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Regeneration of coconut (*Cocos nucifera* L.) from plumule explants through somatic embryogenesis

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Abstract A protocol was developed for coconut regeneration using plumules from mature zygotic embryos as explants, and media with the synthetic growth regulators 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine. Evidence for the regeneration process from these tissues occurring through somatic embryogenesis is presented. The somatic embryos were capable of germination, subsequent development into plantlets and successful transfer to the nursery. The yields were larger, nearly twofold for calli and over tenfold for calli bearing somatic embryos, than those previously reported with inflorescence explants. The present protocol thus represents an improvement in time and yield over previous protocols. Even though plumule explants are not the ideal tissue source due to possible genetic heterogeneity, the improvements made here may be applicable to tissues from mature plants. In addition, micropropagation of coconut using plumules is potentially useful when they are obtained from fruit produced from selected parents of outstanding performance, such as those resistant to diseases.

Key words Coconut · Regeneration · Somatic embryogenesis · Plumule

Abbreviations *6-BAP* 6-Benzylaminopurine · *2,4-D* 2,4-Dichlorophenoxyacetic acid

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Introduction

The coconut palm (*Cocos nucifera* L.) is a very important crop in tropical areas, providing cash and subsistence to smallholders. Most coconut groves worldwide require replanting either because of senescence or because of loss due to diseases such as lethal yellowing in America (Arellano and Oropeza 1995), the lethal diseases in Africa (Eden-Green, 1995), cadang-cadang in Asia (Hanold and Randles 1991) and *Phytophthora*, which is widely spread (Schierer 1970; Joseph and Radha 1975; Quillec and Renard 1984; Franqueville et al. 1991) Unfortunately, improved disease-resistant planting materials are scarce and present propagation methods do not yield sufficient materials to satisfy rapidly growing demands. Therefore, alternative approaches for the propagation of improved planting material must be sought and in vitro cloning via somatic embryogenesis seems to provide the best prospect for the future. Protocols for coconut micropropagation have been developed in various laboratories using different explant sources (Branton and Blake 1983a, b, 1986; Buffard-Morel et al. 1988; Karunaratne and Periyapperuma 1989; Shirke et al. 1993; Verdeil et al. 1994; Blake and Hornung 1995; Hornung 1995a). However, most of the progress has been achieved using inflorescence explants (see Verdeil et al. 1994; Blake and Hornung 1995). When cultured, these explants develop a partly dedifferentiated callus that has been referred to as "calloid" by Brackpool et al. (1986). This is followed by the formation of somatic embryos, which subsequently germinate and eventually form clonal plantlets (Blake 1990; Verdeil et al. 1994; Blake and Hornung 1995). However, only limited success has been achieved and the protocols lack reliability. The present paper reports a protocol for coconut regeneration using plumular tissue, as a source of juvenile tissue, which responds well and more rapidly than immature inflorescences (Hornung 1995b; J. L. C. and C. O., unpublished results).

Materials and methods

Plant material

The fruit were harvested 12–14 months after pollination (except where otherwise indicated) from 15-year-old Malayan Dwarf coconut palms at San Crisanto, Yucatán, México. The fruit were cut transversely with a machete revealing the embryos surrounded by solid endosperm. Embryos were excised from the open nuts using a cork borer (1.6 cm diameter) and placed in distilled water. Under aseptic conditions, the endosperm enclosing the embryo was washed in 70% ethanol for 3 min and rinsed three times with distilled sterile water, washed in a 6% NaClO solution for 20 min and rinsed three times with distilled sterile water. The embryos were excised from the endosperm and washed in a 0.6% NaClO solution for 10 min and rinsed with distilled sterile water three times. Embryos were then either cultured or used for the preparation of plumule explants. Embryos were 5–7 mm long and weighed approximately 100 mg each. The plumules were excised from these embryos under a stereoscopic microscope and placed directly in nutrient medium.

