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Scanning Electron Microscopy and Synchrotron Radiation X-Ray Tomographic Microscopy of 330 Million Year Old Charcoalified Seed Fern Fertile Organs

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Abstract: Abundant charcoalified seed fern (pteridosperm) pollen organs and ovules have been recovered from Late Viséan (Mississippian 330 Ma) limestones from Kingswood, Fife, Scotland. To overcome limitations of data collection from these tiny, sometimes unique, fossils, we have combined low vacuum scanning electron microscopy on uncoated specimens with backscatter detector and synchrotron radiation X-ray tomographic microscopy utilizing the Materials Science and TOMCAT beamlines at the Swiss Light Source of the Paul Scherrer Institut. In combination these techniques improve upon traditional cellulose acetate peel sectioning because they enable study of external morphology and internal anatomy in multiple planes of section on a single specimen that is retained intact. The pollen organ *Melissiotheca* shows a basal parenchymatous cushion bearing more than 100 sporangia on the distal face. Digital sections show the occurrence of pollen in some sporangia. The described ovule is new and has eight integumentary lobes that are covered in spirally arranged glandular hairs. Virtual longitudinal sections reveal the lobes are free above the pollen chamber. Results are applied in taxonomy and will subsequently contribute to our understanding of the former diversity and evolution of ovules, seeds, and pollen organs in the seed ferns, the first seed-bearing plants to conquer the land.

Key words: fossil, anatomy, cellulose acetate peel, Carboniferous, pteridosperms, Swiss Light Source

Introduction

Anatomical preservation of plant fossils is of major significance for our understanding of plant evolution (Taylor & Taylor, 1993). Such permineralization may occur as a result of permineralization or charcoalification processes (Scott, 1990*a*). Initially, preservation plants were studied using thin sections of the rock examined in transmitted light, but this resulted in loss of specimen (due to saw blade) and this technique is now used rarely except to provide information on the permineralization process (e.g., Scott & Collinson, 2003). Permineralized plants are now usually studied by serial sectioning, using the cellulose acetate peel method (Joy et al., 1956). Permineralized assemblages may include charcoalified fossils that can also be studied using peels. However, peeling is time-consuming. It provides results for only a single plane of section for each specimen studied and requires subsequent laborious reconstruction to understand morphology (e.g., Meyer-Berthaud, 1986; Meyer-Berthaud & Galtier, 1986). Small charcoalified plant fossils can be isolated from the rock matrix by acid dissolution. Charcoalified plants preserve excellent morphological and anatomical information (Scott, 2001), but serial sections are required to obtain the necessary information needed for taxonomic and phylogenetic studies (Bateman & Rothwell, 1990; Hilton & Bateman, 2006). The isolated charcoalified fossils may be readily examined using low vacuum scanning electron microscopy (LVSEM) for gross morphology and some aspects of anatomy, but serial sectioning is problematic for brittle charcoal that frequently shatters, resulting in destruction of the specimen. Serial sectioning involves acid isolation followed by embedding and sectioning (Schönenberger, 2005) and has been successfully used in the study of fossil charcoalified flowers (Schönenberger, 2005). To obtain anato-

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mical data from charcoalified plants without destroying specimens, synchrotron radiation X-ray tomographic microscopy (SRXTM) can be employed (Weitkamp et al., 1999; Stampanoni et al., 2002). SRXTM uses monochromatic X-rays, in contrast to conventional computed tomography with polychromatic light (Bird et al., 2008) and has been shown to provide important data on the internal structure of fossil animals and plants (Donoghue et al., 2006; Tafforeau et al., 2006; Friis et al., 2007; von Balthazar et al., 2007; Sutton, 2008). Here, we show that a combination of LVSEM and SRXTM on isolated charcoalified plant fragments yields morphological and digital anatomical data comparable with data obtained from the usual method of peel sections.

