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Chapter 1

Preparation of Tissue Sections for Light-Microscopic Analysis of Phloem Anatomy

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Abstract

Accurate analysis and description of plant tissues often rely on the preparation of high-quality anatomical slides, a task that becomes particularly challenging when dealing with heterogeneous tissues such as phloem, which contains both soft and rigid components. This chapter provides a comprehensive protocol outlining key techniques for the optimal preparation of phloem tissue samples for light microscopy. The protocol encompasses essential steps such as fixation, softening, embedding, sectioning, staining, and mounting, and is adaptable for examining phloem and adjacent tissues in both woody and herbaceous stems and roots. Studying phloem anatomy is crucial for understanding nutrient transport, plant development, and responses to environmental stress, offering insights into both fundamental plant biology and practical applications in agriculture and forestry.

Keywords Bark, Phloem, Sieve element, Structure–function relationship, Sample preparation, Light microscopy, Staining, Dyes, Sectioning

1 Introduction

Botanical histology has been in practice since the dawn of plant microscopy, as shown in some of the classic seventeenth-century works of Grew [1], Malpighi [2], and Van Leeuwenhoek (reviewed by Baas [3]). Yet, producing slides suitable for accurate examination of plant tissues is not trivial, especially for heterogeneous tissues, such as the bark of trees, shrubs, and lianas. The bark typically contains a combination of soft, nonlignified cells, such as the phloem sieve elements and parenchyma, and of hard cells with lignified secondary walls, such as the sclerenchyma (for a detailed, illustrated explanation of each cell type, refer to the IAWA Bark Committee [4]). The term bark is nonspecific and encompasses all tissues outside the vascular cambium, namely the secondary phloem (also known as inner bark), commonly the remnants of primary tissues such as the cortex and primary fiber strands, and the periderm (known as outer bark [5]). Sectioning bark tissues without adequate preparation typically

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results in a collection of fragments, powders, or thick sections where details of the cell types are barely seen and difficult to describe. What follows is the description of some simple preparatory techniques to avoid these outcomes. The focus is on bark, especially the secondary phloem, although the techniques presented here can be easily adopted to any plant tissue or organ.

The methods described here consist of six steps: fixation, softening, embedding, sectioning, staining, and mounting (Fig. 1). The protocol describes the procedure for both large and small samples, with further details on how to proceed with different kinds of samples in the notes section. Fixation is the process of killing cells while preserving the cell structure as close to the living conditions as possible. Since the bark contains numerous nonlignified, fragile cells, which collapse when dried, the best samples are those fixed immediately in the field and conserved later in liquid solutions. Softening is not necessary for all samples. Rather, it makes certain bark samples easier to section. Sample embedding facilitates thin sectioning. After infiltration and hardening, the samples can be fixated and sectioned in a microtome without damage or distortion. The embedding media stabilizes the anatomical structure and cellular organization. In addition to microtome sectioning, hand sectioning can give good results for phloem analysis, especially when fresh material is available. However, for optimal results, the use of either a sliding or a rotary microtome is recommended. Staining is the essential step to make specific structures of the phloem visible. Different from the study of woods, which are typically stained only in safranin or fuchsin, the study of bark yields superior results when sections are double-stained or stained with a metachromatic stain. This is due to the nature of bark, which contains lignified and nonlignified cells. Bleaching is not recommended for barks, as it removes all cell contents, which are important for differentiating conducting and nonconducting phloem [4]. Once stained, the sections are dehydrated, typically in an ethanol series up to absolute ethanol, rinsed in a solvent, and mounted with a natural or synthetic resin.

Anatomical studies of the phloem remain highly relevant, especially for understanding how phloem architecture can limit plant productivity and shape environmental adaptations [6, 7]. The following protocol enables the interested scientist to produce tissue sections for reliable, high-quality quantification of phloem structure.

2 Materials

2.1 Sample Preparation and Fixation

One of four fixatives (FAA, Glutaraldehyde-formaldehyde, paraformaldehyde, CRAF) can be chosen according to sample size and the intended type of analysis (*see* **Note 1**; Fig. 1).

