli were not visible (Fig. 4*F*, *G*).

Formation of somatic embryos and conversion into plantlets Embryogenic calluses (originally from rachillae, stage −6, MYD × MXPT) produced after subculture 3 (Table 4) were transferred to medium III to induce the formation of somatic embryos (SE); 21.4% of these cultured calluses formed SE (Table 4, part *B*). These somatic embryos (Fig. 3) were, in turn, used as explants to determine if the formation of embryogenic calluses could be induced from them; of these, 18.1% formed embryogenic calluses (Table 4 part *C*). As in the previous trials, embryogenic structures from these calluses were used as explants on two subsequent occasions and in each case, fully embryogenic calluses were formed (Table 4 part *C*), such as the one depicted in Fig. 3 after 90 d of culture. These calluses formed SE after 30 d of culture on medium III. The number of SE (globular stage) per callus after 30 d of culture was determined in three batches of ten calluses each; the results were 6.5 ± 4.5, 6.0 ± 3.8, and 5.6 ± 3.6. While still on the calluses (Fig. 5*A*), globular embryos developed into torpedo-shaped embryos (Fig. 5*B*). When the calluses were transferred to medium IV and grown under a 16-h photoperiod, the embryos germinated (Fig. 5*A*, *C*), and on medium V, still under a 16-h photoperiod, they developed into shoots (Fig. 5*D*) and converted to plantlets (Fig. 5*E*, *F*). This cycle could be repeated, as shown in Fig. 6.

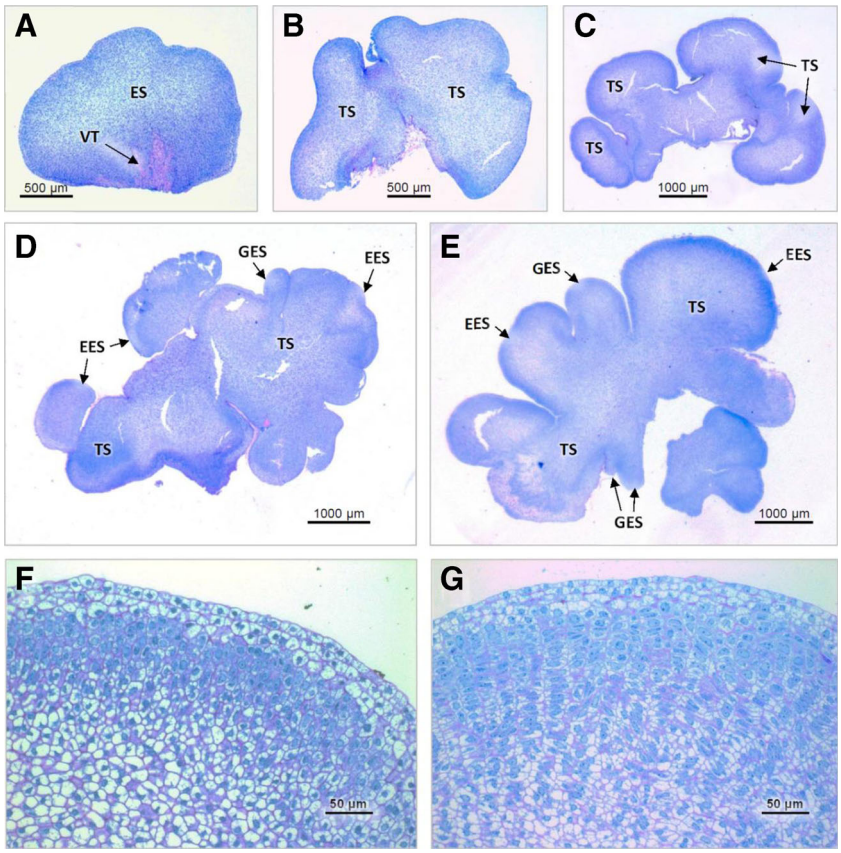

Fig. 4. Histological sections of embryogenic structures and callus from rachilla explants of a Malayan Yellow Dwarf × Mexican Pacific Tall (MYD × MXPT) hybrid palm. *A* An embryogenic structure at day 0. If used as an explant, this type of structure would produce embryogenic callus. *B–E* Callus obtained from an ES explant, forming embryogenic structures after 30 d (*B*), 60 d (*C*), 75 d (*D*), and 90 d (*E*) of culture. *F*, *G* Magnification (×40) of embryogenic callus after 75 (*F*) and 90 d (*G*). *ES* embryogenic structure, *VT* vascular tissue, *TS* translucent structure, *GES* globular embryogenic structure, *EES* elongated embryogenic structure.

