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A NEW METHOD TO OBTAIN GOOD ANATOMICAL SLIDES OF HETEROGENEOUS PLANT PARTS

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SUMMARY

A new method is presented to prepare anatomical slides of plant materials including a combination of soft and hard tissues, such as stems with cambial variants, arboreal monocotyledons, and tree bark. The method integrates previous techniques aimed at softening the samples and making them thereby more homogeneous, with the use of anti-tearing polystyrene foam solution. In addition, we suggest two other alternatives to protect the sections from tearing: adhesive tape and/or Mayer's albumin adhesive, both combined with the polystyrene foam solution. This solution is cheap and easy to make by dissolving any packaging polystyrene in butyl acetate. It is applied before each section is cut on a sliding microtome and ensures that all the tissues in the section will hold together. This novel microtechnical procedure will facilitate the study of heterogeneous plant portions, as shown in some illustrated examples.

Key words: Microtechnique, sectioning, wood, plant preparation, polystyrene foam solution.

INTRODUCTION

Histological studies require high quality slides of sectioned plant parts. Although sectioning techniques have a long history, the sectioning of plant parts that have both rigid and soft tissues, hereafter called heterogeneous plant parts, remains a challenge. Heterogeneous plant parts in general have extremely rigid, sclerenchymatic cells occurring together with soft non-lignified primary-walled cells, causing tissue damage during traditional anatomical procedures. Typical examples are woody tissue with non-lignified parenchyma, lianas with variant secondary growth, and tree bark. Entire taxonomic groups might have such heterogeneous portions, such as the stems of arboreal palms. Thus, in many cases, researchers have conceded the impracticality of conducting anatomical studies on such plant groups using standard botanical techniques (Zimmermann

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& Tomlinson 1965; Tomlinson & Zimmerman 1967; Tomlinson 1990, 2006). To solve this problem, some techniques have used hydrofluoric acid to soften the material, and infiltrating it with celloidin (Duval 1879; Lodewick 1924; Burn 1964). Although this technique has been widely used (Thompson 1918; Cheadle *et al*. 1953; Evert 1960; Zimmermann & Tomlinson 1965, 1968) it has only limited use nowadays because of the toxicity of hydrofluoric acid and the fact that celloidin is no longer easily available; moreover, the technique is expensive, difficult, and time-consuming. Two more recent techniques have been suggested. Kukachka (1977) recommended the use of a 4 % ethylenediamine solution to soften refractory woods, while Carlquist (1982) combined this technique with paraffin embedding. Moreover, Carlquist simplified the technique, eliminating some of the steps and modifying the use of ethylenediamine, suggesting a 10 % solution for thicker portions as well as the 4 % solution, followed by embedding in paraffin for sectioning. By using paraffin embedding, Carlquist (1982) broadened the use of Kukachka's technique from secondary xylem to include seeds and barks.

Besides paraffin, other embedding media are available to mount plant parts for sectioning. Methacrylate copolymers (HistoResin) and polyethylene glycol 1500 are two examples. HistoResin, which is used both in zoological (Shimotsuma & Schoefl 1992; Bancroft & Stevens 1996; Kiernan 1999) and botanical studies (Marcati & Angyalossy 2005; Marcati *et al*. 2006), gives excellent results for detailed analyses of tissues, but its use extends only to rather small blocks (circa in transverse surface area 5 mm^2). Polyethylene glycol (PEG), on the other hand, permits embedding in larger blocks (Rupp 1964). However, tissues mounted with PEG alone tend to tear when sectioned. Therefore, here we suggest a technique that combines three of the aforementioned techniques (Rupp 1964; Kukachka 1977; Carlquist 1982), but adds the use of polystyrene foam solution and, optionally, two additional adhesives: adhesive tape and Mayer egg albumin adhesive. These options are to be used during sectioning and/or mounting procedures, reducing the risk of tearing. The technique we describe has been used in our laboratory extensively and successfully for over 15 years with a variety of plant specimens. We believe this technique will facilitate research on heterogeneous plant portions where studies have been limited for technical reasons.

