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Sporoderm ultrastructure of *Platanus quedlinburgensis* Pacltová emend. Tschan, Denk & von Balthazar from the Late Cretaceous of Germany

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ABSTRACT. The ultrastructure of *in situ* pollen from strongly compressed male capitula of *Platanus quedlinburgensis* Pacltová from the Santonian of Germany was investigated using different staining methods and compared to modern and fossil representatives of Platanaceae and other families. The anthers appear not to have dehisced and are densely packed with pollen, suggesting that the flowers may have become fossilized prior to anthesis. Although the general organization of the pollen wall is identical to that in modern *Platanus*, the relative thickness of the foot layer is larger in the fossil than in modern *Platanus* albeit that the thickness of the foot layer varies markedly in modern species. A conspicuous feature of the fossil pollen is a distinct amorphous to granular layer underlying the endexine, which is more electron dense than the endexine. In uncompressed fossil pollen of the platanaceous genus *Archaranthus* this layer is distinctly granular, whereas in compressed pollen of *Platanus quedlinburgensis* and *Archaranthus* most of the remaining space is filled by a compact dark-staining mass. This mass may partly correspond to the granular layer below the endexine and partly to material of other origin that has been altered during fossilization. A distinct granular layer interior to the endexine is known from a range of *in situ* fossil pollen for which affinities to Platanaceae, Buxaceae, or Hamamelidaceae have been established. A similar layer has been described previously from modern pollen and interpreted in different ways, and has been referred to as "mesine", "membranous granular layer", or "endexine II" by different authors. Because a granular layer below the endexine in modern pollen has mainly been observed in pollen of preanthetic stage, this layer appears to be ephemeral and vanishes upon pollen maturation and final development of the intine. This would explain its preservation in preanthetic fossil pollen.

KEYWORDS: pollen, ultrastructure, foot layer, endexine, granular layer, evolution, fossil preservation

INTRODUCTION

The family Platanaceae has a rich fossil record since the Aptian and *in situ* pollen from extinct and modern members of the family has been reported in a great number of studies (Krassilov 1973, Manchester 1986, Friis et al. 1988, Pigg & Stockey 1991, Crane et al. 1993, Pedersen et al. 1994, Krassilov & Shilin 1995, Magallón-Puebla et al. 1997, Maslova & Kodrul 2003, Maslova et al. 2007, Tschan et al. 2008, Tekleva & Maslova 2011). Furthermore, dispersed pollen of the *Tricolpites-Tricolpopollenites-Tricolporopollenites* group has been assigned to Platanaceae (e.g. Pacltová 1982). Pollen grains of modern Platanaceae are characterized by morphological and ultrastructural features that are widespread among angiosperms, especially among eudicots but they also have several features that make them fairly distinct (e.g. size and tectum ornamentation). Some general features of Platanaceae pollen and possible trends in the group were outlined by Tekleva and Maslova (2004) and

Denk and Tekleva (2006). In view of a much larger diversity of Platanaceae during the Cretaceous and early Cenozoic, detailed investigations of *in situ* pollen grains are crucial in order to better understand the full morphological diversity of Platanaceae pollen.

Pollen grains from staminate heads of *Platanus quedlinburgensis* Pacltová emend. Tschan, Denk & von Balthazar (Santonian, Quedlinburg, Germany) described by Tschan et al. (2008) were previously studied with LM and SEM. In general, they are very similar to modern pollen of *Platanus*.

In this paper, we address the following questions related to the ultrastructure of the pollen of *Platanus quedlinburgensis*: how do ectexine and endexine of the fossil pollen compare to those of other fossil and modern members of Platanaceae and other families? How does the usage of different standard chemicals (osmium tetroxide, uranil acetate, lead citrate) affect the contrast behaviour of exine layers? To what extent can phylogenetic information be extracted from the ultrastructure of pollen?

MATERIAL AND METHODS

Pollen grains were extracted from stamens of the staminate head illustrated by Tschan et al. (2008, pl. XII, A; specimen no. MfN 9323, kept at the Museum für Naturkunde, Berlin). Individual stamens were macerated with Schulze's solution and alkali. The stamens were then divided into several smaller parts for subsequent investigations using scanning (SEM) and transmission (TEM) electron microscopy.

For SEM observations, pollen grains were mounted on a stub (covered with nail varnish) and sputter-coated with gold-palladium. Pollen was observed and photographed using a CamScan SEM at the laboratory of electron microscopy of the biological faculty of Lomonosov Moscow State University (MSU).

For TEM investigations, several protocols were followed:

(1) One part of the material was dehydrated in an ethanol series, dehydrated in acetone, and embedded in Epon mixture according to standard methods (Meyer-Melikyan et al. 2004).

(2) A second part was dehydrated in an ethanol series, stained with uranyl acetate (Ur), dehydrated in acetone, and embedded in Epon mixture according to standard methods (Meyer-Melikyan et al. 2004).

(3) A third part was fixed with 2% osmium (Os), dehydrated in an ethanol series, dehydrated in acetone, and embedded in Epon mixture according to standard methods (Meyer-Melikyan et al. 2004).

(4) A fourth part was fixed with 2% osmium, dehydrated in an ethanol series, stained with uranyl acetate, dehydrated in acetone, and embedded in Epon mixture according to standard methods (Meyer-Melikyan et al. 2004).

