Effects of Organic Nutrients and Hormones on Growth and Development of Tissue Explants from Coconut (*Cocos nucifera*) and Date (*Phoenix dactylifera*) Palms Cultured *in vitro*

By

C. J. EEUWENS

Department of Horticulture, Wye College (University of London), near Ashford, Kent TN25 5AH, England

(Received 26 July, 1977; revised 20 September, 1977)

Abstract

The growth response (increase in weight) of cultured explants of seedling date (*Phoenix dactylifera* L.) and mature coconut (*Cocos nucifera* L. cv. Malayan Dwarf) palms to source and concentration of organic nitrogen, carbohydrate, auxins, cytokinins and gibberellins was examined. Growth was strongly stimulated by the presence of auxins (10^{-7} to 10^{-6} *M*), cytokinins (10^{-6} to 10^{-5} *M*), high concentrations of sucrose (0.2 *M*), and, in the absence of auxin, by organic sources of reduced nitrogen. Higher concentrations of auxin (2,4-D or NAA at 10^{-6} to 10^{-5} *M*) which still promoted growth of Phoenix tissue, proved inhibitory to growth of excised Cocos tissues.

Explants from both palms initiated roots when subcultured on a medium with increased levels of auxin (NAA, 2.5×10^{-6} to 2.5×10^{-5} *M*) and reduced levels of cytokinin (6-BAP, 5×10^{-8} *M*). Shoots excised from these explants continued growth and produced new laterals when subcultured on media with GA_3 (5×10^{-7} *M*) and reduced levels of auxin, cytokinin, and either minerals alone.

Introduction

The present coconut, *Cocos nucifera* L., and oil, *Elaeis guineensis* Jacq., palms are propagated exclusively from seed. Even in the case of the date palm, *Phoenix dactylifera* L., the use of offshoots for vegetative propagation of selected cultivars is extremely limited (Reuveni *et al.* 1972). In view of the considerable genetic variation that exists between individual palms on plantations the development of a rapid method of clonal propagation of elite palms is of prime importance.

Tissue culture techniques may provide a solution to this problem. Embryos excised from seeds of all three palms (Tisserat and Wilson 1954, Rabéchault *et al.* 1970, Guzman and Reuveni and Lilien-Kipnis 1974), and embryoids and callus cultures derived from embryonic and seedling tissue of oil palm have been successfully grown and cultured into plants (Rabéchault *et al.* 1972, Jones 1974). However, as yet, no method of vegetative propagation utilizing tissues from mature palms of proven quality has been developed. With this aim, the growth requirements of cultured tissue explants from Cocos and Phoenix palms are being quantitatively examined.

The present paper reports on some of the main organic nutrient and hormone requirements for optimal growth and root initiation of tissue explants from both palms cultured on a mineral formulation described previously (Eeuwens 1976).

Abbreviations: BMY3, basal medium as described in "Materials and Methods"; macro Y3, macro elements of the Y3 mineral salt formulation (Eeuwens 1976); macro MS, macro elements of Murashige and Skoog's (1962) revised medium; AAM, amino acid mixture (L-glutamine/L-arginine-L-asparagine, 1 : 1 : 1 w/w); 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, 1-naphthylacetic acid; IBA, 4-(indol-3-yl)-butyric acid; IAA, indol-3-yl acetic acid; BTOA, benzothiazole-2-oxyacetic acid; 6-BAP, 6-benzylaminopurine; DMAA, 6 (γ,γ-dimethylallyl-amino) purine; SD8339, 6-benzyl-amino-9-tetrahydro-2-pyranyl-9H-purine; GA_3, gibberellic acid.

Materials and Methods

Origin and preparation of tissue explants. *Phoenix dactylifera* L. palms were raised from seed and grown to the 15 to 20 unfolded-leaf stage in a heated glasshouse (night temperatures not less than 16°C) in the U.K. Soft white tissue near the base of the petioles of the young leaves (75–150 cm long) served as the source of tissue explants.