MATERIAL

Origin and treatment of samples

Samples both from liquid conserved collections or dried materials (*e.g*., from wood collections) can be prepared using this technique. Dry samples need to be re-hydrated in hot water (90 °C) for a few hours. We advise against boiling samples, as this measure can sometimes destroy the more fragile tissues.

We have used our technique with a considerable number of species. However, in this paper, we have chosen seven species belonging to different plant families, and consisting of different portions of the stem, to illustrate the process (Table 1). First, we selected three lianas, two with variant secondary growth, *Pyrostegia venusta* (Bignoniaceae) and *Mascagnia sepium* (Malpighiaceae), and one with regular secondary growth, *Podranea ricasoliana* (Bignoniaceae), which had their complete stem circumference cross-

Table 1. List of species sampled and collection details.

Species with author(s)	Family	Collection site in Brazil	Collector	Habit
Archontophoenix cunninghamiana H. Wendl. & Drude	Arecaceae	São Paulo	Botânico 26	Arboreal
Butia capitata (Mart.) Becc.	Arecaceae	Minas Gerais	Gussella 16	Arboreal
Crescentia cujete L.	Bignoniaceae	São Paulo	Pace 68	Arboreal
Copaifera langsdorffii Desf.	Fabaceae	São Paulo	Marcati 3	Arboreal
Mascagnia sepium (A.Juss.) Griseb.	Malpighiaceae	São Paulo	Pace 67	Lianescent
Podranea ricasoliana (Tanfani) Sprague	Bignoniaceae	São Paulo	Pace 11	Lianescent
Pyrostegia venusta (Ker Gawl.) Miers	Bignoniaceae	São Paulo	Pace 36	Lianescent

sectioned. Second, we selected two arboreal palms, *Archontophoenix cunninghamiana* and *Butia capitata*, which had a portion of their stems cross-sectioned, including cortex and central cylinder. Finally, we selected two trees, *Crescentia cujete* (Bignoniaceae) and *Copaifera langsdorffii* (Fabaceae), from which a portion of their stems containing secondary xylem, thick bark and intervening cambial zone was sectioned.

Ethylenediamine (Kukachka 1977; Carlquist 1982)

Ethylenediamine acts on the cell wall, swelling it, and therefore diminishing its rigidity. By softening the thick walls, heterogeneous plant portions become more homogeneous, facilitating sectioning (Carlquist 1982). A 4–10% ethylenediamine solution is not toxic and can be re-used many times on different materials without losing its softening capacity, although it tends to get darker, eventually impeding visualization of the material inside the jar.

Polyethylene glycol 1500 (Rupp 1964)

Polyethylene glycol (PEG) is suggested rather than paraffin or HistoResin because it is water soluble, better for embedding large samples and tends to soften hard tissues throughout the embedding process.

Mayer albumin adhesive (Mayer 1883)

Mayer adhesive is to be used only on extremely heterogeneous or fragile material. It is useful because it firmly glues the section to the slide, thus fully impeding its fragmentation. Moreover, Mayer albumin adhesive does not stain as much as other commercial adhesives, maintaining a transparent appearance to the slide's background. Mayer albumin adhesive can be purchased or easily prepared by thoroughly mixing the same quantities of glycerine and fresh egg white. It must be stored at low temperatures (2–4 °C), and it expires in approximately three months.

Polystyrene foam solution composition and preparation (Novel)

The polystyrene foam solution we use acts as an anti-tearing agent, and it should be applied on the plant sample in the sliding microtome prior to cutting each section.

The polystyrene foam solution adheres perfectly to PEG and holds the entire section firmly together. This ensures that the sample will not fragment during sectioning and mounting. In fact, since it is insoluble in both water and ethanol, the whole process of clearing, rinsing, staining and dehydrating can be done without any risk of disintegration of the sections. The solution is, however, soluble in butyl acetate, ensuring that the slides will have a perfectly transparent background once rinsed with it.

The foam solution consists of a solution of expanded polystyrene (= solid foam, like the commercial Styrofoam) in butyl acetate. Any solid foam can be dissolved, including those commonly used in packaging, making the polystyrene foam solution very inexpensive.

