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Unfertilized ovary: a novel explant for coconut (*Cocos nucifera* L.) somatic embryogenesis

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Abstract Unfertilized ovaries isolated from immature female flowers of coconut (*Cocos nucifera* L.) were tested as a source of explants for callogenesis and somatic embryogenesis. The correct developmental stage of ovary explants and suitable *in vitro* culture conditions for consistent callus production were identified. The concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) and activated charcoal was found to be critical for callogenesis. When cultured in a medium containing 100 μM 2,4-D and 0.1% activated charcoal, ovary explants gave rise to 41% callusing. Embryogenic calli were sub-cultured into somatic embryogenesis induction medium containing 5 μM abscisic acid, followed by plant regeneration medium (with 5 μM 6-benzylaminopurine). Many of the somatic embryos formed were complete with shoot and root poles and upon germination they gave rise to normal shoots. However, some abnormal developments were also observed. Flow cytometric analysis revealed that all the calli tested were diploid. Through histological studies, it was possible to study the sequence of the events that take place during somatic embryogenesis including orientation, polarization and elongation of the embryos.

Keywords Somatic embryogenesis · Coconut (*Cocos nucifera* L.) · Ovary culture · Histology · Flow cytometry

Abbreviations ABA: Abscisic acid · 2,4-D: 2,4-dichlorophenoxyacetic acid · BAP: 6-Benzylaminopurine · NBB: Naphthol blue black · PAS: Periodic acid Schiff's reaction · CLZ: Cambium-like zone

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Introduction

Coconut (*Cocos nucifera* L.), an out-breeding perennial that is propagated only by seed, exhibits great variation in selected characters. Vegetative propagation of superior palms is a promising possibility for increasing production and homogeneity in coconut lands. Tissue culture remains the only approach to achieve this objective.

Over the past few decades, the problem of cloning coconut has been addressed in a number of research centres worldwide. However, the success has been very limited. This is due to very poor response of coconut tissues to in vitro conditions and thus it is classified as one of the most recalcitrant species to regenerate in vitro (George and Sherrington 1984).

A range of tissues including shoot tip (Weerakoon 2004), roots (Justin 1978), immature inflorescence (Branton and Blake 1983; Verdeil et al. 1994) and tender leaf (Pannetier

and Buffard-Morel 1982; Buffard-Morel et al. 1988) have been used for in vitro culture and different procedures have been adopted. Out of all the explants, the most extensively studied are the immature inflorescence and leaf. A small number of clonal plants have been regenerated from these two explants, through a process of somatic embryogenesis that appears to be the most promising technique for cloning coconut. The time taken for callus initiation in these explants had varied from 4 to 12 months. According to our own experience, defining the most suitable developmental stage for immature inflorescence culture is very difficult and a considerable damage is inflicted to the palm during collection of explants. Hornung (1995b) indicated that the callusing frequency in immature inflorescence explants is also low (usually less than 30%) and the results are not consistent. In the case of leaf explants, low frequency of callusing (about 20%) was obtained from leaves collected from mature palms (Pannetier and Buffard-Morel 1982) and collection of explants also causes damage to the palm.

Due to the poor response of somatic tissues, zygotic tissues such as immature embryo (Karunaratne and Periyapperuma 1989; Fernando and Gamage 2000) and plumule (the shoot apex excised from zygotic embryo) (Hornung 1995a; Chan et al. 1998; Fernando et al. 2003) have also been tested for in vitro plant regeneration. The response of these tissues has shown to be better than that of vegetative tissues, in terms of callus formation and embryogenic capacity (Hornung 1995a; Chan et al. 1998). However, a major disadvantage in using these explants is that they can only produce clones of palms with unknown performance, because of cross-pollination in coconut.

In order to achieve successful callus production in coconut explants, it is also critical to define the most suitable combination of 2,4-D and activated charcoal, two of the essential components in the callus induction medium. The beneficial effects of activated charcoal are attributed to its adsorption of phenols and other growth inhibitory substances. However, it also adsorbs 2,4-D present in the culture medium, leading to undefined conditions with regard to available 2,4-D concentration in the medium (Ebert and Taylor 1990; Verdeil and Buffard-Morel 1995). This in turn could lead to variable tissue performance and non-reproducible results.

