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Exceptional preservation of Miocene pollen: plasmolysis captured in salt?

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ABSTRACT

Exceptionally well-preserved Miocene pollen from the Bochnia salt mine of southern Poland is reported herein. The halite deposits within the salt mine belonging to Late Badenian (Miocene) marine evaporites originated in the Paratethys. Rounded and angular structures are present inside pollen grains. On the basis of the similarity with plasmolyzed pollen grains of modern plants, these structures are considered to represent cytoplasm plasmolyzed in the condensed brine prior to fossilization. Two forms of plasmolyzed cytoplasm (concave and convex) can be observed in modern pollen. Both are distinguished in the investigated fossil material. In porate and colpiate grains the shape of the plasmolyzed cellular content is concave while in inaperturate it is convex. The plasmolysis form depends on the type of apertures and pollen shape. The percentage of pollen with fossilized cytoplasm within individual taxa is a valuable environmental indicator, as it depends on the proximity of the pollen-producing plant assemblages to the depositional setting.

KEYWORDS | Pollen. Salt. Plasmolysis. Exceptional preservation. Miocene.

INTRODUCTION

Since the Middle Ages the Polish part of Carpathian Foreland basin has been known for its deep salt mines. The salt was deposited in the Paratethys, a shallow sea that infilled the Carpathian Foredeep in the Miocene, and is of evaporitic origin. During salt mining frequent discoveries of plant macrofossils have been reported (Zabłocki, 1928; Zabłocki, 1930; Łańcucka-Środoniowa and Zastawniak, 1997). The palynological research, conducted in the Bochnia salt mine situated 40km E of Kraków (Cracow) in southern Poland (Fig. 1), resulted in finding that not only palynomorphs are preserved in salt but, surprisingly, some of them contain internal structures interpreted herein as their fossilized cell content.

Notwithstanding that cytoplasm has an extremely low fossilization potential, several findings of cytoplasm preserved in fossilized plant-cells of different organs,

ages and type of preservation were reported in the literature (Cretaceous spores, Hall, 1971; Pennsylvanian fern spores and pollen in coal balls, Taylor and Millay, 1977; Glauser *et al.*, 2014; Miocene leaves, Niklas *et al.*, 1978; Carboniferous plant cells, Kizilsthein *et al.*, 2003; Cretaceous conifer foliage cells, van der Ham *et al.*, 2003; Devonian charcoalfied mesofossils, Edwards and Axe, 2004; Cretaceous root and bark cells, Wang, 2004; Cretaceous charcoalfied plant debris, Wang and Dilcher, 2005; Eocene stem cells, Wang *et al.*, 2010; Eocene leaf cells, Wang *et al.*, 2014; calcified stem cells from Early Jurassic, Bomfleur *et al.*, 2014). Interesting discoveries of Tertiary pollen (and dinocysts) preserved in amber were presented by De Franceschi *et al.*, 2000; Dejax *et al.*, 2001; Masure *et al.*, 2013. None of these studies however, dealt with the cell content preserved in salt deposits.

The aim of this paper is: i) to report a finding of fossilized cytoplasm in Miocene pollen grains, ii) to



Figure 1. Map of Poland showing the location of the Bochnia Salt Mine (marked with asterisk on the enlarged part).

present a model showing how these cytoplasm could have been preserved and iii) to indicate how the presence of fossilized cytoplasm affects the interpretation of plant assemblages.

MATERIALS AND METHODS

Pollen material was collected from Middle Miocene salt (Late Badenian, 13.4 - 11.1 Ma old according to radiometric data from tuffites (van Couvering *et al.* 1981; Bukowski, 1999; Wieser *et al.* 2000), and nannoplankton investigation, NN6 zone (Gaździcka, 1994; Peryt, 1999; Andreyeva-Grigorovich *et al.*, 2003) from the Bochnia salt mine (Fig. 1). All investigated halite samples contained fossil pollen but the present paper deals solely with the single halite sample where exceptionally well-preserved pollen grains were observed.