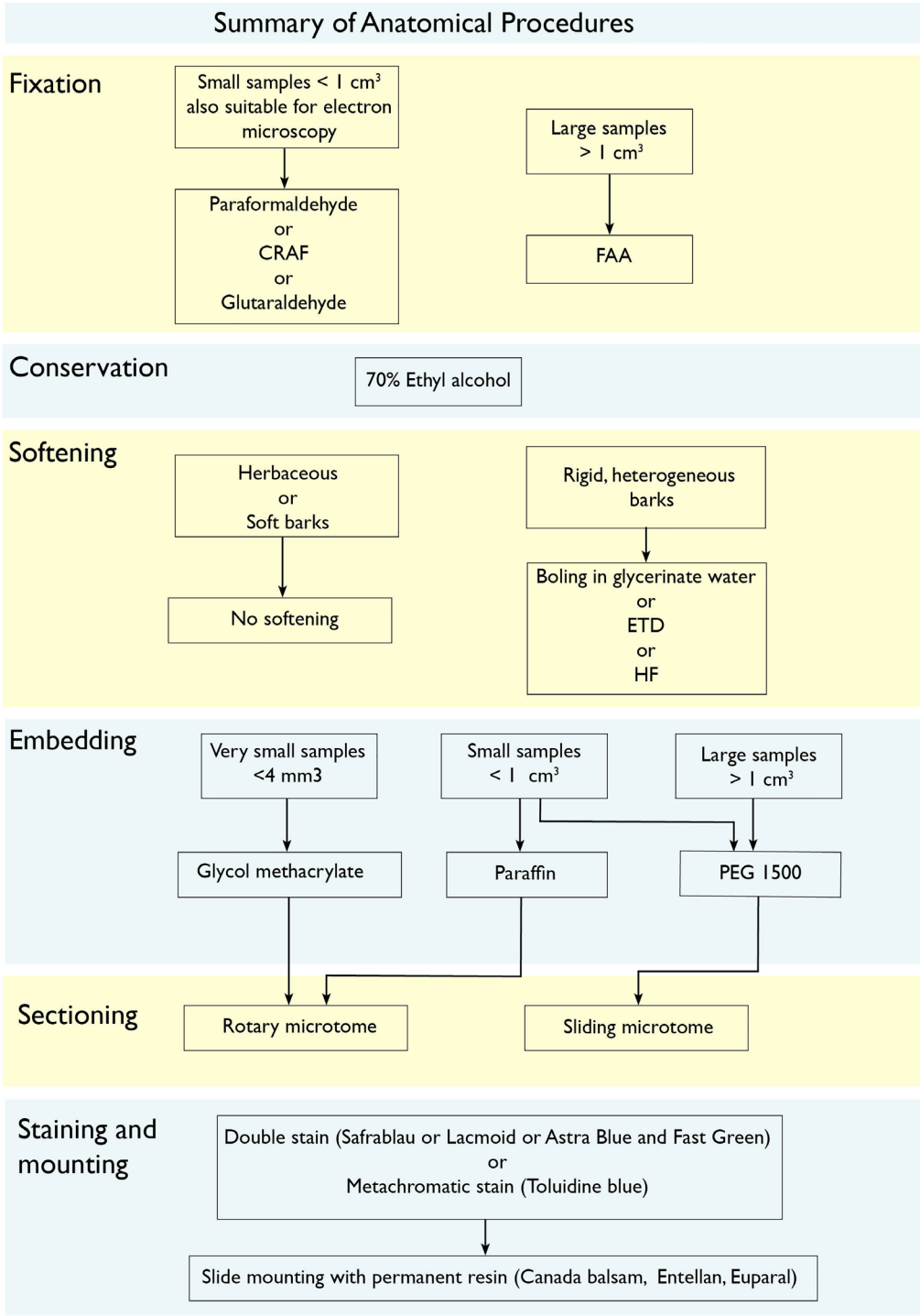

Fig. 1 Flow chart of the sample preparation procedure. *CRAF* chromic acid, acetic acid, formaldehyde, *FAA* formaldehyde, acetic acid, alcohol, *ETD* ethylenediamine, *HF* hydrofluoric acid

