Session a4

PALYNOMORPH WALL CHEMISTRY, STRUCTURE AND ASSEMBLY

Macromolecules in modern and fossil spores, pollen and algae

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Recognizable microfossils, such as pollen, spores and algae, in palynological slides have a long history of use, ranging from stratigraphic markers to entities that provide palaeoenvironmental and palaeoecological information. In most cases, the main underlying reason for their preservation in the fossil record is the fact that they are preserved as acid-resistant organic entities. However, this latter character may significantly affect the fossil record as differences in the chemical composition of these microfossils may bias against those that contain less resistant organic constituents. In order to understand and, possibly, correct for such biases, detailed insights into the chemical composition of both modern and fossil spores, pollen and algae is needed. This study presents an overview of the currently available literature information in combination with new molecular data from modern, sub-fossil and fossil microfossils, including spores, dinoflagellates, and other algae. The new data clearly reveal that the chemical composition of the fossil microfossils is in most cases not representative of that of the original biomacromolecules present in the modern counterparts and that significant chemical changes occur during fossilisation.

MALDI: a new technique with the potential to solve the sporopollenin enigma

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In the forty years of research into the chemical structure of sporopollenin, numerous chemical and instrumental techniques have been brought to bear on this complex biopolymer. Work using solid state ^{13}C- and ^{1}H- NMR and showed the presence of aromatic, aliphatic, and oxygen functionalities. Recently, pyrolysis and GC-Mass spectral analysis have elucidated a number of structural characteristics of sporopollenin. This GC-MS work suggests that two principle components of sporopollenin are fatty acids (C14-C16) and oxygenated cinnamic acid derivatives. We are currently investigating the use of Matrix-assisted laser desorption ionization (MALDI) mass spectrometry as a method of structure elucidation of sporopollenin. A relatively new technique, MALDI mass spectroscopy is a 'soft' technique used to ionize molecules up to several hundred kDa molecular mass. It is especially valuable in the detection and characterisation of biopolymers present in mixtures. We hoped to use this technique to gain insight on the component polymers of sporopollenin. Sporopollenin samples from *Selaginella* megaspores were analyzed following a number of treatment protocols, using different matrices. Additionally, we attempted to derivatise some of these samples by acetylation, followed by MALDI MS analysis. We report here our efforts to date.

Fluorescent staining procedure to identify pollen grains

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Palynological analysis is based on the identification of pollen grains through the distinguishing marks of the external structure, specifically openings and sculptures. Therefore, to point out the exine features, it is often necessary to use acetolysis or dyes.

Acetolytic method destroys all the pollen material with the exception of the exine, this emphasize external structures but may cause misleading in the functional interpretation of some pollen types (HESSE & WAHA, 1989). It is also reported that over-acetolysed grains appear often crumpled and distorted and structural information may be lost (DAFNI, 1992). As regards dyes, several procedures are available in order to stain the exine (KEARNS &INOUYE, 1993).

Observing with light microscope, the internal structure of grains, especially intine and cytoplasm, obstruct a free vision of the sculptures of exine. As a consequence, it results sometimes difficult, or even impossible, to unambiguously characterize small details Therefore, techniques based on light microscopy are sometimes insufficient to detect small details of the external structure of pollen. To overtake this limit several scientists have promoted the scanning electron microscope (SEM) and confocal laser scanning microscope as a necessary tool to observe fine details on pollen surface useful for diagnostic purposes (LANGFORD, 1990; RONNEBERGER, 2002).

Fluorescence microscopy is applied to anatomical and cytological studies of many biological structures because it is much more sensitive than its white light counterpart as a result of the enhanced contrast generated when a light object is viewed against a dark field. Moreover, since fluorescence microscopes are generally combined with a transmitted white light, the same field of a specimen can be observed subsequently with both methods. With this technique, information can be gathered from the two microscopes with an additive process and in a very short time.

The aim of this study was to combine traditional methods of pollen histochemical staining with fluorescence microscopy techniques in order to obtain clearer images of the exine. The methodology proposed herein provided images with enhanced contrast where the sole exine is evidenced without the interference of cytoplasm and pollenkitt, allowing the unambiguous detection of fine details of the exine.