Cocos nucifera L. cv. Malayan Dwarf, palms were grown on plantations in Jamaica. Inflorescences from mature (15–20-year-old) palms were packed in polystyrene containers and flown to the U.K., where they were stored for up to 10 days in the dark at 5 to 10°C until required. Explants were obtained mainly from the stem (rachis) of young inflorescences (inner spathe 10–20 cm long) but also from

branches (rachillae) of even younger inflorescences (inner spathe 2–8 cm long). Each rachilla was composed of numerous flower meristems (mainly male), each enclosed by a bract-like leaf, arranged along a central axis.

Uncontaminated segments of petiole and inflorescence rachis tissue were obtained, without the use of chemical sterilants, by careful removal of enclosing tissues under aseptic conditions. As an added precaution, petiole and rachis, but not rachilla, segments were dipped in aqueous 70% ethanol (15 s) and thoroughly rinsed in sterile distilled water before being cut transversely into slices (2–3 mm thick). Each slice was then cut into explants of uniform size (15–30 mg fresh weight).

Culture media and techniques. All media contained the Y3 mineral salt formulation (Eeuwens 1976) which has the following composition, (m*M*): KNO_3 20, KCl 20, NH_4Cl 10, $NaH_2PO_4 \cdot 2H_2O$ 2, $CaCl_2 \cdot 2H_2O$ 2, $MgSO_4 \cdot 7H_2O$ 1, Na_2-EDTA 0.1, $FeSO_4 \cdot 7H_2O$ 0.05, H_3BO_3 0.05, $MnSO_4 \cdot 4H_2O$ 0.05, KI 0.05, $ZnSO_4 \cdot 7H_2O$ 0.025, $CuSO_4 \cdot 5H_2O$ 0.001, $CoCl_2 \cdot 6H_2O$ 0.001, $Na_2MoO_4 \cdot 2H_2O$ 0.001, $NiCl_2 \cdot 6H_2O$ 0.0001. Except where otherwise stated, the basal medium (BMY3) for all the experiments described below was prepared by supplementation of Y3 minerals with the following organic nutrients and hormones (μM): sucrose 200,000, L-glutamine 685, L-arginine 574, L-asparagine 667, meso-inositol 566, thiamin · HCl 3, pyridoxin · HCl 5, nicotinic acid 8, 6-BAP 5, 2,4-D 0.1.

Borosilicate specimen tubes (2.5 × 6 cm) containing double strength (0.78% w/v) Oxoid No. 3 agar gel (5 ml) were closed with metal caps and autoclaved (120°C, 20 min). Aliquots (5 ml) of double strength liquid nutrient media, adjusted to pH 5.7 with 1 *N* NaOH or HCl, were than sterilized by filtration and mixed with the warm agar in each tube. Individual explants were rinsed in sterile distilled water and placed on the surface of the soft cooled agar gel. Tubes were resealed with autoclaved polypropylene film and transferred to an incubator (31 ± 1°C, dark).

Experimental designs and growth measurements were carried out as described previously (Eeuwens 1976). Explants were "blocked" according to their origin, *i.e.* a particular inflorescence or leaf petiole, and the fresh and dry weights recorded at the end of the culture period (3–6 weeks).

Results and Discussion

Organic nitrogen

The presence of reduced nitrogen, in addition to nitrate, appears to be essential for satisfactory growth of Cocos tissue *in vitro* (Eeuwens 1976). Growth on a medium containing only nitrate nitrogen KNO_3 20 m*M*) was stimulated, not only by NH_4Cl, but also by organic sources of reduced nitrogen. With the exception of L-glutamic acid, each of the six predominant free amino acids present in the liquid endosperm of immature coconuts (Tulecke et al. 1961), acid hydrolysed casein, or a mixture of L-amino acids (AAM), stimulated growth (Tables 1 and 2A). However, only AAM significantly ($p = 0.05$) stimulated growth when added as a supplement (5 m*M* N equivalents) to a medium containing NH_4Cl (10 m*M*) in addition to nitrate (Table 2B). L-glutamic acid, L-aspartic acid, and L-alanine all proved inhibitory.

