IN VITRO CULTURE OF COCONUT ENDOSPERM: CALLUS INDUCTION AND ITS FATTY ACIDS

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Successful induction of callus from coconut endosperm was achieved by using the tissue situated near the micropylar end of a young fruit. For initiation of callus, a high concentration of auxin (20 to 100 ppm) was added to the basal medium containing activated charcoal. Subcultured callus showed a 40-fold increase during culture of three months. Based on the analysis of fatty acid composition, the maturation of endosperm was characterized by an increase in short chain fatty acids (C_8, C_{10}, C_{12}, C_{14}) and a decrease in long chain fatty acids (C_{16}, $C_{18:1}$, $C_{18:2}$). In developing endosperms, proportion of short chain fatty acids was higher in lipids of the antipodal than those of other regions. In the final stage of maturation, around 82% of total fatty acids was short chain fatty acids, while the proportion of long chain fatty acids decreased up to 16%. The fatty acid composition of callus subcultured for six months was comparable to that of the immature endosperm. Lipids were accumulated in callus as globular bodies.

Keywords: ***Callus, coconut, endosperm, fatty acid***

INTRODUCTION

The endosperm tissue of coconut stores a substantial amount of oil which is important as food and for industrial use. Coconut oil is quite distinct from other vegetable oils in physico-chemical nature, because it is a highly saturated oil with a high percentage of lauric acid (C_{12}). Little is known about the mechanism by which the coconut endosperm predominantly accumulates triacylgycerols possessing short chain fatty acids. Elucidation of biochemical mechanism of lipid accumulation would be facilitated if the culture system of coconut endosperm could be established. At present, only limited information is available concerning the induction and maintenance of callus from the endosperm (Fisher and Tsai 1978; Prakash-Kumar et al. 1985). We report here the conditions for successful induction of callus from coconut endosperm and the composition of fatty acids in callus cultures in comparison with that of endosperm tissues at different stages of fruit development.

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MATERIALS AND METHODS

Different ages of fruits were harvested from *Cocos nucifera* L. c.v. San Ramon Tall. Since flowering takes place at intervals of 21 to 25 days, fruits of various stages could be obtained simultaneously from a palm tree. Approximately 6- to 9-mo-old fruits taken from the 9th to 13th bunch were used for the endosperm cultures. The bunches were numbered consecutively starting from the flowering bunch downwards. The fruits were dehusked and surface-sterilized with 80% ethanol. The "soft eye" portion of the endocarp was cut, enough to get tissue pieces at the antipodal, middle and micropylar regions of the endosperm. No further sterilization was done for the isolated tissue pieces. These pieces excluding the endothelial layer were cut into small segments (1 cm^2) and inoculated onto modified Y-3 medium (Branton and Blake 1983; Sugimura and Salvana 1989) containing 20 ppm 2,4-dichlorophenoxyacetic acid (2,4-D), 1 ppm 6-benzylaminopurine (BA), 1 ppm N^6 (2-isopentenyl) adenine (2-ip), 0.25% activated charcoal and 0.2% Gelrite. Cultures were kept in the dark at 28 °C. Subculturing was carried out every 4-6 wk.

For analysis of fatty acids, calli (ca 500 mg dry wt) and endosperm tissue (50-100 mg dry wt) were used. Total lipids were extracted with petroleum ether (5 mL), and esterified with sulfuric acid-methanol according to the method of Chaven et al. (1982). The composition of fatty acid methyl esters was analyzed by GC (Shimadzu GC - 6 AM) : stainless steel column (1.5 m x 4.0 mm i.d.) packed with 30% diethylene glycol succinate on Chromosorb W-AW (60-80 mesh), temperature programmed : injection 200 °C, column 130-180 °C (8 °C/min), detector 250 °C, carrier gas : He (60 mL/min). Identification of fatty acids was done by direct comparison of the retention time with authentic standards. Triplicate analyses were carried out, and average content of each fatty acid was expressed as a percentage of total fatty acids on the basis of the peak area on gas-chromatogram.

