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Ultrastructural characteristics of *Betula pendula* tapetum and pollen grains after rapid freezing and freeze-substitution

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Using rapid fixation and freeze substitution method, the ultrastructure of the cytoplasm, including the membrane system, of *Betula pendula* pollen is well preserved. The cytoplasm of *Betula* pollen appears compact and organelles such as Golgi and RER are not yet demonstrating any secretory activities. The cytoplasm streaming is not established because the pollen grains are not completely hydrated and the microfilaments are still polymerized in bundles. The cytoplasm of the generative cell is well preserved including the cell wall like structure and cytoskeletal complexes present in the cell. Moreover the application of rapid freeze fixation of microspores and pollen grains of *Betula* showed clear details of ultrastructure of the Zwischenkörper (Z) at the aperture sites and the Z-layer (ZL) between the apertures. They are mainly composed of dense helical units that extend from the plasmalemma. Rapid freezing improves to a great extent the fixation of the delicate cell structure of the tapetum. After freeze fixation and substitution it is possible to observe radial helical units similar to those of the Zwischenkörper, throughout the thick coat of tapetal cells.

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The pollen grains of the family Betulaceae (e.g. birch, alder, hazel and hornbeam) represent one of the major causes of pollinosis in Sweden and a large part of the Northern hemisphere (Swoboda et al. 1994). The presence of allergens in *Betula* pollen grains has been tested by using specific monoclonal antibodies (Grote 1991, 1992; Grote et al. 1993, El-Ghazaly et al. 1997). Using conventional chemical fixation, the morphological and histochemical differentiation of *Betula* pollen wall, during dormancy up to anthesis, has been studied (Dunbar & Rowley 1984, El-Ghazaly & Grafström 1995). Other studies concern mainly the morphology and ultrastructure of mature *Betula* pollen grains (e.g., Erdtman et al. 1962, Doyle et al. 1975, Rowley 1981, and Claughner & Rowley 1987).

For better understanding and evaluation of the ultrastructure of *Betula* pollen we used, in the present work, rapid freeze fixation (RF) in liquid propane followed by freeze substitution (FS). Such method is an alternative that has the potential to avoid the artefacts that occur during chemical fixation with the resulting images providing a more realistic representation of cell structures. Ideally RF halts cell processes almost instantly while avoiding the formation of ice crystals detectable at the electron microscopy level. When FS follows RF, the specimens are exposed to a chemical fixative only during dehydration (Lancelle et al. 1986).

MATERIALS AND METHODS

Mature anthers of *Betula pendula* Roth. were collected at the beginning of May 1995 and 1996 in the Stockholm area from

catkins that had just begun to release their pollen. The anthers were hydrated for 30 min in a humidity chamber and then mounted on copper wire loops (3 mm in diameter) pre-coated with formvar 150–200 nm thick. Successively the materials were subjected to rapid freeze-fixation and freeze-substitution as described by Lancelle et al. 1986. As the substitutions were completed, the samples were rinsed in several changes of acetone, followed by methanol. Block staining was then carried out at room temperature in 5% uranyl acetate in methanol for 2 hours. After rinsing in methanol and then in acetone the samples were infiltrated and embedded in Spurr's low viscosity resin. Sections were prepared using a diamond knife with a LKB ultramicrotome, stained for 3 min in Reynolds lead citrate and examined at 80kV in a JEOL TEM 100B and ZEISS EM 906 electron microscope.

RESULTS

Tapetum

Rapid freezing improves to a great extent the preservation of the delicate structure of the tapetum. At an early stage of development, after the microspores are released from the callosic envelope, the tapetal cells are very active. The cells are mono- or binucleate and have a dense cytoplasm (Fig. 1). The high secretory activity of the tapetal cells is presented by secreting pro Ubisch bodies, which pass through the plasmalemma of the tapetal cells and deposit on the inner surface of these cells (Figs. 1, 2). A thick fibrillar material is formed between the tapetal cells and the pollen surface (Figs. 2, 3). It is possible to observe helical radial units through-out the thick coat of tapetal cells

(Fig. 3). These helical units are similar to those of the Zwischenkörper in the microspores and pollen grains (Figs. 4, 5).

Microspores and pollen grain walls

The microspores and pollen grain walls show the general features previously described by El-Ghazaly and Grafström (1995). The walls are characterized by the presence of many microchannels with or without dense material (Figs. 2, 6). The Zwischenkörper is visible as a thick body beneath each aperture (Figs. 4, 5 & 7) and is continuous in the interapertural wall as a thin layer (Z-layer). A thick fibrillar network is observed at the pores of mature pollen grains (Fig. 8). It is similar to the fibrillar-flocculent material described by Dunbar and Rowley (1984) at the pore of *Betula verrucosa* microspores during dormancy.

The pollen grain cytoplasm

Vegetative cell

At the mature stage, the central core of pollen grains contains the vegetative nucleus. Several amyloplasts with one or more starch grains, and lipid bodies are located near the generative cell. The rest of the cytoplasm is disposed at the periphery of the cell near the plasmalemma. We focus our attention particularly in such zones where the Golgi bodies are located (Fig. 9). Mitochondria and plastids seem to have a similar structure but we can distinguish them by the presence or absence of starch. A network of rough endoplasmic reticulum (RER) and free ribosomes is present (Figs. 9, 11). The cytoskeletal apparatus is mainly represented by many bundles of microfilaments (Fig. 10), but microtubules (Mt) are rarely observed. In a few cases the presence of Mt in the vegetative cytoplasm of inactivated (dry) pollen has been reported.

