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Ultrastructure and cytochemistry of **Eucalyptus globulus** (Myrtaceae) pollen grain

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Ultrastructure and cytochemistry of *Eucalyptus globulus* (Myrtaceae) pollen grain

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Abstract
The morphology, ultrastructure and cytochemistry of *Eucalyptus globulus* mature pollen were investigated using light (LM), scanning electron (SEM) and transmission electron microscopy (TEM). The pollen morphology is typically myrtaceous with a suite of characters that allows its distinction from the pollen of other *Eucalyptus* species. The exine consists of a thick endexine and a massive ectexine with hardly distinguishable columellae. The endexine is 2-layered towards the apertural regions where the inner and spongy-granulate layer forms a continuous colpus membrane and a thinner pore membrane. Under the pores the intine is 3-layered forming complex onci. The spindle-shaped generative cell (GC) is deeply undulated and located in a cup-shaped depression of the vegetative cell (VC) nucleus. In the dense VC cytoplasm the main storage reserves are lipid bodies and insoluble carbohydrates in the cytosol, although proteins are also present. The most characteristic feature of the VC cytoplasm is the extremely well-developed rough endoplasmic reticulum (RER), which forms extensive stacks filling large areas of the central cytoplasm. Single RER cisternae are also scattered throughout the cytoplasm, most of them establishing an intimate association with lipid bodies, storage vacuoles, the VC plasmalemma and a few proplastids. The physiological significance of the RER stacks and of the RER cisternae association with other cell components, as well as the structure and function of an endomembrane compartment, found only in the freeze-fixed pollen, are discussed.

Keywords: *Pollen grain, cell structure, vegetative cell, endoplasmic reticulum, storage reserves,* Eucalyptus, *Myrtaceae*

Eucalyptus globulus Labill. (Myrtaceae) is one of the world's most widespread hardwood trees. In Portugal it occupies ca. 20% of the total forest area being the second most abundant, and one of the most important tree species (DGRF, 2006). Due to its social, economical and environmental impacts, *Eucalyptus globulus* has been the object of several genetic, ecological and physiological studies. The pollen biology of *Eucalyptus* has mainly focussed on pollination and pollen grain germination (e.g. Heslop-Harrison & Heslop-Harrison, 1985; Patterson et al., 2004), although some studies on pollen morphology and pollen wall organization have been done in a few species (e.g. Gadek & Martin, 1982; Patel et al., 1984; Heslop-Harrison & Heslop-Harrison, 1985; Zhou & Heusser, 1996; Pickett & Newsome, 1997). Typically, *Eucalyptus* pollen is triangular, isopolar, radiosymmetric, parasyncolpate, with a distinctive apocolpial field. A suite of characters, including grain size, types of apocolpial field and apocolpial edges, surface patterning and its distribution on the mesocolpia and margo colpi, may be used to define and separate a number of *Eucalyptus* pollen types (Pickett & Newsome, 1997).

So far, no detailed ultrastructural and cytochemical studies on the cellular organization of the *Eucalyptus* mature pollen have been published. These studies are important for a better understanding of the reproductive biology of a species and for identification of characteristics that may be of taxonomic and (or) phylogenetic interest. Studies on other plants have shown that the composition and organization of the pollen grains are different among species (e.g. Jensen et al., 1974; Cresti et al., 1975, 1985, 1988, 1990; Van Aelst & Van Went, 1991; Van Aelst et al., 1993; Hess, 1995; Dinis et al.,

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2000). The main differences are in respect to the pollen wall architecture, the number and degree of development of the organelles, the amount and nature of reserve substances, and the generative cell (GC) composition and its association with the nucleus of the vegetative cell (VC). In the present study, the morphology, ultrastucture and cytochemistry of the mature pollen of *Eucalyptus globulus* were investigated using both chemical fixation and rapid freeze-fixation. Our goal is to gain a detailed knowledge of the structural organization of the pollen of this species to better understand its physiology and its performance during shedding, storage and germination.

Material and methods

Plant material and pollen description

Anthers of *Eucalyptus globulus* from the Coimbra Botanical Garden (Coimbra, Portugal) were used. The terminology for pollen description follows Punt et al. (2007).

Scanning Electron Microscopy (SEM)

Mature pollen grains were fixed in 2.5% buffered glutaraldehyde (see below), dehydrated in a graded ethanol series and critical point dried. Then, the grains were mounted on aluminium stubs and sputter coated with a 20 nm layer of gold-palladium prior to examination with a JEOL JSM-5400 at 15 kV.

Transmission Electron Microscopy (TEM)

Chemical fixation. Due to difficulties in preserving the pollen grain ultrastructure, different protocols were used for fixation of dehiscing anthers and isolated mature pollen: i) 2.5% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer (pH 6.8) supplemented with 1 mmol/l calcium chloride. After rinsing with the same buffer, samples were post-fixed in 1% buffered osmium tetroxide (OsO_4) and some were post-stained with 1% aqueous uranyl acetate for 1 h, in the dark, at room temperature; ii) 2.5% glutaraldehyde followed by post-fixation in a mixture of OsO_4 and potassium ferricyanide (OsFeCN; Hepler, 1981); iii) a mixture of 1% glutaraldehyde and 1% OsO_4 in 0.1 mol/l sodium cacodylate buffer as described by Follet-Gueye et al. (2003). Following fixation, the isolated pollen was concentrated and pre-embedded by centrifugation in 1.5% agar. All samples were further dehydrated in a graded ethanol series and embedded in either Spurr's resin or LR-white resin (London Resin, UK).

Freeze-fixation and freeze-substitution. Mature pollen was attached to agar-coated wire loops and freeze-fixed and freeze-substituted according to the procedure of Dinis et al. (2000). After substitution has been completed the samples were rinsed in pure acetone and embedded in Spurr's resin.

For all fixations, sections were cut on a LKB Ultratome NOVA ultramicrotome equipped with a diamond knife, conventionally stained with uranyl acetate and lead citrate, and observed in a JEOL JEM-100 SX at 80 kV.

Cytochemistry

Insoluble polysaccharides were detected in 2 μm thick sections subjected to the periodic acid-Schiff (PAS) reaction. At the ultrastructural level, detection of neutral polysaccharides and other carbohydrates was performed according to the method of Thiéry (1967). For both tests, controls consisted of sections treated in the same way but without periodic acid oxidation. Acidic polysaccharides and glycoproteins were detected with 1% phosphotungstic acid (PTA) in 10% chromic acid (Farrigiana & Marinozzi, 1979), the control consisting of the same treatment but with pH 7.3. Proteins were detected in semithin sections stained with Coomassie brilliant blue, and unsaturated lipids were detected in 0.5 μm thick sections stained with Sudan black B (Bhandari, 1997). At the ultrastructural level, proteins were detected in sections subjected to enzymatic extraction with protease (Dinis et al., 2000). DNA was detected in fresh pollen permeabilized with the agent Triton X-100, which was added to the staining solution (1:10, v/v) consisting of 0.01% DAPI (4,6-diamino-2-phenylindole) in 0.1 mol/l sodium cacodylate buffer, pH 6.8; observations were made under ultraviolet irradiation in an epifluorescence microscope (Nikon Optiphot model XF-EF).