Approximately 1–2 ml of butyl acetate is placed in a small jar, and enough solid foam is cut into small pieces and added to obtain a saturated solution. The polystyrene foam solution must be used the same day it is prepared. Alternatively, a similar solution can be obtained using polyvinyl chloride (PVC) glue dissolved in butyl acetate.

METHODS

All the steps are summarized in a flowchart (Fig. 1).

First Step: *Ethylenediamine softening* (Kukachka 1977; Carlquist 1982, with modifications)

Place the samples in a jar and fill it with a 10 % solution of ethylenediamine. The jar must be closed and placed in a hot oven (approximately 50–60 °C) for up to 4 days, depending on the hardness of the sample. If the samples are particularly fragile, then use the 4 % solution, as recommended by Carlquist (1982), or check their hardness periodically with a razor blade. Following Kukachka (1977) and Carlquist (1982), we do not use hydrofluoric acid for several reasons. It is extremely toxic, and it removes some cell contents such as crystals. Also, given its corrosive characteristics, it can damage the knife over time.

Second Step: *Polyethylene glycol 1500 embedding* (Rupp 1964, with modifications)

After softening with ethylenediamine, rinse the samples in distilled water for at least 2 hours before starting the embedding process. Soak the samples in increasingly concentrated solutions of polyethylene glycol medium in 0.10 increments, beginning with 10 % and reaching 100 %, allowing one day in each solution. This will enhance sample penetration. Since PEG is solid at room temperature with a melting point at 48 °C, the solutions must remain in a hot oven (50–60 °C). It should be stressed that the jars have to be sealed; otherwise, the water will evaporate, increasing the PEG concentrations. If available, a hot vacuum oven can be used for the final step (100 % PEG) to further improve embedding, in which case the jar must be unsealed to allow the evaporation of residual water. Transfer the resulting samples to a paper mould or plastic cup, and fill the mould with fresh, undiluted PEG. The diluted PEG can be reused and kept in an open jar in the hot oven to allow the water to evaporate, thereby restoring PEG to its original state.