Contamination by recent material was excluded by washing the samples thoroughly (and thus discarding the outer, potentially contaminated layer) under running water. To dissolve the NaCl, the sample was treated with hot water several times. Because salt is strongly contaminated with clastic material and anhydrite, the samples were treated with hydrofluoric acid HF and then sieved through a 10 µm sieve. The heavy liquid ZnCl₂ was used to separate

any remaining mineral particles. Organic matter residuum obtained this way is composed of pollen grains, dinocysts, xylem fragments, and amorphous organic matter particles (mainly resin grains and black clasts). As the pollen morphology was found to be distinct enough for a further analysis, there was no need to conduct acetolysis. During palynological analysis it turned out that many pollen grains display its central part partially infilled with yellowish-brown elements (Fig. 2), referred hereinafter as internal structures. The internal structures were observed also in pollen grains from other salt samples from the Bochnia salt mine; however, because only a few grains per sample showed this kind of preservation, it was not possible to perform any quantitative analysis and environmental reconstruction.

In the examined sample 276 pollen grains on one slide have been counted. The grains are generally well preserved, with easily distinguishable details of exine sculpture.

The experiment on the plasmolysis of recent pollen was conducted at room temperature (ca. 20°C) using saturated, ca. 26% NaCl solution. In cases where plasmolysis did not occur, bathing in 90% ethyl alcohol had been conducted before another plasmolysis attempt was made.

The photographs have been taken under a Nikon Eclipse V100 POL microscope with Nikon DS-Fi1 camera. Fossil pollen was observed using a glycerine-gelatin medium, the fresh pollen grains using water and sodium chloride (NaCl) solution.

RESULTS

Twenty one pollen taxa have been identified in the investigated sample. The results of pollen analysis are given in Table 1. Up to 40% of all counted pollen grains, representing 15 out of 21 identified taxa, possess internal structures. Numbers and percentages of grains with internal structures are listed in Table 1. The structures are yellowish to brown, usually spheroid, but also angular in shape, located more or less in the center of grain (Fig. 2). The spheroid and near-spheroid ones are observed in inaperturate, tricolporate and some porate grains (Fig. 2.1-2.5, 2.7, 2.11). In short-axial tricolporate and most of the porate grains the internal structures are of angular shape, either triangular (Fig. 2.8-2.10) or multiangular (Fig. 2.12), with concave sides. The size of the internal structures, in proportion to the cell size, varies, ranging from smaller (Fig. 2.4-2.5, 2.7) to larger, occupying ca. 50% of space inside the grain (Fig. 2.1-2.3, 2.10-2.11).

The function of the pollen wall is to protect its content from drying out, or from mechanical and chemical