Seed ferns (pteridosperms) were the first seed-bearing plants to dominate Earth's vegetation (Taylor & Taylor, 1993). They underwent a major radiation in the mid-Mississippian with the diversification of lyginopterids and the evolution of medullosans (Meyer-Berthaud, 1986; Taylor, 1988; Hilton & Bateman, 2006), groups that became important later in the Pennsylvanian (ca. 318–299 million years ago) (Serbet et al., 2006). Our understanding of this radiation is based mainly on permineralized plant fossils, whose cells were infilled with minerals preserving cell detail (Gordon, 1941; Long, 1960, 1961, 1966, 1975, 1977; Bateman & Rothwell, 1990, Galtier & Rowe, 1991; Hilton & Bateman, 2006). Some data are available from plant compression fossils, but as these are carbon films on rock fewer details are preserved (Millay & Taylor, 1979; Hilton & Bateman, 2006). Discovery of extensive Mississippian charcoalified floras offers the opportunity to further understand the early diversification of pteridosperms. Charcoalified plant fossil assemblages, resulting from wildfires, have been recently considered as fossil lagerstätten or exceptionally preserved fossil assemblages (Glasspool et al., 2006). Wildfire has been an important element of the terrestrial Earth system since vascular land plants colonized the land in the Silurian (420 million years ago) (Scott, 2000; Glasspool et al., 2004; Scott & Glasspool, 2006). Fire became increasingly more frequent during the early Carboniferous (Mississippian, 360–325 Ma) as atmospheric oxygen levels rose (Scott & Glasspool, 2006). This was also a period of rapid land plant diversification (Niklas et al., 1980, 1983) and sediments from the early Carboniferous of the Midland Valley of Scotland contain abundant, diverse, charcoalified plant assemblages (e.g., Scott et al., 1985, 1986; Scott, 1990*a*,*b*; Scott & Glasspool, 2006).

Material and Methods

Specimen Extraction and Preparation

Limestone blocks containing charcoalified plants from the mid-Mississippian (Carboniferous) of Kingswood, Fife, Scotland (National Grid Reference NS 265 864) were sliced into 1 cm thick pieces, and one reference peel (e.g., Fig. 1A) was made of each surface studied. For peeling, surfaces were etched with dilute (10%) hydrochloric acid, washed with water, flooded with acetone, and overlain by a sheet of cellulose acetate (Joy et al., 1956). The result is that the organic cell walls remain upright, free from the rock, and are incorporated into the acetate sheet that is peeled off the surface. A set of peels of each surface was kept for reference. These were photographed using top illumination, with the peel on a white background. In addition, polished thin sections of selected areas were made to provide data on the permineralization process and on the preservation of the plants (e.g., Scott & Collinson, 2003). In the study by Meyer-Berthaud (1986), peel sections of the pollen organs were cut out of the peel and mounted on a glass slide with a cover slip using Canada balsam and photographed by transmitted light microscopy. In this study, selected slabs of block KIN957 were dissolved in dilute (20%) hydrochloric acid. Residues were then sieved using a 180 μm polypropylene sieve. The charcoal residue was further treated with 40% hydrofluoric acid, to remove silica, and neutralized. The cleaned residue was stored in distilled water and sorted in water using a binocular microscope with incident lighting. Specimens were separated using 000 hair brushes and mounted into dry cavity slides. Dry specimens were mounted on 3 mm diameter brass pins using colloidal carbon in isopropanol. We used fossil charcoal from the same lithology and horizon as that for Meyer-Berthaud's (1986) peel-based study to generate details of both external and internal anatomy, using SRXTM and LVSEM.

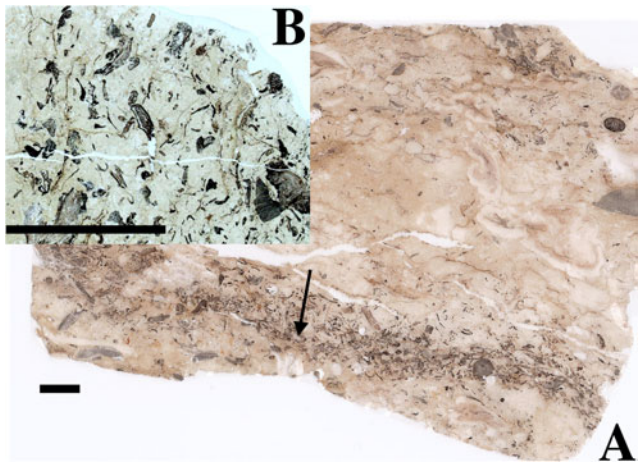

Figure 1. A: Photograph using top illumination of cellulose acetate peel of limestone block containing charcoalified plants (arrow shows charcoalified plant layer) from the late Viséan (mid-Mississippian, Carboniferous) of Kingswood, Scotland (KIN 1019B); scale bar = 1 cm. **B:** Transmitted light micrograph of polished rock thin section of Block KIN 957I showing charcoalified plants; scale bar = 1 cm.

