

Preparation protocols for SEM visualization of charred fossil plants: the case of *Weichselia reticulata* pinnule anatomy

Protocolos de preparación de fósiles vegetales carbonizados para su visualización al MEB: el caso de la anatomía de la pínula de *Weichselia reticulata*

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Abstract: The cosmopolite Mesozoic fern *Weichselia reticulata* presents many peculiarities in the anatomy of its stem as well as in the architecture of its leaves. Previous analyses of the ultrastructure of their pinnule laminas provided some insight on xeromorphic traits. In this work new protocols for the preparation of charred compression foliage specimens embedded in a limestone matrix for their observation under SEM are presented, which enhance the observation of different plant tissues. The preparation with HCl allows for the visualization of the whole lamina, from the upper to the lower epidermis, which had not been done to date. Additionally, collodion, latex, or wax coverings of the fossils before dissolving the matrix with HCl enhance the observation of different structures and prevent the fragmentation of the specimen. These techniques have permitted a better description of the vascular tissues, and a redescription of the stomata, which do not have subsidiary cells as previously described. Moreover, the traits observed suggest a closer systematic relationship of *Weichselia reticulata* with the Marattiales and that this fern lived in areas with fluctuating water availability.

Resumen: El helecho cosmopolita *Weichselia reticulata* presenta muchas peculiaridades en la anatomía de su tallo y en la arquitectura de sus hojas. Análisis previos de la ultraestructura de la lámina de la pínula de este helecho proporcionaron información sobre rasgos xerofíticos. En este trabajo se presentan nuevos protocolos para la preparación de ejemplares de compresiones carbonizadas de hojas de esta especie que se encuentran incorporados en una matriz caliza para su observación con microscopía electrónica de barrido. Estos protocolos mejoran sustancialmente su observación. La preparación del material con ácido clorhídrico permite la visualización de la lámina completa, desde la epidermis superior hasta la inferior, algo que no se había conseguido hasta la fecha. Además, si se cubren los ejemplares con collodion, látex o cera de vela antes de su disolución con ácido clorhídrico, se realzan diferentes estructuras de la lámina y se evita la fragmentación de los ejemplares. Estas nuevas técnicas han permitido una mejor descripción del sistema vascular, así como una mejor observación de los estomas, que no tienen células subsidiarias como se pensaba hasta ahora. Los caracteres observados en la lámina de *Weichselia reticulata* sugieren una relación filogenética más cercana con el orden Marattiales, y que este helecho vivió en zonas con fluctuaciones en la disponibilidad de agua.

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INTRODUCTION

Weichselia reticulata (Stokes & Webb) Fontaine Fontaine has received much attention due to its wide geographic and stratigraphic distribution (see Blanco-Moreno *et al.*, 2018 for a revision) as well as for its peculiar architecture and anatomy. However, many aspects of this plant remain controversial, such as its systematic affinities, *i.e.*, closer to the Marattiaceae (Edwards, 1933; Silantjeva & Krassilov, 2006; Blanco-Moreno *et al.*, 2020) or to the Matoniaceae (Alvin, 1971; Sender *et al.*, 2015), or its habitat, *i.e.*, marsh/coastal vegetation (*e.g.*, Daber, 1968; El-Khayal, 1985), or

xerophyte (*e.g.*, Alvin, 1974; Watson & Alvin, 1996). The most comprehensive study of the anatomy of the pinnules under the scanning electron microscope (SEM) to date was published by Alvin (1974) on charred remains of *Weichselia reticulata* from the Barremian of the Isle of Wight (UK). Other publications such as Florin (1919), Reymanówna (1965), Hill (1990), Lejal-Nicol and Dominik (1990), Collinson (1999), Sender *et al.* (2015), and Abu Hamad *et al.* (2016) contribute with additional data on some parts of the anatomy of *Weichselia*.

The preparation techniques for fossil plants are very varied (see Jones & Rowe, 1999, for a general introduction). Concerning the study of leaves, techniques generally focus on obtaining cuticles that can be studied under the microscope (e.g., Bather, 1908; Kerp, 1990). Cuticles are not well preserved at the locality of Las Hoyas (Diéguez & Meléndez, 2000) and Bernissart (pers. obs.), but the charred specimens from both localities allow for histological analysis. Previous studies of the pinnules of *Weichselia reticulata* used different methods for the extraction of the remains from the matrix: Reymanówna (1965) worked on material from the western Carpathians preserved in shales, she used sequential chemical preparations with H₂O₂, HCl and HF to extract the fossil fragments; on the other hand, the preparation technique used by Alvin (1974) to observe the leaf anatomy of *Weichselia* in material from the Isle of Wight (English Weald) is very simple due to the softness of the matrix (siltstone) where the remains are embedded, which can be grinded with sandpaper, permitting fragments of pinnae and pinnules to be easily extracted mechanically. Preparation in limestone requires more aggressive techniques, either chemical with the use of HCl or HNO₃, or mechanical with needles by the dégagement (extraction) technique.

The main aim of this study is to analyse the ultrastructure of the pinnules of *Weichselia reticulata* from two Early Cretaceous localities, Las Hoyas and Bernissart, in order to propose a more up-to-date reconstruction of the histology of the pinnule lamina of *Weichselia reticulata*, taking advantage of higher resolution SEM machines and comparing it with published works. Additionally, fragments of *Weichselia reticulata* are extracted from the limestone matrix of Las Hoyas using different techniques, testing different protocols for the extraction of the plant material that will provide a higher resolution when visualized under SEM, and permit the observation of all the tissues of the pinnule lamina.

GEOLOGICAL SETTING

The fragments of *Weichselia reticulata* analysed in this work come from two different localities: Las Hoyas, upper Barremian La Huérguina Formation in Spain, and Bernissart, Barremian–Aptian Sainte-Barbe Clays Formation in Belgium. The locality of Las Hoyas is situated in the Serranía de Cuenca, which is part of the southwestern Iberian Range (Fregenal-Martínez & Meléndez, 2016). It consists of a laminated limestone lithosome deposited during the upper Barremian, 126 to 129 Ma (Diéguez *et al.*, 1995; Vicente & Martín-Closas, 2013; Fregenal-Martínez *et al.*, 2017). The locality represents the parastratotype of La Huérguina Formation, interpreted as a freshwater, regional, inland subtropical system of wetlands (Fregenal-Martínez *et al.*, 2017). The facies of this Formation correspond to poorly drained areas, mainly lacustrine, and do not have much influx of allochthonous sediments.

Bernissart is located in the northern part of the Mons basin, 25 km west of Mons, in southwestern Belgium (Baele *et al.*, 2012). This basin corresponds to an East-West subsiding zone where the intrastratal dissolution of the Mississippian (Carboniferous) basement led to the creation of several sinkholes, or natural pits, where Meso–Cenozoic sediments accumulated (Quinif & Licourt, 2012; Spagna *et al.*, 2012). Three Sinkholes have been spotted around Bernissart: North, South, and the Iguanodon sinkholes. The material studied in this work belongs to a -322 m tunnel excavated into the Iguanodon sinkhole. This tunnel corresponds to a Barremian–Aptian (Dejax *et al.*, 2007) lacustrine clay, attributed to the Sainte-Barbe Clays Formation (Cornet & Schmitz, 1898; Cornet, 1927). The environment at the top of this pit has been interpreted as lacustrine (Van den Broeck, 1898) and more recently as lacustrine to swampy (Yans *et al.*, 2012; Schnyder *et al.*, 2009; Spagna *et al.*, 2012).

