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IN VITRO GROWTH OF EMBRYOS AND CALLUS OF COCONUT PALM[1]

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SUMMARY

A medium for optimal growth of embryos of Jamaican Tall and Green Malayan Dwarf varieties of coconut palm was developed. The liquid basal Murashige and Skoog medium was supplemented with coconut milk, IAA and 2IP. Activated charcoal improved embryo growth on agar medium. A single callus line was initiated from solid endosperm and subcultured on basal Schenk and Hildebrandt medium supplemented with 2 mg per l NAA. Attempts at inducing organogenesis in the callus were unsuccessful. No vascular tissue was present. The callus was aneuploid with the chromosome number = 8 (normal 2n = 32).

Key words: coconut; embryo culture; endosperm callus; palm tissue culture.

INTRODUCTION

Since the turn of the century, more than half of the coconut palms (*Cocos nucifera* L.) grown commercially in Jamaica have been killed by lethal yellowing disease (LY). The disease is now affecting coconut and other species of palms throughout the Caribbean region and appears to be present in West Africa (1). The Florida mainland has been affected by LY since 1971 with a devastating effect (2). As a result of this crisis, tissue culture studies were begun in an effort to grow seedlings in vitro and to propagate coconuts vegetatively.

The presence of mycoplasma-like organisms (MLO's) in phloem tissues of diseased palms and the effects of antibiotics on the disease indicate that MLO is the causal agent. Preliminary studies did not show that leafhoppers and planthoppers were vectors of LY (3,4). In an effort to improve LY transmission studies, an in vitro coconut system was sought. Coconut seedlings from tissue culture could provide: (a) material to study more easily the pathogen-host plant relationship with the electron microscope; (b) large numbers of uniform test plants in a relatively small space for experiments; and (c) an in vitro means of culturing the LY causal agent in living tissues.

Some individual trees and certain varieties show resistance to LY. Unfortunately, there is no method to propagate vegetatively such unique genotypes, since propagation is by seed only. Therefore an effort was made to initiate and culture callus of coconut as the first step in developing a method for vegetative propagation.

There has been limited work done in culturing young coconut embryos (5,6), although the only successful work on growing embryos up to young rooted seedlings has used the Makapuno variety of coconut which does not germinate in situ (7). Our study was carried out to produce actively growing embryos of normal coconut varieties as a future experimental tool. To date, there has been no successful vegetative propagation of coconuts, although there has been successful callus culture (8) and plantlet regeneration (9) in African oil palm (*Elaeis guineensis* Jacq.). Although callus formed on the cut surface of coconut explants (10), we report for the first time the induction and subculture of coconut callus.

MATERIALS AND METHODS

Embryos. Fruits of *Cocos nucifera* L. cvs. Jamaican Tall and Green Malayan Dwarf were collected in Fort Lauderdale and split into halves with an axe. The endosperm enclosing the embryo was removed with a knife, and the embryo was extracted in the laboratory. Both young and old embryos were surface sterilized by the following steps: 30 min in 1.5% sodium hypochlorite solution; 15 min in 0.5% sodium hypochlorite solution; three rinses in sterile distilled water; and 1 to

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2 hr in 100 mg per l penicillin-streptomycin or gentamicine solution. Embryos then were transferred directly to media (Fig. 1). Cultures were kept at 24±2° C and 70±5% R.H. under Plant Grow (Sears, Roebuck & Co.) fluorescent lighting (861 lux) for 12 hr per day. For agar culture, embryos were grown individually in 150- by 25-mm tubes containing 20 ml medium. For liquid culture, groups of 10 to 15 embryos were grown in 250-ml flasks containing 100 ml medium. The flasks were agitated gently on a reciprocating shaker (312 oscillations per min). The basal medium of Murashige and Skoog (MS) (11) was supplemented with coconut milk (CM, liquid endosperm from green coconuts), 10 mg per l indole-3-acetic acid (IAA), 20 mg per l indole-3-butyric acid (IBA), 5 mg per l N^6-benzyl-amino-purine or benzyl-adenine, and 2.5 or 5.0 mg per l N^6-r,r-dimethylylamino purine (2IP). Solid media contained 2.5 or 3.6 g per l activated charcoal and were jelled with 6 to 15 g per l agar. Filter-sterilized CM and other organic substances were added to the autoclaved basal medium. The medium had a pH 6.5 to 7.0. There were 70 to 100 embryos in each experiment. Fifteen embryos were selected randomly for measurements of length, width and fresh weight at the start and after 10 wk.