1. FAA solution: Mix formaldehyde, glacial acetic acid, 70% ethyl alcohol in the proportion 1:1:18.
2. Paraformaldehyde solution: Place 15 ml of distilled water on a rotary shaker and add 0.8 g of paraformaldehyde, keeping under rotation for 2 h until complete dissolution. Add 1 N NaOH drop by drop until the solution clears. The solution must be prepared fresh every time.
3. Glutaraldehyde-formaldehyde solution: Dissolve 1 g of paraformaldehyde in 50 ml of distilled water at 60–70°C. Add 1 N NaOH drop by drop until the solution clears. Wait until the solution cools down. Mix this solution with 50 ml of 50% glutaraldehyde diluted in a phosphate buffer with pH 7–7.4 to obtain a final volume of 100 ml. The solution must be prepared fresh every time.
4. CRAF III: Mix 30 ml of 10% chromic acid, 20 ml of 10% acetic acid, 100 ml of formaldehyde, and 850 ml of distilled water. The formaldehyde must be added just before usage. The solution without formaldehyde can be stored for long periods.
5. Water with glycerin: Add 1 volume of 87% glycerin to 10 volumes of water. Use for rehydrating dried samples.
6. Sample containers, vials of various volumes with screw-on lids.
7. Rotary or rocker shaker.
8. Molds: Molds are used for embedding. There are several options for obtaining molds. Nowadays, one can use silicone ice cube trays sold in appliance stores or online. For polyethylene glycol 1500, it is common to make origami (paper-folding) molds that can be discarded after use, but ice cube trays can be used, too.

2.2 Softening

1. Water with glycerin (10:1) prepared as described above.
2. Ethylenediamine (ETD) at 4% or 10%, dissolved in distilled water.
3. Hydrofluoric acid (HF) solution: Dissolve one volume of 1 M HF in two volumes of distilled water. Remember to always pour the acid into the water, not the other way around. Extreme care is essential when handling HF, since it is highly toxic. High-quality plastic (PTFE) containers with screw tops must be used; never glass containers. The solution must always be kept under a fume hood. Thick nitrile gloves are recommended.

2.3 Infiltrating and Embedding

1. Polyethylene glycol (PEG) 1500: Place solid PEG pellets in an open jar in an oven at 60°C to liquify within 5–10 min. Solutions with lower concentrations are made by mixing them with distilled water.
2. Paraffin wax.

3. Ethanol at various concentrations. Mix ethanol with distilled water to obtain concentrations of 50%, 70%, and 90%.
4. Historesin: Glycol methacrylate monomer. Can be purchased as a kit containing a hardener (a derivative of barbituric acid).
5. Historesin mold. These molds are either sold by the provider, or alternatively, one can buy small silicone ice cube trays.

2.4 Sectioning

1. Sliding or rotary microtome.
2. Permanent steel microtome knives or a disposable microtome knife.
3. Polystyrene resin: Dissolve packaging Styrofoam in butyl acetate or xylene to make a saturated solution.
4. Hot plate.
5. Haupt's gelatin adhesive: A blended solution of high-quality gelatin, glycerin, and phenol.
6. Coplin staining jar.
7. Xylol or butyl acetate: To remove paraffin from slides.
8. Ethyl alcohol: mix with distilled water to obtain 50%, 70%, and 90% solutions.

2.5 Stains and Stain Procedures

All these stain solutions can be stored in the fridge for many years.

1. Ferric chloride: Dissolve 2% ferric chloride, 1% tannic acid, and 1% sodium bicarbonate in 50% ethanol.
2. 1% sodium bicarbonate solution: Dissolve the sodium bicarbonate in two steps, first dissolve the sodium bicarbonate in water to 2% (w/v) and then dilute with ethanol to obtain a 1% sodium bicarbonate solution in 50% ethanol.
3. Lacmoid solution: Dissolve resorcin blue in 30% ethanol until saturated. Usually, 0.25 g resorcin blue is used for 100 ml of 30% ethanol. Add a few drops of sodium bicarbonate solution to make it alkaline.
4. Safrablau: Dissolve Astra blue and Safranin O in 50% ethanol. Filter each solution before adding one part Safranin O to nine parts Astra blue (1:9). Different concentrations can be used. In some taxa (e.g., *Ipomoea*, Vitaceae), the lignified tissues do not stain as well as those of other taxa. In these cases, it is recommended to use blends with higher Safranine O (2:8, 3:7, and so forth). It can go to the extreme of dissolving a pinch of Astra blue in pure Safranine O solution.
5. Safranin and Fast Green: Dissolve 1 g of Safranin O in 100 ml of distilled water and 0.5 g of Fast Green FCF in 100 ml of 95% ethanol.

