

Electron tomography of structures in the wall of hazel pollen grains

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ABSTRACT

The three-dimensional structure of channels and bacula cavities in the wall of hazel pollen grains was investigated by automated electron tomography in order to explore their role in the release of allergen proteins from the pollen grains. 3D reconstructions of 100–150 nm thick resin-embedded sections, stabilized by thin platinum–carbon coating, revealed that the channels aimed directly towards the surface of the grain and that the bacula cavities were randomly sized and merged into larger ensembles. The number and the dimensions of the ensembles were quantitatively determined by neighboring voxel analysis on thresholded reconstructed volumes. To simulate the allergen release, allergen proteins were approximated by a hard sphere model of a diameter corresponding to the largest dimension of the known 3D structure of the major birch allergen, Bet v 1, whose amino acid sequence is highly similar to the amino acid sequence of the major hazel allergen, Cor a 1. The analysis of positions where the hard sphere fits into the resolved channels and bacula cavity structures revealed that unbound allergens could freely traverse through the channels and that the bacula cavities support the path of the allergens towards the surface of the grain.

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1. Introduction

Pollen grains (Fig. 1(A)) are male gametophytes of higher plants. Apart from the genetic information they also contain a considerable amount of water-soluble proteins and glycoproteins, which rule the complex reactions of pollen-stigma recognition and fertilization or play a role in plant defense mechanisms (Breiteneder et al., 1989). Unfortunately, some of these soluble glycoproteins initiate the production of IgE antibodies when they come into contact with the mucosa of sensitive individuals, leading to type I allergic symptoms (e.g., rhino conjunctivitis, asthma). Approximately 95% of tree-pollen-allergic patients show IgE binding to the major allergens of trees belonging to the order of Fagales, namely to the birch major allergen (Bet v 1), alder major allergen (Aln g 1), hazel major allergen (Cor a 1) and hornbeam major allergen (Car b 1), whose amino acid sequences were found to be highly similar (Breiteneder et al., 1993). The three-dimensional structure of the major birch pollen allergen, Bet v 1, has been determined by NMR and X-ray crystallography at a resolution of 0.2 nm (Gajhede et al., 1996). According to this data, available in the Protein Data

Bank (Berman et al., 2000), the allergen is a spatially compact molecule whose largest extent slightly exceeds 5 nm.

The outer wall of pollen grains consists of two layers, the soft porous inner layer (the intine) composed predominantly of cellulose, hemicellulose and pectins, and the outer layer (the exine), which is formed by various organic and inorganic substances. Among these is the sporopollenin, a highly resistant biopolymer (Scott, 1994) which is responsible for the high chemical robustness of the exine. In the exine of tree pollen grains there is an embedded layer of bacula cavities, from which numerous narrow channels extend radially to the surface of the grain (Fig. 1(B)). It is assumed that the channels and the bacula cavities contain substances critical for the process of the pollination of the female plant (Heslop-Harrison et al., 1973, 1975).

It has been shown that the allergens are rapidly released from pollen grains upon hydration (Belin and Rowley, 1971; Vrtala et al., 1993). In accordance with this finding, attempts to localize the Bet v 1 protein on ultrathin sections of birch pollen grains by immunogold labeling with monoclonal antibodies were successful only after strictly anhydrous fixations of the pollen grains (Grote, 1992, 1999; Grote et al., 1993). The allergens were found to be localized only in the cytoplasm of the pollen grains, while the intine, exine and the surface of the pollen grains remained unlabeled. In the studies of Vrtala et al. (1993) and Grote et al. (1993), dry birch pollen grains were rehydrated for a short time, briefly centrifuged, anhydrously fixed, and immunogold labeled for the Bet v 1

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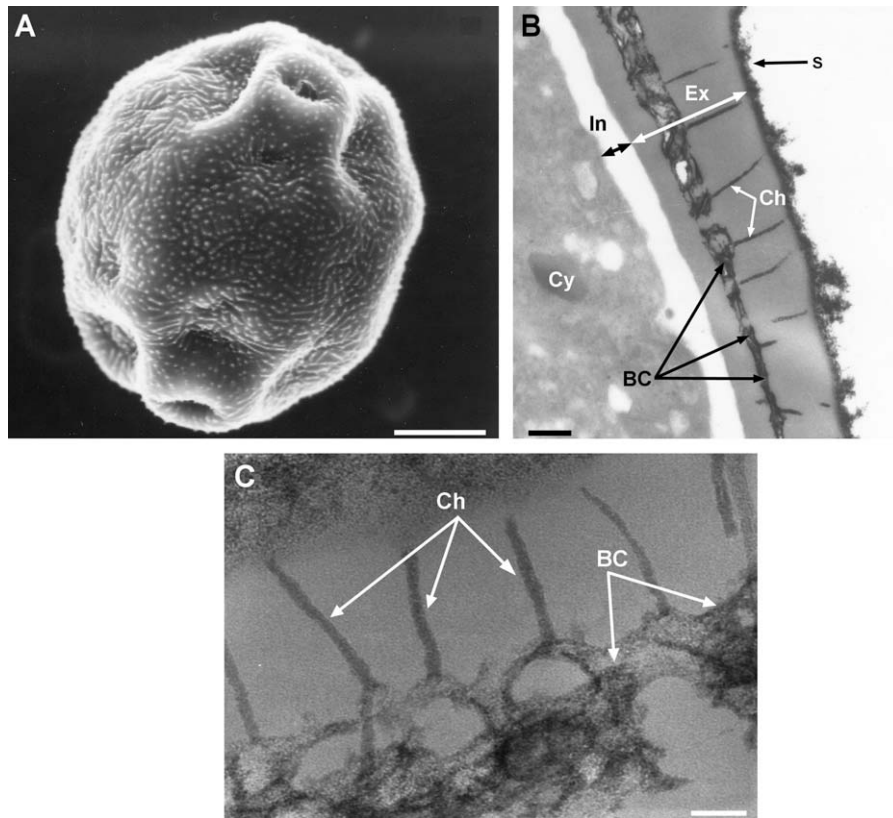


