SOME ASPECTS OF DEVELOPMENT AND CELL WALL PROPERTIES OF THE DESICCATION-SENSITIVE EMBRYOS OF *Encephalartos natalensis* (ZAMIACEAE)

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ABSTRACT

The present investigation can be divided into two main sections: the first dealing with the post-shedding embryogenesis of *Encephalartos natalensis* and the second concerned with the cell wall properties of immature and mature embryos of this species.

Development of the embryo of *E. natalensis* from a rudimentary meristematic structure approximately 700 μm in length, extends over six months after the seed is shed from the strobilus. Throughout its development the embryo remains attached to a long suspensor. Differentiation of the shoot meristem flanked by two cotyledonary protuberances occurs over the first two months, during which peripheral tannin channels become apparent. Tannins, apparently elaborated by the endoplasmic reticulum, first accumulate in the large central vacuole and ultimately fill the channel. By the fourth month of development the root meristem is apparent and procambial tissue forming discrete vascular bundles can be discerned in the elongating cotyledons. Between four and six months, mucilage ducts differentiate, and, after six months when the seed becomes germinable, the embryo is characterised by cotyledons far longer than the axis. Shoot and root meristem cells remain ultrastructurally similar throughout embryo ontogeny, containing small vacuoles, many well-differentiated mitochondria and ER profiles, abundant polysomes, plastids containing small starch deposits and Golgi bodies. Unusually however, Golgi bodies are infrequent in other cells including those elaborating mucilage which is accumulated in distended ER and apparently secreted into the duct lumen directly by ER-derived vesicles. The non-meristematic cells accumulate massive starch deposits to the exclusion of any protein bodies, and only very sparse lipid, features which are considered in terms of the prolonged period of embryo development and the high atmospheric oxygen content of the Carboniferous Period, when cycads are suggested to have originated.

With regard to plant cell walls, the present investigation employed immunofluorescence microscopy and immunocytochemistry to characterise the cell walls of immature and mature embryos of the recalcitrant-seeded *E. natalensis* to determine wall composition and potential changes with development. These techniques, together with cryo-scanning- and transmission-electron microscopy (TEM) were used to analyse potential changes in the cell walls of mature embryos upon desiccation. Immature cell walls appeared to be composed of low- and high methyl esterified epitopes of pectin, rhamnogalacturonan-associated arabinan, and the hemicellulose xyloglucan, while partially-esterified epitopes of pectin appear to have a

punctuate distribution in the wall. Arabinogalactan protein recognised by the LM2 antibody, along with rhamnogalacturonan-associated galactan and the hemicellulose xylan, were not positively localised using immunological probes, suggesting that the embryo of the current species does not possess these epitopes. Interestingly, mature embryos appeared to be identical to immature ones with respect to the cell wall components investigated, implying that these may not change during the protracted post-shedding embryogeny of this species. Analysis of the monosaccharide composition of the walls by gas liquid chromatography complemented the immuno-labelling work. However, there appeared to be abnormally high levels of glucose (Glc), which may indicate the presence of Glc-rich polymers not accounted for by the antibodies used in the current study. Preliminary Glc-normalised data revealed that there may be considerable quantities of arabinose polymers in the wall comparable to that found in desiccation tolerant plants. Drying appeared to induce some degree of cell wall folding in mature embryos, correlating with their possession of wall plasticisers such as arabinose polymers, but this was limited, due to the abundance of amyloplasts, which filled the cytoplasmic space. From the results of this study, it is proposed that the embryo cell walls of *E. natalensis* are constitutively prepared for the flexibility required during cell growth and expansion, which may facilitate the observed moderate cell wall folding in mature embryos upon drying. This, together with an abundant supply of amyloplasts in the cytomatrix may provide sufficient mechanical stabilisation during desiccation even though the seeds of this species are highly desiccation sensitive.

Overall, this study has been a relatively comprehensive coverage of histological and ultrastructural aspects of embryogenesis in *E. natalensis*. This work will form a pivotal basis for future studies, which may ultimately lead to the successful germplasm cryopreservation and *in vitro* production on a commercial scale of these, and other, endangered cycad species. Furthermore, the work on cell walls in this investigation has provided improved comprehension of the responses of seed cell walls to dehydration.

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The work in Chapter 3 of this thesis is presently being prepared by W.R. Woodenberg as the following two independent manuscripts.

Publication 2: Woodenberg WR, Berjak P, Pammenter NW, Driouich A, Farrant JM. Development of cycad ovules and seeds. 3. Immunofluorescence and immunogold labelling of cell walls in the desiccation-sensitive embryos of *Encephalartos natalensis* (Zamiaceae) Dyer and Verdoorn

Publication 3: Woodenberg WR, Berjak P, Pammenter NW, Nguema-Ona E, Farrant JM. Development of cycad ovules and seeds. 4. Monosaccharide composition of cell walls in the mature embryo of *Encephalartos natalensis* (Zamiaceae) Dyer and Verdoorn

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LIST OF CONTENTS

CHAPTER 2

CHAPTER 3

Embryo cell wall properties in relation to development and desiccation in the recalcitrant-seeded *Encephalartos natalensis* (Zamiaceae) Dyer and Verdoorn

CHAPTER 1

INTRODUCTION

1.1. The cycads

Cycads are the most primitive extant spermatophytes (Brenner *et al.* 2003). The members of this family, the Zamiaceae, possess a combination of features that give them the appearance of being a synthetic group of vascular plants (Vashishta 1995). In certain respects its members resemble the ferns and the pteridosperms; in others they resemble the Bennittitales, Pentoxylales, Cordaitales and Ginkgoales (Vashishta 1995). Some cycads bear a resemblance to ferns due to the appearance of the large frond-like leaves (that may be up to 3 m long in *Cycas circinalis* [Vashishta 1995]), but most modern cycads bring to mind palm-trees in having a stout trunk with a crown of pinnate fronds at the apex (Sporne 1965). However, while they may have a general appearance which is readily identifiable by most people, they are usually wrongly linked to ferns and palms (e.g. 'Sago palm'), when, in fact, they are not related to either. Cycads actually constitute a unique assemblage of plants and although they are grouped with the gymnosperms they are considered to be unrelated to any group of living plants (Jones 1993) and may represent a transitional stage between ferns and other gymnosperms (Bhatnagar and Moitra 1996).

Nevertheless, cycads are true gymnosperms in that their seeds are naked and borne on modified leaves, the sporophylls (Bhatnagar and Moitra 1996). These plants are dioecious and vary in height from a few centimetres to several metres (Vovides *et al.* 2003). Cycads are mostly unbranched, but in some species there are occasional adventitious branches. Several species attain a height of 10 to 15 m, e.g. *Dioon spinulosum* and *Microcycas calocoma*, while the tallest of all is *Macrozamia hopei* at 18 m; some have subterranean stems which are short and tuberous and which branch in an irregular manner, e.g. *Stangeria* and *Bowenia* and several species of *Zamia*, *Macrozamia* and *Encephalartos* (Sporne 1965), while one species, *Zamia pseudoparasitica*, is epiphytic (Arnold 1953) on tropical rainforest trees in Panama (Vovides *et al.* 2003).

Members of the Cycadaceae are typically slow-growing and have very long life spans e.g. a 1.8-2 m tall plant of *Dioon* has been estimated to be one thousand years old (Vashishta 1995); a plant of *Macrozamia* takes about 100 years to reach a height of 1 m (Schuster 1932), while *Encephalartos* takes 200-300 years to grow to a height of 1.5-1.75 m (Biswas and Johri

1997). Their slow growth has been suggested to be due to their xerophytic habit (Vashishta 1995), with all species showing strong xeromorphic structures (even those that grow in dense forests).

Some of the xeromorphic features that cycads possess include a heavily cutinised epidermis and deeply sunken stomata (Sporne 1965) and tuberous, underground stems or more usually short and unbranched aerial stems with persistent leaf bases, which, combined with a thick cortex, are considered to insulate the trunk effectively (Bhatnagar and Moitra 1996). These xeromorphic features adapt cycads well against environmental stresses such as drought and fire, as well as protecting against predators and pathogens. It is thought that their secondary chemical compounds may also contribute to their tolerance of such threats (Fosberg 1964; Brenner *et al.* 2003).

Compared with spermatophytes generally, cycads display other unusual and some unique features, e.g. motile antherozoids, cyanobacterial symbionts and elaboration of particular toxins (Ikeno 1896; Osborne *et al.* 1988; Osborne 1995): these, and their morphological features, are considered to be related to their ancient lineage (Vashishta 1995).

1.1.1. Origins

Paleontological investigations have placed cycads in the first diverging group of spermatophytes, the Cycadophytes (Brenner *et al.* 2003). It was believed that the earliest relatives of cycads may have arisen as early as in the Pennsylvanian subdivision of the Carboniferous Period (Norstog and Nicholls 1997; Hermsen *et al.* 2006), approximately 300 million years ago (MYA) (Chaw *et al.* 2005). However, after recent analyses, the identity of many fossil cycads appears doubtful and it has been suggested that these probably represent intermediates in the evolutionary process (Osborne 2002). The contemporary belief is that while cycads have a substantial fossil record dating back at least 70 MYA (Rai *et al.* 2003), the fossil record of the living Cycadales extends only to the Tertiary Period (Brenner *et al.* 2003). However, while the cycads of today are a mere remnant of a family that once dominated the Earth's vegetation (Bhatnagar and Moitra 1996), they still represent the oldest surviving lineage of spermatophytes and hence play a significant role in our understanding of the evolution of morphological characters in plants (Brenner *et al.* 2003).

1.1.2. Diversity and distribution

The extant cycads are restricted to the subtropical and tropical regions of the world with ca 289 species (Whitelocke 2002). However, it is believed that the number of species may reach as many as 400 with the completion of taxonomic studies and when all possible cycad habitats have been explored (Vovides *et al.* 2003).

All extant cycad genera were initially classed in a single family, the Cycadaceae. Later, cycads were divided into three families (Chaw *et al.* 2005) with *Cycas* placed in the suborder Cycadineae, and Stangeriaceae and Zamiaceae assigned to the suborder Zaminiineae (Stevenson 1992).

Currently, the Cycadaceae has one genus (*Cycas*), found along the West African coast, Madagascar, Asia, northern Australia and many islands of the Pacific Ocean. The Stangeriaceae has two genera: *Stangeria* being the one genus in Africa, and *Bowenia* with two species endemic to Australia. The largest family is the Zamiaceae with 202 species in eight genera (Hill *et al.* 2004). The Zamiaceae is also the most diverse cycad family with respect to geographical distribution (Donaldson *et al.* 2003), suggesting that the grouping may have existed before fragmentation of the super continent, Pangaea. *Encephalartos*, which is the only genus of the Zamiaceae occurring in Africa (Goode 2001), is the second largest cycad genus with 65 species (Hill *et al.* 2004), although Cooper and Goode (2004) recently documented 70 species. Thirty eight species of *Encephalartos* are found in South Africa (Donaldson *et al.* 2003). The greatest diversity of cycads is found in Australia (ca 78 species), Mexico (ca 45 species), and South Africa (ca 40 species) (Vovides 2000).

Cycads of today have a fragmented distribution and form an inconspicuous part of the vegetation, which may further allude to their antiquity (reviewed by Vashishta 1995; Bhatnagar and Moitra 1996). However, while cycads have survived for a long period, their numbers have declined steadily in recent years and many species face the risk of extinction (Brenner *et al.* 2003).

1.1.3. Conservation status

Cycads are now a group of global conservation significance and are included in the red data book (Golding and Hurter 2003). While the 1997 IUCN Red List of Threatened Plants placed 12.5% of the world's vascular plants in one of the threatened categories (Walter and Gillett 1998), more than 80% of cycad species were recognised as threatened (Donaldson *et al.*

2003). According to van Schalkwyk (2007), as many as 71% of the critically endangered plants in South Africa are cycads. Eleven of the 38 cycad taxa represented in South Africa are critically endangered, while six are endangered, two are vulnerable and 15 are protected (van Schalkwyk 2007). New species of cycads have also been discovered in Africa, especially in areas of southern Africa to the north of the Limpopo River, but many of these are on the verge of extinction in their natural habitat (Goode 2001; Donaldson *et al.* 2003). Moreover, at least two African species, *Encephalartos woodii* and *E. relictus*, are confirmed as extinct in the wild (Dyer 1965; Osborne 1986).

1.1.4. Reasons for decline

The reasons for the decline in cycad numbers are many and varied. While there are some species that are dying out naturally, two main reasons have been cited for the decline in numbers: 1) the removal of plants from their habitat by traders, landscapers and collectors because of their monetary value and aesthetic appeal; and 2) the disturbance and destruction of natural habitat for the building of dams and roads, agricultural development, commercial forestation, as well as urban and rural housing developments (Osborne 1995; Donaldson *et al.* 2003; Vovides *et al.* 2003).

Other factors, such as the collection of cycads by traditional healers for medicinal purposes and alleged magical properties, and ecological disturbances like fire regime and invasive alien plants have also been implicated in the diminishing cycad populations (Jones 1993). It has also been demonstrated that pollinator survival may be related to the composition and size of cycad populations and that decreasing cycad population sizes could lead to decreasing pollinator populations and *vice versa* (Donaldson 2004). Thus decreasing numbers in specialist cycad pollinator populations may also contribute to the decline of cycad numbers.

Cycads usually display a comparatively high degree of endemism (Dyer 1965; Schneider *et al.* 2003) and while the plants may produce large numbers of seeds, reproductive failure appears to be a concern as there is comparatively little germination in the field with very few seedlings being observed (Forsyth and van Staden 1983; Giddy 1990). According to Tang (1987), about 90% or more of young cycad plants may succumb to stress, competition and attack by predators. In addition, cycads may take up to 15 years to reach sexual maturity (Tang 1990; Vogel *et al.* 1995; Norstog and Nicholls 1997). Hence, cycad populations grow naturally at a relatively slow rate and in order to avert the complete loss of these plants, the majority of cycads are now protected by law (Giddy 1995).

1.1.5. Legislation

Cycads in South Africa are protected under the National Environment: Biodiversity Act of 2004 (van Schalkwyk 2007). Internationally, trade in cycads is censored 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. This implies that no trade is allowed in plants or seed collected from the wild. Trade is restricted to licensed cycad nurseries and, while 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). More than one-third of South African species are subject to the most severe protective legislation, while the rest (including their seeds) are protected by a second class of restrictions (Schlegel 1991).

However, while protective legislation has been implemented, surprisingly little research has been undertaken on cycad seeds in spite of the apparent need for the conservation of these plants (Vorster 1995).

1.1.6. Reasons for lack of research on cycad seeds

Cycads have received little consideration from morphologists, systematists and seed biologists since the major studies by Chamberlain (1935); however, a deficiency of interest cannot be blamed for lack of research. One of the main reasons for the dearth of research on cycad seeds has been a lack of availability of suitable material in sufficient quantities for experimental purposes (Dehgan and Schutzman 1989; Stevenson 1990). The plants are generally rare and usually encountered as prized specimens in botanical gardens (Stevenson 1990), so acquiring seeds for experimental purposes is not easy. In addition, more than one-third of known species are subject to the most severe protective regulations (as mentioned previously), and the remaining, including their seeds, are governed by the second category of restrictions (Schlegel 1991).

It is also extremely difficult to ascertain whether cycad seeds are fertilised or not. In angiosperms, reserve deposition in the seed happens after fertilisation and is accompanied by growth to physiological maturity, at which stage mature angiosperm seeds are shed and, unless dormant, will germinate when conditions are favourable (Bewley and Black 1994). However, cycads are gymnosperms, and in most cases their ovule morphology differs very

little, if at all, from that of the fertilised seed (e.g. Woodenberg 2009). In some gymnosperm species, e.g. *Encephalartos natalensis* Dyer and Verdoorn, the subject of the present study, all the necessary reserves are laid down in the megagametophyte before fertilisation, and when shed the propagules may not contain an embryo (personal observation). This is a challenge in cycad seed research, because unless pollination by hand has been undertaken, there is always great variability in the number of fertilised seeds, or there are low actual seed numbers because of the inefficiency of natural pollination in garden settings (personal observation).

The seeds of most cycad species also need several months to germinate (Dehgan 1983, 1984; Forsyth and van Staden 1983). At least three, and possibly four, simultaneous dormancy mechanisms add to their delayed germination. These include a fleshy sarcotesta that may contain unspecified inhibitors (Brown 1966); a thick and 'stony' sclerotesta; and, in many species, an immature embryo at seed-shed (Dehgan 1983; Dehgan and Johnson 1983; Dehgan and Schutzman 1983; Dehgan and Yeun 1983). Nicolaeva (1977) has referred to such dormancy as "morphophysiological complex dormancy".

Despite – or because – of the aforementioned challenges that have restricted investigations on cycad seed research previously, much research is needed to characterise seed development and for the preservation of cycad germplasm, ultimately for the restoration of cycad populations via improved propagation.

1.2. Seeds

A seed possesses all the information necessary to yield an entire plant of its species. In addition, seeds provide vital intraspecific genetic variability. Hence, they are considered to be the most important propagatory unit of plants (Berjak and Pammenter 2004).

The seeds of most species pass through three main phases of development; the first is histodifferentiation, which is characterised by rapid mitosis, cell development and tissue differentiation (Adams and Rinne 1980). This phase is started by fertilisation – and while it leads to the creation of an embryo and its nutritive tissue in angiosperms – in gymnosperms, here exemplified by the cycads, formation of the nutritive tissue is not a consequence of fertilisation, but is maternal and constituted by the female gametophyte. In angiosperms, histodifferentiation is followed by the second phase, which is characterised by a substantial accumulation of reserves, cell expansion and increasing fresh and dry mass of the seed

(Bewley and Black 1994). The third phase is usually characterised by a deceleration and ultimate termination of dry mass accumulation, with a noticeable decline in fresh mass and is aptly known as maturation drying as the seed may lose up to 90% of the water it originally contained (Adams and Rinne 1980).

The following is a description of the morphological and anatomical aspects of the first two phases of seed development (mainly embryogenesis) across angiosperms and gymnosperms, before a discussion of the third phase and its implications in the present investigation.

1.2.1. Embryogenesis

One of the main differences between early embryogenesis of angiosperms and gymnosperms is that the latter usually display a free-nuclear phase, whereas in angiosperms generally, wall formation follows division of the zygote (Johansen 1950). However, exceptions do occur in both groups. Among the gymnosperms such as *Sequoia*, *Gnetum*, and *Welwitschia*, mitosis of the zygote is followed by cell wall formation (Buchholz 1939; Maheshwari and Vasil 1961), and among the angiosperms *Paeonia* appears to have a free-nuclear phase (Wunderlich 1966).

1.2.1.1.Angiosperms

A characteristic feature of fertilisation in angiosperms is the participation of two male nuclei in what is termed double fertilisation (reviewed by Bewley and Black 1994). In this process, one nucleus exits the pollen tube and fuses with the egg nucleus forming a diploid zygote, while the other fuses with two polar nuclei to give rise to a triploid nucleus (pentaploid 4n + n in *Lilium*). While double fertilisation in this way is unique to angiosperms, a somewhat similar double fusion has now been found in a gymnosperm, *Ephedra* (Friedman 1990), as will be discussed later.

The diploid zygote of an angiosperm develops into an embryo, while the polyploid endosperm cell undergoes a series of mitoses to form a tissue known as the endosperm. This tissue is nutritive in function and will be used by the embryo in further growth towards germination (Bewley and Black 1994). In some seed-types, the endosperm remains a major component and is used by the embryo only at germination (e.g. *Zea mays*); however, in other seed-types, (e.g. *Phaseolus vulgaris*), all of the endospermic nutrients are transferred to the cotyledons of the growing embryo, which therefore become very large (Vijayaraghavan and Prabhakar 1984; Lopes and Larkins 1993).

Two sub-divisions exist in the angiosperms named for the number of cotyledons of their members: The Monocotyledonae (monocotyledons [monocots]) and Dicotyledonae (dicotyledons [dicots]) (reviewed by Bewley and Black 1994), which have one and two cotyledons, respectively, and since embryogenesis has been found to differ between the two divisions, they will be discussed separately here.

Dicotyledons

Schnarf (1929), Johansen (1945) and Maheshwari (1950) recognised five main categories of early stage embryo development among dicotyledons: (1) the Crucifer or Onagrad type; (2) the Asterad type; (3) the Solanad type; (4) the Caryophyllad type; and (5) the Chenopodiad type. For a more detailed review of the five categories the reader is referred to a publication by Sharma (2009); however, an overall summary of dicot embryogenesis reads as follows.

After numerous mitotic divisions of the zygote, a sphere of cells is formed. This is referred to as the globular stage of embryogenesis, which is also characterised by the onset of tissue differentiation. While the outer layer of cells, known as the protoderm in the globular stage, yields the epidermis, the inner cells differentiate to give rise to the procambium and ground meristem of the embryo. Subsequent development by cell division and cell expansion then ensues and the embryo transforms into what is described as the heart stage of embryogenesis. The shape of the heart stage is brought about by the differentiation and elongation of the cotyledonary primordia. The cotyledons grow by further mitoses and expansion of the primordial cells, along with some additional development by cell division and growth of all the other cells of the embryo. The whole embryo consequently elongates, which gives rise to this stage being referred to as the torpedo stage of embryogenesis. After this, the axis tissue continues to differentiate, such that the apical shoot meristem (bearing cotyledons on either side) is evident, as is the radicle, which terminates in the root apical meristem. These two apical meristems separate from one another as a result of division and elongation of the cells between them, while the cotyledons continue to elongate substantially. When these events have occurred, histodifferentiation ceases and the embryo is considered to be fully formed.

Monocotyledons

A number of noteworthy publications by Souéges (see Maheshwari 1950) gave valuable insight into the embryogenesis of various monocots, viz. *Luzula forsteri* (1923); *Poa annua*

(1924); *Sagittaria* (1931) and *Muscari comosum* (1932). From these and other studies, the following information has been formalised.

The early stages of embryogenesis are basically similar in monocots and dicots. The initial mitosis of the zygote forms an apical and basal cell, which are precursors of the embryo proper and the suspensor, respectively. The account below typifies embryogenesis in the Poaceae.

Division of the apical cell results in a sphere of cells, as occurs in dicots, following which the embryo is regarded as being in the globular stage. However, there appears to be no clear demarcation of the sphere of cells of the pro-embryo from the suspensor, since the latter is not stalk-like. This gives a conical shape to the whole pro-embryo. Further mitoses, differentiation and development occur such that, in the late globular stage, the epidermis is distinguishable, along with a group of smaller, compact cells to one side of the pro-embryo. These compact cells are the initials that give rise to a root-shoot continuum forming the embryonic axis. Apart from the suspensor, the rest of the pro-embryo develops into a single cotyledon – known as the scutellum. In the succeeding stage of embryogenesis, the scutellum expands substantially, while differentiation takes place in the axis. Hence, this stage is referred to as the scutellar stage. These events continue into the next stage of embryo development called the coleoptilar stage. In this stage, the axis differentiates to form shoot (plumule) and root (radicle) termini, as well as ensheathing structures around each terminus. The shoot tip becomes ensheathed by the coleoptile (hence the name of the stage), whereas the coleorhiza ensheaths the radicle.

1.2.1.2.Gymnosperms

According to a review by Biswas and Johri (1997), gymnosperm embryogenesis can be divided into three phases:

(A) Pro-embryogenesis: Development from division of the zygote to the stage prior to elongation of the suspensor. There is heterogeneity in the formation of the pro-embryo in gymnosperms and four basic types can be distinguished (in the order of most to least advanced): (i) Gnetum and Welwitschia type, (ii) Ephedra and Sequoia type (iii) Conifer type, and (iv) Cycad and Ginkgo type (the subject of the present study).

(B) Early embryogenesis: This phase, which varies in details depending on the taxona, comprises elongation and proliferation of the suspensor and ends in the formation of a young embryonic mass.

(C) Late embryogenesis: Includes embryo ontogeny after the elongation of the suspensor and the formation of polar meristems, i.e. those of the root and shoot.

Gnetum and Welwitschia type

Early development of the zygote appears to vary in different species of *Gnetum* (Maheshwari and Vasil 1961; Martens 1971). In *G. gnemon* (Madhulata 1960), the zygotes typically occur in pairs. The zygote may form a small protuberance into which the nucleus passes (Lotsy 1899), or it may divide into two cells and one or both cells may form a tube (Fig. 1A). Otherwise, it leads to the formation of a branched tube with the nucleus passing into one of the branches. The tubes have been called either pro-embryonal tubes or suspensor tubes, or primary suspensor tubes. Here they will be called primary suspensor tubes to distinguish them from the secondary suspensor. The tubes become septate, substantially elongated, and coiled, growing into the megagametophyte. The primary suspensor tubes generally grow towards the chalazal end of the megagametophyte; however, some may be observed growing outside of the megagametophyte through the micropylar region. Embryogenesis begins at the tips of a few of these primary suspensor tubes as a small cell is cut off. This cell undergoes division both transversely and longitudinally to give rise to a quartet where subsequent divisions lead to the production of a globular embryo (Figs 1B-E). Cells at the primary suspensor tube end divide and elongate substantially to form a long, coiled secondary suspensor that serves to push the growing embryo deeper in the megagametophyte (Figs 1F-H). In *G. africanum*, the zygote and its products give rise to a row of cells each of which forms a primary suspensor tube.

There is some debate regarding the pro-embryogenesis of *G. ula* (Vasil 1959; Swamy 1973). According to Vasil (1959), division of the zygote is followed directly by wall formation. The two daughter cells elongate forming suspensor tubes, which undergo further divisions. The

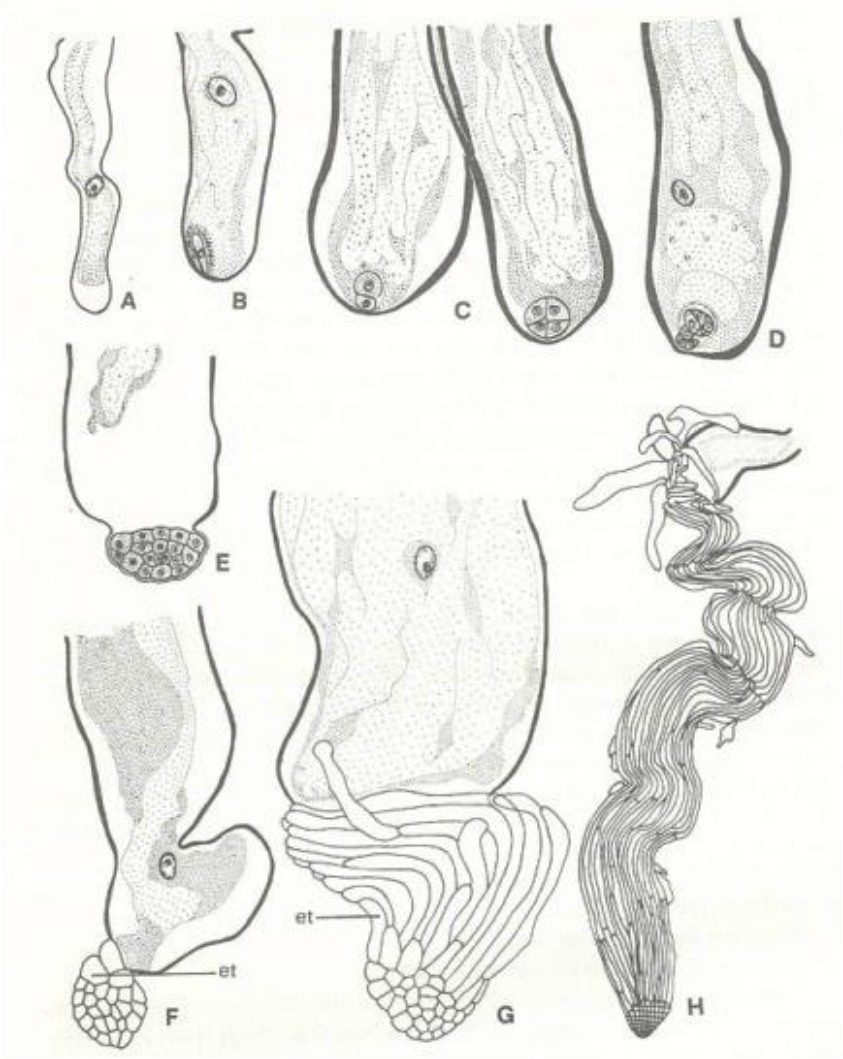

Fig. 1 *Gnetum ula* (et, embryonal tubes). **A** Tip part of primary suspensor tube. **B** Same, demonstrating peculiar cell. **C**, **D**. Peculiar cell divides to form two, four and eight cells; in **D**, the primary suspensor tube nucleus is persistent. **E** Showing a mass of cells produced by the peculiar cell. **F**, **G**. Upper cells of the cellular mass enlarge and undergo division giving rise to a secondary suspensor (embryonal tubes); note the persistent nucleus of the primary suspensor tube. **H**, Young embryo depicting primary suspensor tube with a long, coiled multicellular secondary suspensor. A few cells of the secondary suspensor elongate to yield long tubes that grow on the sides of the primary suspensor tube (after Vasil 1959; Diagrams & captions copyright © Bhatnagar and Moitra 1996; published by V.S. Johri for New Age Publishers, New Delhi, and reproduced by permission [29th November, 2013] of the Rights Department, New Age Publishers)

resultant daughter cells elongate forming a group of uninucleate primary suspensor tubes that grow in different directions within the megagametophyte. However, as Swamy (1973) demonstrated, the zygote in *G. ula* enlarges and undergoes free-nuclear divisions initially. Following the four-nucleate stage, two short processes are seen at one pole of the pro-embryo, which grow towards the chalaza. The tubular processes branch, and at the eight- and 16-nucleate stages the nuclei move into the tubes. This gives rise to a number of uninucleate primary suspensor tubes that penetrate the core of the megagametophyte in the chalazal direction.

According to Swamy (1973), the nucleus of the primary suspensor tube then moves to the tip and undergoes mitosis to form two nuclei of different size. The smaller of the two nuclei gets cut off by a thin wall growing in the direction of the tube. This is known as the 'peculiar cell' because of the various shapes it adopts and the lack of information on its development (Swamy 1973) (Figs 1B-D). However, when the peculiar cell is 'pyriform', it divides to form a globular mass that develops into a mature embryo. Morphologically, therefore, it is a cell of the embryo.

As reviewed by Bhatnagar and Moitra (1996), once the peculiar cell has been cut off by the wall, the primary suspensor tube wall thickens except in the peculiar cell region. The latter becomes rounded and undergoes two divisions producing four cells (Fig. 1C). A subsequent division forms eight cells. Further divisions are irregular giving rise to a mass of cells, the uppermost of which form the secondary suspensor, while compact cells at the tip of the secondary suspensor form the embryo proper (Vasil 1959) (Fig. 1F). The secondary suspensor becomes quite large and has the appearance of a folded plate of cells (Fig. 1H). Some of these cells elongate giving rise to long tubes developing on the sides of the primary suspensor, which ultimately stops functioning. Bhatnagar and Moitra (1996) mentioned that only one or two of the primary suspensor tubes grow to a stage where embryonal cells proliferate, while the rest degenerate.

Those authors also stated that the developing embryo displays a conical mass of cells bearing the shoot apex at the tip, and that the cells on the sides of the shoot tip divide actively to give rise to two cotyledons that cover the central region of the shoot apex. According to the same workers, the root tip differentiates at the opposite end – the root cap cells being contiguous with the relatively large secondary suspensor.

After the shoot and the root apices differentiate, a small extension emerges in the area between the two apices. This is the initiation of the so-called 'feeder' (see Sanwal 1962), which differentiates into epidermis, cortex, vascular bundles and pith. The feeder is a very prominent component of the mature embryo, is usually longer than the hypocotyl, and reportedly functions by absorbing nutrients from the megagametophtyte for the growth and development of the embryo.

In *Welwitschia*, formation of the pro-embryo is not known in much detail; however, it appears similar to *Gnetum* (Pearson 1929; Martens and Waterkeyn 1974). As in *Sequoia* and *Gnetum*, mitosis of the zygote is followed directly by the formation of cell walls. Division of the zygote leads to the production of a long suspensor cell and a small embryonal cell. The embryonal cell undergoes division or cuts off a progression of embryonal suspensors, which elongate while remaining within a cavity in the megagametophyte, sometimes referred to as a prothalial tube (Bhatnagar and Moitra 1996). The single embryonal cell initially undergoes vertical followed by transverse division to give rise to four cells making up two tiers (Martens and Waterkyn 1974). Eight cells are formed in a single tier when the upper two cells undergo anticlinal division. As pointed out by Bhatnagar and Moitra (1996), these eight cells elongate to form embryonal tubes or secondary suspensors, while the lower two cells divide vertically to form a quadrant. These quadrant cells divide by the formation of oblique anticlinal walls, followed by transverse walls which give rise to an inner and outer group of cells.

