

**SOME ASPECTS OF MEGAGAMETOPHYTE DEVELOPMENT
AND POST-SHEDDING SEED BEHAVIOUR OF *Encephalartos
natalensis* (ZAMIACEAE)**

by

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As the candidate's supervisor I have/have not approved this dissertation for submission.

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ABSTRACT

Very little is known about the post-shedding seed behaviour and megagametophyte development of the cycads, the most primitive extant seed-bearing plants, which pre-date the dinosaurs. In the present investigation, seeds of *Encephalartos natalensis* Dyer and Verdoorn were shed with relatively high mean embryo (3.33 g g^{-1}) and megagametophyte ($1.25 \pm 0.16 \text{ g g}^{-1}$) WCs, when the developing embryo consisted primarily of the coiled, elongated suspensor bearing a rudimentary sporophyte at its tip. It was not surprising that these seeds were revealed as desiccation sensitive in the present investigation, as the embryos continued to develop after seed-shed, reaching a germinable size ($\geq 15 \text{ mm}$) only 4 – 6 months after seed abscission from the strobilus.

Maintenance of the seeds in hydrated storage conditions was precluded by the proliferation of fungi, despite the application of the fungicide: Benlate[®]. Some seeds were also found to germinate in hydrated storage, despite the hard physical barrier to germination imposed by the enclosing sclerotesta. Seeds dusted with Benlate[®] and placed in ‘open’ storage in loosely closed paper bags had a longer life-span than those placed in hydrated storage; however, seeds stored in open storage were also overcome by fungi, but only around 18 months after seed-shed. Therefore, while the vigour and viability of the seeds appeared to decline slowly in the months after the embryos reached a germinable size, the life-span of stored *E. natalensis* seeds devoid of fungi is yet to be determined and will be the subject of further research.

The current investigation also combined ultrastructural and viability retention studies to observe the post-shedding behaviour of the storage tissue, the megagametophyte. The cells of the megagametophyte became progressively packed with starch and protein as the two main storage reserves, a limited number of discrete lipid bodies, and occasional mitochondria all of which appeared to be embedded in an homogeneous matrix. When the development of the megagametophyte cells was analysed ultrastructurally, it was found that the unusual matrix was present from the inception of megagametophyte cellularisation, and contained microtubules and numerous very faintly-visible vesicles. Newly-formed megagametophyte cells were thus not highly vacuolated as previously thought, but dominated by an homogeneous matrix.

Enzyme-gold localisation was employed in an attempt to determine the organelles responsible for the deposition of cell wall components during cellularisation of the megagametophyte. It appeared that ER-derived vesicles (and not Golgi-derived vesicles) were the principal

contributors of the primary cell wall components, pectin and xylan. While cellularisation took place over approximately 1 - 2 weeks, subsequent development of the megagametophyte cells involved the accumulation of storage reserves, this phase lasting approximately 8 months - when the seeds were shed whether pollination/fertilisation had recently occurred, or not.

At seed-shed, the cells of the megagametophyte were nucleated and contained a few mitochondria of a metabolically-active appearance. The occurrence of aerobic metabolism in these cells was confirmed by the tetrazolium (TTZ) test. Judging from the TTZ reactivity, the viability of the megagametophyte cells of fertilised seed appeared to decline slowly in the months after seed-shed, in parallel with extension growth of the embryo. The cell layer comprising the external surface of the megagametophyte showed marked ultrastructural differences from the inner cells, and may emerge as having an 'aleurone-like' function. It is, however, possible that the cells of the body of the gametophyte participate actively - at least in the earlier stages of post-shedding seed development - in mobilisation of stored reserves, which must support the development of the embryonic sporophyte.

PREFACE

The experimental work described in this dissertation was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, from January 2006 to December 2008, under the supervision of Professors Patricia Berjak and Norman W. Pammenter.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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W.R. Woodenberg

January 2009

DECLARATION 1 – PLAGIARISM

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DECLARATION 2 – PUBLICATIONS

WOODENBERG, W.R., ERDEY, D., PAMMENTER, N.W. AND BERJAK, P. (2007). Post-shedding seed behaviour of selected *Encephalartos* species. Abstracts from the 5th International Workshop on Desiccation Tolerance and Sensitivity of Seeds and Vegetative Plant Tissues. *South African Journal of Botany* 73:496.

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2.3.3. Prolonged TEM tissue processing protocol	18
2.3.4. Microtomy and microscopy	19
2.3.5. Scanning electron microscopy (SEM)	19
2.3.6. Enzyme-gold localisation and immunocytochemistry	20
2.3.6.1. Enzyme-gold localisation of xylan and pectin	20
2.3.6.2. Immunocytochemistry of microtubules	21
2.3.7. Histochemistry of protein and carbohydrates	21
2.3.7.1. Protein histochemistry	22
Protein histochemistry of wax-embedded sections	22
Protein histochemistry of resin-embedded sections	23
2.3.7.2. Carbohydrate histochemistry	23
Carbohydrate histochemistry of wax-embedded sections	23
Carbohydrate histochemistry of resin-embedded sections	23
2.3.7.3. Lipid histochemistry	24
Lipid histochemistry of wax-embedded sections	24
Lipid histochemistry of resin-embedded sections	24
3. RESULTS	25
3.1. Some aspects of the post-shedding seed behaviour of <i>Encephalartos natalensis</i>	25
3.1.1. The change in embryo and megagametophyte water content in open-stored seeds	25
3.1.2. Change in fresh and dry mass of the embryo and megagametophyte in open-stored seeds	27
3.1.3. Macroscopic observations on embryo development in the	

months after seed-shed	31
3.1.4. Vigour and viability of seeds at various times during open storage	31
3.1.5. The change in embryo and megagametophyte WC of seeds placed in hydrated storage 6 months after seed-shed	31
3.1.6. Change in fresh and dry mass of the embryo and megagametophyte of seeds placed in hydrated storage 6 months after seed-shed	34
3.2. Some aspects of <i>E. natalensis</i> megagametophyte development	39
3.2.1. Structure and ultrastructure of the earliest stage in megagametophyte development observed in this study	39
3.2.2. Structure and ultrastructure of the megagametophyte cells 1 week after the coenocyte was observed	43
3.2.2.1. Enzyme-gold localisation of pectin and xylan during early cell wall development	48
3.2.2.2. Ultrastructure of the cytomatrix	48
3.2.3. Structure and ultrastructure of the megagametophyte cells from 1 to 2 months after the coenocytic stage	55
3.2.3.1. Protein histochemistry	62
3.2.3.2. Carbohydrate histochemistry	62
3.2.3.3. Lipid histochemistry	67
3.2.4. Structure and ultrastructure of the megagametophyte cells from 3 months after the coenocyte was observed to the time of seed-shed	67
3.3. Viability status of the megagametophyte during post-shedding seed development	76
3.3.1. Tetrazolium (TTZ) study	76

3.3.2. Ultrastructural investigations of the megagametophyte during post-shedding seed development	76
3.4. Difference in ultrastructure of the peripheral cell layer of the megagametophyte	81
4. DISCUSSION	88
4.1. Some aspects of the post-shedding seed behaviour of <i>Encephalartos natalensis</i>	88
4.2. Some aspects of <i>E. natalensis</i> megagametophyte development	95
4.3. Concluding remarks and future research	102
5. REFERENCES	105

1. INTRODUCTION

Cycads have been described as the “coelacanths of the plant world”, the “dinosaur plants”, and the “living fossils” (Giddy, 1984; Jones, 1993). These ancient plants have existed for at least 250 million years and are said to be the most primitive extant spermatophytes (Jones, 1993). Allied to their primitive nature, cycads (Gymnospermeae) display many unusual and some unique features in their reproductive systems, cyanobacterial symbiosis and toxins (Osborne *et al.*, 1988; Osborne, 1995) making them one of the most interesting plant groups for botanical research.

1.1. The cycads

The present day cycads are represented by three families - Cycadaceae, Stangeriaceae, and Zamiaceae - with approximately 289 species in 11 genera - *Bowenia*, *Ceratozamia*, *Chigua*, *Cycas*, *Dioon*, *Encephalartos*, *Lepidozamia*, *Macrozamia*, *Microcycas*, *Stangeria*, and *Zamia* (Whitelocke, 2002). However, the cycads of today comprise a diverse, modified remnant of a much larger group of plants which flourished in the Mesozoic Era (Osborne *et al.*, 1988).

1.1.1. Conservation status

Despite representing only a small fraction of today’s plant diversity, cycads are a group of global conservation significance. The 1997 IUCN Red List of Threatened Plants documented 12.5% of the world’s vascular plants in one of the threatened categories (Walter and Gillett, 1998) while as many as 82% of the world’s cycad species were listed as threatened (Donaldson, 2003). A publication in 2007, of a list of critically endangered, endangered, vulnerable and protected species in South Africa, revealed that 71% of the critically endangered plants are cycads (van Schalkwyk, 2007). Of the 38 cycad taxa represented in South Africa, 11 are critically endangered, six are endangered, two are vulnerable and 15 are protected (van Schalkwyk, 2007). This highlights the urgency for the conservation of these evolutionary important plants.

1.1.2. Reasons for decline

Two main reasons have been cited for the decline in cycad numbers: 1) the removal of plants from habitat by traders, landscapers and collectors because of their monetary value and aesthetic appeal; and 2) the disturbance and destruction of habitat during human territorial expansion (i.e. the building of dams and roads, agricultural development and commercial forestation, as well as urban and rural housing development) [Osborne, 1995; Donaldson, 2003]. Collection of cycads by traditional healers for medicinal and reputed magical purposes has also been implicated in the diminishing cycad populations. Therefore, in order to avert the complete loss of these plants, the majority of cycads are protected by law (Giddy, 1995).

1.1.3. Legislation

In South Africa, cycads are protected under the *National Environment: Biodiversity Act* of 2004 (van Schalkwyk, 2007). Internationally, the trade in cycads is monitored by the Convention in International Trade in Endangered Species of Flora and Fauna (CITES). Both *Encephalartos* and *Stangeria* are included in Appendix 1 of the CITES schedules, which means that no trade is permitted in wild-collected plants or seeds. Trade is restricted to artificially propagated (i.e. nursery-grown) specimens. Although CITES permits trade in garden-produced Appendix 1 seeds, The SA Management Authority has instructed the provinces of South Africa not to issue CITES export permits for *Encephalartos* and *Stangeria* seeds on the grounds that it is difficult to distinguish between seed of wild and garden origin (Giddy, 1995). Whilst protective legislation has been passed, surprisingly little research has been done on cycad seeds in light of the obvious need for cycad conservation (Vorster, 1995).

1.1.4. Reasons for lack of research on cycad seeds

Unavailability of cycad seeds in sufficient quantities for experimental purposes has been largely responsible for lack of research (Dehgan and Schutzman, 1989). In addition, more than one-third of known species are subject to the most severe protective regulations, and the remaining, including their seeds, are governed by the second category of restrictions (Schlegel, 1991).

It is also extremely difficult to ascertain whether the seeds of *Encephalartos* and other cycad genera are fertilised or not. In angiosperms, reserve deposition of the seed occurs following fertilisation, so, a developed angiosperm seed has the potential to germinate when conditions are

suitable. However, cycads are gymnosperms, where the ovule morphology may (depending on the species) differ very little, if at all, from that of the fertilised seed.

In some gymnosperm species including the subject of the present study, *Encephalartos natalensis* Dyer and Verdoorn, all the reserves ultimately needed are laid down in the megagametophyte of the ovule prior to fertilisation, and the propagatory units do not always contain a developed embryo (personal observation). This presents a challenge in cycad seed research, in that unless hand pollination has been carried out, there is always variation in the actual seed number available, because fertilised seeds cannot be distinguished from ovules.

Despite – or because – of the above-mentioned challenges which have hindered cycad seed investigations in the past, much research is required in order to preserve cycad germplasm and ultimately to restore natural cycad populations by improved propagation.

1.2. Seeds

The seed, however small, contains all the information needed to produce an entire plant of its species. Moreover, seeds afford invaluable intra-specific genetic variability, and are therefore the most significant propagatory unit of plants (Berjak and Pammenter, 2004).

The seed consists of three major components: an embryonic axis, its nutritive tissue and the protective layers of the seed coat (Bewley and Black, 1994). The nutritive tissue in angiosperms is generally either the endosperm or cotyledon/s, while in gymnosperms it is the megagametophyte. This tissue provides nutrition in the form of lipids, carbohydrates and protein (Bewley and Black, 1994). Lipids are stored as lipid bodies, carbohydrates - as starch or thickened walls, while protein can be found as discrete protein bodies. These nutrients are ultimately utilised by the young plant during the transition from the seed-bound embryo, to a self-sufficient autotrophic organism during embryo development, germination and seedling establishment (Bewley and Black, 1994).

1.2.1. Seed development

Seeds of the majority of species seem to pass through three broad developmental phases. The first phase is characterised by rapid mitosis, cellularisation and histodifferentiation (Adams and Rinne, 1980). This phase is initiated by fertilisation, and in angiosperms, leads to the production

of an embryo and its nutritive tissue, while in some gymnosperm species, fertilisation is not a prerequisite for the formation of the nutritive tissue (outlined above). In angiosperms, cell division ceases in the second phase, when there is a massive accumulation of reserves, which is mirrored by cell expansion and an increase in both the fresh and dry mass of the seed (Leprince *et al.*, 1993). In orthodox seeds, the third developmental phase is characterised by the deceleration and ultimate cessation of dry mass accumulation, along with a marked decline in the fresh mass of the seed (Adams and Rinne, 1980). This phase is therefore known as maturation drying, where the seed can lose as much as 90% of its original water (Adams and Rinne, 1980). Maturation drying results in the decline of seed metabolic activity to low levels, with the seed passing into a state of quiescence or dormancy depending on the species. Seeds which undergo maturation drying are said to display ‘orthodox’ post-harvest behaviour (Roberts, 1973).

1.2.2. Orthodox seeds

Storage of the majority of seeds has largely been facilitated because they show orthodox post-harvest behaviour, i.e. they are dry and will survive predictable durations of storage under defined conditions of relative humidity (RH) and temperature. The storage longevity of orthodox seeds, within limits, is known to increase logarithmically with decreasing water content (Ellis and Roberts, 1980), although there apparently are lower thresholds below which there are no further beneficial effect and, in fact, damage may actually occur (Vertucci and Farrant, 1995; Walters *et al.*, 2005). Orthodox seeds acquire desiccation tolerance relatively early in their pre-shedding development (Bewley and Black, 1994; Vertucci and Farrant, 1995), as a result of the presence and interaction of a suite of mechanisms and processes expressed during development (Pammenter and Berjak, 1999). These include minimisation of vacuolation, intracellular dedifferentiation and metabolic “switch-off”, the presence and optimal operation of antioxidant systems, the presence and operation of putatively protective molecules such as sucrose, and certain oligosaccharides or sugar alcohols, the possession of late embryogenic accumulating / abundant proteins (LEAs), as well as the ability for damage repair on rehydration (Berjak and Pammenter, 2004). It is believed that these mechanisms and processes act together ultimately to give rise to seed ‘orthodoxy’.

However, not all seeds are orthodox. At the other end of the scale are highly recalcitrant seeds which cannot tolerate even a small proportion of loss of tissue water (e.g. *Avicennia marina*, Farrant *et al.*, 1993). Such seeds are categorised as recalcitrant, a term originally introduced by

Roberts (1973) for seeds that cannot be stored at low water contents. Ellis and Hong (1990) defined an intermediate category, to include seeds that can be dried, but not to the same degree as orthodox types. Recalcitrant seeds, or non-orthodox seeds in general, are suggested to be subject to the malfunctioning or absence of one or more of the processes and mechanisms influencing the acquisition of desiccation tolerance (Berjak and Pammenter, 2004).

1.2.3. Non-orthodox seeds

Non-orthodox seeds are shed at high water contents and recalcitrant types remain metabolically active (Berjak and Pammenter, 2008). However, because non-orthodox seeds do not undergo much (if any) maturation drying, there are few clear indications of the maturation status of the seeds before germination commences (Berjak and Pammenter, 2004). Intra- and inter-seasonal variation also occurs within species, especially in those seeds of tropical provenance. For example, Berjak *et al.* (1996) found that the water content of embryonic axes of *Camellia sinensis* seeds varied inter-seasonally from 2.0 ± 0.3 to 4.4 ± 2.4 g H₂O per g dry mass (g g⁻¹). It has also been found that intra-seasonal differences occur in non-orthodox seeds (Chacko and Pillai, 1997), and provenance has been shown to have a marked effect on the degree of recalcitrance, within individual temperate species (Daws *et al.*, 2004; 2006). The variation within non-orthodox seeds highlights the unpredictable nature of the responses of these seeds, which creates numerous difficulties with both experimentation and storage.

Recalcitrant seeds, which must be maintained hydrated, cannot be stored in this condition in the long term (Pammenter *et al.*, 1994). Furthermore, the unpredictable nature of non-orthodox seeds – and particularly recalcitrant seeds – has contributed greatly to the lack of successful storage, even in the medium term. Recalcitrance and true dormancy are seldom, if ever, coexistent, but if embryos are underdeveloped when seeds are shed, an interval of several months may elapse before they are germinable (Berjak and Pammenter, 2004).

Since recalcitrant seeds are desiccation sensitive, storage conditions must be geared to avoid water loss, which requires virtually 100% relative humidity (RH). At the same time, germination (root protrusion) of these seeds in storage should be prevented since there is no exogenous water for germination to be carried out to completion, hence resulting in debilitating water stress (Pammenter *et al.*, 1994). Proliferation of microorganisms, particularly fungi, is another cause for concern. Not only do fungi damage the seeds by degradation, but fungal respiration also produces metabolic water that tends to make the seeds even more metabolically

active (Berjak and Pammenter, 2004). Seed-associated fungi need to be eliminated, or at least their activity minimised, if storage of non-orthodox seeds at high water contents is to be successful.

To optimise the storage viability period of non-orthodox seeds – and particularly truly recalcitrant types – it is necessary to become familiar with the post-harvest behaviour of individual species (Berjak and Pammenter, 2004). This has become apparent through the growing list of species cited by those authors as producing varying degrees of recalcitrance. From the knowledge amassed to date, it is believed that the seed biology of many more species needs to be characterised in fine detail, in order to gain a better understanding of the phenomenon of seed recalcitrance, and also of those non-orthodox types described as showing intermediate behaviour.

Although there has not been a great deal of research on cycad seeds thus far, there was a general belief that they may be recalcitrant (Forsyth and van Staden, 1983; Dehgan and Schutzman, 1989) mainly based on the fact that the seeds are ‘wet’. Woodenberg *et al.* (2007) have since shown that the seeds of *Encephalartos natalensis* and *E. gratus* are recalcitrant after the seeds lost viability when subjected to dehydration. However, more comprehensive assessments are needed to determine details and to develop effective storage protocols for cycad seeds and especially for the long-term germplasm conservation of individual species.

Aside from the need for more research into cycad germplasm conservation, there is also a need to address the paucity of information on cycad embryogenesis and particularly megagametophyte development and reserve deposition at the cellular and ultrastructural levels. Most of the descriptive botany on the megagametophyte was done in the early 1900s (Lang, 1900; Chamberlain, 1906; Smith, 1910; Reynolds, 1924; Sedgwick, 1924). This was before the advent of modern electron microscopes, thus many questions were necessarily left unanswered at the ultrastructural level. Additionally, later studies in the 1960s and '70s (e.g. Maheshwari and Singh, 1967; Singh and Johri, 1972) preceded the common usage of sophisticated methodology, e.g. immunocytochemistry (ICC). The following is a general overview of the knowledge amassed to date on the megagametophyte development of cycads and other gymnosperms.

1.3. The megagametophyte

The female gametophyte of angiosperms is primarily an eight-nucleate, microscopic structure, which bears a single functional gamete (Maheshwari and Singh, 1967). By comparison, the gymnosperm megagametophyte is a relatively large, haploid, multicellular organ, which serves the dual purpose of bearing the female gametes and providing nutrients for the developing sporophyte (the embryo). According to Maheshwari and Singh (1967), the early stages of megagametophyte development seem to follow a similar pattern in cycads, *Ginkgo*, conifers, and *Ephedra*.

1.3.1. Gymnosperm megagametophyte development

The megagametophyte is initiated as a single cell - the megaspore. The megaspore develops from within the nucellar tissue of the young ovule and gives rise to a linear tetrad that consists of three non-functional upper cells (which ultimately degenerate), and one functional megaspore at the chalazal end of the tetrad (Maheshwari and Singh, 1967). The functional megaspore undergoes a number of nuclear divisions with the nuclei lying in a thin, peripheral layer of cytomatrix that surrounds what has been termed the large central vacuole (Maheshwari and Singh, 1967). The number of free nuclei is reportedly characteristic for different species: e.g. Chamberlain (1906) recorded approximately 1000 nuclei for *Dioon edule*.

Early wall formation during the transition from the free-nuclear (coenocytic) to the cellular megagametophyte of cycads was not recorded for *Dioon edule* (Chamberlain, 1906); *Zamia floridana* (Smith, 1910); *Encephalartos* spp. (Sedgwick, 1924) and *Microcycas calocoma* (Reynolds, 1924). This was mainly due to the difficulty of obtaining ovules from young cycad strobili - which are usually concealed by the bud scales of the parent plant. However, cellularisation of the megagametophyte has been observed in some cycads e.g. *Cycas revoluta* (Ikeno, 1898), *Macrozamia reidleyi* (Light, 1924), *M. spiralis* (Brough and Taylor, 1940) and *Encephalartos poggei* (De Sloover, 1964) and other gymnosperms viz. *Torreya californica* (Robertson, 1904); *Ginkgo biloba* (Carothers, 1907) and *Taxus baccata* (Pennel and Bell, 1987).

For the majority of gymnosperms, cellularisation of the megagametophyte seems to proceed as follows: At the end of the last nuclear division, secondary spindles develop such that every nucleus is connected to six neighbouring nuclei (Figs 1.1a,b). The spindles are arranged parallel with the surface of the megagametophyte, while anticlinal cell walls are laid down centripetally

between the nuclei. The laying down of the anticlinal walls gives the megagametophyte a honeycomb appearance (Fig. 1.1d). Each honeycomb cavity is known as an ‘alveolus’ (Sokolowa, 1890), which is sometimes also referred to as a primary prothallial cell (Maheshwari and Singh, 1967; Singh and Johri, 1972).

The alveoli are open-ended toward the megagametophyte interior as they are not separated by periclinal walls from the central vacuole (Maheshwari and Singh, 1967). According to those authors, the alveoli, which appear hexagonal in cross-section (Fig. 1.1d), grow towards the centre of the megagametophyte, with the spindles still connecting adjacent nuclei at the open end (Figs 1.1c,e). Laying down of cell wall material appears to be directed by the persisting spindles. The circumference of the central vacuole decreases as the anticlinal walls of the alveoli encroach inwards. However, some alveoli may close before they reach the centre of the megagametophyte (Figs 1.1c,e,i) [Maheshwari and Singh, 1967].

According to Singh and Johri (1972), an alveolus closes in the following manner (Figs 1.1f,g,h): When a nucleus lags behind, the spindles connecting it with its neighbouring nuclei bend such that the middle regions of the spindles and membranes which have developed on them approach each other. The approaching spindles join, and, therefore, the nuclei previously separated by the closing alveolus become connected by spindles fibres, while the membranes on the new spindle continue inward from the point of closure (Singh and Johri, 1972). Ultimately, the nucleus passes back slightly into the closed alveolus and is ready to initiate division that usually results in cellularisation of the tissue. This is how different alveoli become closed at variable distances from the centre of the gametophyte (Fig. 1.1i).

Some alveoli, particularly at the micropylar and chalazal poles of the megagametophyte, close only a short distance from the megagametophyte periphery, and have been termed ‘precociously’ closed alveoli (Maheshwari and Singh, 1967). According to those authors, while precociously closed alveoli may give rise to archegonial initials at the micropylar end of the megagametophyte, periclinal walls segment other alveoli such that the megagametophyte appears to have rows of radiating cells. As Chamberlain (1906) put it: “*When an ovule [of *Dioon edule*] in which the gametophyte is still spherical is cut through the middle, the gametophyte, as seen with the naked eye, has a beautiful, radiating appearance, looking somewhat like a transverse section of a twig with very fine medullary rays.*” The radiating conformation of the megagametophyte tissue is however, only a transient occurrence that may

be lost due to the laying down of irregular walls as the megagametophyte develops (Singh and Johri, 1972).

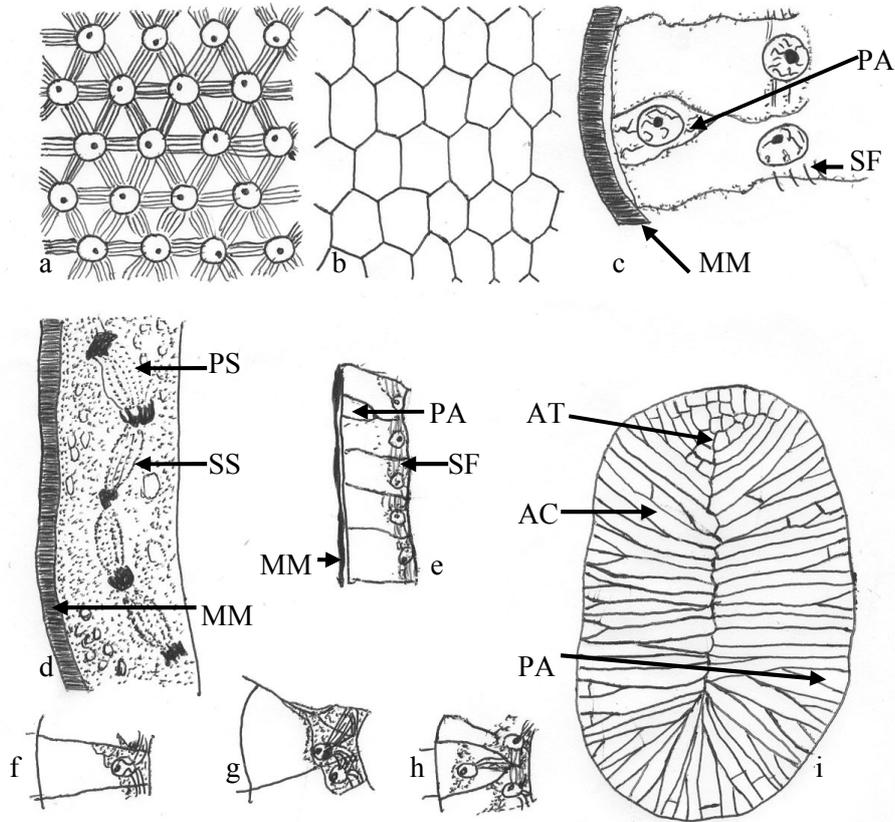


Figure 1.1: Diagrammatic representation of alveolation in the megagametophyte of most gymnosperms. a) arrangement of nuclei after the last free nuclear division; secondary spindles have been formed and each nucleus is connected with six neighbouring nuclei; b) cross section of alveoli; note their hexagonal outline; c) showing young alveoli; one of the alveoli is precociously closed; d) primary as well as secondary spindles connect the telophasic nuclei; e) young alveoli, one of which is precociously closed; f) a growing alveolus with spindles seen at the open end; g) and h) showing the lagging behind of the nucleus with the resultant closing of the alveolus; i) median longitudinal section of the megagametophyte in which alveolation has been just completed; there is a larger number of precociously closed alveoli at the two poles than on the sides; a few alveoli at the micropylar end (AT) are seen in cross section. AC = an alveolus that has reached the centre of the megagametophyte; AT = an alveolus in cross section; MM = megaspore membrane; PA = precociously closed alveolus; PS = primary spindle; SF = spindle at the inner face of the alveolus; SS = secondary spindle. Diagram redrawn after Maheshwari and Singh (1967).

A few minor variations to the mode of megagametophyte development outlined above have been reported for *Actinostrobus pyramidalis* (Saxton, 1913), *Athrotaxis* spp. (Brennan and Doyle, 1956), *Callitris* spp. (Baird, 1953), and *Sequoia* spp. (Looby and Doyle, 1942). However, *Gnetum gnemon* (Sanwal, 1962), *Welwitschia mirabilis* (Martens, 1963) and *Taxus baccata* (Pennel and Bell, 1987) seem to employ a completely different mode of megagametophyte development. In *G. gnemon*, *W. mirabilis* and *T. baccata*, laying down of the cell walls seem to occur by free cell formation - where nuclei become enclosed directly by ingrowing cell walls. Therefore, it is important to note that the mode of development of the megagametophyte does not appear to be uniform across all gymnosperms.

