Ontogeny of the orbicules in *Ilex paraguariensis* A. St. Hil. (Aquifoliaceae)

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Short Title: Ontogeny of the orbicules in Ilex paraguariensis

Abstract

In this work is described the ontogeny of the orbicules in the anthers of *Ilex paraguariensis*. Anthers were fixed in glutaraldehyde and formaldehyde, post-fixed in osmium tetroxide and potassium ferricyanide, and embedded in Spurr's low viscosity resin. A modified Thiéry-reaction was used as standard protocol contrast. The ultrastructural analysis of different stages of the anther development reveals that the pro-orbicules are formed in the periplasmic space of inner tangential and radial walls of the tapetal cells, during the tetrad stage. The pro-orbicules have an electron transparent core surrouded by an electron dense orbicular wall. Later, after the dissolution of the tapetal cell walls, the orbicules grow in a fibrillar glicocalix in the tapetum surface, on plasma membrane depressions. The orbicules in *Ilex* are synthesized in the extracellular medium only and must be homologous to the pollen grain ektexine.

Key Words: orbicules, tapetum, *Ilex*, Aquifoliaceae, ultrastructure

Introduction

The orbicules, also called Ubisch bodies, are granules or particles, with a variable size and shape, formed by the secretory tapetum of the flowering plants (10). They are constituted by sporopollenin, the same biological polymer of the pollen grain exine. Their morphological differences are useful characters for systematics (11). On a sociologic aspect, the orbicules are a source of allergic manifestations, as seen in *Betula* (3). According to a recent revision (10), the orbicules could be involved in the transport of sporopollenin into the anther, in the lysis of the tapetal cells, in the pollen grain dispersion or, just to be a sub-product of the tapetal cellular metabolism. However, the opinion that the orbicules acts as an intermediate for sporopollenin transfer is seen like an absurd as well as the function as sporopollenin storage or surplus (9).

Ilex paraguariensis, the maté, is an Aquifoliaceae that has a secretory tapetum, which produces orbicules concurrently with the pollen wall development. The present work describes some ultrastructural aspects of the orbicular ontogeny in this species and their possible homology and relationship with the pollen wall ontogeny.

Materials and Methods

For transmission electron microscopy, intact anthers of *Ilex paraguariensis* were fixed in 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2 (14), at room temperature, for 24 hours, washed in buffer of same osmolarity and pH (3 washes of one hour each), and post-fixed in a mixture (1:1) of 2% OsO_4 and 0.8% phosphate-buffered $K_3Fe(CN)_6$, for 12 hours, at room temperature and in the darkness (24). After washed in distilled water, the material was dehydrated in a growing series of acetone (30, 50, 70, 90 and 100%), with the addition of 2% uranyl acetate in 70% acetone.

The dehydrated anthers were embedded in the Spurr's low viscosity resin (21), and polymerized inside gelatin capsules in oven at 70°C, for 18 hours. Ultrathin sections

were obtained in a Leica Ultracut UCT ultramicrotome, with a diamond knife.

A modified Thiéry-reaction was used as standard contrasting method (23, 25). Sections collected with a platinum ring were treated with 4% periodic acid (PA; H_5IO_6), for 10 minutes, 0.2% tiocarbohydrazine (TCH) in 20% acetic acid, for 30 minutes, and 1% silver proteinate (SP), for 30 minutes, in the darkness. After this contrast protocol, the sections were contrasted with a long life lead solution (Pb), for 5 minutes (7). Alternatively, ultrathin sections were contrasted in 25% alcoholic saturated solution of uranyl acetate (U), for 45 minutes, and lead solution, for 20 minutes.

Unsaturated lipids were detected using the modified Thiéry reaction (TCH-SP) without oxidation in periodic acid (16). For to detect acidic polysaccharides, the sections were treated with 1% phosphotungtic acid (PTA) in 10% chromic acid (14).