Formation of embryogenic callus from selected coconut palms At the end of the culture period, formation of calluses with necrotic tissue but associated with pearly white tissue, similar to those obtained with explants from rachillae of MYD × MXPT hybrid palm (Fig. 1*A*, *B*), could be observed on the explants of both palms. Most of the explants responding with

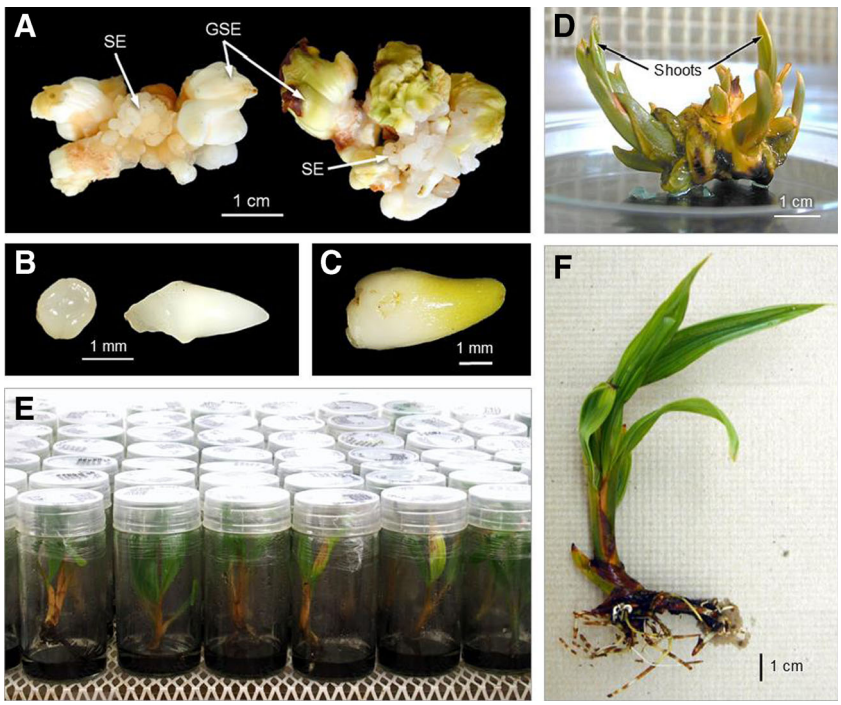

Fig. 5. *A* Calluses with somatic embryos (*SE*) and germinating somatic embryos (*GSE*). *B* Globular and torpedo-shaped embryos, isolated from the callus for illustrative purposes. *C* Germinating embryo, isolated from the callus for illustrative purposes. *D* Shoots developing from GSE of a callus. *E* Batch of coconut plantlets in growth room. *F* A coconut plantlet obtained through SE conversion. The embryogenic calluses producing the development stages shown here were obtained from embryogenic structure explants isolated from embryogenic calluses originally induced from rachilla explants of a Malayan Yellow Dwarf × Mexican Pacific Tall (MYD × MXPT) hybrid palm.