1.4. Mode of development of the endosperm in angiosperm seeds

This developmental event is introduced here, as it was envisaged that parallels might exist with gymnosperm megagametophyte ontogeny. Differences in the mode of development of the endosperm in angiosperms also exist, three main modes of early endosperm development being recognized: nuclear, cellular, and helobial (Lopes and Larkins, 1993; Friedman, 1994). Nuclear endosperm, which is the most common type, develops as follows: a large number of free nuclei are generated when the primary endosperm nucleus undergoes many mitotic waves without cytokinesis, with subsequent cellularisation proceeding from the periphery inwards, as described (above) for most gymnosperm species (Lopes and Larkins, 1993; Friedman, 1994). During cellular endosperm development, mitosis is accompanied by cytokinesis after the first division of the primary endosperm nucleus, followed by further repeated cell divisions (Lopes and Larkins, 1993; Friedman, 1994). Helobial endosperm on the other hand, is said to be formed by an intermediate and uncommon mode of development. The primary endosperm cell undergoes cell division to give rise to two cells of unequal size. One, or sometimes both, of the cells then undergo a series of mitotic divisions, with subsequent development following that of the nuclear-type (Lopes and Larkins, 1993).

Different modes of development of both the gymnosperm megagametophyte and the endosperm of angiosperms thus exist, with a paucity of investigations on cycad megagametophyte ontogeny at the ultrastructural level.

1.5. The present study

The present investigation can be divided into two main sections: one has applications in the conservation of cycad germplasm, while the other is ontogenetic in nature. The present study contributes to cycad germplasm conservation research in terms of a study of the post-shedding seed behaviour of *Encephalartos natalensis* Dyer and Verdoorn, while it also attempts to address the paucity of information in the literature on the ultrastructural development of the megagametophyte (which is functionally the nutrient reserve tissue) of *E. natalensis* seeds.

Encephalartos natalensis is a relatively large cycad (Fig. 1.2) that is endemic to South Africa. It is widely distributed in the province of KwaZulu-Natal (Fig.1.3) from the southern districts of Portshepstone, Howick and Kranskop, to Vryheid in the North (Giddy, 1984). *Encephalartos natalensis* usually has an erect trunk which can be up to 6 m in height and 400 mm diameter. It usually occurs as a solitary, single-stemmed plant, but may form clumps of up to 11 stems when offsets and basal suckers are produced (Giddy, 1984). The glossy, bright green leaves are 1.5 to 3 m in length with a straight midrib. The median leaflets are 150 to 250 mm long and 25 to 40 mm broad, with one to four teeth on one or both margins, and the leaflets are characteristically reduced to a series of prickles towards the base of the midrib (Giddy, 1984; Jones, 1993). Adult plants of *E. natalensis* may produce up to five deep golden-yellow strobili and as many as 250 bright orange-red seeds per female strobilus (personal observation). While it is one of the more common South African cycad species, *E. natalensis* has been classified as protected (van Schalkwyk, 2007).



Figure 1.2: A mature *Encephalartos natalensis* plant growing outside the Elizabeth Sneddon Theatre at the University of KwaZulu-Natal, Durban, Howard College Campus; ruler = 500 mm; inset showing red sarcotesta-enclosed seeds surrounded by the orange strobili bracts; sarcotesta-enclosed seeds were approximately 25 mm wide and 45 mm long.

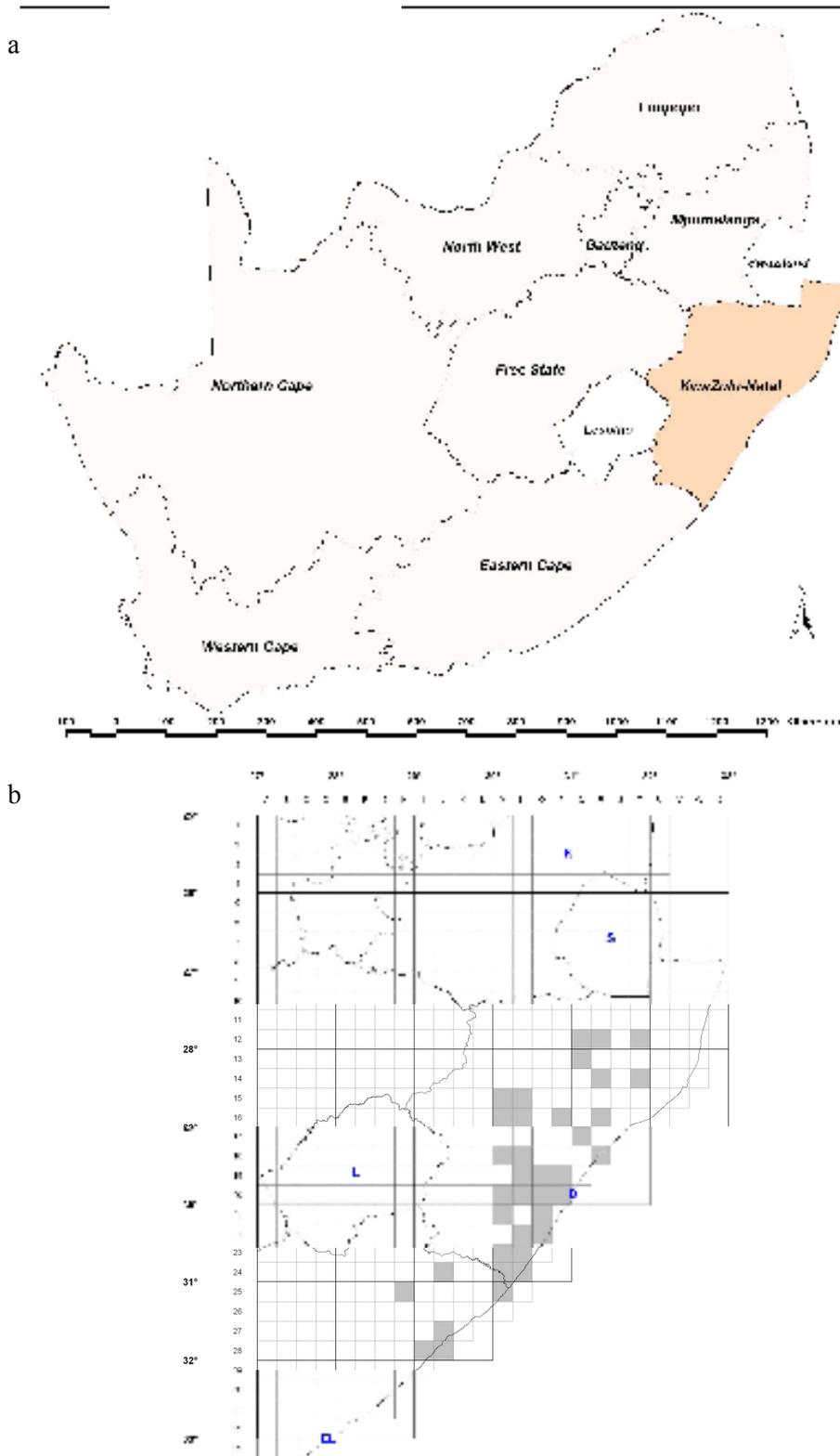


Figure 1.3: a) An illustration of the province of KwaZulu-Natal as a component of South Africa; b) the distribution of *Encephalartos natalensis* in KwaZulu-Natal and the Eastern Cape. (Mapping by S3 Technologies, Pietermaritzburg, KwaZulu-Natal, South Africa).

From the work of Woodenberg *et al.* (2007), it was evident that the seeds of *E. natalensis* seemed to lose both vigour and viability in the months following seed-shed. The present study aimed to determine the longevity of these seeds in storage, and to confirm whether the viability of the seeds decreases or not, in the months after seed-shed. Hence the germination capacity of the seeds was assessed once every 2 months from seed-shed for 18 months.

The two potential reasons for loss of vigour and viability of these seeds have been identified as either a stress caused by the physical obstruction to germination imposed by the hard sclerotesta, or because of a stress caused by water limitation. One of the objectives of the present study was to determine the more likely situation by affording half of the *E. natalensis* seeds hydrated storage conditions, and comparing the water content, vigour and viability of these seeds with those kept in open storage.

Since there is very little information in the literature on the post-shedding development of the cycad storage tissue, the megagametophyte, the present study combined investigations at the ultrastructural level and viability retention using transmission electron microscopy (TEM) and tetrazolium salts (TTZ) respectively.

In addition, the present study also aimed at investigating the mode of development of the megagametophyte tissue including reserve deposition, in order to identify properly the organelles present in the cells, and to document the ultrastructural events leading up to the formation of this unique tissue.

2. MATERIALS AND METHODS

2.1. Seed post-harvest behaviour

2.1.1. Plant material

Seeds of *Encephalartos natalensis* Dyer and Verdoorn were collected from two different plants in Durban, South Africa. One batch of seed (batch A) was obtained from the grounds of the Howard College Campus of the University of KwaZulu-Natal in January 2006 soon after the strobili began seed-shed, while another seed batch (batch B) was obtained from a residential garden in Winston Park during March 2006, 4 months after they were shed from the parent plant. Batch A seeds were derived from strobili that were pollinated naturally – presumably by insect vectors, whilst batch B seeds came from a plant that was hand-pollinated. The collected, sarcotesta-enclosed seeds were transferred in large, black plastic bags to the laboratory, where they were cleaned and placed into storage.

2.1.2. Treatment on arrival in the laboratory

Cleaning the seeds entailed removal of the external sarcotesta layer and decontamination of the underlying surface. The sarcotesta is a relatively soft part of the outer integument of the seed that is prone to decomposition. It was scraped off using a scalpel blade and the seeds were rinsed clean with tap water prior to surface decontamination. The cleaned, sclerotesta-enclosed seeds were surface-decontaminated by soaking in a 2.5% (v/v) solution of sodium hypochlorite (NaOCl) for 10 min, followed by rinsing three times with distilled water. The seeds were then blotted dry using paper towel and a sample of freshly harvested seeds was taken immediately for water content (WC) and germination assessment. The remaining seeds were dusted with Benlate[®] (benzimidazole, 500 g kg⁻¹) to minimise fungal contamination, packaged in brown paper bags ('open' storage) and kept at 16°C until further required.

Four months after being placed into open storage, a subsample of seeds were transferred to hydrated storage conditions to assess the suitability of this storage method for *E. natalensis* seeds. In order to create hydrated storage conditions, seeds were placed in a layer, not more than two seeds deep, on plastic mesh suspended in scrupulously clean (decontaminated using 2.5% [v/v] NaOCl) buckets lined with wet paper towel. The buckets were opened every 3 weeks to prevent anoxia and the build-up of volatiles.

2.1.3. WC and dry mass (g) determination

WC and dry mass of the whole megagametophyte tissue and whole embryo were determined gravimetrically (after drying to constant mass at 80°C for 48 h). The embryo and megagametophyte tissue of each seed was diced (c. 6 mm³ pieces) with a scalpel blade, placed in separate, small, aluminium-foil weighing boats and the fresh and dry mass of each determined using a Mettler MT5 6-place balance. The determination of WC and dry mass was done at bi-monthly intervals after the initial assessment at seed-shed (n = 10), and WC expressed on a dry mass basis (g H₂O per g dry mass), g g⁻¹.

2.1.4. Germination assessment

The ability of the seeds to germinate was determined at bi-monthly intervals (n = 10) from shedding to 18 months after seed-shed (MAS). Seeds were treated in the following manner prior to the assessment of their germinability: The thick, hard outer sclerotesta of each seed was removed by cracking, using a bench-vice and the thin, papery endotesta was peeled-off by hand. The testa-free seeds were then surface-decontaminated by soaking in a 2.5% (v/v) solution of NaOCl for 10 min, followed by rinsing (three times) with sterile, distilled water. The seeds were then aseptically plated onto 0.8% (w/v) water-agar in 100 ml glass jars. The jars were covered with cling-film, sealed with masking-tape, and placed in a growth room at 25°C and maintained under a 14/10 h light (65 µmol m⁻² sec⁻¹)/dark photo-period. Germination was monitored daily, and scored as positive upon radicle emergence from the hypocotyl. At the end of each germination assessment (30 d), ungerminated seeds were bisected to inspect for the presence of an embryo – i.e. to determine whether they had been fertilised or not. Germination percentage was calculated on the basis of the number of fertile seeds.

2.2. Viability status of the megagametophyte during post-harvest seed development

2.2.1. Ultrastructural investigations

2.2.1.1. Transmission electron microscopy (TEM) processing and viewing

A sharp scalpel blade was used to cut small segments (c. 2 mm³) from the megagametophyte tissue of two seeds per sampling interval. These segments were taken from the seeds shortly (days) after shedding, and 3 and 6 MAS.

The samples were fixed overnight in 2.5% (v/v) glutaraldehyde buffered at pH 7.2 with 0.1 M phosphate buffer containing 0.5% (w/v) caffeine. They were then washed in 0.1 M phosphate buffer (3 × 5 min). Primary fixation was followed by post fixation in phosphate-buffered 0.5% (w/v) osmium tetroxide for 1 h, and then washed (3 × 5 min) in 0.1 M phosphate buffer before the commencement of dehydration. Samples were dehydrated in a graded series of ethanol (25, 50, and 75% [v/v] - 2 changes each × 5 min), block stained with a saturated solution of uranyl acetate in 75% ethanol for 45 min, and then dehydrated further (2 × 5 min changes in 100% [v/v] ethanol) followed by 2 × 10 min changes in propylene oxide. The dehydrated samples were then placed in a 1:1 mixture of propylene oxide:resin (Spurr, 1969) in closed tubes on a vertical turntable for 4 h at room temperature, placed in full resin (Spurr, 1969) for 24 h and thereafter embedded in fresh resin for polymerisation at 80°C for 8 h.

2.2.1.2. Microtomy and microscopy

The resin-embedded samples of each seed sampled were sectioned using a Reichert-Jung Ultracut E microtome. Ultrathin sections were cut for TEM analysis, and stained with lead citrate (Reynolds, 1963) for 30 min. A maximum of five sections was viewed on each grid using a Jeol 1010 transmission electron microscope. The resultant micrographs were scrutinised qualitatively for the integrity of cells and their organelles.

2.2.2. Tetrazolium (TTZ) study

The TTZ study was run in parallel with the TEM study. Five, randomly chosen seeds were taken at each TEM sampling interval (i.e. at shedding, 3 and 6 MAS). These seeds were bisected longitudinally with a sharp scalpel blade and submerged immediately in a 1% (w/v) solution of 2,3,5-triphenyltetrazolium chloride (TTZ) in the dark at room temperature for 24 h (method modified after Debeaujon *et al.*, [2000]). Viability was assessed in terms of the presence and degree of red colouration on the cut surface of the seeds, where red colouration was an indication of aerobic respiration and concomitant viability. Images were captured using a Nikon Coolpix 4500 digital camera.

2.3. Megagametophyte development

2.3.1. Collection of material

Ovules were collected from the developing strobilus of a garden-grown *E. natalensis* plant in the Glenwood area, Durban. The ovules were collected weekly, from the earliest sighting of the cone in January 2007, to seed-shed in October 2007. The collected, sarcotesta-enclosed ovules were transferred in small Ziploc[®] bags to the laboratory, where they were processed for microscopy.

2.3.2. Tissue processing for microscopy

In most cases, small cubes (1-2 mm³) were cut out of the *E. natalensis* megagametophyte using a sharp scalpel blade for TEM tissue processing as outlined previously. However, during the first sampling interval, the ovules had a watery consistency which obviated cutting the megagametophyte. The first ovules were therefore processed whole, with extended incubation times detailed below. A sharp scalpel blade was used to trim away the integumentary layers as close to the ovule as possible without inflicting any mechanical damage. In the early, but slightly later, phases of ovule development, where the megagametophyte tissue appeared gel-like, a longitudinal disc was first cut out of the megagametophyte, and then cut into pie-like slices. This was done to minimise the considerable shrinkage or loss of the tissue that was observed during earlier TEM processing trial runs. The 'pie-slices' and whole ovules were also subjected to prolonged incubation and infiltration times as detailed below.

2.3.3. Prolonged TEM tissue processing protocol

The samples were fixed overnight in 2.5% (v/v) glutaraldehyde buffered at pH 7.2 with 0.1 M phosphate buffer containing 1% (w/v) caffeine. They were then washed in 0.1 M phosphate buffer (3 × 5 min). Primary fixation was followed by post fixation in 0.5% (w/v) phosphate-buffered osmium tetroxide for 2 h. The tissue segments were then washed (3 × 5 min) in 0.1 M phosphate buffer before the commencement of dehydration. Samples were dehydrated in a graded series of ethanol (25, 50, and 75% [v/v] - 2 × 10 min) and left in 75% ethanol overnight. The following day, the samples were block-stained with a saturated solution of uranyl acetate in 75% ethanol for 45 min, and then dehydrated further (2 × 10 min changes in 100% [v/v] ethanol) followed by 2 × 10 min changes in propylene oxide. The dehydrated samples were then placed in a 1:1 mixture of propylene oxide:resin (Spurr, 1969) on a vertical turntable overnight at room temperature, placed in full resin (Spurr, 1969) for another 24 h and thereafter embedded in fresh resin for polymerisation at 80°C for 8 h.

2.3.4. Microtomy and microscopy

The resin-embedded samples were sectioned using a Reichert-Jung Ultracut E microtome. Sections, 1 µm thick, were placed on glass slides, stained with 1% (w/v) toluidine blue and viewed with a Nikon eclipse 80i light microscope equipped with NIS Elements F Package imaging software. While ultrathin (gold) sections were cut for TEM analysis and stained with lead citrate (Reynolds, 1963) for 30 min, or zinc iodide-osmium tetroxide (ZIO) [modified after Harris and Chrispeels, 1980] for 2 min. A maximum of five sections was viewed on each grid using a Jeol JEM 1010 transmission electron microscope and *i*TEM Soft Imaging System GmbH imaging software. The following stages were sectioned and viewed: The earliest developmental stage found (approximately 1 week after the strobilus first appeared from the crown of the parent plant [the coenocytic stage]); 1 week after this stage; and 1; 2; 3; 4; 6 and 8 months after this stage (n = 3, replicated twice).

2.3.5. Scanning electron microscopy (SEM)

Scanning electron microscopy was used to determine the presence or not of any substructure in the matrix of megagametophyte cells during the early stages of ovule development when the tissue appeared gel-like. The block face of sectioned, resin-embedded samples was first etched by placing the blocks face-down into a 6.675% solution of potassium methoxide (in a 2:1 mixture of methanol:propylene oxide [Ferey *et al.*, 1986]) for 2 min followed by rinsing for 30 sec in methanol while sonicating. The dried specimens were then sputter-coated with gold in a Polaron E5300 SEM coating unit and viewed with a LEO 1450 scanning electron microscope (n = 3).

2.3.6. Enzyme-gold localisation and immunocytochemistry

2.3.6.1. Enzyme-gold localisation of xylan and pectin

Ultrathin sections of the earliest gel-phase megagametophyte (c. 1 week after the first sighting of the strobilus) were cut using glass knives and collected on 200-mesh nickel grids. For each treatment about five sections were examined (n = 2). The ultrathin sections on nickel grids (hereon referred to as sections) were transferred to a drop of 0.05 M phosphate buffered saline (PBS) pH 7.4 for 5 min at room temperature. The sections were then incubated in blocking solution (PBS containing 10% [v/v] foetal bovine serum [FBS], 1% [w/v] bovine serum albumin [BSA], 0.05% [v/v] Tween-20[®] and 0.2% [w/v] sodium azide), before being transferred to a drop (40 µl) of enzyme-gold complex and maintained in a moist chamber for 30 min.

The moist chamber was created by wetting the filter paper of the Petri dish inside which the sections were incubated. A rolled piece of moistened paper towel was also used to ensure the saturated atmosphere inside the Petri dish and ultimately to prevent precipitate formation during incubation.

The appropriate concentrations of pectinase, from *Rhizopus* sp. (Sigma-Aldrich, Germany), and xylanase, from *Trichoderma viride* (Sigma-Aldrich, Germany), were determined by prior experimentation. The concentrations of the enzymes used were 0.6 mg ml⁻¹ for xylanase and 0.8 mg ml⁻¹ for pectinase. Controls were run in parallel with the sample. The controls included: (1) enzyme only without gold colloid; (2) gold colloid only without the enzyme; and (3) incubation

with the enzyme only first, for the same time as the enzyme-gold complex and then incubation of the same sections with the enzyme-gold complex.

After these incubations, the sections were washed (3×5 min) in a drop of PBS, rinsed (3×10 min) with sterile distilled water, counterstained with lead citrate for 30 min, and viewed with a JEOL JEM-1010 transmission electron microscope (TEM) at 100 kV.

2.3.6.2. Immunocytochemistry of microtubules

Ultrathin sections of the earliest gel-phase megagametophyte (c. 1 week after the first sighting of the strobilus) were cut using glass knives and collected on 200-mesh nickel grids. For each treatment about five sections were examined. The sections were first transferred to a drop of solution 1 (PBS [as prepared in 2.3.6.1.] containing 0.2% [w/v] polyethylene glycol 20 000 [PEG 20 000[®]]) for 5 min at room temperature. The sections were then transferred to a drop of solution 2 (PBS containing 10% [v/v] foetal bovine serum, 1% [w/v] bovine serum albumin [BSA], 0.05% [v/v] Tween-20[®] and 0.2% [w/v] sodium azide), before an overnight incubation in a drop (40 μ l) of anti-acetylated-tubulin mouse ascites fluid (Sigma-Aldrich, Germany) diluted 1:200 in solution 3 (PBS containing 1% [w/v] BSA) for 1 h in a moist chamber at 4°C (the control treatment remaining in solution 3).

The next day, the sections were washed (3×5 min) in a drop of solution 4 (PBS containing 1% [w/v] BSA and 0.05% Tween-20[®]), transferred to a drop (40 μ l) of secondary antibody (goat anti-mouse IgG conjugated with 10 nm gold colloid [Sigma-Aldrich, Germany] diluted 1:10 with solution 5 [950 μ l solution 4 + 50 μ l FBS]) for 1 h at room temperature. The sections were then rinsed (3×5 min) in PBS, fixed in 1% glutaraldehyde for 2 min, and rinsed with double-distilled water (3×10 min). Following this, the sections were counterstained with lead citrate for 30 min and viewed with a JEOL JEM-1010 transmission electron microscope at 100 kV.

2.3.7. Histochemistry of protein, carbohydrates and lipid

Both wax- and resin-embedded sections were used for the histochemical localisation of protein, carbohydrates and lipid. While the resin-embedded samples were processed as outlined above, wax embedded samples were processed as follows:

A central cube (c. 125 mm³) was cut out of the micropylar half of the megagametophyte using a blunt scalpel. These tissue segments were then incubated in a mixture of microtubule-stabilising buffer (MTSB) and dimethyl sulphoxide (DMSO) at room temperature for 15 min. This was followed by fixation with 4% (w/v) paraformaldehyde in MTSB-DMSO mixture for 1 h at room temperature. The tissue segments were then rinsed briefly in MTSB before dehydration.

Dehydration was carried out in a graded ethanol series (30, 50, and 75% [v/v]) diluted in phosphate buffered saline (PBS) for 15 min each, 60 min in 100% ethanol and a further 24 h in fresh 100% ethanol. The tissue segments were then infiltrated in a 37°C oven with mixtures of absolute ethanol plus Steedman's wax (a 9:1 [w/w] mixture of polyethylene glycol [PEG] 400 distearate and 1-hexadecanol [Steedman, 1960]) made up in the proportions of 2:1 (v/v) overnight; 1:1 and 1:2 (v/v), 4 h for each step; followed by two changes of pure wax (30 min each) under vacuum. The tissue segments were then placed individually into fresh wax at 37°C in plastic moulds and infiltrated overnight under vacuum (in a vacuum desiccator) in order to remove the final traces of ethanol from the tissues. Finally the specimens were re-orientated and allowed to polymerise at room temperature.

Sections, 20 µm thick were cut using an American Optical 8209 rotary microtome. The sections were then carefully placed onto Haupt's adhesive-coated slides using a paintbrush before being subjected to histochemical staining.

2.3.7.1. Protein histochemistry

Protein histochemistry of wax-embedded sections

Protein was identified by staining with eosin dye (BDH Chemicals, England) after James and Tas (1984). Sections were dewaxed by placing the slides on a hot tray until the wax was visibly melted, before rinsing briefly (c. 20 sec) in xylene. The sections were then brought to water *via* an ethanol series (100, 75, 50, and 25% [v/v], 3 min in each) and stained with 1 g l⁻¹ eosin dye

for 5 min at room temperature. The slides were then rinsed in distilled water and differentiated (briefly) in 50% (v/v) ethanol, after which dehydration was then completed with the ethanol series, and the sections cleared in xylene, mounted in Permount[®] and viewed with a Nikon eclipse 80i light microscope equipped with NIS Elements F Package imaging software. Protein was identified by means of its staining bright pink-red. A control was set up where 0.1 g l⁻¹ protease from *Streptomyces griseus* Type XIV (Sigma-Aldrich, Germany) prepared in phosphate buffer pH 7.2 was applied to the sections for 2 min at room temperature before the application of eosin.

Protein histochemistry of resin-embedded sections

Before the application of eosin (1 g l⁻¹, as above), 1 µm thick, resin-embedded sections were etched using a 6.675% potassium methoxide (in a 2:1 mixture of methanol:propylene oxide [Ferey, 1986]) for 30 sec, rinsed briefly (10 sec) in methanol and allowed to air-dry. The air-dried slides were then placed on a hot tray at about 60°C, where eosin (20 drops per slide) was applied for approximately 5 min. The stained sections were then rinsed briefly in running tap water, air-dried, mounted in Permount[®] and viewed with a Nikon eclipse 80i light microscope using NIS Elements F Package imaging software.

2.3.7.2. Carbohydrate histochemistry

Carbohydrate histochemistry of wax-embedded sections

Sections, 20 µm thick, were brought to water using the procedures described above, oxidised for 10 min in a 1% (w/v) solution of periodic acid (Hotchkiss, 1948), and washed in running tap water for 5 min. The section-bearing slides were then immersed in a basic fuchsin (Sigma-Aldrich, Germany) substitute for Schiff's reagent (Horobin and Kevill-Davies, 1971) in the dark for 20 min at room temperature, washed in running tap water for 5 min, dehydrated in ethanol as described above, mounted in Permount[®] and viewed with a Nikon eclipse 80i light microscope equipped with NIS Elements F Package imaging software. Carbohydrates were identified by their staining maroon.

Carbohydrate histochemistry of resin-embedded sections

Sections, 1 μm thick, were used. The sections were first etched as described previously for resin-embedded sections, oxidised for 10 min in a 1% (w/v) solution of periodic acid (Hotchkiss, 1948), washed in running tap water for 5 min, immersed in the basic fuchsin substitute for Schiff's reagent (Horobin and Kevill-Davies, 1971) in the dark for 20 min at room temperature, washed in running tap water for 5 min, mounted in Permount[®] and viewed with a Nikon eclipse 80i light microscope equipped with NIS Elements F Package imaging software. Carbohydrates of resin-embedded sections were identified by their staining light pink.

2.3.7.3. Lipid histochemistry

Lipid histochemistry of wax-embedded sections

The method used in this study to visualise lipid histochemically was adapted from the method of McManus (1946) for the staining of lipids in paraffin sections using Sudan Black B (Sigma-Aldrich, Germany). Sections, 20 μm thick, were dewaxed by placing the slides on a hot tray until the wax was visibly melted, before rinsing briefly (c. 20 sec) in xylene. The sections were then brought to 70% (v/v) ethanol by immersion in 100% and then 70% (v/v) ethanol for 3 min each. The sections were stained subsequently for 30 min at room temperature in saturated Sudan Black B in 70% ethanol. The sections were then rinsed in 70% ethanol until the excess dye was removed, washed briefly in running tap water, mounted in a drop of water and viewed within 24 h with a Nikon eclipse 80i light microscope equipped with NIS Elements F Package imaging software. Lipid was identified by means of its staining black. A control was set up where sections were delipidised by immersion in chloroform:methanol (2:1) for an hour at room temperature before the application of Sudan Black B.

