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Mineral Requirements for Growth and Callus Initiation of Tissue Explants Excised from Mature Coconut Palms (*Cocos nucifera*) and Cultured *in vitro*

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Abstract

Growth of stem, leaf, and inflorescence explants from mature coconut (*Cocos nucifera* L.) palms on a new mineral formulation (Y3) was superior to that on the minerals of White, Heller, or Murashige and Skoog. Cell division in the upper part of cultured explants gave rise to a layer of white callus within a month.

The effects of omitting entirely or altering the concentration of individual elements in the Y3 formulation were investigated. It was concluded that growth on White's and Heller's minerals was seriously limited by deficiencies in macro-elements, *i.e.* nitrogen (particularly ammonium), potassium and phosphorus, as well as micro-elements, *i.e.* iron, iodine and molybdenum. The Murashige and Skoog formulation, on the other hand, was deficient only in certain micro-elements (particularly iodine).

Introduction

Many herbaceous species can now be propagated vegetatively using recently developed tissue culture techniques in addition to conventional methods. Unfortunately, special problems have been encountered when these techniques have been applied to certain woody species (King 1974), in particular the palms (Staritsky 1970). Nevertheless, in view of the valuable products of many plantation crops, the clonal propagation of elite individuals with desirable characteristics is of prime importance. This applies particularly to the coconut palm, *Cocos nucifera* L., which can only be propagated from seed.

Cocos nucifera is generally cross-pollinated and very heterozygous; hence variability in both yield (Davis 1969) and disease resistance is considerable. Since this species takes at least four years to reach sexual maturity, and several more years are required before productive capacity can be assessed, breeding programs are extremely time-consuming. Although mature palms do, on rare occasions, produce "bulbils" from inflorescence tissues (Davis 1969), and limited multiplication can be achieved by surgical manipulations on seedling plants (Davis 1969), a reliable method for vegetative propagation of mature palms has yet to be developed.

The possibility that this may be achieved with tissue culture techniques is now being examined. As a result of preliminary investigations a new mineral formulation has been developed (Table 1). In the present paper the mineral requirements for rapid growth and callus initiation on explants derived from mature coconut palms are described.

Abbreviations: W, White's (1943) minerals; H, Heller's (1953) minerals; MS, minerals of Murashige and Skoog's (1962) revised medium; MMS, modified MS minerals; Y3, Y3 minerals; Fe-EDTA, sodium ferric ethylenediaminetetraacetate.

Materials and Methods

Origin of plant tissue. Mature (20–25-year-old) palms of *Cocos nucifera* L. cv. Malayan Dwarf, grown in Jamaica, served as the source of tissue explants. Stem tips, each consisting of a block of stem tissue (15 × 15 × 15 cm), surmounted by the apical meristem, young leaves (less than 30 cm long) and inflorescences were packed with bottles of frozen coconut water in polystyrene containers and flown to England where they were stored for up to ten days in the dark at 5 to 10°C until required.

Preparation of inflorescence explants. Uncontaminated tissue was obtained from young inflorescences, without the use of chemical sterilants, by careful removal of the outer and inner spathes, which completely enclose each inflorescence, under aseptic conditions. The inflorescence stem (rachis) was cut transversely into discs (2–3 mm thick) just above the junction of the inner spathe. Each disc was then cut into cubes (8–12 mg fresh weight), "rachis explants". Branches of the inflorescence (rachillae) with numerous flower primordia were excised, selected for uniformity, and sections (3 mm long, 3–5 mg fresh weight) were cut from approximately 3 mm behind the tip, "rachillae explants".

Preparation of stem and leaf explants. A block of stem tissue (4 × 4 × 4 cm) and the petiole bases of two young leaves (8–20 cm long) from each palm, were surface sterilized in a filtered solution of calcium hypochlorite (2% w/w) for 15 min. Cores of tissue (2 mm diameter, 2–3 cm long) were removed from the block of stem tissue close to the apical meristem, and also from the base of each leaf petiole. The ends of each core were discarded and the remaining tissue cut into short lengths (2–3 mm, 8–12 mg fresh weight), "stem and leaf petiole explants".

Culture techniques and media. Specimen tubes (borosilicate glass, 2.5 × 6 cm), each containing a filter paper (Whatman 42, ashless) bridge were closed with metal caps and autoclaved (120°C, 20 min). Aliquots (10 ml) of liquid medium, sterilized by filtration through "Millipore" filters (final pore size 0.22 μm) were then dispensed into the sterile tubes. Prepared explants were dipped in aqueous 70% ethanol (5 s), rinsed in sterile water (5 s), and transferred to the surface of the filter paper supports (1 explant per tube). The tubes were resealed with autoclaved polypropylene film (Blake 1966) and transferred to an incubator (31 ± 1°C, dark) where they were maintained throughout the culture period (3–6 weeks).