Considering the constraints and limitations experienced with different explants that have already been tested, it was pertinent to look for other suitable explants for in vitro culture with the view of developing a reliable in vitro plant regeneration protocol for coconut. Thus, in this study, the suitability of using unfertilized coconut ovaries (excised from immature female flowers) was tested as a source of explants for coconut somatic embryogenesis. Plant regeneration has been achieved via ovary culture of several crops such as onion (*Allium cepa* L.) (Bohanec et al. 1995; Luthar and Bohanec 1999), sweet potato (*Ipomoea battatas*) (Ruth et al. 1993), lily (*Lilium* crosses) (Van Tuyl et al. 1991), *Tulipa generiana* (Van Creij et al. 2000), *Zea mays* (Tang et al. 2006) and sugar beet (*Beta vulgaris* L.) (Gurel et al. 2000). Even though the main objective of above studies was to obtain haploid plants, regeneration of diploid plants was also observed, indicating the potential use of ovary explants for somatic embryogenesis. The work on non-fertilized female gametophyte culture has been reviewed by Mukhambetzhanov (1997). In vitro culture of unfertilized ovaries of coconut has been reported only once (Griffis and Litz 1997) and except some callus and adventitious root formation, no incidence of somatic embryogenesis has been observed.

This paper reports for the first time successful callogenesis and somatic embryogenesis in unfertilized ovary explants of coconut. Histological studies were undertaken to determine the origin of callus and developmental pathway of the embryogenic structures formed. Since there is a possibility of obtaining haploids with the use of ovary as an explant, flow cytometric analysis was also undertaken to test the ploidy level of callus.

Materials and methods

Experimental material for callus induction

Unfertilized ovaries excised from immature female flowers of adult coconut palms (cultivar Sri Lanka Tall) were used as the explants. For initial studies (data not shown), ovaries obtained from immature inflorescences of −1 to −6 maturity stages were used (the developmental stage of an immature inflorescence was determined by its position within the sequence on the palm. Thus, the most recently opened inflorescence is referred to as 0 stage, while −1 is the next inflorescence to open. In the case of −6, it would open approximately 6 months later. Thus out of the six maturity stages selected, −1 is the most mature stage whereas −6 is the most immature stage). Initial studies (data not shown) indicated that ovaries obtained from inflorescences of −4, −5 and −6 stages (the average lengths being 48, 32 and 15 cm, respectively) responded better to in vitro culture conditions when compared to other maturity stages tested. Thus these three maturity stages were selected for this study. The female flowers (attached to the basal part of each rachilla) were collected from the inflorescence and disinfected with 2% calcium hypochlorite for 12 min followed by four rinses with sterile water. Then the excision of ovaries from female flowers was carried out under a laminar flow cabinet. The sepals and petals in each female flower were removed and the ovary (about 2 mm in size) was dissected out and cultured in vials containing 10 ml of callus induction medium.

Culture conditions

Duplicate experiments designed in three-factor factorial were undertaken to identify the best combination of 2,4-D and activated charcoal (BDH-acid washed) for callusing, in ovaries of −4, −5 and −6 maturity stages. In each experiment, 10 replicate ovaries were used for each treatment. Three different concentrations of 2,4-D (50, 100 and 200 μM) in combination with three levels of activated charcoal (0.1, 0.25 and 0.3%) were tested. Medium 72 (formulated by Karunaratne and Periyapperuma 1989) was used as the basal medium and pH of all media was adjusted to 5.8 before solidifying with 0.8% (w/v) agar. Sucrose was added to the medium at a concentration of 4%. Activated charcoal was added to the medium just before dispensing it in to vials.