Lipid histochemistry of resin-embedded sections

Lipid in resin-embedded sections was localised histochemically using the procedures outlined by Bronner (1975). Sections, 1 μm thick, were used without being etched as described previously for the protein and carbohydrate histochemistry of resin-embedded sections in this study. The slides with sections were placed in 70% (v/v) ethanol for 1 min, stained in a freshly filtered saturated solution of Sudan Black B at 60°C in an oven for 1 h, rinsed in 70% (v/v)

ethanol for 1 min, and washed briefly in running tap water. The sections were then mounted in Permount[®] and viewed with a Nikon eclipse 80i light microscope equipped with NIS Elements F Package imaging software. Lipid was identified on account of it staining dark brown-black.

3. RESULTS

3.1. Some aspects of the post-shedding seed behaviour of *Encephalartos natalensis*

3.1.1. The change in embryo and megagametophyte water content in open-stored seeds

Water contents (WCs) in this chapter are expressed as mean \pm standard error (SE), on a dry mass basis – i.e. g H₂O g⁻¹ dry mass (g g⁻¹). Where the standard error does not accompany the mean value of some embryos, it is because only one embryo was found per 10 seeds in batch A, at each sampling interval, except 2 months after shedding (MAS). Many unfertilised, embryo-less ovules were shed alongside seeds in batch A, and there was no way of telling them apart by superficial inspection. Thus, a sample of 10 ‘seeds’ in batch A did not yield a sample of 10 embryos. Batch B seeds on the other hand, came from strobili that were rigorously hand-pollinated, giving seed yields in excess of about 80% (results not shown).

The WC of *E. natalensis* batch A seeds was assessed from seed-shed to 6 MAS (Fig. 3.1). Seeds were shed with relatively high embryo (3.33 g g⁻¹) and megagametophyte (1.25 \pm 0.16 g g⁻¹) WCs (Fig. 3.1). The WC of the embryo declined markedly ($P < 0.05$) during the first 2 months after seed-shed (from 3.33 to 1.42 \pm 0.07 g g⁻¹), with a more gradual decline during the period from 2 to 6 MAS (from 1.42 \pm 0.07 to 1.13 g g⁻¹). Even though the embryo WC declined in the months from seed-shed, it was consistently higher ($P < 0.05$) than that of the megagametophyte (Fig. 3.1).

The change in WC of the megagametophyte from seed-shed to 6 MAS did not mirror that of the embryo (Fig. 3.1). The decline in megagametophyte WC from seed-shed to 2 MAS was gradual (from 1.25 \pm 0.16 to 0.74 \pm 0.10 g g⁻¹) as opposed to the marked decline in embryo WC. The WC of the megagametophyte apparently increased from 2 to 4 MAS; and then decreased from 4

to 6 MAS (Fig. 3.1). However, the degree of uncertainty in the mean (as shown by the SE) at each sampling point suggested that the apparent fluctuation in megagametophyte WC (Fig. 3.1) might be due to a possible sampling error. There was however, an overall gradual decline ($P < 0.05$) in megagametophyte WC from seed-shed to 6 MAS (from 1.25 ± 0.16 to 0.80 ± 0.10 g g^{-1}).

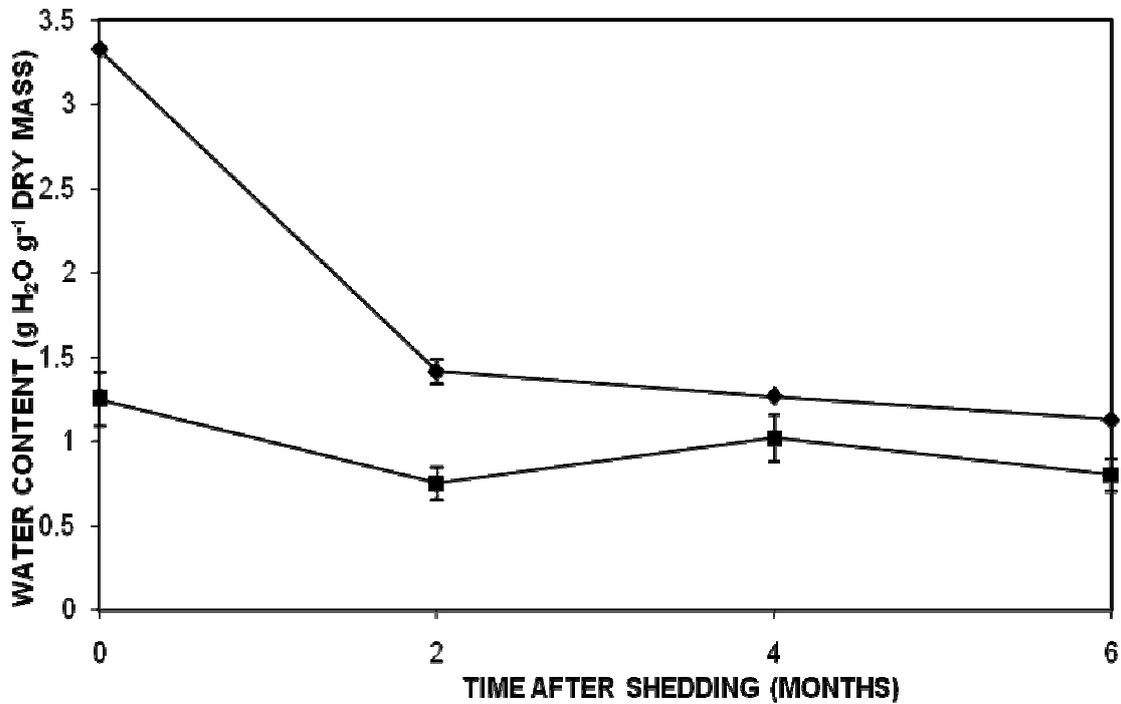


Figure 3.1: Embryo \blacklozenge and megagametophyte \blacksquare WCs of *E. natalensis* batch A seeds assessed at shedding (0) and following open storage for 2, 4 and 6 months at 16°C. $n = 10$; bars indicate standard error of the mean (SE).

The WC of *E. natalensis* batch B seeds was assessed from 4 to 18 MAS (Fig. 3.2). The embryo WC appeared to decline gradually from 4 to 18 MAS (from 2.33 ± 0.22 to 1.17 ± 0.10 g g⁻¹). There was a comparatively substantial degree of uncertainty in the mean of embryo WC at 4 MAS, comparatively little uncertainty from 6 to 12 MAS; however there again appeared to be a greater degree of uncertainty in the mean embryo WC from 14 to 18 MAS (Fig. 3.2). Despite the apparent gradual decline in embryo WC from 4 to 18 MAS, the WC of the embryo remained consistently higher than that of the megagametophyte (Fig. 3.2).

The megagametophyte WC of *E. natalensis* batch B seeds declined slowly (compared with the embryo WC) from 4 to 18 MAS (from 0.79 ± 0.10 to 0.50 ± 0.01 g g⁻¹ [Fig. 3.2]), although this decline was significant ($P < 0.05$). There was relatively little uncertainty in the mean of megagametophyte WCs from 4 to 18 MAS, which suggested that the mean values were reliable estimates of the actual population from which the sample was taken (Fig. 3.2).

3.1.2. Change in fresh and dry mass of the embryo and megagametophyte in open-stored seeds

The change in fresh and dry mass of the megagametophyte tissue of open-stored *E. natalensis* batch A seeds from seed-shed to 6 MAS is shown in Figure 3.3a. Seeds were shed when the megagametophyte had a fresh mass of 4.74 ± 0.30 g and a dry mass of 2.28 ± 0.31 g. The fresh mass of the megagametophyte remained essentially constant over the period from seed-shed to 6 MAS (from 4.74 ± 0.30 to 4.33 ± 0.28 g [$P > 0.05$]), while the uncertainty of the mean at each sampling interval suggested that the slight fluctuation between neighbouring points was due to possible sampling error (Fig. 3.3a). Similar fluctuation between neighbouring sampling points was seen in the dry mass of the megagametophyte (Fig. 3.3a). However, the dry mass of the megagametophyte did not seem to change significantly ($P > 0.05$) from seed-shed to 6 MAS.

The change in fresh and dry mass of embryos of *E. natalensis* batch A seeds, from seed-shed to 6 MAS, is shown in Figure 3.3b. There was an overall, significant increase ($P < 0.05$) in both the fresh and dry mass of the embryo from seed-shed to 6 MAS. The increase in the dry mass of the embryo (80-fold) from seed-shed to 6 MAS, proved to be greater than that of the fresh mass (39-fold). At seed-shed, the fresh mass of the embryo was 0.0036 g while the dry mass was 0.00083 g (Fig. 3.3b), the mass of the embryo being much less than that of the megagametophyte. The embryo fresh mass appeared to increase considerably from seed-shed to 2 MAS (from 0.0036 to 0.10 ± 0.0069 g), and notwithstanding an apparent decrease from

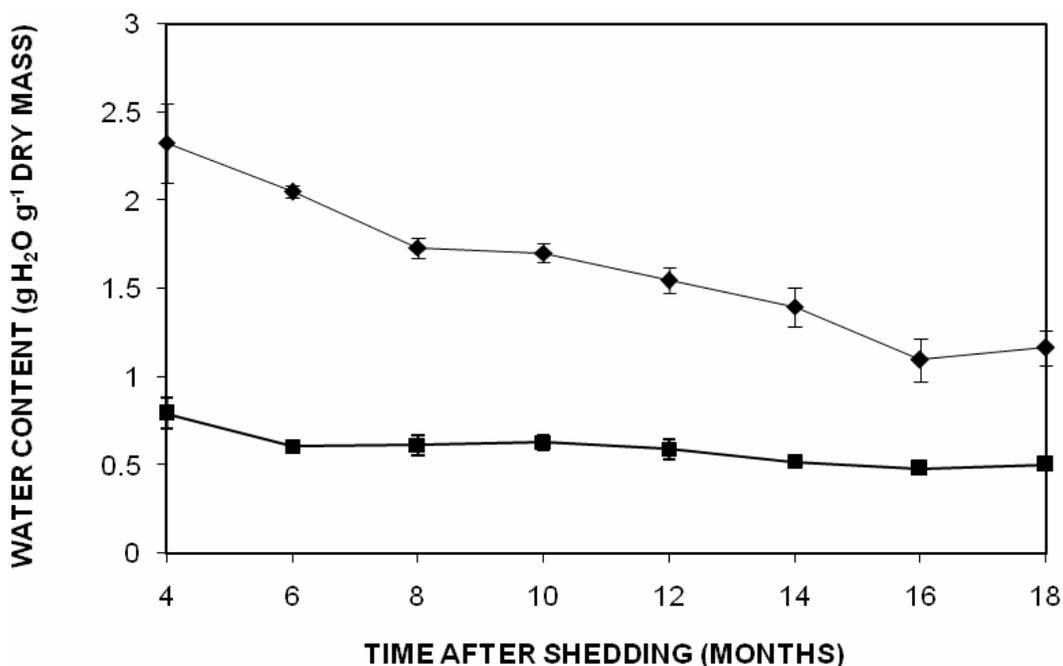


Figure 3.2: Embryo —◆— and megagametophyte —■— WCs of *E. natalensis* batch B seeds assessed from 4 MAS and following open storage for 6, 8, 10, 12, 14, 16 and 18 months at 16°C. $n = 20$; bars indicate SE.

2 to 4 MAS (from 0.10 ± 0.0069 to 0.064 g), increased considerably from 4 to 6 MAS (from 0.064 to 0.14 g). The single embryo found in the batch of 10 seeds sampled at 4 MAS was conspicuously under-developed compared with those at 2 and 6 MAS, which would account for the apparent, but considerable decrease in embryo fresh mass from 2 to 4 MAS (Fig. 3.3b).

The change in dry mass of the embryo showed a similar trend to that of the fresh mass (Fig. 3.3b). However, the differences in dry mass observed between neighbouring sampling points were comparatively less than that seen in fresh mass. The embryo dry mass appeared to increase considerably from seed-shed to 2 MAS (from 0.00083 to 0.041 ± 0.0030 g); decrease somewhat from 2 to 4 MAS (from 0.041 ± 0.0030 to 0.028 g); and then increase considerably from 4 to 6 MAS (from 0.028 to 0.066 g) [Fig. 3.3b]. Despite the apparent fluctuation in the dry mass of the embryo from seed-shed to 6 MAS, there was an overall increase in dry mass from 0.00083 to 0.066 g (Fig. 3.3b).

The change in the fresh and dry mass of the megagametophyte of *E. natalensis* batch B seeds, from 4 to 18 MAS, is shown in Figure 3.4a. At 4 MAS, the fresh mass of the megagametophyte was 3.49 ± 0.11 g; while the dry mass was 2.00 ± 0.12 g. The fresh mass of the megagametophyte appeared to decline gradually over the period from 4 to 18 MAS (from 3.49 ± 0.11 to 2.80 ± 0.08 g [$P < 0.05$]), with a small and insignificant ($P > 0.05$) decline in the dry mass of the megagametophyte over the same period (from 2.00 ± 0.12 to 1.86 ± 0.049 g) [Fig. 3.4a].

Figure 3.4b depicts the change in fresh and dry mass of the embryo of *E. natalensis* batch B seeds, over the period from 4 to 18 MAS. At 4 MAS, the embryo had a fresh mass of 0.023 ± 0.016 g and a dry mass of 0.0065 ± 0.0045 g. Over the 14-month period from 4 to 18 MAS, the fresh mass of the embryo increased significantly ($P < 0.05$) by a factor of 5.7, from 0.023 ± 0.016 to 0.13 ± 0.0093 g, while the dry mass of the embryo increased almost 10-fold, from 0.0065 ± 0.0045 to 0.062 ± 0.0069 g ($P < 0.05$). From Figure 3.4b, there was an apparently greater uncertainty in the mean (based on the size of the SE bars) at each sampling point of the embryo fresh mass relative to the dry mass; however, when SE was calculated as a percentage of the mean, the uncertainty of the mean of fresh and dry mass was similar (results not shown).

When the results shown in Figs. 3.3a,b and Figs. 3.4a,b are compared with the WC data (Figs. 3.1 and 3.2), it is evident that the decrease [$P < 0.05$] in WC of the embryo in the months following seed-shed was not a consequence of decreasing embryo FM (i.e. water loss). This was evidenced by the overall increase in embryo FM [$P < 0.05$] in the months after seed-shed for both

seed batches (Figs. 3.3b and 3.4b) [$P < 0.05$]. The embryo WC of seed batches A and B declined in the months after seed-shed, probably due to the increase in DM [$P < 0.05$] during the post seed-shed growth of the embryo. The increase in embryo fresh and dry mass would most likely come at the expense of the megagametophyte tissue; however, the DM of the megagametophyte did not change significantly [$P > 0.05$] during the storage periods of either seed batch (Figs. 3.3a and 3.4a). This is probably due to the marked size difference of the embryo compared with the megagametophyte tissue, such that a significant change occurring in the embryo would not necessarily mean a similar significant change in the much larger megagametophyte tissue. Similarly, the observed significant decrease [$P < 0.05$] in the megagametophyte WC in the months after seed-shed is most probably due to a loss of water to the surrounding environment, and, to a lesser extent, to the growing embryo.

Overall, when batches A and B are compared, the WC, FM and DM changes are similar. However, the main differences lie in the WC, FM and DM at collection and the initial steep decline in WC of the embryo of seed batch A (Fig. 3.1). These main distinctions would largely be due to the difference in developmental stage at collection (0 and 4 MAS, respectively), as well as differences in the initial storage environments of the seed batches. Seed batch A was taken to the lab shortly after seed-shed and placed in open storage at 16°C, while seed batch B was obtained only 4 MAS - before which the seeds had been left outside with an ambient mean diurnal (summer) temperature of approximately 20 to 25°C.

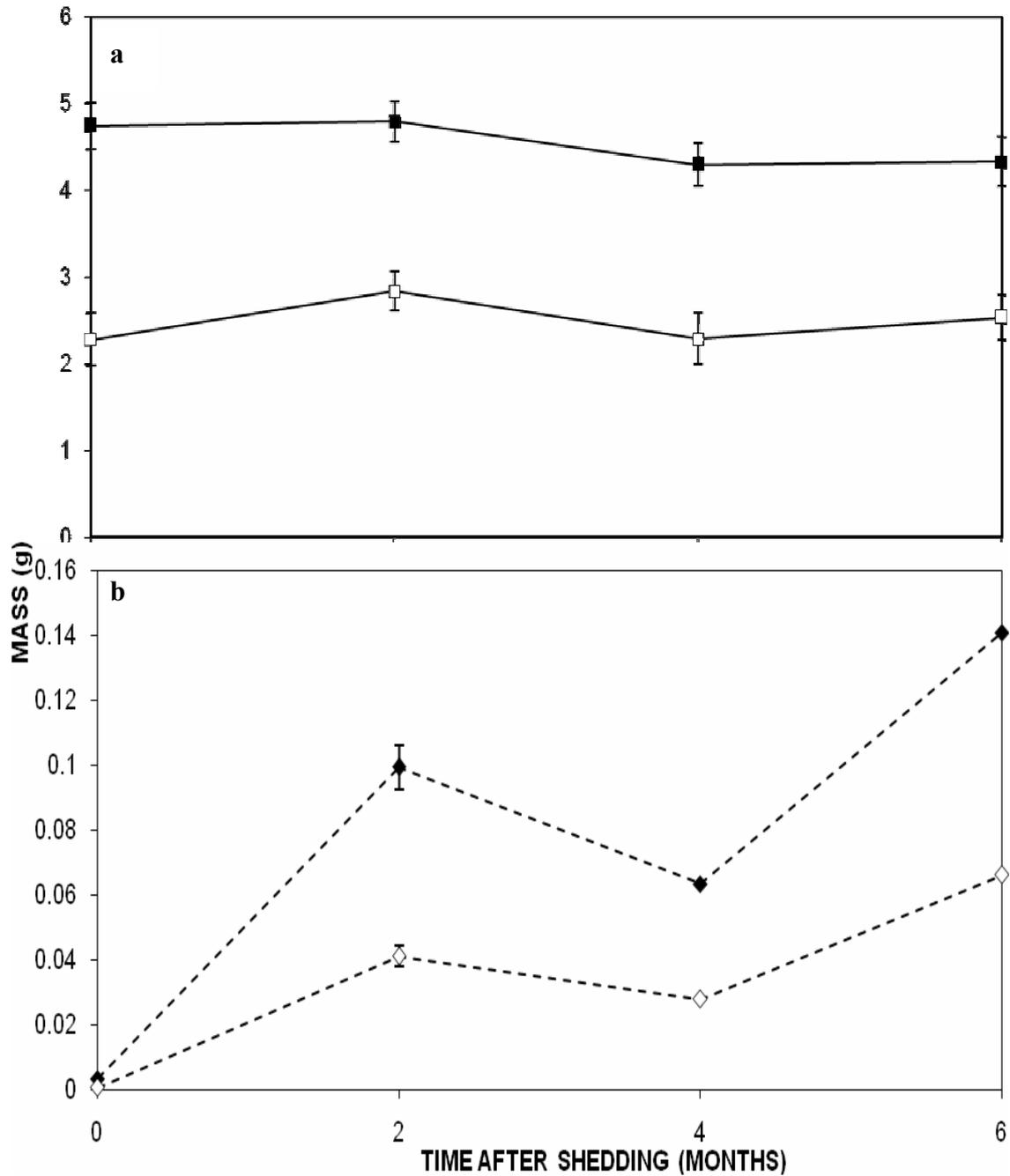


Figure 3.3: a) Fresh mass (FM) —■— and dry mass (DM) —□— of the megagametophyte of *E. natalensis* batch A seeds at seed-shed, and following open storage for 2, 4 and 6 months at 16°C; b) FM - - ♦ - - and DM - - ◇ - - of the embryo of *E. natalensis* batch A seeds at seed-shed and following open storage for 2, 4, and 6 months at 16°C; n = 10; bars indicate SE.

3.1.3 Macroscopic observations on embryo development in the months after seed-shed

The seeds of *E. natalensis* were found to be shed with an incompletely developed embryo that consisted primarily of the coiled, elongated suspensor bearing a rudimentary sporophyte at its tip (Fig. 3.5a). The embryos continued to develop after seed-shed (Figs. 3.5b–d), reaching a germinable size (≥ 15 mm) only 4 to 6 MAS (Figs. 3.5c–d). The suspensor became less prominent with embryo development, being compressed at the micropylar end of the seed from 4 MAS (Fig. 3.5c,d), while the coleorhiza tissue of the embryo developed a red-brown pigmentation at about 4 MAS (as shown in Fig. 3.5c).

3.1.4. Vigour and viability of seeds at various times during open storage

Since the vigour and viability of 4-month stored seeds was very similar for both batches (A and B), the data were combined to give a single graph (Fig. 3.6). At shedding, no germination was observed when the seeds (i.e. fertilised ovules) of *E. natalensis* batch A were set out to germinate (Fig. 3.6). Both germination rate and totality increased with increasing duration in open storage, from 2 to 6 MAS. At 2 MAS, c. 50% of the seeds were capable of germinating within 30 d after removal of the sclerotesta. Seeds stored for 2 months before being set out to germinate also took relatively long to initiate germination (c. 24 d), whereas seeds stored for longer periods initiated germination within 6 to 12 d (Fig. 3.6). Four-month stored seeds initiated germination earlier and displayed a more rapid germination rate and improved totality (c. 90%). All seeds (i.e. 100%) stored for 6 months germinated rapidly, while seeds stored for 8 to 16 months showed a slight decline in viability. Germination totality (viability) of 8 to 16 month stored seeds was found to be in the range of 60 to 80%, while that of 18 month stored seeds was reduced to 25% (Fig. 3.6).

3.1.5. The change in embryo and megagametophyte WC of seeds placed in hydrated storage 6 months after seed-shed

Figure 3.7 compares the WC of embryos and megagametophytes of *E. natalensis* batch B seeds that were placed in hydrated storage (after 6 months in open storage) with seeds of the same batch that were left in open storage. The embryo WC was found to decline from 6 to 8 MAS in both open- and hydrated-stored seeds. The decline in embryo WC from 6 to 8 MAS in seeds stored under hydrated conditions (from 2.05 ± 0.038 to 1.75 ± 0.14 g g⁻¹) was essentially similar ($P > 0.05$) to that of seeds kept in open-storage (from 2.05 ± 0.038 to 1.73 ± 0.057 g g⁻¹)

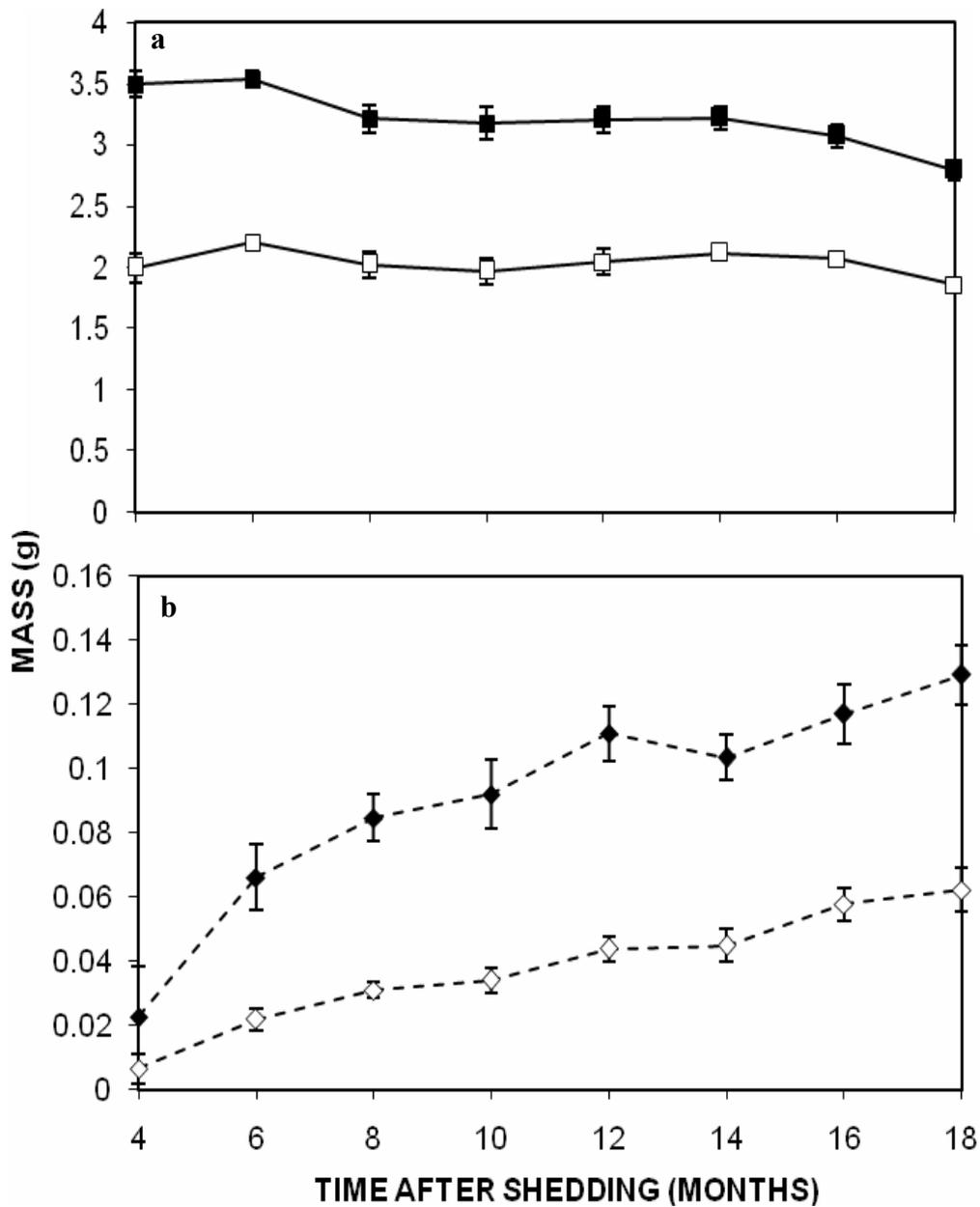


Figure 3.4: a) Fresh mass (FM) —■— and dry mass (DM) —□— of the megagametophyte of *E. natalensis* batch B seeds following open storage for 4, 6, 8, 10, 12, 14, 16 and 18 months at 16°C; b) FM - - ♦ - - and DM - - ◇ - - of the embryo of *E. natalensis* batch B seeds following open storage for 4, 6, 8, 10, 12, 14, 16 and 18 months at 16°C; n = 20; bars indicate SE.

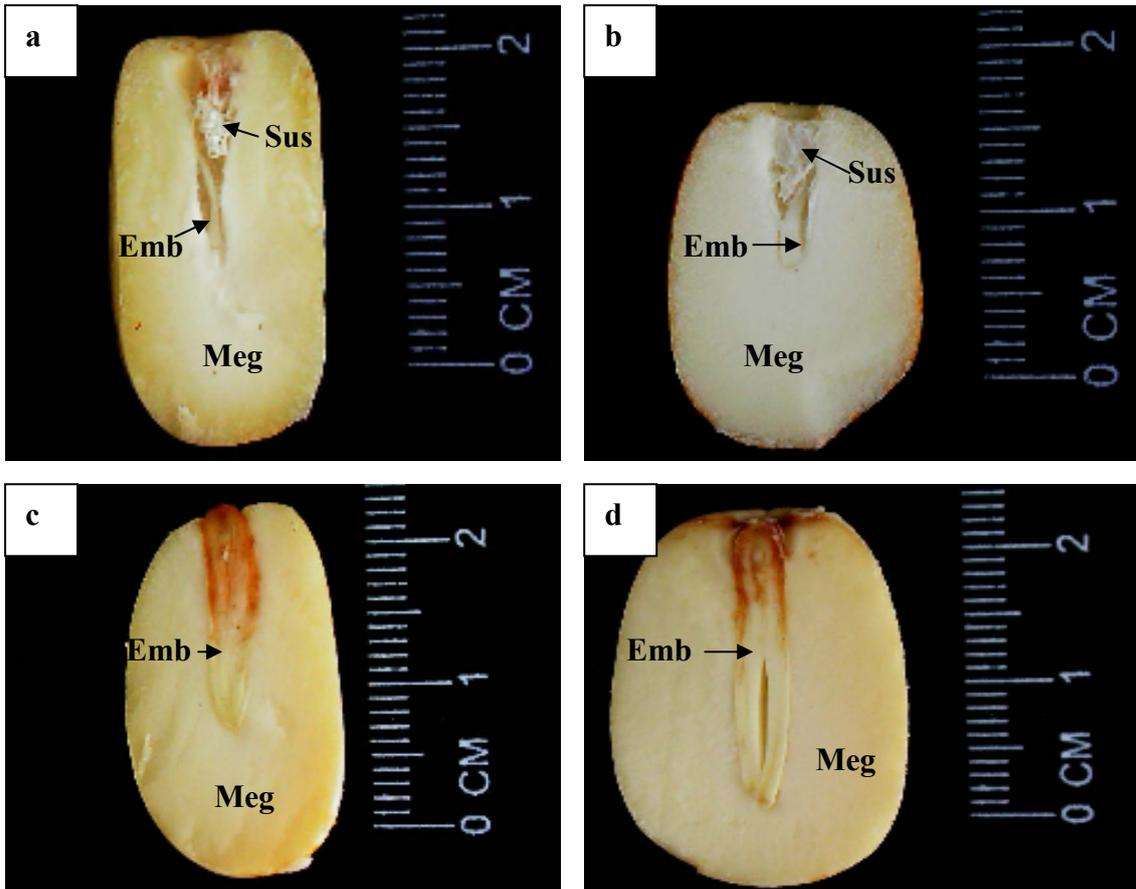


Figure 3.5: Longitudinally bisected *E. natalensis* seeds at various times after seed-shed: a) at shedding, showing tightly coiled suspensor; b) at 2 MAS; c) at 4 MAS; and d) at 6 MAS, showing embryo of germinable size. Meg = megagametophyte; Emb = embryo; Sus = suspensor.