Results

The *Eucalyptus globulus* mature pollen is triangular in polar view (Figure 1A, C-inset) and elliptical in equatorial view (Figure 1B). It is radially symmetric, angulaperturate, 3-parasyncolporate, with a distinctive, slightly arcuate apocolpial field that has broken edges (Figure 1A). The apertures consist of a narrow ectocolpus that is expanded to the apocopial field, and a reasonably circular to lalongate endoporus (Figure 1A, B). The exine surface is rugulate in the centre of the mesocolpia and psilate towards their edges, except in the above mentioned broken edges (Figure 1A). In the apocolpial field it is psilate to microscabrate. Both the annulus and margo are thickened and psilate (Figure 1B). At this stage the

pollen grain is bicellular, the spindle-shaped GC being always very closely associated with the VC nucleus (Figure 1C, D).

Pollen wall

In cross-section the pollen wall shows an outer exine and an inner intine (Figure 1C). In the mesocolpia the exine consists of a thick endexine and a relatively massive ectexine that is composed of a thick tectum, a narrow granular infratectum with hardly distinguishable columellae, and an irregularly thin foot layer, often difficult to distinguish (Figure 2A, E). Depending on the mesocolpia area sectioned the tectum is either smooth (Figure 2B), with rare thin punctae, or verrucate-rugulate (Figures 2A, 4A). Towards the apocolpial field both the infratectum and the foot layer are extremely reduced and at the apocopial field only the thick homogeneous endexine is usually present (Figure 2B). On the other hand, the ectexine is thicker towards the apertural margins as a result of the increase in thickness of both the tectum and, particularly, the foot layer (Figure 2C, D, F, and G). Adjacent to the apertures, the infratectum is absent and the tectum is usually curved completely over the foot layer, interrupting this last layer (Figure 2C, D). The endexine becomes 2-layered towards the apertures (Figure 2D, E) with the outer layer being the homogeneous endexine referred above and the new inner layer being spongy to granulate. As both layers approach the apertures, the outer layer is gradually reduced and disappears completely whereas the inner layer becomes thicker, especially at the ectocolpi (Figure 2C). However, at the endoporal region the inner endexine is also reduced becoming irregularly interrupted, but remaining as a very thin layer at the endoporus (pore membrane; Figure 2D). This pore membrane is Thiéry-negative (Figure 2 D-inset) and shows a similar contrast to that of the exine in sections stained with Sudan black B (Figure 3C). Frequently, it is displaced during processing of the samples due to the partial extrusion of the oncus (e.g. Figure 2G).

The intine is thin and apparently unlayered (Figure 2A, B), except under the apertural regions where prominent lenticular onci are formed (Figure 3). In the interapertural regions the intine is heavily stained after the Thiéry test (Figure 2B) and the PAS reaction (Figure 3A). In each oncus three main intine layers are evident, which react distinctly to the cytochemical tests used. From the outer to the inner surface of the oncus one can distinguish: a relatively thick, electron-lucent layer reacting positively to the Thiéry test (Figure 2F) but negatively to the PTA staining (Figure 2G); a triangular-shaped layer containing numerous plasmatubules radially oriented, which react positively to the tests for proteins (Figure 3B) and the PTA staining (Figure 2G); a thin electron-lucent layer overlying the plasmalemma, which is strongly PAS- (Figure 3A) and Thiéry-positive (Figure 2F) but PTA-negative (Figure 2G). Amorphous material, supposed to be pollenkitt, was found on the exine surface (Figure 2B) and in the minute infratectal cavities, especially in the freeze-fixed pollen (not shown).

The vegetative cell

The vegetative cell (VC) nucleus is cup-shaped and in most sections embraced the GC (Figure 1C, D). In general, it is centrally located and has little internal differentiation.

The VC cytoplasm is dense, with numerous free ribosomes and many, not randomly distributed, organelles (Figure 1C). The mitochondria are abundant and most of them are grouped at the periphery of the cell, although a few were seen lining the VC nucleus (Figure 1D); they are spherical to ovoid and have well-developed cristae. The plastids are in a subsequent layer and contain scarce internal membranes (Figure 5D); they are larger, less electron-dense and less numerous than the mitochondria (Figure 1C). Small starch grains were rarely observed in these proplastids as confirmed by the Thiéry test and the PAS reaction (Figure 3A). The latter produced a positive reaction in the cytosol, mainly at the central region of the VC. Dictyosomes were not apparent, but a number of PTA- and Thiéry-positive small vesicles, of presumably Golgi origin, are present in the cytoplasm (Figure 2G). Sudan black staining showed the lipid bodies to be relatively abundant and located mainly at the central region of the VC (Figure 3C). Under the TEM the lipid bodies show a distinct appearance depending on the fixation technique used. In chemically fixed pollen they are either electron-dense or electron-lucent droplets of variable number and size (Figure 4A-C). In the freeze-fixed pollen they are extracted thus appearing as small clear areas in the cytoplasm. Noteworthy, each lipid body is completely surrounded by a RER cistern (Figure 4A), the ribosomes of which are tightly pressed against the lipid body surface (Figure 4B, C).

The storage vacuoles are located at the central area of the VC cytoplasm (Figure 1C) and also have a different appearance depending on the fixation technique used. In chemically fixed pollen they are roundish in profile and have either their content intact or this is partially or totally extracted, thus presenting heterogeneous electron-density (Figures 1C, 4D). In