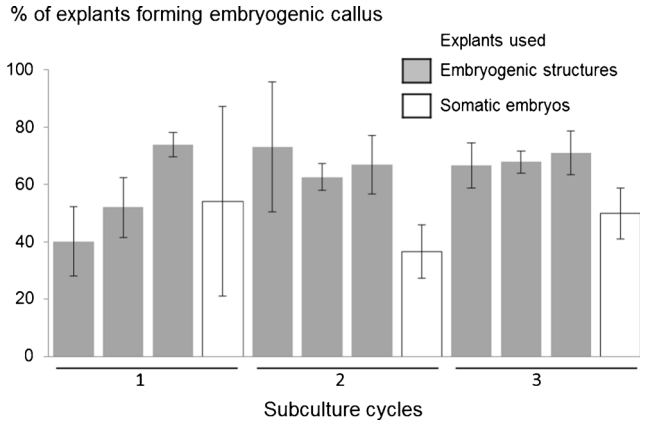

Fig. 6. Subculture cycles for the formation of embryogenic callus. Each cycle consisted of three subcultures of embryogenic structure explants, corresponding embryogenic callus production, and a final step for the induction of somatic embryos, which in turn were finally used as explants for the formation of additional embryogenic callus. Original explants were from rachillae of inflorescences from a Malayan Yellow Dwarf × Mexican Pacific Tall (MYD × MXPT) hybrid coconut palm. Tissues were cultured for 90 d on medium II. *Bars* represent *SD*.

callus formation were from the younger inflorescences tested: stages −8 (10 cm) and −9 (6 cm) from palm 1 and stages −9 (9.8 cm) and −10 (6 cm) from palm 2.

Among the treatments showing a response, the percentages of explants forming callus varied from 0.9 to 44.4% for palm 1 and from 0.9 to 35.5% for palm 2 (Table 5). The data showed numerical differences in callus formation between explants cultured on different media (Table 5), but most of these were not statistically significant, while differences due to developmental stage were statistically significant (Table 6) and the best results were observed with inflorescences at developmental stage III (approximately 10 cm). The embryogenic structures obtained were used as explants, as in the case of MYD × MXPT hybrid palm, and fully embryogenic calluses could be obtained. This was successful for embryogenic structure explants originally from rachilla stages III and IV from palm 1 and from stages II, III, and IV from palm 2 (data not shown). The calluses obtained had several embryogenic structures and no necrotic or spongy tissues (not shown), showing the same development pattern as described for MYD × MXPT hybrid palm in Fig. 3.

Table 5. Callus with embryogenic structures formed from different starting tissues originally obtained from rachilla explants from inflorescences of two MRD × TAGT hybrid coconut palms

Medium	MRD × TAGT 1				MRD × TAGT 2			
	Inflorescence characteristics			Explants forming embryogenic callus (%)	Inflorescence characteristics			Explants forming embryogenic callus (%)
	Position	Size (cm)	Stage		Position	Size (cm)	Stage	
1	−6	39.4	I	0.0a	−7	38.1	I	0.9a
2				0.9a				0.0a
3				0.0a				0.9a
C.V.				49.5				68.4
1	−7	21.9	II	4.6a	−8	20.0	II	5.5a
2				4.6a				7.7a
3				4.6a				6.6a
C.V.				134.6				141.4
1	−8	10.3	III	21.1b	−9	9.7	III	35.5a
2				37.7a				25.5ab
3				44.4a				20.0b
C.V.				102.7				116.0
1	−9	5.9	IV	26.6ab	−10	5.9	IV	13.3a
2				13.3b				15.0a
3				33.3a				13.3a
C.V.				119.5				139.8

Tissues were cultured for 90 d on three media, each prepared with medium I formulation and supplemented with (1) 0.65 mM 2,4-dichlorophenoxyacetic acid (2,4-D), (2) 0.65 mM 2,4-D + 0.01 mM 6-benzylaminopurine (BAP), or (3) 0.65 mM 2,4-D + 0.02 mM BAP. The data shown are averages of values obtained from 60 to 105 repetitions. Values followed by the same *letter* within the same *column* are not significantly different according to Tukey's test ($P \leq 0.05$). Significance and coefficient of variation (C.V.) were determined using a $(\sqrt{x}+1)$ transformation

MRD × *TAGT* Malayan Red Dwarf × Tagnanan Tall

Table 6. Embryogenic callus formation from coconut rachilla explants from inflorescences at different developmental stages of the coconut hybrid MRD × TAGT

Variation source		Explants forming embryogenic callus (%)	C.V.
Culture medium	1	12.3a	139.4
	2	12.5a	
	3	14.2a	
Inflorescence developmental stage[z]	I	0.48d	130.2
	II	5.65c	
	III	30.74a	
	IV	19.28b	

