

## THE USE OF ETHYLENEDIAMINE IN SOFTENING HARD PLANT STRUCTURES FOR PARAFFIN SECTIONING

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**ABSTRACT.** Ethylenediamine has been used as an agent for softening very hard woods prior to sectioning on a sliding microtome. The use of ethylenediamine is recommended for two additional uses: for preparing 1) soft woods in which wide, thin-walled tracheids or vessels tend to collapse during sliding microtome sectioning and 2) plant tissues with sclerenchyma mixed with soft-walled cells (bark, leaves, fruits, etc.) which frequently fail to section well. After softening in ethylenediamine, material is washed, infiltrated, and embedded in paraffin. Preliminary sections are made with a rotary microtome, just exposing the cut surface of the material; this exposed surface is soaked overnight in water. Sectioning is then continued. Sections produced in this fashion are considerably improved. The wood and pith of *Podocarpus ustus*, a parasitic conifer from New Caledonia, is used as an object to demonstrate improvements in sectioning by the ethylenediamine-paraffin method. Thinner sections with minimal tearing, cell collapse, and unevenness are produced. Sections can be handled easily and stained more effectively than unmounted sections. Variations in timing and in treatment are recommended to suit different materials. Ethylenediamine, used with reasonable caution, is much less hazardous than hydrofluoric acid and is more effective in softening plant material. The ethylenediamine method may be used routinely on any material difficult to section because of hardness.

Hydrofluoric acid has traditionally been the fluid used for softening thick-walled lignified cells prior to sectioning. Hydrofluoric acid is, however, dangerous to handle and requires the use of a fume hood; it acts slowly, requiring a month or more for quite woody materials. Kukachka (1977) introduced ethylenediamine as an alternative way of softening excessively hard wood samples before sectioning them on a sliding microtome. Kukachka's method has proved entirely satisfactory in my experience for this purpose. Because diverse plant materials are under study in my laboratory, however, I have applied ethylenediamine to a wide range of objects other than excessively hard wood, and have experienced success.

Very soft woods provide problems in sectioning on a sliding microtome because the wide, thin-walled cells can collapse. These problems are solved by softening such woods with ethylenediamine, embedding them in paraffin, then sectioning them on a rotary microtome. A second series of problems is offered by plant structures in which thick-walled lignified cells occur with thin-walled cells. Bark, pith, leaves with fibrous bundle sheaths, fruits, and seeds all contain such a combination of cells and prove difficult to section with ordinary methods. Typical of the difficulties encountered is the account of Roth (1981), who describes problems in sectioning bark; some of these problems could not be solved satisfactorily.

Using illustrations of wood and pith of *Podocarpus ustus* (Vieill.) Brogn. & Gris, a conifer from New Caledonia notable for its parasitic habit, methods of using ethylenediamine as a softening agent followed by embedding of material in paraffin are described below.

### PROCEDURES

↓ Woods in which tearing of cell walls (Fig. 8), collapse of vessels or tracheids, or obliqueness of cut occur during sectioning may be too soft for sectioning on a

sliding microtome even when an excellently sharpened knife is available. Where tracheids or vessels are thin-walled and relatively wide in diameter, these problems are acute. Lack of support by an embedding medium is the factor primarily responsible for the failures noted above. However, directly embedding a wood, suitably boiled or pickled and then infiltrated, does not prove satisfactory: the wood cells are still too tough and tend to fragment during sectioning. These woods prove amenable to softening by the ethylenediamine method, followed by embedding in paraffin. Three days in 10% ethylenediamine at room temperature (or a week in 4%) has proved optimal in materials I have sectioned. However, variation in treatment time and in strength of solution seems appropriate depending on wall thickness and lignification of a particular wood. Small capped wide-mouth bottles are suitable for this treatment. Kukachka's (1977) method for treatment of excessively hard woods specifies weighting of wood samples with metal weights while they soak in the ethylenediamine. Presumably this counteracts any tendency of wood samples to float. I have omitted weighting because I have not experienced floating in boiled or pickled wood samples.

