

FRM-707
1979

**CULTURE OF LEAF TISSUES OF COCONUT:
DEVELOPMENTS TOWARDS SOMATIC EMBRYOGENESIS**

**S M Karunaratne
Tissue Culture Unit
Coconut Research Institute,
Lunuwila
Sri Lanka**

Table of Contents

	Page
Summary	1
Introduction	3
Abbreviations	4
Materials and Methods	5
Results and Discussion	7
Acknowledgements	34
References	35

**CULTURE OF LEAF TISSUES OF COCONUT:
DEVELOPMENTS TOWARDS SOMATIC EMBRYOGENESIS.**

S M Karunaratne
Coconut Research Institute, Lunuwila,
Sri Lanka

SUMMARY

An investigation to culture leaf tissues of coconut, Cocos nucifera L was conducted with the prime objective of developing a viable technique for clonal propagation. The cabbage or the bud was dissected out from field grown plants, sterilized and leaf tissues were excised under aseptic conditions.

The coconut leaf explants showed no signs of callusing/organogenesis/embryogenesis when cultured in palm tissue culture media developed by other workers. In the medium developed in this laboratory following the broad spectrum tissue culture experiment of de Fossard (1976), about 50% of the cultures produced globular bodies. The number of globules per explant varied from 5-20. They were creamy white, bipolar and resembled the immature zygotic embryo of coconut. They appeared to arise directly from the inner leaf tissue, probably the mesophyll and a few arose from the epidermal tissue. Callus formation never occurred in the cultured explants. Rarely, direct rhizogenesis occurred from the main vascular strands. Leaf tissues explanted from field grown seedlings produced globular bodies consistently but those derived from the bearing palms never exhibited embryogenesis/organogenesis. Even in seedlings, the tender leaves responded differently depending on their developmental maturity. Low concentrations of 2, 4-D and

activated charcoal were also found to be essential for the successful production of globular bodies on leaf explants.

Leaf tissues explanted from various colour forms of var typica and var nana and from plants derived from zygotic embryo cultures also produced globular structures. Those from "suckering palms" merely expanded in culture with no signs of embryogenesis.

Our attempts to achieve sustained growth and germination of the globular structures were unsuccessful. Sporadic germination of the globular bodies occurred in a few cultures but complete plant development was not possible. Adventive root formation and haustorium development were rather common. The globular structures when cultured in the medium formulated (in this laboratory) for germination of the immature zygotic embryos produced a creamy white, compact, fast growing callus. The callus was successfully subcultured.

Techniques were also developed for extracting potential internal factors from the cabbage of coconut and for isolating protoplasts from the coconut leaf.

INTRODUCTION

Coconut is an important perennial crop grown widely in Asia and the Pacific. The palm enters the juvenile phase about 4-7 years after germination and has a productive period of over 40 years. The palm has a single growing vegetative point (apical meristem) and it does not branch nor produces suckers. Hence the palm is propagated exclusively through the seed.

There are no known methods of propagating the coconut palm by vegetative methods. In vitro research of the coconut palm aimed at cloning has a long history dating back to the early 1970s but a sustainable method has not yet been developed for plant regeneration. Plant regeneration has been reported from cultured immature inflorescences (floral meristems) by Branton and Blake (1983) but the regeneration appears to be poor and unproductive (Brackpool, personal communication). Raju et al (1983) also reported plant regeneration from cultured leaf explants but the results are not reproducible (personal communication). Pannetier and Morel (1982) have achieved early stages of plant development from cultured leaf material.

Our investigation aimed at developing technologies for clonal propagation of coconut and identifying some critical factors associated with the process, was commenced in 1984 with financial assistance from United States Agency for International Development (USAID). Although total plant regeneration was unsuccessful, other significant progress has been accomplished. A number of important observations worth investigating further also emerged. The findings are presented in this report and the project is being continued at the Coconut Research Institute utilizing local funds.

Abbreviations

BAP , N⁶ Benzyl Amino Purine ; pCPA , para - Chloro Phenoxy Acetic Acid ; 2,4 -D ; 2, 4-Dichlorophenoxy Acetic Acid ; Y3 ; Eeuwens medium ; IAA ; Indole Acetic Acid ; IBA ; Indole Butyric Acid ; MS ; Murashige and Skoog's ; NAA ; α - Naphthalene Acetic Acid ; NOA ; 2-Naphtloxy Acetic Acid ; PB ; Phosphate Buffer ; PEG ; Polyethylene Glycol ; TBE ; Tris Borate EDTA.

MATERIALS AND METHODS

Leaf segments excised from the apical bud of Cocos nucifera L were used as the experimental material.

The coconut bud consisting of the apical growing point and a few tender leaves was dissected out from 1-2 years old field grown plants and sterilized with a solution of 2% calcium hypochlorite for 10 minutes and then washed with three changes of sterile water. About 2 mm wide leaf segments were then excised from the inner leaves of the bud and placed on culture media and incubated in the dark at 28-30°C. unless otherwise specified.

Culture media:

The culture media tested were, Murashige and Skoog's (1962) and Eeuwens (1978) Y3 with some modifications in the mineral, organic and the phytohormone components and the various combinations of the Broad Spectrum Tissue Culture experiment of de Fossard (1976).

In vitro germination of mature zygotic embryos:

The technique developed by Karunartne et al (1985) was followed to raise seedlings from zygotic embryos in vitro.

Isolation of mesophyll protoplasts:

About one gram of finely segmented tender leaf material was placed in a 5-cm diameter petridish containing the enzyme mixture, (1% pectinase, 2% cellulase, 0.2% driselase and 0.6% mannitol) and incubated on a very slow shaker in the dark for about 4-5 hours. The protoplast suspension cleared of debris was loaded on to a percoll layer and spun

at 1000 rpm for 10 minutes. Protoplasts were then collected from the interphase and washed three times with 0.6M mannitol by repeated centrifugation at 200 rpm for three minutes.

Isolation of potential internal factors from tender tissues (buds) of the coconut:

About 90 g of the coconut bud tissue was homogenised with about 150 ml of TBE buffer in the cold (4°C). The homogenate was spun at 5000 rpm for 5 minutes. 3M ammonium sulphate was added to the supernatant until the final concentration in the mixture was 0.05M and the pellet was removed by centrifugation at 10,000 g for 10 minutes. The pellet was homogenised with TBE + 0.05M ammonium sulphate and washed twice by repeated centrifugation at 10,000 g. The pellet was then homogenised with 20 ml of TB5 in a teflon coated hand homogeniser and stirred for 3 hours in the cold and spun again at 10,000 g for 10 minutes. Nucleic acids were precipitated from the supernatant by a PEG/dextran/NaCl mixture and spinning at 15,000 g for 10 minutes.

