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INDUCTION AND MAINTENANCE OF FRIABLE CALLUS FROM THE CELLULAR ENDOSPERM OF *COCOS NUCIFERA* L.

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A fast-growing friable callus was initiated and maintained by subculture from the portion of coconut endosperm which was initially in contact with the zygotic embryo. Eeuwens' (Physiol. Plant. 36 (1976) 23) Y3 mineral formulation with kinetin (2 mg/l), high 2,4-dichlorophenoxyacetic acid (2,4-D) (50 mg/l), and 1 g/l activated charcoal were used to initiate the callus in the dark. The callus could be subcultured on the basal medium with lowered 2,4-D levels and in the absence of charcoal. The success in obtaining the callus may be partly attributed to the use of explants excised in a sterile condition with the zygotic embryo in situ (no chemical sterilization involved), and the high auxin concentration in the medium. Preliminary cytological studies indicate a high degree of aneuploidy in the callus cells. Also, within the slow-growing callus cells oil globules had accumulated.

Key words: endosperm culture; *Cocos nucifera* L.; friable callus; aneuploidy; oil globules

Introduction

The triploid endosperm tissue of coconut (*Cocos nucifera* L.) is the source of an important edible oil in the tropical countries where the palm is cultivated. The coenocytic liquid endosperm of coconut attains cellular form after the deposition of oil globules as well as other reserve food materials by the characteristic pattern of centripetal cell deposition process during development of the fruit [1]. At maturity, however, the left-over liquid is mostly cytoplasmic in nature.

Although several authors have successfully obtained callus from the endosperm tissue in dicotyledons, reports of success in monocotyledonous species are restricted to members of the Gramineae [2,3]. In the woody monocotyledons, especially the palms, the culture of even vegetative meristematic tissues has been proved to be a rather demanding task [4—7]. Also, the coconut endosperm is considered to be a tissue of limited growth in vivo [1]. Fisher and Tsai [8] have reported the first case of callus formation from coconut endosperm using the 'Malayan Dwarf Orange' cultivar. However, they failed to reproduce the results. We report here the successful induction of callus from coconut endosperm and maintenance of the callus in subculture.

Materials and methods

Immature coconuts (about 6—7 months old) of West Coast Tall cultivar (*C. nucifera* L. cvar. WCT) were harvested from the CPCRI, Kasaragod, farm. The micropylar

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Abbreviations: BA, benzyladenine; BM, basal medium; 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, naphthaleneacetic acid.

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Table I. Combinations of auxins and cytokinins in the basal medium and the growth and color of coconut endosperm cultured in vitro.

	Auxin (mg/l)		Cytokinin (mg/l)		Growth[c]	Color
	2,4-D	NAA	Kinetin	BA		
	0	0	2	0	No callus	—
CI[a]	10	0	1	0	No callus	—
	50	0	2	0	Callus	Yellowish
	10	10	1	0	++	Yellowish
	10	0	1	0	+++	White
	10	0	0	5	+++	White
CS[b]	5	0	0	2	++++	White
	1	0	0.5	0	++++	Snow white
	0.5	0	0.5	0	+++	Snow white

[a]CI, callus induction media.
[b]CS, callus subculturing media.
[c]+, slow growth; ++++, excellent, fast growth.

end of the nuts were cut with a sharp knife to expose the endocarp (shell). Then the surface of the nuts was swabbed with cotton wool soaked in 90% (v/v) ethanol. The 'soft eye' enclosing the zygotic embryo and surrounding soft endosperm, which formed a thin layer of cells within the shell, was scooped out aseptically. This disc, with a diameter of about 1 cm, was used as the initial explant without any further sterilization.

Eeuwens' Y3 mineral formulation [9] was used as the basal medium (BM). It was supplemented with two auxins, 2,4-D and naphthaleneacetic acid (NAA), and two cytokinins, benzyladenine (BA) and kinetin, at different concentrations (Table I). The media were gelled with 0.8% (w/v) agar, and 0.1% (w/v) activated charcoal was incorporated into it after adjusting the pH to 6.0 ± 0.1 with 0.2 M NaOH. The media were dispensed into 25 × 150 mm borosilicate culture tubes (15 ml per tube), plugged with cotton and autoclaved at 1.08 kg/cm^2 for 15 min. The cultures were incubated at 28 ± 2°C in the dark.