The ultrathin sections were examined in a Jeol JEM 1200 EX-II transmission electron microscope at 80 kV.

For light microscopy, semithin sections (0.35 μm) were mounted on glass slides, dried, and stained with Coomassie Blue R-250 (C.I. 42660), for the localization of total protein (20). The slides were examined in a Leitz Dialux 20 EB bright field microscopy.

Results

At the beginning of pollen development, in the tetrad stage, the cortical zone of the tapetal cells presents several organelles, like smooth and rough endoplasmic reticulum, dictyosomes, peroxisomes, and mitochondria (Fig. 1). In this stage, there is formation of a periplasmic space, between the plasma membrane and the inner or radial cell walls of the tapetum, which is filled by an electron transparent material (Fig. 1, star). In this narrow spaces are found small and spherical bodies, with an electron transparent core: the pro-orbicules (Figs. 1-6). The pro-orbicular core has around 100 nm of diameter (Fig. 3), and is enveloped by an amorphous and electron dense layer, with about 30 nm thickness (Figs. 3 and 4). This layer is continuous with another one that covers the outer surface of the plasma membrane (Figs. 3 and 4, asterisks). Endoplasmic reticulum, with an electron dense lumen, has a great proximity with the plasma membrane, in the zones where there are orbicular formation (Fig. 1).

The plasma membrane, under the pro-orbicules, has small invaginations (Figs. 3 and 4, large arrows), containing electron dense substances similar to one present in the pro-orbicular surface. Small and large secretory vesicles, with electron dense appearance, discharge their contents to the tapetal cell surface (Figs. 4 and 6, respectively). Sometimes, an electron dense 'trilamellar line' (black-white-black) is founded in the pro-orbicular core, and frequently split off the core and reach the orbicular wall (Figs. 3, arrow, and 4). Microtubules near to the plasma membrane show a preferential orientation (Fig 2, arrows). More developed pro-orbicules also have a trilamellar or 'membrane' line between the core surface and the outer electron dense layer (Fig. 6, arrow). Furthermore, the pro-orbicular layer is discontinuous and irregular. In general, the outer layer is thinner in the contact zone with the plasma membrane (Fig. 6).

Sometimes, some pro-orbicules has an 'anomalous' development. The orbicular wall forms a tubular growing, with 80 nm in diameter (Fig. 5). This tubule has a 10 nm electron transparent axis, and an electron dense layer, with a radial orientation.

After the full dissolution of the callose, the free microscopes stay in the locular fluid of the anther. The tapetum, without cell walls except in the outer periclinal face, shows a very large amount of smooth endoplasmic reticulum, spread in the cytoplasm (Fig. 7). Tubules of the smooth and rough endoplasmic reticule, which a high electron density, reach the plasma membrane and look like to fuse with it (Fig. 9, small arrow). The tapetal locular surface is covered by a thin and electron dense glicocalix with a protein (Fig. 8, arrow) and lipidic (Figs. 7 and 10, asterisks) composition, which can to involve partially the orbicules. This layer has continuity with the locular substances (Figs. 7 and 10).

The orbicules are found in plasma membrane depressions (Figs. 7, 9 and 10) and have an electron dense core, when contrasted with TCH-SP test (Fig. 7 and 10), with a diameter around 150 to 200 nm. A discontinuous and irregular orbicular wall, up to 200 nm of thickness covers the core. However, the core shows a direct contact with the locular fluid, in the places where the orbicular wall is absent (Fig. 9, large arrow). The same substances that are present in the tapetal glicocalix constitute a thin layer on the orbicular wall (Figs. 9 and 10, asterisks).

The orbicular wall reacts like the pollen grain ektexine under TCH-SP and PTA-chromic acid contrasting methods, and share an identical electron density (Figs. 10 and 11, respectively). The locular substance, also TCH-SP positive, are in contact with the orbicular and ektexine surfaces, and forms thin filaments in the anther locule (Figs. 7 and 10, arrows).

