In Vitro Cell. Dev. Biol.—Plant 42:19–25, January–February 2006
DOI: 10.1079/IVP2005728

1054-5476/06 $18.00+0.00

MORPHOLOGICAL AND HISTOLOGICAL CHANGES DURING SOMATIC EMBRYO FORMATION FROM COCONUT PLUMULE EXPLANTS

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(Received 1 April 2005; accepted 24 October 2005; editor A. Pretová)

Summary

Studies on the development of protocols for the clonal propagation, through somatic embryogenesis, of coconut have been reported for the past three decades, mostly using inflorescence explants, but with low reproducibility and efficiency. Recent improvements in these respects have been achieved using plumular explants. Here, we report a developmental study of embryogenesis in plumule explants using histological techniques in order to extend our understanding of this process. Coconut plumule explants consisted of the shoot meristem including leaf primordia. At day 15 of culture, the explants did not show any apparent growth; however, a transverse section showed noticeable growth of the plumular leaves forming a ring around the inner leaves and the shoot meristem, which did not show any apparent growth. At day 30, the shoot meristem started to grow and the plumular leaves continued growing. At day 45, the explants were still compact and white in color, but showed partial dedifferentiation and meristematic cell proliferation leading to the development of callus structures with a translucent appearance. After 60 d, these meristematic cells evolved into nodular structures. At day 75, the nodular structures became pearly globular structures on the surface of translucent structures, from which somatic embryos eventually formed and presented well-developed root and caulinar meristems. These results allow better insights and an integrated view into the somatic embryogenesis process in coconut plumule explants, which could be helpful for future studies that eventually could lead us to improved control of the process and greater efficiency of somatic embryo and plantlet formation.

Key words: Cocos nucifera; somatic embryogenesis; development.

Introduction

Somatic embryogenesis is the process of formation from somatic cells of structures that follow the histodifferentiation pattern which leads to a body pattern resembling that of zygotic embryos (Emons, 1994), and has been a useful approach for the large-scale micropropagation of plant species, such as coconut, that have no natural vegetative propagation system (Dublin et al., 1991). Somatic embryogenesis studies in coconut have been reported for the past three decades, mostly using inflorescence explants (Eeuwens, 1976; Branton and Blake, 1983; Buffard-Morel et al., 1992; Verdeil et al., 1994). However, the results of such studies have shown low reproducibility and efficiency. On the other hand, recent improvements in these aspects have been achieved using plumular explants, consisting of the shoot meristem surrounded by the leaf primordia as excised from mature zygotic embryos (Hornung, 1995; Chan et al., 1998; Sáenz et al., 1999). Although a plumule-based protocol cannot be used for micropropagation of adult elite genotypes, it is of interest for developmental studies aimed at a better understanding of somatic embryogenesis in coconut. One approach for this is the observation of the development of cells or groups of cells by histological techniques, which has been proven extremely useful in the understanding of embryogenesis in other plant species (Yeung, 1999). In *Hevea brasiliensis*, for example, the adequate time of subculture was determined by histological procedures (Michaux-Ferriere and Carron, 1989). In oil palm, the formation of somatic embryos from perivascular cells of the leaf is well documented (Schwendiman et al., 1990). Alemanno et al. (1997) made a comparison between *Theobroma cacao* L. zygotic embryos and somatic embryogenesis from floral buds, and they found that the somatic embryos lacked starch and protein reserves compared to zygotic embryos.

In the case of coconut, Haccius and Philip (1979) used histological techniques to describe the development of zygotic embryos. In *in vitro* culture of coconut, many observations on the histological changes occurring in explants and derived calluses have been published (Branton and Blake, 1983; Verdeil et al., 1994; Hornung, 1995; Chan et al., 1998). Recently Verdeil et al.,

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(2001) reported on ultrastructural changes occurring during the process of formation of embryogenic cells in immature inflorescences of coconut cultured *in vitro*. They reported that the first changes detected when the calluses were introduced into the embryogenic medium were the closure of the plasmadesmata and callose deposition. The embryogenic cells were characterized by special features: deep invagination of the nuclear envelope, proliferation of dictyosomes, with emission of Golgi vesicles directly related to an increase in cell wall thickness.

