Plant Cell Reports (1994) 13:218–221

Plant Cell Reports

Plant regeneration from cultured immature inflorescences of coconut (*Cocos nucifera* L.): evidence for somatic embryogenesis

Jean-Luc Verdeil, Christine Huet, Frédérique Grosdemange, and Jacqueline Buffard-Morel

ORSTOM-CIRAD/CP Laboratoire de Ressources Génétiques et d'Amélioration des Plantes Tropicales-ORSTOM-911, Av Agropolis. B.P. 5045, 34032-Montpellier, France

Received 19 May 1993/Revised version received 20 August 1993 – Communicated by A.M. Boudet

Abstract. Immature inflorescences of coconut belonging to three different genotypes were cultured on a solid medium supplemented with activated charcoal (2%) and a range of 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations (from 1.5 to 3.5 x 10^{-4}M). Globular white callus formed from immature floral meristems, depending on inflorescence age and 2,4-D concentration. Acquisition of embryogenic competence is described histologically. Somatic embryos presented a functional bipolar organization with a completely differentiated shoot meristem which is reported here for the first time in coconut tissue culture. Embryo maturation allowed reliable plant regeneration of this *in vitro* recalcitrant species. Details are given of exogenous hormonal requirements for the acquisition of embryogenic competence and embryo maturation.

Introduction

The *in vitro* technique for mass production of clonal palms is most advanced with date palm and oil palm (Buffard-Morel et al. 1992). In contrast, coconut palm has remained largely recalcitrant as far as tissue culture is concerned (Ammirato 1983).

Studies on callus, produced from embryos (Gupta et al. 1984), immature leaves (Raju et al. 1984; Buffard-Morel et al. 1992) and inflorescences (Branton and Blake 1984), indicate that regeneration is possible although rather difficult. However, no reliable regeneration process is available.

The reason why coconut is such a recalcitrant species would become more apparent if sufficient attention were paid to patterns of growth and differentiation.

Unfortunately when regeneration has been reported, it was impossible to establish with certitude that somatic embryos were indeed being produced (Krikorian 1989), rather than the occurence of adventitious growth. The aim of the present investigation was to study the early stage of callus formation from immature inflorescences, to elucidate the pattern of growth and differentiation. Evidence is presented for initiation of somatic embryogenesis, differentiation of complete embryos and plant regeneration.

Materials and methods

Plant Material. Immature inflorescences were collected in Côte d'Ivoire from élite palms of the hybrid Malayan Yellow Dwarf x West African Tall (PB 121 hybrid created by CIRAD), West African Tall x Malayan Yellow Dwarf (PB 111 hybrid) and of Malayan Yellow Dwarf. For each plant, a series of four inflorescences were indexed If2, If3, If4, If5 according to their external spathe length (respectively on average 10, 25, 30 and 45 cm, depending on age). The inner spathe of each inflorescence was surface sterilized for 10 minutes in a hypochlorite solution (6% active chlorine) and then removed under aseptic conditions. Spikelets were sliced transversely (1-1.5 mm long), discarding the basal portion bearing female flowers. Each inoculum was then transferred to a borosilicate test tube (24 x 160 mm) containing 20 ml of callogenesis medium.

Callus induction. The basic medium consisted of Eeuwens inorganic nutrients (1976) and Morel and Wetmore vitamins (1951), supplemented with 116.8 mM sucrose, 2,4-D (1.5, 2.0, 2.5, 3.0, 3.5 x 10^{-4}M) and 2 g.l^{-1} of neutralized activated charcoal (Sigma). It was solidified with 7.5 g.l^{-1} agar (Sigma). The pH was adjusted to 4.5 before autoclaving for 20 minutes at 120°C. Inflorescence explants were randomly assigned to auxin treatments. 30 replications were used per treatment (genotype x inflorescence age x 2,4-D concentration). Cultures were incubated in the dark at 27°C ±1°C for 8 months, without subculture.

ANOVA and Newmann (1939) and Keuls test (1952) (significance level: p=0.05) were used for multiple comparisons of means from the callogenesis treatment.

