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Gaëtan Guignard

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Method Article – Title Page

Title	Method for ultrastructural fine details of plant cuticles by transmission electron microscopy
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Keywords	<ul style="list-style-type: none">• Ultrastructure• cuticular sections• fossil and living plants
Co-Submission	<i>Co-Submission</i> Gaëtan Guignard G., 2019. Thirty-three years (1986–2019) of fossil plant cuticle studies using transmission electron microscopy: a review. Review of Palaeobotany and Palynology. DOI 10.1016/j.revpalbo.2019.07.002

ABSTRACT

The dataset corresponds to a review article “Thirty-three years (1986–2019) of fossil plant cuticle studies using transmission electron microscopy: a review” [1]. This method for plant cuticles was adapted from spores and pollen procedure. It already enabled to prepare ultrathin plant cuticle sections of 60-70 nanometers of thickness, in high numbers as a routine, and allowed statistical measurements as any other macro- and/or micro-features. It provides through transmission electron microscope clean and contrasted observations of layers and sublayers of the cuticle, at a magnification over 100,000 times. It has been used efficiently for taxonomy, signature of environment and evolution topics since the end of the nineties [1], however it is detailed here step by step for the first time, and illustrated. It can serve in the future for many purposes, like other taxonomical comparisons, palaeoreconstructions and evolutionary considerations.

Value of the protocol

- This method provides ultrathin plant cuticle sections of 60-70 nanometers of thickness
- Details of layers and sublayers of the cuticle are easily observed
- High numbers of sections allows statistical measurements as a routine

SPECIFICATIONS TABLE

Subject Area	Agricultural and Biological Sciences
More specific subject area	Method for obtaining high numbers of ultra-thin sections of 60-70 nanometers observed with transmission electron microscope, enabling clear magnifications over 100,000 times.
Method name	Method for ultra-thin sections of plant cuticles

<p>Name and reference of original method</p>	<p>This method is adapted from Lugardon's method used for living and fossil spores and pollen, there is no specific name for this method in the papers of Lugardon, parts of it are in the following documents, including thesis:</p> <p>[2] B. Lugardon, Contribution à la connaissance de la morphogénèse et de la structure des parois sporales chez les Filicinées isosporées (Unpublished Thesis), Toulouse University, France, 257 pp. 1971. (in French).</p> <p>[3] C. Arioli, C.H. Wellman, B. Lugardon, T. Servais, Morphology and wall ultrastructure of the megaspore <i>Lagenicula (Triletes) variabilis</i> (Winslow, 1962) Arioli et al. (2004) from the Lower Carboniferous of Ohio, USA, Rev. Pal. Pal. 144 (2007) 231–248. doi:10.1016/j.revpalbo.2006.07.011.</p> <p>[4] L. Grauvogel-Stamm, B. Lugardon, Phylogeny and evolution of the horsetails: Evidence from spore wall ultrastructure, Rev. Pal. Pal. 156 (2009) 116–129. doi:10.1016/j.revpalbo.2008.10.002.</p> <p>[5] C. Wellman, L. Grauvogel-Stamm, G. Guignard, Studies of spore/pollen wall ultrastructure in fossil and living plants: a review of the subject area and the contribution of Bernard Lugardon, Rev. Pal. Pal. 156 (2009) 2-6. https://doi-org.inee.bib.cnrs.fr/10.1016/j.revpalbo.2009.03.010.</p> <p>[6] L. Grauvogel-Stamm, G. Guignard, C. Wellman, Studies of spore/pollen wall ultrastructure in fossil and living plants: a homage to Bernard lugardon, a talented palynologist, Rev. Pal. Pal. 156 (2009) 1. https://doi-org.inee.bib.cnrs.fr/10.1016/j.revpalbo.2009.03.003.</p> <p>[7] M.A. Morbelli, B. Lugardon, Microspore wall organisation and ultrastructure in two species of <i>Selaginella (Lycophyta)</i> producing permanent tetrads, Grana 51 (2012) 97-106. DOI: 10.1080/00173134.2012.691109.</p>
<p>Resource availability</p>	<p>In order to make it easily reproducible, all chemicals and material used may be commonly found in laboratories and do not necessitate here a precise description.</p>

***Method details** Three major difficulties of producing plant cuticle ultrathin sections in high numbers, to allow the observation of a maximum of their fine details by transmission electron microscope, are the proper infiltration of the resin in the material, i.e. the embedding, the contrast and the

cleanliness of the sections. The present method answers to these difficulties especially by the use of very long times of procedures, plus very careful staining and rinsing. It is adapted from Lugardon's method used for living and fossil spores and pollen with Spurr and Epon resin. It is used since the nineties for plant cuticles, mainly for fossils, summarized in all these former papers. As unfortunately the detailed method appears only partially in Lugardon's thesis [2] and in spore – megaspore – pollen articles [3-7], beside of the English text of the present paper, we reproduced here the most interesting method's page hand-written in French by Lugardon with a pencil (Fig. 1), during one of my first stays in Toulouse, also partly hand-written by me for some procedure details. This page contains a little part of the first step (fixation) and the whole long second step of the method (embedding).