MATERIAL AND METHODS

A total of fifty-one specimens consisting of charred compressions of secondary pinnae fragments of *Weichselia reticulata* from the locality of Las Hoyas and Bernissart were included in this study. Fifty of these specimens, all from the locality of Las Hoyas, were prepared using different techniques. When possible, part and counterpart of the specimens were prepared, enabling the study of both sides of the pinnules.

(1) The fossils were treated with 32% HCl that was added with a plastic dropper directly on the pinnae until they became free from the sediment. The fossils were washed with distilled water on to a sieve to collect all the fossil fragments and remove the acid. Six specimens were extracted with this technique.

(2) The pinnae were covered by collodion (Long & Clements, 1934) in liquid form, dissolved in chloroform. The formation of bubbles was avoided by covering the fossils slowly, and any bubbles that formed were broken with a dissecting needle. The collodion was left to dry at room temperature; no heating was possible, as it is an explosive compound. When the preparation was totally dry the rock was treated with 32% HCl, as explained in the first method of preparation, until the fossils covered with collodion became free from the sediment. These were washed with distilled water to remove the acid and a sieve was used so that any remains that had come free from the collodion sheet were collected as well. The collodion sheets with the carbonized pinnules were then immersed in 32% HCl to dissolve any remaining sediment and posteriorly washed with distilled water to remove the acid. Twenty-five specimens were extracted by this method.

(3) Pinnae fragments were covered by latex, and when the preparations were dry, they were treated by the same procedure described for the specimens covered by collodion. Seven specimens were prepared by this technique.

(4) Finally, candle wax was used to cover the pinnae fragments. A lit candle was used to drop hot wax on the specimens and once the fragments of *Weichselia* were completely covered the wax was allowed to cool at room temperature. The same procedure as in the two preceding techniques was used. Twelve specimens were prepared.

Some of these preparations were posteriorly immersed in HF for three hours to dissolve possible silicates. This method was used in 10 fragments of the specimens prepared by the first method and 10 of the collodion covered specimens.

Thirteen specimens were observed under the SEM: one fragment from the locality of Bernissart, embedded in the sediment (Fig. 1E); and twelve from the locality of Las Hoyas with different preparations: two SEM slides with eleven isolated fragments prepared using HCl only (Fig. 1A–1B), two fragments prepared with HCl and covered with collodion (Fig. 1G), six fragments prepared with HCl and covered with collodion, which were coated with gold (Fig. 1F), one fragment covered with wax (Fig. 1C), and one covered with latex (Fig. 1D).

Two different SEM machines were used. All specimens from Las Hoyas except for the gold coated fossils were observed under a FEI QUANTA 200 scanning electronic microscope in ambient atmospheric pressure mode. Gold coated specimens from Las Hoyas, and the specimen from Bernissart were observed under a L-260-MEB in high vacuum pressure mode.

Material examined. Las Hoyas MCCM-LH 38607–38618; Bernissart IRSNB b9502.

Institutional abbreviations. IRSNB, Palaeontology collection at the Royal Belgian Institute of Natural Sciences, Brussels, Belgium; MCCM-LH, Las Hoyas Collection at the Museo de Las Ciencias de Castilla-La Mancha, Cuenca, Spain.

RESULTS

Preparation techniques

The four preparation techniques provided different results which allow for the collection of different anatomical data and show different levels of tissues in pinnules and rachises. Observing these preparations under the SEM, different layers of tissues of the lamina of the pinnules can be identified (e.g., Fig. 2A). This is due to the fact that the pinnules are fragmentary, and they separate into a part and a counterpart when the rock is opened.

Treatment with HCl without covering the fern fragments produces fragmentary pinnules and rachises that can be studied under the stereomicroscope and SEM (Fig. 1A–1B), whereas the covering of the remains by methods 2 to 4 allows to extract complete secondary pinnae, making it possible to observe the side of the fossil that was embedded in the rock (Fig. 1C–1D, 1F–1G). Collodion covering and latex covering

produced similar results macroscopically. However, when latex preparations are observed under the SEM only the vascular tissue can be clearly distinguished, other layers of cells are not well preserved. Candle wax proved to be a good way to avoid the fragmentation of pinnae during the treatment with HCl, but after the treatment the fossils separated from the wax, leaving only traces of carbon, especially of the area of the nerves. The candle wax preparations can be used to study the disposition of the nerves in the pinnules with the stereomicroscope, and to prepare more complete pinnules than those extracted with only HCl. However, this technique is not suitable for study under the SEM, as it forms crystallizations that obscure the details of the tissues (Fig. 2B). The immersion in HF provided cleaner preparations, with no silicate particles. However, one of the immersed collodion preparations produced a CaCO₃ precipitate that made the observation of the anatomy impossible.

The specimen that was observed directly on the sediment also provided good results. Different layers of the lamina were observed on this specimen, although the preservation of the epidermis was rare, and stomata were not observed. Images were clear, however, sediment particles that had been disaggregated from the matrix and been deposited over the fossil sometimes obscured detail and gave the images a 'dusty' appearance (Fig. 2C–2D).

Histology of the pinnules

The pinnule cuticle could only be observed on the upper epidermis and it consists of a very thin membranaceous layer that is very fragmented and is only present at the margins of some pinnules (Fig. 3A). Under this layer the upper epidermis can be distinguished. The cells of both the upper and lower epidermis are very deformed, and it is very hard to identify their shapes, they measure 8–14 µm at the upper epidermis and 20–24 µm at the lower epidermis in Las Hoyas specimens, but can measure up to 35 µm in the specimen from Bernissart (Figs. 2C, 3E). In many cases the cell walls are fused and cracked, making the identification of individual cells impossible. When the sub-epidermal cells are visible, they are slightly interdigitated with each other (Fig. 3B). Under the epidermis there are palisade cells that are ovoid in contour and measure 5–22 µm across their cross-section (Fig. 3C–3E). Only the cell walls are preserved, and the length of the cells cannot be observed in the preparations studied. Under the palisade parenchyma is the spongy mesophyll, with irregular, more rounded cells. These are irregularly distributed, not as tightly packed as the palisade parenchyma, and measure 16–20 µm (Fig. 3F). The conductive tissue is surrounded by rhomboidal elongated cells in the area of the palisade and at its contact with the lower epidermis (Fig. 4A). They vary greatly in size (39–70 µm long by 9–18 µm wide). Other vascular cells can be observed

between the conductive tissue and the rhomboidal cells, these are cylindrical cells measuring 6–14 μm wide (Fig. 4B). The conductive tissue can be seen on the lower part of the vascular bundle, consisting of elongated cells with piths along their walls (Fig. 4C). These conductive cells are very similar in size to the

cylindrical cells mentioned earlier. Tracheids can be identified in the latex and collodion specimens, as well as in the specimen embedded in the sediment. They have annular and scalariform thickenings and measure 6–12 μm wide (Figs. 2D, 4D–4F). Silicate inclusions can be seen in some of the preparations, and they are