(Fig. 3.7). There was however, much greater uncertainty in the mean embryo WC at the 8th month sampling interval of hydrated-stored seeds compared with open-stored seeds (Fig. 3.7). While the embryo WC of open-stored seeds continued to decline from 8 to 10 MAS (from 1.73 ± 0.057 to $1.70 \pm 0.053 \text{ g g}^{-1}$), that of seeds placed in hydrated storage apparently increased (from 1.75 ± 0.14 to $1.85 \pm 0.11 \text{ g g}^{-1}$). However, the increase in embryo WC of seeds placed in hydrated storage proved to be insignificant ($P > 0.05$) when compared with that of open-stored seeds (Fig. 3.7).

While the WC of the megagametophyte remained similar ($P > 0.05$) in open storage from 6 to 8 MAS (0.60 ± 0.005 and $0.61 \pm 0.057 \text{ g g}^{-1}$ respectively), that of seeds placed in hydrated storage increased (from 0.60 ± 0.005 to $0.71 \pm 0.0052 \text{ g g}^{-1}$ [$P < 0.05$]). The megagametophyte WC of seeds 10 MAS in hydrated storage was also higher than that of open-stored seeds; however, it was not significantly different ($P > 0.05$) from that of open-stored seeds (Fig. 3.7).

3.1.6. Change in fresh and dry mass of the embryo and megagametophyte of seeds placed in hydrated storage 6 months after seed-shed

Figure 3.8a shows the megagametophyte fresh and dry mass of *E. natalensis* batch B seeds placed in hydrated storage after 6 months of open-storage, relative to those seeds of the same batch that were left in open storage. While there was a gradual decrease in both fresh and dry mass of the megagametophyte when seeds were left in open storage, there was a significant increase ($P < 0.05$) in both the fresh and dry mass of the megagametophyte 10 MAS of seeds placed in hydrated storage (Fig. 3.8a).

The fresh mass of the megagametophyte of seeds placed in hydrated storage increased from $3.54 \pm 0.069 \text{ g}$ at 6 MAS to $4.84 \pm 0.11 \text{ g}$ at 8 MAS and $4.94 \pm 0.15 \text{ g}$ at 10 MAS. In comparison, the fresh mass of the megagametophyte of open-stored seeds decreased from $3.54 \pm 0.069 \text{ g}$ at 6 MAS to $3.21 \pm 0.11 \text{ g}$ at 8 MAS and $3.18 \pm 0.13 \text{ g}$ at 10 MAS (Fig. 3.8a). The SE bars showed that there was little uncertainty in the mean fresh mass of the megagametophyte at sampling points 6, 8 and 10 months for both open and hydrated storage (Fig. 3.8a), which indicates that the mean of the sample is a reliable estimate of the mean of the actual population from which the sample was taken.

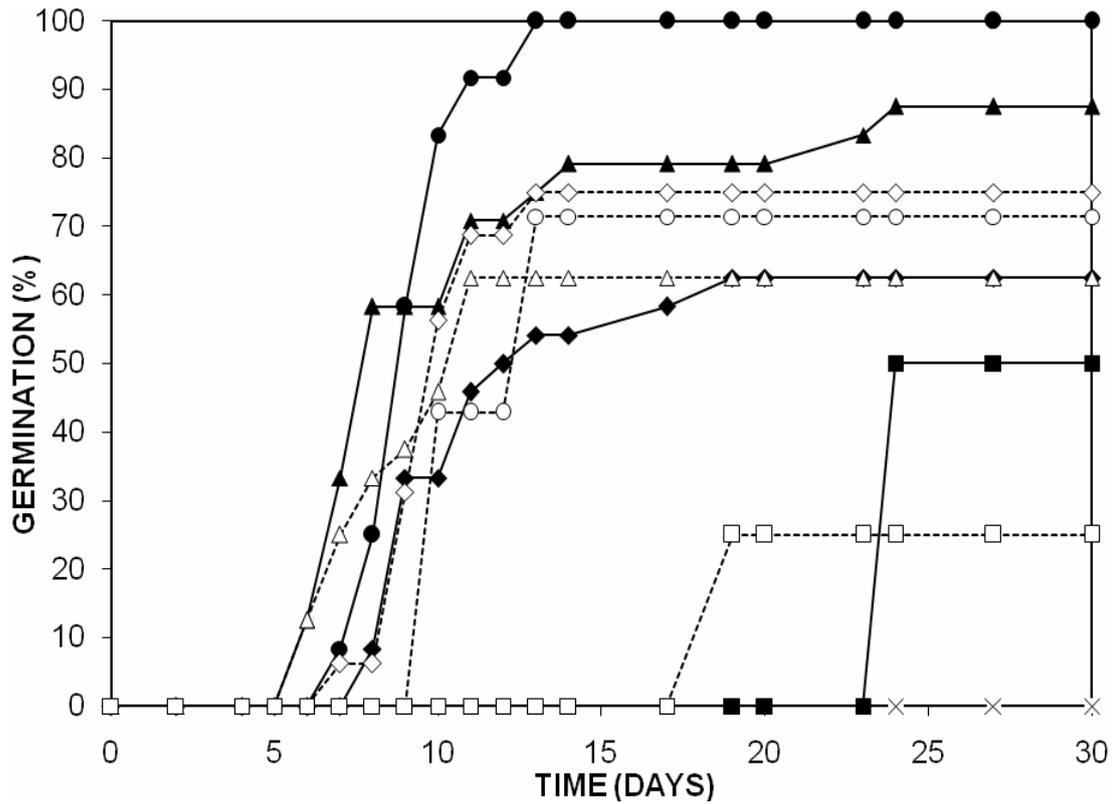


Figure 3.6: Germination of *E. natalensis* seeds assessed at shedding (0 months) and following storage for 2, 4, 6, 8, 10, 12, 14, 16 and 18 months. —x— 0 months; —■— 2 months; —▲— 4 months; —●— 6 months; —◆— 8 months; -◇- 10 months; ---x--- 12 months (not shown); -△- 14 months; -○- 16 months; and -□- 18 months; n = 20.

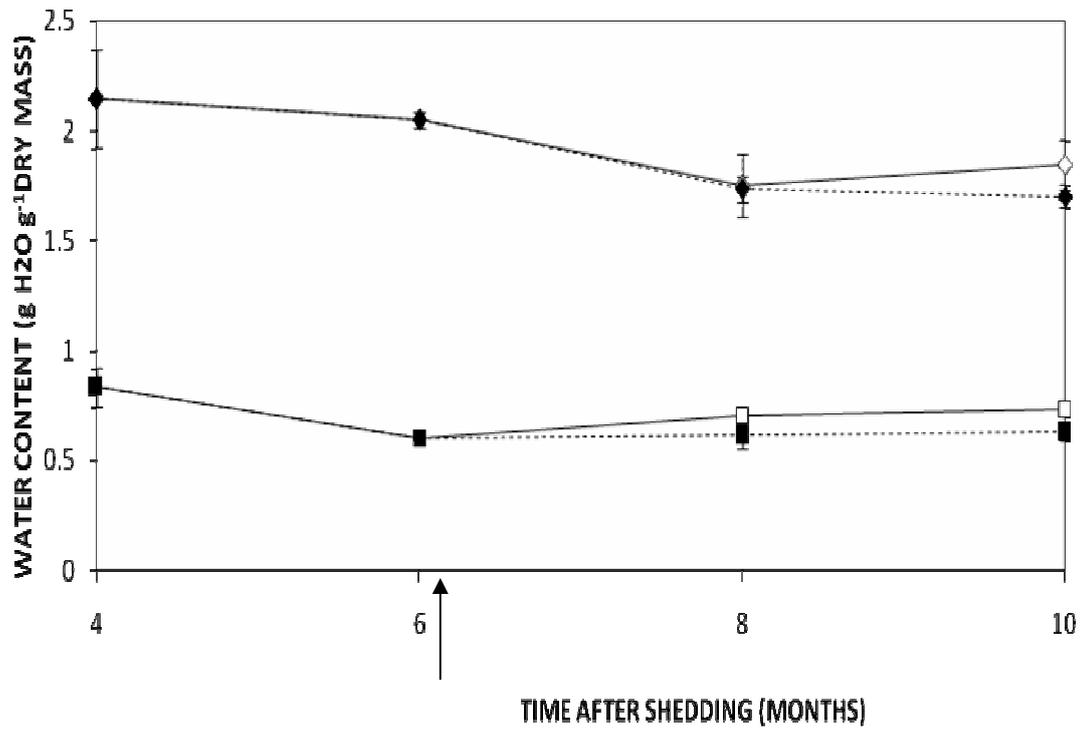


Figure 3.7: Embryo \diamond and megagametophyte \square WC of *E. natalensis* batch B seeds placed in hydrated storage after open storage for 6 months (timing indicated by arrow), relative to embryo \blacklozenge and megagametophyte \blacksquare WC of seeds kept in open storage. $n = 10$; bars indicate SE.

Similarly, while the megagametophyte dry mass of open-stored seeds appeared to decrease from 4 to 10 MAS, the dry mass of the megagametophyte of seeds placed in hydrated storage increased from 2.20 ± 0.041 g at 6 MAS to 2.84 ± 0.064 g at 8 MAS, with a further slight increase to 2.85 ± 0.081 g at 10 MAS (Fig. 3.8a). The SE bars showed that there was little uncertainty in the mean dry mass of the megagametophyte at sampling points 6, 8 and 10 months for both open and hydrated storage, which indicates that the mean of the sample is a reliable estimate of the mean of the actual population from which the sample was taken (Fig. 3.8a).

Figure 3.8b shows the embryo fresh and dry mass of *E. natalensis* batch B seeds that were placed in hydrated storage 6 MAS, relative to those seeds of the same batch that were left in open storage. While there was a gradual increase in both fresh and dry mass of the embryo of seeds left in open storage during the period from 4 to 10 MAS, there was a non-significant increase ($P > 0.05$) in both these parameters of the embryo of seeds placed in hydrated storage 6 MAS (Fig. 3.8b).

The fresh mass of the embryo of seeds placed in hydrated storage increased from 0.07 ± 0.010 g at 6 MAS to 0.11 ± 0.015 g at 8 MAS and 0.15 ± 0.03 g at 10 MAS. In comparison, the fresh mass of the embryo of open-stored seeds increased only slightly from 0.07 ± 0.01 g at 6 MAS to 0.08 ± 0.0073 g at 8 MAS, with a further slight increase to 0.09 ± 0.011 g at 10 MAS (Fig. 3.8b). The SE bars showed that there was some uncertainty in the mean fresh mass of the embryo at sampling points 6, 8 and 10 months for seeds placed in hydrated storage 6 months after seed-shed (Fig. 3.8b), which is possibly due to sampling error.

The dry mass of the embryo of seeds placed in hydrated storage also appeared to increase compared with that of the open-stored seeds (Fig. 3.8b). While the embryo dry mass of open-stored seeds appeared to increase only slightly from 6 to 10 MAS, there was a more marked increase in the dry mass of the embryo of seeds placed in hydrated storage 6 MAS. The dry mass of the embryo of seeds placed in hydrated storage appeared to increase from 0.02 ± 0.0034 g at 6 MAS to 0.04 ± 0.0063 g at 8 MAS, with a further increase to 0.05 ± 0.011 g at 10 MAS (Fig. 3.8b). There was also some uncertainty in the mean dry mass of the embryo at sampling points 6, 8 and 10 months for seeds kept in hydrated storage (Fig. 3.8b), which is due to possible sampling error.

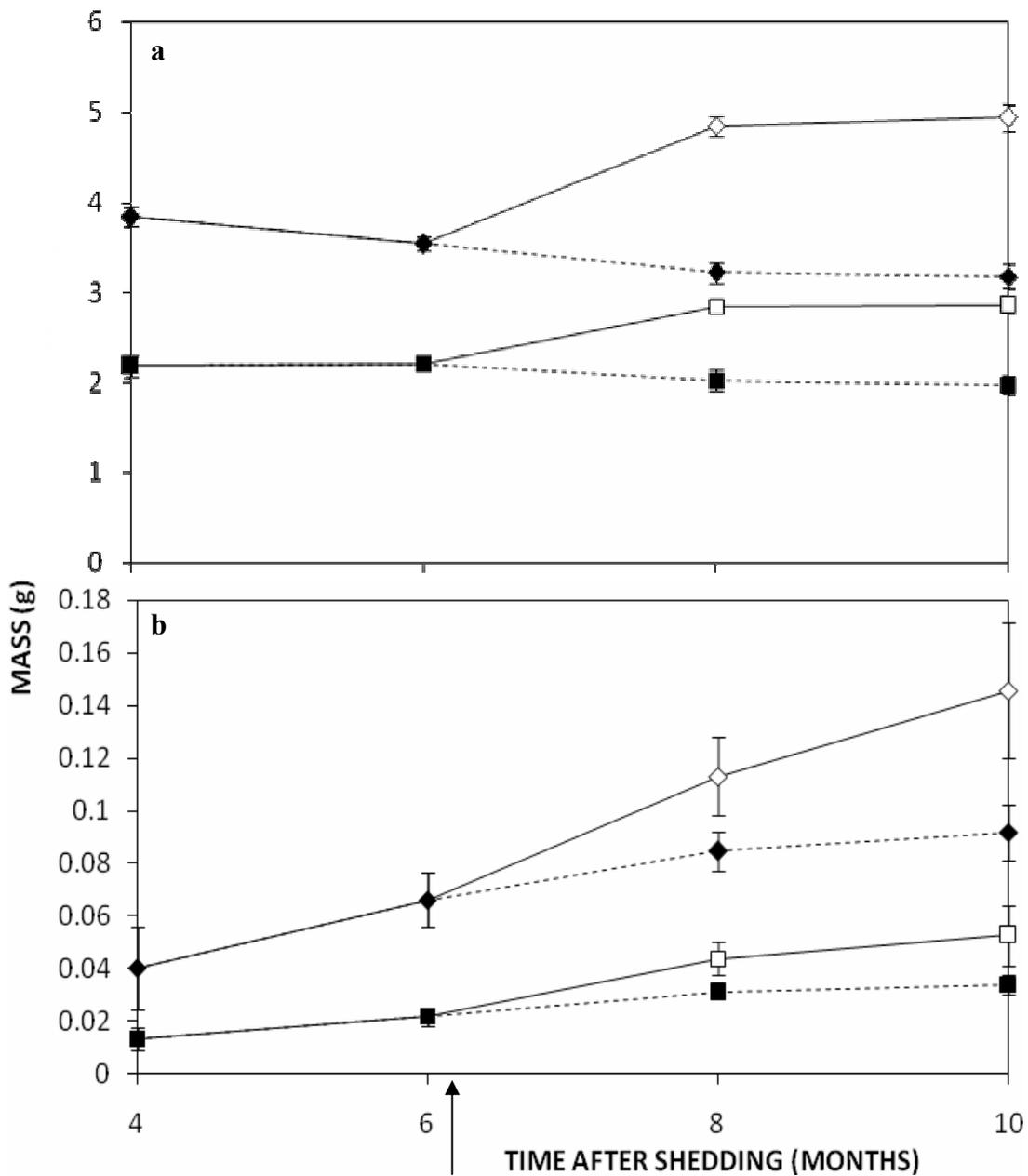


Figure 3.8: a) Fresh mass (FM) —◇— and dry mass (DM) —□— of the megagametophyte of *E. natalensis* batch B seeds placed in hydrated storage after open storage for 6 months (timing indicated by arrow), relative to the FM - - ◆ - - and DM - - ■ - - of the megagametophyte of seeds left in open storage; b) FM —◇— and DM —□— of the embryo of *E. natalensis* batch B seeds placed in hydrated storage after open storage for 6 months, relative to the FM - - ◆ - - and DM - - ■ - - of the embryo of seeds left in open storage. $n = 10$; bars indicate SE.

None of the seeds placed in hydrated storage germinated when set out on water agar after sclerotesta removal, probably as the outcome of fungal contamination (Fig. 3.9a). This occurred despite the surface application of the fungicide, Benlate[®]. Nevertheless, occasional seeds had germinated during the hydrated storage period (Fig. 3.9b) despite the physical barrier imposed by the enclosing sclerotesta. Seeds dusted with Benlate[®] and placed into open storage in loosely closed paper bags had a longer life-span than those placed in hydrated storage, which is attributed to curtailed fungal proliferation under lower relative humidity conditions. However, seeds stored in open storage were ultimately overcome by fungi, but only around 18 MAS. Unfortunately in the present study, no seeds were placed in hydrated storage in disinfected containers immediately upon shedding, and following rigorous decontamination. Hence, the potential of responses and viability retention of the seeds under the strictly controlled hydrated storage conditions generally used for recalcitrant seeds (Berjak and Pammenter, 2004) is not known.

3.2. Some aspects of *E. natalensis* megagametophyte development

The micrographs presented in this section were chosen (after multiple biological and technical repeats) as the best representatives of the present investigation.

3.2.1. Structure and ultrastructure of the earliest stage in megagametophyte development observed in this study

The earliest developmental stage of the megagametophyte identified in this study was that of the coenocyte, which was found to be surrounded by nucellar tissue (Fig. 3.10a). The cells of the nucellus near the coenocyte stained more intensely with toluidine blue compared with the rest of the nucellus, while the cells of the nucellus immediately adjacent to the coenocytic megagametophyte appeared to be degenerated (Fig. 3.10a). The original membrane of the megaspore appeared relatively undulating, and stained dark blue-purple with toluidine blue (Fig. 3.10a,b), and appeared to be made up of honeycomb like chambers when viewed at the transmission electron microscope (TEM) level (Fig. 3.10c).

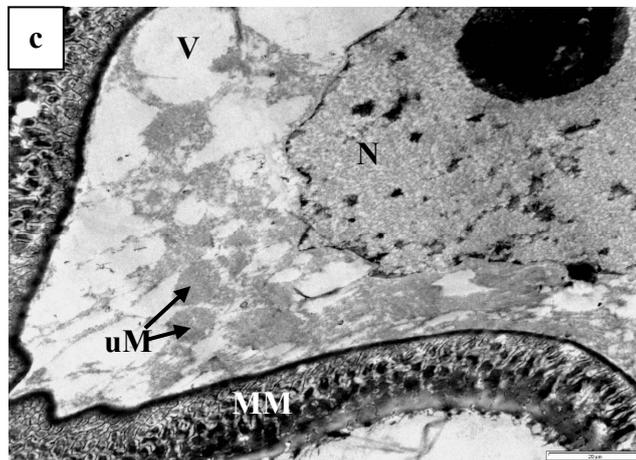
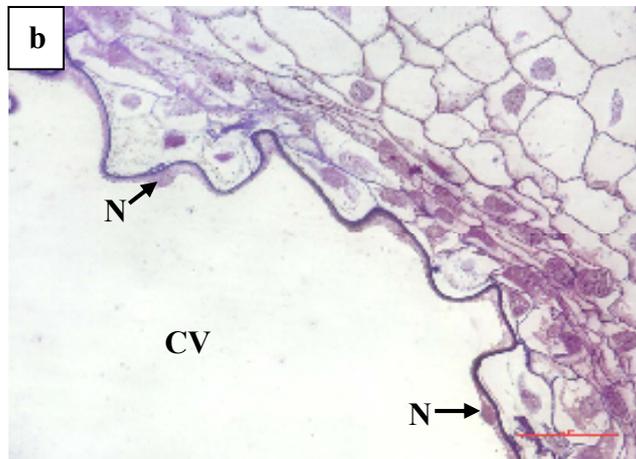
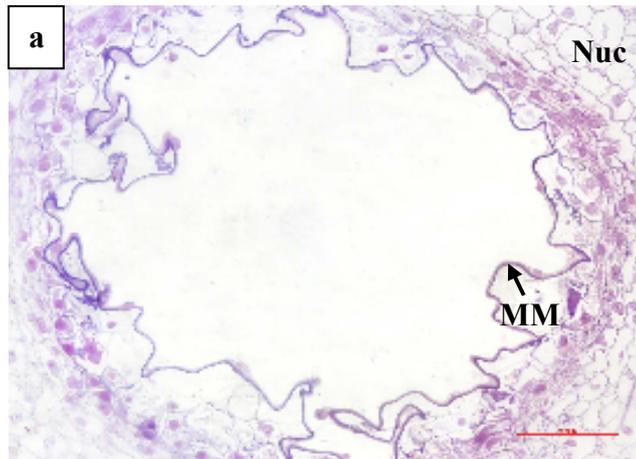
The coenocyte was characterised by a peripheral layer of cytomatrix material containing numerous free nuclei (Fig. 3.10b) and a prominent, large central vacuole (Fig. 3.10a,b). The peripheral layer of cytomatrix contained numerous small vesicles or vacuoles and what

appeared to be undifferentiated mitochondria, while Golgi bodies, endoplasmic reticulum (ER) and cell walls were not seen (Fig. 3.10c).



Figure 3.9: Problems associated with the storage of *E. natalensis* seeds under hydrated conditions: a) contamination of seeds by fungi; and (b) germination of seeds (as indicated by arrows) after 6 months in hydrated storage.

Figure 3.10: The earliest developmental stage of the megagametophyte (the coenocytic stage) is illustrated. a) A cross section through the coenocyte showing the megaspore membrane (MM) and surrounding nucellus (Nuc); section stained with toluidine blue, bar = 100 μm ; b) a cross section of the coenocyte showing free nuclei (N) in a peripheral layer of cytomatrical material, and a large central vacuole (CV); section stained with toluidine blue, bar = 50 μm ; and (c) a transmission electron micrograph of the peripheral cytomatrix showing a nucleus (N), numerous small vesicles or vacuoles (V) and what appear to be undifferentiated mitochondria (uM), while the megaspore membrane (MM) appeared to have numerous honeycomb-like chambers; sections stained with lead citrate and uranyl acetate, bar = 20 μm .



3.2.2. Structure and ultrastructure of the megagametophyte cells 1 week after the coenocyte was observed

It was observed in this study that it took the megagametophyte as little as 1 to 2 weeks to proceed from the coenocytic to the fully cellularised stage (Fig. 3.11a,b). In a cross section midway between the centre and one of either pole of the megagametophyte, the cells appeared parenchymatous, having a relatively high cell:nucleus ratio. However, cross sections through the central region of the megagametophyte revealed relatively small cells near the periphery, and extraordinarily long cells near the centre of the megagametophyte (Fig. 3.11c), in radiating ranks toward the periphery of the megagametophyte (Fig. 3.11c). Such cells at an equivalent stage of development in other gymnosperm species, have been described as being alveolus-like (Sokolowa, 1890). Few nuclei were seen in the central and alveolus-like cells at this stage of megagametophyte development compared with cells of the outer nucellar tissue, the cells comprising the interior of the megagametophyte appearing clear of internal content at the light microscope level (Fig. 3.11).

At the TEM level, it was found that the central and alveolus-like megagametophyte cells were not highly vacuolated as seen in the coenocytic stage, but dominated by an apparently homogeneous cytomatrix: Figure 3.12 shows that various organelles are embedded in this otherwise homogeneous cytomatrix. The thin cell walls that appeared fibrous were flanked by discontinuous membranes and relatively short profiles of ER (Fig. 3.12b) with numerous vesicles in close apposition (Fig. 3.12).

Two main types of vesicles were found: larger ER-derived vesicles some still showing associated polysomes, and fibrous contents; and small, smooth, Golgi-derived vesicles, with darker, homogeneous contents (Fig. 3.12). It appeared as though both the ER-derived vesicles and the small Golgi-derived vesicles were being incorporated into the developing cell membrane with a possible additional role in cell wall formation (Fig. 3.12c). However, there was a relative scarcity of Golgi bodies at this developmental stage.

Figure 3.11: Light microscopy images of the megagametophyte approximately 1 week after the coenocytic stage was observed. a) A cross section showing the megagametophyte as completely cellularised; section stained with toluidine blue, bar = 100 μm ; b) a cross section showing the parenchymateous nature of the cells; section stained with toluidine blue, bar = 50 μm ; and (c) a cross section through the middle of the megagametophyte showing alveolus-like cells (Al); section stained with PAS reagent, bar = 100 μm .