RESULTS

Induction of callus

Using tissues from immature endosperm, white and friable callus was initiated from the explant surfaces after 6 wk of inoculation onto a medium supplemented with 20 ppm 2, 4-D, 1 ppm BA and 1 ppm 2-iP. Preliminary experiments showed that the induction rate ranged from 7 to 52%. This variation might be due to differences in the maturity of endosperm among fruits used. The histological observation of the developing endosperm showed that, around the 6th mo after pollination, a thin coating of a jellylike tissue began to form along the endothelial layer. This tissue was thicker at the antipodal end, and it gradually diminished towards the micropylar end. During successive stages of development up to 9 mo, the endosperm continued to increase in thickness and hardness (Table 1). Complete maturation was accomplished at 10-11 mo. This feature is in accord with the observations described in earlier reports (Baptist 1963; Abraham and Mathew 1963). When endosperm explants were prepared from fruits of different stages, callus formation was observed in those of 6.8- to 9.0-mo-old fruits. The explants from 7.5-mo-old fruits, in particular, formed callus at a higher frequency (Table 1). In addition to the age of the fruit, callus formation was also greatly affected by tissue regions from which explants were taken (Fig. 1). The tissue near to the micropylar end gave callus more frequently than the tissues in the middle or near the antipodal end.

Our previous culture experiments with other sources of coconut explants indicated the requirement of activated charcoal and a high level of auxin for noticeable callus formation (Sugimura et al. 1987; Sugimura and Salvana 1989). Therefore, endosperm tissues were cultured onto media supplemented with 2,4-D, 1-naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA) at concentrations ranging from 20 to 100 ppm in the presence of activated charcoal, but no remarkable differences were observed in the frequency of callus formation (data not shown). Since activated charcoal absorbs a great amount of auxin added to the medium (Ebert and Taylor 1990), the effective dose of auxin is thought to be extremely low. In fact, some of the cultures exhibited callus formation without any addition of phytohormone. However, the use of activated charcoal was essential to prevent tissues from browning.

Calli formed on the endosperm tissues were subcultured onto media with various kinds of auxin. The fresh weight of callus showed a 40-fold increase after 3 mo of culture, either on the phytohormone-free medium or IBA-containing medium (Table 2). For long-term subculturing, the addition

TABLE 1
Callus Induction in the Explants from Different Endosperm Regions of Coconut Fruits at Various Stages

TIME AFTER POLLINATION (mo)	THICKNESS OF ENDOSPERM REGIONS (mm)[a]			FREQUENCY OF CALLUS FORMATION (%)[b]		
	Micropylar	Middle	Antipodal	Micropylar	Middle	Antipodal
6.8	3.4	4.6	5.5	19	4	6
7.5	5.6	6.6	6.8	33	17	5
8.2	7.3	8.2	8.6	6	0	3
9.0	9.2	10.0	10.0	6	0	1

a Average for three fruits per each stage
b Average for three replicates with twenty explants per individual culture

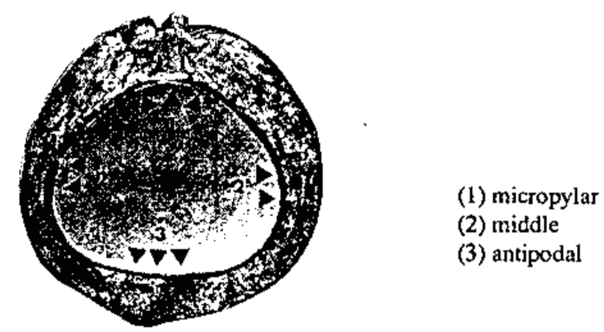

FIGURE 1
Longitudinal section of 7.5-mo-old coconut, showing three different regions of endosperm taken from explant preparation

TABLE 2
Growth of Calli Subcultured for 3 mo on Media Containing Various Kinds of Auxins

AUXIN ADDED	CONC. (ppm)	FRESH WEIGHT (mg)[a]	
		Initial	Final
None		100±5	4325±450
2, 4-D	5	110±12	2235±629
	10	110±0	3272±416
	20	140±35	3669±106
NAA	5	102±10	3307±418
	10	108±13	3383±880
IBA	5	103±5	4156±716
	10	100±7	4290±580

Ten calli were used for each medium. [a]means±S.D.

of activated charcoal was dispensable if the concentration of 2,4-D was below 2 ppm.

Fatty acid composition of fruit

For the analysis of the fatty acid composition in lipids, whole endosperm tissues were collected from the fruits of various stages after pollination. The maturation of the endosperm was accompanied by a progressive increase in the proportions of caprylic (C_8), capric (C_{10}), lauric (C_{12}) and myristic (C_{14}) acids, and by a decrease in those of palmitic (C_{16}), oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids (Fig. 2). The lipids of endosperm at later stages contained saturated short fatty acids (C_{12}, C_{14}) as major components, whereas long chain fatty acids (C_{16}, $C_{18:1}$, $C_{18:2}$) formed large proportions of the lipid extracted from the endosperm of 6.8-mo-old fruits. These changes in the percentage composition of fatty acids during the developing stage of the endosperm implied a substantial accumulation of storage lipids in endosperm cells.