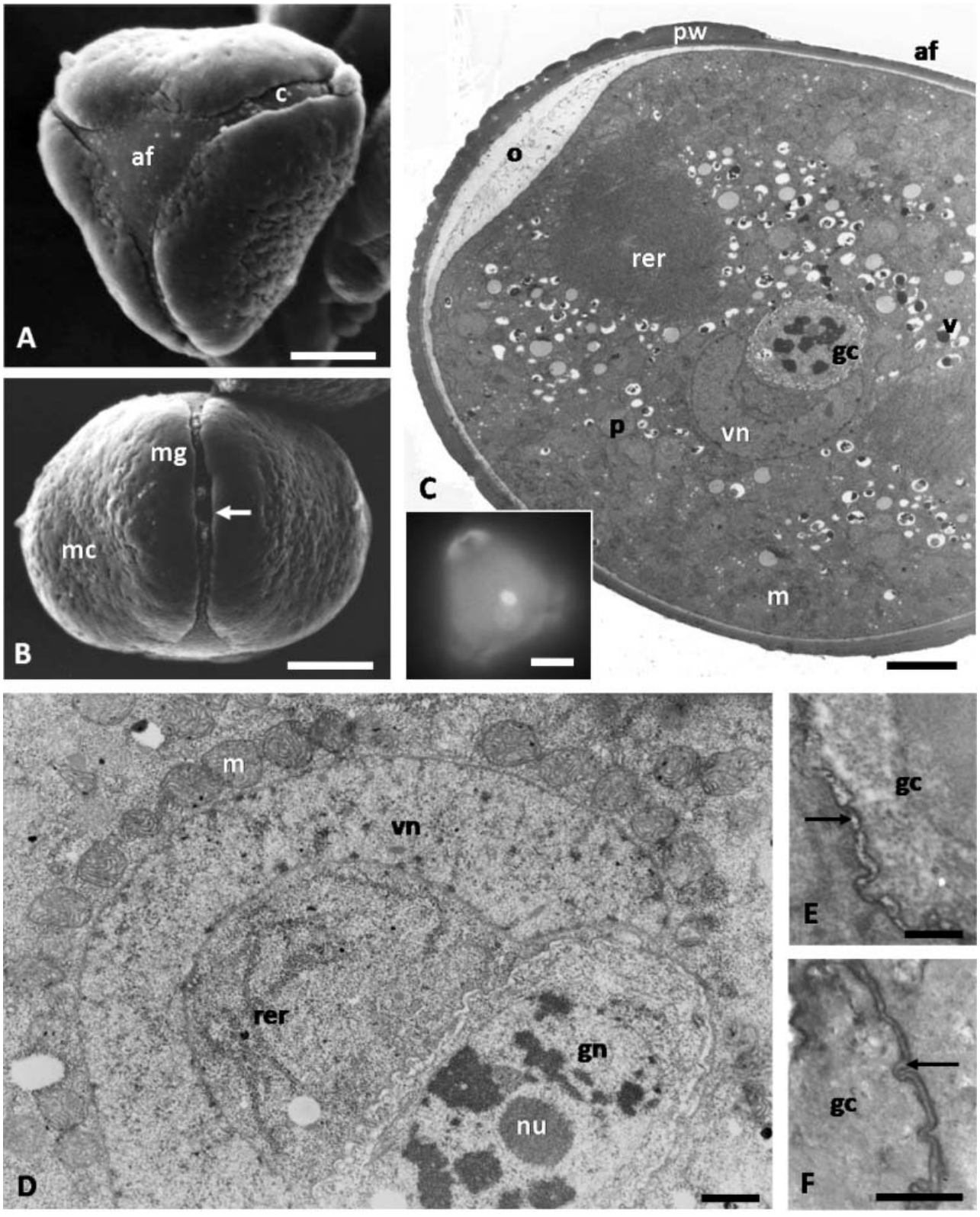

Figure 1. **A-F**. *Eucalyptus globulus* pollen: **A**, **B**. SEM; **C-F**. TEM. **A**. Polar view showing colpi (*c*) fusion and the resultant apocolpial field (*af*). **B**. Equatorial view showing the pore (*arrow*), the thickened and psilate margo (*mg*), and the rugulate mesocolpia (*mc*). **C**. General view showing the close association of the generative cell (*gc*) with the vegetative nucleus (*vn*). RER stacks (*rer*), proplastids (*p*), mitochondria (*m*), and storage vacuoles (*v*) are seen in the VC cytoplasm. The pollen wall (*pw*) is thinner at the apocolpial field (*af*) than at the apertural regions, where prominent lenticular onci (*o*) are formed. **Inset**: DAPI staining showing the highly fluorescent GC nucleus and the less fluorescent VC nucleus. **D**. Detail of the generative cell-vegetative nucleus (*vn*) association. Note the prominent nucleolus (*nu*) within the generative nucleus (*gn*) and mitochondria (*m*) lining the vegetative nucleus (*vn*). **E**. Detail of the generative cell (*gc*) boundary (*arrow*) after

freeze-fixed pollen they are oval in profile and appear uniformly filled with highly electron-dense material (Figure 4E, F). Connections between vacuoles were sometimes seen and each vacuole was usually partially surrounded by one or more RER cisternae (Figure 4D-F). Following the enzymatic extraction with protease the content of these vacuoles was digested (Figure 4G) contrary to the situation in the control experiment (Figure 4H). Another kind of endomembrane compartment identified only in the freeze-fixed pollen is that consisting of vesicular and elongated profiles with a less electron-dense content than that of the storage vacuoles, and a negatively contrasted membrane (Figure 4E, F). Most of these profiles were placed in the vicinity of the RER stacks and among the storage vacuoles. A dark roundish inclusion was frequently seen in most of these membrane profiles (Figure 4F).

The most striking feature of the VC cytoplasm is the extremely well-developed RER. Most of the RER cisternae are densely coated with ribosomes and arranged in huge stacks filling large areas of the central cytoplasm (Figures 1C, 5A). The latter are easily identified even with the LM (Figure 3). Each stack contains up to 80 cisternae and these either run parallel to each other or are concentrically arranged. The cisternae have a much reduced, moderately electron-dense lumen that is positively stained after the Thiéry test (Figure 5B). The control does not show any staining (Figure 5C). At the periphery of each stack, some cisternae are seen interconnected at branch points and with a swollen intracisternal space (Figure 5D, E). Short individual cisternae are also seen throughout the cytoplasm, especially among the mitochondria, these being better identified in samples treated according to Hepler (1981; Figure 4A). Besides a RER cistern underlying the VC plasmalemma, called the cortical ER (Hepler 1981; Figure 4A), others cisternae are closely associated with the above referred lipid bodies and storage vacuoles as well as with a few proplastids (Figure 5D). Irrespective of the fixation technique used, the RER membranes might appear positively or negatively contrasted (cf. Figure 5A with Figures 4E and 5E).

The generative cell

The generative cell (GC) was generally hard to preserve irrespective of the fixation technique used. In LM the GC boundary is clearly identified in sections subjected to the PAS reaction (Figure 3A). At the ultrastructural level the GC is separated from the VC by the plasmalemma of the two cells, which appear deeply undulated and delimiting an electron-transparent periplasmic space (Figure 1D). Both membranes, as well as the sparse granular material contained within the periplasmic space, stained positively after the Thiéry test (Figure 1E) and the PTA staining (Figure 1F). In cross-section, the GC appears roundish (Figure 1C) or spindle-shaped (Figure 3A) and most of it is occupied by a spherical to ellipsoidal nucleus containing dense masses of chromatin and a relatively prominent nucleolus (Figure 1D). The cytoplasm is relatively reduced and contains only a few organelles, including mitochondria, lipid bodies, ribosomes and occasional storage dense vacuoles similar to those of the VC. Plastids were not observed in the GC. Single RER cisternae and a few microtubules were occasionally seen, especially at the elongated portions of the GC (Figure 1D).

Discussion

The *Eucalyptus globulus* mature pollen proved to be difficult to process for electron microscopy. This is largely due to the low permeability of the pollen grain resulting from the presence of a relatively thick massive pollen wall, having specialized onci (see also Heslop-Harrison & Heslop-Harrison, 1985), and a high cytoplasmic density. The latter is attributed to the low pollen water content and the high amount of reserve material, especially insoluble carbohydrates in the cytosol, which contribute to conserve the pollen viability over time (Pacini et al., 2006). Technical difficulties in preserving the pollen grain ultrastructure are likely a reason for the scarcity of studies in this field of research within the Myrtaceae. So far, the present work is the first dealing with the cellular organization of the pollen in a species of *Eucalyptus* and even within the Myrtaceae.

The *Eucalyptus globulus* pollen morphology is typically myrtaceous (cf. Gadek & Martin, 1981, 1982; Patel et al., 1984; Zhou & Heusser, 1996). However, the pollen characteristics of *Eucalyptus globulus* do not fit perfectly into any of the pollen types of *Eucalyptus* defined by Pickett and Newsome (1997). The triangular amb with straight edges, the slightly arched apocolpial field with broken edges, the thickened margo and the surface patterns of the exine (rugulate at the centre of the mesocolpia, microscabrate at the apocolpial field, and psilate at

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the Thiéry test. **F**. Detail of the generative cell (*gc*) boundary (*arrow*) after the PTA staining. Scale bars −5 μm (A, B); 2 μm (C); 15 μm (C inset); 0.5 μm (D); 0.25 μm (E, F).

