AN INVESTIGATION INTO *IN VITRO* CULTURE AND PHYTOCHEMICAL ASPECTS OF SOME MEMBERS OF THE ORDER CYCADALES.

by

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PREFACE

Planning and initial collection of plant material for this project commenced in January 1984. The major portion of the experimental work was carried out in the Departments of Biochemistry and Botany, University of Durban-Westville, over the period December 1985 to December 1986. Additional experimental work was done in the Departments of Biology and Chemistry, University of Natal, Durban, from January 1986 to April 1988. The project was supervised by Professor J. van Staden of the Department of Botany, University of Natal, Pietermaritzburg. Except where the work of others is acknowledged in the text, these studies are the result of my own investigations and have not been submitted in any form to another university for degree purposes.

SIGNED: Roy Osborne.

Roy Osborne, B.Sc. (Hons), M.Sc.
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Amongst the many others who have assisted in various ways, I wish to pay tribute to the following: Professor Mario Ariatti and Mrs. Barbara Huckett, Department of Biochemistry, University of Durban-Westville; Dr. Peter Brain, Natal Institute of Immunology; Professor Pat Berjak and Dr. Norman Pammenter, Department of Biology, University of Natal, Durban; Dr. Jack Cannon, School of Chemistry, University of Western Australia; Professor Paolo de Luca and colleagues, Orto Botanico, Naples; the Directors and staff of the Botanical Research Institute, Pretoria and Durban units and the Sugar Milling Research Institute, Durban, and Dr. R.A. Dyer of the former Institute; Professor Nat Grobbelaar and colleagues, Department of Botany, University of Pretoria; Mr. John G. Hendricks, Statesville, North Carolina, U.S.A.; Mr. Danie Nel, Natal Chairman and Seedbank Officer of the Cycad Society of Southern Africa, and members of the Society and other cycad collectors; Dr. Knut Norstog and his associates at the Fairchild Tropical Garden, Miami, Florida; Professor Moosa Motara, Department of Zoology, University of Durban-Westville; Professors Karl Pegel and David Taylor, Department of Chemistry, University of Natal, Durban; Dr. Colin Rogers, University of Durban-Westville; Professors Antonio and Maria Luiza Salatino, Instituto de Biociências, University of São Paulo, Brazil; Professor Dennis Stevenson, New York Botanical Garden; Dr. Piet Vorster, Department of Botany, University of Stellenbosch; Dr. David Webb, BC Research, Vancouver, Canada; and Mr. Ken Wyman and Mrs. Ann Lambert, Durban Botanic Gardens.

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Finally, I record my appreciation to my wife and family for proof-reading the text and for their patience and understanding throughout this project.

DEDICATION

The work which is reported in this thesis is dedicated with respect to the memory of my father, the late

HARDIE WARWICK OSBORNE

Es tat in nächtlich heil'ger Stund'
der Herr sich durch ein Wunder kund.
Den dürren Stab in Priesters Hand
hat er geschmückt mit frischem Grün :

(Tannhäuser, Act 3, Wagner, Dresden, 1845).

ABSTRACT

The present-day cycads represent the diverse, modified remnants of a much larger group of gymnosperms which flourished in the Mesozoic Era. The approximately 148 surviving species of the Cycadales are sparsely distributed through tropical and sub-tropical floras in a variety of habitats. About one-half of the extant taxa are considered endangered, vulnerable or rare and, because of their scarcity and decorative appeal, have attracted much public interest. Their slow growth rate, the paucity of viable seeds and limited potential for vegetative reproduction severely limit both the natural regeneration and the controlled propagation of cycads.

Over the past 40 years, various attempts have been made to establish *in vitro* systems for cycad culture but none has been successful in establishing a functional protocol for the artificial propagation of these plants. The author has made renewed attempts to establish *in vitro* cultures from a range of haploid and diploid tissues from South African *Encephalartos* and *Stangeria* species. Callus proliferation was readily obtained from most explant sources of most species using a variety of media. Addition of the growth factors 2,4-dichlorophenoxyacetic acid and kinetin in the 10^{-7} to 10^{-6}M range was beneficial but not essential. Culture vessels which allowed relatively free gaseous interchange were advantageous and dark conditions were marginally better than constant light. Explants from cycad taxa which are mesic in habit gave a more rapid response than similar explants from xeric plants.

Attempts to induce any form of differentiation other than, or after, callus formation were unsuccessful in all *Encephalartos* cultures, but two forms of morphogenesis were obtained from *Stangeria*-derived material. Megagametophytic tissue occasionally developed spherical outgrowths analogous to coralloid root primordia. More significantly, primary root cultures after callus formation, subculture and transfer to a light environment, regularly gave rise to meristematic zones and subsequent leaf emergence. This is the first recorded case of *in vitro* morphogenesis of a South African cycad.

The order Cycadales shows several distinctive phytochemical features, principally the presence of the unique methylazoxymethanol glycoside toxins and α-amino-β-methylaminopropionic acid together with some unusual phenolic compounds, flavanoids, carotenoids and cyclitols. *Stangeria* differs from other cycads in at least two phytochemical aspects; the absence of biflavonoids in the leaves and the absence of rhamnose and methylrhamnose in the hydrolysed mucilages. These differences may indicate broader physiological differences which would in turn explain the observed differences in morphogenetic competence of tissues from *Stangeria* and *Encephalartos*. Analyses of various tissues from these and other cycad taxa were performed with respect to moisture, protein, enzyme, toxin and hydrocarbon content. Significant differences, both between organs and between taxa, were noted. The results of peroxidase analyses were particularly important in that high levels of this enzyme correlate with the rapidity of callus formation *in vitro*. Furthermore, a sharp increase in peroxidase activity signals the onset of callogenesis in *Stangeria* megagametophyte cultures.

An important incidental aspect of the phytochemical analyses is that of potential value of these data to the taxonomist. In particular, the leaf wax hydrocarbon profiles appear to be species-specific and are ideally suited to processing by numerical taxonomy computer programmes. It is anticipated that extension of this work will make a significant contribution to the resolution of existing problems in cycad taxonomy and, additionally, provide a means to construct phylogenetic sequences in the order.

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CHAPTER ONE

CYCADS IN PERSPECTIVE

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1.1 Introduction

The present-day group of plants known as cycads* comprise the diverse, modified, remnants of a much larger group of gymnosperms which flourished in the Mesozoic Era, reaching their zenith in the Jurassic Period, some 160 million years ago. Although many palaebotanic records exist (WIELAND, 1906; WORSDELL, 1906; ARNOLD, 1953; HARRIS, 1961; DELEVORYAS, 1975; MAMAY, 1976), the exact origin of the cycads remains uncertain. In essence, it is believed that the Palaeozoic pteridosperms gave rise to two broad sections within the group: the cycadaleans, ancestors of the extant cycads, and the cycadeoidaleans (Bennettitales), which later became extinct. Clearly the cycads, with motile sperm cells like their ancestral ferns, but bearing true embryo-containing seeds like those of modern flowering plants, are a vital part in the botanical evolutionary jig-saw puzzle. How closely related the present cycads are to their forerunners is another point of contention. The popular view is that the extant cycads are relatively unchanged survivors from pre-history ('living fossils', 'coelacanths of the plant world', etc.). Perhaps a better-informed opinion is that these plants constitute a vigorous and successful group which is still evolving and capable of responding even now to changing environmental conditions (ECKENWALDER, 1980). Some further detail of the speculation on the evolution of cycads is given in a popular article by the author (OSBORNE, 1986c which is included in this thesis as Appendix 1).

Although for a major group the number of taxa is relatively small (Section 1.2), there is considerable diversity within these dioecious cone-bearing plants. Amongst the growth forms are the tall, usually unbranched, arborescent species such as the Australian *Lepidozamia hopei*

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*Footnote

The common name 'cycad' refers to all members of the order Cycadales and is derived from *Cycas L.*, the first genus to be described. The Oxford English Dictionary (1978) gives the origin as from *kykas*, as error of *koikas*, the accusative plural of *koix* which is a Greek name for the doum palm of Egypt. Despite this etymology, there is no direct relationship between the palms and the cycads.

Regel (stems up to 18 m) and the South African *Encephalartos transvenosus* Stapf and Burtt Davy (up to 13 m). These arborescent forms, with a terminal crown of frond-like leaves, certainly do have a superficial resemblance to palms. Persistent leaf bases remain when the leaves are shed and lend structural support to the trunk, which is strengthened internally by a complex series of girdling leaf traces. Because of these systems, the amount of secondary development is small in comparison with that of other gymnosperms and woody plants in general. Although concentric and definable growth zones are present in some taxa, these do not constitute seasonal increments and the typical annual rings associated with woody plants are absent. A substantial text has been published on the detailed stem anatomy of extant cycads (GREGUSS, 1968). Apart from the arborescent cycads, there are many taxa in which the stem is partially or completely subterranean. In some of these forms the foliage is nevertheless considerable with leaves reaching up to 3 m in length, as in some specimens of *Encephalartos villosus* Lem. By contrast the appropriately-named Caribbean species, *Zamia pumila* subsp. *pygmaea* (Sims) Eckenwalder, has a subterranean caudex only 25 cm long and has correspondingly small leaves. A particularly unusual cycad is *Zamia pseudoparasitica* Yates in Seemann, which lives as an epiphyte in forest canopies in Costa Rica and Panama.

Apart from the above vegetative features, the cycads have unique reproductive organs and processes (Section 1.6). Add to these an apogeotropic root system with symbiotic Cyanobacteria (Section 1.9). and the presence of a series of exclusive phytotoxins (Section 3.2) and it is clear that this plant group is far from ordinary. The past decade has seen a resurgence of interest in the cycads in botanical and horticultural disciplines. The formation of the Cycad Society (U.S.A.), the Palm and Cycad Societies of Australia and New Zealand, and in particular the Cycad Society of Southern Africa, has met with enthusiastic response and has brought together interactive groups of academics, nurserymen. conservationists and laymen with common interests. A recent bibliography of the living cycads (READ and SOLT, 1986) extends to 167 pages. A popular article, illustrating something of the broad range in current cycad research projects, has been published by the author (OSBORNE, 1986b, and is included in this thesis as Appendix 2). Further testimony to the high level of interest in these plants is the success of the first

International Congress on Cycad Biology in France in 1987. All this activity has not been without its problems, and many cycads must now be considered endangered species (Section 1.4).

1.2 Taxonomy of the Cycadales

A number of proposals have been put forward which attempt to express the relationships between and within the extant and extinct cycads and other gymnosperms. The scheme of PANT (1957) was drawn up on the basis of all available botanical and palaebotanical evidence and remains a useful phylogenetic system. The relative positions of the seven orders of living gymnosperms, in four divisions, is clearly evident (Figure 1). In this scheme, the Cycadales is the only order within the class Cycadopsida of the division Cycadophyta.

GYMNOSPERMS : CLASSIFICATION

Division 1. CYCADOPHYTA

Class 1. Pteridospermopsida

Order 1. Lyginopteridales
Order 2. Medullosales
Order 3. Glossopteridales
Order 4. Peltaspermales
Order 5. Corystospermales
Order 6. Caytoniales

Class 2. Cycadopsida

Order 7. Cycadales

Class 3. Pentoxylopsida

Order 8. Pentoxylales

Class 4. Bennettitopsida (Cycadeoideopsida)

Order 9. Bennettitales (Cycadeoideales)

Division 2. CHLAMYDOSPERMOPHYTA

Class 1. Gnetopsida

Order 1. Gnetales
Order 2. Welwitschiales

Division 3. CONIFEROPHYTA

Class 1. Coniferopsida

Order 1. Cordaitales

Order 2. Coniferales
Order 3. Ginkgoales

Class 2. Ephedropsida

Order 4. Ephedrales

Class 3. Czekanowskiopsida

Order 5. Czekanowskiales

Class 4. Taxopsida

Order 6. Taxales

FIGURE 1: Classification of gymnosperms as proposed in PANT's (1957) modification of ARNOLD's (1948) system. The seven orders boxed are those with present-day representatives.

The taxonomy *within* the Cycadales is of more direct relevance to this project. Here too, the number of taxa at family, tribe, genus and species level has been variously accommodated and revised and early proposals have been substantially modified. In a comprehensive monograph on the subject, SCHUSTER (1932) assigned all representatives to one family, the Cycadaceae. Schuster's work has met with very substantial criticism. JOHNSON (1959) has this to say ".... Schuster so profusely introduces new and profound confusions in taxonomic concepts of every rank and, in nomenclature so blatantly contravenes the rules of priority, and so unreliably cites both synonymy and specimens that the work is quite egregious ....". "Virtually identical plants, even the same specimens, are referred to entirely different species." ".... his arrangement and circumscription .... bear almost no relation to the real affinities ....". "The result is a jumble which almost defies disentanglement." One may sympathise with Schuster to some extent; any attempt at drawing up a cycad classification based largely on herbarium specimens and with reference to live plants cultivated in quite atypical conditions, is unlikely to succeed.

Using parameters such as leaflet structure and the morphology of female cones, JOHNSON (1959) drew up an authoritative reclassification which has formed the basis for all subsequent revisions (Figure 2). Johnson created three families to accommodate the nine known genera and added a tenth genus, *Lepidozamia* Regel, species of which had been grouped previously with *Macrozamia* Miq. Two families, Stangeriaceae and Cycadaceae, contain only single genera, while the family Zamiaceae is split into three tribes to encompass all other genera. STEVENSON (1981) has separated out *Bowenia* Hook. ex Hook. f. into a fourth family, the Boweniaceae. More recently, STEVENSON (1985) has revised the placement of *Dioon* Lindley, introduced two suborders to accommodate the four families, and has included an eleventh genus, *Chigua*, two species of which have recently been found in Colombia (STEVENSON, *pers. comm.*).

In addition to the new genus, *Chigua*, several other species are in the process of description and more are undoubtedly yet to be found. Only the genera *Bowenia* Hook. ex Hook. f., *Lepidozamia* Regel, *Microcycas* (Miq.) A. DC. and *Stangeria* T. Moore are considered botanically complete at this time. *Dioon* Lindley, *Encephalartos* Lehm, and *Macrozamia* Miq. are largely

defined but some changes are anticipated. The genera *Cycas L.* and *Zamia L.* present a number of taxonomic problems and major revision is required. A survery of the current botanically-valid species (OSBORNE, and HENDRICKS, 1985, 1986) reveals a global total of 148 species in which *Encephalartos* and *Zamia* collectively contribute about 60% of the total. The current "World List of Cycads" (OSBORNE, HENDRICKS and STEVENSON, in preparation) is included in this thesis as Appendix 3.

CYCADALES : PRESENT CLASSIFICATIONS

JOHNSON (1959)

order	Cycadales				
families	Stangeriaceae	Cycadaceae	Zamiaceae		
tribes			Encephalarteae	Dioeae	Zamieae
genera	*Stangeria*	*Cycas*	*Lepidozamia*	*Dioon*	*Microcycas*
			Macrozamia		*Ceratozamia*
			Encephalartos		*Zamia*
					Bowenia

STEVENSON (1981)

order	Cycadales					
families	Stangeriaceae	Boweniaceae	Cycadaceae	Zamiaceae		
tribes				Encephalarteae	Dioeae	Zamieae
genera	*Stangeria*	*Bowenia*	*Cycas*	*Lepidozamia*	*Dioon*	*Microcycas*
				Macrozamia		*Ceratozamia*
				Encephalartos		*Zamia*

STEVENSON (1985 and *pers. comm.*)

order	Cycadales					
suborders	Stangerinae		Cycadinae			
families	Stangeriaceae	Boweniaceae	Cycadaceae	Zamiaceae		
tribes				Encephalarteae	Zamieae	
genera	*Stangeria* (1)	*Bowenia* (2)	*Cycas* (17)	*Lepidozamia* (2)	*Microcycas* (1)	
				Macrozamia (14)	*Ceratozamia* (10)	
				Encephalartos (46)	*Zamia* (43)	
				Dioon (10)	*Chiqua* (?) (2)	

FIGURE 2: JOHNSON'S (1959) classification of the Cycadales and STEVENSON'S (1981, 1985 and *pers. comm.*) proposed modifications. Numbers in parenthesis indicate the number of valid species in corresponding genera (OSBORNE, HENDRICKS and STEVENSON, in preparation).

In parallel with many other plant groups, it appears that the taxonomy of the cycads has reached the situation where it is necessary to adopt a much broader perspective. Biochemical, immuno-serological, karyological, palynological and ultra-structural evidence should be collectively and carefully assessed in future revisions. An overall consistency in taxonomic philosophy throughout the order is perhaps the major long-term objective in this respect.

The South African species of *Encephalartos Lehm.* are fairly well defined (DYER, 1965; GIDDY, 1984). A review of *E. eugene-maraisii* Verdoorn currently proposes the establishment of one new subspecies, *E. eugene-maraisii* subsp. *middelburgensis* and two new species, *E. dolomiticus* and *E. dyerianus* (LAVRANOS and GOODE, in preparation). The status of *E.* "reinwaldii/cerinus", *E.* "Msinga" and *E. lebombonensis* "var. Piet Retiefii" requires clarification, which may result in further new species being constituted. The taxonomy of the central, east, and west African species of *Encephalartos* is less well defined and both political and geographic constraints continue to inhibit research in these areas (MALAISSE, F., *pers. comm.*)

1.3 Distribution.

There is ample palaeobotanical evidence to support the view that cycad-like plants were well represented in Mesozoic times. Well-preserved cycadophyte material has been located in strata from the late Triassic to early Cretaceous periods from sources in Siberia, Manchuria, Oregon, Alaska, several islands in the Arctic Ocean, Greenland, Sweden, England, central Europe, India, Australia and the Antarctic continent (ARNOLD, 1953). Significantly, cycads were abundant at the time when the southern part of Africa, South America, India, Antarctica and Australia were all joined in the massive supercontinent of Gondwanaland (DELEVORYAS, 1975). In present times distribution of the extant cycads is limited to the tropical and mild temperate regions in both hemispheres (Figure 3). The highest densities, in terms of proliferation of species, are found in Mexico (34 species in 3 genera), Queensland, Australia (11 species in 4 genera) and South Africa (29 species in 2 genera) (OSBORNE, HENDRICKS and STEVENSON, (in preparation).

Few of the present genera are internationally widespread. *Macrozamia, Lepidozamia* and *Bowenia* are endemic in Australia, while *Cycas* extends from the African coast through the Indian ocean islands to south-east

Asia, Australia and the islands in the western Pacific. *Encephalartos* and *Stangeria* are confined to the African continent. *Zamia* is found from Florida, through the Caribbean and central America to a large part of South America. *Ceratozamia* and *Dioon* have a more restricted distribution in central America, principally in Mexico, while *Microcycas* is confined to Cuba. Mention has already been made of the new species, *Chigua*, found in Colombia.

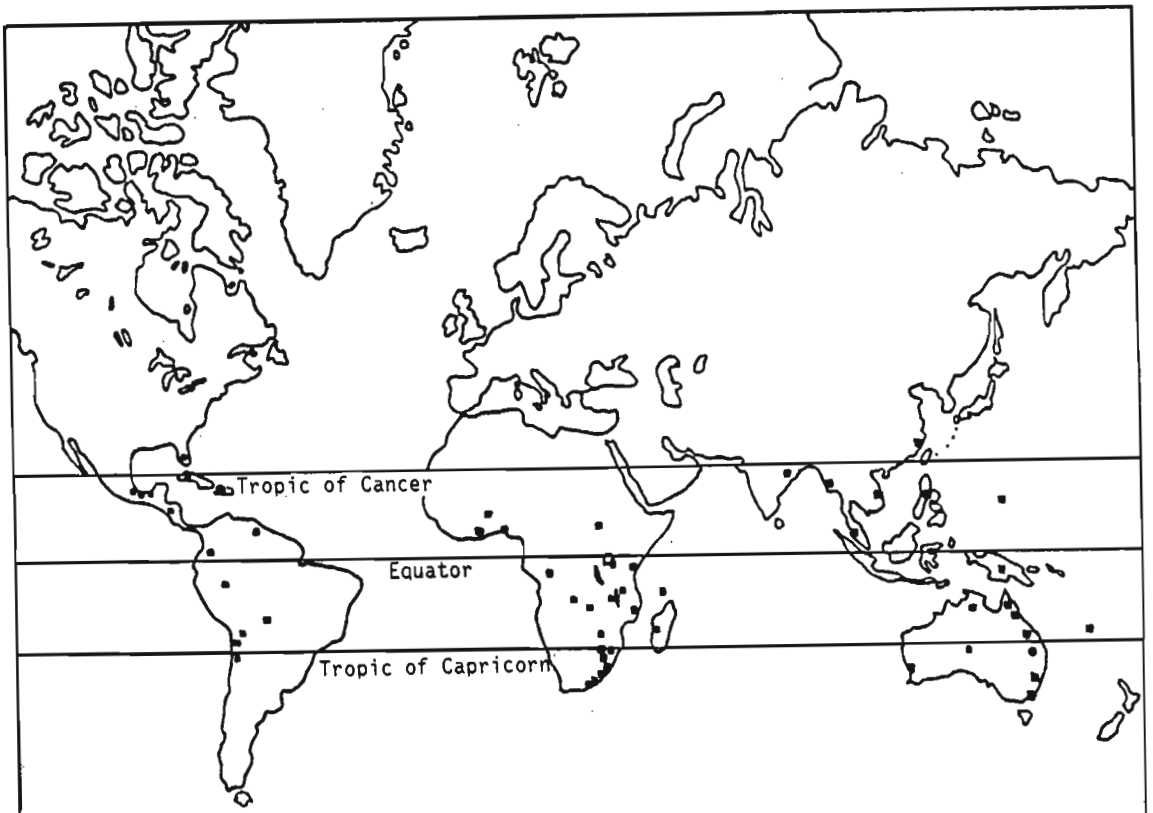


FIGURE 3: The world distribution of cycads. Areas marked indicate sites with the highest frequency of occurrence for the better-known species. After GIDDY (1984).

Within this broad geographical distribution there is a wide variety of local habitats, from the tropical rain-forests of northern Queensland, Australia, and some central American locations, to the fairly arid savannah of some parts of Mexico and the karroid scrub in the Eastern Cape of South Africa. In South Africa, *Encephalartos ghellinckii* Lem. thrives in high-altitude montane grassland in the Drakensberg mountains. As previously stated, *Zamia pseudoparasitica* Yates in Seemann lives as an epiphyte in the central American rain-forests. It is not surprising that many species show pronounced xeric modifications, typified by coriaceous leaves often with spines or prickles, thick waxy cuticles and deeply recessed stomata. Cycads are usually sparsely distributed in their particular associations but in some localities they may be at

least co-dominant. The "Modjadji Palm" forest of *Encephalartos transvenosus* Stapf and Burtt Davy near Duiwelskloof in the northern Transvaal (DYER, 1965), certain Mexican populations of *Dioon spinulosum* Dyer (CHAMBERLAIN, 1919) and stands of *Macrozamia riedlei* (Gaud.) Gardn. in south-western Australia (BURBIDGE and WHELAN, 1982), are examples where cycads approach dominance in the local vegetation.

1.4 Conservation status.

Approximately one-half of the total number of cycad species have been classified as "endangered", "vulnerable" or "rare" by the Threatened Plant Unit (TPU) of the International Union for the Conservation of Nature and Natural Resources (IUCN) (GILBERT, 1984). Prior to the appearance of man, it was the effect of long term climatic changes and short term environmental catastrophes which modified the world's cycad populations: In the last 100 years it has been the activities of man which have placed pressure on the existing stands. Factors contributing to this pressure are man's continuing domestic and agricultural demands on finite land resources, the eradication programme for some toxic species in Australia, activities of the tribal "medicine-men" in some countries and the widespread removal of specimen plants from habitat for horticultural purposes. The conservation problems are exacerbated by the slow growth rate of cycads and the paucity of viable seeds.

In the New World, the Mexican species *Dioon califanoi* De Luca and Sabato and *D. caputoi* De Luca, Sabato and Vasquez-Torres are considered endangered and the Cuban *Microcycas calocoma* (Miq.) A.DC. is at risk (GILBERT, 1984). The worst threat seems to be that of large-scale commercial collection of habitat specimens for export, particularly to the U.S.A. TANG (1983) quotes information of the shipment in 1982 of 25 000 plants of the newly-described *Ceratozamia norstogii* D. Stevenson from Mexico to the U.S.A., resulting in the complete decimation of one of the two known populations of this species. In addition, TANG (1985a) describes an operation in which *Zamia furfuracea* L.f. in Aiton was trucked from Mexico to one Californian nursery at the rate of 30 000 plants per month in 1981. Similarly, between 1979 and 1982, over 145 000 specimens of *Zamia debilis* L.f. in Aiton were exported from the Dominican Republic to the U.S.A. (TANG, 1985a). Japan has also emerged as a major importer of Mexican cycads: in 1981 one shipment alone consisted of 100 000 plants (TANG, 1985a).

In the Orient, the Chinese species *Cycas panzihuaensis* L.Zhou and S.Y. Yang is endangered, *C. micholitzii* Dyer has not been seen in habitat since 1905 and the natural population of *C. hainanensis* C.J. Chen and C.Y. Chen was almost entirely destroyed by a violent typhoon in 1973 (HENDRICKS, J.G., *pers. comm.*)

Of the South African cycads, the Threatened Plant Unit list (GILBERT, 1984) describes three as endangered, seventeen as vulnerable, five as rare and the remaining three as 'insufficiently known' (Table 1). A Natal cycad, *Encephalartos woodii* Sander, is extinct in nature; its unique status is considered separately in this text (Section 1.5).

Much of the trade in cycads results from a combination of collector obsession and business avarice. A number of collectors (often operating through dealers) and some unscrupulous nurserymen find it more expedient to remove mature specimens from habitat, rather than wait the time required to obtain plants through propagation from seed, offsets or division (Section 1.10). This plundering continues despite legislation to the contrary. At the international level, the law is administered by the Convention on International Trade in Endangered species of Wild Fauna and Flora (CITES). Implemented in 1975, CITES lists *Microcycas calocoma, Stangeria eriopus* and all species of *Encephalartos* on "Appendix 1" which means that commercial trade in wild-collected specimens is prohibited. All other cycads fall under "Appendix II" which requires that a permit must be obtained from the local management authority to cover the export of any plants (CITES SECRETARIAT, 1982). However, administration of CITES is not without difficulties; of the 52 countries which possess native cycads, 16 were not signatories to the Convention (CITES SECRETARIAT, 1982). Furthermore, many of the signatories have failed to produce the required annual reports and many of the reports received appear to be incomplete or incorrect. Such defects can seriously reduce the efficacy of the monitoring and control programmes (GILBERT, 1984).

Apart from the international legislation, many countries have attempted to restrict cycad exploitation by means of domestic laws. Fairly stringent legislation exists in Zimbabwe, Australia and South Africa but effective prosecutions are infrequent. In South Africa, most of the relevant legislation is promulgated in various Provincial Conservation Ordinances and is administered by bodies such as the Natal Parks Board

TABLE 1: The conservation status of South African *Encephalartos* and *Stangeria* species

Classification [1]	Species	Locality [2]
Extinct in nature	*E. woodii* Sander	Natal
Endangered (3 species)	*E. cupidus* R.A. Dyer	Transvaal
	E. inopinus R.A. Dyer	Transvaal
	E. latifrons Lehm.	E. Cape
Vulnerable (17 species)	*E. arenarius* R.A. Dyer	E. Cape
	E. caffer (Thunb.) Lehm.	E. Cape
	E. cycadifolius (Jacq.) Lehm.	E. Cape
	E. eugene-maraisii Verdoorn [3]	Transvaal
	E. ghellinckii Lem.	Transkei, Natal
	E. heenanii R.A. Dyer	Swaziland, S.E. Transvaal
	E. horridus (Jacq.) Lehm.	E. Cape
	E. humilis Verdoorn	E. Transvaal
	E. laevifolius Stapf and Burtt Davy	E. Transvaal, Swaziland
	E. lanatus Stapf and Burtt Davy	Transvaal
	E. lebomboensis Verdoorn	N. Natal, S.E. Transvaal, Swaziland, Mozambique
	E. longifolius (Jacq.) Lehm.	E. Cape
	E. ngoyanus Verdoorn	N. Natal, Zululand, S.E. Transvaal, Swaziland
	E. paucidentatus Stapf and Burtt Davy	E. Transvaal, Swaziland
	E. princeps R.A. Dyer	E. Cape, Transkei
	E. trispinosus (Hook.) R.A. Dyer	E. Cape
	E. umbeluziensis R.A. Dyer	Swaziland, Mozambique
Rare (5 species)	*E. altensteinii* Lehm.	E. Cape, Transkei
	E. friderici-guilielmi Lehm.	E. Cape, Transkei
	E. lehmannii Lehm.	E. Cape
	E. natalensis R.A. Dyer and Verdoorn	Natal
	E. transvenosus Stapf and Burtt Davy	N. Transvaal
Insufficiently known (3 species)	*E. ferox* Bertol. f.	Zululand, Natal, Mozambique
	E. villosus Lem.	E. Cape, Natal, S.E. Transvaal Transkei, Zululand, Swaziland, Mozambique
	S. eriopus (Kunze) Baillard	E. Cape, Natal, Transkei, Zululand

[1] GILBERT (1984), see also HALL, DE WINTER, DE WINTER and VAN OOSTERHOUT (1980). Both these classifications suffer from the limitations of being based largely on numerical representations in herbaria.

[2] OSBORNE, HENDRICKS and STEVENSON (in preparation).

[3] The status of this species in currently under review by both LAVRANOS and GOODE (in preparation) and ROBBERTSE, VORSTER and VAN DER WESTHUIZEN (in preparation).

and various regional Nature Conservation Departments (OSBORNE, 1985c). The effective patrolling of large areas is difficult and removal of plants from habitat continues. Furthermore, theft of specimen plants from botanic gardens, and even from private collections seems to have occurred with increasing frequency over the past decade.

1.5 The status of *Encephalartos woodii* Sander.

Because of its extreme rarity and its majestic appearance, *Encephalartos woodii* Sander is much sought after by private collectors and for use in botanical gardens (Plate 1, Figure 4). Only one specimen was ever discovered, the male multi-stemmed clump found in 1895 in the Ngoye Forest, Zululand, by Medley Wood, founder of the Natal Herbarium and Curator of the Natal (later Durban) Botanic Gardens (DYER, 1965). Several schools of thought exist as to the origin of this unique plant. HUTCHINSON and RATTRAY (1933) state that the species is closely allied to *E. altensteinii* Lehm. (from which *E. natalensis* was separated by DYER and VERDOORN in 1951) and to *E. hildebrandtii* A. Braun and Bouché from East Africa. HENDERSON (1945) had no doubt that the plant was a distinct species. DYER and VERDOORN (1966) speculate that the solitary representative of the species is either a relic from a larger population which became extinct or that it arose as a mutant from *E. natalensis*. The latter viewpoint seems to be consistent with that of Medley Wood when he proposed the name 'var. *bispinosa*' (PRAIN, 1914). Each of these views is based on the classical concept of species. The less rigid modern view seems to suggest a series of taxa, in various stages of speciation, from *E. altensteinii* in the Eastern Cape and Transkei, through *E. natalensis* in Natal, *E. lebomboensis* in Northern Natal, *E. manikensis* Gilliland and related types in Mozambique, to various east and central African taxa to the north. If each of these is accepted as a diffuse unit with a number of intermediate forms of indeterminate rank, then *E. woodii* may well be accommodated in the series. A systematic biochemical and karyological survey would be particularly useful in this regard and may also allow speculation as to which taxa represent primitive or advance conditions.

All the trunk and sucker material from the original *E. woodii* clump was removed over the period 1903-1916 (OSBORNE, 1986a). Four plants were re-established in the Durban Botanic Gardens and remain as very

a.

b.

PLATE 1

Encephalartos woodii Sander
Durban Botanical Gardens
April 1985.

(a) One of four large plants from the original stock collected in habitat.

(b) A younger specimen, estimated at 15 years of age, propagated by sucker from the plant in (a) above.

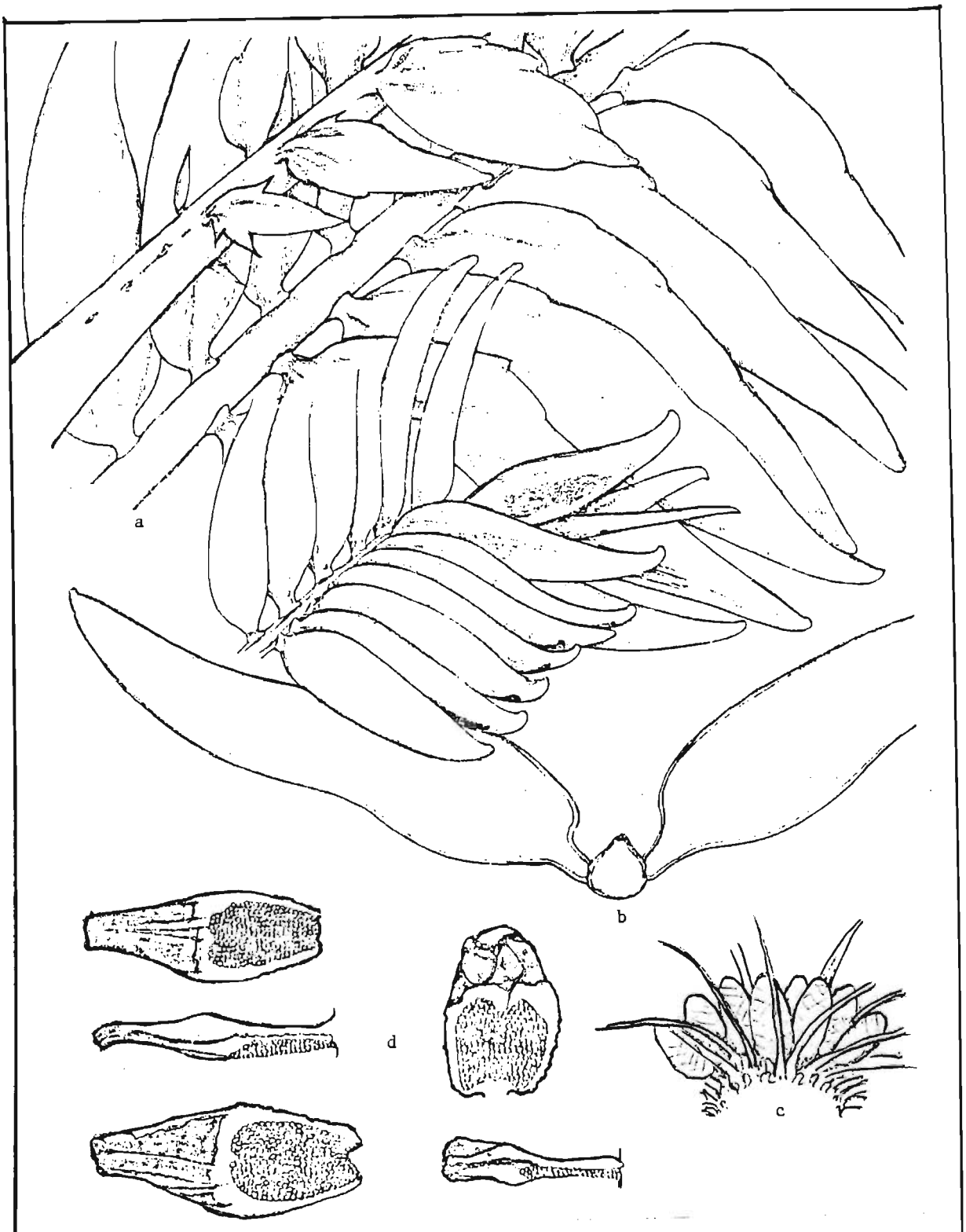


FIGURE 4: Details of leaf structure and male cones of *Encephalartos woodii* (from "Cycads of Africa" by D. Goode, presently in print, and reproduced in reduced scale by kind permission of the author).

a: Lower, mid- and terminal leaf detail.
b: Transverse section of rachis showing attachment of median leaflets.
c: Cluster of male cones at the crown.
d: Details of male cone scales.

impressive specimens at present. One was planted in the grounds of the Union Buildings at Pretoria but was subsequently lost in a transplanting operation (DYER, R.A. *pers. comm.*). Another segment was planted at the Durban home of Medley Wood's associate, Maurice Evans and has since found its way into a private collection in Kloof, Natal (THORPE, E., *pers. comm.*). The final portion to be accounted for was shipped to Kew Gardens and remains on display in their tropical collection (OSBORNE, 1986a). The presence of a large plant, thought to be *E. woodii* (DYER, R.A., *pers. comm.*), in the Old Fort Gardens in Durban, and another large plant thought to be the same species, in a private collection in a garden in the Durban area, remains unaccounted for. Despite numerous excursions into the Ngoye and Mtunzini areas, no other specimen of *E. woodii* was ever located although it is not impossible that one or more plants may exist in some remote position (GARLAND, I., *pers. comm.*). Periodic rumours of a second plant are sometimes heard but are regarded with sceptism; all the 'woodii's' so far investigated have turned out to be rather broad-leaved varieties of *E. natalensis*. Regrettably then the species must now be accepted as extinct in nature.

Fortunately, *E. woodii* produces offsets readily. Careful vegetative propagation has resulted in a fair number of plants being established in other major botanical gardens and private collections throughout the world (OSBORNE, 1986a). Since all these plants represent one male clone, it is not possible to propagate the species from seed.

To avoid the total dependence on continued propagation from the original clone, two avenues may be explored. The first approach is being adopted by Cynthia Giddy at her commercial nursery at Umlaas Road in Natal, and has been described by SPEIRS (1981). Pollen from cones of the existing *E. woodii* stock is used to fertilize *E. natalensis*, the species which seems to be most closely related. Females from this hybrid generation (F_1, 50% *E. woodii*) are back-crossed with more *E. woodii* to give another generation a little closer to pure *E. woodii* (F_2, 75% *E. woodii*). This process is repeated until an almost pure strain is established, e.g. the F_5 generation would have 97% *E. woodii* character. A disadvantage lies in the length of time from one generation to another. Assuming 12 years for each batch to reach maturity, the 5-generation process will take 60 years.

The second possibility forms in large part the motivation for this project. Either haploid cells in the form of *E. woodii* pollen, or diploid tissue from appropriate meristematic regions of *E. woodii* plants, would be used in establishing cultures (Section 1.2). Somatic fusion of the haploid cells may be artificially induced and the resulting tissue, after morphogenesis, may give rise spontaneously to a proportion of female progeny. Alternatively, successful culture of diploid tissue with morphogenesis would provide a substantial number of plantlets which may be used in experimentation, under various chemical treatments or environmental control regimes with the possibility of sex-reversal (Section 1.8), to yield the female phenotype. The production of a female *E. woodii* plant through these means would be of enormous benefit to growers and conservationists alike and would also have major academic value.

1.6 General life-cycle.

The sexual reproduction process in cycads is well-documented; the following general notes are drawn from the texts of CHAMBERLAIN (1919), DYER (1965), PANT (1973), FOSTER and GIFFORD (1974), and GIDDY (1984); this section also forms the basis for a popular paper by the author (OSBORNE, 1986d). It is stressed that the life-cycle and ontogeny described below refer to an entirely hypothetical 'typical cycad'; considerable minor variations occur between different taxa - details of which are beyond the scope of this text.

In nature cycad plants generally produce male or female cones (strobili) seasonally, and it is only at the time of coning that the gender of the individuals can be positively established. Each cone comprises a large number of modified leaves (sporophylls) arranged spirally about a central axis. Each plant can produce several male or female cones at a particular coning 'season'.

The male cone is compact at the time of emergence and elongation of the central axis allows separation of the microsporophylls to reveal numerous pollen-sacs (microsporangia) (Plate 2a, Figure 4). Within each microsporangium at an early stage of development meiosis results in the production of a microspore nucleus, the first cell of the male haploid gametophyte. Division of this nucleus gives a prothallial cell and an

PLATE 2a : *Stangeria eriopus*, Durban Botanic Garden, April 1985. Mature female cone (centre) with a male cone from an adjacent plant (right). The male cone is at the pollen-shedding stage. Part of a leaf is seen on the left.

PLATE 2b : *Encephalartos ferox*, Durban Botanic Garden, April 1985. Two large female cones at a pollen-receptive stage.

antheridial initial which in turn divides to give a generative cell and a tube cell. At this three-celled stage the partially-developed male gametophyte, enclosed within the original spore wall, is released from the microsporangium as a pollen grain (Figure 5). Earlier workers believe the pollen to be wind transported (CHAMBERLAIN, 1919, 1935) but there is now substantial evidence of species-specific insect vectors in pollen transfer (NORSTOG and STEVENSON, 1980; NORSTOG, STEVENSON and NIKLAS, 1986; TANG 1987a). The pollen dehiscence event is usually accompanied by a pronounced temperature increase in the male cone (JACOT-GUILLARMOD, 1958); this thermogenesis following a circadian rhythm (TANG, 1987b) and associated with high rates of starch and lipid consumption (TANG, STERNBERG and PRICE, 1987). It is thought that the heat production results in the volatilization odours which are recognized by the specific insect pollinators (TANG, 1987b; TANG, STERNBERG and PRICE, 1987).

The larger, female cones (Plate 2b) are made up of megasporophylls each of which bears two ovules on its lower surface. Within the nucellar tissue of the ovule, meiosis results in a haploid cell which forms a linear tetrad of megaspores, only one of which develops into the female gametophyte. Numerous divisions take place to give a cellular mass, certain cells at the micropylar end (i.e. opposite the point of attachment to the megasporophyll) become differentiated into archegonial initials. These divide to give a small primary neck cell, which in turn divides to give two or more cells, and a large central cell (Figure 6). Shortly before the fertilisation event, the nucleus of the central cell divides into a ventral canal cell nucleus and a large egg cell, but no wall is laid down between these nuclei. At this time elongation of the female cone axis, particularly at the uppermost zone, allows entry of the pollen grains over a 3 to 4 day period. The pollen grains become trapped in a mucilaginous liquid secreted through the micropyle of the ovule and, as this fluid dehydrates, the microspores are drawn into the pollen chamber, the desiccated gel effectively sealing off the micropylar orifice.

After entry into the ovule, the microspore extends its tube cell into an archegonial chamber arising through degeneration of the neck cells (Figures 5 and 7). At this time the microspore generative cell divides to produce a sterile cell and a terminal spermatogenous cell (body cell) from which two large spermatozoids with numerous cilia differentiate. Fusion of the spermatozoid with the female egg cell constitutes the

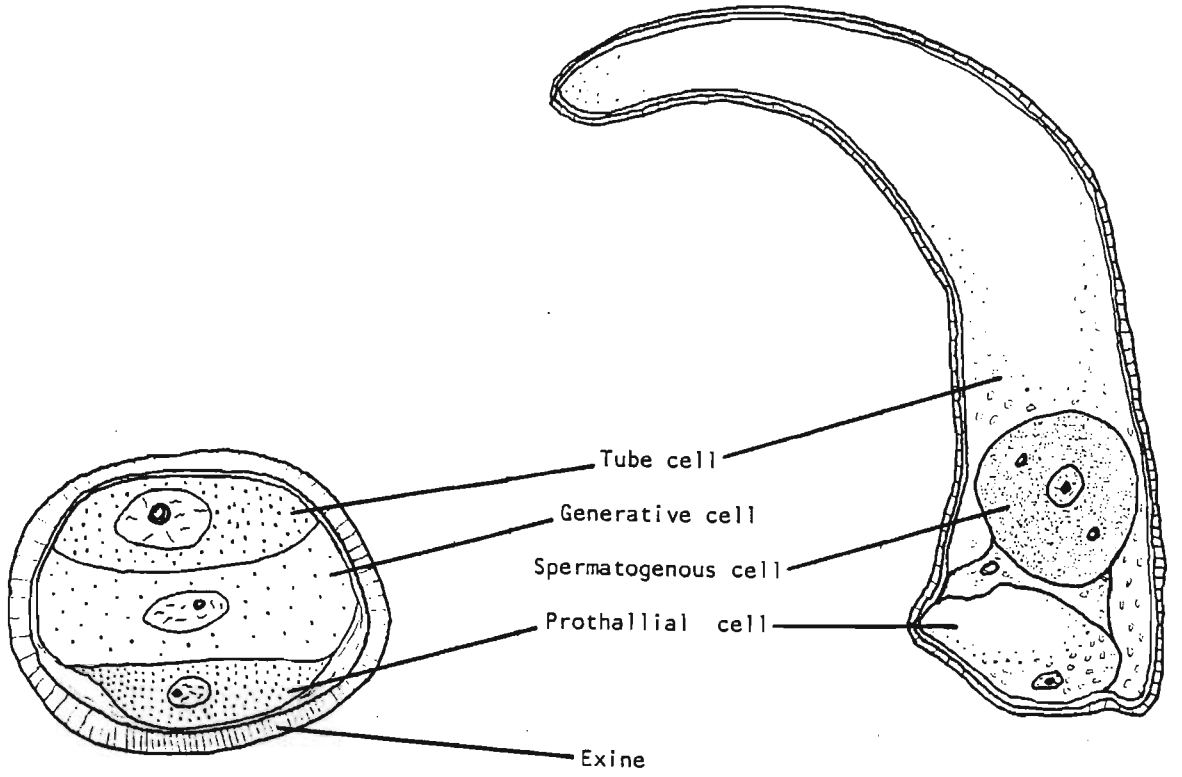


FIGURE 5: Development of the male gametophyte in cycads (much enlarged).
Left: The pollen-grain in transverse section showing the three-celled-stage at the time of dehiscence.
Right: Extension of the pollen tube after pollination; the spermatogenous cell is still to divide to form two motile spermatozoids.

(Redrawn from SWAMY, 1948)

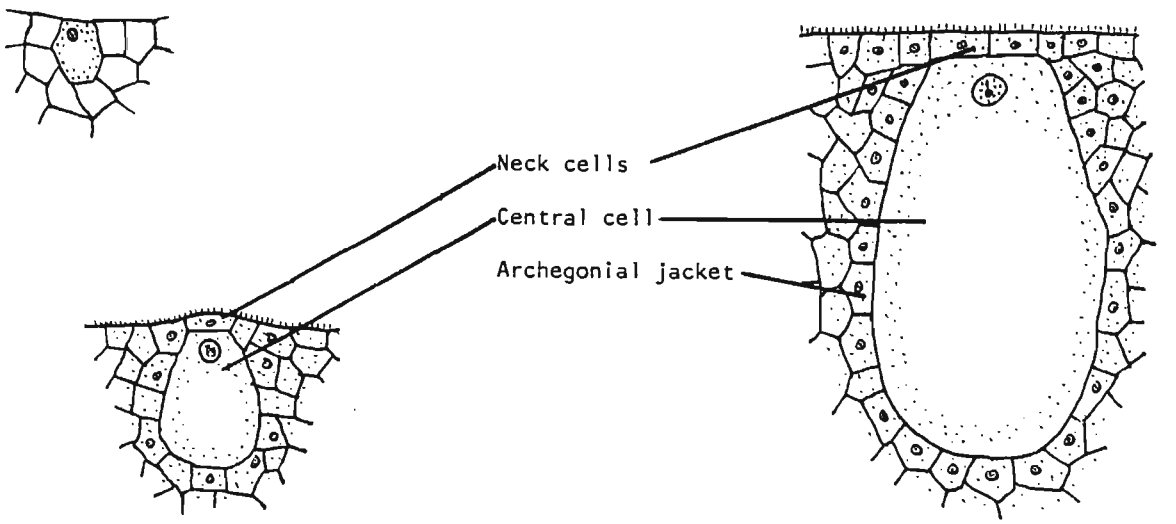


FIGURE 6: Development of the female archegonium in cycads (much enlarged)
Left: An archegonial initial differentiated in the female gametophyte.
Centre: Division into a primary neck cell and a central cell.
Right: The archegonium shortly before fertilisation; the central cell nucleus is still to divide to form the ventral canal cell nucleus and the egg cell nucleus.

(Redrawn from CHAMBERLAIN, 1935).

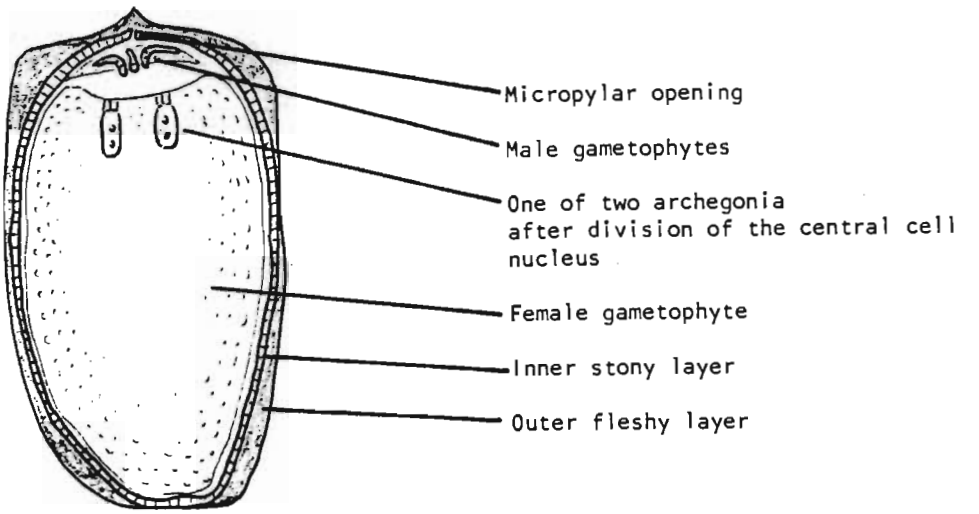


FIGURE 7: Longitudinal section through the ovule shortly before fertilisation (Actual size).

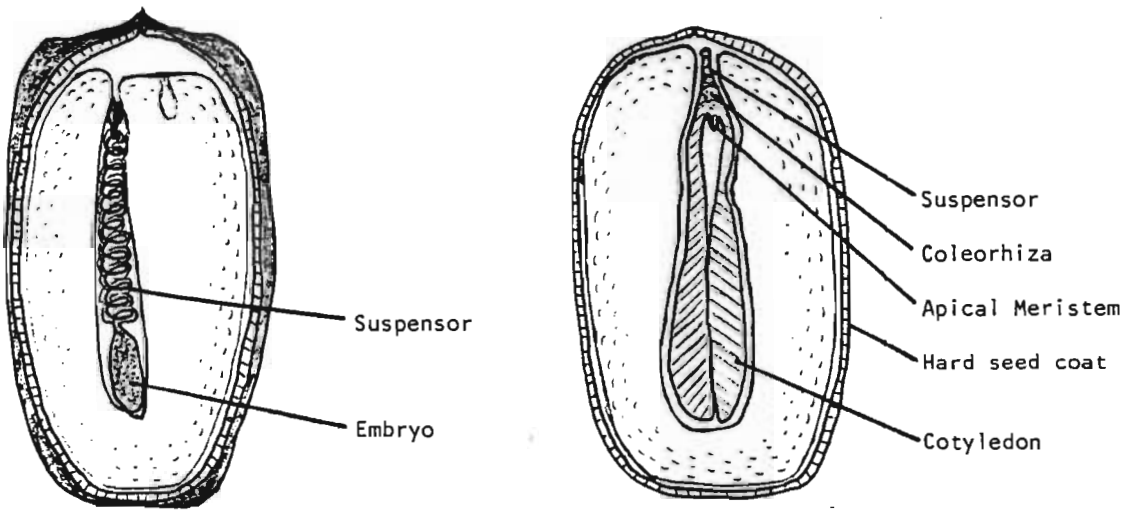


FIGURE 8: Embryology of cycads (actual size).

Left: Early stage of embryo development, at about the time of shedding of the seed, showing the characteristic coiled suspensor.

Right: Later stage of embryo development, shortly before germination.

fertilisation event. The spermatozoids of cycads (about 0,3 mm in diameter) are the largest sperm cells in either the plant or the animal kingdom (NORSTOG, 1980b). The whole process, from ingress of the pollen to fertilisation, takes several months.

The zygote resulting from the fertilization process undergoes numerous successive divisions following which cell walls are laid down. Certain of these cells differentiate into the embryo, with a conspicuous merismatic region from which the functional organs arise, and a posterior region of elongating cells which mark the origin of a massive coiled suspensor unique to the cycads. This is clearly visible in longitudinal section of the seed and is the only true, albeit destructive, method of assessing seed viability (Figure 8). At this stage the cone disintegrates and the megasporophylls bearing the seed are shed. Some of the female cycad cones are the largest known in either living or extinct plants. Individual *Encephalartos* cones may attain a mass of 30 to 40 kg and each bears several hundred seed. Cycad seeds vary widely in colour, shape, size and ornamentation. Such variation seems to be species specific and seed morphology may well provide useful additional evidence for future taxonomic reviews in cycads (OSBORNE, 1988).

After the seed has been shed, the embryo continues to develop for about six months to give a meristematic apex surrounded laterally by two well-developed cotyledons which fuse at their base into a zone of tissue known as the coleorhiza (Figure 8). It is this organ which ruptures the seed coat at the micropylar end and protrudes to give the first external evidence of germination. The cotyledons remain permanently within the seed functioning as haustorial organs until the gametophyte reserve is depleted. The emergent coleorhiza is penetrated by the root tip and later by the plumule from which the first of the true leaves of the new plant arise (Figures 9 and 10).

The sequence of events described above is in general terms similar to that for all higher plants: a dominant sporophytic generation bears specialised sexual organs in which meiosis reduces the chromosome number from the diploid (2n) to the haploid (n) condition; the haploid gametophyte is much reduced and increasingly dependent on the host sporophytic tissue; and fertilisation is accomplished by plasmogamy and karyogamy to restore a diploid zygote, the first cell of the new sporophyte. A generalized life-cycle diagram for cycads is given in Figure 11.

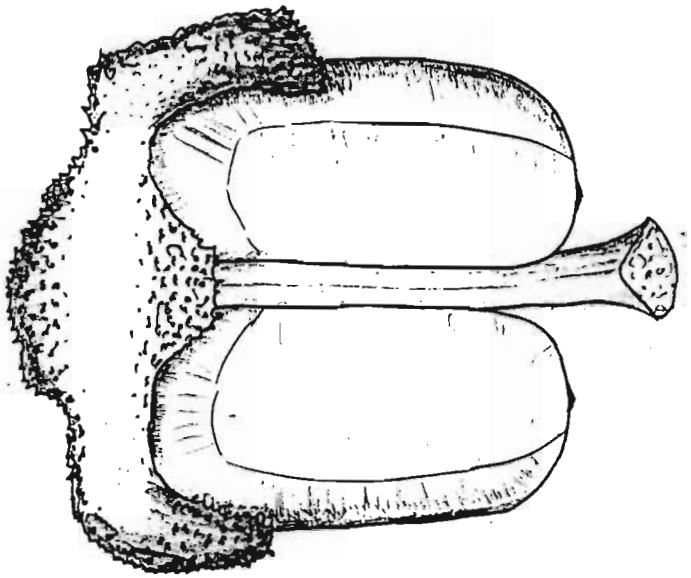
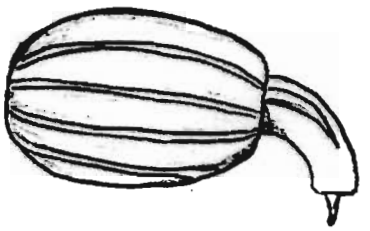


FIGURE 9: Megasporophyll from female cone showing attachment of two ovules. (Actual size).



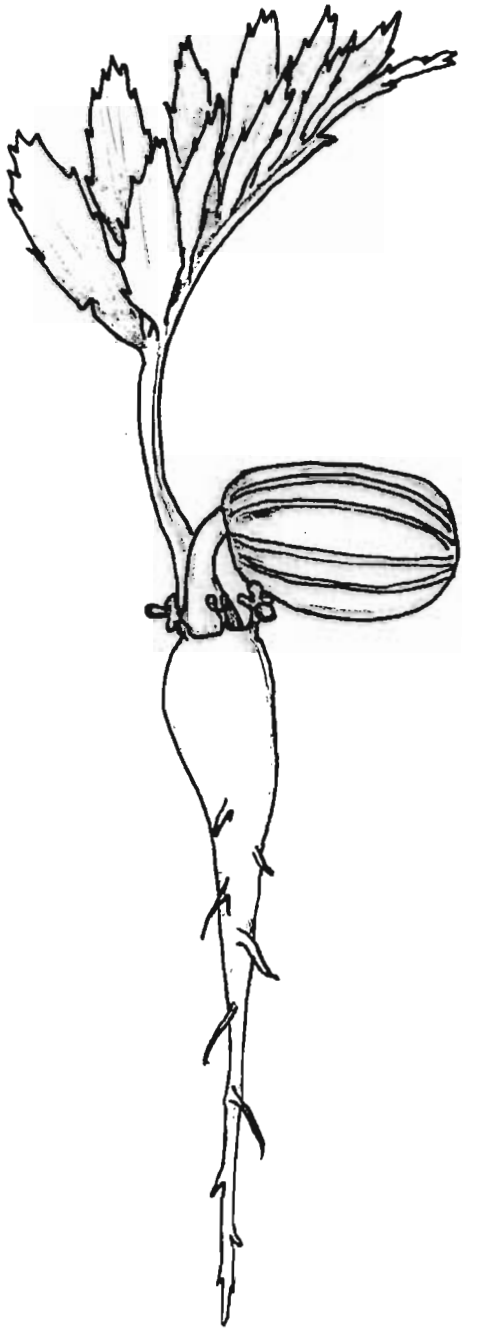


FIGURE 10: Germination of cycads (reduced × ½)

Left: Root tip emerging through coleorhiza.

Right: First-leaf stage with well-developed tap root and evidence of coralloid root development, about 3 months after germination.

(redrawn from GIDDY, 1984).

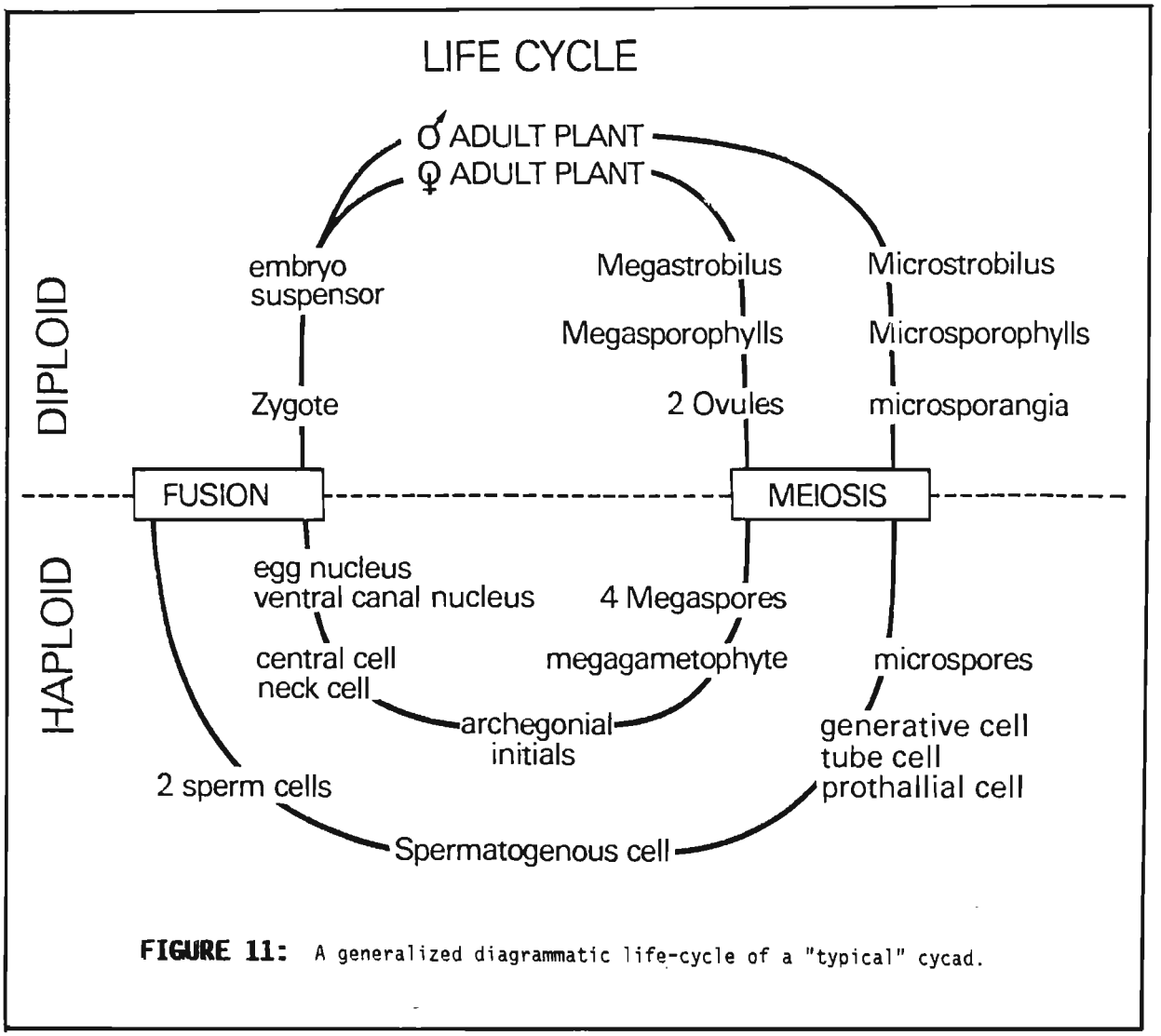


FIGURE 11: A generalized diagrammatic life-cycle of a "typical" cycad.

