Belg. Journ. Bot. **131** (1) : 41-49 (1998)

PREPARATION OF WOOD SPECIMENS FOR TRANSMITTED LIGHT MICROSCOPY AND SCANNING ELECTRON MICROSCOPY

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ABSTRACT. — This paper deals with routine techniques for preparation of wood specimens for light microscopy and scanning electron microscopy as executed at Tervuren and Leuven. In addition to the preparation of macerations, topics covered include: procedures for softening, cutting, clearing, staining, dehydrating, and mounting of the sections.

RÉSUMÉ. — *Préparation de coupes de bois pour les microscopes optique et electronique à balayage.* — Cet article décrit en détail le procédé suivi à Tervuren et Louvain pour la préparation de coupes de bois à l'usage du microscope optique et du microscope électronique à balayage. En plus du procédé de macération, les aspects suivants sont inclus : imbibition et ramollissement du bois, exécution des coupes, traitement, coloration, dessiccation et montage des coupes.

KEY WORDS. — Wood anatomical preparations, light microscopy, scanning electron microscopy, sliding microtome, macerations.

INTRODUCTION

Trees and shrubs characterise by far most of the terrestrial vegetation types. Their lignified tissues contain interesting information for several botanical disciplines such as systematics, ecology, palaeobotany and physiology. The study of wood is also relevant for sciences as archaeology and art history, and even for technological research.

Many papers dealing with techniques used for studying wood anatomy have been published in the past (e.g. BAMBER 1961, ANONYMOUS 1968, ANNERGREN & TREIBER 1979, GARDNER & TAYLOR 1983, KRAHMER *et al.* 1986). In addition, descriptions of techniques are frequently included in textbooks on the structure of wood (e.g. JEFFREY 1917, JANE 1956, NORMAND 1972, GROSSER 1977, SCHWEINGRUBER 1978, Core *et al.* 1979, BAREFOOT & HANKINS 1982) or in general books on plant microtechniques (e.g. SASS 1958, PURVIS *et al.* 1966, SANDERSON 1994). At present, specialised methods are being deve-

TABLE 1

Summary of the procedure for preparation of wood sections for LM

Preliminary preparation	– obtain a wood block with transverse, tangential and radial sides
Softening	– soften the block in a boiling mixture of glycerine/water (1/10 volume ratio) until supersaturation is achieved
Cutting	– cutting angle ca. 15° for moderately hard wood
	– flood knife and wood block with water-glycerine
	– cut sections between 15-30 µm thick
	– verify orientation
	– prevent curling
	– put good sections in basket
Section treatment	– treat with 10% sodium hypochlorite until bleaching (1-3 min.)
	– wash with H_2O
	– treat with 10% acetic acid for 1-2 min.
	– wash with H_2O – stain with 1% safranin for 3-5 min.
	– wash in alcohol series : 25%, 50%, 70%, 96%
	– treat with clearing agent-alcohol mixture
	– treat with clearing agent (2-3 min.)
Mounting	– mount the sections carefully on a slide, add few drops of mounting medium and let the preparations dry with a weight on the coverslip

loped to study the anatomical features of lignified tissue in the context of image analysis, dendrochronology (AMPARADO *et al.* 1990), wood quality investigations, and immuno-fluorescence microscopy.

It is not our intention to give an overview of all the existing techniques or to evaluate them. This paper describes an inexpensive and simple procedure for routine preparation of sections of wood specimens that has proved to be satisfactory for microscopic examination. Most of the methods described are slight adaptations of those techniques long established in general wood anatomical work. This paper could also serve as a quick and easy reference for students whose work requires preparation of wood for light microscopic and/or scanning electron microscopic investigations.

PREPARATION FOR LIGHT MICROSCOPY

Microtomy of wood includes different steps from raw wood to mounted thin sections. These steps are summarised in table 1.