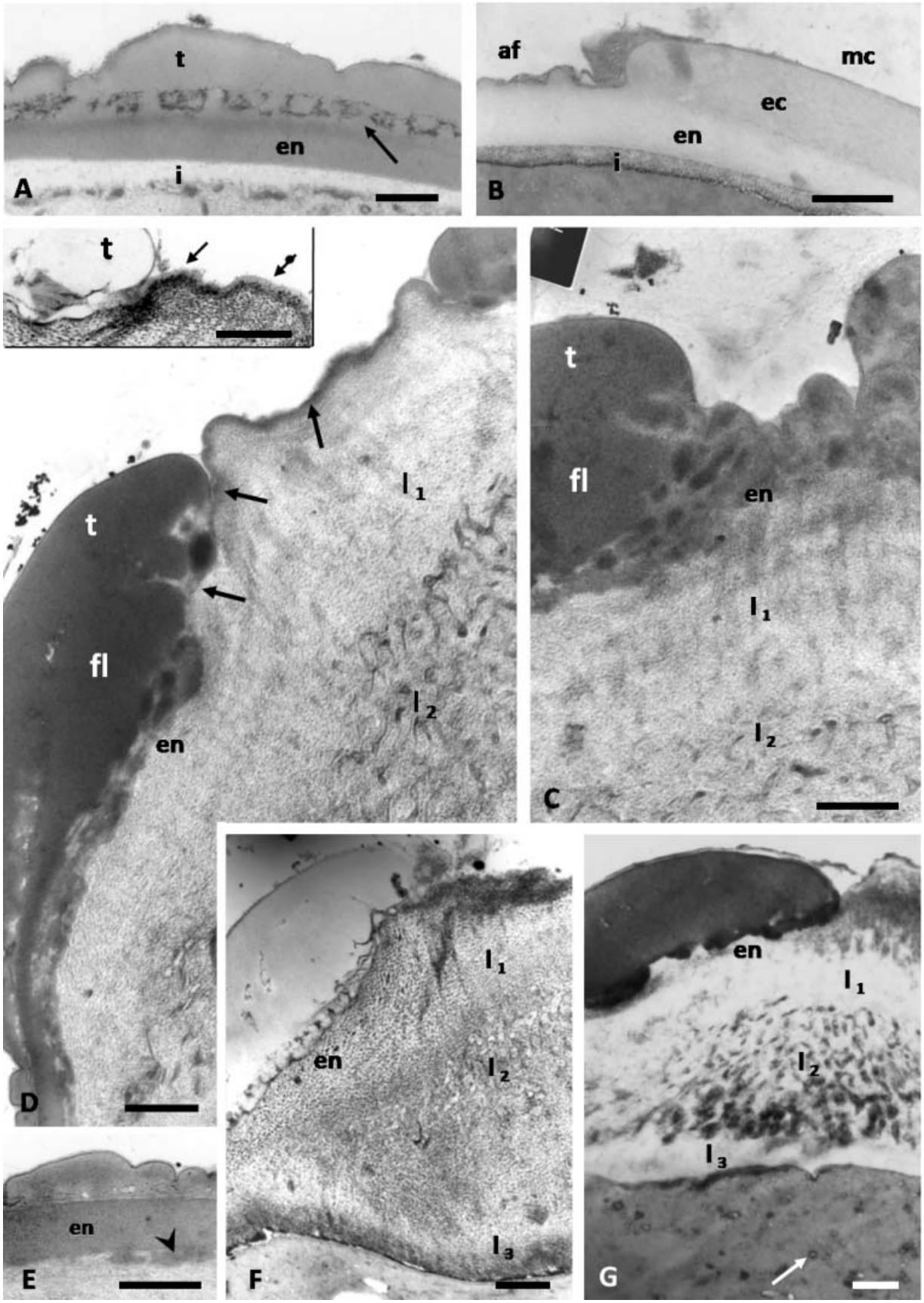

Figure 2. **A-G**. TEM micrographs of *Eucalyptus globulus* pollen grains showing details of the pollen wall. **A**. Section at mesocolpium showing the relatively thin intine (*i*), the thick tectum (*t*) and endexine (*en*), and the irregularly thin foot layer (*arrow*) beneath the

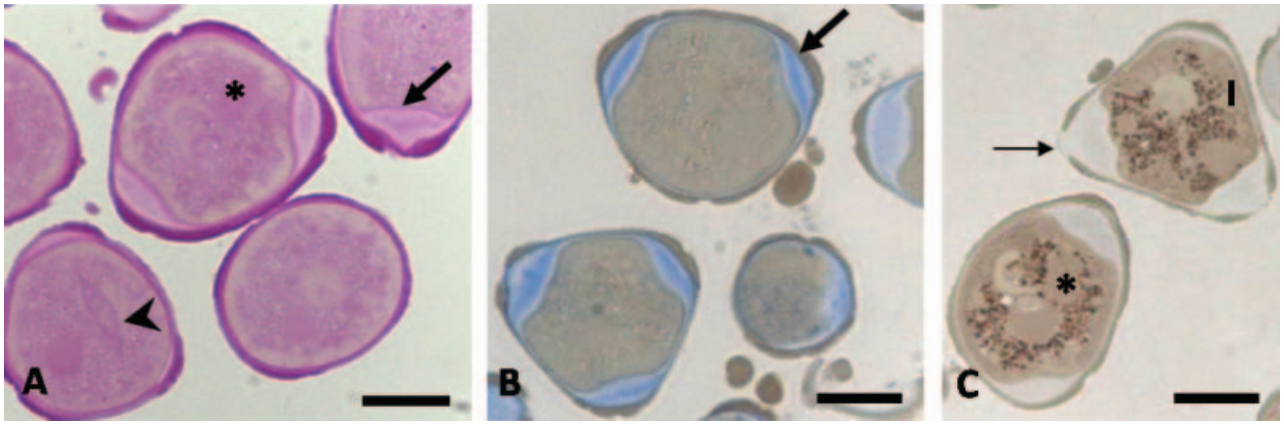

Figure 3. **A-C**. Light micrographs of *Eucalyptus globulus* pollen grains. **A**. PAS reaction. **B**. Coomassie blue staining. **C**. Sudan black B staining. *Thick arrows*=oncus; *thin arrow*=pore membrane; *l*=lipid bodies; *arrowhead*=GC outline; *asterisks*=RER stacks. Scale bars −10 μm (A, B, C).

the margo) are all pollen characteristics that distinguish *Eucalyptus globulus* from other *Eucalyptus* species. As to the pollen wall structure, it does not differ significantly from that of *Eucalyptus phoenicea* (Gadek & Martin, 1982) and *Eucalyptus rhodantha* (Heslop-Harrison & Heslop-Harrison, 1985). The differences detected are most likely related to the different methods used to process and examine the pollen grains. Using LM, Heslop-Harrison & Heslop-Harrison (1985) found a 2-layered intine in the non-apertural regions of *Eucalyptus rhodantha*, the inner layer being cellulosic and the outer one pectic. Such stratification was not evident in *Eucalyptus globulus* pollen examined with the TEM, even though a stronger staining of the intine overlying the plasmalemma was produced after the Thiéry test that may indicate the presence of two intine strata. In *Eucalyptus globulus*, as in *Eucalyptus rhodantha*, the intine is 3-layered under the apertures forming complex onci. The outer layer is pectic and compact, and according to Heslop-Harrison & Heslop-Harrison (1985, 1991) is bordered by a cap or operculum that represents an extension of the foot-layer of the ectexine. The same was reported by Gadek and Martin (1982) who considered the remnant of the foot layer to form a colpus membrane. In this study we show that the inner spongy-granulate endexine, being a continuous layer under the colpus and the pore, is responsible for the formation of both colpus membrane and the thin pore membrane. As showed by Heslop-Harrison & Heslop-Harrison (1985), during the pollen hydration this cap (pore membrane) is disrupted by gelation of the outer pectic intine layer. In the dehydrated mature pollen it possibly contributes, together with the underlying intine layer, to the heat and desiccation pollen tolerance. In the intine middle layer, named protein zone by Heslop-Harrison & Heslop-Harrison (1985), glycoproteins, enzymes and other proteins are stored in labyrinthian tubules derived from plasmalemma evaginations, being expelled during pollen hydration. They have been implicated in the pollen tube emergence, the stigmatic papillae cuticle digestion, and the pollen-stigma recognition process (e.g. Heslop-Harrison & Heslop-Harrison, 1991; Saad-Limam et al., 2005).