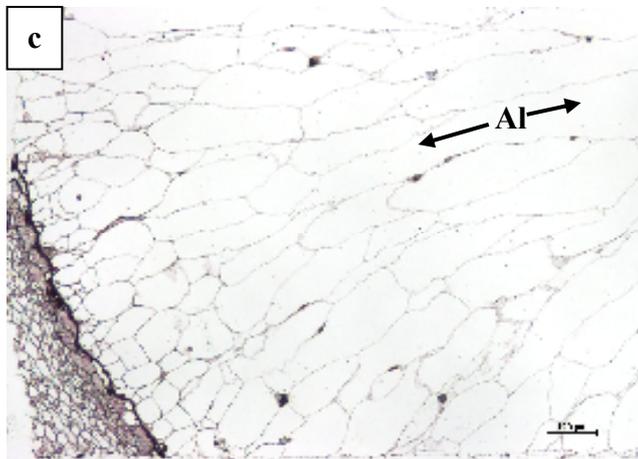
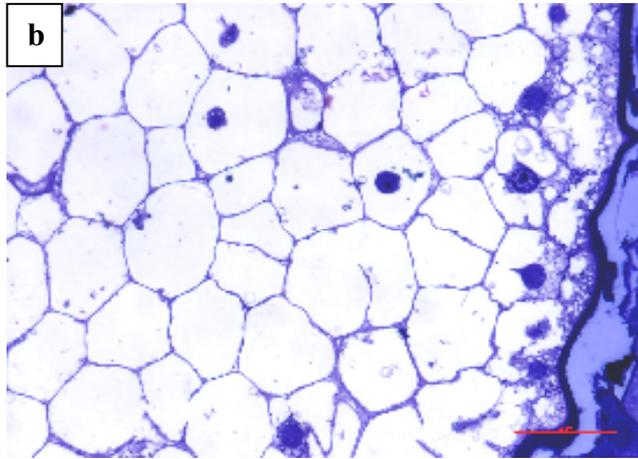
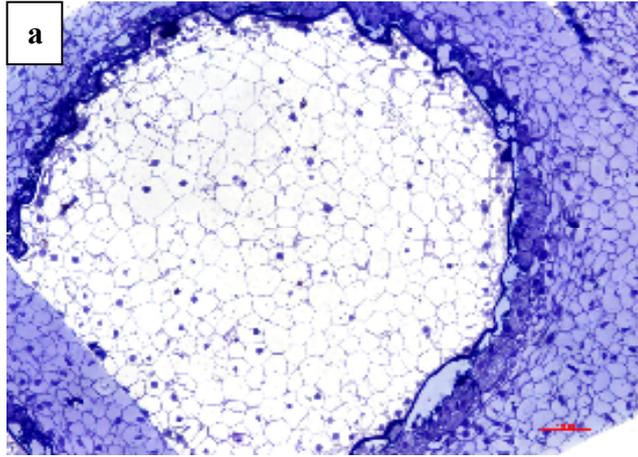
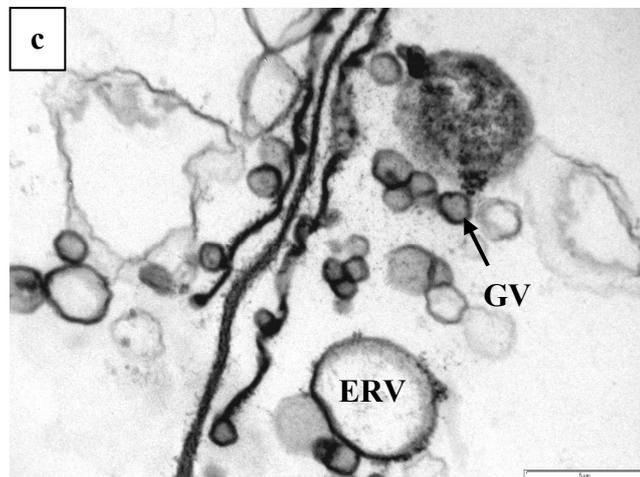
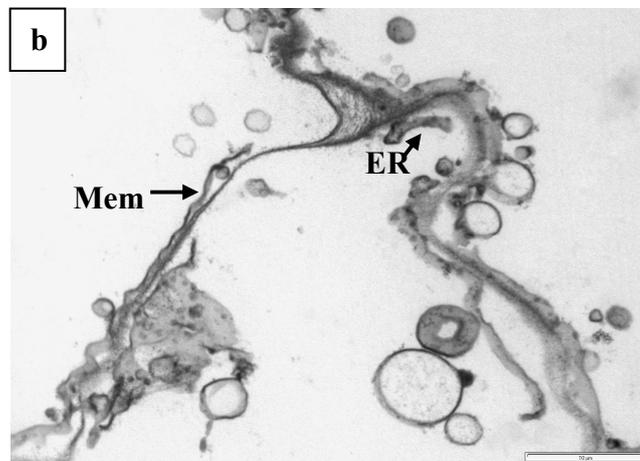
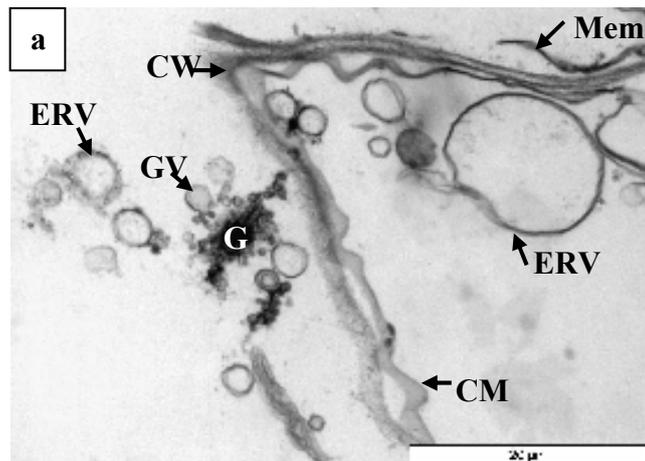


Figure 3.12: The ultrastructure of alveolus-like megagametophyte cells approximately 1 week after the coenocytic stage was observed showing (a) thin primary cell walls (CW) flanked by discontinuous membranes (Mem) and cell membrane (CM), ER-derived vesicles (ERV) and associated Golgi-derived vesicles (GV), within the homogeneous matrix; bar = 20 μm ; b) the walls flanked by short profiles of ER in some places, and discontinuous segments of membrane (Mem); bar = 10 μm ; c) Golgi- (GV) and ER-derived (ERV) vesicles, apparently fusing to form the cell membrane and possibly contributing material to the cell wall; bar = 5 μm . Sections were post-stained with ZIO.



As a caveat, it cannot be precluded at this stage that the homogeneously appearing cytomatrix, overall lack of Golgi bodies and wavy, discontinuous membranes may be artefacts of improper fixation.

3.2.2.1. Enzyme-gold localisation of pectin and xylan during early cell wall development

When enzyme-gold localisation for pectin was undertaken, labelling was found associated with the thin primary cell walls (Fig. 3.13). There was no labelling for pectin in the small, Golgi-derived vesicles (Fig. 3.13) nor did the Golgi bodies label for pectin (Fig. 3.13b). However, labelling was found in some large, ER-derived vesicles (Fig. 3.13b) and a relatively high concentration of label was found in some ill-defined, diffuse structures within the cytomatrix (Fig. 3.13c).

A similar result was obtained when enzyme-gold localisation of the primary cell wall component, xylan, was done (Fig. 3.14). Label for xylan was not found in Golgi bodies nor in the Golgi-derived vesicles (Fig. 3.14b), but it was present in the cell wall (Fig. 3.14a), some ER-derived vesicles (Fig. 3.14b) and in the undefined structures within the cytomatrix (Fig. 3.14c).

While labelling for pectin and xylan was found in the above-mentioned organelles, labelling was not observed in the controls to suggest any non-specific binding (results not shown).

3.2.2.2. Ultrastructure of the cytomatrix

When the apparently homogeneous cytomatrix of the megagametophyte was examined more closely with the TEM, at high magnification and using zinc iodide-osmium tetroxide as a post stain, numerous faintly appearing vesicles could be discerned (Fig. 3.15a). A profusion of what are presumably similar vesicles was found when the surfaces of resin blocks containing material of this developmental stage were etched using potassium methoxide and viewed with the scanning electron microscope (SEM) [Fig. 3.15b]. It can be seen that the vesicles in Figure 3.15b are not artefacts, as the resin-only control did not have any vesicle-like structures (Fig. 3.15c).

Figure 3.13: Enzyme-gold localisation for pectin approximately 1 week after the coenocytic stage was observed. Note (a) label associated with the thin primary cell wall (CW) but not in the small Golgi-derived vesicles (GV); bar = 5 μm ; b) label in some ER-derived vesicles (ERV) and not in the Golgi body (G); bar = 5 μm ; and (c) label in the substructure of the cytomatrix (arrows); bar = 5 μm . Gold particle size = 20 nm.

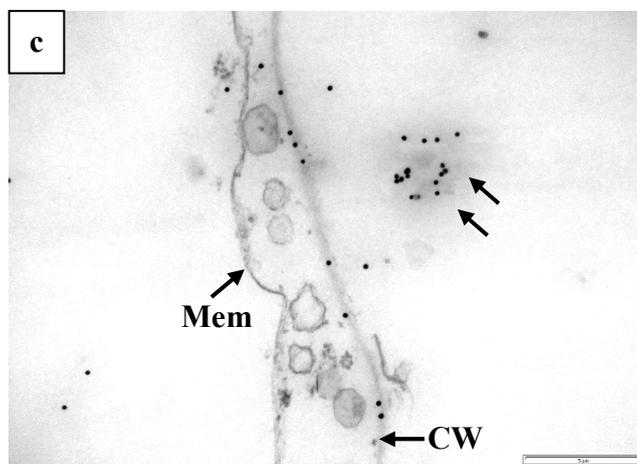
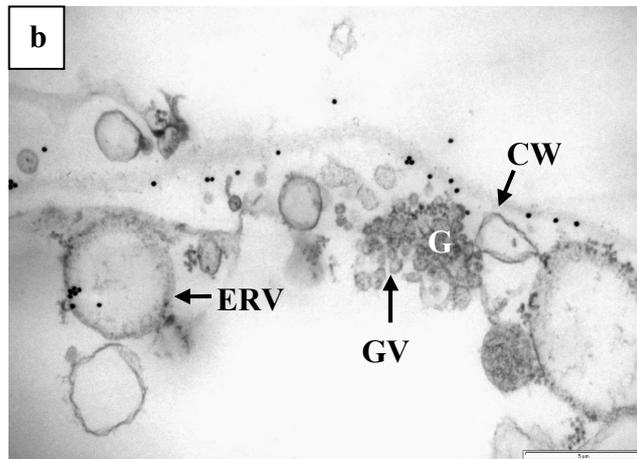
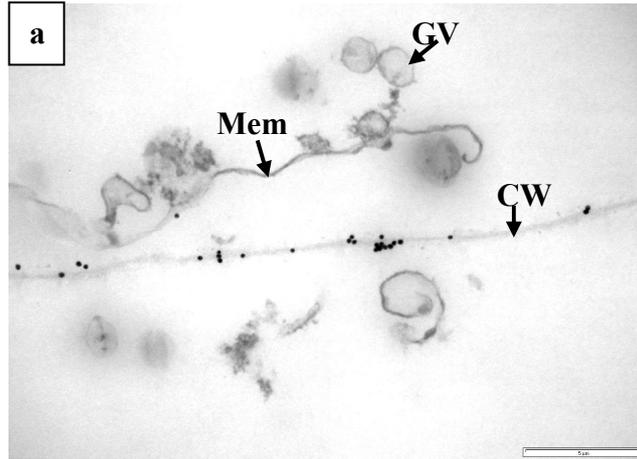


Figure 3.14: Enzyme-gold localisation of xylan approximately 1 week after the coenocytic stage was observed. Note that there is (a) label in the primary cell wall (CW); bar = 5 μm ; b) label in some ER-derived vesicles (ERV) but not in the Golgi body (G) or Golgi-derived vesicles (GV); bar = 5 μm ; and (c) label in the substructure of the matrix (arrows); bar = 5 μm . Gold particle size = 20 nm.

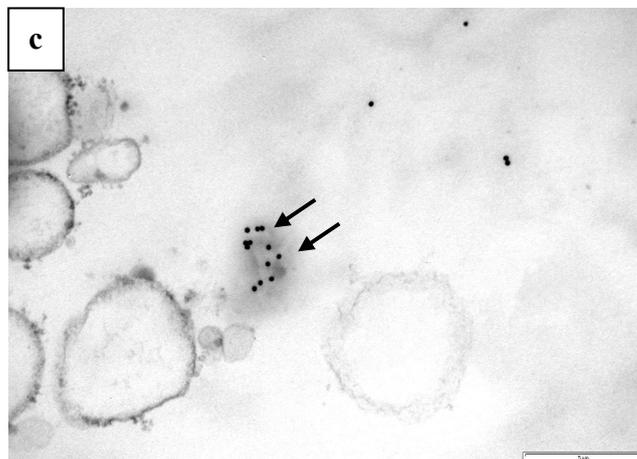
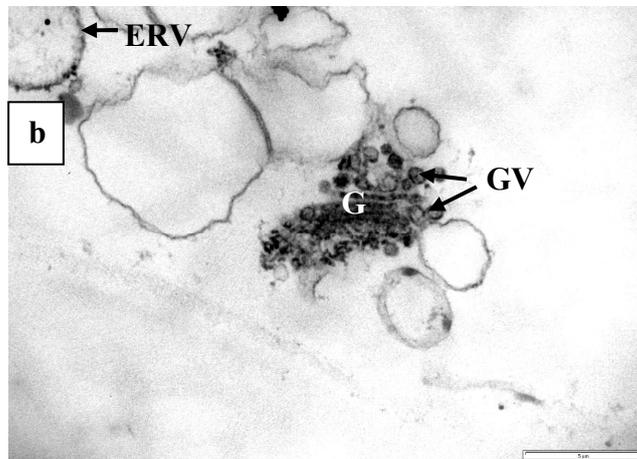
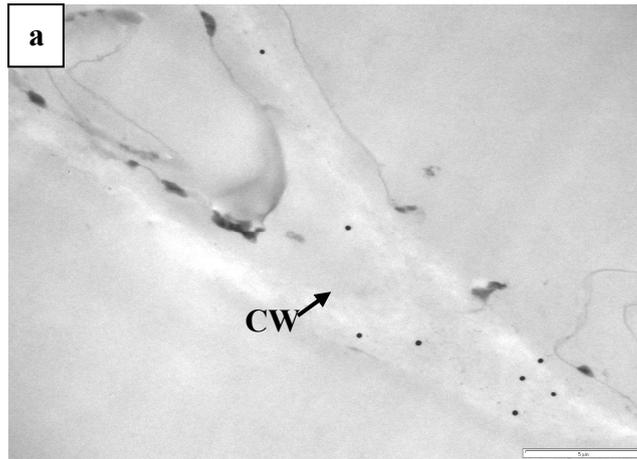
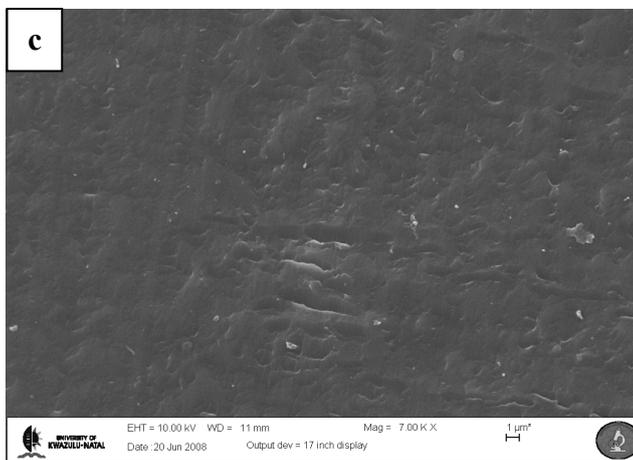
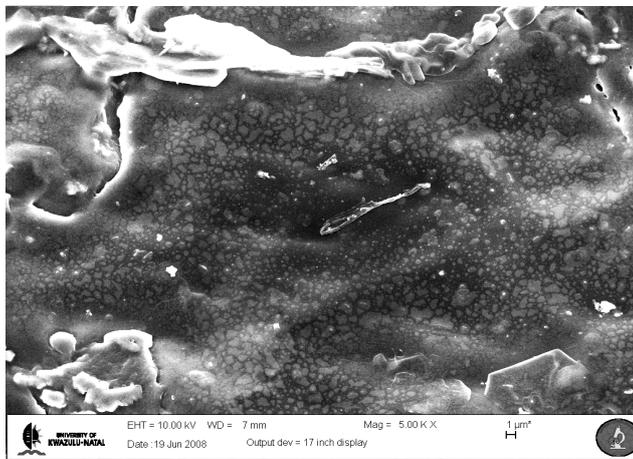
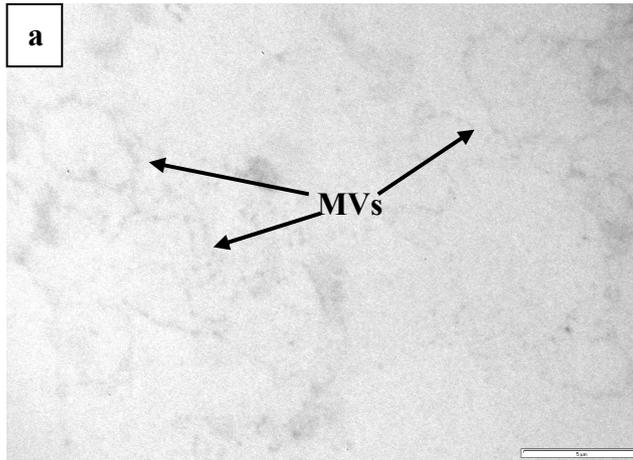


Figure 3.15: Some substructure resolved in the cytomatrix of megagametophyte cells approximately 1 week after the coenocytic stage was observed: a) A transmission electron micrograph of a section post-stained with ZIO showing the faintly visible apparently membrane-bounded vesicles (MVs); bar = 5 μm ; b) a scanning electron micrograph of the smoothed surface of a resin block face, etched using potassium methoxide. Note the profusion of vesicles; bar = 1 μm . c) No vesicles could be discerned when resin-only control block faces were viewed with the scanning electron microscope; bar = 1 μm .



Immunocytochemistry for microtubules utilising gold-tagged anti-acetylated-tubulin, was undertaken to check for the presence of these tubulin-based structures in the cytomatrix of the megagametophyte cells. A relatively uniform distribution of gold-tagged anti-acetylated-tubulin was found throughout the cytomatrix (Fig. 3.16), but absent from vesicles (Fig. 3.16a,b), the mitochondria and Golgi bodies (Fig. 3.16b), and the nucleus (Fig. 3.16c), suggested that the cytomatrix is permeated by a network of microtubules.

3.2.3. Structure and ultrastructure of the megagametophyte cells from 1 to 2 months after the coenocytic stage

Approximately 1 month after the coenocytic stage was observed, faint cellular detail was observable at the light microscope level (Fig. 3.17a), which was not evident at the previous developmental stage, i.e. approximately 1 week after the coenocytic stage. At the TEM level, more consolidated cell walls had formed, relative to the earlier developmental stage, and the cell membrane was now continuous (Fig. 3.17a). There were more Golgi bodies present than in the newly cellularised condition (Fig. 3.17a), and organelles generally were present throughout the cell, not just peripherally as seen 1 week after the coenocytic stage. Long profiles of ER were particularly noticeable at this later stage, producing vesicles of two kinds (Fig. 3.17b,c). One type of ER-derived vesicle was initially double-membrane-bound (Fig. 3.17b), while the other was single-membrane-bound, apparently produced by terminal vesiculation of the ER (Fig. 3.17c). The cytomatrix was now also interspersed with a faintly-staining, granular material that was not seen in the previous developmental stage (Fig. 3.17c).

Two months after the coenocyte was observed, the interior of megagametophyte cells appeared denser at the light microscope level than at the previous stages examined, with many starch-containing amyloplasts now evident (Fig. 3.18a). At the TEM level, the cytomatrix appeared more granular than it did previously (Fig. 3.18b), probably as the result of protein deposition. Considerable ER proliferation was evident near the cell periphery, with many cisternae showing localised luminal dilation (Fig. 3.18c).

Figure 3.16: Immunocytochemistry for microtubules using gold-tagged anti-acetylated-tubulin. Megagametophyte cells 1 week after the coenocyte showing (a) labelling in the cytomatrix but not in vesicles (ERV); arrow indicating two gold particles; bar = 5 μm ; b) labelling in the cytomatrix but not in the mitochondria (M); bar = 5 μm ; and (c) labelling in the cytomatrix but not in the nucleus (N); bar = 5 μm . Gold particle size = 10 nm.

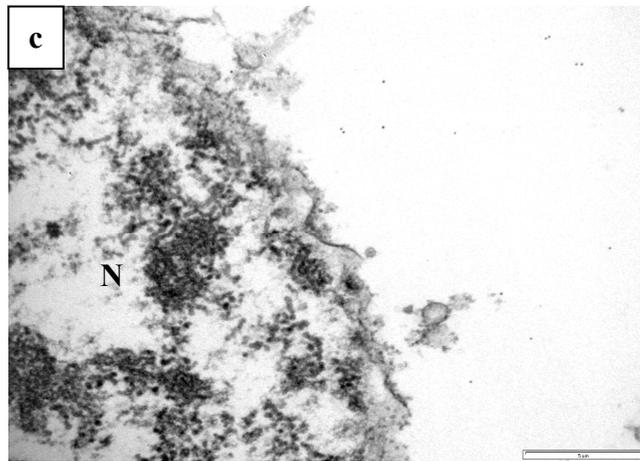
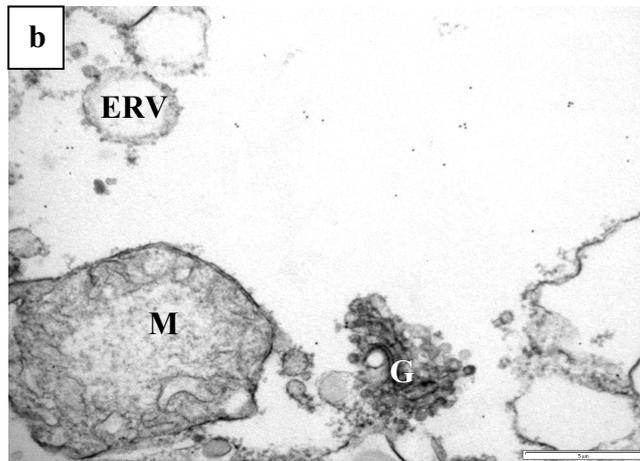
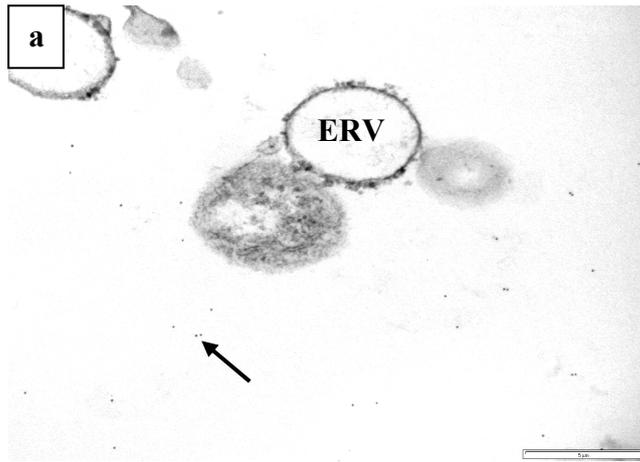


Figure 3.17: Cellular structure and ultrastructure of the megagametophyte 1 month after the coenocyte was observed showing (a) faintly appearing structure throughout the cytomatrix, not just peripherally as observed in the previous stage, while the nucleus (N) was particularly prominent; section stained with toluidine blue, bar = 10 μm ; b) ultrastructure of the cytomatrix showing the thicker cell wall (CW) than in the newly cellularised megagametophyte (cf. Figs 3.12 and 3.13), numerous Golgi bodies (G), mitochondria (M), relatively long profiles of ER and one of the numerous double-membrane-bound ER-derived vesicles (ERV1) observed at this stage; bar = 10 μm ; c) an ER cisterna producing single-membrane-bound vesicles (ERV2) by terminal vesiculation at both ends. Note that the cytomatrix was now characterised by a faintly-contrasted, granular material; bar = 10 μm . Sections shown in (b) and (c) post-stained with lead citrate and uranyl acetate.

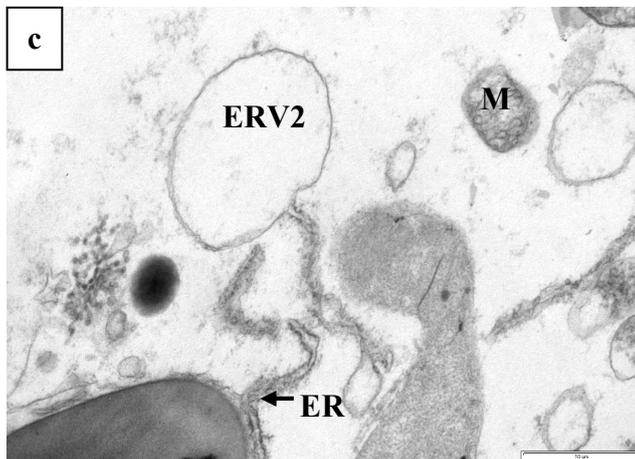
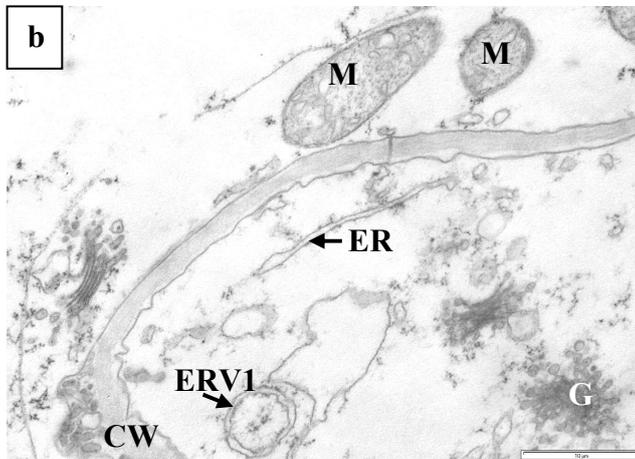
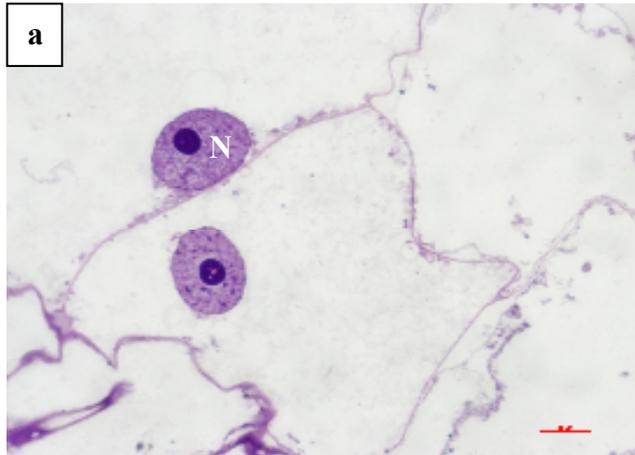
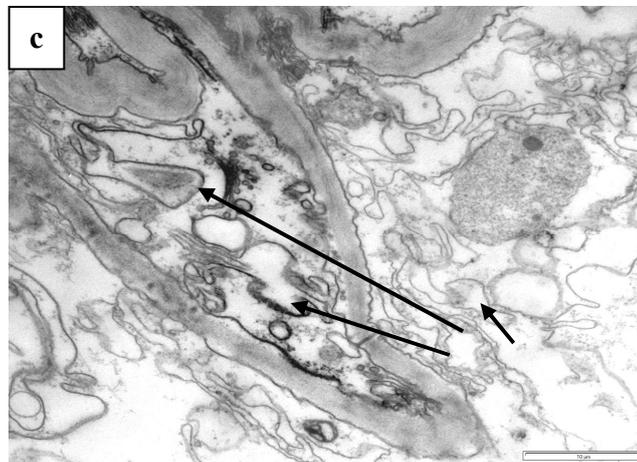
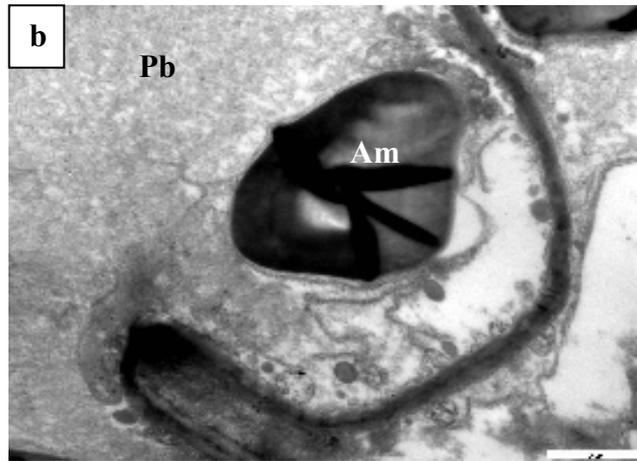
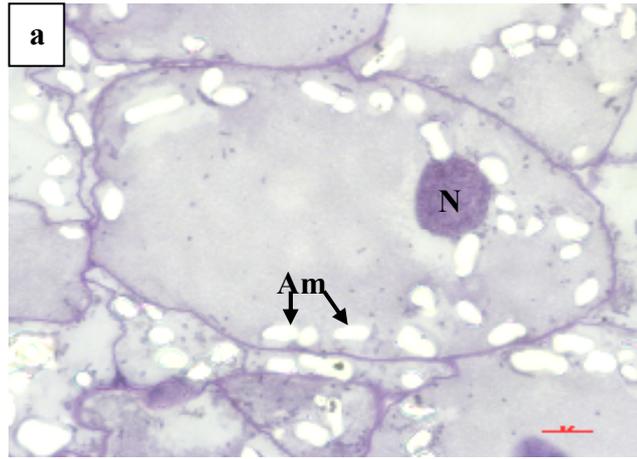


Figure 3.18: Cellular structure and ultrastructure of the megagametophyte 2 months after the coenocyte was observed showing (a) at the light microscope level, the cell interior appearing more dense than it did 1 month prior to this stage, with amyloplasts (Am) frequently evident; section stained with toluidine blue, bar = 10 μm ; b) some regions of the cytomatrix densely packed with a granular material which was tentatively identified (and later confirmed) as protein (Pb); bar = 20 μm ; and c) ultrastructural detail showing regional proliferation of ER in the peripheral regions of neighbouring cells. Many of the ER cisternae showed localised dilation (arrows), bar = 10 μm . Sections in (b) and (c) post-stained with lead citrate and uranyl acetate.