Fig. 1. (A) Secondary electron micrograph of a birch pollen grain coated with a 2 nm thick Pt/C film, which was recorded with a field emission scanning electron microscope S-5000 (Hitachi Ltd., Tokyo, Japan) at 10 kV. (B) Transmission electron micrograph of a 80 nm thick section of a resin-embedded hazel pollen grain, showing part of the wall with channels and bacula cavities, obtained by an EM 410 (Philips, Eindhoven, The Netherlands) at 80 kV. Ex, exine; In, intine; BC, bacula cavities; Ch, channels; Cy, cytoplasm; S, surface. (C) Details of a 350 nm thick exine in a section of resin-embedded hazel pollen grain. The micrograph was recorded at 300 kV in the zero-loss mode by a TEM JEM 3010 (Jeol, Tokyo, Japan) equipped with a Gatan energy filter. Scale bars correspond to 5 μ m (A), 200 nm (B), and 100 nm (C).

protein. After only 1 min of hydration, the Bet v 1 proteins were found in the wall and on the surface of the pollen grains, while the density of labeling in the cytoplasm decreased. The allergens in the pollen wall were often detected in clusters, which indicated presence of preferred pathways of the allergens within the pollen wall (Grote et al., 1993).

Due to the fact that the allergens were also frequently detected close to or in the channels and the bacula cavities, and because the movement of the allergens through the exine is more complicated due to the high content of closely packed sporopollenin biopolymers (Jungfermann et al., 1997), in this study we attempted to elucidate to what extent the bacula cavities and the channels may facilitate the migration of the allergens through the exine by electron tomography of resin-embedded sections of hazel pollen grains.

2. Materials and methods

2.1. Specimen preparation

Our preparation procedures were performed on dry pollen grains to keep the water-soluble allergen proteins in their original places. Hazel pollen was fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 5.7) containing 0.5% of Alcian blue 8GX, postfixed with 1% OsO₄ + 1% lanthanum nitrate in a 0.2 M collidin buffer, subsequently washed with 0.2 M collidin buffer, dehydrated in a graded water/ethanol series, and embedded in Epon. Alcian blue preserves acid mucopolysaccharides and glycoproteins (Behnke and Zelander, 1970; Hayat, 1975) that fill the bacula cavities and the channels, and imparts high contrast to TEM micrographs due

to significant amount of copper (cf. Fig. 1B and C). Approximately 100–150 nm thick sections were conventionally cut with a Reichert Ultracut S ultramicrotome, placed onto bare 1000 mesh Cu-grids and subsequently coated with a ~3 nm thick fine-grained Pt/C layer deposited perpendicular to the sections by electron-beam evaporation (Wepf et al., 1991) in order to prevent charging of sections at low temperature (Braunfeld et al., 1994) and to reduce the electron beam-induced mass-loss/shrinkage. In addition, the Pt/C layer was expected to reinforce the stability of the Epon sections.

2.2. Electron tomography

Specimens for tilt series were investigated at low temperature (~−170 °C) with a Tecnai G2 Polara transmission electron microscope (FEI Company, Eindhoven, The Netherlands) equipped with a Gatan energy filter (GIF 2002, Gatan Pleasanton, USA) and a 2048 × 2048 Gatan CCD camera, operated at 300 kV. Areas of interest were selected according to the orientation of the channel-axis, which at best should be almost parallel to the given tilt axis. The sections were pre-irradiated at a magnification allowing even illumination of the selected grid hole by a total dose of at least 10⁵ electrons/nm² (Braunfeld et al., 1994) in order to minimize potential movements caused by mass-loss/shrinkage during the recording of the tilt series. The automated image acquisition of the tilt series with 1° and 2° angular increments was performed with the acquisition part of the TOM-toolbox (Nickell et al., 2005). Single-axis tilt series were recorded in the zero-loss mode of the energy filter with the energy slit width set to 20 eV; the 1000 mesh Cu-grid allowed for the maximum tilt angle $\alpha_{\max} = |\pm 65^\circ|$. The exposure time was either varied with $1/(\cos \alpha)$

or was kept constant over the entire angular range. The focus was set to $-6\ \mu\text{m}$, at which the first zero of the contrast transfer function (CTF) was located at the spatial frequency of $3.4\ \text{nm}^{-1}$. The total electron microscopic magnification in the CCD plane was $22.9\times$, corresponding to a $1.31\ \text{nm}$ pixel size in the object plane. The pre-irradiated sections allowed collections of tilt series with typical total cumulative doses of $10^5\ \text{electrons/nm}^2$.

2.3. Image processing and three-dimensional reconstructions

Sub-areas have been selected out of the original tilt series, which comprised (i) long channels, (ii) areas where the channels merge into the bacula cavities, (iii) areas where the channels lead to the surface of the grain, and (iv) larger parts of the merged bacula cavities with parts of the channels. Altogether 12 sub-tilt series were subject to the alignment and reconstruction process. The alignment of the sub-tilt series was done by routines programmed in Matlab (MathWorks, Natick, MA, USA) utilizing the conventional cross-correlation scheme, with options similar to the options provided by the IMOD software (Kremer et al., 1996). The logarithm of all projection intensities was taken prior to the computation of the reconstructions because it can be assumed that the acquired images are formed mainly by the scattering contrast due to the specimen preparation technique used. The reconstructions were computed by the weighted back-projection method in the Inspect3D software (FEI Company, Eindhoven, The Netherlands) and by the direct Fourier method in a home-developed C++ programme package, which performs bilinear interpolations in the Fourier space. The Fourier transforms of the images as well as the inverse 3D Fourier transform were computed in Matlab. Finally, the completed 3D reconstructions were low-pass filtered according to the standard resolution criterion for the single-axis tilt series of a thin laterally extended specimen (Rademacher, 1992). The alignment estimates obtained by the sequential cross-correlation were further refined by cross-correlations of the original tilt-series projections with pseudo-projections of the reconstructed volumes computed by a specific home-made C++ programme at the original tilt-series angles. Typically 3–6 cycles of this iterative projection matching were necessary until all detected displacements between the positions of the original projections and the computed re-projections were smaller than 1 pixel ($\sim 1.3\ \text{nm}$). The low-pass filtered reconstructions were finally saved as 8-bit datasets.