The *Eucalyptus globulus* mature pollen possesses the so-called male germ unit (Mogensen, 1992), in which the GC is located in a cup-shaped depression of the VC nucleus, presenting a deeply undulated surface. This last feature, also reported in other taxa (e.g. Cresti et al., 1990; Luegmayr, 1993), reflects a highly specialized interface between the GC and VC that certainly has a significant physiological role, which is as yet unknown. The GC contains very few organelles and storage reserves and has no plastids, which points to the predominantly maternal inheritance of these organelles in *Eucalyptus globulus*. In the VC the plastids lack starch, which supports the prediction

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infratectum. OsFeCN fixation. **B**. Section at the apocolpial field (*af*) -mesocolpium (*mc*) transition showing the stained intine (*i*) and the unstained endexine (*en*) and ectexine (*ec*). Thiéry test. **C**. Section at an endocolpal region showing the thick tectum (*t*) and foot layer (*fl*), the spongy-granulate endexine (*en*) and the two outer intine layers (l_1, l_2) of the oncus. **D**. Section at an endoporal region showing the thick tectum (*t*) and foot layer (*fl*), the spongy-granulate endexine (*en*) and the two outer intine layers (l_1, l_2) of the oncus. The *arrows* point to the endexinous pore membrane. **Inset**: pore membrane (*arrows*) after the Thiéry test. **E**. Section at a mesocolpal region close to an aperture (to the right of figure) showing the homogeneous endexine (*en*) and the beginnings of the spongy-granulate endexine (*arrowhead*). **F**. Oncus after the Thiéry test. **G**. Oncus after the PTA staining (Golgi vesicles – *arrow*). Scale bars −0.5 μm (A - G).

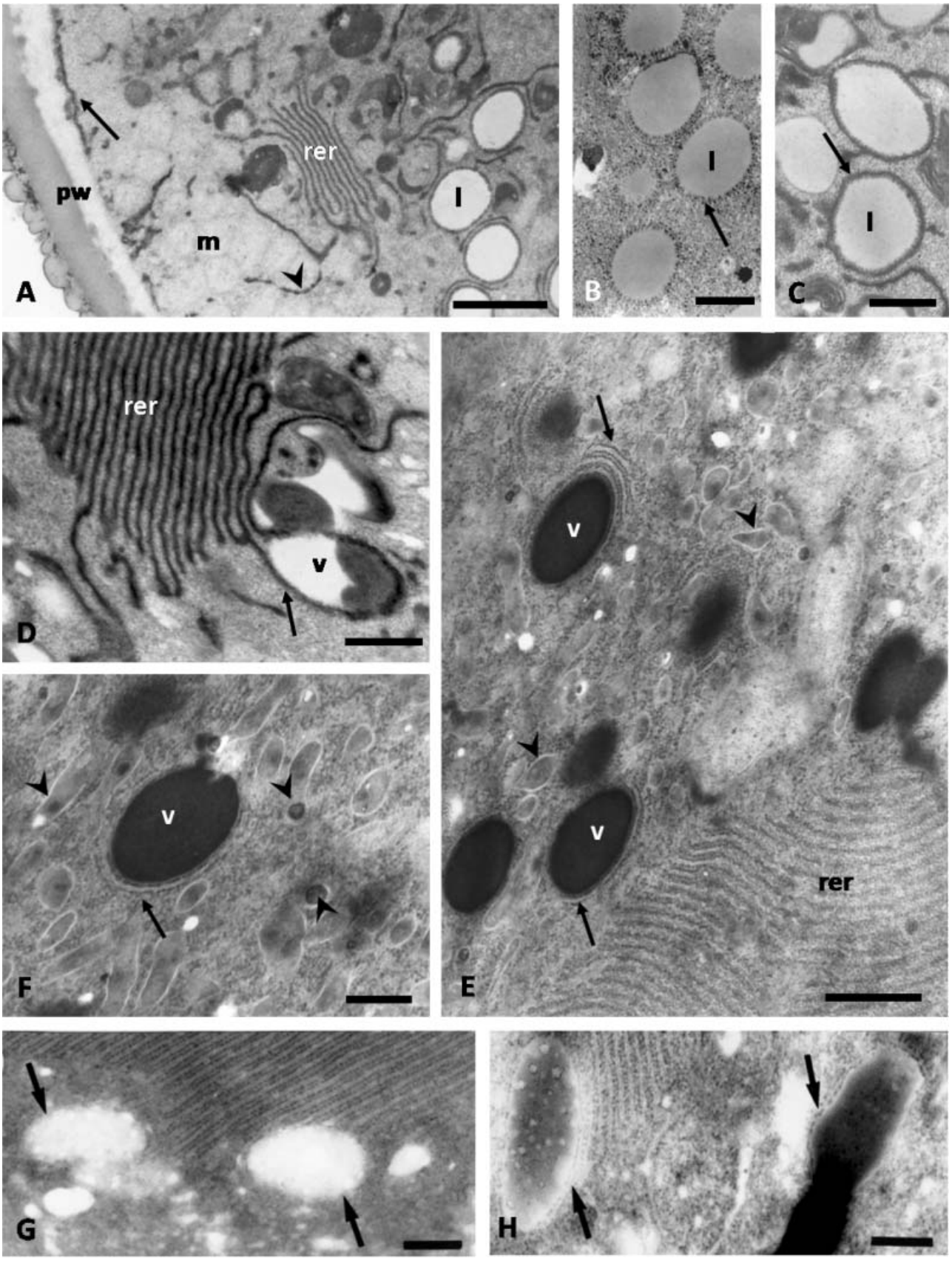

Figure 4. **A-H**. TEM micrographs of *Eucalyptus globulus* pollen grains showing details of the VC cytoplasm. **A-D**. OsFeCN fixation. **E, F**. Freeze-fixation. **G, H**. Glut-OsO_4 fixation. **A**. Periphery of a pollen grain showing aggregation of mitochondria (*m*), the cortical ER (*arrow*),

(Baker & Baker , 1979) that entomophilous species, like eucalypts, have starchless pollen usually of small size. However, this does not mean that *Eucalyptus globulus* pollen has no saccharide reserves. Our study shows abundant insoluble polysaccharides, probably low weight molecules, scattered in the VC cytoplasm. These reserves can be mobilised and interconverted in relation to environmental conditions, which is important to control the internal turgor pressure of the pollen and to maintain its water content within certain limits (Pacini et al., 2006). This water balance is crucial for longer viability of the pollen and higher resistance to environmental stress. Other representative storage reserves in *Eucalyptus globulus* pollen are lipid bodies, which are also characteristic of entomophilous species (Baker & Baker, 1979) and may contribute to the desiccation tolerance of the pollen. Lipids are a convenient form of storing energy and their mobilization originates products that are used as building blocks for membrane formation or as cellular messengers (Piffanelli et al., 1998; Athenstaedt & Daum, 2006). As discussed below, proteins are reserve substances in *Eucalyptus globulus* mature pollen that must also be taken into consideration.