After two or three changes of water at two-hour intervals to remove the ethylenediamine, wood portions were prepared for infiltration. Penetration by paraffin is a problem with woods. Consequently, I carefully slice rectangular chips about  $5 \times 5 \times 1$  mm from the wood samples with a single-edged razor blade after they have been softened and washed. The broad face exposed on each chip is the one from which sections will be cut. The chips prepared for a species are thus of three sorts, so that radial, tangential, and transverse faces will be available. The preparation of chips maximizes paraffin penetration, which would be retarded if wood cubes were embedded. Moreover, the preparation of chips of three kinds insures that the broad face, laid horizontally during the embedding procedure, can be identified easily as the face to be sectioned.

The tertiary butyl alcohol series of Johansen (1940) is employed for infiltration. I have found that porcelain crucibles, as used in chemical laboratories, are ideal. These fit into Coplin jars, which are filled with the infiltrating solutions.

Embedding in a harder grade of paraffin (or commercially prepared paraffin mixture) is recommended. A melting point of 59 to 61 C is preferable. After embedding, the wood chips mounted in paraffin should be sectioned on a rotary microtome. However, I must stress that the sections cut at this stage are usually not satisfactory. A further procedure is necessary. Sections should be cut at first only to expose the surface of the wood chip. When the surface has been exposed by preliminary sectioning, the block or holder bearing the paraffin should be removed from the microtome; it should be placed, inverted, into a beaker or small jar containing a little water (1-2 mm depth). Soaking overnight is recommended. The exposed face of the wood chip imbibes water and the sections produced after this treatment are much improved. Sectioning of paraffin embedded woods without this treatment is likely to be unsuccessful.

Sections 10-15  $\mu$ m thick proved entirely satisfactory. Thickness may be adjusted to the nature of the wood and the structure to be observed. Very thin sections are possible with this method, whereas in microtoming nonembedded woods on a

sliding microtome, sections less than 15  $\mu\text{m}$  thick usually show excessive tearing and unevenness.

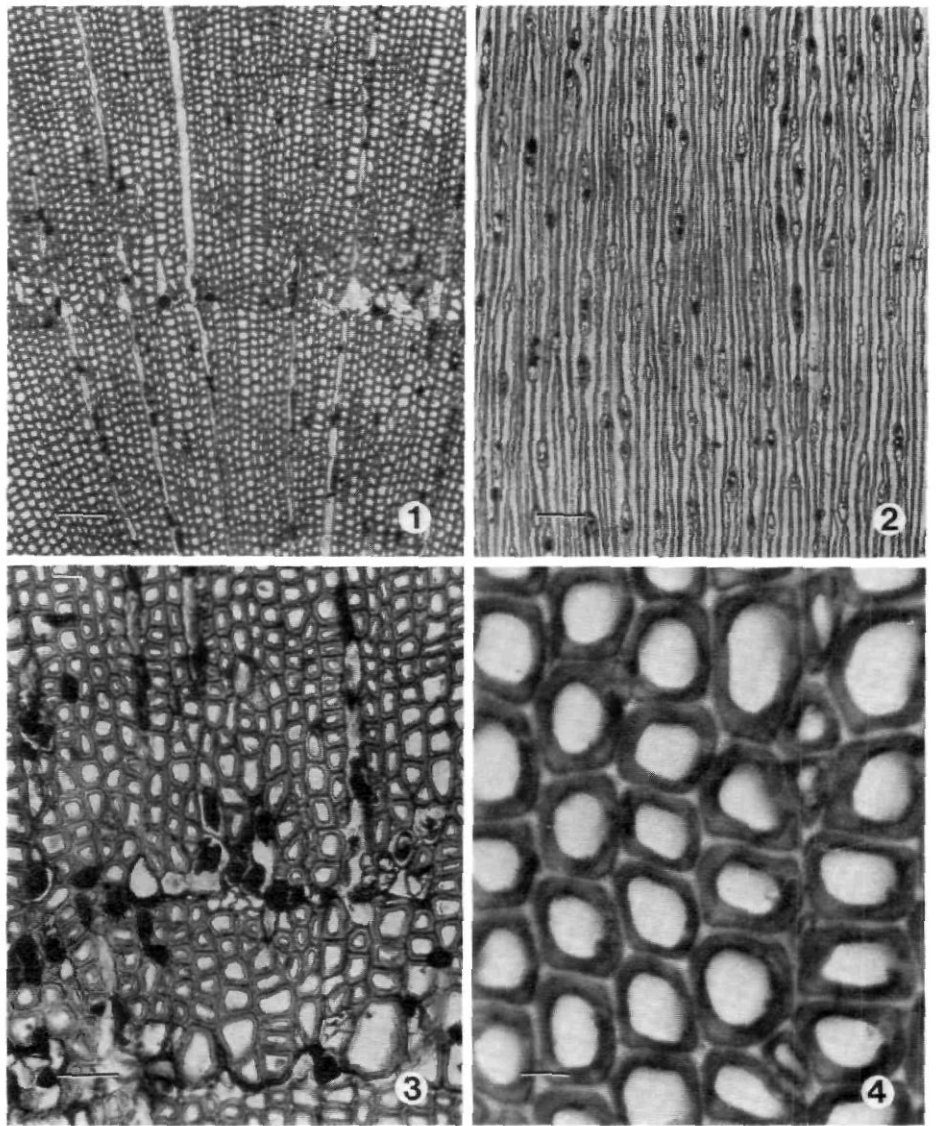
The use of a rotary microtome is recommended for the procedure described above. This has the advantage of yielding ribbons of indefinite length. If difficulty is encountered with the method, a sliding microtome may be tried on the paraffin embedded material. In some instances, this has yielded better sections.