2.4. Estimation of the obtainable resolution of the 3D reconstructions

Due to the fact that (i) the bacula cavities layer extends in a direction roughly perpendicular to the channels, and (ii) the micrographs do not possess any features of known dimensions or symmetry, the standard criterion for specimens with extended geometry (Rademacher, 1992) was used to provide the conservative estimate of the achievable resolution. Because the criterion relates the thickness of the specimen, the number of available projections and the maximum tilt angle used, it was mandatory to evaluate the actual thickness of the specimen after the pre-illumination stage (for example, reduction to about 80% of the original thickness for Epon-embedded, Ti-coated specimens at liquid nitrogen temperature and 300 kV acceleration voltage was reported (Braunfeld et al., 1994)). The measurement of the section thickness in the reconstructions was facilitated by (i) the presence of the Pt/C layer on one side of the section, and (ii) the fact that the layer of bacula cavities spreads through the entire vertical extent of the specimen (see Fig. 4). The thickness of the sections in the reconstructed sub-volumes was found to be in the range from 100 to 120 nm, which resulted in the resolution estimates between 6 and 8 nm for tilt series comprising 115–126 projections in the tilting range $\pm 65^\circ$.

2.5. Volume analysis

The contrast of the reconstructed sub-volumes was inverted in order to facilitate the visualization and a more intuitive interpretation of the results. Due to the complexity of the spatial arrangements of the bacula cavities we decided to use simple thresholding as the segmentation method for the reconstructed volumes. An initial estimate of the thresholding level of each sub-volume $I_{t0} = \mu_{\text{bgvol}} + 3 \times \sigma_{\text{bgvol}}$ was determined from the parameters μ_{bgvol} , σ_{bgvol} of the normal distribution fitted to the distribution of voxel intensities in regions within the reconstructed specimen section, which were located sufficiently far from the electron-dense parts of the sub-volume, so that only background voxels were present in the extracted regions. To find a correct thresholding level, a set of untilted re-projections of the sub-volume thresholded at a range of $I_{t0} \pm 20$ thresholding levels were computed. These re-projections were compared with the original untilted projection thresholded at an intensity level $I_{\text{t-orig0}}$, at which the bimodal histogram of the untilted projection had its minimum. This way the channels in the untilted projection were perfectly extracted out of the surrounding exine, and their shape could be compared with the shape of the reconstructed channels in the re-projections in an overlay image. Since all reconstructed features suffered from the typical reconstruction artifacts arising due to the presence of the missing wedge (arcs), the reconstructed channels are broader in the untilted re-projection than the channels in the original untilted projection when the thresholding level is set too low. Inspecting overlay images created by the thresholded original projection and the individual re-projections thresholded at different intensity levels, a reasonable thresholding intensity level I_t could be set to the level, at which the channel arcs along both sides of the channels disappear.

All voxels having intensity $I_{i,j,k} \leq I_t$ were considered as background voxels V_b and their intensity was set to zero, and the voxels having intensity $I_{i,j,k} > I_t$ were assigned as signal voxels V_s keeping their intensity unchanged. The visualization of the thresholded volumes was performed by creating iso-surfaces with the Amira software (Mercury Computer Systems, San Diego) and by a simple volume-rendering programme developed in our laboratory. However, both the visualization of the modified volumes and visual inspection were ambiguous as to the extent to which the bacula cavities were merged and connected with the channels. Therefore, routines for improved object identification in tomographic volumes were developed in Matlab, which analyze intensities of neighbored voxels in the 6-, 18-, and 26-neighborhood. The technique is analogous to the standard algorithms for object identification in images by 4- and 8-neighborhood analysis described by Rosenfeld and Kak (1982). Two neighboring signal voxels were considered as belonging to a conjoint voxel group if the two voxel cubes were connected by faces or by edges. Each voxel group was labeled and characterized by the number of its voxels (i.e., by the volume of the group) and by its dimensions in each of the x-, y-, and z-directions. As a result of the analysis, a list of all voxel groups in the investigated volume with these specifications was generated.

Due to its compact structure, the molecule of the Bet v 1 protein could be approximated by a hard sphere with a diameter of 4 pixels (equal to $5.24\ \text{nm}$), corresponding to the largest dimension detected in the resolved structure of the Bet v 1 allergen (Gajhede et al., 1996). To estimate the potential allergen pathways, the center of the hard sphere was then placed into every signal voxel. The sphere fits into the resolved bacula cavity only if all voxels of the hard sphere coincide with signal voxels of the investigated volume. This simulation enabled us to create 3D maps showing all possible pathways of the sphere within the reconstructed volume such as Fig. 5A and to estimate the extents of the potential allergen

movements in the *x*-, *y*-, and *z*-direction by the neighboring voxel analysis.

