Ultrastructure and cytochemistry of Miocene angiosperm leaf tissues

(angiosperm paleobotany/cytology/electron microscopy/histochemistry/evolution)

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ABSTRACT Angiosperm leaf fossils ($16.7\text{--}25 \times 10^6$ years by potassium/argon dating) referable to *Zelkova* were isolated from pyroclastic deposits in the Succor Creek Formation, Oregon. These fossils reveal a three-dimensional structure in mesophyll cell layers and vascular bundles, while transmission electron microscopy of fixed tissues reveals well-preserved chloroplasts with grana stacks and starch, as well as nuclei with condensed chromatin. Transmission electron microscopy indicates that cell walls retain a cellulosic microfibrillar architecture, while ultrastructural and cytochemical confirmation is presented for chloroplast starch and the presence of native cellulose. The preservation of cell walls and cytologic structures in fossil materials of this age indicates an unusually mild fossilization process attending the post-depositional maturation of associated sediments. The preservation observed appears to be associated with rapid physical dehydration coupled with endogenous fixation by organic acids (possibly tannins and/or chlorophyll derivatives).

Certain physiochemical conditions may permit the preservation of cytoplasmic remnants in fossil materials of extreme age if autolytic or necrotic processes can be sufficiently reduced or temporarily stopped. In at least a few well-documented cases, the preservation of very delicate cytologic structures has been demonstrated in very ancient plant fossils (1-9). Physical factors attending fossilization may also be sufficiently mild to permit the preservation of biochemical markers that have heretofore been thought too labile so as to preclude their presence in fossil sediments (10-15). While biochemical fidelity in fossils has prompted immunological investigations of problematic fossil taxa,[§] there are no reliable reports of biochemically intact membranes preserved *in situ*. Speculation as to the nature of geochemical factors favoring the preservation of living plant structures indicates that the gradual infiltration of mineral-charged fluids over relatively short time spans may permit the fixation of intracellular structures (16). Few histologic examinations of fossil animal tissues have been attempted (17) other than in cases of cryologic permeation, as in frozen Pleistocene deposits (18, 19). Rehydration of frozen tissue samples taken from the "mummified" remains of mammals dated at from 15,000 to 25,000 years old reveals that muscle and liver tissues may retain some ultrastructural fidelity; however, most tissues are completely or partly replaced by bacterial masses.

The present study attempts to document the preservation of cell ultrastructure in angiosperm leaf tissues. Correlated light and scanning and transmission electron microscopy of *in situ* leaf fossils indicate that some regions of leaf tissues are virtually unaltered and show little or no three-dimensional distortion due to compression. Previous analyses of these specimens reveal a

high level of phytochemical congruence between fossil taxa and their respective nearest living representative (20), while considerations of geochemical profiles indicate that relatively mild physiochemical factors attended post-depositional maturation of associated sediments (21).

MATERIALS AND METHODS

The fossil material investigated in this and previous reports was collected from the Succor Creek Formation, late Hemingfordian or early Barstovian stage, of the Middle Miocene, Oregon. Potassium/argon (K/A) dating of the Succor Creek flora and fauna by Evernden and James (22) gave an age of 16.7×10^6 K/A years, while more recent K/A datings on our material give a range of ages ($36\text{--}25 \times 10^6$ K/A years). Rubidium/strontium dates for the same locality average 22×10^6 R/S years. The incorporation of foreign materials into welded tufts is a major factor in the discrepancies observed in radiogenic datings. On the basis of isotopic and stratigraphic data, the Succor Creek materials used in this study are considered Miocene in age and are at the minimum between 25 and 16.7 million years old. The specimens used in this report were collected approximately 13.2 miles from Jordan Valley along Interstate 95, and some 3.7 mile from Interstate 95 towards Davisville (reference map: Rockville, Oregon-Idaho N4315-W 11700). Leaf tissues appearing a vivid green in color were excavated from lenticular tufts and tuffaceous siltstone and are thought to have been buried as a result of rapidly falling, relatively cool volcanic ash.