1. PRELIMINARY PREPARATION

First a small wood cube of approximately 1-2 cm^3 should be obtained. It is very important that these blocks are perfectly oriented so that the sides are truly transverse, tangential and radial. Precise orientation can be checked with a stereomicroscope or hand lens. When several blocks are being prepared together, each wood sample should be distinctly marked. The best solution for this is by notching or removing one

or more corners of the cube. A record should be kept of these markings. Another possibility is to draw the contours of the samples on a paper or to label each block by a number or a letter. Collection numbers, however, are mostly long numbers and space on the sectioning block is usually limited, so a single digit code can be applied to all surfaces that will not be sectioned. Disadvantageous is that the ink applied to a surface intended for sectioning, may penetrate so deep that it will be visible on the cut sections (KUKACHKA 1978).

2. SOFTENING

It is necessary to soften the wood block in order to obtain the proper condition for sectioning. The method of softening required depends on the kind of wood being used. This can best be decided experimentally. Our method is simple and generally suitable for moderately hard wood.

Swelling of the wood samples in a mixture of glycerine-water is the key to soften wood. Glycerine acts as a plasticizer, like for example ethylene glycol, propylene glycol and other substances, usually applied in the polymer industry. To our knowledge there are no studies on the chemical behaviour of the glycerine entering lignified cell walls, but it can be assumed that the plasticizing effect is obtained through diffusion of the glycerine molecules into the polymer network of the lignin. This act alters considerably the physical properties of wood, without changing the chemical structure of the lignin. The hydroxyl groups of the glycerine are not active enough to break the strong covalent bonds of the lignin macromolecule, but part of the lignin hydrogen bonds and van-der-Waals forces (to which the polymers hardness is due) can be replaced with new physical (hydrogen) bonds between lignin and glycerine. In other words, penetration of glycerine into the lignin makes the interaction among the polymers weaker so that they are more free to move. This results in softening the cell wall lignin matrix and probably also the lignin-polysaccharide complexes.

The softening action can be accelerated by heating the wood because the higher kinetic potential obtained allows the glycerine molecules to penetrate into the polymer network of the lignin easily. Replacement of the air with water also helps a lot, especially in the case of porous hardwoods with tyloses. We recommend that the wood blocks be immersed in a boiling mixture of glycerine and water (1/10 volume ratio) until the wood becomes supersaturated and sinks. However, boiling for a longer period is often recommended to soften the wood still further. The softening process can also be accelerated by immersing the blocks in cold water during some seconds.

Special attention should be paid to the ratio glycerine/water as higher proportions of glycerine increase the boiling temperature and can damage the wood specimen. Because of evaporation, hot water should be regularly added to the boiling mixture to maintain the ratio glycerine/water. Note that only *hot water* should be added because great temperature differences between the boiling mixture and the added water can lead to explosion of the liquid.

Other methods such as treatment with hydrofluoric acid (traditional fluid is 50%) or with 50% glacial acetic acid / 50% hydrogen peroxide for 1 to 2 h are suitable for very hard wood. The latter method will give the outer faces of the wood block a pale or white appearance. Therefore, the blocks should be washed thoroughly for at least 12 h before sectioning, and both softening methods may not be overdone or the wood will become macerated (SANDERSON 1994; for macerations see further). Very hard wood is successfully softened by ethylenediamine (KUKACHKA 1978, CARLQUIST 1982). After soaking in this extremely corrosive chemical, which requires special caution when used, the wood must be washed and stored in 15% glycerol in ethanol for a few days before sectioning. Note that freshly cut wood, especially sapwood, can often be sectioned easily without artificial softening because it is fully swollen and because there are less extractives present.

3. CUTTING

The sliding microtome (Fig. 1) is the standard instrument used for the cutting of sections of large size and even thickness. The hardness of most

woods requires this tool. Compared to other microtomes further advantages linked to sliding microtome are the possibility to produce large and thin sections (SASS & ECKSTEIN 1994), and the comfort to adjust knife and wood cube. Note that both right- and left-handed microtomes exist. Nowadays, in many laboratories disposable microtome blades are used which do not require sharpening. Classical knifes, however, if maintained properly, provide sections having quality as high as sections cut by disposable blades.