In the dehiscent anther stage, the mature orbicules are bodies with around 500 nm in diameter (Fig. 12). They are free particles, and are localized near the anther wall. The orbicular wall is compact, with a clean surface. Between the orbicular wall and core, a trilamellar structure is present and it is similar in structure and localization to one found during the tetrad stage (Fig. 12, arrow).

Discussion

Although the spherical shape of the pro-orbicules in *Ilex* can suggest a participation of cytoplasmic derived vesicles, none similar structure with a same dimension

was observed in the tapetal cytoplasm, near to plasma membrane. In *Ilex*, there is not an intracellular biosynthesis of pro-orbicules, but they are totally synthesized in the extracellular medium, in the periplasmic space, differently of *Platanus acerifolia*, where globular pro-orbicules are formed in the tapetal ER and later, secreted to the cell surface (22). An intracellular synthesis was also reported in the tapetum of *Allium cepa* (13) and *Lilium* (8).

According to Gabarayeva (5), the pro-orbicules has an initial spherical shape because the hydrophobic materials tend to acquire this shape when in contact with a hydrophilic medium. So, in *Ilex*, the orbicular shape could be explained by their composition, where the lipidic materials should be liberated to the periplasmic space, where are 'condensed' as pro-orbicules. The lipidic constitution of the orbicular core was demonstrated by its TCH-SP-positive reaction (unsaturated lipids). However, this reaction is negative in the pro-orbicular stage, suggesting a different composition, perhaps saturated lipids.

The glicocalix in *Ilex* is constituted by proteins and lipids since that it is reactive to Coomassie Blue and TCH-SP tests, respectively. The glicocalix has a important role in the pollen wall development in *Artemisia vulgaris* (6). In *Artemisia*, the glicocalix has a mucopolysaccharidic and proteic composition and it is related with the exine pattern, similar to primexine. In *Ulmus* (17), *Platanus* (22) and *Liriodendron* (6), the glicocalix and primexine terms are synonymous.

It is an interesting idea that the tapetal glicocalix in *Ilex* has a homologous function to primexine, the thick polysaccharidic matrix involving the microspores. Hesse affirms that orbicules and ektexine are homologous structures (9). Possibly, the orbicules are a consequence of physical and chemical conditions like the microscope surface during the pollen wall ontogeny. This homology between the orbicules and the ektexine implies that tapetum and sporogeneous tissues are homologous too, and contain identical genetic informations for sporopollenin synthesis (12). The diversity in the patterns (the ektexine in *Ilex* is intectate and clavate, whereas the orbicules are spherical) could be explained by the absence of a callose wall around the tapetum, and differences in structure and composition of the glicocalix and primexine. In *Ilex*, the primexine is trilayered and its position, thickness, and persistence determine the final form of the ektexine (19). In contrast, the glicocalix is more simplified than the primexine and it disappears with the tapetum maturation and lysis. As the orbicules are specific bodies, used like a taxonomic criteria, the tapetal glicocalix, in other species, can be the essential factor in the definition of the initial shape of the pro-orbicules.

Despite the pro-orbicules are not synthesized into the ER, this endomembrane complex should be the source of many sporopollenin precursors that polymerize themselves on the orbicular wall, due to the abundant smooth ER in the tapetum of *Ilex*. The connections of ER and plasma membrane show that the lumen contents are discharged to the locular spaces, perhaps the periplasm too. In the pollen wall development in *Ilex*, the ER is the main organelle responsible by sporopollenin biosynthesis in the ektexine and endexine (19). It is a logical supposition that the same organelle is involved in the sporophytic and gametophytic domains, represented by tapetum and microspores, respectively. Tubular ER-plasma membrane connections were described during the exine formation in *Dendrobium* (4). In *Liriodendron*, the ER terminals are fused with the plasma membrane of the microspores during the exine ontogeny (6). It was suggested that enzymes mediating sporopollenin polymerization are exchanged between the ER lumen and the extracellular medium. Dickinson, studying the exine development in *Pinus*, *Cosmos* and *Lilium*, concluded that there is not the intracellular sporopollenin synthesis, but only the formation of their precursors (2). The small and large vesicles found in the tapetum of *Ilex* during the tetrad stage can transport these enzymes.