In the case of plumules, there are reports dealing with events occurring during the first days of culture (Hornung, 1995) and the formation of embryogenic callus (Chan et al., 1998). However, these types of studies have shown only partial observations of the somatic embryogenesis process. Therefore, this study presents more complete documentation of morphological and histological events taking place in plumule explants cultured *in vitro*, from the initial callus formation to the somatic embryo formation, including the formation of shoots.

Materials and Methods

Plant materials. The fruits were harvested randomly from different 15-yr-old green Malayan dwarf coconut palms at San Crisanto, Yucatán, México. The age of the fruits was 12–14 mo. after pollination and the embryos within this development period were already mature and did not show visible differences. The fruits were cut transversely with a machete, and cylinders of solid endosperm containing the embryos were excised in the field using a cork borer (1.6 cm diameter) and placed in a 0.6% NaClO solution. Once in the laboratory, under aseptic conditions, the cylinders were washed in 70% ethanol for 3 min, rinsed three times with sterile distilled water, washed in a 6% NaClO solution for 20 min, and rinsed again three times with sterile distilled water. The embryos were excised from the endosperm cylinders, washed in a 0.6% NaClO solution for 10 min and rinsed with sterile distilled water three times. Embryos were 5–7 mm long and weighed *c*. 100 mg each. Two batches of 100 embryos each were prepared for the study. The plumules were excised from these embryos under a stereoscopic microscope according to the procedure described by Chan et al. (1998), and placed directly in nutrient medium.

Culture media and conditions. The protocol used involved two culture stages, each with different media and conditions. All chemicals were supplied by Sigma (St Louis, MO, USA). Each explant was cultured in 35 ml culture vessels containing 10 ml of Y3 medium (Eeuwens, 1976), supplemented with gelrite ($3\,g\,l^{-1}$) and charcoal (acid washed, PCCT) ($2.5\,g\,l^{-1}$). Growth-regulator concentrations were 0.65 m*M* 2,4-dichlorophenoxyacetic acid (2,4-D) for medium I (or as indicated in the text), and 6 μ*M* 2,4-D and 300 μ*M* 6-benzylaminopurine (BA) for medium II. The pH of the medium was adjusted to 5.75 before autoclaving for 20 min at 120°C. For stage I, medium I was used, as were conditions I consisting of incubating the cultures in the dark for 3 mo. at 27 ± 2°C without subculturing. For stage II, medium II was used, as were conditions II consisting of photoperiod (16 h illumination, 45–60 $\mu mol\,m^{-2}\,s^{-1}$ PPFD/8 h darkness) at 27 ± 2°C and subculturing every 2 mo.

Histology. The histological procedures were done according to Buffard-Morel et al. (1992), with slight modifications. Tissue samples were fixed in paraformaldehyde 4% in phosphate buffer (pH 7.2) for 24 h under negative pressure. Samples were dehydrated with different aqueous ethanol solutions of increasing ethanol concentration: 30, 50, 70, 80, 90, 95, and 100%, for 1 h each. This was followed by impregnation with JB-4R resin (Polyscience, USA). Three-micrometer sections were prepared from the resin-impregnated tissues with a microtome (HM 325, MICROM), equipped with knife steel blades. The sections were double-stained with periodic acid-Schiff (PAS) reagent, combined with protein-specific naphthol blue-black. PAS stains starch reserves and walls in pink, while naphthol blue-black specifically stains soluble or reserve proteins dark blue (Fisher, 1968).