Callus subculture and induction of embryogenesis. Calluses were isolated 8 months after inoculation and randomly assigned to test tubes containing a medium composed of a doubled MS formulation supplemented with 87.6 mM sucrose, 2 g.l^{-1} of activated charcoal and 2,4-D: low: (1.5 or 2) x 10^{-4}M, medium: (3 or 4) x 10^{-4}M and high: (4.5 or 5.5) x 10^{-4} M.

Embryo maturation and plant regeneration. Embryogenic callus was maintained on the same basic medium with a gradually reduced 2,4-D concentration during 2 subcultures of 6 weeks (reduction of 4.5 10^{-5}M

Correspondence to: J.-L. Verdeil

at each transfer). Callus was then placed on a medium containing 10^{-5}M 6-benzylaminopurine (BAP) (without auxin and charcoal) in order to achieve embryo maturation.

Caulogenesis occurred on hormone-free medium under a 12h light photoperiod (40 µmol/m^2/s) at 27°C ± 1°C. If the root did not develop, the shoots were placed on a rooting medium, previously described for zygotic embryos (Assy-Bah et al. 1987). The whole experiment was repeated four times and results obtained were similar.

Histological analysis. Histolological methods have been reported in a previous study (Buffard-Morel et al. 1992). The slides were double stained by the periodic acid-Schiff reaction, combined with protein-specific naphthol blue-black (Fisher 1968).

Results

Callogenesis

As reported for many palms (Tisserat 1987), coconut callus production was very slow. The first sign of callusing (floral bud swelling) became evident after 3 months, and the first callus was only obvious 4 months after inoculation. Transfer of the explant during the callusing period produced intense tissue browning. The percentage of explants bearing callus is given in tables 1 and 2. Histological studies showed that globular white callus was formed from male floral meristems (Fig. 1 and 2). A very highly significant influence of 2,4-D concentration on the percentage of explants bearing callus was observed ($F_{4,24} = 8.43^{**}$). A medium 2,4-D concentration (2 or 3 x 10^{-4} M) was optimal for callogenesis from floral areas.

The callogenesis responses of the three genotypes differed with regard to the frequency of explants callus-bearing ($F_{2,24} = 3.93^{**}$). Hybrids PB111 and PB121 gave the best results. Inflorescence age also influenced callogenesis response ($F_{3,24}=40.43^{**}$). If2 and If3 were more reactive than older inflorescences (If4 and If5).

Table 1. Influence of inflorescence age and 2,4-D concentration on the percentage (**) of explants bearing callus after 8 months.

Inflorescence	[2,4-D] x 10^{-4} M				
age *	1.5	2	2.5	3	3.5
iF5	0 a	4 a	5.5 a	3 a	0 a
iF4	0 a	7 ad	9 ae	3 a	0 a
iF3	10 ac	24 bc	22 be	24 b	9 ab
iF2	16 ab	22 bcde	22 bcde	30 bc	14 ab

* inflorescence age increase of 1 month with the index.

** percentage calculated from 90 replications per treatment (inflorescence x 2,4-D concentration).

Table 2. Influence of genotype and 2,4-D concentration on the percentage (*) of explants bearing callus after 8 months.

Genotype	[2,4-D] x 10^{-4} M				
	1.5	2	2.5	3	3.5
NJM	6 ab	16 abc	12 abc	11 abc	4.5 ab
PB 121	5.5 ab	11.2 abc	9 abd	12.5 abc	10.2 abc
PB 111	8 ab	17 ac	23.5 c	20 cd	2.2 b

** = Very highly significant (P=0.01)

* percentage calculated from 120 replications per treatment (genotype x 2,4-D concentration).

Percentages followed by a same letter are not significantly different at 5%.

Callus behaviour during subculture

Preliminary experiments showed that callus precociously isolated from the explants soon turned brown and necrotic whatever the 2,4-D concentration. When callus was isolated after eight months, medium 2,4-D concentrations (3 or 4 x 10^{-4}M) allowed its multiplication. At this stage, the callus comprised a peripheral meristematic zone occasionally exhibiting cambium-like organization. Meristematic cells (Fig. 3) progressively differentiated towards the inside of the zone, into parenchymatous and tracheary cells. Callus multiplication could be maintained for a long period without any noteworthy histological change.