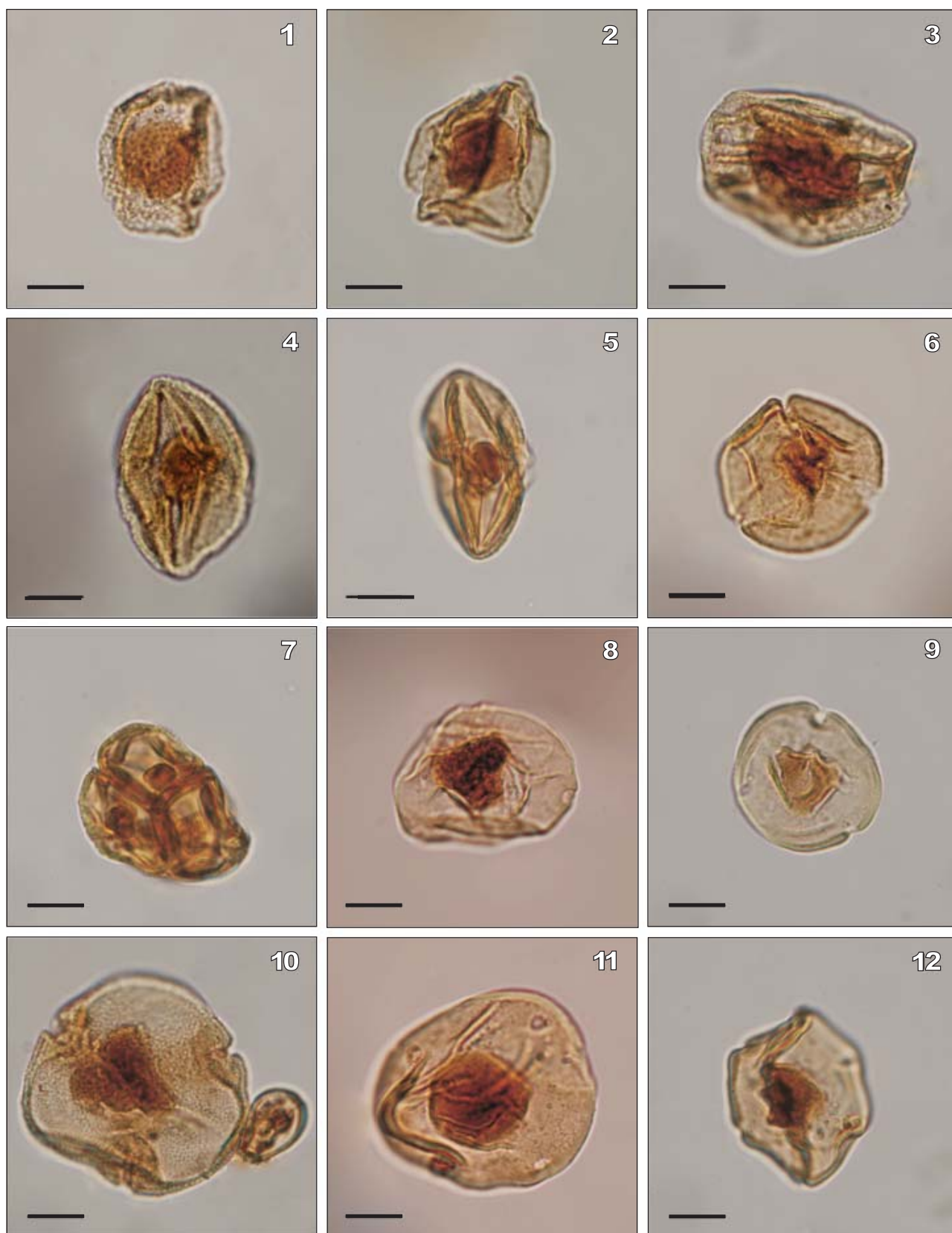


Figure 2. Miocene pollen grains from the Bochnia Salt Mine containing fossilized internal cellular content: 1) *Potamogeton*; 2 and 3) Cupressaceae; 4,5,6) Styracaceae-Fagaceae; 7) Ericaceae; 8) *Engelhardtia*?; 9) *Engelhardtia*; 10) *Tilia* and Cyrillaceae- Clethraceae (small pollen grain); 11) *Carya*; 12) *Pterocarya*. Scale bar 10µm, on all pictures.

destruction. The wall is usually thick, stiff, and covered with waxes. These features make the plasmolysis difficult to carry out. In the course of this study four morphotypes of modern pollen, inaperturate *Taxodium*, triporate *Corylus* and *Betula*, monocolpate *Hyacinthus* and tricolporate *Tilia* and *Rhododendron*, have been subjected to treatment by NaCl solution. In *Taxodium*, *Tilia*, *Corylus*, and *Betula* the plasmolysis occurred immediately after immersion in the brine, while in *Hyacinthus* and *Rhododendron* only some grains experienced plasmolysis, provided that they were previously bathed in ethyl alcohol. The alcohol removes wax from the grain surface. The differences in pollen behavior are difficult to explain. They are probably related to the structure of apertures and wall thickness. The short-axial tricolporate and triporate grains showed a concave

plasmolysis form (Fig. 3.1-3.3, 3.10-3.11). In the case of the monocolpate *Hyacinthus* a distinct cavity in front of the colpus has been observed (Fig. 3.4-3.6), whereas the rest of the pollen contents shrank slightly but remained convex, resembling the grain shape (Fig. 3.5). A similar plasmolysis form has been observed in *Rhododendron* (Fig. 3.9, 3.12). The pollen content in inaperturate *Taxodium* grains shrank significantly taking a generally convex form but its surface remained very irregular (Fig. 3.7-3.8).