The trilamellar structures found in the orbicular core periphery has a similar organization that of immature pollen exine. It is present during the orbicular ontogeny and in the mature orbicules. In *Lilium*, it was observed a 'membranous' structure around the orbicular core (1). This 'membrane' is identical to lamellae in *Ilex*. For us, however, this structure is not a true membrane, but it has the same ultrastructural organization that is found in the trilamellar lines of the young exine. The trilamellar organization of the exine was described by Rowley (15) and Rowley et al (18), and it was called 'tufts'. The 'tufts' would be sporopollenin subunits with around 70-200 nm in diameter. They were described in many species, and show a similar ultrastructural organization.

Conclusions

The tapetal periplasm seems to have an important function in the pro-orbicular synthesis in *Ilex*. The pro-orbicular formation can be the result of precursors and enzymes that accumulate in the periplasmic space and act in the sporopollenin synthesis, during the tetrad stage, when a cell wall is present, and in the tapetal glicocalix, in the free microspores stage, after the tapetal wall dissolution. The association of the tapetal glicocalix with the orbicules is clear and it is inevitable the conclusion that this layer has an important function in the orbicular growing.

However, a question emerges. Why the orbicules are not formed freely in the locular fluid after the tapetal wall dissolution? The answer can to be in the differences of sporopollenin precursor concentration before and after the cell wall loss (where they are confined in the periplasmic space) and in the glicocalix absence. Also, the orbicular core can to have an important role in the wall

initialization. But the final conclusion about this theme needs additional studies and confirmations.

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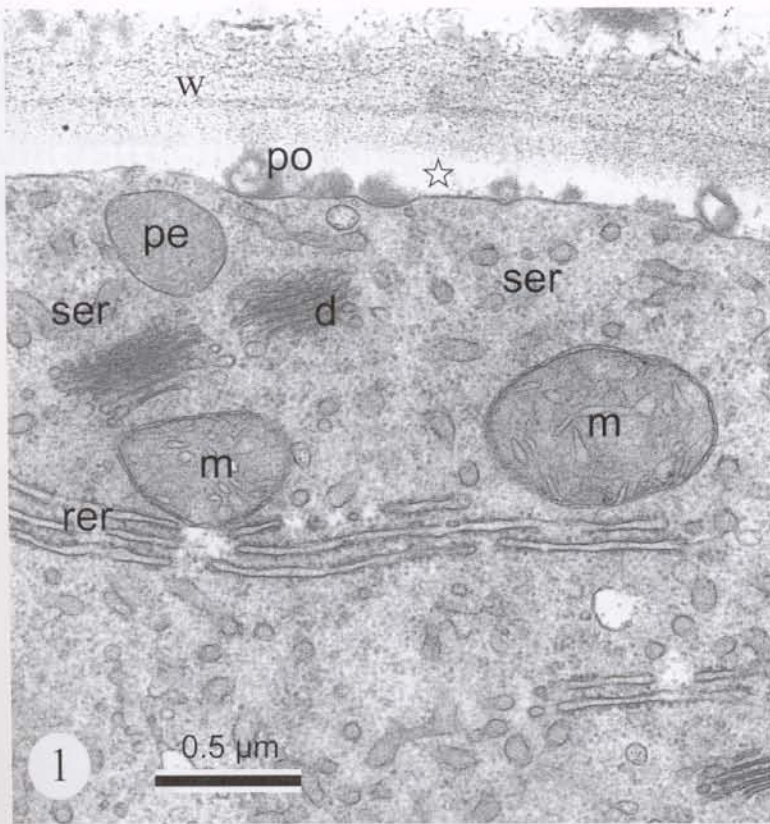
Legend to Figures

Abbreviations: d, dictyosome, ek, ektexine; er, endoplasmic reticule; L, lipid body; m, mitochondria; mi, microspore; o, orbicule; oc, orbicular core; ow, orbicular wall; p, plastid; pe, peroxisome; po, pro-orbicule; rer, rough endoplasmic reticule; ser, smooth endoplasmic reticule; sv, secretory vesicle; ta, tapetum; v, vacuole; w, cell wall. The TEM contrast method employed is indicated into parenthesis.