The supernatant was then dialyzed against 3 portions of PB and NaCl. The presence of proteolytic activity in this fraction was shown by the degradation of radioactive bovine serum albumin, ¹⁴C BSA (see Results).

RESULTS AND DISCUSSION

1. Evaluation of a suitable culture medium:

Random leaf explants, cultured in MS and Y3 media containing activated charcoal, merely expanded with no signs of callusing or organogenesis. Modifications in the mineral and organic components and in auxin and cytokinin combinations also had no favourable effects. In liquid cultures, the tissues remained viable for about 8 months without any further development. Incubation of cultures in the light (8, 16 and 24h photoperiods) and complete withdrawal of activated charcoal from the medium enhanced browning of cultures. Activated charcoal was thus incorporated into all culture media and the cultures were incubated in the dark continuously unless otherwise stated in the text.

The Broad Spectrum Tissue culture experiment of de Fossard (1985), when performed on these leaf explants produced some satisfactory results. In liquid media, the tissues expanded several-fold in surface area and remained viable for about 10-12 months with no apparent signs of differentiation. However, when the media were solidified with 0.8% agar, three out of 81 media compositions namely media nos 44, 45 and 72 induced some developmental changes in the tissues. The changes were apparent 4-6 months after culturing. About 30% of the tissues cultured in the 3 media produced globose structures on the leaf surface without any intervening callus phase. Details of this observation are presented elsewhere in the text. The common feature of the three media was that they all had the same auxin : cytokinin ratio. Mineral salts, amino acids and sucrose levels varied from medium to high. Thus, it is apparent

that the chemical composition, physical state and the auxin : cytokinin ratio of the medium are some of the important factors inducing developmental changes in coconut leaf tissues.

However, the culture period of 4-6 months to exhibit the first developmental change appeared to be too long compared to very short periods frequently reported for other crops.

2. Somatic embryogenesis/organogenesis:

About 30% of the leaf explants cultured in the media, nos. 44, 45 and 72 developed creamy white globular structures after 4-6 months in culture (table 1).

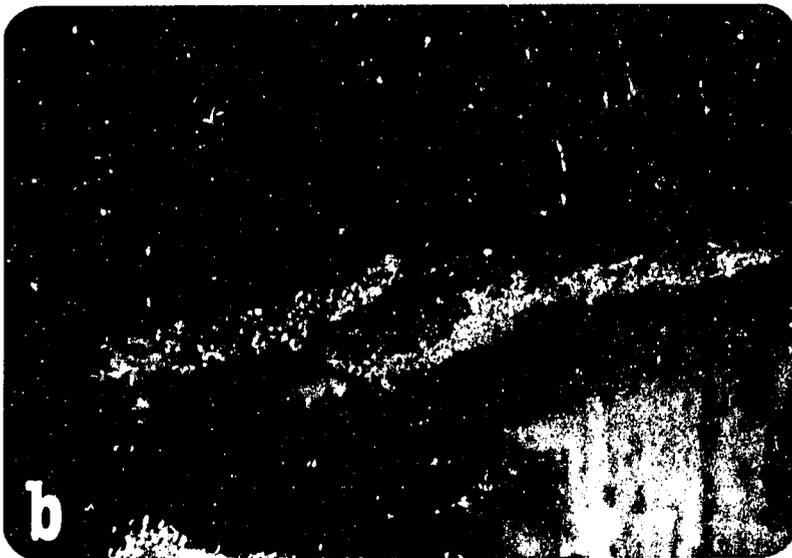
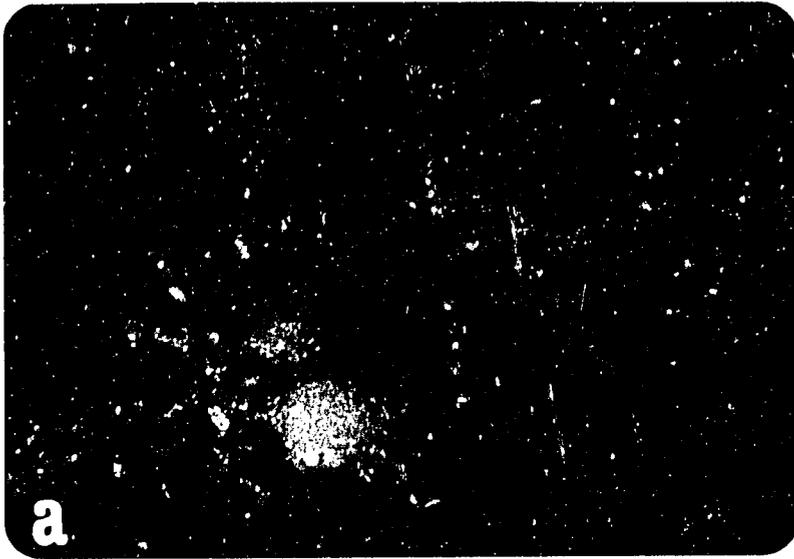
Table 1: Performance of leaf tissues in the media nos. 44, 45, 72

Medium no.	Response (% cultures with globules)	Globules per explant	Duration for 1st appearance (months)
44	24%	1 - 5	5 - 6
45	26%	1 - 5	4 - 5
72	24.2%	1 - 5	4 - 5