Callus. Expanded haustorial tissue (cotyledon) of seedlings, intact embryos, and endosperm (soft jelly to hard mature stages) were collected from Jamaican Tall and Golden Malayan Dwarf coconuts grown in Miami. Material was surface sterilized as above, but antibiotics were not used for endosperm or haustorial tissues. Cultures were kept at 20° to 26° C under cool-white fluorescent lighting (700 to 2100 lux) for 14 hr per day. Agar cultures were grown in 22- by 25-mm screw-capped vials with 10 ml medium. Liquid cultures were grown in 50- or 125-ml flasks with 20 or 50 ml medium, respectively, and were placed on a reciprocating shaker (106 oscillations per min). The basal medium of Schenk and Hildebrandt (SH) (12) was supplemented with 20% CM, 2 to 10 mg per l NAA, and 1 to 2 mg per l 2,4-dichlorophenoxyacetic acid (2,4-D) individually or in combination; or with SH hormones (0.5 mg per l 2,4-D, 2 mg per l p-chlorophenoxyacetic acid, and 0.1 mg per l kinetin). The basal medium of White (13) was supplemented with 1 mg per l IAA and 1 mg per l kinetin. Media were jelled with 6 g per l agar. Material used for histological study was fixed in chromic acid-acetic acid-formalin, and after embedding and sectioning was stained with hematoxylin, safranin and fast green. Chromosome counts were made on material fixed in acetic acid-absolute ethanol (1:3) and stained with acetocarmine (14).

Results and Discussion

Embryos. Much of the preliminary work was devoted to overcoming the incidence of contamination. Growth was inhibited by use of a 10% sodium hypochlorite solution. Reducing the concentration of sodium hypochlorite to an initial 1.5% and a second 0.5% had no apparent effect on subsequent embryo growth. The final treatment with antibiotics further decreased contamination.

Embryo germination in vitro was similar to normal germination except that the haustorium failed to enlarge (Figs. 2–6). The haustorium normally expands and eventually fills up the entire seed cavity. This highly modified cotyledon absorbs endosperm materials and transfers them to the developing seedling. The haustorium surface of cultured embryos usually became darkened and corklike. The cotyledonary sheath and several scale leaves developed (Figs. 4–6). The primary root emerged at the same time as the scale leaves or at a later date (Fig. 4). Root development generally was enhanced by IBA or IAA in medium. Embryos grown in liquid for 6 to 8 wk and then

FIGS. 1–6. Coconut embryos. R = root; H = haustorium; S = scale leaves. Scale lines in mm.
FIG. 1. Initial size. ×1.25.
FIGS. 2,3. After 10 wk in liquid medium. ×1.25.
FIG. 4. After 14 wk on agar medium. ×1.25.
FIG. 5. After 19 wk on agar medium. ×1.0.
FIG. 6. After 18 wk on agar medium. ×1.25.

FIGS. 7–13. Callus derived from endosperm.
FIG. 7. Mitotic metaphase nucleus with eight chromosomes. *Bar* = 20 μm. ×400.
FIGS. 8–11. Projections from callus after 20 wk on agar SH medium plus 2 mg per l NAA. *Bar* = 2.5 mm. Fig. 8, ×5.6; Fig. 9, ×4.0; Fig. 10, ×6.0; Fig. 11, ×6.0.
FIG. 12. Transverse section through a projection showing dense center. *Bar* = 200 μm. ×50.
FIG. 13. Transverse section of callus showing dense center and meristermatic nodule (N). *Bar* = 100 μm. ×80.

transferred to agar had better root growth than those grown continuously on agar. Growth of embryos varied greatly with different media (Table 1). Growth was better in liquid media than on agar. Mean weight increased up to 4.4 times, and mean length increased two to three times that of