Figs. 1-6. Transmission electron micrographies of the pro-orbicules during the tetrad stage (PA-TCH-SP-Pb contrast). Fig. 1. Cortical cytoplasm of a tapetal cell, filled with many organelles. The pro-orbicules appear in the periplasmic space (star). Fig. 2. Detail showing microtubules (arrows) near to plasma membrane depression. Fig. 3. Pro-orbicules on a plasma membrane invagination (large arrow). The orbicular core is split by a trilamellar line (arrow), and it is covered by a fibrillar electron dense layer, continuous to the cell surface (asterisk). The periplasm (star) is electron transparent. Fig. 4. Pro-orbicules on the tapetal plasma membrane. Cytoplasmic vesicles (small arrows) fuse with the plasma membrane. Again, a invagination can be observed under a pro-orbicule (large arrow). Fibrillar material (asterisk) cover the orbicular core. Fig. 5. Anomalous development in a pro-orbicule free in the periplasmic space (stars). The orbicular covering (asterisk) forms a tubular structure with an electron transparent axis (white arrow). Fig. 6. Pro-orbicule on the plasma membrane. A 'membrane' line is found between the core and outer layer (arrow). A secretory vesicle discharges its content to the cell surface (asterisk).

Figs. 7-11. Orbicular maturation during the free microspores stage. Fig. 7. Tapetal cytoplasm containing a lot of ER, with orbicules immersed in the glicocalix (asterisk). The locular content is continuous with the glicocalix and seems to have the same composition. (TCH-SP). Fig. 8. Light micrography of the tapetal cells stained with Coomassie Blue. The glicocalix has a positive reaction (arrow). Fig. 9. Transmission electron micrography of a