The outer layer of cells elongates to give rise to the cap; however, all the cap cells become meristematic in later stages and are added to the embryonal mass. The peripheral cells of the embryonal mass develop and contribute to the secondary suspensor resulting in it becoming relatively large (Martens and Waterkeyn 1974). Those researchers also showed that a feeder exists in the embryo of *Welwitschia*, which appears as a collar or annular bulge in the embryonic axis of the mature embryo, and as Singh and Johri (1972) reviewed, simple and cleavage polyembryony are both regular features in the Welwitschia and Gnetum type.

Ephedra and Sequoia type

Embryogenesis has been studied in only a few species of *Ephedra* (including *E. foliata*; [Figs 2C, E-F]; Khan 1943). Double fertilisation was demonstrated in *E. nevadensis* and *E. trifurca* (Friedman 1990b, 1991), where two sperm cells unite with the egg cell and ventral canal cell

of the megagametophyte, respectively, ultimately giving rise to two pro-embryos (Friedman 1992).

Division of the zygote nucleus occurs *in situ*; the two nuclei separate occasionally and move to the two poles of the zygote. Two more nuclear divisions follow, resulting in eight nuclei (Figs 2A & B). Each nucleus is enclosed in a cytomatrical sheath that radiates strands and appears dense (Lehmann-Baerts 1967) (Fig. 2B). A wall develops around each of the eight nuclei, which is followed by cleavage resulting in eight units. According to Foster and Gifford (1959), this is a precocious type of polyembryony and only one embryo reaches maturity.

A tubular outgrowth then extends from each cell of the pro-embryo (e.g. Khan 1943) (Figs 2C & D). The nucleus divides occasionally before the outgrowth forms, or it may pass into the tube and divide. A transverse wall is formed, giving rise to an embryonal cell and a suspensor cell, which elongates (Fig. 2D). A periclinal mitosis in the embryonal cell is followed by one in the longitudinal plane in the lower cell which gives rise to three cells (Fig. 2E). Subsequent divisions lead to the formation of the embryo-proper and a multicellular secondary suspensor forms contiguous with the suspensor cell (Figs 2F-H). Two cotyledons and a shoot apex are formed at the lower end of the embryo, while a root cap of column (horizontally arranged cells in the central region) and pericolumn (peripheral cells inclined sharply or almost vertically) cells differentiates (Deshpande and Bhatnagar 1961).

Sequoia differs in that there is a total absence of the free nuclear stage – wall formation following the first mitosis of the zygote nucleus (Buchholz 1939). According to the review by Bhatnagar and Moitra (1996), the two independent cellular units that arise from the first mitosis divide again to give rise to four independent units that each put out a tubular extension. The nucleus of each unit divides giving rise to an embryonal cell and an embryonal suspensor, which elongates.

The rest of the development occurs as follows (Bhatnagar and Moitra 1996): Elongation of the suspensor tier thrusts the embryonal tier cells deep into the megagametophyte. Numerous young embryos are formed due to the fertilisation of many egg cells in the same archegonial complex. Cleavage polyembryony is common in *Sequoia* and is brought about by the independent development of embryonal units. It is usually the deep and centrally seated embryo which grows to maturity. The embryonal cells undergo three divisions; the first division is oblique, while the second and third are perpendicular to the first. The next

divisions are irregular giving rise to a group of embryonal cells. The upper cells of the embryonal mass form embryonal tubes, which are elongated cells bearing a thin cytomatrix (Buchholz 1939). The shoot apex differentiates by anticlinal and periclinal

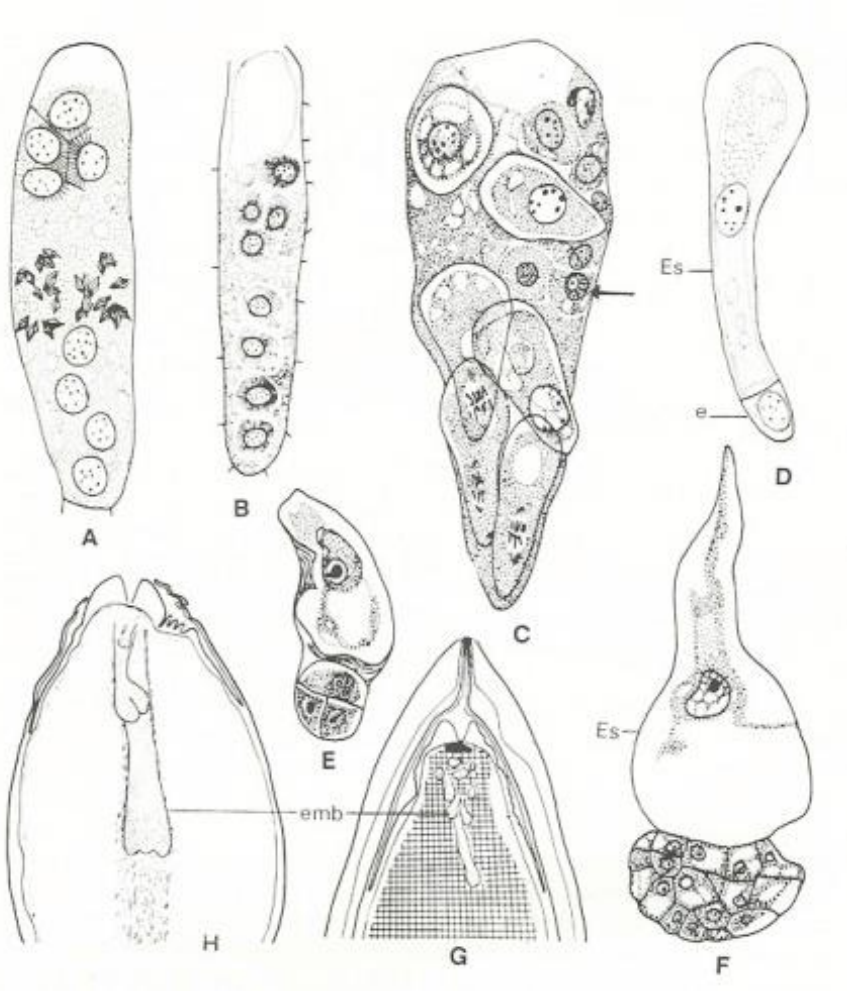

Fig. 2 Pro-embryogenesis in *Ephedra* (e, embryonal cell; emb, embryo; Es, embryonal suspensor). **A** Eight-nucleate pro-embryo, four (from fusion of the ventral canal nucleus and second male gamete) in the upper and four (from zygote) in lower region of the archegonium. Cell walls start to separate the upper four nuclei. **B** At a later stage illustrating eight pro-embryonal units; each nucleus is surrounded by radiating cytomatrix. **C** The embryonal units elongate, while nuclei in three of them divide. Few jacket nuclei are also evident (arrow). **D** A later stage showing elongation of the embryonal suspensor and an embryonal cell. **E** Embryonal cell has divided into three cells. **F-H**. Subsequent stages in embryo development. (**A-B**, **D**, after Lehmann-Bearts 1967; **C**, **E-F**, after Khan 1943; **G**, **H**, after Narang 1956; Diagrams & captions copyright © Bhatnagar and Moitra 1996; published by V.S. Johri for New Age Publishers, New Delhi, and reproduced by permission [29th November, 2013] of the Rights Department, New Age Publishers)

mitoses of the cells near the chalazal end, while the root apex and root cap are formed at the other end of the embryo. The cells between the shoot and the root apices appear elongated and are organised parallel to the longitudinal axis of the embryo. Four cotyledons develop on either side of the shoot apex, while two vascular traces can be found in the hypocotyl region. These vascular traces divide before entering the cotyledons.

Conifer Type

The first mitosis of the zygote is usually intranuclear with the resulting nuclei formed in the nucleoplasm (Camefort 1968) (Figs 3A & B). Subsequent mitoses give rise to four nuclei that move to the base of the archegonium where further synchronous nuclear divisions occur (Bhatnagar and Moitra 1996) (Figs 3C & D). The free nuclei come to lie at the chalazal pole of the zygote which has dense cytoplasm known as 'neocytoplasm' (Camefort 1969). The number of free nuclei prior to wall formation varies e.g., four in *Athrotaxis*; eight in *Pinus*; 16 in *Cephalotaxus*; 32 in *Podocarpus andinus*; and 64 in *Agathis* (Chowdhury 1962).

After wall formation, a lower group of mostly embryonal cells develops, while an upper single layer of cells, the open tier (also known as the primary upper tier), is formed (Dogra 1967) (Figs 3E-G). As stated by Biswas and Johri (1997) in their review, the open tier cells have no walls on their upper side, implying that they are continuous with the cytomatrix of the pro-embryo. All the cells then divide resulting in double the number of lower tier cells (binucleate cells are found in podocarps), which now become known as the embryonal tier. The open tier cells divide transversely to give rise to an upper tier and a lower dysfunctional suspensor tier (usually called the rosette tier). There is no elongation in the cells of the suspensor tier but they divide to give rise to lobes of an evanescent mass of cells called 'rosette embryos'. However, because these masses do not form an embryo, usage of this term seems inappropriate (Doyle 1963; Dogra 1967). The distal embryonal tier usually assumes the function of the suspensor. According to Singh and Johri (1972), the upper tier cells ultimately degenerate, the suspensor elongates, while the cells of the embryonal tier divide to form a mass (Figs 3H & I). The distal cells of the mass elongate, which gives rise to a massive suspensor, known as the secondary suspensor, to which more cells of the embryonal mass are added.

Minor deviations of this basal plan of ontogeny were described for *Actinostrobus*, *Athrotaxis*, *Callitris*, *Cupressus sempevirens*, *Flitzroya*, *Torreya*, and *Widdringtonia* (Doyle 1963).

However, as Singh and Johri (1972) pointed out, these deviations have been resolved interpreting the embryogenesis in all to conform to the basal plan.

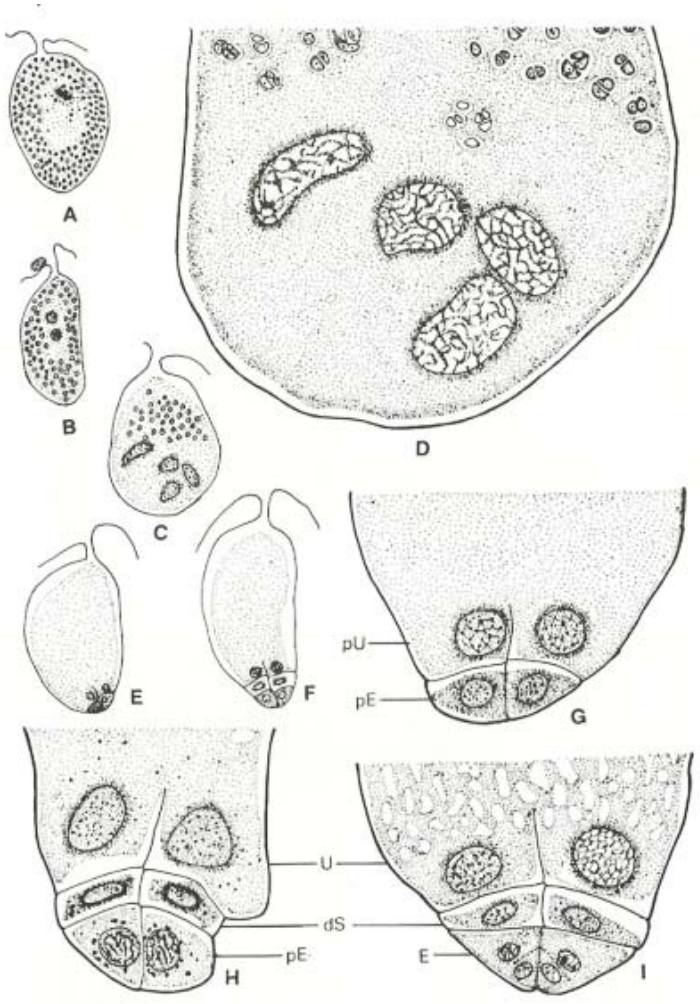

Fig. 3 *Pinus wallichiana* (dS, dysfunctiional suspensor; E, embryonal group; pE, primary embryonal tier; pU, primary upper tier; U, upper tier). **A** Archegonium demonstrating zygote nucleus in metaphase. **B**-**C** Two- and four-nucleate pro-embryos. **D** Portion enlarges from **C** depicting migration of nuclei towards the base prior to final division. **E**, **F** Index figures of **G** and **H**, respectively. **G** Eight-celled pro-embryo arranged into primary upper (pU) and primary embryonal (pE) tiers, of four cells each. **H** Primary upper tier undergoes division internally to give rise to upper (U) and dysfunctional suspensors (dS) tiers; pE tier undergoing internal division. **I** Pro-embryo constituted by an upper (U) and dysfunctional suspensor (dS) and embryonal group (E) of two tiers (after Konar and Ramchandani 1958; Diagrams & captions copyright © Bhatnagar and Moitra 1996; published by V.S. Johri for New Age Publishers, New Delhi, and reproduced by permission [29th November, 2013] of the Rights Department, New Age Publishers)

Embryogenesis in araucarians, on the other hand, appears to differ markedly from the basal plan. The free nuclei persist in the centre of the pro-embryo, with wall formation resulting in a central group of cells enclosed by a peripheral jacket of cells (Eames 1913; Burlingame 1915). The cells proximal to the jacket mature into cap cells; the distal cells develop into the suspensor; while the central cells form the embryonal mass. The cap cells may elongate slightly and appear prominent but they ultimately degenerate (reviewed by Singh and Johri 1972).

Most conifers display both simple and cleavage polyembryony. The latter is usually a result of differing rates in the elongation of suspensor or secondary suspensor components, such that a suspensor cell or a group of them having one or a few embryonal cells separate from the embryo system and grow independently (Singh and Johri 1972).

Cycad and Ginkgo Type

The zygote nucleus divides *in situ* followed by many free-nuclear divisions (Figs 4A-C). The nuclei disperse evenly throughout the entire pro-embryo and on some occasions, evanescent walls appear to develop during the free-nuclear period (Chamberlain 1910; Favre-Ducharte 1956). In later stages, while the free nuclei are distributed evenly in *Ginkgo*, these become concentrated at the base of the pro-embryo in cycads. The upper part of the cycad pro-embryo contains far fewer nuclei in a thin cytomatrix – considered an "advanced" trend over pro-embryos with evenly distributed nuclei (cf. conifers, see Dogra 1992). Subsequently, nuclei at the base of the pro-embryo divide whilst the upper nuclei display signs of degeneration (Bryan 1952).

By the time walls are formed, there are approximately 256 free nuclei in *Ginkgo*, 512 in *Cycas circinalis* (Rao 1963), and 512 or 1024 in *Dioon*. The resultant cells fill the entire pro-embryo in *Ginkgo*; in cycads, however, newly-formed cells are found only in the lower part of the pro-embryo (Fig. 4D) (Biswas and Johri 1997). In *Encephalartos friderici-guiliemi*, *E. villosus* and *Macrozamia spiralis*, segmentation of almost the whole egg cell gives rise to the formation of a primary pro-embryo having a dense, active basal area. In *M. reidlei,* formation of the walls occurs throughout the pro-embryo apart from a small region in the centre that possesses free nuclei. This area disintegrates later on giving rise to a central cavity. In *Cycas*, cellularisation is restricted to the peripheral and basal regions. In *Stangeria*, *Zamia* and *Bowenia*, pro-embryonal cells form basally only (as in conifers; Dogra 1992).

Once walls have been formed, the basal cells divide and function as primary embryonal cells, while the cells of the upper region elongate to give rise to a massive suspensor. The primary embryonal region comprises compact, dense, actively dividing, uniform cells concentrated at the tip (Figs 4E & F). This forms the meristematic cell region of the embryo (Biswas and Johri 1997). These cells continue adding to the elongating suspensor which pushes the embryonal cells deep into the centre of the megagametophyte (Singh and Johri 1972). However, there appears to be no well-defined suspensor in *Ginkgo* apart from a micropylar region of elongated cells (Biswas and Johri 1997).

In *Zamia* and *Cycas* (and probably in other cycad genera) the first layer of embryonal cells elongates slightly to give rise to a noticeable cap around the group of meristematic cells (Figs 1E & F) (Bryan 1952; Maheshwari 1960). The cap cells persist for a while; however, they ultimately degenerate and do not add to the anatomy of the mature embryo. Several young embryos may be found in some seeds, which usually arise when more than one zygote is formed (simple or archegonial polyembryony) (Singh and Johri 1972). Cleavage polyembryony has not been found in the cycads (Biswas and Johri 1997), and while the mature embryo has two cotyledons, three have been found on some occasions (Biswas and Johri 1997).

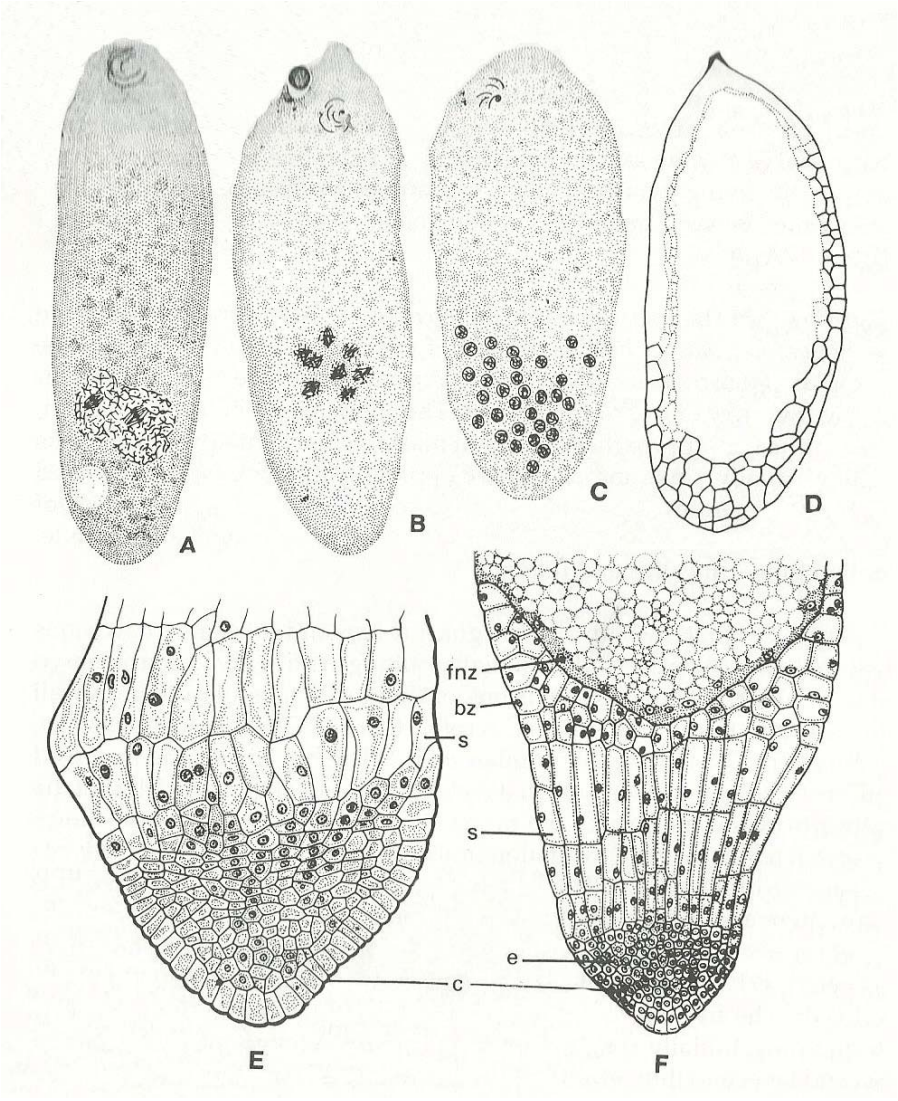

Fig. 4 Illustration of pro-embyogenesis in *Cycas* (**A-E**) and *Zamia* (**F**). **A-C** Two-, eight, and thirty-two nucleate pro-embryos are depicted. Remnants of sperm can still be observed in the upper end of the archegonium. **D** Wall formation has begun at the basal end of the pro-embryo. **E** Later stages demonstrating the outer layer of 'cap cells', embryonic cells and suspensor cells. **F** Developing embryo illustrating free nuclear zone, buffer zone, suspensor, embryonic mass and cap cells. (bz, buffer zone; c, cap cells; e, embryonic mass; fnz, free nuclear zone; s, suspensor). (Diagrams & captions copyright © Bhatnagar and Moitra 1996; published by V.S. Johri for New Age Publishers, New Delhi, and reproduced by permission [29th November, 2013] of the Rights Department, New Age Publishers)

1.2.2. The third phase of seed development

As stated previously, the third main phase of seed development in angiosperms and gymnosperms is known as maturation drying. This phase, which follows histodifferentiation and accumulation of reserves in the angiosperm embryo, is associated with a decline in seed metabolic activity to low levels (Lynch and Clegg 1986) and – depending on the species – the seed usually passes into a state of quiescence or dormancy. Seeds that undergo maturation drying display what has been termed 'orthodox' post-harvest behaviour (Roberts 1973).

Orthodox seeds are, or can be, dried to low water contents, which is a result of the acquisition of tolerance to desiccation relatively early in development, before being shed from the parent plant (Pammenter and Berjak 1999). At low water contents, such seeds may be stored without loss of quality for predictable periods under defined storage temperature and relative humidity conditions, their life-span, within limits, increasing logarithmically with lowered water content of the seed (Ellis and Roberts 1980).

However, not all seeds are tolerant of desiccation. Such desiccation-sensitive seeds do not undergo maturation drying as part of their pre-shedding development; their water contents remain high throughout ontogeny to germination and they do not become metabolically quiescent (Berjak and Pammenter 2008). Since they cannot be desiccated – and their metabolism grades imperceptibly from development to germination at the shedding water content – such seeds are able to be stored only in the short- to medium-term, the period varying with the species (Berjak and Pammenter 2008).

Such seeds are categorised as being recalcitrant – a term coined by Roberts (1973) for seeds that cannot be stored at low water contents. Recalcitrant seeds are most commonly produced by species of tropical trees (Farrant *et al.* 1986, 1993); some aquatic grasses (Probert and Longley 1989); a variety of amaryllids (Sershen *et al.* 2008) and a few woody species from temperate climates e.g. oak and sycamore (Hong and Ellis 1991; Pritchard 1991; Hendry *et al.* 1992). In the context of the present study, seeds of *Encephalartos* spp. have been established as being recalcitrant (Woodenberg et al. 2007; Woodenberg 2009)

While orthodox and recalcitrant seeds display contrasting postharvest behaviour, Ellis and Hong (1990) defined an intermediate group of seeds to include those that can tolerate drying, but not to the same degree as orthodox types. Seeds displaying intermediate post-harvest behaviour may also be chilling-sensitive (especially those of tropical origin), particularly

after dehydration (Ellis and Hong 1990; Hong and Ellis 1996). While there appear to be three distinct categories of postharvest seed behaviour, studies on a wide variety of species, particularly those of tropical and sub-tropical origin, have led to the suggestion that there is open-endedness to the three categories (Pammenter and Berjak 1999). It has therefore been proposed that the post-harvest behaviour of seeds should be viewed as a continuum rather than three discrete entities, with the highest degree of orthodoxy and recalcitrance at either extreme with gradations between the two (Berjak and Pammenter 1997, 2001).

A spectrum of mechanisms has been proposed to confer, or contribute to, the ability of seeds to tolerate desiccation. The lack or ineffectual manifestation of one or more of these mechanisms may govern the relative degree to which seeds of individual species can be dried.

Some of the mechanisms (reviewed by Vertucci and Farrant 1995; Pammenter and Berjak 1999) that have been implicated in conferring desiccation tolerance include the following: Intracellular physical characteristics viz. a reduction in the degree of vacuolation, the quantity and constitution of insoluble reserves accumulated, reaction of the cytoskeleton, and conformation of the DNA, chromatin and nuclear structure; intracellular de-differentiation that results in the minimisation of membrane surface area and presumably also of the cytoskeleton; 'switching off' of metabolism; presence, and effective operation, of antioxidants; accumulation of putatively protective molecules including Late Embryogenesis Abundant proteins (LEAs) and small heat shock proteins (*inter alia*), sucrose and certain oligosaccharides or galactosyl cyclitols; the existence and operation of repair mechanisms upon rehydration (Berjak and Pammenter 2008; Leprince and Buitink 2010). Desiccation tolerance also involves strategies which overcome the mechanical stress associated with a loss of turgor upon desiccation (Walters *et al.* 2002). It is postulated that these mechanisms act collectively to give rise to seed 'orthodoxy'.

While much work has been done investigating the role of various mechanisms in the acquisition of desiccation tolerance in seeds and vegetative tissue (reviewed by Pammenter and Berjak 1999; Berjak 2006; Leprince and Buitink 2010), comparatively little attention has been paid to mechanical stress, particularly in seeds.

1.2.2.1. Mechanical stress

When plant tissue dries, the cytomatrix of the component cells shrinks creating tension between the plasmalemma and the more rigid cell wall (Walters *et al.* 2002). According to those authors, the tension created between the plasmalemma and cell wall can result in tearing of the plasmalemma and thus irreversible damage to the cells of desiccation-sensitive plant tissue.

Support for the idea of such mechanical stress came from a number of ultrastructural studies on desiccation-sensitive seeds which showed severed plasmodesmatal connections, plasmalemma discontinuities, vesiculation of the membrane, and separation of the plasmalemma and cell wall upon drying (e.g., Webster and Leopold 1977; Sargent *et al.* 1981; Fincher and Leopold 1982). However, Wesley-Smith (2001) demonstrated that much of this type of apparent damage associated with desiccation is likely to be due to the characteristics of aqueous aldehyde fixation rather than desiccation damage *per se*. According to that author, when plant material is dehydrated, the aldehydes used in aqueous fixatives cross-link with membrane proteins, causing the plasmalemma (and other membranes) to become relatively rigid, while they do not similarly affect the cell wall, which expands as it becomes hydrated. The use of freeze-substitution on the other hand, presents a superior alternative to aqueous fixation for dehydrated plant tissue – the evidence showing that there is much better preservation of tissues in the dried state (Wesley-Smith 2001). As demonstrated by that same author, membrane damage and plasmalemma-cell wall separation was evident in both desiccation-sensitive and tolerant tissues following aqueous fixation of dried samples; however, when freeze-substitution was employed, the cells appeared shrunken, but displayed varyingly-folded cell walls and similar damage was not evident (Wesley-Smith 2001).

Mechanical stabilisation

Orthodox seeds appear to achieve mechanical stabilisation upon drying predominantly because of extensive subcellular accumulation of insoluble reserves (Webb and Arnott 1982). Interestingly, the timing of maximum reserve accumulation is generally coincident with the attainment of maximal desiccation tolerance in the axes of most seeds (reviewed by Vertucci and Farrant 1995).

Depending on the species, embryos of recalcitrant seeds may accumulate little by way of insoluble reserves, while others accumulate somewhat more. In such seeds the proportion of

water loss tolerated appears to be correlated with the degree of reserve accumulation (e.g. Farrant and Walters 1998; reviewed by Farrant *et al.* 2012). The lack of mechanical stabilisation provided by accumulated insoluble reserves, therefore, could be one factor underlying desiccation sensitivity in recalcitrant seeds.

Mechanical stabilisation has also been found to coincide with flexibility of the cell wall in desiccation tolerant plant tissue (Webb and Arnott 1982; Moore *et al*. 2013). While moderately folded cell walls have been seen in orthodox seeds (Webb and Arnott 1982), in-depth work on resurrection plants has suggested the possible role of cell wall folding in conferring mechanical stabilisation upon dehydration (Vicré *et al.* 1999, 2004a, 2004b; Moore *et al*. 2006, 2007, 2012). It has been proposed that cell wall folding is a strategy exhibited by some plants, which avoids tearing of the plasmalemma from the cell wall during dehydration, thus allowing cell integrity to be maintained (Farrant and Sherwin 1997). It has also been suggested that the extent and manner of folding is dependent upon the chemical composition and structure of the cell wall (Webb and Arnott 1982). However, there have since been very few, if any, studies on the composition of seed embryo cell walls. Such studies that have been done on cell walls of seeds have mostly explored their function as storage reserve sites (reviewed by Buckeridge *et al.* 2000), rather than the compositional and conformational changes during development and upon drying.

Effects of water removal from cell walls

The cell wall is made up of over 60% water (Brett and Hillman 1985), and this essential, although often forgotten, component, is a crucial and integral constituent of the structure and functioning of the wall. As reviewed by Moore *et al.* (2008), one obvious consequence of water removal from the wall is that polymers typically separated in the hydrated state come to be in close association with each other resulting in polymer adhesion. Polysaccharides form non-covalent bonds usually through hydrogen bonding and, upon the removal of water, this bonding is significantly enhanced, causing irreversible interaction between polymers and leading to altered wall biophysical properties and severely impaired growth (Moore *et al.* 2008).

1.3. Cell walls

Early cell walls arose in an aqueous environment and the cell wall is considered to have evolved as a strategy related to osmotic problems (Gerhart and Kirschner 1997). The cell wall

is now one of the major characteristics of plants, the majority of which survive in a terrestrial environment (Popper and Fry 2004). It is apparent that there are dynamic relationships between membrane-bound living cells and their surrounding cell walls, and with each other, possibly through the wall (Kohorn 2000). This extracellular matrix may influence almost every facet of cell function primarily because of its location and physical characteristics (Kohorn 2000).

Cell walls are categorised as primary or secondary. The primary wall is produced throughout cell division and expansion, and material deposited on the primary wall once growth has stopped constitutes the secondary wall (Cosgrove 1997). Neighbouring cell walls sandwich an intercellular layer, the middle lamella, which is apparently continuous with the matrix of the primary cell wall (Moore *et al.* 1986).

Cell walls are dynamic entities that play crucial roles in plant morphology, growth and ontogeny (Albersheim *et al.* 1994; Penell 1998). The main roles of the cell wall are the provision of physical strength to the plant and protection against the external environment (Harholt *et al.* 2010). Walls are also involved in facilitating plant reactions to pathogenic and environmental stresses e.g. osmotic stress (Wakabayashi *et al.* 1997; Fujikawa *et al.* 1999; Kubacka-Zebalska and Kacperska 1999; Stefanowska *et al.* 1999); acclimation to cold (Weiser *et al.* 1990); tolerance of drought (Zwiazek 1991); invasion by pathogens (Boudart *et al.* 1998); wounding (Cardemil and Riquelme 1991); saline stress (Iraki *et al.* 1989) and desiccation (Zwiazek 1991; Ha *et al.* 1997).

The fundamental characteristics of cell walls are determined by their chemical constituents and the three-dimensional organisation of these constituents. Hence, studying the structure of cell walls is fundamental to understanding how they function.

Primary walls are laid down during cell growth, and are required to be both mechanically strong yet sufficiently flexible to allow cell expansion while counteracting cell rupture from turgor pressure. Primary cell walls consist primarily of polysaccharides that can be classified generally as cellulose, the cellulose-binding hemicelluloses, and pectins. The latter two groups of cell wall constituents are often known as matrix polysaccharides. These are produced in Golgi cisternae, while cellulose is assembled at the plasmalemma as paracrystalline microfibrils (Reiter 2002). Apart from polysaccharides, plant cell walls possess an abundance of various proteins. Many of these proteins are thought of as ‘structural’, while others play a role in remodelling the wall (Reiter 2002).

Cellulose is a polymer of glucose residues connected end to end by β (1→4)-glycosidic bonds to form a linear chain. The abundance and ordered arrangement of the hydroxyl groups on each chain permits the side by side hydrogen-bonding of several such chains to give rise to a linear, semicrystalline structure called a microfibril. These microfibrils ultimately comprise the main structural constituent of plant cell walls (Lloyd 2006).

The individual cellulose microfibrils cross-link via another type of carbohydrate polymer, the crosslinking glycans (commonly referred to as hemicellulose). These polymers give rise to a filamentous network united by hydrogen bonds, which extends throughout the cell wall. Around these cross-linked fibrils, which form a structural core, are the pectins, another type of complex carbohydrate. The branched structure of pectins make them highly hydrated, thereby forming a gel matrix around the cellulose (Lloyd 2006).

Lignins and other organic compounds may also be deposited on this matrix conferring mechanical strength and rigidity to the secondary wall (Reiter 1994).

1.3.1. Hemicellulose

Hemicellulose is a term that describes a family of polymers rich in glucose, xylose, or arabinose that, unlike cellulose, have extensive side groups that frequently include xylose, galactose, and fucose. The dicots and monocots vary considerably in the composition of their hemicellulose and complete descriptions appear in various reviews (Carpita and Gibeaut 1993; Reiter 1994; Cosgrove 1997). The structure of the hemicelluloses allows these complex carbohydrates to rest upon the surface of, and possibly intercalate within, the bundles of cellulose, giving rise to a linked matrix.