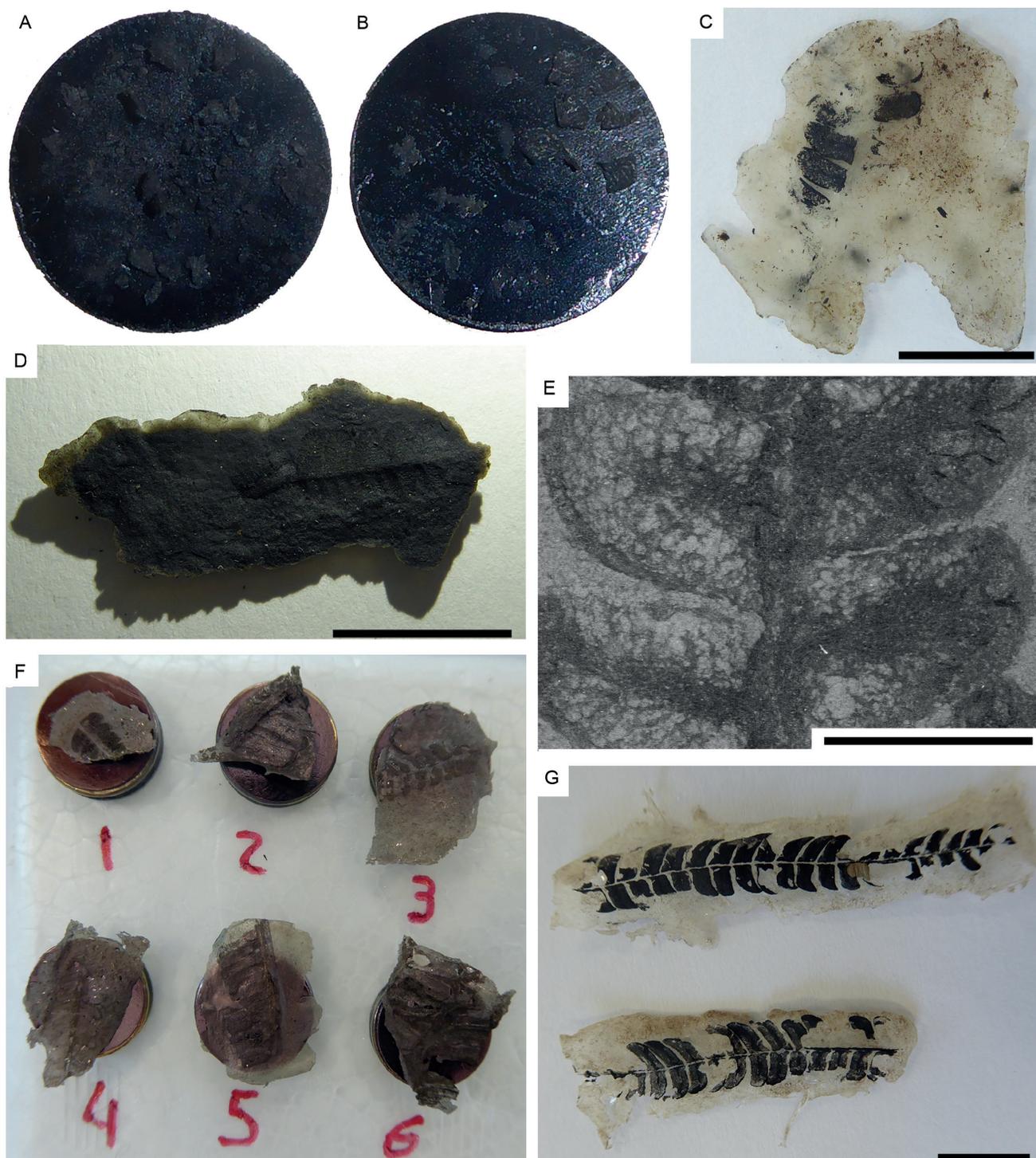


Figure 1. Different preparations for observation under the SEM. **A–B**, SEM slides with fragmentary material extracted directly with HCl (MCCM-LH 38607 and 38608); **C**, specimen extracted by covering with candle wax (MCCM-LH 38609); **D**, specimen extracted by covering with latex (MCCM-LH 38610); **E**, SEM image of IRSNB b9502 embedded in the matrix; **F**, SEM slides with collodion preparations covered with gold (MCCM-LH 38611–38616); **G**, specimens extracted by covering with collodion (MCCM-LH 386017 and 38618); scale bars C, D and G = 1 cm; scale bar E = 2 mm.

located in the inside of the lower epidermis, running parallel to the veins and sometimes disperse in the spongy mesophyll (Fig. 5E). The stomata were only clearly observed in specimens from Las Hoyas and are only present in the lower epidermis. They consist of two guard cells that measure 30–61 μm x 9–18 μm (Fig. 5A–5B). The stomatal aperture is 25–50 μm long, and the total width of the stomatal apparatus is 28–47 μm . Their general orientation is approximately at a 30° angle from the midvein (Fig. 5C). However, some of them appear perpendicular to the general disposition, and are normally associated with circular structures that measure 20–35 μm (Fig. 5D). The circular structures are similar to basal cells in *Danaea* Sm. that bear trichomes, although these are larger, around 50 μm wide.

The margin of the pinnules is rolled towards the abaxial side of the leaf in all specimens. In the preparations of complete pinnules, especially the latex ones, this margin appears to have a different structure, with elongated cells that are similar to the conductive cells from the nerves (Fig. 6A–6B).

The rachis is very similar in all the preparations and only the conductive tissue is preserved (Fig. 6E). One of the fragments shows a tissue under the conductive tissue that could be the cortex of the rachis, however this is not clear. The tracheids can be observed in the rachis are the same as those observed in the pinnules, they have scalariform and annular thickenings and measure 6–10 μm wide (Fig. 6F). The rest of the cylindrical conductive cells are also similar to those observed in the pinnules and measure 14–17 μm x 9 μm .