Tissues were cultured for 90 d on three media, each prepared with medium I formulation and supplemented with (1) 0.65 mM 2,4-dichlorophenoxyacetic acid (2,4-D), (2) 0.65 mM 2,4-D + 0.01 mM 6-benzylaminopurine (BAP), or (3) 0.65 mM 2,4-D + 0.02 mM BAP. The data shown are averages of values from 60 to 105 repetitions. Values followed by the same *letter* within the same *column* are not significantly different according to Tukey's post hoc test ($P \leq 0.05$). Significance and coefficient of variation (C.V.) were determined using a $(\sqrt{x}+1)$ transformation

MRD × *TAGT* Malayan Red Dwarf × Tagnanan Tall

[z] Stage as defined in Table 5

Following the same procedures as for the previous three palms, ten more palms were sampled: another five MRD × TAGT and five MRD × VTT hybrid palms (batch three). In these cases, sampling was also destructive, and inflorescences were selected according to size rather than position. Rachilla explants, about 1500 for each palm, are currently in the culture, and calluses showing the occurrence of embryogenic structures are already forming.

Histological analysis of rachillae used for the study

Histological analyses of rachillae at different stages of development of two hybrids are presented in Fig. 7. Transverse sections of development stage −4 to −6 of inflorescence of MYD × MXPT palm (Fig. 7*A*–*C*) showed a tissue that consisted of floral meristems covered by bracts. Figure 7*C* is representative of the immature stage (−6) that presented the best response for this palm. The transverse sections of rachillae from immature inflorescences of stages −8, −9, and −10 of MRD × TAGT palm 2 are presented in Fig. 7*D*, *E*, *F*, respectively. Figure 7*E* is representative of the immature stage (−9) that presented the best response for this palm. A closer view of the floral meristem (Fig. 7*G*, *H*) showed the meristematic dome formed by a group of meristematic cells with visible nuclei of irregular shape and with nucleoli not clearly defined. The cells also revealed densely stained cytoplasm, indicative of high metabolic activity. Figure 7*I* shows a part

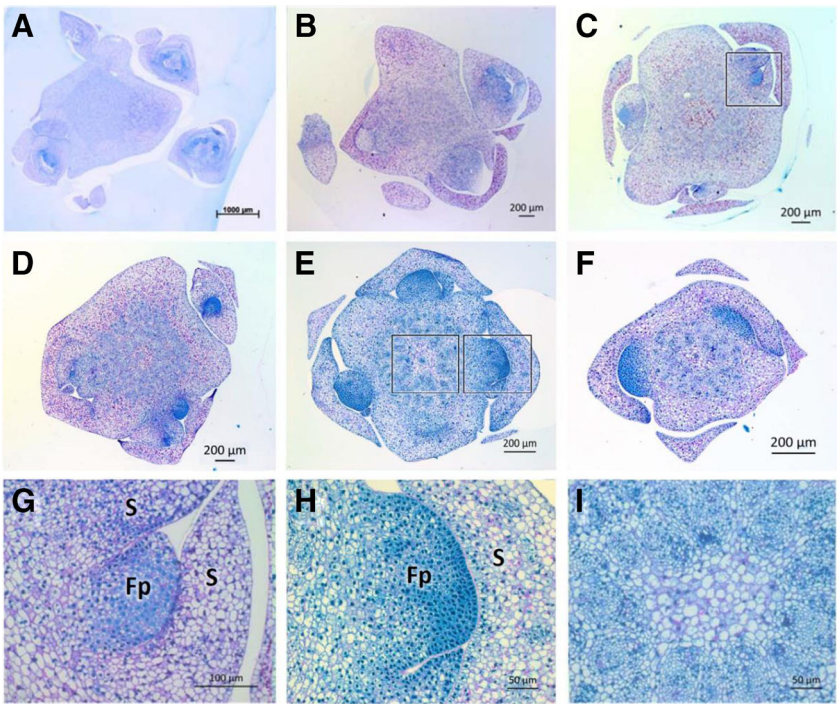

Fig. 7. Histological sections of rachilla explants obtained from immature inflorescences of different developmental stages, excised from two coconut hybrids. *A*–*C* Malayan Yellow Dwarf × Mexican Pacific Tall (MYD × MXPT) hybrid, developmental stages −4 (*A*), −5 (*B*), and −6 (*C*). *D*–*F* Malayan Red Dwarf × Tagnanan Tall (MRD × TAGT) hybrid, developmental stages −8 (*D*), −9 (*E*), and −10 (*F*). *G*, *H* Magnification (×40) of floral meristems corresponding to *box* in (*C*) and *box at right* in (*E*), respectively. *I* Magnification (×40) of center of rachilla corresponding to *center box* in (*E*). *Fp* floral primordia, *S* sepal.

of Fig. 7*E* highlighting the vascular strands at the center of the rachilla transverse section.