1.7 Chromosome numbers and karyology.

Examination of the chromosomes from diploid cycad material is conveniently carried out using actively-growing root tips and young leaflets, while expanding megagametophytes and pollen mitoses provide samples for haploid

chromosome work. Because of the large size of cycad chromosomes it is usually advisable to pre-treat the material chemically prior to fixing, hydrolysis and staining. A number of reagents including α-bromonaphthalene, colchicine, 8-hydroxyquinoline and especially isopropyl-N-phenyl carbamate are employed for this purpose. Aceto-orcein, aceto-carmine and the Feulgen system are used to stain the chromosomes although more sophisticated techniques have applications in detailed morphological analyses.

Within the gymnosperms generally, chromosomes from different taxa are remarkably uniform (KHOSHOO, 1961); this is true too in most cycad genera. Generally consistent diploid (2n) numbers have been reported for *Stangeria* (16), *Ceratozamia* (16), *Encephalartos*, *Macrozamia*, *Lepidozamia*, *Dioon*, *Bowenia* (all 18), *Cycas* (22) and *Microcycas* (26) (SAX and BEAL, 1934; KHOSHOO, 1961; ABRAHAM and MATHEW, 1962; MARCHANT, 1968; STOREY, 1968; SEGAWA, KISHI and TATUNO, 1971; MORETTI, 1982; VOVIDES, 1983). Of these authors, the most extensive work was that of MARCHANT (1968) who surveyed the cycad collection of the Royal Botanic Gardens at Kew and included in his work a detailed cytological study of 16 species of *Encephalartos*. The exception in this pattern of consistency is seen in the genus *Zamia* where variation has been reported both between and within species. NORSTOG (1980a) examined 14 species of *Zamia* and found diploid number of 16 and 18 to occur in most taxa. *Z. acuminata* Oersted from Panama and *Z. muricata* Willd. from Venezuela had 24 chromosomes. In three species, *Z. skinneri* Warcz. from Central America, *Z. loddigesii* Miq. from Mexico and *Z. chigua* Seemann from Colombia, more than one chromosome number was apparent. In particular, the *Z. chigua* material revealed a range from 2n = 22 to 26 (NORSTOG, 1980a, 1981). An analogous situation seems to be present in *Z. paucijuga* Wieland from Mexico where MORETTI and SABATO (1984) report chromosome counts 2n = 23, 25, 26, 27 and 28.

The discussion of chromosome morphology and cycad karyotypes which follows requires some terminology to be introduced. Chromosomes typically have two arms attached at a central point of constriction, the centromere, such chromosomes being called 'biarmed'. Where the arms are equal in length so that the centromere is median, the chromosome is described as 'metacentric' (M). When the arms differ in length, the shape is called 'submetacentric' (S). When there is only one arm, i.e. the centromere is terminal, the structure is described as 'telocentric' (T). The karotype is the combination of these different types of chromosome to

make up the total complement and is usually expressed as xM + yS + zT where x + y + z = 2n, the diploid number. Occasionally the word 'acrocentric' (A) is used to describe a chromosome with a subterminal centromere.

The accompanying table (Table 2) lists most of the known cycad karyotypes, while Figure 12 illustrates the patterns obtained in 10 species of *Encephalartos* with a consistent karyotype (8M + 10S, 2n = 18) (MARCHANT, 1968). A great deal of speculation has been made as to which karyotypes represent primitive or advanced conditions, with processes or centromeric fission and fusion processes being invoked to explain increases and decreases in chromosome numbers respectively (HENDRICKS, 1982). The fission processes lead to a greater proportion of telocentric chromosomes and a karyotype which is described as asymmetric. By contrast, fusion reduces the number of telocentrics and leads to a more symmetric pattern. NORSTOG (1980a) points out that cycads have a long history of geographic isolation and warns against over-zealous attempts at seeking correlations between karyotype and morphological characters. This is particularly true in intergeneric comparisons as nearly all cycad genera show combinations of primitive and advanced morphological characters and diversity in degree of ecological specialisation.

The unique situation in *Zamia* deserves more attention. In *Z. chigua*, which is a tropical arborescent long-lived species where the habitat is unlikely to create strong selection pressures, one may anticipate a primitive status (NORSTOG, 1980a, 1981). The high-numbered asymmetric karyotypes may thus be considered a starting point in *Zamia* evolution and the progression towards reduction and symmetry in karyotype leading ultimately to the Caribbean types, exemplified by *Z. pumila*. Against this argument is the report on *Z. paucijuga* (MORETTI and SABATO, 1984) which has a range of karotypes similar to *Z. chigua*, but is considered an advanced species in view of its subterranean stems and reduced vegetative and reproductive structures. The possibility exists that karyotype changes, in terms of fission and fusion, might be bidirectional (NORSTOG, 1980a), thus making any correlation between karyotype and phenotype even more difficult to access.

Although there is substantial evidence of monoecious character in the Cycadeoidales, an extinct order of Cycadophyta, the present-day cycads

TABLE 2 : Karyotypes of various cycad taxa as reported in the literature.

M - metacentric, S - submetacentric, A - acrocentric and T - telocentric

Genus/species	Diploid Number (2n =)	Karotype				Authors
		M	S	A	T	
Stangeria	16	12	2		2	SAX and BEAL, 1934
	16	10	4		2	MARCHANT, 1968
Bowenia	18	12	6		0	SAX and BEAL, 1934; MARCHANT, 1968
Cycas	22	4	8		10	SAX and BEAL, 1934
Encephalartos	18	8	10		0	MARCHANT, 1968
Dioon	18	8	8		2	MARCHANT, 1968; VOVIDES, 1983
Macrozamia	18	10	6		2	SAX and BEAL, 1934
	18	8	8		2	MARCHANT, 1968; MORETTI, 1982
Lepidozamia	18	8	8		2	MARCHANT, 1968
Microcycas	26	2	2		22	SAX and BEAL, 1934
Ceratozamia	16	12	4		0	SAX and BEAL, 1934
	16	12	2		2	MARCHANT, 1968; VOVIDES, 1983
Zamia *Z. angustifolia* *Z. fischeri* *Z. portoricensis* *Z. pumila* *Z. pygmaea*	16	12	4			SAX and BEAL, 1934; MARCHANT, 1968; NORSTOG, 1980a; VOVIDES, 1983
Z. lindenii *Z. pseudoparasitica* *Z. tuerckheimii*	16	10	4		2	NORSTOG, 1980a
Z. furfuracea *Z. loddigesii* *Z. obliqua* *Z. skinneri*	18	10	2	2	4	NORSTOG, 1980a
Z. loddigesii var. *angustifolia*	18	10	6		2	VOVIDES, 1983
Z. muricata *Z. acuminata*	24	4			10	NORSTOG, 1980a
Z. furfuracea X *Z. loddigesii* ?	27	1	4	6	16	NORSTOG, 1980a
Z. chiqua	22	4	4	2	12	NORSTOG, 1981
	24	2	4	2	16	
	25	1	4	2	18	
	26		4	2	20	
Z. paucijuga	23	5	2	8	8	MORETTI and SABATO, 1984
	25	3	2	8	12	
	26	2	2	8	14	
	27	1	2	8	16	
	28		2	8	18	

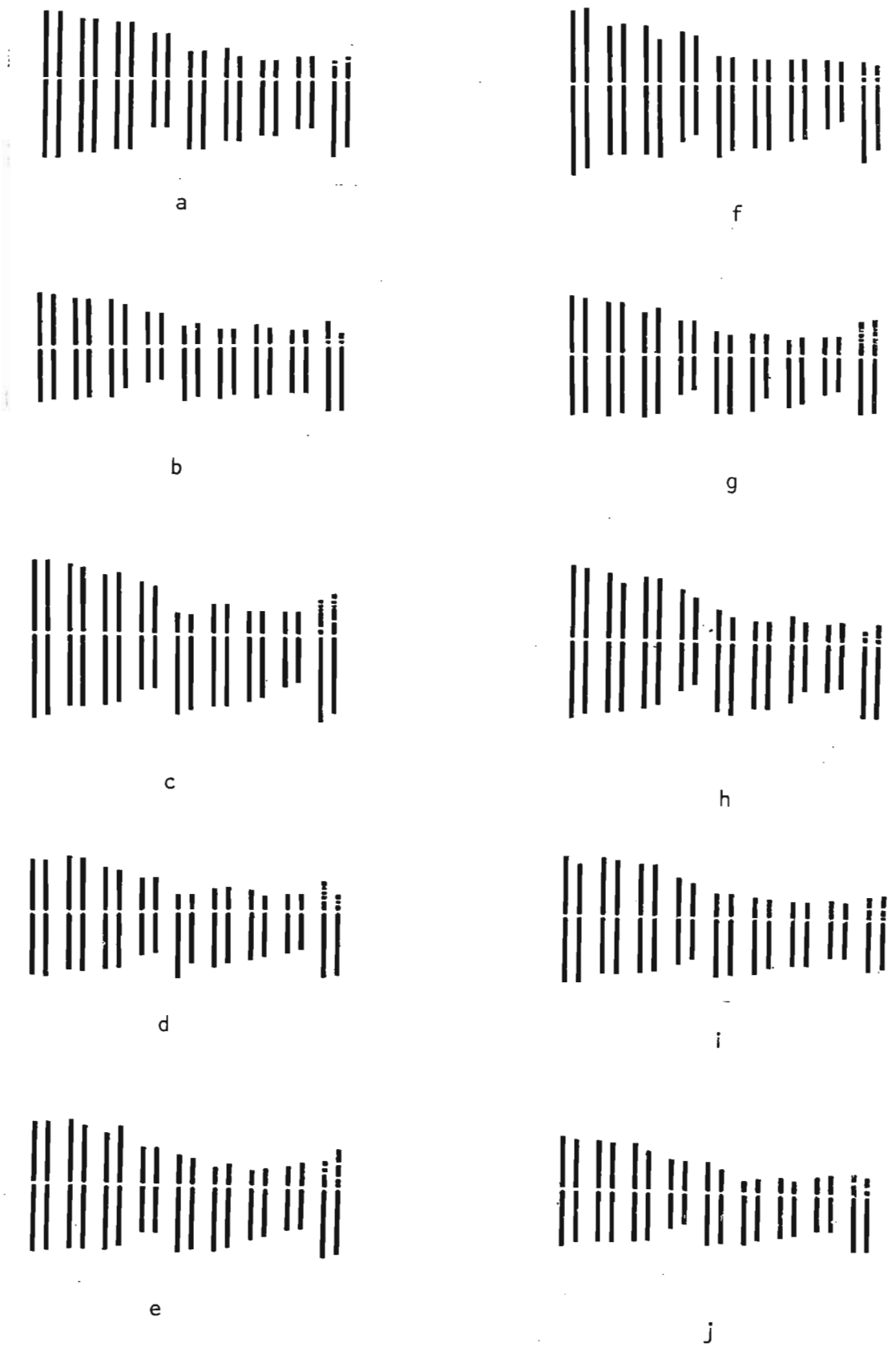


FIGURE 12: Scale drawings of karyotypes of some *Encephalartos* spp. x 900.
a. *E. cycadifolius*, b. *E. umbeluziensis*, c. *E. manikensis*,
d. *E. altensteinii*, e. *E. humilis* f. *E. lehmannii* g. *E. villosus*
h. *E. hildebrandtii* i. *E. laevifolius* j. *E. horridus*.
After MARCHANT (1968).

are uniformly dioecious (CHAMBERLAIN, 1935). The question as to whether one can predict the gender of a particular cycad plant from karyological studies has not been completely resolved. ABRAHAM and MATHEW (1962), in a cytological study of *Cycas pectinata* Griff. (2n = 22), found that one of the submetacentric chromosome pairs showed a satellite attachment to both chromosomes in female nuclei but that only one of the pair was similarly satellited in material from male plants. The authors thus conclude that the chromosome pair which is heteromorphic in the male must be associated with sex expression. According to SHETTY and SUBRAMANYAN (1962), the karyotypes of *Cycas beddomei* Dyer, *C. circinalis* L and *C. revoluta* Thunb. are all similar except for a pair of heteromorphic chromosomes from male specimens of the latter two of the three species. MARCHANT (1968) reports a similar heteromorphic pair from *Stangeria eriopus* (Kunze) Baillard (2n = 16) but was unable to correlate the cytological information with sexual phenotype. The Japanese workers, SEGAWA, KISHI and TATUNO (1971) have reported distinctly-recognisable sex chromosomes in *Cycas revoluta*. A general indication is thus that the male plants are heterogametic with one XY pair of chromosomes while the female are homogametic with an XX chromosome pair, where X represents the satellited chromosome.

MARCHANT's (1968) work on *Encephalartos* does indicate some apparently non-identical chromosome homologues (Figure 12) but the author stresses that slight changes do occur during slide preparation and that final differences do not necessarily mean heteromorphism. Furthermore, the presence of a heteromorphic pair does not necessarily have sexual significance as it may arise from a variety of sources such as satellite suppression. Clearly, a great deal of detailed cytological work is necessary before a final conclusion is reached as to whether recognizable sex-chromosomes are a common feature throughout the Cycadales.

To complete the review on cytological work, two other aspects should be mentioned. One is the phenomenon of somatic reduction, a process in which cells divide without chromosome replication and the chromosome number thus becomes progressively reduced. This occurs sporadically in a number of unrelated vascular plants but seems to be a common feature in the apogeotropic cycad root system. STOREY (1968) found recurrent somatic reduction in 7 species from 6 genera, the reduction often proceeding to a stage where only one or two chromosomes remain in each

cell. These cells become charged with slime and the resulting environment appears conducive to infection by Cyanobacteria. This sequence of stages terminates in establishment of the symbiotic coralloid root system found in all cycads (Section 1.9).

A final aspect in this section refers to the use of more sophisticated cytological techniques. MORETTI (1982) used quinacrine fluorescence analysis to examine chromosomes from 13 Australian cycads in 3 genera. Enhanced fluorescent banding was found at the centromeric region in all species of *Macrozamia* but not in *Lepidozamia* or *Bowenia*. Differences in the banding patterns were observed in different species of *Macrozamia*. It is clear that this and other techniques such as Giemsa banding (SHARMA and SHARMA, 1975) can extend the range of this work. DNA-sequencing investigations have been carried out on pollen from two species of *Encephalartos* (BRANDT and VON HOLT, 1975, 1986) and this also may provide data to extend the current cytological investigations.

1.8 Apparent sex reversals.

Higher plants are divided into three groups insofar as sexuality is concerned. *Dioecious* plants bear male and female reproductive parts on separate individuals; *monoecious* plants have both organs on the same individual but on separate parts of the plant. These two groups are comparatively small in terms of numbers of species. Most plants fall into the third group, the *hermaphrodites*, where each flower has both male and female organs. Cycads are uniformly dioecious in this classification (CHAMBERLAIN, 1935).

The classical concept of sexuality in dioecious plants invokes the occurrence of sex-determining chromosomes, which may or may not be morphologically recognizable. In these terms the gender of each plant is laid down at the time of fertilisation and is immutable. However, the fact that hermaphrodites do occur amongst prevailingly dioecious plants, and that sex changes may be induced or may happen spontaneously in individual plants, requires a re-evaluation of the fundamental principles of plant sexuality. In a recent review, FREEMAN, HARPER and CHARNOV (1980) list 25 plant families comprising more than 50 species where individuals have functioned as males at one time and females at another, or have produced hermaphroditic progeny. Included in this

list are several species with morphologically recognizable sex chromosomes, generally with the male karyotype showing a difference in one chromosome pair.

A number of authors have addressed this apparent conflict between genotypic and phenotypic sexuality (HESLOP-HARRISON, 1972; FREEMAN, HARPER and CHARNOV, 1980; POLICANSKY, 1982; DURAND and DURAND, 1984; LLOYD and BAWA, 1984). A revision of the classical concept seems to be suggested in which the chromosome mechanism is *sex-directing* rather than *sex-controlling*, the normal organogenetic programme being influenced by a number of factors. These factors can be separated into endogenous and exogenous hormone effects on one hand and environmental effects on the other, although it is reasonable to postulate that environmental influences will operate through hormonal action. Amongst the hormones which influence the gender of plants are the cytokinins, gibberellins, auxins, ethylene, and abscisic acid (CHAILAKHYAN, 1979; FREEMAN, HARPER and CHARNOV, 1980; DURAND and DURAND, 1984) and steroid hormones (GEUNS, 1978, 1982). However, it is undesirable to categorise sex hormones in terms of specific responses as the same hormone may have opposite effects according to the plant species under investigation (DURAND and DURAND, 1984). With respect to the environmental factors, it appears that there is a strong tendency for stress situations (e.g. low soil fertility, dry soil, extreme temperatures, low light intensity) to be associated with a change from female to male (FREEMAN, HARPER and CHARNOV, 1980). Undoubtedly the mechanism is hormonal but the adaptive significance may be rationalised in terms of physiological cost. It is less costly for the plant to function as a pollen producer than as a seed producer, thus a change from female to male in terms of stress has a distinct survival value (FREEMAN, HARPER and CHARNOV, 1980; LLOYD and BAWA, 1984).

In an analysis of populations of the Australian cycad *Macrozamia riedlei* (Gaud.) C.A. Gardn. and *Zamia pumila* L. in the Dominican Republic, ORNDUFF (1985) found there was an increase in male coning frequency under adverse conditions and, when competition was lower, a greater proportion of plants bearing female cones. In a survey of *Zamia pumila* populations in Puerto Rico, NEWELL (1985) reported that female plants had a larger number of leaflets per leaf than did the males. Both these observations are consistent with the theory that the physiological cost to the plant is higher when it behaves as a female. This would be especially true in

cycads when one compares the mass of large seed-producing female cones to that of the smaller pollen-bearing male cones. However, these observations do not infer that any sex change takes place in individuals within the populations.

The literature on specific sex changes in individual cycads is limited but it is clear that apparently spontaneous gender reversals do occur and usually appear to be associated with some traumatic incident. SCHUSTER (1932) reports one case where a plant of *Cycas revoluta* Thunb. which was longitudinally bisected, the two pieces going to different places. Apparently the two plants which developed from these sections were of opposite gender. CHAMBERLAIN (1935) mentions a change from female to male with respect to a specimen of *Cycas revoluta* in Australia and also tells of the bud from a female *Cycas circinalis* L. from a Chicago conservatory which eventually coned as a male. MENNINGER (1967) documents two sex change incidents: a specimen of *Cycas circinalis* L. which changed from female to male after being mechanically damaged, and a male of the same species which produced a female cone after severe frost exposure. KEMP (1985) reports a change from male to female in a specimen of *Cycas revoluta* after being transplanted.

An apparently authoritative report of a sex change in *Encephalartos umbeluziensis* R.A. Dyer is given by VAN WYK and CLAASSEN (1981). They followed the history of several specimens, planted in a Pretoria garden, one of which produced a male cone in 1970 but a female cone in 1979. This particular plant was situated in a more exposed position than other similar specimens, and it is possible that a freak spell of sub-zero temperatures in August 1972 may have initiated the change.

Several other incidents of sex reversals in cycads have been reported to the author personally. A female *Zamia* plant is said to have produced a branch bearing a male cone (KOELEMAN, A., *pers. comm.*). A specimen of *Encephalartos villosus* Lem., subjected to particularly dry conditions, supposedly altered from female to male (SWANEPOEL, R., *pers. comm.*). A plant of *Encephalartos latifrons* Lehm., transplanted in 1970 to a farm near East London, bore two successive crops of female cones with viable seed, but following a severe drought at the end of 1983, the plant produced two successive crops of male cones (BURSERY, B., *pers. comm.*).

In attempting to induce sex reversal through transplantation, TANG (1986) moved 97 *Zamia pumila* L. plants of known gender from one site to another. Of the 26 specimens which produced cones within a three year period, none showed any evidence of sex change. Thus, if trauma can induce an alteration in phenotypic sex expression in cycads, the conditions under which it occurs must be fairly restrictive.

Indirectly related to this topic is an observation that the percentage of male and female cycad plants arising from a particular batch of seed may be influenced by the temperature at which the seed is stored during its maturation period (HENDRICKS, J.H., *pers. comm.*).

All the above phenomena can be rationalised in terms of action of hormonal agents or their inhibitors. An observation that gibberellic acid induces maleness in *Zamia* seedlings (NORSTOG, K., *pers. comm.*) adds weight to this and it might be anticipated that cytokinins would have a feminising effect. The cytokinin/gibberrellin ratio appears to be critical in the sex expression of several dioecious plants (CHAILAKHYAN, 1979).

In evaluating the relative genotypic direction and phenotypic expression of plant sex, further complexity is introduced when multiple gene loci are postulated. In extensive work with *Mercurialis annua* L. (Euphorbiaceae). DURAND and DURAND (1984) suggest the existence of three sex genes which would give 64 possible allelic combinations (4^3) and 27 possible genotypes (3^3) ranging from 'supermale' to 'superfemale'. The fact that only one site on one chromosome pair is generally morphologically recognizable when sex chromosomes have been observed microscopically does not preclude the possibility of multiple sex genes being present. If this is the case, then one may speculate that it is the 'supermales' and 'superfemales' which will be constant in sex expression throughout their lives, while the intermediates may be sufficiently influenced by environmental factors so as to show sex reversals.

Experimental work on the influence of environmental factors and exogenous plant growth regulators on the sexual expression of cycads is inhibited by their slow growth rate. *Encephalartos ferox* Bertol. f. requires 12 years to reach sexual maturity (DYER, 1965) although some species of *Zamia* can cone within 3 years from the seedling stage (NORSTOG, K., *pers. comm.*). Assessment of gender by chromosomal morphology has not been

convincingly demonstrated and, as yet, there are no biochemical or serological tests which are sexually diagnostic in cycads. Thus it is unlikely that the precise mechanisms which control sexual expression will be known in the immediate future. The only hope of gender control will be as the result of field trials under various chemical and environmental regimes using fairly large numbers of adult or near-adult plants.

1.9 Coralloid roots.

A common feature in cycads is the formation at an early seedling stage of dichotomously-branching apogeotropic roots. These are initiated terminally on normal lateral roots at various distances away from the main tap root and are found at various depths from the soil surface, often being prominently visible as coralloid masses at the soil surface. These unusual root forms have so far been recorded in 49 cycad species, in all genera except *Microcycas* (GROBBELAAR, 1985). Mention has already been made of the process of somatic reduction in which the chromosomes in the cells of these structures may be reduced to as few as one or two in number (STOREY, 1968; Section 1.7).

The coralloid roots are often, but not always, invaded by various Cyanobacteria which then exist in a symbiotic relation with their host plant. The symbionts are confined to an aerenchymatous zone at the junction of the inner and outer cortex (GRILLI CAIOLA, 1980). They exist as long filamentous strands in which single larger heterocyst cells are regularly placed in chains of smaller vegetative cells (LINDBLAD, HÄLLBOM and BERGMAN, 1985). In early surveys, the colonising organisms were thought to be species of *Anabaena* or *Nostoc* (GRILLI CAIOLA, 1980). However, in an extensive survey of symbionts from southern African cycads, in which 41 cultures were isolated from 31 cycad species, all but one were identified as species of *Nostoc*, the most commonly-occurring being *N. commune* Vaucher (GROBBELAAR, SCOTT, HATTINGH and MARSHALL, 1987). In nodules from *Encephalatros hildebrandtii*, the same authors identified the isolate as a species of *Calothrix*. Significantly too, there was more than one symbiont in a number of the associations.

In common with the situation in many similar plant-Cyanobacterial associations, the cycad symbionts possess the reductase enzyme system which allows for fixation of atmospheric nitrogen. In an adult cycad plant it is believed that a well-established symbiosis can adequately furnish the plants entire nitrogen requirement (GROBBELAAR, N., *pers. comm.*). In field studies with the south-western Australian cycad, *Macrozamia riedlei* (Gaud.) C.A. Gardn., it has been estimated that the symbiosis leads to the fixation of between 1,4 and 8,4 kg nitrogen per hectare per year, depending on the time elapsed in burning cycles (GROVE, O'CONNELL and MALAJCZUK, 1980). Certainly this can be a significant contribution to the overall ecosystem particularly in the situation where the cycad vegetation is the dominant understory in *Eucalyptus* forests on poor soil.

1.10 Conventional methods of propagation.

Cycad plants are usually grown from seed, both in nature and by man. However, seed production and viability varies enormously; even in dense stands the production of fertile seed may be limited (NEWELL, 1983). The main reasons for this appear to be either non-synchrony of the male and female cones or ineffective pollination. Although early workers believed that cycads were generally wind pollinated, the issue has been open to question and there is now increasing evidence that many taxa may be associated with very specific insect vectors (Section 1.6). The problem of effective pollination is exacerbated when the cycad polulation is thinly scattered over large areas. In non-habitat situations, as in plantings in botanic or private gardens, there is little chance of spontaneous pollination and techniques have been developed for artificially-induced pollination (GIDDY, 1984; TANG, 1985b).

In current biological terms, cycad seeds fall into the group known as 'wet' or 'recalcitrant'. This type of seed is shed with high moisture content, is susceptible to desiccation, does not show any true dormancy and has a quantitatively limited storage life (ROBERTS, 1973). For successful germination it is essential that growers are aware of the ongoing maturation processes typical of recalcitrant seed and that storage and sowing times are correctly planned. In an investigation on the germination of *Encephalartos natalensis* Dyer and Verdoorn, it was

found that storage in a moist environment at 20°C, followed by transfer to 30°C when the embryo was mature, lead to improved germination rates (FORSYTH and VAN STADEN, 1983). It is probable that optimal storage and incubation times, temperatures and humidities will vary widely with different species. Germination may sometimes be enhanced by cutting the seed coat or by chemical pre-treatments (BURCH, 1981b; DEHGAN, 1983).

Many cycads produce adventitious offsets or basal suckers which may be used in vegetative propagation (GIDDY, 1984). Division of aerial stems from a branched crown is also possible, as is the separation of multi-headed clumps in those taxa with underground stems (DEHGAN, 1983; GIDDY, 1984). Propagation of some species using leaf-bases with segments of stem attached has been reported (GIDDY, 1984) and further research into this possibility is necessary. While vegetative propagation of cycads is restricted by availability of material, many of the rarer species can be usefully increased in this manner. As an example, one may cite the fact that the entire global population of *Encephalartos woodii*, thought to be some 500 in number (OSBORNE, 1986a) has been propagated from suckers from the solitary specimen of this plant discovered in 1895.

The efforts of the Cycad Society (U.S.), the Cycad Society of Southern Africa and the Palm and Cycad Societies of Australia and New Zealand in public awareness programmes and their establishment of various cycad seedbanks and pollen storage facilities are to be commended. By encouraging propagation from garden-grown material, the pressure on habitat stands from over-zealous collectors will at least be partially relieved.

1.11 Conclusion.

Sections 1.1 to 1.10 of this text represent a necessarily condensed introduction to the biology of the order Cycadales and some of the relevant literature. In essence, these tropical and sub-tropic plants of early evolutionary origin show considerable diversity in vegetative and reproductive morphology and possess several features unique in the plant kingdom. Because of the antiquity of the order and in view of

their relative scarcity and decorative appeal, cycads have attracted much public interest.

In view of the generally slow growth rates, the paucity of viable seeds and the limited potential for vegetative reproduction of cycads, it is evident that a vital contribution could be made to cycad biology if these plants could be successfully propagated by the *in vitro* methods broadly defined as "tissue culture" techniques. Thus a detailed review of the literature specific to this topic is presented in Chapter Two (Section 2.3) of this text. This review also forms the basis of a separate report by WEBB and OSBORNE (in print). The first part of the author's experimental work (Section 2.4) has thus been directed at exploring techniques for the *in vitro* propagation of the South African Cycadales.

CHAPTER TWO

IN VITRO CULTURE OF CYCADS

INDEX TO CHAPTER TWO

2.1 Introduction

The term "tissue culture embodies a broad concept which presently includes the culture *in vitro* of single cells (or even cell components), groups of cells, parts of organs and whole organs. This science is not new; experiments with the culture of plant material *in vitro* commenced early this Century. Notable contributions to the advancement of the science have been made by a large number of researchers including Haberlandt, Robbins, White, Nobécourt, Gautheret, Loo, Ball, Wetmore, Morel, Skoog, Miller, Krikorian, Murashige, Steward, Street, Vasil, Thorpe and their co-workers. A vast literature has been produced on techniques, implications and applications and general overviews of the subject are given in the texts of WHITE (1943, 1963), STREET (1973), MURASHIGE (1974), THOMAS and DAVEY (1975), BUTCHER and INGRAM (1976), REINERT and BAJAJ (1977), CONGER (1981), THORPE (1981) and KRIKORIAN (1982).

Much of the early work on plant tissue culture was done on herbaceous angiosperms and a few bulbous monocotyledons. Many economically-important crops fall outside this group and only fairly recently has there been success with *in vitro* culture of cereal crops, palms, conifers and other woody plants. A brief review of the work in gymnosperms and especially cycads follows (Sections 2.2 and 2.3).

The science of tissue culture has evolved into a recognised and important major discipline with much future potential. Quite apart from the usefulness of *in vitro* techniques as a powerful tool in morpho-genetic and physiological research, there are vital applied benefits. Amongst these can be listed applications such as the propagation of plants which do not readily give viable seed or are particularly slow to germinate, the rapid clonal multiplication to give uniform crops of horticultural and agricultural importance, the possibility of genetic improvement through selection processes in competitive cell culture systems, the elimination of virus and other diseases from infested plant stock, the availability of guaranteed pathogen-free material for international trade, the potential of cryopreservation of valuable germ-plasm and the production of natural plant products *in vitro*. Refinement of the current methodology will be closely associated with any procedures involving recombinant DNA or plant 'genetic engineering'. Numerous problems remain to be solved and opportunities abound for those with imagination and perseverance to contribute further to the theoretical and applied aspects of this exciting field.

2.2 Tissue culture of gymnosperms (other than cycads).

Early work in gymnosperm tissue culture was carried out primarily to investigate various developmental stages and to gain insight into gymnosperm tissue culture was carried out primarily to investigate various developmental stages and to gain insight into gymnosperm morphology. As the methodologies developed, it became clear that the techniques had a significant application to propagation systems. Because of their economical importance, most of the recent work has been done on coniferous timber trees. Within this group of softwoods, the most valuable timber is found in the family Pinaceae and especially the genera *Pinus*, *Picea*, *Abies*, *Pseudotsuga*, *Cedrus* and *Larix*. The history and advances in this field have been reviewed by DURZAN and CAMPBELL (1974), BROWN and SOMMER (1975), DAVID (1982), JOHN (1983) and BONGA and DURZAN (1987).

Most attention has been focussed on the use of seed-derived material, notably embryos, cotyledons and hypocotyls, which can give rise to adventitious budding or somatic embryogenesis with or without an intervening callus stage. The responses obtained vary with the species, the explant material, its physiological condition, the composition of the basal culture medium, addition of growth factors, temperature, light intensity and photoperiod. A review by NORSTOG (1982) provides insight to the work on embryo-derived material.

LA RUE (1936) successfully cultured a large number of excised gymnosperm embryos and showed that plantlets could be raised in the absence of the megagametophyte. Other work soon revealed that the development of embryos *in vitro* did not always follow the pattern *in vivo* and often callus proliferation rather than polarised differentiation occurred. One explanation for this was that in culture situations the embryonic explants had uniform accessibility to nutrients (NORSTOG, 1982). Callus formation on immature embryos of *Pinus* and *Ginkgo* was reported as early as 50 years ago (RADFORTH, 1936; RADFORTH and PEGORARO, 1955; RADFORTH, TRIP and BONGA, 1958). There was evidence that the orientation of the embryo and the photoperiod could have an influence on the growth pattern (BERLYN and MIKSCHE, 1965). When embryos were dissected and the various segments cultured separately, there was evidence of response gradients. Hypocotyl sections from the seed of *Pinus Lambertiana* Dougl. gave a rhizogenic response which increased with distance from the cotyledons (GREENWOOD and BERLYN, 1965).

The discovery of the natural auxins and cytokinins and the synthesis of their analogues provided new impetus to this work. Hypocotyl segments from embryos of *Pinus gerardiana* Wall gave rise to a subculturable callus on a medium supplemented with 4,5 x 10^{-6}M 2,4-dichlorophenoxyacetic acid; some vascular differentiation was present within the callus mass (KONAR, 1974, 1975). Excised embryos and cotyledons from *Pseudotsuga menziesii* (Mirb.) Franco formed callus which became morphogenetic when treated with a solution containing 0,5 - 1,0 mM 6-benzyladenine; subsequent transfer to a medium without cytokinins stimulated growth of the adventitous buds so formed (CHENG, 1975). The buds arose from epidermal or subepidermal layers and primordia became visible after 20 days in culture (CHEAH and CHENG, 1978). The cytokinin 6-benzyladenine was effective in initiating adventitious buds on hypocotyl segments from *Picea glauca* (Moench) Voss (CAMPBELL and DURZAN, 1975, 1976), *Picea abies* (L) Karst. (CHALUPA, 1975) and *Biota orientalis* (L) Endl. (THOMAS, DUHOUX and VAZART, 1977) but subsequent development was inhibited until subculture to media without growth factors (CAMPBELL and DURZAN, 1975; CHALUPA, 1977). Immature embryos from *Pinus radiata* D.Don generated callus on a medium supplemented with auxin and cytokinin and morphogenesis again occurred when the calli were transferred to a hormone-free medium (REILLY and BROWN, 1976). Embryos from *Pseudotsuga menziesii* gave shoots along cotyledonary surfaces, the number of shoots increasing with increasing concentrations of 6-benzyladenine (WINTON and VERHAGEN, 1977). As an alternative route, the cotyledons could be induced to form callus on a medium with auxin and cytokinin, this callus subsequently giving rise to shoots (WINTON and VERHAGEN, 1977). Embryos and hypocotyl segments from *Pinus wallichiana* A.B. Jackson formed callus on a medium containing 5.3 x 10^{-7}M 1-napnthaleneacetic acid; subculture onto a medium supplemented with 4.4 x 10^{-6}M 6-benzyladenine resulted in shoot initiation (KONAR and SINGH, 1980). Excised cotyledons from *Pinus taeda* L. formed buds along the surfaces after 6- 10 weeks in culture and these extended to 5 - 10 mm while still connected to the explant (MOTT, 1981). Callus induced on mature embryos of *Picea sitchensis* (Bong.) Carr. and *Pinus contorta* Dougl. ex Loud. gave shoots on transfer to hormone-free medium (JOHN, 1983). Because of its national timber industry, a great deal of gymnosperm tissue culture work has been carried out in Sweden. Embryos of *Pinus contorta* gave rise to adventitious buds on media containing 10^{-7} to 10^{-4} M 6-benzyladenine; at the higher cytokinin levels, buds were also formed on cotyledons and hypocotyls

(VON ARNOLD and ERIKSSON, 1981). For the initiation phase it was found that a diluted (X 1/4) basal medium was optimal but of the components in the medium, only the concentration of glycine was critical (VON ARNOLD and ERIKSSON, 1981). It was necessary to transfer the explants after about one month to a medium without cytokinins to allow further development of the buds (VON ARNOLD and ERIKSSON, 1981). Similar results were found with *Picia abies* (L) Karst. (VON ARNOLD, 1982) and later work showed that increasing the concentration of agar in the bud-development medium from 0,5 to 2,0% decreased vitrification problems but also reduced shoot growth and rooting potential (VON ARNOLD and ERIKSSON, 1984). A refinement in the technique for bud initiation from *Picea abies* was the use of 2 to 3 hour 'pulse' pre-treatment of the excised embryos in 2,5 to 6,25 x 10^{-4}M 6-benzyladenine at pH 5,5 prior to setting up the cultures (BORNMAN, 1983; VON ARNOLD and ERIKSSON, 1985).

A preincubation period for seven days in diluted hormone-free medium accelerated bud formation and frequency when embryos from *Pinus caribaea* Morlet were innoculated on medium containing 2,2 x 10^{-5}M 6-benzyladenine (WEBB and SANTIAGO, 1983). The use of a medium containing 10^{-5}M 6-benzyladenine was used to initiate shoot formation on embryos, cotyledons and hypocotyl of *Pinus contorta*; subsequent bud development and elongation was enhanced by elimination of hormones, reduction of the concentration of mineral salts, vitamins and sucrose and by the inclusion of charcoal (PATEL and THORPE, 1984). Changes in the concentrations of nuclear proteins, enzymes and reducing substances have been studied in a cytochemical investigation of bud formation from embryos of *Pinus coulteri* D. Don (PATEL and BERLYN, 1983). Despite the consistent pattern of cytokinin-stimulated bud initiation in gymnosperms, morphogenesis is not always achieved. Application of over 100 different auxin and cytokinin treatments to callus from *Pinus strobus* L. failed to induce any morphogenic response (MINOCHA, 1980). Similarly, excised embryos from the unusual Namibian plant, *Welwitschia mirabilis* Hook. f., gave auxin-induced subculturable callus but all attempts at morphogenesis were unsuccessful (BUTTON, BORNMAN and CARTER, 1971).

Quite apart from the process of bud initiation on gymnosperm explants, it was noticed that under some circumstances the material gives rise to embryo-like outgrowths variously described as embryoids, adventive or adventitious embryos, pseudoembryos or somatic embryos). Such structures were noticed on the cotyledons of *Biota orientalis* (L). Endl. and they later

developed into plantlets (KONAR and OBEROI, 1965). Embryos of *Pinus palustris* Mill similarly generated somatic embryos on the cotyledonary surfaces and these too could be excised to form viable plantlets (SOMMER, BROWN and KORMANIK, 1975). Immature embryos from *Picea abies* gave rise to embryogenic callus when cultured for two months in the dark on a medium supplemented with 10^{-5}M 2.4-dichlorophenoxyacetic acid and 10^{-6} to 10^{-5}M 6-benzyladenine or other cytokinin (HAKMAN and VON ARNOLD, 1985). This callus produced numerous small somatic embryos which developed into normal plantlets on subculture under a 16-hour photoperiod on a medium supplemented only with 6-benzyladenine (HAKMAN AND VON ARNOLD, 1985). Both fresh and stored seed of *Pinus lambertiana* was used to provide embryos which developed a mucilaginous embryogenic callus when cultured on a medium containing 1.4×10^{-5}M 2.4-dichlorophenoxy-acetic acid; on transfer to a medium without the auxin but containing 4.4×10^{-7}M 6-benzyladenine, development analogous to that of normal zygotic embryos occurred (GUPTA and DURZAN, 1986).

The use of non-seed-derived material for gymnosperm tissue culture has also received attention. Early work showed that callus could be formed from cambial explants of *Pinus pinaster* Ait. (GAUTHERET, 1934), while callus derived from *Sequoia sempervirens* Endl. could be subcultured with eventual morphogenesis (BALL, 1950). Juvenile and mature needles, brachyblasts (needle fascicles), apical meristems and various cambial regions have been brought into culture with varying degrees of success (JOHN, 1983). Young needles from *Picea abies* produced adventitious buds on a medium supplemented with 5×10^{-6}M 6-benzyladenine and 5×10^{-5}M 1-naphthaleneacetic acid. Outgrowth of the buds was promoted on transfer to diluted medium (x 1/3) without hormones (JANSSON and BORNMAN, 1980). The frequency of this bud initiation was inversely correlated with needle size (JANSSON and BORNMAN, 1981). Bud yield could be increased by initial incubation at reduced temperatures (VON ARNOLD and ERIKSSON, 1979a). Use of 6-benzyladenine initiated callus on needle explants from *Pinus radiata* seedlings: transfer to hormone-free medium allowed formation of meristems which gave shoots after excision and further transfer (REILLY and BROWN, 1976). With the same species, as many as 180 viable shoots could be obtained from needles of a single seedling (AITKEN, HORGAN and THORPE, 1981). Morphogenic callus was initiated from vegetative buds of *Pinus contorta* and *Picea sitchensis* (WEBB AND STREET, 1977).

Vegetative buds of *Picea abies* were induced to form buds and shoots in response to 6-benzyladenine and subsequent transfer to hormone-free medium (VON ARNOLD and ERIKSSON, 1979b).

Of the methodologies used in the work described above, only the somatic embryogenesis route can give rise directly to an entire plant. The techniques which give rise to bud initiation generally require excision, outgrowth and a root-induction process. Rooting may be achieved *in vitro* or with the use of a procedure analogous to that of rooting conifer cuttings in mist-propagation units (JOHN, 1983). The auxin indole-3-butyric acid is favoured by most workers for promoting root induction (JOHN, 1983).

The technique of suspension culture has not yet found wide application in gymnosperms, but some pointers exists for future work. With *Picea abies*, repeated subculture of cell aggregates in suspension lead eventually to the formation of small embryoids (CHALUPA and DURZAN, 1973). Shoot tips of *Pseudotsuga menziesii* provided material for suspension cultures which ultimately gave bipolar embryoids (DURZAN, 1979). With seedling cotyledonary material of the same species, individual protoplasts were isolated and could be multiplied to a callus stage on a fabric support saturated with glutamine-enriched medium (KIRBY and CHENG, 1979). Protoplasts have also been isolated and cultured from the cotyledons of *Pinus pinaster* and they subsequently regenerated cell walls and commenced aggregation into callus structures (DAVID and DAVID, 1979).

With respect to the culture of haploid, rather than diploid, plant material, the gymnosperms provide greater opportunities than the angiosperms. This is largely because of the well-defined micro- and megagametophytic stages in their life cycle and the accessibility of this material in dissection. The first successful pollen culture work was carried out by TULECKE (1957) who was able to initiate haploid callus from mature microspores of various gymnosperms. Microsporophylls from *Pinus* gave a mixed haploid and diploid cellular callus in which some globular embryoids were observed (BONGA, 1974). Microspores from *Pinus resinosa* Ait. subjected to cold treatment and centrifugation gave increased callus proliferation (BONGA and McINNIS, 1975). There has been little indication of any type of regeneration from this material, but it may be that a precise stage in development is critical

to any morphogenic response (NORSTOG, 1982). The female gametophyte from *Ginkgo biloba* gives a variety of outgrowths and subcultureable tissue *in vitro* (ROHR, 1977). Megagametophytes from various species of *Pinus* give callus cultures but, apart from the presence of tracheidal elements, little differentiation has been recorded (NORSTOG, 1982). An exception to this generalisation is the report by KONAR and SINGH (1979) that megagametophytes from *Ephedra foliata* Boiss gave auxin-initiated callus which could give shoots and roots after subculture on a medium supplemented with kinetin. Addition of 6-benzyladenine stimulated more profuse shoot formation and allowed plantlet development.

Publications on the tissue culture of gymnosperms continue to proliferate. A most useful series of reviews is presented in the work of BONGA and DURZAN (1987), the third volume of which deals with *in vitro* case histories of material from coniferous trees.

2.3 Tissue culture of cycads

Prior to the establishment of aseptic plant tissue culture techniques, certain observations had been recorded on the regeneration of viable structures from both haploid and diploid cycad material. DUCHARTE (1888) observed that megagametophytes from infertile seed of *Cycas thouarsii* Gaud., maintained for prolonged periods in germination beds, produced numerous adventitious roots. COULTER and CHRYSLER (1904) reported that whole *Zamia* plants could regenerate from slices of the underground root, an observation which has recently formed the basis for a propagation technique (BURCH, 1981a). It is also common knowledge amongst cycad enthusiasts that when whole cycad leaves are removed from the parent plant and placed in a warm porous medium, as in a 'compost heap' situation, callogenesis and occasional rhizogenesis may be found at the cut leaf base surface. Finally, present methods of vegetative propagation involve regeneration, as in the rooting of lateral branches and basal suckers (Section 1.10).

Much of the early tissue culture work on cycads was directed at observing regeneration of haploid material *in vitro*, and provided important information as to ontogenic development in these plants. LA RUE (1948) cultured megagametophytes of *Zamia floridana* A. DC. (= Z. *pumila* L.) on simple media and found that 'pustular' growth occurred which, in a small number of cases, led to the formation of roots or shoots or both. LA RUE (1954) showed that the same tissue material, grown aseptically on moist sand, could occasionally regenerate small detachable balls of meristematic tissue which he called pseudobulbils. These could be sub-cultured, eventually with the formation of poorly-vascularised roots and leaves. The supply of minerals or carbohydrates did not appear to be beneficial. In the same paper LA RUE (1954) reported the low frequency production of presumably haploid roots and buds from megagametophytes of *Cycas revoluta* Thunb. after two years in culture. Furthermore, immature embryos from both species matured and gave rise to seedlings when fertilised ovules were cultured on moist sand.

NORSTOG (1965) investigated regeneration from both haploid and diploid tissue of *Zamia integrifolia* Ait. (= Z. *pumila* L.) using megagametophytes and embryos respectively on a variety of media which were essentially supplemented modifications of WHITE's (1943) basal medium (Table 3). Cultures were kept at 25°C in darkness except for brief times of examination. Callogenesis was observed in a significant number of two-month-old cultures of megagametophytic explants on media containing auxin, kinetin and organic nitrogen sources. Optimum results were obtained using medium '8' (Table 3) which contained glutamine, asparagine, alanine and adenine in addition to $4,5 \times 10^{-6}$M 2,4-dichlorophenoxyacetic acid and kinetin. In this case, 68% of the half-gametophyte explants formed callus within two months. After another three months there was evidence of root and subsequently leaf initiation. The auxin 2,4-D appeared to be more successful than indole 3-acetic acid when used with kinetin in terms of morphogenetic response. However, elongation of these primordia was inhibited until the explants were transferred to a medium without growth factors. NORSTOG (1965) extended La Rue's earlier work by ascertaining chromosome numbers of the resulting tissue. Regenerated root

TABLE 3 : SELECTED MEDIA USED BY VARIOUS WORKERS IN CYCAD TISSUE CULTURE EXPERIMENTS

Constituents mg per 1000 ml	Basal Medium White (1943)	Modified White's Media		Modified Murashige-Skoog Media	
		"8"[1]	"63"[2]	"59"[3]	"KI"[4]
$MgSO_4.7H_2O$	737	737	730	370	370
Na_2SO_4	200	200	200		
$Ca(NO_3)_2.4H_2O$	288	288	290		
$CaCl_2$				332	332
KCl	65	65	700		
KNO_3	80	80	470	1900	1900
$NaH_2PO_4.H_2O$	13,5	13,5	300		
KH_2PO_4				170	170
NH_4NO_3				1650	1650
H_3BO_3	1,5	1,5	0,5	6,2	6,2
$MnSO_4.4H_2O$	6,7	6,7	3	22,3	22,3
$ZnSO_4.7H_2O$	2,7	2,7	0,5	8,6	8,6
KI	0,75	0,75		0,83	0,83
$Na_2MoO_4.2H_2O$			0,025	0,25	0,25
$CuSO_4.5H_2O$			0,025	0,025	0,025
$CoCl_2.6H_2O$			0,025	0,025	0,025
Fe citrate.$5H_2O$			10		
$Na_2EDTA.2H_2O$				37,3	37,3
$FeSO_4.7H_2O$				27,8	27,8
$Fe_2(SO_4)_3$	2,5	2,5			
$(NH_4)_2$ malate			100		
Asparagine		100	(malic acid adj. to pH5 with NH_4OH)		
Glycine	3	100			
Alanine		100	100	100	400
Glutamine		100	400	400	400
Tyrosine					5
Adenine SO_4		10		10	10
Kinetin		1		1	15
Auxin		1(2,4-D)	10(2,4-D)	1(2,4-D)	10 (NAA)
Ca pantothenate			0,25		
Thiamine HCl	0,1	0,1	0,25	0,4	1
Ascorbic acid			0.25		
Nicotinic acid	0,5	0,5	1,25		
Riboflavin			0,25		
Pyridoxine HCl	0,1	0,1	0,25		
Inositol			50	100	100
Sucrose	20 000	20 000	20 000	20 000	30 000
Bacto-agar	9 000	9 000	9 000	9 000	9 000

[1] NORSTOG (1965); [2,3] NORSTOG & RHAMSTINE (1967); [4] KOELEMAN & SMALL (1982).

and leaf material showed the expected haploid number of 8 chromosomes but nevertheless the structures resembled closely those of similar stages from diploid material, at least in the early development. Use of a medium originally devised for the culture of excised barley embryos promoted the development of somatic embryos from a few explants of both gametophytic and embryonic material of the same *Zamia* species. These embryoids were mostly dicotyledonous and seemed to be analogous to La Rue's pseudobulbils.

Following this work, NORSTOG and RHAMSTINE (1967) excised megagametophytes and juvenile embryos from *Zamia integrifolia* Ait. (=*Z. pumila L.*) and *Cycas circinalis L.* and explored their response to a wide variety of culture media. Both solid and liquid media were used, the latter being held in reciprocating shakers or rotating flasks. To quantify the results, the mass of tissue was recorded at various stages as the experiment progressed. On simple media with no auxin or cytokinin supplements, *Zamia* proembryos grew in essentially a normal fashion although in some instances the usual dicotyledonous development gave way to polycotyledony. On other media containing low concentrations of 2,4-D and kinetin the pro-embryos formed sub-culturable callus. Much more rapid growth occurred on transfer to medium '59' (Table 3) which is a MURASHIGE and SKOOG (1962) medium modified by LINSMAIER and SKOOG (1965) containing amino acids, adenine and $4{,}5 \times 10^{-6}$M 2,4-D and kinetin. On further transfer to media lacking auxin and cytokinin, 'pseudobulbils' appeared which later developed into adventive embryos. This latter type of growth was promoted on media without organic nitrogen supplements and eventually gave rise to 'fronds' about 7 cm long. The resulting plantlets did not develop further and were not transferred to soil. Diploid cultures from *Cycas* pro-embryos were unsuccessful.

In the experiments with haploid material, using media similar to those previously described but relatively high in auxin ($4{,}5 \times 10^{-5}$M 2,4-D), megagametophytic explants from both *Zamia* and *Cycas* could be maintained, sub-cultured, and gave every indication of long-term sustained growth. Abundant undifferentiated tissue appeared

within 60-90 days on both solid and liquid media although the former was more successful. Light and darkness had no obvious affect. As with the diploid experiments, subsequent transfer of the *Zamia*-derived callus to media without auxin and kinetin resulted in the formation of somatic embryos. These structures formed haploid plantlets in minimal media but the plants were not established in soil.

NORSTOG and RHAMSTINE (1967) mention specifically that a decisive factor in induction of embryogenesis is the isolation of cells from organised tissue. Proliferation of small clumps of undifferentiated cellular material led to somatic embryony whereas the use of larger explants favoured direct organogenesis. Another observation by these authors is the importance of nitrogen from amino acids rather than from inorganic sources. It is also evident that the development from embryo to plantlet required a depauperate medium; the authors conclude that the situation *in vitro* is analogous to the usual pattern *in vivo*, where the embryo, initially dependent on its complex organic food reserve, become autotrophic and grows on a largely inorganic substrate. Italian workers (DE LUCA, MORETTI and SABATO, 1979) used haploid explants from the cycads *Ceratozamia mexicana* Brong., *Cycas revoluta* Thunb. and the South African species *Encephalartos umbeluziensis* R.A. Dyer, and explored responses to a wide variety of media under different temperature and light conditions. Verification counts of the resulting tissue confirmed the ploidy status.

Ceratozamia megagametophytes on medium '8' (Table 3) containing $4{,}5 \times 10^{-6}$M 2.4-D and kinetin swelled and became green in a short time. Thereafter the results were somewhat variable; some cultures produced only a crisp white callus, others gave somatic embryos and yet others produced outgrowths, with clearly haploid roots. On a medium high in auxin ($4{,}5 \times 10^{-5}$M 2,4-D) (Medium '63', Table 3), only callus tissue was formed, growing to four or five times its original size in as many months. On a medium without auxin and kinetin, somatic embryogenesis occurred without prior callusing. Some of these embryos progressed through a cotyledonary phase eventually giving rise in one case to a typical circinate leaf with leaflets.

With *Cycas* haploid on medium '8', callusing was followed by 'pseudobulbil' development in relative profusion. However, these spherical nodules were vascularised with tracheids and later shown to be analogous to coralloid root nodules, a normal feature of cycad ontogeny (DE LUCA and SABATO, 1980). *Encephalartos* material on the same medium formed callus without 'pseudobulbil' development. Both *Cycas* and *Encephalartos* megagametophytes produced callus on medium '59' with 4,5 x 10^{-5}M 2,4-D, but there was no indication of subsequent morphogenesis. DE LUCA, MORETTI AND SABATO (1979) conclude that the potential for regeneration appears to be favoured by ambient (25°C) rather than elevated (35°C) temperatures, by diffuse light (50µEm^{-2}s^{-1}) rather than darkness, and by media generally with lower concentrations of auxins, cytokinins and amino acids. There was no report of attempted sub-culturing to different media and it is not clear whether plantlets were grown beyond the stage illustrated by the authors.

DE LUCA AND SABATO (1980) cultivated megagametophyte tissue from *Cycas revoluta* Thunb. on WHITE's (1943) basal medium supplemented with 2,4-D and kinetin at various levels under continuous low-intensity (50µEm^{-2}s^{-1}) light. After three months on the medium with 4,5 x 10^{-6}M levels of each growth factor, a large number of explants had developed coralloid roots from spherical nodules identical to those obtained in earlier work (DE LUCA, MORETTI and SABATO, 1979). No micro-organisms were associated with these root nodules which thus appear to be an inherent feature of cycad root systems and not necessarily a response to a particular symbiotic association. The regeneration of coralloid roots *in vitro* extends an earlier observation that apogeotropic roots are formed when seeds of *Macrozamia riedlei* (Gaud.) Gardn. are grown in sterile conditions (LAMONT and RYAN, 1977). Similar results to those from *Cycas revoluta* were obtained in an experiment with explants from *Macrozamia communis* L. Johnson (DE LUCA, SABATO, BALDUZZI and NAZZARO, 1980). Megagametophyte halves, cultured on WHITE's (1943) medium supplemented with 4,5 x 10^{-6}M 2,4-D and kinetin gave callus formation after two months. About half these calli subsequently gave rise to coralloid root primordia, again free from any micro-organisms. The authors speculate that the cycad coralloid roots represent remnants of primitive pneumatophores which acquired the symbiont in an

evolutionary advance.

Further investigation into regeneration from Z. *pumila* megagametophytes was carried out by RIVERA ROSA (unpublished results). Using Murashige-Skoog medium with various levels of 1-naphthaleneacetic acid and 6-benzyladenine, nodular callus was obtained at different frequencies. With 4.5 x 10^{-7}M levels of each growth factor, half the explants gave nodular callus which in turn gave rise to bipolar dicotyledonous embryoids. These failed to develop further. With 10^{-5}M NAA and 10^{-6}M BA, a friable callus was obtained on 50% of the explants and occasional roots were formed. Some shoot morphogenesis was seen on transfer to hormone-free medium but autonomous plants could not be raised. When entire Z. *pumila* seeds were grown *in vitro*, the germinating root produced a friable callus. If this callus was excised, regeneration occurred from the cut end of the embryo and from the megagametophyte, the latter developing outgrowths which became roots. Roots also occasionally arose from the hypocotyl-cotyledonary area in these circumstances, and normal leaf development was observed in 16% of the explants.

Small cylindrical explants of megagametophyte from the particularly rare Cuban cycad, *Microcycas calocoma* (Miq.) A.DC. were cultivated by PENA and GRILLO (1982) on Murashige-Skoog medium modified by the inclusion of NAA and coconut milk, but lacking potassium iodide. With 10% coconut milk a yellow, friable callus developed and roots were produced when 5 x 10^{-6}M NAA was present. Increasing the auxin level inhibited rhizogenesis and altered the texture of the callus. With 15% coconut milk and 5 x 10^{-5}M NAA, rapid callogenesis occurred with root formation following transfer to medium with reduced NAA levels. At very low or zero NAA concentrations, the formation of pink 'pseudobulbils' was reported. Callus growth could be maintained through five successive passages but growth rates steadily decreased.

Yet another project on megagametophytic tissue was that of LALIBERTE, BERTRAND and VIETH (1983) who used explants from infertile seeds of *Encephalartos villosus* Lem. on a modified WHITE's (1943) medium containing thiamine HCl (0,1 mg l^{-1}), inositol (50 mg l^{-1}), glutamine and asparagine (100 mg l^{-1} each) and adenine sulphate (40 mg l^{-1}) under medium intensity light (150$\mu Em^{-2}s^{-1}$) for a 16-hour photoperiod. Surface nodulation followed callogenesis and was most abundant when the medium was supplemented with 4.5 x 10^{-6}M 2,4-D and 2,3 x 10^{-6}M kinetin. Microscopic studies showed that these meristematic areas occurred internally as well as superficially. Although some tracheid formation was found in the callus, there was no significant morphogenesis. No attempt was made to subculture callus or to promote organised development.

Comparatively little *in vitro* work has been attempted with cycad microgametophytes. LA RUE (1954) carried out experiments with *Zamia pumila* microsporangia and cited earlier reports of cycad pollen germination on a variety of media. Since mature pollen grains were killed during disinfestation processes, unopened microsporangia containing microspores at the uninucleate stage were used. These were cultured on WHITE's (1943) medium with a wide variety of additives. The supplements had little effect; normal pollen development occurred in all cases, with pollen tube formation but without apparent spermatogenesis. While pollen tubes were viable for a prolonged period, growth was limited and the cultures eventually senesced.

In vitro spermatogenesis was achieved when microsporangia of *Encephalartos altensteinii* Lehm. were cultivated on a medium which TULECKE (1957) had previously used for pollen studies on *Ginkgo biloba* L. (DE LUCA and SABATO, 1979). Microspores were in a trinucleate condition at the time of inoculation. After about one week the microsporangia burst open and a mass of pollen tubes appeared. Thereafter spermatogenesis proceeded normally, giving rise to the characteristic large motile spermatozoids after 6 months. There was no difference in observations when the medium was supplemented with 2,7 x 10^{-6}M 2,4-D. In a similar experiment, using the same media, spermatogenesis of *Ceratozamia mexicana* Brong. and *Cycas revoluta* Thunb. was investigated

(DE LUCA, LA VALVA and SABATO, 1980). With the latter species, micro-gametophytes remained at the immature sperm mother stage after 12 months in culture. In the case of the *Ceratozamia*, callogenesis was recorded after 11 months in two of the cultures which had been provided with the 2,4-D supplement. This callus had a superficial periderm and a parenchymatous core but it was not possible to ascertain from which tissue the growth had originated. Chromosomal assessment was not carried out to confirm the presumably haploid nature of the callus.

The amount of published research work on cycad tissue culture, other than that of gametophytic origin, is limited. Possibly the notoriously slow *in vivo* growth rate has influenced workers against the choice of these plants for short-term projects. Fortunately more attention is now being paid to the potential benefits of tissue culture as a propagative tool; this is especially true for cycads where the continued survival of so many taxa is in jeopardy (Section 1.4).

BROWN and TEAS (1966) obtained callus growth from mature embryos of *Zamia floridana* A.DC. (=Z. *pumila* L.) and from the leaf rachis of *Cycas revoluta* Thunb. using Knop's salts and 3% sucrose in solution. Rhizogenesis followed on the embryo-derived callus with some evidence of leaf production later. MUSTOE (1967) reported the production of callus from cambial explants of the same two species.