Though reduced nitrogen appeared less essential for satisfactory growth of Phoenix leaf petiole tissue, growth on nitrate medium was significantly stimulated by the addition of NH_4Cl, casein hydrolysate, or AAM (Table 2A). The presence of inhibitory amino acids, *e.g.* L-glutamic acid, may explain the reduced response of tissues from both palms to casein hydrolysate compared with AAM.

In general, these results and the observations of other workers (Smith and Thomas 1973, Reuveni and Kipnis 1974) suggest that organic nitrogen supplements stimulate growth of cultured palm tissues in the presence of suboptimal levels of inorganic reduced nitrogen. Nevertheless, the addition of AAM, but not NH_4Cl, as a supplement to the standard Y3 mineral formulation (Table 2B) significantly increased growth in fresh weight of Cocos tissues. AAM was, therefore, included in the basal medium (BMY3) used for all other experiments at a concentration of 300 mg/l (5 m*M* N equivalents).

Table 1. *Effects of six amino acids on growth of Cocos inflorescence rachis explants,* (A) *supplied as the sole source of reduced nitrogen and* (B) *supplied as a supplement to the standard Y3 mineral formulation.* Ten replicates per treatment. Four weeks' incubation. Basal medium: BMY3 without reduced nitrogen (NH_4Cl + AAM).

Reduced nitrogen in medium		Mean final weight mg ± SE
Main source (10 m*M* N eq.)	Supplement (5 m*M* N eq.)	Fresh
A. Sole source of reduced N		
None	None	83 ± 5
L-Glutamine	None	443 ± 48
L-Glutamic acid	None	93 ± 6
L-Asparagine	None	155 ± 29
L-Aspartic acid	None	142 ± 15
L-Alanine	None	191 ± 20
L-Arginine	None	212 ± 43
B. Supplement to standard		
NH_4Cl	None	540 ± 104
NH_4Cl	L-Glutamine	592 ± 84
NH_4Cl	L-Glutamic acid	196 ± 44
NH_4Cl	L-Asparagine	417 ± 87
NH_4Cl	L-Aspartic acid	355 ± 78
NH_4Cl	L-Alanine	323 ± 52
NH_4Cl	L-Arginine	674 ± 107

Table 2. *Effects of NH_4Cl, acid hydrolysed casein, and a mixture of* L-*amino acids on growth of Cocos and Phoenix tissue explants,* (A) *supplied as the sole source of reduced nitrogen, and* (B) *supplied as a supplement to the standard Y3 mineral formulation.* AAM — L-glutamine/L-arginine/L-asparagine, 1 : 1 : 1 W/W. Ten replicates per treatment. Six weeks' incubation. Basal medium: BMY3 without reduced nitrogen (NH_4Cl and AAM).

Reduced nitrogen in medium		Mean final weights, mg ± SE			
		Cocos		Phoenix	
Main source (10 m*M* N eq.)	Supplement (5 m*M* N eq.)	Fresh	Dry	Fresh	Dry
A. Sole source of reduced N					
None	None	202 ± 23	33	527 ± 67	98
NH_4Cl	None	844 ± 30	113	853 ± 117	134
Casein hyd.	None	495 ± 46	72	655 ± 62	103
AAM	None	669 ± 49	96	730 ± 113	118
B. Supplement to standard					
NH_4Cl	NH_4Cl	854 ± 41	103	840 ± 86	130
NH_4Cl	Casein hyd.	847 ± 50	106	791 ± 93	130
NH_4Cl	AAM	932 ± 38	114	744 ± 80	124

three amino acids (L-glutamine, L-arginine and L-ine) in the amino acid mixture together comprise 60% (w/w) of the total free amino acid complement of water from young green fruits (Tulecke *et al.* 1961). have been widely used as a source of reduced nitrogen for the initiation of callus cultures from the tissues woody species (White and Gilbey 1966, Winton

whereas dry weight continued to increase up to 0.4 *M* (Table 3). Glucose and fructose, when compared with sucrose on an equimolar (0.2) or an equivalent carbon basis, also supported satisfactory growth of the tissues. The main difference between the palms was the relatively good growth of Cocos tissue on fructose which even exceeded that obtained with sucrose.

