USE OF POLYETHYLENE GLYCOL AS AN EMBEDDING MEDIUM PRODUCES RESULTS SIMILAR TO THOSE OF PARAFFIN WAX EMBEDDING

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Polyethylene glycol (PEG) is a non-carcinogenic, water-soluble polymer of ethylene oxide that has found wide applicability in industry and medicine, and has been used to embed and section small animal and plant tissues. Here we investigate the use of PEG for the rapid embedding of larger plant tissues. Ovaries of *Musa velutina*, *Heliconia psittacorum* and eight other species were embedded with a mixture of PEG 1450 and PEG 4000. It was found that tissues up to 6.5 × 10 mm could easily be embedded and sectioned in PEG. Embedded tissues could be stored at room temperature for up to 5 days with no detrimental effects. Sections were easily cut at 8−15 μm on a rotary microtome. PEG embedding resulted in equal or better tissue differentiation, better retention of cell inclusions, and reduced shrinkage compared with paraffin embedding. The process was also faster, requiring only 3−6 h compared with the 2 days needed for paraffin embedding. PEG is a rapid-embedding medium suitable for use with even large plant tissues.

Introduction

In 1947, Mary E. Carsten (Carsten, 1947) presented an alternative to the common histological technique of embedding tissues with paraffin waxes. She used water-soluble polyethylene glycol polymers (PEGs) for the embedding of human eye tissue. This procedure shortened the processing time and allowed rapid sectioning of embedded material. The PEG embedding method was later adapted for use with botanical samples by van Horne & Zopf (1951) and Walker (1959), who successfully embedded and sectioned stem tissue. These protocols produced embedded material with less shrinkage than with traditional paraffin embedding.

To determine if PEG can be used efficiently for larger plant tissues, we conducted experiments using different concentrations and molecular weights of PEG to embed the ovaries of the plant order Zingiberales, the order including the culinary gingers and bananas. These ovaries range in length from 1.5 to 35 mm (to c.70 mm). The availability of PEG in different molecular weights (Table 1) allowed us to test various infiltration mixtures and times to find the best combination for use with larger tissues.

We tested our procedures primarily on the ovaries of *Heliconia psittacorum* L.f. (Heliconiaceae) and the larger ovaries of *Musa velutina* H.Wendl. & Drude (Musaceae),

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Table 1. Properties of Carbowax[a]

Molecular weight	Form	Melting point (°C)	Solubility, by weight (%)
Carbowax 400	Liquid	4−8	100
Carbowax 1450	Solid	42−46	72
Carbowax 3350	Solid	53−57	67
Carbowax 4400	Solid	53−59	66

[a] Dow Chemical (2019).

and verified the method by embedding and sectioning the ovaries of eight other species. We compared the consistency of the PEG after embedding, and the quality of the resulting sections. We show that larger pieces of tissue, up to c.10 mm, can be embedded and sectioned with good results. Flower parts, such as the androecium, that are not a solid structure can be embedded and sectioned but were difficult to mount, as discussed more fully below.

Methods and results
Polyethylene glycol

Polyethylene glycol is a synthetic, hydrophilic polyether with the molecular formula H−(OCH$_2$CH$_2$)n−OH. It is sold in the USA under the trade name Carbowax™, and many previous publications refer to it by this name (Firminger, 1950; Wade, 1952; Giovacchini, 1958; Zugibe *et al.*, 1958; Jones *et al.*, 1959; Walker, 1959; Riopel & Spurr, 1962; Firminger, 1964). PEG comes in a variety of molecular weights based on the number of repeating oxyethylene groups. The number of these groups determine its form as a liquid or solid (Wade, 1952; Dow Chemical, 2019). It is non-carcinogenic and water-soluble (Dow Chemical, 2019).

The different molecular weights of PEG have different melting and freezing points, as well as different solubilities in water (see Table 1). As its molecular weight increases, the melting and freezing points increase and its solubility in water decreases. No matter the molecular weight, PEG always melts and freezes in a narrow range: 42−46°C for PEG 1450, and 53−59°C for PEG 4000 (Dow Chemical, 2019).

Plant materials

Flowers were collected from botanical gardens and greenhouses in the continental United States and Hawaii (Table 2). Voucher specimen were collected whenever possible. Flowers of *Musa velutina* were collected at Lyon Arboretum, Honolulu, Hawaii. *Heliconia psittacorum* flowers were collected from Waimea Falls Park (now Waimea Valley), Haleiwa, Hawaii.