Discussion

In order to satisfy the growing demand for elite coconut plants, a process is needed for mass propagation such as the one for plumule explants reported by Pérez-Núñez *et al.* (2006) based on the production of embryogenic calluses and their multiplication but that instead uses explant tissues from adult palms that are already bearing fruit and have known measured performance. Thus, the purpose of the present study was to determine whether rachilla explants from immature inflorescences could be used to obtain embryogenic calluses, could be multiplied, and could form somatic embryos that can convert into plantlets.

Hybrid coconut palms were chosen as the source of explants because there is interest in Mexico in hybrid palms, in particular the MRD × TAGT and MRD × VTT hybrids. These hybrids were introduced into Mexico for testing over 10 years ago, and they are very valuable since they were found resistant to LY in a trial in Tabasco, Mexico (Castillo *et al.* 2012), and only a limited number of these palms exist in Mexican territory. From previous attempts to collect immature inflorescences (unpublished data), it was found that the process could be destructive for the palms, and considering the scarcity of the high-value hybrids discussed above, the initial tests described here were carried out with explants from a more readily available hybrid, MYD × MXPT, which is currently produced in Mexico.

In the first trial, the efficacy of 25 different treatments (different combinations of 2,4-D and BAP concentrations) to induce the formation of callus with embryogenic structures (as defined by Pérez-Núñez *et al.* 2006; Sáenz *et al.* 2006) was evaluated on explants from immature inflorescences of this hybrid at three developmental stages. After 90 d of culture, calluses could be obtained consisting of spongy or necrotic tissue with one or two pearly white structures. These calluses were similar to those reported by Verdeil *et al.* (1994) and Hornung and Verdeil (1999). Histological analysis of these pearly white structures revealed the presence of peripheral meristematic tissue, which has previously been associated with callus growth in explants of different species, for example, coconut inflorescences (Verdeil *et al.* 1994, 2001), *Elaeis guineensis* zygotic embryos (Schwendiman *et al.* 1990), and *Coffea canephora* leaves (Berthouly and Michaux-Ferriere 1996).

Considering all of the explants evaluated, the response percentage of callus formation was below 20%, not reaching the yields obtained from plumule explants, which had responses of 40% or higher (Pérez-Núñez *et al.* 2006). Additionally, although the calluses obtained had pearly white structures, these were scarce and associated with spongy or necrotic tissue, contrasting with the calluses covered with white pearly embryogenic structures reported for plumule (Pérez-Núñez *et al.* 2006; Sáenz *et al.* 2006). These initial results could be considered disappointing; however, the obtained pearly white structures resembling embryogenic tissue could be responsive explants once isolated from the rest of the callus tissues, as reported by Pérez-Núñez *et al.* (2006) for plumule-derived tissues. In the present study, calluses were obtained from the isolated pearly white structures that, with successive subcultures, increased in the percentage of calluses forming pearly white structures, without spongy or necrotic tissues and with tissues resembling fully embryogenic callus (as defined by Pérez-Núñez *et al.* 2006).

These apparently embryogenic calluses were subcultured on medium III for induction of SE formation. The SE developed from globular to torpedo-shaped structures that were able to germinate and convert into plantlets, thus showing the embryogenic nature of the calluses obtained from rachilla explants. Therefore, these calluses were considered equivalent to those obtained from plumule explants, which had already been demonstrated to be embryogenic (Sáenz *et al.* 2006).

Somatic embryos were also tested as explants on medium II, and formation of fully embryogenic calluses could be achieved as well. Furthermore, when embryogenic structures from these calluses were used as explants and cultured on medium II, fully embryogenic callus formed again.