In contrast to these findings, Reuveni and Lilien-Kipnis (1974) have reported that altering the sucrose concentration,

Carbohydrate

weight of explants from both palms increased with concentration up to 0.2 *M* and then declined,

Effect of carbohydrate source and concentration on growth and Phoenix tissue explants. Ten replicates per treat- Three weeks' incubation. Basal medium: BMY3, without

	Mean final weights, mg ± SE			
	Cocos		Phoenix	
M	Fresh	Dry	Fresh	Dry
—	15 ± 2	1.1	53 ± 7	3.3
0.05	151 ± 17	14	183 ± 18	16
0.1	259 ± 37	26	216 ± 25	24
0.2	295 ± 40	40	229 ± 25	36
0.4	211 ± 31	46	196 ± 22	45
2	285 ± 30	32	215 ± 25	27
3	300 ± 50	49	196 ± 22	38
2	412 ± 64	40	199 ± 18	25
4	376 ± 58	63	176 ± 11	38

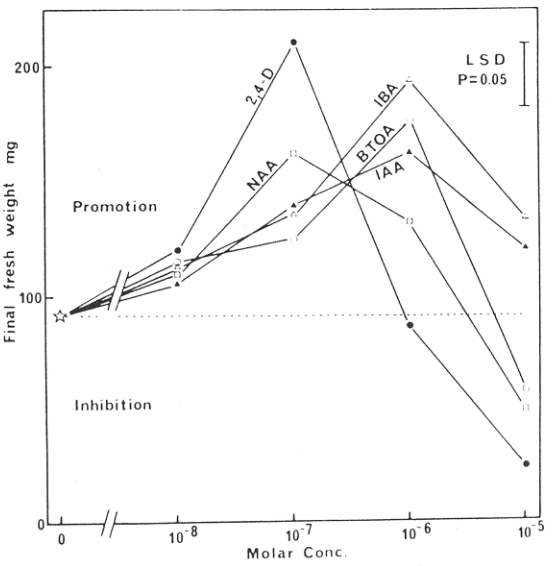


Figure 1. *Effects of five auxins on growth of Cocos inflorescence rachis explants.* Basal medium: BMY3 without 2,4-D and 6-BAP.

Table 4. *Effects of 2,4-D and NAA concentration on growth and survival of Cocos and Phoenix tissue explants.* Ten replicates per treatment. Four weeks' incubation. Basal medium: BMY3, without 2,4-D.

Auxin, *M*		Mean final fresh weight, mg ± SE		% Survival after 4 weeks	
		Cocos	Phoenix	Cocos	Phoenix
None	—	84 ± 11	155 ± 23	100	100
2,4-D	10^{-7}	322 ± 46	358 ± 51	100	100
	10^{-6}	56 ± 6	394 ± 64	0	100
	10^{-5}	28 ± 1	283 ± 36	0	100
NAA	10^{-7}	317 ± 58	258 ± 20	100	100
	10^{-6}	104 ± 24	310 ± 50	100	100
	10^{-5}	40 ± 4	273 ± 22	70	100

Table 5. *Effects of five cytokinins on growth of Cocos and tissue explants.* Ten replicates per treatment. Six weeks Basal medium: BMY3 without 6-BAP.