DISCUSSION

This study deals with pollen grains of Miocene age, exceptionally well preserved in salt. The grains contain

Table 1. Absolute numbers and percentages of taxa in the studied sample and numbers and percentages of pollen grains with fossilized cytoplasm per taxon. Taxa are assigned to plant assemblages according to the nearest extant relatives habitat. Taxa names given after botanical and morphological (in brackets) taxonomy

Plant assemblage	Taxon	Absolute number of grains	% of grains	Absolute number of grains with cytoplasm	% of grains with cytoplasm in respect to absolute number of grains
Aquatic	<i>Potamogeton</i> (<i>Potamogetonacidites</i> sp.)	6	2.2	6	100
Riparian and swamp forest and shrubs	Styracaceae-Fagaceae (<i>Tricolporopollenites pseudocingulum</i>)	21	7.6	18	85.7
	<i>Quercus</i> (<i>Quercopollenites</i> sp.)	6	2.2	5	83.3
	Ericaceae (<i>Ericipites</i> sp.)	8	2.9	6	75
	Cupressaceae (<i>Inaperturopollenites</i> sp.)	74	27.1	54	73
	<i>Engelhardtia</i> (<i>Momipites</i> sp.)	17	6.2	9	52.9
	<i>Ulmus</i> (<i>Ulmipollenites</i> sp.)	8	2.9	4	50
Mixed mesophilous forest	<i>Acer</i> (<i>Aceripollenites</i> sp.)	1	0.3	1	100
	<i>Tilia</i> (<i>Intratrilporopollenites</i> sp.)	1	0.3	1	100
	<i>Carya</i> (<i>Caryapollenites</i> sp.)	2	0.7	1	50
	<i>Castanea</i> - <i>Castanopsis</i> - <i>Lithocarpus</i> (<i>Tricolporopollenites oviformis</i>)	2	0.7	1	50
	<i>Pterocarya</i> (<i>Polyatriopollenites</i> sp.)	4	1.4	2	50
	<i>Sciadopitys</i> (<i>Sciadopityspollenites</i> sp.)	2	0.7	1	50
	<i>Tsuga</i> (<i>Zoanalapollenites</i> sp.)	3	1.0	1	33.3
	Cyrillaceae-Clethraceae (<i>Tricolporopollenites exactus</i>)	1	0.3	0	0
	<i>Myrica</i> (<i>Myricipites</i> sp.)	1	0.3	0	0
	Vitaceae (<i>Vitispollenites</i> sp.)	1	0.3	0	0
Montane coniferous forest	<i>Pinus</i> (<i>Pinuspollenites</i> sp.)	98	35.8	1	1
	<i>Cathaya</i> (<i>Cathayapollis</i> sp.)	16	5.8	0	0
	<i>Picea</i> (<i>Piceapollis</i> sp.)	1	0.3	0	0
	<i>Sequoia</i> (<i>Sequoiapollenites</i> sp.)	3	1.0	0	0

central structures which are considered here to represent their fossilized cell content. Because during maceration the fossil material was treated with HF and ZnCl_2 the structures should be of organic matter; as otherwise, they would be removed during maceration. Also their colour and light refraction in Light Microscope (LM) are similar as those of other organic particles. The central structures are monochromatic and homogenous. The structural examination including Transmission Electron Microscope (TEM) sections has not been done yet, but it is planned. Cell content during fossilization and diagenesis was exposed to unknown environmental parameters. Thus its structure could have been changed significantly (*i.e.* coalified). The discussion presented here is based only on morphology and similarity of central structures to plasmolyzed cell content of fresh pollen. As these structures occur in 40% of grains, and show the same shape for each particular pollen morphotype, it is rather unlikely that they represent artifacts. The pollen grains of *Tilia* from examined material (Fig. 2.10) and *Tilia* found in amber (De Franceschi *et al.* 2000, fig. 5) are similar in the general outline of the cell content. However, in amber the areas in front of pori are convex while in salt are concave. The structure inside the grain preserved in salt is significantly contracted in comparison to that in amber. The shape of the pollen from salt is also similar to the plasmolyzed internal cell content in recent pollen (Fig. 3.11).