The cultures were maintained in dark at 28°C for 3 months without sub-culturing. The percentage of callus production in each treatment was recorded after 10 weeks of culture initiation. Based on the results, the best treatment (i.e. the best combination of 2,4-D, activated charcoal and the maturity stage of ovary) that gave rise to the highest callusing frequency was selected and it was repeated 10 times to check for consistency. The calli were sub-cultured into somatic embryo-induction medium (medium 72 containing 5 μM ABA and 10 μM $AgNO_3$) for 5 weeks followed by somatic embryo maturation medium (medium 72 without any hormones) for 4 weeks. The embryogenic structures obtained were then transferred and maintained in germination medium (modified Eeuwens Y_3 medium) as described by Fernando and Gamage (2000).

Statistical analysis

The percentage callus production of ovaries was calculated from 10 replicates per treatment. The experiment was independently replicated twice. The data were analysed using SAS statistical package (SAS Institute 1999). One-way ANOVA was conducted on the data transformed by arcsine of the square root of the percentage, followed by least significant difference (LSD) test to select the best treatment. Data reported were transformed back to the original scale according to Compton (1994).

Histological analysis

Calli and embryogenic structures (at different developmental stages) formed in these media were sampled for histological analysis. The samples were fixed in FAA (50% ethanol – 10% formaldehyde – glacial acetic acid, 18:1:1) for 72 h. Dehydration was carried out through a graded alcohol series (50–100% ethanol and butanol). After impregnation, samples were embedded in resin, Technovit 7100® (Heraeus Kluzer GmbH, Germany) and allowed to polymerise overnight at room temperature. Sections (3.5 μm thick) were obtained using a microtome (Historange, LKB). Finally, the sections were double stained with periodic acid Schiff's reagent (PAS) and protein-specific naphthol blue black (NBB) (Fisher 1968) as described by Buffard-Morel et al. (1992). The prepared slides were observed under the light microscope (Leitz DMR microscope, Germany).

Flow cytometric analysis

Well-developed calli obtained after 3 months of culture initiation were used for ploidy analysis. Extraction of nuclei and the analysis were done according to the protocol developed for coconut by Sandoval et al. (2003). The ploidy level was determined using a FACScan cytometer (Becton-Dickinson, USA) with an argon laser (15 mV) at 488 nm encompassing an emission range of greater than 590 nm. Calli (approximately 500 mg) were chopped in 2 ml of extraction buffer (Dolezel et al. 1989) containing 3% triton to release the intact nuclei. The suspension containing the nuclei was then filtered through a filter cloth (36 μm mesh size) to eliminate cell debris. The cell nuclei were then stained with propidium iodide (P4170, Sigma) an intercalating fluorochrome, by incorporating 100 μl of a propidium iodide stock solution (at 1 mg ml^{-1}) in 300 μl of filtered nucleus suspension. The solution was left to incubate for 5 min prior to analysis by flow cytometer. Eight callus samples were analysed. Each sample was measured in two replications, using leaves of embryo-cultured Sri Lanka Tall coconut palms as the external standard.

Results and discussion

In vitro culture conditions

Consistent production of callus at a high frequency is important to achieve successful somatic embryogenesis. The explant maturity is known to be crucial for callogenesis and thus it is very important to determine the most suitable developmental stage of explant for in vitro culture.

The results of this study clearly indicated the difference in response of ovaries when cultured in different combinations of 2,4-D and activated charcoal (Table 1).

The F statistic ($df = 26$, $p<0.01$) indicated that there is a significant difference in percentage callus production among different treatments (Table 1). The percentage callus production in ovaries of −4 maturity stage with 100 μM 2,4-D and 0.1% activated charcoal was significantly higher (30%) than all the other treatments tested ($p<0.05$) indicating it to be the best treatment. When this treatment was repeated 10 times, consistent callogenesis was observed and the mean percentage of callus production was 41. The results also revealed that

Table 1 The effect of different combinations of 2,4-D concentrations and activated charcoal on percentage callus production in coconut ovaries at different stages of maturity

Maturity stage of ovary[a]	50[b]			100[b]			200[b]		
	0.1[c]	0.25[c]	0.3[c]	0.1[c]	0.25[c]	0.3[c]	0.1[c]	0.25[c]	0.3[c]
−4	0	0	0	30[d]	0	0	0	5	10
−5	0	0	0	5	0	0	0	0	10
−6	5	0	0	5	0	0	0	0	0

One-way ANOVA is significant with df = 26 at 0.01 level.