Scanning Electron Microscopy

Uncoated specimens were studied using a Hitachi S3000N variable pressure scanning electron microscope (SEM) at

Royal Holloway, University of London, under low vacuum and in backscatter electron mode. The SEM was operated at a pressure of 70 Pa using a working distance of 15–23 mm and accelerating voltage of 20 kV.

Synchrotron Radiation X-Ray Tomographic Microscopy

Synchrotron light is a form of electromagnetic radiation with wavelengths ranging from infrared to hard X-rays. SRXTM in this study was performed at the Materials Science (Stampanoni et al., 2002) and TOMCAT (Stampanoni et al., 2006) beamlines, Swiss Light Source, Paul Scherrer Institut, Switzerland. The X-ray energy was tuned according to the absorption properties of the samples, and monochromatic X-ray beams between 8 and 10 keV have been used. The magnification of the X-ray microscope was ×10–×20. On-chip binning (2×) was chosen to improve the signal to noise ratio resulting in isotropic voxels that ranged in dimensions from 0.7 to 1.48 μm. Projections (501–1501) were acquired equiangularly over 180°, online postprocessed and rearranged into flat and darkfield corrected sinograms. Reconstruction was performed on a 32 node Linux PC farm using highly optimized filtered back projection routines. Slice data derived from the scans were then analyzed and manipulated using AMIRA on a Hewlett Packard PC with 16GB DDR SDRAM at Bristol University. Reconstructions were generated, in the first instance, by threshholding data across a gray scale to obtain the overall three-dimensional (3D) structure. Segmentation of this virtual specimen was then carried out, by interpolation of regions through the data set that equated to structures within the specimens. These regions were then assigned to materials (specific structures) within the virtual specimen. Additional processing was undertaken at Royal Holloway using Avizo 5.0 (Mercury Computer Systems Ltd., Chelmsford, MA, USA) on a Dell Precision PWS690 with 16 GB DDR SDRAM.

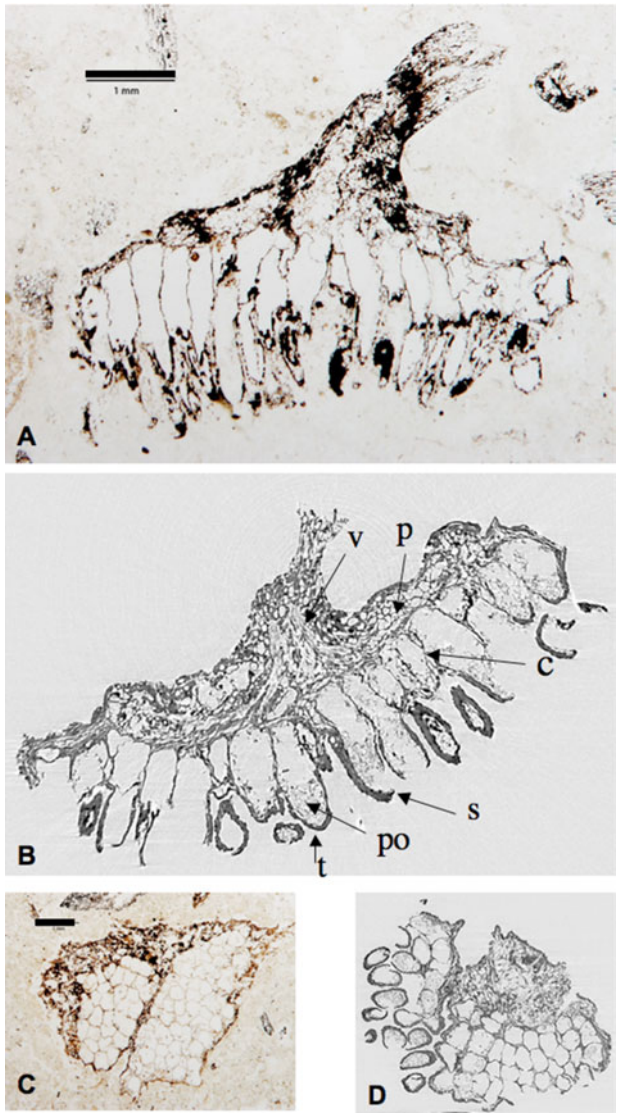

Figure 2. Anatomy of *Melissiotheca* pteridosperm pollen organ from the mid-Mississippian (Carboniferous) of Kingswood, Scotland; scale bar 1 mm for all figures (**A** and **B** same scale; **C** and **D** same scale). **A**,**C:** Transmitted light micrographs of balsam mounted cellulose acetate peel sections of previously described specimens (Meyer-Berthaud, 1986). **A**, longitudinal section (Block KIN 245-F17, holotype); **C**, tangential section (Block KIN245). **B**,**D:** SRXTM sections of specimen (from block KIN 957I spec 1) shown in Figure 3. **B**, digital longitudinal section; **D**, digital tangential section. The longitudinal section shows free tips of the sporangia (s), thin connecting tissue (c), and thick walls to the free tips (t). The sporangia are embedded in parenchymatous tissue (p) that contains a dividing vascular strand (v). Some sporangia contain pollen (po). Note that details of tracheary elements in vascular strand are not resolved.