Ultrathin sections were obtained with an LKB ultratome V. For each protocol (1) – (4), sections were partly studied unstained, and partly stained with lead citrate (Pb) according to Reynolds' (1963) method. Some sections from the same blocks were stained alternatively for 3–5 minutes and 10–15 minutes.

The ultrathin sections were then studied and photographed with Jeol 100 B and Jeol 1011 transmission electron microscopes at the laboratory of electron microscopy of the biological faculty of MSU.

DESCRIPTION

Platanus quedlinburgensis Pacltová emend. Tschan, Denk & von Balthazar (Santonian, Quedlinburg, Germany)

POLLEN MORPHOLOGY

In situ pollen was extracted from strongly compressed male capitula of *P. quedlinburgensis*. The anthers appear not to have dehisced and are densely packed with pollen, suggesting that the flowers may have become fossilized prior to anthesis. Pollen grains are small, tricolpate, semitectate, columellate and prolate to subspheroidal, with a circular to trilobate outline in polar view, and circular to elliptic outline in equatorial view (Plate 1, Plate 2, figs 1, 2). The polar axis is 19.0 (16.5–22.0) µm (SEM, macerated compression fossils), 15.0–18.5 µm (SEM, untreated compression fossils), 11.5–13.5 µm (SEM, untreated three-dimensionally preserved fossils), and 16.0 (12.5–20.0) µm in LM. The exine was reported to be three-layered, about 0.7–1.2 µm thick (LM), with the sexine being 0.67–0.72 µm in SEM (Tschan et al. 2008). The colpus membrane is covered by globular sculptural elements, the tectum is finely reticulate, and the muri are triangular in cross-section.

POLLEN ULTRASTRUCTURE

The exine consists of ectexine and endexine. In the ectexine, the tectum is 0.44 (0.30–0.69) µm thick, the columellae are 0.19 (0.11–0.30) µm high and 0.19 (0.15–0.25) µm wide; the foot layer is 0.54 (0.39–0.69) µm thick. The endexine is darker (more electron dense) than the foot layer, continuous and compact in non-apertural regions, 0.12 (0.05–0.17) µm thick; it is thicker (av. 0.5 µm, range: 0.25–0.83 µm)

and finely lamellate in the apertural regions (Plate 3, figs 3, 4, 6, 7). Below the endexine is a more electron-dense (in stained material) granular or amorphous layer follows that has been interpreted differently in several previous studies (see Discussion). The thickness of this layer is difficult to establish because of the highly compressed pollen grains.

INFLUENCE OF DIFFERENT HISTOCHEMICAL STAINING ON CONTRAST PROPERTIES OF THE POLLEN WALL

The sections were compared after staining with different combinations of chemicals: without any chemicals (Plate 2, fig. 3), with Pb (not shown), Os (Plate 3, fig. 1), Ur (not shown), Os+Ur (not shown), Os+Pb (Plate 2, fig. 1; Plate 3, figs 2, 3), Ur+Pb (Plate 3, fig. 4), and Os+Ur+Pb (Plate 2, fig. 2; Plate 3, figs 5–7; Plate 4). The least useful results were obtained from sections without any staining, although it was still possible to distinguish all principal exine layers (ectexine and endexine, Plate 2, fig. 3); the contrast in these sections was generally low. Staining with Pb gave similar results (not shown), but we could still not distinguish the endexine and the layer below it. The latter two layers were distinct in the sections stained with other combinations of chemicals (Os+Pb, Ur+Pb, Plate 3, figs 2–4). In the pollen grains stained with Ur+Pb (Plate 3, fig. 4) and Os+Pb (Plate 2, fig. 1; Plate 3, figs 2, 3), the endexine lamellation in the aperture region is clearly discernible, whereas with Ur or Os only, the lamellation is almost indiscernible and the contrast between ectexine and endexine is very low (Plate 3, fig. 1). Best results were obtained from the sections stained with all chemicals (Plate 2, fig. 2; Plate 3, figs 5–7; Plate 4). Longer staining appears to improve the contrast (Plate 3, fig. 4, stained and contrasted with Pb for 10 minutes). Generally, thicker sections showed better contrast, especially in case of "partial" staining (e.g., Os only).

DISCUSSION

Comparison of *Platanus quedlinburgensis* to fossil and modern Platanaceae and other plant groups

Denk and Tekleva (2006) analysed pollen characters of modern and fossil Platanaceae and found several characters of diagnostic value: the exine ornamentation, the reticulum pattern near the aperture regions, the thickness of the exine layers, and the endexine structure. In addition, a trend from smaller to larger pollen size is evident from Cretaceous to modern pollen. Pollen of *Platanus quedlinburgensis*, measured by Tschan et al. (2008), is slightly larger than pollen from contemporaneous fossils and more similar to examples from younger deposits and modern pollen. However, the difference is trivial and may in part be due to changes of the pollen size during preservation.