Pollen was collected from anthers at dehiscence and several tests were performed in order to find the best staining and mounting medium for slides. Basic fuchsine stain in glycerol jelly (DAFNI, 1992) was selected. The slides were then observed through an epi-fluorescence microscope. The band pass filter, the dichromatic mirror and the barrier filter of the fluorescence microscope were appropriately selected to get clear view of fluorescent exines in dark background. With this method, the possible presence of fungi and debris on the slide does not interfere with pollen view because they appear not fluorescent.

Our results showed that the combination of staining procedures with fluorescence microscopy is a straightforward method that adds a clear view of fine details to the traditional observation of pollen morphology, without requesting complex preparation of the samples compared with acetolysis or SEM processes. Since this technique is neither costly nor time consuming, its use is very promising in the laboratories of melissopalinology, paleopalinology, aerobiology where routine analyses for pollen classification are performed.

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KEARNS, C. A. & INOUYE, D. W. 1993. **Techniques for Pollination Biologists**. pp. 77-151. University Press of Colorado, Niwot.
LANGFORD, M., TAYLOR, G. E. & FLENLEY, J. R. 1990. Computerized identification of pollen grains by texture analysis. **Rev. Paleo. Palyn**. 64: 197-203.
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Pollen grains transformations after several extraction treatments

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The exceptional pollen grains wall structure and composition make them highly resistant to physical and chemical disturbances. Based in such stability, pollen exine has been used as a tool for several sciences, given rise the Palynology. First application was pollen identification in crude oil, later among the main applications, one could summarize pollen morphology and taxonomy, aerobiology, melissopalinology, palaeopalinology and archaeopalinology. All these sciences are based in specific pollen spectra interpretation from crude oil, fresh or dried plants, air sampling, honey, soil or archaeological remnants. Through this interpretation it could be reconstructed the vegetation that produce these pollen grains and derived from this knowledge the climate and its changes in the past. Despite the high exine resistance, it can be modified depending on the preservation mode and extraction methods from specific samples. Pollen grains could be disturbed by alternative dampness and dryness periods. However, pollen grains with a low percentage of sporopollenine are more susceptible to degradation. Bryant & Holloway (1983). Havinga (1964) analysed several chemicals as exine degradation agents, concluding that bases are specially active. All these facts could affect to final counts and sediment interpretations as Lopez et al. (2003)have stated

We have carried out a study in twenty samples belonging to different pollen types. These samples were taken directly from flowers, fixed and submitted to classical physic-chemical treatments used for cleaning or extracting them from sediments. Later, we have qualified the morphological changes observed in exine and quantified the final pollen concentration for each treatment.

As we supposed, some pollen types were hardly affected by some treatments, other were scarcely affected, and finally, some pollen grains were resistant to most treatments tested. Similar results were obtained from quantitative data, ranging from the loss of a large number of pollen grains to a very few differences from controls. In accordance with our observations, we propose several methods to reduce pollen degradation through the extraction treatment.

HAVINGA, A. J. 1964. Investigations into the differential corrosion susceptibility of pollen and spores. **Pollen et Spores** 6: 621-635.

BRYANT, V. M. Jr. & HOLLOWAY, R. G. 1983. The role of Palynology in Archaeology. Advances in archaeological. **Method and Theory** 6: 191-224.

LÓPEZ SÁEZ, J. A., LÓPEZ GARCÍA, P. & BURJACHS, F. 2003. Arqueopalinología: Síntesis crítica. **Polen** 12: 5-35.

DEX1, a protein required for exine pattern development in *Arabidopsis thaliana*

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We have characterized the T-DNA-tagged *dex1* mutation of *Arabidopsis* that results in defective pollen wall pattern formation and male sterility. Initial light microscopic observations of semi-thin (0.5 µm) sections from buds prepared for conventional transmission electron microscopy (TEM) suggested a wall patterning defect since irregular deposits of wall material surrounded mutant microspores following release from the callose wall instead of the regular pattern of tectum and bacula seen in wild-type (WT) microspores at equivalent developmental stages. Meiosis was unaffected in mutant plants and staining of the callose wall by aniline blue did not differ from that observed in WT microsporocytes. The mutant was named *dex1*, defective in exine pattern formation.