[a]Maturity of ovaries decrease from −4 to −6 stage (i.e. the most immature stage is −6).

[b]2,4-D concentration (μM).

[c]Activated charcoal (%).

[d]Callusing percentage is significantly different from all other treatments according to LSD at 0.05 level.

callusing in unfertilized ovary was more rapid when compared to immature inflorescence explants that could take as long as 8 months for callogenesis (Verdeil et al. 1994).

The well-developed calli consisted of translucent mass of globules in off white colour (Fig. 1a). The calli, sub-cultured on medium containing ABA, gave rise to somatic embryos (Fig. 1b) at a frequency of 50%. This is comparable to the results obtained by Fernando et al. (2003) where coconut plumule has been used as the explant. When transferred to the germination medium, 10% of the somatic embryos gave rise to shoots (Fig. 1c) and a regenerated plantlet is shown in Fig. 1d. The morphological characteristics of these embryogenic structures were found to be similar to those obtained from other coconut explants such as plumule and immature inflorescence (Verdeil et al. 1994; Chan et al. 1998).

Histological analysis

Histological studies showed that at the time of excision, the female flowers at −4 maturity stage contained active

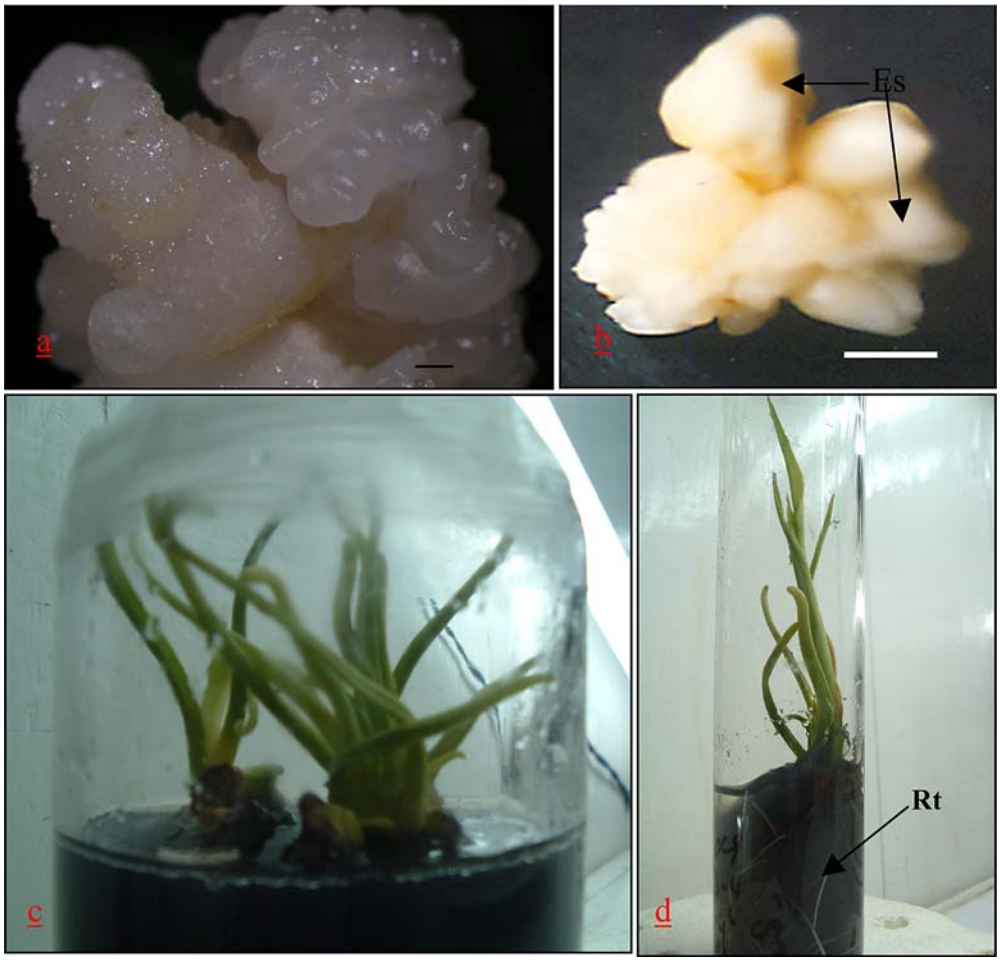

Fig. 1 Morphological aspects of somatic embryogenesis in ovary culture of *Cocos nucifera* L. **a** Callus derived from ovary explants after 10 weeks of culture initiation (bar = 4 mm). **b** Embryogenic structures (Es) developed from calli after sub-culturing into somatic embryo induction medium (bar = 5 mm). **c** Developing shoots in germination medium. **d** A complete plantlet with roots (Rt)