6. Aniline Blue: Dissolve Aniline blue in water to obtain a 1% (w/v) aqueous solution.
7. Toluidine Blue: Dissolve 0.05 g Toluidine Blue in 100 ml of phosphate buffer at pH 6.8.

2.6 Permanent Slide Mounting

1. Microscopy slides and coverslips.
2. Mounting medium, e.g., Canada balsam, Entellan, Euparal (*see* **Note 2**).

3 Methods

3.1 Fixation and Sample Preparation

Fixation of Fresh Samples

1. Subdivide the specimens as soon as possible after collection and place them in a container of suitable size, usually a glass vial with a screw-on lid.
2. Add fixative (FAA, glutaraldehyde-formaldehyde, paraformaldehyde, or CRAF) until the sample is completely submerged.
3. Incubate for 12–24 h at 4°C on a rotary or rocker shaker and, if possible, in a vacuum chamber. The minimum incubation time for herbaceous samples is 2 h. The larger and denser a sample, the longer it should be incubated. Usually, 2 days are enough.
4. Place the sample in 70% ethyl alcohol if samples need to be stored for longer periods.

Preparation of Dry Samples

When dry samples, for example, from a xylarium or a herbarium are used, a rehydration step will be needed. Beware that, in most cases, the quality of the sections will be impaired by the natural collapse of thin-walled cells during the drying process, and no quantitative measurement should be performed on such materials.

1. Suspend samples in water with glycerin.
2. Boil for about 2 h. Sinking of a sample indicates that it has been well rehydrated

3.2 Material Softening

Certain barks are more easily sectioned after treatment with a softening agent. The optimal softening procedure has to be determined by testing for each type of sample. Boiling the sample in water with glycerin is the standard procedure for softening stem or root samples. An alternative is ethylenediamine (ETD) treatment, which swells the lignified cell walls of the sclerenchyma, making the overall tissue more homogeneous and less dense, ultimately allowing better sectioning [8, 9].

The swelling produced using ETD is reversed in the subsequent anatomical procedures [9]. This method has been very effective for some families, such as the Bignoniaceae [10, 11], while it has made the bark detach from the wood in a matter of hours in others, such as in the Malpighiaceae [4]. The highly toxic hydrofluoric acid (HF) is the most drastic method for softening and, therefore, is reserved for very hard materials. These include stem samples from certain hardwoods, for example, from the Sapotaceae [8]. Beware that HF will eliminate silica and crystals.

Boiling in Water and Glycerin

1. Boil your sample in water with glycerin for a period of between a few hours and several days.
2. During the softening procedure, periodically test the sample with a razor blade to know when it has reached its desired level of hardness. Samples will vary in timing and treatment.

Softening with Ethylenediamine (ETD)

1. Place the samples in a jar with a screw-on lid and cover with ETD.
2. Incubate samples for 1–3 days in a ventilated oven at 60°C.
3. During the softening procedure, periodically test the sample with a razor blade to know when it has reached its desired level of hardness. Samples will vary in timing and treatment.

Softening with Hydrofluoric Acid (HF)

Beware of the safety procedures described in "Materials"; HF is extremely toxic.

1. Put the sample in an adequate plastic container and submerge it in the HF solution.
2. Incubate for 3–4 days at room temperature under the fume hood.
3. After the treatment, place the samples in a saturated sodium bicarbonate solution to neutralize the acid.
4. Wash thoroughly with water.

3.3 Infiltrating and Embedding of the Samples

The PEG (Polyethylene Glycol) 1500 Method According to Rupp [12]

1. Place the sample in a reasonably large jar (like a tomato sauce jar) and cover the sample with pure dissolved PEG (*see* **Note 3**). Mark the original level of the pure PEG with a water-resistant pen.
2. Fill the jar with water.
3. Place this solution in a ventilated oven (60°C) until all the water evaporates, meaning the amount of liquid in the jar has reached the level marked in Step 1.