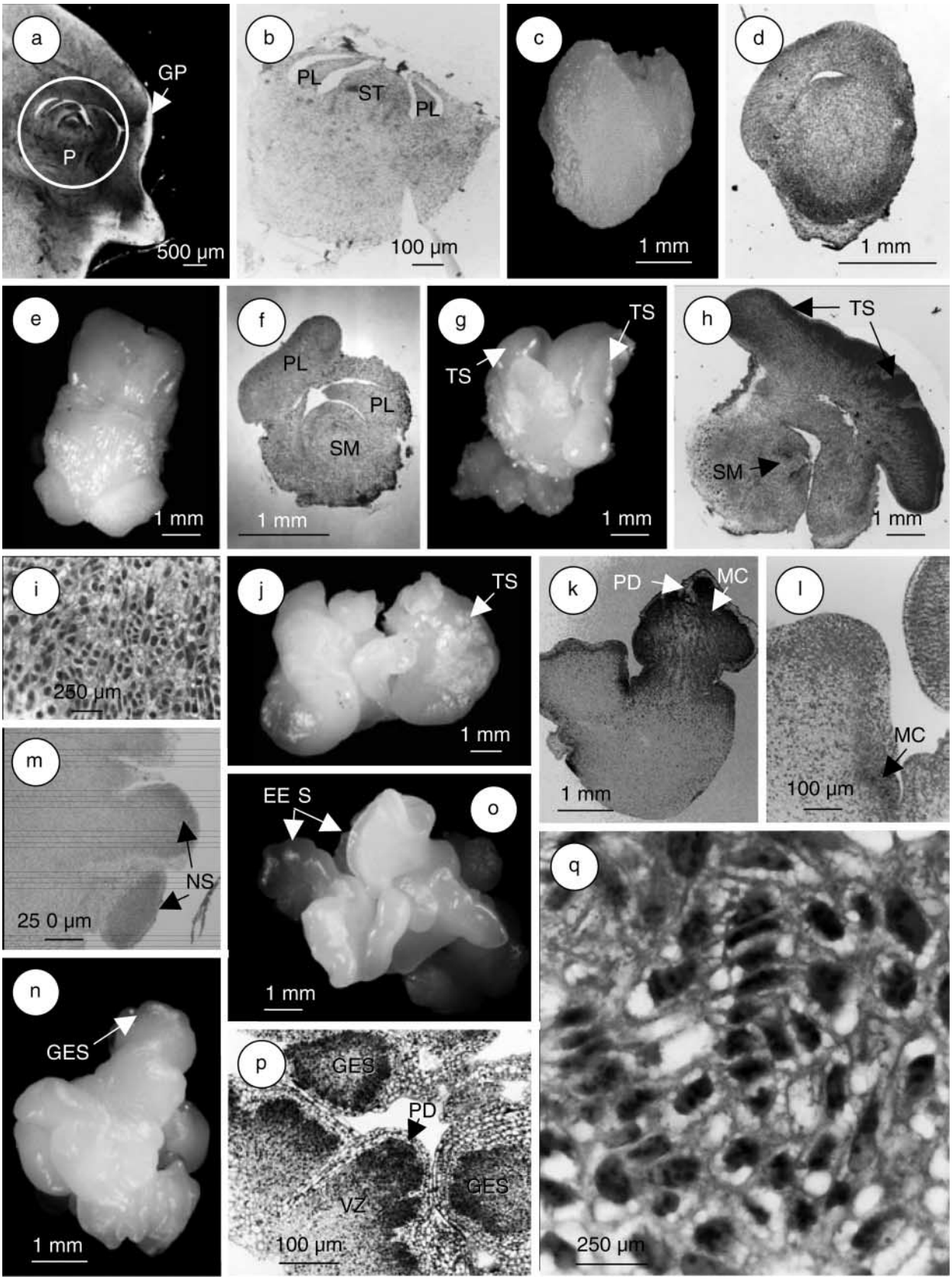
Results

Formation of initial callus. Coconut plumule explants were white in color with a soft consistency and *c*. 1 mm in diameter (Fig. 1*a*), and consisted of the shoot meristem and generally two pairs of plumular leaves, mostly formed by small meristematic cells (Fig. 1*b*). At day 15 of culture, the explant did not show any apparent growth, had a hard consistency, a white color, and a smooth appearance (Fig. 1*c*). However, a transverse section showed noticeable growth of the plumular leaves (particularly the external ones in most cases), which were forming a ring around the inner leaves and the shoot meristem, which did not show any apparent growth (Fig. 1*d*). After 15 d of culture on the callogenesis medium, the explants started growing steadily and forming a callus that will be referred to as initial callus. At day 30 the size of the calluses was *c*. 2–3 mm in diameter, the consistency was hard, and the color was white or beige (Fig. 1*e*). The shoot meristem started to show some growth, and the external plumular leaves continued growing (Fig. 1*f*). At day 45 of culture, the initial calluses showed active cell proliferation leading to the development of structures with a translucent appearance. We will refer to these as 'translucent structures' (Fig. 1*g*).

Initial callus transverse sections showed that the translucent structures formed from the tissues of the external plumular leaves and presented peripheral layers of small cells with meristematic appearance (Fig. 1*h*). That is, the cells had a visible nucleus of irregular shape, with the nucleolus not clearly defined. The cells also revealed densely stained cytoplasm, indicating high metabolic activity (Fig. 1*i*). The shoot meristem structure remained practically unchanged, containing a few meristematic cells.