An ultrastructural comparison of WT and *dex1* anthers from meiosis to released microspore stages was performed (PAXSON-SOWDERS, et al. 1997). Pollen development in the mutant parallels that of WT until the early tetrad stage. In WT plants, the microspore plasma membrane within the callose wall assumes a regular pattern of ridges and valleys. At later stages of development initial sporopollenin deposition occurs on these

ridges, marking the beginning of probacula formation. In contrast the plasma membrane in the mutant at similar developmental stages appears irregular with flattened protuberances; invaginations are rarely seen. Sporopollenin is irregularly deposited on the plasma membrane and does not appear to be anchored to the membrane.

The isolation and characterization of the WT and mutant *dex1* loci have been completed, along with a detailed TEM study of pollen wall development using high-pressure freezing followed by freeze-substitution (PAXSON-SOWDERS, et al. 2001). Genetic, Southern and complementation analyses confirmed the linkage of the *dex1* mutation and a single T-DNA insert. DEX1 is predicted to encode a 99 kDa glycoprotein that is unique to plants. The deduced DEX1 protein sequence is not homologous to other previously characterized proteins, but does exhibit limited sequence and structural similarity to the α-integrin and cadherin families of proteins respectively. In particular DEX1 is predicted to contain a carboxy-terminal membrane-spanning domain and four putative Ca^{+2} binding motifs that resemble the modified EF-hand domains found in the α-integrins. Preliminary biochemical studies indicate that one or more of the motifs do in fact bind Ca^{+2}. Northern-blot analyses indicate that *DEX1* is expressed throughout the plant, including bud, leaf, root and seedling tissue; DEX1 transcripts are absent in *dex1* plants as expected. This wide expression pattern was surprising given that additional morphological differences other than those that occur during pollen development have not been detected.

Polyclonal antibodies have been raised against several different portions of DEX1 expressed in *E. coli* and used in Western blot and immunolocalization experiments. Results from initial experiments indicate that *Arabidopsis* contains several proteins that share epitopes with DEX1, including one or more proteins associated with the tetrad callose wall. The presence of these additional proteins has hampered localization studies with the antibodies. Several different approaches, including epitope-tagging of DEX1 and the use of confocal microscopy on whole mount anther tissue squashes treated with β-glucuronidase to remove the callose wall have produced promising results. The localization of DEX1 during early exine development will be presented along with potential roles for the protein in pollen wall formation.

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Involvement of ER cisternae in the patterning of the pollen exine of *Beta vulgaris* L.

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The formation of the exine is an important event of pollen biology. Sporopollenin is the principal component of the exine, which is synthesized by the tapetum and transferred to the developing microspores (HESLOP-HARRISON, 1968). The main questions concerning the determination of patterning in the pollen wall are open due to the diversity of the results reported, depending on the species and the pollen exine type. It has been reported that the callose, the microspore plasma membrane, plasma membrane glycocalix, primexine and microspore organelles (smooth ER, Golgi vesicles, and microtubule) might be involved in the patterning of the exine (ROWLEY & SKVARLA, 1986; PEREZ-MUÑOZ, et al 1993; FITZGERALD et. al., 1994).

It has been postulated that the determination of exine patterning in angiosperms occurs during early microsporogenesis, when the microspores are surrounded by the special callose wall, and the primexine has not started being deposited (BLACKMORE & BARNES, 1990). In some species, however, pattern determination has been reported to be present already prior to meiosis division, late in the meiotic prophase (SHELDON & DICKINSON, 1983)

In this communication we report the results of our studies on the patterning of exine in *Beta vulgaris* L. during tetrad and young microspores stages, starting from the point when four nuclei have formed after telophase II and are still sharing common cytoplasm. We noted that the first determinants of the exine patterning are present in the post-telophase tetrad cytoplasm, where they are visible as the arrays of RE cisternae laying perpendicularly

to the surface of plasma membrane. The plasma membrane is the most likely structure in conjunction with the ER cisternae to be involved in causing reticulation of exine patterning. Distances between neighboring cisternae are exactly the same as those observed between bacules that are formed in the successive stages of development. The wall of microspores that have been released from the callose wall shows a well consolidated exine of ca. 0.8 µm in thickness, with three distinguishable, electron-dense layers: tectum with spinules, foot layer, and long, thin, radially arranged bacules.