4. Store the sample with pure PEG in a vacuum oven for several hours to days.
5. Transfer to a square paper casting mold.
6. Leave at room temperature until solidified. The finished blocks are trimmed and then sectioned using a sliding microtome.

The Paraffin Method According to Johansen [13]

1. Place the sample in a closed container and cover with a solution of one volume of Tertiary butyl alcohol and one volume 50% ethanol.
2. Incubate at room temperature in a rotary or rocking shaker for 2 h.
3. Replace solution and repeat incubation four times with the following solutions:
   Tertiary butyl alcohol and 70% ethanol.
   Tertiary butyl alcohol and 85% ethanol.
   Tertiary butyl alcohol and 95% ethanol.
   Tertiary butyl alcohol and 100% ethanol.
4. After the final incubation, transfer the sample to pure tertiary butyl alcohol and incubate at room temperature in a rotary or rocking shaker for 12 h.
5. Transfer to a new solution of pure tertiary butyl alcohol and keep it within a closed jar.
6. Incubate in a ventilated oven at 60°C for 2 h.
7. Replace alcohol with the following solutions and repeat incubation for each solution:
   i. 3:1 tertiary butyl alcohol-paraffin.
   ii. 1:1 tertiary butyl alcohol-paraffin.
   iii. 1:2 tertiary butyl alcohol-paraffin.
8. After final incubation, replace the solution with pure liquid paraffin and incubate at room temperature on a rotary or rocking shaker for 4 h.
9. Replace once more with pure liquid paraffin, allowing four more hours on the shaker.
10. Transfer the sample to a paper mold.
11. Once hardened, trim the paraffin block, and section on a rotary microtome as described below.

The Glycol Methacrylate Method

Resin embedding allows for obtaining thin sections (3 µm or less) without tissue distortion, which may be necessary for observing the fragile cells of the phloem [14–16]. However, to obtain a perfect historesin embedding, the samples must be kept small, not exceeding 2 × 2 × 2 mm (8 mm^3). Typically, all resins are purchased as a kit with specific, easy-to-follow instructions provided by the supplier.

Here, we describe what is typical for most of the resins available on the market. An adjustment of the standard protocol, the addition of polyethylene glycol 400 to the resin, facilitates obtaining ribbons from sectioning resin-embedded samples [17]. If possible, do all steps within a vacuum chamber at 4°C.

1. Dehydrate your small samples in an ethanol series up to absolute ethanol.
2. Transfer the samples to a solution of absolute ethanol mixed with the embedding solution (1:1) for several hours up to 1 month, depending on the permeability of the material. Thick sclereids may be nearly impermeable to the glycol methacrylate resin and will need a long time to infiltrate.
3. Transfer the samples to the historesin solution and incubate for several hours up to 1 month. Use a vacuum chamber whenever possible at all steps. Note that some resin kits recommend carrying out the procedure at 4°C.
4. Add the hardener and place the sample in a historesin mold.
5. Incubate for 1 day.
6. Place the solid blocks on a holder and cut with a rotary microtome.
7. Place the sections directly on the slide, dropwise and incubate the slide on a hot plate at 55°C until the water has evaporated.
8. Materials sectioned in resin can be stained with Toluidine blue and left without a coverslip. Toluidine blue tends to fade with time, and without a coverslip, the slides can be re-stained.

3.4 Sectioning

Sectioning of Paraffin- and Resin-embedded Samples

1. Check knife sharpness. Regardless of hand sectioning or microtome sectioning, the key to good sections is a very sharp knife. Either disposable knives or sharp permanent steel knives can be used (*see* **Note 4**).
2. Insert the sample in the sliding or rotary microtome. The knife, sample and all the screws in the microtome need to be very tight.
3. In the rotary microtome, adjust cutting speed and angle, make sections, and use tweezers to hold and collect the sections on microscopy slides.
4. If the sample is embedded in resin, place each section on a drop of water on a slide and place the slide on a hot plate (55°C) and incubate until the water has evaporated. This facilitates attachment to the slide.