Pollen of *Platanus quedlinburgensis* is finely reticulate with a reticulum typical of most Cretaceous and early Cenozoic Platanaceae and modern *Platanus* (cf. Friis et al. 1988, Denk & Tekleva 2006, Tschan et al. 2008, Tekleva & Maslova 2011). Another informative pollen character is the reticulum pattern at the transition between non-apertural and apertural regions; in *Platanus quedlinburgensis* the reticulum is not altered near the colpi, the reticulum breaks up and some of the lumina open towards the colpus membrane (cf. Tschan et al. 2008, pl. X, XIV). This condition is also evident in modern *Platanus* and many fossil Platanaceae. The exine ultrastructure also provides some informative characters, in particular the relative thickness of the foot layer and the endexine structure (Tekleva & Maslova 2004 Denk & Tekleva 2006). The thick foot layer encountered in *P. quedlinburgensis* is uncommon among modern members of *Platanus*. Cretaceous representatives of Platanaceae either had a relatively thin foot layer (for example, *Platananthus hueberi* Friis, Crane & Pedersen) or, more commonly, a thick foot layer (for example, *Platananthus scanicus* Friis, Crane & Pedersen). In *Platanus quedlinburgensis*, the portion of the foot layer in the ectexine is about 0.46 as in several Late Cretaceous platanaceous taxa. In pollen of modern *Platanus*, this ratio is normally smaller but may vary considerably, e.g. 0.2–0.34 in *P. orientalis* L. and up to 0.42 in *P. mexicana* Moric. (Denk & Tekleva 2006).

Below the ectexine, two layers were observed that differ in their electron density throughout the pollen, and in the structure under the apertures. The outer, less electron-dense layer, which is finely lamellar in the aperture regions, unambiguously represents endexine. In contrast, the nature of the inner layer is unclear. The darker layer beneath the endexine proper is clearly

distinct from the endexine by its staining properties. It appears granular in some sections but has no consistent structure and in some cases is detached from the endexine.

Pollen of *Platanus quedlinburgensis* is strongly compressed, hence the structure of the layer below the endexine is disguised. A similar layer has been observed in *Archaranthus*, an extinct genus with affinities to Platanaceae (Maslova & Kodrul 2003, Tekleva & Maslova 2004, 2011) and in *Boguchanthus* (Maslova et al., 2007; Boguchanthaceae). In contrast to *Platanus quedlinburgensis*, both compressed and uncompressed pollen grains of *Archaranthus* were available for sectioning (Plate 4). The endexine of uncompressed pollen is homogeneous; below it, a granular layer is developed, which becomes much thicker in the apertural regions (Plate 4, fig. 2). Compressed pollen grains have an endexine of lower contrast followed by a more or less amorphous darker-staining layer filling most of the pollen lumen in the compressed pollen (Plate 4, fig. 3). This architecture is very similar to that encountered in *P. quedlinburgensis* (compare Plate 2, fig. 1 and Plate 4, fig. 3).

Uncompressed fossil pollen grains of similar appearance but slightly different contrast between the homogeneous outer and granular inner layer were figured, for example, by Friis et al. (1988; Platanaceae), Drinnan et al. (1991; affinities of fossil with Buxaceae), Endress & Friis (1991; Hamamelidaceae), and Pedersen et al. (1994; Platanaceae). One exception, showing distinct lamellate structures interrupted by white lines (cf. Plate 3, figs 3, 4, 6, 7) are pollen grains from a stamen with affinities to Platanaceae figured by Friis et al. (1988, pl. 8, figs 4–7). Here, a relatively thick endexine that consists of smooth lamellae beneath the apertures is followed by a darker-staining granular layer.

The nature of the endexine in ***Platanus quedlinburgensis***

What can the granular electron-dense structure evident in *Platanus quedlinburgensis* and in *Archaranthus* be if not endexine? It is widely accepted that intine is not preserved in fossil material (e.g. Traverse 2007, Bernard et al. 2009), although Zavada (2007) speculated that in fossil pollen a differentially staining inner wall layer, commonly interpreted as endexine, might be a remnant of the intine.

An electron-dense layer ("dense lamellar material") between endexine and intine has previously been observed in palynological studies. Rowley (1959, 1962) called this layer mesine; he recorded its presence in unrelated genera such as *Magnolia*, *Saintpaulia*, *Parkinsonia*, and *Centaurea*. Larson and Skvarla (1961) did not coin a particular name for the "opaque layer on the inner face of the endexine", but stated that this layer was not an artefact of staining. Saad (1963) referred to the same structure as medine. A granular layer between the endexine and intine was termed a "membranous granular layer" (MGL) by El-Ghazaly and Huysmans (2001). This layer was observed in species of basal angiosperms, including magnoliids and monocots, and two more derived eudicots (Betulaceae, Rubiaceae) under the name of MGL, "granular layer" (Kreunen & Osborn 1999) or "inner surface of the nexine" (Dessein et al. 2005). El-Ghazaly and Huysmans (2001) suggested that in several published works MGL had also been identified erroneously as intine or endexine. In their earlier work on *Rondeletia*, El-Ghazaly et al. (2001) questioned whether MGL belongs to the endexine or a different layer of the pollen wall. The MGL is most conspicuous in the late free microspore stage and in young pollen grains when the intine starts developing, and commonly is not visible in mature pollen. MGL has not been reported for *Platanus* (Suarez-Cervera et al. 1995, 2005, Denk & Tekleva 2006), but occurs in *Nelumbo* (Kreunen & Osborn 1999) also belonging to Proteales. Recently, Gabarayeva et al. (2009) called a prominent second layer of endexine "endexine-II". In free microspores of *Trevesia* (Araliaceae), a lamellar endexine with white lines is followed by a prominent coarsely granulate endexine-II on the inner side, which is more electron-dense than both the endexine-I and the intine. The structural and chemical properties of these two layers of endexine are most similar to the characters of layers observed in the present study on *Platanus quedlinburgensis* and *Archaranthus*.