Cytokinin, *M*		Mean final weights, mg ± SE		
		Cocos		Phoenix
		Fresh	Dry	Fresh
None	—	548 ± 40	77	447 ± 79
6-BAP	10^{-6}	601 ± 45	87	688 ± 84
	5×10^{-6}	699 ± 54	103	825 ± 90
	10^{-5}	754 ± 60	105	927 ± 133
	10^{-4}	749 ± 60	98	846 ± 107
Kinetin	5×10^{-6}	583 ± 50	85	599 ± 78
DMAA	5×10^{-6}	639 ± 42	92	716 ± 143
SD8339	5×10^{-6}	682 ± 56	93	838 ± 152
Zeatin	5×10^{-6}	744 ± 58	97	895 ± 165

or replacing sucrose (2%, 0.06 *M*) by a mixture of glucose (1%, 0.05 *M*) and fructose (1%, 0.05 *M*), had no effect on growth of Phoenix seedling tissue explants. The levels of carbohydrate and other culture conditions used in their experiments, however, appear to have been generally sub-optimal.

Growth substances

Growth of Cocos tissue explants was markedly stimulated by auxins (*e.g.* NAA or 2,4-D at 10^{-7} *M*) both in the absence (Figure 1) and in the presence (Tables 4 and 6) of added cytokinin (6-BAP, 5×10^{-6} *M*) and gibberellin (GA_3, 10^{-7} to 10^{-5} *M*). Optimal growth of Phoenix leaf petiole explants was obtained at auxin concentrations (NAA or 2,4-D, 10^{-6} *M*) which were supraoptimal or even inhibitory to the growth of Cocos tissue (Table 4). Though little quantitative data is available, it appears that Cocos tissues are more sensitive to auxin than either Phoenix or Elaeis tissues. The relatively high concentrations (10^{-5} to 10^{-4} *M*) of 2,4-D used in the initiation of callus on Elaeis explants (Rabéchault *et al.* 1972, Smith and Thomas 1973) proved lethal to freshly excised Cocos tissues.

The addition of cytokinins (10^{-6} to 10^{-4} *M*) to a medium containing near optimal levels of auxin (2,4-D, 10^{-7} *M*) stimulated growth still further (Table 5). When compared on an equimolar (5×10^{-6} *M*) basis, kinetin and DMAA were the least effective, and 6-BAP, SD8339 and zeatin were the most effective of the cytokinins tested. By contrast, other workers (Smith and Thomas 1973, Reuveni and

Table 6. *Effect of factorial combinations of 2,4-D, 6-BAP and GA_3* on growth of Cocos and Phoenix tissue explants. Ten replicates per treatment. Four weeks' incubation. Basal medium: BMY3, without 2,4-D and 6-BAP.

Growth substances			Mean final weights, mg ± SE			
			Cocos		Phoenix	
2,4-D *M*	6-BAP *M*	GA_3 *M*	Fresh	Dry	Fresh	Dry
0	0	0	159 ± 37	22	156 ± 46	28
		10^{-7}	101 ± 20	14	209 ± 71	36
		10^{-5}	97 ± 9	13	155 ± 53	30
0	5×10^{-6}	0	147 ± 6	21	252 ± 101	36
		10^{-7}	178 ± 15	23	392 ± 148	64
		10^{-5}	132 ± 20	19	280 ± 104	61
10^{-7}	0	0	288 ± 23	34	329 ± 90	58
		10^{-7}	277 ± 10	31	303 ± 68	56
		10^{-5}	256 ± 31	29	288 ± 103	54
10^{-7}	5×10^{-6}	0	480 ± 31	53	684 ± 377	77
		10^{-7}	443 ± 27	51	584 ± 291	61
		10^{-5}	397 ± 47	46	528 ± 230	69

Table 7. *Composition of culture media used for the in vitro growth and differentiation of Cocos and Phoenix tissue explants.* Basal medium: BMY3, without: macroelements, sucrose and growth hormones.