5. If the sample is embedded in paraffin, place slides upon a hot plate (55°C) and add three drops of Haupt's adhesive and place the paraffin ribbon upon them. Keep for 2 days in a hot paraffin chamber (56°C oven). After that, the slides are treated either with xylol or butyl acetate to remove the paraffin and move to the stains. Because the sections are glued to the slide by the adhesives, they can all be stained simultaneously in a rack in a Coplin staining jar.
6. If using a sliding microtome, start by adjusting the cutting angle. An angle between 15° and 20° gives the best results, even for very dense materials.
7. Adjust the direction in which the knife travels in the sliding microtome towards the block, making it equivalent to between 45° and 90° in relation to the rays. The higher the angle, the lower the impact on the sample; therefore, increase the angle when the bark is harder.
8. Adjust the section thickness to 20 μm on the sliding microtome. However, when the bark or the wood is too hard, make smaller cutting blocks and cut thinner sections (10–15 μm), which will impose less resistance to the knife.

Sectioning of PEG 1500-Embedded Samples

The heterogeneous nature of bark makes it extremely fragile not only while sectioning, but also in the subsequent staining and dehydration procedures. An effective solution to this problem is the use of a polystyrene foam resin placed upon a sample embedded in PEG 1500 [18].

1. In a small vial, place some butyl acetate or xylene and then cut pieces of any packaging Styrofoam and dissolve them in the solvent to make a saturated solution.
2. Install your sample in the microtome and apply some polystyrene foam resin on it with a paintbrush. Let it dry.
3. Perform sectioning. While sectioning, apply a little pressure to the sample with a clean paintbrush to ensure that the sections will not roll up.
4. Transfer sections with a tweezer to a microscope slide or a glass watch with some tap water.
5. Follow the procedures of staining as for other samples.
6. Dehydrate the sections in an ethyl alcohol series (50%, 70%, and 90%, 30 min each step) directly on the slide, so that sample movement is kept minimal.