Commonly encountered hemicelluloses include xylans, xyloglucans, arabinoxylans, mannans, arabinogalactans, glucomannans, and galactoglucomannans (Timell 1964, 1965; Whistler and Richards 1970). Xyloglucan is the main hemicellulose in the primary walls of the majority of higher plants, and typically consist of a 1→4-β-D-glucan backbone bearing 1→6-α-D-xylose moieties on three successive glucose residues (Reiter 2002). Xylans are also the principal noncellulosic polysaccharides of plant cell walls and are particularly copious in secondary cell walls (McCartney *et al.* 2005).

1.3.2. Pectins

Pectins constitute a family of polygalacturonic acids that may differ in their side chains, which are usually arabinose, galactose, or a complex branched organisation of monosaccharides (Cosgrove 1997). Pectins are made up of homogalacturonans (HGs) comprising contiguous, unbranched, 1→4-linked-α-D-galacturonic acid residues intermingled with rhamnose and specific branched polymers that include rhamnogalacturonans (RG-I and RG-II).

A significant functional factor that characterises HGs is their degree of methyl esterification. As a result, HGs are often labelled as low- or high-methylesterified pectins (Jauneau *et al*. 1998). The unbranched and low-esterified HGs may aggregate via calcium bridges which form junction zones that hold them together (Grant *et al.* 1973; Rees 1977; Jarvis 1984; Brett and Waldron 1990). When pectins bind to cations like calcium, the gel stiffens and therefore contributes to the strength of the wall (Lloyd 2006). Calcium-mediated cross-linking may be controlled by the masking of pectic negative charges via the addition of methyl esters (Kačuráková *et al.* 2000).

Pectic fractions characteristically possess a neutral polysaccharide that is mostly covalently attached to the main chain of acidic rhamnogalacturonan (Aspinall 1970). The precise nature of this neutral polysaccharide differs from species to species; however, it has been observed to be either an arabinan or a galactan, or a combination of the two (Aspinall 1970).

Arabinans (and galactans) may function as spatial regulators of the closeness of HG domains (Harholt *et al.* 2010). They are extremely mobile polymers compared with other pectin constituents – their mobility being impacted upon by the hydration of the cell wall. Arabinan and galactan are thus the first polymers to become mobile during the hydration of dry cell walls (Tang *et al.* 1999). Similarly, arabinan and galactan maintain mobility longer than other cell wall polymers during dehydration and appear to fill the cavities caused by the physical rearrangement of cell wall constituents (Harholt *et al.* 2010).

Pectic polysaccharides have been located in every higher plant cell wall investigated (Talmadge *et al.* 1973). They are one of the most fundamental constituents of the cell wall matrix, playing a role in cell adhesion through their gel-like characteristics (Jarvis 1984), and in cell wall architecture (Brett and Waldron 1990; Vian and Roland 1991; McCann et al. 1993; McCann and Roberts 1994) and contributing to the mechanical strength of the cell wall

(Van Buren 1991). Pectins also control the porosity of cell walls (Carpita *et al.* 1979; Baron-Epel *et al.* 1988; McCann and Roberts 1991). Hence, they are considered to be responsible for physical arrest of the enzymes in the apoplasmic space (Jauneau *et al.* 1998).

1.3.3. Cell wall protein

As Kohorn (2000) pointed out, it may not be beneficial to refer to a protein as a "cell wall" constituent, but rather to deal with this large group of secreted proteins from a functional viewpoint. It is conceivable that proteins may extend considerably into the carbohydrate matrix and might even make contact with proteins or carbohydrates on an adjoining cell surface (Kohorn 2000). Hence, to avoid the exclusion of several proteins, that author suggested that it may be best to see the carbohydrates as constituting the cell wall, with proteins being influential visitors.

Traditionally, cell wall proteins have been categorised by their relationship with one or a few of the complex carbohydrates deposited in the walls (Kohorn 2000). These comprise the abundant hydroxyproline-rich glycoproteins (HRGPs; Showalter 1993), proline-rich proteins (PRPs; Showalter 1993), glycine-rich proteins (GRPs, Keller 1993), arabinogalactan proteins (AGPs; Oxley and Bacic 1999; Majewska-Sawka and Nothnagel 2000), wall-associated kinases (WAKs; He *et al.* 1996, 1999), lectins (Herve *et al.* 1999), and expansins (Cosgrove 1997). However, according to Showalter (1993), the list is more extensive, including (amongst others) peroxidases, methyltransferases, galactosidases, glycanases, and proteases.

Arabinogalactan proteins are encoded by a large gene family in an assortment of angiosperms. Up to 90% of the mass of an individual AGP may be composed of carbohydrate that is added in the endomembrane system (Kohorn 2000). Various family members can be expressed in patterns specific to certain tissues. This suggests that AGPs may play pivotal roles in the growth and development of plants. Since AGP types vary in their constituents they tend to have different wall binding capabilities and may well also assist in defining cell location (Roberts 1990; Showalter 1993; Oxley and Bacic 1999). Some AGPs may also contain signals for adding a carboxy-terminal glycosyl phosphatidyl inositol (GPI) anchor, so that upon secretion, AGPs persist on the side of the plasmalemma exposed to the cell wall (Oxley and Bacic 1999; Majewska-Sawka and Nothnagel 2000).

1.3.4. Immunological probes for cell wall research

The primary cell wall has been observed to vary among vascular plants at both monosaccharide and polysaccharide levels (Popper and Fry 2004). As Knox (1992) highlighted, the exact deposition of cell wall polymers influences the anatomy and form of plants. These wall constituents are also imperative for determining the structural and functional characteristics of the plant cell surface. It is thus essential to determine the distribution and localisation of these cell wall components within various tissues and in conjunction with individual cells. In light of this need, the recent production and utilisation of immunocytochemical probes for the recognition of different epitopes of cell wall carbohydrates has been invaluable for the location of these polysaccharides (reviewed by Knox 1997).

A number of immunocytochemical studies have utilised antibodies specific to certain complex polysaccharides to localise their characteristic epitopes not only in different tissues, but in different regions of the cell wall (Moore *et al.* 1986; Vian and Roland 1991; Lynch and Staehelin 1992, 1995; Marty *et al*. 1995; Freshour *et al*. 1996; His *et al*. 1997; Jauneau *et al.* 1997) and cisternal compartments of the Golgi body (Lynch and Staehelin 1992; Zhang and Staehelin 1992). More specifically, immunocytochemical studies of the primary cell wall constituents of plants have been accomplished by the use of polyclonal and monoclonal antibodies specific to purified xyloglucans and pectic polymers (Moore *et al.* 1986; Moore and Staehelin 1988; Northcote *et al*. 1989; Knox *et al*. 1990; Ruel *et al*. 1990). Similarly, antibodies that recognise and bind to certain AGP-associated epitopes have been employed to reveal their cell-type specific location in different plant cells (Stacey *et al.* 1990; Knox *et al.* 1991; Dolan *et al.* 1995; Schindler *et al.* 1995).

Enzyme-gold labelling, a method introduced by Bendayan (1984), has also been used for the localisation of cell wall polysaccharides (Vian *et al*. 1983; Ruel and Joseleau 1984; Benhamou and Oulette 1986; Vian *et al.* 1987; Berg *et al*. 1988; Ruel *et al*. 1990). However, progress has been restricted by the limited supply of sufficiently purified enzymes with restricted specificity (Vian *et al.* 1991).

In combination, however, the results show that the production, secretion, and localisation of cell wall components are controlled in a manner specific to the cell type (Vicré *et al.* 1998).

1.4. The present study

There has been comparatively little research on the ontogeny of desiccation-sensitive (recalcitrant) seeds, with the exception of a few investigations, e.g. on *Quercus alba* (Bonner 1976), *Guilfoylia monostylis* (Nkang and Chandler 1986), *Podocarpus henkelii* (Dodd *et al.* 1989), *Acer pseudoplatanus* (Hong and Ellis 1991) and *Quercus robur* (Finch-Savage 1992; Finch-Savage *et al.* 1992; Grange and Finch-Savage 1992), as well as on *Avicennia marina* (Farrant *et al*. 1992). In the context of the present study, most of the descriptive work on cycad embryos was done in the early 1900s (e.g. Chamberlain 1910) and centred on the early stages of embryogenesis, i.e. development of the pro-embryo. Those investigations also, were undertaken before the advent of modern electron microscopes, while later studies in the 1950s and '60s (Bryan 1952; Favre-Ducharte 1956; Maheshwari 1960; Rao 1963) took place before the possibility of sophisticated methodology like immunocytochemistry (ICC) and immunofluorescence microscopy. Thus many questions pertaining to the ultrastructure and development of cycad embryos remain to be answered.

While there have been few studies on cycad seeds to date, there seemed to be a general understanding that they may be recalcitrant (Forsyth and van Staden 1983; Dehgan and Schutzman 1989) largely due to the fact that the seeds are 'wet'. However, after showing the loss of viability upon drying, Woodenberg *et al.* (2007) have since demonstrated that *Encephalartos natalensis* and *E. gratus* seeds are recalcitrant. This is not surprising as the embryos of the two species continue to develop after the seeds drop from the cones, attaining a size where they are capable of germination only four to six months after seed-shed (Giddy 1984). However, more detailed assessments are required to define details and ultimately to develop useful storage protocols for cycad seeds or germplasm – specifically for the long-term conservation of different species (Berjak and Pammenter 2004). Therefore, understanding cycad seed recalcitrance, and more specifically, post-harvest embryo development, is of major scientific and practical importance: hence the present study.

This investigation can be divided into two main sections: The first concentrates on the histological and ultrastructural development of the *E. natalensis* embryo after seed-shed, while the other is concerned with the composition and conformation of the embryo cell walls of this desiccation-sensitive-seeded species in relation to development and drying. The present study therefore attempts ultimately to make a contribution to cycad embryology and germplasm conservation research using *E. natalensis*, while it also endeavours to address the

paucity of information in the literature on the composition and conformation of cell walls in desiccation-sensitive seeds.

Encephalartos natalensis R.A. Dyer and Verdoorn is a relatively large cycad endemic to South Africa. It has a wide distribution in KwaZulu-Natal in such districts as Port Shepstone, Howick, Kranskop, and Vryheid amongst others (Fig. 5) (Giddy 1984). This species generally has an erect trunk that may be as tall as 6 m, with a diameter of 0.4 m. It grows commonly as a solitary, single-stemmed tree; however, it may produce offsets and basal suckers and thereby form clumps of up to 11 stems (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 female plants of *E. natalensis* may produce up to five deep golden-yellow strobili and as many as 250 bright orange-red seeds per strobilus (personal observation). While it is one of the more common South African cycad species, *E. natalensi*s has nevertheless been classified as protected (van Schalkwyk 2007).

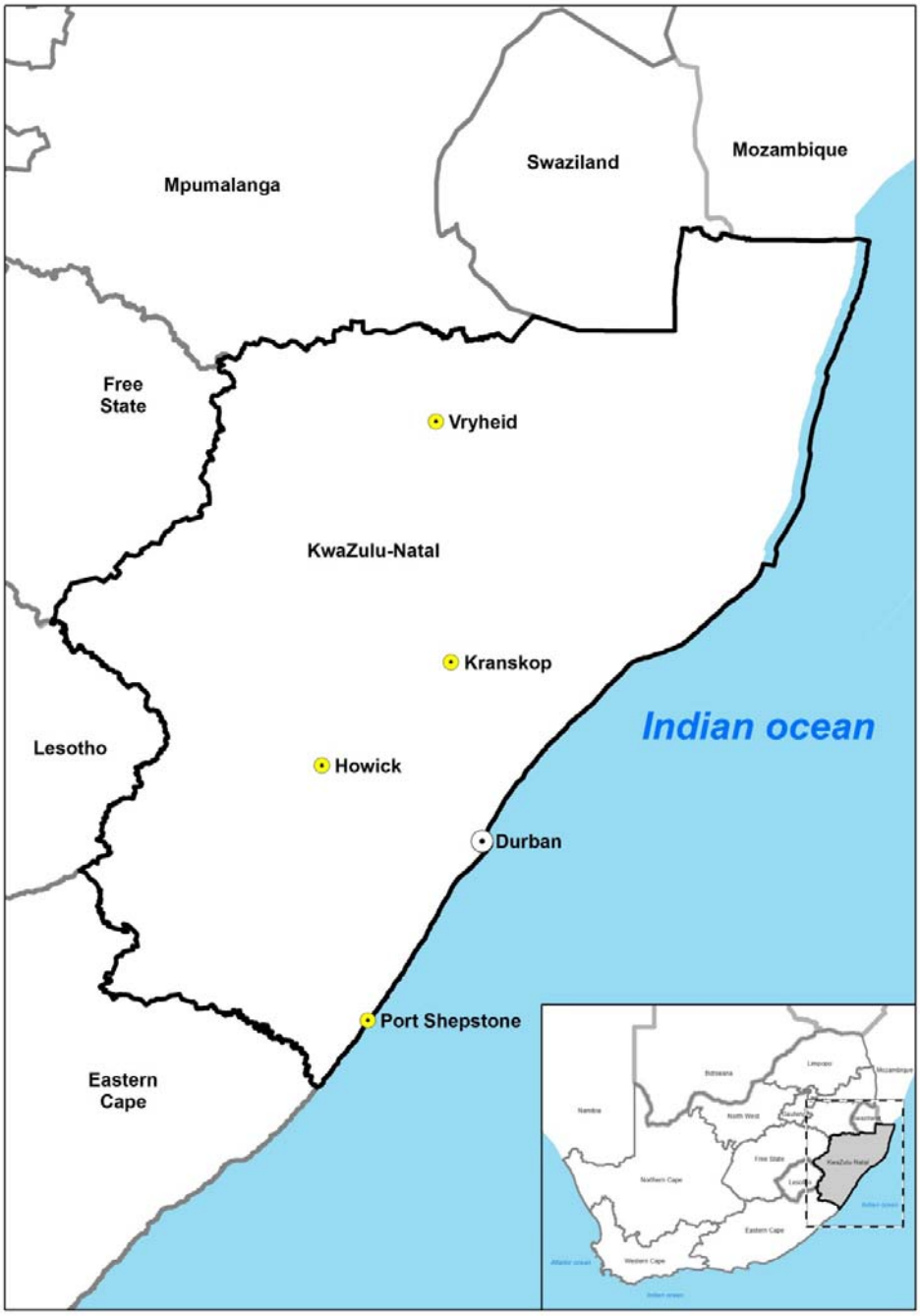

Fig. 5 An illustration of the widespread distribution of districts in KwaZulu-Natal where *E. natalensis* is found (inset: the province of KwaZulu-Natal is illustrated as a component of South Africa). Mapping by S3 Technologies & The Picturebox, Pietermaritzburg, KwaZulu-Natal.

Since development of the *E. natalensis* embryo is largely a post-shedding phenomenon, one of the major objectives of the present investigation was to observe the morphological and anatomical aspects of embryogenesis from seed-shed to six months after shedding – when the seeds were readily able to germinate. To this end, light microscopy and histochemistry were employed to monitor development of cells and tissues, with ultrastuctural parameters being characterised by transmission electron microscopy (TEM).

The present study also aimed at comparing the conformation and composition of the cell walls of mature *E. natalensis* embryos in both the hydrated and dehydrated states. While immunofluorescence microscopy was used to determine whether or not certain epitopes of wall components were present, immunocytochemistry in conjunction with TEM was used to compare the distribution of the epitopes *in muro*, of both immature (at seed-shed) and mature (six months after shedding) embryos.

Aqueous fixation was employed for preparation of hydrated embryos for microscopy, while freeze-substitution was used to preserve the ultrastructure of the embryo in the dried state. The latter was carried out in order to observe whether or not cell wall folding occurred in a dried desiccation-sensitive seed embryo – in this case, that of *E. natalensis*. The impact of desiccation on the ultrastructural integrity of the embryo cells of this species was also assessed using Cryo-SEM, thereby providing verification of whether or not cell wall folding occurred upon drying.

In summation, the present investigation represents the first in-depth microscopy-based study on the post-shedding embryogenesis of a cycad, specifically *Encephalartos natalensis*. Similarly, this study may be the first to shed light on the conformation and composition of cell walls in the embryo of a desiccation-sensitive seeded species in relation to development and drying.

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CHAPTER 2

Development of cycad ovules and seeds. 2. Histological and ultrastructural aspects of ontogeny of the embryo in *Encephalartos natalensis* (Zamiaceae)

Introduction

Cycads (Class Cycadopsida; family Zamiaceae), which are cone-bearing gymnosperms with large, compound leaves, are the most primitive spermatophytes in the world today (Brenner *et al.* 2003). Although the present-day cycads are a mere remnant of a family that once dominated the earth's vegetation in the Mesozoic Era, ~200 million years ago (Bhatnager and Moitra 1996; Pooley 1993), their antiquity renders them a significant role-player in our understanding of the evolution of morphological characters in plants (Brenner *et al.* 2003).

Only 11% of the 36 species of *Encephalartos* in South Africa are categorised as being of least concern (National Red List 2009), most other species being noted in that document as near threatened, vulnerable, endangered, critically endangered or extinct in the wild. However, surprisingly little research has been undertaken on cycad seeds in spite of the pressing need for the conservation of these plants (Vorster 1995). Cycad embryology has received little consideration from morphologists and systematists since the major studies by Chamberlain (1935); however, a deficiency of interest cannot be blamed for lack of research. The reasons for the dearth of research on cycad seeds are, in particular, the lack of availability of suitable research material in sufficient quantities for experimental purposes (Dehgan and Schutzman 1989; Stevenson 1990) and the severe protective regulations that govern the acquisition of cycad seeds (Schlegel 1991), amongst others (Woodenberg *et al.* 2007; 2009).

Despite – or because of – the challenges that have delayed cycad seed research to date, considerable investigation is required for the preservation of cycad germplasm and ultimately, the restoration of cycad populations by means of improved propagation. Fundamental to the improvement of propagation using seeds is a thorough understanding of embryogenesis in cycads. Hence the present investigation was undertaken, which aims to make a contribution to the body of knowledge on cycad embryo ontogeny, using *Encephalartos natalensis* Dyer and Verdoorn (a near threatened species [National Red List 2009]) as a case study.

From published work (reviewed by Bhatnagar and Moitra 1996; Biswas and Johri 1997), it has become apparent that there are similarities between the embryo ontogeny of cycads and

Ginkgo – especially during early embryogenesis. Hence, these two plant groups form what is referred to as the 'Cycad and Ginkgo type' of gymnosperm embryo development. While the reader is referred to the above-mentioned reviews for detailed accounts of embryogenesis in other gymnosperms, the Cycad and Ginkgo type of embryo development is described as follows.

The zygote nucleus divides *in situ* followed by many free-nuclear divisions. The nuclei disperse evenly throughout the entire pro-embryo and on some occasions, evanescent walls appear to develop during the free-nuclear period (Chamberlain 1910; Favre-Ducharte 1956). In later stages, while the free nuclei are distributed evenly in *Ginkgo*, they become concentrated at the base of the pro-embryo in cycads. The upper part of the cycad pro-embryo contains considerably fewer nuclei in a thin cytoplasm – considered to be an "advanced" trend over pro-embryos with evenly distributed nuclei (cf. conifers, see Dogra 1992). Subsequently, nuclei at the base of the pro-embryo divide whilst those in the upper part display signs of degeneration (Bryan 1952).

By the time walls are formed, there are approximately 256 free nuclei in *Ginkgo* (Singh 1978), 512 in *Cycas circinalis* (Rao 1963), and 512 or 1024 in *Dioon* (Chamberlain 1910). The resultant cells fill the entire pro-embryo in *Ginkgo*; however, in cycads, newly-formed cells are found only in the lower part of the pro-embryo (Biswas and Johri 1997). In *Encephalartos friderici-guiliemi*, *E. villosus* and *Macrozamia spiralis*, segmentation of almost the whole egg cell gives rise to the formation of a primary pro-embryo having a dense, active basal area (Sedgwick 1924; Brough and Taylor 1940). In *M. reidlei,* formation of the walls occurs throughout the pro-embryo apart from a small region in the centre characterised by free nuclei (Baird 1939). This area disintegrates later, giving rise to a central cavity. In *C. circinalis*, cellularisation is restricted to the peripheral and basal regions (Swamy 1948); while in *Stangeria* sp., *Zamia* spp. and *Bowenia* spp., pro-embryo cells form only basally (Bryan 1952; Chamberlain 1916; Lawson 1926; as in conifers; Dogra 1992).

Once walls have been formed, the basal cells divide and function as primary embryo cells, while the cells of the upper region elongate to give rise to a massive suspensor. The region designated the primary embryo comprises compact, dense, actively dividing, uniform cells concentrated at the tip (Biswas and Johri 1997). This forms the meristematic cell region of the embryo, the derivatives of which contribute to the elongating suspensor that pushes the mass of embryo cells deep into the centre of the megagametophyte (Singh and Johri 1972).

However, there appears to be no well-defined suspensor in *Ginkgo* apart from a micropylar region of elongated cells (Biswas and Johri 1997).

In *Zamia* and *Cycas* (and probably in other cycad genera) cells of the outermost layer of the embryo elongate slightly to give rise to a noticeable cap around the group of meristematic cells, which probably occurs in other cycad genera as well (Bryan 1952; Maheshwari 1960). The cap cells persist for a while; however, they ultimately degenerate and do not add to the anatomy of the mature embryo (Bryan 1952). A few young embryos may be found in some seeds, which usually arise when more than one zygote is formed (simple/poly-zygotic/archegonial polyembryony) [Singh and Johri 1972]. Cleavage polyembryony (several embryos derived from a single zygote) has not been found to occur in the cycads (Biswas and Johri 1997). As a contrasting example, in the conifer, *Araucaria angustifolia*, both types of polyembryony occur (with mono-zygotic predominating), but generally only one embryo survives (Agapito-Tenfen *et al.* 2011). Generally in cycads, the mature embryo is dicotyledonous, but three cotyledons have been found on some occasions (Biswas and Johri 1997).

From the studies outlined above, it is evident that most of the descriptive work on cycad embryogenesis thus far has centred on the early stages, i.e., development of the pro-embryo. Presently, many questions pertaining to the histology and ultrastructure of cycad embryos in particular remain unanswered. In this regard, the present investigation seeks to provide an in-depth microscopical account of some aspects of the embryo ontogeny of *E. natalensis*. Since development of the embryo of this species is largely a post-shedding phenomenon, one of the major objectives of the present investigation was to observe the morphological and anatomical aspects of embryogenesis from seed-shed to six months after shedding – when the seeds were readily able to germinate. To this end, light microscopy, histochemistry and transmission electron microscopy were employed to monitor development of cells and tissues.

Materials and Methods

Plant material

As a mandatory requirement, application was made and a permit granted for scientific research on a species, *Encephalartos natalensis*, listed as threatened or protected (ToPS) in

terms of the National Environmental Management: Biodiversity Act of South Africa. Male and female *E. natalensis* plants used were accessed on the Howard College Campus (29° 52′ 07.28″ S; 30° 58′ 49.52″ E) of the University of KwaZulu-Natal in Durban. Since it is very easy to mistake futile ovules for fertilised seeds in *E. natalensis* (Woodenberg 2009), and because natural pollination often yields low actual seed numbers in garden settings where male and female cones are far apart (personal observation), hand pollination was undertaken during the period when male *E. natalensis* plants were shedding pollen (i.e. May-June). To this end, a few scales from the top of the female cone were removed using a scalpel blade so that a part the axis of the cone was exposed; freshly-collected, dry pollen was then blown into the opening created in the female cones using a drinking straw. This procedure was performed at three-day intervals as long as fresh pollen was being shed. Following hand pollination, seeds were collected in December of the same year from *E. natalensis* plants, upon the disintegration of the female cone. The seeds were prepared by removal of the sarcotesta with a sharp scalpel blade, and rinsing with water before surface decontamination by soaking in a 2.5% solution of sodium hypochlorite (NaOCl) for 10 min. The seeds were then rinsed three times with distilled water, blotted dry, dusted with Benlate® (benzimidazole, 500 g kg^{-1}), placed in brown paper bags and stored at 16°C until required. Embryos were removed from longitudinally sectioned, newly-shed seeds and monthly from stored seeds for morphological examination and tissue processing. The observations incorporated in the present contribution derive from embryos excised at seed-shed, and from seeds stored for 2, 4 and 6 months.

Imaging of the morphology of intact embryos

Images of the external appearance of whole, intact embryos viewed with a Nikon AZ100 stereo microscope, were captured using NIS Elements D 3.0 imaging software over the 0 – 4 month developmental stages, while mature embryos (6 months after seed-shed) were photographed using a Canon EOS 350D digital camera equipped with a Canon EF 100 mm f/2.8 Macro USM lens.

Tissue processing for microscopy

Processing for light microscopy

For histology, dissected embryo segments (*c.*5x5x5 mm) were fixed overnight in 4% paraformaldehyde buffered at pH 7.2 with 0.1 M phosphate buffer. The segments were then

rinsed briefly in the buffer, dehydrated in a graded ethanol series diluted with phosphate-buffered saline (PBS), after which they were infiltrated under vacuum in a 37°C oven with Steedman's (1960) wax. Sections, 12 µm thick, were cut using an American Optical 8209 rotary microtome, mounted on Haupt's adhesive-coated slides (Jensen 1962) and de-waxed as described below before being subjected to histological staining. Toluidine blue, as a 1% solution (pH unknown), made up as follows: 60ml 1% sodium bicarbonate, 40ml glycerol, 1 g Toluidine blue, was used for routine staining.

Histochemistry

Sections were de-waxed first by placing the slides on a hot tray until the wax melted and then briefly exposing them to xylene. The presence of protein in air-dried sections was tested for by staining with eosin dye (BDH Chemicals, England) according to James and Tas (1984) or mercuric bromophenol blue (MBB: [BDH Chemicals, England]) after Mazia *et al.* (1953), while lipids were visualised histochemically using Sudan Black B (Sigma-Aldrich, Germany) according to McManus (1946). The application of these methods (excluding MBB) to cycad tissues is fully described in Woodenberg *et al.* (2010). For MBB staining, de-waxed sections were treated with mercuric bromophenol blue for 15 min, followed by 20 min in 0.5% acetic acid and brief immersion in distilled water. The sections mounted in distilled water were then viewed immediately with the light microscope. Carbohydrate histochemistry was performed by treating de-waxed sections with Lugol's solution (Jensen 1962) for the detection of starch, which stained dark brown/black. In all cases sections were compared with those not subjected to specific histochemical staining.

Tannins were detected with vanillin-HCl according to Pizzolato and Lillie (1973). Dewaxed sections were immersed for 5 min in a solution containing 10% (w/v) vanillin (Carlo Ertia, Rome) dissolved in a mixture of 100% ethanol and concentrated HCl of equal proportions. Sections were mounted in the same reagent and viewed immediately with the microscope. Tannins stained red.

Ducts, lined by epithelial cells, were checked for the presence of mucilage using a modified (addition of toluidine blue) version of Mace and Howell's (1974) protocol for the staining of mucins. Sections were de-waxed using xylene and treated with a 5% solution of tannic acid in water for 10 min, followed by rinsing briefly in distilled water for *c.* 15 s, after which the sections were flooded with 0.08 M (2%) ferric chloride for 1 min, washed briefly in distilled

water, stained with 1% toluidine blue, rinsed again with water, and viewed microscopically. Mucilage stained pale blue.

Because the regular Mace and Howell (1974) protocol did not yield the characteristic pink stain in the mucilage ducts another histochemical test was carried out to ascertain the presence of acidic polysaccharides, like mucilage. De-waxed sections were treated for 10 min with aqueous Ruthenium red diluted 1:5000. Excess stain was rinsed off with water and the sections viewed. Mucilage stained pink.

Tissue processing for TEM

Small cubes (1-2 mm^3) were excised from embryos using a sharp scalpel blade and subjected to the following infiltration and embedding protocol:

Samples were fixed overnight in 2.5% glutaraldehyde buffered at pH 7.2 with 0.1 M phosphate buffer containing 1% caffeine, washed in 0.1 M phosphate buffer, post-fixed in 0.5% phosphate-buffered osmium tetroxide for 2 h, and washed again in 0.1 M phosphate buffer before dehydration. Samples were dehydrated in a graded ethanol series (25-75%), 2 x 10 min each, and left in 75% ethanol overnight. The samples were then block-stained with a saturated solution of uranyl acetate in 75% ethanol for 45 min, dehydrated further in 100% ethanol, followed by 2 x 10 min changes in propylene oxide. The dehydrated samples were then placed in a 1:1 mixture of propylene oxide:epoxy resin (Spurr 1969), rotated 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.

Microtomy and microscopy

The resin-embedded samples were sectioned using a Reichert-Jung Ultracut E microtome. Sections, 1 μm thick, were stained with 1% toluidine blue and viewed with a Nikon Eclipse 80i light microscope equipped with NIS Elements F Package imaging software. Ultrathin sections were cut for TEM analysis and stained with lead citrate (Reynolds 1963) for 10 min. A maximum of five sections was viewed on each grid using a Jeol JEM 1010 transmission electron microscope and iTEM Soft Imaging System GmbH imaging software. The following stages were sectioned and viewed: at seed-shed and 2, 4 and 6 months after this stage (n = 6).

Results and discussion

At the time of seed-shed, the embryo of *Encephalartos natalensis* was found to be a rudimentary structure subtended by a long, coiled suspensor (Fig. 1a). The embryo was observed to undergo considerable growth and development in the months after seed-shed (Figs 1a-d) eventually reaching its full length and becoming naturally germinable six months later (Fig. 1d). Immature embryos at seed-shed are not unique to *E. natalensis* having been found in other cycads such as *Cycas rumphii* (De Silva and Tambiah 1952) and *C. revoluta* (Dehgan and Schutzman 1989). However, according to the latter authors, other cycad taxa e.g. *Zamia fischeri* and *Z. loddigesii* germinate readily upon seed-shed, which is an indication that they are in a much more advanced stage of development compared with *E. natalensis*. Similarly, Vorster (1995), noticed that some, but not all, of *E. transvenosus* and *E. manikensis* seeds may germinate shortly after they are shed (see also Grobbelaar 1990).

It is not surprising that the seeds of *E. natalensis* are unable to germinate immediately they are shed as microscopy in the present study revealed that the embryos at this time were composed only of numerous undifferentiated, meristematic cells (Figs 2a-f). The embryo tissue, as is typical of meristems, had no intercellular spaces. The cells were bounded by thin walls, had prominent nuclei, along with numerous small vacuoles, amyloplasts, mitochondria of active appearance, and short profiles of endoplasmic reticulum (ER), while few Golgi bodies were seen (Figs 2b-f).

The suspensor tissue, on the other hand, appeared translucent at low magnification (Fig. 2a), and was composed of elongated, highly vacuolated cells that contained a few amyloplasts (Fig. 2b). According to Chamberlain (1919), cycad suspensors are the longest in the plant kingdom and function to thrust the embryo into the corrosion cavity of the megagamatophyte. In addition, it has been suggested that the suspensor may provide nutrients and growth regulators for the embryo during the early phases of embryogenesis (Beers 1997; Schwartz *et al.* 1997; Wredle *et al.* 2001). The suspensor was observed in the present investigation to remain attached to the growing embryo right through the six month period (Figs 1a–d); although at six months after seed-shed it appeared shrivelled and dehydrated (Fig. 1d).