Generative cell

The generative cell is located in the central part of the pollen and it is delimited from the vegetative cell by two membranes. In the space between the membranes electron dense material can be observed forming a cell wall like structure (Fig. 12). A few mitochondria and numerous ribosomes are also present. There are no connections between the vegetative and generative cytoplasm. The generative nucleus contains aggregations of chromatin. Microtubules are occasionally aggregated as bundles in the cytoplasm (Fig. 12).

DISCUSSION

The technique of rapid freezing followed by freeze-substitution has become an extraordinary alternative to the conventional specimen preparation methods in pollen ultrastructural studies (Lancelle et al. 1987, Lancelle & Hepler 1992, Lancelle et al. 1996, Tiwari & Polito 1988, Tiwari 1994, Cresti et al. 1987, Cresti et al. 1990, Theunis et al. 1991, De Win et al. 1996, Martinez & Wick 1991).

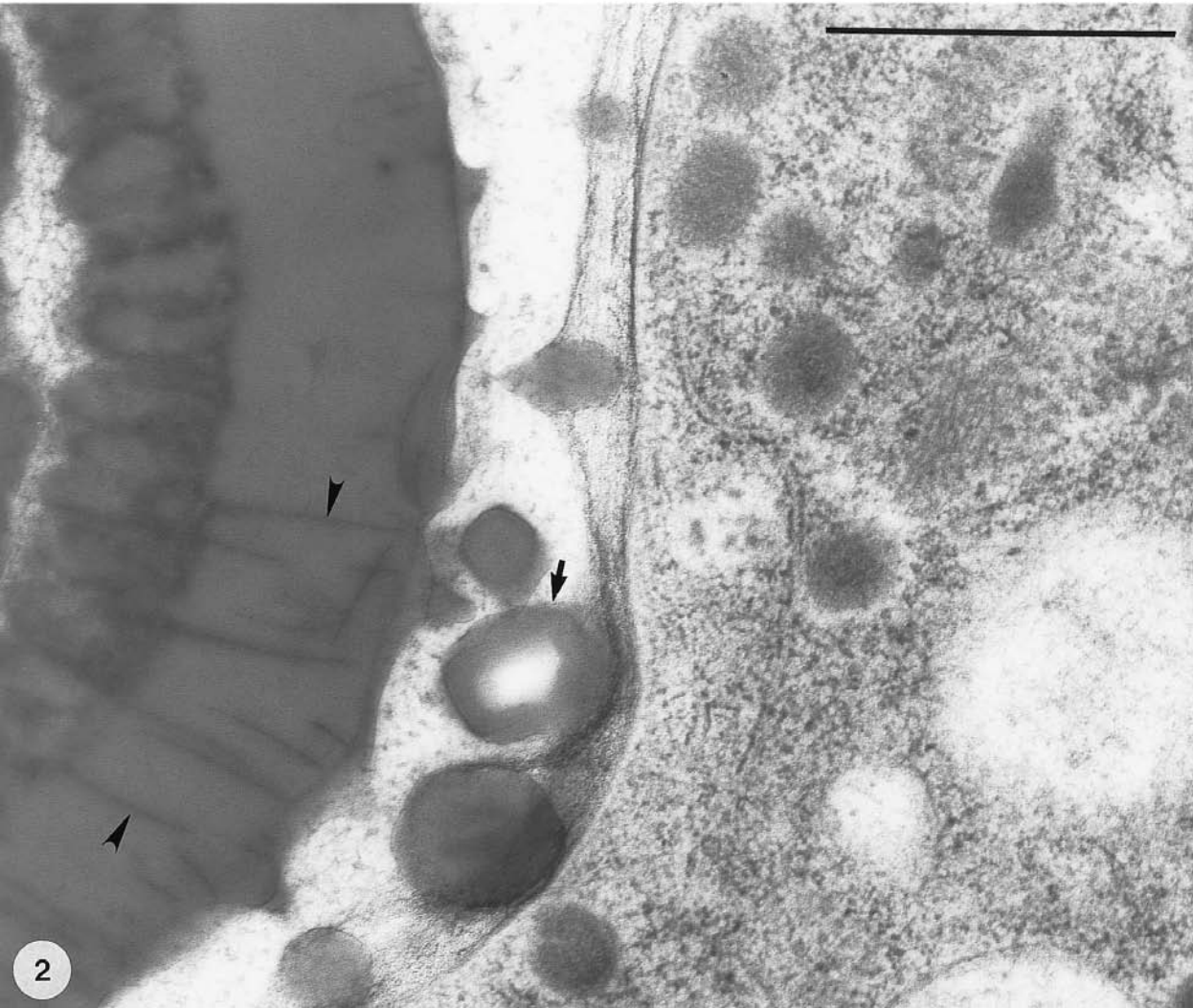
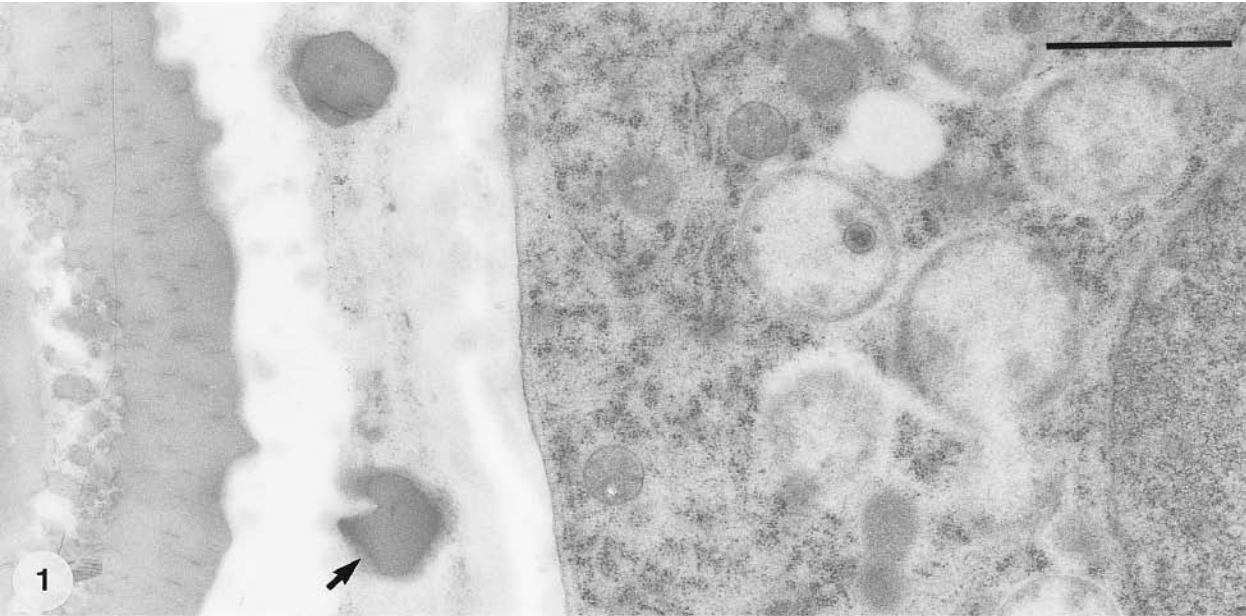
In the present work we used *Betula* pollen just after a brief hydration in moist chambers but without sowing in the artificial and optimised medium where the pollen are completely hydrated and successively germinate. We are consequently preserving more of the natural conditions of the pollen. In fact after sowing pollen grains in a liquid medium, the proteins and other substances held in the intine are released (Heslop-Harrison & Heslop-Harrison 1988, Que & Tang 1988). However, in our treatment the complex synthetic machinery of the pollen is not completely reactivated and also the membrane systems are still probably in the transition phase from gel to liquid crystalline status (cf. Heslop-Harrison 1987, Crowe et al. 1989, Pierson & Cresti 1992, Taylor & Hepler 1997).