Culture media and conditions

All chemicals were supplied by Sigma except charcoal which was supplied by Reactivos y Productos Químicos Finos. Each explant was cultured in 35-ml culture vessels containing 10 ml of Y3 medium (Eeuwens 1976), to which gelrite (3 g l^{-1}) and charcoal (2.5 g l^{-1}) were added. Growth regulator concentrations were 0.1 mM 2,4-dichlorophenoxyacetic acid (2,4-D) for medium I (or as indicated in the text), and 1 μM 2,4-D and 50 μM 6-benzylaminopurine (6-BAP) for medium II. The pH of the medium was adjusted to 5.75 before autoclaving for 20 min at 120°C. The cultures were incubated in medium I in the dark for 6 months (or as indicated in the text) at 27±2°C without subculturing and then transferred to medium II under illumination (45–60 μmol m^{-2} s^{-1} PPFD) at 27±2°C, subculturing every 3 months.

Plantlet acclimatization

Plantlets were transferred to black polyethylene bags containing a mixture of compost, sand and soil (2:1:1, wt:wt:wt) and covered with a transparent polyethylene bag for acclimatization in a glasshouse.

Histology

Tissue samples were fixed in formalin-acetic acid-alcohol (FAA) for 24 h under negative pressure. The tissue was dehydrated in sequenced aqueous ethanol solutions (70%, 95%, 100%) for 30 min in each step. Tissue samples were impregnated with JB-4 resin (Polyscience). Sections of 5 μm were prepared from the resin-impregnated tissue and stained with toluidine blue.

Statistical analysis

Statistical analysis was performed on the binomial data using the chi-square goodness-of-fit test.

Results

Effect of 2,4-D concentration on callus formation

The response of plumule explants to culture was studied in media containing a range of 2,4-D concentrations (0.001, 0.01, 0.1, and 1 mM) without cytokinin. Responses were observed over a 6-month period of culture ($n = 20$ per treatment). The explants became necrotic and did not show any other response at the highest 2,4-D concentration. No response was observed at the lowest 2,4-D concentration. With 0.01 mM 2,4-D, shoot development was observed without callus formation. With 0.1 mM 2,4-D after 3 months of culture, 40% of the plumules began developing callus (Fig. 1a). Histological cross-sections demonstrated meristematic centers in callus tissue (Fig. 1b). The medium containing 0.1 mM 2,4-D was designated medium I. To determine more precisely the optimum 2,4-D levels, a narrower range of 2,4-D concentrations was then tested (Table 1) and the best callus formation response was again observed with 0.1 mM 2,4-D. Therefore, this concentration seems to be optimal for callus formation from the plumule explants used.

Table 1 Effect of 2,4-D concentration on differentiation of coconut plumule explants after 3 months of culture without subculturing ($n = 20$). Values followed by different letters vary significantly ($P < 0.05$)

2,4-D concentration (mM)	Type of response	Response (%)
0.01	Germination	100 c
0.03	Germination	60 c
0.06	Callus	20 a
0.1	Callus	40 b
0.3	Necrosis	100 c
0.6	Necrosis	100 c
1	Necrosis	100 c

Formation of calli bearing embryogenic structures

Callus cultured in medium I (without subculturing) for an additional 3 months (i.e., 6 months after initiation of the culture) formed calli bearing embryogenic structures (Fig. 1c). Histological sections of these calli showed the occurrence of embryogenic cells (Fig. 1d), proembryos (Fig. 1e), and embryos (Fig. 1f).

To evaluate the effect of subculture frequency on the formation of calli bearing embryogenic structures, different subculture protocols were compared during a

6-month period. These included subculturing every month, no subculturing and subculturing once at different times (after 1, 2 or 3 months). The results (Table 2) show that undisturbed cultures on medium I produced a higher proportion of explants developing into calli bearing embryogenic structures. With subculturing, the percentage of explants developing into calli bearing embryogenic structures decreased. The earlier the explants were subcultured, the greater the reduction. Based on the results presented above, five batches of plumules (average number per batch = 74, total number of plumules = 370) were cultured in medium I without subculturing. Yields obtained were:

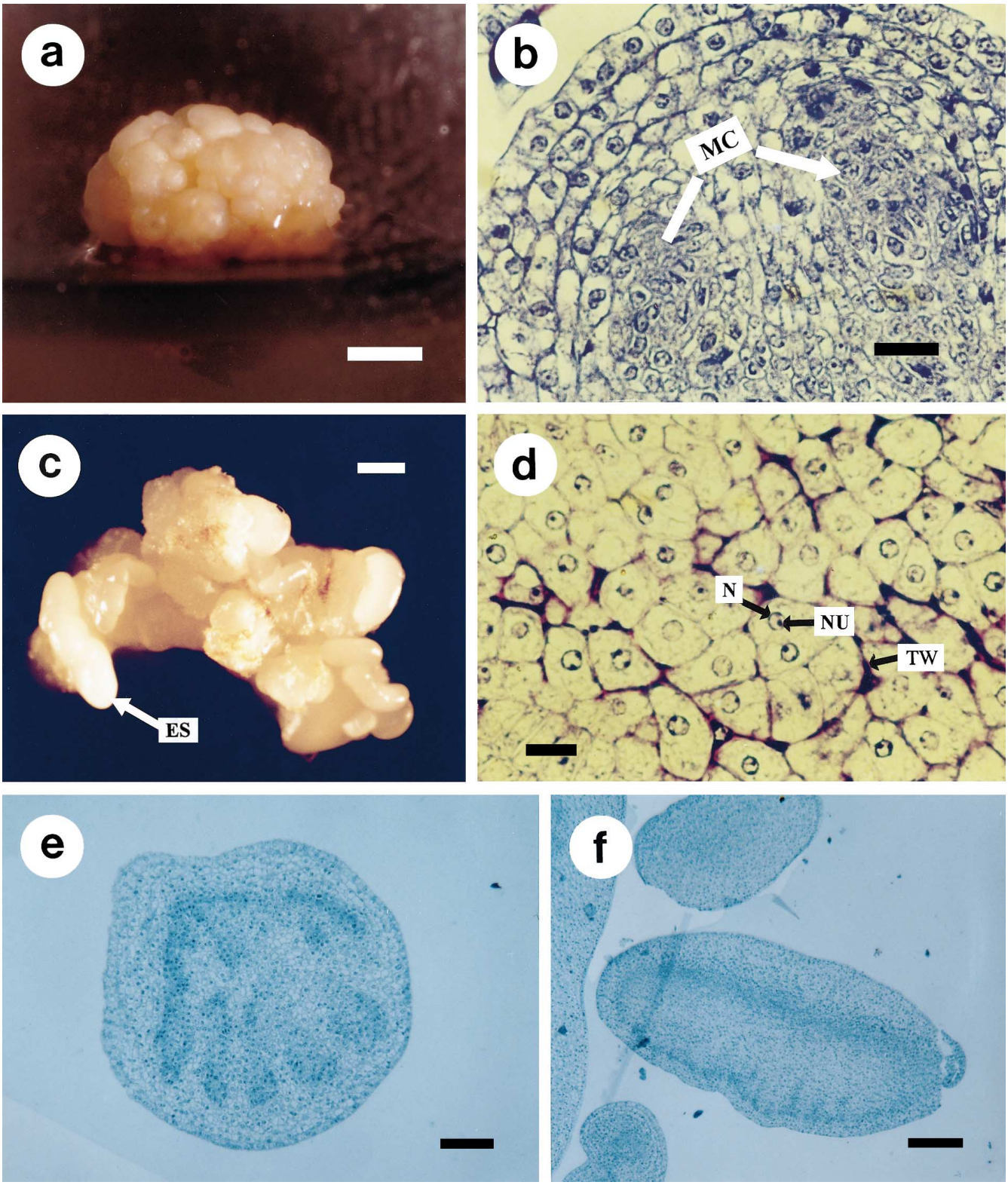

Fig. 1 **a** Callus tissue developed from plumule explants after 3 months of culture in medium I (0.1 mM 2,4-D) (*bar* 2 mm). **b** Histological cross-section showing meristematic centers (*MC*) in callus tissue (*bar* 27 μm). **c** Calli bearing embryogenic structures (*ES*) developed from callus tissue further cultured in medium I for another 3 months (or 6 months from initiation of the culture) (*bar* 1 mm). **d** Embryogenic cells, nucleus (*N*), nucleolus (*NU*) and thick wall (*TW*) (*bar* 10 μm). **e**, **f** Proembryos (**e**, *bar* 100 μm) and embryos (**f**, *bar* 200 μm) in histological sections of calli bearing embryogenic structures depicted in **c**