A fairly extensive project on the potential of cycad tissue culture for propagation was carried out by HENSON (1980) at the micropropagation unit of the Royal Botanic Gardens at Kew. Using relatively large explants, up to 3 cm in length, from young leaves and other parts of the plant, including ovules, mega- and micro-sporophylls, coralloid roots and seedling hypocotyls, and using mainly Murashige-Skoog media with a range of concentrations of auxins, cytokinins and gibberellic acid, Henson grew callus from 35 cycad species. Callus formed readily with explants form *Ceratozamia*, *Stangeria* and *Zamia* without exogenous growth factors, but either NAA or IAA was needed for callus initiation in *Cycas*, *Dioon*, *Encephalartos*, *Lepidozamia* and

Macrozamia. Whilst the absolute concentrations were not critical, good results were generally obtained with $5,3 \times 10^{-6}$ M NAA and $4,5 \times 10^{-7}$ M BA. Callus morphology and colour were variable and independent of explant origin or growth regulator application. Friable callus proliferated on sub-culture but did not differentiate. A slower-growing, more compact callus, which developed in about half the species tested, gave rise to spherical structures analogous to LA RUE's (1954) pseudobulbils. These were most readily obtained in cultures kept in the dark for several months and did not necessarily require any exogenous growth substances. The structures comprised an orange-brown epidermis and a parenchymatous core with internal tracheidal development. Rhizogenesis was observed from the spherical structures obtained from young *Zamia* leaves in a few instances, but plantlets were not successfully regenerated.

Another major project with a propagative bias was that undertaken by South African workers (KOELEMAN and SMALL, 1982). Stem and root explants from *E. cupidus* R.A. Dyer, *E. eugene-maraisii* Verdoorn, *E. inopinus* R.A. Dyer, *E. lanatus* Stapf and Burtt Davy, *E. lebomboensis* Verdoorn, *E. lehmannii* Lehm., *E. natalensis* R.A. Dyer and Verdoorn, *E. paucidentatus* Stapf and Burtt Davy and *E. transvenosus* Stapf and Burtt Davy were used. The aim was to develop a protocol generally applicable for clonal propagation of the rarer cycad species. Callogenesis was achieved in each of the nine species tested, requiring about two to four months for stem explants and an additional two months for root tissue. Optimum results were obtained using medium 'K1' (Table 3) which is a MURASHIGE-SKOOG medium containing amino acids amides and supplemented with $5,4 \times 10^{-5}$ M NAA and $7,0 \times 10^{-5}$ M kinetin. Explants from mesic species generally grew faster than those from xeric habitats. Of the species tested, *E. lebomboensis* was the most vigorous while *E. lanatus* gave the poorest response. KOELEMAN (*pers. comm*) subsequently reported that no organogenesis occurred in this series of experiments.

Extensive use of *in vitro* culture techniques has been made by WEBB and colleagues of Queen's University, Kingston, Canada (WEBB, 1981, 1982a, 1982b, 1983, 1984; WEBB and DE JESUS, 1982; WEBB, NEVAREZ and DE JESUS, 1984). Work was initially concerned with the assessment of light on cycad apogeotropic root nodule development. Embryos from several taxa were cultured in darkness on a medium based on WHITE's

macronutrients with MURASHIGE-SKOOG micronutrients, and the resulting tissues then exposed to different light regimes. A general conclusion was that light stimulated nodule development but inhibited both primary and secondary root initiation and elongation. If leaf initiation did occur, this followed much later. Partial or complete cotyledonary excision or removal of the gametophyte suppressed growth (WEBB, 1982a) as did the reduction or dilution of nitrate in the medium (WEBB, *pers. comm.*) Light-induced root nodules had the same anatomy as algal free nodules from soil-grown plants (WEBB, 1983) but there has not yet been any attempt at establishing the cycad-cyanobacterium symbiosis *in vitro*.

Using mature embryos from *Zamia pumila*, WEBB, RIVERA STARSZAK and MATOS (1983) report on callus formation and differentiation on MURASHIGE-SKOOG medium with different NAA and BA supplements. The auxin was necessary for callus initiation while the cytokinin appeared to affect the frequency of callogenesis and the morphology of the product. Good results were obtained with $4,5 \times 10^{-6}$ M NAA and BA. Friable callus, with poor regenerative potential, was produced when the NAA/BA ratio was high. More compact callus development when the BA level was increased and this yielded roots, shoots and occasional embryo-like structures resembling the somatic embryos reported by NORSTOG and RHAMSTINE (1967). Although there was considerable leaf elongation *in vitro*, autotrophic plants could not be established.

Continuing the studies on *Z. pumila*, MONNIER and NORSTOG (1984) cultivated immature embryos and their separate embryo, suspensor and egg membrane components on various media modified from a barley embryo culture formulation, essentially to compare development *in ovulo* and *in vitro*. Maximum growth arose at the embryo base and after two months protruberances identifiable as cotyledons appeared, but these had a more callus-like appearance than the comparative stage *in vivo*. The suspensor and the egg membrane were not required for embryo development *in vitro* except in particularly immature embryo systems. Further work was carried out by the same authors (MONNIER and NORSTOG, 1986) in order to compare the *in vitro* growth of *Zamia* embryos excised at different stages in their development. Embryos excised shortly after fertilization gave an initial extension of the suspensors followed by enlargement of the embryo to a club-shaped structure with callus-like cellular appearance.

Embryos excised two months later were still undifferentiated but rapidly developed two cotyledons in culture. When these embryos were longitudinally bisected, each regenerated a bipolar structure. Mature, differentiated embryos did not have this potential for regeneration. The authors propose three stages of *in ovulo* development : an early unprogrammed phase is followed by a time when inductive stimuli cause programmed organogenesis; the final phase is a period in which cell lineages are no longer labile.

2.4 Experimental : materials and methods

2.4.1 Sources of plant material

Seeds, seedlings and vegetative material used in the tissue culture work described in this Chapter and for the biochemical studies described in Chapter 3, were obtained from a large number of sources. In each case, care was taken to ensure that the material was correctly identified; where any doubt existed as to the validity of the name, the material was rejected. Seeds and young seedlings of *Encephalartos* and *Stangeria* species were obtained from members of the Cycad Society of Southern Africa either directly or via the Society's central seedbank. Seeds of the Australasian cycads, *Bowenia, Cycas, Lepidozamia* and *Macrozamia*, were supplied by the Palm and Cycad Society of Australia. Seeds from the New World genera, *Ceratozamia, Dioon* and *Zamia*, were sent from the seedbank of the Cycad Society (U.S.) and from Fairchild Tropical Garden, Florida, U.S.A. Vegetative material (leaves, roots, etc.) and samples from reproductive organs (cone-scales, cone-axes, pollen, etc.) were made available by the Durban Botanic Gardens, the Durban unit of the Botanic Research Institute and by cycad enthusiasts with large plant collections of known provenance, including Mr G.C. Cox, Mr. R. Heerman, Mr. R.E. Levitt and Mr. H.E. Wohlberg, all of the greater Durban area.

2.4.2 Nursery procedure

Seeds were germinated in seed trays containing a friable, humus-rich potting soil in which the seeds were placed horizontally and buried to approximately one-third of their depth. After the hypocotyl emerged from the micropyle, the seeds were transferred individually to black plastic nursery bags, 200 mm x 100 mm x 55 mm, containing humus-rich garden loam supplemented with a commercial 3:2:1 (25) granular fertiliser at the rate of 250 g per 50 kg. Watering was carried out so as to maintain moist conditions. Pest control was accomplished by the occassional use of a commercial systemic insecticide. Seeds and seedlings were kept in a shade house covered with "50%"shade cloth and at ambient temperature conditions.

2.4.3 Disinfection procedures

The disinfection procedures adopted in this project generally employed ethanol and hypochlorite solutions as surface sterilants; these procedures being modified slightly dependent on the nature of the material.

Fresh seeds were freed from their fleshy sarcotestae and washed thoroughly in water. The seeds were then immersed for 4 minutes in 70% ($^{v}/_{v}$) ethanol containing a few drops of "Teepol" wetting agent, washed with sterile distilled water and placed for 30 minutes in a 20% ($^{v}/_{v}$) solution of "Jik" (3,5% NaOCℓ when packed, Reckitt Household Products), also containing a few drops of wetting agent. The disinfected seeds were then rinsed three times in sterile distilled water, allowing 5 minutes soaking time between each change. The sclerotestae were then cracked with sterile pliers and the intact megagametophytes subjected to a further hypochlorite treatment and washing process as described above. Seeds treated in this manner were then aseptically dissected under a laminar-flow hood to give embryos or sections of megagametophytic tissue.

Primary root tissue was obtained from seedlings grown as described in Section 2.4.2. The seedlings were carefully removed from their

containers, freed from the bulk of the soil medium and washed in running tap water. After removal of the leaves and lateral rootlets, the primary roots were scrubbed with a soft bristle brush to remove adherent soil particles together with the epidermal layer. The cleaned roots were then immersed in 70% ethanol and 20% "Jik" with water rinses as described above. Aseptic dissection then provided cubes or cylinders of root tissue for transfer to culture.

Cycad leaves were found to give the most difficulties with regard to disinfection. The combined effect of a waxy cuticle, covering of leaf hairs and deeply-recessed stomata necessitated a fairly rigorous treatment. However, the young, actively-growing leaflets, which offered the best potential for growth *in vitro*, were prone to damage during the disinfection process. Thus the fine balance between incomplete disinfection and necrotic damage of the leaf tissue was not easily obtained. The procedure adopted was as follows. The intact leaflets were washed with tap water containing 1% "Teepol", swabbed briefly with toluene and chloroform in succession, immersed for 4 minutes in 70% ethanol, rinsed in sterile distilled water, immersed in 20% "Jik" for 30 minutes, rinsed in sterile distilled water, immersed for 30 minutes in 0,5% mercuric chloride solution, rinsed in sterile distilled water, immersed again in 70% alcohol for 4 minutes and finally rinsed three times with sterile distilled water. Despite this rigorous treatment, fungal infections were frequent and between 50 and 100% of leaflet explant cultures showed contamination within 3 weeks. This problem does not seem to have been addressed by other workers. For instance, HENSON (1980), strongly favoured the use of cycad leaflets as an explant source but did not describe his disinfection procedure. It is speculated that the deeply-recessed stomata in cycad leaflets (BAIJNATH, NAIDOO and RAMCHARUN, 1980) act as reservoirs for microbial infection but the possibility of natural fungal endophytes cannot be eliminated.

Other cycad tissues used in this project, e.g. leaf petioles, cone scales and cone axes, were disinfected by the sequential treatment with ethanol and hypochlorite solutions as previously described. These sources of tissues, as with the seed and root material, resulted in sporadic contamination by bacteria and fungi, but the infection frequency (generally less than 5%) was considered acceptable.

2.4.4 Choice and preparation of media

Mention has been made in Section 2.3 of several specific media used for the *in vitro* culture of cycad material; these being essentially modifications of WHITE's (1943) medium and that of MURASHIGE and SKOOG (1962), as detailed in Table 3. Table 4 gives details of four other widely-used media: the original MURASHIGE and SKOOG (1962) (MS) formulation and the subsequent LINSMAIER and SKOOG (1965) modification; the SCHENK and HILDEBRANDT (1972) (SH) medium; the medium used for cell suspension cultures developed by GAMBORG, MILLER and OJIMA (1968) (B5) and that used for pollen grain work by NITSCH and NITSCH (1969) (NN-69). These media reflect only a few of the wide number of formulations developed and modified over the last 50 years. Since the nutritional requirements of plants *in vitro* cannot be unconditionally extrapolated to culture requirements *in vitro*, almost all work involves an empirical process of media selection and optimization. Thus the usual procedure involves the use of one or more of the more "popular" media as a starting point with progressive modification of individual components on the basis of experimental results, until near-optimal growth is obtained. Often the macro-nutrients of one formulation are combined with the micro-nutrients of another and even the organic supplements of a third. Another modification, often applied to mineral-rich media like the MS formulation, is the use of half - or quarter-strength mixtures. Finally, it must be noted that the medium often requires modification for different stages of growth - i.e. initiation, proliferation and morphogenesis. In order to provide a basis for initial medium selection, it is useful to review briefly the roles of particular constituents.

Macro-nutrients

The levels of each of the macronutrient elements (N, P, K, Ca and Mg) and the "incidental" elements (S, Cl and Na) for the various formulations discussed, are shown in Table 5. A detailed discussion of the function of each element is beyond the scope of this text; such information is readily obtained from reviews on plant nutrition (MURASHIGE, 1973; CLARKSON and HANSON, 1980). Besides their specific major structural and functional purposes, the elements may contribute to buffer capacity and osmotic potential, act in ion-balance roles and cations may serve as Lewis acids.

TABLE 4 : FOUR MEDIA USED IN TISSUE CULTURE EXPERIMENTS

Constituents [1] mg per 1000 ml	"MS"[2]	"SH"[3]	"B5"[4]	"NN-69"[5]
$MgSO_4.7H_2O$	370	400	250	185
$CaCl_2.2H_2O$	440	200	150	166
KNO_3	1900	2500	2500	950
KH_2PO_4	170			68
$NH_4H_2PO_4$		300		
$NaH_2PO_4.H_2O$			150	
$(NH_4)_2SO_4$			134	
NH_4NO_3	1650			720
H_3BO_3	6,2	5,0	3,0	10,0
$MnSO_4.4H_2O$	22,3	10 * [6]	10 * [6]	25
$ZnSO_4.7H_2O$	8,6	1,0	2,0	10
KI	0,83	1,0	0,75	
$Na_2MoO_4.2H_2O$	0,25	0,1	0,25	0,25
$CuSO_4.5H_2O$	0,025	0,2	0,025	0,025
$CoCl_2.6H_2O$	0,025	0,1	0,025	
$Na_2EDTA.2H_2O$	37,3	20	37,3	37,3
$FeSO_4.7H_2O$	27,8	15	27,8	27,8
Glycine	2,0[7]			2,0
Folic acid				0,5
Biotin				0,05
Thiamine.HCl	0,1[7]	5,0	10,0	0,5
Nicotinic acid	0,5[7]	5,0	1,0	5,0
Pyridoxine.HCl	0,5[7]	0,5	1,0	0,5
Inositol	100	1000	100	100
Sucrose	30000	30000	20000	20000
Bacto-agar	6000	6000	6000-8000	8000

Notes: (1) Auxin and cytokinin are added according to specific requirements.

(2) MURASHIGE and SKOOG (1962).

(3) SCHENK and HILDEBRANDT (1972).

(4) GAMBORG, MILLER and OJIMA (1968).

(5) NITSCH and NITSCH (1969).

(6) * These media list this as the monohydrate, $MnSO_4.H_2O$.

(7) The LINSMAIER and SKOOG (1965) modification of the MS formula omits nicotinic acid, pyridoxine and glycine, and increases the thiamine level to 0,4 mg ℓ^{-1}.

TABLE 5 : MACRO-ELEMENTS PRESENT IN VARIOUS CULTURE MEDIA

	ELEMENT SUPPLIED	Formulation (refs. given in text, see also Tables 1 & 2); all figs. as mg per 1000 ml (Figures in parenthesis represent the corresponding molar concentrations)								
		White's basal	"8"	"63"	"59"	"KI"	"MS"	"SH"	"B5"	"NN-69"
ESSENTIAL	N (ex NH_4^+)	-	-	21 ($1,5 \times 10^{-3}$)	289 ($2,1 \times 10^{-2}$)	289 ($2,1 \times 10^{-2}$)	289 ($2,1 \times 10^{-2}$)	37 ($2,6 \times 10^{-3}$)	28 ($2,0 \times 10^{-3}$)	126 ($9,0 \times 10^{-3}$)
	N (ex NO_3^-)	45 ($3,2 \times 10^{-3}$)	45 ($3,2 \times 10^{-3}$)	99 ($7,1 \times 10^{-3}$)	552 ($3,9 \times 10^{-2}$)	552 ($3,9 \times 10^{-2}$)	552 ($3,9 \times 10^{-2}$)	346 ($2,5 \times 10^{-2}$)	346 ($2,5 \times 10^{-2}$)	258 ($1,8 \times 10^{-2}$)
	N (total)	45 ($3,2 \times 10^{-3}$)	45 ($3,2 \times 10^{-3}$)	120 ($8,6 \times 10^{-3}$)	841 ($6,0 \times 10^{-2}$)	841 ($6,0 \times 10^{-2}$)	841 ($6,0 \times 10^{-2}$)	383 ($2,8 \times 10^{-2}$)	374 ($2,6 \times 10^{-2}$)	384 ($2,7 \times 10^{-2}$)
	P	3 ($1,0 \times 10^{-4}$)	3 ($1,0 \times 10^{-4}$)	180 ($5,8 \times 10^{-3}$)	39 ($1,2 \times 10^{-3}$)	39 ($1,2 \times 10^{-3}$)	39 ($1,2 \times 10^{-3}$)	81 ($2,6 \times 10^{-3}$)	34 ($1,1 \times 10^{-3}$)	16 ($5,0 \times 10^{-4}$)
	K	65 ($1,7 \times 10^{-3}$)	65 ($1,7 \times 10^{-3}$)	547 ($1,4 \times 10^{-2}$)	783 ($2,0 \times 10^{-2}$)	783 ($2,0 \times 10^{-2}$)	783 ($2,0 \times 10^{-2}$)	965 ($2,5 \times 10^{-2}$)	965 ($2,5 \times 10^{-2}$)	387 ($9,9 \times 10^{-3}$)
	Ca	49 ($1,2 \times 10^{-3}$)	49 ($1,2 \times 10^{-3}$)	49 ($1,2 \times 10^{-3}$)	120 ($3,0 \times 10^{-3}$)	120 ($3,0 \times 10^{-3}$)	120 ($3,0 \times 10^{-3}$)	54 ($1,4 \times 10^{-3}$)	41 ($1,0 \times 10^{-3}$)	45 ($1,1 \times 10^{-3}$)
	Mg	73 ($3,0 \times 10^{-3}$)	73 ($3,0 \times 10^{-3}$)	72 ($3,0 \times 10^{-3}$)	36 ($1,5 \times 10^{-3}$)	36 ($1,5 \times 10^{-3}$)	36 ($1,5 \times 10^{-3}$)	40 ($1,6 \times 10^{-3}$)	25 ($1,0 \times 10^{-3}$)	18 ($7,6 \times 10^{-4}$)
INCIDENTAL	S	143 ($4,5 \times 10^{-3}$)	143 ($4,5 \times 10^{-3}$)	141 ($4,4 \times 10^{-3}$)	55 ($1,7 \times 10^{-3}$)	55 ($1,7 \times 10^{-3}$)	55 ($1,7 \times 10^{-3}$)	56 ($1,7 \times 10^{-3}$)	71 ($2,2 \times 10^{-3}$)	32 ($1,0 \times 10^{-3}$)
	Cl	31 ($8,7 \times 10^{-4}$)	31 ($8,7 \times 10^{-4}$)	334 ($9,4 \times 10^{-3}$)	212 ($6,0 \times 10^{-3}$)	212 ($6,0 \times 10^{-3}$)	212 ($6,0 \times 10^{-3}$)	97 ($2,7 \times 10^{-3}$)	72 ($2,0 \times 10^{-3}$)	80 ($2,2 \times 10^{-3}$)
	Na	67 ($2,9 \times 10^{-3}$)	67 ($2,9 \times 10^{-3}$)	198 ($8,6 \times 10^{-3}$)	-	-	-	-	25 ($1,1 \times 10^{-3}$)	-

NOTE: The nitrogen figures do not include contributions from the amino acids present in "8", "63", "59" and "KI".

Nitrogen is supplied in three forms: inorganic ammonium ions, as from ammonium nitrate, dihydrogen phosphate, sulphate and malate; nitrate ions, as from calcium, potassium and ammonium nitrate; and organic nitrogen compounds such as amino-acids, other amines and amides and more complex nitrogenous substances. A drift in pH during culture may often be ascribed to the absorption of NH_4^+ or NO_3^- with the consequent release of protons or anions into the medium. Variation on the nature and quantity of nitrogen used is clearly seen in Table 5. WHITE's (1943) medium has the low total N of $3,2 \times 10^{-3}$M while the MS medium is $3,9 \times 10^{-2}$M. Nitrate is consistently supplied at levels greater than that provided by the ammonium ion, which is sometimes considered inhibitory (MURASHIGE, 1973). The low nitrogen in WHITE's medium is offset in the "8" and "63" modifications with amine supplements. Phosphorus is employed in all formulations as the dihydrogen phosphate anion, $H_2PO_4^-$, supplied as its potassium, ammonium or sodium salt, and has usefulness in buffering capacity apart from being a source of the element. Medium "63" has the highest level of phosphorus, at $5,8 \times 10^{-3}$M but the use of the sodium salt provides the high cationic level of $8,6 \times 10^{-3}$M which may be disadvantageous. Potassium is supplied as the nitrate, the dihydrogen phosphate or the chloride, with levels from $1,7 \times 10^{-3}$M in WHITE's formulation to $2,5 \times 10^{-2}$M in both "SH" and "B5". Use of the chloride anion at $9,4 \times 10^{-3}$M in "63" may create adverse effects. Calcium is present from nitrate or chloride sources and varies from $1,0 \times 10^{-3}$M to $3,0 \times 10^{-3}$M. Magnesium is consistently supplied from Epsom salts and the quantities used yield the cation at between $7,6 \times 10^{-4}$M and 3.0×10^{-3}M.

A comparison of the various media shows that the MS formulation is most "nutrient-rich" with WHITE's medium at the other end of the scale. An interesting quantification of this is obtained in comparison of the total macronutrient levels; MS provides $9,3 \times 10^{-2}$M, SH has $6,3 \times 10^{-2}$M and WHITE's $1,7 \times 10^{-3}$M. A further comparison of general interest is that of the N:P:K ratios; MS has 50:1:17, SH provides 11:1:10 and WHITE's shows 32:1:17. Whilst this may be useful reference information, it is not appropriate to select any one medium as being superior to another on this basis; such decisions can be made only on the basis of experimental observations.

Micro-elements

The levels of micronutrients supplied in the various media described are shown in Table 6. Less variation is seen in both the element levels supplied and the range of source compounds used. Micro-elements are required by plants for specific structural purposes (e.g. metalloproteins or structural chelates), participation in redox reactions via electron transfer, and by the catalytic role of the transition metals. They may also serve as Lewis acids.

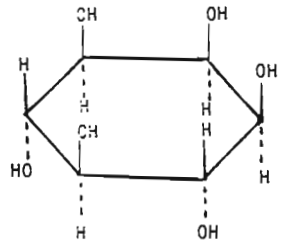
The iron level is variable and a range from $1,2 \times 10^{-5}$M to $1,0 \times 10^{-4}$M is seen. Older literature refers to the use of ferric citrate and ferric sulphate as iron sources but the more recent formulations consistently use ferrous sulphate with a stoichiometric equivalent of EDTA. It should be noted that some of this EDTA will inevitably 'migrate' in the prepared medium to those trace metals with higher complex-formation constants (Co, Cu and Zn) but, since these are present at much lower concentrations, the net loss is small. Use of the complexing reagent inhibits undesirable precipitation reactions in the medium.

Carbohydrates

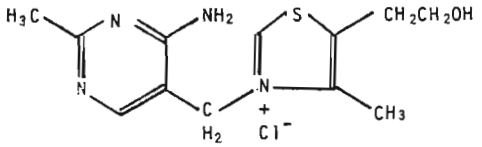
In all formulations quoted, carbon is supplied as sucrose at $5,8 \times 10^{-2}$M to $8,8 \times 10^{-2}$M (20-30 g l^{-1}). The sucrose serves the three-fold purpose of being a source of elemental carbon, providing an energy-rich substrate for the heterotrophic metabolism *in vitro*, and has the major influence on osmotic potential of the medium. The monosaccharides glucose, arabinose, mannose, ribose and xylose, and the hexol carbohydrate, sorbitol, have sometimes been used in media but without any apparent general advantage. The substance *myo*-inositol (1), one of the four naturally-occurring isomers of hexahydroxycyclohexane, has been used widely in media. Thought to be involved with the synthesis of phospholipids, cell wall pectins and cytoplasmic membranes, and a constituent of the vitamin B complex, it is often incorporated into media at levels from $5,6 \times 10^{-4}$M to $5,6 \times 10^{-3}$M (100-1000 mg l^{-1}).

TABLE 6 : MICRO-ELEMENTS PRESENT IN VARIOUS CULTURE MEDIA

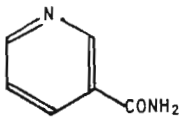
ELEMENT SUPPLIED	Formulation (refs. given in text, see also Tables 1 & 2); all figs. as mg per 1000 ml (Figures in parenthesis represent the corresponding molar concentrations)								
	White's basal	"8"	"63"	"59"	"KI"	"MS"	"SH"	"B5"	"NN-69"
Fe	0,70 ($1,2 \times 10^{-5}$)	0,70 ($1,2 \times 10^{-5}$)	1,67 ($3,0 \times 10^{-5}$)	5,58 ($1,0 \times 10^{-4}$)	5,58 ($1,0 \times 10^{-4}$)	5,58 ($1,0 \times 10^{-4}$)	3,01 ($5,4 \times 10^{-5}$)	5,58 ($1,0 \times 10^{-4}$)	5,58 ($1,0 \times 10^{-4}$)
Mn	1,65 ($3,0 \times 10^{-5}$)	1,65 ($3,0 \times 10^{-5}$)	0,74 ($1,3 \times 10^{-5}$)	5,50 ($1,0 \times 10^{-4}$)	5,50 ($1,0 \times 10^{-4}$)	5,50 ($1,0 \times 10^{-4}$)	2,91 ($5,8 \times 10^{-5}$)	2,91 ($5,8 \times 10^{-5}$)	6,16 ($1,1 \times 10^{-4}$)
Zn	0,61 ($9,4 \times 10^{-6}$)	0,61 ($9,4 \times 10^{-6}$)	0,11 ($1,7 \times 10^{-6}$)	1,95 ($3,0 \times 10^{-5}$)	1,95 ($3,0 \times 10^{-5}$)	1,95 ($3,0 \times 10^{-5}$)	0,23 ($3,5 \times 10^{-6}$)	0,45 ($7,0 \times 10^{-6}$)	2,27 ($3,5 \times 10^{-5}$)
B	0,27 ($2,4 \times 10^{-5}$)	0,27 ($2,4 \times 10^{-5}$)	0,09 ($8,0 \times 10^{-6}$)	1,10 ($1,0 \times 10^{-4}$)	1,10 ($1,0 \times 10^{-4}$)	1,10 ($1,0 \times 10^{-4}$)	0,89 ($8,1 \times 10^{-5}$)	0,53 ($4,8 \times 10^{-5}$)	1,77 ($1,6 \times 10^{-4}$)
I	0,57 ($4,5 \times 10^{-6}$)	0,57 ($4,5 \times 10^{-6}$)	–	0,63 ($5,0 \times 10^{-6}$)	0,63 ($5,0 \times 10^{-6}$)	0,63 ($5,0 \times 10^{-6}$)	0,76 ($6,0 \times 10^{-6}$)	0,57 ($4,5 \times 10^{-6}$)	–
Mo	–	–	0,010 ($1,0 \times 10^{-7}$)	0,10 ($1,0 \times 10^{-6}$)	0,10 ($1,0 \times 10^{-6}$)	0,10 ($1,0 \times 10^{-6}$)	0,040 ($4,1 \times 10^{-7}$)	0,10 ($1,0 \times 10^{-6}$)	0,10 ($1,0 \times 10^{-6}$)
Cu	–	–	0,006 ($1,0 \times 10^{-7}$)	0,006 ($1,0 \times 10^{-7}$)	0,006 ($1,0 \times 10^{-7}$)	0,006 ($1,0 \times 10^{-7}$)	0,051 ($8,0 \times 10^{-7}$)	0,006 ($1,0 \times 10^{-7}$)	0,006 ($1,0 \times 10^{-7}$)
Co	–	–	0,006 ($1,0 \times 10^{-7}$)	0,006 ($1,0 \times 10^{-7}$)	0,006 ($1,0 \times 10^{-7}$)	0,006 ($1,0 \times 10^{-7}$)	0,025 ($4,2 \times 10^{-7}$)	0,006 ($1,0 \times 10^{-7}$)	–



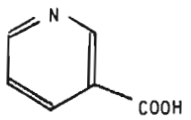
(1) MYO-INOSITOL



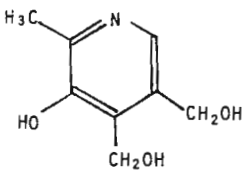
(2) THIAMINE



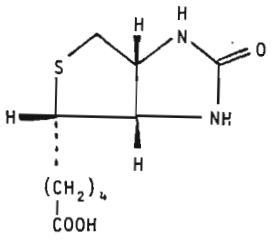
(3) NICOTINAMIDE



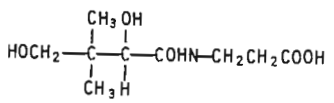
(4) NICOTINIC ACID



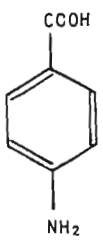
(5) PYRIDOXIDE



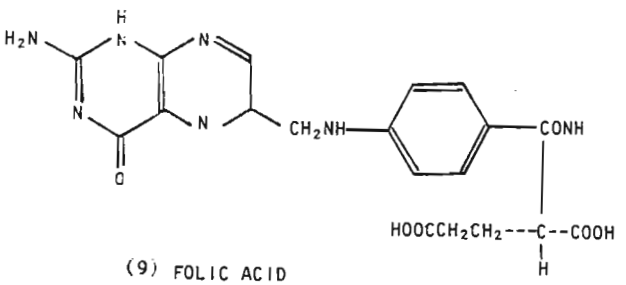
(6) BIOTIN



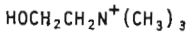
(7) PANTOTHENIC ACID



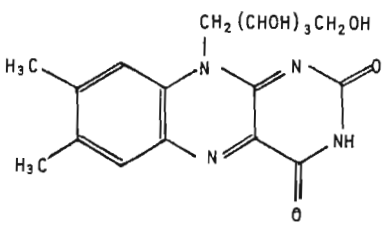
(8) PARA-AMINOBENZOIC ACID



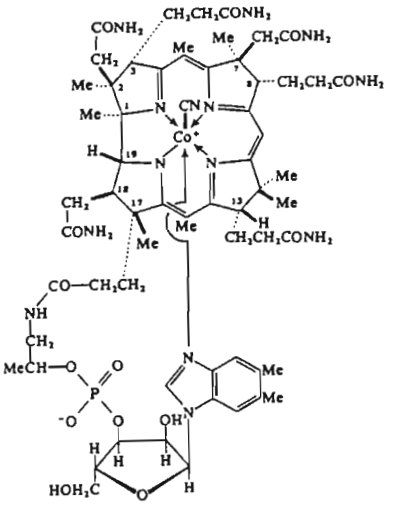
(9) FOLIC ACID



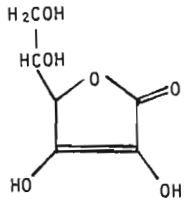
(10) CHOLINE



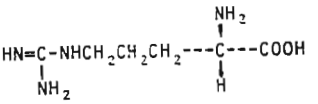
(11) RIBOFLAVINE



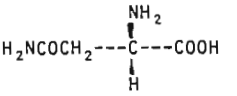
(12) CYANOCOBALAMIN



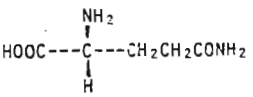
(13) L-ASCORBIC ACID



(14) L-ARGININE

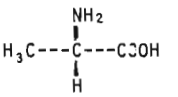


(15) L-ASPARAGINE

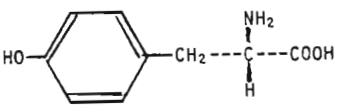


(16) L-GLUTAMINE

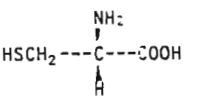
(17) GLYCINE



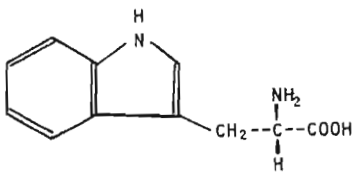
(18) L-ALANINE



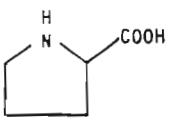
(19) L-TYROSINE



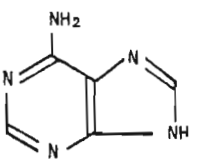
(20) L-CYSTEINE



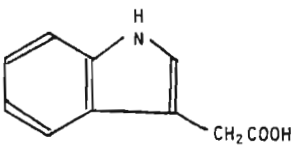
(21) L-TRYPTOPHANE



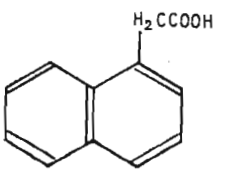
(22) L-PROLINE



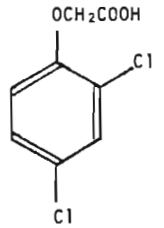
(23) ADENINE



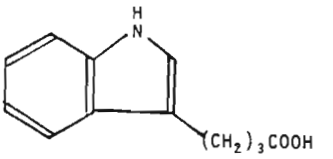
(24) INDOLE-3-ACETIC ACID
IAA



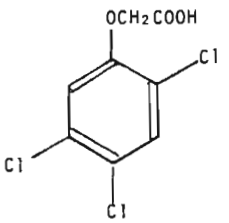
(25) 1-NAPHTHALENEACETIC ACID
NAA



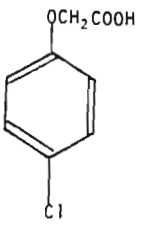
(26) 2,4-DICHLOROPHENOXYACETIC ACID
2,4-D



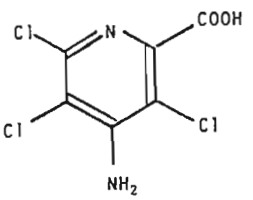
(27) INDOLE-3-BUTYRIC ACID
IBA



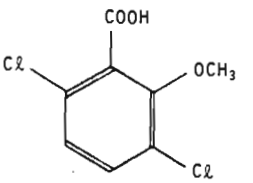
(28) 2,4,5-TRICHLOROPHENOXYACETIC ACID
2,4,5-T



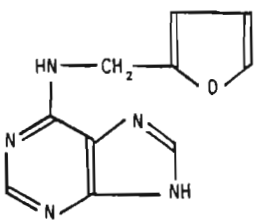
(29) 4-CHLOROPHENOXYACETIC ACID
CPA



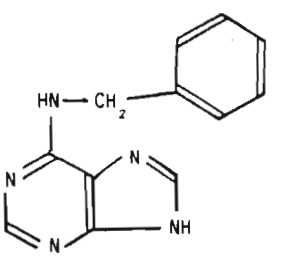
(30) 4-AMINO-3,5,6-TRICHLOROPICOLINIC ACID
PICLORAM



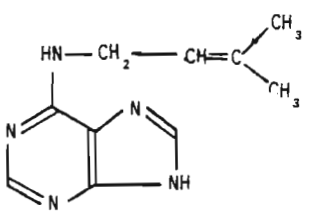
(31) 3,6-DICHLORO-O-ANISIC ACID
(DICAMBA)



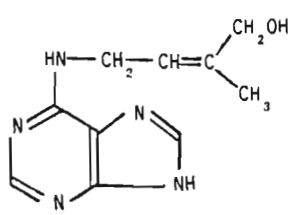
(32) KINETIN, KN



(33) 6-BENZYLADENINE, BA



(34) ISO-PENENYLADENINE, 2iP, IPA



(35) ZEATIN

Vitamins

In their work with tobacco callus culture, LINSMAIER and SKOOG (1965) demonstrated that many water-soluble vitamins are *not* essential; indeed some of these compounds are growth inhibitors. One of the more important plant vitamins is thiamine (2) (vitamin B_1) which is involved with the co-enzyme cocarboxylase, essential in the KREBS cycle, and also with the transketolase enzyme in the pentose shunt pathway in carbohydrate metabolism. Thiamine is generally supplied as the hydrochloride, at levels from 3×10^{-7}M to 3×10^{-5}M. The usefulness of other B-group vitamins remains equivocal and synergistic interactions further complicate matters. Despite this, most formulations include nicotinamide (3) (niacin) or nicotinic acid (4) which are important co-enzymes in biological dehydrogenase reactions, and pyridoxine (5) (one of the vitamins B_6), supplied as the hydrochloride, and which is an essential co-enzyme in amino acid metabolism. These three components may be incorporated at levels from 10^{-7}M to 10^{-5}M. Less commonly used are biotin (6) (vitamin H), pantothenic acid (7), as its calcium salt, and p-aminobenzoic acid (8). The latter is a part of the structure of folic acid (9), itself occasionally used in culture media. Choline (10) is another constituent of the vitamin B complex and occasionally used in its chloride form. Riboflavin (11) (vitamin B_2) is rarely used in view of its possibly inhibitory effects (LINSMAIER and SKOOG, 1965). Finally, among the B-group vitamins, the unusual cobalt-containing natural product, cyanocobalamin (12) (vitamin B_{12}), has infrequently been added to media at very low concentrations.

Among the other vitamins it is only vitamin C that has an application in *in vitro* culture. A commonly-observed event in tissue cultures is the progressive darkening of explants and callus, due to secretion and oxidation of phenolic substances. The oxidation products are often toxic to the tissue and severe inhibition or necrosis usually results. The phenolic metabolites also appear to inactive auxins with obvious consequences. Ascorbic acid (13) (vitamin C), especially in combination with citric acid, may often alleviate this problem (MURASHIGE, 1974). Various other natural and synthetic anti-oxidants have been used for the same purpose. Activated charcoal has also been utilised, but this material is not specific in its adsorption properties and may remove essential growth factors from the medium in addition

to the toxic metabolites. There is no panacea to the phenolic problem which continues to plague many workers, especially those dealing with woody explant material.

Supplementary organic compounds

Many of the earlier media contained organic supplements in the form of materials such as coconut milk, yeast extract, casein hydrolysate, tomato juice, orange juice, banana puree and other more exotic mixtures. Useful as they may have been, these supplements result in undefined media with consequent difficulties in exact reproducibility. Largely as the result of isolation and identification of the active constituents in such mixtures, current media are more fully defined; the previously-used extracts, juices and suspensions have been replaced by specific amino acid mixtures and other organic compounds.

Amino acids and amides have been shown to be beneficial in many cases. Perhaps the most frequently-used amino acids and amides are arginine (14). asparagine (15), glutamine (16) and glycine (17) (MURASHIGE, 1973). The first two of these are reported to be stimulatory in gymnosperms like *Picea abies* (STEINHART, STANDIFER and SKOOG, 1961). Other amino acids sometimes used are alanine (18), tyrosine (19), cysteine (20), tryptophane (21) and proline (22). Only the L-isomers of the optically-active compounds are effective. The D-form is usually without effect and sometimes negates the value of the L-isomer when racemic mixtures are used (MURASHIGE, 1974). Amino acid supplementation must be done with caution as these compounds can be quantitatively and qualitatively inhibitory to some tissues. The purine, adenine (23), is very commonly added to media, usually in the form of its sulphate. Adenine, vital in nucleotide biosynthesis, appears to be more necessary in morphogenesis stages than in callus initiation or proliferation (MURASHIGE, 1974). Despite the voluminous literature on vitamins and other organic supplements, their use remains disputed. LETHAM (1967) claimed that if the appropriate cytokinin was used, the only organic compounds necessary would be sucrose, inositol and an auxin, with thiamine as a 'desirable extra'.

Growth regulating substances

The selection of the growth regulating auxins and cytokinins is one of the most critical aspects of any plant tissue culture project. Different phytohormones bring about different responses, concentrations must be within specific limits and synergistic and antagonistic interactions add to the complexity. Where large explants are used, or where an active meristem is included in the explant, there may be sufficient endogenous phytohormones for sustained growth. When smaller explants are used, or when specific morphogenetic responses are sought, then an exogenous supply becomes essential. The classical experimentation of SKOOG and MILLER (1957) demonstrated clearly that an auxin-cytokinin interaction critically controls morphogenesis. In general, relatively high concentrations of cytokinin favour shoot initiation and repress rooting while high auxin levels stimulate rooting and inhibit shooting. In development of the MS medium, MURASHIGE and SKOOG (1962) used auxin and cytokinin at 2,0 and 0,2 mg l^{-1} for callus cultures and increased these to 4,0 and 2,5 for shoot differentiation. Rooting was induced by transfer to a medium without phytohormones. Manipulation of the auxin-cytokinin ratio to achieve the desired growth response is now standard technique in *in vitro* work. It is also noted that well-established callus cultures sometimes undergo a spontaneous change manifested by a lack of dependence on further exogenous hormone supply, such cultures being described as habituated.

Among the auxins used in tissue cultures are the naturally-occurring compound indole-3-acetic acid (24) (IAA) and a large number of related synthetic substances such as 1-napthaleneacetic acid (25) (NAA), 2,4-dichlorophenoxyacetic acid (26) (2,4-D), indole-3-butyric acid (27) (BA), 2,4,5-trichlorophenoxyacetic acid (28) (2,4,5-T), 4-chlorophenoxyacetic acid (29) (CPA), 4-amino 3,5,6-trichloropicolinic acid (30) ('Picloram') and 3,6-dichloro-o-anisic acid (31) ('Dicamba'). Of these the first three are most commonly used, 2,4-D and NAA being preferred to IAA on account of better stability. The cytokinins are all derivatives of adenine (aminopurine) and include 6-furfurylaminopurine (32) (kinetin, KN), 6-benzylaminopurine (33) (BA), 6-γ-γ-dimethylaminopurine (34) (isopententyladenine, 2iP) and the naturally-occurring 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine (35) (zeatin, Z). Other plant

regulating substances are ethylene, abscisic acid and the gibberellins which are less commonly used for *in vitro* work. Of the latter group, the most important compound used is gibberellic acid (GA_3).

On the basis of the information summarized in the preceeding paragraphs, it was felt that the media of SCHENK and HILDEBRANT (1972) (SH) and medium 59 of NORSTOG and RHAMSTINE (1967) provided a useful starting point. Initial experimentation showed these media to be generally satisfactory for both haploid and diploid cycad material and hence an elaborate multi-factorial experiment in optimising the level of each component was not considered necessary.

Preparation of media

All media in this project were prepared freshly in one-litre batches. The pH was adjusted to 5,8 by addition of 0,2 M KOH. Bacteriological grade agar (BioLab) was used at 7,5 g ℓ^{-1} as the solidifying agent. Media were dispensed into the appropriate containers and sterilized by autoclaving at 103,4 k Pa (121°C) for 20 minutes.

The pH of the medium is important insofar as solubility and agar-gelling properties are concerned, quite apart from its effect on tissue growth and development. At a pH of 4,0 the agar fails to gel while at a pH of 7,0 incipient precipitation of the least soluble salts (calcium sulphate, K_{sp} 2,45 x 10^{-5}, and magnesium ammonium phosphate, K_{sp} 2,5 x 10^{-13}; both at 25°C) commences. The effect of autoclaving and storage on pH-adjusted SH media is shown in Table 7.

TABLE 7 : The effect of autoclaving and short storage periods on the pH value of pH-adjusted SH media.

Initial pH value	pH value immediatedly after autoclaving	pH value one week after autoclaving
4,0	4,6	4,6
5,0	5,0	5,0
6,0	5,8	5,8
7,0	6,7	6,6

It is evident that at pH values less than 5, the pH drift is upwards while at pH values greater than 5, the drift is downwards. Further storage does not greatly affect pH values. From the above results it appears reasonable to continue the practice of adjusting pH to 5,8.

2.4.5 Choice of culture containers

A wide variety of differently-shaped glass and plastic containers is available for use in plant tissue culture and insufficient attention is often paid to the selection of container and closure. Minor influences on *in vitro* responses could arise from the chemical nature of the container, usually borosilicate glass or polycarbonate plastic, and from the volume and surface area of the medium in relation to headspace volumes. The major effect appears to be associated with the relative rates of gaseous exchange between the internal headspace and the external atmosphere. In containers with restricted gas exchange it may be anticipated that 100% relative humidity is maintained, that oxygen depletion may occur and that carbon dioxide ethylene and other volatile substances may accumulate. These circumstances may lead to growth inhibition; in general an hermetically sealed container is more detrimental to culture growth than a container allowing gaseous exchange (DE PROFT, MAENE and DEBERGH, 1985).

In order to test the effect of the container on the growth of haploid and diploid cycad tissue *in vitro*, a series of 69 explants of megagametophytic tissue and 48 cubes of primary root tissue from *Encephalartos natalensis* were grown in SH medium supplemented with $4,5 \times 10^{-6}$M 2,4-D and kinetin in three different types of containers. The response, in terms of callogenesis within 50 days, is shown in Table 8.

Inspection of these results shows that the frequency of callus formation is clearly related to the type of container. The best response is shown by cultures in Erlenmeyer flasks with cotton-wool closures, hence this type of container was used wherever possible in subsequent experimentation.

TABLE 8 : The effect of container on callus formation from megagametophyte and primary root explants of *Encephalartos natalensis*.

(A) Megagametophyte explants

Culture vessel	Closure	Number of explants	% showing callus within 50 days
Universal bottles, 28mls.	Rubber seal and screw cap	23	0%
Tissue culture tubes, 40mls.	"Magenta" ribbed plastic closures; "Parafilm" seal	23	26%
Erlenmeyer flasks, 25mls.	Cotton wool bung, foil cap and masking tape seal	23	70%

(B) Primary root explants

Culture vessel	Closure	Number of explants	% showing callus within 50 days
Tissue culture tubes, 40mls.	"Magenta" ribbed plastic closures; "Parafilm" seal	24	58%
Erlenmeyer flasks, 25mls.	Cotton wool bung, foil cap and masking tape seal	24	71%

2.4.6 Choice of culture conditions

Cultures on media containing sucrose should bypass the need for light; many species are routinely cultured in the dark. However, light does play a role in inducing morphogenesis. MURASHIGE (1974) reviewed light requirements in terms of photoperiodic, intensity and spectral quality parameters. He makes a general recommendation of continuous 100 $\mu E\ m^{-2}s^{-1}$ fluorescent light for establishment and maintenance stages followed by an increase in intensity to 300 to 1000 $\mu E\ m^{-2}s^{-1}$ for morphogenesis. It is noted in this respect that the daylight quantum flux measurement at solar noon is typically about 2000 $\mu E\ m^{-2}s^{-1}$ (Lambda Instrument Corporation, Brochure B-777).

Tissue culture experiments are usually kept at constant controlled temperatures in the 20-28°C range but, where controlled temperature facilities are not available, ambient temperatures often suffice. A diurnal temperature cycle may be used, especially in association with controlled photoperiods in growth cabinets. An incidental advantage of a controlled fluctuating temperature cycle is the effect in promoting a more rapid gaseous exchange.

In this project, unless otherwise stated, cultures were initiated in the dark at 25 ± 2°C and then transferred after 2-3 weeks to a controlled environment cabinet. The latter was held at 25 ± 2°C under either continuous light or under a 16/8 hour photoperiodic cycle. The light was provided by a combined bank of fluorescent tubes and incandescent bulbs at mean intensities from 30 to 100 uE $m^{-2}s^{-1}$.

2.4.7 Cytological examination

Since explants from well-defined haploid and diploid cycad tissues were used in this project, it was considered adviseable to monitor the chromosomal status of cultures. Small segments of tissue were removed from the bulk of the material and fixed overnight in Carnoy's fluid (ethanol : chloroform : acetic acid :: 6:3:1). After rinsing in distilled water, the samples were softened in 1M hydrochloric acid for 2,5 hours at room temperature and rinsed well with water. Small sub-samples were squashed in 1% aceto-orcein in glacial acetic acid for 30 minutes on a glass slide and the preparation carefully flattened under a cover slip. After visual microscopic examination, representative groups of metaphase chromosomes were counted and photographed using a Zeiss compound photomicroscope with Kodak Pan 2415 technical film which was subsequently processed with D163 developer (1:3) for 5 minutes at 20°C.

2.5 Experiments with haploid material

2.5.1 Megagametophyte cultures

Segments from megagametophytes from *Stangeria eriopus* and 4 species of *Encephalartos* were subjected to a variety of pre-treatments and cultured

on solid media with different growth factor supplements under different environmental conditions. The results obtained are summarized in Tables 9, 10 and 11.

With *Stangeria eriopus* (Table 9), vigorous growth of a soft, white friable callus was readily obtained in 25-30 days (Plate 3). No significant difference was found in the performance of longitudinally or transversely bisected megagametophytes or 5mm cubes of the internal tissue. The selection of the container had a major effect on callogenesis, the explants in Universal bottles giving a consistently poorer response than those in tissue culture tubes or Erlenmeyer flasks (Section 2.4.5 and Table 8). The presence, absence or intensity of light did not affect callus formation, but the original explants in light conditions became green and presumably photosynthetic. Cytological examination confirmed a consistent n = 8 haploid chromosome number (Plates 4a & b) in the callus material. Culture of the explants on a medium containing 0,02% colchicine somewhat inhibited the rate of growth and resulted in the appearance of cells with diploid, tetraploid and octaploid chromosome complements. The material could not tolerate the higher concentration of 0,05% colchicine and became necrotic.

The megagametophyte-derived callus from *Stangeria eriopus* could be maintained in subculture over several passages at 3-4 week intervals. Results of several experimenta in this regard are summarized in Table 10. In general, callus growth continued and was unaffected by the strength or nature of the medium employed and the presence, absence or nature of growth factor supplements. However, in six explants the presence of a number of small, compact "spherical structures" was seen (Plate 5a). These structures attained about 5mm in diameter. A transverse section revealed a central parenchymatous zone within a cambial layer surrounded by peripheral cork cells (Plate 5b). There was no evidence of any vascular development. These structures appear to be analogous to the coralloid root nodules reported to have arisen in several other cycad megagametophyte culture experiments (DE LUCA & SABATO, 1980: DE LUCA, SABATO, BALDUZZI & NAZZARO, 1980) and also in cycad seedling and embryo cultures, particularly at low to medium light intensities (WEBB, 1981, 1982b, 1983, 1984: WEBB & DE JESUS, 1982: WEBB, NEVAREZ & DE JESUS, 1984) (Section 2.3). No further differentiation was observed, either on the

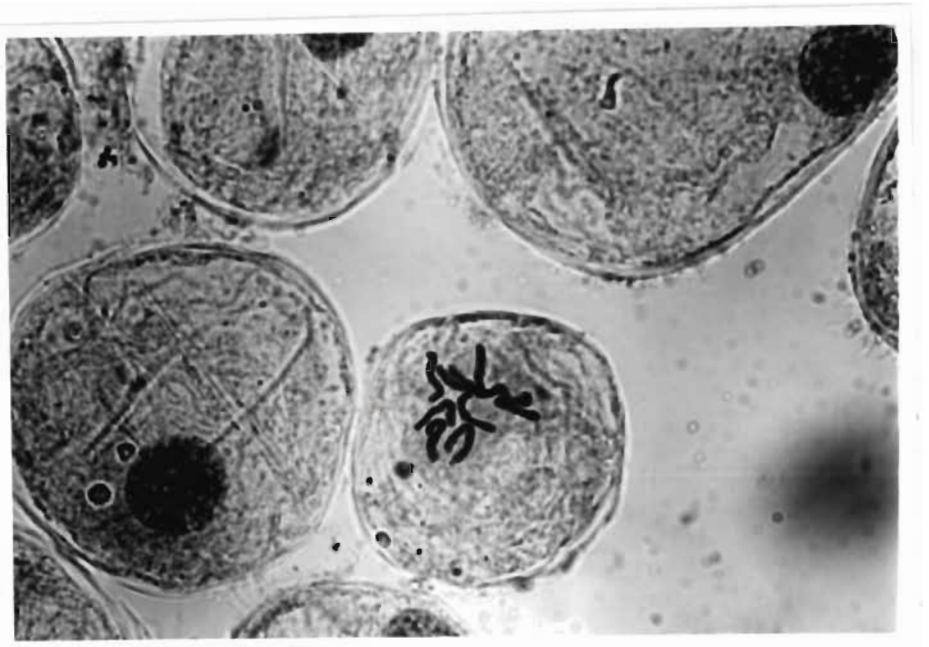
TABLE 9 : *In vitro* culture of megagametophyte explants from *Stangeria eriopus*

Explant	Number of explants	Culture medium	Container	Light regime	Observations
5mm cubes	13	SH, $4,5x10^{-6}$M 2,4-D and KN	50ml Erlenmeyers	Dark	Vigorous callus growth in 25-30 days
Transverse ½-megas.	6	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Vigorous callus growth in 25-30 days
Transverse ½-megas.	6	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	100µE $m^{-2}s^{-1}$, constant	Greening of explant, vigorous callus growth
Transverse ½-megas.	12	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Vigorous callus in 25 days = n = 8
Transverse ½-megas.	12	as above + 0,02% colchicine	25ml Erlenmeyers	Dark	Less vigorous callus, n = 8, 16, 32, 64.
Transverse ½-megas.	12	as above + 0,05% colchicine	25ml Erlenmeyers	Dark	All cultures necrotic
Longitudinal ½-megas.	12	SH, $4,5+10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Vigorous callus growth in 25-30 days
Longitudinal ½-megas.	5	59, no growth factors	Universal bottles	Dark	Limited callus growth in 30 days
Longitudinal ½-megas.	5	59, $1,4x10^{-6}$ 2,4-D; $1,4x10^{-5}$ KN	Universal bottles	Dark	Moderate callus growth in 30 days
Longitudinal ½-megas.	5	59, $1,4x10^{-6}$ IAA; $1,4x10^{-5}$ 2iP	Universal bottles	Dark	Moderate callus growth in 30 days
Longitudinal ½-megas.	5	59, no growth factors	Universal bottles	30µE$m^{-2}s^{-1}$, constant	Greening of explant, moderate callus growth
Longitudinal ½-megas.	5	59, $1,4x10^{-6}$ 2,4-D; $1,4x10^{-5}$ KN	Universal bottles	30 E$m^{-2}s^{-1}$, constant	Greening of explant, moderate callus growth

Notes: SH, medium of SCHENK and HILDEBRANDT (1972); 59, medium of NORSTOG and RHAMSTINE (1967); 2,4-D, 2,4-dichlorophenoxyacetic acid; KN, kinetin; IAA, indole-3-acetic acid; 2iP, isopentenyladenine; n = chromosome number observed in cytological examination; megas., megagametophytes.

PLATE 3

Prolific crumbly white callus growth on an explant of megagametophytic tissue from *Stangeria eriopus* seed after 42 days on SH medium supplemented with $4,5 \times 10^{-6}$M 2,4-D and kinetin and grown in the dark at ambient temperatures (22-25°C). The explant was taken from the micropylar end of the megagametophyte as evidenced by the dark area of the micropylar orifice. Scale × 2.



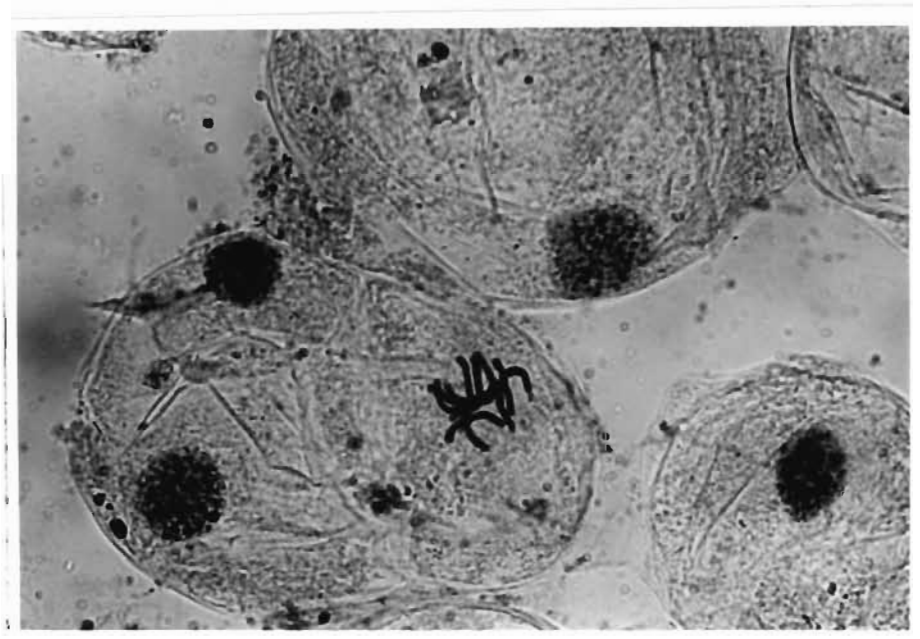


PLATE 4a & b

Photomicrographs of chromosomes from callus tissue cultured from *Stangeria eriopus* megagametophytic tissue. Squash preparation with aceto-orcein stain. The haploid (n = 8) chromosome number is evident in both early (upper picture) and late (lower picture) metaphase stages. Scale x 400.

TABLE 10 : Subculture of megagametophyte-derived callus from *Stangeria eriopus*

Number of explants	Culture medium	Container	Light regime	Observations
11	SH, no growth factors	25ml Erlenmeyers	$50\mu Em^{-2}s^{-1}$, 12 hr photoperiod	Continued callus growth; "spherical structures" on one explant in 60 days
6	½-strength SH, no growth factors	25ml Erlenmeyers	$50\mu Em^{-2}s^{-1}$, 12 hr photoperiod	Continued callus growth
5	SH, 5-hour pre-treatment of explant with $4,5x10^{-4}$M BA	25ml Erlenmeyers	$50\mu Em^{-2}s^{-1}$, 12 hr photoperiod	Continued callus growth; "spherical structures" on one explant in 60 days
5	SH, 5-hour pre-treatment of explant with $3x10^{-4}$M GA_3	25ml Erlenmeyers	$50\mu Em^{-2}s^{-1}$, 12 hr photoperiod	Continued callus growth; "spherical structures" on two explants in 60 days
5	SH, $4,5x10^{-6}$M BA	25ml Erlenmeyers	$50\mu Em^{-2}s^{-1}$, 12 hr photoperiod	Continued callus growth; "spherical structures" on two explants in 60 days
5	SH, $4,5x10^{-5}$M BA	25ml Erlenmeyers	$50\mu Em^{-2}s^{-1}$, 12 hr photoperiod	Continued callus growth
5	SH, $3x10^{-6}$ M GA_3	25ml Erlenmeyers	$50\mu Em^{-2}s^{-1}$, 12 hr photoperiod	Continued callus growth
5	SH, $3x10^{-5}$ M GA_3	25ml Erlenmeyers	$50\mu Em^{-2}s^{-1}$, 12 hr photoperiod	Continued callus growth
7	59, 0,1% activated charcoal	50ml Erlenmeyers	$50\mu Em^{-2}s^{-1}$, 12 hr photoperiod	Continued callus growth
6	59, No growth factors	50ml Erlenmeyers	$30\mu Em^{-2}s^{-1}$, constant	Continued callus growth
6	59, $1x10^{-5}$M 2iP	50ml Erlenmeyers	Dark	Continued callus growth

Notes: SH, medium of SCHENK and HILDEBRANDT (1972); 59, medium of NORSTOG and RHAMSTINE (1967); BA, 6-benzyladenine; GA_3, gibberellic acid; 2iP, isopentenyladenine

PLATE 5a : Megagametophytic tissue of *Stangeria eriopus* showing the development of spherical structures analogous to coralloid root nodules. Scale x 3.