Formation of embryogenic callus. At day 60 of culture, the calluses were *c*. 4–5 mm diameter. The translucent structures were better defined, showing an ear-like shape (Fig. 1*j*). Transverse sections showed that the body of the callus was a compact structure formed by centers of densely stained small meristematic cells, located on the periphery of the callus, but below a layer of unstained cells forming the protoderm (Fig. 1*k*). At this stage, the meristematic cells started grouping into meristematic nodules (Fig. 1*l*). After 60 d, these meristematic nodules evolved into nodular structures (Fig. 1*m*). At day 75, the nodular structures became pearly globular structures on the surface of translucent structures of the calluses (Fig. 1*n*). From these structures, somatic embryos eventually form and are referred to as globular embryogenic structures (Fig. 1*n*).

By day 90 of culture, the calluses were *c*. 5–7 mm in diameter and the globular embryogenic structures were larger (with a compact consistency and a smooth appearance, easily distinguishable from the rest of the callus). The globular embryogenic structures were numerous, covering most of surface of the callus that was not in contact with the medium gel (Fig. 1*o*). Transverse sections showed that these structures consisted of meristematic cells surrounded by a protoderm. Some globular embryogenic structures became larger and others seemed to fuse with each other and acquired an elongated appearance. Inner tissues of the callus consisted mostly of parenchyma cells and vascularized zones (Fig. 1*p*). The cells forming the globular embryogenic structures had nuclei of irregular shape, the nucleoli scarcely visible, and the cytoplasm dark-stained (Fig. 1*q*).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Formation and germination of somatic embryos. Once the globular embryogenic structures were formed, the calluses were transferred to medium II under photoperiod conditions. Before 30 d after transfer to medium II, formation of somatic embryos occurred from the globular embryogenic structures, some forming clumps (Fig. 2*a*). Early somatic embryos were observed as globular white translucent structures of 1–2 mm diameter, with a few layers of meristematic cells (Fig. 2*b*). Generally, there were 3–6 globular somatic embryos per callus. Thirty days after transfer to medium II, somatic embryos differentiated further, forming a well-defined meristematic zone, and provascular bundles became visible, resembling those of zygotic embryos (Fig. 2*c*). Some of these somatic embryos contained starch reserves in their cortical parenchyma. At day 45 after subcultivation, it was possible to observe the presence of the germinative pore and the coleoptile in the somatic embryos (Fig. 2*d*). Transverse sections showed that the shoot meristem and the root meristem were beginning to define (Fig. 2*e*). After 45 d, the somatic embryos differentiated into a more mature, elongated stage. They were 3–6 mm long and a coleoptile was present (Fig. 2*f*, *g*). By day 60, they had further developed into larger embryos with the appearance of the leaf primordium (Fig. 2*h*, *i*).

Two months after transfer to medium II, the coleoptile was green and it was possible to observe the occurrence of the cotyledon and scutellum (Fig. 2*j*). The shoot meristem was completely differentiated and more than one plumular leaf was present (Fig. 2*k*, *l*). At this stage, embryos started germinating (Fig. 2*m*) and forming shoots (Fig. 2*n*). It was also possible to observe that some well-formed somatic embryos did not germinate. About 10–15% of the somatic embryos could produce well-formed shoots. Some embryos showed irregular development with aberrant structures. The most common of these were fused embryos and embryos with fused leaves (Fig. 2*o*), both types generally without roots. After germination, other embryos formed haustorial-like tissue (Fig. 2*p*).

Discussion

A schematic representation of the events that occur during the development of coconut callus and embryogenic callus from plumule explants is depicted in Fig. 3. The origin of the formation of callus can be traced according to the changes of the plumular leaves, particularly the external ones. During the first 15 d of culture they grew and coalesced, apparently becoming one single structure that surrounded the shoot meristem and the other leaves. At this stage, at the periphery of these fusing leaves, a meristematic zone developed, showing layers of small densely stained cells that apparently formed from provascular cells. As time elapsed, this structure comprising the external leaves grew and developed into a callus at 30–45 d of culture. This growth was assured by the multiplication of the cells in the meristematic zone that progressively became more extensive during the following weeks.