tapetal ER-plasma membrane connection in a cell invagination (arrow). The glicocalix (asterisk) shows a similar electron density to ER lumen content. (U-Pb). Fig. 10. Transmission electron micrography of orbicules and immature pollen grain ektexine contrasted with TCH-SP. The orbicular wall and ektexine share an identical electron density and both are in contact with the tapetal secreted substances, more abundant in the glicocalix (asterisk). Fig. 11. Transmission electron micrography of the orbicules and ektexine contrasted with PTA-chromic acid. Fig. 12. Orbicules in the dehiscent anther. (U-Pb).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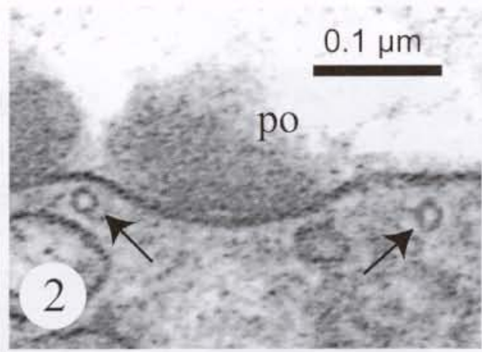
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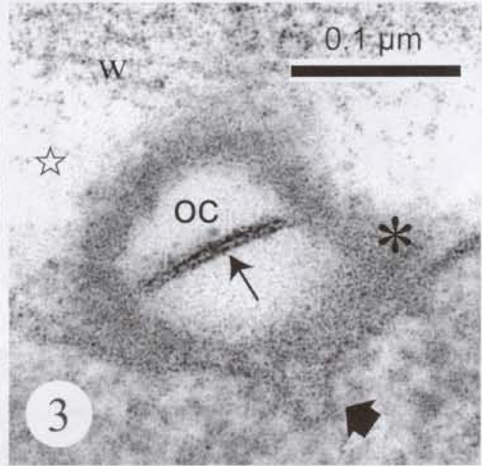
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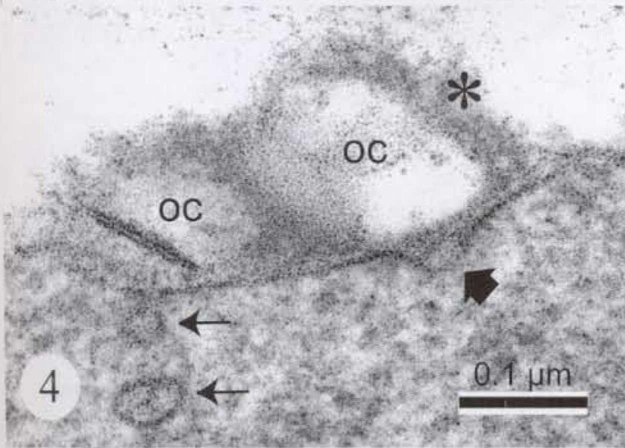