In order to introduce briefly the plant cuticle, it is a very thin organic material covering leaves as commonly found in fossils, but "also present in fruits, stems, flowers and embryos as revealed in living plants" [1]. More in details, The cuticle of a plant (= cuticular membrane) may be divided into two regions: an outer cuticle proper in contact with the atmosphere in its outermost part, and in its innermost part an inner cuticular layer in contact with the cell wall of the subjacent cell (very rarely present in fossil material). The outer cuticle proper can be divided in two layers: an outermost A1 polylamellate layer possibly subdivided into A1U (= upper zone or sublayer, which can be straight or wavy) and A1L (= lower zone or sublayer). Just below an inner A2 granular layer can occur, divided in some cases into A2U (= upper zone or sublayer) and A2L (lower zone or sublayer). The inner cuticular B can be divided in two layers: fibrilous or spongy B1 in its outer part and granular B2 in its innermost part. The presence or absence of all these layers and sublayers depends of course on the taxa observed.

The method follows three steps, the two first ones being unusual as very long:

1. Fixation [appendix A, detailing with colour photos some steps of the embedding: fixation, possible use of different kinds of plants (fossil, but also living or dried), last step after the polymerization of the resin block at 60° C.]

Samples are firstly fixed during at least 3 weeks (actually, according to discussions with Lugardon, for spores and pollen there is no maximum duration, up to several years do not make any change in the results and these are for some taxa even better) at room temperature (20-23 °C) in paraformaldehyde (buffer solution: solution A, NaOH 2.52% in 100 ml H₂O; solution B, NaH₂PO₄ 2.26% in 100 ml H₂O; final solution: 17 ml A + 83 ml B; add 4 gr paraformaldehyde, diluted at 60° C and pH 7.2-7.4, adjusted with NaOH. Although pH is not relevant for fossil plants, it is very important for living plant cells, this is essential as it enables cuticles comparisons between fossils and living plants using the same method.

2. Embedding (appendix A)

The one week embedding step is adapted for cuticles (for fossil and living plants) with Epon resin (the best resin so far for an adequate hardness equilibrium between the resin and cuticle). Following the fixation in paraformaldehyde, the cuticles are dropped in the subsequent solutions and others:

- = **Day 1** (Monday) rinsing in H₂O 10 min (2 times). Osmium tetroxide OsO₄ 1% in Sodium-Phosphate buffer 24 hours, room temperature (20-23 °C);
- = **Day 2** (Tuesday) rinsing in H₂O 10 min. ethanol CH₃CH₂OH 50° 10 min (2 times), ethanol 70° 10 min + 1 hour (1 time each), ethanol 95° one overnight, room temperature (20-23 °C);

- **Day 3** (Wednesday) ethanol 100 °C 1 hour (4 times). Propylene oxide 1 hour (4 times). 2/3 Propylene oxide and 1/3 Epon Resin (A 1/3 + B 2/3) 1 overnight;
- **Day 4** (Thursday) 1/3 Propylene oxide and 2/3 Epon Resin 3 hours. Only Epon Resin (A 1/3 B 2/3) 1 overnight, room temperature (20-23 °C);
- **Days 5-6-7** (Friday to Sunday) drop of the cuticles in only Epon Resin + hardener in the moulds 60 °C 3 days (72 hours);
- **Day 8** (next Monday) Epon resin blocks are ready. In order to avoid humidity, it is better to keep them in a box with silica gel, room temperature (20-23 °C).

3. Sectioning and staining [appendix B, detailing with colour photos some steps of the sectioning of the blocks of resin containing the plant samples, staining of the grids, finally the observation of the plant cuticles with the transmission electron microscope TEM.]