A high-quality knife, accurately sharpened, is very important for cutting good sections. Several automatic knife-sharpening machines are on the market, but it is possible to sharpen knives by hand. However, hand sharpening is very time consuming and requires extensive experience. Sharpening involves removing sufficient metal to replace the rounded cutting edge by moving the knife over a hard surface with abrasive and also polishing the remade edge, with or without abrasive, on a softer surface to give a crisp cutting edge. For more information on knife sharpening, see e.g. ANONYMOUS (1968), CLAYDEN (1971) or GRAY (1972). A blade is sharp when it can cut a hair held in the hand (SCHWEINGRUBER 1978). However, the only true test is the quality of a section as it is cut. The condition of the knife is best judged by microscopic examination at a low magnification (50 x).

For cutting wood it is customary to use a 'C' microtome knife. This wedge-shaped or 'universal' knife is easier to use than other types. A planoconcave 'A' knife or biconcave 'BL' knife is suitable for softer material. Very hard wood is best sectioned with a 'D' knife, which has a massive profile, but this knife is difficult to resharpen. All knifes should be cleaned and dried after use.

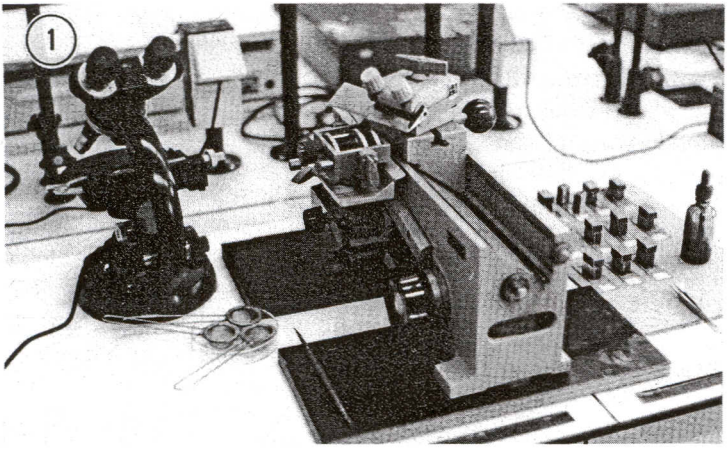
It is important that the sliding microtome runs smoothly, otherwise the entire sledge with the knife may skip while pushed by hand, which can be dangerous for the worker. Therefore, all bearing surfaces and screws should always be cleaned thoroughly after use and oiled using special oil that is fine, light and pure (for instance Micron). To prevent dust the microtome should also be covered when not in use.

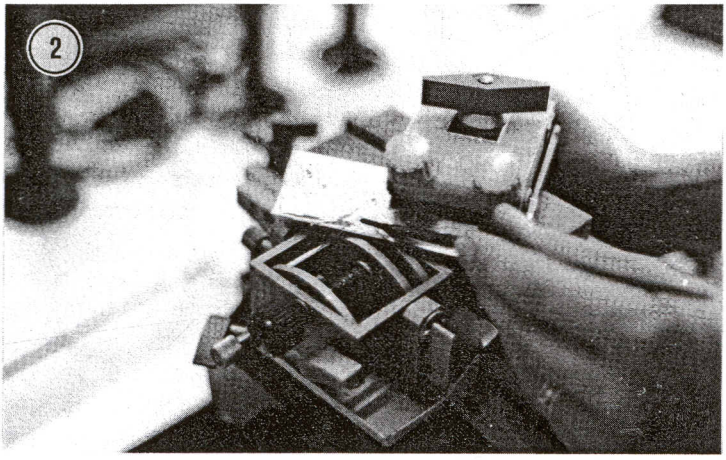
Heat plays an important role in the softening process. Therefore the blocks should be sectioned while they are still hot and fully swollen. If the knife skips on the block during sectioning or if the resulting sections have an uneven thickness, the wood is still too hard and should be reheated. Check also all clamps for tightness, and the condition of the sectioning knife. A slightly dull knife may skip whereas a perfectly sharp knife will cut good sections. Nicks in the knife will cause scores in the sections.