The mineral components of four media, *i.e.* White's (1943), Heller's (1953), Murashige and Skoog's (1962), and Y3 (Tables 1, 2 and 3), supplemented with the following organic additions (mg/l): sucrose, 45,000; meso-inositol, 100; thiamin HCl, 0.5; pyridoxin HCl, 0.05; calcium pantothenate, 0.05; nicotinic acid, 0.05; biotin, 0.05; kinetin (6-furfuryl amino purine), 0.22; gibberellic acid, 0.038; and 2,4-dichlorophenoxyacetic acid, 0.022, were used. All culture media were prepared with glass-distilled water and adjusted to pH 5.5 by the addition of a few drops of 1 *M* sodium hydroxide.

Experimental design and growth measurements. Randomized block designs were used throughout. Explants were "blocked" according to their origin, *e.g.* a particular inflorescence rachis disc, a selected group of rachillae from the same inflorescence, or a particular stem or leaf core. Initial weights (fresh and dry) were obtained from a sample of explants (usually four) from each block at the beginning of each experiment and the final weights from the remaining explants at the end of the culture period.

Measurements of the initial weights of several samples of explants indicated that the value of the variance coefficient (standard deviation/mean) for a particular block was never greater than 10%. Initial fresh weights of explants in each block could, therefore, be estimated with reasonable

Table 1. *Composition of the Y3 mineral formulation, specially developed for the culture of Cocos nucifera tissues.*

Macro-elements		Micro-elements	
Component	Concentration mg/l	Component	Concentration mg/l
KNO_3	2020	Fe-EDTA	3[illegible].5
KCl	1492	$MnSO_4 \cdot 4H_2O$	11.2
NH_4Cl	535	KI	8.3
$NaH_2PO_4 \cdot 2H_2O$	312	$ZnSO_4 \cdot 7H_2O$	7.2
$CaCl_2 \cdot 2H_2O$	294	H_3BO_3	3.1
$MgSO_4 \cdot 7H_2O$	247	$CoCl_2 \cdot 6H_2O$	0.24
		$Na_2MoO_4 \cdot 2H_2O$	0.24
		$CuSO_4 \cdot 5H_2O$	0.16
		$NiCl_2 \cdot 6H_2O$	0.024

Table 2. *Concentrations of macro-nutrient ions in mineral formulations used in the culture of Cocos nucifera explants.*

Mineral formulation	Macro-nutrient ion, m*M*							
	K^+	Na^+	Ca^{2+}	Mg^{2+}	NH_4^+	NO_3^-	SO_4^{2-}	H_2PO_4	Cl^-
W	1.7	3.0	1.2	3.0	—	3.2	4.4	0.14	0.9
H	10.0	8.0	0.5	1.0	—	7.1	1.0	0.9	11.0
MS	20.0	0.2	3.0	1.5	20.0	39.4	1.6	1.25	6.0
Y3	40.0	2.0	2.0	1.0	10.0	20.0	1.0	2.0	34.0

Table 3. *Concentrations of micro-elements in mineral formulations used in the culture of Cocos nucifera explants.*

Mineral formulation	Micro-element, μ*M*									
	Fe	B	Mn	Zn	I	Cu	Co	Mo	Ni	Al
W	13	25	30	10	4.5	—	—	—	—	—
H	4	16	4.5	3.5	0.06	0.12	—	—	0.13	0.23
MS	100	100	100	30	5	0.1	0.1	1.0	—	—
Y3	50	50	50	25	50	1.0	1.0	1.0	0.1	—

Table 4. *Growth of stem, leaf, and inflorescence explants on four mineral salt formulations.*

Source of explants	Mean initial fresh weight mg	Mean final fresh weight mg ± SE W	H	MS	Y3
Stem	11.8	65 ± 4	77 ± 3	194 ± 15	279 ± 25
Leaf	9.3	44 ± 4	41 ± 4	184 ± 13	329 ± 37
Inflorescence:					
Rachis	8.1	62 ± 4	75 ± 8	261 ± 23	401 ± 27
Rachilla	4.5	35 ± 3	70 ± 17	349 ± 32	486 ± 22

precision. Moreover, the blocking procedure enabled much of the variation due to differences in origin and size to be eliminated statistically.