Results

Example 1: Pollen Organ

Melissiotheca (family Lyginopteridaceae) is a pteridospermous pollen organ that was described from the Kingswood flora by Meyer-Berthaud (1986) based on transmitted light microscopy of cellulose acetate peel sections of charcoalified material. The pollen organ is a pedicellate synangium with 50–150 sporangia, embedded in a basal, lobed parenchymatous cushion. The sporangia are fused along their proximal half but are free distally. The holotype is a longitudinally sectioned specimen (KIN245-F17) (Fig. 2A). Other specimens (such as that shown in Fig. 2C) were used to provide supplementary details on the organization of the pollen organ. While the peel method may provide excellent results on the anatomy, increasing charcoalification causes cell walls to become brittle and more difficult to peel, and details of the fine scale anatomy may not be resolved. Etching with hydrochloric acid may disturb the thinner upstanding cell walls making good peel sections difficult to obtain. Section-

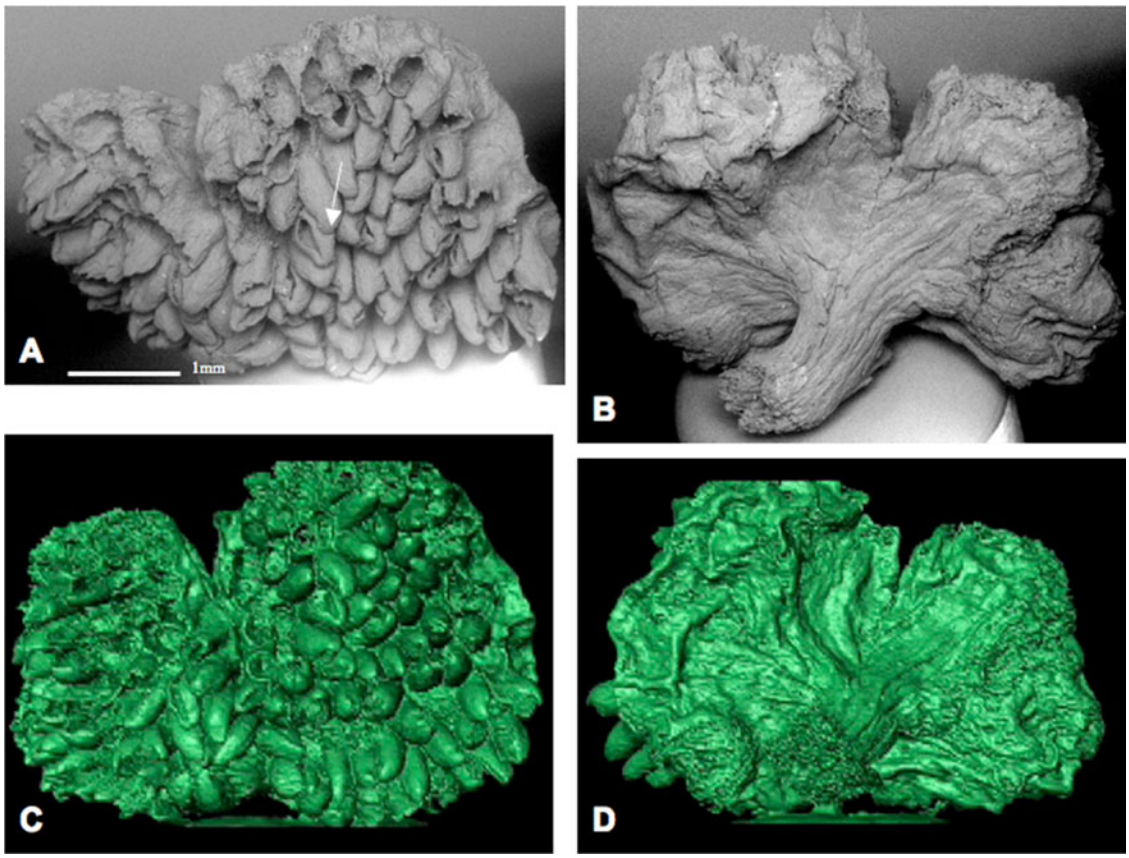

Figure 3. General morphology of *Melissiotheca* pteridosperm pollen organ from the mid-Mississippian (Carboniferous) of Kingswood, Scotland (from block KIN957I spec1); scale bar = 1 mm for all figures. **A**,**B:** LVSEM of whole pollen organ. **A**, distal face showing more than 100 free sporangia (arrow); **B**, proximal surface showing stalk and two lobes. **C**,**D:** SRXTM digital reconstructions. **C**, proximal face; **D**, distal face.

ing of these charcoalified specimens that have been acid released from the limestone matrix is difficult as the cells may be brittle and attempted sectioning (fracturing) of the specimen may result in total destruction.