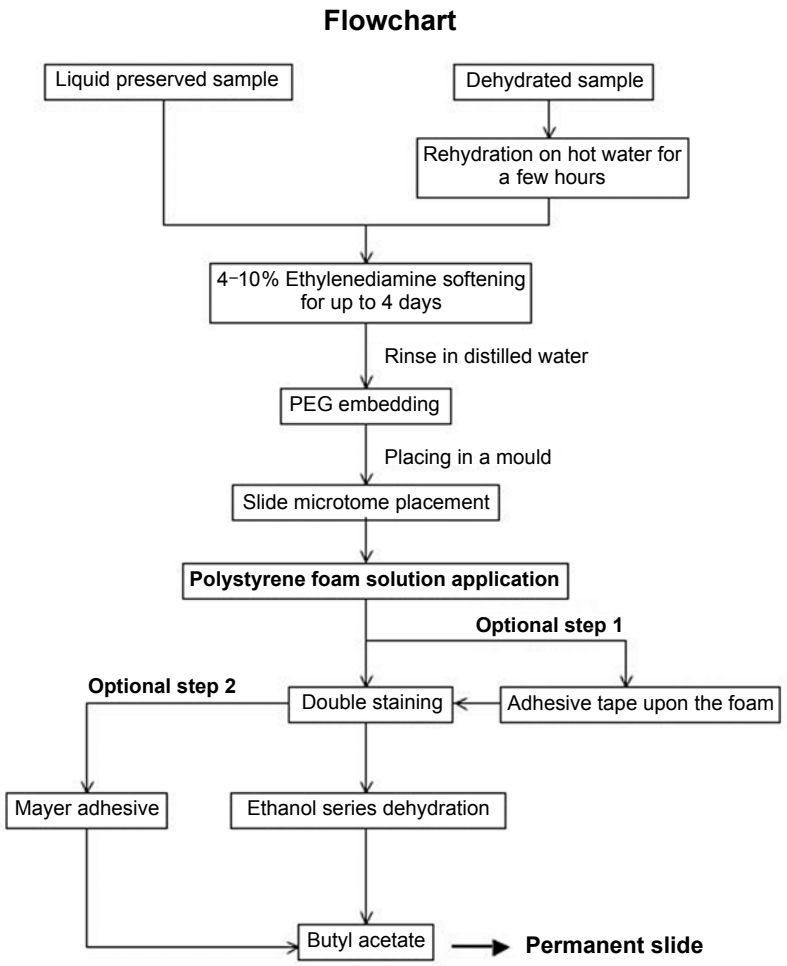

Figure 1. Flowchart with all the steps needed to obtain good slides from a heterogeneous plant part. Including optional steps.

Third Step: *Preparing and positioning the blocks for sectioning*

The blocks have to be perfectly cubic to fit in the sliding microtome (Fig. 2A, B). Trim them with a razor blade, polishing the excess PEG from the sample. If the sample is very small, more PEG should be left around the sample to provide support. Sectioning provides best results when the rays are at an angle of 45–75° to the knife.

Fourth Step: *Using the polystyrene foam solution as anti-tearing agent to section the samples (**Novel**)*

Trim the specimen with the sliding microtome until the surface of the sample is exposed. Apply the polystyrene foam solution to the sample's exposed surface with a paintbrush prior to each new section being cut (Fig. 2A). Wait until the polystyrene solidifies, or fan for a few seconds to accelerate the process, and then section the block.

