Plant Cell, Tissue and Organ Culture **72:** 281–284, 2003.

Research note

Histological analysis of plant regeneration from plumule explants of *Cocos nucifera*

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Received 1 May 2002; accepted in revised form 30 September 2002

Key words: callogenesis, coconut, histology, plumule culture, somatic embryogenesis

Abstract

Plant regeneration was achieved from plumules excised from mature zygotic embryos of a local coconut cultivar (Sri Lanka Tall). A detailed histological study was undertaken to gain a better understanding of the cellular changes that occur during plant regeneration from plumule tissues. This study led to the identification of the cellular origin, specific cell characterization and development pattern of embryogenic calluses. It also revealed that abscisic acid induces plant regeneration through somatic embryogenesis. The presence of incomplete somatic embryos that lacked shoot poles was also observed.

Abbreviations: ABA – abscisic acid; BA – 6-benzylaminopurine; 2,4-D – dichlorophenoxyacetic acid; NBB – naphthol blue black; PAS – periodic acid Schiff's reaction, Y3 – Eeuwens Y3 medium

Tissue culture is a promising technique for vegetative multiplication of coconut palms (*Cocos nucifera* L.) and many research projects have been directed towards the development of a clonal propagation method for coconut. However, only a limited success has been achieved in plant regeneration due to highly recalcitrant nature of coconut tissues to *in vitro* conditions (Fernando and Gamage, 2000). Out of various explants tested, plumules have shown the best response to *in vitro* culture (Hornung, 1995; Chan et al., 1998). Thus, plumule culture holds a considerable potential as a model system for developing a clonal propagation method for coconut. The objective of the present study was to assess the plant regeneration potential of plumules excised from a local coconut cultivar (Sri Lanka Tall). A detailed histological study was also conducted to gain a better understanding of the cellular changes that take place during the regeneration process.

Plumules were excised from 12 to 14-month-old mature zygotic embryos of coconut that were precultured for 15–17 days in Y3 liquid medium (Eeuwens, 1978) supplemented with 0.1 μM 2,4-D, 5.0 μM BA, 6% (w/v) sucrose and 0.25% (w/v) activated charcoal (Fluka, Catalogue No. 05105). The plumules were crushed with a scalpel and cultured in 28-ml screw-capped vials containing 10 ml of culture medium 72 (Karunaratne and Periyapperuma, 1989) supplemented with 24 μM 2,4-D, 4% (w/v) sucrose. Cultures were incubated for 10 weeks at 30±1 °C in the dark. Callogenesis was repeated four times each time with 15 replicates.

Callus separated from the original explant, was subcultured in 28-ml screw-capped vials containing 10 ml of medium 72 supplemented with 16 μM 2,4-D and 4% (w/v) sucrose before transferring to somatic embryo induction medium. Somatic embryogenesis was induced by subculturing of callus in 28-ml screw-capped vials containing 10 ml of medium 72 supplemented with 5 μM ABA (filter-sterilized) and 6% (w/v) sucrose for 5 weeks. Maturation of somatic embryos was achieved by transferring them to

medium 72 (devoid of hormones) for 4 weeks. All the media used for callogenesis and somatic embryogenesis were supplemented with 0.8% (w/v) agar and 0.25% (w/v) activated charcoal (Phamacos, Catalogue No. 06393 A). Somatic embryo germination was achieved in Y3 solid medium (0.8% (w/v) agar). Regenerated shoots were maintained in Y3 solid medium for 4–6 months by subculturing at 4-weekly intervals (Fernando and Gamage, 2000). Cultures during somatic embryo maturation and germination were maintained in 100-ml flasks containing 50 ml of medium. Cultures were maintained at 30±1 °C in the dark. Regenerated shoots were maintained at 16-h photoperiod (intensity 17 μmol m^{-2} s^{-1}). Plant regeneration was repeated three times each time with 20 replicates.

For histological analysis, cultures were sampled at weekly intervals, starting from culture initiation. Three samples of each material were fixed in FAA (50% ethanol–10% formaldehyde–glacial acetic acid, 18:1:1) for 72 h. Then the samples were dehydrated through a graded alcohol series (50–100% ethanol and 50–100% butanol), impregnated with methylmethylacrylate and embedded in the resin, Technovit 7100® (Heraeus Kulzer GmbH, Germany). The resin was allowed to polymerize for 2 h at room temperature. Sections (3.5–4.0 μm thick) were obtained using an ultramicrotome (Historange, LKB). Finally, the sections were double-stained with periodic acid Schiff's reaction (PAS) and protein-specific naphthol blue black (NBB).

