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Abstract
In the early stages of a previous study of fossil spores of Botryopteris globosa and B. americana from the Carboniferous (Phillips & Rosso, 1970), considerable difficulty was encountered in replicating and cleaning spore materials and associ- ated debris from the carbon-platinum spore replicas. After numerous attempts to clean these carbon films, the techniques presented in this paper proved ef- ficient and reliable for transmission electron microscope examination of Botry- opteris spores found in calcified coal ball fossils.

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THE PREPARATION OF CARBON-METAL REPLICAS FOR THE STUDY OF CARBONIFEROUS COAL BALL FOSSILS: REPLICATION AND CLEANING PROCEDURES[1]

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ROSSO, S. W., WILLIAMS, M. G., HORNER, H. T., JR. & MORELAND, J. 1976. The preparation of carbon-metal replicas for the study of Carboniferous coal ball fossils: replication and cleaning procedures. *Trans. Amer. Micros. Soc.*, 95: 112–115. A modified technique is explained for obtaining good carbon-metal replicas of coal ball fossils for use with electron microscopy.

In the early stages of a previous study of fossil spores of *Botryopteris globosa* and *B. americana* from the Carboniferous (Phillips & Rosso, 1970), considerable difficulty was encountered in replicating and cleaning spore materials and associated debris from the carbon-platinum spore replicas. After numerous attempts to clean these carbon films, the techniques presented in this paper proved efficient and reliable for transmission electron microscope examination of *Botryopteris* spores found in calcified coal ball fossils.

Coal ball specimens from European and midwestern U. S. localities often contain well-preserved stems, roots, leaves, and sporangia of early vascular plants that flourished during the Carboniferous. Prior to 1953, paleobotanists used liquid nitrocellulose compounds for replication techniques in examining coal ball materials (Stewart & Taylor, 1965). Lacey (1953) introduced a cellulose acetate sheet film technique that has become widely used by many paleobotanical laboratories throughout the world. Preparation of specimens for this sheet film technique involves: (1) slicing of rough specimens with a diamond saw; (2)

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polishing the surface with grinding abrasives; and (3) etching the polished surface with dilute HCl if the fossil matrix is calcified or with hydrofluoric acid (HF) if the matrix is siliceous. Etching removes a small amount of the mineral matrix from the surface of the coal ball. The insoluble cell walls that are exposed by this process can then be bonded to cellulose acetate by the use of acetone solvent. When the acetate sheet is dry, it is then "peeled" or stripped from the fossil surface. Sizable fossil specimens can thus be easily examined by means of light microscopy, and the "peels" can be stored indefinitely without deterioration of quality.

Materials and Methods

The *Botryopteris* specimens described in this study were loaned from the research collection of Dr. Tom Phillips, University of Illinois, Urbana. These specimens are identical with the *B. globosa* specimens of Phillips & Rosso, 1970.

Replication Procedures for the Electron Microscope

Coal ball specimens containing *Botryopteris* sporangia were first polished and then etched at varying intervals ranging from 15 to 30 sec in a 4% HCl v/v solution. The etched surface was carefully washed with distilled water by directing the water flow onto nonsporangial portions of the specimens. After draining most of the water from the specimen, several rinses of absolute acetone were applied. These acetone rinses were allowed to remain on the specimen for approximately 3 min each in order to properly remove all traces of water from the fossil surface. A feasible alternative, if time permits, is to air-dry the specimens for several hours.

First Stage Acetate Replication

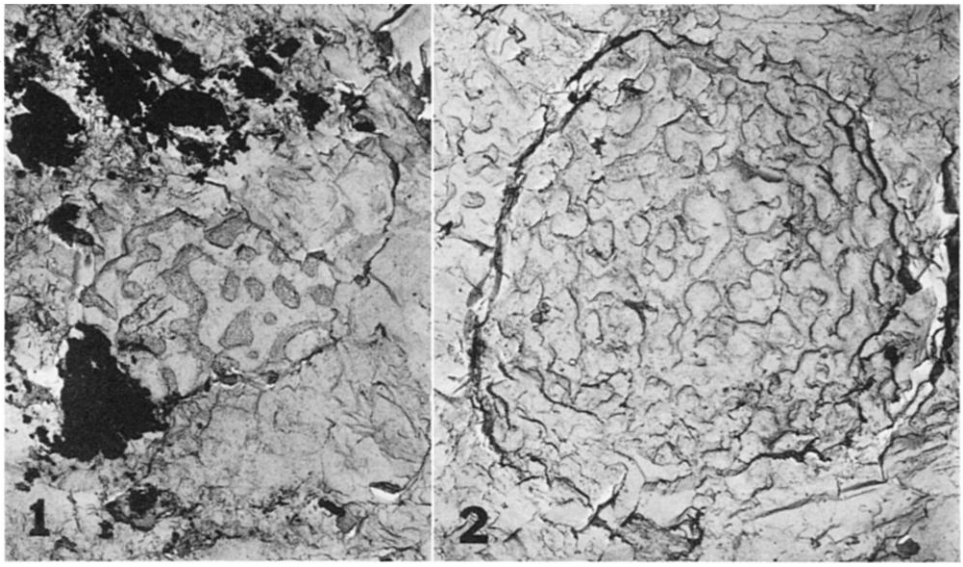
Once the surface is thoroughly dry, it is again flooded with a copious amount of absolute acetone. A piece of replication tape slightly larger than the sporangium is gently placed over the specimen. Care is taken to avoid application of pressure on the tape as it is lowered on the specimen. Immediately following application of the tape, several drops of acetone are added to its upper side. This particular step enhances replication by apparently aiding the inner tape surface to become more flexible and "mold" more effectively to the etched fossil surface.