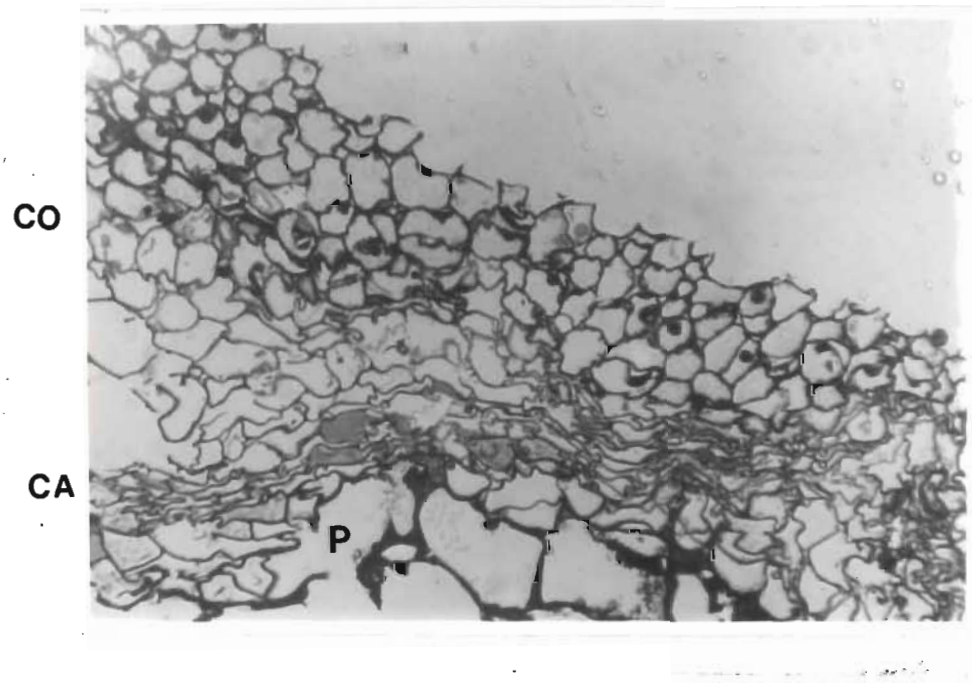


PLATE 5b : Transverse section through the outermost tissues from the above structure. A cambial zone (CA) separates the outer cork layer (CO) from the internal parenchyma (P). Toluidine blue stain. Scale x 200.

spherical structures or the remaining tissues.

Results with cultures from *Encephalartos* megagametophytes are recorded in Table 11. No significant difference was noticed with respect to the two different basal media employed, but the growth factor supplements did appear to influence callus production. Combinations of 10^{-6} and 10^{-7}M 2,4-D and kinetin, and NAA and 6-benzyladenine, generally resulted in vigorous callus growth in 40-50 days. Indoleacetic acid and isopentenyl-adenine supplements did normally not promote callogenesis. In the various pre-treatment tests, the solvent dimethylsulphoxide appears to be a useful vehicle to facilitate hormone absorption by the explant (BLAKELY, BLAKELY and GALLOWAY, 1986); the solvent itself having no deleterious affect. In these pre-treatment experiments the two auxins 2,4-D and NAA promoted callogenesis, while the auxin IAA and the three cytokinins tested did not result in callus growth. Tests to evaluate the performance with different levels of sucrose showed that the levels of 2 - 5% were satisfactory, but that an increase to 10% inhibited callus formation. As found with the *Stangeria* explants, the use of universal bottles lead to unsatisfactory results. In the evaluation of light influences, it appears that the presence, absence or intensity of light does not affect callus production, but does cause the original explant to become photosynthetic. The addition of colchicine seems to inhibit the rate of callus growth, but unlike the situation in *Stangeria*, did not result in any multiplicities of chromosome number. There was no evidence of any type of differentiation or morphogenesis in the *Encephalartos* megagametophyte cultures.

2.5.2 Microgametophyte cultures

An initial attempt at establishing *in vitro* cultures of cycad microspores was carried out using pollen collected, at the time of dehiscence, from the male cones of *Encephalartos caffer*. After disinfection by sequential treatment with alcohol, hypochlorite and sterile distilled water, the pollen, in suspension in sterile distilled water, was innoculated onto solid SH media supplemented with various combinations of 2,4-D, NAA, kinetin and 6-benzyladenine. Cultures were incubated at 25°C in both light and dark conditions. No evidence of any development was seen

TABLE 11 : *In vitro* culture of megagametophyte explants from various *Encephalartos* spp.

Species	Explant	No. of explants	Culture medium	Container	Light regime	Observations
E. natalensis	¼-megas.	23	SH, $4{,}5\times10^{-6}$M 2,4-D & KN	Universal bottles	Dark	No response in 50 days
E. natalensis	¼-megas.	23	SH, $4{,}5\times10^{-6}$M 2,4-D & KN	Tissue culture tubes	Dark	26% of explants with callus in 50 days
E. natalensis	¼-megas.	23	SH, $4{,}5\times10^{-6}$M 2,4-D & KN	25ml Erlenmeyers	Dark	70% of explants with callus in 50 days
E. natalensis	Transverse ½-megas	6	59, 24-hr pretreatment in 5% DMSO	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening within 40 days
E. natalensis	Transverse ½-megas	6	59, 24-hr pretreatment in 5% DMSO with 3×10^{-4}M IAA	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening within 40 days
E. natalensis	Transverse ½-megas	6	59, 24-hr pretreatment in 5% DMSO with 2×10^{-4}M 2,4-D	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening, with callus on all explants within 40 days
E. natalensis	Transverse ½-megas.	6	59, 24-hr pretreatment in 5% DMSO with 3×10^{-4}M NAA	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening, with callus on all explants within 40 days
E. natalensis	Transverse ½-megas.	6	59, 24-hr pretreatment in 5% DMSO with 2×10^{-4} KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening within 40 days
E. natalensis	Transverse ½-megas.	6	59, 24-hr pretreatment in 5% DMSO with 2×10^{-4} BA	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening within 40 days
E. natalensis	Transverse ½-megas.	6	59, 24-hr pretreatment in 5% DMSO with 2×10^{-4} 2iP	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening within 40 days
E. natalensis	Transverse ½-megas.	6	59, $4{,}5\times10^{-6}$M 2,4-D & KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening, with callus on all explants within 40 days

TABLE 11 - continued

Species	Explant	No. of explants	Culture medium	Container	Light regime	Observations
E. natalensis	Transverse ½-megas.	6	59, $4,5x10^{-6}$M 2,4-D & KN sucrose level raised to 5%	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening, with callus on all explants within 40 days
E. natalensis	Transverse ½-megas.	6	59, $4,5x10^{-6}$M 2,4-D & KN sucrose level raised to 10%	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening within 40 days
E. natalensis	Transverse ½-megas.	6	59, $4,5x10^{-6}$M IAA & 2iP	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening within 40 days
E. natalensis	Transverse ½-megas.	6	59, $4,5x10^{-6}$M IAA and 2iP sucrose level raised to 5%	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening, callus on 2 explants within 40 days
E. natalensis	Transverse ½-megas.	6	59, $4,5x10^{-6}$M IAA & 2iP sucrose level raised to 10%	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening within 40 days
E. natalensis	Transverse ½-megas.	6	59, $4,5x10^{-6}$M 2,4-D and $4,5x10^{-7}$M KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening, callus on 3 explants within 40 days
E. natalensis	Transverse ½-megas.	6	59, $4,5x10^{-7}$M 2,4D and $4,5x10^{-6}$M KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening, callus on all explants within 40 days
E. natalensis	Transverse ½-megas.	6	59, $4,5x10^{-6}$M NAA & BA	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening, callus on all explants within 40 days
E. natalensis	Transverse ½-megas.	6	59, $4,5x10^{-6}$M NAA and $4,5x10^{-7}$M BA	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening, callus on all explants within 40 days
E. natalensis	Transverse ½-megas.	6	59, $4,5x10^{-7}$M NAA and $4,5x10^{-6}$M BA	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening, callus on 3 explants within 40 days
E. natalensis	Transverse ½-megas.	6	59, $4,5x10^{-6}$M IAA and $4,5x10^{-7}$M 2iP	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening within 40 days
E. natalensis	Transverse ½-megas.	6	59, $4,5x10^{-7}$M IAA and $4,5x10^{-6}$M 2iP	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Greening within 40 days

TABLE 11 - continued

Species	Explant	No. of explants	Culture medium	Container	Light regime	Observations
E. villosus	Longitudinal ½-megas.	30	59, various supplements	Universal bottles	Light and dark conditions	All explants become necrotic
E. lebombo-ensis	½-megas.	90	SH, various pretreatments and various supplements	Universal bottles	Dark	Sporadic callus formation in some cultures in 50 days
E. pterogonus	Transverse ½-megas.	10	59, $4,5 \times 10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100 \mu Em^{-2}s^{-1}$ constant	Vigorous callus in 40 days n = 9
E. pterogonus	Transverse ½-megas.	10	as above + 0,01% colchicine	Tissue culture tubes	$100 \mu Em^{-2}s^{-1}$ constant	Moderate callus in 40 days n = 9
E. pterogonus	Transverse ½-megas.	10	as above + 0,02% colchicine	Tissue culture tubes	$100 \mu Em^{-2}s^{-1}$ constant	Slight callus in 40 days n = 9

Notes: SH, medium of SCHENK and HILDEBRANDT (1972); 59, medium of NORSTOG and RHAMSTINE (1967);
2,4-D, 2,4-dichlorophenoxyacetic acid; KN, kinetin; DMSO, dimethylsulphoxide;
IAA, indole-3-acetic acid; NAA, 1-naphthaleneacetic acid; BA, 6-benzyladenine;
2iP, isopentenyladenine.

over a 3-month period; microscopic examination showed the presence of intact, ungerminated, microspores.

Although TULECKE (1957) managed to induce proliferation and callus development in pollen cultures from *Ginkgo biloba* L., most workers have had difficulties using pollen grains for *in vitro* cultures. It may be that pollen material is susceptible to necrosis during the disinfestation treatment (LA RUE, 1954). Most current work on haploid male tissue culture from angiospermous material has favoured the use of whole anthers for the induction of androgenesis (REINERT and BAJAJ, 1977). By analogy, in cycads, results have been reported from microsporangia rather than microspores. (DE LUCA and SABATO, 1979; DE LUCA, LA VALVA and SABATO, 1980). Although in their work the focus of attention has been on development of pollen tubes, nuclear divisions and spermatogenesis, callus initiation was observed in at least two cases from *Ceratozamia mexicana* when the medium was supplemented with 2,4-D. Thus attempts were made to establish microspore cultures using unopened microsporangia rather than the pollen collected after dehiscence.

In the case of *Encephalartos ferox* and *E. woodii*, the unopened microsporangia were collected from male cones at a period approximately one week prior to dehiscence. The microsporangia were disinfected by treatment with alcohol and hypochlorite solutions and innoculated onto various media as indicated in Table 12. Microscopic examination of the material showed that, at the time of collection and innoculation, most of the spores were in a mononucleate condition (Plate 6a); a small number were in the more advanced two-celled stage with the small prothallial cell separated from the larger antheridial cell. All cultures responded by showing germination to give a mass of interwoven pollen tubes within 2-3 weeks, the culture having an almost callus-like appearance on superficial inspection. Microscopic examination showed the pollen tubes to be variously extended in length and width, but generally unbranched, with granular cytoplasmic contents (Plate 6b). A large number of the microspores had not germinated but had developed to the three-celled stage.

Subculture of the above pollen-tube masses allowed continued extension. No evidence of spermatogenesis or any other form of morphogenesis was observed.

TABLE 12 : *In vitro* culture of cycad microgametophytes

Species	Number of cultures	Culture medium	Container	Light regime	Observations
E. ferox	4	SH, no growth factors	Universal bottles	Dark	Germination of microspores and development of mass of pollen tubes in 2-3 weeks
E. ferox	4	SH, $4{,}5\times10^{-6}$M 2,4-D and KN	Universal bottles	Dark	
E. ferox	4	SH, $4{,}5\times10^{-5}$M NAA	Universal bottles	Dark	
E. ferox	4	½-strength SH	Universal bottles	Dark	
E. woodii	19	SH, $4{,}5\times10^{-6}$M 2,4-D and KN	Universal bottles	Dark	
subcultures from *E. woodii*	9	59, 0,1% activated charcoal	Universal bottles	$100\mu Em^{-2}s^{-1}$ constant	Continued growth of pollen tubes

Notes: SH, medium of SCHENK and HILDEBRANDT (1972); 59, medium of NORSTOG and RHAMSTINE (1967);
2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, 1-naphthaleneacetic acid;
KN, kinetin.

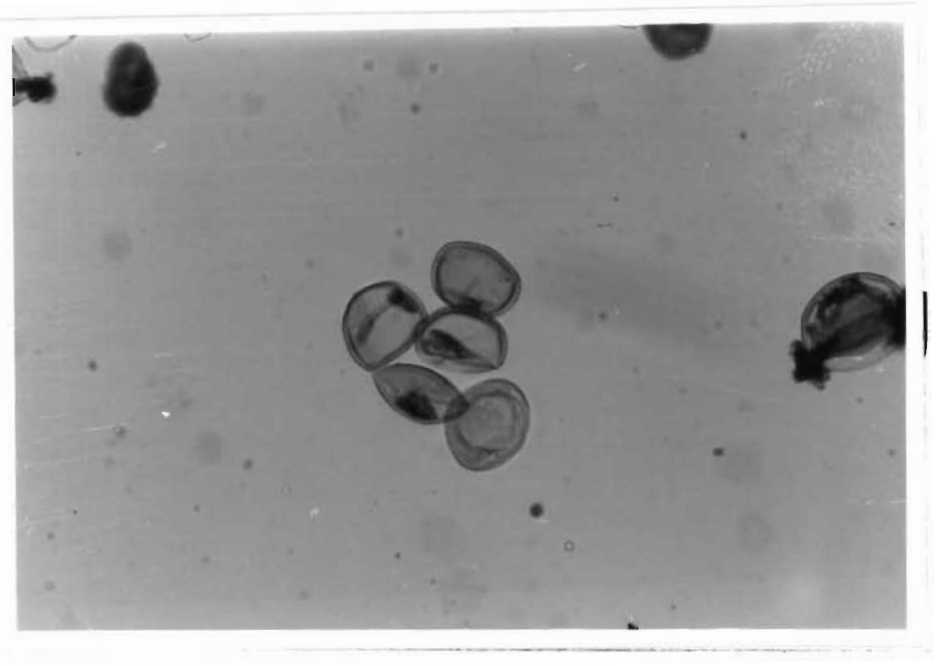


PLATE 6a

Microspores of *E. woodii* at the time of culture initiation. The mononucleate condition and peripheral cytoplasm are clearly evident. Scale x 350.

PLATE 6b

Pollen tubes arising from germinating microspores of *E. woodii* after 35 days on SH medium with $4{,}5 \times 10^{-6}$M 2,4-D and kinetin. Aceto-orcein stain. Scale x 33.

2.6 Experiments with diploid material

2.6.1 Embryo cultures

As is the case in other recalcitrant or "wet" seeds, the embryo in cycad seeds shows more-or-less uninterrupted development from the time of fertilisation until germination (Section 1.6). Thus seed collected at the time of shedding from the parent cones contains immature embryos while seed dissected just prior to germination will reveal fully-developed embryos. Embryos at different stages were aseptically removed from surface-sterilised seeds of three species of *Encephalartos* and *Stangeria eriopus*, subjected in some cases to hormone pre-treatments, and cultured on a variety of media in different containers under different conditions. The results are summarised in Table 13.

In general, immature embryos continued essentially normal development for a period of about 6 weeks (Plate 7a) but thereafter became necrotic. On the other hand, mature embryos gave a variety of responses, dependent on the pre-treatment, medium, container and environmental conditions. Without pre-treatment and with no growth factors, the embryos again continued essentially normal growth (Plate 7b), although in some cases the cotyledons became highly coiled (Plate 7c). Prolific crumbly off-white callus arose from mature embryos of *E. natalensis* and *E. villosus* after 2 - 3 weeks on both the SH and the "59" media when low auxin and cytokinin supplements were present (Plate 7d). In common with the situation in many other gymnosperms (Section 2.2), the callus developed along the cotyledonary surfaces. Cytological examination of 5 such cultures from *E. villosus* confirmed its consistent diploid (2n = 18) status.

The conditions which produced the highest frequency of callogenesis in these embryo cultures were similar in many respects to those which gave the best results with the megagametophyte cultures (Section 2.5.1). There was no significant difference in performance of the explants on the two different basal media. Cytokinin pre-treatment offered no particular advantage but an auxin/cytokinin supplement was essential for callus development. Concentrations in the range 10^{-6} to 10^{-5} M were satisfactory in this respect but an increase in the 6-benzyladenine level above 10^{-5}M resulted in necrosis. Sugar concentrations in the 2 - 5%

TABLE 13 : *In vitro* culture of cycad embryos

Species	Embryo condition	No. of explants	Culture medium	Container	Light regime	Observations
E. natalensis	intermediate and mature	4	SH, 4-hr pre-treatment in $2,5x10^{-4}$M BA	Universal bottles	Dark	Callus along suspensor in 3 explants after 2 months
E. natalensis	intermediate and mature	4	SH, no pre-treatment	Universal bottles	Dark	Highly coiled cotyledons and callus at suspensor in 2 explants after 2 months
E. natalensis	intermediate and mature	4	SH, 4-hr pre-treatment in $2,5x10^{-4}$M BA	Universal bottles	$50\mu Em^{-2}s^{-1}$ 12/12 hr photoperiod	All cultures necrotic
E. natalensis	intermediate and mature	4	SH, no pre-treatment	Universal bottles	$50\mu Em^{-2}s^{-1}$ 12/12 hr photoperiod	Continuous normal growth with greening of cotyledons in 1 explant
E. natalensis	mature	4	SH, $4,5x10^{-6}$M 2,4-D and KN	50ml Erlenmeyers	Dark	Vigorous callus on cotyledons in 3 explants after 20 days
E. natalensis	mature	4	SH, $4,5x10^{-6}$M 2,4-D and KN	50ml Erlenmeyers	$100\mu Em^{-2}s^{-1}$ constant	Vigorous callus on cotyledons in 2 explants after 20 days
E. natalensis	mature	4	59, $4,5x10^{-6}$M 2,4-D and KN	50ml Erlenmeyers	Dark	Vigorous callus on cotyledons in all explants after 20 days
E. natalensis	mature	4	59, $4,5x10^{-6}$M 2,4-D and KN	50ml Erlenmeyers	$100\mu Em^{-2}s^{-1}$ constant	All cultures necrotic
E. natalensis	mature	8	SH, $4,5x10^{-6}$M 2,4-D and BA	25ml Erlenmeyers	$100\mu Em^{-2}s^{-1}$ 16/8 hr photoperiod	Callus on cotyledons of 5 explants after 4 weeks
E. natalensis	mature	8	SH, $4,5x10^{-6}$M 2,4-D; $4,5x10^{-5}$M BA	25ml Erlenmeyers	$100\mu Em^{-2}s^{-1}$ 16/8 hr photoperiod	Continued growth of all explants

TABLE 13 : continued

Species	Embryo condition	No. of explants	Culture medium	Container	Light regime	Observations
E. natalensis	mature	8	SH, $4,5 \times 10^{-6}$M 2,4-D; $2,2 \times 10^{-4}$M BA	25ml Erlenmeyers	$100 \mu Em^{-2}s^{-1}$ 16/8 hr photoperiod	All cultures necrotic
E. natalensis	mature	8	SH, $4,5 \times 10^{-6}$M 2,4-D; $4,5 \times 10^{-4}$M BA	25ml Erlenmeyers	$100 \mu Em^{-2}s^{-1}$ 16/8 hr photoperiod	All cultures necrotic
E. paucidentatus	immature	6	SH, ½-hr pretreatment in $2,5 \times 10^{-4}$M BA	25ml Erlenmeyers	$50 \mu Em^{-2}s^{-1}$ 12/12 hr photoperiod	Continued development in all explants for 6 weeks; thereafter necrotic
E. villosus	mature	5	59, $1,4 \times 10^{-6}$M 2,4-D; $1,4 \times 10^{-5}$M BA; 2% sucrose	25ml Erlenmeyers	Dark	Vigorous callus on cotyledons in 4 explants after 3 weeks
E. villosus	mature	5	59, $1,4 \times 10^{-6}$M 2,4-D; $1,4 \times 10^{-5}$M BA; 2% sucrose	25ml Erlenmeyers	$100 \mu Em^{-2}s^{-1}$ constant	Vigorous callus on cotyledons in 3 explants after 3 weeks
E. villosus	mature	5	59, $1,4 \times 10^{-6}$M 2,4-D; $1,4 \times 10^{-5}$M BA; 5% sucrose	25ml Erlenmeyers	Dark	Vigorous callus on cotyledons in all explants after 3 weeks
E. villosus	mature	5	59, $1,4 \times 10^{-6}$M 2,4-D; $1,4 \times 10^{-5}$M BA; 5% sucrose	25ml Erlenmeyers	$100 \mu Em^{-2}s^{-1}$ constant	Vigorous callus on cotyledons in 4 explants after 3 weeks
E. villosus	mature	5	59, $1,4 \times 10^{-6}$M 2,4-D; $1,4 \times 10^{-5}$M BA; 10% sucrose	25ml Erlenmeyers	Dark	Slight callus growth in 4 explants after 3 weeks
E. villosus	mature	5	59, $1,4 \times 10^{-6}$M 2,4-D; $1,4 \times 10^{-5}$M BA; 10% sucrose	25ml Erlenmeyers	$100 \mu Em^{-2}s^{-1}$ constant	Slight callus growth in 1 explant after 3 weeks
S. eriopus	immature	6	SH, $4,5 \times 10^{-6}$M 2,4-D and KN	Universal bottles	Dark	Continued normal development in all explants for 6 weeks; thereafter necrotic

Notes : SH, medium of SCHENK and HILDEBRANDT (1972); 59, medium of NORSTOG and RHAMSTINE (1967);
2,4-D, 2,4-dichlorophenoxyacetic acid; KN, kinetin; BA, 6-benzyladenine

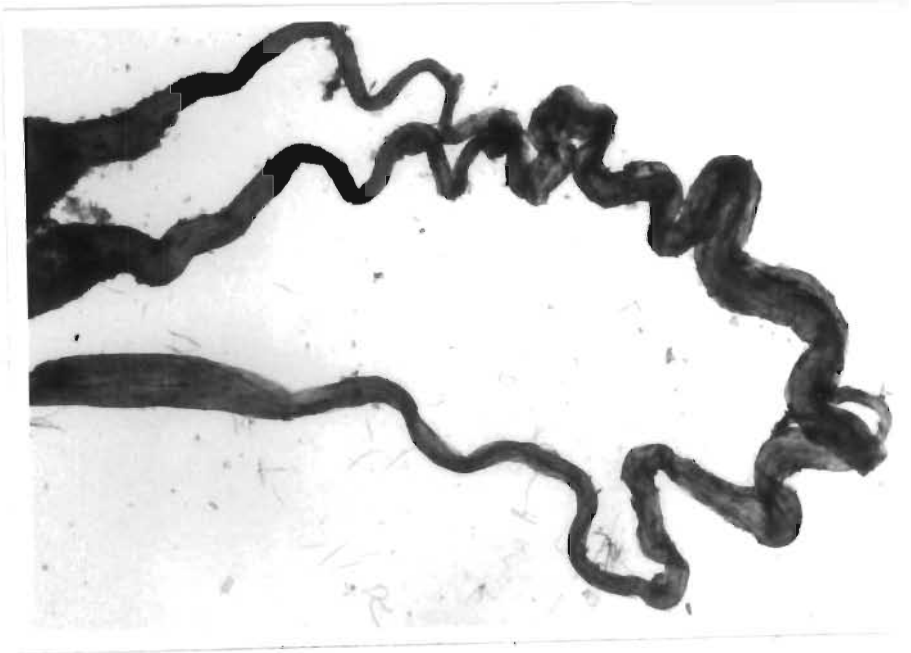


PLATE 7a

Development of a juvenile embryo of *Encephalartos paucidentatus* on SH medium, with no growth factors, after six weeks in culture. The pro-embryo (lower left), the massive coiled suspensor and two egg membranes are well defined. Aceto-orcein stain. Scale X 6.

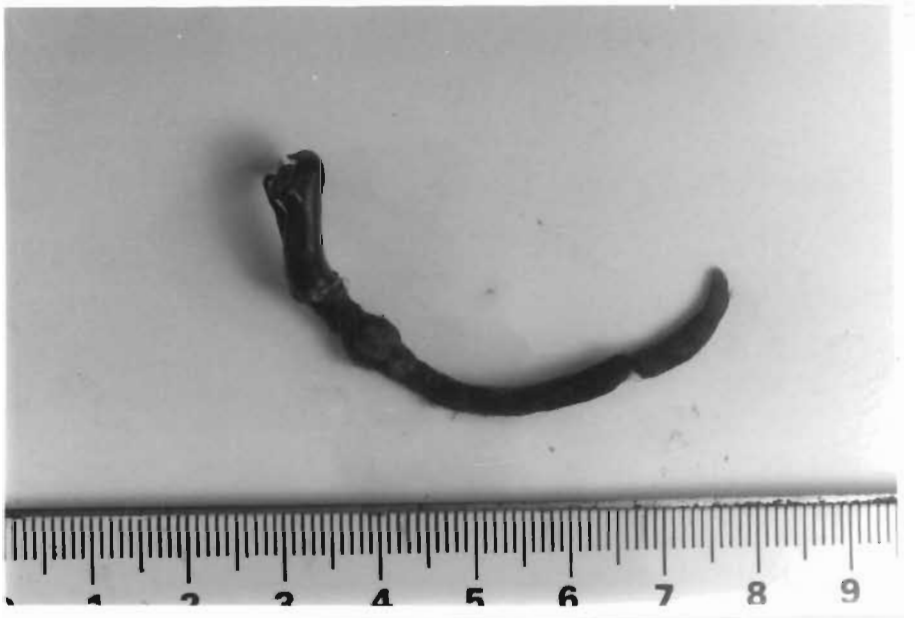


PLATE 7b

Development of a mature embryo of *Encephalartos natalensis* on SH medium, with no growth factors, after two months in culture. Behaviour *in vitro* is analagous to that *in vivo*.

PLATE 7c

Development of an intermediate-stage embryo of *Encephalartos natalensis* on SH medium, with no growth factors, after two months in culture. Pronounced curvature of the cotyledons is apparent. Scale X 8.

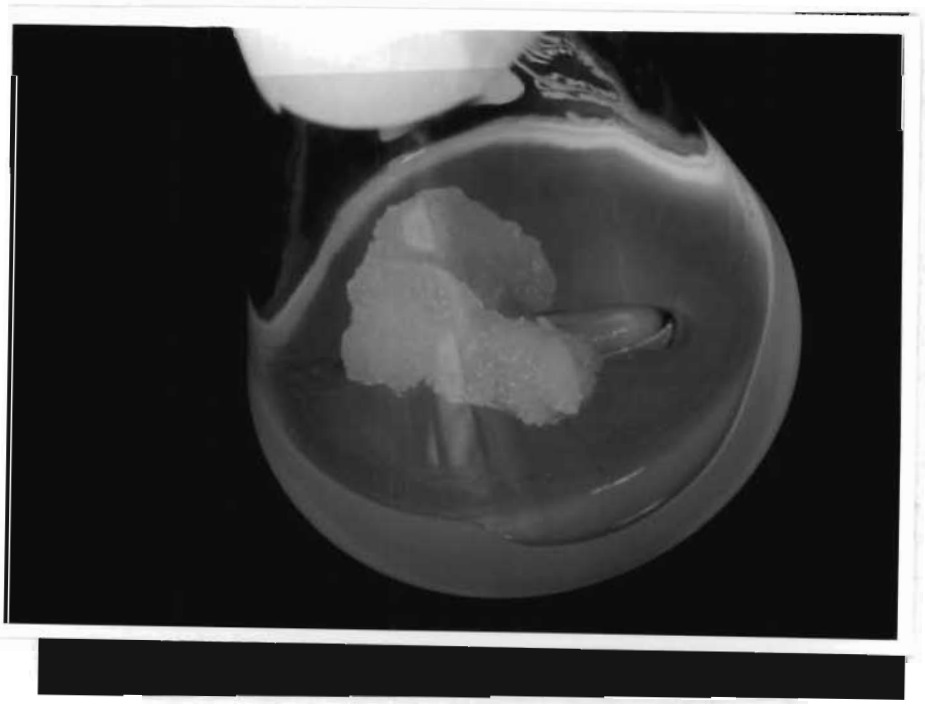


PLATE 7d

Vigorous callus growth occurring on the distal (cotyledonary) end of a mature embryo of *Encephalartos villosus* after 40 days on medium '59' supplemented with $1,4 \times 10^{-6}$M 2,4-D and $1,4 \times 10^{-5}$M BA and grown in the dark at 22°C. Scale X 2.

range were satisfactory, but 10% was inhibitory. The use of Erlenmeyer flasks gave better results than use of Universal bottles. The frequency of callogenesis was higher in the dark than in constant light or under a diurnal photoperiod. However, explants exposed to light conditions became green and presumably photosynthetic with possible long term advantages. No evidence was seen of any differentiation in any of the cultures.

To investigate the response to sub-culture, explants from the more vigorous callus from *E. natalensis* and *E. villosus* were transferred to fresh medium with various growth factor supplements, as shown in Table 14. When 2,4-D and kinetin in concentrations from 10^{-7} to 10^{-6} were used, callus proliferation continued. The absence of growth factors inhibited this continued development but several of the *E. villosus* subcultures on media without hormones gave rise to green areas of tissue with possible meristematic activity. These later became necrotic. Concentrations of 2×10^{-5}M cytokinin, gibberellic acid and vitamin D_3 all resulted in necrosis of the cultures.

TABLE 14 : Analysis of responses obtained in subculture of embryo-derived callus from *E. villosus* and *E. natalensis* on medium '59' with different growth factor supplements.

Species	Growth factor supplements	Number of cultures	Observations
E. villosus	no growth factors	10	Some greening, possibly meristematic
	$4{,}4 \times 10^{-6}$M 2,4-D and KN	5	Continued callus proliferation
	$4{,}5 \times 10^{-7}$M 2,4-D; $4{,}5 \times 10^{-6}$M KN	5	Continued callus proliferation
	$4{,}5 \times 10^{-6}$M 2,5-D, $4{,}5 \times 10^{-7}$M KN	5	Continued callus proliferation
	2×10^{-5}M KN	2	No response
	2×10^{-5}M BA	3	No response
	2×10^{-5}M 2iP	2	No response
	2×10^{-5}M GA_3	2	No response
	2×10^{-5}M Vitamin D_3	5	No response
E. natalensis	no growth factors	8	No response

2.6.2 Primary root explant cultures

In the first few months after germination of cycad seeds, a well-defined primary tap-root is established (Figure 10). Depending on the species and the growing conditions, this attains somewhat carrot-like dimensions within one to three years. In cross section, roots at this stage show a broad, well-defined central vascular zone, usually with a tetrach arrangement of xylem elements, to the outside of which is an abundant parenchymatous cortex. In view of the abundance of this tissue, the ease with which it can be surface-sterilized, and the relative absence of endogenous micro-organisms, it was logical to explore this material as an explant source. This extends the previous work of KOELEMAN and SMALL (1982).

Cubes of primary root tissue (5mm-10mm sides) were aseptically dissected from roots of 21 cycad species, each cube containing some vascular tissue. These explants were cultured on a variety of media with different hormone supplements, sometimes after specific pre-treatments, in different culture vessels and exposed to different light regimes. Due to the scarcity of certain root samples, several of the treatments could be carried out only in small replicate numbers; interpretation of the results must take this limitation into account. The parameters tested and the responses obtained are presented in Table 15 (*E. natalensis*), Table 16 (other *Encephalartos* spp.), and Table 17 (other cycad genera). Table 18 gives an overview of the root explant experimentation. A vigorous fast-growing callus is quite readily obtained in fairly high frequency (>50%) from most root explants under most conditions within 20-30 days (Plate 8). There was little evidence of fungal or bacterial contamination. The callus was generally initiated in the root cambial zone but spread rapidly over the entire cut surface of the explant. It was usually crisp, off-white in colour and comprised large vacuolated cells with pronounced nuclei.

The size of the explant in the cubes seemed to be important. Most cubes were in the range 5mm-10mm per side and these gave the generally successful callus growth described above. However, smaller cubes, such as 3mm per side, did not give any response, even after several months. These observations are consistent with the comment by MURASHIGE (1974) that the explant size is often critical in callus initiation. In evaluating the response to the position in the root from which the explant is

TABLE 15 : *In vitro* culture of primary root explants from *Encephalartos natalensis* seedlings

Origin of explant	No. of explants	Pre-treatment conditions	Culture medium	Contalner	Light regime	Observations
Top 1/3rd	6	-	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Vigorous callus in 5 explants in 15 days
Top 1/3rd	18	-	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Vigorous callus in 11 explants in 21 days
Mid 1/3rd	6	-	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Vigorous callus in all explants in 21 days
Mid 1/3rd	17	-	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Vigorous callus in 13 explants in 21 days
Bottom 1/3rd	6	-	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Moderate callus in 5 explants in 30 days
Bottom 1/3rd	16	-	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Moderate callus in 6 explants in 30 days
Top 2/3rds	27	-	SH, $4,5x10^{-6}$M 2,4-D and KN	Universal bottles	Dark	Moderate callus in 4 explants in 40 days
Top 2/3rds	27	-	SH, $4,5x10^{-6}$M 2,4-D and KN	Universal bottles	$50\mu Em^{-2}s^{-1}$ 12/12hr photoperiod	Moderate callus in 1 explant 40 days
Top 2/3rds	4	-	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Vigorous callus in all explants in 23 days
Top 2/3rds	3	-	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Vigorous callus in 1 explant in 30 days
Top 2/3rds	2	-	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$ constant	Vigorous callus in 1 explant in 30 days
Top 2/3rds	3	-	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	No response in 50 days
Top 2/3rds	6	-	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$30\mu Em^{-2}s^{-1}$ constant	Vigorous calls in all explants in 25 days

TABLE 15 continued ....

Origin of explant	No. of explants	Pre-treatment conditions	Culture medium	Container	Light regime	Observations
Top 2/3rds	2	-	59, $4{,}5\times10^{-6}$M 2,4-D and KN	Tissue culture tubes	100$\mu Em^{-2}s^{-1}$ constant	Vigorous callus in 1 explant in 30 days
Top 2/3rds	6	-	59, $4{,}5\times10^{-6}$M 2,4-D and $4{,}5\times10^{-7}$M KN	Tissue culture tubes	30$\mu Em^{-2}s^{-1}$ constant	No response in 50 days
Top 2/3rds	6	-	59, $4{,}5\times10^{-7}$M 2,4-D and $4{,}5\times10^{-6}$M KN	Tissue culture tubes	30$\mu Em^{-2}s^{-1}$ constant	No response in 50 days
Top 2/3rds	6	-	59, $4{,}5\times10^{-6}$M NAA and BA	Tissue culture tubes	30$\mu Em^{-2}s^{-1}$ constant	Vigorous callus in all explants in 25 days
Top 2/3rds	6	-	59, $4{,}5\times10^{-6}$M NAA and $4{,}5\times10^{-7}$M BA	Tissue culture tubes	30$\mu Em^{-2}s^{-1}$ constant	No response in 50 days
Top 2/3rds	6	-	59, $4{,}5\times10^{-7}$M NAA and $4{,}5\times10^{-6}$BA	Tissue culture tubes	30$\mu Em^{-2}s^{-1}$ constant	No response in 50 days
Top 2/3rds	6	-	59, $4{,}5\times10^{-6}$M IAA and 2iP	Tissue culture tubes	30$\mu Em^{-2}s^{-1}$ constant	No response in 50 days
Top 2/3rds	6	-	59, $4{,}5\times10^{-6}$M IAA and $4{,}5\times10^{-7}$M 2iP	Tissue culture tubes	30$\mu Em^{-2}s^{-1}$ constant	No response in 50 days
Top 2/3rds	6	-	59, $4{,}5\times10^{-7}$M IAA and $4{,}5\times10^{-6}$M 2iP	Tissue culture tubes	30$\mu Em^{-2}s^{-1}$ constant	No response in 50 days
Top 2/3rds	6 + 6	-	59, $4{,}5\times10^{-6}$M 2,4-D and KN sucrose to 5% and 10%	Tissue culture tubes	30$\mu Em^{-2}s^{-1}$ constant	No response in 50 days
Top 2/3rds	6 + 6	-	59, $4{,}5\times10^{-6}$M IAA and 2iP sucrose to 5% and 10%	Tissue culture tubes	30$\mu Em^{-2}s^{-1}$ constant	No response in 50 days
Top 2/3rds	6	2×10^{-4}M 2,4-D in 5% DMSO, 24 hrs.	59, no growth factors	Tissue culture tubes	30$\mu Em^{-2}s^{-1}$ constant	No response in 50 days
Top 2/3rds	6	2×10^{-4}M NAA in 5% DMSO, 24 hrs.	59, no growth factors	Tissue culture tubes	30$\mu Em^{-2}s^{-1}$ constant	No response in 50 days

TABLE 15 continued.....

Origin of explant	No. of explants	Pre-treatment conditions	Culture medium	Container	Light regime	Observations
Top 2/3rds	6	$2x10^{-4}$M IAA in 5% DMSO, 24 hrs.	59, no growth factors	Tissue culture tubes	$30\mu Em^{-2}s^{-1}$ constant	No response in 50 days
Top 2/3rds	6	$2x10^{-4}$M KN in 5% DMSO, 24 hrs.	59, no growth factors	Tissue culture tubes	$30\mu Em^{-2}s^{-1}$ constant	No response in 50 days
Top 2/3rds	6	$2x10^{-4}$M BA in 5% DMSO, 24 hrs.	59, no growth factors	Tissue culture tubes	$30\mu Em^{-2}s^{-1}$ constant	No response in 50 days
Top 2/3rds	6	$2x10^{-4}$M 2iP in 5% DMSO, 24 hrs.	59, no growth factors	Tissue culture tubes	$30\mu Em^{-2}s^{-1}$ constant	No response in 50 days
Top 2/3rds	3	$2x10^{-4}$M BA, 4 hrs.	SH, no growth factors	25ml Erlen-meyers	Dark	Callus in 1 explant in 26 days
Top 2/3rds	2	$2x10^{-4}$M BA, 4 hrs.	SH, no growth factors	25ml Erlen-meyers	$30\mu Em^{-2}s^{-1}$ constant	Callus in 1 explant in 30 days
Top 2/3rds	3	$2x10^{-4}$M NAA, 4 hrs.	SH, no growth factors	25ml Erlen-meyers	Dark	Callus in all explants in 15 days
Top 2/3rds	2	$2x10^{-4}$M NAA, 4 hrs.	SH, no growth factors	25ml Erlen-meyers	$30\mu Em^{-2}s^{-1}$ constant	Callus in 1 explant in 19 days
Top 2/3rds	4	$4x10^{-4}$M NAA, 4 hrs.	SH, no growth factors	Universal bottles	Dark	Callus in 3 explants in 21 days
Top 2/3rds	4	$1x10^{-3}$M NAA, 4 hrs.	SH, no growth factors	Universal bottles	Dark	Callus in 2 explants in 18 days
Top 2/3rds	20	$4x10^{-4}$M NAA, 4 hrs.	MS, $4,5x10^{-6}$M BA	Universal bottles	Dark	Callus in all explants in 25 days
Top 2/3rds	2	$2x10^{-4}$M NAA and BA, 4 hrs.	SH, no growth factors	25ml Erle-meyers	Dark	Callus on 1 explant in 22 days

TABLE 15 continued....

Origin of explant	No. of explants	Pre-treatment conditions	Culture medium	Container	Light regime	Observations
Top 2/3rds	3	$2x10^{-4}$M NAA and BA, 4 hrs.	SH, no growth factors	25ml Erlenmeyers	$30\mu Em^{-2}s^{-1}$ constant	Callus in all explants in 25 days
Top 2/3rds	12	-	SH liquid medium; explants on filter paper bridge	Universal bottles	Dark	Callus in 6 cultures in 21 days

Notes : SH, medium of SCHENK and HILDEBRANDT (1972)
MS, medium of MURASHIGE and SKOOG (1962)
NAA, 1-naphthaleneacetic acid
BA, 6-benzyladenine, 2iP, isopentenyladenine
59, medium of NORSTOG and RHAMSTINE (1967)
2,4-D, 2,4-dichlorophenoxyacetic acid,
IAA, indole-3-acetic acid KN, kinetin
DMSO, dimethylsulphoxide

TABLE 16 : *In vitro* culture of primary root explants of other *Encephalartos* species

Species	No. of explants	Culture medium	Container	Light regime	Observations
E. altensteinii	4	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 2 explants in 25 days
E. caffer	8	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 2 explants in 22 days
E. caffer	3	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 3 explants in 20 days
E. caffer	2	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Callus in 2 explants in 25 days
E. caffer	3	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 3 explants in 26 days
E. caffer	2	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Callus in 1 explant in 25 days
E. cycadifolius	4	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 1 explant in 50 days
E. cycadifolius	3	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 3 explants in 26 days
E. cycadifolius	2	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Callus in 2 explants in 28 days
E. cycadifolius	3	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	No response in 50 days
E. cycadifolius	2	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	No response in 50 days
E. ferox	4	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 1 explant in 21 days
E. ferox	3	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 3 explants in 26 days
E. ferox	2	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Callus in 2 explants in 25 days
E. ferox	3	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 3 explants in 26 days
E. ferox	2	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Callus in 2 explants in 25 days

continued.....

TABLE 16 continued....

Species	No. of explants	Culture medium	Container	Light regime	Observations
E. friderici-guilielmi	4	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 3 explants in 37 days
E. friderici-guilielmi	3	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 1 explant in 14 days
E. friderici-guilielmi	2	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	No response in 50 days
E. friderici-guilielmi	3	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	No response in 50 days
E. friderici-guilielmi	2	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	No response in 50 days
E. ghellinckii	4	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	No response in 50 days
E. horridus	4	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 3 explants in 23 days
E. horridus	3	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 3 explants in 14 days
E. horridus	2	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Callus in 2 explants in 20 days
E. horridus	3	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	No response in 50 days
E. horridus	2	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	No response in 50 days
E. lanatus	4	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 3 explants in 48 days
E. lanatus	3	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 1 explant in 14 days
E. lanatus	2	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	No response in 50 days
E. lanatus	3	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	No response in 50 days
E. lanatus	2	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Callus in 2 explants in 20 days
E. lebomboensis	5	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 5 explants in 21 days
E. lehmannii	4	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 1 explant in 42 days

Continued.....

TABLE 16 continued....

Species	No. of explants	Culture medium	Container	Light regime	Observations
E. longifolius	4	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 4 explants in 28 days
E. longifolius	21	MS, $4,5x10^{-6}$M BA; explants pre-treated with $4x10^{-4}$M NAA for 4 hours	Universal bottles	Dark	Callus in all explants in 25 days
E. longifolius	3	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 3 explants in 18 days
E. longifolius	2	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Callus in 2 explants in 14 days
E. longifolius	3	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 2 explants in 14 days
E. longifolius	2	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Callus in 2 explants in 25 days
E. ngoyanus	5	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	No response in 50 days
E. transvenosus	4	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 4 explants in 21 days
E. trispinosus	4	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 4 explants in 32 days
E. villosus	4	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 4 explants in 38 days
E. villosus	3	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 3 explants in 23 days
E. villosus	2	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Callus in 2 explants in 20 days
E. villosus	3	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 3 explants in 18 days
E. villosus	2	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Callus in 2 explants in 25 days
E. woodii (lateral root from mature plant)	5	SH, $4,5x10^{-6}$M 2,4-D and KN	Universal bottles	Dark	No response in 50 days
	8	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 7 explants in 40 days

NOTES : SH, medium of SCHENK and HILDEBRANDT (1972)
59, medium of NORSTOG and RHAMSTINE (1967)
MS, medium of MURASHIGE and SKOOG (1962)
2,4-D, 2,4-dichlorophenoxyacetic acid
NAA, 1-naphthaleneacetic acid
KN, kinetin
BA, 6-benzyladenine

TABLE 17 : *In vitro* culture of primary root explants of cycads other than *Encephalartos*

Species	No. of explants	Culture medium	Container	Light regime	Observations
Stangeria eriopus	14	SH, $4,5x10^{-6}$M 2,4-D and KN	Universal bottles	Dark	Callus in all explants in 24 days
Stangeria eriopus	23	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 16 explants in 25 days
Stangeria eriopus	4	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 2 explants in 25 days
Stangeria eriopus	3	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Callus in all explants in 25 days
Stangeria eriopus	3	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	No response in 50 days
Stangeria eriopus	2	59, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Callus in 2 explants in 30 days
Bowenia serrulata	12	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 1 explant in 21 days
Bowenia spectabilis	10	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 6 explants in 29 days
Zamia furfuracea	8	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 1 explant in 21 days
Zamia pumila	15	SH, $4,5x10^{-6}$M 2,4-D and KN	Tissue culture tubes	Dark	Callus in 1 explant in 25 days

Notes : SH, medium of SCHENK and HILDEBRANDT (1972) 59, medium of NORSTOG and RHAMSTINE (1967)
2,4-D, 2,4-dichlorophenoxyacetic acid KN, kinetin

TABLE 18 : Analysis of responses by primary root explants to various parameters

A. Origin of explant in the root

Position	No. of explants tested	% showing callus formation in 50 days	Average time to show callogenesis (days)
Top one-third	24	66	20
Middle one-third	23	83	21
Bottom one-third	22	50	30

B. Choice of basal medium

Basal medium	No. of explants tested	% showing callus formation in 50 days	Average time to show callogenesis (days)
Schenk & Hildebrandt (1972)	238	53	25
Norstog & Rhamstine's 59 (1967)	51	53	23

C. Choice of container

Container	No. of explants tested	% showing callus formation in 50 days	Average time to show callogenesis (days)
25ml Erlenmeyers	90	65	28
Tissue culture tubes	187	49	23

D. Light regime

Light condition	No. of explants tested	% showing callus formation in 50 days	Average time to show callogenesis (days)
Constant dark	170	56	24
Constant light ($100\mu Em^{-2}s^{-1}$	65	46	23

E. Response by species

Species	No. of explants tested	% showing callus formation in 50 days	Average time to show callogenesis (days)
E. altensteinii	4	50	25
E. caffer	18	61	23
E. cycadifolius	14	43	35
E. ferox	14	79	25
E. friderici-guilielmi	14	29	31
E. ghellinckii	4	0	-
E. horridus	14	57	19
E. lanatus	14	43	33
E. lebomboensis	5	100	21
E. lehmannii	4	25	42
E. longifolius	14	93	23
E. natalensis	20	65	26
E. ngoyanus	5	0	-
E. transvenosus	4	100	21
E. trispinosus	4	100	32
E. villosus	14	100	26
S. eriopus	49	71	25

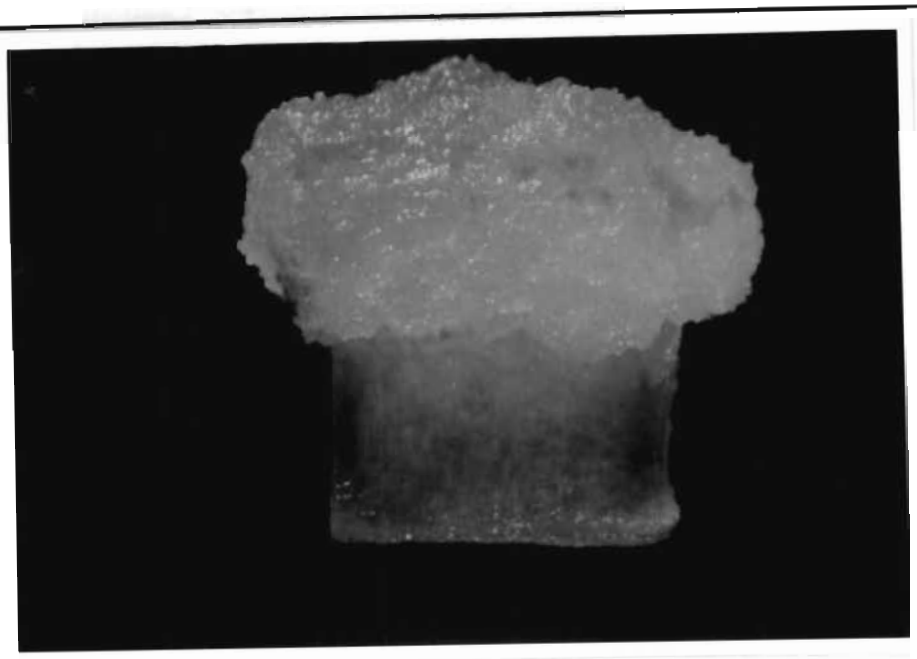


PLATE 8

Extensive callus growth occurring on the upper surface of *ca.* 5 mm cube from primary root tissue of one-year old *Encephalartos natalensis* after 37 days in culture on SH medium supplemented with $4,5 \times 10^{-6}$M 2,4-D and kinetin and grown in the dark at 22°C. Scale × 6.

dissected, there was no significant difference between central and peripheral explants. Transverse cylinders of tissue gave a response similar to that of cubes of tissue. There was, however, a difference with respect to explant position relative to the long axis of the root. A higher frequency of callogenesis in a shorter time period was observed in explants from top and particularly the middle one-third of the root, as compared to a lower frequency and longer time requirement in explants from the bottom one-third (Table 18a). It was noted in dissection that the upper root material was firmer in texture, and contained a more well-defined cambium, than the lower root tissue. Furthermore the possibility exists of a hormone gradient along the root axis. It is shown later that the peroxidase activity in cycad roots is consistently higher in the upper root tissue than in the lower tissue (Section 3.6); this may well comprise part of a growth-response control mechanism. Analogous examples are readily found in the literature; such as a decline in regenerative response down the length of tobacco stems, differences in growth responses of leaf explants of different ages and a polarisation in regenerative ability of *Lilium* bulb scales (MURASHIGE, 1974).

As with the cultures of megagametophytes and embryos, the selection of basal medium had no material effect on callus formation by root explants (Table 18b). However, as in the aforementioned cases, the choice of culture container was important. The use of 25ml Erlenmeyer flasks gave rise to a higher frequency of callus formation, although requiring somewhat more time, than the explants in tissue culture tubes (Table 18c). The possible explanations for this phenomenon have already been stated (Section 2.4.5) and confirm previous results (Table 8). As with the embryo cultures, the root explants gave a somewhat better response in dark conditions than in the light (Table 18d). The presence of both an auxin and a cytokinin appeared beneficial; optimal results were found when $4,5 \times 10^{-6}$M 2,4-D and kinetin were used in combination. Other concentrations and different hormone combinations were generally unsuccessful in promoting callogenesis, with the exception of the $4,5 \times 10^{-6}$M NAA and 6-benzyladenine combination (Table 15). A "pulse" pre-treatment of the explant with relatively high auxin and cytokinin concentrations was unsuccessful with 24-hour exposure, but some response was noted when the treatment time was reduced to 4 hours (Table 15). The use of NAA appeared to lead to the best results in the series of pre-treatments, but this offered no particular advantage over culture onto medium incorporating lower auxin and cytokinin levels.

In the analysis of callus-production responses by the different species of cycads tested, there is evidence that the fast-growing species of mesic origin (e.g. *E. ferox*, *E. lebomboensis*, *E. longifolius*, *E. natalensis*, *E. transvenosus* and *E. villosus*) respond more quickly and at a higher frequency, than species which are adapted to more xeric habitats (e.g. *E. cycadifolius*, *E. friderici-guilielmi*, *E. ghellinckii*, *E. lanatus* and *E. lehmannii*). This observation is consistent with the report by KOELEMAN and SMALL (1982) who found root explants of *E. lebomboensis* the most responsive of 9 species tested, *E. lanatus* being the least successful.

Cytological examination of a sample of the callus derived from a lateral root explant of *Encephalartos woodii* showed a diploid (2n = 18) chromosome complement in each of 8 cells with clear metaphase stages (Plate 9). This provides useful evidence of the status of this species;

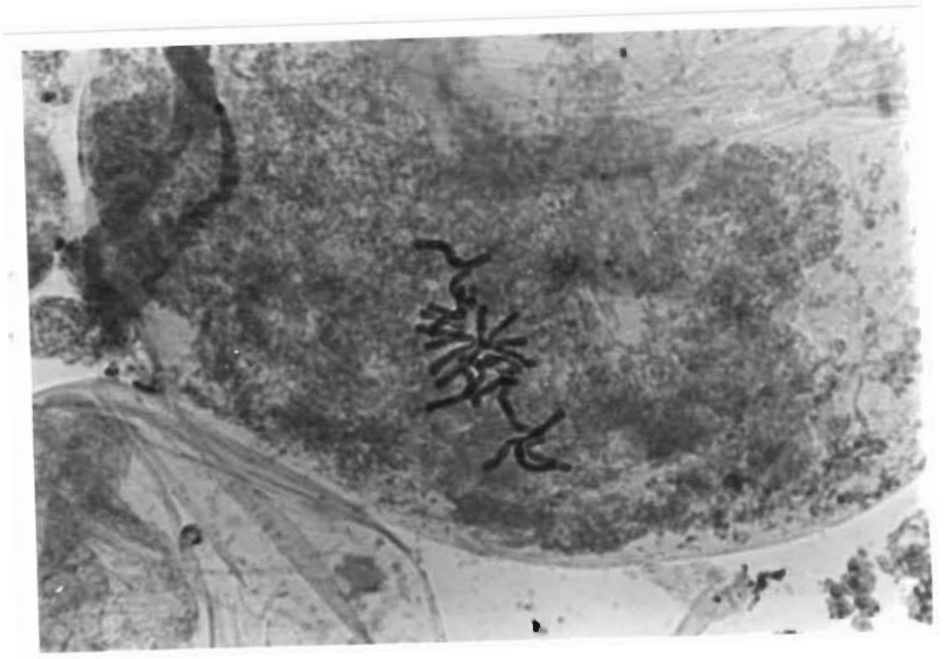


PLATE 9

Photomicrograph of chromosomes in callus tissue arising from a lateral root explant of *E. woodii*. The diploid number (2n = 18) is evident. Squash preparation in aceto-orcein, scale x 400.

the observed (2n = 18) number, which is characteristic of the genus as a whole, is likely to represent the ploidy condition of the parent material. This finding thus discounts speculation (OSBORNE, 1986a) that *E. woodii* may be aneuploid or polyploid variant of *E. natalensis* or another closely-related species.

Subculture of primary root-derived callus

Callus derived from primary root explants of *E. natalensis* and other *Encephalartos* species was subcultured to various media in different containers and under different light regimes as shown in Table 19. In these transfers it was found essential to include at least a portion of the original explant material in the subcultures; where this was not done, the subcultured material failed to continue growth. The subcultures

TABLE 19 : Subculture of primary root-derived callus from various cycad species

Species	Number of subcultures	Culture medium	Container	Light regime	Observations
E. natalensis	14	SH, no growth factors	25ml Erlenmeyers	$50\mu Em^{-2}s^{-1}$, constant	Continued callus growth
E. natalensis	10	SH, explants treated with $2x10^{-4}M$ BA for 4 hours	25ml Erlenmeyers	$50\mu Em^{-2}s^{-1}$, constant	Continued callus growth
E. natalensis	16	SH, $4,5x10^{-6}$ 2,4-D and KN	Universal bottles	Dark	Necrotic in 60 days
E. natalensis	7	SH, $4,5x10^{-5}M$ BA	25ml Erlenmeyers	$100\mu Em^{-2}s^{-1}$, constant	Continued callus growth
E. natalensis	9	MS, $4,5x10^{-6}M$ BA	25ml Erlenmeyers	Dark	Continued callus growth
E. natalensis	10	59, $4,5x10^{-6}M$ 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Continued callus growth
E. natalensis	20	SH, 0,5ml 10^{-5} to $10^{-3}M$ BA added to callus	25ml Erlenmeyers	$100\mu Em^{-2}s^{-1}$, constant	Necrotic in 30-90 days
E. various spp.	13	SH, no growth factors	25ml Erlenmeyers	$50\mu Em^{-2}s^{-1}$, constant	Continued callus growth
E. various spp.	12	SH, explants treated with $2x10^{-4}M$ BA for 4 hours	25ml Erlenmeyers	$50\mu Em^{-2}s^{-1}$, constant	Continued callus growth
E. various spp.	10	SH, no growth factors	25ml Erlenmeyers	$100\mu Em^{-2}s^{-1}$, constant	Continued callus growth
E. various spp.	7	59, $2,5x10^{-6}M$ KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Continued callus growth
E. various spp.	7	59, no growth factors	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Continued callus growth
E. various spp.	18	SH, $4,5x10^{-6}M$ 2,4-D and KN	Tissue culture tubes	$100\mu Em^{-2}s^{-1}$, constant	Continued callus growth
Bowenia spectabilis	7	SH, $4,5x10^{-6}M$ 2,4-D and KN	25ml Erlenmeyers	$100\mu Em^{-2}s^{-1}$, photo-period	Continued callus growth
Stangeria eriopus	15	SH, $4,5x10^{-6}M$ 2,4-D and KN	25ml Erlenmeyers	Dark	Continued callus growth
Stangeria eriopus	15	SH, $4,5x10^{-6}M$ 2,4-D and KN	25ml Erlenmeyers	$100\mu Em^{-2}s^{-1}$, constant	Apical meristem and leaf regeneration in all cultures

Notes : SH, medium of SCHENK and HILDEBRANDT (1972)
MS, medium of MURASHIGE and SKOOG (1962)
2,4-D, 2,4-dichlorophenoxyacetic acid
59, medium of NORSTOG and RHAMSTINE (1967)
BA, 6-benzyladenine
KN, kinetin

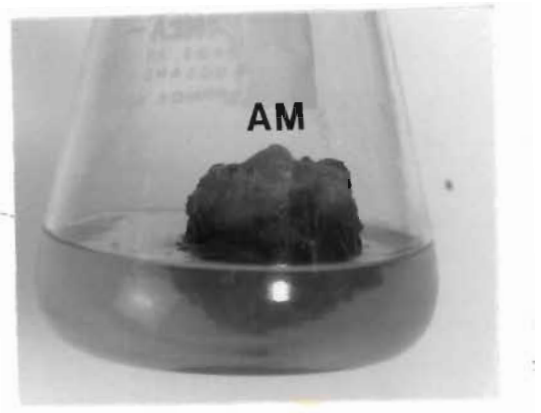
with portions of the original material generally showed continued callus growth which could be maintained over several passages at about 3-monthly transfer intervals. There was little difference in performance with respect to the presence or absence of hormone supplements or the light conditions employed. Cultures in Universal bottles failed to continue growth after about 60 days. Treatment with 6-benzyladenine at various concentrations during the transfer produced no change in development. Spot applications of higher levels of the cytokinin directly onto the callus resulted in necrosis after 30-90 days, somewhat dependent on the concentration used. There was no evidence of any type of differentiation in any of the cultures.

In the case of *Stangeria eriopus*, differentiation was consequent on sub-culture. Thirty primary root tissue explants which gave rise to a fairly compact callus on SH medium with 4,5 x 10^{-6}M 2,4-D and kinetin were subcultured after 25 days onto similar medium in 25ml Erlenmeyer flasks. Fifteen of these subcultures were exposed to constant ($100\mu Em^{-2}s^{-1}$) light while the remainder were maintained in darkness. Each of the subcultures in the light environment developed a small green meristematic zone within 14 days (Plate 10a) followed by the emergence (Plate 10b) and expansion (Place 10c) of a typical circinate leaf. This pattern of morphogenesis was consistent in all cultures after transfer to the light environment, but did not occur in any of the flasks held in the dark. Attempts to re-establish plantlets in axenic conditions were unsuccessful due to necrosis following fungal infections.

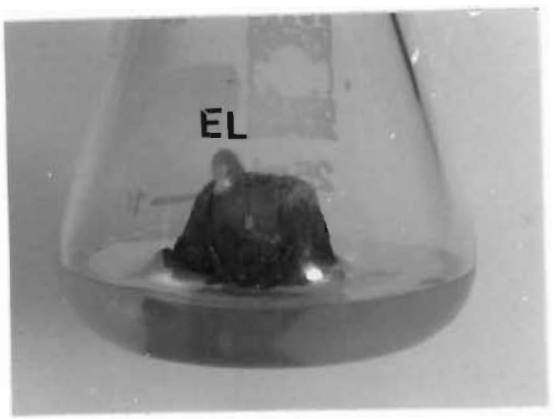
The above is the first instance of *in vitro* morphogenesis in a South African cycad. In view of the significance of these results, this experimentation has been separately reported (OSBORNE and VAN STADEN, 1987) in a paper which also comprises Appendix 4 to this thesis.