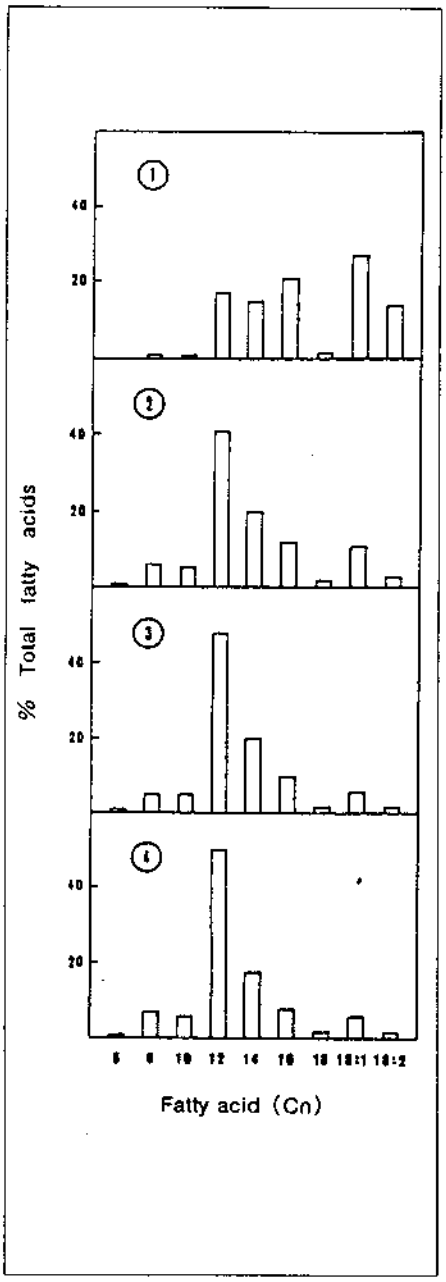

FIGURE 2
Fatty acid composition of total lipids extracted from endosperm of coconut fruits harvested at various stages. (1) 6.8-, (2) 7.5- (3) 9.6-, (4) 10.3-mo-old fruit.

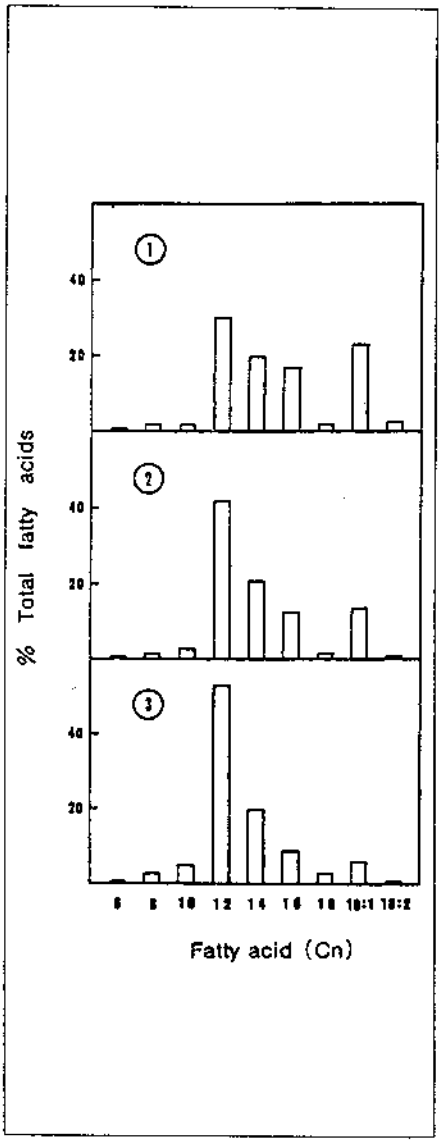

FIGURE 3
Fatty acid composition of total lipids extracted from different endosperm regions of a 7.5-mo-old fruit of coconut. (1) micropylar; (2) middle; (3) antipodal

In addition to analysis of whole endosperm, fatty acid composition was also evaluated by using different regions of endosperm taken from 7.5-mo-old fruits. There was a high proportion of long chain fatty acids in lipids of the micropylar region compared with those of other regions (Fig. 3). In contrast, a proportion of short chain fatty acids increased in the antipodal region. This indicates that endosperm maturation within a fruit was not synchronous, developing progressively from the antipodal to the micropylar region.

Fatty acid composition of callus

Short chain fatty acids were identified from endosperm calli. Judging from fatty acid composition, callus subcultured for 6 mo was comparable to the immature endosperm of 6.8-mo-old fruits (Fig. 4). The callus culture was composed of tiny and compact cells (9 - 17 μ m) containing lipid bodies stained with Sudan III (Fig. 5). Prolonged subculturing of callus for over 18 mo resulted in a reduction in short chain fatty acids, whereas unsaturated long chain fatty acids ($C_{18:1}$, $C_{18:2}$) increased markedly.