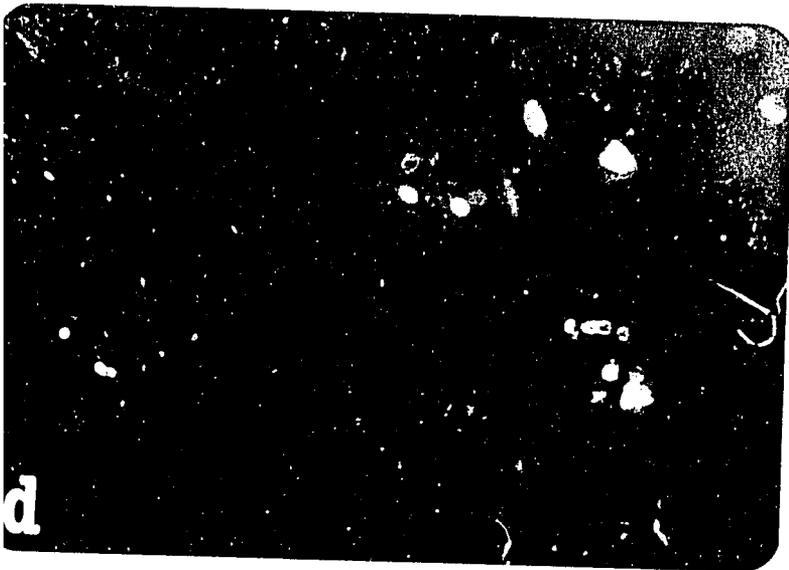
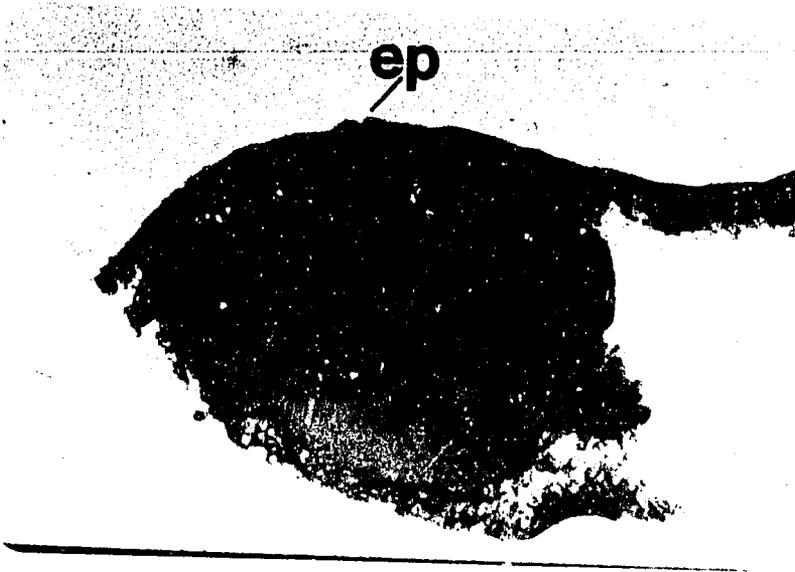
Response is expressed as a percent of the total number of leaf explants cultured.

The number of globules per explant varied from 1-5 and appeared to originate directly from inner tissues of the leaf, probably the mesophyll (figure 1 a, b, c). Occasionally, a few developed on the epidermal tissue. Very few pseudo callus tissues consisting of filaments of large hyaline cells were observed in the vicinity

Figure 1. Development of globular bodies from cultured leaf explants.



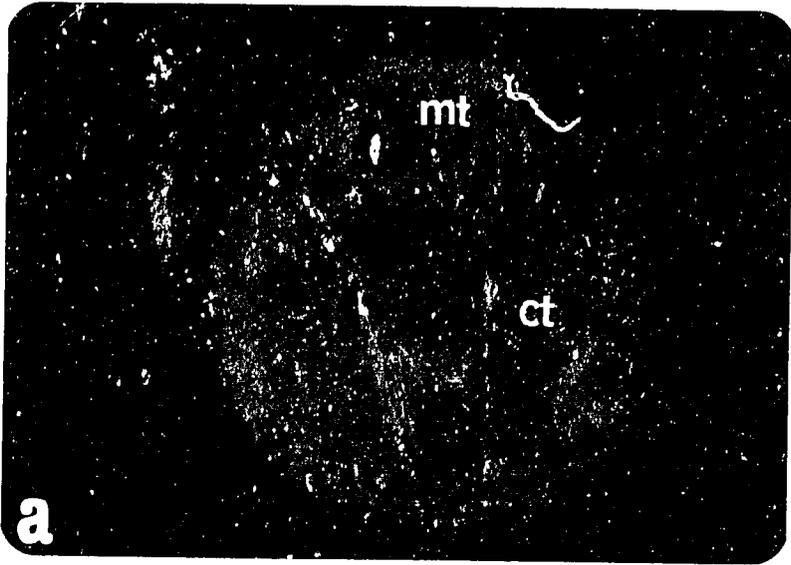
Contd.....

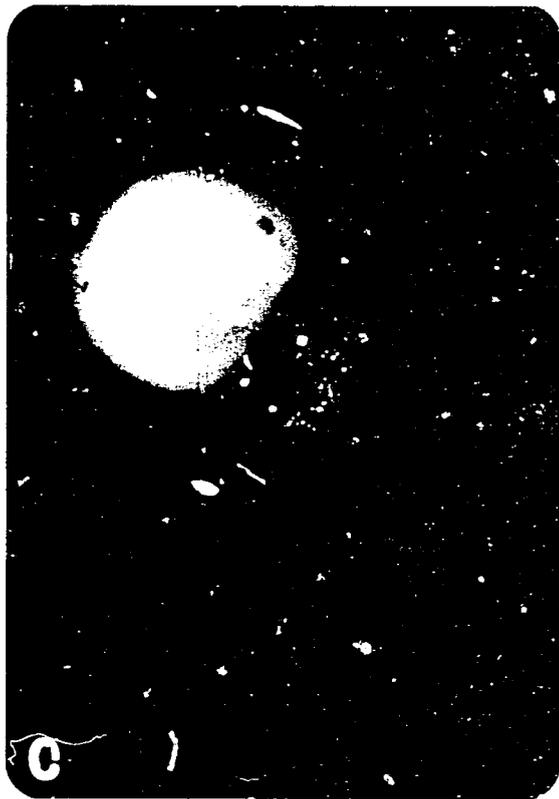


Globules a) on the leaf epidermis . b) emerging from the cut surface . c) within the leaf epidermis (ep) . d) showing weak point of attachment (p) to the leaf surface .

of the globular bodies (figure 1b) and all attempts to subculture these were unsuccessful. Eeuwens (1978) also reported formation of filamentous cells in cultured coconut tissues. The globular bodies when examined closely were observed to be very loosely attached to the leaf tissue (or rather just sitting on the epidermis) and were easily detachable from the mother tissue by the slightest mechanical disturbance (figure 1,d). This may perhaps be attributed to a single cell origin in the mesophyll/epidermal tissue. During early stages of development and whilst still within the leaf tissue, it was roughly spherical, demarcated by an epidermis - like structure and consisted of a compact mass of cells with scattered vascular elements (figure 1 c). Gradually it enlarged and acquired distinct polarity and emerged out of the leaf explant, either through a weakened point in the epidermis or through the cut surface. When sectioned, a cotyledon like structure and an apical meristematic region were seen as in the case of the early zygotic embryo (figure 2 a, b). Tracing the initial stages of development of the globular embryo-like structures was a difficult task. This probably indicates that the initial growth phase of the globule which lasts 3-4 months (ie. before emergence from leaf surface) is a very slow phase, and that this is followed by a very rapid growth phase during which it emerges out of the leaf explant. The zygotic embryo also exhibits a slow growth phase initially (Haccius and Philip, 1979). In addition to the embryo-like structures various neoformations were also observed in these cultures (figure 2 c, d, e). Rarely, roots developed directly from the main vascular strands of the explant and their attachment was observed to be very firm unlike the case in globular bodies.