2.6.3 Leaflet explant cultures

The ready availability of leaf material, particularly of the rarer species of cycads, coupled with the indications from the work of HENSON (1980) that cycad leaf explant material offers good potential for *in vitro* culture, made it important to explore this avenue further. Explants of approximately $1cm^2$ were dissected from unblemished, surface-sterilized, juvenile and mature leaflets of 6 species



(a) Appearance of apical meristem (AM), two weeks after subculture and transfer to a light environment.



(b) Emergence of a circinate leaf (EL), one week subsequent to (a) above.

(c) Pronounced extension of the leaf petiole, two weeks subsequent to (b) above.

PLATE 10 : Light-induced morphogenesis from a primary root explant of *Stangeria eriopus* on SH medium supplemented with 1 mg ℓ^{-1} each of 2,4-D and kinetin. Scale x 2.

of *Encephalartos* and from *Stangeria eriopus*. These were set on different media in a variety of containers as shown in Table 20.

Despite a vigorous disinfection treatment, between 50 and 100% of the leaflet explant cultures showed fungal infection within 3 weeks. It is thought that the deeply-recessed leaf stomata act as reservoirs holding fungal spores and these cavities escape penetration by the disinfecting reagents (Section 2.4.3). Increasing the concentration of the reagents and the use of longer exposure times resulted in necrosis of the leaf tissue itself. This infection problem is not mentioned in HENSON's (1980) report.

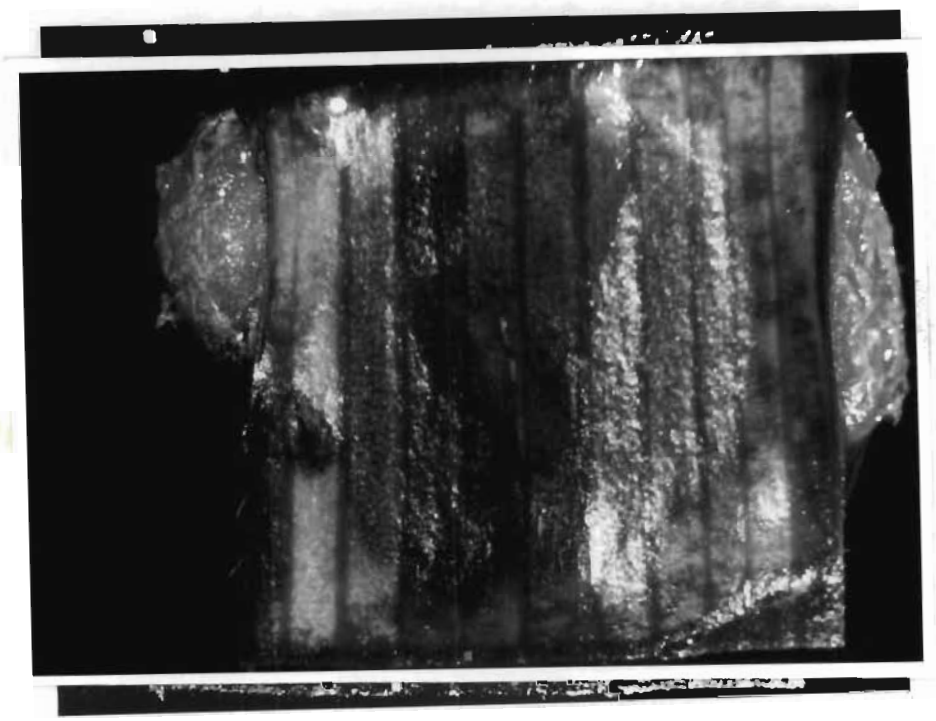


PLATE 11

Callus initiation on longitudinally-cut surfaces of a leaflet of *of Encephalartos woodii* after 37 days in culture on SH medium containing 5 x 10^{-5}M NAA and KN. Scale x 10.

TABLE 20 : *In vitro* culture of cycad leaflets

Species	No. of explants	Culture medium	Container	Light regime	Observations
E. natalensis (juvenile leaf)	10	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 5 explants in 70 days Remaining 5 cultures fungus-infected
E. natalensis (mature leaf)	20	SH, $4,5x10^{-6}$M 2,4-D and KN	Universal bottles	Dark	No response in 11 explants in 100 days Fungal-infection in 9 cultures
E. natalensis (seedling leaf)	10	MS, $4,5x10^{-6}$M 2,4-D and KN	Petri-dishes	Dark	Callus in 4 explants in 45 days Remaining 6 cultures fungus-infected
E. woodii (juvenile leaf)	4	SH, $4,5x10^{-6}$M NAA	25ml Erlenmeyers	Dark	Callus in 1 explant in 30 days Remaining 3 cultures fungus-infected
E. woodii (juvenile leaf)	4	SH, $5x10^{-5}$M NAA and KN	25ml Erlenmeyers	Dark	Callus in 1 explant in 30 days Remaining 3 cultures fungus-infected
E. woodii (juvenile leaf)	28	SH, various 2,4-D and KN combinations	Universal bottles	Dark	All cultures fungus-infected
E. woodii (juvenile leaf)	16	SH, various NAA and BA combinations	25ml Erlenmeyers	Dark	All cultures fungus-infected
E. woodii (juvenile leaf)	25	SH, no growth factors, explants pre-treated with 0 to $1x10^{-4}$M NAA for 4 hours	25ml Erlenmeyers and Universal bottles	Dark	All cultures fungus-infected
E. woodii (juvenile leaf)	50	59, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark and $100\mu Em^{-2}s^{-1}$, constant	No response in 12 explants in 100 days Remaining 38 cultures fungus-infected
E. cupidus (mature leaf)	15	MS, $4,5x10^{-6}$M BA	25ml Erlenmeyers and Universal bottles	Dark	No response in 9 explants in 100 days Remaining 6 cultures fungus-infected

TABLE 20 continued....

Species	No. of explants	Culture medium	Container	Light regime	Observations
E. eugene-maraisii (mature leaf)	12	MS, $4,5x10^{-6}$M BA	25ml Erlenmeyers	Dark	No response in 11 explants in 100 days Remaining culture fungus-infected
E. lehmannii (mature leaf)	25	SH, various NAA and BA combinations	25ml Erlenmeyers	Dark	No response in 18 explants in 100 days Remaining 7 cultures fungus-infected
E. inopinus (mature leaf)	25	SH, various NAA and BA combinations	25ml Erlenmeyers	Dark	No response in 13 explants in 100 days Remaining 12 cultures fungus-infected
Stangeria eriopus (mature leaf)	20	SH, $4,5x10^{-6}$M 2,4-D and KN	Universal bottles	Dark	No response in 18 explants in 100 days Remaining 2 cultures fungus-infected

Notes : SH, medium of SCHENK and HILDEBRANDT (1972)
MS, medium of MURASHIGE and SKOOG (1962)
59, medium of NORSTOG and RHAMSTINE (1967)
2,4-D, 2,4-dichlorophenoxyacetic acid
NAA, 1-naphthaleneacetic acid
BA, 6-benzyladenine

In those instances where fungal infection was absent, only the explants from juvenile leaflets or from seedling leaves had the potential for callogenesis; no callus formation occurred on any explants from mature, fully-expanded leaves. Callus formation was favoured by the incorporation of the auxins 2,4-D or NAA at low levels in the medium, and arose between the upper and lower epidermis along the cut surfaces of the explant (Plate 11). The callus required a longer period in initiation, and was more compact in appearance and less vigorous in growth than callus from megagametophyte, embryo or root tissues. In the limited experimentation on subculturing this material, callus proliferation could not be maintained; all explants with callus becoming necrotic in about 2 months.

2.6.4 Other explant cultures

In addition to the use of embryos, roots and leaflets as diploid tissues for *in vitro* culture, it was considered useful to explore other sources. Explants from leaf petioles, leaflet petiolules, leaf bases, cone axes and sporophylls of 5 species of *Encephalartos* were dissected and set on SH medium with various supplements, in either Universal bottles or Erlenmeyer flasks and incubated in the dark at 25°C as indicated in Table 21. Callogenesis occurred in varying frequency in all explants except those of the leaflet petiolules. The absence of any response with the latter explants may be due to the comparatively small explant size since leaf petioles, of similar ontogeny, did give at least some evidence of callus formation. The optimum performance in terms of frequency of callus development and time for this to occur, was found with explants from the cone axes and microsporophylls of *E. ferox* and *E. woodii*.

Those explants which had given rise to callus were subcultured on SH medium supplemented with $4,5 \times 10^{-6}$ M 2,4-D and kinetin. In all cases the callus continued to develop for a period of 1-2 months but subsequently became necrotic. No evidence of any form of differentiation was observed.

TABLE 21 : *In vitro* culture of other diploid tissues from *Encephalartos*

Species	Explant source	No. of explants	Culture medium	Container	Light regime	Observations
E. caffer	male cone axis	18	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	No response in 70 days
E. ferox	male cone axis	6	SH, no growth factors	Universal bottles	Dark	No response in 70 days
E. ferox	male cone axis	4	SH, $5x10^{-5}$M NAA	Universal bottles	Dark	Callus in 1 explant in 21 days
E. ferox	microsporophylls	4	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in all explants in 14 days
E. ferox	microsporophylls	4	SH, $5x10^{-5}$M NAA	25ml Erlenmeyers	Dark	Callus in all explants in 14 days
E. latifrons	leaf petioles	3	SH, $4,5x10^{-6}$M 2,4-D and KN	Universal bottles	Dark	Callus in 2 explants in 40 days
E. latifrons	leaf petioles	3	SH, $5x10^{-5}$M NAA	Universal bottles	Dark	No response in 70 days
E. natalensis	leaflet petiolules	30	SH, $4,5x10^{-6}$M 2,4-D and KN	Universal bottles and 25ml Erlenmeyers	Dark	No response in 70 days
E. woodii	leaf bases	8	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in 4 explants in 30 days
E. woodii	male cone axis	11	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in all explants in 21 days
E. woodii	microsporophylls	11	SH, $4,5x10^{-6}$M 2,4-D and KN	25ml Erlenmeyers	Dark	Callus in all explants in 21 days

Notes : SH, medium of SCHENK and HILDEBRANDT (1972) 2,4-D, 2,4-dichlorophenoxyacetic acid
NAA, 1-naphthaleneacetic acid KN, kinetin

2.7 Suspension cultures

A brief investigation was made into the feasibility of establishing viable suspension cultures from cycad material. Vigorously-growing haploid callus derived from *Stangeria eriopus* megagametophytic tissue (Section 2.5.1) was used as the starting material, and two procedures were used to initiate the suspension cultures.

In the first procedure, approximately 1g portions of the callus were macerated in a Dounce glass homogenizer in small quantities of the B5 liquid medium of GAMBORG, MILLER and OJIMA (1968). The crude suspensions were diluted with further portions of the medium and maintained on an orbital shaker. After 48 hours, the coarse aggregates were removed by filtration through 100µm nylon mesh, and the resulting suspensions subcultured to fresh medium. After five days aliquots from the cultures were plated onto solid media in Petri dishes and subsequently examined under an inverted microscope.

In the second procedure, similar quantities of the callus were suspended in liquid SH medium and incubated with cellulase and pectinase enzymes on a shaking table. After 48 hours, the cultures were centrifuged, washed and resuspended in fresh medium . Aliquots were withdrawn over a 14-day period and set on solid medium of the same composition in Petri dishes. In both cases outlined, further subcultures to fresh liquid media were made at intervals and the progresses monitored by all density counts. Both the mechanical and the enzymatic preparations gave rise to suspensions with single cells and aggregates of up to 10 cells. These did not proliferate during the two week period; all cell density counts on subculturing were simply related to solution dilution factors. Plating out of aliquots on solid media appeared to promote cell division and growth; within about 2-3 weeks aggregates of 100 cells were visible. There was no evidence of any organization within these cell clusters which subsequently slowly became necrotic.

2.8 Conclusion

Attempts have been made to establish *in vitro* cultures, using various haploid and diploid cycad source material, on various media with

different hormone supplements and exposed to different environmental conditions. Effective surface disinfection of leaflets proved difficult but other explant material was conveniently disinfected with sequential 70% ethanol and 0,75% sodium hypochlorite treatments. Callus production was readily achieved from most explant sources of most cycad species tested. Crisp white callus generally arose within twenty to thirty days on cut surfaces of these explants. There was no particularly significant difference in the responses on MURASHIGE-SKOOG (1962), NORSTOG and RHAMSTINE (1967) and SCHENK and HILDEBRANDT (1972) basal media. Addition of an auxin and a cytokinin appeared beneficial but not essential; inclusion of 2,4-D and kinetin in the 10^{-7} to 10^{-6}M range gave a generally satisfactory response. The selection of culture vessels was important; containers which allowed a relatively free gaseous exchange lead to a higher frequency of callogenesis than containers where gaseous exchange was inhibited. Dark conditions appeared to give a marginally greater success rate than constant light. Cycad taxa which are mesic in natural habitat gave more rapid responses at higher frequencies than their xeric counterparts. Differences in responses between juvenile and mature leaflet explants implied that physiological age of the explant tissue is important. Similarly, differences in response from explants taken at various positions in primary root tissue may imply the existence of endogenous hormone gradients in the root.

Attempts to induce any form of differentiation on the callused explants met with little success. Subculture to media with different hormone supplements, and environmental changes, both failed to induce any form of morphogenesis in all cultures from *Encephalartos*. Some degree of success was achieved with *Stangeria*-derived cultures. Subculture of megagametophytic callus lead occasionally to the formation of spherical structures which may be coralloid root primordia. Subculture of callused *Stangeria* primary root explants to SCHENK and HILDEBRANDT (1972) medium with $4,5 \times 10^{-6}$M 2,4-D and kinetin, associated with simultaneous transfer from a dark to a constant light environment lead to the appearance of meristematic zones and subsequent leaf appearance. This is the first known report of *in vitro* morphogenesis of a South African cycad. It is clear that the aim of establishing a

protocol for the clonal propagation of cycads by *in vitro* techniques, is an ambitious one. Some considerable effort has resulted in only a very modest degree of success, and a large element of serendipity makes the design of any project of this nature somewhat "open-ended". It is possible that future advances in tissue culture techniques, perhaps associated with a much wider understanding of the physiological role of plant growth regulators and environmental interactions, may lead to a more predictable control over morphogenesis.

In order better to understand the physiology of cycads, the section of work which follows was directed primarily at exploring at least some aspects of cycad phytochemistry. Comparative chemical and biochemical analyses both of different organs and between different taxa may provide evidence of fundamental differences which may in turn relate to different *in vitro* responses. Thus the question as to why *Stangeria* root explants give rise to meristematic zones in culture when the identical material from other cycads does not, may be answered in terms of inherent biochemical differences.

Apart from the obvious benefits of establishing *in vitro* propagation systems, researches combining this work with phytochemical investigations may yield clues to the mechanism of sex-expression and hence offer a means of sex control. The creation of a female clone of *Encephalartos woodii* may eventually be possible. *In vitro* systems may also lead to a better understanding of the cycad-cyanobacterial root symbiosis and to an assessment of conditions influencing pollen viability. Yet a further application would be the establishment of haploid cultures of all cycad taxa for the purposes of detailed karyological work. It is important that further projects of this kind are encouraged. The value of systems for the propagation of endangered plants remains unquestionable.

CHAPTER THREE

CYCAD PHYTOCHEMISTRY

INDEX TO CHAPTER THREE

3.1 Introduction

Despite the significant evolutionary position of the Cycadales, little work has been done to investigate the phytochemistry of the order until fairly recently. Impetus here was provided by the discovery of the first of a series of unique toxins in cycad seeds in the 1940's (section 3.2). More recently, some investigations have been made into cycad phenolics, flavonoids, carbohydrates and other compounds (section 3.3). Although most of this work is founded on chemo-taxonomic objectives, the data contribute to our knowledge of cycad physiology and further relate to developmental morphology. In the experimental section of this Chapter (sections 3.4 to 3.10), the author reports on various phytochemical investigations both of different organs and between different taxa. Integration of these various researches will undoubtedly result in a better understanding of the fundamental factors controlling morphogenetic competence *in vitro*.

3.2 Cycad phytotoxins

Cycads have been used as a source of edible starch in different parts of the world for many years, particularly in circumstances when normal food supplies have been restricted. The 'arrowroot' starch, generally obtained from rhizomes of *Marantha*, can also be extracted from *Zamia* roots while 'sago' starch, usually derived from the stem of certain palms, is also harvested from *Cycas*, *Zamia* and *Macrozamia* plants (THIERET, 1958; WHITING, 1963). The Cape Hottentot people produced a palatable form of bread from the stems of certain *Encephalartos* species (DYER, 1965), a process which gave rise both to the vernacular name 'broodboom' and to the genus name (*en* = within, *kephali* = head, *artos* = bread).

Although the vegetative parts of certain species can provide a source of starch, this harvest is invariably destructive to the plant. By contrast, the seed crop presents a food source which is both readily and repeatedly collectable. However, it is in the starch-rich megagametophytes where cycad toxins are present at their highest concentrations. It is quite remarkable that many indigenous people in different parts of the world have apparently quite independently found out how to detoxify

the seeds. All 'recipes' are based on steeping the crushed seeds in water which allows degradation and extraction of the toxins (WHITING, 1963; OSBORNE, 1985a). Improperly detoxified or untreated seed have caused severe illness and sometimes death to humans and to livestock. Interesting circumstantial evidence of cycad toxins was recorded by the botanist Banks, who accompanied Captain Cook's expedition. In 1770 a number of the crew suffered from a violent fit of vomiting and two of the ship's hogs died after eating seed of *Cycas media* R. Br. (THIERET, 1958). Another notable case was that of General J.C. Smuts and his Commando who were incapacitated for several days during the South African War of 1899 - 1902 when they resorted to cycad seed for emergency food supplies (REITZ, 1969), the plant responsible believed to be *Encephalartos longifolius* (Jacq) Lehm. (DYER, 1965). The high post-war incidence of amyotrophic lateral sclerosis and associated conditions such as Parkinsonism and Alzheimer's disease amongst the Chamorro peoples on the Marianas islands of Guam and Rota has been ascribed to the accumulated effect of many years' consumption of *Cycas circinalis* seeds in which complete removal of the toxin is never certain (PALEKAR and DASTUR, 1965; SPENCER, NUNN, HUGON, LUDOLPH, ROSS, ROY and ROBERTSON, 1987). Evidence that it is the cycad seed which is responsible is provided by the comparatively low incidence of the aforementioned diseases on the neighbouring island of Saipan, where the cycad populations have been cleared for agricultural purposes, and by the declining frequency of the diseases as the Chamorro population has become westernized and dietary habits have changed. (SPENCER, NUNN, HUGON, LUDOLPH, ROSS, ROY and ROBERTSON, 1987).

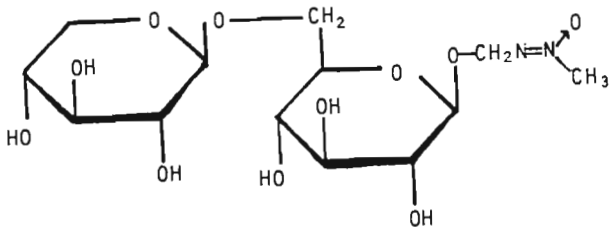
The outer fleshy layer and/or the megagametophyte of the South African species *Encephalartos cycadifolius*. *E. eugene-maraisii*, *E. ferox*, *E. horridus*, *E. lehmannii*, *E. longifolius* and *E. villosus* have either proved to be acutely toxic when administered to rabbits or have been suspected or known to be poisonous when ingested by man (STEYN, VAN DER WALT and VERDOORN, 1948). Toxic and carcinogenic effects have been observed in rats fed with various parts of the female cones of *E. umbeluziensis*, *E. villosus*, *E. lebomboensis* and *E. laevifolius* (TUSTIN, 1974). The kernels of *E. lanatus* have also been recognized as being toxic and carcinogenic (TUSTIN, 1983).

It is not only the cycad seeds which are toxic. Grazing by stock on

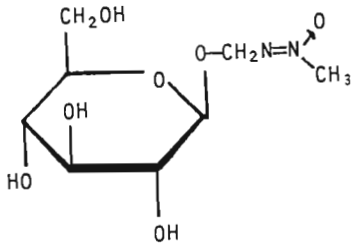
leaves of certain *Macrozamia* species in Australia and *Zamia* species in the Americas has resulted in the partial or total paralysis of the hind legs, a condition known as the 'wobbles' or 'staggers'. Whilst this rarely kills the animals directly, they are unable to find food and water and perish as a result. The Australian Government has embarked on quite extensive eradication programmes to remove the offending species, particularly *Macrozamia moorei* F. Muell. but fortunately a specific reserve has been set aside to prevent the species becoming extinct (OSBORNE, 1985a). The active principles fall into two groups, the methylazoxymethanol glycosides and at least one non-protein amino acid. Of these two, the former have received considerable attention while the latter has more recently been reinvestigated following earlier reports.

The methylazoxymethanol glycosides

COOPER (1940) obtained a crystalline substance, which he named macrozamin, from the seeds of the Australian cycad *Macrozamia spiralis* (Salisb.) Miq. This compound was shown to comprise a methylazoxylmethanol unit linked to a primeverose sugar residue (LYTHGOE and RIGGS, 1949; LANGLEY, LYTHGOE and RIGGS, 1951), (Figure 13, structure 36). Macrozamin was isolated from South African species *Encephalartos lanatus* and *E. transvenosus* (ALTENKIRK, 1974). A similar toxin, cycasin (Figure 13, structure 37) consists of the same aglycone unit linked to D-glucose and was found initially in *Cycas revoluta* Thunb. (NISHIDA, KOBAYASHI and NAGAHAMA, 1955) and *C. circinalis* L. (RIGGS, 1956; KORSCH and RIGGS, 1964). Although macrozamin and cycasin are the major cycad toxins, several closely-related substances, the neocycasins (Figure 13, structures 37a to 37g) have been isolated from *Cycas* species; all share the common methylazoxymethanol aglycone with a β-glycosidic linkage to a variety of different sugar residues (NAGAHAMA, 1964). Cycasin and macrozamin have now been found in all ten cycad genera but not in any other plants (DE LUCA, MORETTI, SABATO and SINISCALCO GIGLIANO, 1980; MORETTI, SABATO and SINISCALCO GIGLIANO, 1981a, 1983). Macrozamin is generally more abundant than cycasin, occurring in levels from about 0,2 to 5% by fresh weight in mature seeds. The range in concentrations is genus dependent and thus has chemotaxonomic significance. In the South African cycads, *Encephalartos* seed have 2,09 to 2,86% macrozamin while *Stangeria eriopus* contains 4,70% (MORETTI, SABATO and SINISCALCO GIGLIANO, 1983).



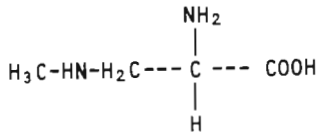
(36) MACROZAMIN *
= (Methyl-ONN-azoxy) methyl
or Methylazoxymethanol
β - primeveroside



(37) CYCASIN
= (Methyl-ONN-azoxy) methyl
or Methylazoxymethanol
β-D-glucopyranoside

(37a)	NEOCYCASIN A	=	Methylazoxymethanol β-laminaribioside
(37b)	NEOCYCASIN B	=	Methylazoxymethanol β-gentiobioside
(37c)	NEOCYCASIN C	=	Methylazoxymethanol β-laminaritetraoside
(37d)	NEOCYCASIN D	=	Methylazoxymethanol β-laminaritrioside
(37e)	NEOCYCASIN E	=	Methylazoxymethanol β-cellobioside
(37f)	NEOCYCASIN F	=	Cycasin 6-O-β-laminaribioside
(37g)	NEOCYCASIN G	=	Cycasin 3-O-β-gentiobioside

* Note : In (36) and (37) above, the trivial names are followed by a full chemically-descriptive name defined by IUPAC rules, and an abridged alternative. The names β-primeverosyloxyazoxymethane for (36) and β-D-glucosyloxyazoxymethane for (37) are also encountered in the literature.



(38) α-AMINO-β-METHYLAMINOPROPIONIC ACID
= β-N-methylamino-L-alanine, BMAA.

FIGURE 13 : Structure and nomenclature of cycad phytotoxins.

Whilst references to the biosynthesis of these natural products are lacking, there has been some work on the toxic action. The glycosides are hydrolysed soon after ingestion by vertebrate animals by the action of the enzyme β-glycosidase in the gut; it is the resulting aglycone and its subsequent decomposition products which are toxic. Evidence for this is provided by the fact that cycasin is non-toxic when administered orally to germ-free rats (SPATZ, SMITH, McDANIEL and LAQUEUR, 1967) and is similarly without ill-effect when injected directly into the bloodstream (SEELY, FREED, SILVERSTONE and RIPPERE,

1985). Oxidative degradation of the aglycone methylazoxymethanol gives methylazoxyformaldehyde, methylazoxycarboxylic acid and the methyldiazonium ion as principle metabolites; the carcinogenicity is thought to arise from the methylating action of one or more of these units on DNA residues (KIKUCHI, KARASAWA, SUZUKI and HOPFINGER, 1982). It is of interest to note that certain insects which feed on cycads, notably the hairstreak butterfly *Eumaeus atala florida* Roeber which eats *Zamia* foliage, accumulate fairly large quantities of the toxins internally (ROTHSCHILD, NASH and BELL, 1986). It is believed that the compounds remain in the glycosylated form and are therefore harmless to the insect but a valuable defence mechanism; on ingestion by a predator, the molecules are hydrolysed and exert their toxic influence (ROTHSCHILD, M., *pers comm.*).

α-amino-β-methylaminopropionic acid

The compound α-amino-β-methylaminopropionic acid (synonym β-N-methylamino-L-alanine or BMAA, Figure 13, structure 38) was first isolated from seeds of *Cycas circinalis* L. (VEGA AND BELL, 1967). It was later shown to be widespread in free or bound form in seeds and leaves throughout the genus but not in any other cycad genera (DOSSAJI and BELL, 1973). Biological activity of this toxin is confined to the L-isomer which is toxic to chicks, rats and mice (VEGA, BELL and NUNN, 1968) and causes neurological disorders to macaque monkeys (SPENCER, NUNN, HUGON, LUDOLPH, ROSS, ROY and ROBERTSON, 1987). These authors speculate that it is the amino acid derivative, rather than the azoxy compounds, which is responsible for the cycad-associated neurological diseases in the Pacific Islands.

3.3 Other phytochemical aspects

While the major biochemical interest in cycads has been focussed on their phytotoxins (Section 3.2), a number of other phytochemical investigations have been carried out and many of these are significant in application to cycad taxonomy. The older work is summarized in the review of THIERET (1958), which includes a discussion of the somewhat limited uses of cycad material as sources of fibres, gums and oils. An entry into the current phytochemical literature of the group is provided by HEGNAUER (1986).

Phenolic compounds and flavanoids

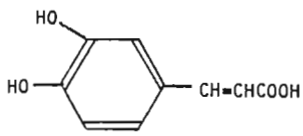
In a survey of leaves of 22 species of cycads, WALLACE (1972) found evidence of caffeic, protocatechuic, p-coumaric, p-hydroxybenzoic, ferulic and vanillic acids (Figure 14, structures 39-44) in all examples. Since these compounds are ubiquitous in the pteridophytes, gymnosperms and angiosperms, their presence is of limited significance.

Sinapic acid (Figure 14, structure 45) occurred in *Dioon spinulosum* Dyer and *Ceratozamia mexicana* Brongniart and possibly other taxa while 2,4-dihydroxybenzoic acid (Figure 14, structure 46) was found in two species of *Encephalartos* and in *Bowenia serrulata* (W. Bull) Chamberlain. Syringic acid (Figure 14, structure 47) was tentatively identified in *Ceratozamia mexicana*. The presence of sinapic and syringic acids in cycads indicates that the distribution of these compounds in plants is wider than thought previously (WALLACE, 1972).

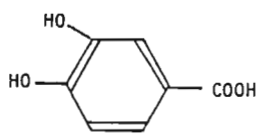
The biflavonoid chemistry of cycads has attracted a certain amount of attention, principally because of the taxonomic implications. A survey of biflavones in the leaves of 82 species has shown that the pattern of occurrence of amentoflavone, hinokiflavone (Figure 15, structures 48-49), their methyl ethers and other derivatives is consistent within most genera (DOSSAJI, MABRY and BELL, 1975). Leaf biflavones are confined to the outer periclinal wall and anticlinal walls of epidermal cells and their presence is believed to contribute to the plant's defense against microbial and predator attack (GADEK, QUINN and ASHFORD, 1984). The biflavonoid distribution in the seed testae is quite different to that in the leaves. In an analysis of 8 species, GADEK (1982) found that representatives from *Macrozamia* are characterised by the occurrence of cupressuflavone and amentoflavone derivatives while *Cycas* species contained only the amento-flavone-based compounds. *Encephalartos*, *Lepidozamia* and *Zamia* samples gave only trace amounts of biflavonoids. The occurrence of cupressuflavone (Figure 15, structure 50) is unusual outside the gymnosperm families Cupressaceae and Araucariaceae. An unusual feature is the complete absence of biflavonoids in *Stangeria* (DOSSAJI, MABRY and BELL, 1975).

Carotenoids

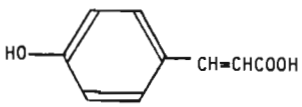
The highly-coloured seed coats of cycads contain simple carotenoid mixtures. The bright yellow seed coat of *Cycas revoluta* has zeaxanthin as the major component with smaller amounts of cryptoxanthin and β-carotene (Figure 16,



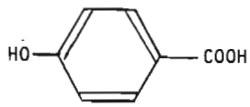
(39) CAFFEIC ACID



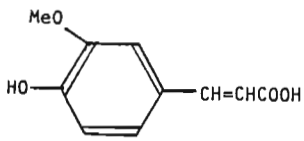
(40) PROTOCATECHUIC ACID



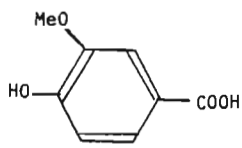
(41) p-COUMARIC ACID



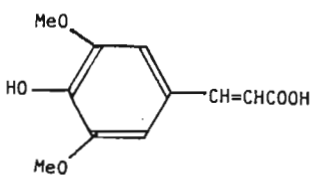
(42) p-HYDROXYBENZOIC ACID



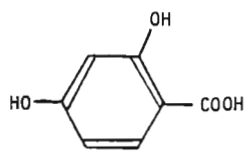
(43) FERULIC ACID



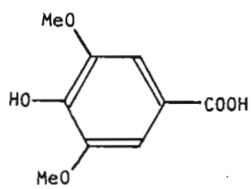
(44) VANILLIC ACID



(45) SINAPIC ACID

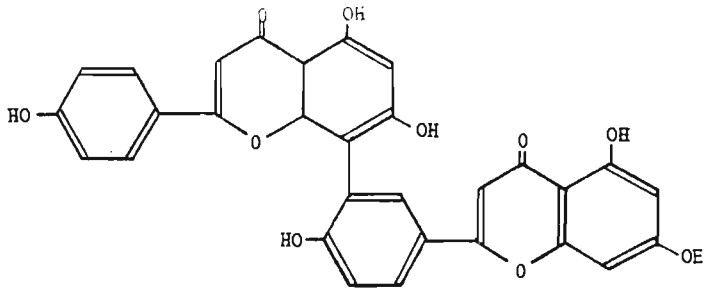


(46) 2,4-DIHYDROXYBENZOIC ACID

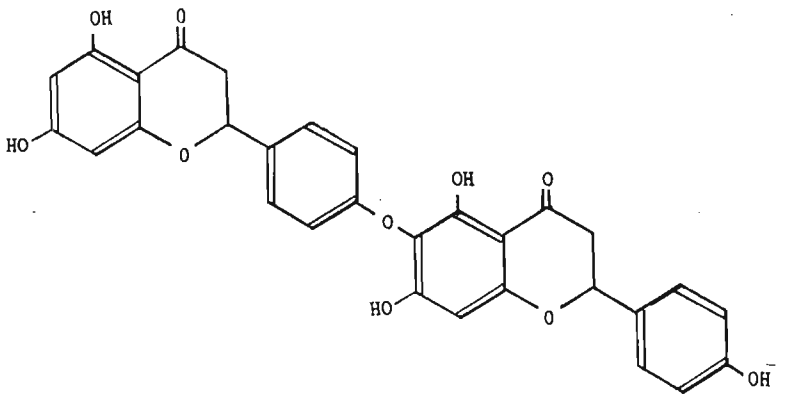


(47) SYRINGIC ACID

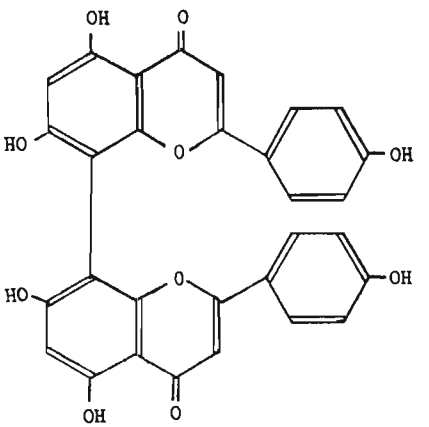
FIGURE 14 : Some simple phenolic compounds isolated from cycads.



(48) AMENTOFLAVONE

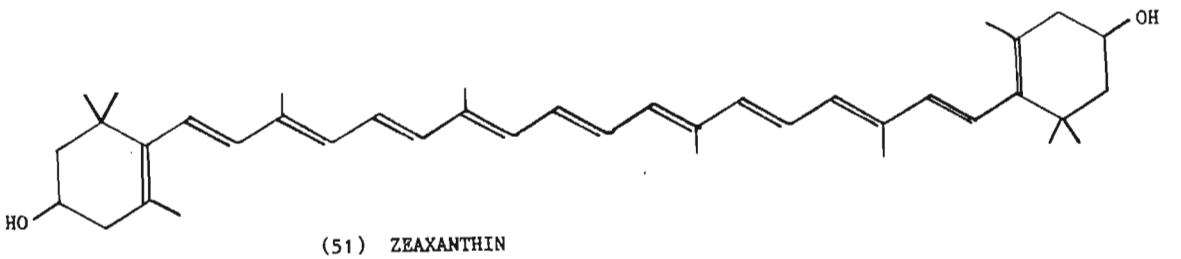


(49) HINOKIFLAVONE

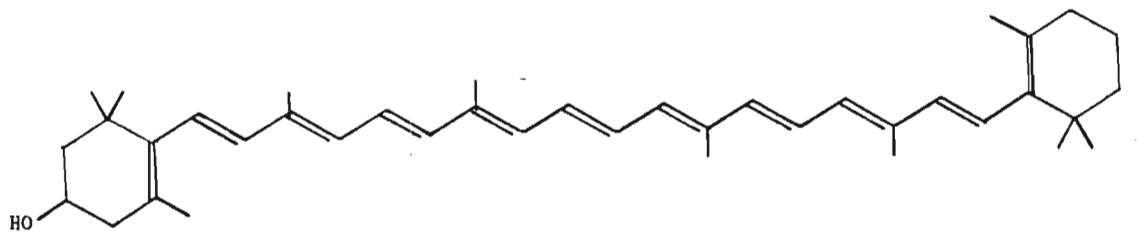


(50) CUPRESSUFLAVONE

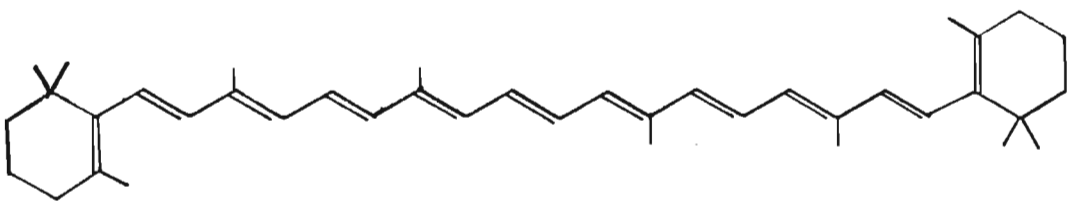
FIGURE 15 : Some biflavonoids isolated from cycads.



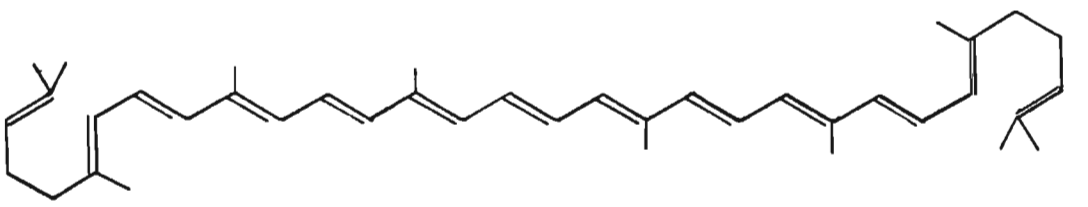
(51) ZEAXANTHIN



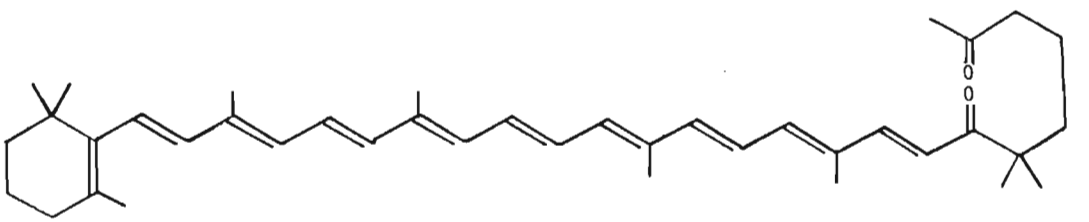
(52) CRYPTOXANTHIN



(53) β-CAROTENE



(54) LYCOPENE



(55) SEMI-β-CAROTENONE

FIGURE 16 : Some carotenoids isolated from cycads.

structures 51-53) (BOUCHEZ, ARPIN, DERUAZ and GUILLUY, 1970). The seed coat of *Zamia* has lycopene (Figure 16, structure 54) as the principal pigment while extracts from *Encephalartos*, *Dioon* and *Macrozamia* contained a mixture of unsubstituted mono- and dihydroxy- β- carotenes (BAUMAN and YOKOYAMA, 1976). An attractive red-brown colouration, characteristic of emergent *Ceratozamia* leaflets, has been ascribed to the presence of semi-β-carotenone (Figure 16, structure 55), an unusual secocarotenoid found previously only in the fruits of the citrus relative, *Murraja exotica* (CARDINI, GINANNESCHI, SELVA and CHELLI, 1987).

Carbohydrates

All cycads have a well-developed system of mucilage ducts and excision of the leaf rachis or cone peduncle, or injury to the caudex allow collection of the exudate. This mucilage consists of a complex polysaccharide which may be hydrolysed to its component sugars. The exudate from a female cone of *Encephalartos longifolius* yielded fucose, rhamnose and 3-O-methyl rhamnose, arabinose, xylose, galactose, mannose, glucuronic acid with its 4-O-methyl ether (Figure 17, structures 56-62) (STEPHEN and DE BRUYN, 1967).

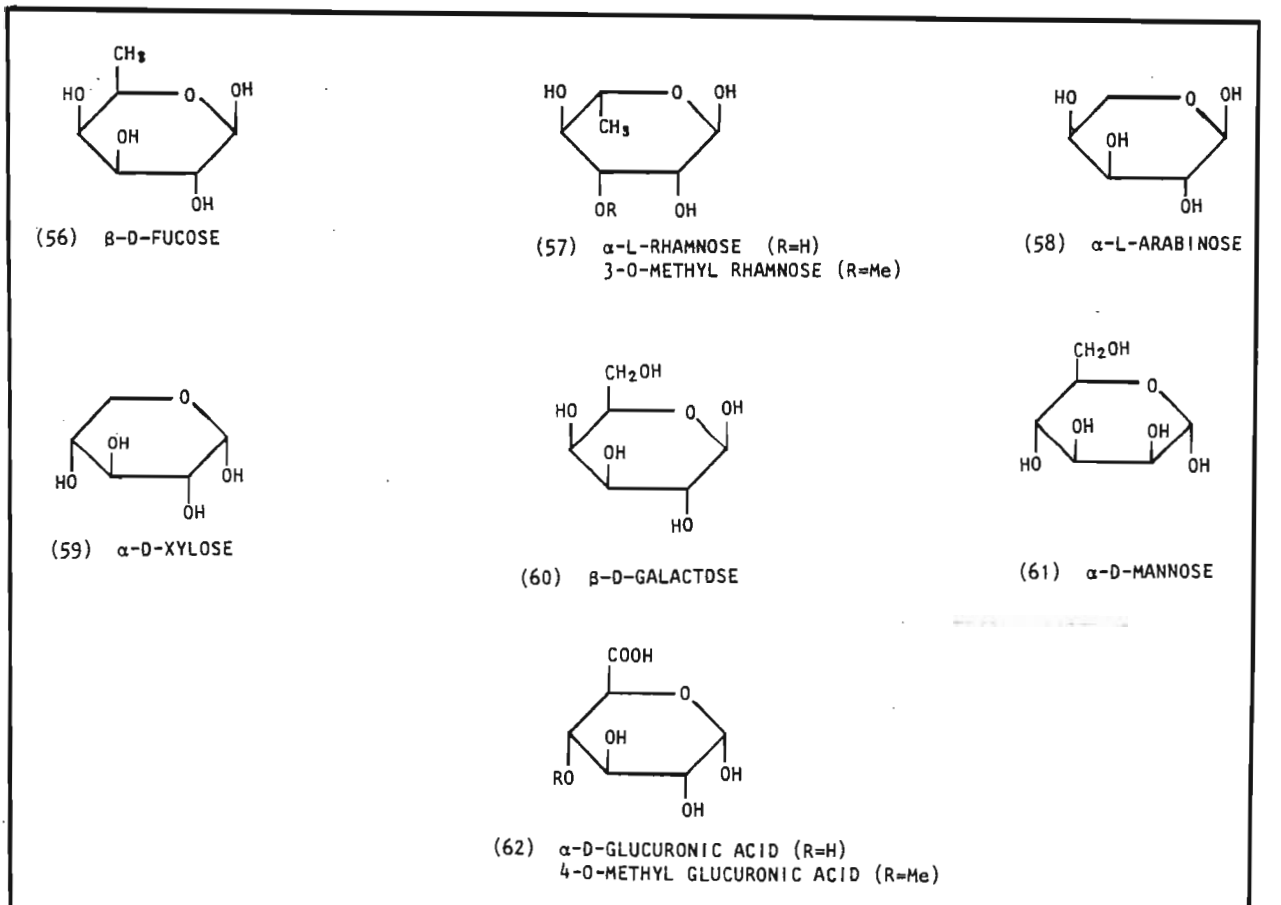


FIGURE 17 : Monosaccharides obtained on hydrolysis of cycad mucilages.

In a survey of hydrolysed mucilages from excised leaf raches of 21 cycad species DE LUCA, MORETTI, SABATO and SINISCALCO GIGLIANO (1982) showed the taxonomic usefulness of these data at a generic level. The African and Australasian genera contain mainly arabinose and galactose while the American genera have higher proportions of fucose and galactose in *Dioon*; rhamnose and arabinose in *Ceratozamia*; rhamnose, fucose and methylrhamnose in *Zamia*; and galactose and methylrhamnose in *Microcycas*. *Stangeria* is unusual in having virtually no rhamnose or methylrhamnose. *Lepidozamia* and *Encephalartos* are the only genera which show similar patterns. With *E. longifolius* at least, the monosaccharide pattern is not significantly affected by plant age, sex or environment (SINISCALCO GIGLIANO, 1980). A more comprehensive survey of the genus *Encephalartos* has shown a more-or-less identical monosaccharide pattern in 14 species investigated (MORETTI, SABATO and SINISCALCO GIGLIANO, 1981b). Analyses of the exudates from cones of three species of *Encephalartos* have highlighted certain structural features (STEPHENS and STEPHEN, 1988). One is the occurrence in varying amounts of 3-O-methyl-L-rhamnose which, together with the parent L-rhamnopyranose unit, can comprise more than 20% of the total carbohydrate composition. Another is the high proportion of acidic units, notably D-glucuronic acid and its related 4-methyl ether. Sequential degredation of the polysaccharide has lead to the postulated structure shown in Figure 18.

Cyclitols

The occurrence of polyhydroxycyclohexanes (cyclitols) is common in gymnosperms. In the cycads, the compounds myo-inositol, sequoyitol and pinitol (Figure 19, structures 63-65) have been found. In particular, sequoyitol has been isolated from leaves of several species of *Cycas*, *Encephalartos*, *Lepidozamia* and *Ceratozamia* (PLOUVIER, 1965) and it is extracted together with macrozamin from the seeds of *Macrozamia riedlei* (Gaud.) C.A. Gardn. (CANNON, RASTON, TOIA and WHITE, 1980).

Enzymes

Despite the wide application of enzymological and serological techniques in biological investigations, little attention has been paid to their use in cycad research. MERRIAM (1974) studied the *Zamia* populations in Florida using isoenzyme banding systems and found the peroxidases to provide useful 'fingerprints'. An indication was that the Florida *Zamias* comprise one

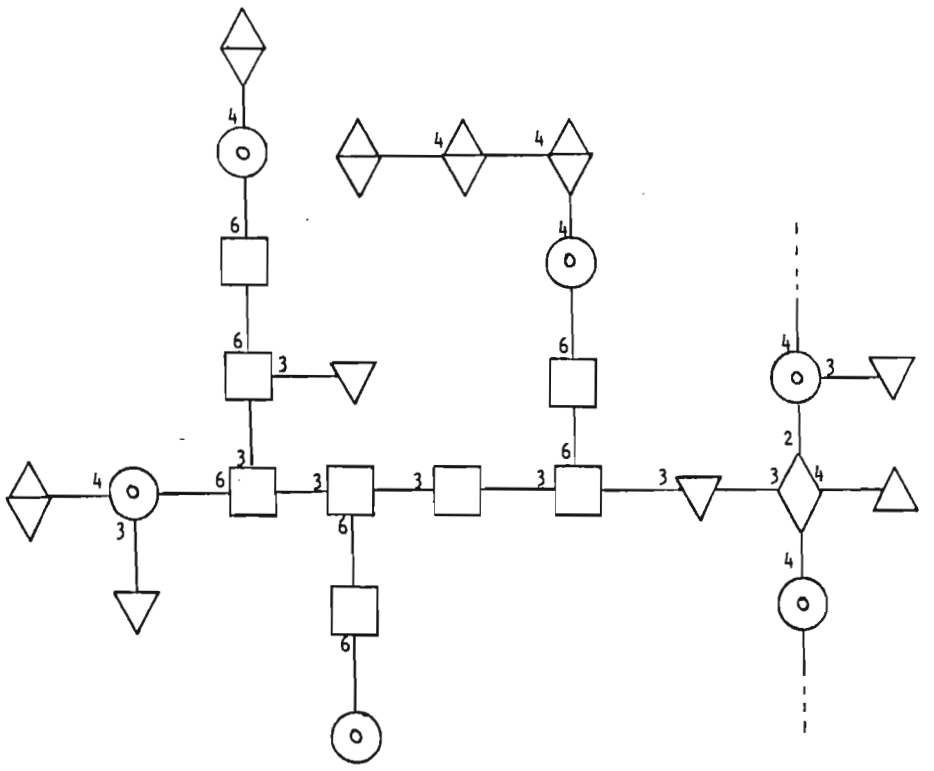


FIGURE 18 : A proposed structure for the polysaccharide in *Encephalartos* cone exudate. Numbers indicate linkage configurations where known; legend as below (STEPHENS and STEPHEN, 1988).

α-L-RHAMNOSE

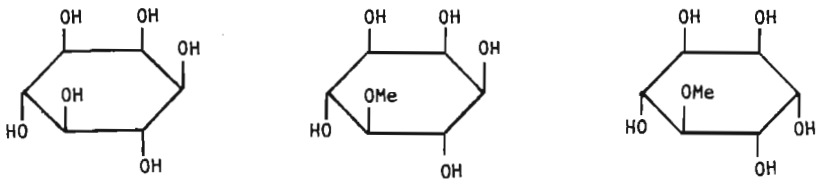
β-D-MANNOSE

α-L-ARABINOSE

β-D-GALACTOSE

β-D-XYLOSE

β-D-GLUCURONIC ACID



(63) MYO-INOSITOL (64) SEQUOYITOL (65) D-PINITOL

FIGURE 19 : Cylitols isolated from cycads.

polymorphic species. A study of the peroxidase isoenzymes in the seeds of *Cycas circinalis* showed a difference in the enzyme pattern at different stages during seed maturation (PENA, GRILLO and PEREX, 1983).

Other compounds

Mention of other compounds isolated from cycads is scattered sparsely in the literature. The bibliography of READ and SOLT (1986) and the review by HEGNAUER (1986) are useful reference sources. The reviews by OSBORNE (1986b, Appendix 2) and OSBORNE, GROBBELAAR and VORSTER (in preparation), Appendix 5) may also be consulted in this regard.

3.4 Analysis of moisture content

3.4.1 Materials and methods

An appropriate mass of the sample material (*ca.* 2 g) was cut into smallish portions and oven dried at 105°C to constant mass, which was generally achieved within 12 hours. The percentage loss in mass is reported as moisture although this is more correctly "moisture and volatiles".

3.4.2 Results and discussion

Although the purpose of the moisture determinations was primarily to enable expression of other analytical results on a dry basis, the data also provided some inherently useful information. The results are recorded in Table 22.

ROBERTS (1973) classifies seeds into two major groups described as *orthodox*, normally desiccated prior to dispersal and with moisture contents reduced to the 10-15% range, and *wet* or *recalcitrant* seeds which retain high moisture levels and in which the embryo continues to develop. In this classification the cycad seeds fall into the recalcitrant group. The analyses recorded show that cycad megagametophytes at the time of seed fall have moistures in the 41 - 55% range and that partial dehydration to 32 - 42% occurs during storage. However, the enclosed embryos appear protected from desiccation and retain relatively high moisture levels (*ca.* 64%). This indeed would be the anticipated situation in all recalcitrant seeds as any significant dessication of the continuously developing embryo itself would lead to loss of viability.

TABLE 22 : MOISTURE CONTENT IN CYCAD MATERIAL

CYCAD SPECIES	SAMPLE MATERIAL USED	NUMBER OF SAMPLES	MEAN MOISTURE CONTENT, %
E. lebomboensis	mature fresh seed, sarcotesta	1	73,5
	mature fresh seed, megagametophyte	1	43,8
E. natalensis	3-year old seedling, leaf	1	60,5
	6-year old plant, emergent leaflet	1	81,1
	6-year old plant, emergent leaf rachis	1	87,7
	mature plant, mature leaflet	2	49,5
	3-year old seedling, primary root	2	91,8
	mature fresh seed, sarcotesta	1	57,3
	mature fresh seed, megagametophyte	1	54,6
	mature stored seed, megagametophyte	10	41,8
	mature stored seed, embryo	4	64,2
E. villosus	fresh infertile seed, megagametophyte	2	41,6
	mature stored seed, megagametophyte	1	36,8
	mature stored seed, embryo	1	64,4
E. woodii	mature plant, emergent leaflet	2	72,0
	mature plant, emergent leaf rachis	2	88,4
	mature plant, lateral roots	2	76,2
S. eriopus	mature fresh seed, megagametophyte	2	50,0
	mature stored seed, megagametophyte	1	32,2
	callus culture ex megagametophyte	2	92,1
Cycas revoluta	mature plant, mature leaflet	1	58,4
Cycas thouarsii	mature plant, mature leaflet	1	67,6
Dioon edule	mature plant, mature leaflet	1	43,9
Lepidozamia peroffskyana	3-year old seedling, leaflet	1	70,4
Zamia furfuracea	3-year old seedling, leaflet	1	65,4

It is also reasonable to assume that any moisture deficit in the mega-gametophyte would be reversed by imbibition processes prior to germination. The fact that germination occurs naturally during the wet summer periods reinforces this hypothesis.

The findings outlined above appear to be consistent with a report by FORSYTH and VAN STADEN (1983) where *Encephalartos natalensis* seed were found to have 48% moisture when shed and, when exposed after storage to moist germination-inducing conditions, rapidly imbibed water to establish a final moisture content of 66%.
Another point of interest is the change in moisture content of leaf material from the time of emergence to maturity. The figures from *E. natalensis* show the emergent leaflet and rachis with moisture levels of 81,1 and 87,7% respectively while a fully-mature leaflet yields 49,5%. Fully-expanded seedling leaves appear to be intermediate in this range. The moisture content may thus be a useful indicator of leaflet physiological age and hence callogenic and morphogenic competence.

3.5 Analysis for soluble protein content

3.5.1 Materials and methods

The determination of soluble protein is an important routine biochemical test. A commonly-used technique is the spectrophotometric method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951) which makes use of the Folin and Ciocalteau reagent. A known mass of fresh material (e.g. 1,00 g) was ground in a mortar with a suitable volume (e.g. 9,0 mls) of pre-cooled (4°C) extraction buffer (0,076 M Tris, 0,005 M citric acid, pH 8,65; POULIK, 1957). The macerate was centrifuged at 50 000 x g for 30 minutes at 4°C. The clear supernatant solution was used for the soluble protein determinations, the peroxidase analyses (Section 3.6) and for serological work (Section 3.8). For the soluble protein determination the extract was diluted x 5, with buffer solution, prior to analysis.

A series of standard solutions providing 10 - 20 µg protein was prepared using bovine serum albumin (Merck Art. 12018). The alkaline reagent comprised 1 ml 1% $CuSO_4.5H_2O$ and 1 ml 2% potassium sodium

tartarate to which 100 ml 2% Na_2CO_3 in 0,1 M NaOH was added. Sample aliquots (e.g. 0,1 ml. equivalent to 2 mg fresh material in the example) were made up to a volume of 1,0 ml. Standard solutions were similarly treated. The alkaline reagent (5,0 ml) was added and the mixture left for 10 minutes, after which 0,5 ml dilute (1:2) Folin reagent was added. The absorbance was read at 660 nm in 1 cm glass cells after 30 minutes against a reagent blank. The protein amounts were derived from a calibration curve and expressed as mg total soluble protein per g fresh weight of sample material.

3.5.2 Results and discussion

The protein analysis figures are recorded in Table 23 together with the corresponding peroxidase figures (Section 3.6), thus also allowing expression of peroxidase/protein ratios. The soluble protein contents vary widely. Emergent leaves and 1 - 2 year old seedling leaflets show generally lower levels than fully-expanded leaves or those from older seedlings and mature plants. Thus accumulation of soluble protein appears to be a combined function of leaf and plant age. There is no particular correlation between protein levels and taxonomic rank or plant habit. The highest leaf protein amount recorded (200 mg g^{-1}) was that from a mature leaf from a mature plant of *Dioon edule*. High protein contents were also observed in megagametophytes and cone scales, in particular the microsporophylls from *Encephalartos woodii* provided 232 mg g^{-1}. Generally low soluble protein concentrations (< 10 mg g^{-1}) were found in seed sarcotestae, embryos and roots. In the primary roots there is some evidence of a protein gradient from top to bottom. The material in culture does not appear to show any significant protein accumulation at callus-growth stages.

There are few references with which to compare these results. The 'seed kernels' of *Cycas revoluta* are said to contain 12 - 14% crude protein and 66 - 70% starch on a fresh weight basis, while the stem of this species has 9,15% protein on a dry basis (THIERET, 1958). In an Australian cycad, *Macrozamia riedlei*, the megagametophyte on dry basis is reported as containing 13% protein and 60,7% starch. No details have been published on the composition of South African cycads.

3.6 Analysis of peroxidase enzyme activity

3.6.1 Materials and methods

Determination of total soluble peroxidase activity

Preparation of the sample material is described in Section 3.5.1. The clarified extract was again diluted x 5 with buffer solution prior to analysis.

The assay for total soluble peroxidase activity follows a method modified from that of GASPAR, BERVILLE and DARIMONT (1974). Aliquots of 0,1 ml of this solution (equivalent to 2 mg material in the example given) were added to 8,0 ml phosphate buffer (pH 6,1), 1,0 ml o-phenylenediamine (1% m/v) and 1,0 ml hydrogen peroxide (0,2 volumes) and the absorbance read at 449 nm after exactly 2 and 5 minutes on a Beckman Model 24 spectrophotometer in 1 cm glass cells against a reference solution containing buffer and organic substrate only. The difference between the two absorbance values was used in calculations. A calibration curve was prepared following the identical procedure and using amounts of 0,02 to 0,50 µg horseradish peroxidase (ref. 1.11.1.7, BDH) as the standard enzyme. Results are expressed in terms of µg HRP equivalent per g fresh weight of material, i.e. in p.p.m. by mass.

Quantitative analysis of peroxidase isoenzymes by starch gel electrophoresis

Preparation of the sample material is described in Section 3.5.1, but in this case the centrifugation of the abstract was unnecessary.

The technique used follows the method of BRAIN (1986). A gel was prepared by suspending 20 g of hydrolysed starch (Sigma S-4501) in 200 ml gel buffer (as for extraction buffer, above; POULIK, 1957) and heated over a flame until a viscous solution resulted. This was de-aerated under vacuum, cast into a perspex frame (internal measurements 21 x 11 x 0,5 cm) and chilled to a firm consistency. The sample solutions were absorbed onto pieces of thick filter paper (5 x 5 mm) which were inserted into slits (*ca.* 15 per frame) cut across the gel 2 cms from the cathodic edge. The gel assembly was mounted in a conventional horizontal electrophoresis tank containing electrode buffer (0,05 M NaOH, 0,30 M H_3BO_3; POULIK, 1957) and run at a starting current

of 25 mA until the buffer front, indicated by a brown line in the gel, had advanced about 60 mm in the anodic direction (*ca.* 4 hours). The gel assembly was chilled briefly and then sliced horizontally by means of a length of surgical nylon under tension. The half remaining in the frame was stained by flooding with detecting reagent (15 ml water, 0,3 ml glacial acetic acid, 0,05 g benzidene, 1 drop 100 vol. H_2O_2; BRAIN, 1986) for 15 minutes. Gels were then photographed and/or careful sketches made to record the positions of the various anodic (+ve) or cathodic (-ve) bands. The electrophoretic mobility of each band was then calculated as a fraction of the distance travelled by the buffer front.

3.6.2 Results and discussion

Total soluble peroxidase activity

The results are recorded together with the soluble protein analyses in Table 23.

For the *in vivo* situation, the peroxidase levels are higher in leaves than in other parts of the plant. In *Encephalartos*, the fast growing species of mesic origin (e.g. *E. transvenosus*, *E. natalensis*) have higher seedling leaf peroxidase concentrations than slower growing taxa with xeric modifications (e.g. *E. lehmannii*, *E. gnellinckii*). It is noted that those species with the higher leaf peroxidase levels are those which responded most rapidly in terms of callogenesis from root explants (Section 2.6.2). There is also a clear indication that leaf peroxidase activity falls as the plants get older (e.g. *E. natalensis*, *S. eriopus*) but a comparison of juvenile and mature leaf samples from the same specimen (*E. natalensis*) does not show this effect. There is no clear evidence that the enzyme concentrations are influenced by the plant's gender (*E. natalensis*, *E. lebomboensis* var. *Piet Retiefii*). In the limited evaluation of the leaf peroxidase levels in the different genera, it seems that *Cycas*, *Dioon* and *Lepidozamia* are higher in enzyme concentration than *Encephalartos* which is in turn richer in peroxidase that *Stangeria* and *Zamia*.

Relatively high levels of the enzyme are seen in some mature seeds where

TABLE 23 : Total soluble peroxidase activity and total soluble protein in cycad material.