The occurrence of a peripheral meristematic zone associated with callus growth has also been observed in explants of *C. nucifera* inflorescences (Verdeil et al., 1994, 2001), *Elaeis guineensis* zygotic embryos (Schwendiman et al., 1990) and *Coffea canephora* leaves (Berthouly and Michaux-Ferriere, 1996). In these species, callus formation from vascular bundle cells has also been reported (Schwendiman et al., 1990; Verdeil et al., 1994; Berthouly and Michaux-Ferriere, 1996). In plumular explants, callus growth derived mostly from the changes in the external leaves and only to a lesser extent from those in the other leaves. In the case of the shoot meristem, practically no changes were observed.

The meristematic zone gave rise to the formation of the meristematic nodules by 60–75 d, from which nodular or globular embryogenic structures developed by 75–90 d. Occurrence of these types of structures is well documented in *E. guineensis* (Schwendiman et al., 1990), *H. brasiliensis* (Michaux-Ferriere and Carron, 1989) and inflorescence explants of *C. nucifera* (Verdeil and Buffard-Morel, 1995). The globular embryogenic structures were isolated from each other and had a layer of protoderm around them. Due to increasing abundance, the globular embryogenic structures coalesced into structures with elongated appearances. When observed in detail, the cells of the globular embryogenic structures showed a nucleus with an irregular morphology. Verdeil et al. (2001) reported that the nuclei of the embryogenic cells in coconut callus derived from inflorescence explants changed from a round shape to an irregular shape (with invaginations) when the callus was transferred to a medium with a higher concentration of 2,4-D. In the present case, the release of 2,4-D from the activated charcoal may have produced alteration in the 2,4-D level that could similarly affect the morphology of the nucleus from plumular cells.

Eventually, somatic embryos formed from the globular embryogenic structures when the calluses were transferred to medium II (containing a 100-fold lower 2,4-D concentration plus BA) and photoperiodic light conditions. As previously reported by Chan et al. (1998), evidence obtained in the present study supports a pluricellular origin for somatic embryo formation, as no unicellular embryo formation was observed. This contrasts with previous reports providing evidence of the unicellular path for embryo formation in coconut callus obtained from

Fig. 1. Formation of initial callus and embryogenic callus from coconut plumule explants. *a*, The coconut plumule explants; *b*, histological cross section of the plumule explant; *c*, a developing initial callus after 15 d of *in vitro* culture; *d*, a transverse section showing noticeable growth of the plumular leaves; *e*, an initial callus already formed after 1 mo.; *f*, a cross section of this callus, showing growth and fused external plumular leaves, which continued growing; *g*, a developing embryogenic callus after 45 d showing the formation of translucent structures; *h*, a cross section of this callus presenting peripheral layers of small cells with meristematic appearance in the translucent structures; *i*, magnification of *h*; *j*, callus after 60 d; *k*, cross section of this callus; *l*, magnification of the meristematic zone; *m*, after 60 d, these meristematic centers evolve into nodular structures; *n*, embryogenic callus after 75 d; *o*, embryogenic callus on day 90 *p*, cross section of this callus showing the meristematic nodules and vascularized zone; *q*, magnification of the meristematic nodules showing nuclei of irregular shape. Abbreviations: P, plumule; GP, germinative pore; SM, shoot meristem; PL, plumular leaf; TS, structures with a translucent appearance; PD, protodermis; MC, meristematic centers; NS, nodular structures; GES, globular embryogenic structures. EES, embryogenic structures of elongated appearance; VZ, vascular zones.