Following transfer to media containing low 2,4-D (1.5 or 2 x 10^{-4}M), callus produced more defined white, translucent, pearly or convoluted structures. During subculture, they underwent complex organogenesis including leafy structure differentiation, flower-like structure formation or embryo-like development. The embryo-like structures were characterised by the development of haustorial tissue and sometimes roots, but no shoots or visible buds were found.

Initiation of somatic embryogenesis:

Acquisition of real embryogenic competence was observed when callus was subcultured on high 2,4-D (4.5 or 5.5 x 10^{-4}M) for one to four cycles (two months each) (Fig. 4). It appeared in approximately 10 % of the isolated calluses of all genotypes studied. When callus developed favourably, the peripheral meristematic zone became discontinuous and partially disorganized. Individualization of densely cytoplasmic cells (Fig.5), interspersed with large vacuolized and degenerative cells, gave the zone heterogeneous appearance. The isolated, densely cytoplasmic cells had cytological features consistent with descriptions of embryogenic cells (Halperin and Wetherell 1964; Schwendiman et al. 1990): high nucleus-cytoplasm ratio, a central enlarged nucleus with a prominent densely stainable nucleolus, small vacuoles, and a cytoplasm intensely stained by naphthol blue black, indicating high soluble protein concentration. Single embryogenic cells were often delineated by a thickened outer wall, clearly separating them from the non-embryogenic tissues. Some of these isodiametric cells contained insoluble protein bodies that may be storage proteins, since they disappear with further proembryo development.

Generally, polarized mitotic divisions of isolated single embryogenic cells formed proembryos directly. The cell wall surrounding each proembryo was thicker than the internal cell walls (Fig. 6). However, embryogenic cells could divide to form a proembryonal cell complex composed of several proembryo units delineated by a