Figure 2. Zygotic and "somatic" embryos of coconut.





a) An immature zygotic embryo. b) "Somatic" embryos on leaf explants (in section) showing the enlarging cotyledon (ct) and the meristematic region (mt). c), d), e) Neoformations on leaf explants.

3. Optimisation of culture conditions:

a) Hormones

Instead of the normal auxin mixture of IAA, IBA, NAA, NOA, 2, 4-D and pCPA in the three culture media, each auxin was tested separately at the low concentration of 1.0 μm and at the total low concentration of all auxins, 6.0 μm . The full media nos. 44, 45 and 72 were used as the control treatments. From the results of this experiment, it is clear that the formation of globular structures had occurred in leaf cultures only when 2, 4-D was present in the medium, either alone or in combination with other auxins (table 2).

Table 2: Effect of different auxins on leaf cultures

Auxin	Concentration, μm	Response (Cultures with globules)		
		44	45	72
IAA	1.0 , 6.0	-	-	-
IBA	1.0 , 6.0	-	-	-
NAA	1.0 , 6.0	-	-	-
NOA	1.0 , 6.0	-	-	-
2, 4-D	1.0	-	-	-
2, 4-D	6.0	++	++	++
p CPA	1.0	-	-	-
p CPA	6.0	-	-	*
Control (all 6)	total 6.0	+++	+++	+++

+++, 25-30% ; ++, 10-11% ; *Rhizogenesis (poor and inconsistent) ; -, leaf expansion but no globule formation

When supplemented alone at 6.0 μm level however, the response was lower than in the control which consists of a mixture of 6 auxins at a total concentration of 6.0 μm . This experiment shows that 2, 4-D is one important auxin inducing morphogenetic characters in coconut leaf tissue and that more than one auxin is perhaps needed to enhance the process.

In the following experiment, each and every auxin in the mixture was tested in combinations of two while maintaining the total auxin concentration at 6.0 μm as shown in the table 3.

Table 3: Effect of incorporating auxin pairs into leaf culture media.

Treatment	Response	Treatment	Response	Treatment	Response
2, 4-D/NAA	+	NAA/IAA	-	IAA/pCPA	-
2, 4-D/IAA	.	NAA/NOA	-	NOA/IBA	-
		NAA/IBA	-		
2, 4-D/NOA	-	NAA/pCPA	-	NOA/pCPA	-
2, 4-D/IBA	+	IAA/NOA	-	IBA/pCPA	-
2, 4-D/pCPA	-	IAA/IBA	-	Control (45)	++

Total auxin concentration is 6.0 μm , ++, globule formation in 20-25% of cultures; +, 8%.

The results of this experiment confirmed that 2, 4-D is essential for response in leaf cultures. It's pairing with other auxins however had no significant effect on cultures. In the subsequent experiment, each auxin was deleted from the mixture while maintaining the total auxin concentration in the medium at 6.0 μm . This experiment produced inconsistent results which were difficult to interpret.

A similar approach to the above was then followed to investigate the cytokinin component of the three media. Instead of the mixture of two cytokinins, BAP and Kinetin at the total concentration of 20 μm , the two were incorporated separately into three media at 10, 20 and 30 μm levels. A mixture of BAP and kinetin at a total concentration of 30 μm was also tested. The results are presented in table 4.

Table 4: Response of leaf cultures to cytokinins

Cytokinin	Concentration, μm	Response		
		44	45	72
BAP	10	++	++	++
BAP	20	++	++	++
BAP	30	-	-	-
Kinetin	10	-	-	-
Kinetin	20	-	-	-
Kinetin	30	-	-	-
BAP + Kinetin	15 + 15	-	-	-
Cytokinin-free	0	-	-	-
BAP + Kinetin (control)	10 + 10	++	++	++

++ ; 23-26% of cultures produced globular structures., + , 3%

It was found that, BAP at concentrations 10-20 μm was as equally effective as the control which consisted of a mixture of 10 μm kinetin and 10 μm BAP in inducing morphogenetic features. However, kinetin when used alone at 20 μm completely arrested "somatic

embryogenesis" in leaf explants. This shows that kinetin in the presence of BAP has no inhibitory effects. Complete withdrawal of cytokinins from the culture media also had no beneficial effect.

In the next series of experiments, the effect of incorporating high 2, 4-D into culture media was tested. The auxin mixture in the medium was replaced by high 2, 4-D concentrations ranging from 8-200 μm . In the presence of cytokinin in the medium, high 2, 4-D had no beneficial effect on cultures. However, when the cytokinin component was completely removed, the situation changed dramatically. About 50% of the cultures developed globular bodies compared to 25-30% recorded for the controls. Further, the medium no. 72 was found to be superior to medium nos. 44 and 45 (table 5).

Table 5: Effect of high concentrations of 2, 4-D on leaf cultures.

2, 4-D Concentration, μm	P.C. Somatic embryogenesis		
	44	45	72
* 8.0	++	++	+++
* 12.0	++	+	+++
* 16.0	+	++	+++
* 20.0	+	+	++
24.0	+	-	+
30.0 to 100	-	-	-
200	-	-	-
Control	++	++	++

++ ; 25 - 30% ; +++ 45 - 50% of cultures ; *, no: of globular structures per explant increased up to 20 where as in the control it was 1-5

The number of globules per explant also increased upto 20. The effective 2, 4-D concentration ranged from 8-20 μm . At concentrations above 30 μm , the cultures turned brown rapidly and died. The use of considerably low levels of 2, 4-D for coconut leaf cultures, in this investigation is noteworthy. Jennet Blake (1983) reported the use of a high level of 2, 4-D (10^{-4} - 10^{-3} mol) to induce a subculturable callus in coconut tissue. Tisserat (1980) recorded 30 mg/l 2,4-D for organ culture of Phoenix dactylifera.