Cycad species	Sample material used (Number of samples if > 1)	Mean peroxidase µg g^{-1} fresh weight	Mean protein mg g^{-1} fresh weight	µg peroxidase per mg protein
E. altensteinii	mature plant, mature leaf	21,3	122	0, 175
E. arenarius	3-yr old seedling, leaf	11,6	151	0,077
E. ferox	5-yr old plant, mature leaf	34,3	175	0,196
E. ghellinckii	3-yr old seedling, leaf	17,4	134	0,130
E. lebomboensis	mature male plant, mature leaf	41,3	100	0,413
	fresh seed, infertile, sarcotesta	3,4	37,5	0,091
	fresh seed, infertile, megagametophyte	3,4	119	0,028
E. lebomboensis	mature male plant, mature leaf	39,0	52	0,750
var. Piet Retief	mature female plant, mature leaf	50,0	101	0,495
E. lehmannii	2-yr old seedling, leaf	12,2	48,8	0,250
E. longifolius	6-month old seedling, upper half of primary root	0,88	15,6	0,056
	6-month old seedling, lower half of primary root	0,48	11,9	0,040
	3-yr old seedling, leaf	30,3	90	0,337
	callus tissue ex culture from primary root	128	25,8	4,96
E. natalensis	1-yr old seedling, upper half of primary root (2)	11,4	8,4	1,36
	1-yr old seedling, lower half of primary root (3)	3,4	6,0	0,567
	1-yr old seedling, leaf	63	16,0	3,94
	3-yr old seedling, leaf (2)	36,9	97	0,380
	6-yr old plant, emergent leaf (2)	30,5	18,1	1,68
	6-yr old plant, petiole (2)	11,8	4,4	2,68
	mature male plant, juvenile leaf	13,6	98	0,139
	mature male plant, mature leaf (2)	19,5	88	0,221
	mature female plant, mature leaf (3)	22,0	84	0,262
	mature fertile seed, megagametophyte	37,8	86	0,440
	mature fertile seed, embryo	3,8	3,4	1,117
E. princeps	3-yr old seedling, leaf	34,8	116	0,300
E. transvenosus	2-yr old seedling leaf	74	45	1,64
E. villosus	2-yr old seedling, leaf	28,3	23,7	1,19
E. woodii	mature male plant, mature leaf	26,0	184	0,141
	male cone, microsporangia	10,9	110	0,099
	male cone, microsporophylls	16,0	232	0,070
	male cone, central axis	23,3	28,0	0,832

TABLE 23 : Total soluble peroxidase activity and total soluble protein in cycad material. (Continued)

Cycad species	Sample material used (Number of samples if > 1)	Mean peroxidase $\mu g\ g^{-1}$ fresh weight	Mean protein $mg\ g^{-1}$ fresh weight	µg peroxidase per mg protein
S. eriopus	2-yr old seedling, upper half of primary root (2)	1,1	8,1	0,136
	2-yr old seedling, lower half of primary root (2)	0,66	7,2	0,092
	2-yr old seedling, leaf (2)	25,8	25,5	1,01
	mature plant, mature leaf (2)	10,2	52	0,196
	immature fertile seed, sarcotesta	-	8,9	-
	immature fertile seed, megagametophyte (3)	0,12	79	0,002
	callus tissue ex culture from megagametophyte (2)	60,9	14,4	4,23
Cycas revoluta	mature plant, mature leaf (2)	165	51	3,24
	mature seed, sarcotesta	6,1	38,1	0,160
	mature seed, megagametophyte	11,2	93	0,120
Cycas thouarsii	young plant, leaf	94	111	0,847
Dioon edule	mature plant, mature leaf	62	200	0,310
Lepidozamia peroffskyana	3-yr old seedling, leaf	69	69	1,00
Zamia furfuracea	young plant, leaf (2)	26,6	62	0,429

the activity is located in the megagametophytic tissue rather than the embryo (*E. natalensis*) or sarcotesta (*Cycas revoluta*). The enzyme may increase in concentration as a function of maturity and fertilisation as the immature (*S. eriopus*) and infertile (*E. lebomboensis*) megagametophytes have particularly low levels.

Root peroxidase levels are generally low and there appears to be a gradient from the upper to the lower parts of the roots (*E. natalensis*, *E. longifolius*, *S. eriopus*). This may be associated with the growth responses reported previously (Section 2.6.2) where explants from the upper one-third of the primary roots showed more rapid callogenesis than those from the lower portions.

Particularly high peroxidase concentrations were found in fast-growing callus *in vitro*. In the case of *S. eriopus* megagametophyte cultures, there is an approximately 500-fold increase while the root explants of *E. longifolius* show almost a 200-fold increase. This aspect of massive enzyme increase is examined in greater detail in Section 3.7.

Observations on the correlation between peroxidase level and *in vitro* growth are found in the literature. Leaf segments of *Cichorium intybus* L. with different endogenous peroxidase concentrations showed a correlated variation in growth potential and organ-forming ability (VASSEUR and LEGRAND, 1972). With callus tissue from *Populus tremuloides* Michx. peroxidase activity is closely and positively related to growth rate; there is also evidence that exogenous auxin and cytokinin applications have different qualitative and quantitative effects on the peroxidase enzyme system (WOLTER and GORDON, 1975). The peroxidase activity in stems of *Nicotiana tabacum* L. increases basipetally (i.e. with increasing age of internodal segments) (THORPE, TRAN THANH VAN and GASPAR, 1978). These workers also point out the relation between peroxidases and auxin metabolism; changes in the enzyme pattern are manifestations of the differentiation derepression and repression mechanisms. Further evidence is becoming available from recent *in vitro* studies on several plant species. For instance, the passage of stem tissue of *Actinidia chinensis* L. to callus is accompanied by an increase in total soluble peroxidase (HIRSCH and FORTUNE, 1984).

Studies of the isoperoxidase levels in gymnosperms have been limited, but a recent paper by PATEL and BERLYN (1983) details the cytochemical events which occur in cultured embryonic explants of *Pinus coulteri* D. Don. It was found that peroxidase was strongly localised in regions of growth and differentiation, especially in developing vascular strands. The only reference to peroxidase in cycads is an observation that there is increased enzyme activity during germination of seeds of *Cycas circinalis* (PENA, GRILLO and PEREZ, 1983).

Quantitative analysis of peroxidase isoenzymes

In view of the usefulness of plant enzyme "fingerprints" to the taxonomist (SMITH, 1976), a brief investigation into this field was considered worthwhile. A preliminary survey using crude extracts from a few cycad plants and well-known enzyme-detecting spot tests, failed to detect any significant quantities of alcohol dehydrogenase, esterase, acid and alkaline phosphatase, leucine amino peptidase or malate dehydrogenase. The benzidine test however, readily showed peroxidase in all samples. A further test on a starch gel system showed that anodic peroxidases were present in all cycad material

samples and that a cathodic response was very much less common. Leaf extracts gave the best 'zymograms' and hence the electrophoresis method was employed to compare leaf peroxidases from different individuals and from different taxa.

The results are shown in diagrammatic form in Figures 20a and 20b. It is emphasized that many of the bands were diffuse and hence the exact position and electrophoretic mobility were difficult to ascertain. Notwithstanding this, it is clear that the peroxidase enzyme patterns can provide useful input for the taxonomists. Polymorphism *within* species appears to be absent, indeed several species give apparently identical patterns; this is not surprising with closely-related species *E. natalensis* and *E. lebomboensis* but it is unexpected that *E. trispinosus* gives the same pattern. The presence of cathodic bands in *E. villosus*, *E. ngoyanus* and *E. caffer* is consistent with their phylogeny and it is remarkable that a strong cathodic band is also seen in *Stangeria eriopus*; the only common feature amongst these four taxa being a subterranean habit.

In the exotic cycads examined, an apparently identical pattern is shared by the three *Macrozamia* species and *Lepidozamia hopei* and it is significant that the latter genus was previously incorporated in the former. The variation in the two *Zamia* species examined is consistent with reports on variation in chromosome number and karyotype within the genus (NORSTOG, 1980a, 1981). It is interesting to note that starch gel electrophoresis has been used to examine polymorphism in *Cycas rumphii* (LEIBENGUTH, 1984). Leaf material from 48 plants was examined for peroxidase and esterase activity and the author reports poor resolution and weak reproducibility of the peroxidase pattern but it was noted that peroxidase activity in the older leaves is increased together with an increase in isozyme complexity. The esterase pattern revealed considerable dissimilarity from one plant to another but there was some evidence of polymorphism in certain bands. The author suggests that this may indicate some genetic variability which may have evolutionary potential.

These preliminary tests show the usefulness of the peroxidase isozyme system in taxonomic work. The starch gel medium offers a rapid and inexpensive analytical system and although the resolution is often limited, a trial run using a polyacrylamide gel system did not indicate that an alternative matrix could improve resolution.

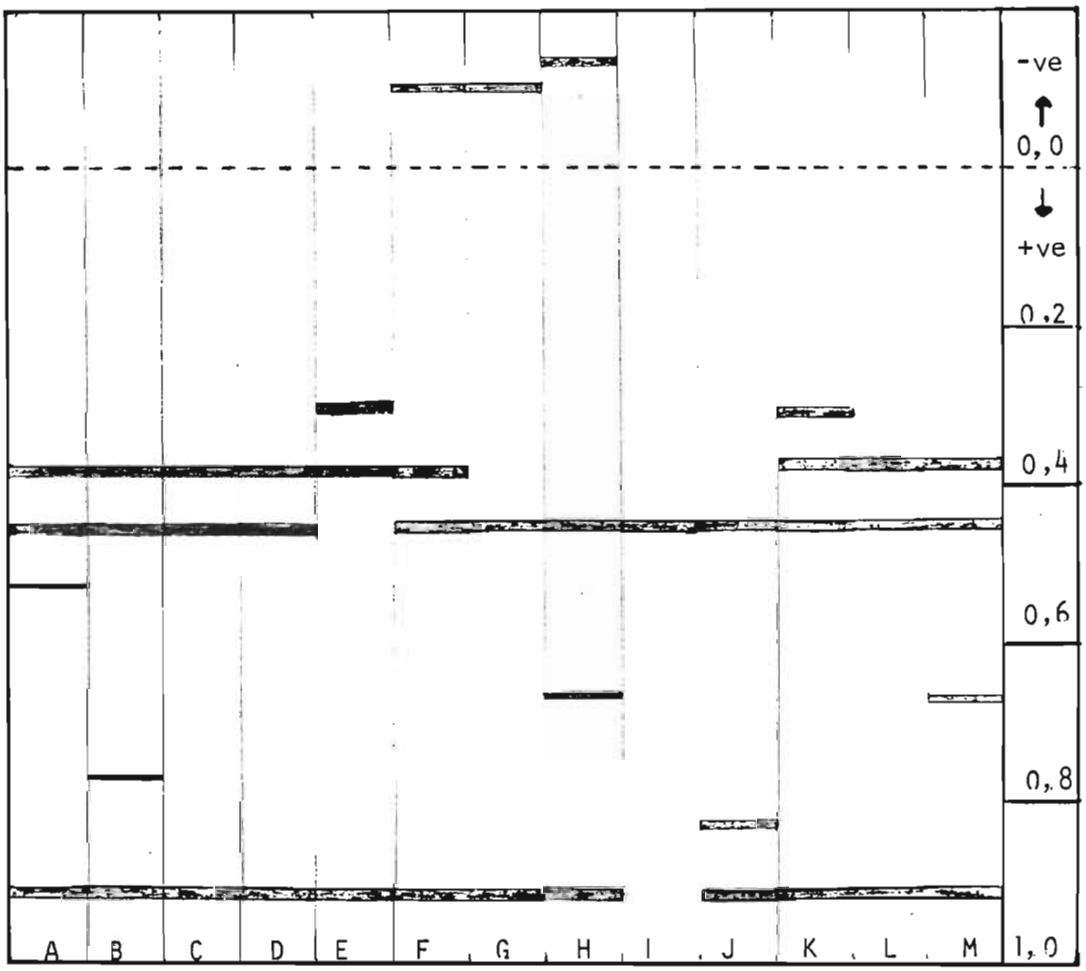


Figure 20a: Diagrammatic representation of zymograms obtained in starch gel electrophoresis of leaf extracts from various species of *Encephalartos*. A. *E. longifolius*, B. *E. altensteinii*, C. *E. natalensis*, D. *E. lebomboensis*, E. *E. transvenosus*, F. *E. villosus*, G. *E. ngoyanus*, H. *E. caffer*, I. *E. ferox*, J. *E. friderici-guilielmi*, K. *E. lehmannii*, L. *E. trispinosus*, M. *E. horridus*.

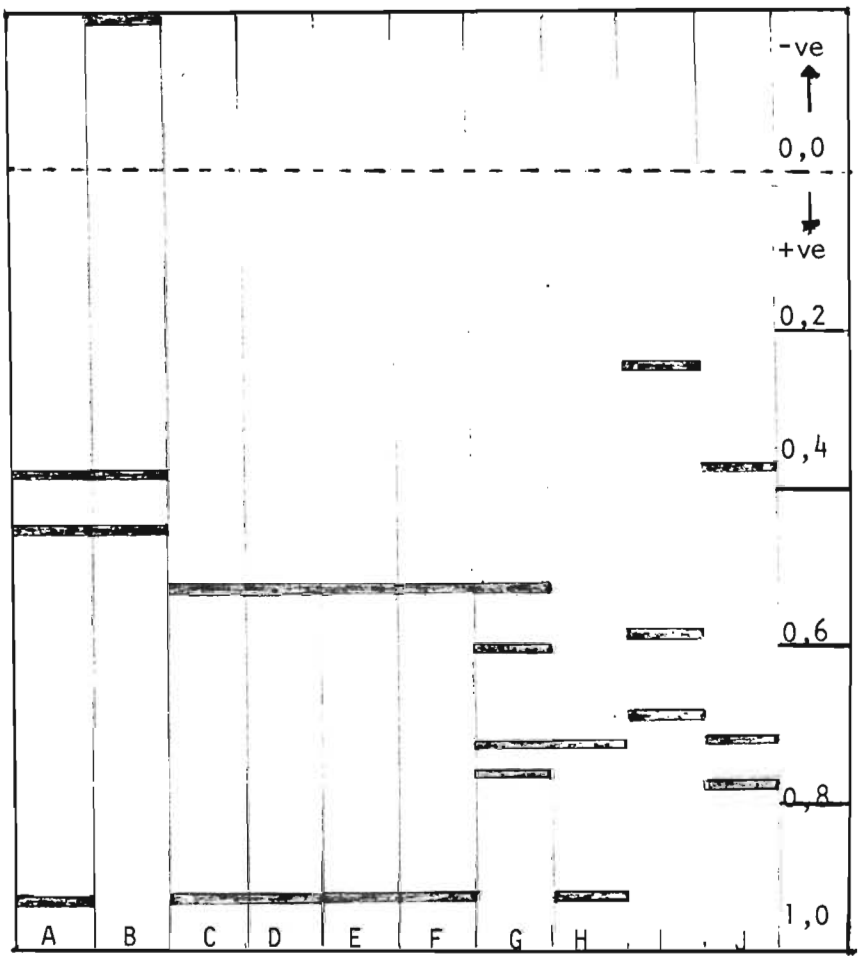


Figure 20b: Diagrammatic representation of zymograms obtained in starch gel electrophoresis of leaf extracts from various cycads. A. *Encephalartos natalensis*, B. *Stangeria eriopus*, C. *Macrozamia moorei*, D. *M. communis*, E. *M. miquelii*, F. *Lepidozamia hopei*, G. *Bowenia spectabilis*, H. *Cycas normanbyana*, I. *Zamia pumila* var. *pumila*, J. *Z. furfuracea*.

3.7 Biochemical changes during callogenesis from *Stangeria eriopus*

3.7.1 Materials and methods

Because of the ease and reproducibility with which callus growth could be obtained from megagametophyte cultures of *Stangeria eriopus* (Section 2.5.1), this explant material was selected for a brief investigation into the changes in fresh weight, dry weight, soluble protein and peroxidase activity, which occur prior to and during callogenesis *in vitro*.

Transversely bisected megagmetophytes were aseptically transferred to preweighed culture vessels containing SH medium supplemented with $4,5 \times 10^{-6}$ M 2,4-D and kinetin, 48 such cultures being prepared and reweighed to establish the initial fresh mass of each explant. All cultures were incubated in the dark at 25°C.

At intervals of 0, 7, 14, 21, 28, 35, 42 and 49 days, sets of six cultures were removed and processed as follows. The explants were removed, washed free from residual medium and weighed to determine fresh weight at the time of harvest. A sample (*ca.* 0,3g) from each culture was oven dried to constant mass at 105°C to determine moisture content and hence dry mass at the time of harvest. The fresh and dry mass increase from the time of inoculation could then be estimated. A further sample (1,00 g) was ground in a mortar with 9,0 mls of pre-cooled (4°C) extraction buffer (0,076 M Tris, 0,005 M citric acid, pH 8,65, POULIK, 1957) and the resulting mixture centrifuged at 50 000 x g for 30 minutes at 4°C. Aliquots from the clarified extract were taken for total soluble protein and total soluble peroxidase determinations as described in Sections 3.5.1 and 3.6.1.

3.7.2 Results and discussion

The data obtained in this experiment are presented in Table 24 and are shown in graphical form in Figures 21a - f.

It is clear that several significant physiological changes occur in the explant tissue prior to macroscopic evidence of callogenesis, the latter event being evident from 42 days of the day of inoculation. There is an almost linear increase in fresh weight over the first four

TABLE 24 : Variation in fresh weight, dry weight, total soluble protein and peroxidase activity of megagametophyte explants from *Stangeria eriopus* in culture.

PRIMARY DATA

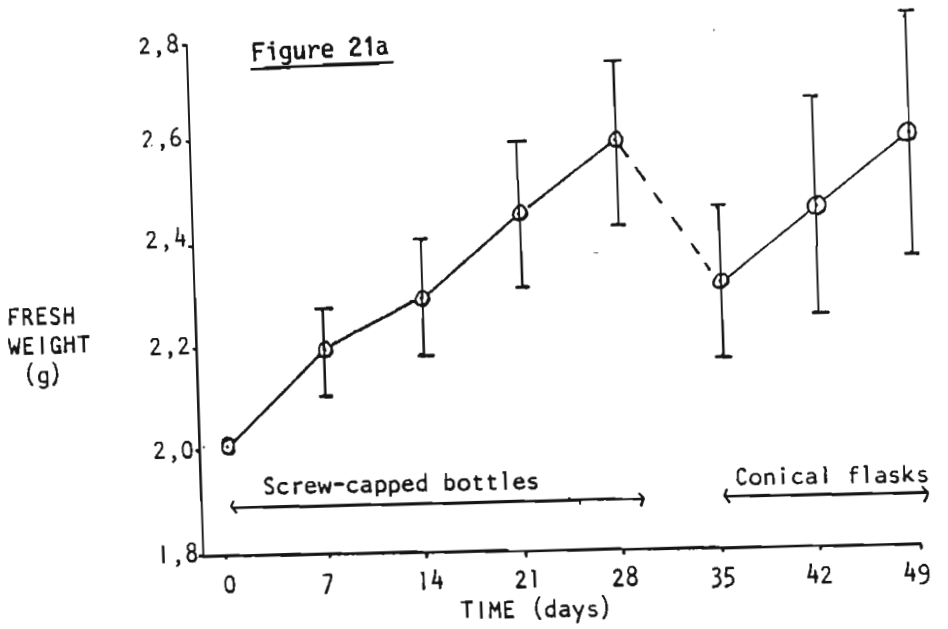
Time (days)	Fresh weight at start (g)	Fresh weight at end (g)	Moisture (g/100g fresh weight)	Total soluble protein (mg g^{-1} fresh weight)	Peroxidase activity (µg g^{-1} fresh weight)
0	2,10 ± 0,37†	2,10 ± 0,37	48,6 ± 3,3	83,1 ± 7,3	8,9 ± 11,9
7	1,96 ± 0,48	2,12 ± 0,47	54,2 ± 5,9	42,1 ± 4,0	33,8 ± 40,4
14	1,87 ± 0,43	2,13 ± 0,47	60,4 ± 5,0	63,6 ± 9,4	5,3 ± 2,1
21	1,95 ± 0,54	2,42 ± 0,68	57,4 ± 4,4	64,9 ± 3,2	22,7 ± 14,3
28	1,96 ± 0,33	2,52 ± 0,40	60,4 ± 1,9	62,6 ± 5,4	8,0 ± 4,4
35	2,09 ± 0,34	2,33 ± 0,39	63,4 ± 1,9	65,9 ± 4,6	22,4 ± 9,0
42	1,87 ± 0,15	2,28 ± 0,19	61,3 ± 3,7	60,1 ± 6,6	94,2 ± 61,4
49	2,01 ± 0,44	2,58 ± 0,55	61,7 ± 3,8	58,4 ± 11,3	77,5 ± 33,2

DERIVED DATA

Time (days)	Adjusted fresh* weight end (g)	Adjusted dry* weight at end (g)	Moisture (g/100g dry weight)	Total soluble protein (mg g^{-1} dry weight)	Peroxidase activity (µg g^{-1} dry weight)
0	(2,00 initial)	(1,03 initial)	95 ± 12	162 ± 11	18,1 ± 25,7
7	2,18 ± 0,08	0,99 ± 0,10	121 ± 30	93 ± 14	77,7 ± 99,9
14	2,28 ± 0,11	0,90 ± 0,08	159 ± 36	161 ± 21	13,9 ± 6,5
21	2,44 ± 0,14	1,03 ± 0,07	137 ± 25	154 ± 20	55,4 ± 37,5
28	2,58 ± 0 16	1,02 ± 0,04	153 ± 12	158 ± 13	19,8 ± 10,3
35	2,31 ± 0,14	0,84 ± 0,04	174 ± 13	180 ± 6	62,0 ± 25,5
42	2,45 ± 0,21	0,94 ± 0,07	160 ± 25	160 ± 22	248 ± 155
49	2,59 ± 0,23	0,99 ± 0,05	163 ± 25	152 ± 15	204 ± 89

* Proportionally adjusted to an initial explant weight of 2,00 g.

† The range is indicated throughout as ± one standard deviation.



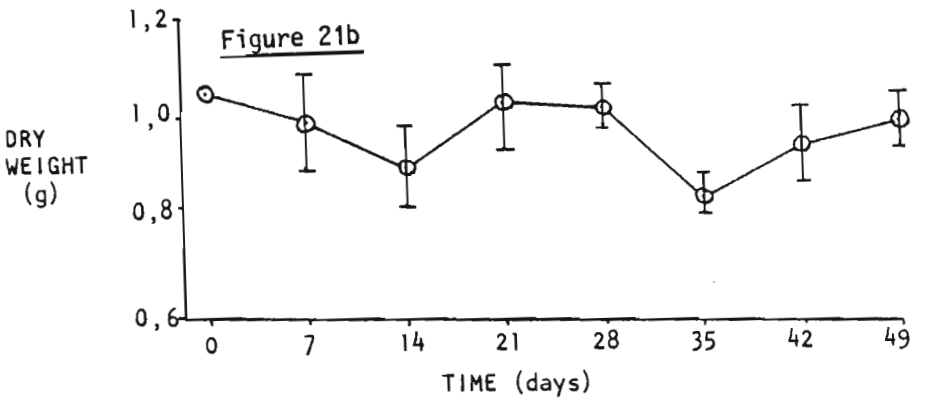
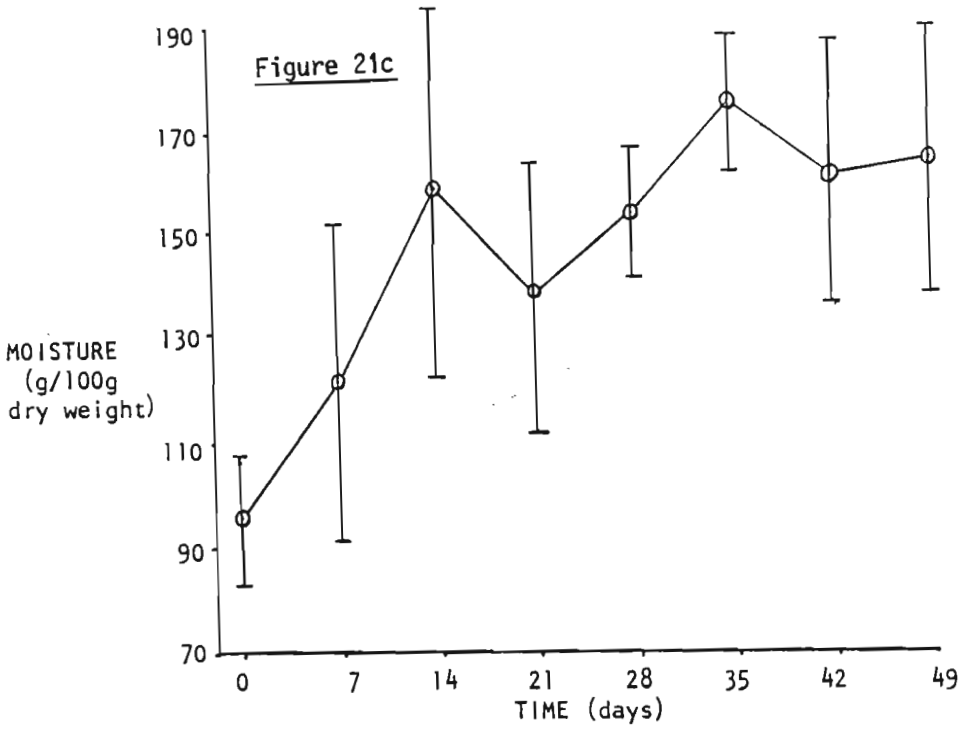


Figure 21a and b : Variation in fresh and dry weight of megagametophyte explants from *Stangeria eriopus* in the dark on SH medium with 4,5 x 10^{-6}M, 2,4-D and KN. Vertical bars represent +/- one standard deviation.



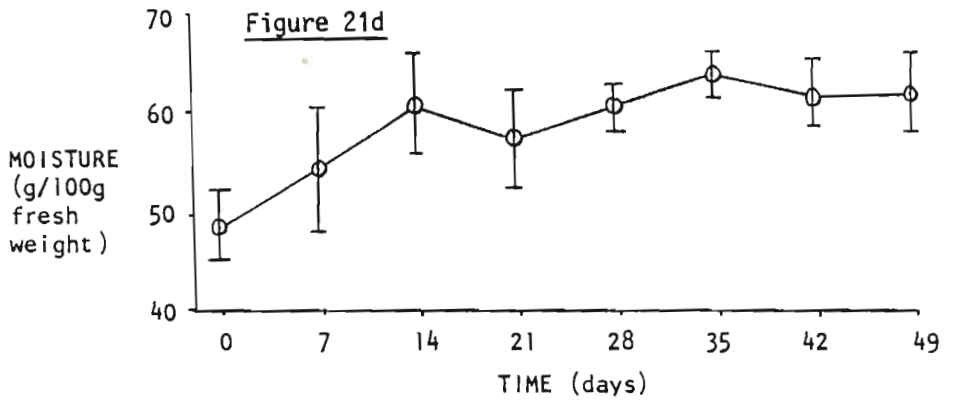
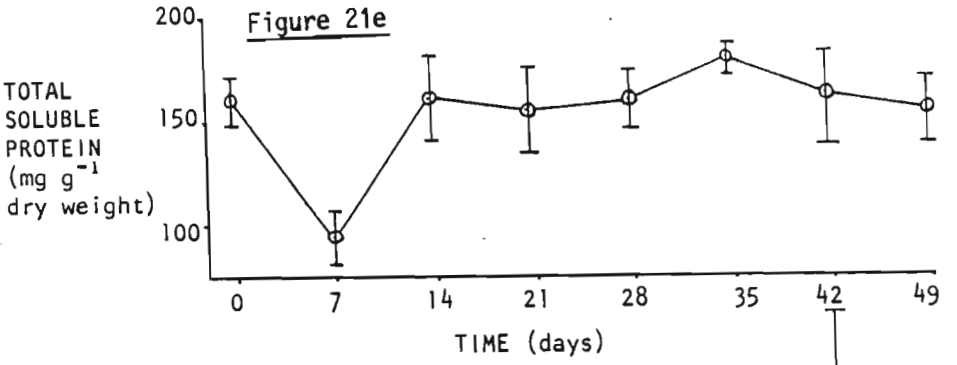


Figure 21c and d : Variation in moisture content of megagametophyte explants from *Stangeria eriopus* in the dark on SH medium with 4,5 x 10^{-6}M 2,4-D and KN. Vertical bars represent +/- one standard deviation.



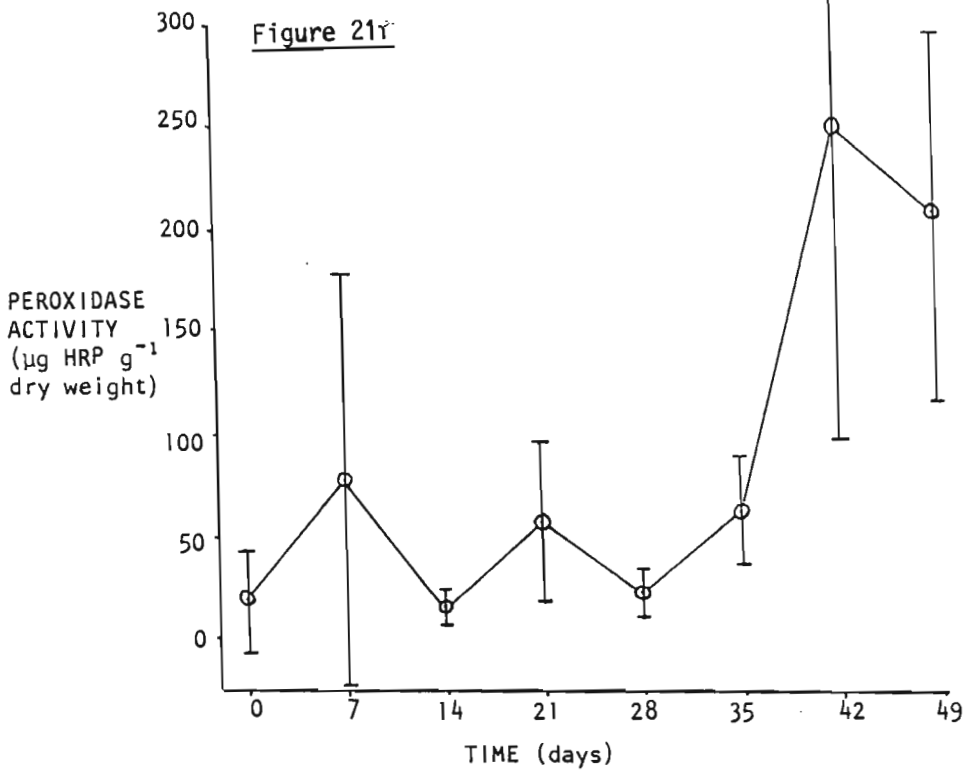


Figure 21e and f : Variation in total soluble protein and peroxidase activity of megagametophyte explants from *Stangeria eriopus* in the dark on SH medium with 4,5 x 10^{-6}M 2,4-D and KN. Vertical bars represent +/- one standard deviation.

weeks (Figure 21a) and this increase may be ascribed to an increase in moisture content (Figure 21c). By contrast, the dry weight shows an initial decline with a recovery later (Figure 21b). These effects are analagous to seed behaviour when moisture is imbibed and food reserves are utilised in the period just before germination.

An interesting and entirely fortuitous observation is associated with the choice of culture container. Due to limitations of glassware supply, two types of containers had been used in the experiment. The "early" cultures were contained in 28 ml screw-capped "Universal" bottles while the "later" explants were held in 25 ml conical flasks with cotton-wool bung closures. Figure 21a shows that the fresh weight increase of explants in the screw-capped containers is considerably more advanced than those in the conical flasks. This effect is quite different to that previously observed in megagametophytes from *Encephalartos* (Section 2.4.5) where use of Universal bottles inhibited callus growth altogether. In the case of *Stangeria*, it appears that use of the Universal bottles may result in an initially favourable response, but this is not sustained. There is about a three-week time delay for the explants in the conical flasks to reach the same stage as those in the screw-capped bottles. It is speculated that the restricted gaseous exchange in the screw-capped containers results in the headspace having constant 100% relative humidity while the conical flasks undergo slight desiccation with a concomitant slight but significant increase in water deficit in the medium. Other influences arising from differences in the gaseous exchange rates, such as carbon dioxide and ethylene accumulation, and restriction of oxygen supply, may have an effect (Section 2.4.5).

The total soluble protein shows an initial sharp decline (Figure 21e), possibly due to the utilisation of readily-available protein reserves. This fall is restored within two weeks after which the soluble protein stays more-or-less constant. The peroxidase activity is widely-variable as shown by the large standard deviations (Figure 21f). However, a significant increase in the enzyme level does occur from 28 to 42 days, the period just before visible evidence of callus formation. This confirms the earlier report (Section 3.6.2) where both haploid and diploid callus show dramatic increases over the initial peroxidase levels. Again, the situation is parallel to that of the

seed, where large increases in enzyme activity are a fundamental aspect of the germination process.

3.8 Serological investigations

3.8.1 Materials and methods

Fresh seed of *E. natalensis*, derived from plants in the Kranskop area of Natal, were supplied to the Natal Institute of Immunology where a quantity of the antiserum was prepared using standard techniques. These involved preparation of the crude seed extract and emulsification with Freund's complete adjuvant after which a rabbit was injected intramuscularly at four sites at several intervals over a six-week period. At the end of this period the antiserum was obtained after clotting of a blood sample from a test bleed.

Double-diffusion serology

An Ouchterloni double-diffusion plate (10 cm x 10 cm) was prepared using 0,8% agarose in barbital buffer (0,05 M barbitone, pH 8,6). Extracts from various cycad seeds were prepared by grinding approximately 0,5 g fresh megagametophytic material in about 2 ml barbital buffer and centrifuging the product at 9000 x g for 5 minutes. The *E. natalensis* antiserum was placed in the central wells and the test samples in the peripheral wells. The reaction was allowed to proceed for at least 6 hours at ambient temperature and the resulting plates examined over a light box to ascertain the nature of any precipitin reactions.

Immuno-electrophoresis

Immuno-electrophoresis plates (10 cm x 10 cm) were prepared using 0,8% agarose in barbital buffer (0,05 M barbitone, pH 8,6). Wells were cut into the matrix half-way along the plate (relative to the cathode-anode axis). Test samples of various cycad seeds were prepared as described above, aliquots of the clarified suspension being placed in the sample wells. The plates were electrophoresed, using the barbital solution as an electrode buffer, at a starting current of 25 mA and running for 5 hours.

Channels were then cut into the matrix parallel to the current direction, about 15 mm from the sample wells, and these troughs loaded with antiserum prepared as described previously. The nature and extent of the antiserum-antigen reaction was assessed by viewing over a light box after about 6 hours.

3.8.2 Results and discussion

Double-diffusion serology

The results are shown diagrammatically in Figure 22.

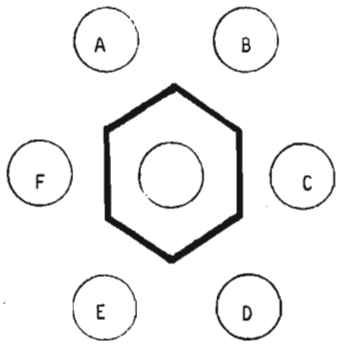
Although only a few taxa have been examined, it is clear that the responses obtained correlate with the present taxonomy at the family level. The *E. natalensis* (Kranskop) antiserum gives the expected response with a fresh extract from the same batch of seed, confirming the validity of the method. A similar intense single band was obtained with other samples of *E. natalensis* and indeed with all species of *Encephalartos* tested. Other representatives from the Zamiaceae (*Macrozamia*, *Lepidozamia*, *Ceratozamia* and *Zamia*) all showed a similar band of lower intensity. The response from *Stangeria* (Stangeriaceae) was quite different; a double faint banding pattern being seen. No reaction was found in any of the samples of *Cycas* (Cycadaceae), again indicating significant difference from the *Encephalartos* protein.

Following the initial experiment with extracts from the cycad megagametophytes, a similar test was run using the same antiserum with extracts from cycad leaflets in the peripheral wells. Thirteen species of *Encephalartos* and five exotic cycads were tested in this manner. No reaction was seen in any of these samples. The inference drawn is that the leaflet proteins differ markedly from those in the seeds. Application of the methodology is thus limited to comparisons of antiserum and antigen derived from similar organs.

Immuno-electrophoresis

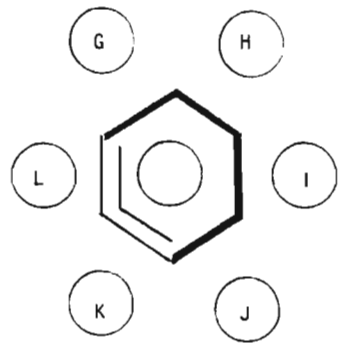
The results are shown diagrammatically in Figure 23.

While the double-diffusion serological tests gave correlations with taxa at the family level, the immuno-electrophoresis patterns appear



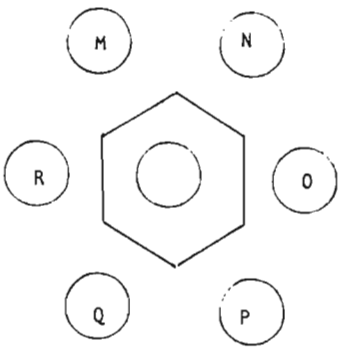
GROUP 1

A. *E. natalensis*, Kranskop area.
B. *E. natalensis*, Msinga area.
C. *E. natalensis*, B.R.I., Durban.
D. *E. villosus*, E. Cape
E. *E. villosus*, Durban.
F. *E. ferox*
E. natalensis, (Kranskop) anti-serum in central well



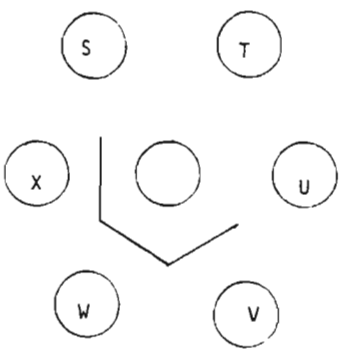
GROUP 2

G. *E. natalensis*, Kranskop area.
H. *E. paucidentatus*,
I. *E. lanatus*
J. *E. trispinosus*
K. *Stangeria eriopus*, Umzinto area.
L. *Stangeria eriopus*, B.R.I., Durban.
E. natalensis (Kranskop) anti-serum in central well.



GROUP 3

M. *Macrozamia heteromera*
N. *Macrozamia lucida*
O. *Macrozamia moorei*
P. *Macrozamia platyrachis*
Q. *Lepidozamia hopei*
R. *Lepidozamia peroffskyana*
E. natalensis (Kranskop) anti-serum in central well.



GROUP 4

S. *Cycas calcicola*
T. *Cycas media*
U. *Cycas revoluta*
V. *Ceratozamia mexicana, Belize*
W. *Zamia furfuracea*
X. *Zamia latifolia*
E. natalensis (Kranskop) anti-serum in central well.

FIGURE 22: Double-diffusion serological tests with cycad seed proteins showing antiserum-antigen precipitation reactions in samples with similar proteins.

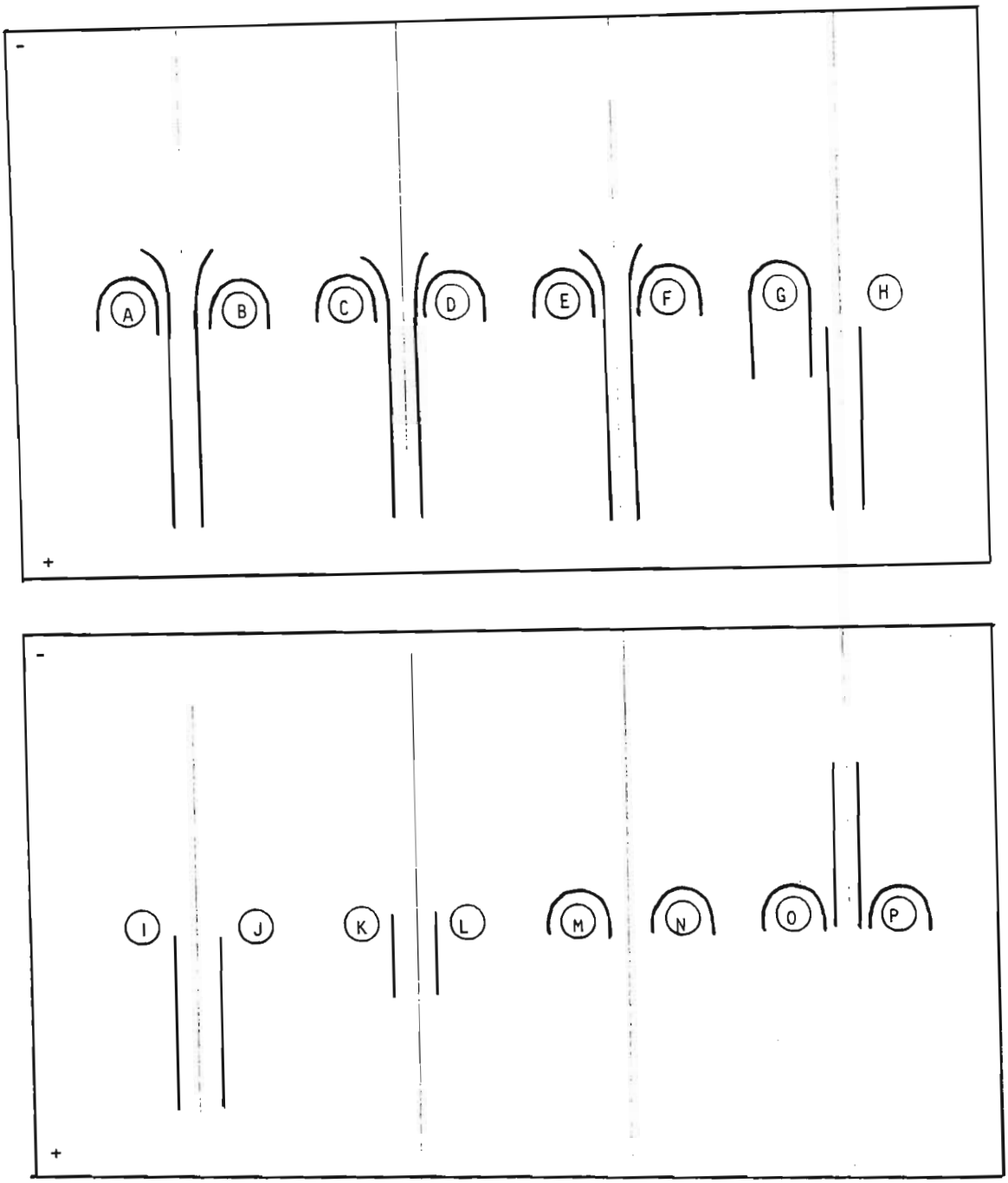


FIGURE 23: Immuno-electrophoresis of some cycad seed proteins against *E. natalensis* antiserum. A. *E. natalensis*, B. *E. ferox*, C. *E. ngoyanus*, D. *E. paucidentatus*, E. *E. trispinosus*, F. *E. villosus*, G. *Stangeria eriopus*, H. *Macrozamia heteromera*, I. *M. moorei*, J. *M. platyrachis*, K. *Lepidozamia hopei*, L. *L. peroffskyana*, M. *Cycas calcicola*, N. *Ceratozamia mexicana*, O. *Zamia furfuracea*, P. *Z. latifolia*.

to be consistent at the genus level. In the genera tested, each gave a characteristic profile although it was not possible to distinguish between *Cycas* and *Ceratozamia*. There was no separation into discreet bands along the direction of current travel as is usually observed with higher plant proteins (SMITH, 1976); thus it may be inferred that cycad proteins are somewhat less complex with greater homogeneity within each genus. The results with the two species of *Zamia* are unusual in that the reacting protein is largely cathodic; all other genera show anodic migration.

Whilst the serological results are useful in a confirmation of groupings at the family and genus level, in the examples tested there was no evidence of individuality at the species level. This line of investigation was not therefore persued.

3.9 Cycad toxins

3.9.1 Materials and methods

The method used was modified from that of MORETTI, SABATO and SINISCALCO GIGLIANO (1981). A 1,0 g fresh weight sample of the material was ground in a mortar with 9,0 ml of 50% aqueous ethanol and a portion of the resulting suspension centrifuged in an Eppendorf tube at 9000 x g for 10 minutes. For the qualitative tests, 10µℓ aliquots of the clear extracts were applied to a 10 x 10 cm T.L.C. silica gel plate (Merck Art. 5554) which was developed in one dimension using n-butanol-acetone-water (4:5:1) as the solvent system. Samples of pure macrozamin kindly supplied by Dr J Cannon of the University of Western Australia and Dr. P. Casorio of the University of Naples, were used for reference purposes. After drying off the residual solvent, the T.L.C. plates were sprayed with aniline-diphenylamine-acetone-phosphoric acid regent (1 ml:1 g:50 ml:8,5 ml) and heated at 80°C for 15 minutes. Simple sugars and glycosides, including macrozamin, adopt a blue-grey colour on this treatment.

3.9.2 Results and discussion

A representation of the results from the TLC plates is shown in Figure 24. The method is a convenient and rapid technique which

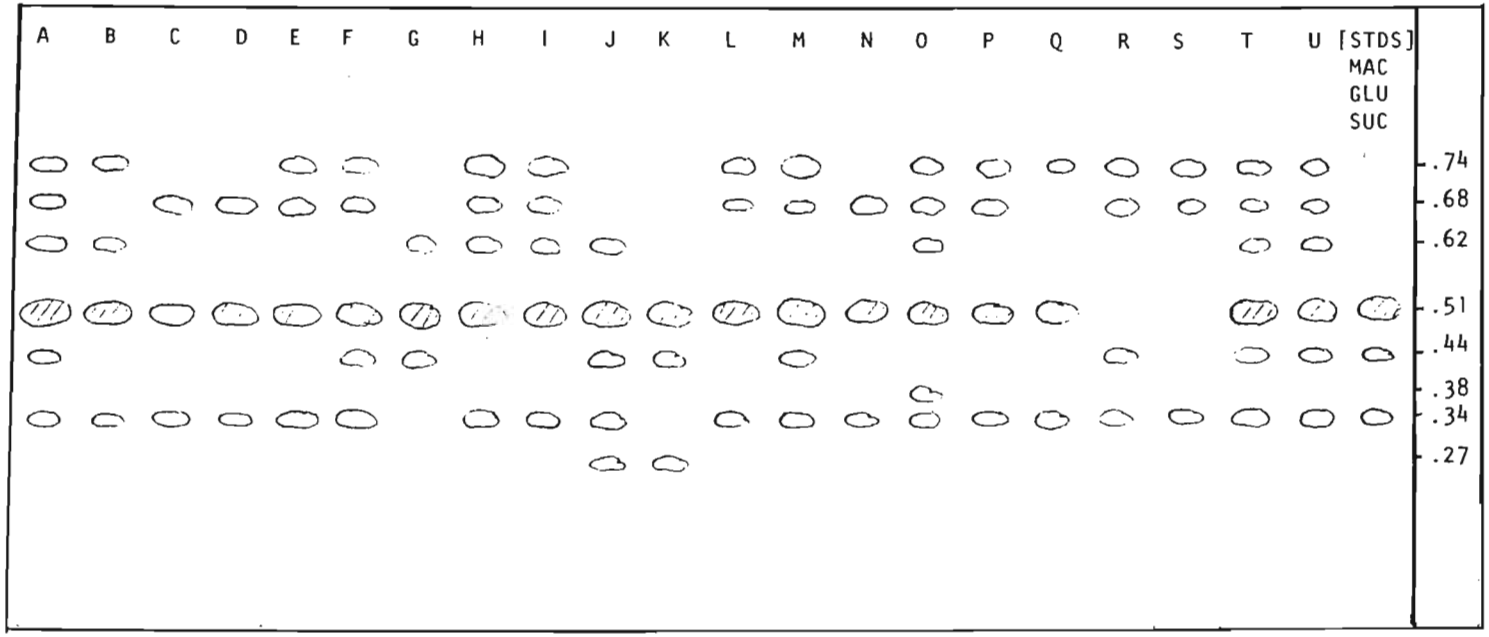


FIGURE 24: TLC analysis of cycad seed extracts. A. *E. natalensis*, Kranskop area, after 3 months' storage, B. *E. natalensis*, Msinga area, after 3 months' storage, C. *E. natalensis*, B.R.I., Durban, fresh megagametophyte, C. *E. natalensis*, B.R.I., Durban, after 6 months' storage, E. *E. villosus*, Durban, fresh megagametophyte, F. *E. villosus*, Durban, after 6 months' storage, G. *E. lebomboensis*, Westville, after 3 months' storage at 0°C, H. *S. eriopus*, Umzinto area, fresh megagametophyte, I. *S. eriopus*, Umzinto area, after 6 months' storage, J. *S. eriopus*, B.R.I., Durban, fresh megagametophyte, K. *S. eriopus*, B.R.I., Durban, after 3 months' storage at 0°C, L. *Macrozamia heteromera*, after 6 months' storage, M. *M. moorei*, after 6 months' storage, N. *M. platyrachis*, after 4 months' storage, O. *Lepidozamia peroffskyana*, after 6 months' storage, P. *Cycas calcicola*, after 4 months' storage, Q. *C. revoluta*, U.N. Pietermaritzburg, fresh sarcotesta, R. *C. revoluta*, U.N. Pietermaritzburg, fresh megagametophyte, S. *C. revoluta*, U.N. Pietermaritzburg, after 3 months' storage, T. *Zamia furfuracea*, after 4 months' storage, U. *Z. latifolia*, after 4 months' storage, MAC, authentic macrozamin, GLU, glucose, SUC, sucrose. Solvent system n-butanol-acetone-water, 4:5:1.

detects macrozamin at levels greater than about 0,1% by fresh weight of material. The toxin (R_F 0,51) was found in nearly all samples tested and did not appear to be affected by short term storage at ambient or cold conditions. On the basis of colour intensity, the *Stangeria* seed contains more macrozamin than any of the *Encephalartos* seed samples. This is consistent with the finding of MORETTI, SABATO and SINISCALCO GIGLIANO (1983) who report *Encephalartos* seed as having, 2,09 - 2,86% macrozamin and *Stangeria eriopus* as having 4,70%. Of the various samples tested, only the megagametophytes of *Cycas revoluta* failed to show the presence of macrozamin, although the toxin was present in the sarcotesta of the same seeds. Either sucrose (R_F 0,34) or glucose (R_F 0,44) or both sugars, could be seen in the extracts and there appears to be some degree of change in sugar distribution on storage.

Several unidentified spots with R_F values in the range 0,62 - 0,74 appeared on the chromatograms. It is possible that these represent cycasin or various neocycasins. Cycasin, being a monosaccharide glycoside, would be expected to exhibit a higher R_F value than macrozamin, a disaccharide glycoside, but no standard material was obtainable to establish this.

An attempt to quantify the toxin concentrations by HPLC techniques, using a conventional C-18 reverse phase column, was unsuccessful. No response at all was obtained with the standard macrozamin sample. This may have been because the instrument used recorded response by refractive index of the eluant; if the alternate ultra-violet system had been available the response may have been detected. Indeed, this has been employed successfully in the simultaneous determination of cycasin and its degradation products (YAGI, TADERA and KOBAYASHI, 1980).

3.10 Analysis of leaf wax hydrocarbons

NOTE: The work described in this section refers to results of a joint project carried out in association with Professors Antonio and Maria Luiza Salatino of the Instituto de Biociências, University of São Paulo, São Paulo, Brazil, over the period August 1986 to April 1988.

3.10.1 Materials and methods

Fresh leaf material was collected from different habitat and garden grown plants of as many *Encephalartos* species as possible, and from *Stangeria eriopus*. Samples were taken from mature unblemished leaves of adult plants where no doubt existed as to their existing taxonomic status. Voucher specimens of the leaf samples were deposited at the herbarium of the University of Durban-Westville. Only the median leaflets were used in the analysis. Samples were also collected from seedling plants and juvenile leaves for comparison. The selected leaf portions were oven-dried at 50°C for approximately 72 hours.

The epicuticular waxes were extracted by means of three successive immersions of 30 seconds in chloroform. The extracts were combined and the solvent evaporated. The crude residue was fractioned in a column (30 x 1,5cm) of silica gel (Merck, 70-230 mesh), the alkane fraction being eluted by 30ml of petroleum ether (boiling range 30-60°C). The purity of the alkane fraction was monitored by TLC using silica gel 60 G plates (Merck) with fluorescein indicator and visualized under long wave ultra-violet light. The presence or absence of olefinic hydrocarbons was assessed by comparing the above plates with those obtained under identical conditions but using silica gel impregnated with 0,1M silver nitrate.

Analyses of the constituent normal and branched-chain alkane homologues were performed on a CG-37 gas chromatograph (CG-Instrumentos Cientificos Ltda) which is similar in capability to the current Varian instrumentation. The column of stainless steel (2m x 3,2mm internal diameter) was packed with 1% OV-101 on Gas Chrom. Q. Nitrogen was used as the carrier gas. Injector and detector temperatures were set at 280°C and the column temperature ramped from 160 to 280°C at 4°C min^{-1}. A flame ionizer detector system provided input to a CG-300 processor-integrator (CG-Instrumentos Cientificos Ltda). Identification of the alkane homologues was based on comparison of peak retention times with those of authentic samples of linear alkanes. Peaks with retention times intermediate between two successive linear homologues were ascribed to branched chain alkanes since there was no evidence of an olefins being present in the extracts. A cluster analysis and phylogenetic evaluation of the data was performed using the NTSYS numerical taxonomy analytical

system (Applied Biostatistics Inc.). The SIMINT routine was used to compute taxonomic distances which were in turn analysed using an unweighted pair-group method in the SAHN programme. This resulted in the production of a matrix which could be displayed in the form of a phenogram by use of the TREE routine. Goodness of fit of the clustering analysis was measured by processing the tree matrix through the COPH routine to give a cophenetic value matrix which could be compared with the original taxonomic distance matrix by use of the MXCOMP routine. The manipulations involved in the various aspects of these analyses are described in detail by ROHLF, KISHPAUGH and KIRK (1971) and SNEATH and SOKAL (1973).

3.10.2 Results and discussion

The analytical data obtained from the various samples are shown in Tables 25, 26, 27 and 28 and Figures 25, 26, 27 and 28. In nearly all cases the contribution of the n-alkane homologue far exceeds the total contribution from the corresponding isomers. Thus, for clarity in the graphical representations, only the n-isomer figures are plotted. Similiarly, the discussion of results is conveniently limited to a consideration of the n-alkane profiles.

Seedling and mature leaves

Table 25 shows a comparison of the alkane distribution in seedling and mature plant leaves of seven species of *Encephalartos*. The n-alkane profile for four of these pairs, selected as representative, is given in Figure 25. With *E. ngoyanus*, the rather irregular profile for the seedling leaves differs markedly from the "skewed-normal" pattern in the mature leaves. The reverse trend is seen in *E. lanatus*. In *E. ferox* and *E. villosus* there is considerable similarity between the juvenile and adult leaf patterns. There is therefore little overall consistency between the profiles of seedling and mature leaves. This statement must be qualified by the constraint that the seedling and mature leaf samples tested, although from the same species, are not necessarily from the same clone. Except in the case of *E. ferox*, the results obtained in these tests are not generally consistent with the situation in Angiosperms where younger leaves usually bear a higher proportion of short-chain homologues (FABOYA, OKOGUN and GODDARD, 1980; BAKER and HUNT, 1981;

TABLE 25 : A COMPARISON OF THE LEAF WAX ALKANE DISTRIBUTION IN SEEDLING AND MATURE *Encephalartos* PLANTS

Alkane homologue by carbon atom number (normal/isomers)[1]	Percentage abundance of alkane homologues by species in seedling and mature plant leaves													
	E. ferox		*E. lanatus*		*E. longifolius*		*E. natalensis*		*E. ngoyanus*		*E. transvenosus*		*E. villosus*	
	S[2]	M(2)	S	M(1)	S	M(1)	S	M(9)	S	M(2)	S	M(1)	S	M(3)
c17n	0	0	0	0	1	0	0	0	0	0	0	0	0	0
c17i	0	0	0	0	0	0	0	0	0	0	0	0	0	0
c18n	2	0	0	4	6	5	0	1	0	1	0	2	0	tr
c18i	0	0	0	tr	1	0	0	tr	0	0	0	0	0	0
c19n	6	tr	1	20	13	18	3	3	1	7	3	5	tr	tr
c19i	1	tr	tr	2	5	1	0	tr	0	tr	0	tr	tr	0
c20n	6	1	3	20	12	16	4	7	1	20	3	4	1	4
c20i	2	tr	1	9	6	7	1	2	tr	6	1	1	tr	tr
c21n	4	1	2	3	7	7	3	4	3	18	2	4	tr	2
c21i	1	tr	1	3	8	2	1	2	tr	8	tr	tr	tr	tr
c22n	2	1	0	1	4	2	2	3	3	8	1	4	tr	1
c22i	0	1	0	1	6	0	0	tr	tr	2	0	1	0	0
c23n	10	4	11	6	9	19	16	8	21	5	9	22	1	1
c23i	0	1	0	0	3	tr	tr	tr	0	1	0	2	tr	0
c24n	2	2	7	2	1	3	2	5	5	4	2	7	tr	1
c24i	0	tr	2	1	0	0	1	1	3	0	1	0	0	0
c25n	5	3	9	5	3	5	22	7	15	7	8	11	1	1
c25i	0	1	3	1	0	tr	0	2	0	0	1	2	tr	0
c26n	1	4	7	tr	1	2	2	5	4	4	2	3	1	1
c26i	0	tr	4	1	0	0	0	tr	2	0	0	0	tr	0
c27n	2	3	7	1	1	1	4	5	4	3	2	5	1	1
c27i	0	tr	4	tr	tr	0	tr	1	1	0	2	tr	tr	0
c28n	5	4	11	7	3	8	7	11	16	3	6	8	2	2
c28i	0	tr	3	tr	tr	tr	tr	tr	1	tr	1	0	tr	0
c29n	11	10	4	2	2	1	10	9	2	3	3	6	5	6
c29i	0	0	0	0	0	0	0	0	0	0	0	0	0	0
c30n	5	8	2	1	2	1	7	10	1	3	2	3	3	4
c30i	0	0	2	1	0	0	0	0	tr	0	tr	0	0	0
c31n	25	28	3	2	2	1	9	7	3	2	3	6	33	37
c31i	0	0	1	0	tr	0	tr	tr	2	0	1	tr	0	0
c32n	2	14	tr	1	1	tr	2	3	1	1	18	1	5	7
c32i	0	0	0	0	0	0	0	0	0	0	0	0	0	0
c33n	8	18	5	2	2	1	3	2	5	1	4	3	43	31
c33i	0	0	0	0	0	0	0	0	tr	0	18	0	0	0
c34n	0	1	1	1	0	0	0	1	2	tr	3	0	1	1
c34i	0	0	0	0	0	0	0	tr	tr	0	0	0	0	0
c35n	0	1	2	2	0	0	0	tr	3	0	4	0	2	1
c35i	0	0	0	0	0	0	0	0	0	0	1	0	0	0
c36n	0	0	tr	0	0	0	0	0	1	0	0	0	0	0
c36i	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Notes: 1. "n" represents the normal or unbranched homologue, "i" represents the total isomeric contribution
2. "S" represents seedling leaves, "M" leaves from mature plants, the numbers being an average composition over the number of samples indicated in parentnesis.