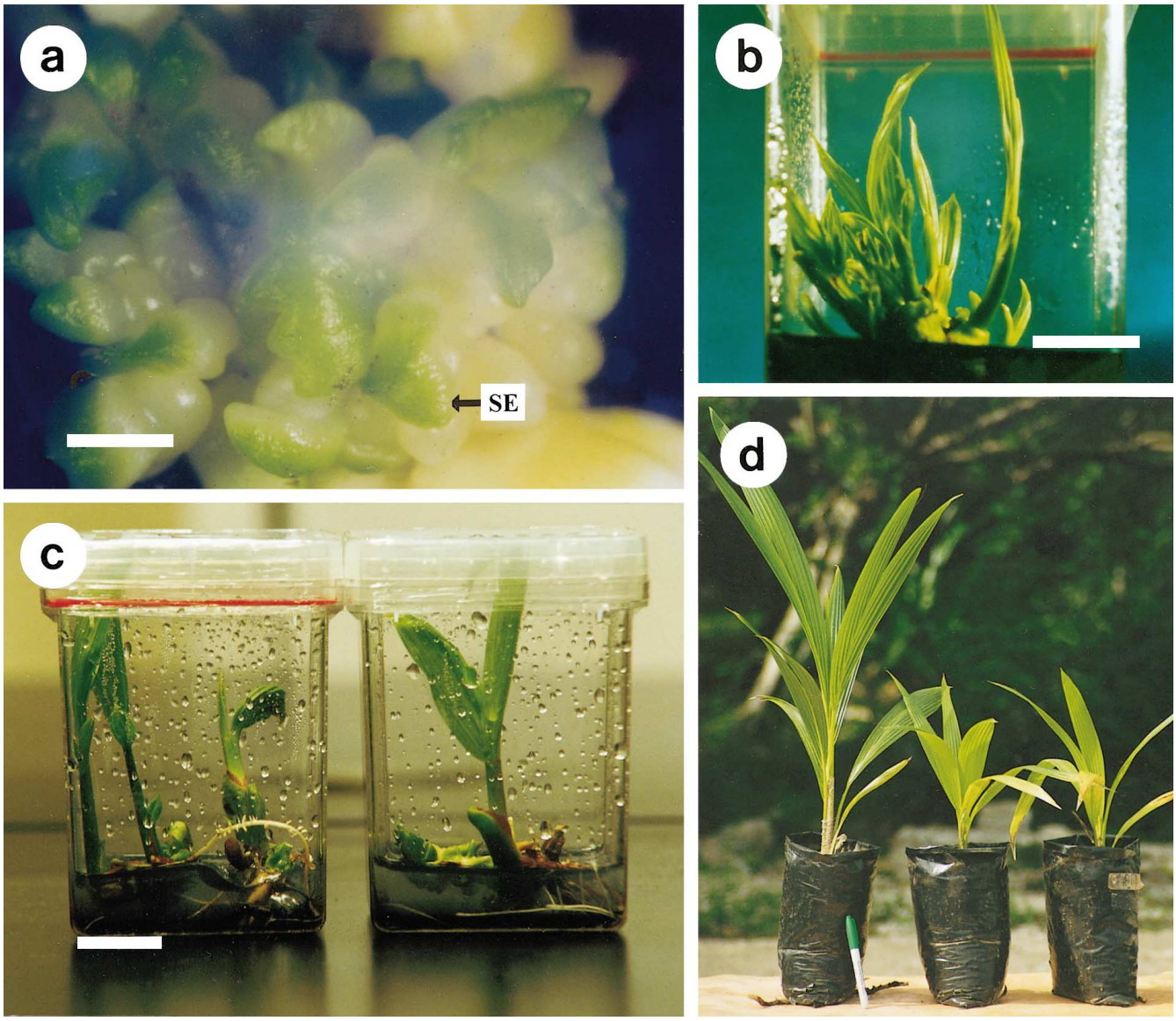

Fig. 2 **a** Somatic embryos (*SE*) developed in calli bearing embryogenic structures after 3 months of culture in medium II (1 µM 2,4-D and 50 µM 6-BAP) and a photoperiod of 12 h light/12 h dark (*bar* 4 mm). **b** Clumps of shoots formed as somatic embryos germinate (*bar* 20 mm). **c** Single shoots excised from clumps of shoots developed into individual plantlets (*bar* 20 mm). **d** Plantlets in the nursery after acclimatization in a greenhouse (*pen* 14 cm)

Table 2 Effect of subculturing on the formation of calli bearing embryogenic structures from plumule explants after 6 months of culture in medium I (0.1 mM 2,4-D, $n = 20$). Values followed by different letters vary significantly ($P < 0.05$)

Treatment		Calli bearing embryogenic structures (%)
One single subculture after	1 month	17 b
	2 months	22 b
	3 months	34 a
No subculturing		39 a
Subculturing every month		22 b

57% (±8.1 SD) of the plumules produced callus after 3 months of culture, and 40.6% (±16.9 SD) produced callus bearing embryogenic structures after six months of culture.