A leaf was carefully removed from the ash sediment and placed under nitrogen. The upper portion was maintained for scanning electron microscope analysis: the specimen was glued to a stub and then carbon, gold-palladium coated. The lower part of the leaf was fixed and embedded for transmission electron microscopy as follows: The light-brown leaf was placed into a fixative containing 2% (vol/vol) glutaraldehyde, 1% (wt/vol) tannic acid, and 50 mM cacodylate buffer at pH 7.2. At first the leaf floated, so it was swirled gently and intermittently for the first 15 min. At the end of this period, sufficient fixative had penetrated the tissue so that the leaf settled to the bottom of the fixation vial. The leaf was in the fixative for 45 min at 25°, then transferred to 4° for 20 hr. After washes in buffer, the sample was postfixed in 2% OsO₄ in 50 mM cacodylate buffer, pH 7.2, for 3 hr at 4°. Then the sample was rinsed once in buffer as described above, then once in deionized water. The sample was dehydrated with ethanol and infiltrated with

Abbreviations: K/A years, years by potassium/argon dating; PATAG, periodic acid-thiocarbohydrazide silver-staining method (23).

[§] Schmid, R. S., Wolniak, M. & Vreeland, V. J. (1976) *Electron Microscopy and Immunochemistry of Prototaxites* (Botanical Society of America, Lawrence, KA), p. 31 (abstr.).

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FIG. 1. (Legend appears at bottom of the following page.)

Spurr's Epoxy resin. Sections were made with a Rotch OM-U2 Ultramicrotome and transferred to copper grids for conventional examination and to stainless steel grids for cytochemical tests. Material on the grids was post-stained with 2% aqueous uranyl acetate followed by Reynolds lead citrate. For cytochemical analysis of carbohydrates the periodic acid-thiocarbohydrazide silver-staining (PATAG) method was used (see ref. 23). Vouchers of *in situ* fossils and scanning and transmission electron microscopy specimens are stored at The New York Botanical Garden and the Department of Botany, University of North Carolina, Chapel Hill, NC.

RESULTS

Morphology and ultrastructure

In situ fossil leaves were mechanically removed from associated rock matrices (Fig. 1 A–C) and prepared for scanning electron microscopy (Fig. 1 D–F) and transmission electron microscopy (Figs. 1 H–K and 2). A typical angiosperm leaf anatomy is discernable at both the light and electron microscopy levels with a cuticle external to the epidermis (Figs. 1 D–G and 2 C and D). Fractured leaves show tracheids and palisade/spongy mesophyll (Fig. 1 E and F), while correlated light and transmission electron microscopy reveals exceptionally well-preserved cell walls and some cytoplasmic organelles (Fig. 1 G–J). Structures clearly identifiable as chloroplasts are present in all cells examined. Typical chloroplast stroma/grana arrangements of the thylakoids are observed in negative contrast (Figs. 1 K and 2 A). Definitive bimolecular leaflet staining of the membranes is lacking. Starch bodies in chloroplasts are exceptionally well preserved, and the PATAG cytochemical test for carbohydrates substantiates the preservation of the original polysaccharide (Figs. 1 K and 2 A and B).

A structure interpreted as a heterochromatic nucleus is depicted in Fig. 2 B and E. Nuclear pores and the typical inner and outer reflections of the nuclear membrane are not resolved although, like chloroplasts, an electron-dense layer surrounds this body. Plasmodesmata channels are clearly preserved (Fig. 2 B) and the membranes appear only as electron-dense single layers. Membranes or profiles of membranes were not preserved in other cytoplasmic structures, namely, the Golgi apparatus, endoplasmic reticulum, plasma membrane, and vacuolar membrane. In contrast, the cell walls were well preserved (Figs. 1 K and 2 B) and produced positive reactions with the PATAG cytochemical test for carbohydrates. Cellulose microfibrils (see below) are well preserved and the cell walls show characteristic cross-hatching or lamination (Fig. 2 F). Both upper and lower surfaces of the leaf have a characteristic cuticle (Fig. 2 C and D) with prominent cuticular projections.