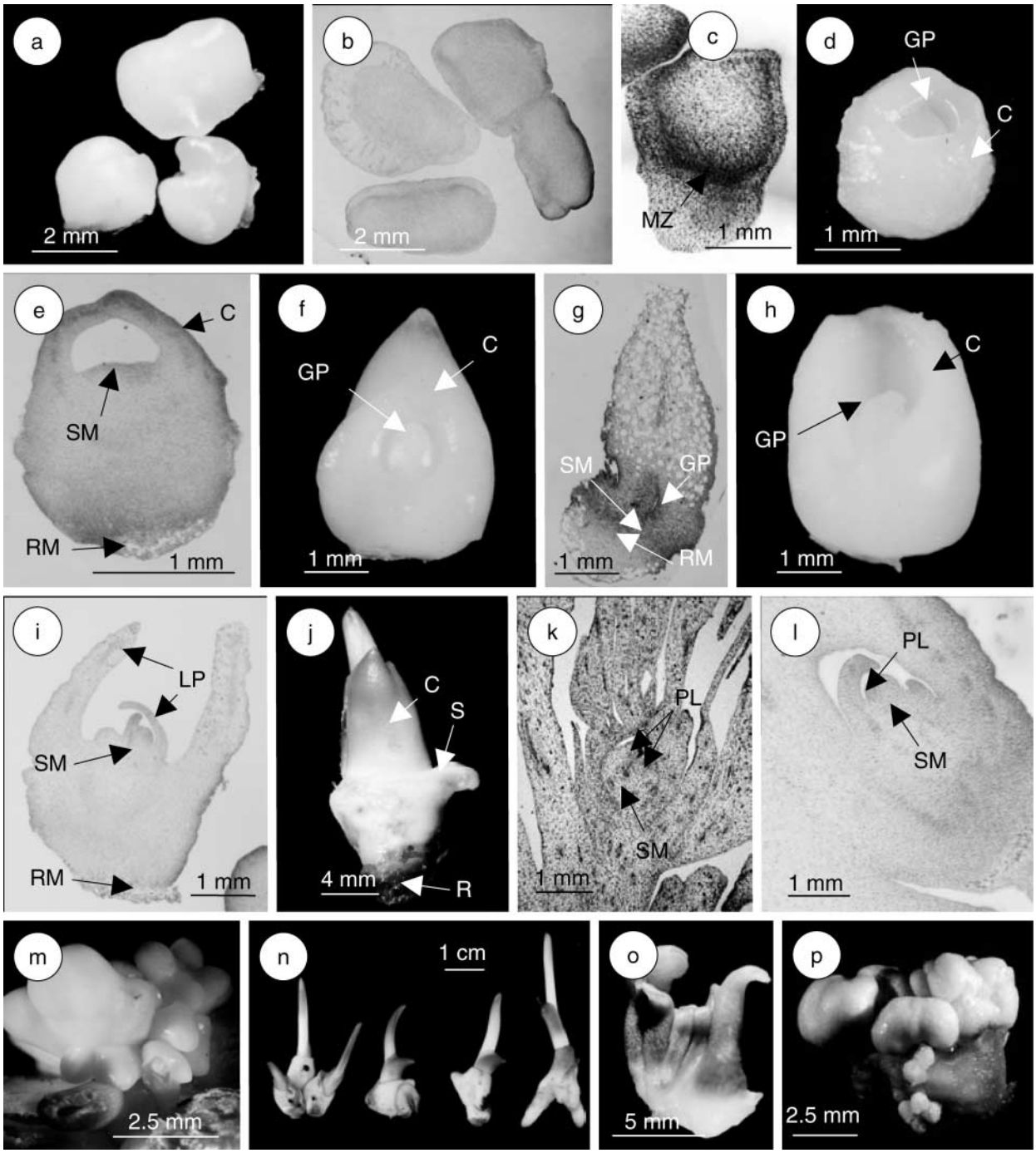

FIG. 2. Formation and germination of somatic embryos from coconut plumule explants. *a*, Somatic embryos; *b*, cross section of somatic embryos; *c*, a well-differentiated somatic embryo, resembling zygotic embryos; *d*, somatic embryo showing the germinative pore and the coleoptile; *e*, transverse section of *d*; *f*, somatic embryo differentiated into torpedo stage; *g*, transverse section of *f*; *h*, somatic embryo, further developed; *i*, cross section of a germinated-somatic embryo; *j*, germinated somatic embryo; *k*, *l*, magnification of the shoot meristem; *m*, clump of germinating somatic embryos; *n*, shoots from these clumps of somatic embryos; *o*, aberrant somatic embryos showing fused leaves and (*p*), haustorial-like tissue. Abbreviations: MZ, meristematic zone; GP, germinative pore; SM, shoot meristem; RM, root meristem, C, coleoptile; LP, leaf primordium; S, scutellum; PL, plumular leaf.

inflorescence explants (Verdeil et al., 1994; Verdeil and Buffard-Morel, 1995) and from plumule explants (Chan et al., 1998). The sequence of events leading to complete somatic embryos capable of germination was the same as that for the zygotic embryo: individualization of a protoderm, individualization of provascular strands and meristematic areas giving rise to the shoot meristem first and further to the root meristem (Haccius and Philip, 1979).