Several important conclusions can thus be drawn from this investigation at this juncture. Firstly, as mentioned earlier, 2, 4-D is one of the crucial factors inducing morphogenetic changes in coconut and that the presence of other auxins and cytokinins is not required if an appropriate concentration of 2, 4-D is used in the medium. Secondly, the effective range of 2, 4-D in the medium is very short, from about 8-20 μm . Outside this range, the decline in performance of the cultures was rather sharp. Thirdly, the 2, 4-D range is most effective only when incorporated into the medium 72. This probably indicates that there is another yet unknown crucial factor in the medium 72 and that it enhances morphogenesis only when present in combination with 2, 4-D within the effective range.

b) Chemically undefined substances

Incorporation of chemically undefined substances such as yeast extract (0.1 to 1.5 g/l), casein hydrolysate (0.025 to 0.1 g/l) and coconut milk (10. to 200 ml/l) had no significant effect on leaf cultures. Globule formation occurred in a few cultures but the

results were inconsistent. The medium pre-conditioned by growth of the zygotic embryo of coconut completely suppressed the growth of leaf cultures, perhaps due to high contents of phenolics released into the medium by the growing embryo.

Thus, the culture medium which induces optimum response in leaf cultures consists of mineral salts and growth factors of medium 72 and 2, 4-D at 16-20 μm (modified 72, table 6).

Table 6: Composition of medium 72

Constituents	Concentration
Macronutrient elements (mmol)	
NH_4NO_3	20
KNO_3	20
NaH_2PO_4	2
CaCl_2	3
MgSO_4	3
Micronutrient elements (μmol)	
H_2Bo_3	150
MnSO_4	100
ZnSO_4	40
CuSO_4	1.5
Na_2MoO_4	1.0
CoCl_2	1.0
KI	5.0
FeSO_4	100
Na_2EDTA	100
Na_2SO_4	650

Constituents	Concentration
Auxins (μ mol)	
2, 4-D	12-20 μ m
Growth Factors (μ mol)	
Inositol	600
Nicotinic acid	40
Thiamine. HCl	40
Biotin	1.0
γ -Ca-Pantothenate	5.0
Pyridoxine. HCl	6.0
Riboflavin	10
Ascorbic acid	10
Amino acids (μ mol)	
L - Cysteine. HCl	120
Glycine	50
Sucrose (m mol)	120

4. Response of leaf tissues explanted from different sources

Leaf tissues were excised from seedlings of different varieties and forms of coconut, bearing palms and from embryo-cultured plants and cultured in the modified 72 medium in the usual manner. The seedling materials of typica and nana showed no morphogenetic differences in culture (table 7).

Table 7: Performance of leaf tissues derived from various types of planting materials

Source of leaf tissue	Response
Typica (seedlings)	++
Nana form pumila (seedlings)	++
" eburnea (seedlings)	++
" regia (seedlings)	++
Suckers (from "suckering palms", a sucker in place of the inflorescence)	-
Embryo - cultured plants	+
Bearing palms (typica)	-

++ globose structures in 45 - 52% of cultures

+ globose structures in less than 10% of cultures

The overall performance of the tissues explanted from embryo-cultured plants was poor and this may be attributed to the poor yield of viable explants. The leaf tissues derived from bearing palms never produced morphogenetic effects in culture. According to Pannetier and Morel (1982) only the callus formation (in low percent) is possible from bearing coconut palm tissues. In the case of oil palm also, the performance was reported poor when the leaf tissues derived from bearing palms were cultured (Ahee et al,1981). The poor performance was attributed to the slightly high degree of maturity of the explants. However, in our investigation nodule

formation (or callusing) never occurred even in the extremely tender leaf tissues of bearing palms of age 10-20 years. The age of the palms used by Pannetier and Morel (1982) for their experiments is not clear.

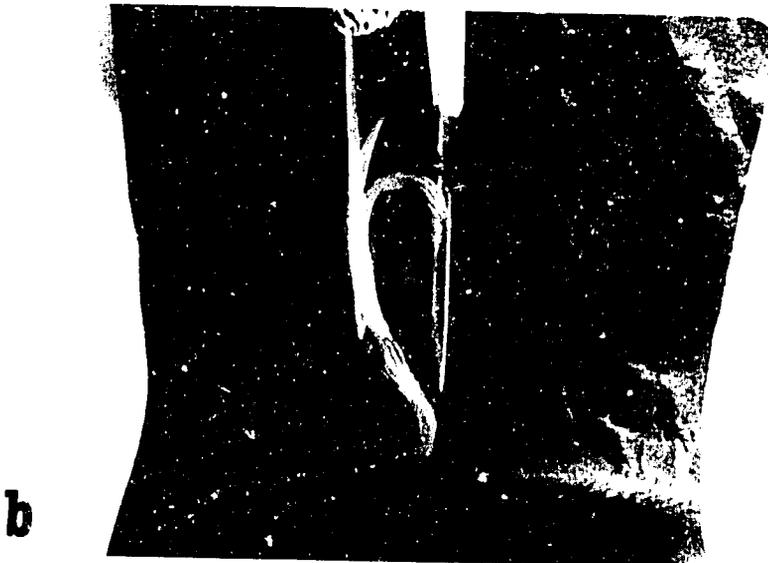
Poor morphogenetic potential of the bearing palm tissues was evident not only in leaf cultures, but in the apical meristem cultures as well. When the apical meristem was cultured, the rudimentary leaves slightly unfurled and remained inactive for more than 12 months without any further development. In contrast, under identical culture conditions, the apical meristem of young seedlings developed into a completely normal plant (figure 3). Plant multiplication however, was unsuccessful. Callus proliferation from the apical meristem was poor in both cases.

5. Leaf maturity and morphogenetic potential:

Coconut seedlings and adult palms always possess a range of leaves of differing maturity in the crown. The leaflets towards the base of each rachis are more tender and are generally used for culturing. The coconut palm thus appears to yield a range of culture material year-round.

Using a large number of 1-year old seedlings, leaves at various stages of development were removed for explanting (a single plant normally does not yield all the stages). The leaves were then cut into 1-cm wide segments starting from the leaf base. Explants from each segment were then cultured separately on modified 72 medium. The results of this investigation are summarised in table 8.

Figure 3. Meristem culture of coconut.



a) Meristem of a bearing palm after 12 months in culture (x 25); b) Meristem of a 2-yr old seedling developing into a seedling in the same medium (6 months).

Table 8: Effect of leaf maturity on development of globular structures

leaf stages* Distance from apical meristem, cm	p.c.c. explants producing globules					
	i	ii	iii	iv	v	vi
S-1(basal)	0	0	100	87	33	0
S-2	0	0	78	90	60	0
S-3	-	0	60	33	57	0
S-4	-	25	66	50	50	0
S-5	-	0	50	20	0	0
S-6(apical)	-	-	-	33	0	0

Globule formation is expressed as a percent of the total number of explants cultured. *, i to vi, Arbitrary stages from youngest excisable leaf in increasing sizes.