2.6. Alignment of partially overlapping 3D reconstructions

We intended to determine of the spatial arrangement of the bacula cavities layer and the extents of the potential allergen pathways in a larger scope. However, we could not find a sufficiently precise alignment of projections of large unbinned sub-areas ($\sim 1.8 \times 1 \mu\text{m}$), as judged from the size and the number of misalignment artifacts in the reconstructions. However, three of the 12 selected sub-areas were chosen so that they partially overlapped and each pair of the overlapping sub-areas had one common channel, as schematically depicted in Fig. 2. It was therefore possible to join the individual low-pass filtered, thresholded reconstructions into a single volume. To estimate the amount of overlap of two reconstructed sub-volumes in *x*-, *y*-, and *z*-direction, the parts of the sub-volumes that were surely located in the overlapping region, i.e., within the hatched areas in Fig. 2, were extracted from both neighbored sub-volumes and subjected to three-dimensional cross-correlation. The mutual displacement of the two sub-areas was derived in the standard way from the position of the maximum of the cross-correlation function. Subsequently, all planes of one of the cross-correlated sub-volumes were shifted according to the position of the maximum of the cross-correlation function, and the intensity values of voxels in the overlapping region were averaged. Slightly different displacement estimates were later obtained by the IMOD's Join programme, which works with projections of the extracted parts of the reconstructed sub-volumes. However, the slightly different alignments did not result in any significant differences in the results of the neighboring voxel analysis.

3. Results

3.1. The channels

All reconstructed channels seem to originate in the upper part of the bacula cavities layer in the form of broad funnels, which gradually merge into long tubes with a diameter of 20–30 nm. These tubes run directly towards the surface of the pollen grain without any branching, interruption or a significant change in diameter (see Fig. 3C and D), fully in accordance with all previous TEM observations (Fig. 1B, C and 3C). The channels end either on one of the surfaces of the section (i.e., running out of the section,

see the *y*-*z* planes in Fig. 4A and B) or about 40–50 nm before reaching the outer surface of the pollen grain (Fig. 3C). Reconstructions of sub-areas with little electron-dense material contained empty channels with only a thin layer of electron-dense material covering the walls (Figs. 3C, D and 4C, D). In the reconstructions of sub-areas with high content of electron-dense material the reconstructed channels appeared almost completely filled with staining material (Fig. 4A and B). The full channels appear oval, prolonged in the normal direction to the section (i.e., *z*-direction at $\alpha = 0^\circ$) while the thin layer of the staining material in the empty channels fades out in this direction (see Fig. 4C and D) as a consequence of the limited range of the tilt angle. Therefore, we assume that the channels are tubes having nearly circular cross-sections with the diameter equal to the minor axis of the oval.

3.2. The bacula cavities

The layer of the bacula cavities within the exine appears partially discontinuous, having a thickness ranging from 60 to 300 nm in the radial direction. Four of the 12 reconstructed sub-volumes with the highest content of electron-dense material in the bacula cavities were subjected to the quantitative volume analysis. It appeared that there were a large number of independent groups of signal voxels in each reconstructed sub-volume. The most of them were very small—the spatial extent of 95% of the cavities did not exceed 26 nm in *x*-, *y*-, and *z*-direction. On the other hand, there was always one major agglomeration of signal voxels in each reconstructed sub-volume, which spread through its substantial part and included 73–85% of all signal voxels detected in the sub-volume. In the merged volume, the largest agglomeration of signal voxels comprised only 61% of all detected signal voxels but still extended more than 1 μm across the section. Some numerical results of the analysis of the investigated sub-volumes are summarized in Table 1, the visualization of the largest continuous group of signal voxels found by the neighboring voxel analysis is presented in Fig. 5C. The channels emerging from the bacula cavities layer were always part of the major signal voxel agglomeration in all investigated sub-volumes.

The neighboring voxel analysis using the maps of voxels where the virtual hard sphere of the 5.24 nm diameter could be placed (see Fig. 5A) showed that the space accessible by the hard sphere movements in the bacula cavities was limited. Although thousands of small individual voxel groups were located in each investigated sub-volume, the neighboring voxel analysis revealed that the electron-dense channels can hold the hard sphere along their whole length and that the largest groups of voxels found in the bacula cavities extended to more than 500 nm (see Fig. 5B). Unlike in the case of the neighboring signal voxels analysis of the thresholded sub-volumes, there is no major group of signal voxels, usually a few voxel groups with at least one extent greater than 100 nm were detected. The merging of the partially overlapping sub-volumes did not reveal any improvement, the largest signal voxel groups detected in the individual sub-volumes stayed intact. The numerical results of the neighboring voxel analysis on the maps of the accessible positions of the virtual hard sphere of the 5.24 nm diameter in the four investigated sub-volumes are summarized in Table 2.

3.3. The Pt/C layer

The Pt/C layer could be recognized in the reconstructed sub-volumes as a discontinuous, rather sparse layer of fine grains. Due to its presence the actual course of the coated exine surface could be followed and the thickness of the specimen could be assessed more easily. The other (uncoated) surface of the section could be recognized only occasionally.

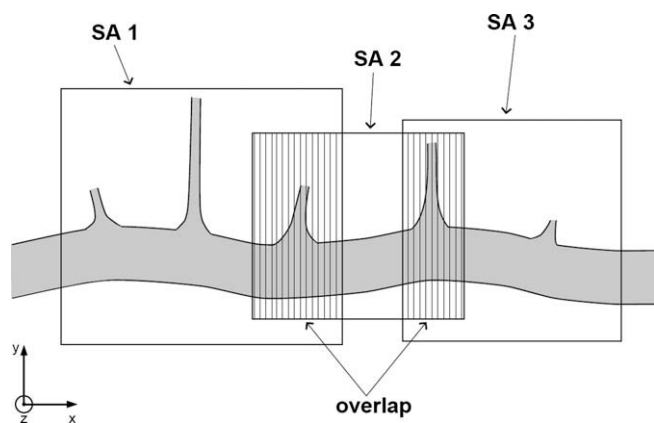


Fig. 2. Schematic illustration of three partially overlapping sub-areas in the untitled projection. The hatched areas indicate the overlapping regions. SA, sub-area.