This phenomenon, referred to as secondary somatic embryogenesis, has been reported previously for different species (Vasic *et al.* 2001; Maximova *et al.* 2002), including coconut (Pérez-Núñez *et al.* 2006). Secondary somatic embryogenesis has also been reported to help retain embryogenic competence during prolonged culture times, for as long as 10 yr in different species (Baker and Wetzstein 1995; Schavemaker and Jacobsen 1995; Martinelli *et al.* 2001).

Developmentally, the formation of embryogenic callus, either from embryogenic structures or somatic embryo explants, was very similar to the process described previously in which embryogenic callus formed from plumular explants (Sáenz *et al.* 2006). During the first month, a callus formed that started showing ear-shaped structures (referred to as translucent structures by Sáenz *et al.* 2006). During the second month, these translucent structures increased in number and histologically, they showed the occurrence of peripheral meristematic tissue. During the following days of culture, globular structures appeared on the surface of the translucent structures. Later, elongated structures were visible, and at day 90, the translucent structures were covered partially by globular structures but mostly by elongated structures. Histologically, these structures also showed the presence of peripheral meristematic tissue. According to the morphological development and histological observations, both globular and elongated structures can be considered as embryogenic structures, as

described by Sáenz *et al.* (2006). At the end of this 90-d period, the obtained callus appeared as fully embryogenic callus, which developed SE on the surface of the embryogenic structures when the callus was transferred to medium III.

Taken together, these findings show that the process of obtaining plantlets derived from rachilla explants is developmentally and functionally comparable to that reported for plumular explants (Pérez-Núñez *et al.* 2006; Sáenz *et al.* 2006), as both involve the formation of embryogenic callus, SE, conversion of these to plantlets, and, most importantly, the fact that in both cases, embryogenic callus can be multiplied consistently by using embryogenic structures or SE as explants.

This new knowledge was then applied to generate embryogenic calluses from rachilla explants obtained from two palms of the more valuable MRD × TAGT hybrid, and the results were positive, obtaining calluses with a few pearly white structures that after isolation and subsequent subcultures of these structures, fully embryogenic calluses were obtained as in the case of the first hybrid palm (MYD × MXPT).

Next, the number of fruit-bearing palms tested was extended with five more MRD × TAGT hybrid palms and five MRD × VTT hybrid palms. Once again, calluses with pearly white structures were formed from the explants from these palms. These structures were isolated and cultured new calluses are currently under development.

It is interesting that, for these two palms as well as for the MYD × MXPT palm, the younger inflorescences were more responsive than the more mature explant sources. This has also been observed for other species such as *Triticum aestivum* (Benkirane *et al.* 2000) and *Euterpe edulis* (Guerra and Handro 1998) and palms *Areca catechu* (Karun *et al.* 2004) and *Bactris gasipaes* (Steinmacher *et al.* 2007). In the present study, it was also noticed that most of the explants responding with callus formation were from inflorescences about 10 cm long, irrespectively of their relative position in the palm but with similar morphological and histological development. This suggests that size, together with morphological development, is probably a more convenient reference for sampling than inflorescence position in the plant, the usual parameter considered. Accordingly, for the last batches of palms sampled, size (about 10 cm long) was used as a reference to select the inflorescence for obtaining explants, and the results support this decision.

Conclusions

The results presented here show that fully embryogenic callus can be obtained from rachilla explants, through a stepwise process of subculturing the white pearly structures, scarce at the beginning and becoming more abundant in the new calluses formed, to finally obtain fully embryogenic calluses. The results also indicate that embryos can be obtained from these calluses, that embryos themselves can be used as explants to produce new embryogenic callus, and that this sequential process can be carried out repeatedly and reproducibly. Furthermore, embryos obtained through this process were able to germinate and convert to plantlets.

These results could be the basis for further studies to develop a protocol for massive propagation of coconut using rachilla explants from immature inflorescences therefore allowing the cloning of fruit-bearing palms of known traits and measured performance. As shown, the present protocol is already being used to produce and multiply embryogenic calluses from the very valuable MRD × TAGT and MRD × VTT hybrid palms.

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