Results

Growth of all tissue explants, irrespective of their origin, was considerably greater on the specially developed Y3 (Table 1) than on the W, H or MS mineral formulations (Table 4). Significantly more growth than on the standard W and H minerals was obtained when either the micro- or macro-element components of these formulations were replaced by those of the Y3 minerals (Table 5). In contrast, substitution of the macro-elements in the MS formulation by Y3 macro-elements gave no increase in growth. However, when the MS macro-elements were combined with the Y3 micro-elements growth equal to that on the complete Y3 minerals and superior to that on any other combination of macro- and micro-elements was obtained.

Following a series of preliminary tests the effects of source and concentration of inorganic nitrogen were examined in a factorial experiment (Figure 1) in which the nitrogen was supplied as ammonium (NH_4Cl) and nitrate (KNO_3). The potassium concentration was maintained constant (40 m*M*) by suitably adjusting the amount of KCl present. Either ammonium or nitrate alone supported little growth and resulted in rapid browning of the tissues. In contrast to the nitrate medium, which remained at a pH value close to 5.5, the pH of the ammonium medium drifted towards acidity. The addition of nitrate to media containing only ammonium nitrogen, both increased growth and reduced the drift in pH (Figure 1). The first-order interaction between ammonium and nitrate was highly significant (*i.e.* at $p = 0.001$) indicating that the effects of the individual ions on growth were not simply additive. The optimum concentration of ammonium and nitrate (NH_4Cl 10 m*M*, KNO_3 20 m*M*) resulted in healthy cultures which browned only slightly at the base.

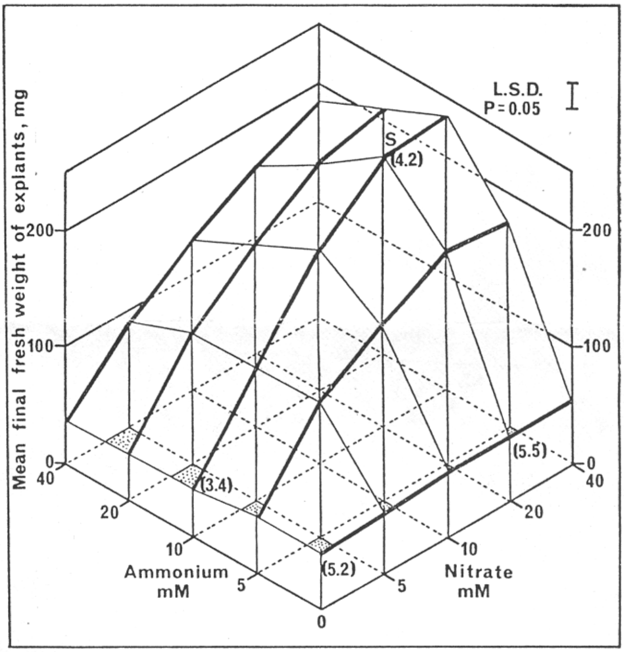

Figure 1. *Effects on growth of inflorescence rachis explants of altering the nitrogen concentration and ammonium/nitrate balance in the Y3 mineral formulation.* Mean initial fresh weight = 10.1 mg, figures in brackets give final pH, S = standard formulation.

Table 5. *Effects on growth of inflorescence rachis explants of factorial combinations of macro- and micro-element components of four mineral formulations.* Values indicate mean final fresh weight as mg ± SE. Mean initial fresh weight = 10.0 mg.

Micro-element component	Macro-element component W	H	MS	Y3
W	57 ± 7	81 ± 10	237 ± 28	215 ± 24
H	58 ± 5	75 ± 7	168 ± 18	225 ± 28
MS	60 ± 5	92 ± 9	209 ± 22	222 ± 26
Y3	64 ± 7	99 ± 7	301 ± 26	312 ± 29

The effects of altering the concentrations of other macro-elements were examined with a modified medium in which the level of nitrogen was maintained constant (NH_4NO_3 10 m*M*, KNO_3 10 m*M*), and the levels of potassium (KCl), magnesium ($MgSO_4$), calcium ($CaCl_2$), and phosphorus (NaH_2PO_4) were varied in factorial combinations. If the variation in less essential elements, *i.e.* sodium, chlorine and sulphur, can be disregarded, then the results

Table 6. *Effects on growth of inflorescence rachis explants of varying the concentrations of potassium* (KCl), *phosphate* (NaH_2PO_4), *calcium* ($CaCl_2$) *and magnesium* ($MgSO_4$) *in the otherwise standard Y3 mineral formulation in factorial combinations.* Values indicate mean final fresh weight in mg. Mean initial fresh weight = 10.4 mg, p = probability of significance, N.S. = not significant at $p = 0.05$, LSD = least significant difference ($p = 0.05$).