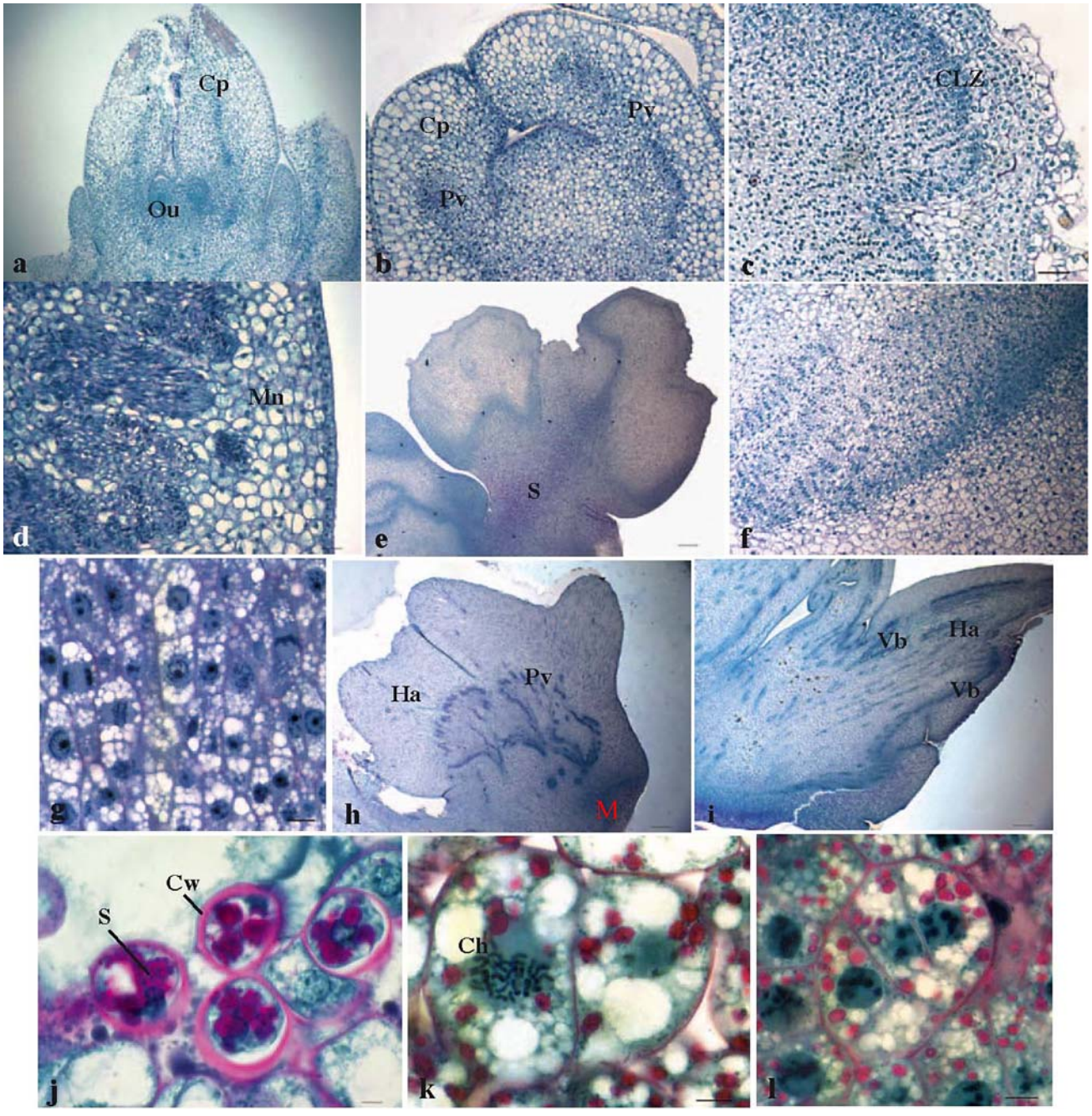

Fig. 2 Histological sections of somatic embryogenesis in ovary explants of *Cocos nucifera* L. **a** Explanted ovary at –4 stage of maturity. Note the differentiating ovule (Ou) at the base of the carpel (Cp) (bar = 59 μm). **b** Ovary 1 week after inoculation. Note the formation of new meristematic cells originated from provascular cells (Pv) of the carpel (bar = 59 μm). **c** Formation of cambium-like zone (CLZ) (bar = 29.5 μm). **d** Fragmentation of the CLZ. Note the formation of meristematic nodules (Mn) (bar = 30 μm). **e** Bipolar orientation of somatic embryo. Note the accumulation of starch (S) at proximal end (bar = 240 μm). **f** Differentiation of the cells in the CLZ into the meristematic area (bar = 59 μm). **g** The cells in the meristematic area with high mitotic index (bar = 29.5 μm). **h** Polarisation of the embryo forming the haustorial tissue (Ha) and the M zone (bar = 240 μm). Note the formation of provascular strands (Pv) in the haustorial tissue (bar = 240 μm). **i** Elongation of the polarised embryo. Note the differentiated vascular bundle (Vb) (bar = 240 μm). **j** Embryogenic cells. Note the thick cell wall (Cw) and starch reserves (S) (bar = 6 μm). **k** Pro-embryo at two-celled stage. Note the active nucleus with chromosomes (Ch) in metaphase (bar = 39 μm). **l** Pro-embryo at four-celled stage (bar = 39 μm)

meristematic cells that give rise to the ovule (Fig. 2a). The initial stage of callogenesis in ovary explants was indicated by the formation of new meristematic cells originated from provascular cells of the carpel (Fig. 2b). Further cell division towards the epidermis of the carpel resulted in the formation of a highly meristematic region, referred to as the cambium-like zone (CLZ) (Fig. 2c). Periclinal cell division of the peripheral cells of the CLZ and the