The mean percentage of plumules that produced callus was 55.2 (the range being 53.3–57.1%). Morphological observations revealed that 27.6% (the range being 25–30%) of the calluses subcultured on medium containing ABA produced somatic embryos. Maturation of the somatic embryos took place in hormone-free medium. When transferred to Y3 medium, 4.8% (the range being 4.3–5.5%) of cultures were able to produce complete plants that could be transferred to soil. The others often showed the development of haustorial tissues, roots or fasciated shoots.

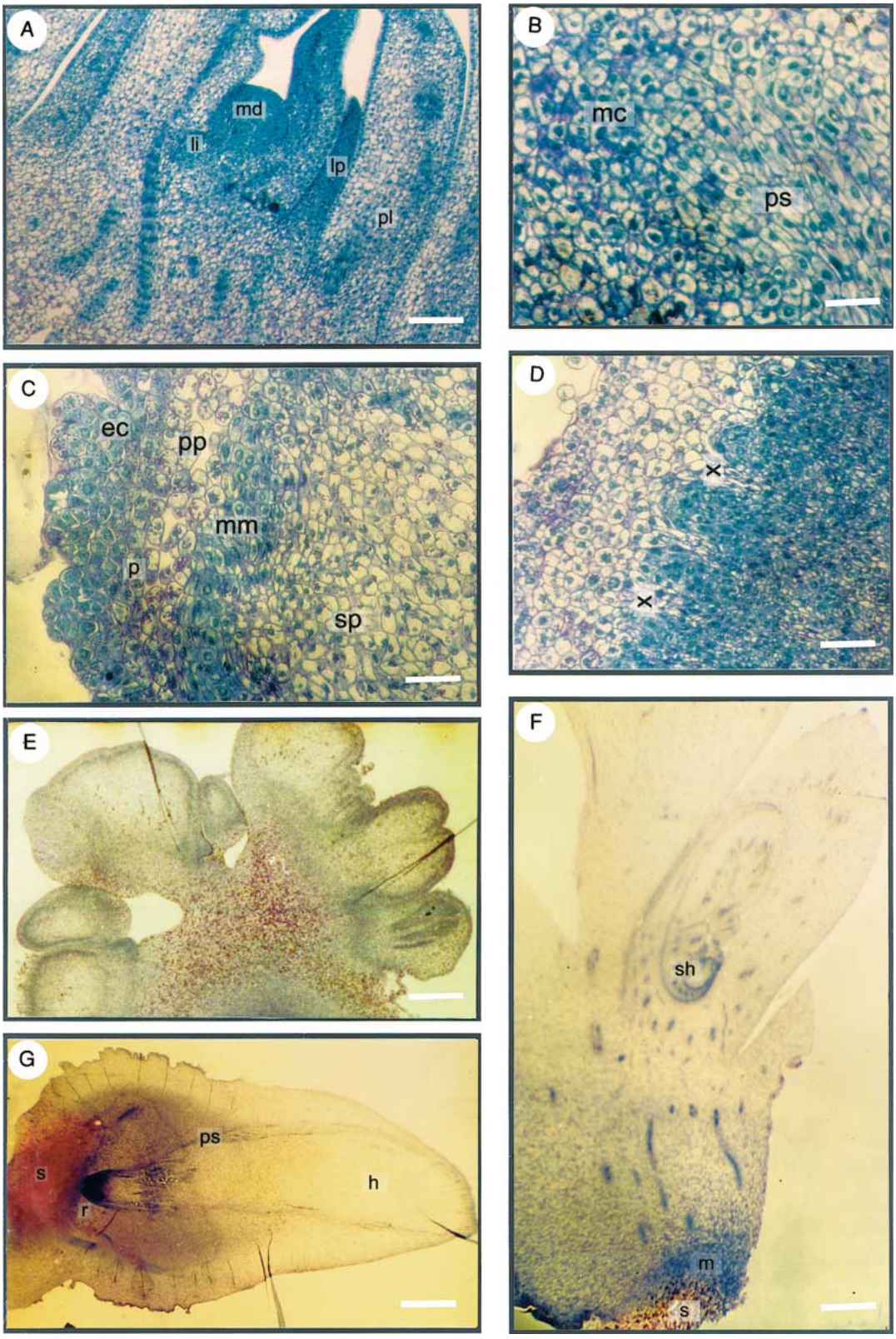
Histological studies showed that at the time of excision, plumules contained active meristematic domes that give rise to leaf initials. Each meristematic dome was surrounded by leaf initials, leaf primordia and preliminary leaves (Figure 1A). The histological observations of tissues fixed 1 week after culturing in the callusing medium revealed that new meristematic cells were formed by the division of provascular cells of preliminary leaves (Figure 1B). The meristematic cells formed by continuous division of provascular cells gave rise to peripheral meristematic masses. This peripheral meristematic zone ensures callus growth. Despite the difference in the type of explant, callus formation from vascular bundles has been reported in various monocotyledonous plants, particularly in leaf explants of coconut (Buffard-Morel et al., 1992) and oil palm (Schwendiman et al., 1988).