The most characteristic feature of the *Eucalyptus globulus* mature pollen is the extremely well-developed RER. Extensive RER stacks, like those in *Eucalyptus globulus*, have been reported in the pollen of several species of the Scrophulariaceae (Jensen et al., 1974) and of many other unrelated families (e.g. Cresti et al., 1975, 1985, 1988; Van Aelst & Van Went, 1991; Luegmayr, 1993). These RER stacks have been correlated with the arrest of metabolic activities in the mature pollen, since during pollen activation the metabolism in the VC resumes and the stacked cisternae becomes free in the cytoplasm (Jensen et al., 1974; Cresti et al., 1975, 1985; Ciampolini et al., 1988). Similarly to the dry mature seeds (Bergfeld & Schopfer, 1984), the RER stacks in the mature pollen are likely induced by the protoplasmic dehydration, which leads to metabolic arrest. The different water content in the distinct mature pollen may thus be the answer for why some pollen grains contain such extensive stacked RER whereas others do not. These RER stacks constitute large protein-synthesizing machinery in the mature pollen, which remains available for the subsequent pollen germination and tube growth.

According to some authors (e.g. Jensen et al., 1974; Cresti et al., 1990; Hess, 1995) the stacked cisternae may also represent a storage site of nutrient materials. In *Eucalyptus globulus* the lumen of the stacked cisternae was positively stained after the Thiéry test indicating the presence of abundant neutral polysaccharides and other carbohydrates (Thiéry, 1967). However, the storage of saccharides in stacked cisternae has not been reported, except for cotton pollen in which ER pockets were considered to store proteins and possibly carbohydrates (Fisher et al., 1968). The RER is the site of protein synthesis and where most of them initiate glycosylation. An interesting hypothesis is that the referred stained materials are highly glycosylated proteins (probably gametophytic glycoproteins or most likely their precursors) involved in the pollen-pistil recognition and interaction. It is known that many of these glycoproteins are allergens and some of them were already immunolocalized in the lumen of RER cisternae of *Zygophyllum fabago* (Castells et al., 2002) and several species of Oleaceae (Rodríguez-García et al., 1995; Alché et al., 2002). The same may be true for *Eucalyptus globulus* pollen since it is also considered as a potential cause of respiratory allergic diseases (Galdi et al., 2003).

Besides the stacked RER, many single cisternae are scattered throughout the VC cytoplasm establishing a preferential close association with other cell components. These intimate associations merit further attention, especially those of the RER with lipid bodies and storage vacuoles, because of their physiological meaning. Single lipid bodies encircled completely or partially by RER cisternae have been reported in the mature pollen of several species (e.g. Jensen et al., 1974; Cresti et al., 1975, 1988, 1990; Ciampolini et al., 1988; Van Aelst & Van Went, 1991; Van Aelst et al., 1993) but the physiological role of this association is still controversial. It has been proposed that the RER cistern encircling each lipid body acts as a physical barrier preventing the aggregation and coalescence of lipid bodies (Frandsen et al., 2001; Murphy, 2001). This hypothesis has been put forward as a result of the

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and a small RER stack (*rer*). Short individual cisternae (*arrowhead*) are seen throughout the cytoplasm and surrounding lipid bodies (*l*). **B**, **C**. Intimate association of lipid bodies (*l*) with RER cisternae (*arrow*). The typical RER staining is absent in **B**. **D**. RER stack (*rer*) with one cistern intimately associated (*arrow*) with a storage vacuole (*v*). **E**. Storage vacuoles (*v*) in the vicinity of a RER stack (*rer*), some of which are surrounded by a single RER cistern (*arrows*). Note the vesicular and elongated profiles (*arrowheads*) with a negatively stained membrane and a less electron-dense content than that of the storage vacuoles. **F**. Detail of the negatively stained membrane profiles, some of which contain a dark roundish inclusion (*arrowheads*). A storage vacuole (*v*) is surrounded by a RER cistern (*arrow*). **G**. Storage vacuoles (*arrows*) after the enzymatic digestion with protease. **H**. Control to the enzymatic digestion with protease showing electron-dense storage vacuoles (*arrows*). Scale bars −1 μm (A); 0.5 μm (B - E); 0.25 μm (F, G, H).