The scarcity of ultrastructural data dealing with dry (or semidry) pollen probably derives from the technical difficulties encountered at the moment of fixation of this desiccated material. The cytoplasm of *Betula* pollen appears compact and organelles such as Golgi and RER are not yet demonstrating any secretory activities. The cytoplasm streaming is not yet established because the pollen grains are not completely hydrated and the microfilaments are still polymerized in bundles. The GC cytoplasm is well preserved including the cell wall like structure and cytoskeletal complexes present in the cell.

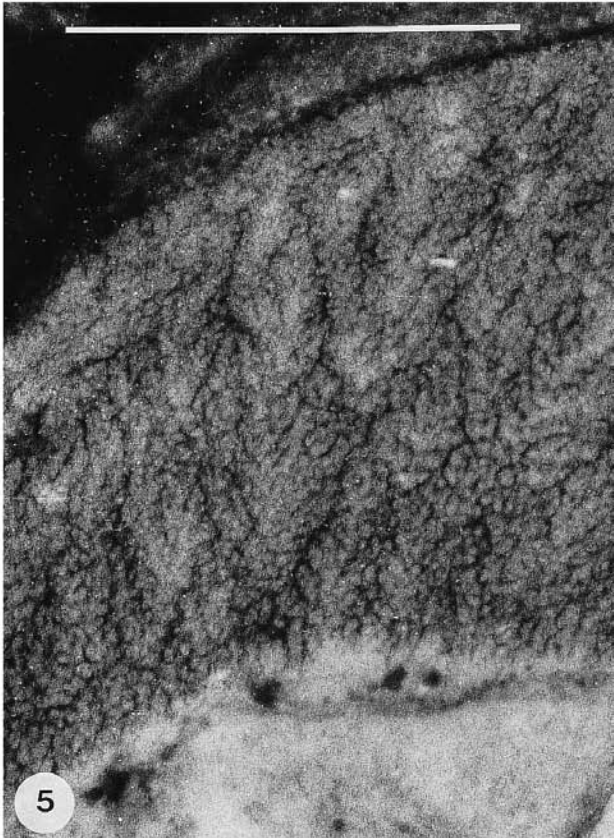
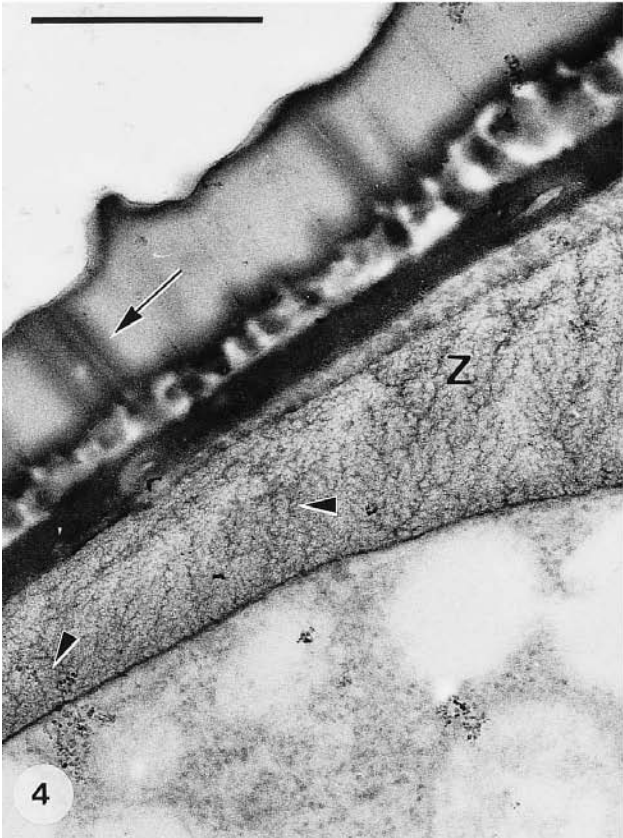
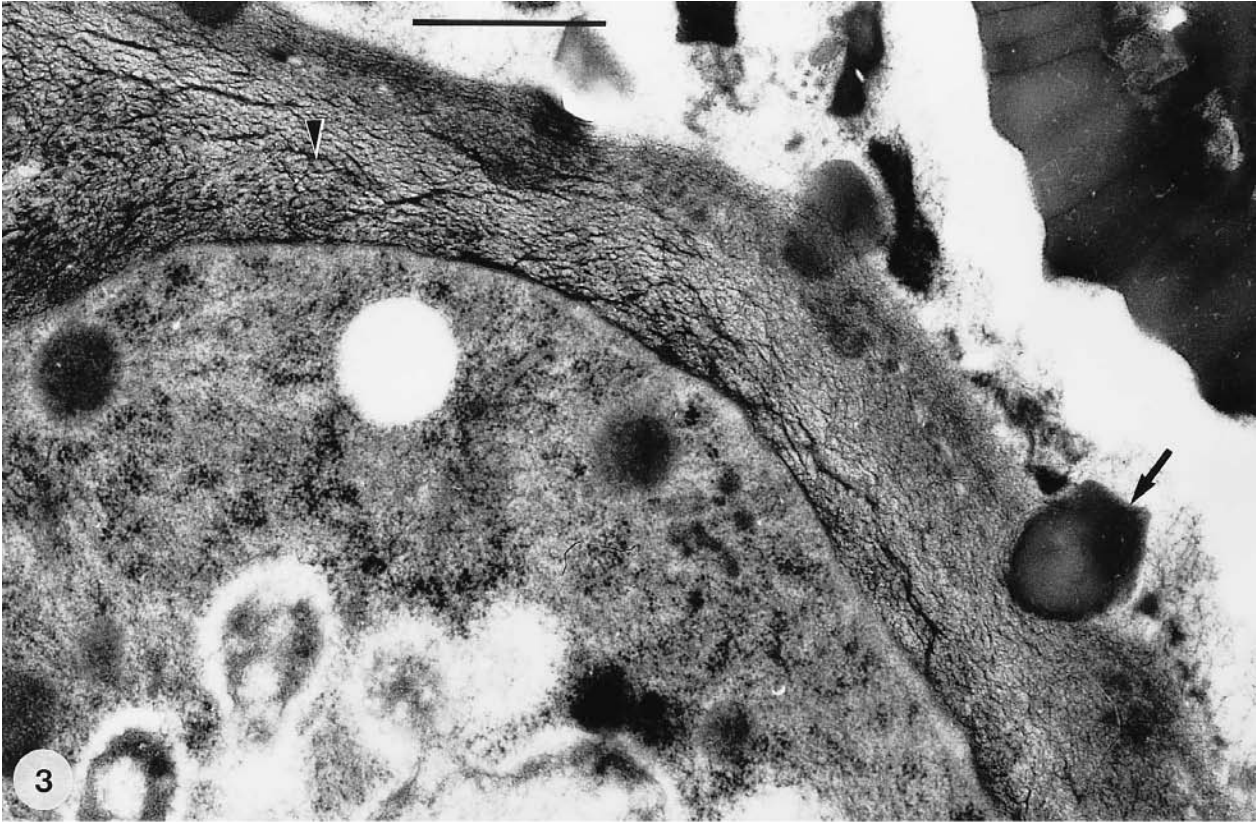
Moreover the application of rapid freeze fixation of microspores and pollen grains of *Betula pendula* showed clear details of ultrastructure of the wall. It is easy to differentiate the ultrastructure of the Zwischenkörper (Z) at the aperture sites and the Z-layer (ZL) between the apertures. They are mainly composed of dense helical units that extend from the plasmalemma. In chemical fixation the ultrastructure of (Z) is, in most cases indistinct (El-Ghazaly & Grafström 1995). Rapid freezing improves to a great extent the fixation of the delicate cell structure of the tapetum. After freeze fixation and substitution it is possible to observe radial helical units similar to those of the Zwischenkörper, throughout the thick coat of tapetal cells. In chemical fixation the coat of the tapetal cells appears compact and structure-less (Unpublished data). Charzynska et al. (1990) suggested that Zwischenkörper in *Secale* may facilitate the diffusion of solutes from the tapetum to the microspore. The early development of Zwischenkörper at the sites of the apertures in close contact with the tapetum may be in favour of this suggestion. A recent study on tapetum and microspores of *Betula* by Cryo-SEM (El-Ghazaly & Rowley: in press) demonstrate possible routes for transfer of materials between tapetal cells and developing microspores. Moreover, the presence of helical filamentous units within Zwischenkörper, as showed by freeze substitute fixation, add new information in favour of a transportation function.