Generally, the wood samples are clamped in the microtome so that the softest tissues (rays or bands of parenchyma) are parallel to the movement of the knife. However, in our experience, it is sometimes better for the knife to move obliquely across the specimen, i.e. neither radially nor tangentially. For softwoods the knife should first meet the early wood; for hardwoods it should first meet the late wood. Obviously attention must be paid to ensure that the blocks are correctly oriented so that good sections are obtained.

The knife is adjusted so that the horizontal tilt (this is the angle between the blade and the horizontal plane) is 15° for hardwoods and approximately 8° for softwoods. For the cutting angle, small values of 10° to 30° are recommended for very soft and very hard material, and high values of 50° to 85° for medium hard material. These angles are only approximate, and must be varied somewhat to suit the specimen. It is very important that the knife is firmly secured in its holder and that all clamping screws and clamping levers are tight.

Both knife and block should be flooded with a mixture of water and glycerine. The knife should carry as much solution as possible on its upper surface. The fragile sections will then be buoyed up by the liquid and will not drag along the knife. The knife is moved in its slide with a steady firm movement of one hand, while a small brush held in the other hand is placed gently on the block to prevent the sections from curling (Fig. 2). Care should be taken not to push the section on the knife as this may cause tearing. Practice and skill are required to accomplish this task. Using a small brush, the section is then





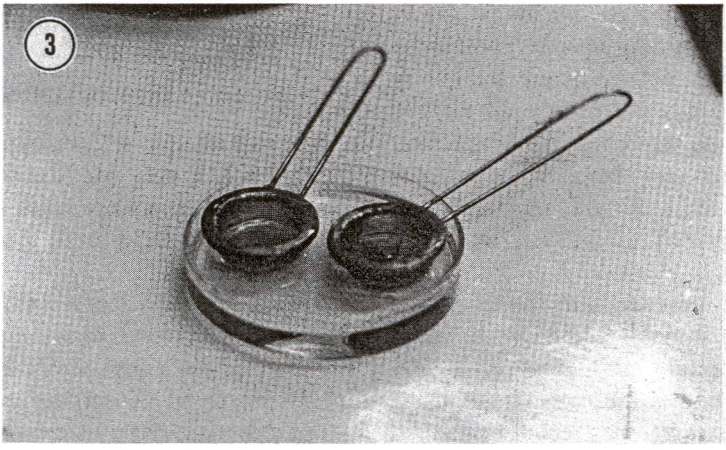


FIG. 1. — The sliding microtome, suitable for cutting wood sections.

FIG. 2. — Cutting the section (note the use of the brush to prevent section curling).

FIG. 3. — Baskets used for section treatment.

placed on a slide and observed under a light microscope to verify the orientation. If the section is good, it is put in a small basket (Fig. 3). The baskets resemble the water strainers for taps. They can be hand made from plastic or metal net. The use of baskets has the following advantages: (1) a lower quantity of treating products is needed, (2) a quicker treatment of the thin sections is possible, and (3) no damage is done to the sections during the treatment. Three or four transverse and longitudinal sections of the same wood sample may be placed in the same basket.

Sections of approximately 10 μm do reveal most details well and produce photographs of admirable clarity, but thicker sections (15-30 μm) are entirely usable for many other purposes. For example, thicker sections allow observations of entire perforation plates and give a better view for the context of a particular cell (CARLQUIST 1988).