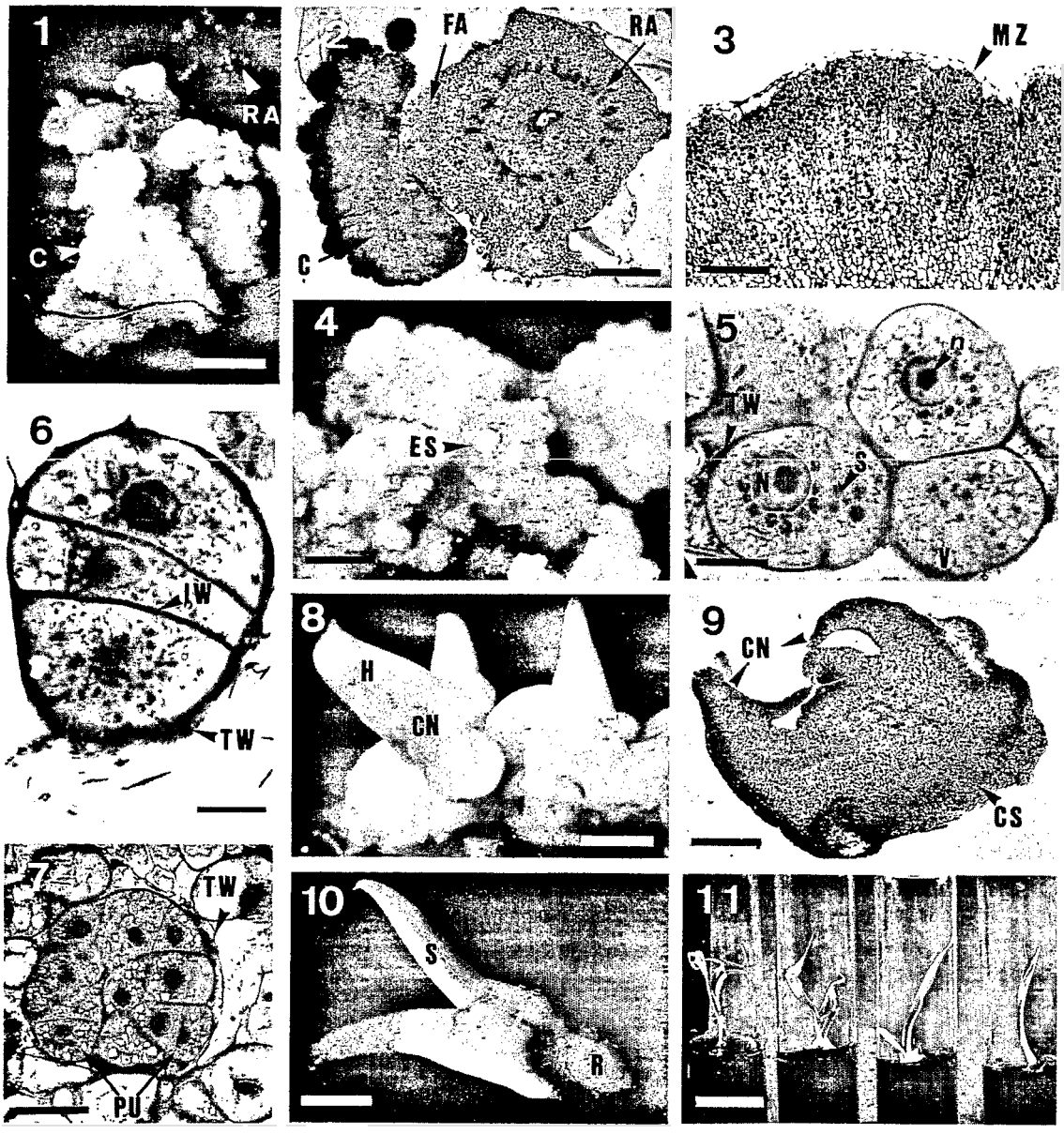

Fig 1 to 11 : Coconut regeneration from somatic embryogenesis.
(1) Globular white callus (C) (RA = Rachillae) (bar = 5mm).
(2) Histological cross section of an inflorescence explant bearing a callus; C = callus; FA = Floral Area (bar = 160µm).
(3) Callus proliferation ensured by partial fragmentation of the peripheral meristematic zone (MZ) (bar = 55µm).
(4) Embryogenic callus; ES = Embryogenic structure (bar = 4 mm).
(5) Embryogenic cells; N = nucleus; n = nucleolus; S = starch grain; v = vacuoles; TW = thickened cell wall (bar = 20µm).
(6) Proembryo formed by segmenting division in a single embryogenic cell; TW = thickened outer cell wall, IW = internal cell wall (bar = 12.5µm).
(7) Proembryonal cell complex composed of several proembyo units (Pu); TW = thickened outer cell wall (bar = 50µm).
(8) Somatic embryo clump; H = haustorium; CN = cotyledonary notch (bar = 4.5mm).
(9) Cross section of a somatic embryo below the cotyledonary notch (CN); SM = shoot meristem; CS = cells containing starch reserve (bar = 900µm).
(10) Single somatic embryo; S = shoot; R = root (bar = 5mm).
(11) *In vitro* coconut plantlets (bar = 33mm).

thickened outer wall (Fig. 7). This type of indirect embryogenesis has been described in carrot as poly-embryogenic cleavage (Haccius 1978).

Proembryo maturation and plant regeneration

Proembryo maturation began when the 2,4-D concentration was gradually reduced during three subcultures. Further maturation was only achieved on a medium containing BAP. Single embryos were generally obtained, but some of them could give rise to a second generation of embryos by proliferation at their root pole (Fig 8). A transverse section just below the cotyledonary notch revealed a well-structured shoot meristem (Fig. 9). Twenty percent of regenerated embryos grew into whole plants. Direct embryo conversion (Fig. 11) was obtained after transfer to hormone-free medium. Simultaneous development of a shoot and root (Fig. 10) in the absence of hormones was evidence for the presence of a functional and organized embryonic axis. In this case, vascular connection between shoot and root was observed after manual dissection of some germinated embryos.

Discussion and conclusions

Our histological studies have confirmed that callus is a proliferation of the immature floral meristem. Such callus, named calloid by Branton and Blake (1984), has been described in different monocotyledonous cultures including taro (Nyman et al. 1983), orchid, and wheat (Wernicke and Milkovits 1986). As with most plant species (Ammirato 1983), the success of the real embryogenic competence reported here depended on the presence of a high level of 2,4-D.