Due to the preserving properties of salt (*e.g.* Caputo *et al.*, 2011), the presence of fossilized cell content in Miocene halite deposits should not be surprising. Palynological investigations confirmed that the presence of salt in the sediment provides a good chance for pollen preservation. The experiment made by Campbell and Campbell (1994) showed that the growth of salt crystals, that envelope the grains, may help to preserve them thanks to mechanical stabilization.

The Paratethys, where the investigated material was deposited, buried and fossilized, was a shallow basin (30–40m deep, Bukowski, 1994), where evaporites were formed according to a drawdown salina basin model (Babel, 2004). On the basis of the bromine content in halite (20–67ppm) it can be stated that the concentration of the primary Badenian salt brine was usually low and only incidentally higher (221ppm) (Garlicki and Wiewiórka, 1981; Garlicki *et al.*, 1991). Halite crystallization occurs when brine concentration is ten to eleven times higher than in seawater (Warren, 2006). In this context a low brine concentration should be understood as reaching at least 35%. This is comparable with values reached in the Dead Sea.

The term ‘plasmolysis’ was introduced by de Vries (1877) and defined as “the separation of the living

protoplasmic envelope from the cell wall, caused by external water-withdrawing solution” (after Oparka, 1994). Oparka (1994) recapitulated the knowledge about the process and studied structural and ultrastructural changes, which occur during plasmolysis and their implications for the functioning of the cell. He distinguished several plasmolysis forms – the ways in which cytoplasm retracts from the cell wall. Two plasmolysis forms can be taken into consideration when studying Badenian pollen material: convex and concave. Convex plasmolysis means that the protoplast separates from the smaller transverse walls of each cell, thus forming a symmetrical protoplast with convex ends (Oparka, 1994). Concave shapes exist in concave pockets formed along longitudinal cell walls (Oparka, 1994). The form of plasmolysis depends on protoplasmic viscosity and the strength of bindings between the plasmic membrane and cell wall (Lee-Stadelmann *et al.*, 1984). The contraction of cytoplasm is believed to be repeatable for tissues of the same age however it can be changed *e.g.* by wounding (Sachs, 1972) or can depend on ionic balance of the plasmolyticum, the solution that causes plasmolysis (Lee-Stadelmann *et al.*, 1984). As Oparka (1994) studied the plasmolysis using plant tissues, his observations were made on elongated, frequently angular cells. The pollen grains are rounded or elongated, very rarely angular thus the definitions concerning “longitudinal” or “smaller transverse walls” probably cannot be fully adopted here. Although some studies documented the effect of osmotic potential on pollen viability (*e.g.* Reddy and Goss, 1971; Lopes Loguerio, 2002), no experiments, so far, were focused exclusively on the plasmolysis process in pollen.

According to the results of experiments performed on modern material, it is stated that the plasmolysis in pollen is a complex process, controlled by many factors. These are presumably: the pollen shape, type of apertures and their arrangement on the grain surface, pollen wall thickness as well as a distribution of mechanical stress inside the grain. Since apertures are an adaptation to pollen germination, the exine thickness inside apertures is much smaller than in interporium/intercolpium. Due to this feature and properties of pollen wall (see results chapter), the plasmolyticum enters the grain via apertures. The pollen content contracts in front of the pores or colpi giving the plasmolysis a concave form. Such forms are visible in modern triporate *Corylus* (Fig. 3.2–3.3) and *Betula* grains, and also in short-axial tricolporate *Tilia* pollen (Fig. 3.11). Internal structures of angular shape observed in fossil pollen (Fig. 2.8–2.10, 2.12) theoretically fit in this plasmolysis form because concave walls occur in front of the apertures. In *Engelhardtia* (Fig. 2.8–2.9) and *Tilia* (Fig. 2.10) the shape of the structure is triangular, while in hexaporate *Pterocarya* six concave sides are distinguishable (Fig. 2.12). Although internal structures show an angular shape quite frequently, in most of the