Chemistry

Solvent extractions, spectral characteristics, and cytochemistry permit the identification and localization of various organic constituents and confirm previously reported chemical profiles. X-ray diffractograms of pulverized and washed leaves show $2\theta^\circ$ Bragg peaks at 22, 17, and 15, and are identical to spectra of cellulose standards. Similarly, Raman spectra of fossil material, as well as fresh leaf preparations, show $\Delta V(\text{cm}^{-1})$ shifts at 1100 and between 500 and 300. Leaf tissues placed in cuprammonium solutions break apart; however, the quantity of fossil material available for study precluded a viscometric determination. Optical birefringence, spectral characteristics, and chemical properties of fossil leaves confirm the presence of a cellulose or cellulose-like component in cell walls, and are consistent with ultrastructural observations. PATAG staining and I_2KI reactions of the fossil material also indicate the presence of true starch localized in cytoplasmic residues.

Thin-layer chromatography on silica gel (40:10:1, vol/vol, hexane/ether/methanol) of the ether-soluble acids released by methanolic KOH hydrolysis indicates the presence of mono-, di-, and trihydroxy acids (20). Two series of monohydroxy acids were detected; the high molecular weight hydroxy acids, 10,16-dihydroxyhexadecanoic acid and 9,10,18-trihydroxy-octadecanoic acid, were predominant. Leaves from which various "waxy" acids were extracted reveal, upon microscopic examination, a poorly defined cuticular layer, and these compounds are therefore thought to be located within the cuticular complex (see Figs. 1 E and 2 C and D).

DISCUSSION

Ultrastructural and chemical analyses of Miocene angiosperm leaves confirm the presence of typical higher plant organelles. On the basis of transmission electron microscopic characteristics and cytochemistry, some structures are interpreted as the remnants of chloroplasts and nuclei. Chemical studies indicate that some protoplasmic structures are not solely artifacts of preservation or inorganic replacements, but rather the remnants of the original protoplasm that retain many biochemical characteristics of extant cells. The Succor Creek flora is stratigraphically placed within the Late Hemingfordian to Early Barstovian (equivalent to Late Burdigalian to Vindobonian in European terminology), on the basis of associated mammal fossils (24). Potassium/argon dates vary from 16.7×10^6 K/A years (22) to between 25 and 36×10^6 K/A years (20, 21). Part of the age discrepancy is the result of rock alteration and the incorporation of detrital components in these tuffaceous deposits. For the purposes of this discussion, we accept the minimum age for the Succor Creek flora as between 16.7 and $25 \times$

FIG. 1 (on preceding page). (A) Fossil leaf of *Zelkova* *in situ*. Rectangle denotes area from which sample was taken for transmission electron microscopy. ($\times 2.1$.) (B) Fossil leaf (left) removed from the ash. Note imprint of leaf. Rectangle same as designated in A. ($\times 1.2$.) (C) Lower portion of the same fossil leaf shown in A and B. This sample is fixed and embedded in Spurr's resin. The two parallel lines to the left of the midrib indicate the region chosen for light and transmission electron microscopy. The area in the rectangle of the upper half of the leaf was prepared for scanning electron microscopy. ($\times 3.0$.) (D) Scanning electron micrograph of a cross section from the upper half of the leaf complementary to the region sampled for transmission electron microscopy. Note the prominent midrib, epidermis, and mesophyll. ($\times 110$.) (E) Scanning electron micrograph of epidermal tissue cut normal to the long axis of the leaf. Note waxy layer over epidermis. ($\times 140$.) (F) Scanning electron micrograph of the xylem elements, demonstrating the characteristic spiral secondary wall thickenings. ($\times 900$.) (G) Nomarski light micrograph montage of a thick cross section from the basal portion of the leaf indicated by the parallel lines in C. Vascular tissue is sectioned obliquely (to the left of center), and the epidermis and mesophyll tissues are clearly discerned. The same idioblast region is visible in an adjacent ultrathin section for transmission electron microscopy in H. ($\times 660$.) (H) Transmission electron micrograph of cross section of leaf from region demonstrated at the light microscopical level in G. Note swollen idioblast cell. Area denoted in rectangle is enlarged in I. Two adjacent cells in the mesophyll depicted in H. ($\times 9700$.) (J) Transmission electron micrograph of longitudinal section through secondary spiral thickenings of the xylem. Compare with F. ($\times 6100$.) (K) Ultrastructure of a mesophyll cell. Note the parietal chloroplast, patterns of stroma and grana thylakoids, and densely stained starch grain. This section was subjected to cytochemical analysis for carbohydrates (24); the silver clearly is localized over the starch. Note the variation in staining of the inner and outer layers of the cell wall microfibrils. ($\times 24,000$.)