It should be noted that all the observations of mesine, MGL, and endexine-II were made on modern pollen grains and, to our knowledge, no such structures have been documented amongst fossil pollen. We face the problem of an interpretation of layers that cannot be traced in their development. Tekleva and Maslova (2004) reported pollen of

the extinct Platanaceous genera *Archaranthus* and *Chemurnautia* as having a two-layered endexine, with the inner part clearly being granular throughout the pollen and the outer one finely lamellar beneath the apertures. In the Cretaceous *Platananthus potomacensis* Friis, Crane & Pedersen, the endexine was described as "mostly homogeneous, but the inner part is finely granular around the aperture" (Friis et al. 1988). In *Aquia brookensis* Crane, Pedersen, Friis & Drinnan and *Hamatia elkneckensis* Pedersen, Friis, Crane & Drinnan, endexine was described as granular in the non-apertural region, "becoming clearly layered" under the apertures; in *Platananthus hueberi* Friis, Crane & Pedersen and in *P. scanicus* Friis, Crane & Pedersen, the endexine was described as "laminate to granular" (Friis et al. 1988). In conclusion, there is some similarity in the layer structure previously described as endexine in fossil Platanaceae – it includes a granular structure, which is most evident on the inner surface of the pollen wall and beneath the apertures where it is thicker and in many cases loosely arranged. In non-apertural regions, this structure may appear homogeneous. Overall, the granular structure of this layer ("endexine") is quite uniform among Platanaceae and also some (unrelated) fossil pollen with similar sporoderm ultrastructure (*Archamamelis* of Endress & Friis 1991; *Spanomera* of Drinnan et al. 1991, *Boguchanthus* of Maslova et al. 2007). If we do not consider the innermost electron dense layer in the material studied here to be endexine, then we should admit that for other fossil Platanaceae the granular structure evident below the aperture may in part be material other than the endexine.

The closest match to the aforementioned types of endexine is encountered in the endexine of *Trevesia* (Apiales, Araliaceae) described by Gabarayeva et al. (2009). For the time being, we hypothesize that the granular structures encountered in the fossil sporoderms described herein correspond to the endexine-II of Gabarayeva et al. (2009).

Effect of different fixation, staining, and post-staining and thickness of sections

Factors such as the developmental stage of the pollen, the preservation and any subsequent diagenesis of the specimens can influence the appearance of the spore or pollen wall at the ultrastructural level and, therefore, may influence the ability of the wall to absorb different stains and, in some instances, to reveal delicate structures such as lamellae. Thus, it is necessary to examine sections of varying thickness and treatments with different concentrations and combinations of stains for varying periods of time (Taylor 1999, Ellis 2007). In our material, thicker sections indeed showed better contrast, although in some other cases (e.g. some sections of *Archaranthus*), thicker sections masked the fine lamellations in the apertural regions.

We used different combinations of standard stains (osmium tetroxide, Os, uranyl acetate, Ur, lead citrate, Pb) to test their influence on the accentuation of pollen wall layers in electron imagery. In our material, sections stained with all three stains gave the best results. Sections stained with either Os or Ur and contrasted with Pb also resulted in well-contrasted images showing delicate structures such as lamellations. However, here, good results were achieved only by increasing the time of staining (Pb) or by using thicker sections (Os+Pb). All other combinations did not result in satisfying images.

In the present study, the sections were stained with uranyl acetate in 70% ethanol before embedding, although we saw no difference between the present results and those of similar material stained with aqueous uranyl acetate (Maslova & Tekleva, unpublished).

Interestingly, pollen treated only with Ur and Pb without fixing with osmium tetroxide, showed rather good results, particularly when stained for a longer time (Plate 3, fig. 4; 10 minutes), whereas for sections stained with Os, Ur and Pb, timing prolonged interval of staining did not make a considerable difference.

Some other methods mentioned in the literature may be advantageous if standard methods are not sufficient. Imaging of specimens with inherently low contrast can be improved by staining first with lead citrate for 1 minute followed by uranyl acetate for 5 to 10 minutes and then a final 1-minute stain with lead citrate (Daddow 1983). In addition, contrast can be improved in weakly contrasted sections by using a smaller objective aperture and/or working at a lower accelerating voltage. The trade-off in working at a lower accelerating voltage is the decrease in resolution; however, decreased resolution will probably not be an

issue when working with lower magnification (Ellis 2007).

No single, universal staining protocol is optimal because not all grains are preserved at the same developmental stage and under the exact same preservational conditions. Structural details (e.g. fine lamellations) or different layers of the wall can be masked or remain unresolved. Thus, it is important to use a range of stain concentrations with varied staining periods (Taylor 1999). Furthermore, in order to be reproducible, exact protocols describing the histochemical staining used must be provided when studying the fine structure of fossil pollen and spores (Taylor et al. 1996).

CONCLUSIONS

The layer observed inside the endexine of *Platanus quedlinburgensis* and *Archaranthus* pollen probably represents a structure that has previously been called mesine, a membranous globular layer, and in particular endexine-II. Regardless of its nomenclature, it has been shown, based on modern material, that both the endexine and the layer interior to the endexine are more or less ephemeral and partly involved in the formation of the intine (Rowley 1962). A distinct granular layer beneath the endexine proper, and very similar to the layer observed in the present study, has been documented in several fossil taxa belonging to basal eudicots (e.g. Platanaceae, Buxaceae) and more derived Saxifragales (Hamamelidaceae; possibly also in other Saxifragales, such as *Cercidiphyllum*, cf. Zavada & Dilcher 1986) and modern groups (Apiales, Araliaceae, Gabarayeva et al. 2009). If the structure observed in fossil pollen indeed corresponds to endexine-II according to Gabarayeva et al. (2009), a possible explanation for its fine preservation may be that the male flowers producing the fossil pollen were commonly fossilized before anthesis. Endexine-II in modern plants is well-developed only in pollen grains before anthesis.