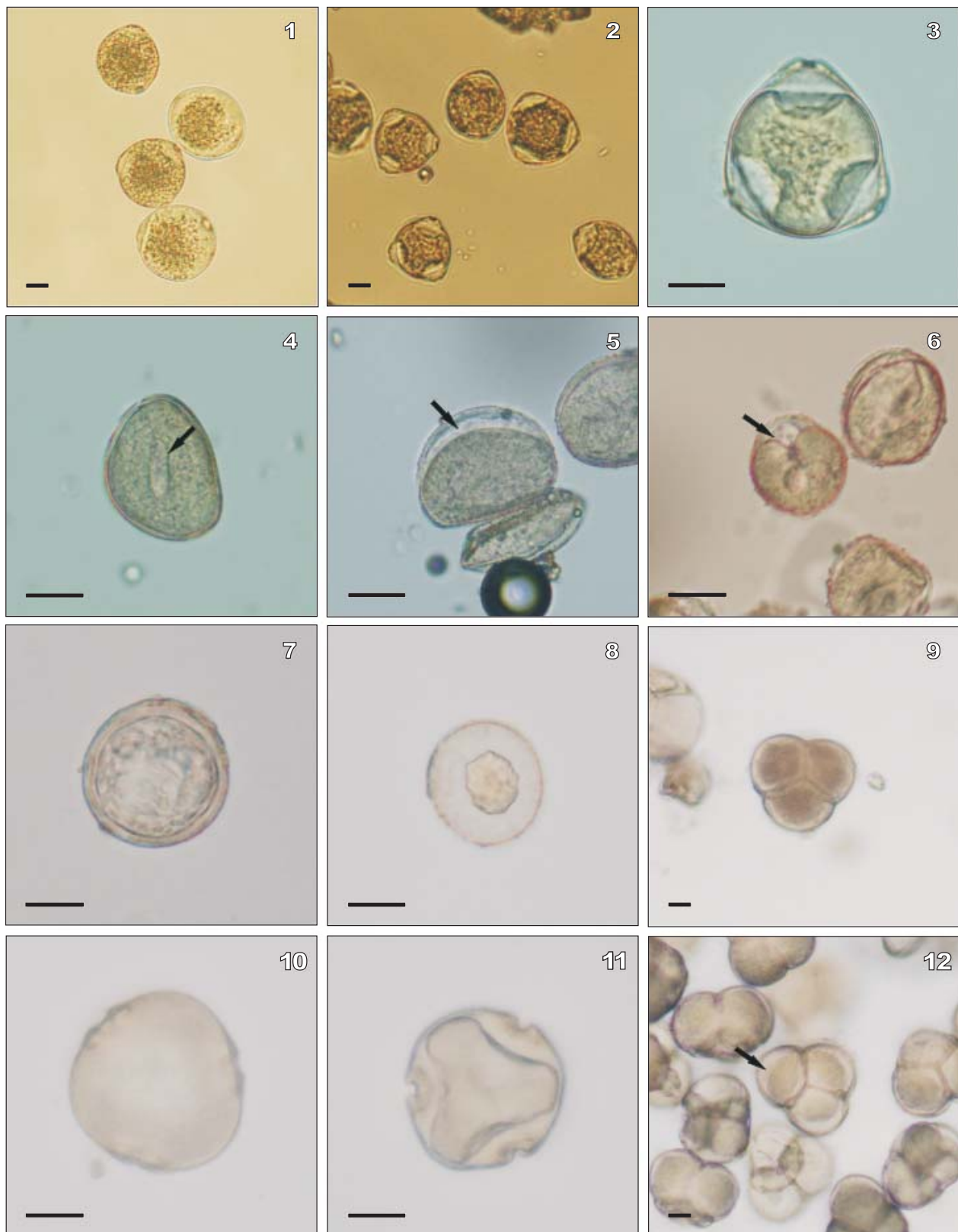


Figure 3. Recent non-plasmolyzed and plasmolyzed pollen grains: 1) non-plasmolyzed *Corylus*. 2 and 3) plasmolyzed *Corylus*; 4) non-plasmolyzed *Hyacinthus*, arrow indicating colpus; 5) plasmolyzed *Hyacinthus*, arrow indicating convex cellular content resembling the grains shape; 6) plasmolyzed *Hyacinthus*, arrow indicating cavity in the cytoplasm, in front of the colpus. 7) non-plasmolyzed *Taxodium*; 8) plasmolyzed *Taxodium*; 9) non-plasmolyzed *Rhododendron*; 10) non-plasmolyzed *Tilia*; 11) plasmolyzed *Tilia*; 12) plasmolyzed *Rhododendron*, arrow indicating convex cellular content resembling the grains shape. Scale bar 10µm, on all pictures.

examined fossil pollen grains the structures are apparently of spheroidal form and seem to represent a convex form of plasmolysis. In practice, they are indeed spheroidal in inaperturate grains of Cupressaceae (Fig. 2.2-2.3) and *Potamogeton* (Fig. 2.1). In tricolporate grains (excluding short-axial *Tilia*) the structures are spheroidal in equatorial view. In polar view they turn out to be concave because in front of the colpi cavities are present (Fig. 2.9). These cavities are shallower and less pronounced in comparison to the porate grains. Taking into account observations on plasmolysis in modern *Hyacinthus* pollen (see results chapter), the important role of apertures as a factor controlling plasmolysis shape cannot be overstated.

Therefore, the following interpretation of the plasmolysis process in the Badenian pollen can be proposed. In inaperturate pollen the brine penetrated the grains via soaking through the wall. The plasmolyticum inflow was equal on each pollen side which resulted in an evenly contracted cellular content. In tricolporate and porate grains the solution attacked the internal cellular content in a more “targeted” way causing its rapid contraction, first in front of the apertures. However, tricolporate grains are elongated (excluding short-axial ones) and their apertures are relatively large in contrast to flat zonoporate ones. These features