In contrast to the peel method, details of both external morphology and internal anatomy can be obtained from a single specimen released from the rock matrix by acid using SEM and SRXTM. With SEM, acid released specimens of *Melissiotheca* clearly show the basal parenchymatous cushion bearing more than 100 sporangia on the distal face (Fig. 3A). In addition, the dichotomy producing two main lobes can be clearly seen (Fig. 3B). Rendered reconstructions from SRXTM data also clearly show these features (Fig. 3C,D) and are comparable to SEM. SEM also shows the cellular detail of the sporangia, the occurrence of pollen in some of the sporangia, and detail of the vascular tissue in the stalk; however, currently none of these can be resolved on the rendered reconstructions.

A major benefit of SRXTM is that sections from the same specimen can be made in multiple planes (Fig. 2B,D). These digital sections are directly comparable to those made from multiple specimens with the traditional peel technique (Fig. 2A,C), although there is a current limitation to the SRXTM technique where, for theoretical pixel sizes in the order of 0.35 μm, the best spatial resolution currently reachable is 0.75 μm. In the digital longitudinal section, details of the stalk with a dichotomizing vascular strand are visible (Fig. 2B) though high-magnification details, such as characteristic thickenings on the vascular tissue, are currently not discernible. The sections also show the numerous sporangia embedded in a parenchymatous cushion (Fig. 2B). It is possible to identify the number of layers of parenchyma that constitute the cushion. SRXTM also has the advantage of being able to measure all dimensions of each sporangium and to render them in 3D so that their shape can clearly be seen. The thinner cell walls, where the sporangia are fused, and the thicker cell walls, where they are free, are evident (Fig. 2B), and SRXTM is advantageous in that there is no physical breaking of the cell walls during preparation. The occurrence of pollen within the sporangia is also recognizable (Fig. 2B, po). While resolution of SRXTM is not yet sufficient to allow details of pollen morphology or organization to be seen, specimens with pollen can be identified and carefully chosen for destructive