Medium	Development	Varied components in culture media				
		Macro elements	Sucrose *M*	NAA *M*	6-BAP *M*	GA_3 *M*
A	Rapid growth of explant	Y3	0.2	2.5×10^{-7}	5×10^{-5}	—
B	Root initiation	Y3	0.2	2.5×10^{-6}	5×10^{-8}	—
C	Root growth	Y3	0.1	5×10^{-8}	5×10^{-8}	5×10^{-7}
D	Root growth	$\frac{1}{4}$MS	0.2	5×10^{-8}	5×10^{-8}	5×10^{-7}

...is 1974) have reported that kinetin was more effective ...either 6-BAP or zeatin in stimulating explant and callus ...th of Phoenix and Elaeis.

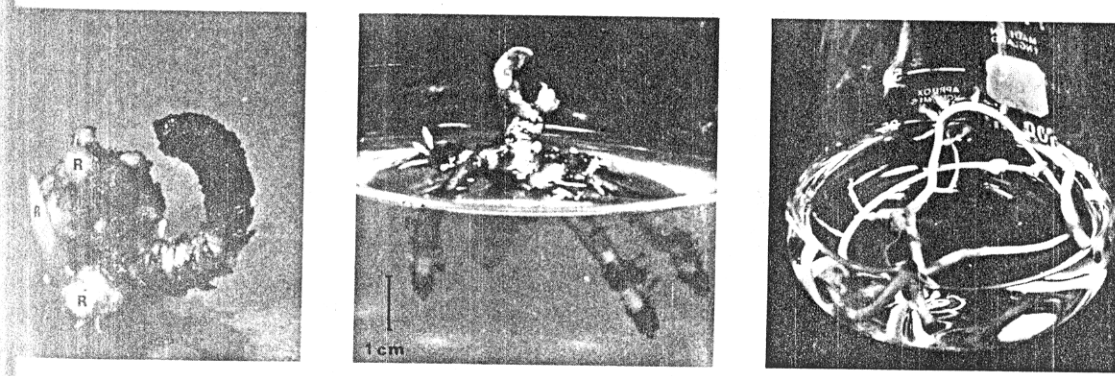
...examination of the effects of factorial combinations of ... (2,4-D), cytokinin (6-BAP) and gibberellin (GA_3) on ...th (Table 6) indicated that the positive interaction be... auxin and cytokinin was highly significant ($p = 0.001$). ... concentrations (10^{-7} *M*) of GA_3 had no significant ...t on growth but higher levels (10^{-5} *M*) appeared to be ...ly inhibitory.

Other growth factors

During short culture periods (3 to 6 weeks) neither meso... (100 to 400 mg/l), nor individual vitamins ...min · HCl, pyridoxin · HCl, nicotinic acid, calcium ...othenate and biotin, tested at 0.05, 0.5 and 5 mg/l) had ...significant effect on the amount of growth obtained. ...ever, it is likely that some of these factors, particularly ...o-inositol and thiamin would be required during pro...ged culture periods.

Explant development and organogenesis

...a result of the above investigations a medium (Table ... has been developed which supports satisfactory growth of tissue explants from both Cocos (inflorescence, leaf petiole, and stem) and Phoenix (leaf petiole and stem) palms. As reported previously (Eeuwens 1976), increases in weight were mainly due to growth of the original explant with very little callus proliferation in spite of the wide range and concentration of growth substances tested. Attempts to sub-culture small pieces of callus separated from the original explant tissue have so far failed. Nevertheless, Phoenix leaf petiole explants and Cocos inflorescence rachilla explants (Figure 2a) have initiated roots within 6 weeks when sub-culture, after 2 weeks on medium A, onto a medium (Table 7B) with increased levels of auxin (NAA 2.5×10^{-6} to 2.5×10^{-5} *M*). Root initiation was not prevented by the presence of high levels of cytokinin (6-BAP, 5×10^{-6} *M*) or low levels of sucrose (0.1 *M*) but occurred more frequently when high levels of auxin were combined with high sucrose (0.2 *M*) and reduced cytokinin (6-BAP, 5×10^{-8} *M*) levels. Further development of the root initials (Figure 2b) was favoured by the presence of low concentrations of gibberellic acid and by a reduction in the concentration of auxin and either minerals or sucrose (Table 7, media C and D). Isolated roots excised from cultured explants of both palms have continued growth and produced laterals (Figure 2c) when sub-cultured on liquid static media (Table 7, media C and D). So far roots have not been initiated on stem (Cocos and Phoenix) or inflorescence rachis (Cocos) explants which lack leaf or