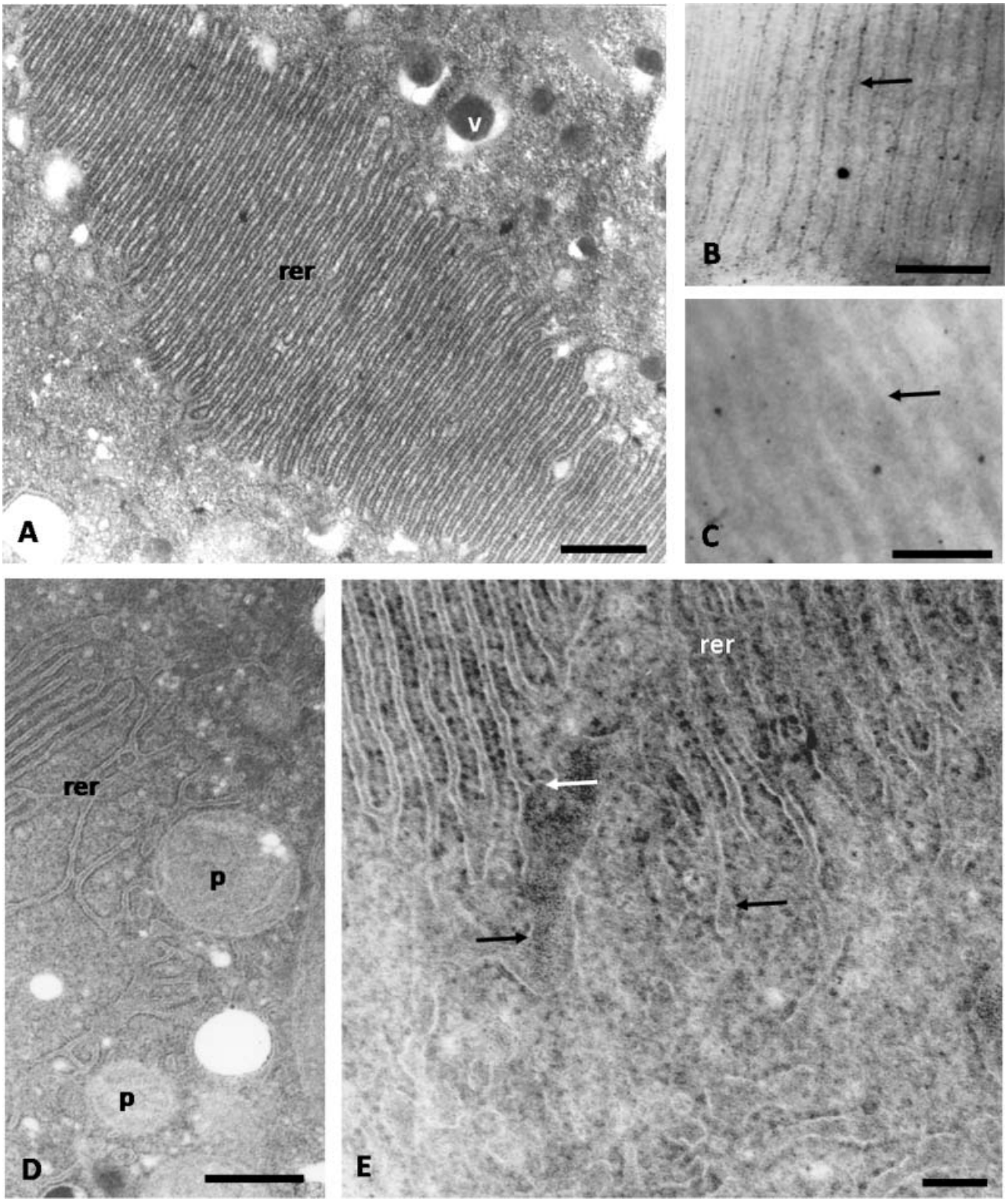

Figure 5. **A-E**. TEM micrographs of *Eucalyptys globulus* pollen grains showing details of the RER in the VC. Chemical fixations. **A**. Higher magnification of a RER stack (*rer*). **B**. RER cisternae with the lumina positively stained (*arrow*) after the Thiéry test. **C**. Control to the Thiéry test showing unstained RER cisternae (*arrow*). **D**. RER network closely associated with proplastids (*p*). **E**. Periphery of a RER stack (*rer*) showing cisternae interconnected at branch points and with swollen intracisternal spaces (*arrows*). Note the negatively contrasted membranes. Scale bars −0.5 μm (A, D); 0.25 μm (B, C, E).

absence of oleosins in pollen grains (Piffanelli et al., 1998) contrary to the situation in seeds (Hsieh & Huang, 2004). However, genes encoding oleosins on the storage lipid bodies of *Arabidopsis* pollen were recently described (Kim et al., 2002), which contradicts the above proposal. Possibly, the RER-lipid bodies association represents a mechanism by which the storage lipid bodies acquire the oleosin coat. The presence of ribosomes on the cistern membrane tightly pressed to the lipid body surface supports this hypothesis. As the RER-lipid bodies association persists throughout pollen release, it may also facilitate the direct mobilisation of lipid bodies, required upon pollen germination, by the action of lipases and hydrolases (Murphy, 2001). Oleosins have been assumed to act as docking and (or) activating proteins for these newly synthesized lipases and hydrolases (Athenstaedt & Daum, 2006).

So far, the RER-storage vacuoles association found in *Eucalyptus globulus* has not been reported in other pollen. These vacuoles closely resemble the protein bodies in *Ledebouria* pollen (Hess, 1995) and the protein storage vacuoles/reticulum in *Michelia figo* (Dinis et al., 2000). As also showed by Dinis et al. (2000), their appearance is different following chemical fixation and freeze-fixation. This is due to the total or partial extraction of the vacuole content during the chemical fixation, since the proteins in the storage vacuoles are alcohol soluble and therefore are dissolved during dehydration. The enzymatic digestion with protease indicates that the storage vacuoles in *Eucalyptus globulus* mature pollen consist mostly of proteins, too. We use the term protein storage vacuole instead of protein bodies (Hess, 1995) following the recent distinction established between the two structures (Herman & Larkins, 1999). Due to technical procedures in preparing the pollen grains, protein reserves have not usually been considered in the mature pollen (see e.g. Nepi & Franchi, 2000). However, a few studies show that they are a class of important storage reserves in pollen (Hess, 1995; Dinis et al., 2000), which likely provide building blocks for rapid growth upon germination. The RER-storage vacuoles association in *Eucalyptus globulus* may facilitate the transfer of products (namely proteins) synthesized in the RER to the vacuoles or the mobilization of the storage reserves during the pollen germination and tube growth.

The endomembrane compartment found only in the freeze-fixed pollen is probably an intermediate or extra site for the storage of reserve material. Its preservation occurring only in the freeze-fixed pollen indicates that it is a delicate structure, which likely represents an anastomosing endomembrane compartment due to the polymorphism and disposition of its membrane profiles and the proximity of the profiles in relation to each other. Also, the close proximity between the membrane profiles and the swollen cisternae in the periphery of each RER stack suggests that the former derive from the RER. However, the profiles were bounded by a negatively contrasted membrane that lacked ribosomes. Possibly, this is an ER subdomain that lost the ribosomes and actually stores proteins. As is known, all storage proteins are initially synthesised on the RER and remain either in the ER or are transported through the endomembrane system to distal sites (Herman & Larkins, 1999). This endomembrane compartment is distinct from the above referred protein storage vacuoles, being closer to the protein bodies. This assumption, as well as the presumable phytic nature of the dark inclusion found in most of the membranous profiles, needs further confirmation.

Conclusions

The *Eucalyptus globulus* mature pollen proved to be difficult to process for electron microscopy, several adjustments having to be made to the conventional techniques. This is largely due to the pollen wall characteristics, which are most likely related to the environmental conditions under which this species grows. Also, pollen organization and features, especially those concerning storage reserves, most likely contribute to longer viability of the pollen and higher resistance to environmental stress. Many studies have revealed the involvement of hydration and cytoplasmic reserves in pollen viability, germinability and vigour (reviewed by Pacini et al., 2006). According to Heslop-Harrison & Heslop-Harrison (1985, p. 155) ''such an ability (of *Eucalyptus* pollen) to resist high temperatures must reflect primarily special properties of the protoplast of the VC''. Thus, it seems that the objective of such properties is to guarantee the pollen viability in time and sufficient vigour for rapid germination. The high development of the RER and its intimate association with other cell components are striking characteristics of the VC cytoplasm that merit further attention. Future studies should focus on the RER behaviour during pollen maturation and germination to better understand its dynamics and the physiological meaning of its close association with other cell components.

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References

Alché, J., Castro, A. & Rodríguez-García, M. (2002). Localization of transcripts corresponding to the major allergen from olive pollen (*Ole e* I) by electron microscopic non-reactive in situ RT-PCR. *Micron*, *33*, 33–37.

Athenstaedt, K. & Daum, G. (2006). The life cycle of neutral lipids: Synthesis, storage and degradation. *Cell. Molec. Life Sci.*, *63*, 1355–1369.

Baker, H. & Baker, I. (1979). Starch in angiosperm pollen grains and its evolutionary significance. *Am. J. Bot.*, *66*, 591–600.

Bhandari, N. (1997). *Staining techniques – a manual*. New Delhi: Ultimate Prints.

Bergfeld, R. & Schopfer, P. (1984). Transitory development of rough endoplasmic reticulum aggregates during embryo maturation in seeds of mustard (*Sinapis alba* L.). *Eur. J. Cell Biol.*, *34*, 27–33.

Castells, T., Arcalís, E., Morano-Grau, S., Bayo, J., Elvira-Rendueles, B., Belchí, J., Seoane-Camba, J. & Suárez-Cervera, M. (2002). Immunocytochemical localization of allergenic proteins from mature to activated *Zygophylum fabago* L. (Zygophyllaceae) pollen grains. *Eur. J. Cell Biol.*, *81*, 107–115.

Ciampolini, F., Moscatelli, A. & Cresti, M. (1988). Ultrastructural features of *Aloe ciliaris* pollen: I. Mature grain and its activation in vitro. *Sex. Pl. Reprod.*, *1*, 88–96.

Cresti, M., Pacini, E., Sarfatti, G. & Simoncioli, C. (1975). Ultrastructural features and storage function of *Lycopersicum peruvianum* pollen. In D. Mulcahy (Ed.), *Gamete competition in plants and animals* (pp. 19–28). Amsterdam: North Holland Publ. Co.