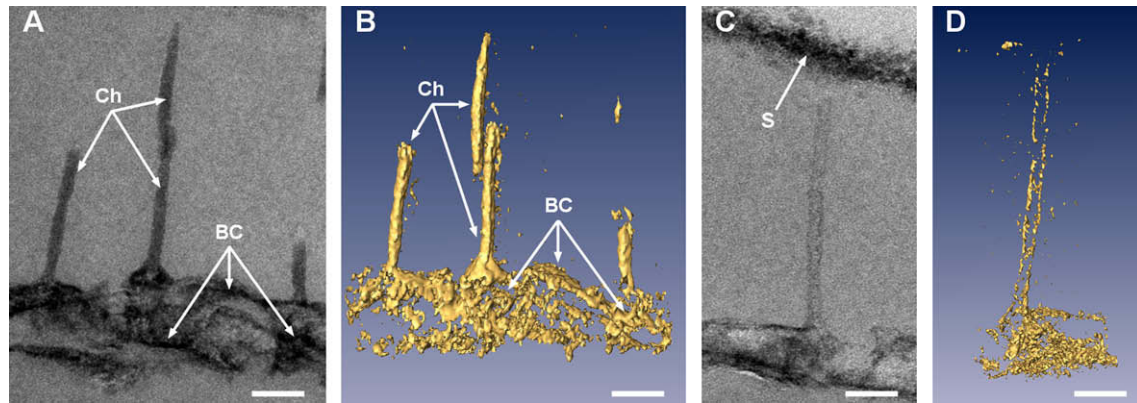


Fig. 3. (A) A sub-area with high content of electron-dense material selected from the untilted projection. (B) A surface-rendered view (Amira software) at the corresponding thresholded and surface-rendered reconstruction. The channels end at one of the section surfaces. (C) The zero-tilt projection of an “empty channel”, (D) its thresholded, surface-rendered 3D reconstruction (Amira software). The electron-dense material on the wall of the channel vanishes at about 50 nm from the surface of the grain. Ch, channels; BC, bacula cavities. Scale bars correspond to 100 nm.

Table 1

Results of the neighboring signal voxel analysis in four reconstructed sub-volumes and in the volume created by merging of three partially overlapping sub-volumes.

Sub-volume number	Sub-volume dimensions $x \times y \times z$ (nm)	Number of independent voxel groups	Largest voxel group	
			Extents $x \times y \times z$ (nm)	Percentage from the total volume of all signal voxels (%)
1	724 × 798 × 724	4324	432 × 598 × 724	82.5
2	583 × 520 × 583	2806	223 × 500 × 582	85
3	763 × 378 × 763	2994	225 × 369 × 545	72.5
4	397 × 389 × 397	1011	379 × 198 × 397	85
Merged 1 + 2 + 3	763 × 798 × 1618	7862	267 × 667 × 1070	61

Table 2

Results of the analysis of the accessible positions of a virtual hard sphere with a diameter of 5.24 nm in four reconstructed sub-volumes and in the volume created by merging of three partially overlapping sub-volumes.

Sub-volume number	Sub-volume dimensions $x \times y \times z$ (nm)	Number of independent voxel groups	Extents of the largest group $x \times y \times z$ (nm)	Number of groups with one extent >100 nm
1	724 × 798 × 724	1770	197 × 514 × 524	7
2	583 × 520 × 583	977	200 × 494 × 415	8
3	763 × 378 × 763	1039	181 × 232 × 202	8
4	397 × 389 × 397	479	90 × 161 × 57	6
Merged 1 + 2 + 3	763 × 798 × 1618	722	170 × 368 × 109	13

3.4. Radiation damage of the specimen

The stained parts of the specimens showed higher resistance to the beam electrons than the coated exine. As a result, the electron-dense stained channels running slightly sagittally across the section, which were expected to end due to the microtome cutting at the top or bottom surface of the section, were found to protrude out of the specimen section in the 3D reconstructions. The electron-dense stain of the channels (elongated in the direction of the missing wedge) protrudes as much as 15 nm out of the surrounding exine on the top surface (see Fig. 4A). The height of the protrusion at the bottom (uncoated side) of the section could not be estimated.

4. Discussion

4.1. Reliability of specimen preparation

Pollen grains present a very special and difficult specimen to prepare. Due to their dehydrated state (water content may be re-

duced down to 30%) and the hydrophobic and extremely resistant sporopollenin-composed outer layer (the exine), freezing techniques have not been very promising. Recently, Ripper et al. (2008) succeeded in cryo-fixation of pollen grains by high-pressure freezing in 1-hexadecene + freeze substitution, after which the morphology of protoplasmic organelles of the pollen grain was much better preserved than in the chemically fixed grains. However, the impact of cryo-fixation to the pollen exine has not been discussed by this study and remains still questionable. We suppose that the microanatomy of bacula cavities is much the same after both cryo and chemical fixation in both dry and wet pollen grains due to the specific chemical composition of pollen exines.

4.2. Electron tomography and interpretation of three-dimensional reconstructions

Our preparatory TEM observations of sections with different thicknesses revealed that ~350 nm thick sections would be most advantageous for our studies because in contrast to thin sections (Fig. 1(B)), the ~350 nm thick sections can span the entire spatial