3.2.3.1. Protein histochemistry

Using mature megagametophyte specimens, the dense, granular material in the cytomatrix was positively identified as protein on account of its positive reaction with eosin (James and Tas, 1984) in sections prepared by wax embedding (Fig. 3.19a), and dark pink in resin-embedded sections (Fig. 3.19b) that were etched prior to the application of eosin dye. While protein bodies stained with eosin, the carbohydrate-containing amyloplasts were contrasted, as they did not stain. When the protein in wax-embedded sections was first digested using protease before application of eosin, no, or very little staining of protein was observed (Fig. 3.19c). From Figure 3.19b, it is evident that there are at least two types of protein bodies formed in the megagametophyte, based on their physical appearance. One type appeared densely packed, quite possibly ER distended with accumulated protein, while the other type appeared less dense or interspersed with clear regions. However, apparent continuity could be seen between some of the regions of dense and less dense protein deposition (arrows, Fig. 3.19b). This possibly indicates localised accumulation of different types of protein within discrete regions of ER cisternae (Fig. 3.18c), or different stages in the deposition process.

3.2.3.2. Carbohydrate histochemistry

Carbohydrate histochemistry on mature megagametophyte tissue using Periodic acid-Schiff (PAS) reagent resulted in only the starch-containing amyloplasts staining in wax-embedded sections (Fig. 3.20a), and resin-embedded sections (Fig. 3.20b) that were etched prior to the application of PAS reagent. The granular material previously identified as protein did not stain with PAS reagent (Fig. 3.20b). When the non-stained section in Figure 3.20c is compared with Figure 3.20b it can be seen that only the amyloplasts stained for carbohydrates.

Figure 3.19: Protein histochemistry, using eosin, of the mature megagametophyte showing (a) protein staining bright red in a 20 μm thick (originally wax-embedded) section; bar = 100 μm ; b) protein staining as various intensities of pink in a 1 μm thick resin-embedded section. Note the apparent regions of continuity (arrows) between protein accumulations showing different staining intensity or disposition; bar = 50 μm ; and (c) no, or very little, protein reaction with eosin in a 1 μm thick, control, resin-embedded section where protein was digested using protease before application of the stain; bar = 100 μm .

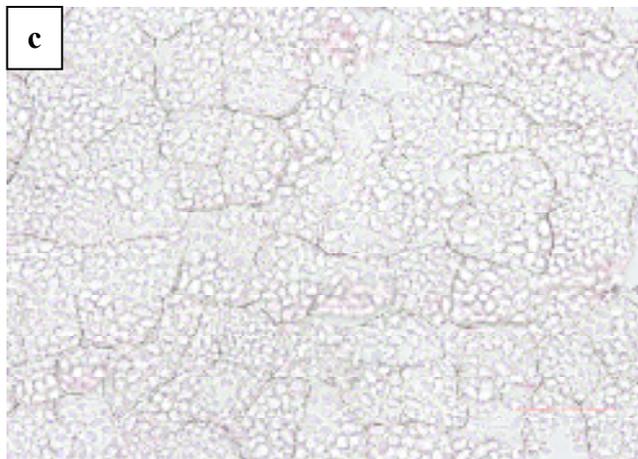
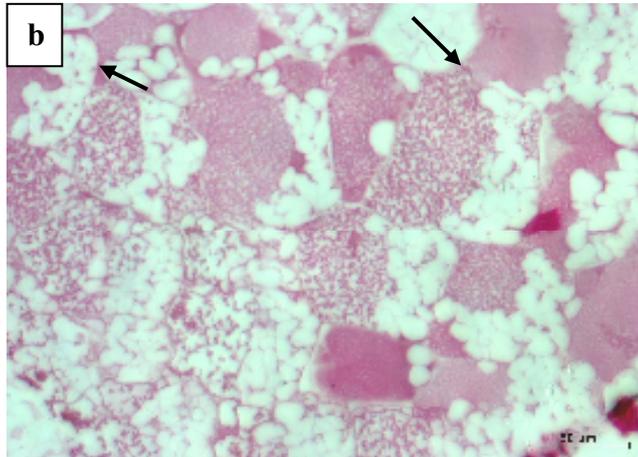
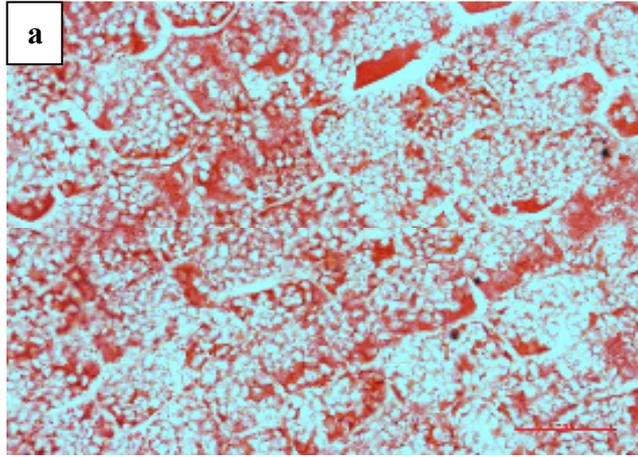
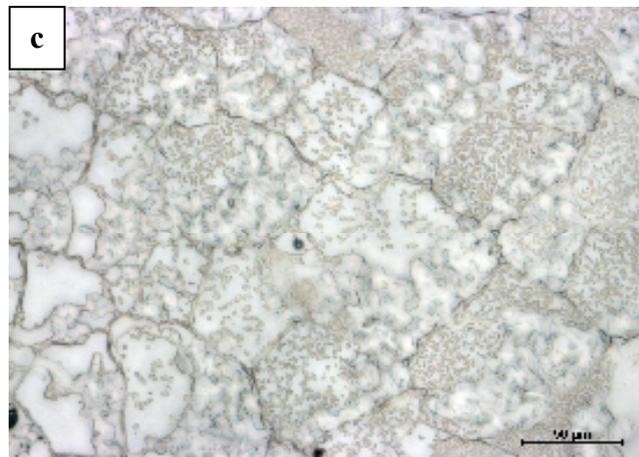
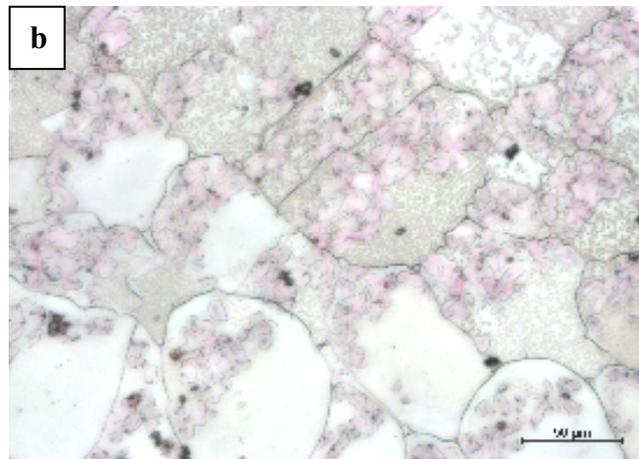
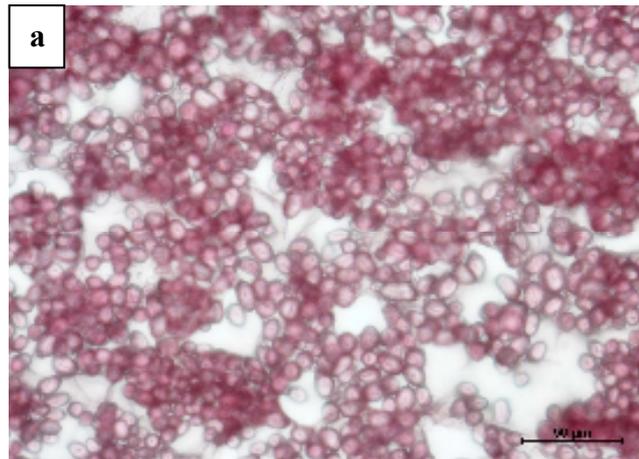


Figure 3.20: Carbohydrate histochemistry, using PAS reagent, of the mature megagametophyte showing (a) amyloplasts staining maroon in a 20 μm thick (originally wax-embedded) section; bar = 50 μm ; b) amyloplasts staining pale pink in a 1 μm thick resin-embedded section; bar = 50 μm ; and (c) a 1 μm thick, control, resin-embedded section showing the original colour of the material before application of the stain. Note that the amyloplasts stained light pink only after application of the stain; bar = 50 μm .



3.2.3.3. Lipid histochemistry

The results obtained for the histochemical localisation of lipid in the mature megagametophyte of *E. natalensis*, using Sudan Black B, are shown in Figure 3.21. When a resin-embedded section was stained for lipid, the peripheral cell layer of the megagametophyte showed substantial lipid deposits, compared with other cells of the body of the megagametophyte, where only discrete, limited lipid content was indicated (Fig. 3.21a). The amyloplasts and nuclei did not stain. When previously wax-embedded sections were treated with the Sudan stain, numerous, relatively small, discrete lipid bodies were seen scattered throughout the cells of the megagametophyte (Fig. 3.21b). In a control treatment, where sections were first delipidised using chloroform:methanol (2:1) before application of the Sudan stain, very few locations staining with Sudan Black B were seen (Fig. 3.21c).

3.2.4. Structure and ultrastructure of the megagametophyte cells from 3 months after the coenocyte was observed to the time of seed-shed

Figure 3.22 shows an increased quantity of starch as evident from the development of the amyloplasts in the cytomatrix of megagametophyte cells 3 months after the coenocyte was observed compared with the situation a month previously (cf Fig. 3.18a). Large protein bodies and amyloplasts persisted in the cytomatrix (Fig. 3.22b), while lipid bodies were still being produced by relatively long profiles of ER (Fig. 3.22c).

Four months after the coenocyte was observed, there was a further apparent increase in starch accumulation within the amyloplasts (Fig. 3.23a), while the relatively large protein bodies, lipid bodies and various organelles had not changed qualitatively between the 3rd and 4th month of megagametophyte development (Fig. 3.23b). Mitochondria were not frequent: however, the ultrastructure of those mitochondria that were observed, characterised by dense matrices and well-developed cristae (Fig. 3.23c), suggested intense, if localised, metabolic potential.

Six months after the coenocyte was observed (Fig. 3.24), the only notable difference was an increase in the amount of amyloplast starch in the cells (Fig. 3.24a). The amyloplasts and large protein bodies dominated the cytomatrix (Fig. 3.24b), while long profiles of ER and occasional mitochondria still remained (Fig. 3.24c).

Figure 3.21: Lipid histochemistry, using Sudan Black B, of the mature megagametophyte showing (a) the peripheral cell layer of the megamatophyte staining dark brown-black with the Sudan stain in a 1 μm thick resin-embedded section with speckles of similar staining (arrows) in the otherwise homogeneous regions of underlying cells; bar = 100 μm ; b) discrete, small lipid bodies staining black/grey in a 20 μm thick (originally wax-embedded) section; bar = 100 μm ; inset showing lipid bodies at higher magnification; bar = 50 μm ; and (c) a 20 μm thick, control, wax-embedded section where very few lipid bodies were seen stained after delipidisation using chloroform:methanol (2:1); bar = 100 μm .

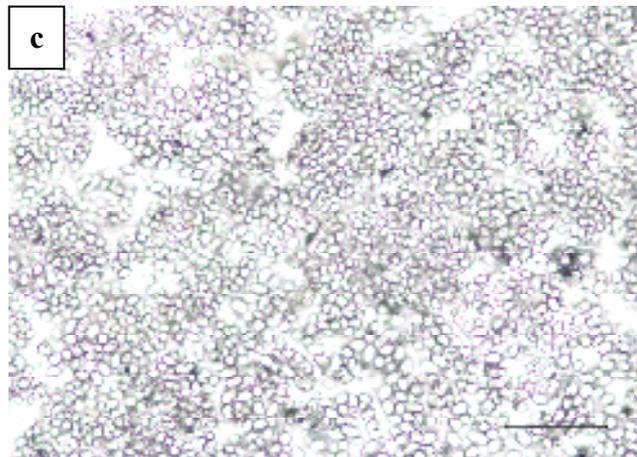
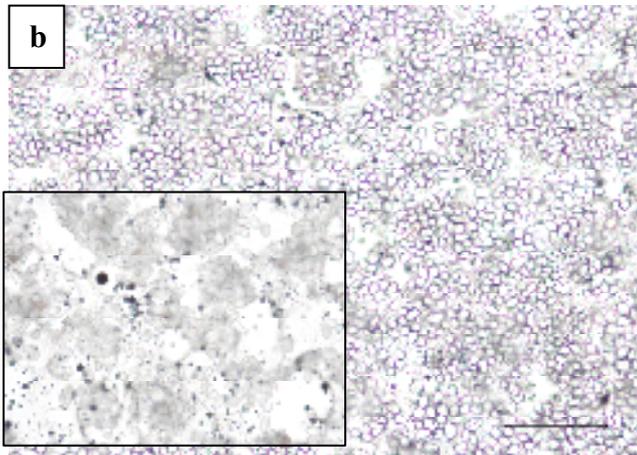
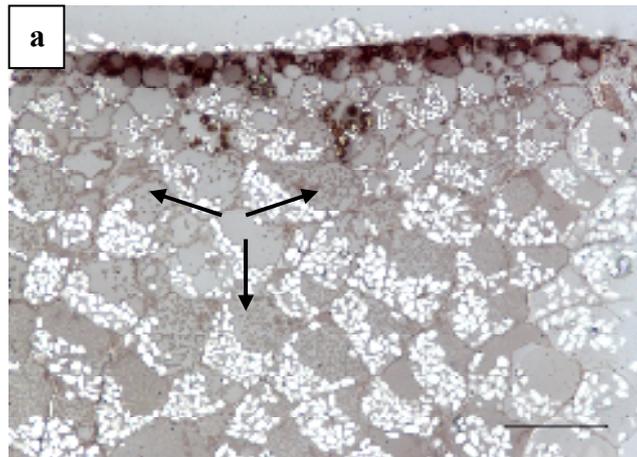


Figure 3.22: Cellular structure and ultrastructure of the megagametophyte 3 months after the coenocyte was observed. Note that (a) relatively more starch had accumulated in the amyloplasts (Am) than 1 month earlier; section stained with toluidine blue, bar = 10 μm ; b) ultrastructure of the cells with relatively large protein bodies (Pb) and amyloplasts (Am); bar = 50 μm ; c) discrete, small lipid bodies (L) that were apparently being produced by relatively long profiles of ER; bar = 10 μm . Sections in (b) and (c) post-stained with lead citrate and uranyl acetate.

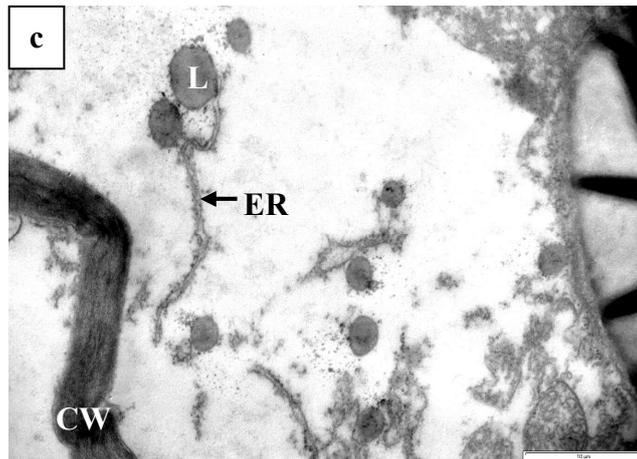
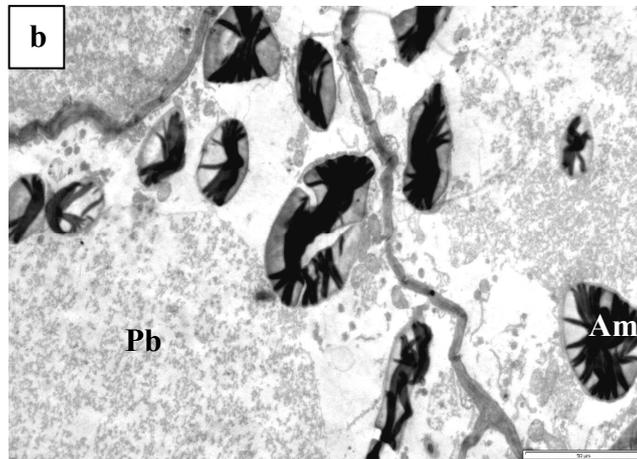
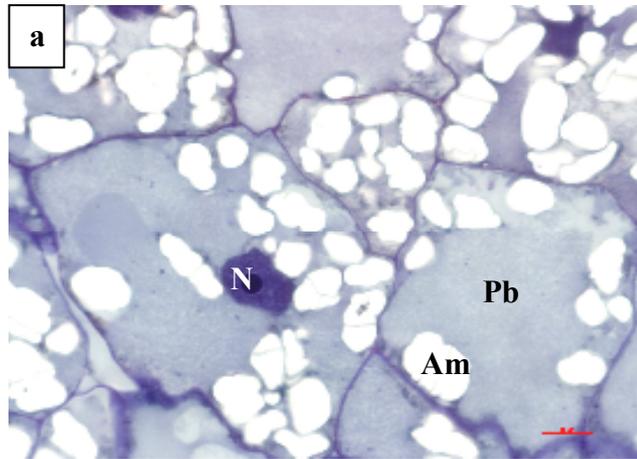


Figure 3.23: Cellular structure and ultrastructure of the megagametophyte 4 months after the coenocyte was observed. Note that (a) the cell interior had as many (if not more) starch-containing amyloplasts (Am) than it did 1 month prior to this stage; section stained with toluidine blue, bar = 10 μm ; b) ultrastructure of the cells with relatively large protein bodies (Pb) and amyloplasts, while mitochondria (M) occurred in the homogeneous cytomatrix; bar = 50 μm ; c) Mitochondria possessed dense matrices and well-defined cristae and lipid bodies (L) had remained small and discrete bar = 10 μm . Sections in (b) and (c) stained with lead citrate and uranyl acetate.

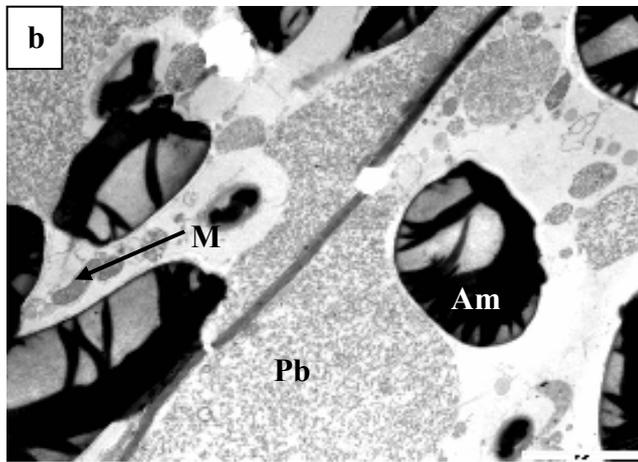
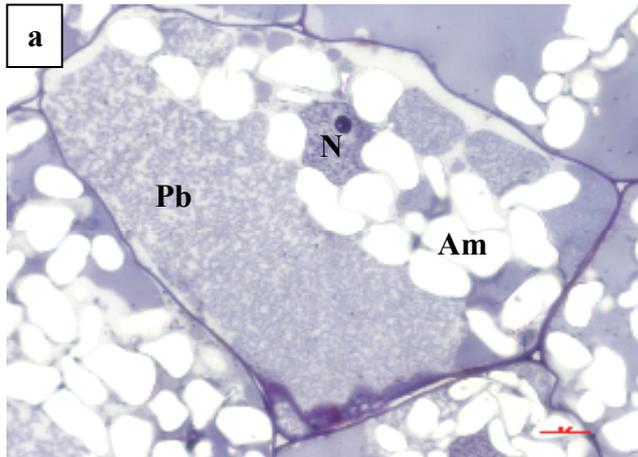
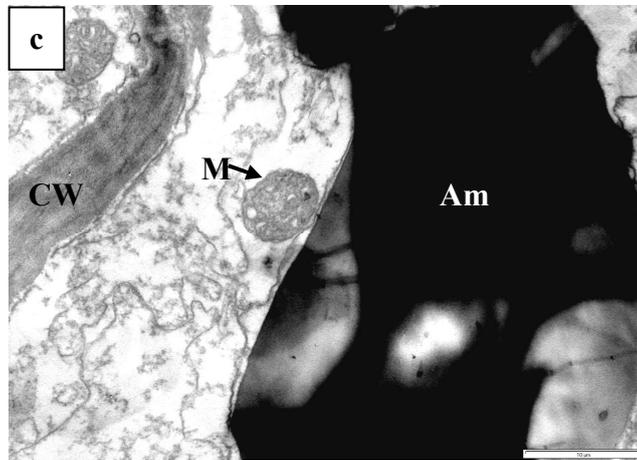
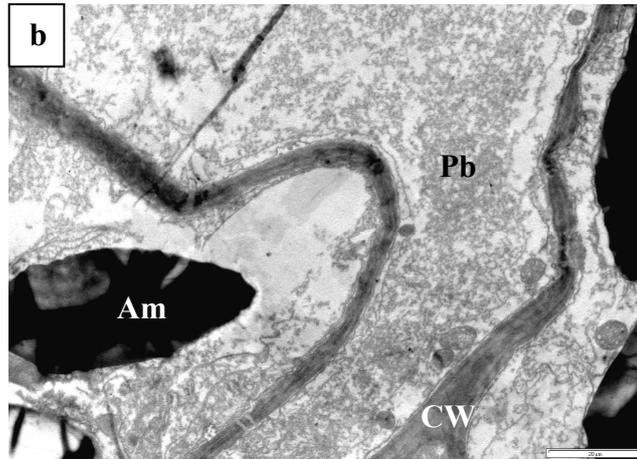
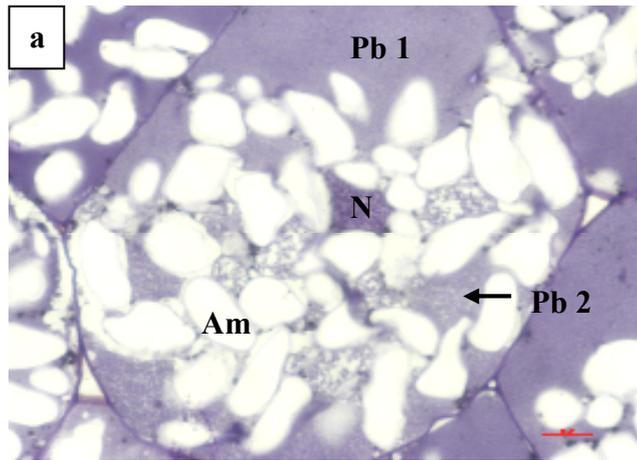


Figure 3.24: Cellular structure and ultrastructure of the megagametophyte 6 months after the coenocyte was observed. Note that (a) the cell interior contained considerable starch within amyloplasts (Am) and possibly more of these organelles than previously, while there was apparent persistence of the two forms of protein body (Pb1 and 2) based on protein density; section stained with toluidine blue, bar = 10 μm ; b) ultrastructure of the cells with relatively large protein bodies (Pb) and amyloplasts (Am); bar = 20 μm ; c) long profiles of ER, and mitochondria (M) characterised by dense matrices and well-defined cristae occurred within localised regions of the cytomatrix; bar = 10 μm . Sections in (b) and (c) stained with lead citrate and uranyl acetate.



Eight months after the original coenocytic stage, when the seeds were ultimately being shed from the parent plant, the cells of the megagametophyte were seen to be crammed with starch-containing amyloplasts and protein bodies (Fig. 3.25a), to such an extent that other organelles were not seen and the occasional nucleus visualised appeared ‘squashed’ (Fig. 3.25b).

3.3. Viability status of the megagametophyte during post-shedding seed development

3.3.1. Tetrazolium (TTZ) study

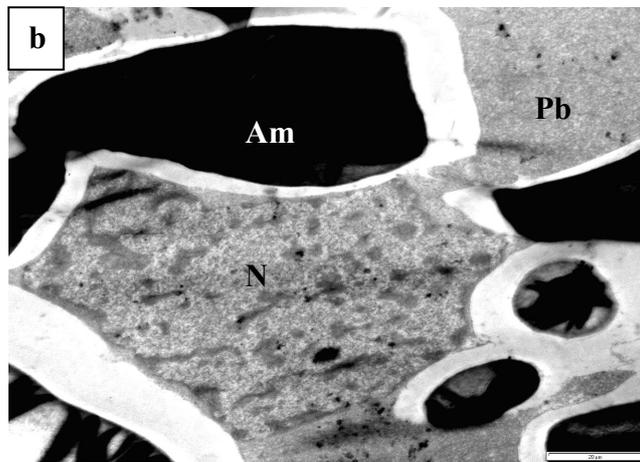
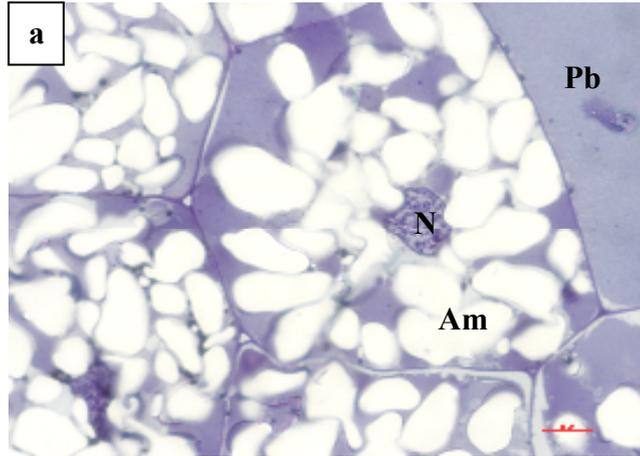
The viability of the megagametophyte tissue of fertilised seeds (Fig. 3.26) and unfertilised, ‘futile’ ovules (Fig. 3.27) was assessed by staining with 2,3,5-triphenyl tetrazolium chloride (TTZ). Tetrazolium acts as the terminal acceptor in the electron transport chain during respiration, developing a characteristic red-pink colour in tissue undergoing aerobic respiration (i.e. a metabolically-active / living tissue).

Judging from the reactivity with tetrazolium, the megagametophyte of seeds (Fig. 3.26a) and shed, futile (unfertilised) ovules (Fig. 3.27a) was still metabolically active soon (days) after being shed from the parent plant. Three-month-old ovules (Fig. 3.27b) and seeds (Fig. 3.26b) showed less intense and somewhat more patchy tetrazolium staining than at shedding, indicating that although diminished, metabolic activity was still taking place in the megagametophyte. However, there was variability, with little TTZ reactivity in some ovules and seeds. The megagametophyte of 6-month-old seeds (Fig. 3.26c), showed very little, if any, tetrazolium reactivity. However, inconsistently, some of the unfertilised, ‘futile’ ovules (Fig. 3.27) showed positive TTZ reactivity, indicating continuing respiratory metabolism, in particular, the archeogonial regions stained bright red with tetrazolium.

3.3.2. Ultrastructural investigations of the megagametophyte during post-shedding seed development

The megagametophyte cells of newly-shed seeds were nucleated (judging from the few instances where this body was able to be visualised), and packed with starch and protein, the two principal storage reserves (Fig. 3.28a). A relatively small number of discrete lipid bodies, and occasional mitochondria (Fig. 3.28c) could be seen where their visualisation was not precluded by the masses of stored reserves. At seed-shed, the mitochondria were of a

Figure 3.25: Cellular structure and ultrastructure of the megagametophyte 8 months after the coenocyte stage. Note that (a) the cell interior appeared to be crammed with starch-containing amyloplasts (Am) and protein bodies (Pb); section stained with toluidine blue, bar = 10 μm ; b) ultrastructure of a cell showing the nucleus (N) compressed between the abundant amyloplasts (Am) and protein bodies (Pb); section stained with lead citrate and uranyl acetate, bar = 20 μm .