Longitudinal sections of callus formed after 10 weeks of culture (Figure 1C) showed that the callus was highly heterogeneous and consisted of embryogenic cells (originated from the division of protodermal cells), protodermis, primary parenchyma, peripheral meristematic masses and secondary parenchyma formed by the division of the meristematic masses.

Histologically, the first stage of development of embryogenic nodules was characterized by the breaking up of the meristematic masses (Figure 1D). Active cell division in the discontinuous zone led to the formation of protuberances composed of meristematic cells followed by the formation of epidermis. This sequence of events provided evidence for the multicellular origin of somatic embryos (Figure 1E).

Histological analysis revealed that plant regeneration from callus treated with ABA occurred through somatic embryogenesis (Figure 1F). Establishment of the shoot pole preceded the root pole differentiation from M zone. The haustorial tissues located opposite the stem meristem contained starch reserves. How-

Figure 1. (A) Longitudinal section of a plumule at excision: li – leaf initials; lp – leaf primordia; md – meristematic dome; pl – preliminary leaves (bar=127 μm). (B) Meristematic cell formation by division of provascular cells: mc – meristematic cells; ps – provascular strands (bar=52 μm). (C) Longitudinal section of a callus, formed 10 weeks after culturing: mm – meristematic masses formed by the division of provascular cells; ec – highly embryogenic cells formed by the division of protodermal cells; p – protodermis; pp – primary parenchyma; sp – secondary parenchyma (bar=52 μm). (D) Fragmentation of the meristematic masses (x) (bar=52 μm). (E) Embryogenic structures of multicellular origin (bar=544 μm). (F) Longitudinal section of a bipolar somatic embryo, cultured in the germination medium for 1 week: m – M zone; s – starch; sh – shoot pole (bar=347 μm). (G) Longitudinal section of an incomplete somatic embryo: h – haustorium; ps – provascular strands; s – starch; r – root pole (bar=1.2 mm).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ever, many of the somatic embryos sectioned were incomplete, as they contained a prominent haustorium with vascular strands and root pole but lacked a shoot pole (Figure 1G).

Successful plant regeneration in coconut using ABA with or without osmotically active agents was reported by Samosir et al. (1999). However, histological analysis was not described. Histological analysis of embryogenic cells, proembryos and embryos (obtained by decreasing the level of 2,4-D and applying BA) was reported by Chan et al. (1998) but the sequence of all the events that occur during somatic embryogenesis was not described.

The present study enabled to confirm that treating callus with ABA resulted in plant regeneration through somatic embryogenesis. These plants were successfully acclimatized and several of them were transferred to the field. Even though plant regeneration was consistent, the number of plants regenerated was low. The low plant regeneration frequency ($<5\%$) observed in the present study could be due to the presence of incomplete somatic embryos.

Acknowledgements

We gratefully acknowledge the financial assistance provided by the Institut de Recherche pour le Developpement (IRD), Montpellier, France, the Council for Agricultural Research Policy, Sri Lanka (CARP grant 12/419/315), National Research Council, Sri Lanka (NRC Grant 00-21) and Coconut Research Institute, Sri Lanka. We are thankful to Dr D T Wettasinghe for useful comments.

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