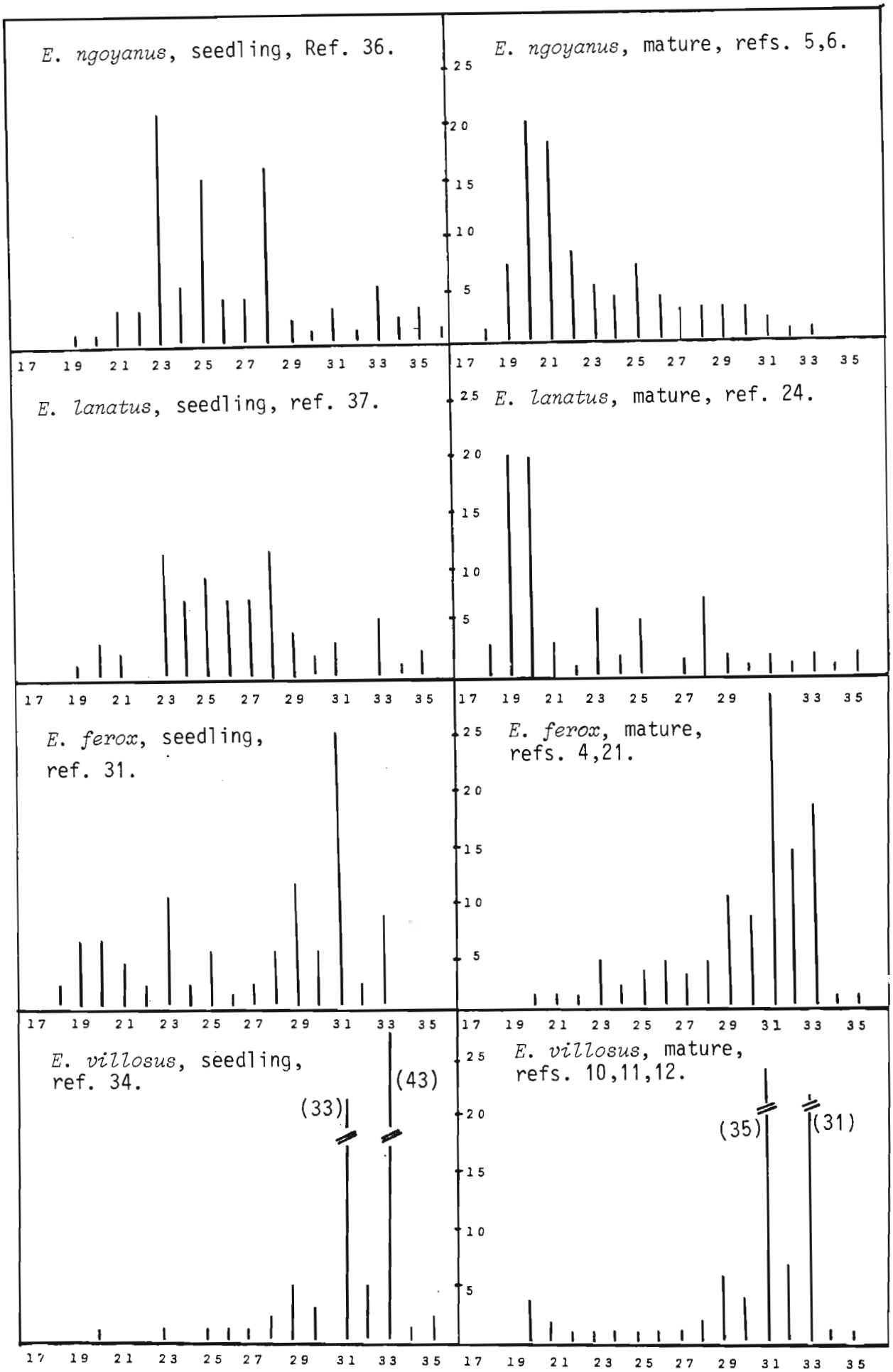


FIGURE 25 : LEAF WAX n-ALKANE PROFILES FOR SEEDLING AND MATURE *ENCEPHALARTOS* PLANTS. The percentage contribution of each alkane (y-axis) is plotted against the alkane carbon atom number (x-axis).

SALASOO, 1983).

Pure species and their hybrids

Table 26 and Figure 26 show the data relating to analyses from two sets of parent species and their hybrids, which occur naturally in overlapping distribution areas. In both cases the hydrocarbon profile of the hybrid is intermediate in character between the patterns in the parents. This intermediacy is quantified in terms of the fairly high correlation coefficients between the hybrid data and the arithmetic mean of results of the two parents. The correlation is higher in the *E. horridus* X *E. lehmannii* hybrid (0,96) than in the *E. horridus* X *E. longifolius* hybrid (0,81). In the latter cross, the profile is much closer correlated with the *E. horridus* parent (0,90) than with the *E. longifolius* parent (0,57). It is tempting to evaluate the hybrid profiles in terms of dominant and recessive gene contributions from the parents but the intermediacy in the hybrid results would seem to indicate a multiple-gene system is operative. There is insufficient evidence available at this stage to speculate further in this regard.

Localities

Table 27 and Figure 27 give the data from the analyses of leaves of mature *Encephalartos natalensis* plants in different localities throughout the distribution area for the species. In the plants from several localities (Ngeli Forest, Kranskop and Msinga) there is a bimodal alkane profile. The lower-carbon-homologue series is centred at about C-20 while the higher alkanes are distributed nore-or-less normally about the C-30 peak. This bimodal distribution is less obvious in the Eston, Kloof gorge, Jollivet and Vryheid samples while the Melmoth sample is irregular.

The conclusion infered is thus that a certain amount of variation occurs within at least this species. Since only one sample from each locality was tested, it is not possible to say whether the variation is indeed locality-specific. Furthermore, it is not known whether similar infraspecific variation occurs in other *Encephalartos* species. The data available do not preclude the possibility of polymorphism within *E. natalensis*.

TABLE 26 : A COMPARISON OF THE LEAF WAX ALKANE DISTRIBUTION IN SOME *Encephalartos* SPECIES AND THEIR HYBRIDS

Alkane homologue by carbon atom number (normal/isomers)[1]	Percentage abundance of alkane homologues in the taxa indicated						
	E. horridus Ref. 47[2]	*E. longifolius* Ref. 29	Arithmetic mean Refs. 47,29	*E. horridus* x *E. longifolius* Refs. 54, 64	*E. lehmannii* Ref. 46	Arithmetic mean Refs. 47, 46	*E. horridus* x *E. lehmannii* Ref. 58
c17n	2	0	1	tr	2	2	1
c17i	tr	0	tr	0	tr	tr	0
c18n	6	5	6	2	6	6	2
c18i	1	0	1	1	1	1	0
c19n	13	18	16	8	13	13	9
c19i	2	1	2	3	2	2	1
c20n	16	16	16	14	26	21	25
c20i	6	7	7	5	12	9	10
c21n	4	7	6	5	8	6	7
c21i	2	2	2	3	4	3	4
c22n	1	2	2	3	2	2	2
c22i	1	0	1	2	1	1	1
c23n	1	19	10	2	1	1	2
c23i	tr	tr	tr	2	0	tr	0
c24n	1	3	2	2	2	2	3
c24i	0	0	0	2	1	1	0
c25n	1	5	3	5	2	2	2
c25i	tr	tr	tr	1	2	1	1
c26n	2	2	2	3	2	2	2
c26i	tr	0	tr	1	2	1	2
c27n	2	1	2	3	tr	1	2
c27i	0	0	0	tr	tr	tr	0
c28n	4	8	6	6	2	3	3
c28i	0	tr	tr	0	tr	tr	0
c29n	4	1	3	6	1	3	2
c29i	0	0	0	0	0	0	0
c30n	11	1	6	10	3	7	6
c30i	0	0	0	0	0	0	0
c31n	8	1	5	9	2	5	4
c31i	0	0	0	0	0	0	0
c32n	7	tr	4	4	2	5	6
c32i	0	0	0	tr	0	0	0
c33n	4	1	3	3	1	3	3
c33i	0	0	0	0	0	0	0
c34n	tr	0	tr	tr	0	tr	0
c34i	0	0	0	0	0	0	0
c35n	tr	0	tr	tr	0	tr	0
c35i	0	0	0	0	0	0	0
c36n	0	0	0	0	0	0	0
c36i	0	0	0	0	0	0	0

Correlation coefficients

Arithmetic mean Refs. 47,29 / *E. horridus* x *E. longifolius* Refs. 54, 64	0,81	
E. horridus Ref. 47 / Arithmetic mean Refs. 47,29	0,90	
E. longifolius Ref. 29 / Arithmetic mean Refs. 47,29	0,57	
Arithmetic mean Refs. 47, 46 / *E. horridus* x *E. lehmannii* Ref. 58	0,96	
E. horridus Ref. 47 / Arithmetic mean Refs. 47, 46	0,95	
E. horridus Ref. 47 / *E. horridus* x *E. lehmannii* Ref. 58	0,86	

Notes: 1. "n" represents the normal or unbranched homologue, "i" represents the total isomeric contribution
2. The reference number refers to the collection number for the material and corresponds to herbarium voucher specimen numbers.

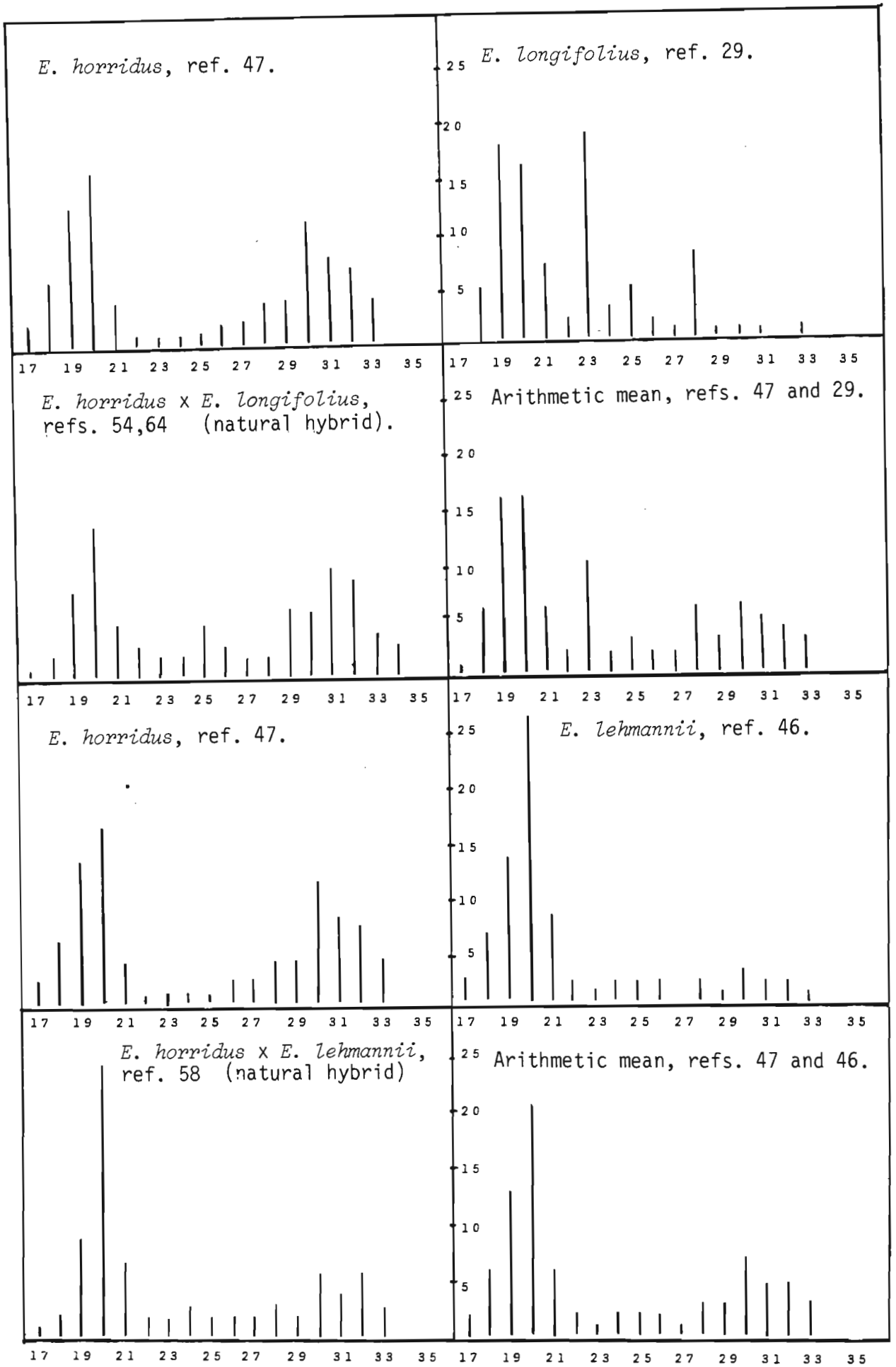


FIGURE 26 : LEAF WAX n-ALKANE PROFILES FOR SOME *Encephalartos* species and their hybrids.

TABLE 27 : A COMPARISON OF THE LEAF WAX ALKANE DISTRIBUTION OF *ENCEPHALARTOS NATALENSIS* FROM DIFFERENT LOCALITIES

Alkane homologue by carbon atom number (normal/isomers)[1]	Percentage abundance of alkane homologues by samples from localities indicated									
	Male Msinga Ref. 1	Male Malagata Ref. 2	? Eston Ref. 3	Male Melmoth Ref. 14	Male Vryheid Ref. 15	Female Kloof Ref. 16	Female Jollivet Ref. 18	? Ngeli Ref. 60	Female Kranskop Ref. 61	Arithmetic Average (of 9)
c17n	0	0	0	0	0	0	0	1	tr	0
c17i	0	0	0	0	0	0	0	tr	tr	0
c18n	1	0	tr	tr	0	tr	1	3	1	1
c18i	0	0	0	0	0	0	tr	1	tr	tr
c19n	2	1	2	tr	3	2	4	7	4	3
c19i	0	0	0	tr	0	tr	tr	1	1	0
c20n	9	4	7	2	7	2	4	16	8	7
c20i	4	1	1	1	1	1	2	7	3	2
c21n	9	4	7	3	2	3	2	4	2	4
c21i	8	2	3	tr	2	tr	1	3	1	2
c22n	4	2	7	3	2	2	2	1	1	3
c22i	1	0	0	1	0	tr	1	1	tr	1
c23n	4	3	9	20	15	10	7	1	1	9
c23i	0	0	0	0	0	1	1	0	tr	tr
c24n	6	4	10	6	6	4	4	2	3	5
c24i	tr	0	tr	3	0	3	2	1	tr	1
c25n	5	5	10	14	6	9	7	2	3	7
c25i	1	0	1	2	6	2	3	tr	1	2
c26n	7	7	9	3	4	4	5	4	6	6
c26i	0	0	1	0	0	0	2	tr	tr	tr
c27n	6	7	8	3	3	6	5	4	7	9
c27i	0	0	1	1	3	1	1	0	0	1
c28n	8	16	8	13	13	11	9	9	14	11
c28i	0	0	0	1	0	0	0	0	0	0
c29n	6	11	6	3	7	12	16	8	10	9
c29i	0	0	0	0	0	0	0	0	0	0
c30n	9	16	4	1	11	10	9	11	15	10
c30i	0	0	0	0	0	0	0	0	0	0
c31n	6	11	4	3	7	9	7	8	11	7
c31i	0	0	0	1	0	tr	0	0	0	0
c32n	3	5	1	1	3	3	2	3	4	3
c32i	0	0	0	0	0	0	0	0	0	0
c33n	1	1	1	6	2	4	2	1	2	2
c33i	0	0	0	0	0	0	0	0	0	0
c34n	tr	0	0	2	0	1	tr	tr	tr	tr
c34i	0	0	0	1	0	0	tr	0	0	0
c35n	0	0	0	3	0	tr	tr	tr	tr	tr
c35i	0	0	0	1	0	0	0	0	0	0
c36n	0	0	0	1	0	0	0	0	0	0
c36i	0	0	0	2	0	0	0	0	0	0

Notes : 1 : "n" represents the normal or unbranched homologue, "i" represents the total isomeric contribution
2 : The reference number refers to the collection number for the material and corresponds to herbarium voucher specimen numbers.

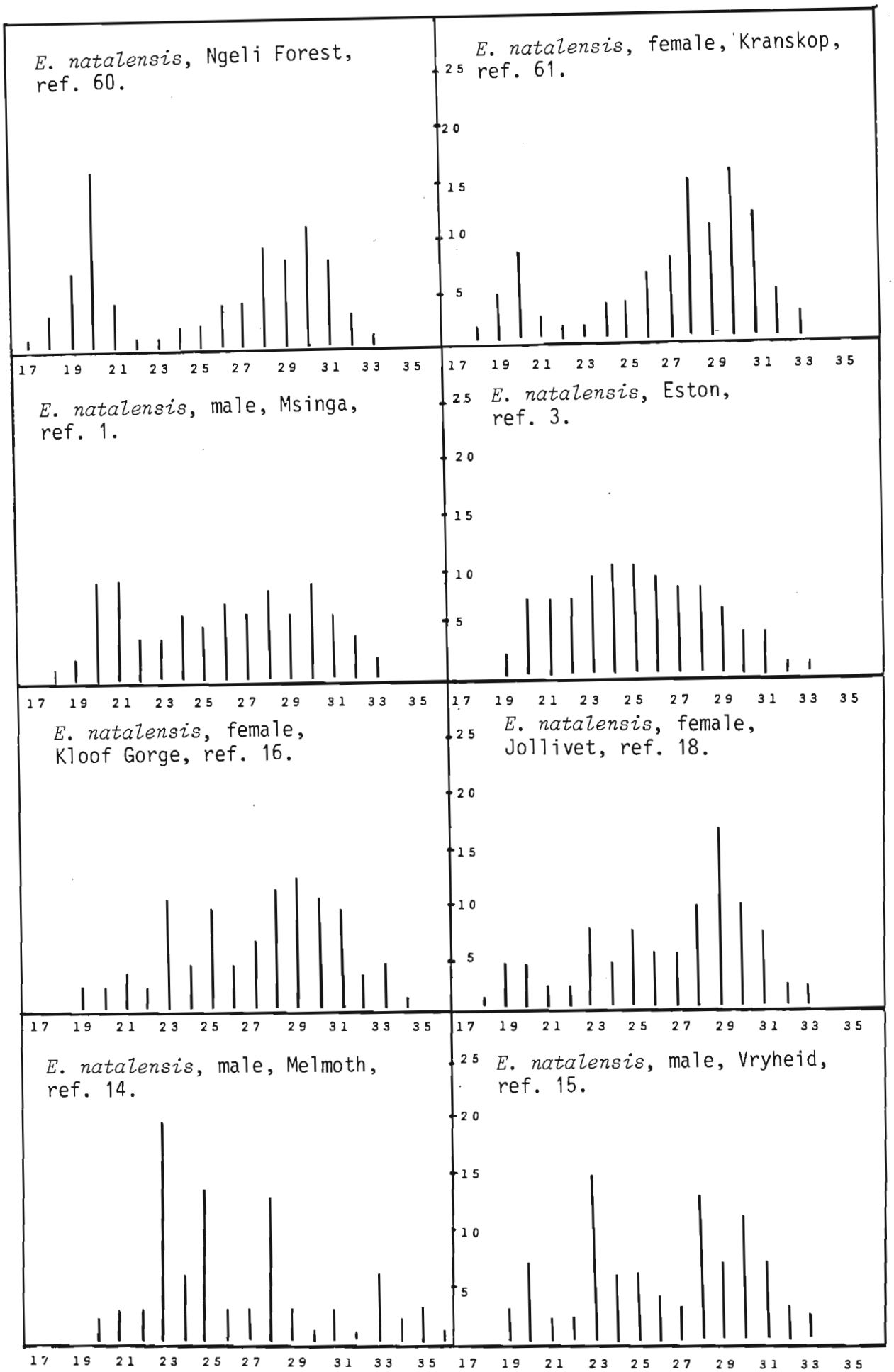


FIGURE 27 : LEAF WAX n-ALKANE PROFILES FOR *E. natalensis* PLANTS FROM DIFFERENT LOCALITIES.

Inter-specific variation

Details of the leaf wax alkane analyses for 24 *Encephalartos* taxa are given in Tables 28 and Figure 28. Inspection of the data shows that a wide range of variations occurs from one taxon to another. The variation between taxa appears to be wider than that within *E. natalensis* (see previous paragraph) and hence there is a potential usefulness of these data for taxonomic evaluation.

The bimodal distribution between lower and higher carbon homologues occurs widely throughout the genus, with the C-20 and C-30 fractions being approximate centres for the two groups. In the higher homologue zone there is a bias towards odd-numbered C-atom homologues, this tendency exemplified best by *E. ferox* and *E. villosus* where the C-31 and C-33 alkanes are particularly prominent. An explanation for this tendency is found in the biosynthesis of alkanes.

Biosynthesis of alkanes

The production of plant waxes is a consequence of a series of enzymically-controlled, and hence genetically-determined, biosynthetic steps. The metabolic pathways for alkane biosynthesis are fairly well known (KOLATTUKUDY, 1976; HARWOOD and RUSSELL, 1984). A first stage involves the elongation of ubiquitous saturated fatty acid molecules such as palmitic acid ($CH_3(CH_2)_{14}COOH$) and stearic acid ($CH_3(CH_2)_{16}COOH$) by successive C-2 unit additions, the product thus retaining an even-C number. The second stage is a decarboxylation process which yields an odd-C alkane. This biosynthetic route is clearly consistent with the typical occurrence of high proportions of C-29, C-31 and C-33 alkanes in epicuticular waxes of a large number of higher plants. The process is consistent with the analytical results obtained from leaf wax samples from *E. ferox* and *E. villosus* as described in the previous paragraph.

An alternative pathway which involves a condensation of two fatty acids followed by reductive decarboxylation, the so-called "acceptor-donor" condensation mechanism (KOLATTUKUDY, 1976), has since lost favour (HARWOOD and RUSSELL, 1984).

In some higher plants, there are significant quantities of even-C alkanes.

TABLE 28 : A COMPARISON OF THE LEAF WAX ALKANE DISTRIBUTION OF DIFFERENT SPECIES OF *Encephalartos*

Alkane homologue by carbon atom number (normal/isomers)	Percentage abundance of alkane homologues based on average composition per taxon							
	E. arenarius Ref. 22	*E. cupidus* Ref. 28	*E. eugene-maraisii* (Waterberg) Ref. 25	*E. eugene-maraisii* (Middleburg) Refs. 26,48	*E. dolomiticus* (sensu LAVRANOS and GOODE) Ref. 27	*E. ferox* Refs. 4,21	*E. friderici-guilielmi* Refs. 43,44	*E. ghellinckii* Refs. 41,42, 65,66
c17n	0	0	0	tr	0	0	tr	tr
c17i	0	0	0	0	0	0	0	0
c18n	0	0	1	2	0	0	2	2
c18i	0	0	0	1	0	0	1	1
c19n	tr	tr	4	9	4	tr	9	12
c19i	0	0	tr	2	tr	tr	4	4
c20n	2	2	5	16	6	1	14	19
c20i	tr	tr	2	5	2	tr	9	9
c21n	3	3	7	6	3	1	7	8
c21i	tr	2	1	3	1	tr	12	10
c22n	1	5	4	2	1	1	3	3
c22i	0	1	1	2	tr	1	3	3
c23n	12	26	20	5	9	4	2	2
c23i	tr	5	0	0	0	1	2	2
c24n	2	9	4	3	3	2	2	2
c24i	tr	0	2	2	2	tr	2	2
c25n	6	17	10	5	7	3	3	3
c25i	tr	5	2	2	2	1	2	1
c26n	1	6	1	3	5	4	2	3
c26i	1	0	0	2	3	tr	1	2
c27n	1	3	1	2	6	3	2	2
c27i	tr	2	0	1	2	tr	1	2
c28n	16	6	12	6	9	4	5	3
c28i	tr	2	1	1	0	tr	tr	1
c29n	4	1	2	4	5	10	5	3
c29i	0	0	0	0	0	0	0	0
c30n	13	1	2	5	12	8	5	2
c30i	0	0	1	tr	tr	0	0	0
c31n	12	1	3	6	9	28	5	2
c31i	0	0	1	1	0	0	0	0
c32n	8	tr	1	4	5	14	1	tr
c32i	0	0	0	0	0	0	0	0
c33n	9	2	3	4	2	18	1	tr
c33i	0	0	tr	0	0	0	0	0
c34n	2	0	2	tr	1	1	tr	0
c34i	0	0	tr	0	0	0	0	0
c35n	3	0	5	1	1	1	0	0
c35i	0	0	0	2	0	0	0	0
c36n	2	0	2	1	0	0	0	0
c36i	0	0	0	0	0	0	0	0

TABLE 28 (continued)....

Alkane homologue by carbon atom number	Percentage abundance of alkane homologues based on average composition per taxon							
	E. horridus Ref. 47	*E. lanatus* Ref. 24	*E. latifrons* Ref. 23	*E. lebomboensis* Ref. 7	*E. lebomboensis* var "Piet Ketlefii" Refs. 8,9	*E. lehmannii* Ref. 46	*E. longifolius* Ref. 29	*E. manikensis* Ref. 50
c17n	2	0	0	0	0	2	0	0
c17i	tr	0	0	0	0	tr	0	0
c18n	6	4	tr	0	0	6	5	1
c18i	1	tr	0	0	0	1	0	0
c19n	13	20	1	4	1	13	18	4
c19i	2	2	0	0	0	2	1	0
c20n	16	20	3	12	11	26	16	6
c20i	6	9	1	2	3	12	7	2
c21n	4	3	3	9	8	8	7	2
c21i	2	3	1	6	6	4	2	tr
c22n	1	1	3	4	3	2	2	1
c22i	1	1	0	0	0	1	0	0
c23n	1	6	22	4	3	1	19	1
c23i	tr	0	2	0	0	0	tr	0
c24n	1	2	7	5	3	2	3	1
c24i	0	1	0	0	0	1	0	0
c25n	1	5	13	5	4	2	5	2
c25i	tr	1	4	0	0	2	tr	tr
c26n	2	tr	5	6	4	2	2	3
c26i	tr	1	0	0	0	2	0	1
c27n	2	1	2	6	5	tr	1	4
c27i	0	tr	0	0	0	tr	0	tr
c28n	4	7	14	6	7	2	8	10
c28i	0	tr	0	0	0	tr	tr	0
c29n	4	2	4	7	8	1	1	10
c29i	0	0	0	0	0	0	0	0
c30n	11	1	5	6	11	3	1	21
c30i	0	1	0	0	0	0	0	0
c31n	8	2	5	8	10	2	1	18
c31i	0	0	0	0	0	0	0	0
c32n	7	1	1	7	7	2	tr	7
c32i	0	0	0	0	0	0	0	0
c33n	4	2	4	5	9	1	1	6
c33i	0	0	0	0	0	0	0	0
c34n	tr	1	0	4	tr	0	0	0
c34i	0	0	0	0	0	0	0	0
c35n	tr	2	0	0	2	0	0	0
c35i	0	0	0	0	0	0	0	0
c36n	0	0	0	0	0	0	0	0
c36i	0	0	0	0	0	0	0	0

TABLE 28 (continued)....

Alkane homologue by carbon atom number	Percentage abundance of alkane homologues based on average composition per taxon							
	E. manonii Ref. 51	*E. natalensis* ex Table 27 (mean of 9)	*E. ngoyanus* Refs. 5,6	*E. paucidentatus* Ref. 49	*E. princeps* Ref. 45	*E. transcendens* Ref. 20	*E. velutinus* Refs. 10,11,12	*E. woodii* Ref. 19
c17n	0	0	0	1	1	0	0	0
c17i	0	0	0	tr	0	0	0	0
c18n	1	1	1	2	3	2	tr	2
c18i	tr	tr	0	1	tr	0	0	0
c19n	8	3	4	10	11	5	tr	4
c19i	3	0	tr	3	1	tr	0	tr
c20n	19	7	20	12	16	4	4	4
c20i	5	2	6	5	6	1	tr	2
c21n	3	4	18	3	4	4	2	1
c21i	2	2	8	2	2	tr	tr	tr
c22n	1	3	8	2	1	4	1	2
c22i	tr	1	2	1	1	1	0	0
c23n	1	9	10	2	2	22	1	8
c23i	0	tr	1	2	0	2	0	0
c24n	2	5	4	3	3	7	1	4
c24i	0	1	0	2	2	0	0	2
c25n	2	7	7	4	4	11	1	7
c25i	tr	2	0	2	2	2	0	2
c26n	3	6	4	5	5	3	1	4
c26i	tr	tr	0	2	2	0	0	0
c27n	5	9	3	6	4	5	1	7
c27i	0	1	0	1	0	tr	0	0
c28n	7	11	3	7	5	8	2	15
c28i	0	0	tr	0	0	0	0	0
c29n	9	9	3	7	5	6	6	20
c29i	0	0	0	0	0	0	0	0
c30n	10	10	3	6	7	3	4	3
c30i	0	0	0	0	0	0	0	0
c31n	12	7	2	6	6	6	37	5
c31i	0	0	0	0	0	tr	0	tr
c32n	4	3	1	2	3	1	7	1
c32i	0	0	0	0	0	0	0	0
c33n	3	2	1	1	2	3	31	2
c33i	0	0	0	0	0	0	0	0
c34n	tr	tr	tr	tr	tr	0	1	1
c34i	0	0	0	0	0	0	0	0
c35n	0	tr	0	0	0	0	1	2
c35i	0	0	0	0	0	0	0	0
c36n	0	0	0	0	0	0	0	1
c36i	0	0	0	0	0	0	0	0

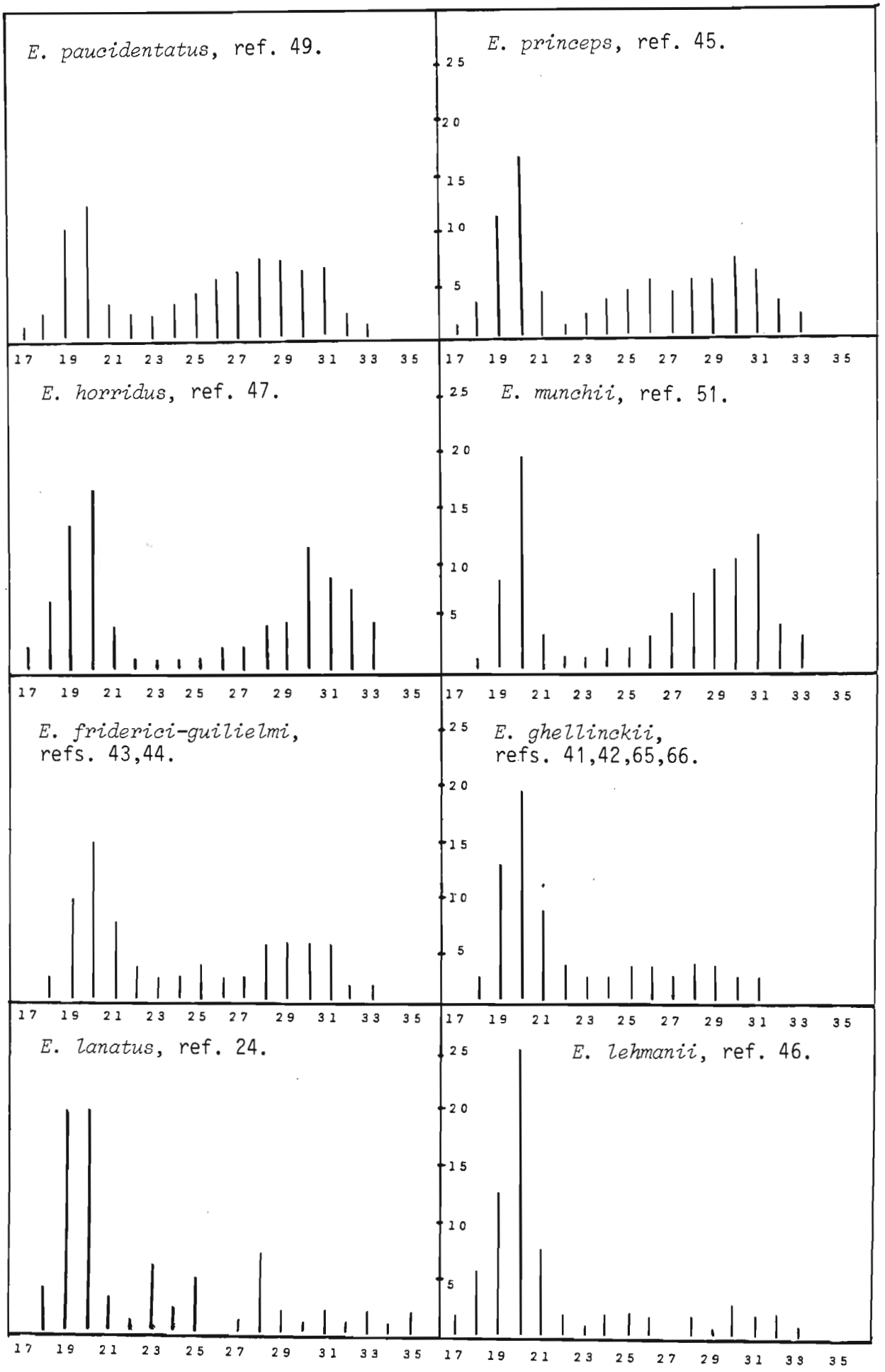


FIGURE 28 (continued): LEAF WAX n-ALKANE PROFILES OF *Encephalartos* SPECIES.

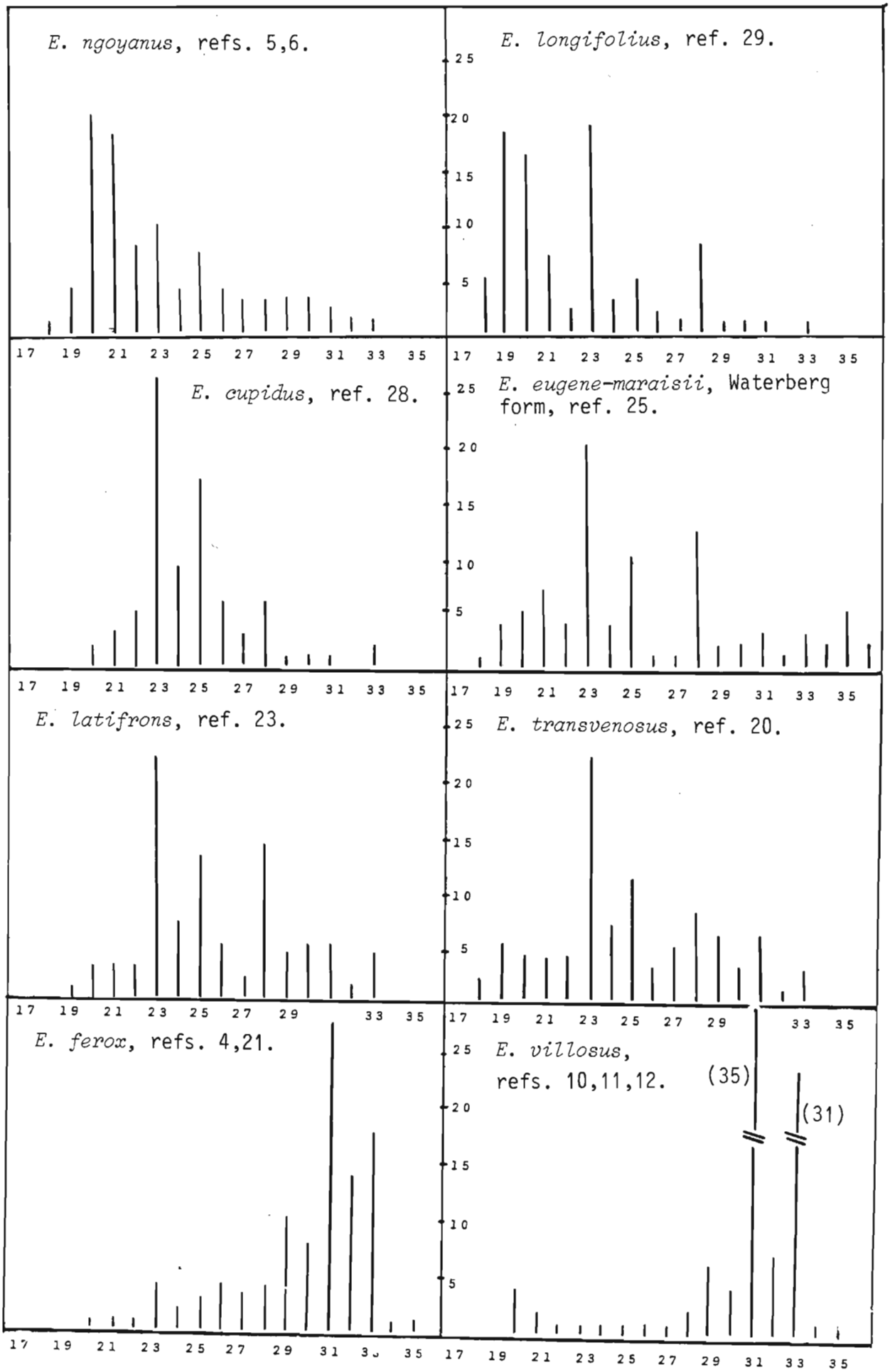


FIGURE 28 (continued): LEAF WAX n-ALKANE PROFILES OF *Encephalartos* SPECIES.

An explanation for their occurrence may lie in the loss of one carbon unit by means of an α-oxidation process at some undefined stage in the biosynthetic sequence (KOLATTUKUDY, 1976; HARWOOD and RUSSELL, 1984). This type of process may account for the common occurrence of large proportions of even-C alkanes in *Encephalartos* species other than *E. ferox* and *E. villosus*.

The bimodal distribution of alkanes which occurs so commonly in *Encephalartos* is not readily accounted for in biosynthetic terms. It may be speculated that two competing biosynthetic routes occur, one of which terminates at about C-20 and the other at C-30 alkane units. Alternatively, the possibility of the same process operating on different precursors cannot be eliminated.

The alkane production processes in lower plants are not well known, but a general indication exists that algae, mosses and ferns have somewhat greater concentrations of lower alkane homologues (C-15 to C-23), and that the ratio of odd-C to even-C compounds is closer to unity, than in the Angiosperms (STRANSKY, STREIBL and HEROUT, 1967). Thus, if the bimodal alkane profile in *Encephalartos* is associated with two separate biosynthetic mechanisms, it may be that the route leading to the lower homologues is that which occurs in lower plants while the production of higher homolgues follows an Angiosperm biosynthetic route. Clearly there is an opportunity for useful research work to test this hypothesis but such a project is beyond the scope of the current thesis.

Taxonomic implications

The taxonomic usefulness of leaf wax analyses is well documented and has been focussed in particular on alkane distribution patterns in Angiosperms (EGLINTON, GONZALES, HAMILTON and RAPHAEL, 1962; KOLATTUKUDY, 1976; SMITH, 1976). Development of modern gas chromatography techniques has greatly facilitated data collection. Whilst there is usually considerable correlation between hydrocarbon profiles and taxonomic rank, the influences of factors such as physiological age of the material, seasonal effects, soil type and other local environmental influences must not be discounted (STRANSKY, STREIBL and HEROUT, 1967). The variation of alkane profiles between juvenile and mature *Encephalartos* leaves, and the variation with

one species from different localities, have already been shown to be significant in the preceding paragraphs.

Despite the above constraints, it is believed that the leaf-wax alkane distribution in *Encephalartos* has considerable taxonomic value. Figure 29 gives a dendrogram constructed on the basis of unweighted pair-group analysis of taxonomic distances calculated from the alkane distributions in 24 *Encephalartos* taxa (see Section 3.10.1).

Inspection of the dendrogram reveals many relationships which are consistent with morphological similarities, but there are a number of instances where the alkane-based relationships differ markedly from those anticipated on morphological grounds.

In view of the prevalence of the C-31 and C-33 alkanes in *E. ferox* and *E. villosus* (as previously discussed) the separation of these from the remaining taxa is not surprising. These two species are similar in sharing mesic habitats and having well-developed subterranean caudices. However, they differ substantially in leaf and cone morphology and are not considered to be closely related.

A group of mesic plants of arborescent habit, with relatively few adaptations, comprises *E. natalensis*, *E. lebomboensis*, *E. manikensis* and *E. woodii*. These occur in juxtraposition in the dendrogram. On morphological grounds, it may be anticipated that *E. longifolius*, *E. paucidentatus* and *E. transvenosus* would be included but the alkane data indicate otherwise. The presence of *E. arenarius* and *E. dolomiticus* in this area would not be expected on morphological evidence.

A second group which is usually considered to be related comprises *E. horridus*, *E. lehmannii* and *E. princeps* (and *E. trispinosus* on which data are not available). These occur in the dendrogram within the same broad area but together with several other species not considered to be related. Within this zone of the dendrogram, the close relation of *E. friderici-guilielmi* and *E. ghellinckii* is consistent with some degree of morphological similarity coupled with an unusual degree of frost tolerance.

The positions of the various taxa within the *E. eugene-maraisii* complex

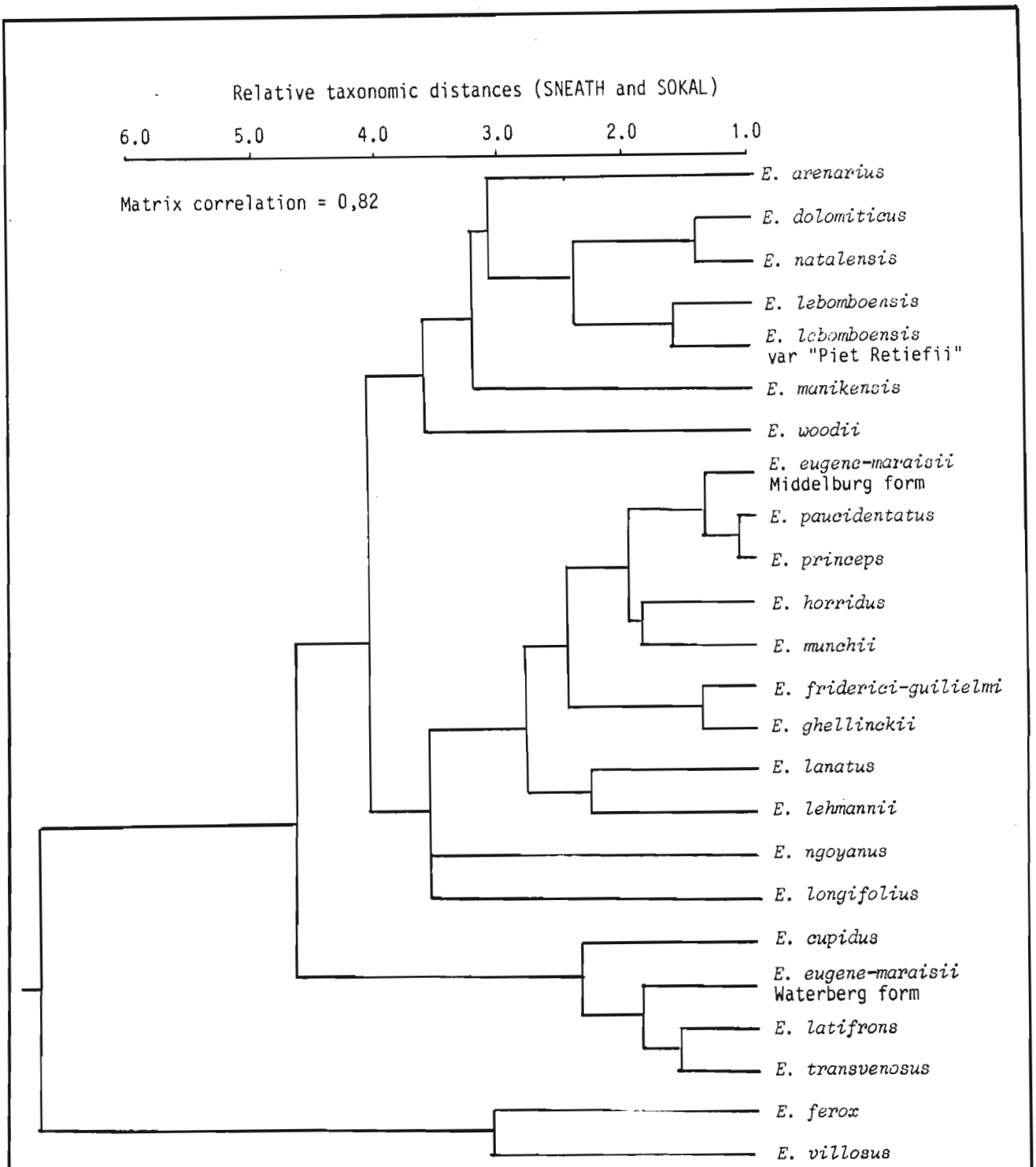


FIGURE 29 : A dendrogram constructed on the basis of unweighted pair-group analysis of taxonomic distances calculated from the alkane leaf wax composition of 24 *Encephalartos* taxa.

deserves mention. The Wolkberg form (*E. dolomiticus* sensu LAVRANOS and GOODE), the Middelburg form and the Waterberg form are evidently quite different in terms of alkane profiles. This may lend support to a current proposal that *E. eugene-maraisii* should be separated into four distinct taxa (LAVRANOS AND GOODE, 1988).

In summary, the evidence collected so far from approximately one-half of the various *Encephalartos* taxa is seen to provide much useful evidence to the taxonomists. This author, in conjunction with Professor A and M.L. Salatino, will continue to collect and process leaf wax alkane data until a fully-representative "library" of alkane profiles is available. It is anticipated that an important publication will arise from this work and, in the longer term, similar surveys may be conducted on other genera within the Cycadales.

3.11 Conclusion

The experimental work in this section has explored a number of facets of cycad phytochemistry with an emphasis on the South African species.

Moisture analyses of the seed show that at the time of shedding, a relatively wet embryo is surrounded by relatively dry megagametophytic tissue within the sclerotesta. The latter is encased in turn within a relatively wet fleshy layer. On storage, both the outer fleshy layer and the megagametophyte loose moisture, this process being reversed by imbibition prior to germination. This behaviour is consistent with the character of the broad group described as recalcitrant seeds. Moisture contents of other tissues show a general tendency to decrease with age.

The soluble protein analyses of various tissues vary widely. A tendency for accumulation of soluble proteins with physiological age was evident. High levels are present in some reproductive structures but not in embryos or sarcotestae. There is an indication of a protein gradient from the top to the bottom of primary roots.

Peroxidase enzymes are higher in cycad leaves than other parts of the plants. Higher peroxidase activities are shown by leaves of plants from mesic habitats than from those with xeric adaptations, and the

levels decline with leaf age. Tissues with higher peroxidase levels give a more rapid callogenesis response *in vitro* than corresponding tissue with lower enzyme levels. A general indication emerges of a correlation between peroxidase activity and growth potential, consistent with reports in the literature. This is undoubtedly part of the complex sequence of interactions between various growth regulating substances and specific enzymes. Further evidence of this correlation is provided by the sharp increase in peroxidase activity of *Stangeria* megagametophytes *in vitro* which occurs just prior to visible signs of callus formation.

In general, it is clear that organ composition varies widely. Whilst this variation is not unexpected, it is significant in terms of explaining the responses of different organs in culture. More comprehensive analyses may lead to a more rational basis on which material could be selected for *in vitro* experiments. Data on the distribution of endogenous plant growth regulators would be especially useful in this respect.

An incidental benefit arising from the phytochemical work is seen in its value in taxonomic applications. In this respect, the author's experimental work shows some parameters to be more useful than others. The thin layer chromatography of cycad toxins, the seed protein serology and immuno-electrophoresis data reinforce the present separations at family and genus levels but have little value at the species level. Peroxidase electrophoresis provides a measure of separation between species but techniques suffer from limitations in resolution and reproducibility.

Of the various parameters explored, the analyses of cycad leaf wax hydrocarbons provided the most potentially-useful data for taxonomic evaluation. At least within the genus *Encephalartos*, each species provides a unique hydrocarbon profile. It is anticipated that use of these techniques, in conjunction with numerical taxonomy computer programmes, could contribute much to resolution of existing taxonomic problems and could in addition allow the construction of a phylogenetic sequence for the order.

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APPENDICES

ON THE EVOLUTION OF CYCADS

by Roy Osborne

WHAT really did happen "in the beginning" of life on earth is a puzzle which is likely to remain unsolved, at least in our lifetimes. Careful examination and re-examination of all the evidence by a great many trained minds has resulted in a story of what *might* have happened. Over the last century this story has been progressively modified and becomes steadily more convincing.

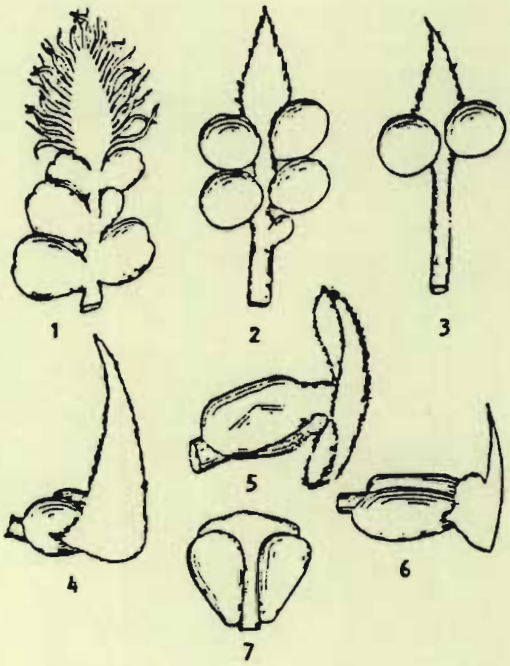

FIG. 1:
Series of female sporophylls, showing reduction from its leafy condition to the reduced sporophyll of the most compact one:
1. *Cycas revoluta*
2. *Cycas circinalis*
3. *Cycas media*
4. *Dioon edule*
5. *Dioon spinulosum*
6. *Macrozamia miguelii*
7. *Zamia floridana*
(Chamberlain, 1919).

The first plants abundant on this planet were undoubtedly fairly simple algae which grew in the oceans up to three billion years ago. Their photosynthetic ability resulted in the production of oxygen which slowly built up to 1%, 5% and 10% in the earth's atmosphere. This increase in oxygen had two far-reaching consequences: the potential for life outside the marine environment and the establishment of the protective ozone layer high up in the atmosphere which could screen out lethal ultra-violet radiation and allow organisms on land to survive. The stage was thus set for the first of the land plants, the liverworts, mosses amd primitive ferns, to make their appearance. But these early plants all retained one trait from their marine ancestors, an actively swimming male sperm cell and the constraint that water is essential in the reproductive process. Later, with the advent of the seed-bearing plants, the motile spermatozoid became redundant and (except for a few strange anachronisms like *Ginkgo biloba*) water was no longer essential in reproduction.

Somewhere in the complex evolutionary pattern between the great groups of 'ferns' and 'seed-plants' came the cycads, which do have motile sperm cells like their primitive ancestors but bear seeds similar to those of the most advanced flowering plants.

Dr Knut Norstog of the Fairchild Tropical Garden draws an analogy between the evolutionary sequence in plants and animals; in his comparison the cycads are the 'reptiles' of the plant world since it is the reptiles that span the great gap between the amphibian restricted to its watery habitat and the birds and mammals with

internal fertilisation. Norstog and his colleagues are presently carrying out detailed electron microscopy studies on the sperm cells of mosses, ferns and cycads, and believe that the fundemental differencs and similarities could shed new light on plant evolution.

Within the cycads characters range from the primitive to the advanced. Take for instance the way in which the leaflets of *Cycas* uncoil – very much like the circinate character of the fern fronds. In *Cycas* too, the very loosely-organised collection of modified seed-bearing leaves (see Fig. 1) is clearly earlier in evolutionary time than the compact cone we know in *Encephalartos*. Yet the prominent midrib in the *Cycas* leaflet is considered advanced. Wind-pollination is considered primitive and insect or bird-pollination advanced; obviously animal-pollinated plants cannot pre-date the animals themselves! But the experts cannot seem to agree just how cycads are pollinated so that does not help much. Consideration of features like the anatomy of the stem and the structure of leaf stomata all add more evidence. The overall conclusion is that cycads usually have a mixture of some relatively-advanced and some relatively-primitive characteristics – the situation is not as simple as was first thought.

In explaining this apparent paradox some of the most useful evidence comes from the fossil records. Figure 2 shows a 160-million year old cycad leaf called *Zamites*, a name implying probable relationship with the present *Zamia* genus. Since the age of rock strata are fairly accurately assessed, the age of any fossil specimen trapped within may be estimated. But not all fossils are perfect specimens; often they are poorly preserved and consist of incomplete fragments rather than whole plants. The latter difficulty has frequently resulted in fossil leaves, stems and other parts of the same plant being given different names at first. But painstaking work by many dedicated palaeobotanists has allowed the theoretical reconstruction of the plants and forests of many millions of years ago. Figure 3 shows some examples of these reconstructed species.

FIG. 2:
a 160-million year old fossilized frond named *Zamites fenconis* found in France.
(Case, 1982).

Well-preserved cycad fossils have been found, often abundantly, in Mesozoic rocks from Siberia, Manchuria, Oregon, Alaska, Greenland, Sweden, England and Central Europe, India, Australia and South Africa. The fossil record from this part of the world shares the same problems of incompleteness and fragmentation as do most other areas. The seed-fern (pteridosperm) foliage, which preceded the cycads, is quite well represented by the so-called *Dicroidium* flora,

examples of which have been found from the upper Umkomaas valley and 'Little Switzerland' in Natal, Rouxville in the O.F.S. and Dordrecht in the Eastern Cape. Later and more typical cycad foliage called *Zamites* and *Dictyozamites* comes from the geological 'Uitenhage' series, and good examples are displayed at the Port Elizabeth and Kingwilliamstown museums and the Bernard Price Institute for Palaeontological

vegetation described as the Devonian Flora was almost entirely destroyed by a major glaciation in the Southern Hemisphere at the end of the Palaeozoic era, about 250 million years ago. But the cold was followed by a long period of warm and balmy years, the Mesozoic era. Mean temperatures were about 10°C higher than at present and the climate was more or less the same worldwide. It was at this time that the cycads

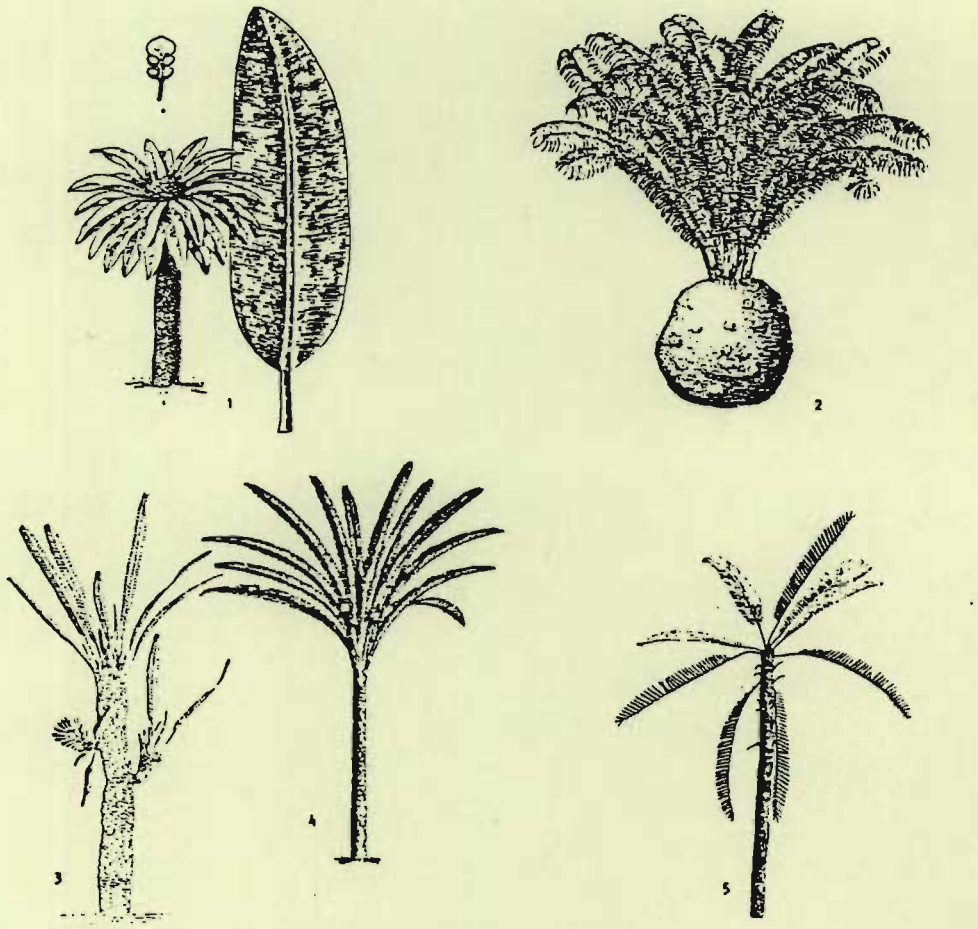

FIG 3: Hypothetical reconstructions of early cycads from fossil records.
1. *Bjuvia simplex* (Florin, 1933); 2. *Cycadeoidea* (Delevoryas, 1971); 3. *Williamsonia sewardiana* (Sahni, 1932); 4. *Williamsonia gigas* (Williamson, 1870); 5. *Leptocycas gracilis* (Delevoryas & Hope, 1971).

Research at Wits University. The tireless efforts of workers like Professor Alex du Toit, Dr Edna Plumstead and Dr Heidi Anderson have done much to sort out the complexities of the early cycad flora of Southern Africa.

Another factor which may shed some light on cycad evolution is the earth's climate. For instance, it is now known that the lycopod type

rose to abundance in the world's vegetation; just as in the animal world the dinosaurs were then at zenith. But the Mesozoic cycads were not the same as our present cycads, an important and often overlooked point. Indeed there were two broad types, the cycadaleans (ancestors of our present-day cycads) and the cycadeoidaleans (sometimes called the Bennettitales) which later

became extinct. These two groups differed so much in their reproductive habits, and in other ways, that it is thought that both groups evolved *independently* from a much earlier ancestor in the seed-ferns.

A final factor in the cycad equation is that of the geography of the world of the late Palaeozoic and Mesozoic eras. This was the time when the southern part of Africa, South America, India, the gradient to the coast – the great escarpment took its present form and the central karroo became the arid basin we know now.

Throughout this time the cycads were changing – the present world distribution provides an excellent example of how a group of plants evolved from presumably one common ancestor and, as a result of geographical isolation and consequent climatic and other differences,

FIG. 4:
Proposed continental re-grouping for Gondwanaland.
(Trustwell, 1970).

Antarctica and Australia were all joined to form the massive supercontinent of Gondwanaland (see Figure 4). At the time when the cycads had reached their zenith, great cracks appeared in the land and thousands of tons of basalts welled up and spread over the surface. The South Atlantic ocean gradually widened, starting by a separation of the Falkland tip from the Agulhas escarpment fairly late in the Mesozoic era. On the eastern side, the Mascarene Plateau reached across Tanzania–Kenya through Madagascar to India – a giant dinosaur trail which later became disrupted; only 65 million years ago India separated and rafted up to collide with Asian landmass. To the south, Antarctica formed a bridge between South America and Australia until about 50 million years ago. As recently as 25 million years ago the African continent had assumed its present position, but major uplifts were still to follow. These raised the interior plateaux by more than 1 800m and increased underwent a whole series of very marginal changes which collectively gave rise to different genera: *Encephalartos* and *Stangeria* in Africa, *Cycas* in the Indian ocean Islands, *Macrozamia, Bowenia* and *Lepidozamia* in Australia, and *Zamia, Ceratozamia* and *Microcycas* in the New World. Futhermore, *within* each of these genera, there was the possibility of further speciation under the influence of local environmental conditions. In *Encephalartos* for example, we can postulate a fairly close relationship between *E. altensteinii, E. natalensis, E. woodii (?), E. lebomboensis, E. manikensis* and *E. gratus. E. princeps* is thought by Dr Dyer to be the first (hence the name) in a group including *E. lehmannii, E. trispinosus* and *E. horridus*. Clearly related are *E. villosus* and *E. umbeluziensis, E. ghellinckii* and *E. cycadifolius; E. arenarius* and *E. ferox; E. caffer* and *E. ngoyenus*. And so on.

It is to be considered something of a miracle that the cycads survived through so many and such

extreme climatic and geographical changes. Those that survived did so by virtue of their adaptability – the ability to change through genetic processes, the elimination of weaker plants in competition with the stronger, the 'survival of the fittest'. Thus the cycads are not really some sort of hangers-on from the coal ages, but, as Dr James Eckenwalder of the University of Toronto puts it . . . 'a vigorous and successful modern group of plants, still evolving and capable of responding to changing environmental conditions.'

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CYCAD RESEARCH IN THE 80'S

BY ROY OSBORNE

Although this newsletter is not set out to be an in-depth scientific journal, it is clear that many of our readers are deeply interested in current technical projects involving cycads and thus it is appropriate to highlight some of the cycad-related research work which has been carried out over the past few years. This article is not intended to be a comprehensive review of the literature, indeed only about one-half of the total number of 'cycad' publications is mentioned. To avoid making this too involved, I have not for instance covered the current work on palaebotany such as that of Delevoryas (1982). I have also not included specific comments on the many more popular-style articles such as those featured in the magazines of the four cycad societies, nor the several excellent cycad articles published in Fairchild Tropical Garden Bulletins. Nevertheless, this article does show something of the great deal of scientific work currently in progress.