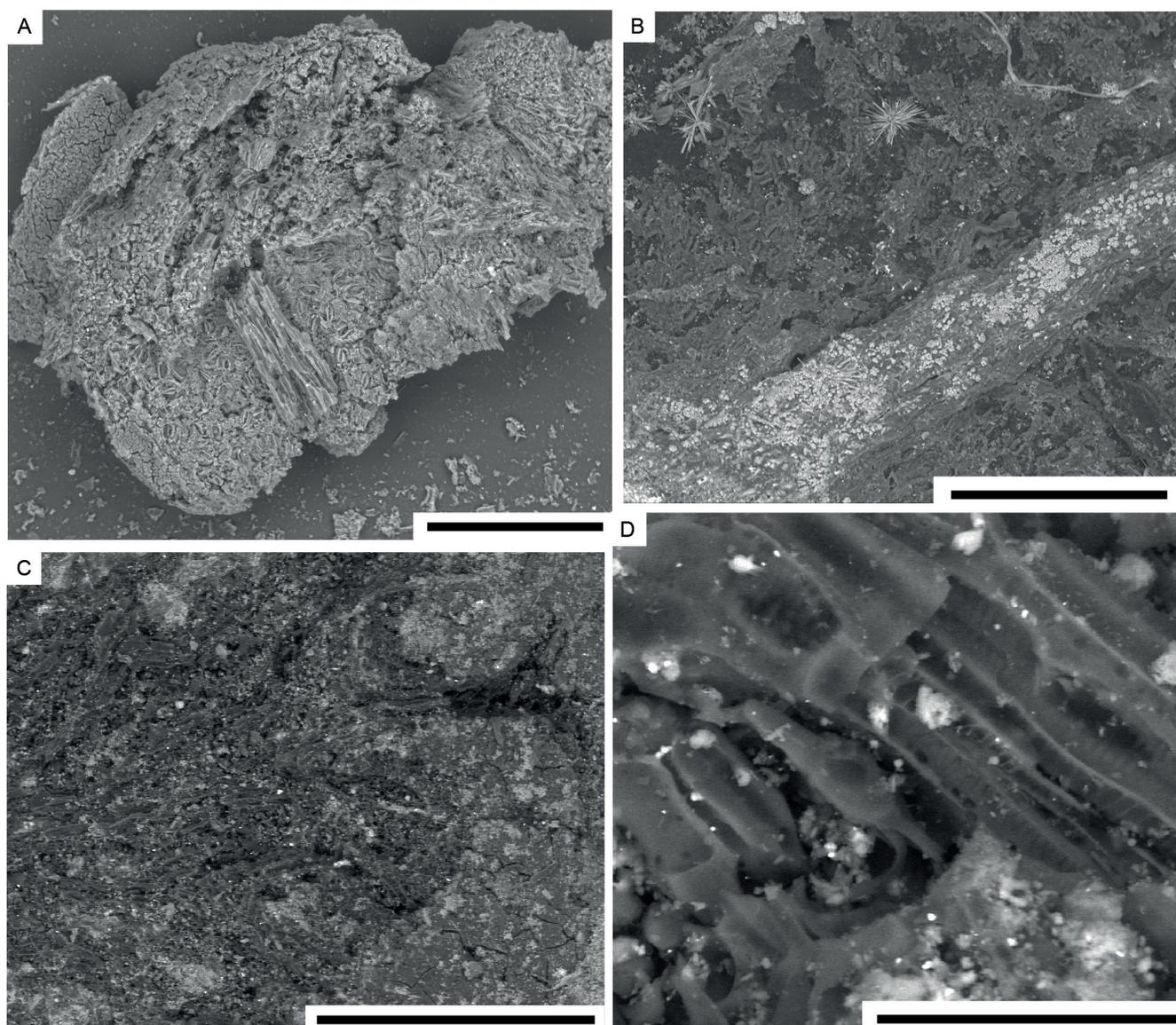


Figure 2. SEM images of obscuring elements. **A**, overview of the different tissues of the pinnule lamina (MCCM-LH 38608); **B**, wax preparation showing crystallizations (MCCM-LH 38609); **C–D**, 'dusty' appearance of pinnules embedded in the matrix (IRSNB b9502); **C**, upper epidermis and palisade parenchyma; **D**, vascular tissue showing scalariform pits; scale bar A–C = 500 μm ; scale bar D = 50 μm .

Venation pattern

The latex and candle wax preparations allow for the detailed study of the venation pattern of the pinnules (Figs. 1C, 6B). The type of reticulation according to the

classification by Wagner (1979) is simple reticulate, with no free included veinlets. It is intrasegmental, costal (Fig. 6D), although the apical pinnules generally

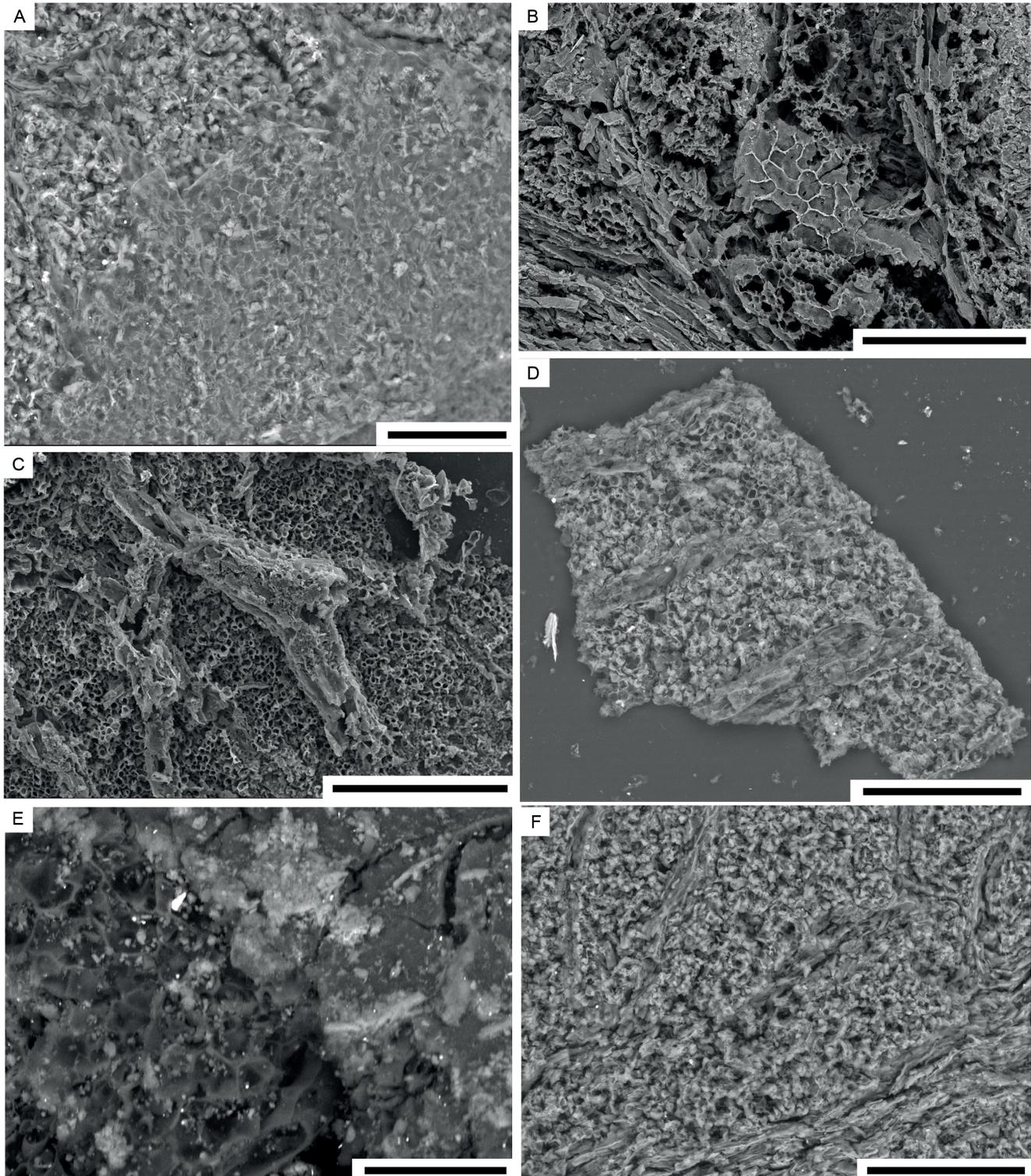


Figure 3. SEM images. **A**, cuticle, upper epidermis and veins (MCCM-LH 38617); scale bar = 100 μm; **B**, upper epidermis preserving the morphology of the cells and underlying parenchyma (MCCM-LH 38615); scale bar = 200 μm; **C–D**, palisade parenchyma and veins; **C**, MCCM-LH 38611; scale bar = 300 μm; **D**, MCCM-LH 38608; scale bar = 200 μm; **E**, large upper epidermis cells and underlying parenchyma of the Bernissart specimen (IRSNB b9502); scale bar = 50 μm; **F**, mesophyll and veins (MCCM-LH 38617); scale bar = 200 μm.