Difference between pollen fixed with rapid freezing and conventional chemical fixation

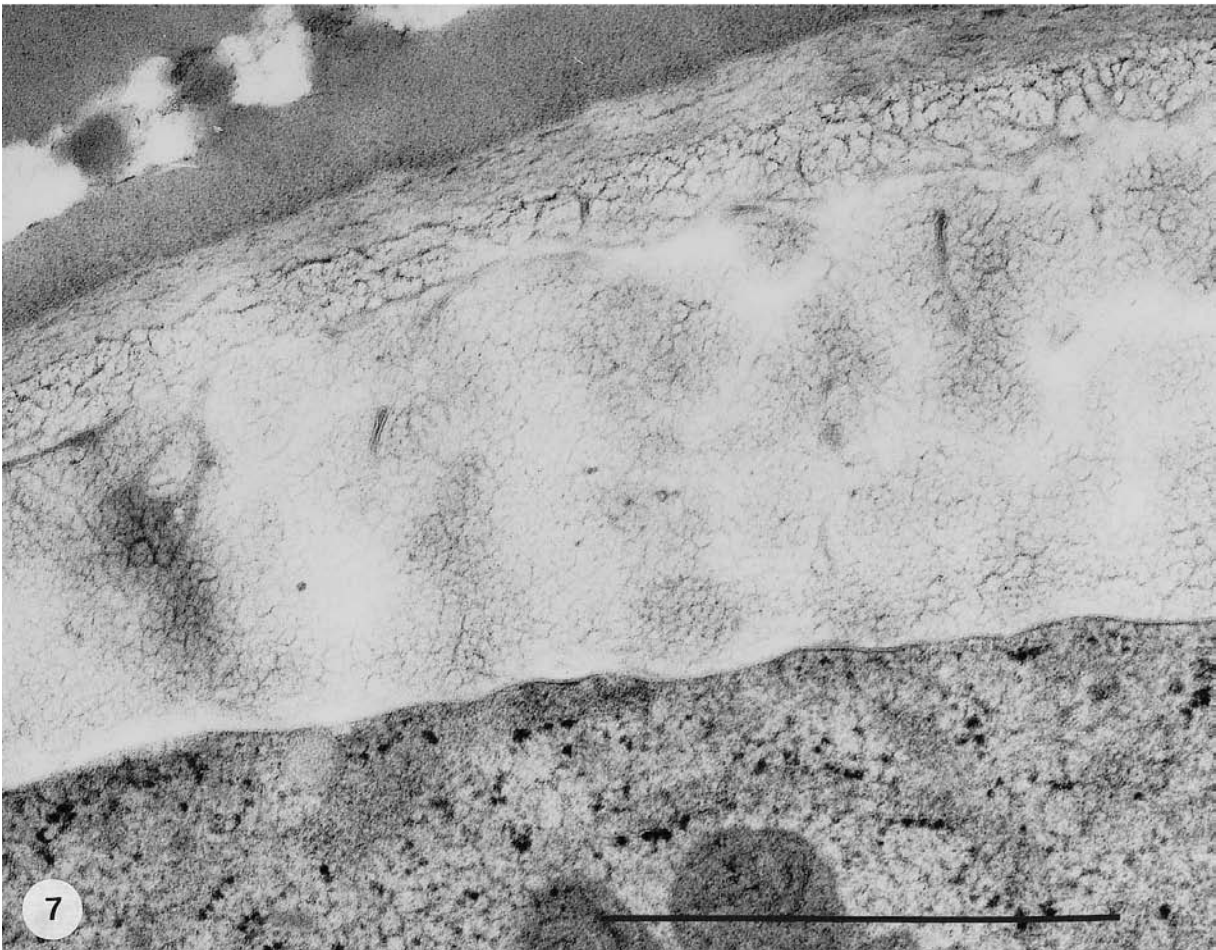
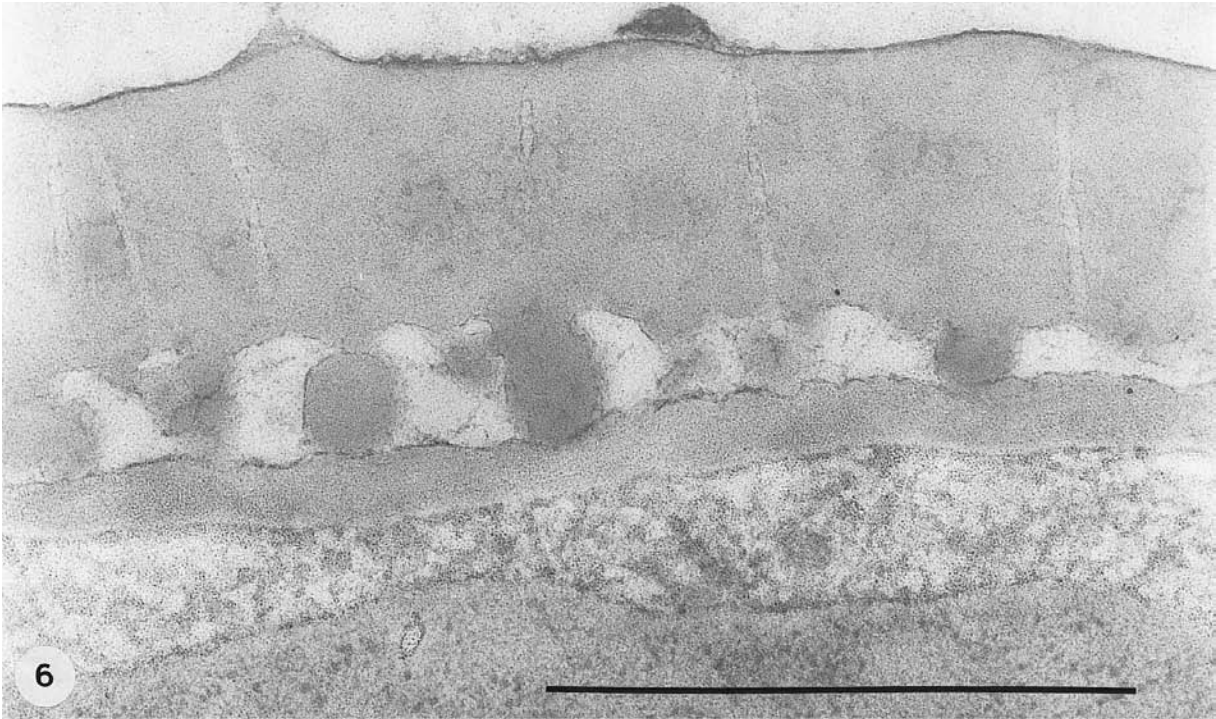
Many papers in the field of pollen development and pollen wall ultrastructure were based on material fixed chemically (Dahl 1986, Suarez-Cervera et al. 1995, Dickinson & Potter 1976). In spite of the significant results obtained from these