anticlinal cell division of the inner parenchyma cells ensured the growth of the callus. Similar callus formation from vascular bundles has been reported in plumule (Chan et al. 1998; Fernando et al. 2003), immature inflorescence (Verdeil et al. 1994) and leaf (Buffard-Morel et al. 1992) explants of coconut.

Histological analysis also revealed that somatic embryos from ovary explants could be formed either by a unicellular or a multicellular pathway. In the multicellular pathway, the first stage of development of embryogenic nodules was indicated by the fragmentation of the CLZ (Fig. 2d) and these nodular structures developed further and formed proembryos of multicellular origin. These proembryos later gave rise to fully developed somatic embryos. The first sign of bipolar orientation of the somatic embryo was indicated by the accumulation of starch reserves in the cells at the base of the globular embryo (that give rise to the root pole later) and the presence of highly meristematic cells that give rise to the shoot meristem (Fig. 2e and f). A prominent nucleus (with distinct nucleolus) in the middle of the cell was a characteristic feature of these meristematic cells. Cells at different stages of division could be observed in this meristematically active area (Fig. 2g). In the polarised somatic embryo, the haustorium is located at the distal end whereas the meristematic zone (M zone) and the shoot meristem could be seen at the proximal end (Fig. 2h). With elongation of the bipolar somatic embryo, the provascular strands in the haustorial tissues were differentiated further (Fig. 2i). A similar multicellular developmental pathway has already been described for somatic embryos obtained from immature inflorescence (Verdeil et al. 1994; Verdeil and Buffard-Morel 1995) and plumule (Chan et al. 1998; Fernando et al. 2003) explants of coconut.