Stages i and ii yielded very few explants and they merely expanded in culture without any apparent signs of callusing or organogenesis. In contrast all the leaf tissues excised from the first 1 cm long segment of stage iii produced globular structures. However, even in this leaf the further the segment from the leaf base, the less responsive the tissue became. Similarly, the morphogenetic potential of the tissue declined as the leaf elongated further.

Thus it is clear that the coconut leaf has the highest morphogenetic potential at a stage which is probably associated with the most rapid growth phase of the leaf.

Leaf maturity is considered an important factor in inducing somatic embryogenesis of many plants, particularly, in cereals (Wernicke and Brettel, 1980; O'Hara and Street, 1978; Saalbach and Koblitz, 1978). In the case of coconut, the perennial palm, the situation appears to be very critical and complex and our observations probably partly explains the difficulty in developing a sustainable method for plant regeneration starting from the leaf tissue. Firstly, the younger leaves although more closer to the apical meristem are less responsive to culture conditions (Table 8). This appears to be associated with some kind of a lag phase in the growth of the leaf. The younger leaves of this phase do not respond to culture. Secondly, due to this lag there is no gradation in the developmental potential of leaves but a brusque transition. Hence, a chance of excising more than one leaf at a favourable stage of development from a single bud, never occurs in coconut. These two factors collectively limit the availability of suitable material for culturing. To worsen the situation, the palm produces on an average, about 12 leaves annually and the availability of material becomes still more critical.

5. Studies on further development' of the globular structures derived from cultured leaf tissues:

In an effort to improve growth of the globose embryo-like structures, the cultures were subjected to various treatments. Some of the treatments tested and the results obtained are summarised in the table 9.

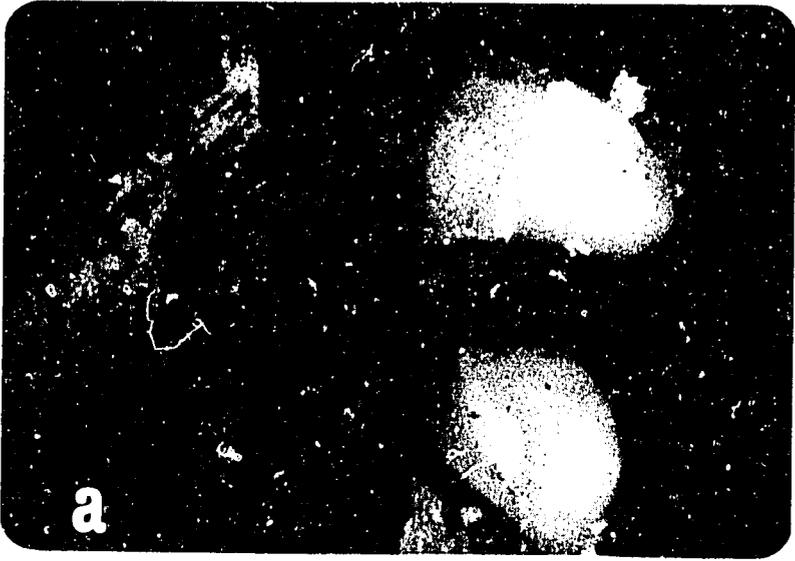
Table 9: Effects of different culture treatments on growth and development of globular structures

Treatment	Response
1. Repeated transfers to media of same composition.	Enhanced globule formation
2. Gradual transfer to low auxin (20 μ m) and auxin-free medium	Globule formation slightly enhanced
3. Gradual transfer to low auxin and high cytokinin (upto 30 μ m BAP) media	Sporadic germination, adventive root formation more frequent
4. Gradual transfer to auxin free but high cytokinin media (upto 30 μ m BAP and kinetin)	- do -
5. Transfer to the germination medium of coconut zygotic embryos (Karunaratne et al 1985)	Haustorium development, adventive roots developed from root pole
6. Transfer to media with low inorganic nitrogen	No further developments
7. Transfer to media with high organic nitrogen (asparagine, glutamine, arginine, upto 100 mg/l and their combinations)	Turned brown
8. Low and high sucrose and inositol (1/2 and 1 $\frac{1}{2}$ of normal concentration)	No further developments
9. Transfer to MS medium of normal, 1/2 and $\frac{1}{4}$ strengths	Turned brown
10. Exposure to low light intensity (1000 lux, 8, 16, 24 photoperiods)	Slight greening
11. Liquid medium of same composition, on filter paper bridges.	Browning

These results clearly show that the culture conditions conducive to growth and development of the somatic embryos of other species in general have no favourable effect on growth of embryo-like structures originated from coconut leaf tissues. Sporadic germination of the globose structures occurred under certain culture conditions but they did not develop into complete plants (figure 4 c, d). Very often adventitious roots developed from the globose structures during its course of further development (figure 4 b) and this was found to be highly unfavourable for subsequent shoot development. Haustorial tissue also developed in these cultures. (figure 4 a, b).

The reasons for poor growth of globose embryo-like structures is not clear at this stage. Raju et al (1983) also appear to have difficulties in reproducing the plant regeneration they accomplished from leaf cultures (personal communication). Germination of immature zygotic embryo of coconut in vitro was thus attempted with a view to develop a favourable system for sustained growth of the embryo like structures. Zygotic embryos, about 6-9 months postanthesis, when cultured in the leaf culture medium, with pCPA (10 μ m) in place of 2, 4-D germinated into minute seedlings (figure 5 a). Strangely, under identical culture conditions the globose structures developed into creamy white fast growing callus tissues (figure 5 b). The callus was easily subcultured. Part of the globular body developed into a haustorial tissue and it never produced callus (figure 5 c). This indicates that the culture conditions conducive to growth and development of the zygotic embryo and that of the somatic embryo-like structures are different in coconut.

Figure 4. Various developmental stages of the "somatic embryo".





a) Cotyledon developing fast; b) Development of shoot (s), roots (r) and haustorium (h);
c) , d) Sprouting.