Cresti, M., Mulcahy, D. & Mulcahy, G. (1985). Ultrastructure of *Nicotiana alata* pollen, its germination and early tube formation. *Am. J. Bot.*, *72*, 719–727.

Cresti, M., Milanesi, C., Tiezzi, A., Ciampolini, F. & Moscatelli, A. (1988). Ultrastructure of *Linaria vulgaris* pollen grains. *Acta Bot. Neerl.*, *37*, 379–386.

Cresti, M., Milanesi, C., Salvatici, P. & Van Aelst, A. (1990). Ultrastructural observations of *Papaver rhoeas* mature pollen grains. *Bot. Acta*, *103*, 349–354.

Dinis, A., Santos Dias, J. & Mesquita, J. (2000). Ultrastructure of the mature pollen of *Michelia figo* (Lour.) Spreng. (Magnoliaceae). *J. Submicrosc. Cytol. Pathol.*, *32*, 591–601.

Direcção-Geral dos Recursos Florestais (DGRF). (2006). *Inventário florestal nacional (2005-2006) – resultados preliminares Julho 2006*. Lisboa: Min. Agric. Desenvol. Rur. Pesc. *www.dgrf.min-agricultura.pt*.

Farrigiana, T. & Marinozzi, V. (1979). Phosphotungstic acid staining of polysaccharides containing structures on epoxy embedded tissues. *J. Submicrosc. Cytol.*, *11*, 263–265.

Fisher, D., Jensen, W. & Ashton, M. (1968). Histochemic studies of pollen: Storage pockets in the endoplasmic reticulum (ER). *Histochem. J.*, *13*, 169–182.

Follet-Gueye, M., Pagny, S., Faye, L., Gomord, V. & Driouich, A. (2003). An improved chemical fixation method suitable for immunogold localization of green fluorescent protein in the golgi apparatus of tobacco bright yellow (BY-2) cells. *J. Histochem. Cytochem.*, *51*, 931–940.

Frandsen, G., Mundy, J. & Tzen, J. (2001). Oil bodies and their associated proteins, oleosin and caleosin. *Pl. Physiol.*, *112*, 301–307.

Gadek, P. & Martin, H. (1981). Pollen morphology in the subtribe Metrosiderinae of the Leptospermoideae (Myrtaceae) and its taxonomic significance. *Aust. J. Bot.*, *29*, 159–184.

Gadek, P. & Martin, H. (1982). Exine ultrastructure of Myrtaceous pollen. *Aust. J. Bot.*, *30*, 75–86.

Galdi, E., Perfetti, L., Calcagno, G., Marcotulli, M. C. & Moscato, G. (2003). Exacerbation of asthma related to *Eucalyptus* pollens and to herb infusion containing *Eucalyptus*. *Monaldi Arch. Chest Dis.*, *59*, 220–221.

Hepler, P. (1981). The structure of the endoplasmic reticulum revealed by osmium tetroxide-potassium ferricyanide staining. *Eur. J. Cell Biol.*, *26*, 102–110.

Herman, E. & Larkins, B. (1999). Protein storage bodies and vacuoles. *Pl. Cell*, *11*, 601–613.

Heslop-Harrison, J. & Heslop-Harrison, Y. (1985). Germination of stress-tolerant *Eucalyptus* pollen. *J. Cell Sci.*, *73*, 135–157.

Heslop-Harrison, J. & Heslop-Harrison, Y. (1991). Structural and functional variation in pollen intines. In S. Blackmore & S. H Barnes (Eds.), *Pollen and spores: Patterns of diversification* (pp. 331–343). Oxford: Clarendon Press.

Hess, M. (1995). High-pressure freeze fixation reveals novel features during ontogenesis of the vegetative cell in *Ledebouria* pollen: An ultrastructural and cytochemical study. *Biochem. Cell Biol.*, *73*, 1–10.

Hsieh, K. & Huang, A. (2004). Endoplasmic reticulum, oleosins, and oils in seeds and tapetum cells. *Pl. Physiol.*, *136*, 3427–3434.

Jensen, W., Ashton, M. & Heckard, L. (1974). Ultrastructural studies of the pollen of subtribe Castilleiinae, subfamily Scrophulariaceae. *Bot. Gaz. (Chicago)*, *135*, 210–218.

Kim, H., Hsieh, K., Ratnayake, C. & Huang, A. (2002). A novel group of oleosins is present inside the pollen of *Arabidopsis*. *J. Biol. Chem.*, *277*, 22677–22684.

Luegmayr, E. (1993). The generative cell and its close association with the endoplasmic reticulum of the vegetative cell in pollen of *Cyrtandra pendula* (Gesneriaceae). *Protoplasma*, *177*, 73–81.

Mogensen, H. L. (1992). The male germ unit: Concept, composition, and significance. *Int. Rev. Cytol.*, *140*, 129–147.

Murphy, D. (2001). The biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Progr. Lipid Res.*, *40*, 325–438.

Nepi, M. & Franchi, G. (2000). Cytochemistry of mature angiosperm pollen grain. *Pl. Syst. Evol.*, *222*, 45–62.

Pacini, E., Guarnieri, M. & Nepi, M. (2006). Pollen carbohydrates and water content during development, presentation and dispersal: A short review. *Protoplasma*, *228*, 73–77.

Patel, V. C., Skvarla, J. J. & Raven, P. H. (1984). Pollen characters in relation to the delimitation of Myrtales. *Ann. Mo. Bot. Gard.*, *71*, 858–969.

Patterson, B., Gore, P., Potts, B. & Vaillancourt, R. (2004). Advances in pollination techniques for large-scale seed production in *Eucalyptus globulus*. *Aust. J. Bot.*, *52*, 781–788.

Pickett, E. & Newsome, J. (1997). *Eucalyptus* (Myrtaceae) pollen and its potential role in investigations of Holocene environments in southwestern Australia. *Rev. Palaeobot. Palynol.*, *98*, 187–205.

Piffanelli, P., Ross, J. & Murphy, D. (1998). Biogenesis and function of the lipidic structures of pollen grains. *Sex. Pl. Reprod.*, *11*, 65–80.

Punt, W., Hoen, P. P., Blackmore, S., Nilsson, S. & Le Thomas, A. (2007). Glossary of pollen and spore terminology. *Rev. Palaeobot. Palynol.*, *143*, 1–81.

Rodríguez-García, M., Fernández, M., Alché, J. & Olmedilla, A. (1995). Endoplasmic reticulum as a storage site for allergenic proteins in pollen grains of several Oleaceae. *Protoplasma*, *187*, 111–116.

Saad-Limam, S., Nabli, M. & Rowley, J. (2005). Pollen wall ultrastructure and ontogeny in *Heliotropium europaeum* L. (Boraginaceae). *Rev. Palaeobot. Palynol.*, *133*, 135–149.

Thiéry, J. (1967). Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. *J. Microsc.*, *6*, 987–1018.

Van Aelst, A. & Van Went, J. (1991). The ultrastructure of mature *Papaver dubium* L. pollen grains. *Acta Bot. Neerl.*, *40*, 319–328.

Van Aelst, A., Pierson, E., Van Went, J. & Cresti, M. (1993). Ultrastructural changes of *Arabidopsis thaliana* pollen during final maturation and rehydration. *Zygote*, *1*, 173–179.

Zhou, M. & Heusser, C. (1996). Late-glacial palynology of the Myrtaceae of southern Chile. *Rev. Palaeobot. Palynol.*, *91*, 283–315.