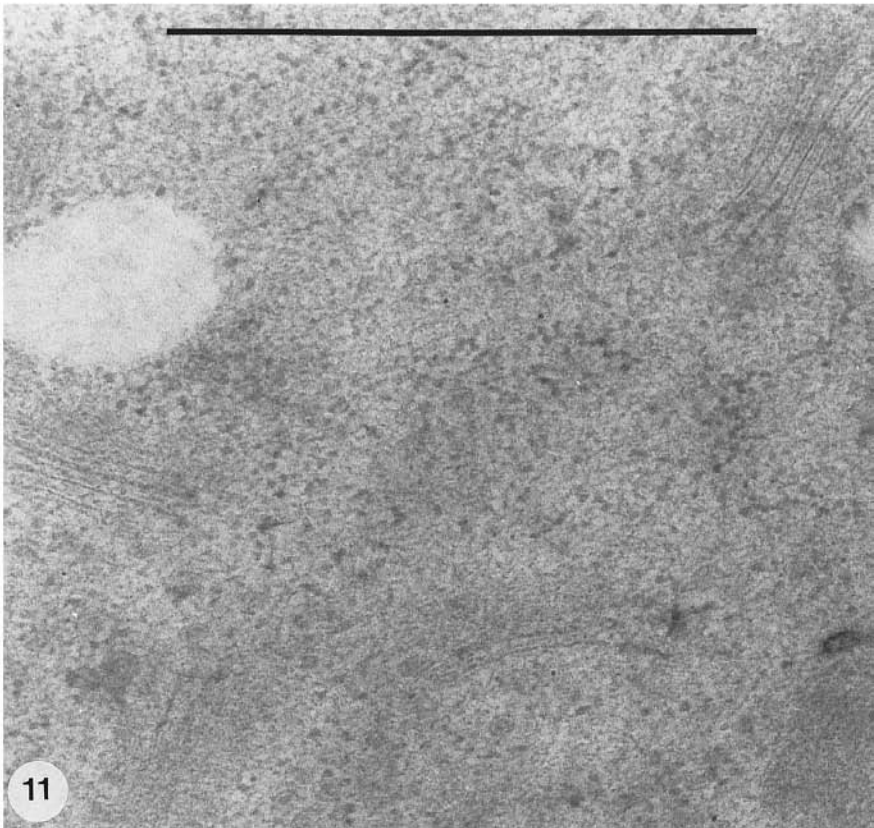
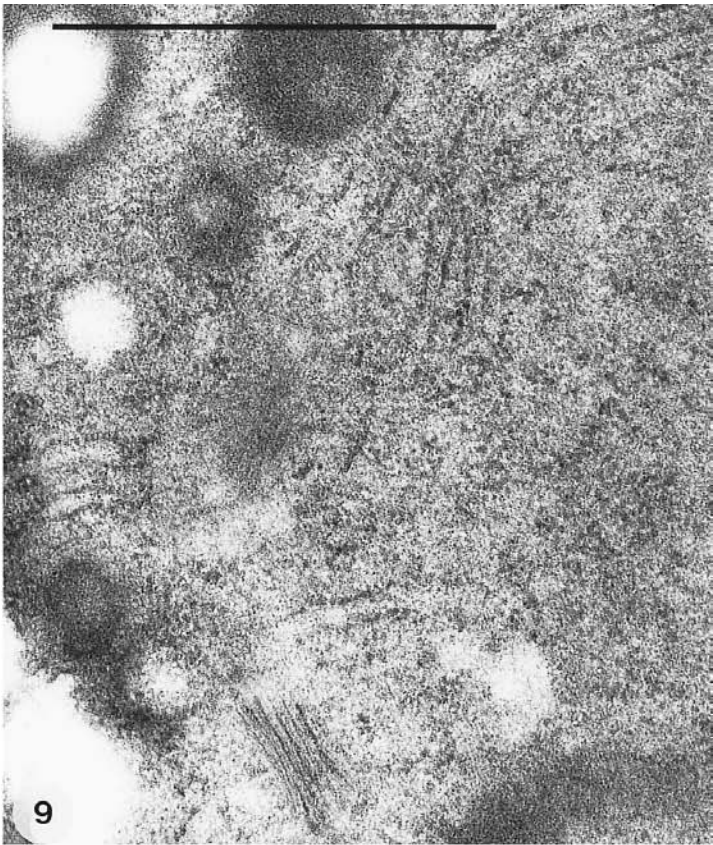
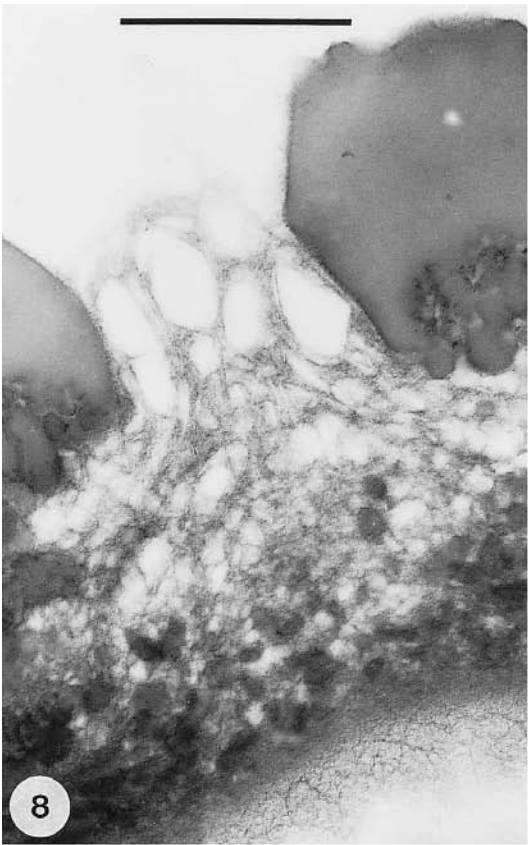
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