While embryos in the present investigation seemed to be at the same developmental stage when they were shed from the parent plant, their subsequent development appeared to be asynchronous in the months after seed-shed. Dehgan and Schutzman (1989) observed similar asynchrony in the ontogeny of *Cycas* seeds stored at 22°C, while embryos of cold-stored

Fig.1 The external appearance of *E. natalensis* embryos at various stages of post-shedding seed development is illustrated. **a** At seed-shed where the embryo (E) is miniscule and attached to a long, coiled suspensor (S). **b** The now-ovoid embryo (E) two months after seed-shed at the end of the suspensor (S). **c** Shows the torpedo shape of the embryo still attached to the suspensor (S) in the fourth month after seed-shed. **d** The mature embryo (E), which is considerably larger than it was at seed-shed, still attached to a dried, shrivelled suspensor; bar = 2 mm

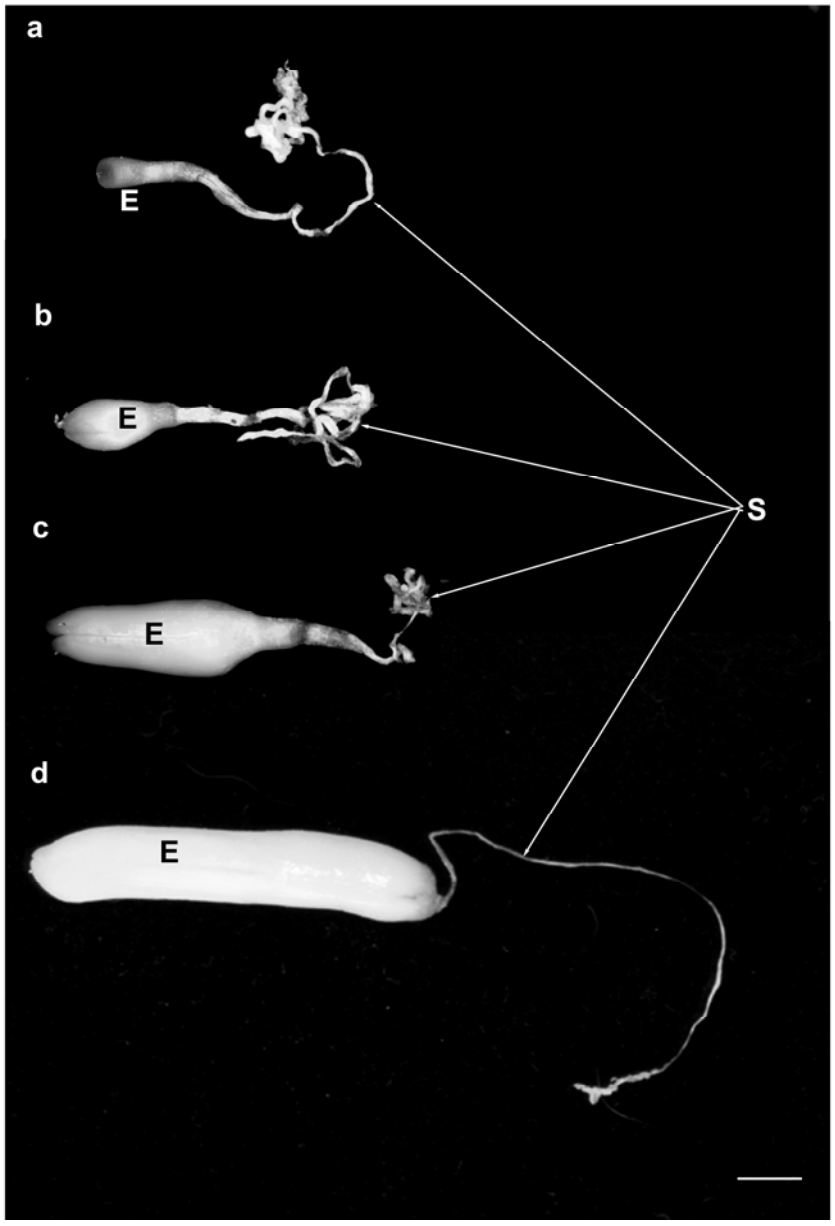
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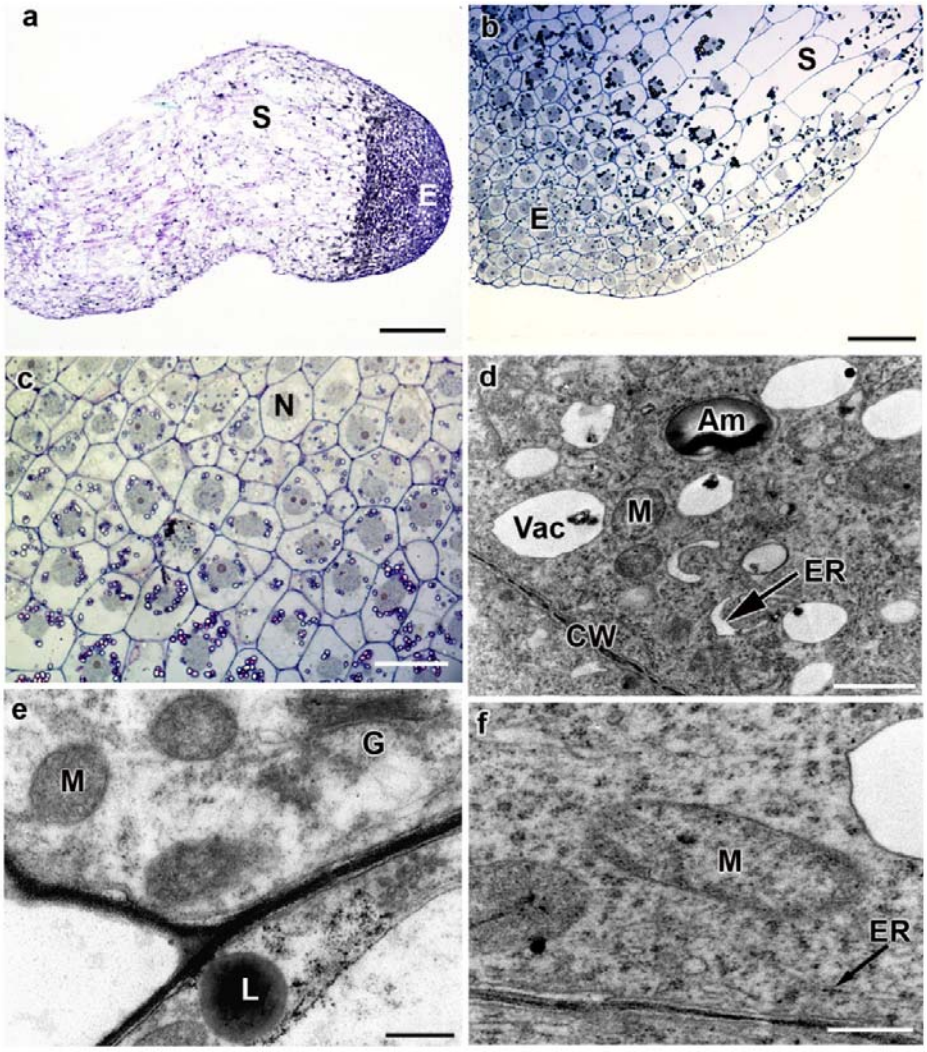
Fig. 2 Histology and ultrastructure of the embryo shortly after seed-shed. **a** The embryo (E) stained darkly with toluidine blue compared with the suspensor (S); bar = 500 μm. **b** The cells of the suspensor (S) can be seen to be much longer and with a considerably more translucent cytomatrix compared with the cells of the embryo (E); bar = 100 μm. **c** The structure of the embryo cells is depicted. These cells are isodiametric, compact, with no intercellular spaces, and have a relatively large nucleus (N) to cell area ratio; bar = 50 μm. **d** Illustrates the ultrastructure of the embryo cells, having relatively thin cell walls (CW), small amyloplasts (Am) and vacuoles (Vac), abundant mitochondria (M) and crescentic, partially-dilated profiles of the endoplasmic reticulum (ER) scattered in the cytomatrix; bar = 1 μm. **e** Mitochondria (M) displayed dense matrices and well-defined cristae, while only a few Golgi bodies (G) and lipid bodies (L) occurred; bar = 0.5 μm. **f** Short profiles of endoplasmic reticulum (ER) were also prevalent between mitochondria (M) and other organelles in the peripheral region of the cytomatrix, which exhibited an abundance of polysomes; bar = 0.5 μm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(5°C) seeds displayed equally well-developed embryos over the same 24 week period. Asynchronous embryo development may be a strategy used by plants to lower the risk of losing an entire crop of seeds under non-conducive environmental conditions at the time when some seeds are ready to germinate. Given the asynchronous nature of embryo development in the current species, micrographs currently presented are of the most representative embryos at each stage.

The embryos of the next stage observed in this study (i.e. two months after seed-shed) had undergone a measure of morphological, histological and cellular differentiation (Figs 3a-f). The acropetal region observed at the earlier stage had remained meristematic, now constituting the shoot meristem, while two flanking cotyledonary protuberances were now evident (Fig. 3a). These developing cotyledons appeared to have originated from two groups of cells, one on either side of the shoot meristem as described previously for *Ginkgo biloba* (Lyon 1904), and, as Saxton (1910) suggested, is probably the case for all gymnosperms. As illustrated by Chamberlain (1919), differentiation of the cotyledonary protuberances of cycads seems to occur as follows: The rapid growth of the undifferentiated embryo becomes somewhat retarded acropetally, while growth of the region around it is accelerated. This causes what that author describes as a depression surrounded by a ring of cotyledonary tissue. The cotyledonary ring is not fully complete and consists of two equally crescentic parts that nearly touch each other at their ends (Chamberlain 1919).

In the current study of embryos two months after seed-shed, while the cotyledonary protuberances were a major feature, a few tannin channels were also now evident near the periphery of the embryo growing in the direction of its longitudinal plane (Figs 3a-c). The structures presently termed tannin channels have been seen in previous studies on cycad germplasm, and while they have been referred to as tannin cells in most reports (Dorety 1909; 1919; Sanchez-Tinoco and Engelman 2004; Saxton 1910), they have also been called canals (Saxton 1910), tannin idioblasts, and gold cells (Vovides 1991; Vovides ***et al.*** 1993, respectively).

The tannins in the current investigation appeared amorphous in wax-embedded embryo material (e.g. Figs 3a & b); but was sometimes condensed or perhaps precipitated in material embedded in resin (e.g. Fig. 3c). Sanchez-Tinoco and Engelman (2004) have also observed two manifestations of tannin in the seed coat of another cycad, *Ceratozamia mexicana*. This may be an indication of two types of tannins or other phenolic compounds, or it may indicate

poor penetration into the tannins by resin. From the present study, the latter explanation is considered to be more likely, as the dual appearance of the tannins was consistent with the embedding medium used.

Non-tanniniferous embryo cells at two months after seed-shed contained large vacuoles as illustrated in Figs 3c, d & e, while relatively small amyloplasts, few discrete lipid bodies and Golgi bodies, mitochondria with dense matrices, and short profiles of ER were a common feature of the cytomatrix (Figs 3d & e), but generally few Golgi bodies were observed. Mitochondria exhibited well-developed cristae and relatively dense matrices and clusters of polysomes were a consistent feature (Fig. 3f). These intracellular features are congruent with the on-going metabolism that accompanies embryogenesis. The cell walls now also appeared more substantial than the previous stage and nascent intercellular spaces (not illustrated) were sometimes observed indicating that the embryo was no longer composed of meristematic cells only.

In the early stages of channel formation, the tannin appeared to be contained within vacuoles in cells also showing the occurrence of amyloplasts and nuclei (Fig. 4a). Subsequently, the channels elongated considerably (Figs 4b & d), although there was no evidence of prior coenocyte formation, as was observed by Zobel (1985) in shoots of *Sambucus racemosa*, an angiospermous species. The contents of the tannin channels were amber/brown-coloured in unstained sections (see Figs 12a & e-g); and light to very dark blue in sections stained with toluidine blue (Figs 4c & d). Transmission electron microscopy confirmed tannin accumulations within vacuoles (Figs 4e-h) following internalisation of vesicles containing small condensations (Fig 4g). There was evidence of the vesicles being ER-derived (Fig. 4f), suggesting the origin of the tannins to be from the endoplasmic reticulum. This is in agreement with views that tannins originate in the ER prior to being accumulated intravacuolarly (Zobel 1985; Rao 1988; Evert 2006).

The embryo at the next stage, i.e. four months after seed-shed (Figs 5-7), consisted of a shoot and root meristem that appeared to be separated by a short hypocotyl region (Fig. 5a). When viewed in cross section, the root meristem displayed radiating rows of cells (Fig. 5b), which suggested that new meristematic cells in this tissue are formed mostly by anticlinal mitoses from centripetal initials. The root meristem appears to be endogenous in the *E. natalensis* embryo (as it is in *Dioon spinulosum*; [Dorety 1919]), since it is surrounded by differentiated cells that are interspersed with tannin channels (Figs 5a & b). Those differentiated cells had

Fig. 3 Some histological and ultrastructural aspects of the embryo at two months after seed-shed are shown. **a** Two cotyledons (C), which are seen in the early stages of their development, flank the shoot meristem (SM) of the embryo, while the cells of the suspensor (S) show no visible change compared with the earlier stage. A few tannin channels (TC) were now apparent near the periphery of the embryo; bar = 500 μm. **b** The tannin channels (TC) appeared to develop parallel with the longitudinal plane of the embryo, while the procambium (PC), appearing as bands of elongated meristematic cells, was also now apparent; bar = 100 μm. **c** Demonstrates a tannin channel (TC) in cross-section, in which the contents appear condensed peripherally, while non-tanniniferous cells display darkly-stained nuclei (N) and large vacuoles (Vac); bar = 50 μm. **d** The cytomatrix between the vacuoles (Vac) typically displayed long profiles of endoplasmic reticulum (ER), small amyloplasts (Am), mitochondria (M) and occasional lipid bodies (L) near the cell wall (CW); bar = 0.5 μm. **e** Occasional Golgi bodies (G) were seen in the periphery of the cytomatrix where other organelles like the mitochondrion (M) illustrated, were found; bar = 0.5 μm. **f** Mitochondria (M) had relatively dense matrices and clearly-defined cristae, and clusters of polysomes were apparent; bar = 0.5 μm

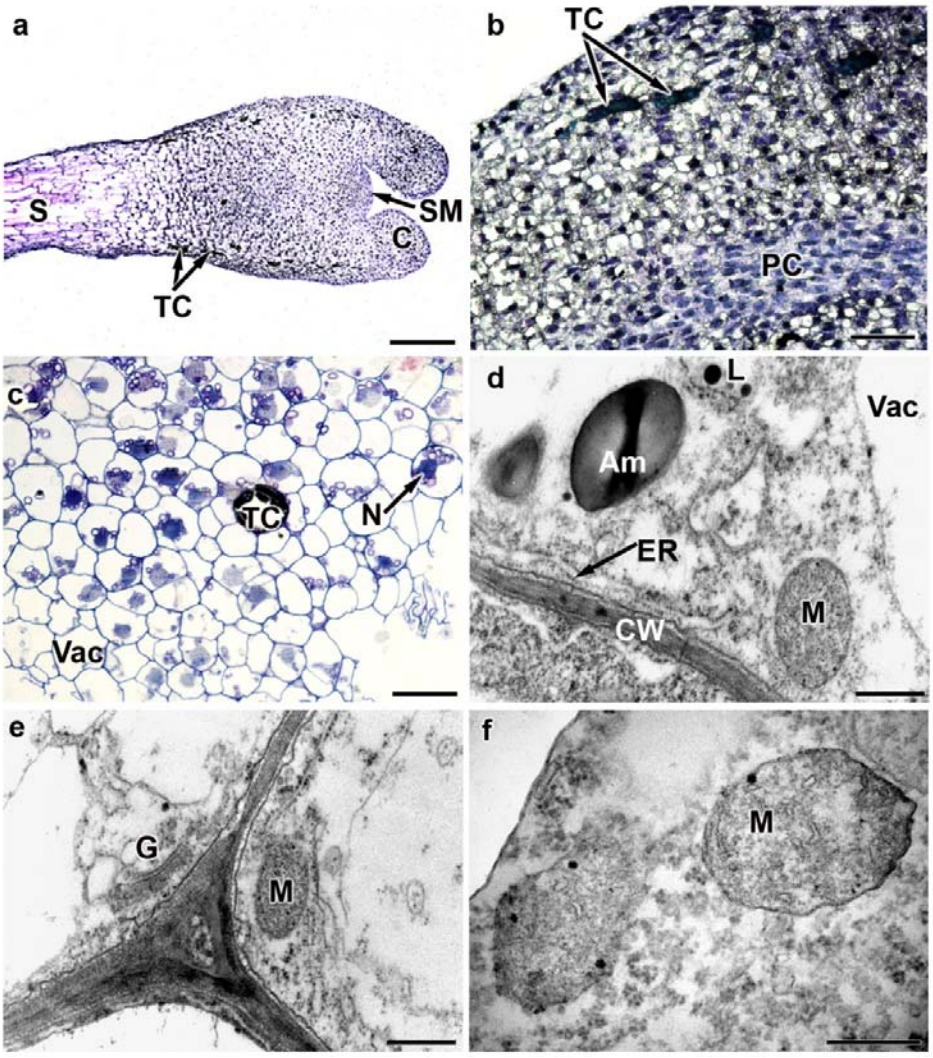
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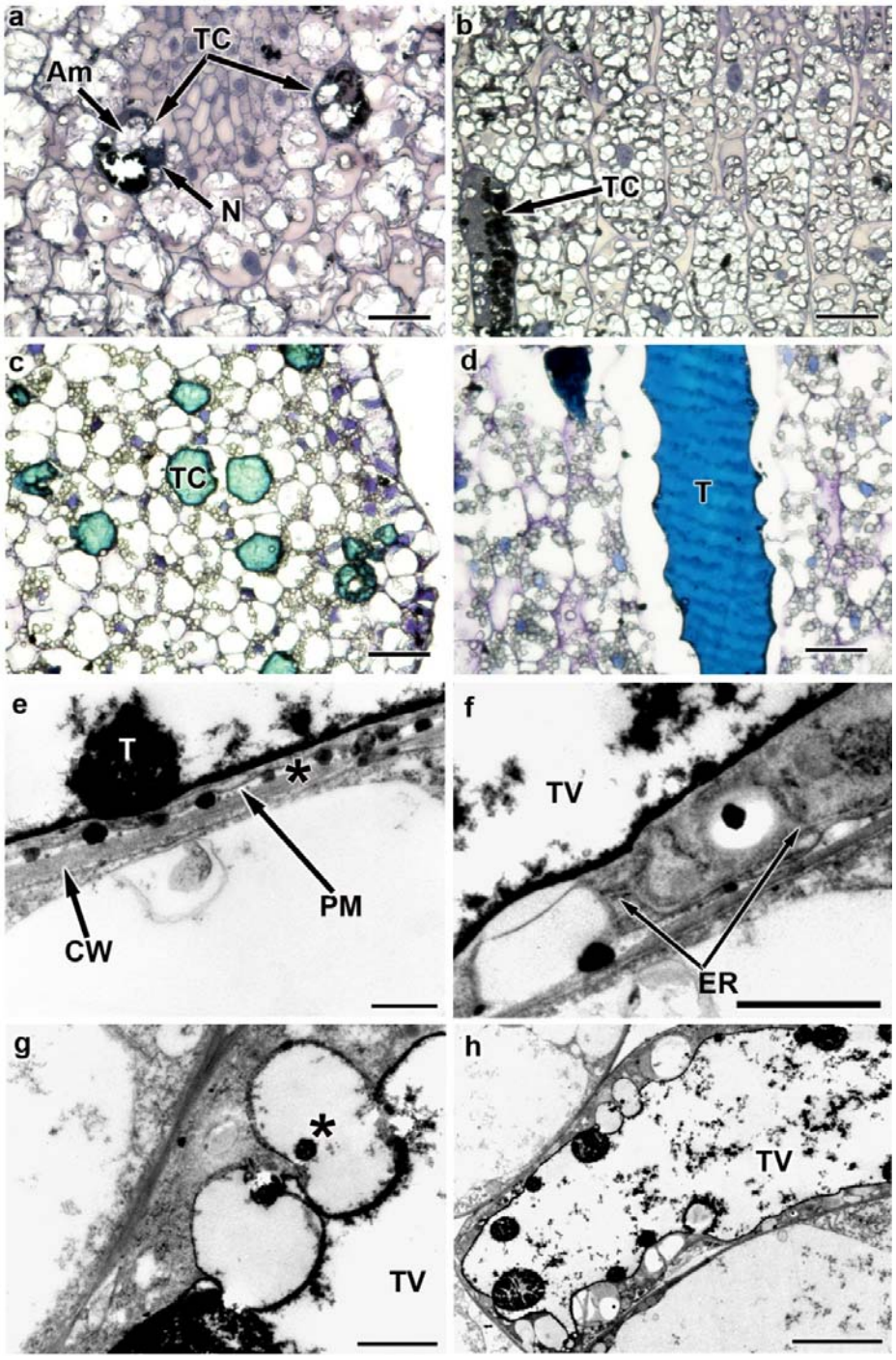
Fig. 4 Optical and electron micrographs demonstrating tannin channels. **a** A cross section showing early stages of tannin channel (TC) formation with the tannins apparently contained in vacuoles. Amyloplasts (Am) and nuclei (N) can also be resolved; bar = 50 µm. **b** Longitudinal section of a resin-embedded embryo showing a tannin channel (TC) during development. This channel appears considerably longer than the surrounding cells and contains darkly-staining deposits and a few amyloplasts; bar = 50 µm. **c** Tannin channels (TC) in a cross section of wax-embedded embryo tissue at a later stage of development, displaying relatively uniform tannin content filling the entire channel; bar = 50 µm. **d** A tannin channel sectioned longitudinally showing tannin drawn away from the walls, while no organelles can be discerned; bar = 50 µm. **e** Early stages of tannin channel formation showing tannin (T) accumulations in the tonoplast and small, electron-opaque deposits (asterisk) between the plasmalemma (PM) and cell wall (CW); bar = 0.5 µm. **f** Nascent vesicles apparently originating from the endoplasmic reticulum (ER, arrowed) and incorporating small electron-opaque deposits can be seen between the tannin vacuole (TV) and plasmalemma; bar = 1 µm. **g** ER-derived vesicles bearing small, electron-opaque deposits (asterisk) appeared to be internalised by the tannin vacuole (TV); bar = 1 µm. **h** A large, central tannin vacuole, the predominant organelle in a developing tannin channel, is illustrated; bar = 5 µm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considerably larger, multifaceted amyloplasts (Figs 5c & d) than seen in the previous stages. Similar amyloplasts have been observed previously in the megagametophyte of *E. natalensis*, where they were in such abundance that other organelles and the nuclei appeared exceedingly compressed (Woodenberg *et al.* 2010). The differentiated cells were also highly vacuolated (Figs 5d-f) which further compressed the ground cytomatrix, in which long profiles of ER, the occasional lipid bodies (Fig. 5e) and mitochondria (Fig. 5f) occurred. A striking feature of these cells also, was the abundance of polysomes (Figs 5e & f), contributing further to the dense appearance of the cytomatrix.

The region of differentiated cells between the root meristem and suspensor has been observed previously in other cycad embryos and has been called either the coleorhiza (Chamberlain 1910; 1919; Dehgan and Schutzman 1989; Hooft 1970) or root cap (Chavez *et al.* 1995; Saxton 1910). Regardless of the nomenclature, this tissue reportedly acts as a hard sheath that protects the root meristem from injury when the germinating embryo penetrates the woody sclerotesta (Chamberlain 1919; Sporne 1965).

While some cellular differentiation was seen in the hypocotyl region of the embryo four months after the seeds had been shed, the cells of the root and shoot meristems remained undifferentiated (Figs 6a-d) and appeared essentially similar to the cells of the embryo at seed-shed, i.e. small, compact cells each containing a prominent nucleus (Figs 6c & d), many small vacuoles and plastids showing only relatively small starch deposits (Fig. 6d). Profiles of ER and a few lipid bodies were seen (Fig. 6e). There were numerous mitochondria (Fig. 6f), but only occasional Golgi bodies (Fig. 6g). One of the most striking features of the meristem cells was frequency of both cytomatrical and membrane-associated polysomes (Fig. 6h), attesting to intensive protein synthesis. Although the ultrastructure of the cells of both the root and shoot meristems remained essentially consistent across the developmental stages of the embryos, the overall size of these meristems had increased as the embryos developed (Fig. 6a, cf. Fig. 3a). In the case of the meristematic region of the shoot, it is probable that the cellular proliferation was partially directed towards the considerable growth and development of the cotyledons (Fig. 6a) which ultimately became far longer than the axis of the embryo itself.

While the cotyledons were observed to be separate from each other (Fig. 5a) at this stage of ontogeny, in some sections they could be seen to be in close contact, at least laterally (Fig. 7a). In cross section, there appeared to be some seven vascular bundles (Fig. 7a) forming an

Fig. 5 Showing the histology and ultrastructure of the embryo four months after seed-shed. **a** Longitudinal section demonstrating the root meristem (RM) and shoot meristem (SM), the latter flanked by the two cotyledons (C), which are longer than at the previous stage. The procambium (PC) and tannin channels (TC) can be seen in each cotyledon; bar = 500 µm. **b** A cross-section of the embryo depicting the root meristem (RM) with radiating rows of meristematic cells; bar = 200 µm. **c** Tannin channels (TC) appeared to have condensed, poorly-preserved contents in cross-section in resin-embedded material, while the surrounding cells can be seen to be dominated by amyloplasts; bar = 50 µm. **d** The non-meristematic cells were extensively vacuolated (Vac) and typified by large amyloplasts (Am) each containing a considerable amount of starch, while other organelles and the nucleus (N) occurred in the dense cytomatrix; bar = 5 µm. **e** A few lipid bodies (L) as well as numerous long and short profiles of endoplasmic reticulum (ER) were apparent in the dense cytomatrix; bar 0.5 µm. **f** Mitochondria (M) were a common feature of the cytomatrix, its density being ascribed to the occurrence of many polysomes (arrowheads); bar = 20 µm

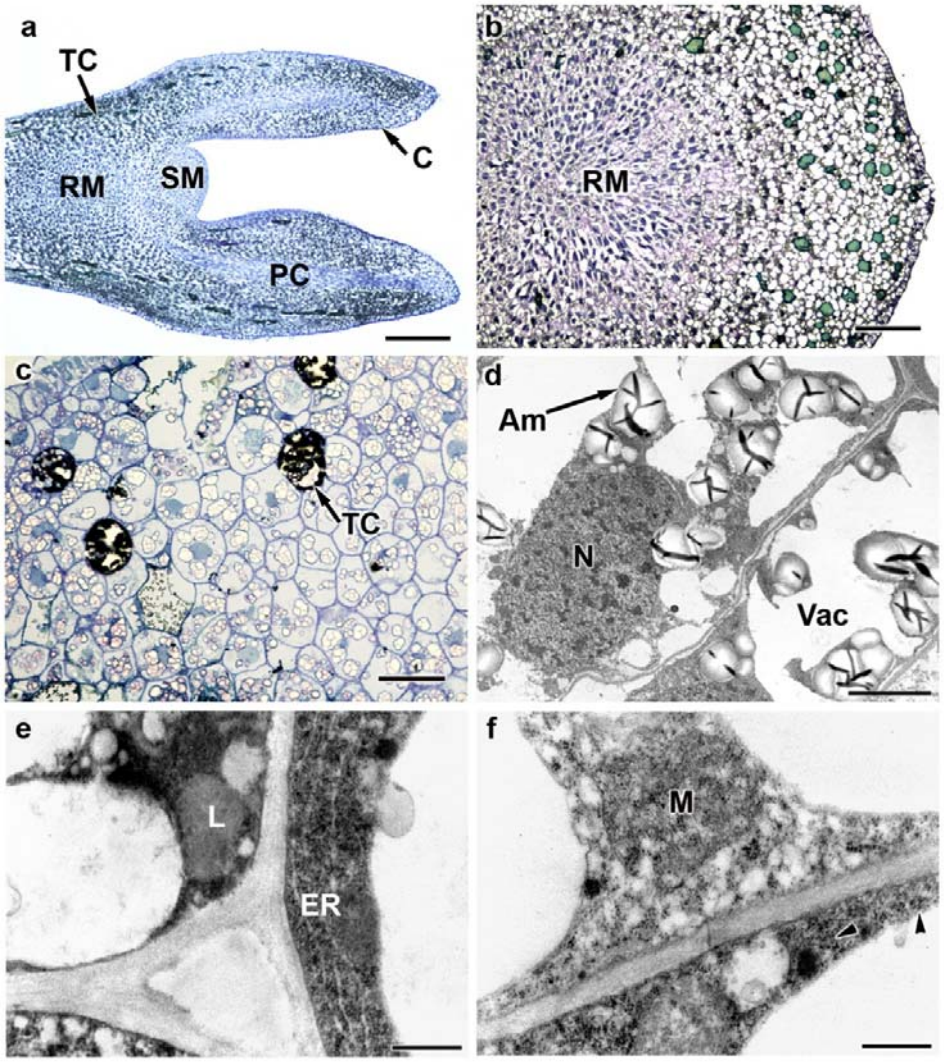
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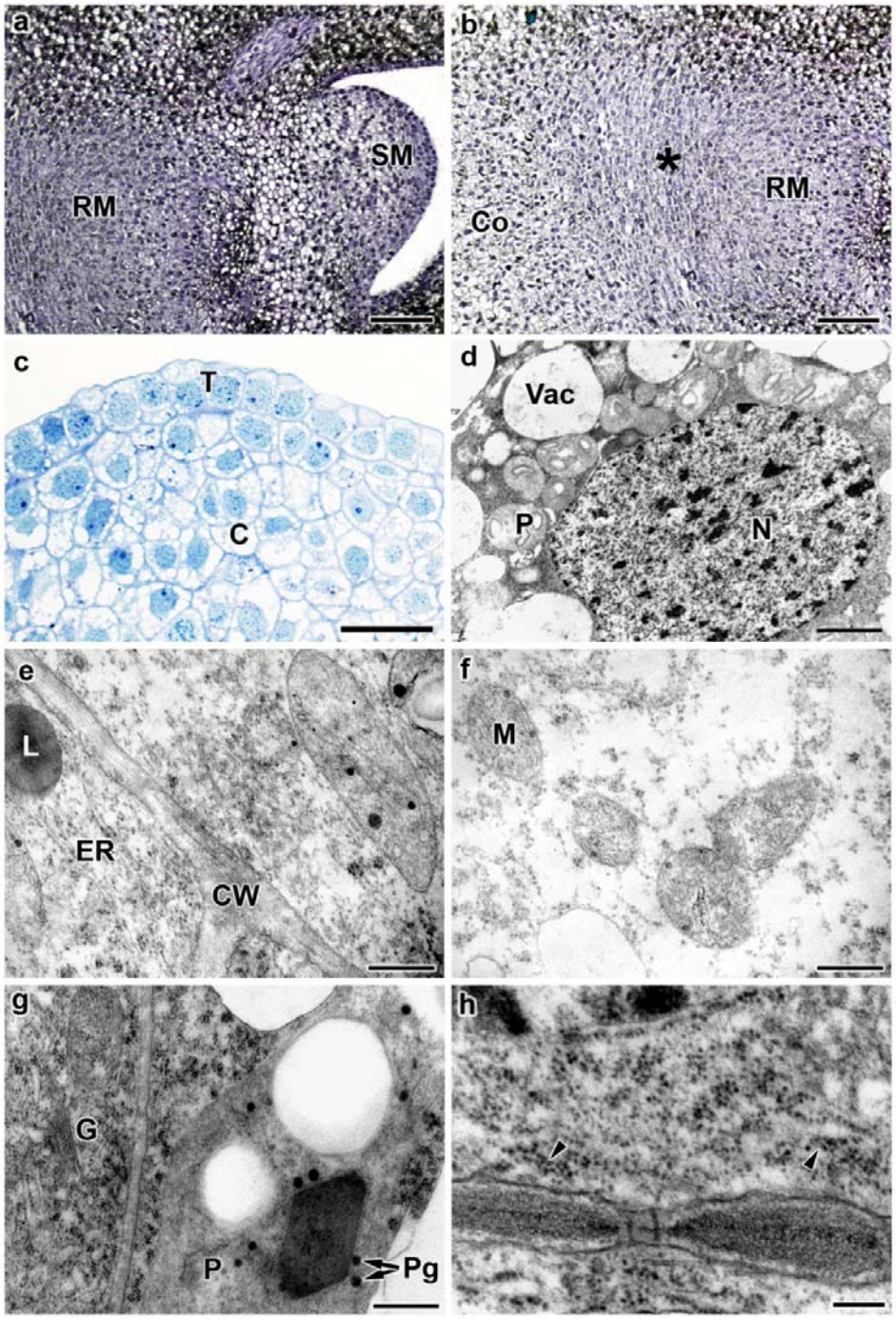
Fig. 6 Structure and ultrastructure of the meristematic regions of the embryo four months after seed-shed. **a** Longitudinal section of the root meristem (RM) and shoot meristem (SM), which appeared to be separated from each other by a region of larger, vacuolated cells; bar = 200 μm. **b** The root meristem (RM) in longitudinal section displayed a gentle arc (asterisk) of meristematic cells between its centre and coleorhiza region (Co); bar = 200 μm **c** A resin-embedded shoot apex sectioned longitudinally illustrating the meristem, in which the peripheral tunica (T) overlies the corpus (C); bar = 50 μm. **d** Cells of both root and shoot meristems displayed similar ultrastructure that seemed unchanged from the previous stage, typified by a relatively large nucleus (N), numerous small vacuoles (Vac) and plastids (P) containing only small starch deposits; bar = 2 μm. **e** As is typical for meristems, no intercellular spaces were evident at cell wall (CW) junctions, while a few, relatively darkly-staining lipid bodies and short profiles of endoplasmic reticulum (ER) were seen; bar = 5 μm. **f** Mitochondria (M) were numerous and showed well-defined cristae and dense matrices; bar = 0.5 μm. **g** Golgi bodies (G) also occurred, particularly near the cell periphery, while plastids (P) displayed varying numbers of relatively small, electron-translucent starch inclusions and scattered plastoglobuli (Pg); bar = 0.5 μm. **h** The cytomatrix was typified by the occurrence of polysomes, many of which were clearly ER-associated (arrows); bar = 0.2 μm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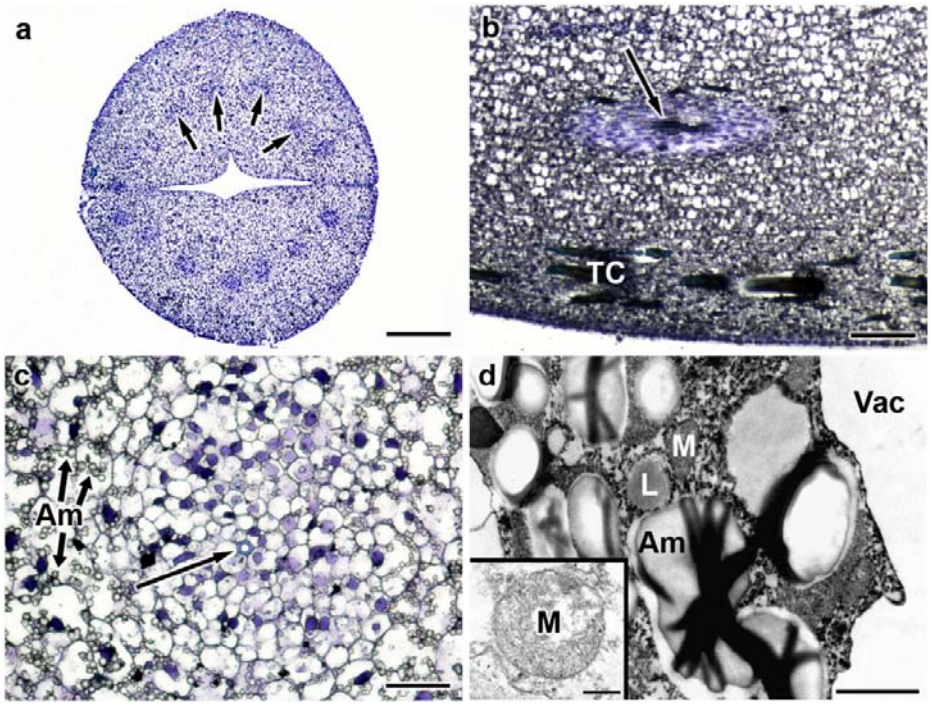
arc in the central region of each cotyledon. Longitudinal or tangential sections revealed that one or two tracheids had now differentiated centripetally in the developing vascular tissue (Figs 7b & c). These tracheids, when viewed in cross section had much thicker walls than the surrounding cells and displayed clear lumina (Fig. 7c), suggesting that they may be potentially functional at this stage in the transport of water contributing to cotyledon expansion during intra-seminal embryo ontogeny. Presently, however, this is only conjectural, as the tracheids that develop in this stage may become fully operational only during and after germination, and seedling establishment.