FIG. 2. (Legend appears at bottom of the following page.)

10⁶ years. The documentation of cytoplasmic structures, by means of light microscopy, in older material (1) indicates that the geologic environment attending fossilization is a more significant factor in determining the preservational state than is the absolute age of the specimen.

Various cellular structures have been reported in very ancient fossils, e.g., starch grains in Pennsylvanian gametophytes (1); unicellular Precambrian algae, phloem tissues (3, 4), nuclei (5), and chloroplast-like bodies in Eocene *Spirogyra* (6); and cytoplasmic remnants in Eocene angiosperm leaf tissues (7). In all but a few cases (8, 9), these reports have been based upon light microscopy and confirmation of ultrastructural features is lacking. Regrettably, many modes of fossilization preclude the use of transmission or scanning electron microscopy, and laboratory-simulated fossilization (25) has shown that granules and/or other cellular inclusions can be induced to take on the appearance of "organelles." The occurrence of phenomena that regularly produce organelle-like structures severely limit the reliability of any technique other than transmission electron microscopy.

The fossils used in this study were preserved in rapidly falling volcanic ash; the inorganic matrix appears to have behaved in part as a physical desiccant that prevented or reduced microbial degradation. The presence of cellulose in the Miocene leaves suggests that an unusual volcanic ash environment contributed to the stabilization of cytologic and chemical features. Usually the anaerobic fermentation of cellulose and pectins into aliphatic acids makes native cellulose a transient constituent in recent sediments. However, reports of cellulose microfibrils in Devonian plants (26), fossil *Chara* (27), and fossil algae (28) indicate that bacterial fermentation may be prevented under some conditions. Provided that thermal gradients are small, cellulose may remain intact over considerable periods of time.

The geochemical mechanisms allowing the preservation of cellular ultrastructure appear to have involved: (a) rapid burial without heat damage; (b) rapid dehydration of the leaf tissue with accompanying cell shrinkage and damage to the plasma and vacuolar membranes; (c) subsequent release of flavanols and tanniferous compounds into the protoplasm, which could have served as endogenous "fixatives" of cell ultrastructure; and (d) gradual permineralization of some cellular cavities before possible microbial or excessive enzymatic degradation could have taken place.

It certainly now seems clear that some details of cellular ultrastructure, once preserved, can survive hundreds of thousands or even millions of years. Still more ancient fossils with excellent preservation of cellular ultrastructure may be discovered. In our opinion, older volcanic ash deposits may yield such fossils and should be carefully examined. Equally useful

would be analyses of recent burials in ash (e.g., Kilauea Iki Eruption of 1959, Hawaii) to determine whether or not the relatively short-term preservation of cell ultrastructure is equal to that of Miocene fossil leaves.

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FIG. 2 (on preceding page). (A) Details of a chloroplast in a mesophyll cell. Note the prominent starch grain as well as the grana thylakoids, which are imaged in negative contrast in the fossil specimen. ($\times 78,000$.) (B) Two adjacent mesophyll cells connected by plasmodesmata. Note the prominent "nuclear-like" structure in the upper cell. Outlines of the chromatin-like material are visible. Chloroplast starch is very electron dense (PATAG staining). ($\times 12,000$.) (C) Upper epidermal cell wall and cuticle. Note microfibrillar cell walls of two adjacent cells (lower part of photograph) as well as the particulate inner cutinized layer and the homogeneous outer cuticle. Compare with scanning electron micrograph shown in Fig. 1E. ($\times 11,000$.) (D) Lower epidermal cell demonstrating irregular wall thickenings and undulations associated with the base of trichomes. ($\times 11,000$.) (E) Mesophyll cells; the one to the right has a prominent nucleus-like body with "chromatin." ($\times 10,000$.) (F) High magnification view of the microfibrillar patterns in the secondary wall thickenings of the xylem. The staining pattern and morphology appears identical with extant cell walls. ($\times 50,000$.)