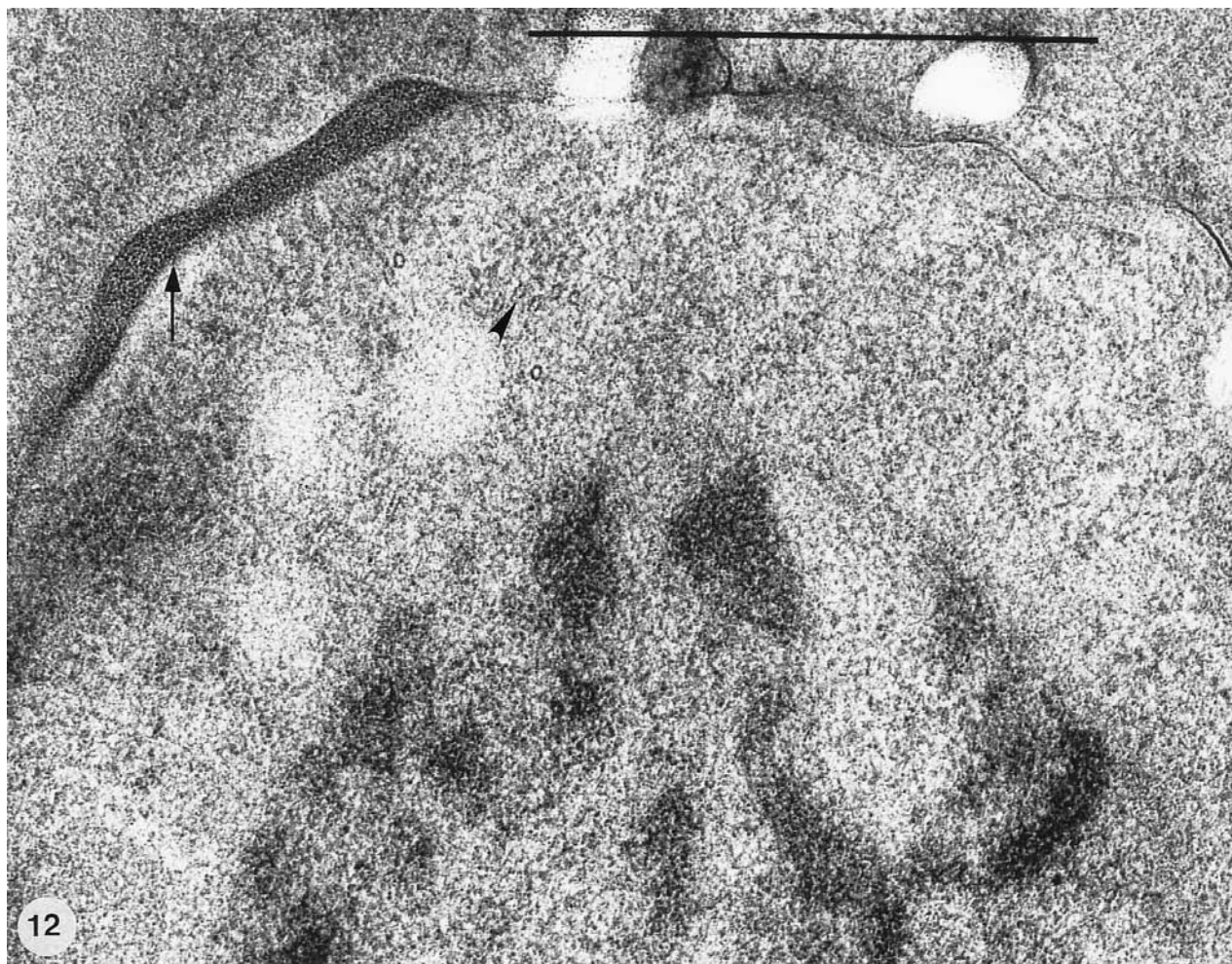
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Figs. 1–12. TEM micrographs of *Betula pendula*. Scale bars in all Figs. – 1 μm except Fig. 10 (10 μm).