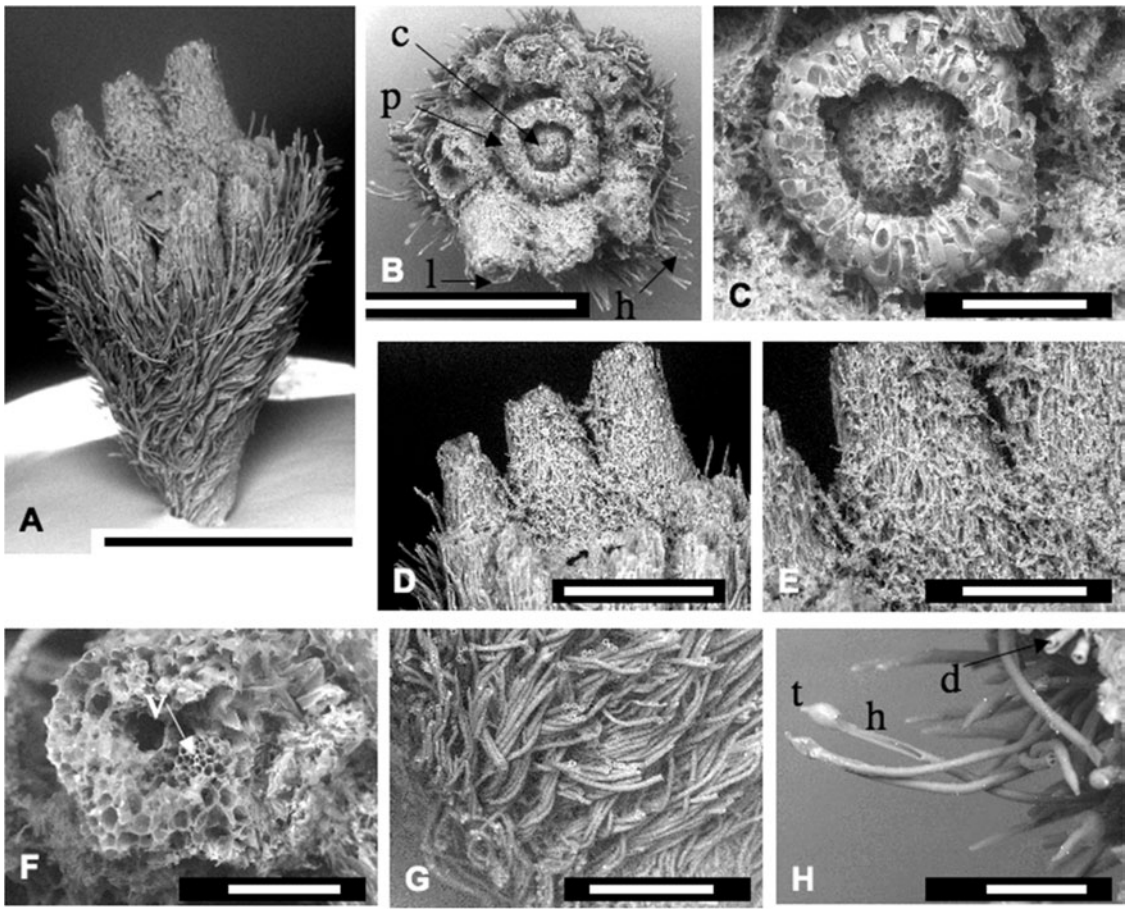

Figure 4. SEM of charcoalified pteridosperm ovule from the mid-Mississippian (Carboniferous) of Kingswood, Scotland (from block KIN957I spec 2). **A:** Lateral view of whole ovule; scale bar = 1 mm. **B:** Top view showing free integumentary lobes (l) with external glandular hairs (h), pollen chamber (p), and central column (c); scale bar = 1 mm. **C:** Detail of **B** showing cellular detail of the pollen chamber; scale bar = 20 μm. **D:** View of the internal surface of the free integumentary lobes; scale bar = 500 μm. **E:** Detail of **D** showing hairy surface on the inner part of the integumentary lobes; scale bar = 200 μm. **F:** Broken tip of a free integumentary lobe showing position of vascular tissue (v); scale bar = 100 μm. **G:** Details of external glandular hairs showing bundles of spirally arranged hairs; scale bar = 200 μm. **H:** Detail of external hairs with glandular tips (t). Broken hairs show hollow center (h) and occasional internal division (d); scale bar = 100 μm.

analysis, negating the necessity to destroy more specimens than absolutely necessary.

Example 2: Ovule

Relatively few pteridospermous ovules have been described from the Late Viséan (mid-Mississippian, Carboniferous). Only two taxa (*Sphaerostoma* and *Physostoma*) have been recognized from the Pettycur locality (near Kingswood), which has been intensively studied for over 100 years (Scott et al., 1984). Until now no ovules have been reported from the Kingswood deposit, despite the abundance of pollen organs (Scott et al., 1986). The ovule described here is a unique specimen of a typical hydrasperman-type ovule (Lyginopteridaceae) with a distinctive pollen chamber. It is 2 mm long and 1.25 mm at its widest. It is relatively small for Carboniferous ovules, which may be 3–15 mm long (Long, 1960 et seq.). This ovule probably represents a new taxon that will be formally described elsewhere.

SEM reveals detail of the morphological features of the ovule including the eight integumentary lobes (Fig. 4A,B), whose inner and outer surfaces are covered in different types of hairs. SRXTM allows the internal anatomy to be seen (Fig. 5A–C). In the digital transverse section, it is clear that the eight integumentary lobes are free above the pollen chamber (Fig. 5A) but united below the pollen chamber (Fig. 5B). In the digital longitudinal section, the nucellus and central column can be seen (Fig. 5C). The cellular structure of the surface of the pollen chamber is clearly seen with SEM (Fig. 4C). The broken tips of the