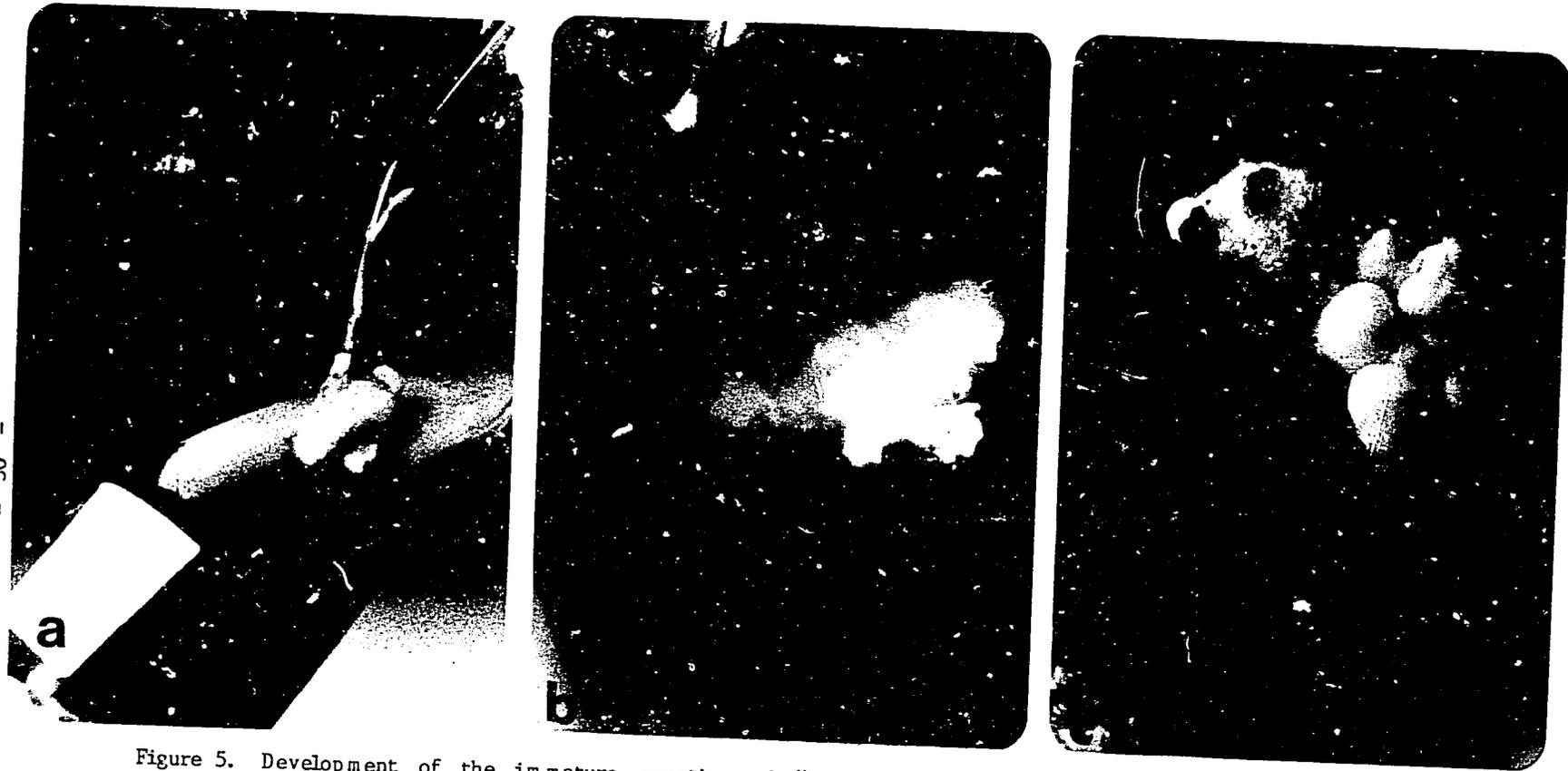


Figure 5. Development of the immature zygotic and "somatic" embryos in the germination medium.
a) Immature zygotic embryo germinating (b) "Somatic embryo" developing into a callus
c) Haustorial tissue of the somatic embryo enlarging. All three, cultured in the same medium.

7. Isolation of potential internal factors from coconut buds:

The differences in the responses of zygotic embryos and somatic embryo-like structures to the same medium led us to the obvious conclusion that specific internal factors were significantly affecting the phenomenon. Some very preliminary attempts were made in this direction, rather as a tentative beginning to gain practical experience for later work which we now propose to take up. They were carried out during a short stay at the University of Paris (Physiologie de la Differentiation Cellulaire - Prof. A. Kooor).

Instead of a search for 'embryogenic' proteins by an exhaustive electrophoretic screening of proteins (Ferdinando et al 1987) we opted for a search of proteolytic enzymes following the observations that in many other systems key proteins do exhibit some sort of proteolytic activity. Extracts of coconut buds (see materials and methods) showed characteristic profiles of proteins after DEAE column chromatography (figure 6). What appeared most promising to us was however, the presence of protease inhibitors. This was as far as we could go. We hope to take up this approach more systematically in the near future if material conditions will permit.

Thus in conclusion, direct development of somatic embryo-like structures from coconut leaf tissues is now possible. However, sustained growth of the embryo-like structures leading to plant development is unsuccessful. Instead, it produces a fast growing subculturable callus and it may be worth attempting somatic embryogenesis from these tissues and from neoformations for a viable propagation method.