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PLATES

Plate 1

SEM micrographs of staminate capitulum of *Platanus quedlinburgensis* (specimen MfN 9323) and *in situ* pollen displaying reticulate tectum typical of *Platanus*. LM images from the same collection were presented by Pacltová (1982, pl. 6, figs. 8–10) and Tschan et al. (2008)

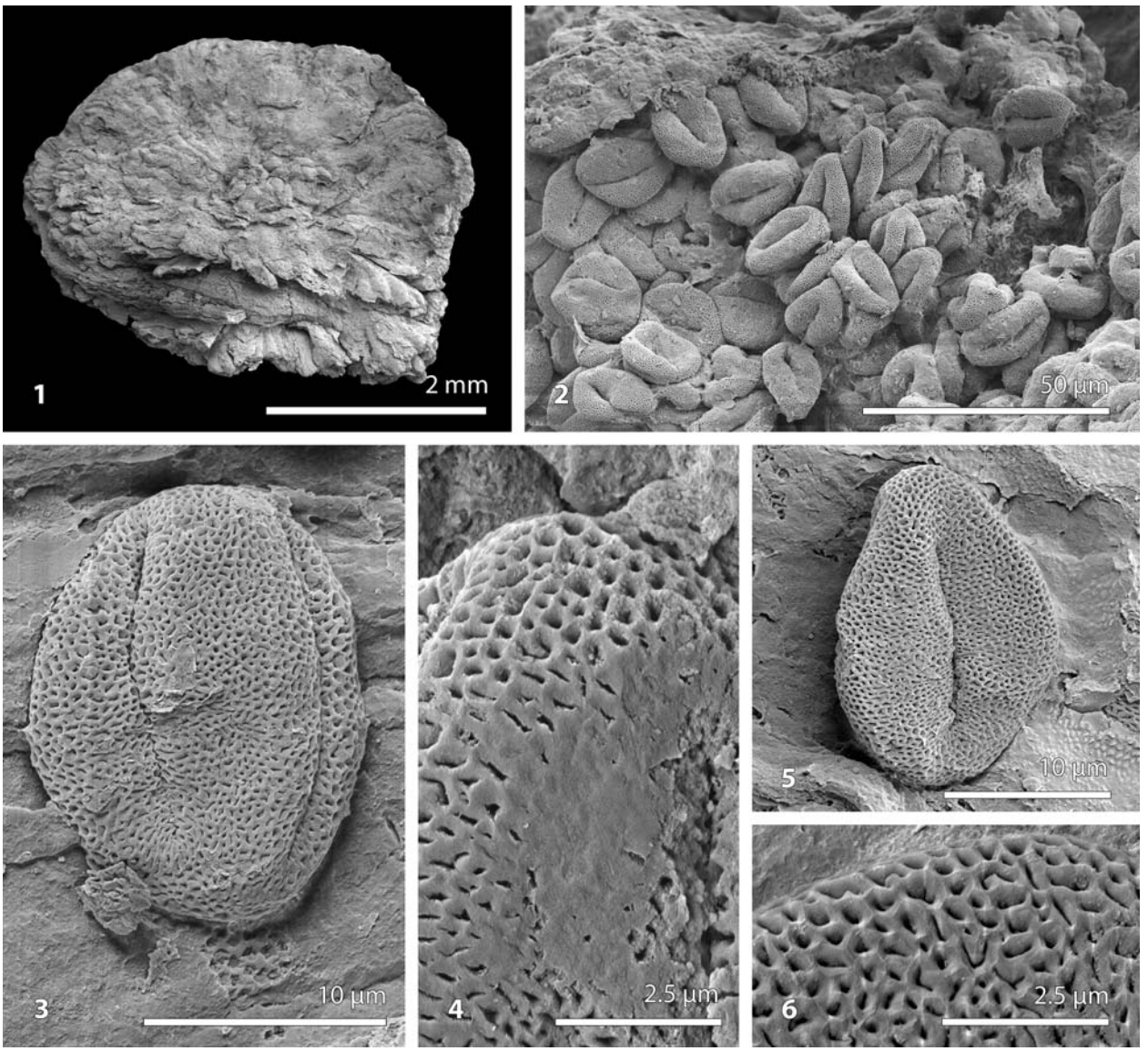
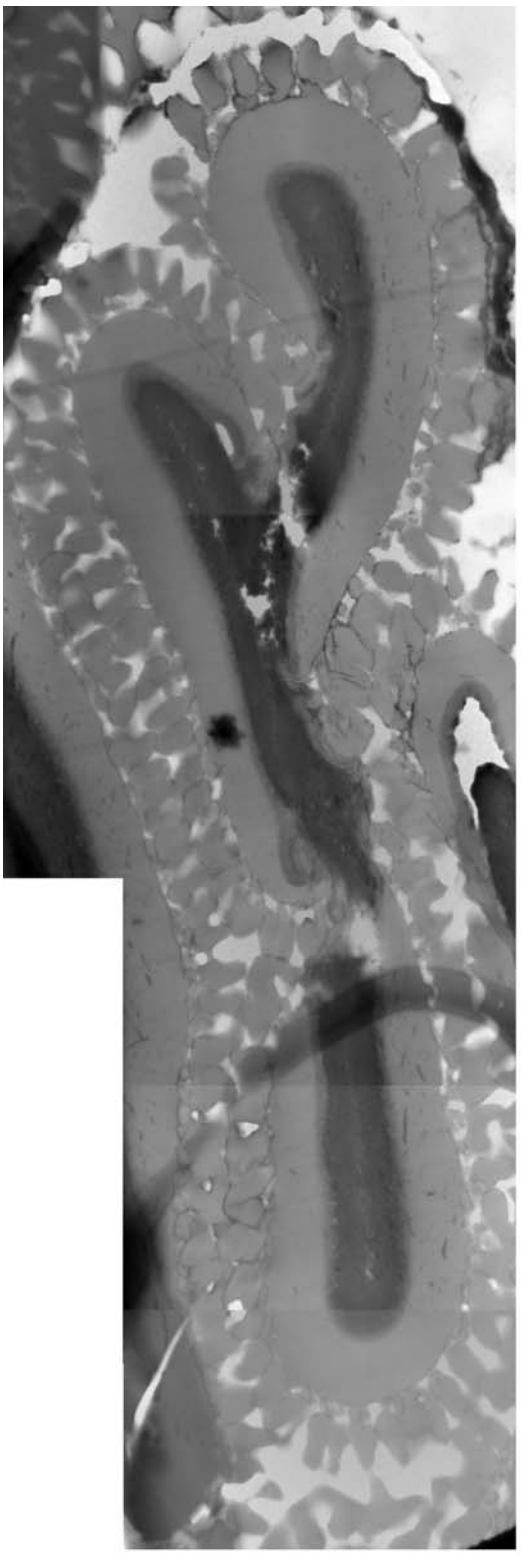