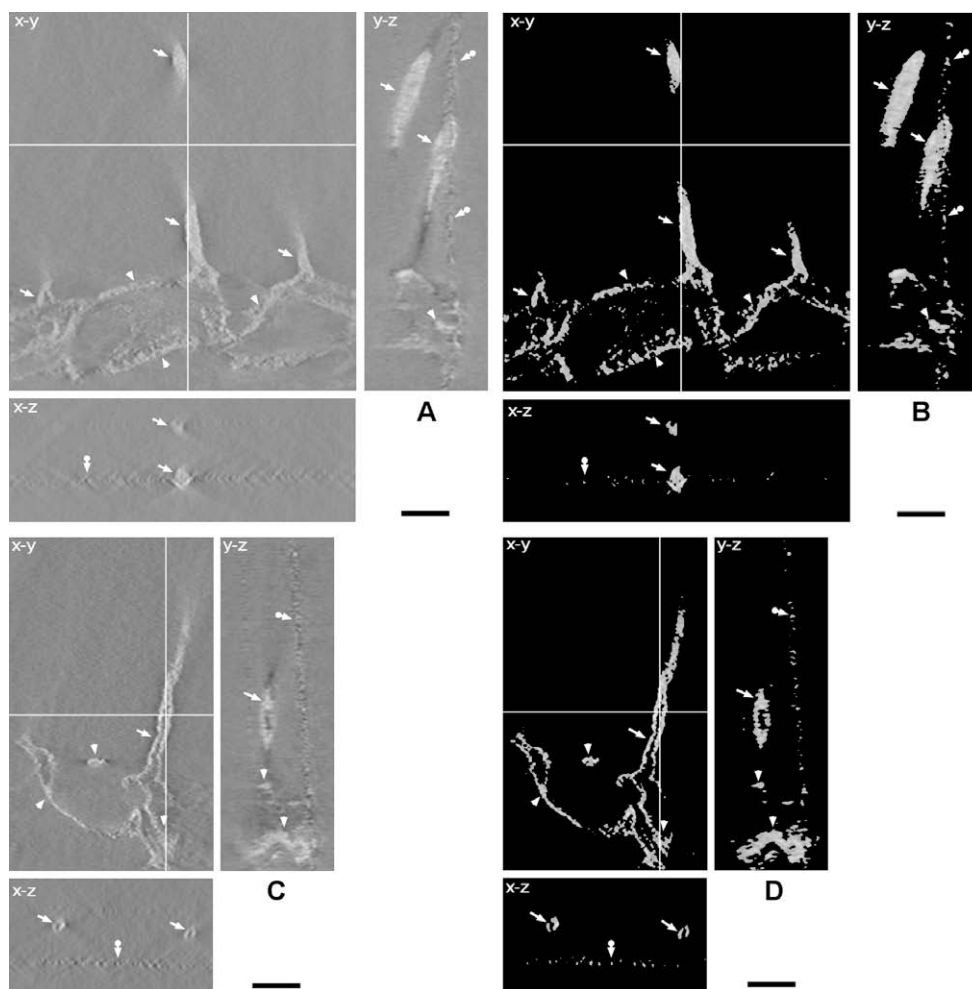


Fig. 4. (A and B) Cross-sections through a contrast-inverted sub-volume with high content of electron-dense material shown in Fig. 3B, (A) before thresholding, (B) after thresholding. The channels end slightly above one of the section surfaces. (C and D) Cross-sections through a contrast-inverted sub-volume containing less electron-dense material, (C) before thresholding, (D) after thresholding. Arrows show cuts through the channels, arrowheads cuts through the bacula cavities. Arrows with ball ends point to Pt/C layer, i.e., its position at the top surface of the specimen. Thin white lines indicate the position of the x - y , x - z , y - z cross-sections in the sub-volumes. Scale bars correspond to 50 nm.

arrangement of the bacula cavities and are more likely to contain channels in their full lengths (Fig. 1(C)). Unfortunately, we have experienced problems with the stability of the ~ 350 nm thick uncoated sections during our earlier tomography attempts, particularly at the attempts to obtain focus of the sections at high tilting angles. Therefore we were forced to reduce the thickness of the sections down to 100–150 nm. In addition, we attempted to increase the stability of the sections by evaporation of a ~ 3 nm thick Pt/C layer onto their top surface. As judged from the variation of the mean intensity of the projections throughout the acquired tomogram, the adopted measures made the specimen sufficiently stable for a high-dose tomogram acquisition after the pre-illumination at low magnification. At the same time, the presence of the thin Pt/C layer did not substantially affect the quality of the tomograms recorded at 300 kV accelerating voltage, as can be seen in the cross-sections through the reconstructed volumes in Figs. 4 and 6.

However, the electron beam-induced movements in the illuminated part of the specimen during the tomogram acquisition were still significant and prohibited thus a sufficiently precise alignment estimation of projections of large sub-areas, which was essential for the investigation of the potential pollen movements at a larger scale. Therefore, we split the large area of interest to three smaller ones, as schematically depicted in Fig. 2, reconstructed each sub-

area independently, and joined the partially overlapping sub-areas by three-dimensional cross-correlation.

The iterative projection matching method, adopted for the refinement of the initial alignment estimates as obtained by the sequential cross-correlation, significantly improved the quality of the three-dimensional reconstructions. As a result, the number as well as the magnitude of the arc-like features emerging from the electron-dense structures decreased, while the shape of the channels in the cross-sections approximately perpendicular to the channel-axis became more compact (see Fig. 6). Still, the achieved improvement was not sufficient for an artifact-free reconstruction of the Pt/C grains on the top of the section in Figs. 4A and 6B. A more sophisticated method for the alignment refinement like the one presented by Cantele et al. (2007) would probably lead to even fewer and less pronounced artifacts. In that study the large volume reconstructed after the preliminary alignment was divided into many neighboring small cubes, whose edges were approximately equal to the thickness of the specimen section. Then the reconstruction was carried out independently from local projections extracted from the original projections corresponding to the position of each cube, and subsequently the position and the angular orientation of each local projection were iteratively refined in several cycles by projection matching. This approach resulted in a substantial increase of the quality of the reconstructions. We adopted the