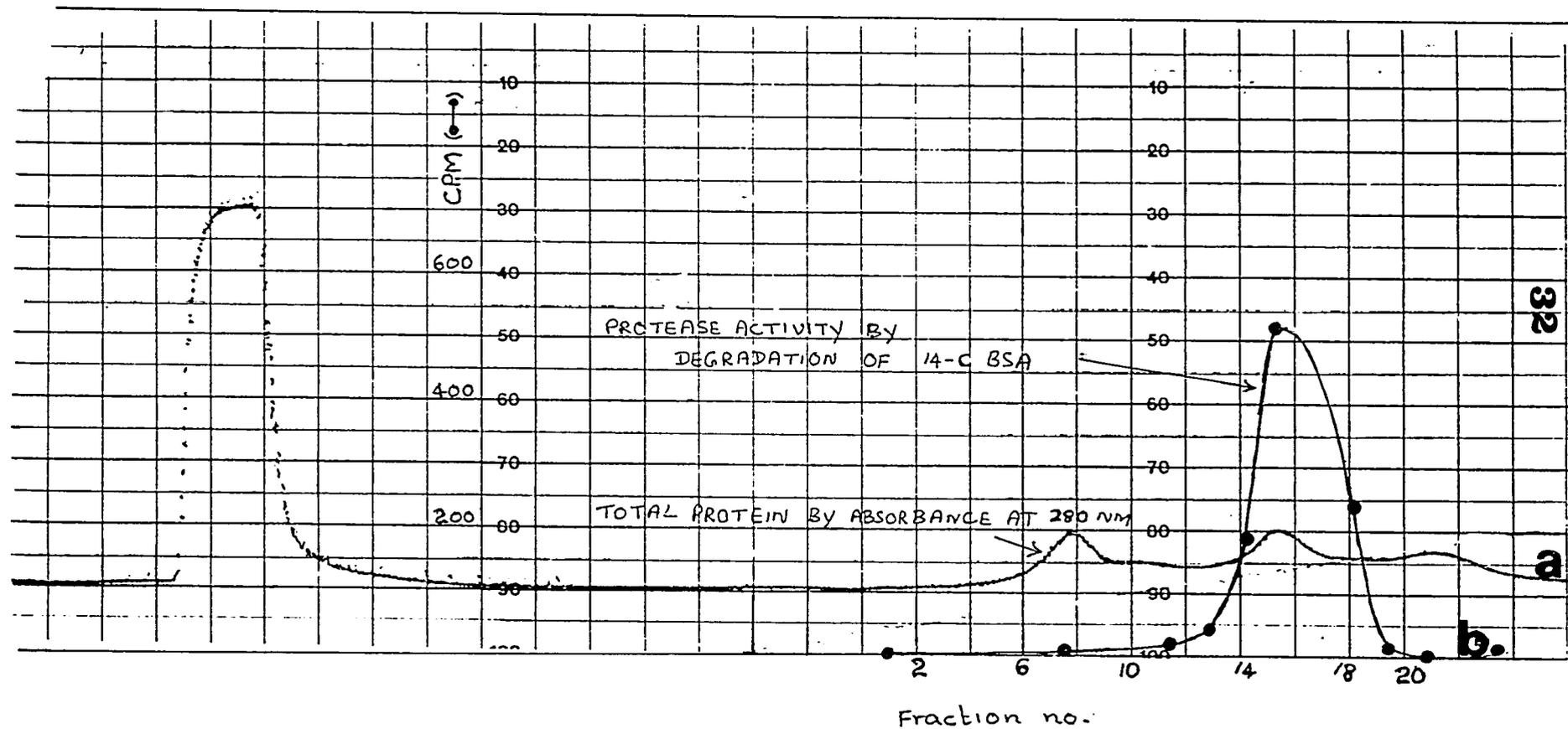


Figure 6. Protein profiles of coconut bud extract a) after DEAE column chromatography, b) after degradation with 14C-BSA radioactivity.

Secondly, the coconut palm possesses a large number of extremely tender leaves but only a very few yields viable explants. Physiological maturity of the leaf, at the time of excision is critical. An investigation leading to isolation of internal factors from these tissues may also be worth attempting as the requirements for germination of the two types of embryos are different.

ACKNOWLEDGEMENTS

I am most grateful to United States Agency for International Development (USAID) for supporting this research project. Assistance tendered by USAID office in Sri Lanka and Natural Resources, Energy and Science Authority of Sri Lanka (NARESA) is also acknowledged.

Special thanks are also due to Ms C K Zoysa and the staff of the Tissue Culture Unit for the technical assistance tendered throughout the project. Prof. A Kovoov of the University of Paris (Physiologie de la Differentiation Cellulaire) deserves special thanks for the consultancy services provided purely on an honorary basis and for the opportunity given to me to conduct some experiments in his laboratory. Finally, my thanks to the Coconut Research Board and the Director of the Coconut Research Institute for the permission granted to conduct this investigation at the Coconut Research Institute.

REFERENCES

1. Ahee, J; Arthuis, P ; Cas, G ; Duval, Y ; Guenin, G ; Hanower, J ; Hanower, P ; Lievoux, D ; Lioret, C ; Malaurie, B ; Pannetier, C ; Raillot, D ; Varechon, C Zuckerman, et al. (1981) La multiplication vegetative in vitro du palmier a huile par embryogenese somatique. *Oleagineux* 39 : 113-115.
2. Branton, Richard ; Blake, Jennet (1983): A lovely clone of coconuts. *New Scientist* 26 May 554-557.
3. Blake, Jennet (1983): Tissue culture propagation of coconut, date and oil palm. In, *Tissue culture of trees* Ed John. H. Dodds. Croom Helm London Ltd.
4. Eeuwens, C.J.(1978): Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera* L) and cultured in vitro. *Physiol Plant* 36: 23-28
5. Eeuwens, C.J. ; Blake, Jennet (1977): Culture of coconut and date palm tissue with a view to vegetative propagation. *Acta Hort* 78; 277-284
6. Ferdinando, de. ; Hulme, J. ; Hughes, W.A.(1937): Oil palm embryogenesis: A biochemical and morphological study. In , *Experimental manipulation of ovule tissues* Ed G.P. Chapman , S.H. Mantell, R W. Daniels. Longman.
7. Fossard de R.A.(1976): Tissue culture for plant propagators pp 132-149. The University of New England Printery, Australia.
8. Haccius, Barbara; Philip V.J. (1979) Embryo development in Cocos nucifera L: A critical contribution to a general understanding of palm embryogenesis. *Pl Syst Evol* 132 : 91-106
9. O'Hara, J.F. ; Street, H.E. (1978) Wheat callus culture : the initiation, growth and organogenesis of callus derived from various explant sources. *Ann. Bot.* 42 : 1029-1038

10. Karunaratne, S. ; Kurukulaarachchi, C. ; Gamage, C.K.(1985): A report on the culture of embryos of dwarf coconut, Cocos nucifera L var nana in vitro. Cocos 3: 1-8
11. Murashige, T. ; Skoog, F. (1962): A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum 15: 473-497
12. Pannetier, C. ; Buffard-Morel, J. (1982): First results of somatic embryo production from leaf tissues of coconut, Cocos nucifera L. Oleagineux 37 : 352-353.
13. Raju, C.R. ; Prakash Kumar, P. ; Chandramohan, Mini ; Iyer R.D. (1984): Coconut plantlets from leaf tissue cultures. J. Plant. Crops 12: 75-78
14. Saalbach, G. ; Koblitz, H. (1978): Attempts to initiate callus formation from barley leaves Pl. Sci. 13: 165-169
15. Tisserat (1980): A histological study of development of adventive embryo in organ cultures of Phoenix dactylifera L. Ann.Bot. 46: 465-472
6. Wernicke Wolfgang; Brettel Richard (1980) Somatic Embryogenesis from Sorghum bicolor leaves. Nature 287: 138-139