caused the solution inflow to be more evenly distributed, resulting in more spheroidal shapes of the fossilized cellular contents within tricolporate grains (in comparison to angular shapes in porate ones). An exception to the presented model is the triporate grain of *Carya* (Fig. 2.11) in which the internal structure is rounded. To summarize it should be stated that not only the number and size of the apertures affected the fossilized shape of the internal cellular content but also the grain’s shape played a role. Probably due to other processes occurring during fossilization (e.g. dehydration), the internal structures are much more shrunken and never connected to the grain’s wall, in contrast to modern plasmolyzed pollen content. However, the general shape remains the same (for comparison: *Taxodium*, Figs. 2.2; 3.8; *Tilia*, Figs. 2.10; 3.11; *Engelhardtia*, Fig. 2.8; *Corylus*, Fig. 3.2-3.3).

Internal structures have been found in ca. 40% of counted grains. The frequency of pollen grains containing these structures varies between individual taxa, ranging from 0 to 100% (Table 1). A question arises here why in some taxa the fossilized cellular content is preserved, while in others is absent or very rare. Little is known about how long the cellular content can survive in dispersed pollen. However, the average pollen longevity is estimated to be of the order of several days (Dafni and Firmage, 2000, and references therein). After several days the pollen wall becomes degraded by bacteria and phycomycetes (Elsik, 1966, 1971; Goldstein, 1960). Therefore the degradation

of the cytoplasm in natural environments is quite fast. Cytoplasm can remain within the pollen grain for a longer time, if a preservation agent is present. Examples of the preservation of pollen grain’s content are known e.g. from amber (De Franceschi *et al.*, 2000; Dejax *et al.*, 2001), from bulk sediments of permafrost (Willerslev *et al.*, 2003) or from Holocene lake deposits (Bennett and Parducci, 2006). In the two latter cases the extraction of DNA from pollen grains was even possible.