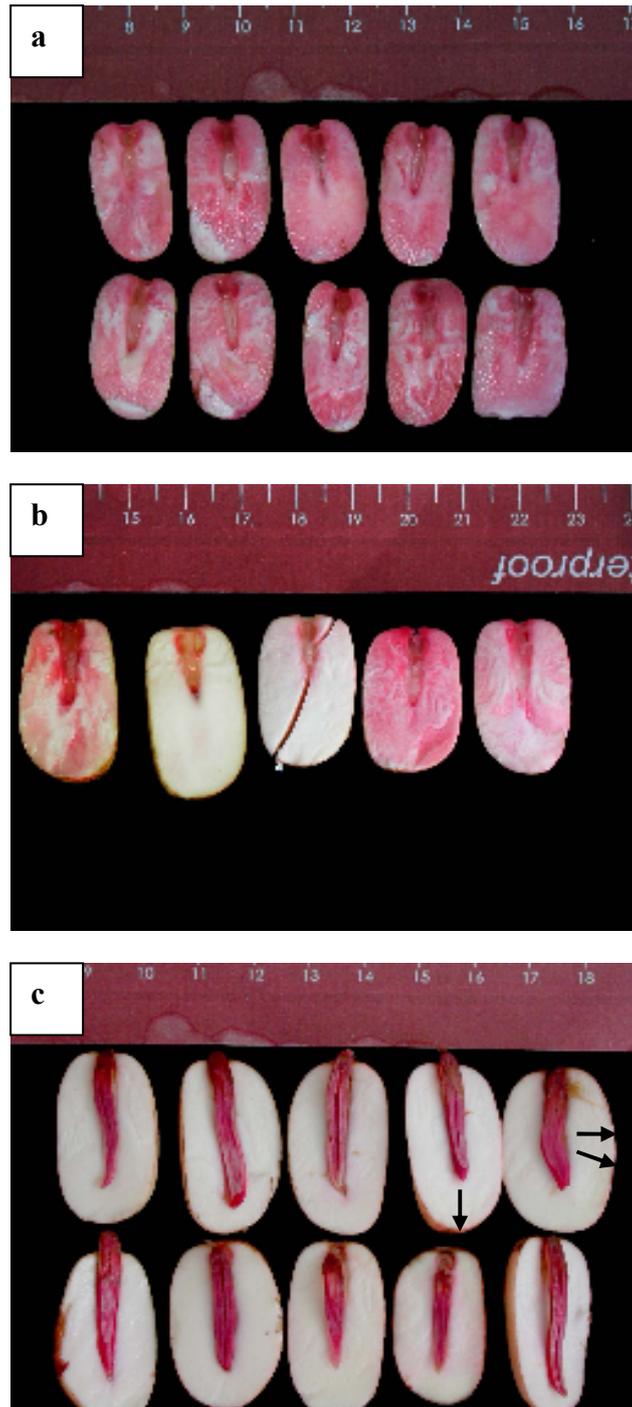


Figure 3.26: Reactivity to tetrazolium (TTZ) salts of the megagametophyte of fertilised seed, at various times after seed-shed: a) at seed-shed; b) at 3 MAS; and (c) at 6 MAS, arrows indicate thin peripheral band of tetrazolium staining.

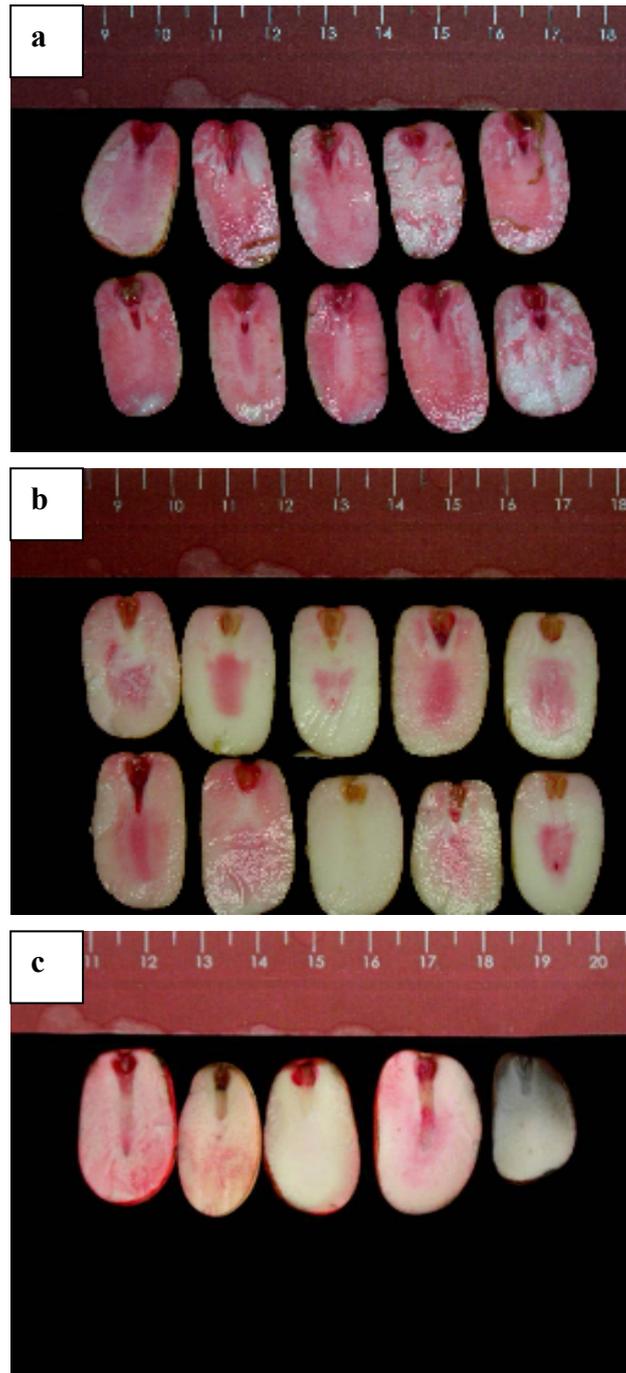
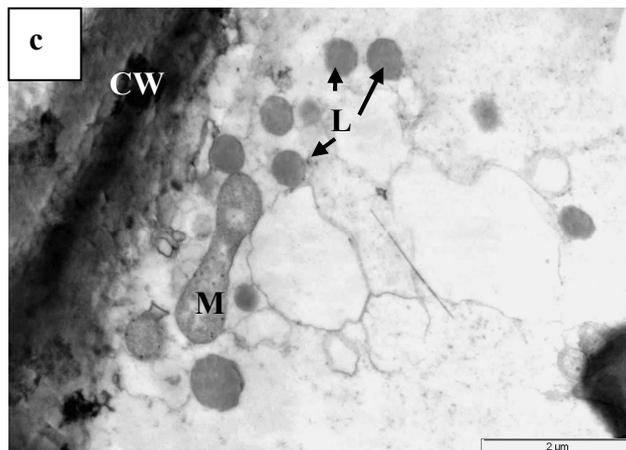
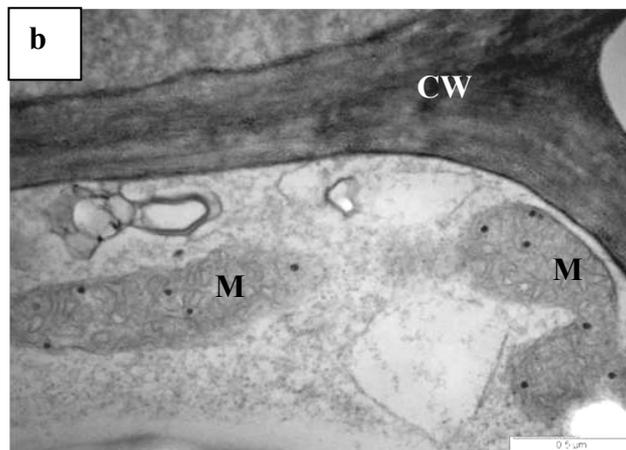
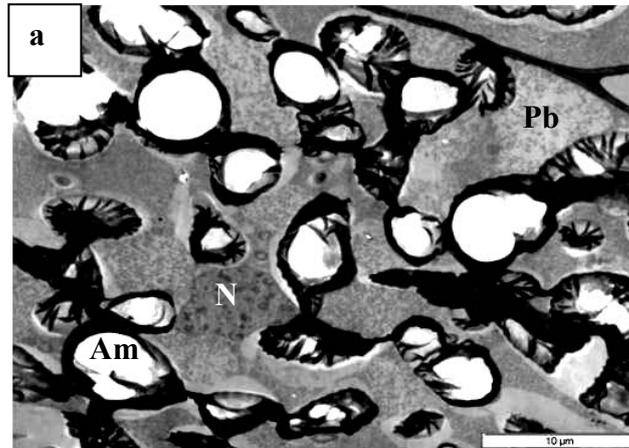


Figure 3.27: Reactivity to tetrazolium (TTZ) salts of the megagametophyte of unfertilised, futile ovules, at various times after seed-shed: a) at seed-shed; b) at 3 MAS; and (c) at 6 MAS.

Figure 3.28: Ultrastructure of the megagametophyte cells at seed-shed. Note (a) that the cells were packed with starch-containing amyloplasts (Am) and protein (Pb); bar = 10 μm ; b) mitochondria (M) with clearly defined cristae and dense matrices; bar = 0.5 μm ; and c) discrete lipid bodies (L), bar = 2 μm . Sections stained with lead citrate and uranyl acetate.



metabolically active appearance with clearly defined cristae (Fig. 3.28d) and lipid bodies in their vicinity (Fig. 3.28c).

At 3 MAS, the cells of the megagametophyte started to show some signs of deterioration which are presumed to be associated with utilisation of their content by the developing embryo (Fig. 3.29a). Discernible nuclei were infrequently seen (not shown), there appeared to be utilisation of the protein in some areas (Fig. 3.29b), no rough ER nor Golgi bodies were found, and the mitochondria appeared distorted with poorly defined cristae and pale matrices (Fig. 3.29c,d).

At 6 MAS, no discernible nuclei, endoplasmic reticulum or Golgi bodies occurred (Fig. 3.30a), where reserve utilisation appeared not (yet) to have occurred, the cells were dominated by large amyloplasts and tightly-packed protein bodies (Fig. 3.30a). Remnants of lipid bodies in some megagametophyte cells, illustrated in Figure 3.30b, were indicative of utilisation of this reserve. Few discernible mitochondria were found at this stage after seed-shed, and the mitochondria that were found were characterised by diminished cristae and pale matrices, especially in the centre of the organelle (Fig. 3.30c).

3.4. Difference in ultrastructure of the peripheral cell layer of the megagametophyte

The peripheral cell layer appeared very different from the cells of the body of the megagametophyte (Fig. 3.31). These peripheral cells were packed with lipid bodies as opposed to starch and storage protein (Fig. 3.31a,b). There was also far less accumulation of starch in these peripheral cells than in the cells of the body of the megagametophyte (Fig. 3.31a,b). Some of the plastids within these cells contained membranous stacks (Fig. 3.31c), which was suggestive of thylakoids.

Figure 3.29: Ultrastructure of the megagametophyte cells at 3 MAS. Note (a) and (b) signs of cellular deterioration presumably associated with mobilisation, and utilisation of their contents by the developing embryo; bar = 10 and 1 μm respectively; and (c) the disfigured appearance of one of the few mitochondria (M) found at this stage after seed-shed. The mitochondria had ill-defined cristae and pale matrices; bar = 0.5 μm . Sections were stained with lead citrate and uranyl acetate.

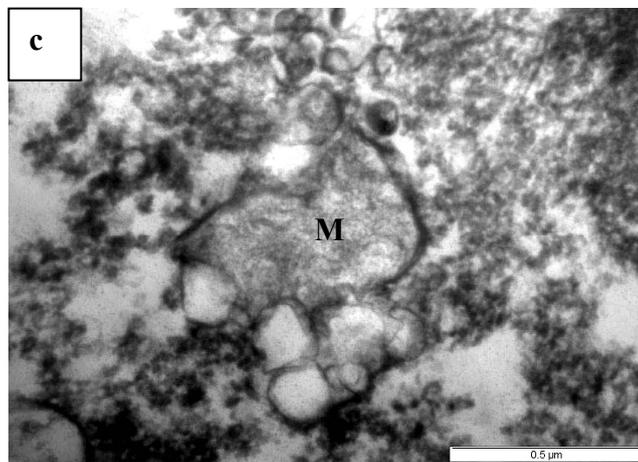
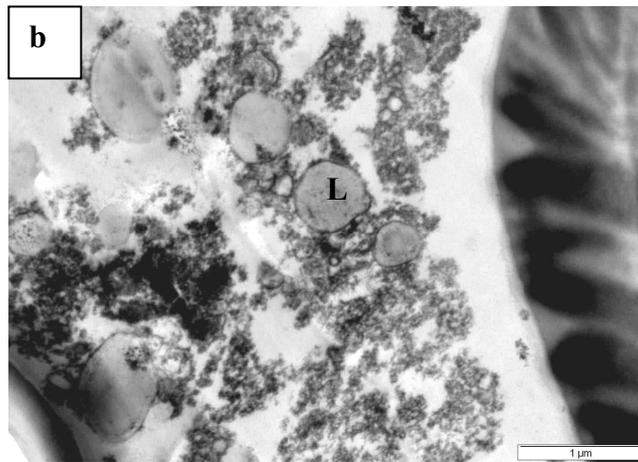
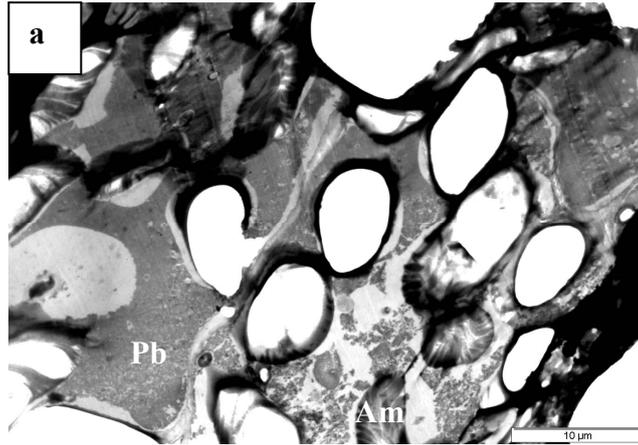


Figure 3.30: Ultrastructure of the megagametophyte cells at 6 MAS. a) Occasional cells did not show obvious starch and protein depletion; bar = 20 μm . In such cells (b) some lipid bodies (L) were observed and other structures appeared to be the remains of protein bodies (arrows); bar = 0.5 μm ; and (c) the mitochondria (M) were of less active appearance than at seed-shed, displaying small cristae and electron-opaque matrices, bar = 0.2 μm . Sections were stained with lead citrate and uranyl acetate.

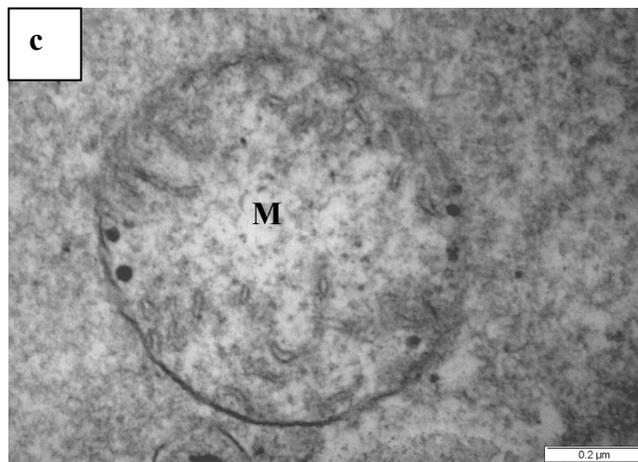
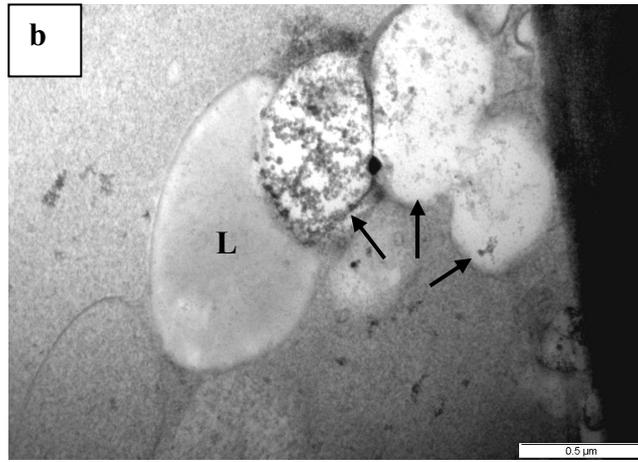
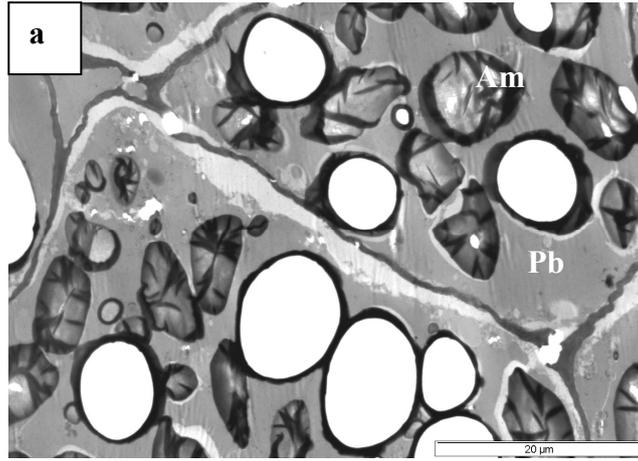
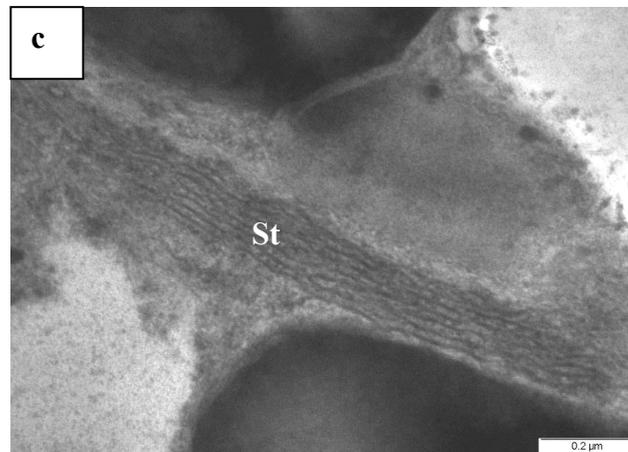
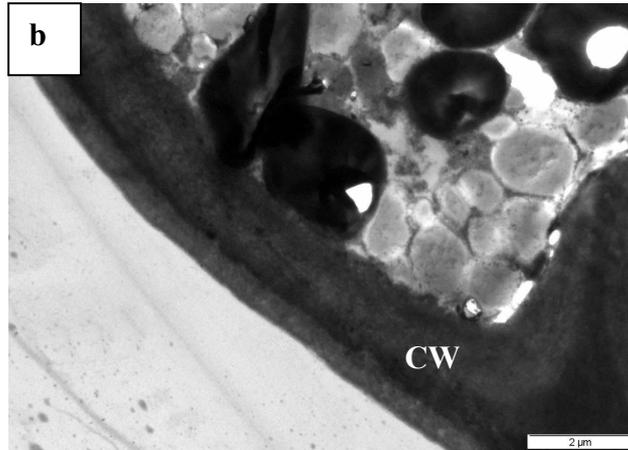
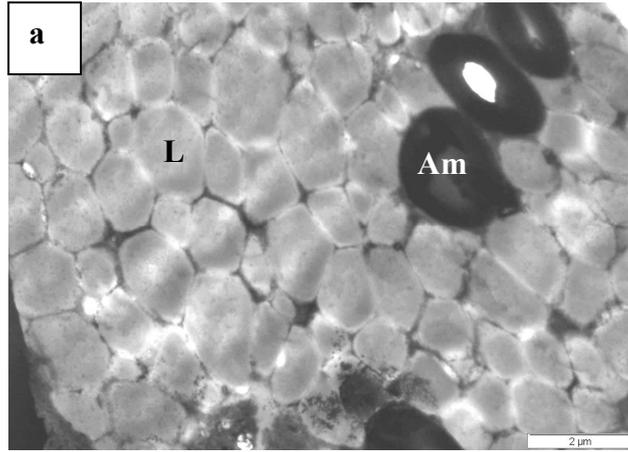


Figure 3.31: Ultrastructure of the peripheral cell layer of the megagametophyte at 6 months after seed-shed. Note that (a) the cells were packed with lipid bodies (L) and relatively small amyloplasts (Am); bar = 2 μm ; b) the outer cell wall (CW) was thickened and appeared cuticularised; bar = 2 μm ; and c) the amyloplast plastids often contained membranous stacks (St), suggestive of thylakoids; bar = 0.2 μm . Sections were stained with lead citrate and uranyl acetate.



4. DISCUSSION

4.1. Some aspects of *E. natalensis* megagametophyte development

The earliest developmental stage of the megagametophyte in this study was characterised by a large central vacuole and a thin peripheral layer of cytomatrix containing many free nuclei, small vesicles or vacuoles and what appeared to be undifferentiated mitochondria. This corresponds with the findings of Pettitt (1977), who observed undifferentiated mitochondria in the cytomatrix of cycad and *Ginkgo biloba* ovules at the coenocytic stage of development. However, Stewart and Gifford (1967), observed mitochondria with distinct cristae in the megaspore cytomatrix of *G. biloba*. The significance of undifferentiated mitochondria at the coenocytic stage is not immediately apparent, as the coenocyte should presumably be highly metabolically active in order for development to proceed. The undifferentiated appearance of the mitochondria at this stage may possibly be artefactual, as a consequence of improper fixation, as the coenocytic stage is a notoriously difficult stage of development to preserve (Maheshwari and Singh, 1967).

The megaspore membrane of the coenocyte was relatively thick and appeared to be made up of honeycomb like chambers. Pettitt (1966; 1977), reported that the megaspore membrane of gymnosperms is a multiple structure that differs between species; is made up of contributions by both the megagametophyte and sporophyte; and is comprised principally of the polymer sporopollenin.

According to Maheshwari and Singh (1967) the coenocytic stage is the most common developmental stage of the ovule at which most descriptive studies start. This is because the preceding stages, from the megaspore mother cell to the linear tetrad, normally occur when the ovules are concealed in the crown of the parent plant. The developmental stages preceding the coenocytic megagametophyte have, however, been observed in a few cycads: In *Zamia floridana*, the megaspore mother cell yields a linear tetrad (Smith, 1910); in *Macrozamia spiralis* (Brough and Taylor, 1940) and *Cycas rumphii* (De Silva and Tambiah, 1952), a linear triad is formed when the upper dyad fails to divide; whilst in *Encephalartos poggei* (De Sloover, 1961), tetrads are encountered more often than triads. Whether a linear triad or tetrad is formed, the lower-most cell becomes the functional megaspore and it is this cell which enlarges and undergoes rapid free-nuclear division to give rise to the coenocyte (Maheshwari and Singh, 1967).

While the earliest stage of megagametophyte development viewed in this study was the free-nuclear coenocyte, the next sampling interval 1 week later showed a completely cellular megagametophyte. Extraordinarily long cells were observed at this stage in the central plane of the ovule, akin to the alveolar cells described by Sokolowa (1890). The megagametophyte of *Encephalartos natalensis* therefore appears to follow a similar mode of cellularisation (i.e. by alveolus formation) to other cycads viz. *Cycas revoluta* (Ikeno, 1898), *Macrozamia reidleyi* (Light, 1924), *M. spiralis* (Brough and Taylor, 1940) and *Encephalartos poggei* (De Sloover, 1964).

The newly formed cells of the megagametophyte interior appeared essentially clear of internal content at the light microscope level, except for the occasional nucleus visible. Newly formed cells of the megagametophyte of *Dioon* (Chamberlain, 1906) and *Ginkgo biloba* (Carothers, 1907) were reportedly filled with transparent “cell sap” by those authors, while in *Taxus cuspidata* (Sterling, 1948), they were said to possess large vacuoles and a lightly staining cytomatrix. In the present study, large vacuoles were not seen in the first cells of the megagametophyte. Instead, the cells were dominated by an apparently homogeneous cytomatrix material in which various organelles (e.g. discontinuous membranes, relatively short profiles of ER, the occasional Golgi body and numerous vesicles) were seen. The organelles were mostly concentrated at the periphery of the cells, in close apposition to the developing primary cell wall, where it is suggested that they were probably involved in wall formation.

While mention has been made of the involvement of nuclei and phragmoplastic spindles in the development of the anticlinal walls during cellularisation of the megagametophyte (Carothers, 1907; Light, 1924), very little attention has been paid to the organelles involved in the deposition of cell wall components (Maheshwari and Singh, 1967; Singh and Johri, 1972). According to Light's (1924) account of *Macrozamia reidleyi*, cell plates were laid down between adjacent nuclei interconnected by phragmoplastic spindles. However, while the cell plate-like concatenation of vesicles was seen in electron-microscopic investigations of *Taxus baccata* ovules, no phragmoplastic spindles seemed to be involved in wall formation (Pennell and Bell, 1987). Those authors found evidence to suggest that the developmental path of the anticlinal walls was marked out by ER cisternae as opposed to the phragmoplastic spindles of normal cytokinesis.

Golgi bodies have largely been implicated in the synthesis and deposition of primary cell wall components during cytokinesis (Pickett-Heaps and Northcote, 1966; Moore and Staehelin, 1988; Moore *et al.*, 1991; Driouich and Staehelin, 1997). Golgi-derived vesicles ostensibly carry the precursors of wall components, which are added to the developing wall by a process of exocytosis (Pickett-Heaps and Northcote, 1966). However, those authors alluded to the fact that in some instances, the origins of the vesicles were not apparent. In the present study, Golgi bodies were particularly scarce during initial cellularisation of the megagametophyte when primary cell walls were being laid down. Both Golgi-derived and ER-derived vesicles were observed being incorporated into the developing cell membrane – with a possible involvement in cell wall formation.

Immunocytochemistry and enzyme-gold localisation techniques have been used successfully to localise specific components of cell walls in several plant species (Fultcher *et al.*, 1976; Moore *et al.*, 1986; Benhamou and Côté, 1992; Lynch and Staehelin, 1992; Sutherland *et al.*, 1999) and to identify the organelles responsible for their deposition (Lynch and Staehelin, 1992). For example, Lynch and Staehelin (1992), working on clover root tips, found xyloglucan labelling in both the cell wall and Golgi-derived vesicles, thereby implicating those vesicles in the deposition of xyloglucan in the walls.

However, when enzyme-gold localisation for the primary wall components, xylan and pectin was done in the present investigation, label was not found in Golgi stacks nor Golgi-derived vesicles, but in some ER-derived vesicles. This result suggested that ER- (and not Golgi-) derived vesicles were the most likely contributors of xylan and pectin to the developing wall, which was surprising considering that (to the knowledge of this author) ER-derived vesicles have never been implicated in the development of the primary cell wall in gymnosperms. In a study on the development of the barley endosperm, Bosnes and Olsen (1992) observed fusion of ER-derived vesicles during the initial stage of primary cell wall formation and suggested that the associated polysomes probably represent transcripts that participate in the formation of the cell wall. This reiterates the notion of Staehelin (1997), that the endoplasmic reticulum is “*the most versatile and adaptable organelle of eukaryotic cells*”.

Even more surprising than the apparent involvement of ER-derived vesicles in the formation of cell walls in the megagametophyte of *E. natalensis*, was the occurrence of a relatively high concentration of label in some ill-defined, diffuse structures within the cytomatrix. Zinc iodide-osmium tetroxide (ZIO), first used by Maillet (1962) for neurohistochemical studies, was thus

employed as it has also been shown to accentuate visualisation of the endomembrane system in plant cells (Hawes, 1991). The ZIO post-staining treatment revealed numerous, faintly-appearing vesicles within the cytomatrix. The presence of these cytomatrix vesicles seemed to be confirmed when a profusion of vesicular structures was found in the cells of the megagametophyte at the SEM level. It is proposed that these vesicles may well be the same ill-defined structures that labelled strongly for pectin and xylan.

These vesicles may ultimately fuse with ER- and Golgi-derived vesicles before their contents are incorporated into the developing cell wall, or their contents may be incorporated into the developing wall independent of the ER- and Golgi-derived vesicles. It is suggested that while the membrane component of these three vesicle types may give rise to the cell membrane, their inner contents may be incorporated into the developing cell wall. Furthermore, the presence of these faintly-appearing vesicles also suggested that the cytomatrix had a substructure that was not resolved adequately with lead citrate and uranyl acetate post-staining. For clearer resolution of these vesicles, future studies at the TEM level should incorporate the ZIO staining step to the specimens during processing – i.e. prior to embedding – as done by Machado and Gregório (2001).

The substructure of the cytomatrix was then investigated further to check for organisation in the form of microtubules. The organised nature of the cytomatrix was confirmed by the positive localisation of these structures using immunocytochemical techniques. Microtubules are purportedly responsible for the control of cytokinesis; cell wall insertion; intracellular motility; and generally for organisation within the cytomatrix (Tiwari *et al.*, 1984). Although microtubules were positively localised in the cytomatrix in the current investigation, they were not sufficiently concentrated near the cell walls or nuclei to suggest that the walls were laid down on cell plates. The path of development of the primary cell walls in this study appeared to be marked out by ER of short profile as well as discontinuous membranes, which are probably derived from ER and Golgi vesicles and which presumably join with each other to give rise to the cell membrane. At present, however, further observations are needed to confirm (or not) the implication of microtubules in spatial control.