		NaH_2PO_4, m*M*				Main effects and significant ($p = 0.05$) interactions	
		10 m*M* KCl		40 m*M* KCl			
$CaCl_2$ m*M*	$MgSO_4$ m*M*	0.5	2.0	0.5	2.0	Factors	p
0.5	0.5	229	309	339	409	K	0.001
	2.0	244	302	323	440	P	0.001
						Ca	0.001
						Mg	N.S.
2.0	0.5	293	300	413	516		
	2.0	313	332	369	421		
			LSD = 67			Ca, Mg, K	0.05

Table 7. *Effects on growth of inflorescence rachis explants of omitting individual micro-elements from the Y3 mineral formulation.* Mean initial fresh weight = 10.4 mg.

Element omitted	Mean final fresh weight mg ± SE
None (control)	517 ± 41
Boron	512 ± 32
Manganese	489 ± 24
Zinc	449 ± 41
Nickel	442 ± 28
Copper	441 ± 33
Molybdenum	422 ± 37
Cobalt	415 ± 40
Iron	332 ± 45
Iodine	304 ± 25

(Table 6) indicate that an increase in the concentration of potassium (10 to 40 m*M*), phosphorus (0.5 to 2.0 m*M*) and to a lesser extent calcium (0.5 to 2.0 m*M*) stimulated growth significantly. By contrast, the effect of altering the concentration of magnesium (0.5 to 2.0 m*M*) was not significant though there was evidence for a second-order interaction (significant at $p = 0.05$) between magnesium, calcium and potassium.

The omission of individual micro-elements from the standard Y3 mineral formulation indicated that during a six-week culture period without transfer certain micro-elements, in particular iron and iodine, could become growth-limiting factors (Table 7). Growth was significantly stimulated, and tissue browning reduced, when the concentration of iodine alone in the Y3 or MS mineral formulations was increased from 5 to 50 μmol/l (Table 8). Moreover, complete substitution of the other micro-elements in the MS minerals by those in the Y3 formulation resulted in increased growth irrespective of the associated iodine concentration (Table 8). This suggests that the levels of other micro-elements besides iodine may limit growth on the standard MS minerals.

Growth on the standard W, H, and to a lesser extent MS, formulations was stimulated by the addition of macro-element (N, P, K) and/or micro-element (Fe, I, Mo) supplements (Table 9). By contrast, growth on the standard Y3 formulation was not significantly affected or, if anything, slightly reduced by these supplements.

Cell division in the upper part of cultured explants resulted in a layer of white callus consisting of large vacuolated cells (Figure 2A). By contrast, cells near the base of the explants remained small and became filled with dense contents which stained heavily with safranin (Figure 2B). The frequency of these cells increased during the culture period and appeared to be associated with tissue maturation and browning. The callus continued to proliferate slowly when the entire growing explant was transferred to fresh nutrient medium at two-week intervals. However, attempts to subculture small pieces of tissue excised from the callus layer alone have so far failed.

Table 8. *Effects of iodine* (KI) *concentration on growth of inflorescence rachis explants on MS, modified MS* (MMS) and *Y3 mineral formulations.* Mean initial weights of explants: fresh 10 mg, dry 1.4 mg. MMS = MS macro-elements + Y3 micro-elements.

	Mean final fresh and dry weight, mg ± SE					
	MS		MMS		Y3	
Iodine μ*M*	Fresh	Dry	Fresh	Dry	Fresh	Dry
5	272 ± 15	30	347 ± 21	35	331 ± 24	32
50	344 ± 38	38	393 ± 37	41	424 ± 29	38

Table 9. *Effects of micro- and/or macro-element supplements on growth of inflorescence rachis explants on four mineral formulations.* Mean initial weights of explants: fresh 10.4 mg, dry 1.5 mg. A = micro-element supplement (KI, 50 μM; Fe-EDTA, 50 μM; Na_2MoO_4, 1 μM), B = macro-element supplement (KNO_3, 20 mM; NH_4Cl, 10 mM; NaH_2PO_4, 1 mM).