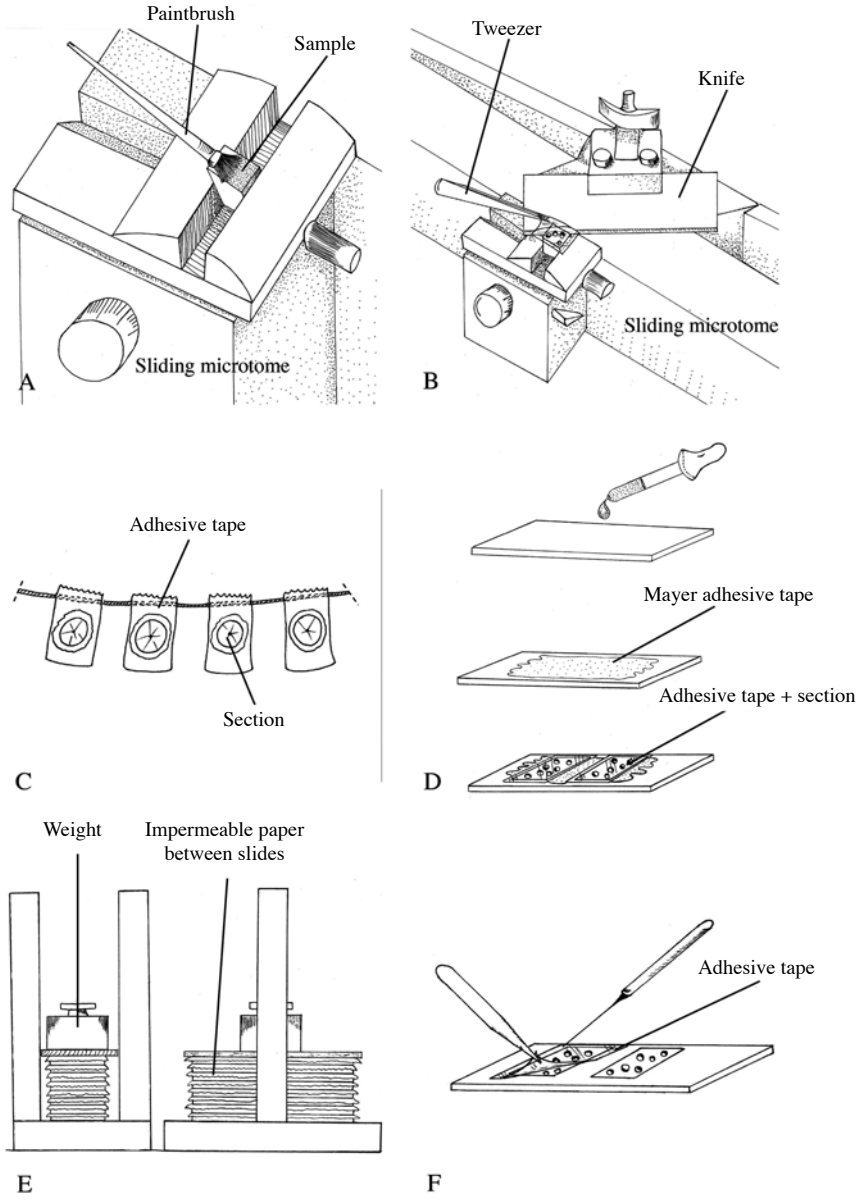

Figure 2. Six-step process for preparing plant material. – A: With a paintbrush, apply polystyrene solution on the exposed surface of the sample. – B: With one hand, hold adhesive tape with tweezers, with the other hand, slide the knife for sectioning. – C: After sectioning the material, all other steps can be postponed as long as necessary. – D: Spread one drop of Mayer's adhesive on extremely heterogeneous plant material, and put the sections on a slide treaded with the adhesive. – E: Slides can be piled up for 72 hours to await fixation. Put a piece of impermeable paper between each pair of slides; otherwise slides may stick to each other. – F: Remove the adhesive tape after rinsing with butyl acetate.

Optional steps: *Other anti-tearing supports to obtain good sections (**Novel**)*

a) Optional step 1: Using an adhesive tape to aid sectioning

The application of an adhesive tape to the resin surface further improves the process of sectioning and mounting. The section is made while holding one side of the tape (Fig. 2B) and sectioning with the other hand. All the adhesive tapes with sections can be hung from a line for as long as needed (Fig. 2C), even days, until the next step, when adhesive tape + section can be put on a slide.

b) Optional step 2: Fixing sections on the glass slides (Mayer 1883)

For extremely heterogeneous tissues, such as arboreal palm stems, monocot stems in general, or even large portions of secondary phloem, fixing the section to the slide may be necessary. However, gelatin adhesives, such as those of Haupt (1930) and Bissing (1974), frequently do not provide sufficient adhesion. Therefore, we suggest the adhesive used by Mayer (1883), which consists of glycerinate egg albumin. As the concentration of albumin increases, the strength of adhesion increases.

Drop a little of the Mayer adhesive on the glass slide and spread it equally until it becomes sticky (Fig. 2D). Afterwards, place the section on the slide (Fig. 2D) and cover it with an impermeable paper. This paper strip is used to separate each pair in a stack of slides (Fig. 2E), avoiding the slides to stick to each other. The stack of slides will be kept still for approximately 72 hours to allow section fixation.

Note that the section should be stained before being placed in the Mayer adhesive (Fig. 1). Moreover, since the albumin is water soluble, let the stained sections dry naturally for 20 minutes before gluing them in the Mayer adhesive.

The next step for sections that were glued in Mayer adhesive is the final step, when butyl acetate is added to dissolve both the polystyrene and/or the adhesive tape from the section (Fig. 1, 2F).

Fifth Step: *Clearing and staining the sections*

Once the sections are fixed to the anti-tearing supports (polystyrene solution with or without adhesive tape), all other anatomical procedures can follow without danger of damaging the tissues. If the section starts to fold during dehydration procedures, put a piece of toilet paper over it with a drop of water, impeding the section to move. If adhesive tape was used, simply affix the end of the tape to the glass slide. The sections glued to the slides can be cleared in 10–20% sodium hypochlorite, which removes some of the cell contents. After rinsing in distilled water, sections can be double-stained using any staining process commonly used in plant anatomy. It should be stressed that certain materials should not be cleared, as the structure may be too fragile and may be destroyed. For analysis of phloem, clearing can remove the contents of sieve elements and companion cells, which are critical to an evaluation of their functioning.