Fig. 1. Part of pollen grains wall and tapetal cell at a late microspore stage. The Ubisch bodies (arrow) are embedded in a thick fibrillar layer outside the plasmalemma. $\times 24,000$.

Fig. 2. Part of tapetal cell and late microspore. The tapetal cell cytoplasm appears active in secreting and producing Ubisch bodies (arrow). The microspore wall is traversed by numerous microchannels that are filled with electron dense material (arrowheads). $\times 48,000$.

Fig. 3. Tapetal cell at late microspore stage. The thick coat of the tapetal cell is distinguished by radial helical units (arrowhead). The Ubisch bodies (arrow) are nested in its inner surface (arrow). $\times 25,000$.

Fig. 4. Young pollen grain. The tectum is interrupted by translucent microchannels bordered by electron dense zones (arrow). A part of a Zwischenkörper (Z) and Z-layer are characterised by the inclusion of helical radial units (arrowhead). $\times 30,000$.

Fig. 5. Magnified part of Fig. 4, emphasizing the helical units in the Zwischenkörper. $\times 60,000$.

Fig. 6. Young pollen grain. Microchannels are evident and are not filled with electron dense material (cf. Fig. 2). Note the development of a periplasmic zone between the exine and the plasmalemma. $\times 78,000$.

Fig. 7. Part of wall and Zwischenkörper of a young pollen grain. A well-preserved plasmalemma is visible. Note the helical units in Zwischenkörper $\times 69,000$.

Fig. 8. A network of fibrillar material is located in one of the pores of a mature pollen grain. $\times 30,000$.

Fig. 9. Peripheral layer of cytoplasm with lipid droplets and a network of RER. $\times 57,000$.

Fig. 10. Microfilaments bundles (arrowhead), a plastid and lipid globules in the cytoplasm of a pollen grain. $\times 85,000$. Scale bar – 10 μm .

Fig. 11. Part of a pollen grain showing a peripheral layer of cytoplasm. Golgi bodies without any secretory activities and free ribosomes are present.

Fig. 12. Generative cell surrounded by the vegetative cell. The generative cell wall complex is formed by the plasmalemmas of the generative and vegetative cells. Between the two membranes a layer of electron-dense material is present (arrow). Microtubules, often aggregate in bundles, are seen in the generative cell (arrowhead). $\times 77,500$.

studies, several questions regarding exine pattern and function still remain to elucidate. Chemical fixatives penetrate and react relatively slowly and cannot preserve all cellular components. Certain structures are simply too labile to be adequately preserved by chemical conventional fixation. Such fixation influences the diffusion properties of the membranes and results therefore in alterations of shape, volume, and organelles of the cell. Furthermore, dehydration of fixed samples results in dimensional changes (shrinkage). Rapid freezing may be the only way to stabilize them (McCully et al. 1985, Studer et al. 1989). Rapid freeze fixation for transmission electron microscopy proved to be an adequate technique to study the delicate tapetal tissue and the wall, the plasmalemma and cell contents of the microspores and pollen grains. This technique for transmission electron microscopy is improving and providing fine imaging and permits the study of cell organelles, membranous system and cytoskeletal components in greater detail than before (Tanaka 1981, Tanaka & Naguro 1981, Barnes et al. 1985, Blackmore & Barnes 1986, Studer et al. 1989).

Rapid freezing techniques, however, are limited in application since the zone in which adequate cryofixation is obtained only occasionally exceeds the dimensions of one plant cell (Dahmen & Hobot 1986).

In conclusion, using RF-FS methods the ultrastructure of the cytoplasm, including the membrane system, of *Betula* pollen is well preserved and with small improvements changes (e.g. using an appropriate embedding medium polymerised at low temperature), can be suitable for more specific detection of well preserved allergens. The starch grains seem to be the main site where the allergens (Bet v 1) are located (El-Ghazaly et al. 1997) but their presence outside the pollen cytoplasm has also been reported (for references see: El-Ghazaly et al. 1995, Schäppi et al. 1997).

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