Callus was fixed in 3:1 (v/v) ethanol/glacial acetic acid and squashed in iron-acetocarmine for cytological investigations [10].

Results and discussion

Over 30% of the explants (18 out of 50 explants) exhibited callus initiation from the cut ends of the endosperm after 4 weeks of incubation on the BM supplemented with 50 mg/l of 2,4-D and 2.0 mg/l of kinetin. The explants on media containing 0 or 10 mg/l auxin did not show any signs of growth, though the endosperm tissue remained white for over 2 months. Our earlier experiments on the culture of coconut tissue in vitro had indicated the requirement of high levels of the auxin 2,4-D for any noticeable growth response [7]. Hence, the endosperm explants were cultured on BM supplemented with two different concentrations of 2,4-D for callus initiation. Although a relatively high level of 2,4-D was essential for the initiation of callus, lower auxin levels could sustain good growth during subculture (Table I). Also, the incorporation of activated charcoal was not essential while subculturing. The degree of friability and growth of the callus was better on the low-auxin media (Fig. 1) when compared to those on high auxin-containing media.

The zygotic embryo was visible to the unaided eye at the time of explantation

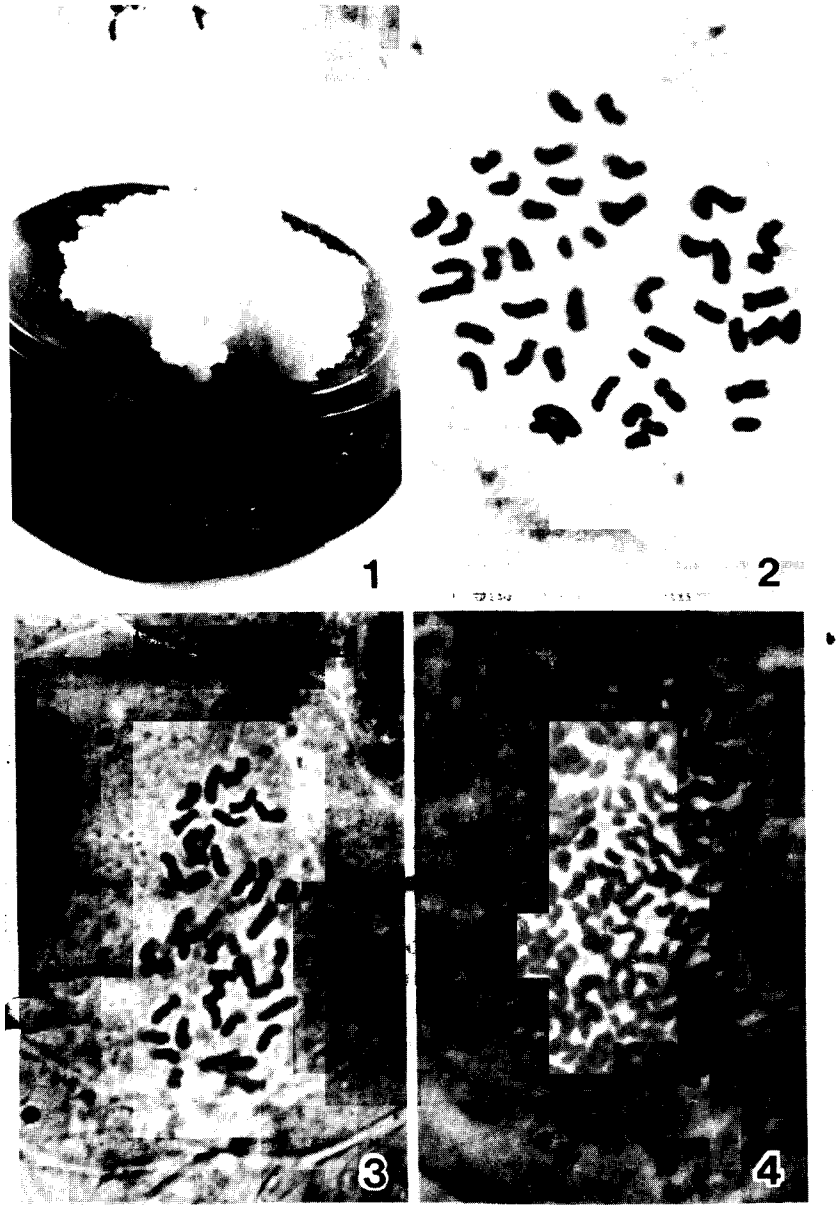

Fig. 1. Coconut endosperm callus subcultured on Eeuwens' Y3 minerals supplemented with 1.0 mg/l 2,4-D; 0.5 mg/l kinetin and 0.5 g/l activated charcoal (× 1.2).

Figs. 2—4. Acetocarmine squash of coconut endosperm callus showing varying chromosome numbers: 45 in Fig. 2, 41 in Fig. 3, and over 175 in Fig. 4 (× 2500).

as a white spherical mass about 1 mm in diameter. A 4-fold expansion was observed in the size of the embryo after 7 weeks of incubation. Initially, the portion of the endocarp explanted along with the endosperm and embryo exuded a considerable amount of tannins into the medium. However, the endocarp tissue did not show any

cell proliferation. Later, the senescent endocarp could be separated from the growing endosperm. Also, the proliferating endosperm was separated from the zygotic embryo during the first subculture. The separated embryos were free of callus, and could be maintained in culture without callus formation indicating that the cells of the embryo did not contribute to the observed callus formation.

The callus was subcultured at intervals of 8—10 weeks. Approximately 100 mg (0.5 cm^3) of the callus was needed for the successful establishment of subcultures. The fresh weight of the callus was observed to increase from 0.1 g to 1.0 g in 30 days during the second subculture.