Final Step: *Mounting*

With the sections on the slide (with or without the adhesive tape), wash them with an ethanol series, from 10% to 100%. After rinsing the section twice in 100% ethanol, rinse it in butyl acetate to dissolve the resin and unglue the adhesive tape. If the sections

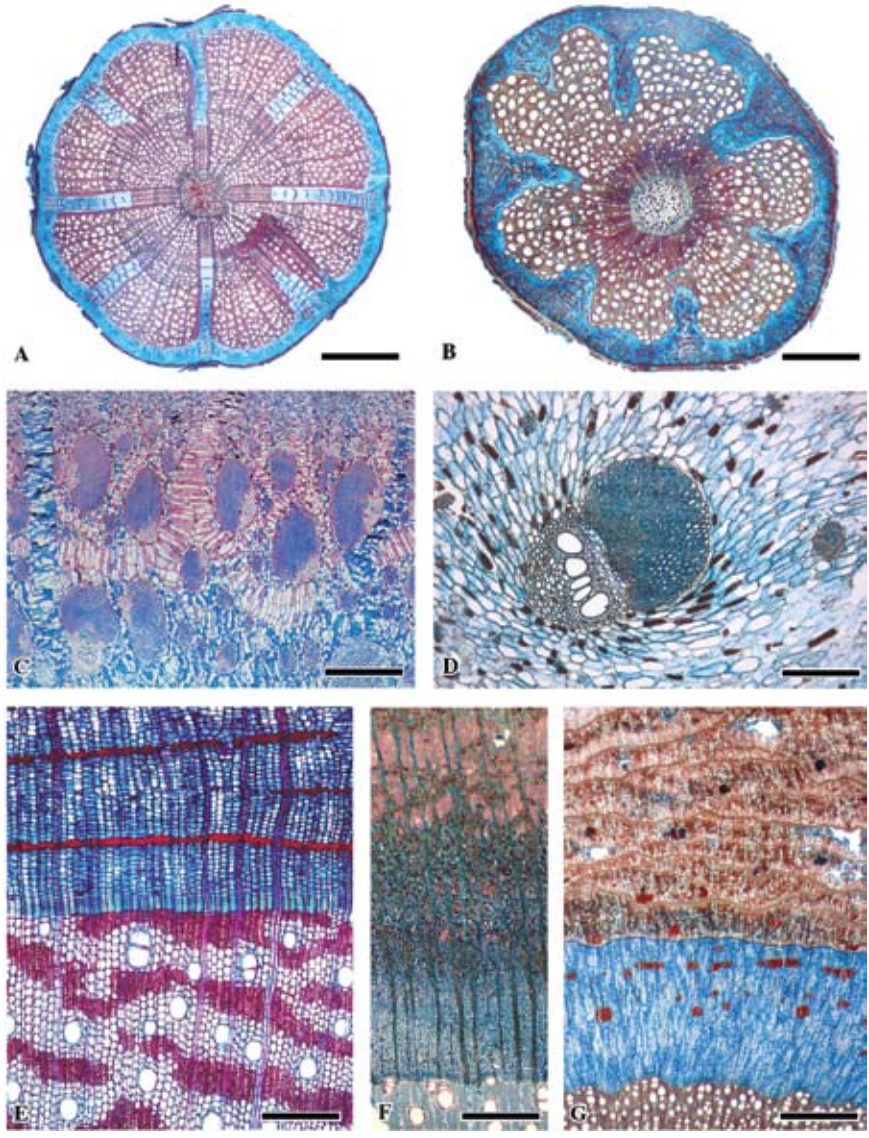

Figure 3. Stem cross sections. – A: *Pyrostegia venusta*, liana with variant secondary growth. Entire stem with eight phloem wedges. – B: *Mascagnia sepium*, liana with variant secondary growth. Entire stem with seven phloem arcs and islands of non lignified parenchyma embedded in the xylem. – C: *Archontophoenix cunninghamiana*, arboreal palm showing the limits between the cortex and the central cylinder, an extremely heterogeneous portion. – D: *Butia capitata*, arboreal palm. Vascular bundle with primary xylem and phloem and a cap of highly lignified fibers surrounding the phloem, in a matrix of parenchyma. – E: *Crescentia cujete*, tree, stem showing xylem, cambium and phloem perfectly preserved. – F: *Copaifera langsdorfii*, tree, stem displaying xylem, cambium and a large portion of its bark. – G: *Podranea ricasoliana*, liana showing xylem, cambium, phloem and a large rhytidome. — Scale bars for A: 4 mm; B: 5 mm; C: 1 mm; D: 200 μm; E: 700 μm; F: 400 μm; G: 500 μm.