present dichotomizing free venation (Fig. 6C). The width of the veins ranges from 40 μm to 168 μm . In a complete pinnule, measurements were taken to

observe differences in the width within the pinnule: veins measure 72–104 μm at the base, 40–75 μm in the middle part, and 79–108 μm near the margin.

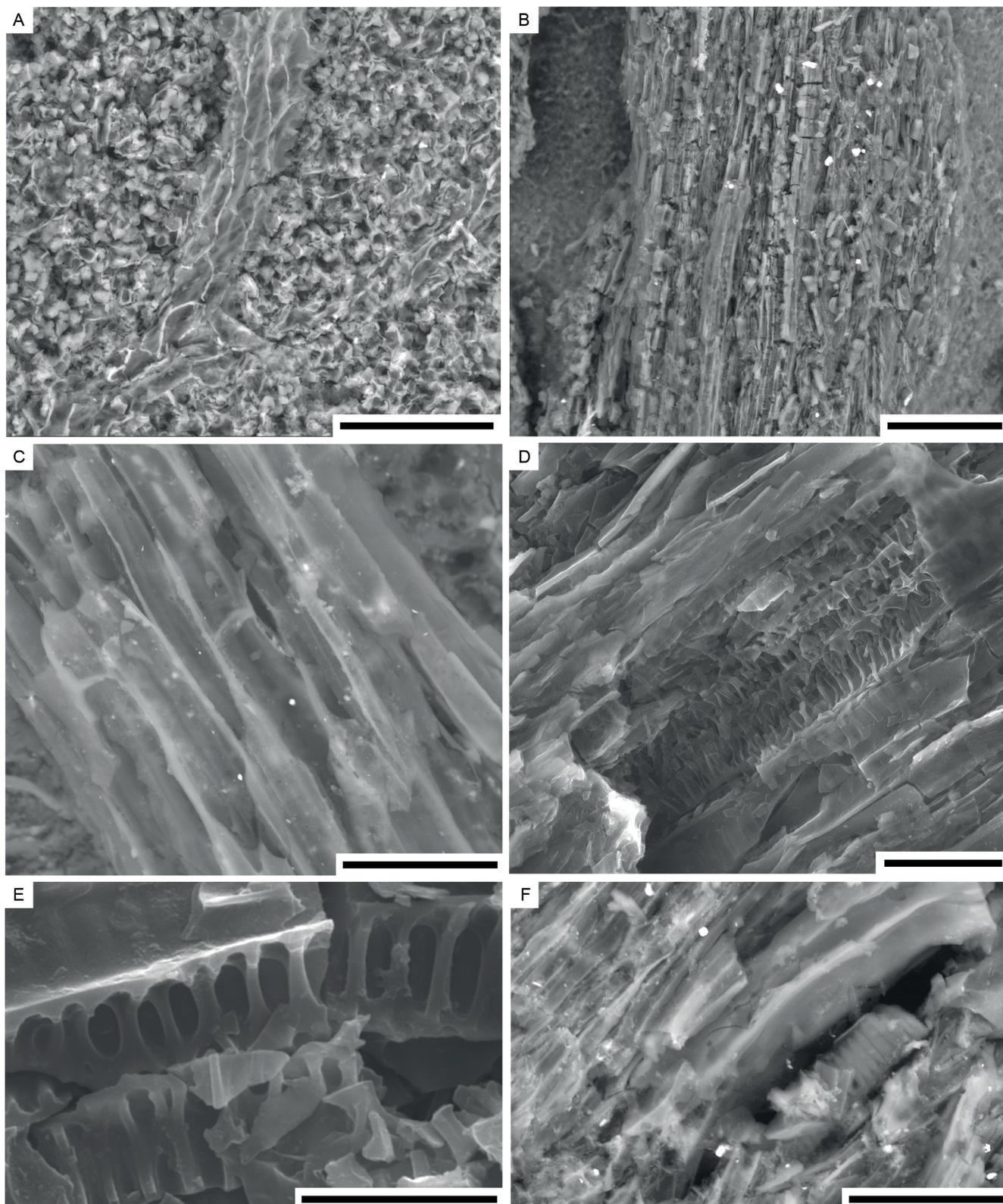


Figure 4. SEM images of the vascular system. **A**, conductive tissue (MCCM-LH 38617); scale bar = 100 μm ; **B**, cylindrical cells of the vascular system (MCCM-LH 38618); scale bar = 100 μm ; **C**, conductive cells, probably phloem (MCCM-LH 38608); scale bar = 50 μm ; **D–E**, tracheids with scalariform thickenings; **D**, MCCM-LH 38608; scale bar = 20 μm ; **E**, MCCM-LH 38611; scale bar = 10 μm ; **F**, tracheid with annular thickenings (MCCM-LH 38608); scale bar = 50 μm .

DISCUSSION

Comparison with previously published specimens from other localities

The results obtained in the present study (summarized in Fig. 7A as a simplified reconstruction) agree with the general reconstruction of Alvin (1974) (redrawn in Fig. 7B). However, the stomata seen in the preparations of *Weichselia* pinnules from Las Hoyas (no clear stomata could be seen on the specimen from Bernissart) show

no subsidiary cells. In some of the preparations, the guard cells are broken and only the thickened cell walls that surround the stomatal pore are preserved. In these cases, only the contour of the peripheral part of the guard cell can be seen. This preservation could be what Florin (1919), Alvin (1974) and Collinson (1999) interpreted as subsidiary cells surrounding the guard cells. Additionally, the size of the stomata of the specimens from the Isle of Wight is larger than the specimens from Las Hoyas, measuring 50–85 μm