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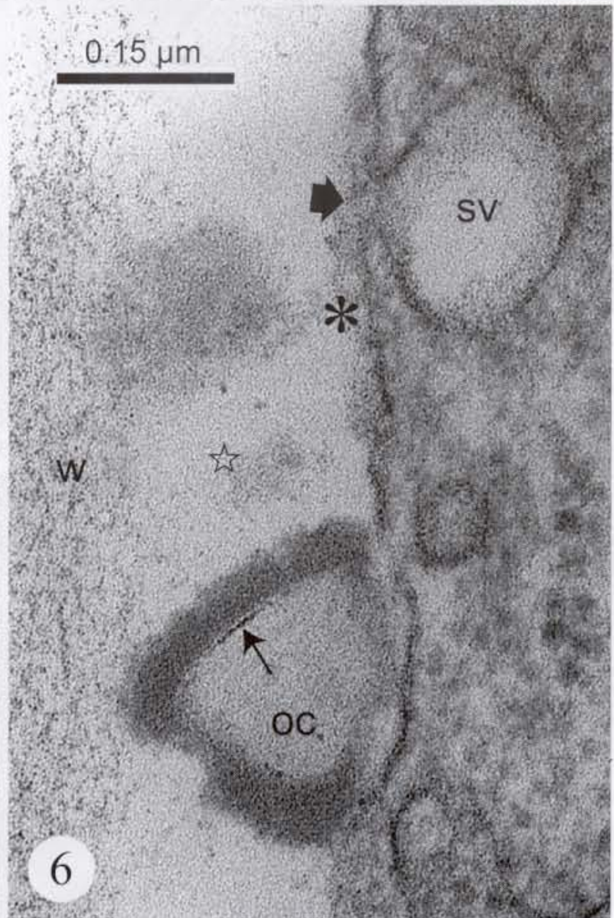
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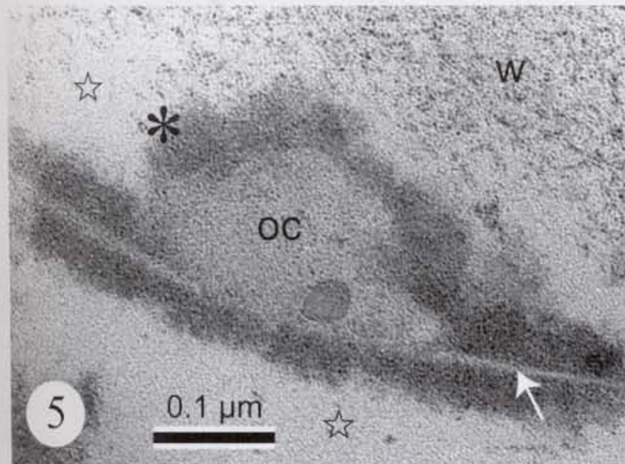
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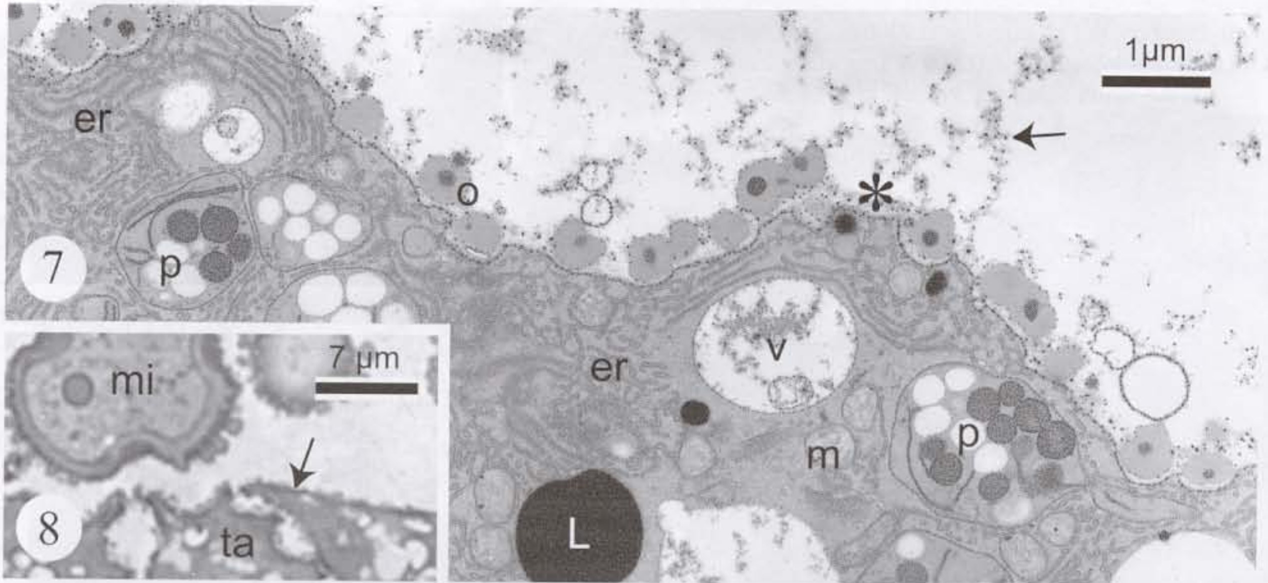
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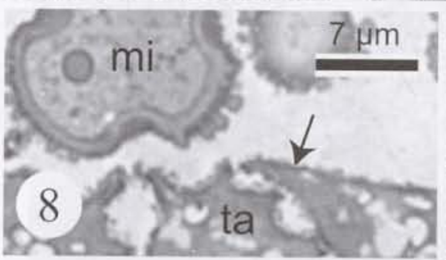
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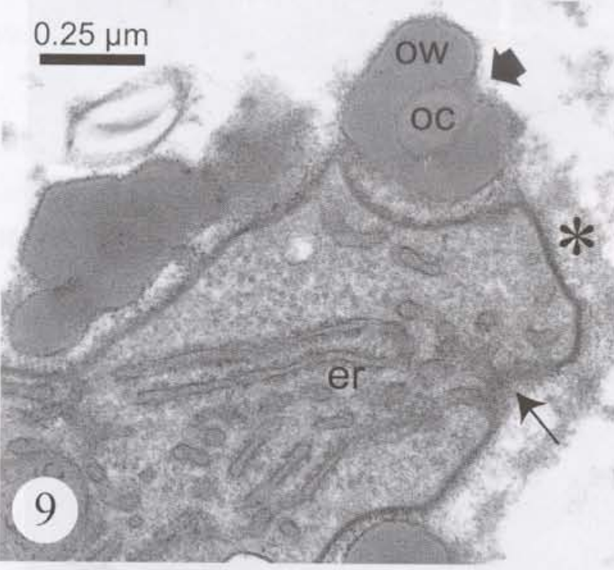
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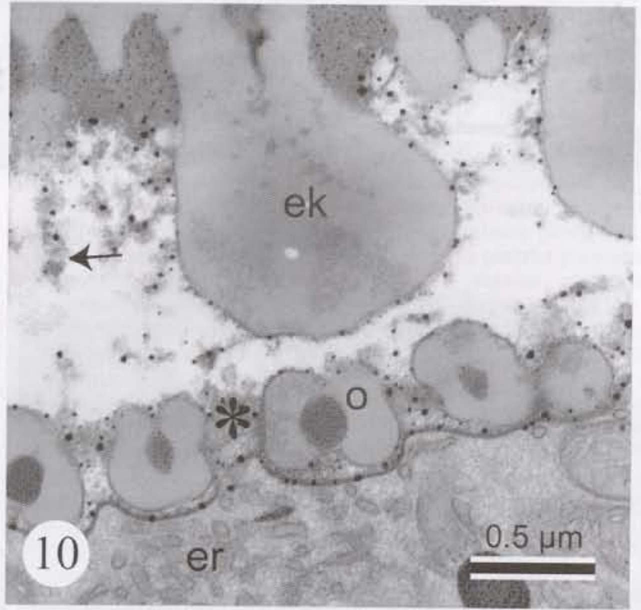
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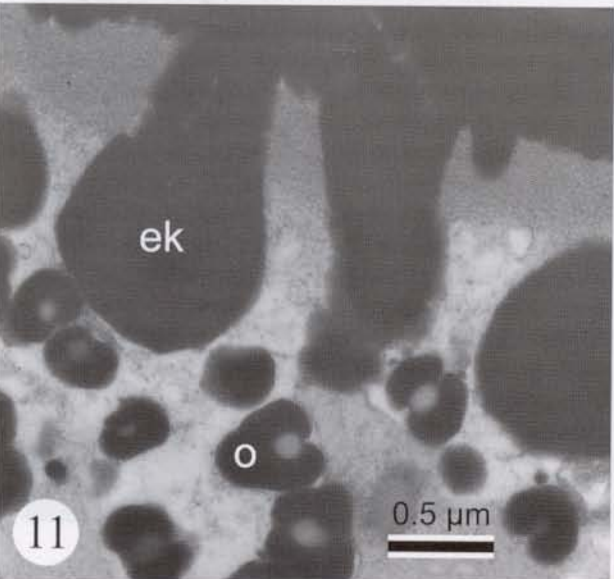
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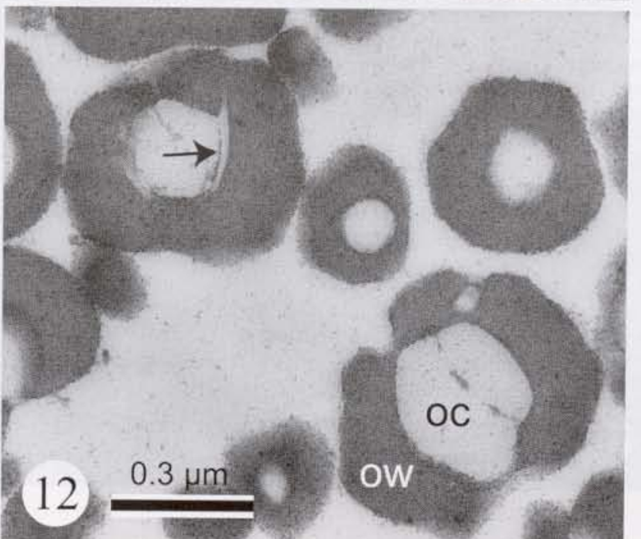
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