1
2 mm
2
50 µm
3
10 µm
4
2.5 µm
5
10 µm
6
2.5 µm

Plate 2

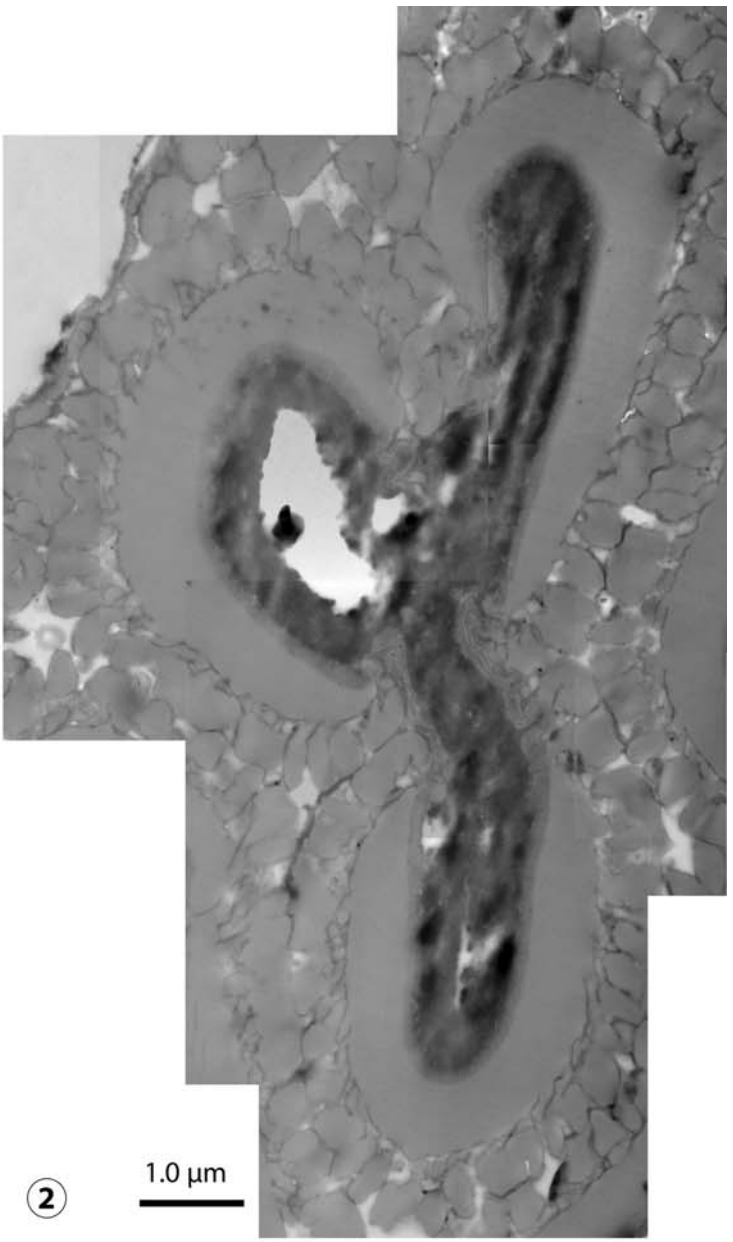
TEM-sections of strongly compressed pollen grains of *Platanus quedlinburgensis* Pacltova emend. Tschan et al.