Sometimes, curling of sections can be very troublesome. Especially cuts from dense woods show a decided tendency to curl or, if the wood has not been adequately softened, to roll up tightly. Since curled sections are difficult to mount, a satisfactory way of dealing with the sections is to glide them from the knife to a slide turning them upside down and allowing them to remain on the slide for a minute or so before removal. Sometimes curling can be avoided by transferring the thin sections from alcohol to water for a few seconds. Another good method to prevent curling is described by KUKACHKA (1978): the sections are placed between glass slides, clamped at the ends with paper clips or clothes-pegs and heated in distilled water for several hours.

When mineral inclusions are abundant in a wood sample, it is sometimes difficult to make good sections, especially transverse ones. Removing the silica bodies and/or crystals using hydrofluoric acid is sometimes recommended. It will also prolong the life of the knife blade (for more detail see KUKACHKA 1978).

4. SECTION TREATMENT

The following steps describe the treatment of the sections. These steps include bleaching, staining, dehydrating and clearing before mounting on slides.

The principal aim of bleaching is to increase brightness of the wood sections. It is performed by oxidising chemicals, which convert or stabilise the chromophoric groups of the cell wall lignin. Along with this, some extractives and ash compounds or cytoplasmic debris from the protoplasts have to be removed. Therefore bleaching can additionally be regarded as a purification process (FENGEL & WEGENER 1984). Bleaching is particularly needed for thicker slides (25-30 μm). As for staining, it also improves the contrast of the image and makes fine structures of the cell wall visible. Staining is especially recommended when the sections are pale coloured and treated with sodium hypochlorite and acetic acid, otherwise they will be too transparent for microscopic examination.

The different steps in section treatment can easily be carried out by moving the basket from one solution to the other. When several sections are being processed at one time, it is important to agitate the solution or basket to keep all the sections exposed to the solution. Before transferring the basket to another solution, it can be placed on some absorbing paper to avoid mixing of the solutions.

The sections in a numbered basket are:

- treated with 10% sodium hypochlorite (domestic bleach is also good) until they are totally bleached (this usually takes only 1 to 3 min);
- washed with water until the bleaching odour disappears;
- treated with 10% acetic acid (for 1-2 min);
- washed with water to remove the acetic acid;
- immersed in a 10% alcoholic solution of 1% safranin (1 g/100 ml) for approximately 3-5 minutes (time depends on the nature of wood, not on the thickness); safranin is generally recommended to stain wood sections because it is highly effective in highlighting the lignified walls;

- washed in 25% alcohol to remove the excess safranin, and dehydrated in respectively 50%, 70%, and 96% alcohol (if the excess stain is not washed off, an irremovable precipitate will be produced in the sections);
- treated with an equal mixture of clearing agent (active ingredients = blend of aliphatic hydrocarbons 100%; it is a substitute for the more dangerous xylol, trade name Parasolve, Bioclear, ...) and alcohol (100%);
- treated with clearing agent (for approximately 3-5 min). When the solution appears milky, the sections are not completely dehydrated and should be returned to alcohol.

5. Mounting

The sections are taken with tweezers and are moved carefully up and down in the clearing agent to remove any remaining dust particles. The transverse, tangential and radial sections are then placed on a clean glass slide. It is customary to mount the transverse section with its rays at right angles to the long axis of the slide, and the longitudinal sections with the longitudinal elements in a similar direction. This facilitates observations of the wood sections. Two or three drops of Coumarone or Caedax (synthetic resins) are placed on the sections and a coverslip of appropriate size is lowered on them. Other permanent mounting media are for example Canada balsam, Euparal and Entellan. Canada balsam is prone to yellowing and oxidising with age. The synthetic resins, however, stay clear for many years. Surplus resin should be cleared from the edges. Difficulty may be encountered in keeping the three sections in position. It is desirable to use only enough resin to fill the space below the coverslip.

Before being stored, the slides should be labelled and left lying flat with a weight (approximately 50 gr.) on the coverslip while the resin hardens during several days. The pressing is necessary especially for photography, because it is essential that sections lay perfectly flat in a mounting layer of even thickness. The weights also prevent the formation of air bubbles while the slide is drying.