In the unicellular pathway, some highly embryogenic cells (Fig. 2j) present in the protoderm (the periphery of the CLZ) gave rise to proembryos. These embryogenic cells have a very high mitotic activity and they are characterized by the presence of high starch and protein reserves, high nucleus to cytoplasm ratio, mitotically active nucleus (with a prominent nucleolus) in the middle of the cell. The presence of starch reserves in embryogenic cells has been reported previously in coconut (Verdeil and Buffard-Morel 1995) and oil palm (Schwendiman et al. 1988) tissue cultures. The single embryogenic cells were clearly separated from non-embryogenic cells by a thick cell wall and production of each proembryo was a result of mitotic divisions within an isolated, single embryogenic cell. Proembryos at two-celled (Fig. 2k) and four-celled (Fig. 2l) stages could be observed. However, further development of these proembryos was not observed. Similar observations on a unicellular origin of somatic embryos have been made in immature inflorescence explants of coconut (Verdeil et al. 1994, 2001).

After sub-culturing the somatic embryos (originated from multicellular pathway) into the germination medium, further differentiation of shoot meristem and haustorial tissues occurred. A well-developed haustorium is characterized by the presence of stomata in the epidermis, parenchyma cells with very low nucleus to cytoplasm ratio, prominent intercellular spaces, presence of many vascular bundles and calcium oxalate crystals (idioblasts) in some cells (data not shown). Initially, the shoot meristem consisted of a mass of meristematic cells which later differentiated into the meristematic dome, spear leaves, pith and provascular bundles (data not shown). This developmental pattern of the somatic embryo was found to be comparable to that of zygotic embryo (Verdeil and Buffard-Morel 1995). The formation of incomplete somatic embryos that lack shoot poles has been accounted for the low plant-regeneration frequency (4.8%) observed in plumule culture of coconut (Fernando et al. 2003).

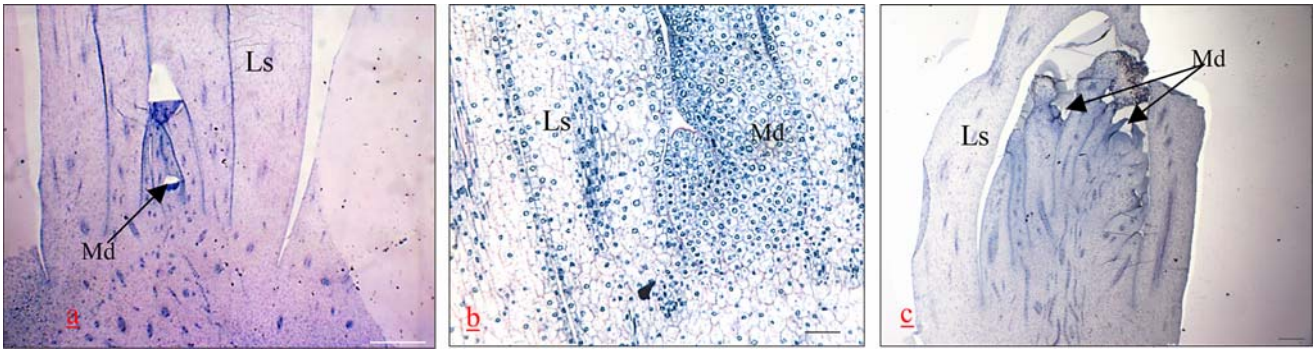

Fig. 3 Histological sections of somatic embryo germination and shoot development in ovary culture of *Cocos nucifera* L. **a** Normal developing shoot; the meristematic dome is covered by several leaf sheaths (Ls) (bar = 120 μm). **b** Close view of a normal developing shoot (bar = 29.5 μm). **c** Multiple shoot consisting of several meristematic domes (bar = 240 μm)