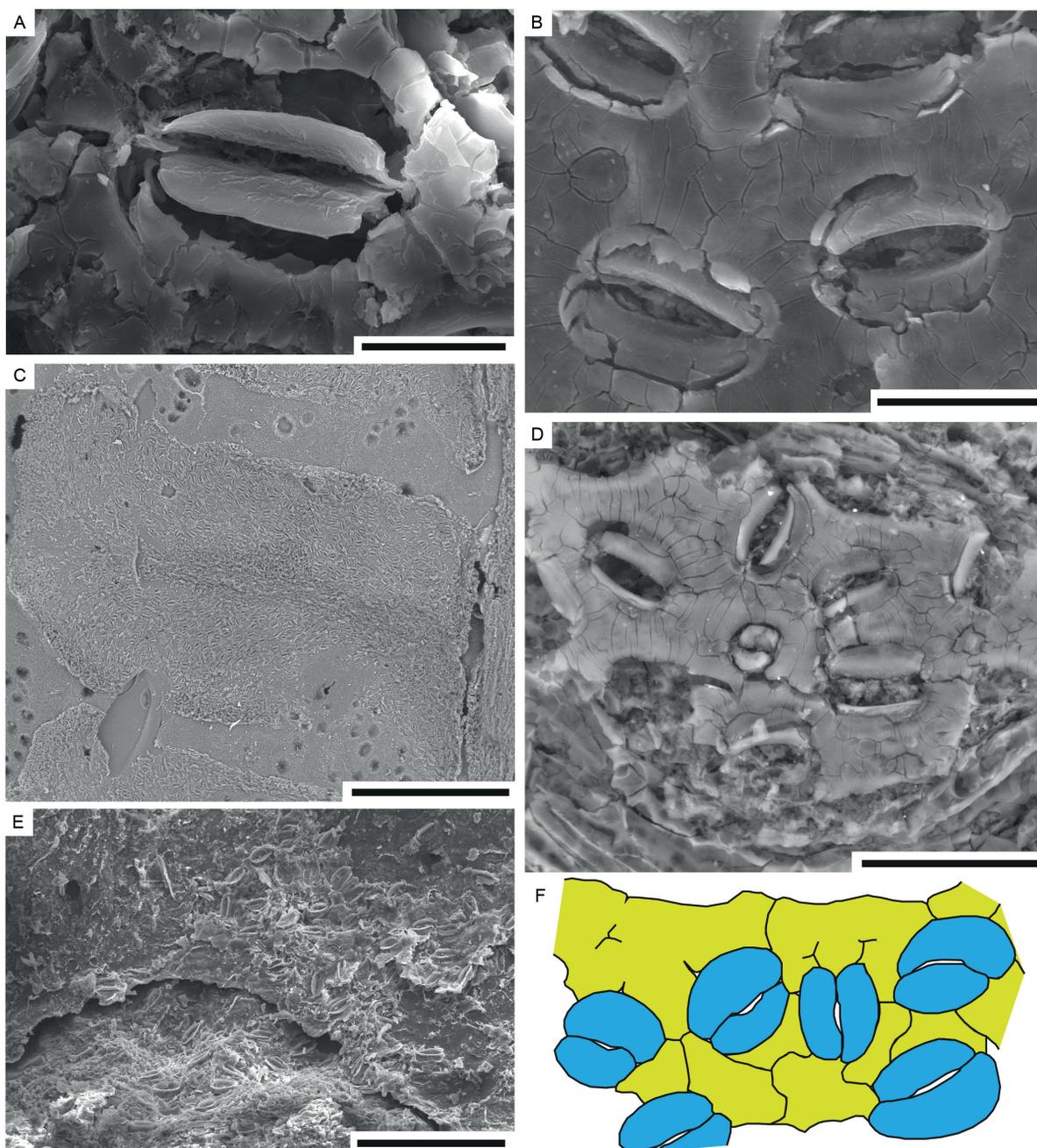


Figure 5. SEM images of stomata and lower epidermis. **A**, stomata with broken guard cells, MCCM-LH 38616; scale bar = 30 μm ; **B**, stomata and fused cells of the lower epidermis, MCCM-LH 38608; scale bar = 50 μm ; **C**, lower face of a pinnule covered with stomata (MCCM-LH 38618); scale bar = 1 mm; **D**, stomata surrounding possible basal cell (MCCM-LH 38608); scale bar = 100 μm ; **E**, internal face of the lower epidermis with stomata and silicate inclusions (MCCM-LH 38616); scale bar = 300 μm ; **F**, drawing modified from Alvin (1971) showing the disposition of the epidermal cells in relation to the stomata.

(Alvin, 1974) where Las Hoyas specimens measure 30–61 μm and are generally around 46 μm .

The scalariform thickenings observed in the tracheids are also present in both pinnules and rachises in the English material (Alvin, 1974) but not in the material from the Barremian of Jordan (Abu Hamad *et al.*, 2016).

This could be due to the fact that the pinnules from Las Hoyas and Bernissart show a better preservation, and that the details of tracheids and parenchyma cells in the material from Jordan are obscured by crater-like structures interpreted as pyrite (Abu Hamad *et al.*, 2016). The width of the tracheids is also slightly smaller