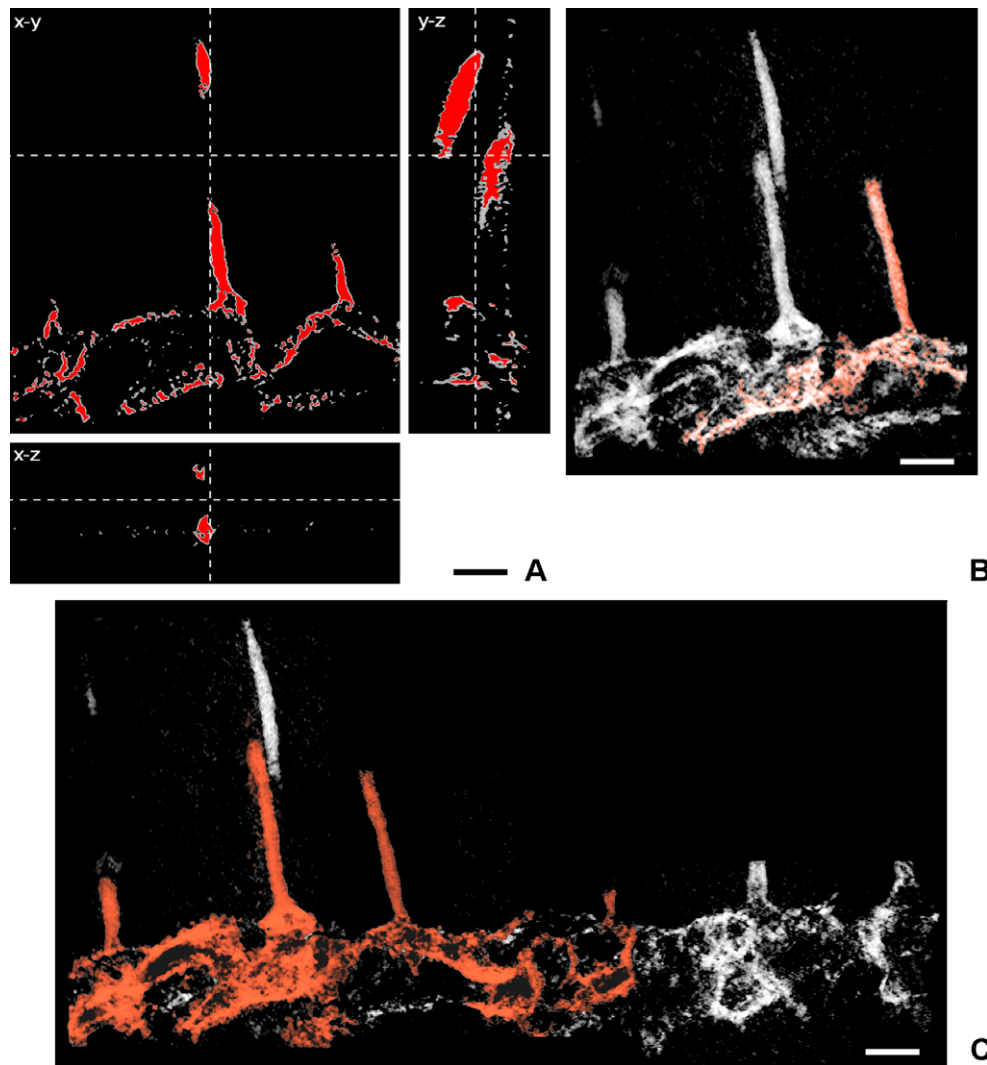


Fig. 5. Results of the analysis of the accessible positions of a virtual hard sphere with a diameter of 5.24 nm within the resolved structures in the reconstructed sub-volume shown in Figs. 3B and 4A, B. (A) The red areas indicate locations where the hard sphere fully fits into the structures resolved by thresholding. (B) The largest detected continuous group of voxels, which can be accessed by the hard sphere, is indicated by red areas embedded within the reconstructed structures shown in gray levels. Scale bars in (A and B) correspond to 50 nm. (C) The largest continuous group of signal voxels found in the volume created by merging of three partially overlapping sub-volumes is shown as the red area. Scale bar corresponds to 100 nm.

more simple strategy, which partially proved to be sufficient for single-axis tilt series of resin-embedded specimens at low temperatures. However, a finer division of the volume of interest into smaller portions than we selected might further increase the quality of the reconstructed volumes.

There are several factors that may substantially influence the final judgement of the structural properties of the reconstructed specimens. Firstly, it must be taken into account that the image contrast of the cavities and channels arises mainly due to the significant amount of copper atoms of Alcian blue. The 3D reconstructions thus basically show the spatial distribution of this dye that binds to the mucopolysaccharides, which may naturally fill the bacula cavities and the channels to an arbitrary extent. Secondly, reconstructions are always hampered by the presence of artifacts. The standard sequential cross-correlation method, as offered e.g., by the Inspect3D and IMOD programme, was therefore followed by several loops of cross-correlation matching of re-projections with the original tomogram projections. In planes perpendicular to the tilting axis, arcs emanating from each channel became smaller in comparison with the standard sequential cross-correlation

method. However, the time required was incomparably higher. The reconstruction artifacts were further reduced by the application of the resolution limiting low-pass filter in combination with thresholding of the contrast-inverted sub-volumes at a high intensity level. Indeed, this action has explicit effect on the results of the quantitative voxel analysis. Because low-pass filtering blurs the potentially sharp edges of the reconstructed structures, voxels within the reconstructed objects located near the edges obtain intensities lower than the threshold intensity and are consequently excluded from the quantitative analysis. The thresholding intensity level must be (i) set at least so high that the majority of the reconstruction artifacts are prevented to participate in the quantitative analysis and (ii) it must be higher than the intensity of majority of voxels in sub-volume regions containing no electron-dense material. Therefore, the first estimate of the minimum thresholding level could be obtained from the parameters of the normal distribution μ_{bgvol} , σ_{bgvol} fitted to the distribution of intensities in regions located within the reconstructed specimen section that are free of electron-dense material. Consequently, the channels in the untilted re-projections of sub-volumes thresholded at the