...2. *Initiation and subculture of roots.* (a) Root initiation at basal end of Cocos inflorescence rachilla explant (Medium B, Table 7), R... (b) Root development on subcultured Cocos inflorescence rachilla explant (Medium D, Table 7). (c) Subculture of roots excised ...Phoenix leaf petiole explant (Medium C, Table 7).

meristem tissue. Reuveni and Lilien-Kipnis (1974) have also reported the occasional production of roots on cultured axillary buds and "tips" of Phoenix offshoots. However, no pattern of response to treatment could be established and attempts to sub-culture both these roots and roots excised from seedlings were unsuccessful.

The initiation of roots on palm inflorescence explants cultured *in vitro* has not been reported previously. The possibility that young inflorescences from Cocos and other palms, with their abundance of flower meristems, may serve as a suitable source of explants for vegetative propagation is now being investigated.

Grateful acknowledgement is made to the Ministry of Overseas Development, U.K., for financial support and the Coconut Industry Board, Jamaica, for supplying plant tissue. Appreciation is also expressed to Dr. Jennet Blake for valuable discussions and to Mrs. Elizabeth Campling for technical assistance.

References

Cutter, V. M. & Wilson, K. S. 1954. Effects of coconut endosperm and other growth stimulants upon the development in vitro of embryos of Cocos nucifera. — Bot. Gaz. 115:234–240.

Eeuwens, C. J. 1976. Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (Cocos nucifera) and cultured in vitro. — Physiol. Plant. 36: 23–28.

Guzman, E. V. de, 1971. The growth and development of coconut 'Macapuno' embryo in vitro. I. The induction of rooting. — Philipp. Agric. 53: 65–78.

Jones, L. H. 1974. Propagation of clonal oil palm by tissue culture. — Oil Palm News 17: 1–8.

Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. — Physiol. Plant. 15: 473–497.

Rabéchault, H., Ahée, J. & Guénin, G. 1970. Recherches sur la culture in vitro des embryons de palmier à huile (Elaeis guineensis Jacq.). IV. Effets de la teneur en eau des noix et de la durée de leur stockage. — Oléagineux 23: 233–237.

— Martin, J. P. & Cas, S. 1972. Recherches sur la culture des tissus de palmier à huile (Elaeis guineensis Jacq.). — *Ibid.* 27: 531–534.

Reuveni, O. & Lilien-Kipnis, H. 1974. Studies of the in vitro culture of date palm (Phoenix dactylifera L.) tissues and organs. — Pamphlet No 145, Volcani Inst. Agric. Res., Israel.

— Adato, Y. & Lilien-Kipnis, H. 1972. A study of new and rapid methods for vegetative propagation of date palms. — Date Grower's Inst. Rep. 49: 17–24.

Smith, W. K. & Thomas, J. A. 1973. The isolation and in vitro cultivation of cells of Elaeis guineensis. — Oléagineux 28: 123–127.

Tulecke, W., Weinstein, L. H., Rutner, A. & Laurencot, H. J. 1961. The biochemical composition of coconut water (coconut milk) as related to its use in plant tissue culture. — Contrib. Boyce Thompson Inst. 21: 115–128.

White, P. R. & Gilbey, S. N. 1966. Sources of nitrogen for sprout tissue culture. — Physiol. Plant. 19: 177–186.

Winton, L. L. 1972. Annotated bibliography of somatic cell and callus cultures. — Genet. Physiol. Notes 16: 1–19. Inst. Paper Chem. Appleton, Wisconsin, U.S.A.