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SHELDON, K. M. & DICKINSON, H. G. 1983. Determination of pattering in the pollen wall of *Lilium henry*. **J. Cell Science** 63: 191-208.

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Merging concepts: the role of self-assembly in the development of pollen wall structure

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The belief that self-assembly of components plays an important role in the production of exine structure and sculpture is not new. However, in recent years, a number of attempts have been made to define the mechanisms that may be involved in the generation of species distinct patterning. These mechanisms must unite what is known about the ultrastructural aspects of exine development with an understanding of the macromolecular interactions likely at the scale considered. Here we analyse established details of exine development from a perspective that favours the integration of self-assembly. We isolate those intervals in development in which genomic control is exercised and offer a number of scenarios which show how self assembly can build upon a genetic basis to give rise to the basic pollen exine structure.

Conventional and novel modes of exine patterning in Araceae

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Ultrastructure, chemistry and ontogeny of the angiosperm pollen wall, known in all details for many taxa, follow generally the same modes, which are reported in practically all botanical or palynological text books. However, it was to our high surprise that some angiosperms, especially various members of the subfamily Aroideae (Araceae), do not fit to this commonly accepted, conventional scheme, but perform novel modes of exine patterning.

In many Aroideae principal features are deviating from the conventional mode with respect to the timing of the respective developmental stages, of the chemical outer wall composition, and of the fine structure of

pollen wall strata and substrata. Taken together they represent spectacular novel features of exine formation, stratification and patterning.

In details:

1.- The chemical composition of the outermost exine layer: while sporopollenin so far known is always present, at least as a thin layer or covering only parts of pollen grains, it is absent in most of the genera in Aroideae, where a polysaccharidic (protein) layer is present (WEBER et al. 1998, WEBER et al. 1999).

2.- Callose is (usually) present in all angiosperms, but absent (at least in *Arum*) during tetrad formation (ANGER 2001).

3.- A primexine matrix, responsible for a sporopollenin ektexine, is present in angiosperms (if not within a compound pollen the inner walls are lacking an exine), but absent at least in *Arum* (ANGER 2001).

4.- Time table of formation of ektexine, endexine and intine: in contrast to the conventional time sequence, in *Arum* the outer exine layer is formed after the endexine, and not before, and exclusively by the tapetum without any primexine matrix. Hence, also the interaction with the tapetum differs from the conventional mode.

5.- The endexine, in the past often questioned for most monocots, is a regular feature in Aroideae. Moreover, it is to a high degree spongy, and not compact or at best lamellate as in other angiosperms (WEBER et al. 1998).

6.- The presence of a highly regular ornamentation pattern is typical for most angiosperm pollen, even many "psilate" pollen show under high SEM magnification a symmetric pattern. However such a symmetric pattern is absent in some "lower" Aroideae (e.g., at least in *Anubias*) (HESSE et al. in prep.).

In sum, the most characteristic deviations from the conventional mode in Aroideae are: there is no callose formation, no primexine matrix formation and no sporopollenin ektexine. The lack of callose and the lack of a primexine matrix may be the reason for the novel pattering mode rather than its consequence. This is supported by the lack of a sporopollenin exine in inner monads of compound pollen, where likewise callose is absent during tetrad stages.

However, the fundamental question should not be overlooked: Why do most genera of Aroideae lack an elaborated "common" sporopollenin exine? Why is even the most common outer exine chemical, sporopollenin, absent? It looks that a major change in aroid evolution took place at the point when the family "went unisexual" (Simon MAYO, pers. comm.). The hypothesis is, that ephemeral spathes (at least the upper portion which correlates with the male portion of the spadix) and absence of sporopollenin are the consequence of an adaptive syndrome for a short pollination "time window", where short-lived pollen, rapid germination of pollen tube and brief receptivity of stigma all work together.

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