While tracheids could be unequivocally identified in the cotyledons at this stage, other than the procambium, the majority of the cotyledonary cells were similar to the differentiated cells of the hypocotyl region, with a notable abundance of amyloplasts (Figs 7c & d), polysomes (Fig. 7d) and well-developed mitochondria (inset, Fig. 7d). The starch deposited in these amyloplasts, and in those of the axis cells had to have been built up from hydrolysed starch reserves of the megagametophyte (Woodenberg *et al.* 2010) since all contact with the female strobilus was lost very early in embryo ontogeny. The starch was progressively accumulated seemingly without dimunition in the developing embryo. It is probable that it functions as the carbohydrate reserve during germination and seedling establishment, rather than during *in ovulo* growth of the embryo which is suggestedly sustained by continuous importation from the megagametophyte. While the content of the few discrete lipid bodies found in the embryo cells may also be utilised at germination, protein bodies such as those which were common in the megagametophyte cells of this species (Woodenberg *et al.* 2010) were entirely absent from the cells of the embryo (see later), despite the frequency of polysomes (Fig. 7d).

The predominance of starch in the mature embryo is in agreement with observations on *Dioon edule* (Chamberlain 1910) and whole seeds of *Ginkgo* (Singh *et al.* 2008). However, it is in marked contrast to most other gymnosperm species, e.g., conifers, which typically accumulate lipid as the primary storage reserve (Ching 1966; Gifford 1988; Krasowski and Owens 1993). However, according to Haines (1983), some *Araucaria* species viz. *A. angustifolia*, *A. hunstei*, *A. araucana* accumulate mostly carbohydrates, while carbohydrates constitute the total food storage component of *A. bidwillii* seeds. Apart from the situation in *Araucaria*, the preponderance of starch as a major storage product in the embryo appears to be a comparatively primitive trait, with lipid- and protein-dominant embryos representing the more advanced condition amongst the gymnosperms. In terms of the basal evolutionary position of the cycads in gymnosperm phylogeny, it could be suggested that starch was the

Fig. 7 Some aspects of the histology and ultrastructure of the cotyledons at four months after seed-shed are demonstrated. **a** Cross section showing the orientation of vascular bundles (arrows) in each of the two cotyledons; bar = 500 μm. **b** A tangential section through a vascular bundle, which appears to be made up primarily of procambial tissue with one or two tracheids (arrow) apparent. Tannin channels (TC) were located peripherally; bar = 200 μm. **c** At higher magnification, occasional tracheids (arrow) can be seen in the developing vascular bundle. The solitary tracheid has a typically thickened wall, compared with the surrounding cells, in which amyloplasts (Am) can clearly be resolved; bar = 50 μm. **d** Cells contained numerous, relatively large amyloplasts (Am) and vacuoles (Vac), many polysomes, a few lipid bodies (L) and mitochondria (M) with well-defined cristae (inset; bar = 0.5 μm); bar = 1 μm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first reserve to be accumulated generally in embryos of seed plants of ancient lineage. The accumulation of massive starch deposits initially in the gametophyte of *E. natalensis* (Woodenberg *et al.* 2010) with subsequent sucrose transport into, and starch synthesis in, the developing embryo is biochemically far less complicated than would be a system involving lipids (pers. comm. M. Black, King's College, London).

Furthermore, the predominance of starch as opposed to lipid as the major storage reserve in the *E. natalensis* embryo is not surprising given the relatively long period (*c*. 12 months) of embryo development from fertilisation to germination. Lipids are known to be vulnerable to oxidation (McDonald 2004), which may lead to a loss of viability during the protracted development of the embryo in the current species. This may have been especially important considering that cycads are suggested to have originated in the Carboniferous Period (Schwendemann *et al.* 2009) when atmospheric oxygen concentrations may have been as high as 35% (Berner and Canfield 1989). A further salient feature may be that seeds of *E. natalensis* and other species (Woodenberg *et al.* 2007) and those of *Araucaria angustifolia* (Farrant *et al.* 1989); *A. hunsteinii* (Pritchard and Prendergast 1986), *A. araucana* (Royal Botanic Gardens Kew 2008) and *A. bidwillii* (Del Zoppo *et al.* 1998) are desiccation-sensitive, thus requiring them to remain hydrated throughout development to germination. It is suggested that lipid would be more prone to oxidative degradation in metabolically active tissue which remains hydrated for relatively prolonged periods, than in, desiccation-tolerant (orthodox) seed tissues of e.g. most of the conifers.

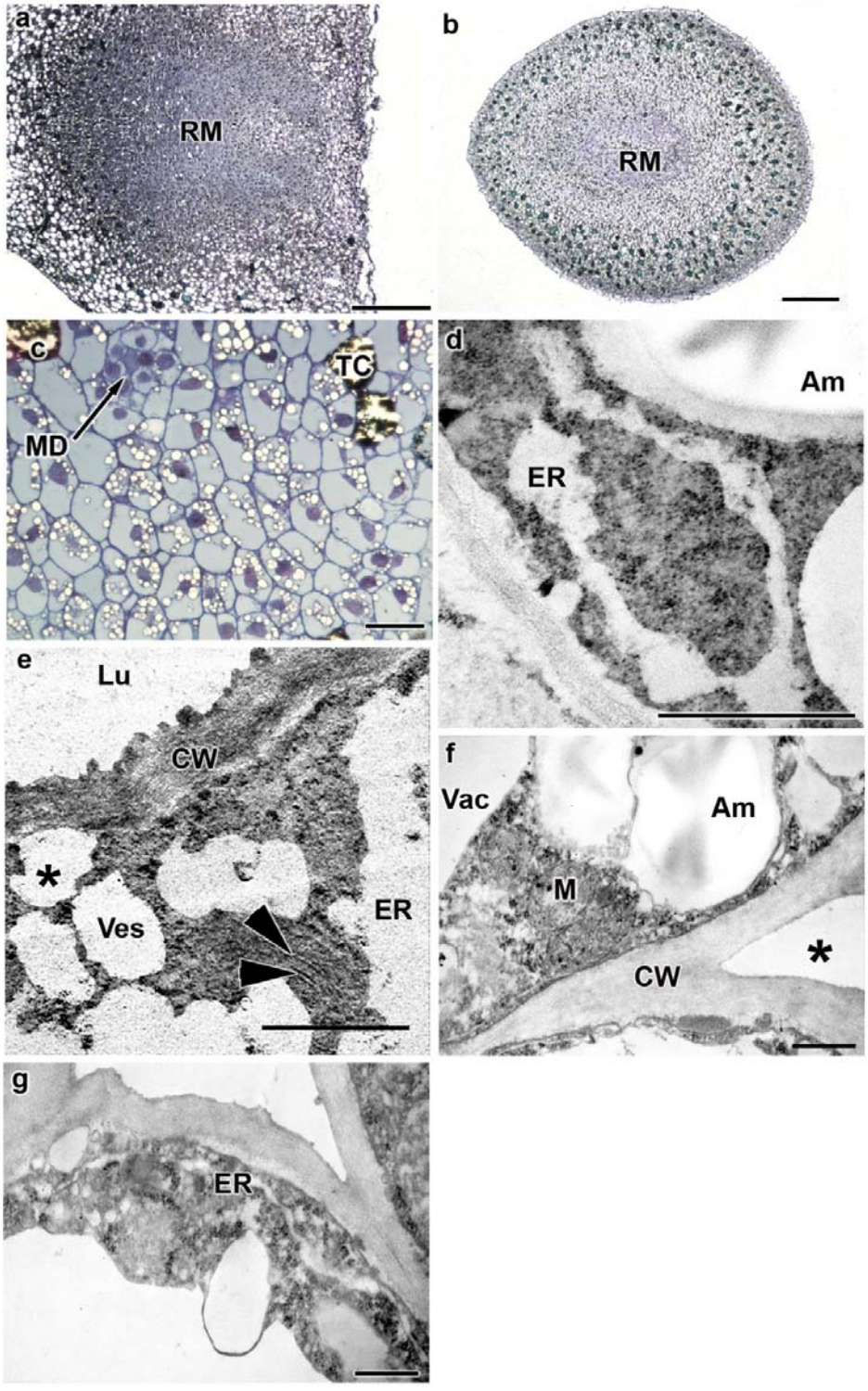
At six months after seed-shed, the root meristem region of the embryo had increased in overall size (Figs 8a & b). A conspicuous feature of the embryo at this stage was the development of the mucilage ducts (Fig. 8c). These ducts were lined by epithelial cells and while the one demonstrated in Fig. 8c appears to have retained its contents, the lumina of most appeared clear (illustrated later). The evidence that these mucilage ducts were lined by intact cells suggested that they may have been formed schizogenously, i.e. by separation of parenchyma cells from each other (Esau 1953; Evert 2006). According to those authors, after a few divisions, the cells that separate from each other come to line the duct, effectively forming an epithelium. Duct-lining epithelial cells have been described as producing hydrophilic mucilaginous polysaccharides intracellularly before transferring the secretion into the duct (Romberger 1993): however, in *E. natalensis* embryos it is suggested that they elaborate glycoproteins/mucopolysaccharides, as has been suggested for fruit tissue of *Mangifera indica* (Joel and Fahn 1980). The epithelial cells in the current study were

characterised ultrastructurally by numerous profiles of variously-distended ER (Figs 8d & e) that appeared to give rise to many vesicles (Fig. 8e). Some of those vesicles appeared to fuse with the plasmalemma (Fig. 8e) and are suggested to be involved in the secretion of mucopolysaccharides into the lumen of the mucilage duct. Since Golgi bodies were observed extremely infrequently in the epithelial cells, it appeared that the endoplasmic reticulum is the intracellular system involved in the manufacture and secretion of mucopolysaccharides in the current species. The apparent non-involvement of Golgi bodies in mucilage secretion in developing *E. natalensis* embryos was an unexpected feature: however, it is in keeping with observations on early cellularisation of the megagametophyte in this species, where ER-derived vesicles were found to elaborate and export cell wall material without intermediary involvement of Golgi bodies, which were rarely observed (Woodenberg *et al.* 2010). Core glycosylation of nascent polypeptides/proteins is an ER-mediated process, and it could be argued that this process suffices in the production of mucopolysaccharide mucilage in embryos of *E. natalensis*. Golgi bypass in secretory pathways has been described and debated for animal tissues (Grieve and Rabouille 2011) and described for maturing pumpkin seeds (Hara-Nishimura *et al.* 1998) and storage protein in rice endosperm (Takahashi *et al.* 2005). Although unusual, Golgi bypass in plants seems likely (Hawes 2005), but is not uncontestable (Tian *et al.* 2013). However, to our knowledge no such studies have been carried out on seeds of plants of ancient lineage, as represented by the cycads.

According to the review by Bhatnagar and Moitra (1996), mucilage ducts in cycads may act as water reservoirs, as a xerophytic characteristic. At this stage also, six months after the *E. natalesis* seeds were shed, intercellular spaces had developed in the hypocotyl tissue (Fig. 8f) the cells of which were characterised by many amyloplasts and large vacuoles, mitochondria, ER and numerous polysomes (Figs 8f & g).

Another conspicuous feature of the embryo six months after seed-shed was the appearance of developing leaf primordia (Figs 9a & b). While the shoot meristem appeared generally similar to that of the earlier developmental stage, leaf primordia that were not seen previously could now be discerned in some cases as slight bulges, and in others as substantial structures, associated baso-laterally with the shoot meristem in longitudinal and cross section (Figs 9a & b, respectively). The development of these primordia coincided with the time the seeds were able to germinate readily (Woodenberg 2009), i.e. when the embryos could be considered to be mature.

Fig. 8 Illustrates the structure and ultrastructure of the hypocotyl region of the embryo six months after the seeds were shed. **a** The root meristem (RM) in longitudinal section, which stained densely with toluidine blue appears very broad in relation to the width of the embryo; bar = 500 μm. **b** The hypocotyl in cross-section demonstrating the central position of the root meristem (RM); bar = 500 μm. **c** Mucilage ducts (MD) lined by a layer of epithelial cells were observed for the first time at this stage of development. These ducts were positioned between the meristem and tannin channel (TC) regions of the hypocotyl; bar = 50 μm. **d** Ultrastructure of mucilage duct epithelial cells displayed many, variously-distended lengths of endoplasmic reticulum (ER) amongst relatively small amyloplasts (Am); bar = 1 μm. **e** Numerous vesicles (Ves) were also a common feature of the cytomatrix and were sometimes observed to join with the plasmalemma (asterisk), while undistended (arrowheads) and a grossly-distended profile of the endoplasmic reticulum (ER) are also depicted. The surface of cell walls (CW) facing the lumen (Lu) displayed small, dark protrusions as illustrated; bar = 1 μm. **f** The hypocotyl region other than the root meristem had relatively large intercellular spaces (asterisk), while the cells, bounded by relatively thin walls (CW) contained numerous large vacuoles (Vac) and abundant amyloplasts (Am) and mitochondria (M); bar = 10 μm. **g** The cytomatrix also contained abundant profiles of endoplasmic reticulum (ER) and polysomes; bar = 0.5 μm.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Apart from the developing leaf primordia, the cells of the shoot meristem (Figs 9c & d) appeared unchanged from that seen at the 4-month developmental stage, with the clear appearance of the tunica and corpus regions (Fig. 9c, cf. Fig. 6c). Some indications of the plane of division of these cells could be seen (Fig. 9c). While most of the tunica cells of the shoot meristem seemed to undergo periclinal divisions, some cells in this external layer were observed to divide anticlinally (Fig. 9c). Both anticlinal and periclinal planes of division were observed in the sub-apical cells of the corpus (Fig. 9c).

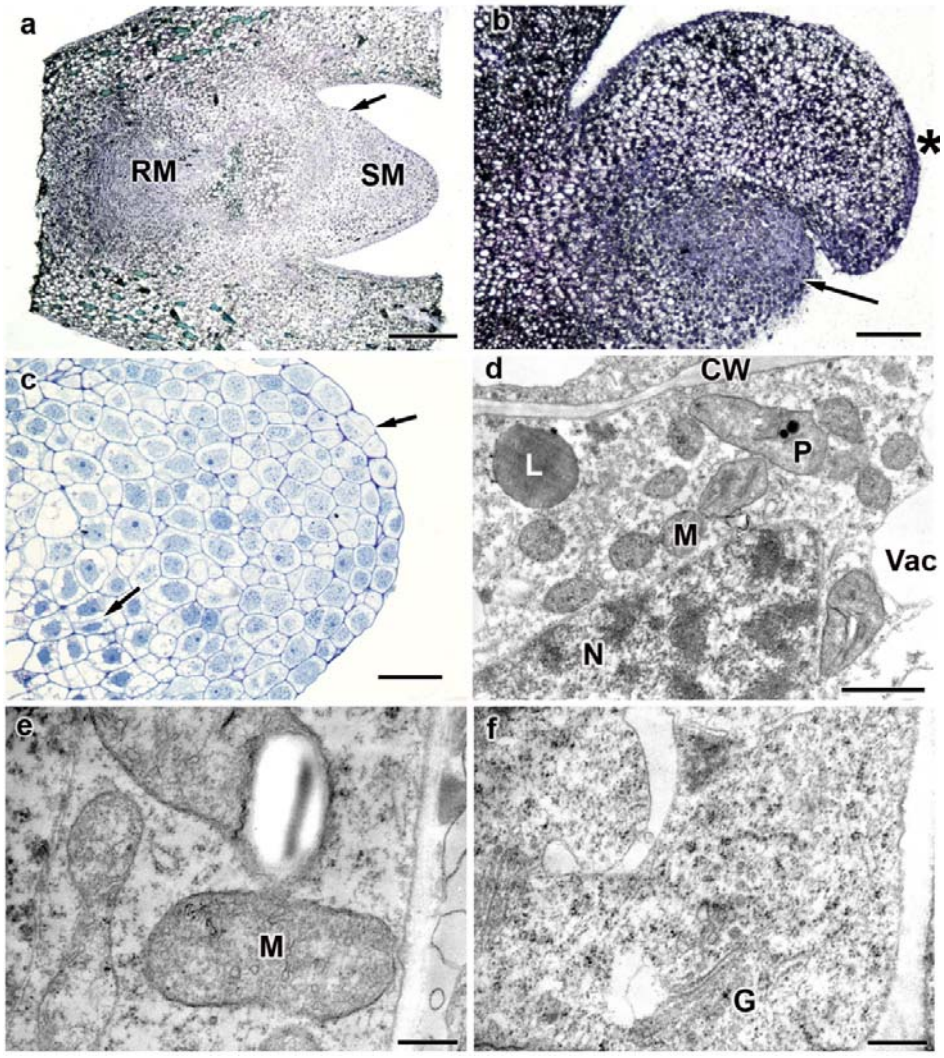
Cells of the root and shoot meristems were essentially similar at six months after seed-shed (Fig. 9d) compared with previous stages of ontogeny. The cells were dominated by large nuclei, while plastids containing only a little starch and small vacuoles were common and occasional lipid bodies occurred (Fig. 9d). Mitochondria were prominent (Fig. 9e) and Golgi bodies were far more common in the cells of both root and shoot meristems (Fig. 9f) than in cells of all other regions of the embryonic axes where their occurrence was sparse. As in the earlier developmental stages, polysomes were abundant (Fig. 9f).

The most striking feature of the embryos in the sixth month after seed-shed, was the differentiation which had occurred in the cotyledons (Figs 10a-d). Numerous mucilage ducts lined by cells which stained pink with toluidine blue and having translucent contents had formed in the central region of each cotyledon (Figs 10a & b), while the primary vascular tissue was also prominent toward the adaxial surface of the cotyledon in cross section (Fig. 10a). This arrangement contrasts with that observed in *Microcycas calocoma* (Dorety 1909), where cotyledonary traces were found to alternate with mucilage ducts. Each of the primary vascular bundles observed in the present study was surrounded by a few tannin channels (Figs 10a & b), while more of these structures were abundant in the peripheral regions of each cotyledon (Fig. 10a).

More tracheids also appeared to have differentiated in the primary vascular tissue when compared with the previous stage (Fig. 10c) although this vascular tissue was still dominated by procambial cells (Fig. 10d). This indicated that although the embryo was at a stage of ontogeny considered to be mature, the vascular tissue appeared still to be in an immature state overall and may well be equipped to be fully functional only later on in development, i.e. during germination and subsequent stages of seedling growth.

The cotyledons were seen in the earlier stages to develop as discrete structures (ref. Figs 5a, 6a & 7a). However, in the mature embryos they were tightly adpressed as demonstrated by

Fig. 9 Histological and ultrastructural situation of the shoot and root meristems six months post-shedding. **a** Longitudinal section of the root meristem (RM) and shoot meristem (SM) with what appears to be the initiation of a leaf primordium (arrow); bar = 500 µm. **b** Shoot meristem in cross-section (arrow) in relation to a well-developed leaf primordium (asterisk); bar = 200 µm. **c** Isodiametric cells of the shoot meristem with newly-divided cells (arrowed) in the tunica (T) and corpus (C) are apparent; bar = 50 µm. **d** The ultrastructure was consistent with previous stages, with meristem cells showing a relatively large nucleus (N) with numerous plastids (P) containing little accumulated starch, vacuoles (Vac), lipid bodies (L), and dense mitochondria (M) in the cytomatrix that was bound by a relatively thin cell wall (CW); bar = 1 µm. **e** The cytomatrix contained mitochondria (M) with dense matrices and well-defined cristae; bar = 0.5 µm. **f** Golgi bodies (G) were also seen in the cytomatrix as were numerous polysomes; bar = 0.5 µm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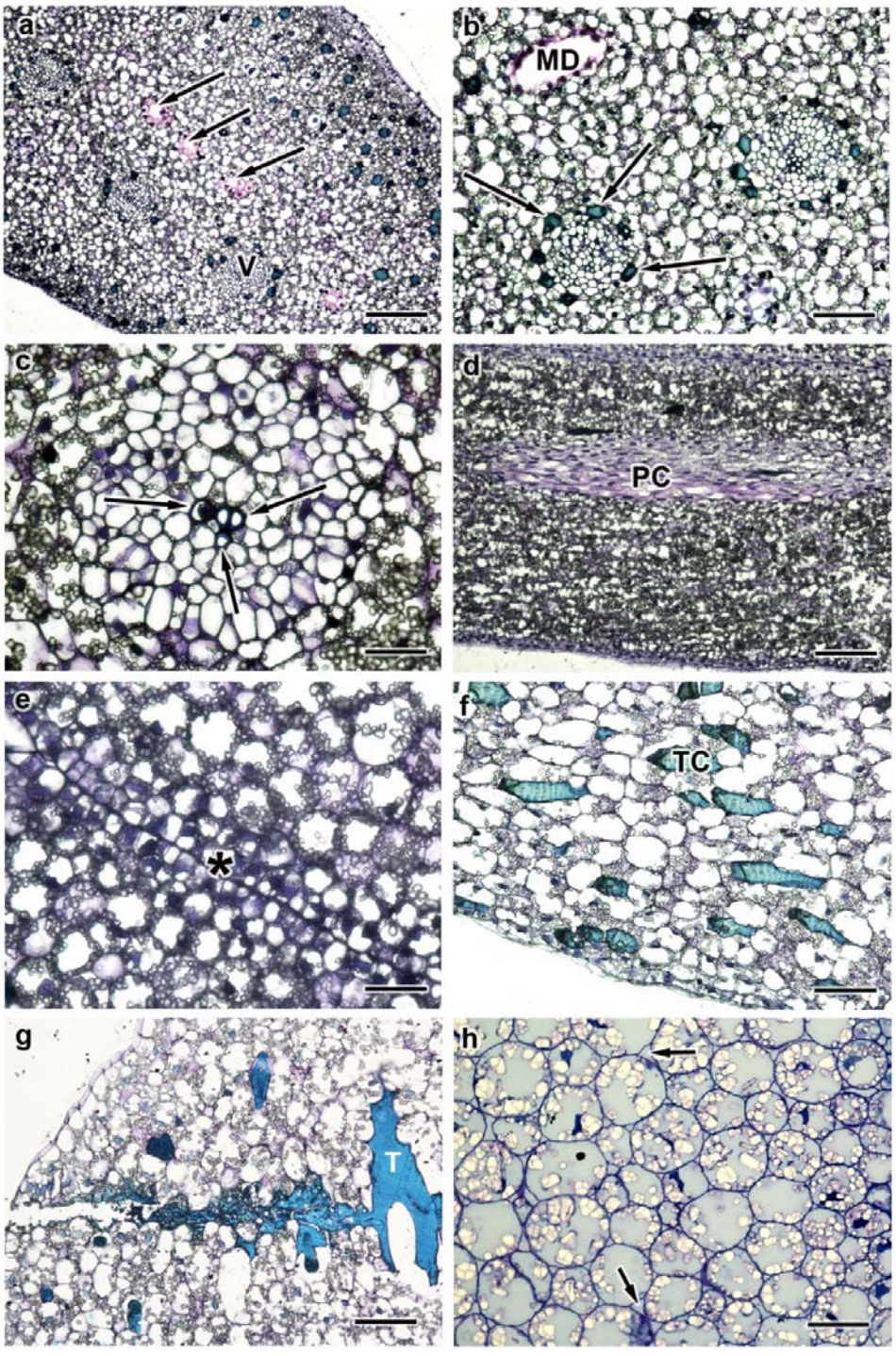
the close association of the adjacent adaxial epidermal layers of the two cotyledons (Fig. 10e). Such closely-associated cotyledons have also been seen in studies on other cycad embryos (Dorety 1909; 1919; Saxton 1910; Webb *et al.* 1984), and it was suggested by those authors that the cotyledons were fused. According to Dorety (1909) for example, when the cotyledons of *Microcycas calocoma* were examined microscopically, no trace could be found of the characteristic seam expected if the adaxial epidermal layers had become adpressed. Although cotyledon fusion may appear to have occurred in the cycad species investigated by those authors, the seam alluded to by Dorety (1909) was evident in the present investigation on *E. natalensis* embryos. In this regard, Monnier and Norstog (1986) found that while the cotyledons of *Zamia* sp. remained closely adpressed *in situ*, they grew separately when embryos were cultured *in vitro*. Those authors proposed that the cotyledons of that species may be closely associated in the seed because of the pressure exerted on them by the surrounding megagametophyte tissue. This is probably also true for *E. natalensis*, as bisected seeds showed the embryo enlarging in a tight space provided by the corrosion cavity in the centre of the comparatively large and fairly rigid megagametophyte tissue (Woodenberg 2009).

Cotyledons of mature embryos examined in the present study, had tannin channels with contents that consistently displayed oblique striations when longitudinal sections were viewed (Fig. 10f). While these may be artefacts of sectioning, they occurred repeatedly in a similar pattern, which may be an indication of an underlying ridging of the channel wall. On a few occasions where the embryo had appeared to be injured before fixation, tannin from the surrounding channel(s) seemed to infiltrate the wound (Fig. 10g). This is suggestive of the manner in which the tannins may function in the prevention of pathogen entry upon wounding (Wood 1967; Goodman *et al.* 1967), and/or provision of antioxidant activity (Esau 1953), as wounding is known to be associated with a surge of reactive oxygen species (ROS) (Bolwell 1999). The position revealed by the present investigation of tannin channels near the periphery and vital tissues of the embryo, viz. the vascular tissue and shoot meristem, are suggested to provide defence against external threats including pathogen invasion and physical trauma. The cotyledonary ground tissue in which intercellular spaces were evident, was comprised of starch-containing, vacuolated, rounded parenchymatous cells (Fig. 10h).

In the current study, the embryo was found to produce only two cotyledons. This situation has been observed in some other cycads e.g. *Microcycas calocoma* (Dorety 1909), *Zamia* spp. (Monnier and Norstog 1984), *Encephalartos friderici-guilielmi* (Saxton 1910), while

Fig. 10 Histology of the cotyledons six months after seed-shed. **a** Cross section displaying the position of numerous mucilage ducts (arrows) in the central region of the cotyledon, while three vascular bundles (V) and many blue-staining tannin channels can be seen; bar = 200 µm. **b** Demonstrating a relatively large mucilage duct (MD) with a translucent cavity lined by epithelial cells, and developing vascular tissue each surrounded by a few tannin channels (arrows); bar = 100 µm. **c** A vascular bundle in cross section displaying a small cluster of thick-walled tracheids (arrows), rather than the solitary structures seen four months after seed-shed; bar = 50 µm. **d** The procambium (PC) was seen in longitudinal sections to be composed of relatively elongated, thin-walled cells, the cytomatrix of which stained mauve to pink with toluidine blue; bar = 100 µm. **e** The two cotyledons were closely adpressed in some places (asterisk); bar = 50 µm. **f** Tannin channels (TC) near the peripheral region of the cotyledons displayed striations as illustrated in this longitudinal section; bar = 100 µm. **g** Tannins (T) presumably from tannin channels appeared to have seeped into a wound that was presumably created before fixation; bar = 100 µm. **h** Showing a cross section of numerous cells that were packed with amyloplasts, and were rounded with concomitant development of intercellular spaces (arrows); bar = 50 µm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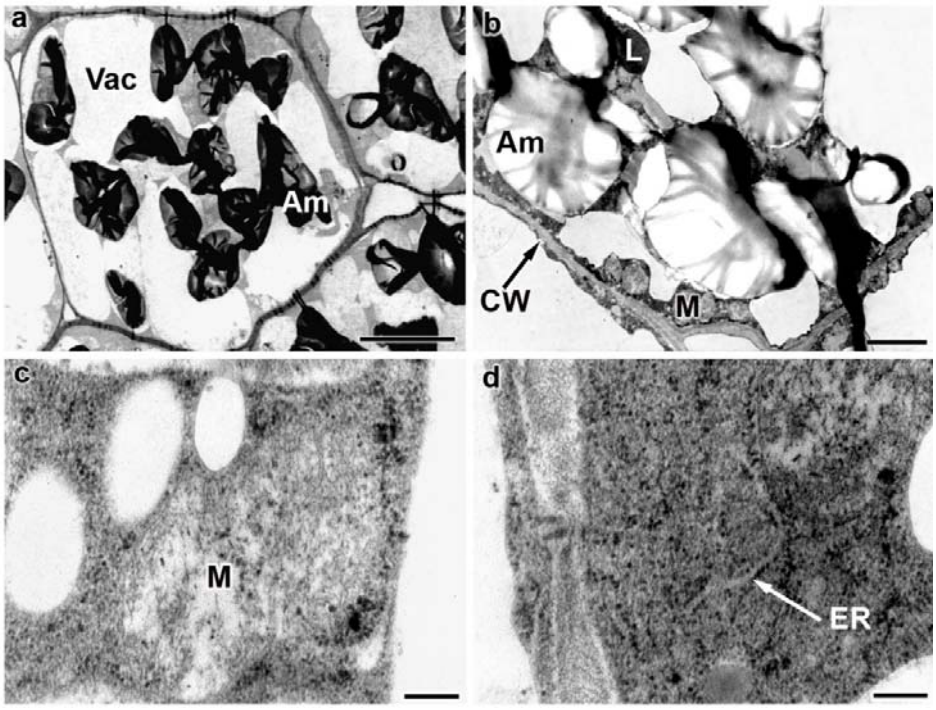
Dorety (1919) found that *Dioon spinulosum* could have from two to four cotyledons (although the number was commonly two). In addition, Dorety (1908) demonstrated that while *Ceratozamia* species may appear to have one cotyledon, the second cotyledon is in fact present, but its growth is substantially inhibited in that genus.

Aside from vascular tissue, resin ducts and tannin channels, the cells of the cotyledons in the current study were dominated by large amyloplasts containing substantial amounts of starch, vacuoles, a considerable number of mitochondria, the limited occurrence of lipid bodies (Figs 11a & b) and only occasional Golgi bodies. Together, the vacuoles and bulky amyloplasts compressed the ground cytomatrix and other organelles mostly against the cell periphery (Fig. 11b). The compression of the polysome-rich ground cytomatrix was so great that although the mitochondria and ER (Figs 11c & d) could be resolved, the effect was almost one of negative staining. While the general intracellular situation of the cotyledonary ground tissue was essentially similar to that in the mature megagametophyte cells of *E. natalensis*, what was remarkably different was the absence of the dense protein bodies which were prevalent in the latter tissue (Woodenberg *et al.* 2010). The absence of similar substantial protein deposits in the embryo tissues was confirmed by the lack of specific staining histochemically (see below).