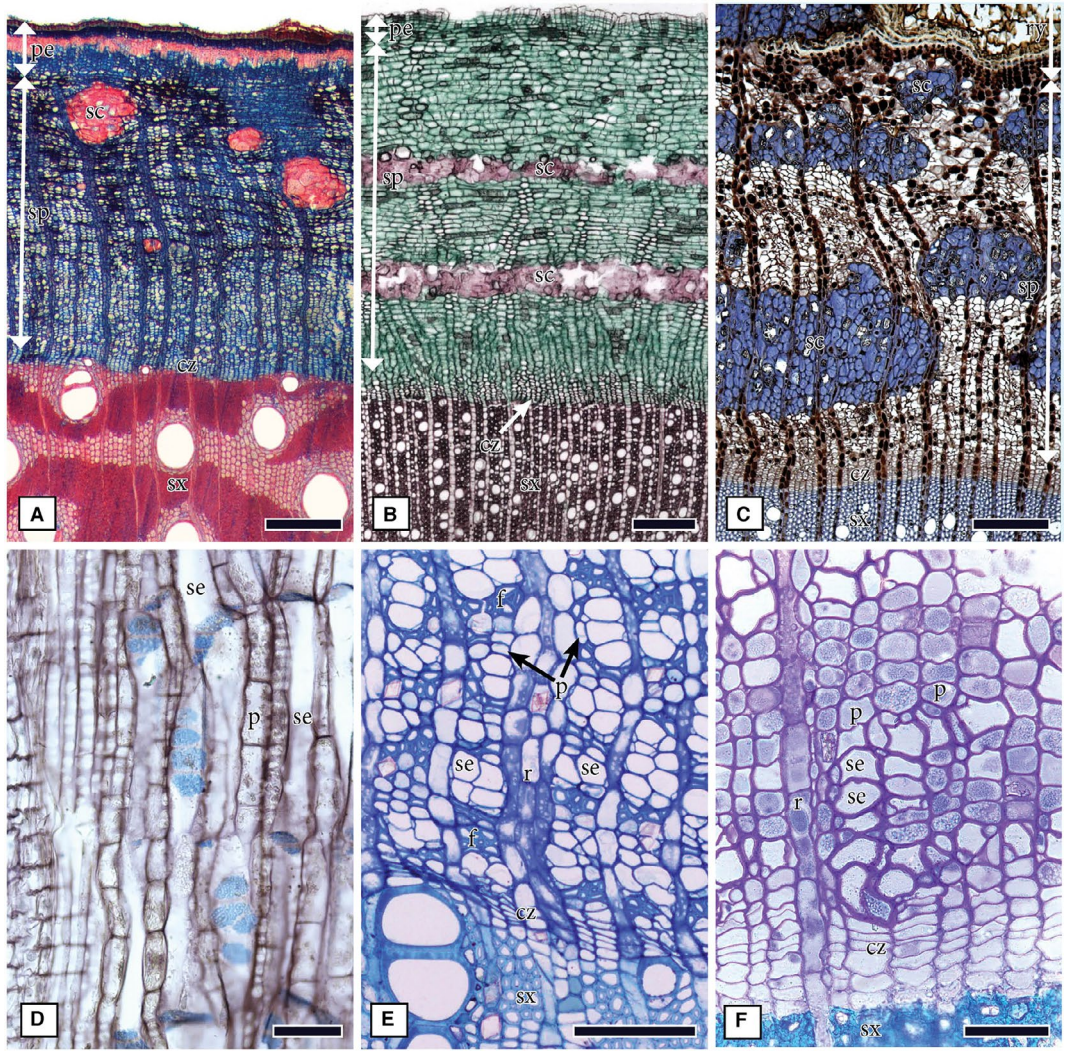

Fig. 2 Photomicrography of barks. All images show transverse sections, except for **D**, which is a longitudinal radial section. (**A**) *Caesalpinia ferrea* (Leguminosae), embedded in PEG 1500, sectioned using a sliding microtome with a polystyrene foam resin, stained with Safrablau, and mounted in Canada Balsam. Lignified tissues stain red and nonlignified tissues blue. (**B**) *Robinsonella discolor* (Malvaceae), sectioned unembedded using a sliding microtome, stained in safranine and fast green (by courtesy of Teresa Terrazas). Lignified tissues stain in red and nonlignified tissues in green. (**C**) *Crataegus intricata* (Rosaceae), stained with ferric chloride and resorcin blue (lacmoid). Lignified tissues stain in blue and nonlignified tissues in brown. (**D**) *Fraxinus americana* (Oleaceae), stained with ferric chloride and resorcin blue (lacmoid), evidencing the pores at the sieve areas, stained in bright blue*. (**E**) *Luehea divaricata* (Malvaceae), embedded in glycol methacrylate, sectioned at the rotary microtome, and stained with toluidine blue. Mounted in Canada Balsam, which reduces the metachromasia. (**F**) *Leucaena leucocephala* (Leguminosae), embedded in glycol methacrylate, sectioned at the rotary microtome, and stained with toluidine blue. Unmounted, with just tap water and a coverslip. Samples shown in **C**, **D** come from the Dr. Ray Evert Collection, which was imaged in collaboration with Carmen R. Marcati and Veronica Angyalossy. Sx = secondary xylem, cz = cambial zone, sp = secondary phloem, pe = periderm, sc = sclereid, ry = rhytidome, se = sieve tube element, p = axial parenchyma, f = fibers, r = rays. Scale bars: **A** = 300 μm, **B**, **C** = 200 μm, **D** = 40 μm, **E** = 100 μm, **F** = 50 μm

7. Remove the Styrofoam resin by carefully washing the sections with butyl acetate or xylene. Note that after the removal of the Styrofoam resin, the sample is very fragile.
8. Carefully add mounting media and the coverslip.

3.5 Stains and Stain Procedures

Ferric Chloride and Resorcin Blue (Lacmoid)

This combination of stains is one of the best for phloem studies. It stains the nonlignified tissues brown, the lignified tissues blue (Fig. 2C), and the callose of the sieve areas bright blue, making it very easy to locate sieve elements and pores of the sieve areas (Fig. 2D).