1–3. Sections of whole pollen grains

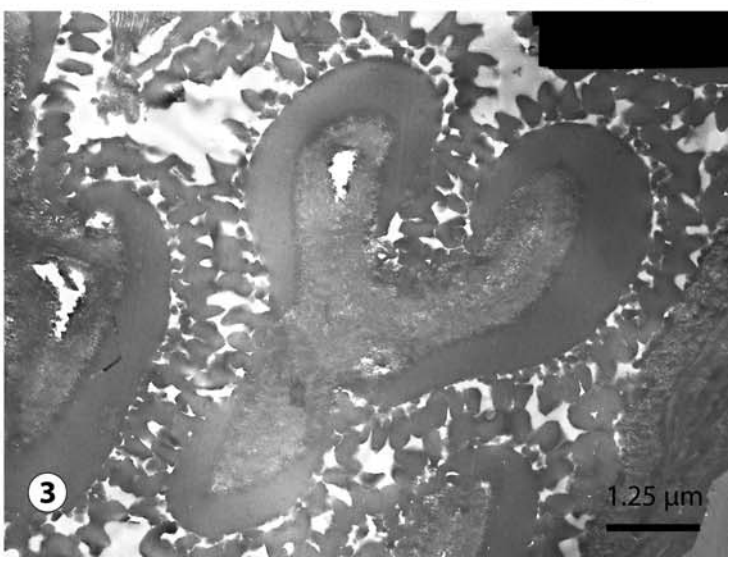
1. Material stained with Os and Pb
2. Material stained with Os, Ur, and Pb
3. Material without staining. Note that the layer below the endexine is lighter than the endexine



1



2



3

Plate 3

TEM-sections of strongly compressed pollen grains of *Platanus quedlinburgensis* Pacltova emend. Tschan et al. treated with various stains

1. Os staining; part of the sporoderm, in which the endexine layer is barely discernible
2. Os+Pb staining; non-aperture to aperture region
3. Os+Pb staining; aperture region, in which the lamellar endexine is evident
4. Ur+Pb staining; aperture region
5. Os+Ur+Pb staining; non-aperture region

6–7. Os+Ur+Pb staining; aperture region

1–6. Arrow heads indicate endexine, asterisk indicates the electron-dense layer beneath the endexine