In the current study, mucilage ducts were positively identified by staining with a modified version of the protocol of Mace and Howell (1974) for the location of mucins. Initial results using the standard protocol outlined by those authors yielded no staining in the schizogenously-formed ducts; however, indications of their contents were obtained serendipitously when toluidine blue was applied to sections already stained using the Mace and Howell (1974) method (Fig. 12a). The ducts displayed pale blue staining when this was done indicating that they might not be empty. Staining of these structures was also evident when Ruthenium red was employed for the detection of acidic polysaccharides typical of mucilage (Fig. 12b), where the epithelial cells stained red and the duct contents appeared a light pink. The constituents such as amyloplasts and vacuoles of other cells remained unstained. These two results seem to complement one another and strongly suggest that the structures investigated are, in fact, mucilage ducts.

Fig. 11 Ultrastructure of the cotyledonary cells six months after seed-shed. **a** Cells were dominated by abundant, relatively large amyloplasts (Am) and vacuoles (Vac); bar = 5 μm. **b** Ultrastructure of one of those cells demonstrating the size of the abundant, starch-filled amyloplasts (Am) compared with the mitochondria (M) shown compressed in the peripheral cytomatrix and the few lipid bodies (L) that were present in the cells; bar = 20 μm. **c** The mitochondria (M) that were present had well-defined cristae and patchily-dense matrices; bar = 0.2 μm. **d** Relatively short profiles of endoplasmic reticulum (ER) were also prevalent in some areas of the dense cytomatrix, which displayed an abundance of polysomes as seen in other stages of development; bar = 0.2 μm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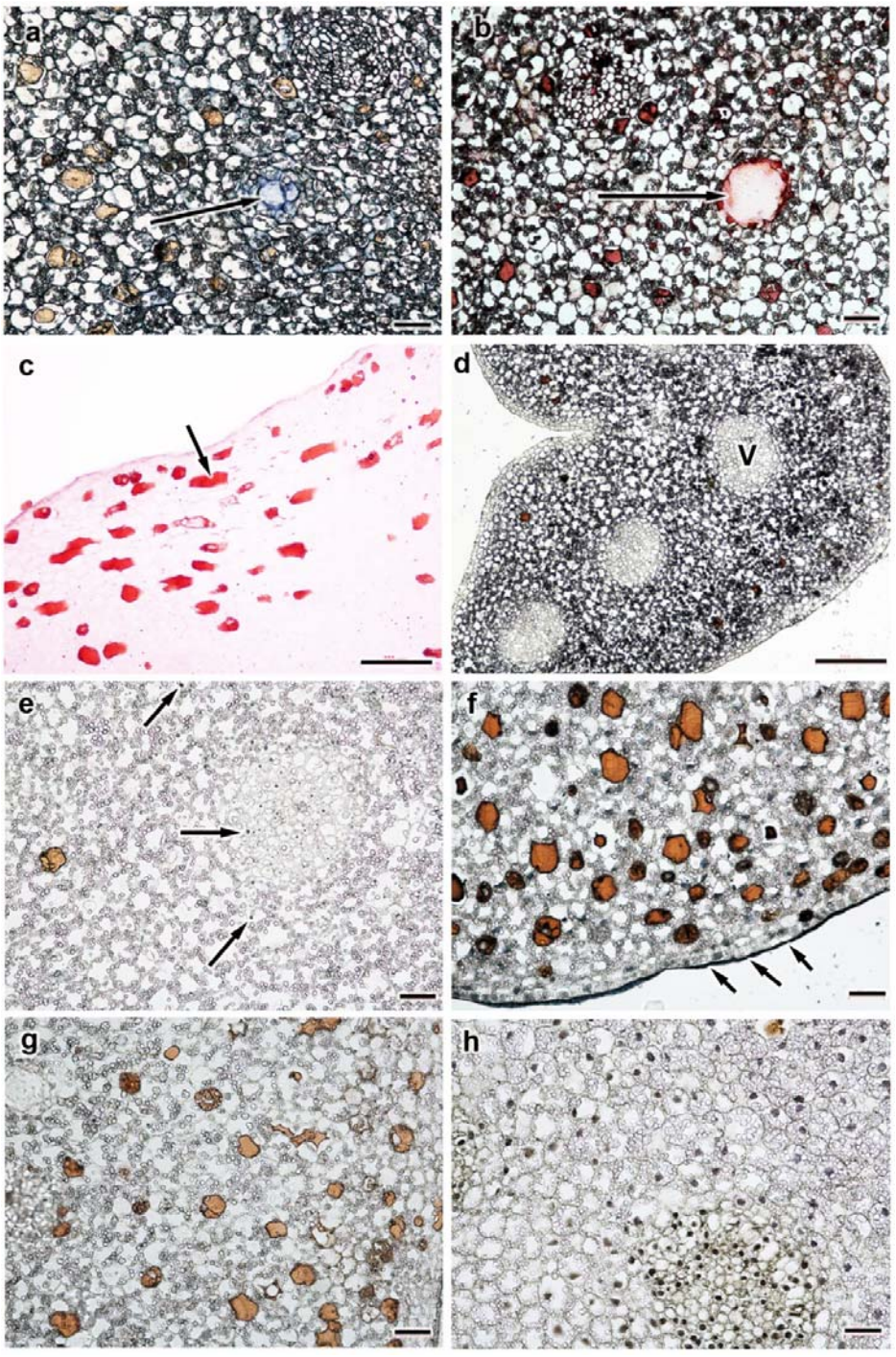
When vanillin-HCl was applied, positive bright red staining indicated the presence of tannins in discrete locations (Fig. 12c), serving to confirm that these are in all likelihood, the tannin channels. The amyloplasts, which constituted a large proportion of the contents of the majority of cells tested positive for starch with the use of Lugol's stain (Fig. 12d). As indicated by transmission electron microscopy, lipid was confirmed to be sparsely present in the cells as a few, very small, discrete entities when Sudan Black B was used (Fig. 12e); however, use of that stain also indicated the presence of a layer of lipid, probably wax, covering the exterior of the embryo (Fig. 12f). Effectively, this would constitute a cuticle and be a further adaptation (additional to the relatively impermeable sclerotesta) to retard the loss of water from these embryos, which have been shown to be desiccation-sensitive (Woodenberg *et al.* 2007; 2009).

Protein histochemistry in the present investigation yielded an unexpected interesting result. According to Woodenberg *et al.* (2010), many of the vacuoles of the megagametophyte cells of *E. natalensis* displayed considerable amounts of a relatively dense, granular material that stained positively for protein when eosin dye was used. When the same stain was utilised in the current study, vacuoles did not stain positively (Figs 10g & h), and no similar dense, granular material was observed with the TEM or light microscope. However, some staining for protein did occur, but confined to the nuclei, when mercuric bromophenol blue was utilised (Fig. 12h). The embryo appears, therefore, not to accumulate any significant amount of reserve protein as protein bodies compared with the megagametophyte, but it suggestedly obtains the required amino acids directly from the protein reserves of the megagametophyte tissue. From the prevalence of polysomes throughout embryogenesis, there can be no doubt that active protein synthesis is on-going. However, this presumably is directed towards synthesis of structural proteins of e.g. membranes and the cytoskeleton, as well as of enzymes.

Concluding remarks

The present investigation has revealed some important aspects of the post-shedding development of the *E. natalensis* embryo that further understanding of the recalcitrant post-shedding behaviour of these seeds (Woodenberg 2009). Seeds of this species are shed from the parent plant when the embryo is rudimentary and decidedly physiologically immature; its development has been shown to be a continuous process after seed-shed that ultimately

Fig. 12 Histochemistry of secondary metabolites and storage products accumulated in the mature embryo of *E. natalensis* (Toluidine blue was used only on the section in 'a'). **a** Contents of mucilage ducts (arrow) appeared faintly blue when toluidine blue was applied to the sections after staining for mucins with the tannic acid/ferric chloride protocol of Mace and Howell (1974), while the non-stained, orange/brown colouration of tannin channels is also evident; bar = 50 µm. **b** The contents of mucilage ducts stained pale pink (arrow) with the epithelial cells staining a deeper pink to red when Ruthenium red was used for the detection of acidic polysaccharides, while tannin channels stained darkly; bar = 50 µm. **c** The contents of tannin channels stained red (arrow) with vanillin-HCl while the rest of the tissue did not stain, indicating the localised presence of tannins; bar = 200 µm. **d** Amyloplasts in most of the tissue stained black when Lugol's solution, which localises carbohydrate (by reaction with potassium iodide/iodine; Jensen 1962) was employed, while vascular tissue (V) did not display similar staining; bar = 200 µm. **e** Scattered, small, discrete bodies of lipid were observed to stain darkly (arrows) with Sudan Black B; bar = 50 µm. **f** The use of Sudan Black B also yielded a thin film of darkly staining substance, presumed to be cuticular wax, on the external surface of the epidermis (arrows); bar = 50 µm. **g** There was no staining which would have indicated the presence of protein bodies when eosin dye was used; bar = 50 µm. **h** A similar, negative result was had when mercuric bromophenol blue was used to localise protein, although the nuclei stained prominently; bar = 50 µm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culminates in germination. Histochemistry in this study showed that the major storage product accumulated in the embryo is starch, which is accumulated abundantly in the many amyloplasts of non-meristematic cells, while lipid is accumulated in only minor quantities and reserve protein accumulation is negligible. This information, along with that revealed about the megagametophyte (Woodenberg *et al.* 2010), may be important for the future design of biotechnological protocols for the *in vitro* growth of immature *E. natalensis* embryos that were observed in the present study at the time of seed-shed. Immature embryos of this species may provide a suitable explant for the cryopreservation of this species, the seeds of which are currently unstorable in the long-term.

The current investigation has also increased understanding of the secondary metabolites accumulated in the embryo of this species, particularly mucilage and tannin. Mucilage ducts were found to be a common feature of mature embryos in this species, which, along with the possession a tough sclerotesta external to the seed, and a waxy layer on the surface of the embryo may contribute to water retention in the months after seed-shed (Woodenberg 2009). These characteristics are suggested to contribute to the relative longevity (one to two years) in storage under conditions precluding dehydration, compared with recalcitrant seeds of other species which is generally a matter of days to months (Pammenter *et al.* 1994).

Apart from mucilage, the other major secondary metabolite found in the embryo of the current species was tannin, accumulated in tannin channels. These may be analogous to the resin canals of most conifers, gum canals of *Welwitschia*, and laticifers of *Gnetum* and some angiosperm species (Esau 1953; Romberger 1993). The purported anti-microbial and wound-healing properties of tannins may also account for the use of parts of cycads in traditional medicine in South Africa (e.g. Osborne *et al.* 1994), and according to Vashishta (1995), the 'resin' obtained from *Cycas rumphii* is apparently applied to malignant tumours. The use of tannins from cycads for medicinal purposes therefore warrants further investigation, and if verified, would justify research on *in vitro* culture for their production, as further depletion of the naturally-occurring plants cannot be countenanced.

Further study is also encouraged on the early stages of wall formation in the embryo of *E. natalensis*. As Woodenberg *et al.* (2010) demonstrated, - initial cellularisation of the megagametophe of this species seemed to occur without the involvement of Golgi bodies. Similarly, very few Golgi bodies were evident in the early embryo in the present investigation. It will therefore be intriguing to see whether or not a similar mode of primary

cell wall development occurs during the initial cellularisation of the *E. natalensis* embryo. This would entail seed collection immediately, and shortly after, fertilisation (the dynamics of which would require to be ascertained), and adaptation of preparative technology for microscopy of the minute nascent structures.

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CHAPTER 3

Embryo cell wall properties in relation to development and desiccation in the recalcitrant-seeded *Encephalartos natalensis* (Zamiaceae) Dyer and Verdoorn

Introduction

Plant cell walls are dynamic assemblages that play important roles in plant morphology and ontogeny (Albersheim *et al.* 1994; Penell 1998). The main functions of the cell wall are to provide plants with mechanical strength and to act as a barrier against the external environment (Harholt *et al.* 2010). In this regard, cell walls are involved in facilitating essential plant responses to osmotic stress (e.g. Wakabayashi *et al.* 1997); acclimation to cold (e.g. Weiser *et al.* 1990); drought stress (e.g. Zwiazek 1991); pathogenic attacks (e.g. Boudart *et al.* 1998); wounding (e.g. Cardemil and Riquelme 1991); stress associated with salinity (e.g. Iraki *et al.* 1989a); and drying (e.g. Zwiazek 1991).

According to Reiter (2002), the functional properties of cell walls may be influenced both by their composition and three-dimensional organisation of those components within their structure. Plant cell walls therefore do not possess the exact same chemical composition i.e. plant genera may have their own "signature" wall composition; and it has become apparent that the chemical composition of the cell walls of an individual plant can change with development (reviewed by Sarkar *et al.* 2009). Thus, knowledge of the composition and structure of cell walls is fundamental to understanding the functions of cell walls (Reiter 2002).

Cell walls are classified broadly as primary or secondary. Primary walls are laid down during cytokinesis and expansion, while material deposited on the primary wall after the cessation of growth comprises the secondary wall (Cosgrove 1997). Neighbouring cell walls are divided by an intercellular layer known as the middle lamella, which is apparently continuous with the primary cell wall matrix (Moore *et al.* 1986).

All plant cell walls are comprised of cellulose microfibrils, which form the main framework of the wall, and a matrix phase (consisting of hemicellulose and pectin) forming cross-links amongst the microfibrils, filling the space between the fibrillar frame-work (reviewed by Sarkar *et al.* 2009).

Hemicelluloses are a family of polymers that have abundant glucose, xylose, or arabinose (Talmadge *et al.* 1973). Unlike cellulose, they have extensive side chains that often include xylose, galactose, and fucose. Commonly found hemicelluloses include xyloglucans, xylans, arabinoxylans, arabinogalactans, mannans, glucomannans, and galactoglucomannans (Timell 1964; Timell 1965; Whistler *et al.* 1970). Dicots and monocots have been found to vary considerably in their composition of hemicellulose – descriptions of which have been reviewed comprehensively (Carpita and Gibeaut 1993; Reiter 1994; Cosgrove 1997).

Pectins, on the other hand, are a group of polygalacturonic acids that may differ in their side chains, which are typically arabinose, galactose, or a complex branched organisation of monosaccharides (Cosgrove 1997). The precise nature of the neutral side-chains differs from plant to plant being either an arabinan, galactan, or a combination of the two (Aspinall 1970). Pectins are made up of homogalacturonans (HGs), which consist of contiguous, unbranched, α 1-4-linked α-D-galacturonic acid residues intermixed with rhamnose and specific branched polymers that include rhamnogalacturonans (RG-I and RG II).

A significant functional aspect that characterises HGs is their amount of methylesterification of the galacturonic acid's carboxylic function. As a consequence, HGs are frequently characterised as low- or high-methylesterified pectins (Jauneau *et al.* 1998). The unbranched and low-esterified HGs may aggregate via calcium bridges giving rise to junction zones that hold them together (Grant *et al.* 1973; Rees 1977; Jarvis 1984; Brett and Waldron 1990). This binding stiffens the gel matrix of the wall and contributes strength to the wall (Lloyd 2006).

Ascertaining the composition of cell walls has thus become important for the comprehension of plant development and their responses to biotic and abiotic stresses. In this regard, the aims of the current work are twofold: 1) to analyse the chemical composition of immature and mature embryos of *E. natalensis* to see how the walls change during post-shedding development of these desiccation sensitive seeds; and 2) to assess the effects of drying on the composition and conformation of mature embryo cell walls of this species. To this end, immunofluorescence microscopy in conjunction with immunogold labelling and biochemistry were employed to characterise the chemical composition of the pectin and hemicellulose components of the wall, while TEM and cryo-SEM were used to gauge the conformation of cell walls in hydrated and dehydrated mature embryos. To the knowledge of the authors, this appears to be the first study to characterise the cell wall composition of a desiccation-sensitive embryo in relation to development and drying.

Materials and Methods

Plant material

Collection of seeds was made after issue of the appropriate permits, as *Encephalartos natalensis* is listed as threatened or protected (ToPS) in accordance with the National Environmental Management: Biodiversity Act of South Africa. Upon receipt of the permit, *E. natalensis* seeds were collected from a plant at the University of KwaZulu-Natal, Howard College Campus (29° 52□ 07.28□ S; 30° 58□ 49.52□ E), Durban, as soon as a few seeds had been shed in December (summer). Immature embryos were excised from a subsample of these seeds shortly after seed-shed and processed for microscopy, while the rest of the seeds were decontaminated with a 2.5% sodium hypochlorite solution, rinsed in water, dusted with Benlate® (benzimidazole, 500 g kg^{-1}), and placed into 'open' storage in brown paper bags at 16°C. Another subsample of mature embryos was excised (hypocotyl tissue) from the batch of seeds stored for six months. Small cubes (1-2 mm^3) were cut out of the embryonic axes using a sharp scalpel blade before subjecting them to prolonged infiltration and resin embedding as outlined below.

Other plant material used in the present study for positive controls included leaf tissue from a *Zea mays* seedling, root tissue of *Daucus carota*, and pericarp tissue of *Lycopersonicon esculentum*.

Prolonged tissue processing for TEM

Excised embryo material was fixed for 18-24 h in 2.5% glutaraldehyde buffered at pH 7.2 with 0.1 M phosphate buffer containing 1% caffeine, washed in 0.1 M phosphate buffer, prior to dehydration in a graded ethanol series (25-75%) [2 × 10 min each] and left in 75% ethanol overnight. Samples were dehydrated further with 100% ethanol, and then 2 × 10 min treatments with propylene oxide. These samples were subsequently placed in a mixture containing equal amounts of propylene oxide and epoxy resin (Spurr 1969), which was placed on a vertical turntable for 24 h at room temperature, incubated in full resin (Spurr 1969) for a further 24 h before embedding in fresh resin and polymerisation at 80°C for 8 h.

Freeze-substitution of dried embryo samples

Mature, excised embryos were air-dried at room temperature for two weeks to an average water content of 0.4 g g^{-1} (dry mass basis). Samples of 1-2 mm^3 were taken from these dried

embryos and plunged into liquid propane (-190°C) for fixation using a Leica CPC cryo-fixation instrument as described by Wesley-Smith (2001). The protocol used by that author was subsequently modified as follows. Liquid propane-cooled samples were transferred to a -80°C solution of 0.1% tannic acid in acetone where they were kept overnight; followed by three days in 2% anhydrous glutaraldehyde in acetone (-80°C). The vial containing the samples was subsequently transferred to -20°C for a further three days, before being placed in a container of dry ice at room temperature. Once the dry ice had sublimed and the samples equilibrated to room temperature, they were subjected to two rinses in acetone (5 min each) and infiltrated with increasing concentrations of LR White in acetone (1:3; 1:1; 3:1; and 1:0) for 8-12 h each, before polymerisation at 50°C for 6 h.

Microtomy and microscopy

Embedded samples were sectioned using a Reichert-Jung Ultracut E microtome. Sections, 1 µm thick, were collected on glass slides before treatment for immunofluorescence microscopy outlined below. Viewing of immunofluorescent-treated sections was achieved with a Nikon Eclipse 80i light microscope equipped with epifluorescence and NIS Elements F Package imaging software. For TEM, copper/gold ultrathin sections were cut using the same microtome, placed on 200 mesh nickel grids, treated as described below for immunogold labelling, and viewed with a Jeol JEM 1010 transmission electron microscope and iTEM Soft Imaging System GmbH imaging software. A maximum of five and minimum of three sections were viewed from each of three grids.

Immunofluorescence microscopy

Immunofluorescence microscopy was employed to characterise the chemical composition and distribution of the embryo cell walls in the hydrated and dried conditions. The antibodies for the immunofluorescence microscopy included JIM5 and JIM7, which recognise relatively low and high methyl esterified homogalacturonans (HG) [Knox *et al.* 1990] respectively; LM2, which recognises arabinogalactan protein (Smallwood *et al.* 1996); LM5, which recognises galactan (Jones *et al.* 1997); LM6, which labels arabinan (Willats *et al.* 1998); LM7, which recognises relatively low methyl esterified HGs from non-blockwise de-esterification (Clausen *et al.* 2003); LM11, which recognises xylan and arabinoxylan (McCartney *et al.* 2005), and LM15 which recognises xyloglucan (Marcus *et al.* 2008). The antibodies were used during the following procedure.

Section-containing slides were flooded with Tween Tris-Buffered Saline (TTBS) for 5 min at room temperature, before incubation with a solution of 3% milk in TTBS for 30 min. The sections were then rinsed (3 × 3 min) in TTBS and incubated overnight at 4°C in a primary antibody solution diluted 1:10 with TTBS. The next day, sections were retrieved from 4°C and allowed to warm up to room temperature, subjected to a (3 × 3 min) wash in TTBS, and then incubated for one hour in the dark with the secondary antibody diluted 1:20 in TTBS. The sections were kept in the dark at room temperature, rinsed (3 × 3 min) in distilled water and viewed immediately with the microscope. Specificity of the label was assessed by examination of control sections in which either the primary or secondary antibody was omitted, while negative reactions were tested by treating tissues that had previously been shown to give a positive reaction.

Immunocytochemistry

The protocol used in this study was described by Hu and Rijkenberg (1998). Section-bearing grids were placed on a drop of 0.01 M phosphate-buffered saline (PBS) (pH 7.4), containing 0.2% polyethylene glycol (PEG) 20,000 (Fluka, Buchs, Switzerland) for 5 min. Thereafter, the sections were incubated for 30 min in a drop of blocking solution made up of 0.01 M PBS (pH 7.4), 10% foetal bovine serum (FBS) [Delta Bioproducts, Kempton Park, South Africa], 1% BSA [Sigma, St. Louis, Missouri, USA], 0.05% Tween 20 (Sigma) and 0.2% sodium azide (Fluka). They were then incubated in 40µl of primary antibody (rat IgG, Plantprobes, Leeds, UK) in 0.01 M PBS (pH 7.4) (1:20) in a moist chamber, overnight at 4°C. Grids were then washed (3 × 5 min) in 0.01 M PBS (pH 7.4), containing 1% BSA and 0.05% Tween 20, and immersed for 1 h in a drop of 10 nm colloidal gold-conjugated goat antiserum to rat immunoglobulin (GAR-gold antibody [Sigma] diluted at 1:10 in washing solution. Finally, the sections were fixed with 1% glutaraldehyde for 2 min, rinsed with sterile distilled water, counterstained with uranyl acetate and examined with a JEOL JEM-1010 transmission electron microscope (TEM) at 100 kV. Specificity of the label was assessed by examination of control sections in which either the primary or secondary antibody was omitted.

Cryo-SEM

Dehydrated and hydrated mature embryo samples were fixed in nitrogen slush under vacuum, transferred to a stage, fractured, sublimated briefly and viewed with a Qurom PP3000T Technology (UK) Cryo-SEM.

Cell wall biochemistry

Cell wall isolation and fractionation

Cell wall material was extracted from frozen *E. natalensis* embryos and fractionated according to a protocol modified from Nguema-Ona *et al.* (2012). Briefly, the embryos were ground, under liquid nitrogen using a mortar and pestle, to a fine powder. After boiling in 80% ethanol for 20 min, insoluble material was washed in methanol: chloroform (1:1) for 24 h, thereafter the residue was washed in methanol before air drying. The dry material, referred to as alcohol insoluble residue (AIR), was de-starched using a combination of thermostable α-amylase, amyloglucosidase and pullulanase (all from Megazyme). De-starched AIR was then chemically fractionated with hot water, 50 mM cyclohexane-1,2-diamine tetra-acetic acid (CDTA), and 4 M KOH. Chemically extracted fractions were dialysed (3.5 kDa cutoff dialysis tubing) against deionised water (48 h at 8°C), and freeze dried before compositional analyses were conducted.

Monosaccharide composition analysis by gas chromatography

A gas liquid chromatography method (see Nguema-Ona *et al.* 2012) was used to determine the monosaccharide content of cell wall residues and fractions. Approximately 1-3 mg of wall residue or fractionated material was hydrolysed (2 M trifluoroacetic acid (TFA), 110°C, 2 h) and the liberated monosaccharides converted to methoxy sugars using 1 M methanolic HCl at 80°C for 24 h. Silylation was performed at 80°C (20 min) to produce trimethyl-silyl-glycosides which were dissolved in cyclohexane. The derivatives (trimethylsilyl methyl ester methyl glycosides) were separated and analysed in a gas chromatograph (VARIAN CP 3800) coupled to a flame ionisation detector, using a 30 m × 0.25 mm (i.d.) CP-Sil 5 CB column (Agilent). The oven temperature program was stabilised at 120°C for 2 min, ramped at 10°C/min to 160°C, then at 1.5°C/min to 220°C and finally at 20°C/min to 280°C. Myo-inositol (0.5 µmol) was used as the internal standard. Derivatives were identified based on their retention time and quantified by determination of their peak areas. Monosaccharides (from Sigma-Aldrich) were used as standards to determine the retention time of the nine main monosaccharides found in plant cell walls. The sugar composition was expressed as mole percentage of each monosaccharide. Error bars in the histograms represent the standard deviation SD of the mean of four biological/technical samples.

Results and discussion

Since the seeds of *Encephalartos natalensis* are shed at an early stage of embryogenesis (Woodenberg *et al.* 2014), it was decided to analyse the cell walls of two developmental stages: at seed-shed (immature stage) and six months after seed-shed (mature stage). While these two stages are characterised by differences at the subcellular level, e.g. typical meristematic ultrastructure in the immature stage versus subcellular domination by starch-containing-amyloplasts in the mature stage, results from the current investigation suggested that there are no differences with respect to some key cell wall components (Figs 1a-p).

In order to corroborate the immunofluorescence microscopy results of the present study, numerous controls were employed (Appendix A; Figs 8a-d). Negative controls suggested that the cells of the embryo possessed some faint autofluorescence in the cytomatrix and nuclei (Figs 8a-c) and although autofluorescence was present, it was noticeably faint compared with fluorescence in cell walls from the binding of fluorescent-tagged primary antibodies to their respective target molecules. Positive controls demonstrated that the antibodies which yielded no fluorescence in the cell walls of the *E. natalensis* embryos (LM2, LM5 and LM11; Figs 1e-h; m; n), were operational (Figs 8d-f, respectively).

Cell wall composition of immature and mature hydrated embryos

Cell wall fluorescence was observed in embryos from both stages of development when both JIM5 (Figs 1a & b) and JIM7 (Figs 1c & d) antibodies were used. This indicates the possible presence of poorly and highly methyl-esterified epitopes of homogalacturonan (HG), respectively. This is not unexpected as HG is believed to be a ubiquitous component of the cell wall in most, if not all, plants (Talmadge *et al.* 1973). It has been proposed that HG is synthesised in a highly methyl-esterified form in the Golgi body before being exported to the cell walls where it is subsequently de-esterified by the action of pectin methyl esterases (PMEs) [Zhang and Staehelin 1992]. According to Jarvis (1984), HG with reduced methyl-esterification can associate by calcium cross-linking. Such an association promotes the formation of supramolecular pectic gels, which are important in controlling the porosity and mechanical properties of cell walls and contribute to the maintenance of intercellular adhesion (Knox 1992; Carpita and Gibeaut 1993).

In contrast to the results obtained for JIM5 and JIM7, when the antibodies LM2 and LM5 were employed, cell walls did not display fluorescence (Figs 1e & f; g & h, respectively),

suggesting an absence of arabinogalactan protein and (1→4)-β-D-galactan, respectively. Arabinogalactan protein and (1→4)-β-D-galactan reportedly act as wall plasticisers (Ha *et al.* 1997; Lamport 2001; Lee *et al.* 2005), which aid wall folding upon desiccation (Moore *et al.* 2013). The apparent lack of these plasticising molecules in cell walls of *E. natalensis* embryos is commensurate with their desiccation sensitivity. Galactans are also known to have water-binding capabilities (Guinel and McCully 1986), which would be a potential advantage to the embryos of *E. natalensis*. The appearance of galactan has also been correlated with an increase in the firmness of cotyledons in pea (McCartney *et al.* 2000). This has led to the proposal that galactans may play a role in cell wall strengthening. Hence the cell walls of the *E. natalensis* embryo appeared to lack certain wall strengthening, plasticising, and water-retentive molecules that are usually present in desiccation-tolerant plant tissues.

On the other hand, when cell wall fluorescence was observed in the embryo after utilisation of LM6 (Figs 1i & j), it indicated that arabinan is likely to be present in the embryo cell walls of the current species. According to Jones *et al.* (2003), arabinan is another wall plasticiser that increases cell wall flexibility by diminishing strong interactions between HG chains. The cell walls of the immature and mature embryo of *E. natalensis* may therefore not be devoid of plasticising molecules, suggesting that they may ultimately have some flexibility to allow for cell expansion during growth of the embryo.

While the fluorescence provided by JIM5, JIM7 and LM6 appeared to have a relatively uniform distribution within the wall, the LM7 antibody produced unique labelling. Fluorescence with LM7 appeared to be reduced to a few randomly dispersed regions of the cell wall (Figs 1k & l). The punctuate distribution of the LM7 epitope found in this study is in contrast to other studies, where this molecule usually has a precise and consistent location at corners of intercellular spaces and at the junction between cells (Willats *et al.* 2001). The results of the present study implied that partially methyl-esterified epitopes of HG derived from non-blockwise de-esterification may be present in a few, relatively small, isolated regions of the cell wall or that they were being deposited into the wall by nearby secretory vesicles.

While the LM7 antibody produced fluorescence in small, discrete areas of the cell wall, use of LM11 yielded a result similar to the LM2 and LM5 antibodies i.e., no visible fluorescence of the cell walls (Figs 1m & n). Therefore, the hemicellulose xylan may be absent from the walls of the *E. natalensis* embryo. This result can be expected as xylan is thought to be a

major component of secondary walls and would not be found normally in the middle lamella or primary cell walls. However, while xylan may appear to be absent from the cell walls of *E. natalensis*, the hemicellulose xyloglucan appeared to be present. This was suggested to be the case after the LM15 antibody gave rise to bright fluorescence in the walls as depicted in Figures 1o & p. One of the main functions of xyloglucan is to confer strength to the cell wall by binding cellulose microfibrils together (Moore *et al.* 1986; Fry 1989). It may therefore form an important part in the maintenance of cell wall integrity upon environmental stresses such as drying.

In muro localisation of cell wall components

Immunogold labelling performed in the present study showed that the JIM5 epitope may be restricted to the middle lamella of the embryo cell wall (Figs 2a & b), while that of JIM7 appeared to occur in both the middle lamella and primary cell wall (Figs 2c & d). Similar distribution patterns of those molecules have been reported in studies on other plants. A few studies have demonstrated poorly esterified HG in cell wall junctions and the middle lamella (Knox *et al.* 1990; Rihouey *et al.* 1995), while other studies have demonstrated highly esterified HG throughout the cell wall layers (Schindler *et al.* 1995; Jauneau *et al.* 1997).