The paraffin sections of woods are mounted on slides. I strongly recommend the use of Bissing's solution (Bissing 1974). Wood sections tend to adhere to slides less well than sections of other material; Bissing's solution promotes more reliable adherence. Wood anatomists generally stain wood sections with safranin. Hematoxylin or other counterstains may be used in addition. Paraffin sections of woods mounted in this way can, in comparison to unmounted sections, be sensitively and easily stained. Moreover, the thinner sections obtained from paraffin embedded woods can be stained more intensely without obscuring details. Thus differentiation among wall layers with the aid of a counterstain is often superior to results obtained with the thicker sections obtained by sectioning nonembedded wood on a sliding microtome.

For materials other than woods (bark, leaves, fruits, seeds, *etc.*), the ethylenediamine softening technique followed by embedding in paraffin is strongly recommended. Although the illustrations here are drawn mostly from wood, the potential application to plant portions other than secondary xylem is unlimited. Good sections of bark and leaves have been produced routinely in my laboratory by means of this method. Any material containing sclereids shows marked improvement in my experience. Because materials other than wood tend to have a mixture of hard and soft cells, one must cut portions suitable for sectioning before treating the material with ethylenediamine. Attempting to subdivide the material after it has been softened may result in crushing of the tissue rather than slicing it cleanly. Treatment of leaves rich in sclereids for periods up to two months in ethylenediamine has yielded good sections; less problematic leaves can be treated for shorter periods. A 4% ethylenediamine solution is recommended; a stronger solution makes control of softening time more difficult.

Materials other than woods are washed, infiltrated, and embedded as described above for wood. Preliminary sectioning to expose the surface of the material, followed by soaking of paraffin embedded material in water, then sectioning of the paraffin block as described above for wood results in marked improvement of sections, with less compression, tearing and cell collapse.