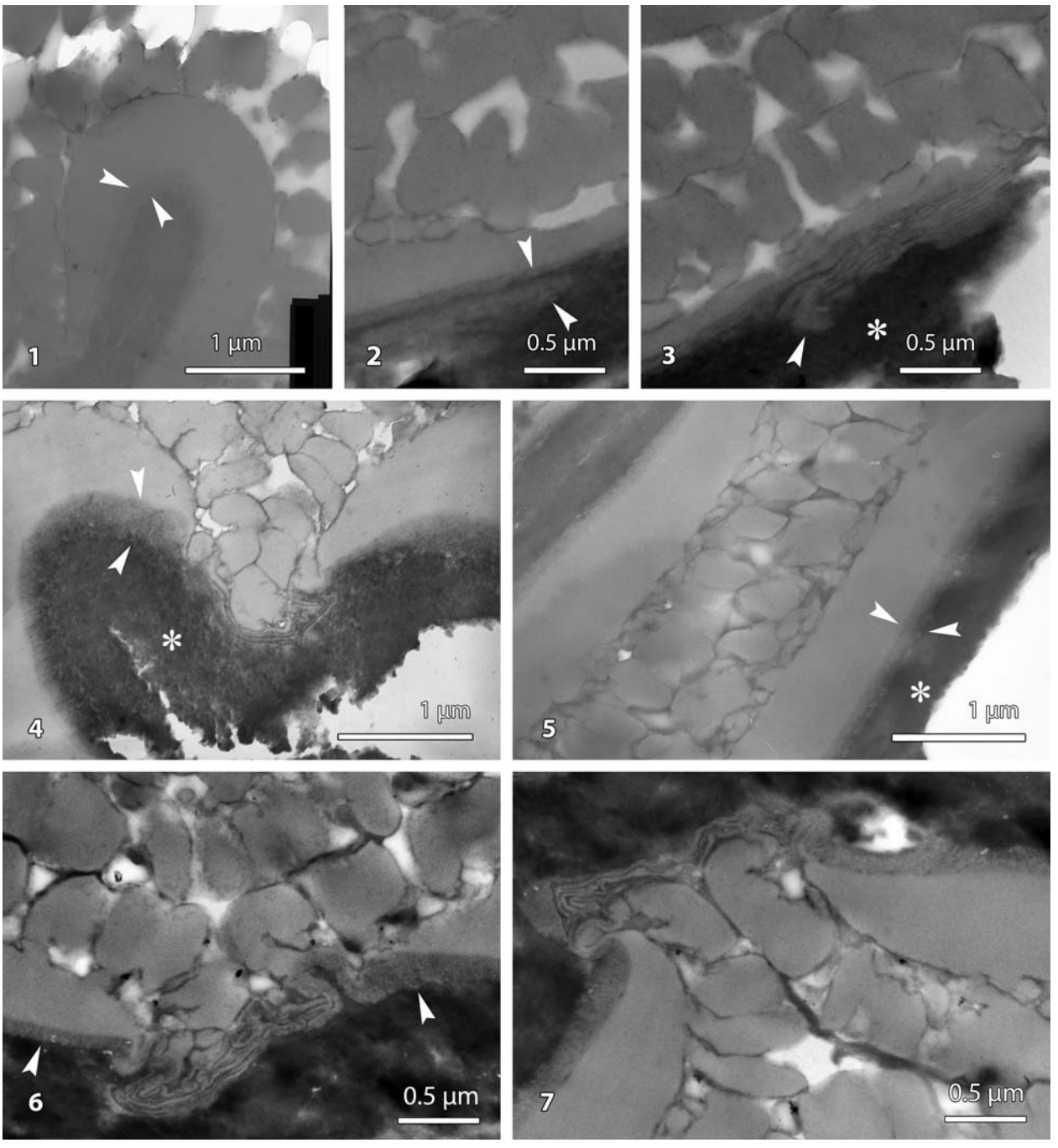

1
1 µm
2
0.5 µm
3
0.5 µm
4
1 µm
5
1 µm
6
0.5 µm
7
0.5 µm

Plate 4

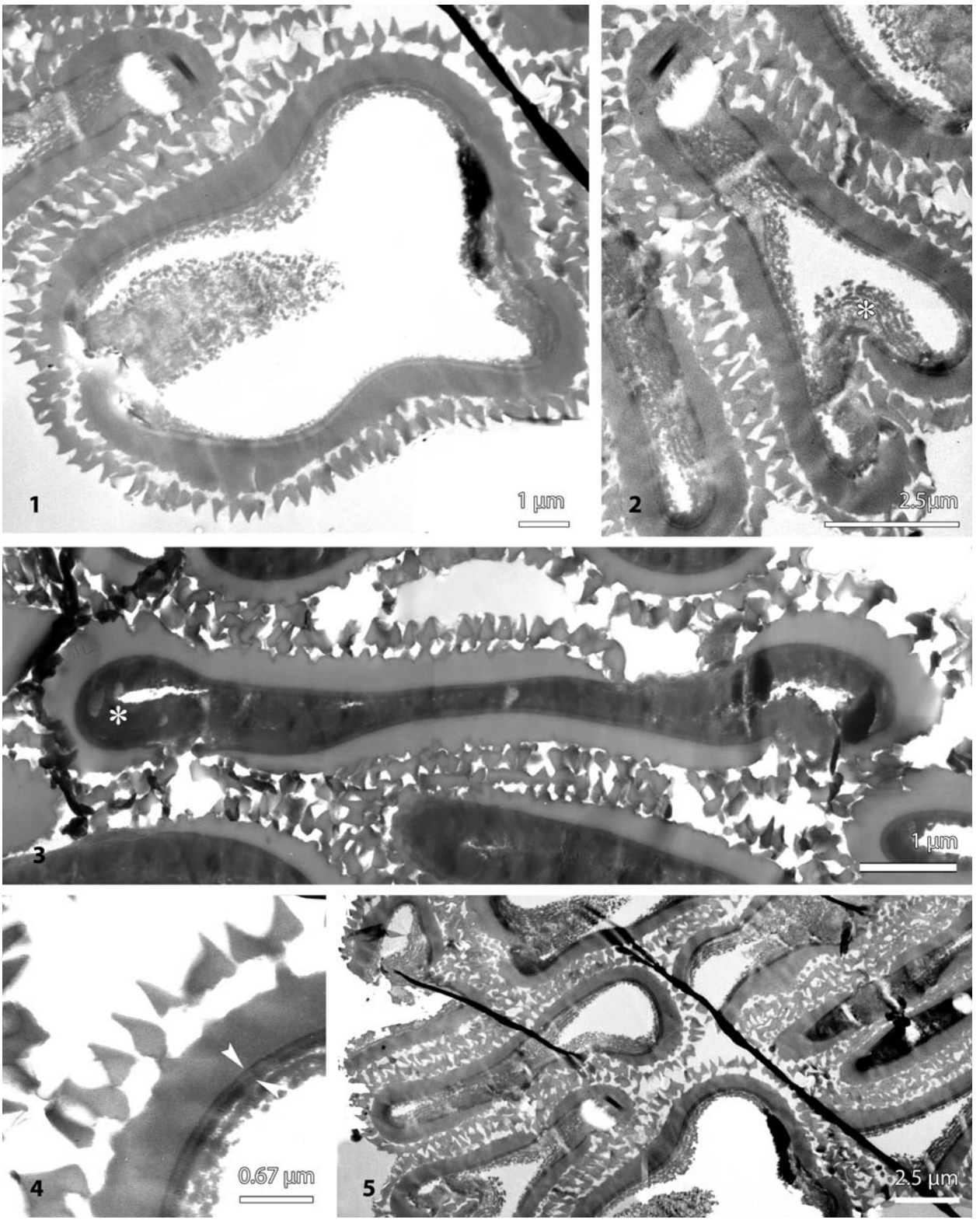
TEM-sections of pollen grains of *Archaranthus krassilovii* Maslova & Kodrul

1–2. Uncompressed pollen, sections of the whole grain, a two-layered "endexine" is clearly evident

3. Compressed pollen, section of the whole grain, two layers of the "endexine" are discernible, asterisk indicates the inner part of the endexine, perhaps not belonging to the endexine proper

4. Uncompressed pollen, non-apertural region, arrow heads indicate outer layer of the endexine or endexine proper (according to the present study)

5. Section through part of the anther


1
1 µm
2
2.5µm
3
1 µm
4
0.67 µm
5
2.5 µm