Mineral supplement	Mean final fresh and dry weight, mg ± SE							
	W		H		MS		Y3	
	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
None	58 ± 6	20	98 ± 14	14	310 ± 34	35	502 ± 33	46
A	66 ± 12	12	126 ± 12	18	346 ± 30	40	497 ± 57	46
B	216 ± 22	22	282 ± 31	27	342 ± 42	35	467 ± 60	39
A + B	307 ± 24	29	318 ± 30	32	393 ± 35	41	433 ± 27	37

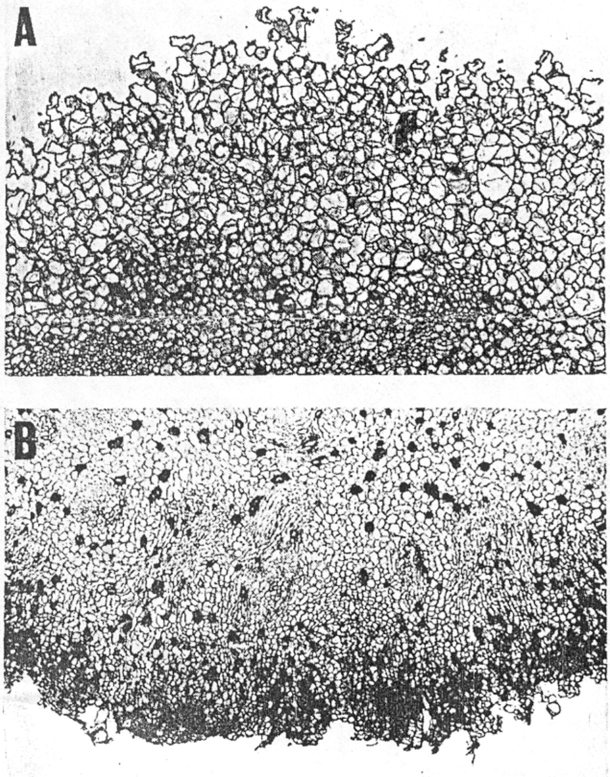

Figure 2. *Transverse section through an inflorescence rachis explant after two weeks in culture.* A: Upper part of explant with callus; B: Base of explant with large numbers of small cells heavily stained with safranin.

Discussion

Growth of excised coconut tissues *in vitro* was greatly influenced by the mineral composition of the medium. Perhaps the most important factors contributing to the observed differences in growth were the source and concentration of inorganic nitrogen. A requirement for both a reduced form of nitrogen (ammonium) and high concentrations of nitrate could be clearly demonstrated. There is evidence that ammonium can only serve as the sole source of nitrogen at pH values close to neutrality (Sheat *et al.* 1959) though uptake of the ion is not seriously impaired at much lower pH values (Street 1966). Thus, the poor growth of coconut tissues supplied only with ammonium may have resulted from the low initial pH (5.5) of the medium and the rapid drift towards even more acid conditions.

It has been suggested that, at low pH values, ammonium may either interfere with the uptake of some essential inorganic element or, alternatively, enhance the leakage of essential nitrogenous metabolites (Street 1966). Since the addition of nitrate to media containing only ammonium nitrogen, both reduced the drift in pH and stimulated growth, it may check or reverse some of the deleterious effects of ammonium under weakly acidic conditions.

Data presented by other workers suggest that the presence of ammonium nitrogen is essential for the culture of other monocotyledonous species (Kaul and Sabharwal 1972), including the date palm, *Phoenix dactylifera* L. (Reuveni and Lilien-Kipnis 1974). In contrast, the mineral composition of the medium appears to be less critical in the case of the oil palm, *Elaeis guineensis* Jacq. (Rabéchault *et al.* 1972, Smith and Thomas 1973), where no consistent differences were observed between the rates and amount of growth on media with and without ammonium.

The absence of ammonium nitrogen and the low levels of nitrate in the W and H formulations appeared to be the main reasons for poor growth on these minerals compared with that on the more concentrated MS and Y3 formulations. Nevertheless, the results indicated that low levels of other macro-elements (K, P and Ca) and micro-elements (Fe, I and Mo) and perhaps the balance between calcium, magnesium and potassium ions, were also contributing factors.

A deficiency in iodine induced by the high chlorine concentration (Hannay 1956) of the Y3 formulation did not appear to be the only cause of the unexpectedly high (50 μM) iodine requirement of coconut tissues. Growth on the standard MS mineral formulation, which contain comparatively low levels of chlorine, was also significantly stimulated by the addition of iodine.

In view of the complicated interactions that exist, not only between individual mineral elements, but also between the numerous other variables regulating growth, the development of a truly optimal mineral formulation may be a practical impossibility. Nevertheless, the Y3 formulation (Table 1) represents an approximation to this, and has proved superior to all other mineral formulations, so far tested, for the *in vitro* culture of coconut tissues. A requirement by cultured coconut tissues for a concentrated mineral salt solution with high levels of potassium and iodine might, to some extent, be an adaptation to its natural habitat on soils frequently exposed to sea-water and/or sea-spray. However, the Y3 formulation has also proved satisfactory for the culture of tissues from several other species, *e.g. Phoenix dactylifera* L. and *Carica papaya* L., which grow in very different habitats.

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