In contrast to JIM5 and JIM7, immunogold labelling with the LM6 antibody indicated that arabinan may be present in the primary cell wall and absent from the middle lamella (Figs 2e & f). Immunogold labelling with LM7 produced results that were similar to the immunofluorescence microscopy in mature embryos only (Figs 2g & h). In immature embryo cell walls, fluorescent labelling gave a sparse, punctuate distribution whereas there seemed to be no cell wall labelling with use of the immunogold technique (Fig. 2g). This occurred despite a few extra independent repeat studies using this antibody to check for technical errors. In mature embryo material, as was the case with immunofluorescence labelling with LM7, gold labelling was evident in localised areas of the cell wall (Fig. 2h) confirming the findings that partially methyl-esterified epitopes of HG are probably present in certain discrete regions of the *E. natalensis* embryo cell walls. This change in labelling pattern

Fig. 1 Immunofluorescent labelling of *E. natalensis* embryo cell walls. Uniform labelling was evident when JIM5 was used on **a** immature and **b** mature stages of ontogeny. Similar labelling occurred when JIM7 was employed on **c** immature and **d** mature stages. When LM2 was utilised, no fluorescence was evident in both **e** immature and **f** mature stages of development. The use of LM5 yielded a similar result displaying no fluorescence in the walls of **g** immature and **h** mature developmental stages. The LM6 epitope, on the other hand, displayed fluorescence in the cell walls of both **i** immature and **j** mature embryos. Fluorescent labelling appeared punctuate (arrows) when LM7 was utilised on **k** immature and **l** mature stages. LM11 yielded no cell wall fluorescence in both **m** immature and **n** mature developmental stages. Labelling with LM15 is apparent in both **o** immature and **p** mature stages. Bar = 20 μm (a-d; g; j-m; n); 50 μm (e; h; i; o; p); 100 μm (f)

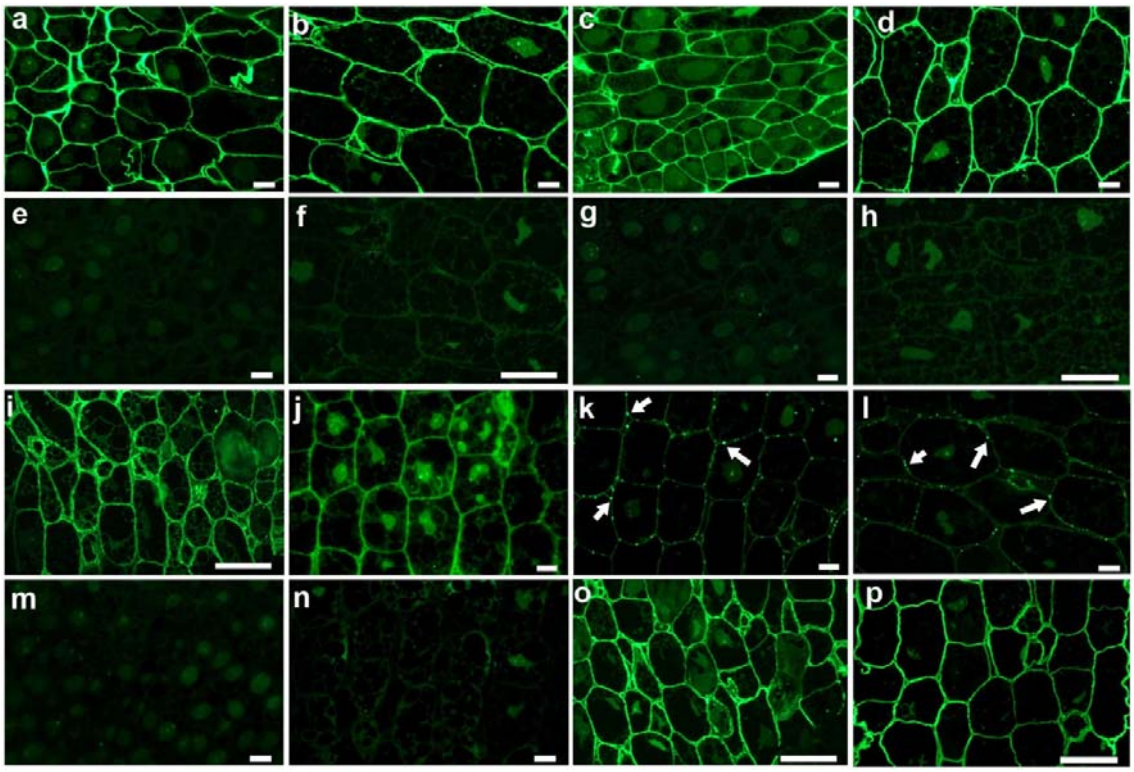
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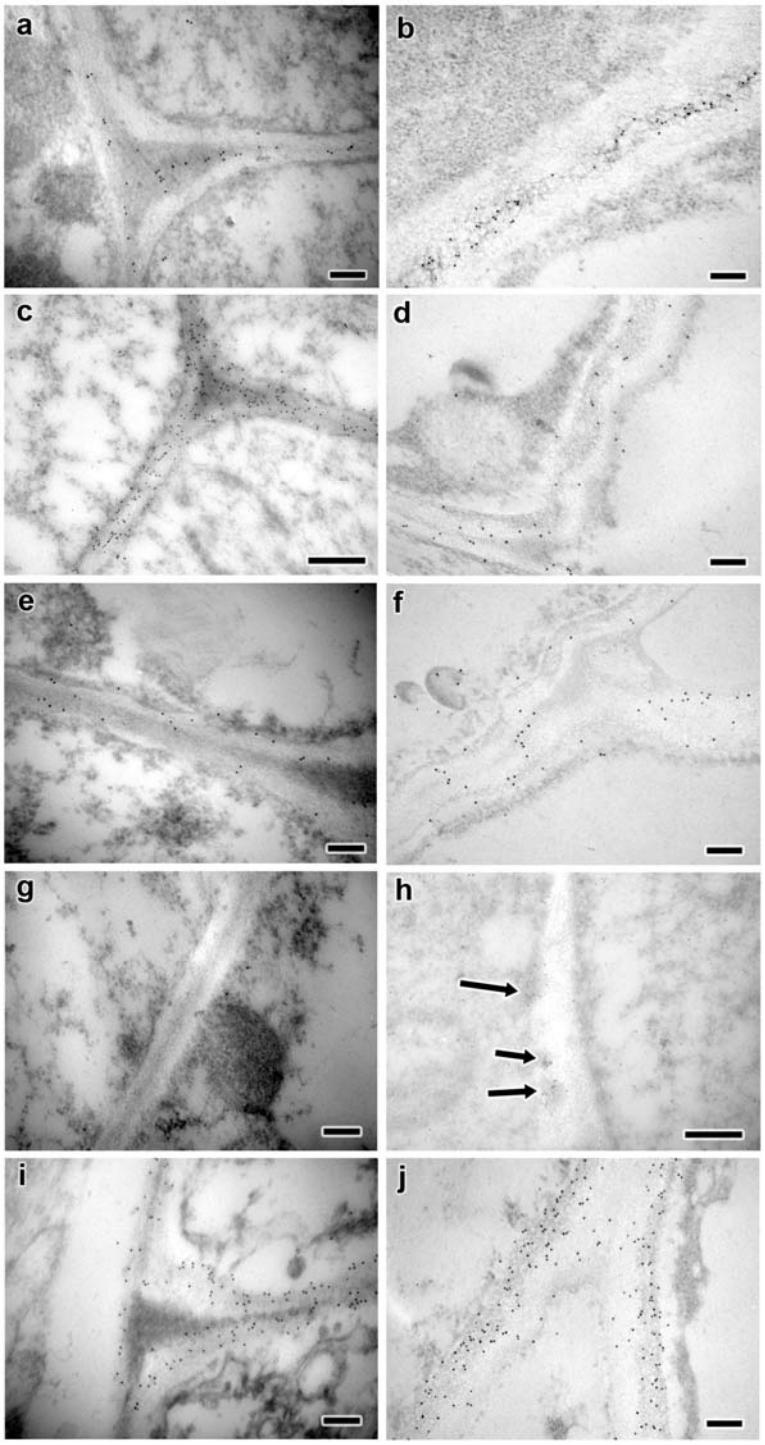
Fig. 2 Immunogold labelling of embryo cell walls with probes that gave positive reactions in the immunofluorescence work. Labelling for JIM5 was evident in the **a** middle lamella and not primary cell wall of immature embryos and **b** middle lamella and cell wall junctions of mature ones. JIM7 labelling appeared uniform throughout the cell wall layers in both immature and **d** mature embryos. Immunogold label was apparent in the primary cell wall and absent from the middle lamella when LM6 was utilised on **e** immature embryos, while a few gold particles can also be seen in the middle lamella of **f** mature embryos. No or very little label was noticed on the cell wall when LM7 was used on **g** immature embryos, while Label occurred in certain randomly distributed cell wall areas (arrows) that stained darker than the rest of the cell wall when LM7 was used on **h** mature embryos. Immunogold particles attached to LM15 can be seen adhering to sites in the primary cell wall layer and not the middle lamella of **i** immature embryos, while **j** few particles can be seen also in the middle lamella region in mature embryos. Gold particles = 20 nm (a-f & i; j); 10 nm (g; h). Bar = 0.5 µm (a; b; d; e; g-j); 5 µm (c; f)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suggests a possible change to the LM7 epitope on maturation of the embryo, which might have influenced antibody binding when a gold (vs fluorescent label) is attached.

The localised distribution of partially esterified HG differed considerably from the distribution of xyloglucan, which appeared to be found mostly in the primary cell wall layer (and not the middle lamella or cell junctions) of immature and mature embryos, as demonstrated by labelling with LM15 (Figs 2 i & j). This implied that xyloglucan is probably present in the primary cell wall and not the middle lamella – contrasting with the distribution of the JIM5 epitope of the present study.

Monosaccharide composition of destarched alcohol insoluble residue (AIR)

Biochemical analysis of the cell wall monosaccharide composition was performed in parallel with the immunofluorescence and immunogold labelling work. This was done in order to substantiate the data and quantify the cell wall components.

Monosaccharide composition of destarched AIR material of mature *E. natalensis* embryo was determined by gas chromatography analysis of the supernatant obtained after trifluoroacetic acid (TFA) hydrolysis of AIR. TFA hydrolyses AIR and yields two fractions: a TFA-soluble fraction which contains non-cellulosic polysaccharides including pectins, hemicelluloses, cell wall glycoproteins such as arabinogalactan proteins, and sometimes amorphous cellulose; the TFA insoluble fraction mainly containing crystalline cellulose, which was not analysed in this study. Examination of the monosaccharide composition of the destarched AIR (Fig. 3a) of the current species revealed an abundance of Glc (ca 80%), which would indicate a predominance of Glc-enriched polymers. Such a high level of Glc is unusual in AIR, especially after the α-amylase/ amyloglucosidase digestion. It is well known that in many plant tissues, starch accumulates and is also hydrolysed with AIR by TFA. This often leads to such a high level of Glc after analysis of the TFA-soluble fraction. In the present study, starch was digested extensively in order to remove it before the analysis of AIR. Composition analysis of AIR before removal of starch was also performed in the current investigation, and in this condition, the level of Glc was much higher compared with destarched material. Two possibilities have to be considered: either not all the starch had been digested, and this was reflected in the composition analysis; or there was another Glc-enriched polymer present. Woodenberg *et al.* (2014) have shown that the embryo cells of *E. natalensis* accumulate large quantities of starch almost to the exclusion of other storage products and organelles, which may account for the unusually high Glc content in the present study; however, the Glc seen

here could also be part of xyloglucan, callose, mixed-linked glucan (MLG) or amorphous cellulose. Nevertheless, a much lower amount of Glc would be expected.

Apart from Glc, the destarched AIR also contained a significant amount of Ara, Gal, Xyl, Rha and GalUA (Figs 3a & b). The relative proportion of destarched AIR was also normalised without the Glc to make the composition more readable (Fig. 3b). The most striking feature of the Glc-normalised data was the substantial amount of Ara (ca 55 mol %, Fig. 3b). This is surprising as desiccation sensitive vegetative tissues have been found to accumulate considerably less arabinose polymers than desiccation tolerant types (Farrant *et al.* 2012; Moore *et al.* 2013).

Overall, the composition of Ara, Gal, Xyl, Rha and GalUA suggested that pectin- and hemicellulose-associated polysaccharides may be present in the AIR (Fig. 3b). The combination of Rha and GalUA suggested the occurrence of rhamnogalacturonan I (RG-I) (Schols and Voragen 1996); the presence of Ara and Gal can be indicative of arabinan and galactan chains associated with RG-I, or to type II arabinogalactan often related to arabinogalactan proteins (AGPs). Supporting the presence of AGPs, was the presence of all minor sugars reported to be incorporated into AGPs, such as Fuc, GlcUA and Rha. However, the lack of information on cycad cell composition in the literature encourages careful interpretation of this data.

In the present investigation, three cell wall polysaccharide-enriched fractions were extracted sequentially i.e. with hot water, 50 mM cyclohexane-1,2-diamine tetra-acetic acid (CDTA), and 4 M KOH (Figs 3c-e, respectively). Usually, starch-derived Glc would be removed before commencement of the sequential extraction process; however, the three fractions were found to be highly-enriched in Glc (Figs 3c-e). It is tempting to speculate that there was still some starch in those fractions, but theoretically, most of the starch should have been removed by this stage.

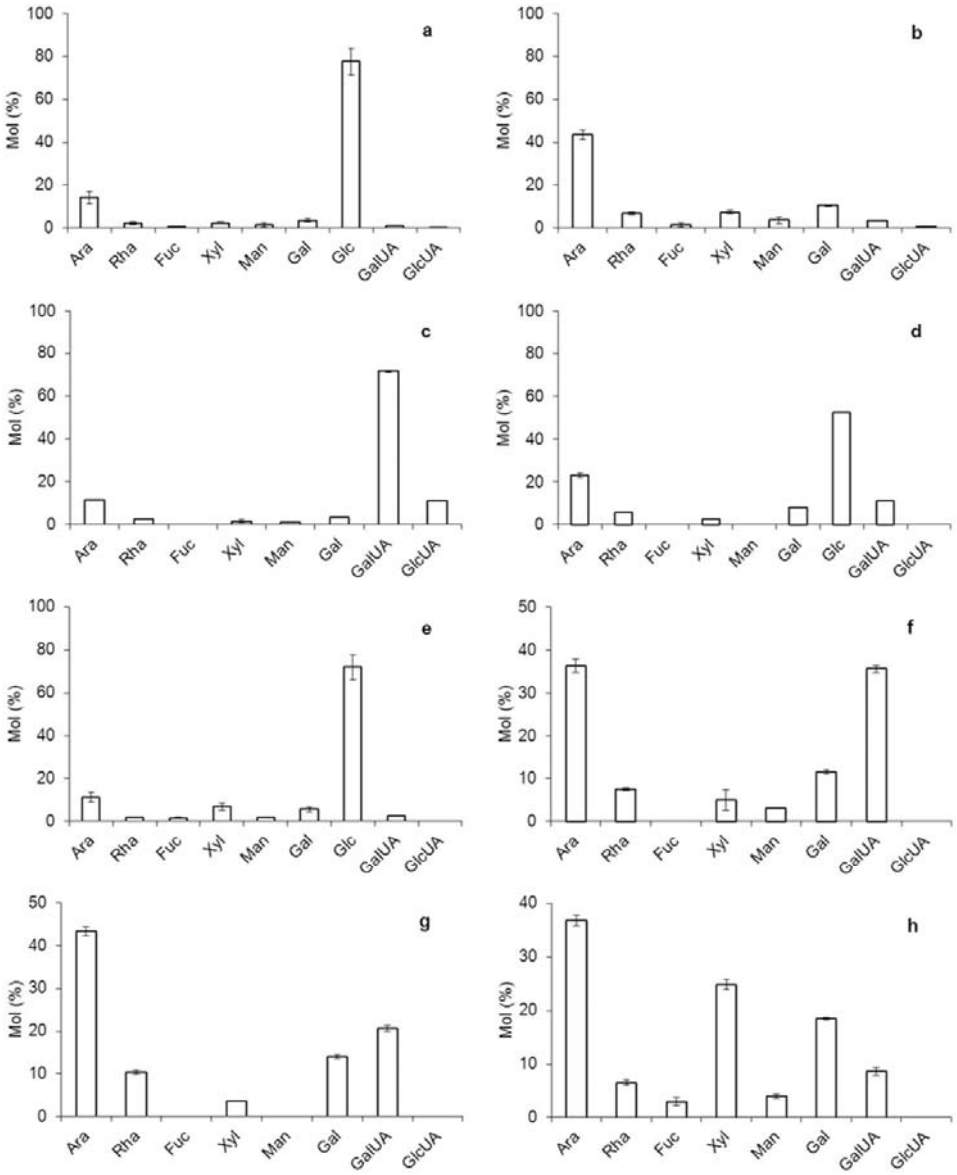
The immunolabelling work of the present study suggested the presence of xyloglucan in the cell walls. In higher plants, the xyloglucan ratio Glc/Xyl is often conserved (Bacic *et al.* 1988). A Glc/Xyl ratio of 4:3 is found in most cases, although sometimes a ratio of 5:2 may be found; however, a ratio of 5:1, 6:1, or 8:1 was found here in *E. natalensis* destarched AIR (Fig. 3a) and hemicellulose-enriched fractions (4 M KOH, Fig. 3e). Clearly, there appeared to be an extra amount of Glc that is not yet attributable to any cell wall polysaccharide.

When the fractions were analysed individually, the water-extracted one (Fig. 3c) showed that, in addition to Glc, other monosaccharides were present viz. Ara, GalUA, Gal and Rha, and then Xyl and Man. The CDTA-extracted fraction contained almost the same monosaccharides (Fig. 3d). The composition of these three fractions depleted of Glc is also presented (Figs 3f-h). Interestingly, the ratio between these monosaccharides was conserved. Water-extracted and CDTA-extracted fractions are known to be pectin-enriched material differently bound into the wall. Note first that the ratio of GalUA/Rha was 7:1 in the water-extracted fraction (Fig. 3f) and 2:1 in the CDTA-enriched fraction (Fig. 3g). Together this implied that both fractions may contain a rhamnogalacturonan I (RG-I) backbone (the stoichiometry of RG-I backbone being 1:1), while the remaining GalUA consisted of homogalacturonan (HGs; polymer of α-1,4-GalUA). Together these compositions showed that there may be more water-extractable HGs than CDTA-extractable HGs in the embryo cell walls of *E. natalensis*. However, the composition does not tell us if there were more or less methyl-esterified HGs. This is in agreement with the immunolabelling work of the current study, which showed fluorescence when the antibodies JIM5, JIM7 and LM7 were used (cf. Figs 1a-d; k-l, respectively).

In higher plants, the RG-I backbone is often found to be branched with galactan or arabinan chains (Harholt *et al.* 2010). Both water- and CDTA-extracted fractions were found to be enriched in Ara in this study (Figs 3f & g, respectively), which corresponds with the cell wall fluorescence after labelling with the LM6 antibody that recognises (1→5)-α-L-arabinan chains associated with RG-I.

In both these fractions, there was also a significant amount of Gal. However, there appeared to be no (apart from the walls of epithelial cells lining mucilage ducts, cf. Fig. 6c) label with the LM5 antibody for (1→4)-β-D-galactan chains of RG-I, as well as with the LM2-anti AGP antibody. Note that LM2 recognises an AGP-associated epitope that contains GlcUA (Smallwood *et al.* 1996). There are many other anti-AGP antibodies recognising AGP-associated epitopes (see Moller *et al.* 2007). This raises the possibility that the Gal observed in all the fractions of the current investigation comes from AGPs. It also suggests indirectly, that part of the Ara was also coming from AGPs. To validate this possibility, future studies should employ JIM13 or JIM14 antibodies, which recognise other epitopes of AGP. In addition, LM6 was also found in some cases to recognise AGP-associated epitopes. Trying anti-galactomannan or galactoglucomannan antibodies may also be useful to account for Gal detected in this investigation.

Fig. 3 Monosaccharide composition of destarched AIR material of mature *E. natalensis* embryos determined by gas chromatography analysis. **a** The monosaccharide composition is dominated by Glc, which accounts for ca 80% of the monosaccharides present. **b** Relative proportion of the monosaccharides from destarched AIR material normalised without Glc. Monosaccharide composition of cell wall fractions from destarched AIR extracted with **c** Water; **d** CDTA; and **e** 4M KOH. All three extracts display a dominance of Glc over other monosaccharides. Monosaccharide composition of the same cell wall fractions from destarched AIR normalised without Glc as extracted with **f** water; **g** CDTA; and **h** 4M KOH. Error bars represent the standard deviation SD of the mean of four biological/technical samples. Monosaccharide codes are as follows: arabinose (Ara); rhamnose (Rha); fucose (Fuc); xylose (Xyl); mannose (Man); galactose (Gal); glucose (Glc); galacturonic acid (GalUA); glucuronic acid (GlcUA)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GalUA
GlcUA

Finally, the 4 M KOH-extracted fraction also showed an almost similar composition to the two previous fractions, except that the level of Xyl was much higher (Fig. 3g). This suggested that the same polymers observed in the two previous fractions were present, since the ratio between Ara, Rha, Gal and GalUA appeared to be the same. Further comment will therefore not be made on those sugars; however, the higher level of Xyl confirmed that it was a hemicellulose-enriched fraction. In higher plants, Xyl occurs abundantly in xyloglucans and xylans (Reiter 2002; McCartney *et al.* 2005). Thus, this Xyl was probably incorporated in xyloglucans as immunofluorescence microscopy in the present study suggested that xylan was not present in the embryo cell walls of *E. natalensis*. However, as observed earlier, there was still a significant extra amount of Glc which was probably derived from other polymers (galactoglucomannan, glucomannan, amorphous cellulose). Nevertheless, the immunolabelling work involving LM15 seemed to confirm the presence of xyloglucan. Note that in some plant species, the xyloglucan can be galactosylated and fucosylated; in others species, xyloglucan is only partially arabinosylated. Future studies should therefore attempt to analyse further the xyloglucan present in the embryo of *E. natalensis*.

Effects of drying on cell wall conformation

In the current study, the desiccation-sensitive mature embryos of *E. natalensis* were dried to a lethal water content of ca 0.4 g g^{-1} on a dry mass basis to see whether or not cell wall folding would occur (Figs 4a-e). Cryo-SEM (Figs 4a & b) was employed to compare the cell walls of hydrated versus dehydrated embryos without the potential introduction of artefacts that may arise through fixation and embedding with aqueous chemicals (Figs 4c & d). Cells appeared turgid before drying and had walls of a relatively straight appearance (Fig. 4a) compared with dehydrated samples in which the walls appeared convoluted (Fig. 4b), indicating that the walls probably do not remain straight upon drying. The degree of undulation seemed to have been influenced by the abundant supply of amyloplasts in the cytomatrix, which appeared to have restricted cell shrinkage and concomitant wall undulation.

Moderately-folded cell walls have been observed previously in dried, freeze-substituted, desiccation-sensitive cortical cells of jackfruit axes (Wesley-Smith 2001). Although there are many other studies on subcellular responses to drying of desiccation-sensitive seeds most did not concentrate on walls (Devey 1988; Berjak *et al.* 1989; Dewar 1989; Kioko 2002). Additionally, many of these studies used aqueous fixation and so the characteristics observed,

particularly plasmalemma withdrawal, may have been artefacts of rehydration (*inter alia* of walls) during fixation, and wall folding would not have been observed.

Cell walls of *E. natalensis* embryos in the present study consisted of a middle lamella and primary cell wall only, and it seems reasonable to expect that these cell walls are not rigid, as the primary cell wall is generally required to be sufficiently flexible to allow for cell expansion (Reiter 2002). The cell wall convolution seen in the current investigation is also not dissimilar in appearance from what was called 'wall folding' in the cells of a variety of desiccation-tolerant seeds (see Webb and Arnott 1982). In that study wall folding also appeared to be restricted by the abundant amyloplasts and storage vacuoles depicted in those cells (as seen in the present investigation).

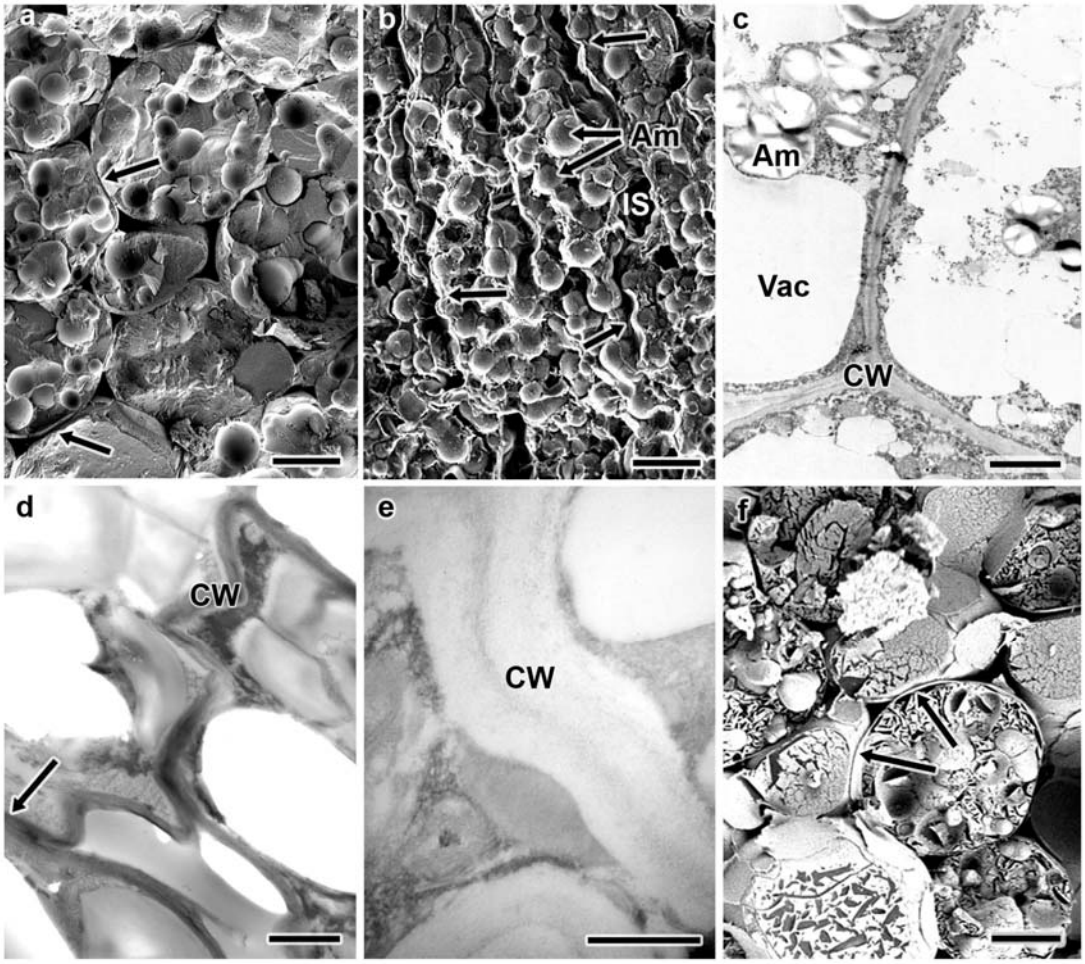
Although wall folding in embryos of *E. natalensis* is not as extensive as is demonstrated in desiccation-tolerant plant material (e.g. Vicré *et al.* 1999; Moore *et al.* 2006), there appeared to be an enlargement of intercellular spaces (Fig. 4b) and plasmolysis was notably absent in dehydrated embryo cells processed for Cryo-SEM. Enlargement of intercellular spaces upon drying has also been seen in dry desiccation-tolerant seeds prepared by anhydrous methods (Webb and Arnott 1982) and may not necessarily indicate damage upon drying.

In some areas, extensive cell wall folding was apparent in the embryo of *E. natalensis* as evidenced by a comparison of hydrated, conventionally-fixed embryo samples (Fig. 4c) with dehydrated, freeze-substituted material (Fig. 4d). While wall folding may have been restricted by the abundant amyloplasts, it appeared to be extensive in some places producing 'hairpin' convolutions (or folds) without rupture of the cell wall (Fig. 4d), which was indicative of the potential of these walls to fold.

When Webb and Arnott (1982) processed dry, orthodox seeds using aqueous fixation methods, they observed turgid cells with straightened cell walls. Dehydrated *E. natalensis* embryo tissue fixed via conventional means produced moderately wavy cell walls (Fig. 4e) similar to that seen in the study by Webb and Arnott (1982).

Although some wall folding was evident in the present investigation, it was unclear whether it was a controlled process or an uncontrolled collapse upon cell shrinkage. Cryo-SEM of embryo samples dried to a water content of ca 1 g g^{-1} dry mass, i.e. just above the water content where 50% viability is lost, revealed relatively straight cell walls (Fig. 4f). This

Fig. 4 Cryo-SEM and TEM of hydrated versus dehydrated *E. natalensis* embryonic axes. **a** Cell walls (arrows) appeared straight and cells took on a turgid appearance in hydrated cells viewed with Cryo-SEM; bar = 20 µm. **b** Convolutions (arrows) that seemed to be restricted by abundant amyloplasts (Am) were evident in the cell walls of dehydrated embryo tissue, producing enlarged intercellular spaces (IS); bar = 10 µm. **c** TEM image of conventionally-fixed and embedded, hydrated embryo cells demonstrating relatively straight cell walls (CW), large vacuoles and amyloplasts; bar = 2 µm. **d** Dried embryo samples that were freeze-substituted displayed highly convoluted walls (CW) in some areas with hairpin-like bends (arrow); bar = 2 µm. **e** Dehydrated samples that were processed via conventional fixation methods displayed moderately undulating walls (CW); bar = 0.5 µm. **f** Embryos dried to a water content just above lethal levels displayed relatively straight walls (arrows); bar = 20 µm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suggested that the cell wall folding observed in the *E. natalensis* embryo may not be as a result of a controlled process but a consequence of cell shrinkage.

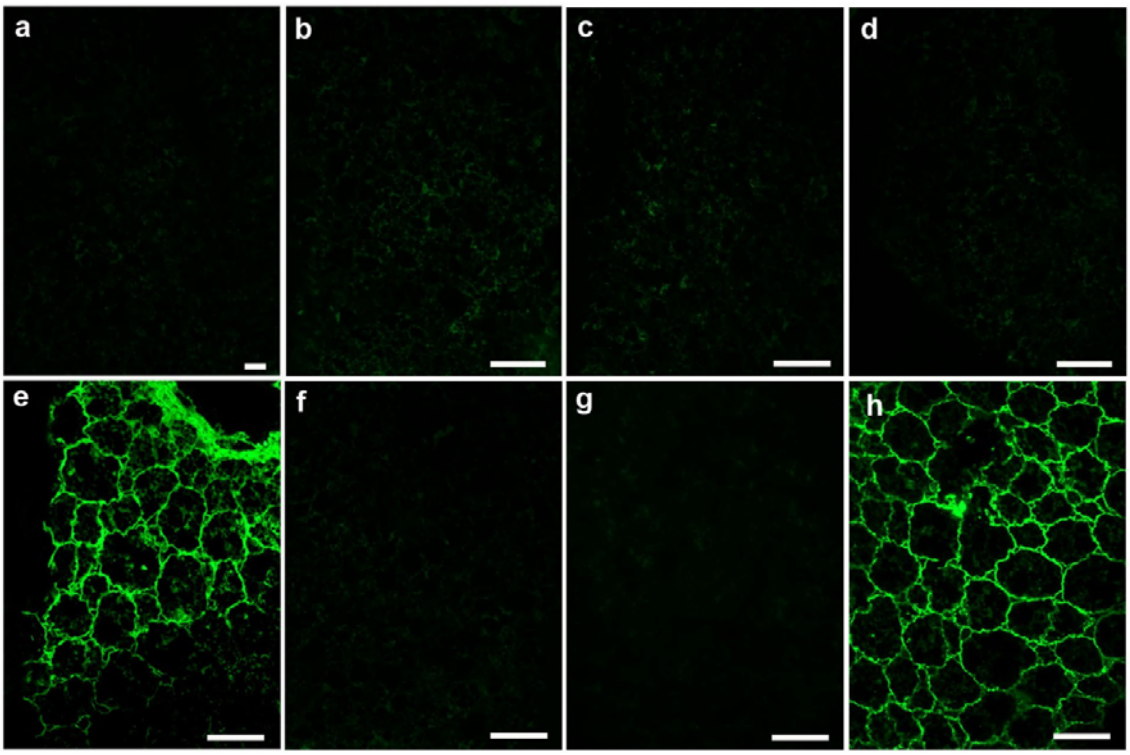
Based on their observations of the walls of dry, orthodox seeds, Webb and Arnott (1982) proposed that cell wall folding may be dependent on the chemical make-up of both the cell wall and cytomatrix, i.e., the nature and amount of accumulated reserves. While Woodenberg *et al.* (2014) have confirmed that the embryo cells of *E. natalensis* are packed with numerous starch-containing amyloplasts, the current study analysed the pectic and hemicellulosic components of the cell wall of dried *E. natalensis* embryos to increase understanding of the relation between chemical composition of the cell wall and its ability to fold upon desiccation.

Cell wall composition of dried mature embryos

While JIM5, JIM7, LM6 and LM15 epitopes appeared to be present in hydrated embryo cell walls of *E. natalensis*, when the embryos were dried to a water content of about 0.4 g g^{-1} on a dry mass basis and fixed via freeze-substitution, fluorescence microscopy results were suggestive of an alteration in the chemical make-up of the walls upon drying (Figs 5a-h). Use of the JIM5 and JIM7 antibodies did not result in visible fluorescence of the cell walls (Figs 5a & b, respectively) and LM2 and LM5 epitopes appeared to remain unchanged in their absence of expression (Figs 5c & d) compared with hydrated embryo cell walls. However, the LM6 epitope seemed to be present in the cell walls of dried embryos by virtue of the bright fluorescence that was displayed (Fig. 5e). In contrast, LM7 and LM11 appeared to be absent as no fluorescence was observed (Figs 5f & g, respectively), whilst use of the LM15 antibody gave rise to intense fluorescence (Fig. 5h) as was the case in hydrated embryo cell walls. Therefore, the results from the fluorescence microscopy of dried embryos fixed by freeze-substitution indicated that poorly, highly and partially methyl-esterified epitopes of HG were no longer present in the cell walls after the embryo had been dried, whilst expression of arabinan and xyloglucan appeared unchanged by dehydration.