Curling of the initial acetate replica is prevented by "backing" it with a piece of tape of the same size. This second piece of tape is added after flooding the upper surface of the initial replica with acetone ca. 2 min after it is placed on the fossil surface. This is considered to be an important part of the procedure in that a curled replica does not provide a uniform surface for later carbon-metal replication.

Second Stage Replication with Carbon-Platinum

The acetate replica is allowed to dry in situ for several hours before stripping it from the specimen. It is then taped to a clean glass slide, impression side upward, and simultaneously shadowed with a carbon-platinum film to a thickness of approximately 30 nm at an angle of 30°. The coated replica is then cut into 3 mm sq pieces which are subsequently placed into an absolute acetone bath until the acetate has dissolved. At this point, the platinum-carbon film will float freely on the acetone surface. Four changes of acetone usually insure total removal of the acetate from the carbon film.

The films are transferred to distilled water through a graded series of acetone-water mixtures (95% acetone; 50% acetone; 25% acetone; 100% water). A graded series of solvent and water is not necessary in all cases, but in certain instances it



FIGS. 1, 2. *Botryopteris globosa* spore. Fig. 1. Uncleaned second stage replica film showing small portion of surface structure. × 2,000. Fig. 2. Cleaned second stage replica film showing surface ornamentation. × 2,000.

prevents damage to the replica films by agitation from diffusion streaming created by acetone-water interaction.

Regardless of the degree of etching, many relatively intact spores adhere to the impression side of the initial acetate replicas (Fig. 1). These become attached to the carbon-platinum coating and remain in situ when the acetate is dissolved away. If these extraction spores are sufficiently stable in the electron beam, they may yield valuable information (see also Figs. 15, 18, Phillips & Rosso, 1970); however, when subjected to the electron beam their ornamentation may be altered considerably. The basic procedures just described for the carbon-metal replication are essentially those of Bradley (1961).

Cleaning Procedures

Of the many chemical agents that were tested in order to clean spore and coal ball debris from the second stage replicas, a sulfuric acid-sodium dichromate glassware cleaning solution (16ml conc. H_2SO_4; 10ml H_2O; 1.2gm $Na_2Cr_2O_7$; see Wadsworth, 1939) yielded the best results and eventually, after the cleaning technique had been perfected, the least time consuming. The cleaning procedure is outlined as follows. (1) Place a porcelain Color Reaction Plate (also called a Spot Test Plate) with wells approximately 8 mm deep on a controlled temperature hot plate set at approximately 50 C. (2) Fill two or three of the wells with sulfuric acid-sodium dichromate solution. The other wells should be filled with distilled water. (3) Using a fire-polished glass rod with a small, rounded tip ca. 1–2 mm in diameter (slightly smaller than the replica), the carbon-metal film is transferred to the cleaning solution. Care should be exercised to adjust the solution temperature so that the specimen will not be mechanically damaged by strong convection currents in the heated solution. (4) The optimum cleaning time of the *Botryopteris* films was usually less than 10 min. Determination of the optimum cleaning time is made by subjecting specimens to 3, 5, 10, and 15 min

intervals in the acid solution. Intervals greater than 10 min may produce severe etching of the carbon-metal replicas. Once the solution changes color from orange-red to black, the specimen is then transferred into another well containing fresh acid solution. (5) Following the acid bath, specimens are washed for several minutes in at least three successive wells containing distilled water. (6) After the final wash, specimens are transferred and stored indefinitely in a sterile distilled water bath. Whenever specimens are to be examined, they are placed on HCl-cleaned 100 and 200 mesh copper grids (Fig. 2). In order to prevent possible specimen contamination during the cleaning procedure, all dishes, pipettes, and transfer rods should be cleaned in the acid-dichromate solution prior to use.

Discussion

Our previous experience in replicating coal ball specimens for light microscopy provided at least one pitfall for electron microscope replication procedures, that is, we used acetate sheet film 0.003 inch in thickness such as that used in most paleobotanical "peel" techniques. Initially, it was found that an undissolvable impurity incorporated into the first stage acetate sheet caused the second stage carbon-metal film to adhere firmly. This impurity could not be dissolved and, as a result, the second stage replica was severly wrinkled. This defect became apparent only when the carbon film was transferred into the water bath following absolute acetone treatment to remove the first stage replica. Use of electron microscope grade replication tape corrected this problem.

Hess & Blair (1972) cleaned freeze-etch replicas effectively with various cleaning solutions such as sulfuric acid maintained at 50 C followed by a sodium hypochlorite (Clorox or Purex) wash and a sulfuric acid-dichromate solution for 6 to 12 hr. They found that the latter was more effective and, in some instances, did not require a postwash in sodium hypochlorite. Juniper et al. (1970) incorporated a chromic acid bath for 30 min to 1 hr; however, they found that their solution tended to burst entire spores.

In our studies of the effectiveness of a sulfuric acid-dichromate solution on removing spore and debris materials from replication films, we found that prolonged immersion at room temperature was relatively ineffective while a heated solution cleaned sufficiently in less than 10 min (Fig. 2).

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