Pollen transported by water (in rivers) or by the wind will be exposed to mechanical damage and enhanced microbial degradation and will suffer a higher rate of decay of its internal cell content. In this sense, taphonomic alterations, including transport, should be taken into account to explain selective cytoplasm preservations. Thus, it could be expected that the cytoplasm that underwent plasmolysis could be preserved only if fresh pollen fell directly into concentrated brine. After burial in salt it may have persisted there for millions of years under stable, anaerobic conditions that are toxic for most decomposing microorganisms. Following this reasoning, it can be proposed that taxa with a high percentage of grains showing preserved internal structures represent plants growing directly on the shore of the depositional area. Taking into account both the percentage data of pollen with fossilized internal contents (Table 1) and the knowledge about Neogene European plants (e.g. Mai, 1995), the following plant assemblages could be proposed to grow near the depositional site: i) an aquatic vegetation dominated by *Potamogeton* (all grains with fossilized cytoplasm), ii) riparian-swamp trees and shrubs (at least 50% of grains with fossilized cytoplasm) including Cupressaceae, Styracaceae-Fagaceae, *Quercus*, Ericaceae, *Engelhardtia*, and *Ulmus*; iii) a mixed mesophilous forest, represented by rare pollen grains with or without cytoplasm, including *Acer*, *Carya*, *Castanea-Castanopsis-Lithocarpus*, *Pterocarya*, Cyrillaceae-Clethraceae, *Myrica*, *Sciadopitys*, *Tilia*, *Tsuga*, *Vitaceae*; and iv) a montane coniferous forest (pollen grains frequencies depending on the type of adaptations to wind transportation, occasionally with internal structures) including *Pinus*, *Cathaya*, *Picea*, and *Sequoia*. In general terms this model of plant communities corresponds to that known from numerous studies (e.g. Kovar-Eder *et al.*, 2008; Ważyńska *et al.*, 1998). Therefore, the hypothesis is proposed that the probability of cytoplasm preservation depends on the distance from the source plant to the depositional site.

Further examination of central structures should be performed in order to study their anatomy. The use of some new methods could reveal interesting results, as was shown by Glauser *et al.* (2014). Raman spectroscopy allowed the authors to reject the hypothesis proposed by Taylor and Millay (1977), which was based only on morphological

observation, concerning the presence of presumed nuclei in Pennsylvanian pollen and spores preserved in carbonaceous coal balls. It is not possible to compare this finding with the results reported herein (calcite vs. halite as preservative agent), but this example shows that some new data can be expected after the detailed structural studies are performed.

CONCLUSIONS

Pollen grains are frequently documented in rocks of different ages, however the remains that are usually preserved are only the empty “envelopes” and the preservation of cell contents is rarely observed. This is why, the finding of pollen with internal organic structures, preserved in 13Ma Badenian halite deposits, is exceptional. The organic matter was preserved within pollen grains due to the preservative properties of salt. Although the anatomy of the central structures has not yet been studied, their characteristic and homogeneous shapes bring them in relation with plasmolyzed pollen content and would exclude an artificial origin. The similarities between fossil and recent (plasmolyzed) pollen cell content has been also documented herein. Two forms of central structures, concave and convex, have been observed in the fossil pollen studied. Experiments made with modern, morphologically similar pollen grains revealed very similar shapes of plasmolyzed cytoplasm, despite the fact that fossilized cell contents were significantly shrunken. Based on these facts, it is concluded here that the central structures represent a mass of plasmolyzed cytoplasm preserved in condensed brine and later fossilized.

The presence of preserved cellular contents, in around 40% of grains, brings an insight on pollen producing plant communities. The percentage of pollen grains with preserved internal content is very different between taxa and varies from 0% to 100%. The cytoplasm is short-living and the prolonged transport might result in its decay and its consequent absence from the fossil material. Thus the observed differences in frequency of appearance are interpreted as a result of different distances from pollen producing plants to the depositional site. The shorter the distance was, the more likely the preservation of cellular content would occur. This percentage gives, therefore, some indication of which plant community the given pollen taxon might have been produced in.

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