were not glued with Mayer adhesive, extreme care is needed when adding the butyl acetate because it will dissolve the anti-tearing agents, leaving the sections completely loose for a short period of time. If the section is glued to the adhesive tape, hold one of its ends with tweezers, and, with another set of tweezers, gently detach it from the section (Fig. 2F). Once the section has been released, put a drop of synthetic resin (*e.g.* Canada balsam) over the section and cover it with a cover-slip.

RESULTS AND DISCUSSION

As can be seen in Figure 3, this technique allowed us to produce good quality, perfectly sectioned samples. The use of the polystyrene foam solution, sometimes combined with an adhesive tape and Mayer adhesive, avoided tissue damage or folding during sectioning and mounting. In the lianas *Pyrostegia venusta* (Fig. 3A) and *Mascagnia sepium* (Fig. 3B), the presence of variant secondary growth produces a pattern of soft primary-walled secondary phloem cells intermingling with portions of stiff secondary xylem. In lianas, the mixture of large portions of tissues with very different hardness often makes the phloem detach from the secondary xylem during sectioning or mounting. With our technique, however, entire stems up to 5 cm could be sectioned and mounted without any damage to the phloem-cambium-xylem connection (Fig. 3A, B).

Good results were also obtained for an even more challenging material: the arboreal palms *Archontophoenix cunninghamiana* (Fig. 3C) and *Butia capitata* (Fig. 3D). Monocotyledons in general possess vascular bundles with primary xylem and phloem associated with fibers, all embedded in a matrix of parenchyma cells. Such vascular bundles are usually very rigid, while the parenchymatous matrix is very fragile (Fig. 3C, D). Using traditional sectioning methods, the parenchymatous matrix is commonly destroyed. With the use of our technique, however, including the optional step of fixing the section on the slide with Mayer adhesive, it was possible to obtain perfect slides.

Even more challenging is bark, which is composed of conducting and nonconducting secondary phloem, and periderm. Bark is usually very thick and extremely heterogeneous. In fact, the bark usually combines very soft, primary walled cells, such as sieve elements and parenchyma, associated with highly lignified tissues, such as fibers and sclereids (Fig. 3E, G). Moreover, its nonconducting portion tends to lignify (Fig. 3F), and when associated with one or multiple phellogens (cork cambia), forms an extremely fragile zone (Fig. 3G). However, even for these materials, we could obtain very good slides.

CONCLUSIONS

Our technique aims to provide an alternative to traditional methods of sectioning heterogeneous plant parts. Moreover, our technique will be useful for cases in which the entire cross sections of the stem are needed to provide a good basis for comparative analyses (*e.g.*, Pace *et. al.* 2009; Lima *et al.* 2010).

While our technique is slow to implement, it is uncomplicated, and all the materials are inexpensive. The main advantage of this technique is that it can be used for a range of materials, from small to thick stems, always with satisfactory results. Apart from

the use of polystyrene foam solution, the other techniques described in this paper were already known (Mayer 1883; Rupp 1964; Kukachka 1977; Carlquist 1982), but they had not been previously combined.

After having tested several types of resins, we adopted polystyrene foam solution because it is cheap and can be applied easily and rapidly. Furthermore, the fact that it is insoluble in water-based solutions allows staining and rinsing of the sections without risks of fracturing them. The polystyrene is removed only when butyl acetate is added, immediately before mounting. Up to this point, the section is relatively robust, but extreme care is needed at this point because the section is loose for the first time, and this is the only moment in which tearing can occur. When such damage occurs frequently, the fixing of sections to the slide with glycerinate egg albumin, will resolve this problem.

We believe that this technique will help plant anatomists expand their studies to plant groups and plant parts that are interesting by their heterogeneity, but were thus far neglected because of microtechnical problems.

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