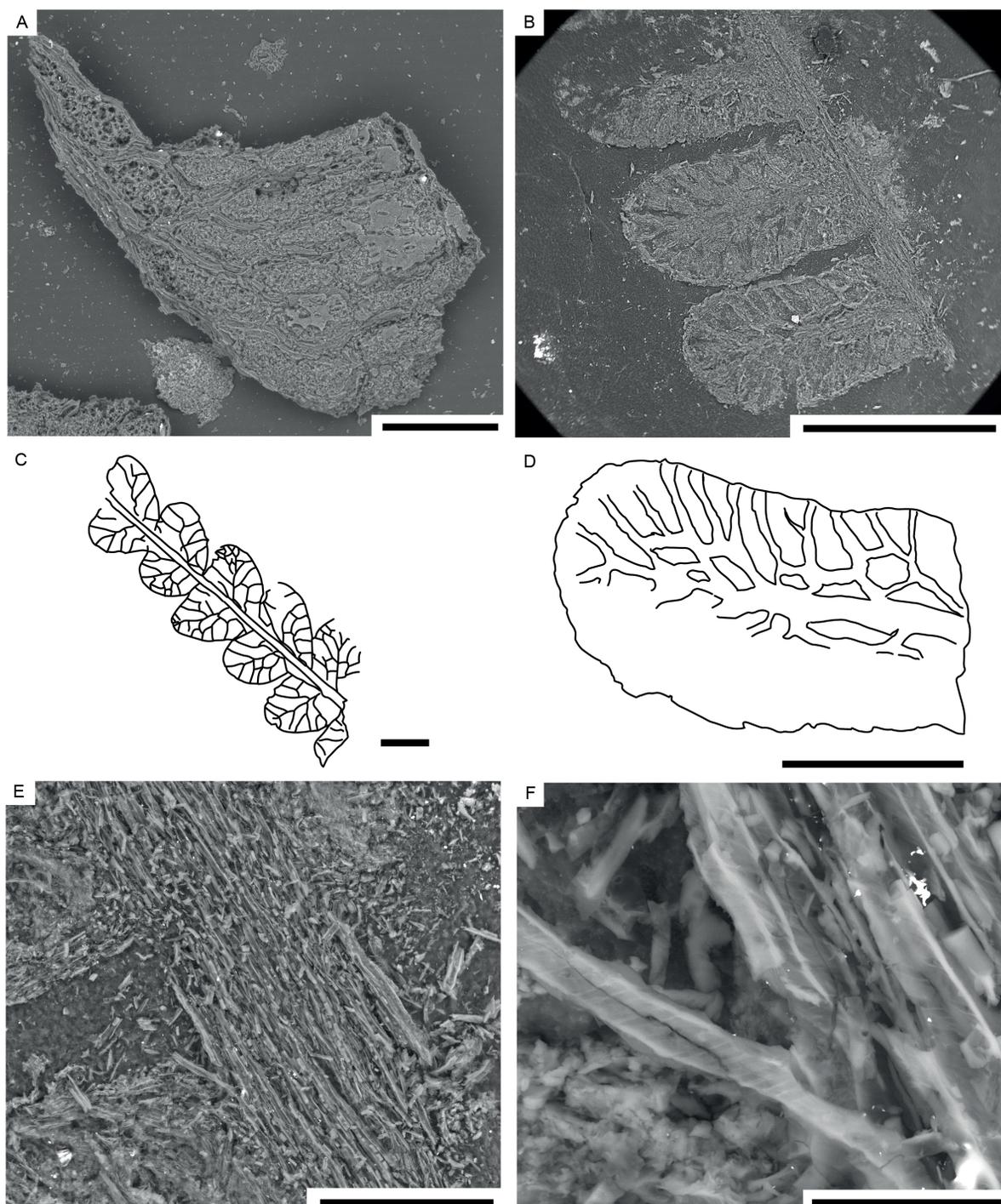


Figure 6. Venation and rachis ultrastructure. **A**, SEM image of the venation and thickening of the margin of the pinnule (MCCM-LH 38608); scale bar = 500 μm ; **B**, SEM image of the thickening of the margin of the pinnule and venation clearly visible in latex specimen (MCCM-LH 38610); scale bar = 2 mm; **C**, drawing of the venation of the apical portion of a pinna (MCCM-LH 14037a); scale bar = 1 mm; **D**, drawing of the simple reticulate, intrasegmental, costal venation of a mature pinnule prepared with latex (MCCM-LH 38610); scale bar = 1 mm; **E**, SEM image of the vascular system at the pinna rachis (MCCM-LH 38610); scale bar = 300 μm ; **F**, SEM image of the tracheids with scalariform thickenings in the pinna rachis (MCCM-LH 38610); scale bar = 50 μm .

(6–12 μm) in Las Hoyas and Bernissart specimens, than the material described by *Abu Hamad et al. (2016)*, which ranges 10–20 μm . This difference in size could be due to the fact that all the tracheids measured in this work are broken, except for one measuring 10 μm , and also that the pinnules from Jordan are slightly larger in general size than the ones from Las Hoyas and Bernissart (*Blanco-Moreno et al., 2018*). The fact that the rachises from Las Hoyas in most cases only preserve the conductive tissue could be a result of the charring of the pinnae (*McParland et al., 2007*), a phenomenon that can also be observed in the burnt rachises from Jordan (*Abu Hamad et al., 2016*).

Comparison with Recent ferns and systematic implications

The systematic position of *Weichselia* has been very discussed, and it has mainly been included in families Marattiaceae or Matoniaceae (*Edwards, 1933; Alvin, 1971*), based on different interpretations of the fertile structures (*Sender et al., 2015; Silantieva & Krassilov, 2006*). The results of the general histology of the pinnule of *Weichselia reticulata* herein obtained show distinct characters that suggest affinities with the Marattiaceae, and some features that are shared with the Matoniaceae.

Epidermal features suggest a closer affinity to Marattiaceae. Although epidermal cells are fused and cracked in the specimens studied due to burning,

obscuring their relationship with the guard cells, *Alvin (1971, fig. 1)* (Fig. 5F) depicted their disposition from better preserved specimens. The disposition corresponds to type 1 in *Thurston (1969)*, which can be found in families Ophioglossaceae, Marattiaceae, Osmundaceae, Thyrsopteridaceae and Culcitaceae. Additionally, the circular structures observed in association with stomata in the lower epidermis, which are very similar to trichome bearing basal cells in *Danaea (Rolleri et al., 1991)*, also suggest a close affinity to Marattiaceae.

The thickenings observed in the margin of the pinnules of *Weichselia* are very similar in their structure to the ones present in *Matonia pectinata*, where the margins are strongly recurved (*Seward, 1899*). *Harris (1981)* suggested that the pinnules in *Weichselia reticulata* could have been recurved due to the process of charring, however, the observed thickenings, and the presence of this type of margin in other ferns, argues against this. It must be noted that recurved margins are quite frequent in ferns (including Marattiales), although the precise ultrastructure of these thickenings has not been published.

The cellular types and elements observed within the lamina of *Weichselia reticulata* pinpoint peculiarities that are rarely present in fern taxa. For example, the palisade parenchyma, which is clearly observed in the specimens herein studied, is rare in ferns, and it is absent in *Matonia pectinata* R. Br. (*Seward, 1899*) and most Marattiaceae except for *Angiopteris Hoffm.* (*Lavalle,*