The cell multiplication process in the endosperm callus reported here is an induced phenomenon, while the cellular endosperm formation in vivo involves 'cell deposition' and only rare centripetal cytokinesis [1]. The successful induction and maintenance in subculture of a fast-growing friable callus now proves the potential of this 'tissue of limited growth' to grow indefinitely. The success of this experiment may be partly due to the excision of the initial explant in an already sterile condition (without any chemical sterilization of the actual explant), the presence of the zygotic embryo, and the high auxin concentration in the medium, although the possibility of reduced availability of free auxin as a result of adsorption of 2,4-D onto the charcoal cannot be ruled out. However, the physiological and biochemical factors that bring about this deviation in the pattern of growth warrant further investigation.

Endosperm, in general, is a triploid tissue in most of the angiosperms. The haploid chromosome number in coconut is $n. = 16$. We recorded chromosome numbers of 41 to over 175 in the endosperm callus (Figs. 2—4). Fisher and Tsai (1978) have reported a chromosome number of 8 in a single callus line obtained from immature solid endosperm of 'Golden Malayan Dwarf' coconut. They concluded that one or more aneuploid endosperm cells proliferated in a rare, chance occurrence [8]. In our study, close to 30% of the cells had chromosome numbers between 41 and 46. However, the relative frequencies of the various chromosome numbers have not been estimated in the present study. The observed inconsistency in the ploidy may not be entirely ascribed to the presence of high levels of 2,4-D in the medium. The numerical aberrations in the chromosome number of this callus could be a natural phenomenon, because varying chromosome numbers of 16—384 have also been reported in coconut endosperm cells in vivo [11,12].

Attempts to induce organ differentiation from the callus by varying the auxin concentrations in the BM have not been successful. This could be partly due to the aneuploid nature of the majority of the cells.

Preliminary histochemical observations (sudan III staining) have shown the presence of abundant oil globules in the callus cells that are maintained for over 8 weeks on the same medium without subculturing. The fast-growing cells were without oil globules in them. This is in accord with the observation that secondary metabolites accumulate in slow-growing or stationary phase cells only [13]. Hence the possibilities of using this system for large scale extraction of the edible coconut oil or other metabolites like amino acids and cytokinins which are known to occur in this tissue [14] are worth exploring.

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