Embryo maturation

It has previously been shown that somatic embryo formation is favored by reducing the auxin concentration and including a cytokinin in the culture medium (Verdeil et al. 1994). Therefore, calli bearing embryogenic structures were transferred to medium II, with the same formulation of medium I except that the concentration of 2,4-D was reduced to 1 µM 2,4-D and 50 µM 6-BAP was added. The cultures were incubated under a 12-h photoperiod (45–60 µmol m^{-2} s^{-1} PPFD) (see below) at 27±2°C. Somatic embryos developed after approximately 3 months (Fig. 2a) and some commenced germinating.

Effect of illumination on somatic embryo formation

Preliminary observations in our laboratory indicated that illumination may affect somatic embryo formation from calli bearing embryogenic structures. To test this, two illumination conditions were studied, a photoperiod of 12 h light/12 h dark and darkness. The proportion of calli forming embryos was greater when cultured in a 12-h photoperiod (Table 3). The percentage of calli forming embryos in darkness was nearly four-fold lower. Seven batches of calli bearing embryogenic structures (average number per batch = 28, total number of calli = 199) were cultured for 3 months under the 12 h/12 h photoperiod in medium II without subculturing. A total of 54.3% (±5.6 SD) of the calli showed embryo formation.

Table 3 Effect of illumination on somatic embryo formation in calli bearing embryogenic structures after 3 months of culture in medium II (1 μM 2,4-D and 50 μM 6-BAP). The results (%) are significantly different ($P<0.05$)

Condition	Number of calli bearing embryogenic structures	Number of calli bearing embryos	%
Photoperiod 12 h/12 h	66	37	56
Darkness	33	5	15

Embryo conversion

Cultures of calli bearing embryos were subcultured onto medium II and kept under a 12 h/12 h photoperiod. Subculturing was carried out every 3 months. Under these conditions, embryos continued germinating and clumps of shoots started developing within 3–6 months (Fig. 2b). Each callus developed an average of 11.5 shoots (±4.5 SD, *n* of calli=23). Single shoots excised from the clumps of shoots developed into individual plantlets (Fig. 2c), with approximately one plantlet from every two shoots.

Acclimatization

After subculturing every 3 months for two or three times, the plantlets were transferred to a container with a cover that allowed gas exchange for acclimatization. One month later, the cover was removed. A batch of the most developed plants have been transferred to the open environmental conditions in our nursery and have continued to produce new leaves (Fig. 2d).

Based on the results presented above, a protocol for the regeneration of coconut from plumule explants is proposed (Fig. 3).