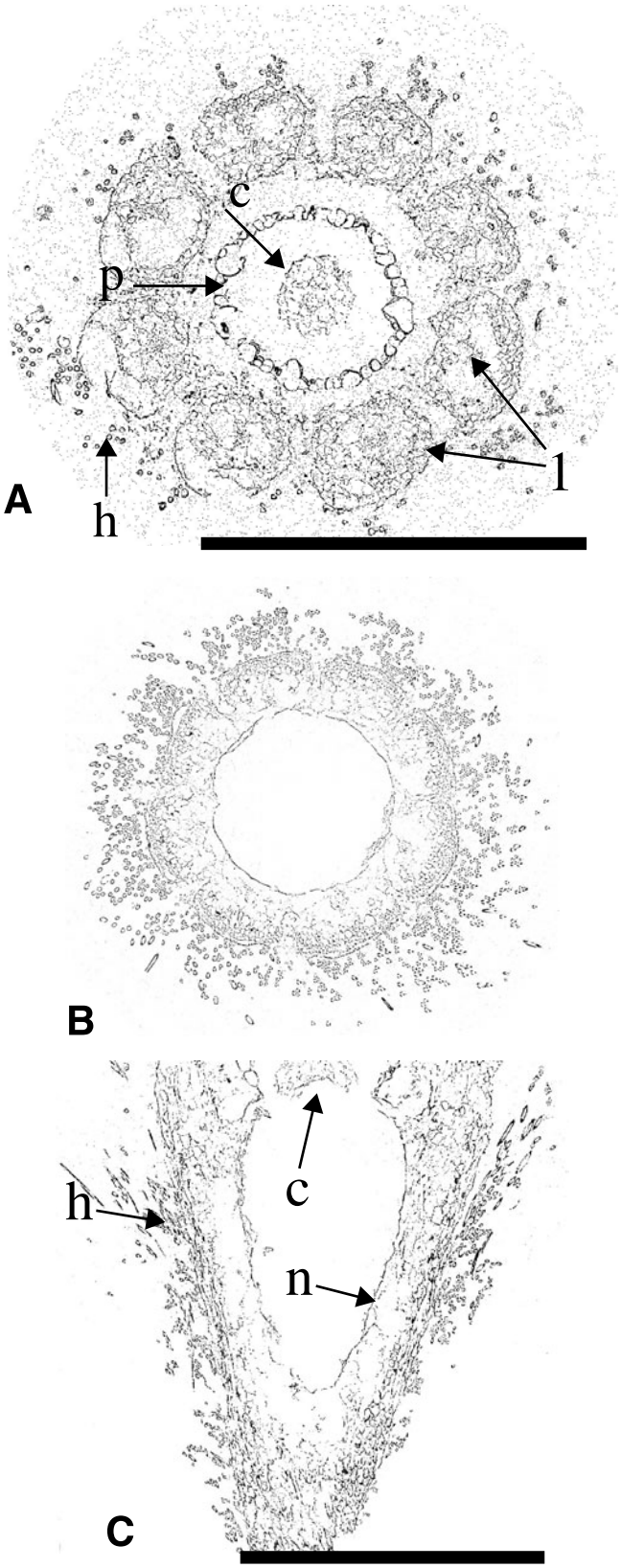

Figure 5. SRXTM of charcoalified pteridosperm ovule (shown in Fig. 4) from the mid-Mississippian (Carboniferous) of Kingswood, Scotland (from block KIN957I spec 2); scale bar = 1 mm. **A:** Digital transverse section showing free integumentary lobes (l), central column (c), pollen chamber (p), and hollow external glandular hairs (h). **B:** Digital transverse section below pollen chamber, showing unlobed integument with external hairs, at same scale as **A**. **C:** Digital longitudinal section showing outer glandular hairs (h), central column (c), and nucellus (n).

integumentary lobes show the position of the vascular strand (Fig. 4F).

A very important character is the presence of dense, external, beautifully preserved spirally arranged glandular hairs (Fig. 4A). The spiral arrangement can be seen particularly well in movies of SRXTM transverse slices (see Supplementary Material at journals.cambridge.org/mam). In our specimen, SEM shows that the hairs are not only spirally arranged but also occur as intertwined clusters (Fig. 4G), and this intertwining is also visible on the rendered digital reconstruction (Fig. 6). SRXTM shows that the hairs are hollow (Fig. 5A), also revealed by broken hairs in SEM where occasional internal dividing walls can also be seen (Fig. 4H). The hairs characteristically end in glandular tips (Fig. 4H). Hairs on the external integumentary surface are a common feature in early Carboniferous (Mississippian) ovules (Gordon, 1941; Barnard & Long, 1973; Long, 1975).