Discussion

For better induction of endosperm callus, the micropylar tissues of earlier-developing endosperm were suitable as a source of explant. Abraham and Mathew (1963) observed that frequency of cell division was higher in the region near to the micropyle, gradually decreasing towards the antipodal at an early stage of endosperm development. This pattern seems to correspond well with the differences in the callus induction rate among different regions, suggesting that callus formation is initiated from highly meristematic cells of the endosperm.

Callus initiation could be achieved consistently using a medium supplemented with phytohormones in the presence of activated charcoal. The occasional formation of callus was also observed even when explants were cultured onto the basal medium without phytohormones. Furthermore, the addition of phytohormones was dispensable for long-term subculture of calli which had been formed by culturing onto the media with or without phytohormones. A possible explanation can be made that coconut endosperm cells have potential capacity for proliferating themselves without the aid of exogenous phytohormones.

Histochemical observation showed that coconut endosperm cells have enormous lipid bodies (Murakami and Sugimura 1987). While short chain fatty acids are the characteristic constituents of storage lipids, long chain fatty acids are derived mainly from the membrane lipids of endosperm cells. Therefore, short chain fatty acids are useful markers for monitoring lipids stored in endosperm cells. Based on fatty acid analyses and histological observations, the accumulation of storage lipids was limited at the initial stage of endosperm development, and was followed by a sharp increase at progressive stages. At the final stage of maturation, around 82% of total fatty acids was occupied by short chain fatty acids, while long chain fatty acids decreased up to 16%. There were quantitative differences in storage lipids among tissue regions of endosperm taken from a 7.5-mo-old fruit. Proportion of short chain fatty acids was around 72% in the antipodal but 50% in the micropylar tissue. Considering callus induction rates at this stage, the micropylar tissue might consist of proliferative cells in which storage lipids do not accumulate fully.

Prakash-Kumar et al. (1985) reported that there were no lipid bodies in subcultured endosperm callus of coconut. On the contrary, we found the accumulation of storage lipids which were identified by analysis of fatty acids, as well as by histochemical assay. Fatty acid composition of callus subcultured for 6 mo showed different composition of fatty acids, compared with endosperm tissues at various stages. The long-term culture may allow formation of callus which is composed of highly proliferative cells producing less amount of storage lipids.

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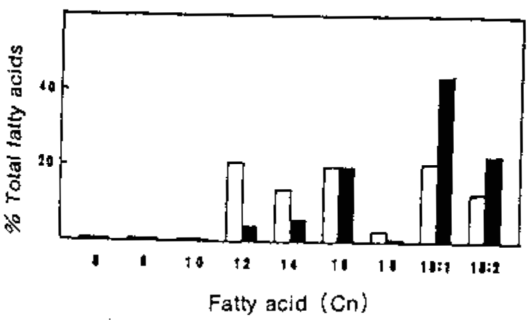

FIGURE 4
Fatty acid composition of total lipids extracted from endosperm callus subcultured for different duration. ☐ : subcultured for 6 months; ■ : subcultured for 18 months

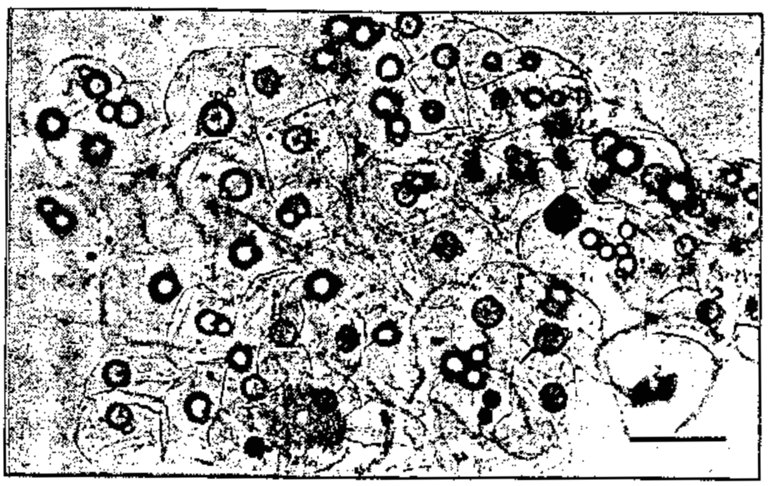

FIGURE 5
Oil globules in cultured cels of coconut endosperm callus (bar: 10 μm)