A possible explanation for the inability of some antibodies to yield positive results in freeze-substituted material could be the phenomenon of steric hindrance (see Roth 1982). The cell walls may have changed conformation when dried and/or exposed to cryogenic temperatures such that sites usually available for tagging with the primary antibody in the hydrated cell wall are masked by the crumpled conformation of the cell wall in the dehydrated state. Results from aqueously-fixed, dehydrated embryos appeared to provide strength for the idea

Fig. 5 Immunofluorescence microscopy of various epitopes of pectin and hemicellulose in the cell walls of dry *E. natalensis* embryos fixed via freeze-substitution. **a** Cell walls do not display fluorescence when JIM5 was used; bar = 20 µm. **b** No cell wall fluorescence is evident in a section treated with JIM7; bar = 50 µm. **c** Similarly, the use of LM2 did not yield fluorescence in the cell walls; bar = 50 µm. **d** The LM5 epitope appeared to be absent from the walls; bar = 50 µm. **e** Bright fluorescing, undulating cell walls can be seen when LM6 was used; bar = 50 µm. **f** LM7 did not yield any fluorescence in the cell walls; bar = 50 µm. **g** No cell wall fluorescence is apparent after LM11 was employed; bar = 50 µm. **h** Cell walls with an undulating conformation fluoresce intensely when LM15 was applied; bar = 50 µm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of steric hindrance (Figs 6a-h). Dried embryo material fixed in aqueous medium displayed results similar to that of hydrated embryos: JIM5 and JIM7 epitopes showed fluorescence in the cell walls (Figs 6a & b, respectively) implying that poorly and highly esterified epitopes of pectin were present.

In the current study, LM2 and LM5 epitopes seemed to be absent from parenchyma cells since no fluorescence was observed when those antibodies were employed (Figs 6c & d, respectively); however, some faint labelling occurred in the walls of epithelial cells that lined mucilage ducts (Fig. 6c). This suggested that arabinogalactan proteins are not absent entirely from the embryo cell walls of *E. natalensis*, but that they may be a feature of the epithelial cells lining mucilage ducts, while galactans are not present.

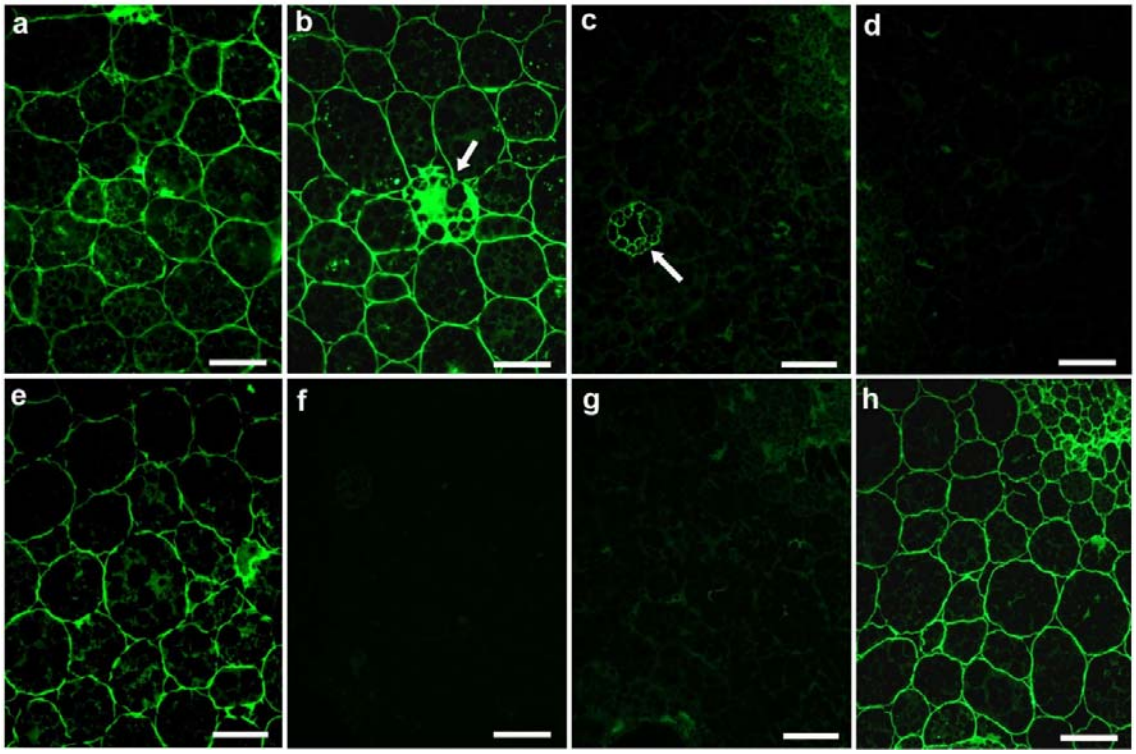
On the other hand, arabinan appeared to be present throughout the cell walls as LM6 yielded fluorescence in the cell walls (Fig. 6e). The embryo cell walls of *E. natalensis* therefore do contain some wall plasticising molecules, which may account for the convoluted appearance of cell walls seen in dehydrated tissues.

While arabinan was apparently present, partially esterified epitopes (recognised by LM7) and xylan seemed to be absent from the walls as exemplified by the absence of fluorescence when LM7 and LM11 antibodies were used (Figs 6f & g, respectively); while the LM15 epitope, xyloglucan, appeared to be present when the use of its corresponding antibody yielded fluorescent cell walls.

Immunogold labelling of dried, aqueously-fixed embryo material (Figs 7a-e) also showed similar results to that of hydrated embryos. Label was seen when both JIM5 and JIM7 antibodies were used (Figs 7a & b, respectively), indicating that these epitopes are present in both the middle lamella and primary cell wall; however, label with JIM5 occurred throughout the wall not just in the middle lamella and cell junctions as seen previously. This implied that either more molecules of this epitope were being produced and that the distribution was spread to the primary cell wall too in order to confer strength, or that leaching of the JIM5 epitope had taken place during the drying and rehydration process.

The antibody LM6 produced label that bound mostly to the primary cell wall and nearby cytomatrix (Fig. 7c) suggesting that arabinan might be concentrated in those regions. The cell walls were mostly clear of label when LM7 was employed; however, a few vesicles that were presumably derived from Golgi bodies displayed some label (arrow, Fig. 7d), implying that

Fig. 6 Immunofluorescence microscopy of various pectin and hemicellulose epitopes in dried, aqueously-fixed embryos of *E. natalensis*. **a** Cell walls display bright fluorescence when JIM5 was utilised; bar = 50 µm. **b** JIM7 yielded a similar result with walls as well as tannin (arrows) fluorescing brightly; bar = 50 µm. **c** Use of the LM2 antibody resulted in faint fluorescence in the walls of epithelial cells that line mucilage ducts only (arrow); bar = 50 µm. **d** LM5 yielded no visible fluorescence in the cell walls; bar = 50 µm. **e** Bright fluorescing cell walls are apparent when LM6 was used; bar = 50 µm. **f** No cell walls can be seen fluorescing after treatment involving LM7; bar = 100 µm. **g** Cell walls do not demonstrate fluorescence when LM11 was utilised although faint fluorescence can be seen in the cytomatrix; bar = 50 µm. **h** Use of LM15 gave rise to cell walls that fluoresced brightly in both parenchyma cells and those of the vascular tissue; bar = 50 µm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partially methyl-esterified epitopes of HG may have been incorporated into the wall. As evidenced in hydrated embryos, LM15 produced label in the primary cell wall layer and considerably less label was seen in the middle lamella (Fig. 7e). Hence, the results of the dried, aqueously-fixed embryos are indicative of an unchanged expression upon drying of xyloglucan, arabinan, as well as poorly, highly and partially methyl-esterified epitopes of HG in the cell walls of *E. natalensis*. They also imply that arabinogalactan protein, galactan, and xylan are probably not produced as a consequence of drying.

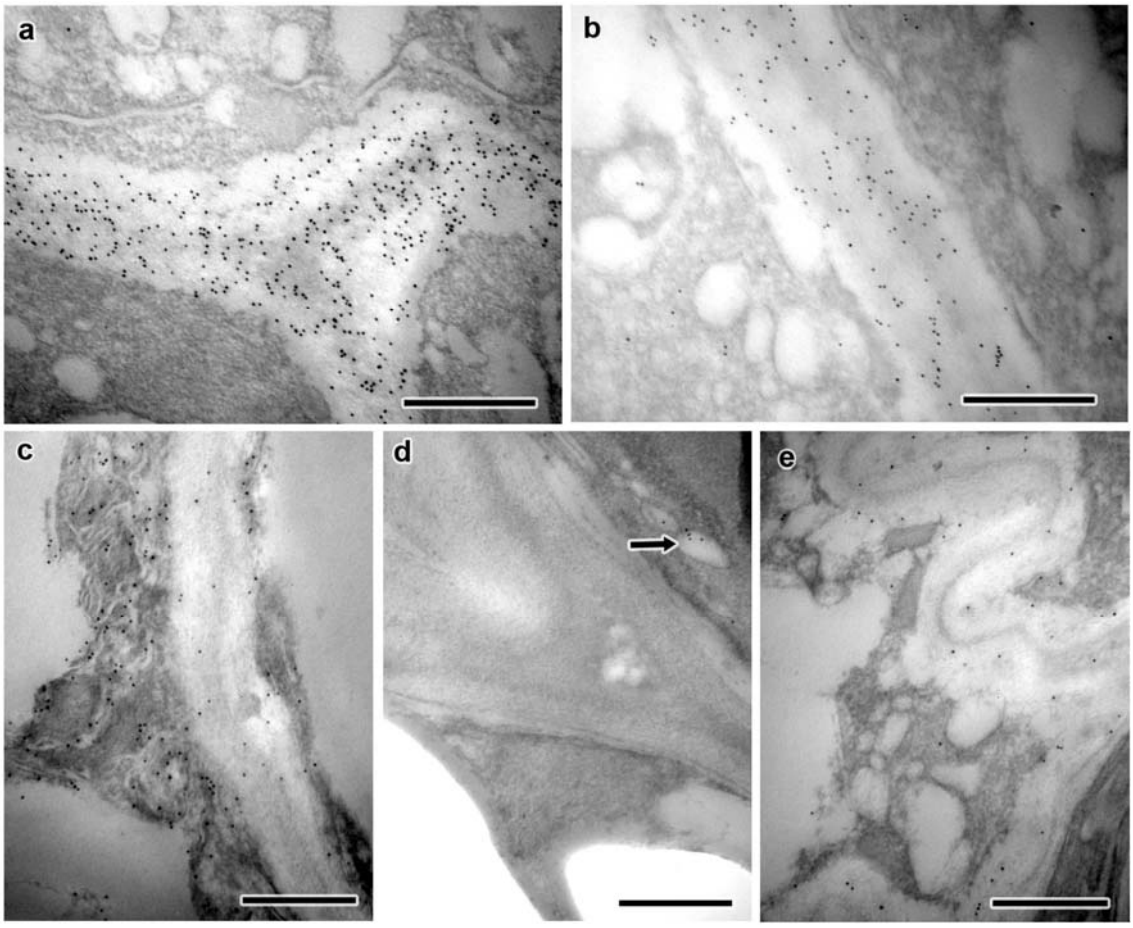
Concluding comments

It appears that the two sets of data (immunolabelling and biochemical analysis) are complementary. They provide an interesting, preliminary description of embryo cell wall composition in *E. natalensis*. While the immunolabelling work has shown the presence and positioning within the cell wall of different epitopes of pectin and hemicellulose, the biochemical analysis of monosaccharides has revealed that there may be more cell wall components (Glc-enriched polymers) that are present in the embryo that future research should explore.

Moreover, the biochemical work provided quantitative data revealing potentially high concentrations of arabinose polymers in Glc-normalised graphs (ca 55 mol % of non-cellulosic polysaccharides; Fig. 3b) that warrants further investigation. Such a large quantity of arabinose polymers is comparable with the cell walls of desiccation tolerant vegetative plant tissue (Moore *et al.* 2013) and orthodox seeds (Navarro *et al.* 2002; Dourado *et al.* 2004; Gomes *et al.* 2009). However, it has been shown that in the desiccation tolerant seeds of *Arabidopsis*, arabinose polymers accumulated in the wall may also function as storage reserves for utilisation during germination (Gomes *et al.* 2009). In that study, the major subcellular storage reserves were lipid and protein and so the arabinose polymers of the wall were proposed to provide a dual role in conferring wall flexibility and being a source of carbohydrates needed for germination.

However, in *E. natalensis* embryos are dominated by the accumulation of large starch-containing amyloplasts (Woodenberg *et al.* 2014), which would naturally fulfill the carbohydrate needs of the germinating embryo. Although no study has been performed on *E. natalensis* seeds to confirm whether or not cell wall arabinose polymers are utilised during germination, the main carbohydrate source would probably come from the abundant amyloplasts. However, such a high concentration of arabinose polymers in the cell wall of *E.*

Fig. 7 Immunogold labelling of xyloglucan and various epitopes of pectin in the cell walls of dried, aqueously-fixed *E. natalensis* embryos. **a** Label can be seen in both the middle lamella and primary cell wall layers when JIM5 was employed; bar = 0.5 µm. **b** A similar demonstration of labelling is apparent when for JIM7; bar = 0.5 µm. **c** Some label is evident in the cell wall while many gold particles are found also in the cytomatrix close to the cell wall when LM6 was used; bar = 0.5 µm. **d** LM7 yielded an absence of gold label from the cell wall while a few Golgi-derived vesicles (arrows) display some labelling; bar = 0.5 µm. **e** Primary cell walls show some gold label while the middle lamella appears to be free of label when LM15 was used; bar = 0.5 µm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natalensis could also mean that they are constitutively prepared for cell wall folding upon drying.

Because the cells of the *E. natalensis* embryo contained an abundance of amyloplasts, cell shrinkage was not marked. Therefore, high arabinose polymer levels would not be required for the purpose of extensive wall folding in this species, but the possession of substantial amounts of this polymer may coincidently make the walls amenable to folding.

Similarly, the accumulation of abundant starch-containing amyloplasts in cells of the *E. natalensis* embryo may function primarily as storage reserves required for the protracted post-seed-shed ontogeny and germination of this species (Woodenberg *et al.* 2014). However, as evidenced by the current study, the amyloplasts may also fulfill a secondary role in the provision of mechanical stabilisation during dehydration, by preventing excessive cell shrinkage.

Interestingly, although cells of the *E. natalensis* embryo seem to have mechanical stabilisation provided by both constitutive cell wall flexibility and substantial accumulation of subcellular reserves, the embryo of this species is highly sensitive to desiccation (Woodenberg *et al.* 2007). This is in contrast to the subcellular situation in the highly recalcitrant seeds of *Avicennia marina* (e.g. Farrant *et al.* 1997), which does not accumulate much by way of insoluble reserves. *Encephalartos natalensis* therefore appears to be an exception to the general belief that the degree of reserve accumulation in recalcitrant seeded species tends to be commensurate with the amount of water loss tolerated (e.g. Farrant and Walters 1998; reviewed by Farrant *et al.* 2012).

The cryo-SEM and freeze-substitution work reported in the present study suggested that the walls of the *E. natalensis* embryo may have some degree of folding upon dehydration that is comparable with previous reports on desiccation-tolerant plant material. The fact that the walls displayed some form of folding is not surprising as the cell walls were found to consist of a middle lamella and primary cell wall only, i.e. the walls were not abnormally thick and should theoretically be flexible, at least to allow for cell expansion during embryo development. While wall plasticising molecules such as arabinogalactan protein recognised by the LM2 antibody and galactan appeared to be absent from the cells (apart from the walls of epithelial cells lining mucilage ducts), the walls of *E. natalensis* embryo were apparently not devoid of plasticising molecules, as arabinan was present in hydrated immature, mature, and dehydrated mature tissue. Similarly, while xylan was not positively labelled in this

investigation, the embryo of *E. natalensis* may well accumulate this hemicellulose in xylem walls (see Woodenberg *et al.* 2014). Future research should look at the ability and extent of cell wall folding in desiccation-sensitive embryo cells that have fewer large amyloplasts (e.g. meristematic cells of the immature embryo of this species) to see whether or not the cells can fold to a similar extent as desiccation-tolerant plant tissues.

From the results of the present study and other works, it is tempting to speculate that the potential of walls to fold (which is determined by their composition and structure) may be a strategy to avoid membrane damage on drying; however, the concept deserves an extension of the interest currently being paid to it. Additionally, results from this study have reinforced the belief that seed recalcitrance is a consequence of the malfunction or absence of many interacting mechanisms that allow for desiccation tolerance (Pammenter and Berjak 1999). Further research is thus needed to elucidate why *E. natalensis* seeds lose viability upon dehydration.

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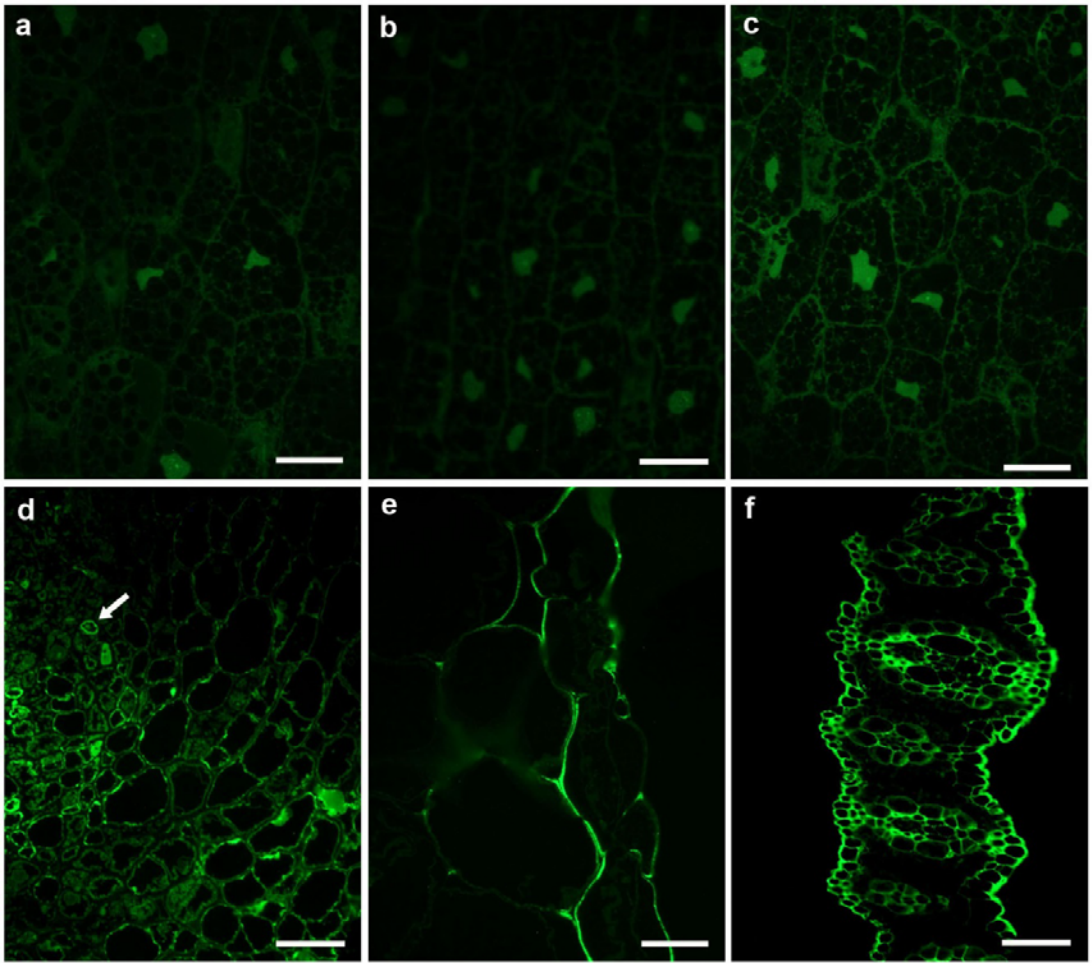
Appendix A

Fig. 8 Demonstrates fluorescence microscopy of negative and positive controls used to verify results. **a** Some autofluorescence appeared to be present in the cells with faint fluorescence evident in the nuclei and certain areas of the cytomatrix; bar = 50 µm. **b** A similar picture can be seen in a primary antibody only control; bar = 50 µm. **c** Fluorescence was somewhat more intense in the nuclei and cytomatrix when the protocol was performed with the secondary antibody only; bar = 50 µm. **d** Relatively intense fluorescence was evident in the root tip cell walls [especially in the xylem walls (arrowed)] of *Daucus* spp. when the LM2 antibody was employed; bar = 50 µm. **e** Epidermal and subepidermal cell walls display fluorescence when the LM5 antibody was used on the pericarp tissue of *Lycopersonicon* spp.; bar = 50 µm. **f** Intense fluorescence is apparent when the LM11 antibody was used on *Zea mays* leaf tissue; bar = 100 µm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CHAPTER 4

Overview and concluding comments

The present investigation has revealed some important aspects of the post-shedding development of the *E. natalensis* embryo that furthers understanding of the recalcitrant behaviour of these seeds (Woodenberg 2009). The seeds of this species are shed from the parent plant when the embryo is rudimentary and physiologically immature, and its development has been shown to be a continuous process after seed-shed that ultimately culminates in germination. This is in contrast with orthodox seeds where full embryo development has usually occurred by the time they are shed in a quiescent or dormant state following maturation drying (Adams and Rinne 1980). Much of the development of the embryo of the cycad, *E. natalensis*, was found to occur after seed-shed and since these developmental processes would require the degree of hydration facilitating ongoing metabolism, it is clear why the seeds of this species do not tolerate desiccation.

While cells continued to show signs of ongoing metabolism throughout embryogenesis – exemplified by mitochondria of active appearance, abundant polysomes and profiles of ER – the current investigation has revealed the details of development that occur in the months after seed-shed, viz. cellular differentiation during the ontogeny of cotyledonary protuberances; procambial strands giving rise to vascular tissue; development of tannin-containing channels and mucilage ducts; while reserve accumulation, principally starch, also emerged as a post-shedding phenomenon. So while recalcitrant seeds of most species are notoriously short-lived (Pammenter *et al.* 1994), *E. natalensis* seeds require a protracted period (ca six months) of post-shedding development before they reach a maturity level commensurate with germinability.

In fact, the seeds of *E. natalensis* when maintained in dry storage (i.e. at ambient relative humidity) at 16°C, have been found to remain viable for up to two years after they have been shed (personal observation). The present work has revealed possible explanations for this unusually long period of viability of these desiccation-sensitive seeds. For example, mucilage ducts, which are suggested to act as water reservoirs (reviewed by Bhatnagar and Moitra 1996), were a common feature of mature embryos in this species, and the waxy layer on the surface of the embryo, which, along with the possession of a tough sclerotesta external to the megagametophyte in which the embryo is contained, may contribute to water retention in the months after seed-shed (Woodenberg 2009). These structures are suggested to act together to

minimise water loss in the months after seed-shed – thus facilitating ongoing metabolism and post-shedding ontogeny in these desiccation-sensitive seeds.

Another salient feature of the *E. natalensis* embryo that may play a role in its ability to remain viable for a relatively protracted period could be the accumulation of considerable deposits of starch in amyloplasts as opposed to lipid bodies as the chief storage reserve. The latter reserve type is known to be susceptible to oxidation (McDonald 2004), which may well have led to a loss of viability during the long period of embryogenesis in the current species. This is suggested to have been of particular importance during the Carboniferous Period when cycads are believed to have originated (Schwendemann *et al.* 2009). Reports suggest that during that Period, atmospheric oxygen concentrations may have been as high as 35% (e.g. Berner and Canfield 1989), and thus the propensity for oxidative damage would have been considerably greater than it is today. In accumulation of starch as the major storage reserve of the embryo, *E. natalensis* shows commonality with other recalcitrant-seeded gymnosperms viz. *Araucaria angustifolia* (Farrant *et al.* 1989); *A. hunsteinii* (Pritchard and Prendergast 1986), *A. araucana* (Royal Botanic Gardens Kew 2008) and *A. bidwillii* (Del Zoppo *et al.* 1998). It is proposed that lipid in such seeds, which remain hydrated and metabolically active for relatively extended periods, would be more prone to oxidative degradation than would be the case in desiccation-tolerant (orthodox) seeds of e.g., many of the conifers.

While there are comparatively few studies that have analysed the storage reserves of embryo tissue of gymnosperm seeds, the predominance of starch as the main embryo storage reserve could be viewed as an ancestral trait, while lipid- and protein-rich embryos seem to represent the more advanced state in gymnosperms (Ching 1966; Gifford 1988; Krasowski and Owens 1993). Since cycads are regarded as the most primitive group of gymnosperm, it would be interesting to speculate that starch was the initial reserve to be accumulated in embryos of the first spermatophytes. In *E. natalensis*, for example, an importation of sucrose from the megagametophyte and its conversion to lipid would seemingly be a much more sophisticated biochemical process compared with that involving starch (pers. comm. M. Black, King's College, London).

While on the subject of reserve accumulation, another interesting observation in the current investigation was the apparent absence of dense protein bodies that were prevalent in the megagametophyte tissue of this species (Woodenberg *et al.* 2010). This was observed despite

the preponderance of polysomes in the cytomatrix of the embryo cells throughout ontogeny. The embryo of *E. natalensis* therefore appears to obtain its amino acid quota directly from the protein reserves of the megagametophyte. However, the abundant polysomes indicate ongoing active protein synthesis, presumably directly for the production of membrane-component and structural proteins and enzymes, which would negate the need for storage of protein in protein bodies in the embryo. In the light of the current work on reserve accumulation in the embryo of *E. natalensis*, future studies are encouraged on reserve accumulation in other gymnosperm and angiosperm embryos to further understanding of how this relates to post-harvest behaviour, germination rates, and evolutionary status.

While the current investigation has afforded information on reserve accumulation in the *E. natalensis* embryo, it has also revealed some important aspects of secondary metabolite production – namely of mucilage and tannins – in the embryo of this species. For example, initial stages of tannin channel formation suggested that tannins are accumulated in vacuoles of nucleated cells that also display amyloplasts. Tannins appeared to accumulate by the internalisation of small electron-opaque deposits within vesicles, which are suggestedly derived from the endoplasmic reticulum. This is in keeping with proposals made in studies on other plants (Zobel 1985; Rao 1988; Evert 2006), that tannins originate in the ER prior to being accumulated intravacuolarly. In the current investigation, subsequent elongation of tannin-accumulating cells appeared to occur without prior formation of a syncytium as was reported by Zobel (1985) for shoots of the angiosperm, *Sambucus racemosa*.

Clues with regard to the function of tannin in the current species became apparent in a few occasions when the embryo had apparently been wounded before fixation. In those instances, tannin(s) from the nearby channel(s) seemed to penetrate the wound. This suggests the manner in which tannins may function to prevent pathogen entry upon physical injury (Goodman *et al.* 1967; Wood 1967), and/or their role in providing antioxidant activity where and when needed (Esau 1953), since wounding is reported to be accompanied by increased levels of reactive oxygen species (ROS) (Bolwell 1999). In this regard, the present study also highlighted the positioning of tannin channels near the periphery and vital tissues of the *E. natalensis* embryo, where they are suggested to provide defence against pathogens. Moreover, attention was drawn in this study to the use of parts of cycads in traditional medicine (e.g. Osborne *et al.* 1994), where tannins may be the active principles. Further investigation is thus also encouraged on the efficacy of tannins from cycads for medicinal

purposes, with the possibility of their production via *in vitro* cultivation of e.g. callus from selected explants.

As far as the other secondary metabolite, mucilage, is concerned, ultrastructural observations in the present work suggested that the endoplasmic reticulum (as opposed to Golgi bodies) is the chief subcellular component responsible for the manufacture and secretion of mucopolysaccharides in the embryo of *E. natalensis*. Although this was an unexpected observation, it was in keeping with an earlier study on cellularisation in the megagametophyte of this species (Woodenberg *et al.* 2010). In that study, ER-derived vesicles were observed to elaborate and deposit cell wall material without the involvement of Golgi bodies, which were rarely observed. In this regard, further research should also be undertaken to investigate the intracellular pathways implicated in primary wall formation during the initial cellularisation of the *E. natalensis* pro-embryo, as it will be interesting to see whether or not a similar mode of Golgi-bypass (Hara-Nishimura *et al.* 1998; Hawes 2005; Takahashi *et al.* 2005) occurs.

With regard to the cell walls, the current work appears to be the first in-depth characterisation of the apoplast in a cycad embryo. It also appears to be the first in-depth study on the walls of a desiccation-sensitive embryo in relation to development and dehydration. Cell walls of immature embryos of *E. natalensis* appeared to be characterised by both poorly- and highly-methyl-esterified epitopes of the pectin, homogalacturonan (HG); partially-methyl-esterified epitopes of HG recognised by the antibody, LM7; rhamnogalacturonan-associated arabinan; and the hemicellulose, xyloglucan; while arabinogalactan protein (AGP) recognised by LM2 antibody, rhamnogalacturonan-associated galactan, and the hemicellulose, xylan, were apparently absent.

These results demonstrated that the cell walls of the *E. natalensis* embryos may not be dissimilar from those of other plants in their possession of poorly- and highly-methyl-esterified HG (Talmadge *et al.* 1973); however, they seem to differ from cell walls of other plants in the punctuate distribution of partially-esterified epitopes of HG recognised by the LM7 antibody as opposed to the accumulation of this epitope at the corners of intercellular spaces and junctions between cells in other plant tissue (Willats *et al.* 2001). This suggested that the LM7 epitope may have been localised in vesicles close to the wall or in those that were being incorporated into the wall in the present study.

As far as pectin side-chains are concerned, there appeared to be an absence of certain wall plasticisers, viz. arabinogalactan protein as would be recognised by the LM2 antibody, and rhamnogalacturonan-associated galactan; however, the walls were not devoid of plasticisers as rhamnogalacturonan-associated arabinan appeared to be present. This implied that the walls may have some degree of flexibility, which is expected given that embryos in these recalcitrant seeds were undergoing continuous growth and development in the post-shedding phase.

Xyloglucan appeared to be the predominant hemicellulose in the *E. natalensis* embryo, while xylan was undetected with LM11. This could be expected as xylan is usually a component of the secondary cell wall, as opposed to the primary cell wall and middle lamella layers that were prevalent in embryos in the current investigation.

Interestingly, the cell wall components analysed in this study did not appear to change during the six month post-shedding development of the embryo. However, while the current investigation made use of a number of cell wall antibodies, the biochemical work in this study revealed that there are other wall components that future research should explore. For example, the high concentration of glucose (Glc)-rich polymers identified in this study was of particular significance. Future research is needed to elucidate whether this is due to various Glc-rich polymers e.g. galactoglucomannan, glucomannan, and/or amorphous cellulose, or whether it is due to starch contamination. If those Glc-rich polymers are not localised in the wall, it may imply contamination of the cell wall fraction in this study by starch which is a dominant feature in the cytomatix of mature *E. natalensis* embryo cells (Woodenberg *et al.* 2014). Furthermore, if there is contamination by starch, future research will involve developing suitable protocols for complete de-starching of *E. natalensis* embryo cell wall fractions prior to analysis.

Similarly, the high concentrations of arabinose polymers in the present study in Glc-normalised data, also warrants further investigation. Future studies should employ a range of antibodies for the detection of other epitopes of AGP to confirm whether or not these molecules are present in the embryos of *E. natalensis*. The current study suggested that the LM2 epitope may be absent; however, it may emerge that other epitopes of AGP are present and may contribute to the high concentration of arabinose found in this study. Given the basal position of cycads in spermatophyte phylogeny, a thorough analysis of cell walls in these plants could be important towards an understanding of cell wall evolution in plants.

In the present study, however, the absence of AGP and rhamnogalacturonan-associated galactan did not appear to be significant with regard to drying in mature embryos, as the walls appeared sufficiently flexible and were afforded enough mechanical stabilisation by the occurrence of the abundant amyloplasts in the cytomatrices of the cells. In this particular study, only mature embryos were subjected to drying. It will be intriguing to see the responses of the walls of immature embryos of *E. natalensis* where there would be less mechanical stabilisation from amyloplasts, which are fewer and smaller than in the mature stage of embryogenesis (Woodenberg *et al.* 2014).

Since there are very little data, other than the work on resurrection plant cell walls (reviewed by Farrant *et al.* 2012; Moore *et al.* 2013), to compare with the current findings, future research is encouraged to investigate cell wall properties in embryos across a range of cycad genera. Similarly, investigations on embryo cell walls across a spectrum of orthodox- and recalcitrant- seeded species could prove important in elucidating plant cell wall evolution and improve understanding of the relationship between cell wall composition and the responses of cell walls to desiccation.

Overall, this study has been relatively comprehensive in coverage of the ultrastructural and histological aspects of embryogenesis of *E. natalensis*. This work will form a pivotal basis for future studies, which may lead to an improved understanding of the responses of seed cell walls to desiccation – and also, through the understanding gained about the intricacies of embryogenesis – ultimately, result in the successful germplasm cryopreservation and *in vitro* production on a commercial scale of these, and other, endangered cycad species.

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