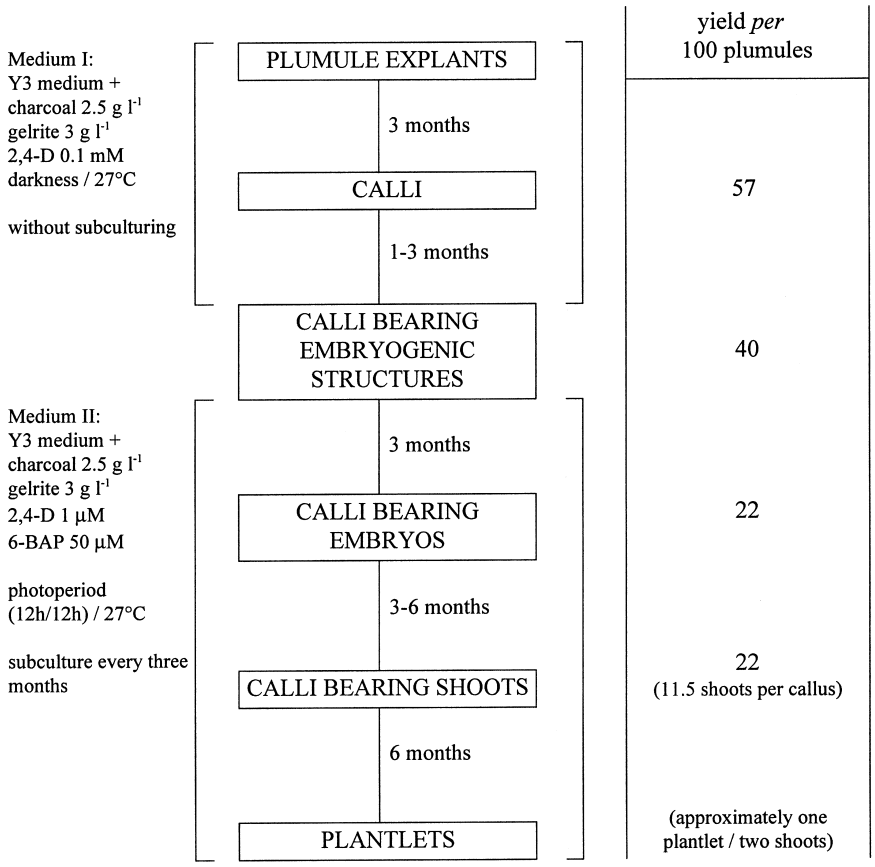

Fig. 3 Protocol for the regeneration of coconut plantlets from plumule explants through somatic embryogenesis

Discussion

The present study describes the regeneration of coconut plants using plumule explants. This source of juvenile tissue has been shown previously to respond well and more rapidly than immature inflorescence explants in terms of callus formation and the development of embryogenic capacity (Hornung 1995b; J. L. C. and C. O., unpublished data). Callus formation was obtained with or without cytokinins, but required auxin (2,4-D) at an optimum concentration of 0.1 mM 2,4-D. These calli developed meristematic centers, indicative of embryogenic capacity and of a multicellular pathway for embryo formation according to Verdeil and Buffard-Morel (1995). In addition, individualization of embryogenic cells was observed, indicative of a unicellular pathway of embryo formation (Verdeil and Buffard-Morel 1995). This occurred less often than the formation of meristematic centers. By keeping the cultures at the same auxin concentration (0.1 mM), the calli developed embryogenic structures. A greater proportion of plumule explants developed into calli bearing embryogenic structures when the cultures were undisturbed and no subculturing was practised. Calli bearing embryogenic structures produced somatic embryos when the auxin concentration was reduced a hundredfold and cytokinin was added (50 μM 6-BAP), performing better under illumination (12 h photoperiod) than in the dark. Keeping cultures in these conditions and subculturing every 3 months allowed embryos to germinate and the resulting shoots eventually developed into plantlets. Based on these results, a protocol for the regeneration of coconut from plumule explants is proposed (Fig. 3). Following this protocol, different batches of cultures were tested and the performance was found to be reproducible.

In addition, the results showed that with plumule explants, shorter times were required to obtain calli (2–3 months) and calli bearing somatic embryos (7–9 months) than those previously reported with inflorescence explants (8 months and 14–20 months, respectively; Verdeil et al. 1994), and the yields were larger (nearly twofold for calli and over tenfold for calli bearing somatic embryos) than those reported with inflorescences (Verdeil et al. 1994). Acclimatization has been successful and plantlets are doing well in open environmental conditions, continuing to produce new leaves.

Although for practical purposes, the efficiency of the present protocol is still far from adequate, its performance is an improvement in time and yield over previous protocols and may be useful as a model for research with which further knowledge could be derived for advancing protocols using other explants, such as inflorescences. One advantage of inflorescences as an explant source is that the performance of the individual from which an explant is derived can be determined at the time of harvesting, allowing cloning of selected individual palms. This is not the case for plumules and this poses a constraint for the practical application of the technique. However, micropropagation of coconut using plumules is potentially useful when the explants are obtained from fruit produced from selected parents of outstanding performance. This is the case of nuts of varieties resistant to diseases which are produced from selected parents (such as the Maypan hybrid) of which only small amounts are available in countries affected by these diseases, and micropropagation from plumules could be used to multiply the output from conventional propagation. This is currently otherwise impossible but extremely desirable.

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