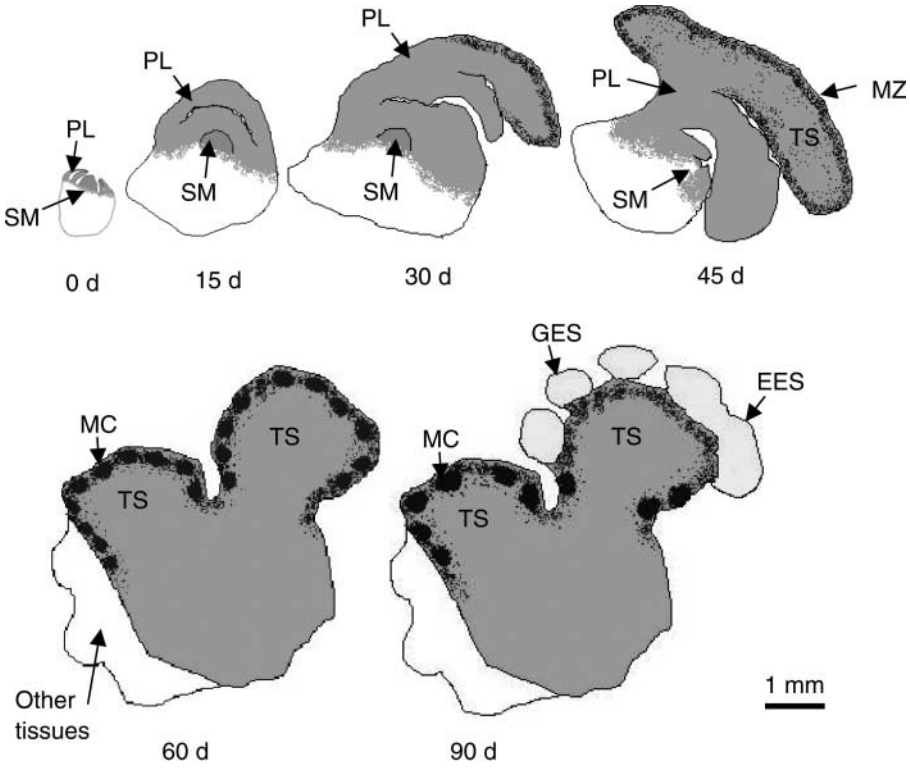

FIG. 3. Schematic representation of the changes that occur during the formation of initial callus and embryogenic callus from plumule explants cultured *in vitro*. At day 15, the coalescence of the external plumular leaves (PL) is depicted. At day 30 the PL have grown and formed an initial callus. At day 45 the formation of a meristematic zone (MZ) near the periphery of this callus is observed. The callus forms translucent structures (TS). At 60 d the formation of meristematic centers (MC) in the TS is observed. At 75–90 d the formation of globular (GES) and elongated (EES) embryogenic structures appeared. White areas correspond to other tissues. SM, shoot meristem.

Most well-formed somatic embryos were capable of germination and conversion, whereas those showing irregular development with aberrant structures did not germinate. Germination started after 30 d of culture in medium II, and the development of the shoots occurred after 3 mo. of culture. At this stage, the presence of roots was not observed; however, histological studies showed that the root meristem was already well formed. Finally, with the formation of roots after 3 mo. of culture, whole plantlets were formed.

The present study allows better insights and an integrated view (as depicted in Fig. 3) into the somatic embryogenesis process in coconut plumule explants that could be helpful for improvement in future studies. For instance, we can relate current limitations of the process, such as the low germination of embryos, with occurrence of aberrant development (shoot meristem absence, fused cotyledons, etc.), and consequently focus our attention on these particular aspects to overcome the limitations. In addition, this study can broaden our understanding of the process of embryogenesis. For instance, to relate the histomorphological development with gene expression, particularly of genes involved in the signaling process of somatic embryogenesis, such as the somatic embryogenesis receptor-like kinase gene (SERK) already reported in other species (Schmidt et al., 1997; Baudino et al., 2001; Hecht et al., 2001). The resulting knowledge could eventually lead us to improved control of the process and greater efficiency of somatic embryo and plantlet formation.

ACKNOWLEDGMENTS

A.A. thanks Instituto Nacional de Investigaciones Agrícolas y Pecuarias (INIFAP) and CONACYT (Ref. 119335) for the financial support for his PhD studies of which the present paper is a part. This project was supported by CONACyT-SISIERRA (Project number 990130).

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