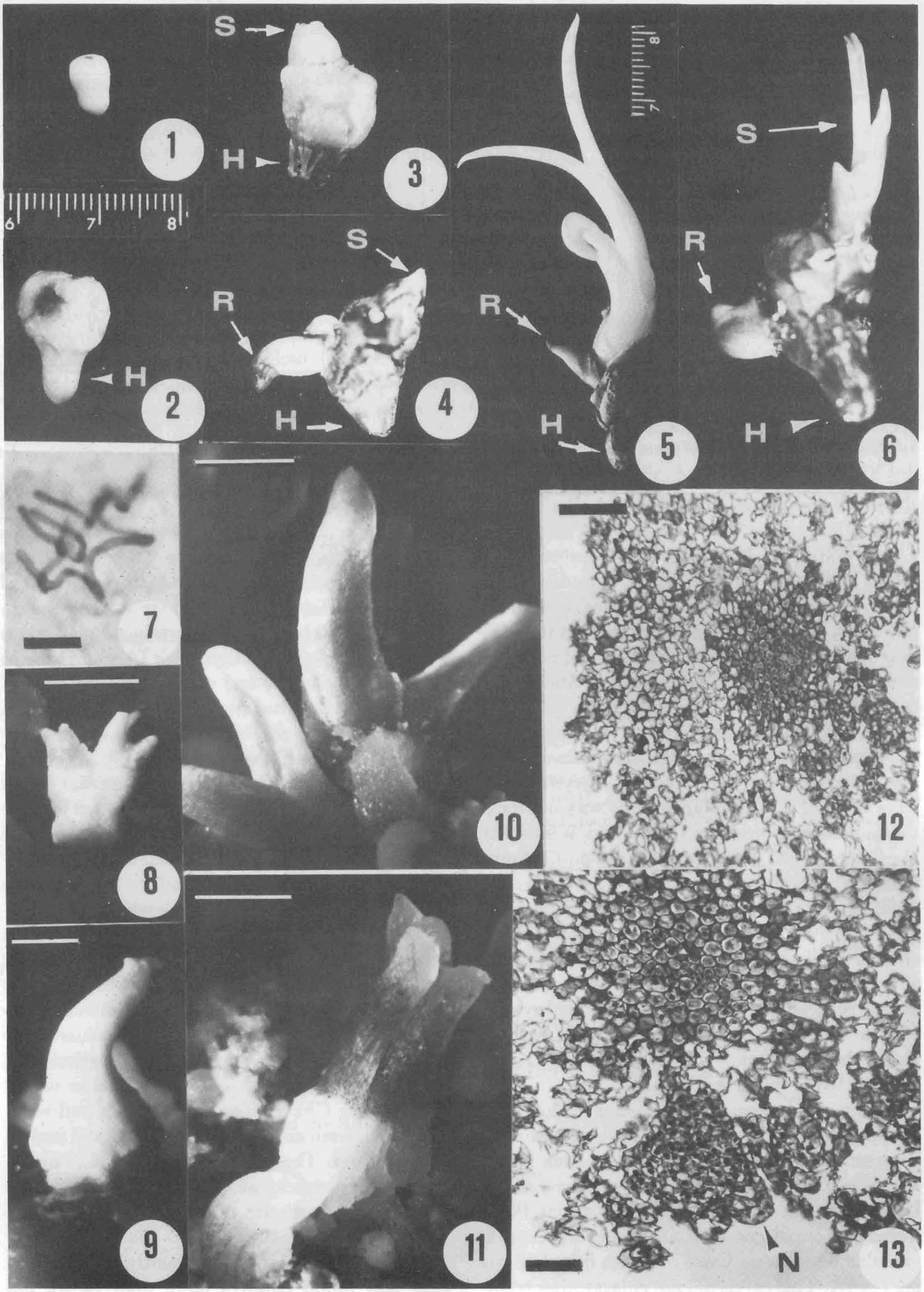

TABLE 1

GROWTH EFFECT OF MEDIA ON EMBRYO CULTURES OF COCONUT PALMS (*COCOS NUCIFERA* L.) IN 10 WEEKS

Culture Medium[a]	Mean Initial Fresh Weight[b]	Mean Fresh Weight at Harvest	Mean Initial Fresh Size	Mean Fresh Size at Harvest
Basic+IBA+IAA+2IP+BA+CM+CC+A			Length/Width	Length/Width
	g	*g*	*mm*	*mm*
1. Basic+20+0+0+5+250+3.6+10	0.161	A 0.30	9.30/5.00	A 12.50/7.00 A
2. Basic+20+0+0+5+250+0+0	0.155	B 0.36	9.40/4.90	C 13.70/7.80 BC
3. Basic+20+0+5+0+250+3.6+15	0.160	A 0.32	9.30/4.90	B 13.00/7.60 B
4. Basic+20+0+5+0+250+0+0	0.158	D 0.46	9.40/5.00	D 14.70/8.00 C
5. Basic+20+0+5+0+250+3.6+8	0.158	C 0.40	9.20/4.90	C 13.90/7.80 BC
6. Basic+20+0+5+0+250+0+0	0.160	D E 0.48	9.10/4.80	D 15.00/8.40 D
7. Basic+0+10+2.5+0+250+3.6+8	0.159	D E 0.48	9.40/4.90	E 15.50/8.50 D
8. Basic+0+10+2.5+0+250+0+0	0.161	G 0.69	9.40/5.00	I 24.00/9.10 E
9. Basic+0+10+2.5+0+400+3.6+8	0.156	E 0.50	9.20/4.80	F 16.50/8.60 D
10. Basic+0+10+2.5+0+400+0+0	0.160	F 0.60	9.10/5.00	H 21.00/9.00 E
11. Basic+0+10+2.5+0+250+2.5+6	0.159	F 0.58	9.50/4.90	G 18.00/8.90 E
12. Basic+0+10+2.5+0+250+0+0	0.158	G 0.71	9.30/5.00	J 26.20/9.50 F