1. Place the sections in 1% tannic acid for 5–10 min.
2. Wash well in distilled water.
3. Transfer to 2% ferric chloride for 5 min.
4. Wash well in distilled water. At this point, the coloration should be medium to dark gray. Repeat the staining if needed.
5. Transfer the sections to a 1% sodium bicarbonate solution. The coloration should change from gray to brown.
6. Transfer the sections directly to the lacmoid solution.
7. Leave the sections in lacmoid for 12–18 h or more at room temperature.
8. Transfer the section to a sodium bicarbonate solution in 50% ethanol for a few minutes
9. Move to the ethanol dehydrating procedures of mounting (Step 2 in Sect. 3.6).

Safrablau

A solution of Astra blue and Safranin O in 50% ethanol generally yields excellent results for staining bark. This solution is called Safrablau. The lignified tissues stain red and the nonlignified tissues blue (Fig. 2A).

1. Stain sections with Safrablau solution for 5–10 min.
2. Wash well with distilled water and then move to the mounting procedure (see Sect. 3.6).

Safranine and Fast Green FCS

This is one of histologists' preferred staining methods. It stains the lignified tissues in red and the nonlignified tissues in green (Fig. 2B).

1. Stain in 1% Safranin O for 1–12 h.
2. Wash well in distilled water and then dehydrate with an ethanol series up to 95% ethanol. No more than 10 min is needed in each ethanol rinsing step.

3. Counterstain in 0.5% Fast Green FCS for 5–30 s.
4. Wash well in 96% ethanol and move to the permanent mounting procedures (Sect. 3.6).

Aniline Blue

This stain enables the visualization of callose under ultraviolet fluorescent light.

1. Stain sections in 1% aqueous Aniline blue for 1 h.
2. Wash gently with 50% glycerin.
3. Counterstain in 1% aqueous Eosin Y for 15–20 min.
4. Move to the mount procedures (Sect. 3.6). Note that Aniline blue typically fades with time [19].

Toluidine Blue

This metachromatic stain is especially recommended for sections embedded in glycol methacrylate resin, although it also works well on thicker sections obtained from other embedding methods. Typically, the color change is less clear or lost if the material is mounted in permanent resins such as Canada balsam (Fig. 2E). Therefore, it is recommended to have at least some slides unmounted that can be re-stained and observed fresh when needed (Fig. 2F).

1. Place the samples in the Toluidine Blue solution for several minutes.
2. Wash well in distilled water.
3. Either observe the samples freshly stained by placing some drops of 50% glycerin and a coverslip on the slide or move to the permanent mounting procedure (Sect. 3.6), knowing that the color shift will decrease over time.

3.6 Permanent Slide Mounting

1. If not already mounted on a slide, place the sections in the center of a microscope slide using delicate forceps.
2. Dehydrate the samples with an ethanol series and incubation times of 10 min up to absolute ethanol.
3. Wash in absolute ethanol several times to ensure that no water is present. If any water residue is left, it will react with the solvent, creating numerous tiny bubbles that will impair tissue observation.
4. Wash the samples in butyl acetate or xylene under a fume hood. Butyl acetate is less toxic than xylene and toluene and is therefore recommended here.
5. Add a few drops of any permanent mounting media, such as Canada Balsam, Euparal, etc. (*see* **Note 2**), and coverslip. Be careful when adding the coverslip as sections are easily damaged.

4 Notes

1. Bulky samples are preferably fixed in FAA, which has a strong penetrating ability. For small samples (<1 cm^3), basic, noncoagulant fixatives are more adequate and will yield good results for electron microscopy when the objective is the study of organelles such as mitochondria, nucleoplasm and vacuoles.
2. Consider that some mounting media age better than others. Canada balsam is a natural oleoresin obtained from the bark of the balsam fir *Abies balsamea* (Pinaceae) and has been used since at least 1830 [19, 20]. It is one of the best mounting media, since it endures the passage of time without crystallizing. Permount, for instance, should be avoided as it is nonarchival [21].
3. Slower methods can be employed for harder, less permeable specimens, allowing 24 h each in increasing concentrations of PEG, from 20%, 30%, and 40% up to pure PEG [18].
4. To sharpen permanent steel knives, there are two alternatives: either the use of abrasive solutions sold by knife sharpening manufacturers or, as a cheaper alternative, a recently proposed waterproof sandpaper sharpening method [22].

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