TAXONOMY AND SYSTEMATICS

Dr. Dennis Wm. Stevenson (1981,1985) of Columbia University in New York has proposed significant classification changes within the Cycadales on the basis of known and suspected relationships in the order. In his current hypothesis, the order is divided into two sub-orders, the Stangerineae (families Stangeriaceae and Boweniaceae) and the Cycadineae (families Cycadaceae and Zamiaceae). The family Zamiaceae is split into sub-families Dioooideae *(Dion)* and Zamioideae with tribes Zamieae *(Ceratozamia, Microcycas, Zamia)* and Encephalarteae *(Encephalartos, Lepidozamia, Macrozamia)*.

The rather confused taxonomy of the genus *Zamia* has been reviewed by James Eckenwalder (1980) who consolidated 35 taxa from the West Indies into the single species *Zamia pumila*, a proposal which has met with some dissention and may become modified. New species in the genus are *Z. fairchildiana* and *Z. pseudomonticola* (Gomez, 1982), *Z. inermis* (Vovides & Rees, 1983a) and *Z. splendens* (Schutzman, 1984). Schutzman and Vovides (1985) have collaborated on a systematic study of the broad concepts of *Z. loddigesii* and *Z. furfuracea*.

An impressive amount of taxonomic research has been carried out by the Italian team headed by Prof. Paolo De Luca at the University of Naples, with associates Sergio Sabato, Aldo Moretti and Mario Vazquez-Torres and others. The efforts of about 15 years field work in Mexico have resulted in six new *Dion* species being described: *D. califanoi* (1979), *D. caputoi* (1980a), *D. rzedowskii* (1980b), *D. merolae* (1981a), *D. holmgrenii* (1981b) and *D. tomasellii* in two varietal forms (1984). Two varieties of *D. edule*, var. *edule* and var. *angustifolium* are now recognized (1982).

The Italian school was responsible for changing the name from 'Dioon' back to the original *Dion* (1982), a move opposed by Andrew Vovides and Nancy Moreno (1983) but resubstantiated by De Luca, Sabato and Stevenson (1984). The work on *Dion* has very recently been summarised by Sabato and De Luca (1985) in a most informative paper on distribution, ecology and morphology of the genus in which three natural groups are recognized and which includes a key to the genus.

Four new species of Mexican *Ceratozamia* have been described: *C. hildae* (Vovides and Rees, 1980), *C. kuesteriana*, closely allied to *C. zaragozae*, re-discovered by Aldo Moretti and colleagues (1982), *C. norstogii* (Stevenson, 1982) and *C. microstrobila* (Vovides and Rees, 1983b) although the rank of the latter species is under review. Future reclassification of several taxa, presently varieties of *C. mexicana*,

is imminent.

A comprehensive work on the distribution (with maps) and ecology of the New World cycads has been published by Balduzzi, De Luca and Sabato (1982) and includes a discussion on the origin of these cycads on the basis of fossil records and continental drift. A pertinent observation for growers is that most *Dion* species normally favour dry conditions, *Zamia* is consistently heat-loving while *Ceratozamia* is restricted to areas where moisture is high.

Whilst there has thus been substantial work on the New World cycads, little has been published on the African or Australasian species. Two new *Cycas* species are added: *C. panzhihuaensis* (Cheng *et al*, 1980) and *C. guizhouensis* (Lan & Zou, 1983), both from China.

The relatively new science of karyotyping, characterisation of the individual chromosomes and chromosome pairs, has proved to have useful taxonomic significance in cycad systematics. Dr. Knut Norstog of Fairchild Tropical Garden has been the leader in this area of research and has shown that the situation is not as simple as was first thought. Dr. Norstog (1980, 1981) investigated the chromosome numbers of 14 species of *Zamia* and found considerable variation. In *Zamia chigua*, considered to be one of the primitive members of the genus, the chromosome number varies from 22 to 26. Less variation is observed with the chromosome numbers of West Indian *Zamias* and *Z. pseudoparasitica* which are thought to be more advanced. Andrew Vovides (1983) is also active in the field of *Zamia* karyotyping while Moretti and Sabato (1984) make reference to chromosome evolution in presenting evidence for the advanced status of *Z. paucijuga*. John Hendricks (1982) discusses aspects of chromosome evolution in the broader concept within the order as a whole.

Aldo Moretti (1982) has carried out a chromosome study of several Australian cycads using a fluorescence-staining technique which showed clear differences between *Macrozamia* on one hand and *Bowenia* and *Lepidozamia* on the other. This observation supports the taxonomic separation of *Lepidozamia* as a genus distinct from *Macrozamia* with which it had previously been included. Additional evidence in this connection is given later in this text. However, Moretti also found that *Macrozamia communis* and *M. pauli-guilielmi* subsp. *pauli-guilielmi* are karyotypically similar despite their present classification into the Macrozamia and Parazamia sections of the genus respectively,

Even more detailed in the microscopic sense is the painstaking mapping of the information on the genes themselves. Buran Kurdi-Haidar and co-workers (1983) from the American University of Beirut have reported on the DNA sequencing in the genome of *Cycas revoluta*.

Work in the cycad taxonomy continues and it is hoped that some of the loose ends in the *Cycas* and *Zamia* genera will be tied up soon. Some long-known species like *Encephalartos* 'archeri' from Voi in Kenya are yet to be properly written up and several 'new' species like the *Encephalartos* 'msinga' from Northern Natal/Kwazulu will be described in forthcoming literature.

POLLEN GENESIS AND DISPERSAL

Because of their unique evolutionary position, the cycads continue to attract interest in developmental studies and this is especially true with respect to pollen grain ontogeny. Paolo de Luca, Vincenzo la Valva and Sergio Sabato (1980) have investigated the process of sperm formation in *Ceratozamia mexicana* and *Cycas revoluta*, while the French worker, Jean-Claude Audran (1981), similarly reported details of pollen grain development in *Ceratozamia mexicana*. Osama Terasaka (1982) from Tokyo has reported his results on the development of the nucleus inside pollen grains of various gymnosperms including *Cycas revoluta* and *Bowenia serrulata*, while Stuchlick and Moncada (1983) have written up the pollen morphology of Cuban gymnosperms, including *Zamia* and *Microcycas*. Michael Zavada (1983) from Indiana University has published a report on the pollen wall development of *Zamia floridana*. A current report by Dehgan and Dehgan (1985) on the pollen morphology of

cycads provides valuable insights into taxonomic relationships, and supports current re-classification proposals within the order.

Karl Niklas has been involved in a joint project with Knut Norstog (1984) in which they make detailed measurements of pollen transport in aerodynamic terms from the source to the female cone surface. The implications of their findings in the reproduction of *Cycas*, *Dion* and *Zamia* are discussed. Little work has yet been done on the insect vector aspect in cycad pollination, one publication of relevance being a description by Gary Breckon and V.N. Ortiz (1983) on the pollination of *Zamia pumila* through the agency of fungus gnats. There is a real need for more studies on the relative importance of wind and insect factors in pollination of cycad species in habitat.

LEAF MORPHOLOGY, ANATOMY & PHYSIOLOGY

Baijnath, Naidoo and Ramcharuy (1980) from the University of Durban-Westville in Natal have used electron microscopic techniques to examine in detail the leaf surfaces of several cycad species, while Koeleman, Robbertse and Eicker (1981) from the University of Pretoria have produced an extensive paper on the anatomy of leaflets of South African *Encephalartos* species, together with an identification key based on leaf anatomy. Leaf surface morphology has also been investigated by Professor Bijan Dehgan and Bart Schutzman (1984) of the University of Florida. Karatela and Gill (1984) from Bendel State University in Nigeria have similarly reported on the leaf surface character of their local species, *Encephalartos barteri*.

The detailed examination of certain structures within cycad cells has been carried out by Dr. David Webb (1982a) of Queen's University in Canada, who reported on the differences in plastid structures between light-grown and dark-grown seedlings of *Zamia floridana*. The structure of plastids from leaves of *Macrozamia moorei* received the attention of Bonatti and Sabato (1984).

Investigations into the structure of the ovule include the report on storage cells in the ovule of *Cycas revoluta* by Rene Rohr (1980), while Japanese workers, Hiraoka, Wada and Takada (1981) have found evidence in the same species of interaction between the nucleus and cytoplasm by means of an intranuclear canal system.

Dennis Stevenson, whose taxonomic work was referred to earlier, has also been concerned with growth and development studies of cycads, demonstrating the radial growth pattern in 8 genera (1980a) and reporting on leaf bud and subsequent leaf growth processes (1981); on the basis of the latter findings, the recommendation was made to separate out the Boweniaceae as a family apart from the Zamiaceae, a proposal which is supported by evidence from chemical constituents (see later).

Little work has been published on cycad physiology, one study of interest being the investigations into the photochemical properties of *Cycas circinalis* and *C. beddomei* by Prabhakar and Rao (1981, 1984).

SEED MORPHOLOGY, DISPERSAL AND GERMINATION

Professor Bijan Dehgan and his coworkers in Florida have been very active in research into cycad seed morphology and its implications in dispersal and evolution. In 1983 Dehgan and Yuen showed the buoyancy in sea-water of seeds of *Cycas rumphii* and *C. thouarsii*, a property arising from an internal spongy layer within the seed. Because of their flotation it is probable that these seeds can travel from one island to another in Indian Ocean currents. Another interesting topic covered by Dehgan and Johnson (1983) has been the effect of sulphuric acid followed by gibberellic acid, on seed germination. When *Zamia floridana* seeds were treated in this manner, a remarkable 90% germination was found within 6 weeks.

Seed germination in cycads is dependent on the interaction of time, moisture content, humidity and temperature. C. Forsyth and 'Hannes' van Staden (1983) of the University of Natal's Pietermaritzburg campus have investigated this interaction in *Encephalartos natalensis*. Optimum germination (90%) was found when the seeds were stored in

moist conditions for 3 months at 20°C and then incubated at 30°C. The process of germination seems to be associated with enzymes known as peroxidases which were studied by Esperenza Pena and co-workers (1983) at the University of Havana in *Cycas circinalis* seeds.

Seed dispersal by animals presents an important avenue for research. Allan Burbidge and Robert Whelan (1982) have studied and quantified the transportation of *Macrozamia riedlei* seed by the possums, nocturnal marsupials of Australia.

ROOT MORPHOLOGY AND CORALLOID ROOTS

The formation and development of cycad root nodules or coralloid roots has been an area of much research. De Luca and Sabato (1980) and Schneider (1984) have studied the situation in *Cycas revoluta*. Numerous publications from the team lead by Dr. David Webb of Queen's University have explored the interaction between light and root nodule development in such plants as *Bowenia serrulata* (1981), *Zamia floridana* (1982b), *Z. pumila* (1983a), *Macrozamia communis* (1983b), *M. diplomera* (1983c), *Dion edule* (1984), *Cycas revoluta, Encephalartos altensteinii* and *Zamia furfuracea* (1984 ref. 99). The different nodulation traits in different taxa could provide additional criteria for taxonomic decisions. Webb (1982c) has also reported on the effect of the gametophyte and cotyledons on root growth of *Zamia floridana* embryos, partial or complete excision of these organs reducing both primary and secondary root elongation.

Within the root nodules are the Cyanobacteria (previously blue-green algae), *Nostoc* as reported by Grilli Caiola (1980) and *Anabaena* as detailed by Cheng Zhu (1982) with respect to *Cycas revoluta* nodules. Extracts from the coralloid roots of this species appear to have anti-viral properties, at least with respect to the control of tomato viruses, an interesting observation from Rao and co-workers (1984). Another physiological aspect has been the seasonal change in phenolic substances; Obukowicz and colleagues (1981) of the University of Wisconsin believing that these compounds may serve as a natural defence mechanism against unde sirable microorganisms. The Swedish workers, Lindblad, Hällbom and Bergman (1985) are currently exploring the metabolic activity of *Zamia* coralloid roots in relation to the heterocyst cells in the filamentous strands of Cyanobacteria.

Dennis Stevenson (1980b) has researched the growth of cycads with subterranean stems, using *Zamia pumila* and *Stangeria* as typical examples. In both these plants, the stem and root progressively contract as new growth occurs at the apex, effectively pulling the stem underground.

TISSUE CULTURE

The propagation of viable plantlets by means of tissue culture techniques may well be the key to protection of endangered cycad species. Dr. Nicolas Henson (1980) working at Kew Gardens, investigated 35 species and found that the establishment of callus growth from tissue explants is relatively easily obtained. Furthermore, promising indications of the induction of shoot and leaf growth from callus stages was obtained in some *Zamia* cultures. Arthur Koeleman and Professor J.G.C. Small (1982) managed to obtain quite vigorous callus growth from stem and root tissue of several *Encephalartos* species, but subsequent organogenesis was disappointing.

The differentiation stage from callus to plantlet appears to be controlled by a critical auzin-cytokinin interaction. David Webb and colleagues working at the University of Puerto Rico have investigated the influence of the auxin, NAA, the cytokinin, BAP, and the amino acid, L-glutamine, on the *in vitro* development of *Zamia pumila* embryos. In this work by Webb, Rivera, Starszak and Matos (1983), callus growth led to either shoot or root development, and embryo-like structures were formed depending on the treatment programmes employed. However, plantlets capable of growing in soil were not obtained.

Esperenza Pena and Emma Grillo (1982) of Havana University have cultured the particularly endangered species *Microcycas calocoma* to a callus stage with some evidence of root formation. In Montreal, Sylvie Laliberte, Charles-

Bertrand and Joachim Vieth (1983) report on callus formation and some degree of differentiation in megagametophyte cultures from *Encephalartos villosus*. Michel Monnier has collaborated with Knut Norstog (1984) studying the development of immature embryos of *Zamia* in culture.

Like much research, there is an element of good luck in this field. With sufficient work it is almost certain that a suitable technique for the propagation of cycads *en masse* by tissue cloning will be perfected.

CHEMICAL CONSTITUENTS

The toxic constituents of cycads have been further explored. Jack Cannon and colleagues (1980) from the University of Western Australia have determined the crystal structure of the toxic compounds macrozamin and sequoyitol from *Macrozamia riedlei*. De Luca, Moretti, Sabato and Siniscalo Gigliano (1980) have reported on the ubiquity of cycasin and the latter three authors (1981, 1983) have since shown both toxins to be present in all cycad genera but not in other plants. Macrozamin is generally more abundant than cycasin, occurring at levels from 0.2% in *Cycas cairnsiana* to about 5% in *Bowenia spectabilis*, the different concentration being largely genus dependent. The evidence now available supports the previously-mentioned proposal to separate *Bowenia* from the Zamiaceae; indeed, *Bowenia* may be closer to *Stangeria* than originally thought. Professor R.C. Tustin (1983) from the Onderstepoort Veterinary Research Unit has published details of the toxicity and carcinogenicity of the South African cycads, especially *Encephalartos lanatus*, while George Hoffman and R.W. Morgan (1984) comment on the implication of cycad materials as a source of foodstuff.

Professor de Luca's team (1982, ref. 21, has investigated the sugar composition of the hydrolysed mucilage from different cycads and has found that there is also a broad range of differences between Australasian, African and American genera. In their analyses, *Lepidozamia* appears to be quite distinct from *Macrozamia*, reinforcing the justification for its separate generic status.

P.A. Gadek (1982) of the University of New South Wales explored the class of compounds known as biflavonoids which accumulate in the brightly-coloured testae of cycad seeds as ripening occurs. Again, different genera have different chemical patterns. Interestingly the compound cupressuflavone, previously known in other gymnosperms like the Cupressaceae, Auracariaceae and Podocarpaceae, was found in two *Macrozamias*, *M. macdonnellii* and *M. communis*. Gadek, Quinn and Ashford (1984) later reported on the biflavonoids in cycad leaves where it is thought to act as a deterrent to leaf-eating insects and microbial invasion.

Other chemical substances recently isolated from cycads include some unusual fatty acids from *Cycas revoluta*, found by Japanese workers from Toru, Takagi and Y. Itabashi (1982), certain proteins which occur in the pollen tube of *Cycas armstrongii*, demonstrated by J.M. Pettit (1982) from the British Museum and the novel storage globulin named macrozim, isolated from the seeds of *Macrozamia communis* by R.J. Blagrove, Lilley and Higgins (1984) from the CSIRO in Australia.

ECOLOGY

The effect of spontaneous or accidental fires on cycads in habitat has been considered by various Australian scientists. T.S. Grove, A.M. O'Connell and N. Malajczuk (1980) from the CSIRO in Wembley, Western Australia, have investigated the effects of burning cycles on growth, nutrient content and rate of nitrogen-fixation in populations of *Macrozamia riedlei* and found that fire stimulates leaf growth and coralloid root activity. This plant makes a significant contribution to the biosystem of eucalyptus forests where it fixes about 35 kg of nitrogen per hectare in each 5-7 year interval between burnings. June Dolva and John Scott (1982) have studied the effect of fire on the mealybug/cycad association and found both respond favourably to increased fire frequency. A fascinating paper by J.M. Beaton (1982) discussed the fire aspect with respect to Australian aboriginal cycad "farming", while Beth Gott (1982) has written on

the usage of *Macrozamia moorei* roots by the indigenous people of South Australia.

Another traumatic incident to plants in habitat is the effect of severe cold. Rita Hummel (1984) from the University of Florida has published a paper on the freezing tolerance of cycads.

Occasional reports of spontaneous sex-changes in cycads continue to appear. Van Wyk and Claassen (1981) have produced the most thorough such report with respect to *Encephalartos umbeluziensis*, and Maans Kemp (1985) records a male to female change in *Cycas revoluta*. In all cases, these sex reversals appear to be associated with some traumatic environmental incident. A current publication by Ornduff (1985) deals with the topic of sex ratios in cycads, reporting on field studies on *Macrozamia riedlei* and *Zamia pumila*.

Hungarian workers, Borhidi and Muniz (1980), have discussed the separation in geological time of Cuba from the continental landmass, and the consequent evolution of an endemic flora including *Microcycas;* a similar and broader theme is found in the paper by Balduzzi *et al* mentioned previously. More recently, Elenevskii (1984) from Moscow University, has reviewed features of Cuban flora and includes a discussion of their indigenous cycads.

Sandra J. Newell, now at the Indiana University of Pennsylvania, has published papers (1983 , 1985) arising from her earlier field studies on *Zamia pumila* in habitat in Puerto Rico, in which interesting aspects of leaf morphology and reproduction are discussed.

CONCLUSION AND ACKNOWLEDGEMENTS

There is a surprising amount of research work currently in progress and it is clear that the centres of such projects are in the U.S.A and Europe. Countries like South Africa and Australia have the real benefit of large endemic cycad populations which provide ideal opportunities for many projects, the outcome of which could add significantly to our knowledge of the plants. It is hoped that academics and teachers will thus motivate students in this direction more in the future than in the past.

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THE WORLD LIST OF CYCADS *

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Taxonomy is that discipline of botany which provides a rational basis for the naming of plants. It is an objective science in that set procedures work toward developing a meaningful systematic genealogy. However, problems relating to the selection of criteria of rank result in subjective judgement and opinion; this is especially true in the order Cycadales.

Most cycad species names originated from studies of isolated plants or habitats. When descriptions of these species were later compared on a broader regional basis, many appeared to be synonomous or less differentiated than others, thus names and classifications had to be reassigned. This process may repeat as often as justified, the most recent properly-published names and ranks being accepted as valid.

Apart from the as yet unresolved confusion of the early literature over the identification of some taxa, there are also problems of judgement and of opinion. In addition, there are known but undescribed new species and there are difficulties arising from situations when botanically invalid names applied by collectors to taxa that differ from or have not been reconciled with valid taxa. Another important factor is that some cycad habitats have yet to be fully explored and the plants properly classified. Fortunately, *Dioon*, *Encephalartos* and *Macrozamia* are largely defined although some changes are anticipated. Only *Bowenia*, *Lepidozamia*, *Microcycas* and *Stangeria* are considered taxonomically complete at this time.

* At the time of submission of this thesis, this paper is currently under consideration for publication in the *Memoirs of the New York Botanical Garden*.

Major advances have recently been made in *Zamia* taxonomy by information from chromosome karyotyping. Biochemical studies too are being used to define genera and give promise of differentiation at lower taxonomic rank. Other modern analytical techniques may have application in future years. Taxonomy in its purest form reflects evolution; the best advances will be made by combining the knowledge from the many relevant interacting scientific disciplines.

Our list of validly-named cycad species was first published in ENCEPHALARTOS (No. 3, September 1985), Journal of the Cycad Society of Southern Africa. A supplementary list followed in a subsequent issue of the same Journal (No. 5, March 1986). The list which now follows is a further update where several changes have been made on the basis of recent information and itemizes the currently valid taxonomic ranking at or below the species level to the best of our existing knowledge. It is not a listing of all validly published species, nor does it represent our opinion as to 'good' species or suitable classification. Indeed, our opinions and those of many others are at considerable variance from this list, particularly in assignments in the genus *Cycas*.

Work is proceeding toward an improved cycad taxonomy, though perhaps not of the scope nor at the rate which might be desired. In the interim, it is recommended that labelling of specimen plants is supplemented with any additional appropriate information.

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Note: An asterisk against the taxon given indicates that the publication is currently *in print*.

BOWENIA (2 species)	Australia
B. *serrulata* (W. Bull) Chamberlain	Queensland
B. *spectabilis* Hook. ex Hook. f.	Queensland
CERATOZAMIA (10 species)	Mexico, Guatamala, Belize
C. *euryphyllidia* V-Torres, Sabato & D. Stevenson	Veracruz
C. *hildae* G. Laundry & M. Wilson	San Luis Potosi & Queretaro
C. *kuesteriana* Regel	Tamaulipas
C. *latifolia* Miq.	San Luis Potosi & Queretaro
C. *matudae* Lundell	Chiapas, N. Guatemala
C. *mexicana* Brongn.	Pueblo, Veracruz
C. *miqueliana* H.Wendl.	Veracruz
C. *norstogii* D. Stevenson	Chiapas
C. *robusta* Miq.	S. Mexico Guatemala & Belize
C. *zaragozae* Medellin-Leal	San Luis Potosi
* CHIGUA (2 species)	South America
* C. *bernalii* D. Stevenson	Columbia
* C. *restrepoi* D. Stevenson	Columbia
CYCAS (24 species)	
C. *armstrongii* Miq.	
C. *baguanheensis* L.K. Fu & S.Z. Cheng	China
C. *basaltica* C.A. Gardn.	Western Australia
C. *cairnsiana* F. Muell	Queensland, Australia
C. *calcicola* J.R. Maconochie	N. Territory, Australia
C. *chamberlainii* W.H. Brown & Kienholz	
C. *chevalieri* Leandri	Vietnam

C. circinalis L.	Asia, Africa
subsp. *circinalis*	
forma *circinalis*	
forma *glauca* (Miq.) Schuster	
forma *gothanii* Schuster	
subsp. *madagascariensis* (Miq.) Schuster	Madagascar
forma *madagascariensis*	
forma *trigonocarpoides* Schuster	
subsp. *papuana* (F.Muell) Schuster	New Guinea
subsp. *ruiminiana* (Porte) Schuster	Philippines
var. *ruiminiana*	
var. *curranii* Schuster	
forma *curranii*	
forma *apertorum* Schuster	
forma *maritima* Schuster	
var. *scratchleyana* (F.Muell.) Schuster	New Guinea
subsp. *vera* Schuster	
var. *vera*	
var. *beddomei* Dyer	India
C. guizhouensis K. Lan & R. Zou	China
C. hainanensis C.J. Chen & C.Y. Chen	Hainan Island, China
C. media R. Br.	
var. *media*	N. Territory, Australia
var. *furfuracea* (W.V. Fitzg.) Schuster	Western Australia
var. *lane-poolei* (C.A. Gardn.) Schuster	Western Australia
C. micholitzii Dyer	
var. *micholitzii*	Vietnam, China
var. *simplicipinna* Smitinand	S.E. Asia

C. *nathorstii* Schuster	Sri Lanka
C. *normanbyana* F. Muell	Queensland, Australia
C. *panzhihuaensis* L. Zhou & S.Y. Yang	China
C. *pectinata* W. Griff	Widespread, S.E. Asia
C. *pruinosa* J.R. Maconochie	Western Australia
C. *revoluta* Thunb.	Japan, Ryukyu Is.
var. *revoluta*	
var. *robusta*	
C. *rumphii* Miq.	S.E. Asia & Pacific Is.
subsp. *rumphii*	
var. *rumphii*	
var. *subinclusa* Schuster	
forma *subinclusa*	
forma *palauica* Kaneh	Palau
forma *papuana* (F. Muell.) Kaneh	New Guinea
forma *seemannii* (A. Braun) Kaneh	Fiji
subsp. *zeylanica* Schuster	Sri Lanka
C. *szechuanensis* C. Chen & L.K. Fu	China
C. *siamensis* Miq.	S.E. Asia
subsp. *siamensis*	
subsp. *balansae* (Warburg) Schuster	Vietnam
C. *taiwaniana* Carruth.	China, Taiwan
C. *undulata* Desf.	
C. *wadei* Merrill	Philippines

DIOON (10 species)	Mexico, Honduras
D. *califanoi* De Luca & Sabato	Oaxaca
D. *caputoi* De Luca, Sabato & V-Torres	Puebla
D. *edule* Lindley	
var. *edule*	Nuevo Leon, San Luis Potosi, Tamaulipas

var. *angustifolium* (Miq.) Miq.	Nuevo Leon, Tamaulipas, San Luis Potosi
D. holmgrenii De Luca, Sabato & V-Torres	Oaxaca
D. mejiae Standley & L.O. Williams	N. Honduras
D. merolae De Luca, Sabato & V-Torres	Chiapas
D. purpusii Rose	Oaxaca
D. rzedowskii De Luca, Moretti, Sabato & V-Torres	Oaxaca
D. spinulosum Dyer	Vera Cruz, Oaxaca
D. tomasellii De Luca, Sabato & V-Torres	
var. *tomasellii*	S.W. Coast, Mexico
var. *sonorense* De Luca, Sabato & V-Torres	N.W. Coast, Mexico

ENCEPHALARTOS (46 species)	Africa:
E. altensteinii Lehm.	E.Cape, Transkei
E. arenarius R.A. Dyer	E. Cape
E. barteri Carruth. ex Miq.	
subsp. *barteri*	Benin, Ghana, Nigeria, Sudan, Togo
subsp. *allochrous* L.E. Newton	Nigeria
E. bubalinus Mellville	Tanzania, Kenya
E. caffer (Thunb.) Lehm.	E. Cape
E. chimanimaniensis R.A. Dyer & Verdoorn	Mozambique, Zimbabwe
E. concinnus R.A. Dyer & Verdoorn	Zimbabwe
E. cupidus R.A. Dyer	Transvaal
E. cycadifolius (Jacq.) Lehm.	E. Cape
E. eugene-maraisii Verdoorn	Transvaal
E. ferox Bertol f.	Zululand, N. Natal, Mozambique
E. friderici-guilielmi Lehm.	E. Cape, Transkei

E. ghellinckii Lem.	Transkei, Natal
E. gratus Prain	Malawi, Mozambique
E. heenanii R.A. Dyer	Swaziland, S.E. Transvaal,
E. hildebrandtii A. Braun & Bouché	
var. *hildebrandtii*	E. Africa
var. *dentatus* Melville	Tanzania, Uganda
E. horridus (Jacq.) Lehm.	E. Cape
E. humilis Verdoorn	E. Transvaal
E. inopinus R.A. Dyer	Transvaal
* *E. ituriensis* Bamps & Lisowski	Zaire
E. laevifolius Stapf & Burtt Davy	E. Transvaal, Swaziland
E. lanatus Stapf & Burtt Davy	Transvaal
E. latifrons Lehm.	E. Cape
E. laurentianus De Wild.	Angola, Zaire
E. lebomboensis Verdoorn	N.Natal, S.E. Transvaal, Swaziland, Mozambique
E. lehmannii Lehm.	E. Cape
E. longifolius (Jacq.) Lehm.	E. Cape
E. manikensis Gilliland	Mozambique, Zimbabwe
E. marunguensis Devred	Zaire
E. munchii R.A. Dyer & Verdoorn	Mozambique
E. natalensis R.A. Dyer & Verdoorn	Natal
E. ngoyanus Verdoorn	Zululand, N. Natal S.E. Transvaal, Swaziland
E. paucidentatus Stapf & Burtt Davy	E. Transvaal, Swaziland
E. poggei Ascherson	Angola, Zaire
E. princeps R.A. Dyer	E. Cape, Transkei
E. pterogonus R.A. Dyer & Verdoorn	Mozambique
E. schmitzii Malaisse	Zaire, Zambia

* *E. sclavoi* (Authors and locality to be advised)	
E. septentrionalis Schweinfurth	Zaire, Sudan, Uganda Central Af. Rep.
E. tegulaneus Melville	Kenya
E. transvenosus Stapf & Burtt Davy	N. Transvaal
E. trispinosus (Hook.) R.A. Dyer	E. Cape
E. umbeluziensis R.A. Dyer	Swaziland, Mozambique
E. villosus Lem.	← E. Cape, Natal, S.E. Transvaal Transkei, Zululand, Swaziland, Mozambique
* *E. voiensis* (Authors and locality to be advised)	
E. woodii Sander	(Extinct in nature)

LEPIDOZAMIA (2 species)	Australia:
L. hopei Regel	Queensland
L. peroffskyana Regel	N.S.W. & Queensland

MACROZAMIA (14 species)	
M. communis L.A.S. Johnson	N.S.W.
M. diplomera (F.Muell.) L.A.S. Johnson	N.S.W.
M. fawcettii C. Moore	N.S.W.
M. heteromera C. Moore	N.S.W.
M. lucida L.A.S. Johnson	Queensland & N.S.W.
M. macdonnellii (F. Muell. ex Miq.) A.DC.	Central Australia
M. miquelii (F. Muell.) A.DC.	Queensland & N.S.W.
M. moorei F. Muell.	Queensland & N.S.W.
M. pauli-guilielmi W. Hill & F. Muell.	
subsp. *pauli-guilielmi*	Queensland
subsp. *flexuosa* (C.Moore) L.A.S. Johnson	N.S.W.
subsp. *plurinervia* L.A.S. Johnson	Queensland & N.S.W.
M. platyrachis F.M. Bailey	Queensland
M. riedlei (Gaud.) C.A. Gardn.	S.W. Australia

M. *secunda* C. Moore	N.S.W.
M. *spiralis* (Salisb.) Miq.	N.S.W.
M. *stenomera* L.A.S. Johnson	N.S.W.
MICROCYCAS (1 species)	Cuba
M. *calocoma* (Miq.) A.D.C.	W. Cuba
STANGERIA (1 species)	South Africa
S. *eriopus* (Kunze) Baillard	E. Cape, Natal, Transkei & Zululand.
ZAMIA (43 species)	North, Central, South America, and West Indies
Z. *acuminata* Oersted ex Dyer	Nicaragua, Panama.
Z. *amblyphyllidia* D. Stevenson	Puerto Rico, Cuba, Jamaica
Z. *amplifolia* Hort. ex Masters	Colombia
Z. *angustifolia* Jacq.	Bahamas, Cuba
Z. *boliviana* (Bongn.) A. DC.	Bolivia
Z. *chigua* Seemann	Colombia, Panama
Z. *cupatiensis* Ducke	Colombia, Brazil
Z. *fairchildiana* L.D. Gomez	Costa Rica, Panama
Z. *fischeri* Miq.	Querétaro, San Luis Potosi, Veracruz
Z. *furfuracea* L. fil. in Aiton	Veracruz
Z. *herrerae* Calderon & Standley	El Salvador, Honduras, Guatemala, Chiapas
Z. *inermis* Vovides, Rees & V-Torres	Veracruz
Z. *integrifolia* L. fil. in Aiton	Florida, Bahamas, Cuba
Z. *jirijirimensis* R.E. Schultes	Colombia
Z. *lawsoniana* Dyer	Oaxaca
Z. *lecointei* Ducke	Brazil

Z. *lindenii* Regel ex André	Ecuador
Z. *lindleyi* Warsz. ex A. Dietrich	Panama
Z. *loddigesii* Miq.	Mexico
Z. *manicata* Linden ex Regel	Colombia, Panama
Z. *montana* A. Br.	Colombia
Z. *monticola* Chamberlain	Veracruz
Z, *muricata* Willd.	Venezuela
Z. *obidensis* Ducke	Brazil
Z. *obliqua* A. Br.	Colombia
Z. *paucijuga* Wieland	W. Mexico
Z. *picta* Dyer	Guatemala, Mexico
Z. *poeppigiana* Martius & Eichler	Peru
Z. *portoricensis* Urban	Puerto Rico
Z. *pseudomonticola* L.D. Gomez	Costa Rica
Z. *pseudoparasitica* Yates in Seemann	Panama, Costa Rica
Z. *pumila* L.	Caribbean (widespread)
Z. *purpurea* Vovides, Rees & V-Torres	Veracruz
Z. *pygmaea* Sims	W. Cuba & Isla da Pinas
Z. *roezlii* Linden	Colombia
Z. *skinneri* Warsz. ex A. Dietrich	Nicaragua, Panama Costa Rica, Guatemala
Z. *spartea* A.DC.	Oaxaca
Z. *splendens* Schutzman	Mexico
Z. *sylvatica* Chamberlain	Mexico
Z. *tuerckheimii* J. Donnell Smith	Guatemala
Z. *ulei* Damm.	Brazil
Z. *verschaffeltii* Miq.	Mexico
Z. *wallisii* A. Br.	Colombia

NOTES

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In Vitro Regeneration of *Stangeria eriopus*

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Additional index words. tissue culture, cycads, primary root explants

In South Africa, the indigenous cycads comprise 28 species of *Encephalartos* (Zamiaceae) and *Stangeria eriopus* (Kunze) Baillard (3), the latter species being the sole representative of the Stangeriaceae. Present cycad populations are under severe pressure from combined effects of agricultural and urban development, exploitation by the tribal "medicine-men", activities of plant collectors, and demands from scientific and educational institutions. In addition, the relatively slow growth rate, limited potential for vegetative propagation, and the restricted supply of viable seed further exacerbate the delicate conservation status of these dioecious plants (2).

Although considerable attention has been given to the use of haploid material, usually the female gametophyte in cycad tissue culture, there are few reports on *in vitro* culture of somatic tissue. Callus has been derived from leaflet explants of both *Encephalartos* and *Stangeria* (4) and from stem and root tissue of *Encephalartos* (5), but no significant organogenesis was obtained in any of the reported studies. Regeneration of vegetative material has been achieved using primary root tissue from *Stangeria eriopus* seedlings and is reported here.

Fresh seeds of *Stangeria eriopus* were germinated and seedlings established in plastic bags containing a loam soil. After 18 months, five healthy plants were freed from the soil and the primary roots (≈100 mm long and 15 mm in diameter at the widest point) excised. These organs then were scrubbed to remove superficial dirt, allowed to stand in running water (30 min), and disinfected by sequential immersion in 70% ethanol (4 min), 20% Jik (commercial 3.5% NaOCl solution) containing 1% Teepol (30 min) and 1% $HgCl_2$ containing 1% Teepol (30 min). After thorough rinsing and soaking in sterile distilled water (2 hr), the roots again were treated with hypochlorite and rinsed three times with sterile distilled water.

Segments (≈5 mm cubes), each of which included vascular and cortical tissue, then were dissected aseptically from the upper two-thirds of the roots and transferred individually to 25-ml flasks containing the medium of Schenk and Hildebrandt (7) supplemented with 4.5×10^{-6} M each of 2,4-D and kinetin. The medium was solidified with 0.6% Bacto-agar. Thirty cultures were established in this manner and they were placed in a dark cupboard at 25° ± 2°C for 30 days. At the end of the initial culture period, 15 cultures were transferred to fresh medium of the same composition and placed in a growth cabinet under constant light (≈100 $\mu mol \cdot s^{-1} \cdot m^{-2}$) and temperature (25° ± 0.2°).

After about 25 days of dark culture, no contamination had occurred. Compact white callus had formed on the upper surface of all explants. On subculture and transfer to the light environment, each explant developed a small green meristematic zone within 14 days followed by emergence (Fig. 1) and expansion (Fig. 2) of a typical circinate leaf. This pattern of morphogenesis was consistent in all cultures after transfer to light, but did not occur in any of the flasks remaining in the dark. This is, to our knowledge, the first report of in vitro morphogenesis in a South African cycad. In separate experiments, we have been unable to obtain a similar response from any species of *Encephalartos*. Thus, the success with *Stangeria* may be associated with an inherent regenerative capacity of the root material; it has been reported that *Stangeria* plants may be obtained from root cuttings (6), a phenomenon not known in other cycads. It is also of interest that the American *Zamia* cycad long has been recognized for the regenerative potential of its underground tuberous root and stem tissue (1).

Fig. 1. Light-induced regeneration of a vegetative bud (arrow) following initial callogenesis on a primary root explant of *Stangeria eriopus*.

Fig. 2. Emergence and extension of the first leaf; 2 weeks subsequent to the stage in Fig. 1.

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SOUTH AFRICAN CYCAD RESEARCH - PROGRESS AND PROSPECTS*

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ABSTRACT

The order Cycadales occupies a key position in the botanical hierarchy and shows many unusual and some unique features. With 28 species of Encephalartos and the only representative of Stangeria, South Africa is similar to Mexico and Australia in the richness of its cycad flora. A resurgence of interest in this plant group is demonstrated by the extent of recent and current research projects into their taxonomy, morphology, propagation and biochemistry. Numerous other research opportunities remain to be explored.

UITTREKSEL

Die Cycadales is 'n orde wat vele ongewone en enkele unieke kenmerke vertoon en gevolglik 'n sleutelposisie in die planthierargie beklee. Met 28 Encephalartos spesies en die enigste verteenwordiger van Stangeria inheems in Suid-Afrika, verskil Suid-Afrika min van Meksiko en Australie wat die rykheid van sy broodboomflora aanbetref. Hernude belangstelling in hierdie plantgroep word in die omvang van die onlangse en huidige navorsing oor hul taksonomie, morfologie, voortplanting en biochemie weerspieel. Desondanks wag talle ander navorsingsgeleenthede op ontginning.

KEYWORDS: Cycadales, cycads, Encephalartos, Stangeria.

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INTRODUCTION

The present-day cycads comprise the diverse, modified remnants of a much larger group of plants which flourished in the Mesozoic Era, reaching their zenith in the Jurassic Period. Of the 132 valid species in the Cycadales,[1,2] South Africa has 29 species in two genera (Encephalartos Lehm. and Stangeria T. Moore).[3] South Africa, Mexico (approx. 28 species in 3 genera) and Australia (approx. 23 species in 4 genera) collectively account for about 60 percent of the world's cycad flora. Yet in a recent bibliography of cycad literature, in which 1713 references were listed, only 103 mention Encephalartos and 19 deal with Stangeria.[4] A current resurgence of local interest in these plants may address this imbalance. In this text, we wish to review current knowledge of the South African cycads and outline some preliminary results from certain projects presently underway.

TAXONOMY

Due very largely to the excellent work of Drs R.A. Dyer and I. Verdoorn, the taxonomy of the South African cycads has been fairly well defined.[5-8] In essence, this country has 28 species of Encephalartos (Zamiaceae) and Stangeria eriopus (Kunze) Baillard, the sole representative of both the genus and its family (Stangeriaceae) (Table 1). The status of several Encephalartos taxa is currently under review (Vorster, unpublished results). Of particular interest is the taxonomic position of several small populations which are clearly related to larger groups of defined species from which they have become geographically isolated.

Despite its fairly well-defined taxonomy, there has as yet been only one attempt to establish phylogenetic relationships within Encephalartos.[9] It is speculated that the ancestral cycad stock was arborescent and mesic and that all adaptations to harsher environments represent advances.[10] In this sense E. altensteinii Lehm., E. lebomboensis Verdoorn, E. natalensis Dyer & Verdoorn and E. transvenosus Stapf & Burtt Davy are examples of primitive species. By contrast, those with subterranean caudices (e.g. E. ngoyanus Verdoorn and E. villosus Lem.); those with much reduced leaflets (e.g. E. cycadifolius (Jacq.)Lehm. and E. ghellinckii Lem.) and the highly-armed species in arid areas (e.g. E. horridus (Jacq.) Lehm. and E. trispinosus (Hook) Dyer) must be considered relatively more advanced. It is anticipated that much of the research presently in hand will allow construction of a more rigorous phylogeny of the genus.

CONSERVATION

Of the South African cycads, the Threatened Plant Unit (TPU) of the International Union for the Conservation of Nature and Natural Resources (IUCN) lists three as endangered, seventeen as vulnerable, five as rare and three as "insufficiently known" (Table 1).[11] This classification is a revision of that of local workers [12] and suffers from the same limitations of being based largely on numerical representation from herbarium specimens. A Natal species, Encephalartos woodii Sander, is extinct in nature, only one multi-trunked specimen of this plant ever being discovered and all portions being transplanted to botanical gardens for safekeeping.[5,13] Pressure on existing cycad populations arises from the combined effects of man's continuing domestic and agricultural demands on finite land resources and the widespread removal of plants from habitat by unscrupulous dealers or collectors, despite legislation intended to prevent this. There is also some evidence that significant numbers of Stangeria eriopus have been removed for use by tribal herbalists (Cunningham, pers.comm.). Thefts from public and private gardens also occur with dismaying frequency, prize specimens commanding exhorbitant prices on local and overseas black markets. Conservation problems are exacerbated by the slow growth rate, paucity of viable seeds and the limited potential for vegetative propagation.

Legislation to control the exploitation, sale and transportation of cycads exists at an international level in terms of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES)[14] and locally in terms of the various national and regional nature conservation laws.[15] The "Modjadji Palms" (E. transvenosus) in the Letaba area are additionally and effectively protected in terms of the authority of the Rain Queen of the Balobedu tribe;[5] furthermore both this forest and the type specimen of E. natalensis near Monteseel in Natal have been proclaimed National Monuments.[16]

The activities of the Cycad Society of Southern Africa in creating a public awareness of the necessity for conservation, and in establishing seedbank and pollen exchange facilities, together with the operations of public and private cycad nurseries, have an effect of reducing collector demand on habitat specimens. The principle of "conservation through cultivation" making plants available through authorised channels, may well be at least as effective as attempts at discipline through legislation. The establishment of cycad reserves and scientifically-managed collections in botanical gardens offers much hope for the maintainance of cycad gene-pools in the long term.

ECOLOGY

South African cycads are found in a variety of habitats, ranging from rain-forests to karroo scrub and from coastal woodlands to high-altitude grasslands.[3,6] Although their distribution is fairly well documented, there has been no detailed work published on their ecology. A quantitative survey of existing populations would provide

invaluable data for conservation planning and would allow for better evaluation of their conservation status. Studies on the population dynamics could quantify and integrate with aspects such as sex-ratios, seed production, seed dispersal, plant regeneration and growth rate. In addition, the effect of spontaneous and deliberate fires on the grassland species may confirm speculation that burning cycles promote vegetative growth and induce coning. A further interesting aspect pertaining to cycads with subterranean caudices relates to how the position of the stem apex is maintained; speculation is made that contactile roots operate together with stem compaction and possibly progressive decomposition of the stem base (Grobbelaar, unpublished results).

MORPHOLOGY

The gross morphology of the South African cycads has been thoroughly described and forms the basis for their present taxonomy.[5] A study of leaf surface characters of Encephalartos and Stangeria describes differences in leaf cell morphology, stomatal arrangement, leaf hairs and cuticular wax deposit patterns.[17] Some of these characters appear to be species-specific and possibly sex-related in Stangeria. An investigation into the comparative anatomy of Encephalartos leaflets describes the stomatal distribution, and the arrangment of sclerenchyma, pallisade, vascular tissues and resin canals.[9] E. eugene-maraisii and E. cupidus leaflets are unusual in having bilateral symmetry with stomata and pallisade layers both adaxially and abaxially. The remaining 26 species can be grouped on the basis of leaf anatomy and plant habitat. The characters described are used to create a species key and allow speculation on the phylogeny of the genus.

Preliminary SEM studies on cycad pollen morphology have indicated characteristic differences at the genus level (Fig. 1)[18] confirming earlier reports by Wodehouse[19] and Van Zinderen Bakker.[20] Cycad pollen microspores are boatshaped, monosulcate and bilaterally symmetrical, measuring about 26 - 37 micron by 14 - 23 micron, with an exine surface which varies from psilate to foveolate and fossulate.[18-21] Since the characteristics of the sexual reproductive structures in plants are usually highly conserved, any differences in the pollen morphology of different species may be particularly significant. A survey of Encephalartos pollen morphology is presently under way at the University of Pretoria. A similar survey with respect to the seed kernel (sclerotesta) morphology is being planned; preliminary results here too indicate that this aspect merits further investigation.[22]

REPRODUCTIVE BIOLOGY

Because of the antiquity of the order, much attention has rightly been focussed on the reproductive biology of the Cycadales. The absolute dioeciousness of the plants, the ontogeny of the microgametophyte with the ultimate release of large motile spermatozoids, the development of a substantial megagametophyte and the embryology of cycads, have all been well-documented.[23-26] Most of the work in this regard has been done on non-African species.

Although early assumptions were made that cycads were wind pollinated, recent studies on the meso-American Zamia species have shown that beetles may be obligatory pollen vectors at least in this genus.[27-29] This work calls to mind the early observations on insect pollinators in South African Encephalartos species.[30-32] The most likely pollinators of our local cycads appear to be the elongate weevils of the subfamily Rhyncophorinae; the activities of these host-specific beetles undoubtedly resulting in a more efficient disperal than wind distribution.[33] A thorough investigation into the role of insects in pollination of South African cycads remains to be done. In passing, it is noted that the noble idea of re-establishing nursery-raised plants in habitats from which the particular species had previously disappeared, may be futile unless the pollination vector is simulanteously re-introduced.

Significant temperature increases by male Encephalartos cones, and the associated release of volatile odours, at the time of pollen release is uncommon but not unique in plants, and is clearly associated with insect pollination. A male cone from Encephalartos altensteinii reached a temperature of 17 deg. C above ambient shortly after removal from the parent plant.[34-35] Similar heating has been noticed in male cones of E. transvenosus (15 deg. C. above ambient)(Grobbelaar, unpublished results) and E. woodii (7 deg. C. above ambient)(Osborne, unpublished results). A detailed investigation has since shown that thermogenesis is widespead throughout the Cycadales.[36] Temperature rises follow a circadian rhythm, reaching maxima in the late afternoon or early evening and lasting for 1 to 5 hours. Thermogenesis is coincident with the time of maximum cone axis elongation and pollen release, the site of heating being located in the cone sporophylls. Cones of Stangeria are exceptional in not showing any significant heating.

The pollination and fertilisation processes in Encephalartos and Stangeria are not well known. It is thought that a relatively long period elapses between the time of pollination of a female cone and the event of ovule fertilisation. Little is known of the site of the microspore at that time or the germination process. Grobbelaar (unpublished results) notes that large interspecific differences are found in the length of the micropylar tubes in Encephalartos. A useful report on spermatogenesis in Encephalartos altensteinii shows the development of the gametes to be similar to that in other cycads, resulting ultimately in two ciliate spermatozoids some 150 - 180 micron in diameter.[37]

Chromosome counts from Encephalartos and Stangeria cells indicate consistency in the diploid number 2n=18 and 2n=16 respectively.[38] A count from root tissue of Encephalartos woodii gave 2n=18, showing that the surviving clone of this species is not an artefact of chromosome multiplicity.[39] Whilst detailed karyotype analyses of the meso-American cycads has been given attention, largely in elucidation of the variable chromosome numbers within the genus Zamia,[10,40-41] there has been little such work on the African cycads. A karyotype survey of these taxa could contribute to their phylogeny and could also provide answers as to whether or not these plants have recognizable sex chromosomes, a contentious issue at present.[42] The work of Mogford,[43-44] who used fluorescence techniques to find the position of heterochromatin in chromosomes from root tip cells of E. caffer and E. lehmannii, could be usefully extended in this direction.

The presence or absence of sex-directing chromosomes must be viewed in relation to the reports on spontaneous gender changes in mature cycad plants. This unusual occurrence has been recorded in both exotic and South African species. The most authoritative report details the history of several specimens of Encephalartos umbeluziensis in a Pretoria garden, one of which produced a male cone in 1970 but a female in 1979.[45] This plant was the most exposed of a group during a freak sub-zero temperature spell in 1972. A specimen of E. villosus subjected to particularly dry conditions is said to have changed from female to male (Swanepoel, pers. comm.). A plant of E. latifrons, transplanted in 1970 to a farm near East London, bore two successive crops of female cones with viable seeds but, following a severe drought in 1983, then produced two successive crops of male cones (Bursey, pers. comm.). Consistent with similar observations in exotic cycads, all these sex changes appear to be associated with some traumatic event; the phenomenon may be rationalised in hormonal terms and on the basis of higher physiological cost to the plant in production of large, seed-bearing female cones. In similar context, a recent study of the sex ratios of Encephalartos transvenosus in the Modjaji Nature Reserve, provides evidence that the availability of water may influence the proportion of plants bearing female or male cones in a given year.[46] However,a dilemma arises in reconciling an environmental influence with a genotypically-controlled process, especially if sex-chromosomes are shown to be present in cycads; thus the mechanism of gender manifestation deserves research attention. Elucidation of this mechanism could lead to the artificial control of the gender of propagated plants and a particularly significant application would be the possible re-creation of a female clone of Encephalartos woodii.[47]

A further area of cycad biology to be explored locally is that of interspecific hybridisation. Work accomplished on the meso-American cycads has shown the diagnostic value of this to the taxonomists.[48] In South Africa, a number of natural Encephalartos hybrids have been recorded.[49] Their relative scarcity is ascribed to discreet geographical distribution patterns and the non-synchrony of coning times, rather than to physiological reproductive barriers. This viewpoint is reinforced by the comparative ease with which artificial interspecific hybrids can be obtained.

Another topic of interest is the recalcitrant or "wet" nature of cycad seeds. The embryo in these propagules shows continuous development from fertilisation through to germination. Thus the long-term storage time for cycad seeds is limited and the embryos are subject to necrosis through desiccation. Storing Encephalartos natalensis seeds in moist conditions until the embryo is mature, followed by incubation at 30 deg C, gives satisfactory germination frequencies,[50] but little is known generally of the optimal conditions for post-harvest embryo development and for seed germination. Similarly, little is known of the best storage conditions for cycad pollen or its longevity. Information of this sort would be invaluable in upgrading the few existing cycad seedbank and pollen-bank facilities.

CORALLOID ROOT STUDIES

A common feature in cycad plants is the formation at an early seedling stage of dichotomously-branching apogeotropic roots which often later appear as coralloid masses at soil level. These unusual root forms have been found in 49 species, in all genera except Microcycas which was not tested.[51] The structures are usually, but not always, invaded symbiotically in the cortical regions by filamentous nitrogen-fixing Cyanobacteria.[52] In a study of the symbionts in African cycads, 41 cultures were isolated from 31 cycad species and all but one were identified as Nostoc species, most often N. commune Vaucher.[53] In E. hildebrandtii Braun & Bouche, an East African cycad, the isolate was a species of Calothrix. However, both the role of and the induction process of coralloid roots require further study; little is known of cycad-cyanobiont specificity or the details of the nitrogen metabolism in these systems. Work continues at the University of Pretoria on various aspects of the root symbiotic associations.

In pioneering TEM studies on cycad coralloid roots, large crystalline inclusions were observed in cells from Encephalartos altensteinii and Macrozamia communis.[54] These apparently proteinaceous structures do not occur in coralloid roots from Cycas circinalis, C. revoluta or Dioon edule. TEM studies on the cyanobiont in situ and after isolation have followed.[55] Similar studies have since been carried out at the University of Pretoria on several South African cycad species using both TEM and SEM techniques (Joubert et al., in press; Chang et al., in press).

TISSUE CULTURE

The delicate conservation status of many of the South African cycads is clear motivation for attempting to establish protocols for in vitro propagation systems. Callus growth was quite readily obtained on leaflet explants of most cycads, including Encephalartos and Stangeria, on a variety of artificial media.[56] Callogenesis was similarly easily induced on stem and root tissue segments from nine Encephalartos species.[57] However, in both these studies, no further morphogenesis could be promoted. Experiments with a wide variety of both haploid and diploid tissue explants from various Encephalartos

species have been carried out on several media with different auxin and cytokinin supplements (Osborne, unpublished results). Vigorous callus production was again obtained in many cases but no further differentiation could be promoted. Some encouraging results have been obtained from primary root tissue cultures of Stangeria where callus formation can progress to light-induced emergence of meristematic zones, buds and leaves in sequence.[58] Despite the necessarily empirical nature of this experimentation, further efforts could prove rewarding.

BIOCHEMISTRY

All cycad seeds contain fairly large quantities of unusual substituted azoxy compounds which are acutely and chronically toxic. A notable result of this was when General J. C. Smuts and many of his commando were incapacitated for several days during the South African War of 1899-1902 after resorting to cycad seed for emergency food supplies.[59] The plant responsible is believed to be Encephalartos longifolius.[5] The fleshy sarcotesta and/or the starch-rich megagametophyte from E. cycadifolius, E. eugene-maraisii, E. ferox, E. horridus, E. lehmannii, E. longifolius and E. villosus have either proved acutely toxic to rabbits or have been suspected or known to be poisonous to man.[60] Toxic and carcinogenic effects have been observed in rats fed on female cone portions from E. umbeluziensis, E. villosus, E. lebomboensis and E. laevifolius.[61] The megagametophyte of E. lanatus has also been reported as toxic and carcinogenic.[62]

The compound macrozamin (methylazoxymethanol β-primeveroside)(Fig 2), first found in the Australian Macrozamia, has been isolated from E. lanatus and E. transvenosus.[63] This compound, together with the closely-related cycasin (methylazoxymethanol β-D-glucoside)(Fig 2), has now been found in all cycad genera in concentrations which are genus-dependent, but neither has been found in any other plant group.[64-66] Macrozamin is generally more abundant that cycasin, occurring at 2,09 - 2,86% in Encephalartos seeds and at 4,70% in Stangeria eriopus.[66] Further extraction and quantification of minor related azoxy compounds may extend the chemotaxonomic usefulness of these toxins, the biosynthesis of which remains to be explored.

Recent attention has been focussed on the compound α -amino-β-methylaminopropionic acid (synonym β -N-methylaminopropionic acid or BMAA). This compound has been isolated from seeds of Cycas [67] and has been implicated in the etiology of human amyotrophic lateral sclerosis and other neuromotor disorders such as Parkinsonism and Alzheimer's diseases.[68] Whether this compound is present in South African cycads remains to be seen.

The biflavonoid chemistry of cycads has been partially researched and appears to be useful taxonomically at least at the genus level.[69-71] An unusual feature is the complete absence of biflavonoids from the leaves of Stangeria.[69]

All cycads contain mucilage canals and excision of leaves or cones allows collection of the exudate. Hydrolysis of this material and gas-chromatography of the monosaccharides has provided data which correlate with genera.[72] Encephalartos monosaccharides comprise mainly arabinose and galactose and are similar in this respect to the sugars from the Australian genus Lepidozamia. Stangeria is once again exceptional in not having detectable amounts of rhamnose or methylrhamnose. Local workers have shown that the hydrolysed exudate from the cone of Encephalartos longifolius comprises fucose, rhamnose and 3-O-methyl rhamnose, arabinose, xylose, galactose, mannose, glucuronic acid and its 4-O-methyl ether.[73] With this species, the monosaccharide pattern is not affected by plant age, sex or environment.[74] A more comprehensive survey of the genus Encephalartos has revealed a similar pattern in 14 species investigated.[75] Current work at the University of Cape Town on Encephalartos cone exudates has highlighted the high proportions of rhamnose and its methyl ether, and D-glucuronic acid and its methyl ether.[76-77] These authors have used sequential degradation techniques which enable postulation of the structure and linkages in the polysaccharide.

A preliminary survey of Encephalartos leaf waxes indicates that species-specific profiles may be readily obtained using gas-chromatographic techniques.[78] In Angiosperms, the biosynthetic route typically leads to hydrocarbons with odd carbon atom numbers, usually C_{29} and C_{31} molecules. In some Encephalartos species there appear to be high proportions of alkanes with even-numbered carbon atoms. Thus this area of work is important both for its taxonomic and its biosynthetic relevance. A comprehensive survey of cycad leaf hydrocarbons is presently being carried out.

Of the various enzymes present in plants, the peroxidase group is readily examined by electrophoretic methods. Crude leaf extracts from cycad leaves give characteristic "fingerprints" of several, mainly anodic, bands (Osborne, unpublished results). Preliminary results suggest that polymorphism within Encephalartos is absent and that the peroxidase zymograms may contribute useful data in interspecific relationships. Serological and immuno-electrophoresis work on cycad seed proteins is useful in confirming groupings at the family and genus levels (Osborne, unpublished results). Opportunities exist in amino acid sequencing analyses; some work has been accomplished using Encephalartos pollen as a source of histone proteins.[79-80]

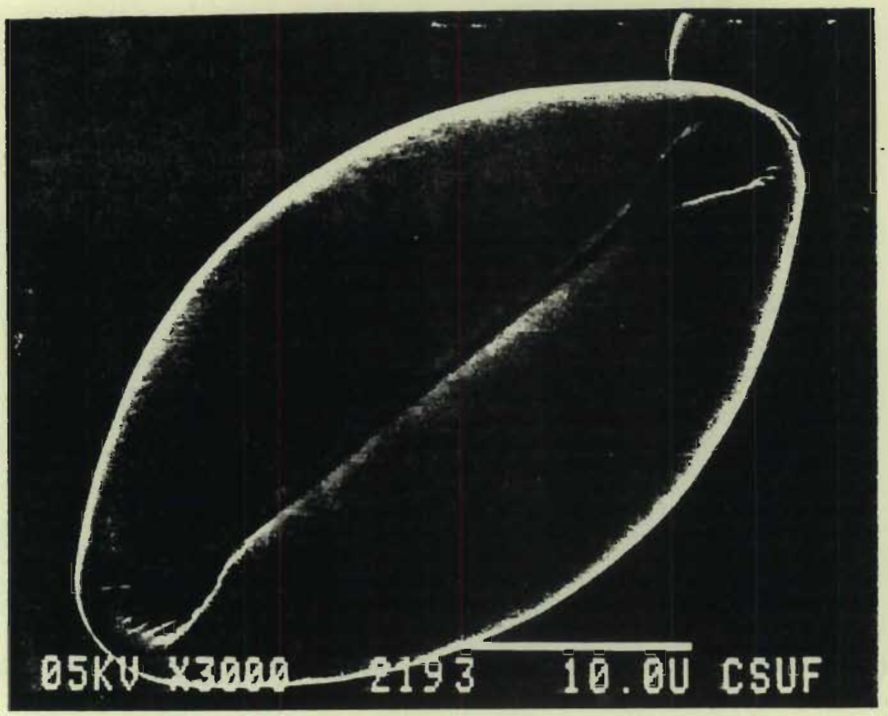
The South African cycads have been neglected in studies by plant physiologists. In view of the early origin of the group, it is anticipated that a C-3 photosynthetic mechanism will be found to operate, but experimental confirmation is required. Studies on mineral nutrition and deficiency symptoms would provide useful information to growers.

CONCLUSION

The South African cycads have quite deservedly been admired and appreciated by botanists, horticulturists and laymen in view of the decorative appeal and the rarity status of these plants. Increasing

attention is now being paid to various aspects of their taxonomy, morphology, propagation and biochemistry. In most cases this work is at a fairly preliminary stage and many avenues of research are yet to be explored. Exciting opportunities and challenges, many of an interdisciplinary nature, await cycad researchers in the future.

Note : Table I and Figure 2 of this paper are not reproduced in this Appendix. The reader is referred to the corresponding details in the main text; Table I corresponds to Table I (page 11) while Figure 2 corresponds to Figure 13 (page 121).



(a)

(b)

FIGURE 1 : SEM photomicrographs of cycad microspores, x 3000, scale bar 10μ.[18] (a) *Encephalartos arenarius* showing smooth exine surface and regular colpus (b) *Stangeria eriopus* showing pitted exine and irregular colpus.

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