Several notes regarding the use of ethylenediamine should be made. Commercial strength ethylenediamine gives off smoky fumes when unsealed. Whenever a dilute solution is prepared from the stock solution, the use of a fume hood or other equivalent arrangement is recommended. Dilute solutions of ethylenediamine do not give off fumes so noticeably, but care should be taken not to inhale vapors for prolonged periods. Although not as seriously dangerous to the skin as hydrofluoric acid, ethylenediamine may cause a skin irritation in some individuals if the solution is not washed from the hands with reasonable promptness. Ethylenediamine solution acquires a brownish color when plant materials are soaked in it. This color



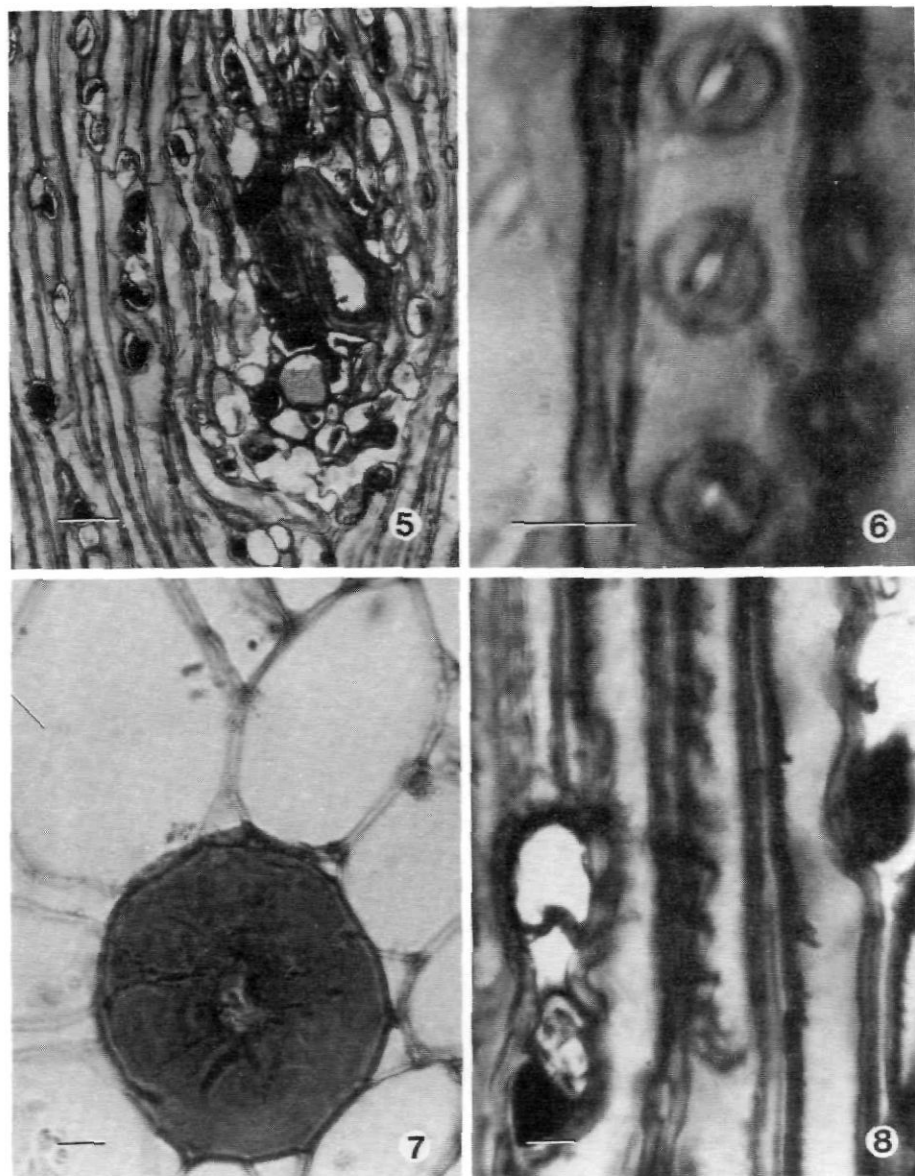
FIGS. 1-4. Wood sections of *Podocarpus ustus*, Carlquist 15596a, cut with ethylenediamine-paraffin embedding method.