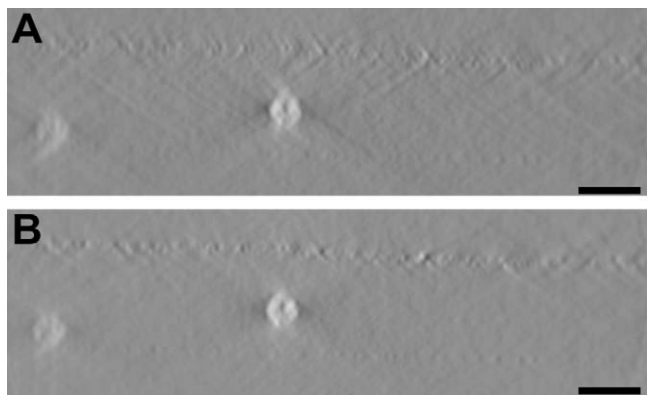


Fig. 6. Improvement of the quality of a three-dimensional reconstruction achieved by iterative projection matching. A selected x - z -cross-section (perpendicular to the tilting axis) through a contrast-inverted sub-volume, which cuts the channels approximately perpendicularly, is shown in (A) after the initial cross-correlation alignment, and in (B) after three loops of the iterative projection matching. Scale bars correspond to 50 nm.

intensity level $\mu_{\text{bgvol}} + 3 \times \sigma_{\text{bgvol}}$ still appeared broader than in the original projection. With increasing thresholding intensity level, the channels in the untilted re-projections become gradually thinner until they reached the diameter of the channels in the thresholded original untilted projection, which could be clearly observed in the overlay images. This level was then set as the final thresholding intensity level I_t . The Pt/C grains on the exine surface were excluded from the calculation of the re-projections by a simple reduction of the extents of the sub-volume in the z -direction. The Otsu thresholding method (Otsu, 1979), frequently used in the image analysis, yielded a lower thresholding intensity value in all cases when applied to our reconstructed sub-volumes.

The prolongation of reconstructed structures in the direction of the missing wedge may cause that adjacent independent cavities are detected as one merged cavity. This effect is partially reduced by the fact that the intensity of a reconstructed structure generally decreases with the prolongation in the direction of the missing wedge. Thresholding thus frequently excludes voxels located within the elongated specimen features from the quantitative analysis.

The high number of very small groups of signal voxels detected by the quantitative analysis may be also placed to debit of several other effects. Firstly, small groups of signal voxels may still appear as a consequence of noise or some small misalignment. Secondly, some cavities may be only partially filled and are thus detected as a group of independent cavities instead of one larger group. Small groups of signal voxels may also represent segments of bacula cavities reaching into the cut section from the neighboring exine layers and the small grains of the Pt/C layer.

The lack of the electron-dense material in the channels within the range of about 40–50 nm from the outer surface of the pollen wall was typical for our TEM observations of sections of hazel pollen grains. The effect is most likely caused by the washing-out of the Alcian Blue from the outermost parts of the channels by the collidin buffer, as suggested by Whiteman (1973). However, in few cases we found the grain surface layer clearly delineated and joined by the channel outlets (see Fig. 7).

5. Conclusion

We have investigated only small portions of the whole bacula cavities layer. As every single part of the bacula cavities layer is unique, it is impossible to generalize conclusions about the structure of the entire layer around the pollen grain. However, all of our TEM observations up to date indicate that the architecture of the bacula

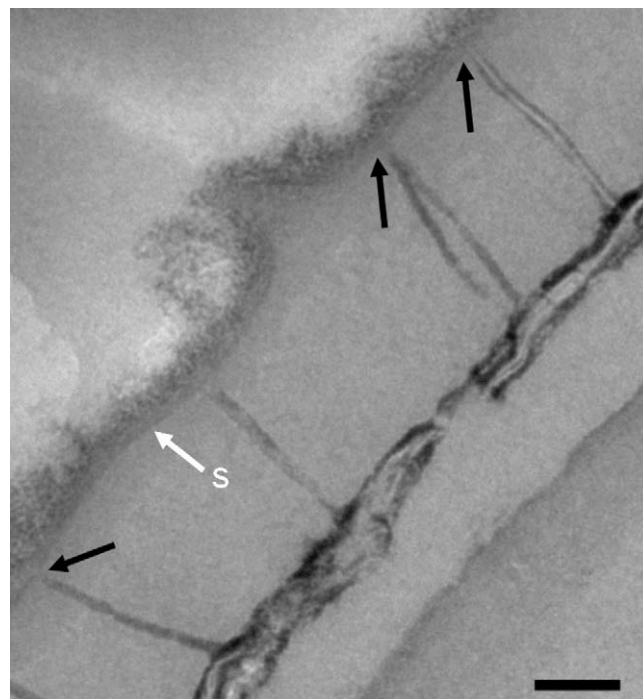


Fig. 7. Transmission electron micrograph of a 150 nm thick section of the wall of resin-embedded hazel pollen grain recorded by a Tecnai G² Sphera 20 (FEI Company, Eindhoven, The Netherlands) at 120 kV. The surface layer (S), which is joined by the channel outlets (black arrows), is clearly delineated. Scale bar corresponds to 200 nm.

cavities layer anywhere in the whole grain wall is basically similar to the structure in the reconstructed sub-volumes, i.e., that the bacula cavities layer is composed of many small cavities, which may form large agglomerates.

Considering the release of the allergens from pollen grains through the channels and the bacula cavities, the results of the performed quantitative analysis clearly show that the allergens may easily traverse through the channels and that the dimensions and the architecture of the bacula cavities also support their movement towards the surface of the pollen grains. We have not found the expected direct long-distance linking of the positions where a hard sphere of ~5 nm diameter could be positioned in the bacula cavities that would explain the very fast release of the allergen proteins after hydration. However, the results of the quantitative analysis strongly depend on the thresholding level, which was set high in order to suppress the typical reconstruction artifacts. Moreover, the hard sphere is only an approximation of the non-spherical shape of the allergen protein, which may freely rotate and thus could pass through tighter bottlenecks than the hard sphere. Therefore the quantitative results that we have obtained should be considered as a first approach results.

Last but not least, the interpolation and volume-rendering software developed in our laboratory is available on request from the corresponding author.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found – in the online version – at doi:10.1016/j.jsb.2009.03.008.

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