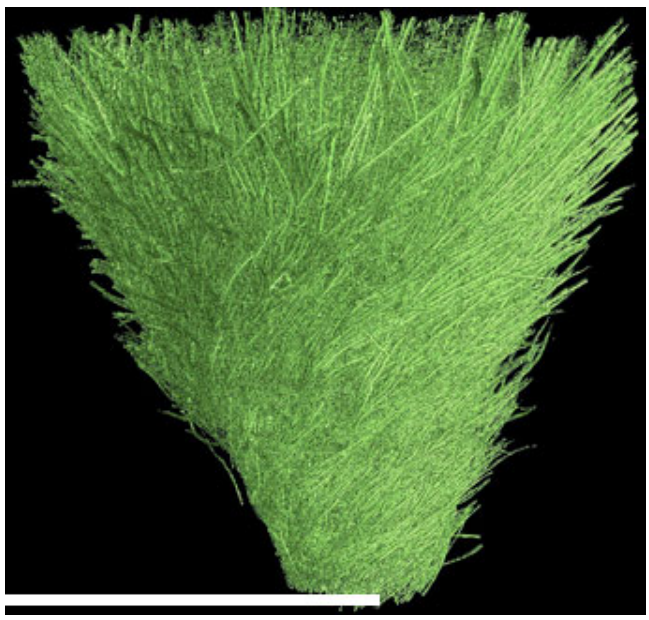

Figure 6. Reconstructed digital image (SRXTM) of ovule showing spirally arranged glandular hairs. The integumentary lobes are missing as these were not originally scanned. Scale bar = 1 mm.

However, in all published examples these are interpreted as straight, as in *Salpingostoma* (Gordon, 1941), *Dolichosperma* (Long, 1975), and *Tantallosperma* (Barnard & Long, 1973) and are usually shown as being solid. Because of their small diameter, it may be difficult and time consuming to follow individual hairs in peel sections, so that their spiral arrangement, hollow centers, and glandular tips may not be accurately reconstructed. SEM shows that the inner surface of the free tips of the integumentary lobes are covered with a fine mat of hairs of a different nature than on the external surfaces (Fig. 4D,E). Owing to their very small size, these may be missed entirely in studies of peel sections, especially with highly charred specimens that are very brittle.

Discussion

Our study of *Melissiotheca* has confirmed most of the observations made by Meyer-Berthaud (1986) but has shown the value of combining SEM and SRXTM of charcoalified pollen organs to generate the basic description of the taxon. Using SEM and SRXTM in combination provides both morphological and anatomical data on a single specimen in a faster, nondestructive way. Therefore, a specimen dissolved from the rock may be designated as a holotype. Data can be used to identify appropriate material for destructive sampling to obtain additional details of the finer anatomy of the specimen.

Our study of the charcoalified ovule demonstrates that the ability to define the organization of the integumentary lobes, pollen chamber, and central column, as well as the nature of the glandular hairs from the same specimen, makes SRXTM a particularly powerful technique for the study of charcoalified ovules and especially of unique specimens. Lupia (1995) has shown, however, that charring of other fertile organs, such as flowers, causes shrinkage, a fact that must be taken into account when comparing specimens preserved as calcareous permineralizations with those preserved as charcoal, especially if dimensions are critical for taxonomic assignment.

Conclusions

Dissolving the Kingswood limestone has resulted in the discovery of a range of fertile organs of seed ferns. These are preserved as charcoal and yield exquisite morphological detail. LVSEM has allowed detailed gross morphological data to be obtained from the specimens, very difficult to achieve using the standard serial peel sectioning technique, even with laborious reconstruction of peels into 3D models. SRXTM has allowed the nondestructive study of the internal anatomy of charcoalified specimens in multiple planes while retaining the original specimen intact. This is impossible to achieve from traditional peel sections that reduce the specimen to serial sections in a single plane. Data from SRXTM can be used to make digital reconstructions of the specimens from which the shapes of individual internal components (such as sporangial cavities) can be discerned. The beamline at the Swiss Light Source is undergoing continual development. Currently limitations exist in the maximum size of the specimen that can be imaged but the ability to stack images has meant an increase in specimen size from a few to 10 mm. Likewise an improvement of the computer software has allowed a reduction of scan time. It is anticipated that new developments will enhance not only the quality of data but also the range of specimens that can be studied in future. In combination SEM and SRTXM improves upon the information available from the usual peel technique, especially for small, rare, or unique charcoalified fossils. These data will be invaluable for future taxonomic and diversity studies.

Acknowledgments

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