In addition, as a caveat, further research is required using freeze-substitution as opposed to gluteraldehyde fixation to ensure that the peculiar ultrastructure seen 1 week after the coenocyte was not a feature of improper fixation – i.e. artefactual, as this stage of ontogeny is notoriously difficult to preserve (Maheshwari and Singh, 1967).

The cell walls of the megagametophyte appeared more consolidated 1 month after the coenocytic stage was observed, relative to the earlier developmental stage. There was an increase in the number of Golgi bodies and ER, and the organelles were now spread throughout the cytomatrix, as opposed to being located peripherally in the previous stage. This seemed to represent a change in focus in the megagametophyte from cell wall development to the development of the rest of the cell and quite possibly initiation of reserve accumulation. The ER appeared particularly active at this stage, producing two kinds of vesicles: one kind initially double-membrane-bound, and the other, single-membrane-bound, which seemed to be produced by terminal vesiculation as illustrated by Lamb and Berjak (1981).

The ER continued to proliferate during development from 1 to 2 months after the coenocytic stage, with numerous cisternae displaying localised luminal dilation. Several starch-containing amyloplasts were seen at this stage, and there appeared to be an accumulation of a granular material in the cytomatrix and in some of the dilated ER cisternae. Histochemical staining of mature megagametophyte (at seed-shed) sections revealed that the granular material was likely to be protein; the amyloplasts contained carbohydrate, presumably starch; and that there was a limited occurrence of small, discrete lipid bodies. The two principal storage reserves in the megagametophyte of *E. natalensis* were thus confirmed to be starch and protein, with lipid contributing a minor component. While the megagametophyte tissue of *Stangeria eriopus* (Lang, 1900), *Dioon edule* (Chamberlain, 1906) and *D. spinulosum* (Dorety, 1919) was also seen to have starch in large quantities, the low levels of lipid observed in the present investigation were surprising as gymnosperm species are said to have characteristically fat-storing seeds (Dodd *et al.*, 1989). According to Tillman-Sutela and Kauppi (Berjak pers. comm.),¹ the mature megagametophyte cells of species of conifer store significant amounts of lipid.

The protein found in this study appeared to be of two kinds. One type was apparently densely packed, quite possibly in ER distended with accumulated protein, while the other type appeared less dense or interspersed with clearer regions. Continuity was also apparent between some of the regions of denser and less dense protein deposition. This possibly indicates localised accumulations of different types of protein within regions of ER cisternae, or different stages in the deposition process.

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The next stage of megagametophyte development, approximately 3 months after the coenocytic stage, revealed an increase in the quantity of starch as evidenced by the development of the amyloplasts, while lipid bodies were now seen being produced at the terminal ends of long ER profiles. Therefore, it seems that during reserve accumulation in the megagametophyte of *E. natalensis*, starch and protein are accrued first (in amyloplasts and protein bodies respectively), followed by a limited quantity of lipid (in discrete bodies).

From 4 to 8 months after the coenocytic stage was observed, the most noticeable feature of the megagametophyte cells was the increase in quantity of starch as seen by the increase in size of the amyloplasts. The quantity of starch amongst the abundant protein increased to such an extent that the visibility of other organelles was largely obscured, and the occasional nucleus seen appeared irregularly compressed. This was in contrast to the findings of Dodd and co-workers (1989), where reserve accumulation in *Podocarpus henkelii* did not seem to obscure cytomatrical ultrastructure. Mitochondria in the current study were infrequently seen; however, when they were observed, they were characterised by well-defined cristae and dense matrices, suggesting intense, but localised metabolic potential.

The seeds of *E. natalensis* were shed approximately 8 months after the coenocytic stage was observed. The cells of the megagametophyte were apparently still metabolically active upon seed-shed. This was confirmed by a positive tetrazolium test on the megagametophyte of both fertilised seed and unfertilised, futile ovules. The tetrazolium viability test is reportedly a rapid and good measure of seed quality / viability (Kulik and Yaklich, 1982).

The degree of development and ultimately the ultrastructural integrity of mitochondria can also be used to gauge the metabolic status of cells (e.g. Kioko *et al.*, 2006). The ultrastructure of the megagametophyte cells at seed-shed was found to be in agreement with the result of the tetrazolium test as mitochondria appeared metabolically-active, possessing dense matrices and well-developed cristae. The cells of the megagametophyte were still nucleated and showed an overall maintenance of integrity even after the seeds were shed from the parent plant.

However, judging from the tetrazolium reactivity, the viability of the megagametophyte cells of both fertilised seed and unfertilised, futile ovules appeared to decline in the months after seed-shed. This too, was in line with ultrastructural observations in the megagametophyte cells of seeds 3 months after seed-shed, showing generally featureless mitochondria, and where apparent, poorly defined cristae and a pale matrix, while nuclei were rarely seen. In addition,

those cells had a more deteriorated overall appearance compared with cells at seed-shed, presumably mostly due to the utilisation of reserves by the developing embryo.

Based on the findings of the tetrazolium study, the decline in viability of the megagametophyte cells appeared to culminate in a complete loss at 6 months after seed-shed. Interestingly, even at this stage, there were regions of the megagametophyte where only a little depletion of reserve material seem to have had occurred, and occasionally apparently intact, although probably inactive mitochondria could be seen.

At 6 months after seed-shed, the megagametophyte of fertilised seed and unfertilised, futile ovules displayed contrasting tetrazolium reactivity. While there was a complete loss of reactivity in the megagametophyte of seeds, some reactivity was evident in the megagametophyte of some unfertilised, futile ovules, especially around the archegonia. This seemed to suggest that there was still some continued, albeit localised, respiratory metabolism in these otherwise futile ovules 6 months after being shed.

Two possible explanations have been identified to account for this reactivity in the ovules: (1) there may have been systemic fungi in the ovules which were actively respiring aerobically; or (2) unfertilised ovules may have retained metabolic activity after seed-shed, especially around the gamete-containing archegonia in the eventuality of post-shedding fertilisation. This suggests a further, interesting line of investigation – i.e. for how long after shedding might ovules be receptive to pollen with resultant fertilisation?

The cell layer comprising the external surface of the megagametophyte showed marked ultrastructural differences from the inner cells. These peripheral cells were packed with lipid bodies and relatively small amyloplasts as opposed to large amyloplasts and protein bodies of the underlying cells, some of the plastids in these cells contained membranous stacks akin to the thylakoids of chloroplasts. Arguably the most obvious function of a lipid-rich peripheral cell layer might be to protect the seed against loss of water as the seeds of *E. natalensis* have been suggested, and shown to be desiccation-sensitive (Forsyth and van Staden, 1983 and Woodenberg *et al.*, 2007 respectively), but since a thin band of tetrazolium stain was seen at the extreme periphery of the megagametophyte of seeds 6 months after seed-shed, this cell layer may still emerge as having an ‘aleurone-like’ function as in the endosperm of angiosperms (Lopes and Larkins, 1993). It is, however, possible that the cells of the body of the megagametophyte also participate actively – at least in the earlier stages of post-shedding seed development – in

mobilisation of stored reserves, which must support the development of the embryonic sporophyte.

4.2. Some aspects of the post-shedding seed behaviour of *Encephalartos natalensis*

The most important form of cycad reproduction is *via* the synthesis of seeds; however, cycad seeds collected from field and garden sources are often found outnumbered by unfertilised, seed-resembling ovules (personal observation; Osborne *et al.*, 1992; Donaldson, 1995) due to deficient natural pollination. Cycads were originally thought to be anemophilic (Lawson, 1926; Chamberlain, 1935) despite an earlier report (Marloth, 1914) about the entomophilous nature of some *Encephalartos* species. It has since been shown (Norstog *et al.*, 1986; Tang, 1987; Donaldson *et al.*, 1995; Donaldson, 1997; Wilson, 2002) that the pollination of many, and possibly all, cycads is entomophilic. However, with the depletion of natural cycad populations, and the scattering of these dioecious plants in gardens around the world, artificial pollination is often necessary to ensure a high yield of seeds (Osborne *et al.*, 1992). This was evident in the present study, with relatively low (c. 10%) seed yields from naturally-pollinated strobili (batch A), and superior seed yields (>80%) from those that were rigorously hand-pollinated (batch B).

The WC of batch A seeds, which were insect-pollinated, was assessed from seed-shed to 6 MAS. Seeds were shed with relatively high mean embryo (3.33 g g^{-1}) and megagametophyte ($1.25 \pm 0.16 \text{ g g}^{-1}$) WCs, which is typical of recalcitrant seeded-species (Roberts and King, 1980; Hong and Ellis, 1998). However, it has been shown (King *et al.*, 1981; Wood *et al.*, 2000) that not all seeds shed at high WCs are recalcitrant. A more reliable characteristic of recalcitrance is a loss of viability at relatively high WCs upon drying (King and Roberts, 1980).

Although Forsyth and van Staden (1983) alluded to the fact that *Encephalartos natalensis* produces 'wet' seeds and that they may be desiccation-sensitive, they did not test the desiccation sensitivity of the seeds so being able to categorise them unequivocally as being recalcitrant. This left the Seed Information Database of the Royal Botanic Gardens Kew, Millennium Seed Bank, which is presently the best collection of information on ecological and evolutionary aspects of seeds (Berjak and Pammenter, 2008), undecided about whether cycad seeds are recalcitrant or not (Liu *et al.*, 2008). However, *Encephalartos natalensis* seeds were shown to be desiccation-sensitive, i.e. recalcitrant, by Woodenberg *et al.* (2007), after the seeds lost viability at relatively high WCs when subjected to a desiccation trial. Recalcitrant seeds of many species are notoriously short-lived in storage (Chin and Roberts, 1980; King and Roberts,

1980; Farrant *et al.*, 1989; Berjak *et al.*, 1989; Bonner, 1990; Pammenter *et al.*, 1994); however, the life-span of *E. natalensis* seeds in open storage had not been determined.

It was observed in the present investigation, that whilst the embryo and megagametophyte WC of open-stored *E. natalensis* seeds declined in the months after seed-shed, the seeds remained in a relatively highly hydrated state throughout the 18-month storage period (batch B, Fig. 3.2). The decline in megagametophyte WC can probably be accounted for by the slow loss of water to the external sclerotesta and atmosphere. This was evidenced by the fact that only the decrease in fresh mass from 4 to 18 MAS was statistically significant.

On the other hand, the decline in embryo WC may also be attributed to the substantial increase in dry mass (2-fold, relative to fresh mass) as the embryos grew, such that the amount of water per gram of dry mass decreased. There was also continuous development of the embryos after seed-shed, with both the fresh and dry mass continuing to increase even after the embryos had reached a germinable size 4 to 6 MAS. This ongoing embryo development typifies recalcitrant seeds, which are always metabolically active and show continuous accumulation of dry mass until seed-shed (Hong and Ellis, 1990; Farrant *et al.*, 1992; Tompsett and Pritchard, 1993; Finch-Savage and Blake, 1994; Fu *et al.*, 1994; Lin and Chen, 1995), continuing through seed-shed to germination (Berjak *et al.*, 1984; 1989; Pammenter *et al.*, 1984). At the other extreme, orthodox seeds undergo a cessation of dry mass accumulation and enter a state of metabolic quiescence upon maturation drying before the seeds are shed (Kermode, 1990; Bewley and Black, 1994).

Although it is difficult to pin-point the timing of the transition from seed development to germination in recalcitrant seeds, it has been suggested that germinative events are initiated on, or soon after, seed-shed (Berjak *et al.*, 1984; 1989; Pammenter *et al.*, 1984). However, in the current study, when the seeds of *E. natalensis* were set out to germinate immediately after seed-shed, no germination had occurred within 30 d, presently taken as the 'cut-off' time in this study, as fungal infection invariably occurred after this period. There was little doubt that the seeds were unable to germinate immediately upon seed-shed because of the incompletely developed, virtually rudimentary embryos.

De Silva and Tambiah (1952) proposed a reason to account for the incompletely developed embryo of most cycad species at seed-shed. According to those authors, an immature, or incompletely developed, embryo would have been advantageous to those species reliant on the

ocean for dispersal e.g. *Cycas rumphii*, since such long-distance dispersal requires seeds to remain viable for a considerable period. For instance, crossing the Indian Ocean would provide sufficient time for the embryos within seeds impermeable to seawater to reach maturity.

According to Vorster (1995), cycad seeds may take from 1 month to almost 6 years (*Cycas rumphii*) after sowing to germinate; however, not all cycad species shed their seeds with an incompletely developed embryo. It has been reported (Hooft, 1970; Vorster, 1995) that most, if not all, *Zamia* species shed their seeds with fully developed embryos capable of germination soon after seed-shed, while the seeds of others e.g. *Encephalartos transvenosus* and *E. manikensis*, are shed with a fully developed embryo (Vorster, 1995).

However, even with a fully developed embryo at seed-shed, other concomitant dormancy mechanisms are known to contribute to the delayed germination of cycad seeds (Dehgan and Schutzman, 1989). According to those authors, dormancy mechanisms include a fleshy sarcotesta, which apparently contains unspecified inhibitors, and a thick, woody sclerotesta in addition to the under-developed embryo of some species at seed-shed. Nicolaeva (1977) has referred to such dormancy contributed by several factors as “morphophysiological complex dormancy”.

Working on the seeds of *Zamia floridana*, Hooft (1970) discovered that the sarcotesta and sclerotesta were impermeable to water uptake and interfered with germination for 5 to 6 months after seed-shed. Noting that seeds of *Zamia* spp. are shed with fully developed embryos (Vorster, 1995), removal of the sarcotesta shortened germination time to 50 to 60 d, whereas removal of both the external seed coverings permitted immediate germination (Hooft, 1970).

It has also been reported that the germination of cycad seeds may also be enhanced by scarification of the sclerotesta using sulphuric acid (H_2SO_4) for various lengths of time (Dehgan, 1983; 1984; Dehgan and Johnson, 1983). However, scarification treatments would be ineffectual in facilitating germination of seeds with incompletely developed embryos immediately upon seed-shed, as was shown in the current study when newly shed seeds were unable to germinate after both the sarcotesta and sclerotesta were removed.

Treatment of seeds containing incompletely developed embryos with gibberellic acid (GA_3) has been shown to improve germination in some cycad species (Dehgan and Johnson, 1983), which has been attributed to the breakdown of physiological dormancy (Khan, 1975; Lewak, 1985).

During the present investigation GA₃ was not employed, as the objective was to assess the post-shedding seed longevity and behaviour as opposed to improvement of germination *per se*. However, future studies will entail investigations on the germinative response of *E. natalensis* seeds with incompletely developed embryos to GA₃, as, to the knowledge of this author, such studies have not previously been carried out on the seeds of *Encephalartos* species.

In the present study, while no seeds were capable of germinating immediately after seed-shed, about 50% were capable of germinating within 30 d after removal of the sclerotesta at 2 MAS. Seeds stored for 2 months also took relatively long to initiate germination (c. 24 d), whereas seeds stored for longer periods initiated germination within 6 to 12 d. Thus, the seeds of *E. natalensis* were found to exhibit the timing of germination-related processes characteristic of seeds classed in group C (very slow germination after shedding) of the model proposed by Berjak *et al.* (1989).

Four-month stored seeds initiated germination earlier and displayed a more rapid germination rate and improved totality (c. 90%) compared with seeds stored for less time after seed-shed, while the highest viability (i.e. 100%) was seen at 6 MAS. Thereafter, the viability of the seeds decreased somewhat to the region of about 60 to 80% at 8 to 16 MAS, while 25% of 18-month stored seeds were still viable. This result seemed to suggest that the optimal time after seed-shed for the germination of *E. natalensis* seeds is around 4 to 6 months, where most of the embryos are fully developed and capable of germinating vigorously.

While the reduced levels of vigour and viability seen before the optimal storage period for germination are ascribed to the under-developed nature of the embryo at seed-shed, the reduction in seed vigour and viability after storage periods longer than 6 MAS is probably a result of stress. Germination of the embryos was probably impeded by the thick sclerotesta during open storage where no extraneous water was provided, and the slightly slower rate of germination observed from 8 to 16 MAS may be indicative of the need for repair prior to germination.

In comparison with the majority of other recalcitrant-seeded species, which have a shelf-life in open storage of weeks to a few months (e.g. Chin and Roberts, 1980; King and Roberts, 1980; Berjak *et al.*, 1989; Farrant *et al.*, 1989; Bonner, 1990; Pammenter *et al.*, 1994), the seeds of *E. natalensis* in this study were relatively long-lived. This is suggested to be a consequence of the under-developed embryo at shedding, which must undergo post-shedding development (for

about 6 months) before the initiation of germination (Berjak *et al.*, 1989), as well as the maintenance of a relatively high WC in the months after seed-shed as the consequence of a thick sclerotesta that is largely impervious to water.

While the seeds in the present investigation were relatively long-lived, the vigour and viability of 18-month stored seeds was reduced considerably compared with 8- to 16-month stored seeds. It is still uncertain whether this is an indication of the characteristic life-span of *E. natalensis* seeds being exceeded or not, as many *E. natalensis* seeds were observed to have germinated in sand when sown 24 MAS (personal observation, results not shown).

The cause for the ultimate loss of vigour and viability of hydrated recalcitrant seeds was proposed by Pammenter and co-workers (1994). Those authors suggested that because hydrated recalcitrant seeds are metabolically active and undergo germination-associated changes in storage, they sooner or later (depending on the rate of these changes) require water additional to that present in the seed at shedding. As exogenous water is not available during open storage, the *E. natalensis* seeds are suggested to have suffered mild, but increasingly prolonged, water stress, which ultimately led to the death of the embryos by uncontrolled free-radical-mediated oxidative damage (Pammenter *et al.*, 1994).

Berjak and co-workers (1989) have suggested that recalcitrant seeds be kept as close to their shedding WC as possible to maximise their longevity in storage. However, in the present study, seeds were not placed in hydrated storage at seed-shed and following rigorous decontamination, but only after 6 months of open storage. Therefore, the potential of responses and viability retention of the seeds under the strictly controlled hydrated storage conditions generally used for recalcitrant seeds (Berjak and Pammenter, 2004) were not determined.

When *E. natalensis* seeds were placed in hydrated storage after 6 months in open storage, both the embryo and megagametophyte WCs appeared to increase relative to those of seeds left in open storage at 10 MAS. However, the increased embryo and megagametophyte WCs were not significantly different from those of open-stored seeds.

Similarly, the apparent increase in the embryo fresh and dry mass of hydrated-stored seeds was not significantly different from the embryo fresh and dry mass of open-stored seeds. Even though the WC, fresh and dry mass of embryos of hydrated-stored seeds were not significantly different from those of open storage, the apparent increases, along with the degree of

uncertainty in the mean, suggest that there may have been a significant difference if there was little or no experimental error.

While there was no significant difference in the embryo fresh and dry mass of hydrated-stored seeds relative to open-stored seeds at 8 and 10 MAS, the difference in these parameters for the megagametophyte was significantly different. However, this did not seem to have an impact on the WC of the megagametophyte at 10 MAS, as it was not significantly different from that of open-stored seeds. It would be expected that the fresh mass of the megagametophyte would increase in the months after seeds were placed in hydrated storage since seeds are hygroscopic (Roberts, 1972; Justice and Bass, 1978), with their WC usually coming to equilibrium with that of the surrounding atmosphere. However, in the present investigation, surprisingly the dry mass of the megagametophyte was also found to increase. Thus, the apparent increase in WC of the megagametophyte was not in direct proportion to the significant increase in fresh mass observed when seeds were placed in hydrated storage. It is suggested that the increase in dry mass of the megagametophyte may be explained by the unavoidable inclusion in the calculations, of the dry mass of fungi, which were also seen to affect the germination of the seeds adversely. Viability of the seeds after 2 months in hydrated storage was affected to such an extent that no germination occurred before the seeds were overwhelmed by fungi.

Even orthodox seeds are reported to harbour a variety of fungi at seed-shed (McLean and Berjak, 1987), which resume vigorous activity when the seeds become wet (Christensen and Kaufmann, 1969, 1974). Although comprising different species, a spectrum of fungi has been identified, consistently associated with recalcitrant seeds of many species in South Africa (Mycock and Berjak, 1990; Sutherland *et al.*, 2002). This was also evident in the present study, as fungal proliferation in hydrated storage was rife despite the application of Benlate[®].

Benlate[®] is reportedly a systemic fungicide which, even when applied to the surface of seeds as dust, is able to reduce fungal infections within seeds (Maude, 1983). The active ingredient, benzimidazole, has reputedly reduced fungal species of the genera, *Fusarium* (Vidhysekaran, 1983; Nakagawa and Yamaguchi, 1989; Champawat, 1990; Hawara and Kannaiyan, 1992; Wilson *et al.*, 1993); *Penicillium* (Wilson *et al.*, 1993) and *Aspergillus* (Gupta *et al.*, 1993) in seeds. In the present study, however, no fungal identification was undertaken (which will be remedied in future investigations), but surface application of Benlate[®] was ineffective in curbing fungal activity.

Forsyth and van Staden (1983) did not report any fungal proliferation during their experiments involving the hydrated storage of *E. natalensis* seeds. This may highlight the point made by Berjak and Pammenter (2004) that the quality of seeds at the outset is important. Seeds in the present study may have been of inferior quality in terms of contamination when shed, compared with those used in the study of Forsyth and van Staden (1983). Those authors found that storing the seeds in hydrated conditions at 20°C promoted embryo growth, where the embryo could mature relatively quickly and, on transfer to a suitable incubation temperature (30°C), could germinate within 3 weeks (Forsyth and van Staden, 1983).

In the present investigation, while no germination occurred after 2 months in hydrated storage without the seeds being overwhelmed by fungal proliferation, protrusion of the hypocotyl and germination earlier, during the course of hydrated storage was, observed in some cases. The hypocotyl was able to protrude despite the physical barrier imposed by the sclerotesta. This suggests that even with the eradication of seed-associated fungi, the seeds of *E. natalensis* cannot be stored in hydrated conditions for extended (months to years) periods. This is not only a problem with *E. natalensis* seeds, but recalcitrant seeds in general (Berjak and Pammenter, 2004).

4.3. Concluding remarks and future research

Several interesting findings emerged from the current investigation. The first was that a study on the post-shedding seed behaviour of *E. natalensis* needs to start at least 6 months before seed-shed, because, at least in the *ex situ* situation, the strobili require hand-pollination to obtain a good yield of seed (>80%) on which to conduct experiments. Since fungi were found to proliferate in hydrated storage despite decontamination treatment using sodium hypochlorite, followed by surface application of a fungicide, future studies should also develop appropriate decontamination treatments from as early on in seed development as possible. Anti-fungal measures should be attempted preferably before pollination, as fungal spores that give rise to systemic fungal infections in the seeds may well be drawn into the ovule along with the pollen grains as has been shown for the desert gymnosperm, *Welwitschia mirabilis* (Whitaker *et al.*, 2008).

Another finding of the present study was that the seeds of *E. natalensis* were shed with relatively high mean embryo (3.33 g g⁻¹) and megagametophyte (1.25 ± 0.16 g g⁻¹) WCs, when the embryo was rudimentary and incapable of germination. The embryos continued to develop

after seed-shed, reaching a germinable size (≥ 15 mm) only 4 to 6 months after seed-shed. It will be of interest to explore the use of gibberellic acid in hastening embryo maturation and overcoming the effective dormancy as achieved by Dehgan and Johnson (1983) for *Zamia floridana*, for application in the horticultural industry.

Although *E. natalensis* has been found to produce recalcitrant seeds (Woodenberg *et al.*, 2007), which are characteristically short-lived (Chin and Roberts, 1980; King and Roberts, 1980; Farrant *et al.*, 1989; Berjak *et al.*, 1989; Bonner, 1990; Pammenter *et al.*, 1994), the current contribution showed that a study on the post-harvest seed behaviour of *E. natalensis* needs to be longer than 18 months in order to record the lifespan of the seeds in open storage. This may well have implications for *in situ* as well as *ex situ* conservation of endangered cycad species in general.

It would also be of interest to compare and contrast the storage lifespan of seeds that have an intact sarcotesta, with those that have this external layer removed, as the sarcotesta has been shown to be inhibitory to germination (Dehgan and Schutzman, 1989). In the present investigation, *E. natalensis* seeds were found to germinate in hydrated storage, which precludes storage of these seeds in a hydrated environment. However, the seeds were put into hydrated storage only 6 months after seed-shed and not immediately upon shedding as recommended by Berjak and co-workers (1989) for recalcitrant seeds. It will be informative to ascertain characteristics of the storage lifespan of sarcotesta-enclosed *E. natalensis* seeds that are placed in hydrated conditions immediately after seed-shed.

While the current study has characterised the post-harvest seed behaviour of *E. natalensis* and supports the notion that the seeds of this species are recalcitrant, the post-shedding seed behaviour of many *Encephalartos* species remains a mystery. It has been reported (Vorster, 1995) that *Encephalartos manikensis* and *E. transvenosus* produce seed with fully developed embryos at seed-shed. It will therefore be intriguing to assess, compare and contrast the post-harvest behaviour of as many species of *Encephalartos* as possible and to see whether or not they produce desiccation-sensitive seeds. This will be of paramount importance if suitable storage regimes are to be devised for the long-term conservation of cycads, in line with Target viii of the Global Strategy for Plant Conservation (of the Convention on Biodiversity) which advocates that 60% of all threatened plants should be in accessible *ex situ* collections by 2010.

Ultrastructural and viability assessments in the current investigation showed that the cells of the megagametophyte were nucleated and contained a few apparently-active mitochondria at seed shed. The metabolically active status of these cells was confirmed by the tetrazolium (TTZ) test. While the viability of the megagametophyte cells of fertilised seed appeared to decline slowly in the months after seed-shed, the archegonia of shed, futile ovules appeared to remain metabolically active even 6 months after abscission from the strobilus. Future research should thus aim to find out whether the shed, futile ovules would still be receptive to post-shedding, artificial pollination.

Since the megagametophyte tissue of seeds was apparently metabolically active during the early stages of post-shedding seed development, this tissue could participate actively in the mobilisation of stored reserves during embryo growth. In addition, the cell layer comprising the external surface of the megagametophyte showed marked ultrastructural differences from the inner cells, and may emerge as having an 'aleurone-like' function.

While the peripheral cells of the megagametophyte were dominated by lipid bodies, the cells of the body of the megagametophyte became progressively more packed with starch and protein as the two main storage reserves, with a limited number of discrete lipid bodies, and occasional mitochondria, all of which appeared to be embedded in a homogeneous cytomatrix. It was found that the unusual cytomatrix was present from the inception of megagametophyte cellularisation, and apparently contained microtubules, and numerous very faintly-visible vesicles. While further observations are needed to confirm (or not) the implication of microtubules in spatial control of the organelles during wall formation, the faintly-visible vesicles may appear more distinct if future studies employ zinc iodide-osmium tetroxide (ZIO) as a block stain instead of a post-stain as done in the current investigation.

It was apparent from this study that cellularisation of the *E. natalensis* megagametophyte occurred over a period of about 1 to 2 weeks, with the rest of the 8-month-long ovule development on the parent plant dominated by reserve accumulation whether pollination/fertilisation had taken place or not. With the use of enzyme-gold localisation, it appeared as though ER-derived vesicles (and not Golgi-derived vesicles) were the principal contributors of the primary cell wall components, pectin and xylan during cellularisation. However, this finding is contrary to the popular understanding (Pickett-Heaps and Northcote, 1966; Moore and Staehelin, 1988; Moore *et al.*, 1991; Driouich and Staehelin, 1997) that Golgi bodies are responsible for the synthesis and deposition of the primary cell wall components.

Also, because of the relative scarcity of Golgi bodies and peculiar homogeneous appearance of the cytomatrix during the initial stages of megagametophyte ontogeny, future research must employ freeze-substitution techniques to fix material as opposed to the conventional gluteraldehyde fixation used in this study. This approach should resolve whether the relevant findings of the present study are artefactual, or whether the synthesis and deposition of primary cell wall components is indeed, almost unique in *E. natalensis*, thus meriting further investigations on early megagametophyte ontogeny across a spectrum of cycad species.

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