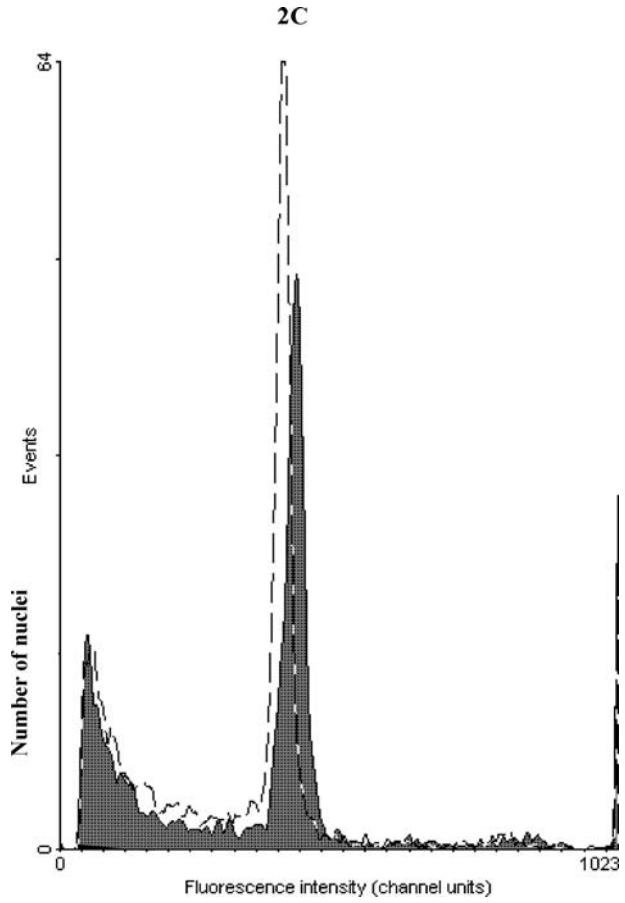

Fig. 4 Histogram (in *grey*) of fluorescence intensity of nuclei isolated from calli (derived from coconut ovary) after staining with propidium iodide. The fluorescence pulse was connected on 1024-channel analyzer. Results represent measurements of about 5,000 individual nuclei. Mature leaves (*dashed line*) were used as diploid control. Coefficient of variation (CV) was 4.44%. C: DNA content in arbitrary units. CV: Coefficient of variation

In contrast, most of the ovary-derived somatic embryos that were analyzed were complete with shoot and root poles. However, it was not possible to obtain histological sections with the root and the shoot pole in the same plane due to the large size of the embryo. Many of the shoots analyzed were normal with the meristematic dome covered by several leaf sheaths (Fig. 3a and b). However, some fused shoots and multiple shoots (Fig. 3c) were also observed.

Recently, Perez-Nunez et al. (2006) reported a highly efficient system of plant regeneration via somatic embryogenesis in plumule explants of coconut in which 100,000 somatic embryos could be produced from a single plumule explant. This has been achieved by multiplication of embryogenic callus and secondary embryogenesis. However, the use of plumules from zygotic embryos obtained by cross-pollination will preclude the application of this protocol for cloning of palms with known agronomic traits (Perez-Nunez et al. 2006). If the above approach can be applied to unfertilized ovary explants, it will be possible to develop an efficient protocol to clone palms with known performance. Thus, future research will be directed towards inducing callus multiplication and secondary embryogenesis in unfertilized ovary explants with a view to develop a micropropagation protocol for mass propagation of coconut, a growing need of the industry.

Flow cytometric analysis

Flow cytometric analysis revealed that all the tested calli were diploid ($2n = 2x = 32$) (Fig. 4) indicating that the origin of the calli is from diploid tissues. This is further supported by histological evidence where callus was shown to originate from the carpel of the ovary. It was important to undertake flow cytometric analysis since there is a possibility of obtaining haploids with the use of ovary as an explant. Furthermore, mutations which could occur during the process of plant regeneration could affect the ploidy level. Thus, the genetic stability of the ovary-derived calli needed to be confirmed by flow cytometry.

Conclusion

This study indicated the feasibility of developing an in vitro plant regeneration protocol via somatic embryogenesis with the use of unfertilized ovary of coconut as the explant. Suitable culture conditions could be developed for consistent callogenesis and complete somatic embryos that gave rise to normal shoots could be obtained from ovary-derived callus. Through histological studies, it was possible to study the sequence of the events that take place during somatic embryogenesis including orientation, polarization and elongation of the embryos.

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