[a] Basic = nutrient medium; IBA = indole-3-butyric acid (mg per l); IAA = indole-3-acetic acid (mg per l); 2IP = N^6-r,r,-dimethylylamino-purine (mg per l); BA = N^6-benzylamino-purine (mg per l); CM = coconut milk (ml per l); CC = charcoal (g per l); A = agar (g per l).

[b] Average of 15 embryos. Means flanked by a common letter are not significantly different at the 5% level.

the initial embryo after 10 wk in culture (Table 1, media 8 and 12).

There was no browning of tissues on liquid media during the culture period, presumably because intact and uninjured embryos were used. However, preliminary work showed that the addition of relatively high concentrations of activated charcoal to agar media noticeably promoted embryo growth. This finding was similar to that of Wang and Huang (15) who grew embryos of *Caryota* and *Mascarena* palms. Preliminary work also indicated that CM promoted embryo growth. This effect of CM from green nuts was similar to the response reported by Cutter and Wilson (6). De Guzman, del Rosario and Eusebio (7) found CM inhibitory for embryo growth of the Makapuno variety of coconut. Varietal differences and the maturity of CM are the likely reasons for this observed difference. In our study CM was used in all media and charcoal was used in all agar media.

The successful growth of vigorous embryos in vitro presents a useful tool for studies of coconut palm physiology and will be utilized in future work on lethal yellowing disease.

Callus. All attempts at inducing callus from whole or halved embryos were unsuccessful, in contrast to the reported successful callus induction in embryos of oil palm in the presence of 2,4-D and kinetin (8). Cubes of haustorial tissue formed a small amount of callus adjacent to vascular bundles on basal SH with 5 and 10 mg per l NAA. This callus did not enlarge after transfer to fresh medium, nor could it be subcultured after separation from the original explant. This was similar to findings of Apavatjrut and Blake on coconut stem explants (10). A single explant of solid, but not yet fully mature, endosperm (Golden Malayan Dwarf) produced callus on basal White's agar medium with IAA and kinetin after 8 wk in culture and was successfully subcultured. All other attempts at new callus induction using endosperm of various ages and on a variety of media were unsuccessful.

Optimal growth of the friable-to-nodular callus occurred on basal SH agar medium with 2 to 5 mg per l NAA, and this medium was used for routine subculturing. This single clone of endosperm-derived callus has been maintained for over 4 yr with subculturing every 8 to 12 wk. The subcultured callus doubled in size in 3 to 6 wk, although individual subcultures were quite variable. Quantitative measurements of growth were not made because of this variability; however, increase in callus volume was usually better in liquid cultures. All attempts at organogenesis by transferring callus to basal SH medium or varied media using CM and/or kinetin, with and without auxin, were unsuccessful both in liquid and on agar cultures. The callus was composed of undifferentiated parenchyma cells with scattered, more compact meristematic regions (Fig. 13). There was no vascular tissue.

Finger-like projections frequently developed in agar and liquid cultures more than 12 wk old

(Figs. 8-11). These white embryoid-like structures did not contain vascular tissues or organized meristems (Fig. 12). Their outer surfaces were smooth but lacked a defined epidermis. Occasional unorganized meristematic nodules were present which were similar to those in callus.

The chromosome number of the callus was eight (with occasional counts of seven) as determined from cells in mitotic metaphase and anaphase (Fig. 7). Although the callus had a chromosome complement equal to one-fourth the normal somatic number of 32, there is no published evidence indicating that *Cocos* is a tetraploid species derived from a base chromosome number of eight. Other cocosoid palms have $n = 16$ or 15 (16). Previous studies of coconut endosperm development have shown that high polyploid ($10n = 160$), triploid ($3n = 48$), diploid ($2n = 32$), and abnormal aneuploid nuclei are regularly produced (17,18).

We conclude that one or more aneuploid endosperm cells proliferated in a rare chance occurrence. Work is now in progress to multiply the chromosome number of the callus so that it might be more amenable to plantlet regeneration. It would be especially interesting to see if plants produced from such a callus would be similar to the original Golden Malayan Dwarf coconut.

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