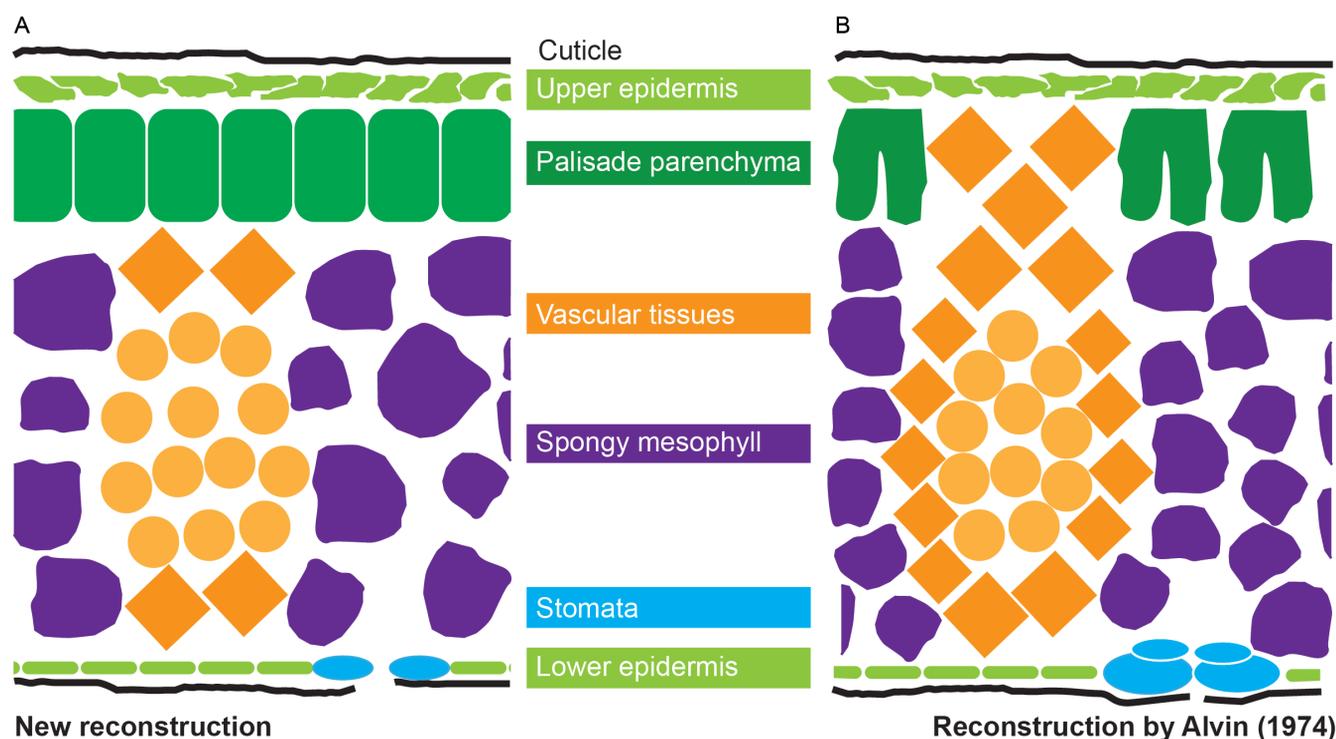


Figure 7. Simplified diagrams of the reconstruction of the pinnule lamina of *Weichselia reticulata*. **A**, reconstruction based on the results of this work; **B**, reconstruction based on the diagram published by *Alvin (1974)*: including the palisade-like cells, the protected vascular system which reaches to the upper epidermis, and the subsidiary cells of the stomatal apparatus.

2005), suggesting a closer affinity to the latter family. Interestingly, both *Matonia pectinata* and Marattiaceae have minute rods in the spongy mesophyll next to the lower epidermis (Seward, 1899), which are similar to structures in the spongy mesophyll in *Weichselia* which have been identified as silicate inclusions (Fig. 5E), although in the case of *Weichselia* they very frequently run parallel to the veins, while in the other groups they are dispersed in the spongy mesophyll (Seward, 1899; Lavalle *et al.*, 2008). Possible mucilage canals, essential elements that are present in the pinnule lamina of the Marattiaceae (Smith *et al.*, 2006), were identified in the stem of *Weichselia reticulata* (Blanco-Moreno *et al.*, 2020), however, they have not been observed in the present analysis.

Focusing on the vascular system, venation in ferns has a systematic value (Wagner, 1979). Reticulate venation can be found in many groups of ferns, however most families only show one reticulate pattern, except for Dryopteridaceae and Thelypteridaceae which have both simple and complex intersegmental venations. Within the Marattiaceae, reticulate venation is intrasegmental and complex (Wagner, 1979). In the Matoniaceae reticulate venation is intrasegmental and simple (Wagner, 1979), and costal in *Matonia* R. Br. ex Wall (Kato, 1998), as it occurs in *Weichselia reticulata* and other fossil genera that have been assigned to the Matoniaceae such as *Phlebopteris* Brongn., *Piazopteris* Lorch, and *Matonidium* Schenk (Van Konijnenburg-van Cittert, 1993). Another similarity with *Matonia* is the protection of the vascular tissue, surrounded by rhomboidal elongated cells. However, *Matonia* presents few tracheids, and phloem is scarce (Seward, 1899) which is rare, as most fern species have numerous vessels and tracheids (Carlquist & Schneider, 2001), including *Weichselia*.

Another diagnostic character is the absence of endodermis in Marattiaceae (extant and fossil) and *Ophioglossum* L. (Bower, 1926), which is present in every vascular strand in other ferns (Priestley & Radcliffe, 1924). The Belgian stem material of *Weichselia* does not preserve the endodermis (Alvin, 1971; Blanco-Moreno *et al.*, 2020). In the case of the fragmented charred pinnae from Las Hoyas and Bernissart, the endodermis has not been identified in the pinnules nor rachises. It must be noted that studies on extant fern petioles show that when these are burnt, they break at the connection of the cortex and the stele (McParland *et al.*, 2007), making the endodermis hard to identify in charred remains.

The epidermal features and peculiarities in the ultrastructure of the lamina of *Weichselia reticulata* suggest an affinity with the Marattiaceae, however, the venation and some of the vascular elements are more similar to the Matoniaceae. The absence of endodermis, although it has not been confirmed and preservation biases cannot be ruled out, would also strongly indicate a phylogenetic relationship with Marattiales in general.

Autecological and climatic insights

The study of the ultrastructure of the pinnules of *Weichselia reticulata* provides some insights on the habitat where these ferns grew. Traits such as the morphology of the pits in the tracheids have been demonstrated to give a signal on the variation in the humidity of the habitat of the plant: scalariform pits, such as those present in *Weichselia*, are related to habitats with changing humidity, while circular pits are more related to uniformly humid areas where there are not strong fluctuations in water availability (Carlquist & Schneider, 2001). Although Las Hoyas was located in an arid climate belt and Bernissart in a warm humid belt (Hay & Floegel, 2012), the pits observed on the specimens from localities are scalariform, suggesting that this species lived in habitats with strong water availability fluctuations in the vicinity of both localities. Other traits such as venation density, trichome density, and stomatal density also correlate with climate, mainly with temperature and humidity (Kessler *et al.*, 2007). This is very interesting for the study of local scale differences between localities or even within the same locality in a temporal scale. Future research will focus on these traits in order to provide some approximations to possible differences in climate in the Barremian of Europe.

Collodion coating for SEM observation: future prospects

This is the first time that remains prepared using collodion have been observed under the SEM. This technique allows for a perfect visualization of plant fossil material with no interference of the collodion. The positive results obtained open the possibility of observing plant remains embedded in thick rocks that could not be directly inserted in the SEM machines, without fragmentation of the material. Additionally, the specimens can be coated with gold and observed with higher resolution in high vacuum pressure mode. The use of collodion to avoid the fragmentation of plant remains when extracted from their sediment matrix had been previously tested, for example, in transfers of stems of early plants that were posteriorly bleached and observed under the optic microscope (Posthumus, 1923), and in transfers of angiosperm compressions (Dilcher, 1974). This suggests non charcoalified compressions can also be successfully separated from the matrix by this method, and that collodion coating could be used to prepare a wide range of plant specimens for their observation under SEM with a high resolution. It must be noted that the exhaustive results obtained in this paper were only possible through the combination of fragmented specimens (separated from the matrix using acid only), where all tissue layers could be observed in greater detail, and unfragmented specimens, where the relative position of the lamina elements could be identified.

CONCLUSIONS

The study of the anatomy of charred pinnules from two Lower Cretaceous localities, Las Hoyas and Bernissart, has proven greatly informative on the reconstruction of the lamina of *Weichselia reticulata*. An absence of subsidiary cells in the stomata has been detected and the vascular system has been described in a more precise way: with scalariform pits and less rhomboidal cells protecting the vascular tissues than previously interpreted. These characters suggest *Weichselia* lived in a habitat with changing humidity in both localities, which was not as arid as previously thought. The analysis of the traits observed has also provided novel insights on the systematic assignment of the species and, as already observed in the anatomy of the stems, suggests a closer affinity to Marattiales. The new protocols for the preparation of fossil material for its observation under SEM allow for the use of a precise methodology which enhances the observation of each target organ/tissue/structure. Future analyses on more specimens from both localities, and from other localities throughout the chronostratigraphic and geographical range of the species will provide comparative data on stomatal, trichome and venation density, and on vessel pit type, which will help to elucidate if *Weichselia reticulata* inhabited habitats with different climatic conditions.

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