At first 1 µm sections (cut with diamond knife) of the Epon resin blocks allow to select the best sections thanks to glass slides warmed at 100 °C in the oven (to stick the sections on glass) and observed with light microscope. Selected parts containing potential informative data (of ordinary epidermal cell cuticles, subsidiary and guard cell cuticles of the stomatal apparatuses...) are then cut in 60-70 nanometers sections also with diamond knife, deposited on copper grids (preferentially uncoated, i.e. devoid of Formvar as this film may bring opaque and/or dirty areas after the staining), dried during a few days (in order to avoid breakings especially with uncoated copper grids), then stained with both:

- uranyl acetate (7% in methanol 15 min), previously filtered, and in the dark (as it is very sensible to the light); then rinsed vigorously in order to avoid unclear areas and crystals on the cuticle sections (with a rapid movement of the pinset perpendicular to the surface of the water) in three 100% methanol baths (in order to avoid breakings of the sections especially with uncoated copper grids); between each bath the drops of liquid (containing potential "dirty" parts) still attached to the copper grids are dried with the edge of a filter paper;
- lead citrate (aqueous 6% solution 20 min) in a petri dish with NaOH pellets (lead citrate being very sensible to CO₂), then rinsed also vigorously (in order to avoid black dots and opaque areas) in one NaOH (one small drop) + water solution, finally rinsed also vigorously in three 100% water baths; between each bath the drops of liquid (containing potential "dirty" parts) still attached to the copper grids are also dried with the edge of a filter paper.

In order to avoid any "dirty" small parts, the water is freshly distilled and freshly filtered.

Method validation

The method allows, for fossil plants belonging to very various taxa observed until now (Coniferales, Ginkgoales, Czekanowskiales, Pteridospermales, Cycadales...) and from various geological periods [8-18, 21-28], also for living plants as angiosperms and gymnosperms [19-20], a very satisfying observation of the cuticles through transmission electron microscope. These sections are very flat (the efficient infiltration of resin makes no disruption between cuticle and Epon resin, mainly thanks to the long embedding procedure of one week), high numbers of sections can be obtained as a routine, over a few hundreds for some taxa, enabling statistical measurements. XLSTAT 2019.1 and XLSTAT3D Plot were used efficiently (Addinsoft (2019); XLSTAT statistical and data analysis solution. Long Island, NY, USA. Moreover the contrast of the cuticle contents through the staining steps is also very efficient and allows to distinguish all layers and sublayers described above. With very careful rinses at a maximum of steps, the sections are in their high majority devoid of "dirty" parts, enabling high magnifications over X 100,000 and high quality electron micrographs (examples in Fig. 2). It is also available for fossil spores with some personal papers published together with Lugardon [29-33] and reproductive organs with cuticles [34].

Captions of figures and appendices

Figure 1. Hand-written embedding method part, by Lugardon.

It is all translated in English in this paper. Some additions also in French are from the author of the present paper, added during discussions.

Figure 2. Examples of transmission electron microscopy fossil cuticle details of layers and sublayers.

OEC = ordinary epidermal cell cuticle, GC = guard cell cuticle, ECM = extracuticular material over the cuticle. A. wavy polylamellae of A1 layer, outermost part of the cuticle, just below extracuticular material. Granules of the A2 layer are below, and extracuticular material over the A1 layer; *Pseudofrenelopsis gansuensis* (Coniferales, Cheirolepidiaceae), Mesozoic (Lower Cretaceous), OEC. B. straight lamellae of A1 layer, making in this case in two sublayers: A1U (U = upper) sublayer (or zone) with straight polylamellae and A1L (L = lower) sublayer (or zone), with fewer lamellae oriented more oblique; *Ginkgoites ticoensis* (*Ginkgoales*), Mesozoic (Lower Cretaceous, Aptian), OEC. C-D. Granules of A2 and fibrils of B1 layers mixed and making various schemes; *Ginkgoites ticoensis* (*Ginkgoales*), Mesozoic (Lower Cretaceous, Aptian), OEC. E-H. Fibrils of B1 layer with very various degrees of concentration, making a spongy aspect; the fibrils are more or less mixed with delicate granules; *Brachyphyllum garciarum* (Coniferales, Araucariaceae affinities), (Early Cretaceous), GC. I-J. Very homogeneous granules of B2 layer, lowermost part of the cuticle; *Pseudofrenelopsis gansuensis* (Coniferales, Cheirolepidiaceae), Mesozoic (Lower Cretaceous), OEC.

For other photos of these cuticle taxa, see [21] for *Ginkgoites ticoensis*, [25] for *Pseudofrenelopsis gansuensis*, [28] for *Brachyphyllum garciarum*.

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Declaration of interests:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix B. Some steps of the sectioning, staining and observation of the plant cuticles, for transmission electron microscopy

To appear in colour

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TEPI technique Legendre 1990

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