If a permanent slide is not necessary, an alternative quick method is to mount sections temporarily in glycerine (either pure or with 50% distilled water). Mounting the sections into glycerine improves their transparency to light and it enhances the minute structure (e.g. pits). This is probably due to the plasticizing effect of the glycerine on the cell wall polymers. Such phenomenon is observed in different carbohydrate polymers when treated with plasticizers like propylene glycol and others. Glycerine jelly may also be used for semi-permanent slides.

Note that there is a very convenient system to store permanent slides in aluminium holders (for instance Thomas-Brown micro slide filers). These holders are of the standard size and may be filed, like cards, in a file cabinet.

6. Macerations

The purpose of macerations is to separate the plant cells by dissolving the middle lamella, which acts as a cementing layer. For comparative purposes, macerations are essential to obtain quantitative data on the length of vessel elements, tracheids and fibres.

Several methods can be used and are described for instance by Sass (1958 : Jeffrey's method), Franklin (1937, 1945), and Van (1967). These methods are rather similar but use different acids for dissolving the middle lamella. However, the procedure of Franklin (1945) is preferred by Purvis *et al.* (1966), Normand (1972) and us because of the less harmful reagents.

Slivers about the size of half of a match stick in length are cut longitudinally with a razorblade. Normand (1972) recommended boiling the slivers first in water until they become supersaturated and sink, but this softening step is not necessary if the slivers are taken from wood that has been softened already. The slivers of wood are immersed in a 1/1 mixture of glacial acetic acid and hydrogen peroxide and heated at 60°C for 1 or 2 days until they become white in appearance. The hardness can be tested any time during the operation by taking a small quantity and tearing it apart with needles; the cells should separate at a touch. Then, the slivers are rinsed thoroughly

with water, stained with safranin, and dehydrated with alcohol in the same manner as the sections (see above).

A small quantity of a sliver is placed on a slide. The cells are gently separated with needles and imbedded with Kaiser's glycerol gelatine. A coverslip is placed on top and a slight pressure is applied to further disperse the cells. It is important to take a small quantity for preparation on the slide, otherwise the cells obscure one another and observation will become difficult.

PREPARATION FOR SCANNING ELECTRON MICROSCOPY

Light microscopes are the stand-by instruments of wood anatomists even in these days of electron microscopy and ultraviolet microspectroscopy. However, the great advantage of the SEM over the LM and TEM is that, having a vastly greater depth of field, the subject is viewed in its three-dimensional structure and more detailed observations are possible due to the higher resolution. Several very good descriptions for the preparation of wood for SEM are published by e.g. EXLEY *et al.* (1974, 1977), KUČERA (1981), and NAGAI *et al.* (1994). Our method is summarised below.

- Softening of the wood samples is similar to the one described above for LM.
- The wood is cut by the sliding microtome with correct orientation. Possibly, final cuts are made by hand with single-edge razor blades because these cuts give very clean cut surfaces. A new razor blade should be used for each cut. Another method is to split wood samples; these surfaces however, are rough and usually do not allow observation into the cell lumen.
- The wood blocks are put in baskets and bleached with sodium hypochlorite or household bleach (15%) for ca. 60-90 min until the surface has lost colour.
- The samples are washed in distilled water to remove the bleaching agent.
- Next the samples are dehydrated in alcohol (50-70-96%).
- Let the samples dry at room temperature during 1 night or more. Critical point drying of the wood blocks is also possible. In our experience, however, the latter method gives no better results and therefore we do not recommend it.
- Finally the blocks are mounted on stubs using electrically conducting paste and are coated with gold in a high-vacuum evaporator during 2.5-3 min.

ACKNOWLEDGEMENTS

The research of S. Jansen and E. Smets is supported by grants from the Fund for Scientific Research — Flanders, F.W.O. project 2.0038.91 (scanning electron microscope) and project G.0143.95 (general research).

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Revised manuscript received 8 April 1998.