A critical concentration of 2,4-D in the medium disrupted the organization and highly regulated functioning of the peripheral meristematic zone normally ensuring calloid growth. This new level of disorganization allows changes in the morphogenic competence of surviving cells via acquisition of embryogenic characters.

The sequence of histological events- cell separation, wall thickness increase and internal segmenting divisions leading to proembryos- provided further evidence for the single cell origin of somatic embryos.

Our findings on somatic embryogenesis in coconut can support the concept of a single cell origin of the embryos, either directly or after the formation of a proembryogenic cell mass (Haccius 1978), as also proposed for numerous monocotyledonous species, particularly graminaceous species (Lu and Vasil 1985).

It is difficult to determine whether all the embryos obtained here followed this pattern of development. It is possible that coconut somatic embryos are of multicellular origin (Verdeil et al. 1989), as described for oil palm where the unicellular process always aborts (Schwendiman et al. 1990).

A decrease in 2,4-D concentration followed by addition of BAP was a key to complete differentiation of bipolar embryos in coconut. The frequent absence of a shoot apex in somatic embryo might be associated with the use of prolonged auxin treatments (without cytokinin) to initiate somatic embryogenesis. It seems similar to the detrimental effect of prolonged auxin exposure in carrot (Halperin and Wetherell 1964) and soybean somatic embryos (Lazzeri et al. 1987).

We have established the first reproducible *in vitro* system for coconut regeneration, *via* somatic embryogenesis leading to three different clones. The main objective of our future research is to determine the conditions of embryoid multiplication in order to ensure mass ramet production.

Acknowledgements. The authors thank the coconut breeding station "Marc Delorme" Port Bouët (Côte d'Ivoire) for providing the plant material. This study was performed in the framework of a joint research programme between ORSTOM and CIRAD/CP.

References

Ammirato PV (1983) In: Evans DA, Ammirato PV, Yamada Y (eds) Handbook of Plant Cell Culture, Macmillan, New-York vol 1, 82-123

Assy-Bah B, Durand-Gasselin T, Pannetier C (1987) Plant Gen Resources Newslet 71:4-10

Branton RL, Blake J (1984) In: Pushparajah E, Chew Poh Soon (eds) Proc. Int. Cocoa/Coconuts. Kuala Lumpur, pp.1-8

Buffard-Morel J, Verdeil JL, Pannetier C (1992) Can J Bot 70:735-741

Eeuwens CJ (1976) Physiol.Plant 36:23-28

Fisher DB.(1968) Histochemie 16:92-96

Gupta PK, Kendurka SV, Kulkarni VM, Shirgurkar MV, Mascarenhas AF (1984) Plant Cell Rep 3:222-225

Haccius B (1978) Phytomorphology 28:74-81

Halperin W, Wetherell DF (1964) Am J Bot 51:274-283

Krikorian AD (1989) In: Dhawan V (ed) Applications of biotechnology in forestry and horticulture, Plenum Press, New York London, pp119-144

Keuls M. (1952) Euphytica 1:112-122

Lazzeri PA, Hildebrand DR, Collins GB (1987) Plant Cell Tiss Org Cult 10:197-208

Lu CY, Vasil IK (1985) Amer J Bot 72:1908-1913

Morel G & Wetmore RM (1951) Amer J Bot 38:141-143

Newmann D (1939) Biometrika 31:20-30

Nyman LP, Gonzales CJ, Arditti J (1983) Ann Bot 51:279-286

Raju CR, Prakash Kumar P, Chandramohan M, Iyer RD (1984) Journal of Plantation Crops 12:75-81

Schwendiman J, Pannetier C, Michaux-Ferrière (1990) Oléagineux 45:409-418.

Tisserat B. (1987) In: Bonja J.M., Durzan D.J. (eds) Cell and Tissue Culture in Forestry, M. Nijhoff Dordrecht. pp.338-356

Verdeil JL, Huet C, Grosdemanges F, Rival A, Buffard-Morel J (1989) Oléagineux 45:465-469

Wernicke W, Milkovits L (1986) Protoplasma 131:131-141