FIG. 1. Transection; band of traumatic parenchyma cells visible in middle of photograph. Marker: 100  $\mu\text{m}$ .

FIG. 2. Tangential section, lightly stained with safranin. Marker: 100  $\mu\text{m}$ .

FIG. 3. Transection showing groups of traumatic parenchyma cells and a few traumatic tracheids in a background of normal tracheids. Marker: 50  $\mu\text{m}$ .

FIG. 4. Transection showing staining differentiation in tracheids. Marker: 10  $\mu\text{m}$ .



FIGS. 5-8. Wood and stem sections of *Podocarpus ustus*, Carlquist 15596a prepared with ethylenediamine-paraffin embedding method (except Fig. 8).

FIG. 5. Tangential section. An atypical ray (related to branching), both sclereids and parenchyma occur. Marker: 50  $\mu$ m.

FIG. 6. Piceoid pits, characteristic of this species, from a radial section; dark outline of pits results from counterstaining with fast green FCF. Marker: 10  $\mu$ m.

FIG. 7. Sclereid from transection of pith, surrounded by thin-walled parenchyma (safranin used without counterstain). Marker: 10  $\mu$ m.

FIG. 8. Tangential section produced by sectioning unsoftened wood on a sliding microtome. Note shredding of tracheid wall and tearing of ray cell walls. Marker: 10  $\mu$ m.

can obscure the objects placed in it. If opacity of the solution is not a drawback, however, ethylenediamine solution can be saved and reused, effecting an economy.

### RESULTS

A small dried stem of *Podocarpus ustus* (Figs. 1-8) was boiled and sectioned both by the usual sliding microtome method and by the method described above in order to offer a suitable object for illustration in this paper. The woody cylinder of this stem was about 5 mm in diameter. Stems of this diameter or less often provide problems because they cannot be gripped firmly by the holder of a sliding microtome; such difficulties are obviated by embedding in paraffin. Sections of this stem cut on a sliding microtome were superficially satisfactory. Examination under the microscope, however, revealed a high frequency of tears in tracheid walls and disruptions in ray parenchyma walls (Fig. 8). Sections thinner than 20  $\mu\text{m}$  showed excessive tearing. The pith of the *Podocarpus ustus* stems contained sclereids which did not section cleanly and which were torn loose from the parenchyma in which they are embedded.

With the softening and paraffin embedding technique, the stem of *Podocarpus ustus* was sectioned at 12  $\mu\text{m}$  (Figs. 1 and 2). There were no areas uneven in thickness in the sections, nor were tears or cell wall collapse more than minimal (Fig. 4). Ray cells were little displaced at worst. A band of parenchyma (Fig. 1, center), probably a response to drought or injury in the plant's environment, contained thin-walled cells which caused a separation in nonembedded material. The parenchyma band (Fig. 3) did not break, and cell walls of the band remained intact. The tracheids did not collapse or tear; secondary walls stained distinctively at this thickness (Figs. 4 and 6). Sclereids in an atypical ray (Fig. 5) sectioned well despite being associated with thin-walled cells.

Isolated thick-walled sclereids in the pith (Fig. 7) sectioned easily without breakage or displacement of thin-walled pith cells nearby.

### DISCUSSION

The method described above has now been applied to several different problems. Wide thin-walled tracheids of two genera of vesselless angiosperms, *Belliolum* (Carlquist 1982a) and *Exospermum* (Carlquist 1982b) sectioned well. Wide thin-walled vessels, often a sectioning problem, proved amenable to this method in *Trimenia* (Carlquist, unpublished). Leaves of Bruniaceae, notable for strands of fibers which do not section well with ordinary techniques, provided much better sections after softening (Carlquist, unpublished).

Some swelling of thick-walled cells could be observed in some materials. This swelling was judged to be minimal and probably less than occurs, in my experience, when materials are softened with hydrofluoric acid. The ethylenediamine method, therefore, can be recommended with only minor reservations for a wide range of plant materials difficult because of hardness of lignified cell walls.

### REFERENCES

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