Flowers were fixed in formalin−acetic acid−alcohol (FAA), comprising 95% EtOH (53%), H$_2$O (37%), glacial acetic acid (5%) and formalin (5%) (Berlyn & Miksche, 1976). Long-term

Table 2. Species used in the present study to test and verify polyethylene glycol as an embedding medium[a]

Species	Family	Voucher (herbarium[b])	Accession no.	Approximate size of embedded tissue (height [mm] × width [mm])
Main testing species				
Heliconia psittacorum L.f.	Heliconiaceae	*Kirchoff* 89-258 (BISH)	*Waimea* 82p942	6.5 × 10
Musa velutina H.Wendl. & Drude	Musaceae	*Kirchoff* 88-144 (BISH)	*Lyon* L67.0284	6.5 × 4
Species used to verify the method				
Alpinia elegans (C.Presl) K.Schum	Zingiberaceae	*Nagata* 3085 (HLA)	*Lyon* L-82.0098	5 × 6
Alpinia galanga (L.) Willd.	Zingiberaceae	*Nagata* 3543 (E)	*Lyon* L-83.0505	4 × 3
Globba marantina L.	Zingiberaceae	*Kirchoff* 84-20 (FTG)	*FTG* 71-348	1.5 × 1.7
Hedychium flavescens Carey ex Roscoe	Zingiberaceae	*Kirchoff* 91-269 (BISH)	*Lyon s.n.*	4 × 4
Hedychium gardnerianum Sheph. ex Ker Gawl.	Zingiberaceae	NA	*UNCG s.n.*	4 × 3
Kaempferia pulchra Ridl.	Zingiberaceae	*Nagata* 3662 (HLA)	*Lyon* L87.326	3 × 2
Trimezia longifolia (Link & Otto) Christenh. & Byng	Iridaceae	*Kirchoff* 89-239 (BISH)	*Waimea* 74p532	3 × 2
Vellozia sp.	Velloziaceae	NA	*Duke s.n.*	4 × 4

Duke, Duke University Greenhouses, Durham, North Carolina, USA; FTG, Fairchild Tropical Garden, Miami, Florida, USA; Lyon, Harold L. Lyon Arboretum, Honolulu, Hawaii, USA; NA, not applicable; *s.n.*, *sine nomine* (without an accession number); UNCG, University of North Carolina Teaching Greenhouse; Waimea, Waimea Valley, Haleiwa, Hawaii, USA.
[a] For each species, the material embedded was ovary.
[b] Herbarium codes follow Thiers *et al.* (continuously updated).

storage was in Kew fluid: 95% EtOH (53%), H_2O (37%), glycerol (5%) and formalin (5%). Infiltration and embedding methods were similar for all species, but photographs were taken of only *Musa velutina* and *Heliconia psittacorum*.

PEG infiltration

Specimens for infiltration were removed from Kew fluid and, following Smithson *et al.* (1983), washed in buffer. Unlike Smithson *et al.* (1983), we were not working on immunochemistry and therefore used Sorensen's phosphate buffer at pH 7.0 (readily available in our laboratory) for c.5 min. Infiltration was carried out through a graded series of PEG 1450 and PEG 4000 (Table 3), with adjustments to the latter steps based on the size of the tissue. Separate beakers of PEG 1450 and PEG 4000 were melted in a vacuum oven at 55°C and 15–20" Hg of vacuum to provide stock solutions for infiltration. We found this temperature and pressure to be optimal given the melting points of PEG 1400 and 4000. These settings did not denature the PEG nor destroy the structure of the ovaries.

Table 3. Polyethylene glycol (PEG) infiltration process

Relative percentage of PEG and distilled water	Duration of immersion (h)	
	Smaller tissues	Larger tissues
30% PEG 1450/70% distilled water	0.5	0.5
50% PEG 1450/50% distilled water	0.5	0.5
100% PEG 1450	1	2
95% PEG 1450/5% PEG 4000	1	3

To ensure that there was no water in the vial during the last two steps of infiltration, the specimens were moved to new vials before being transferred to 100% PEG 1450 (see Table 3). This precaution may not have been necessary as we added water back to the infiltration mixture at the time of embedding to prevent cracking as the PEG hardened.

Embedding

Immediately before embedding, room temperature (c.24°C) ultrapure water in the amount of 3% by volume was added to the embedding mixture (Bosman & Go, 1981). We also tested the addition of 6% water by volume, but under these conditions the block became too soft to section. Ultrapure water was used because it was readily available in our laboratory. Although we did not try tap water, we have no reason to believe it could not be used.

Embedding was carried out in polyethylene molding cups (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA; catalogue no. 70176), more commonly used for JB-4 embedding, or in aluminium weighing dishes (Fisher Scientific, Pittsburgh, Pennsylvania, USA; 08-732-101).

Storage of embedded material

The PEG-embedded material could be sectioned after it had set at room temperature for at least 8 h, and it remained viable for sectioning up to 5 days after embedding. After that time, the PEG tended to dry out and crack or absorb too much water from the air. The amount of time that the blocks were viable depended on the humidity and temperature, which varied with the time of year. We obtained the best sections when the embedded material was left to cure at room temperature overnight before sectioning.

Mounting and sectioning of blocks

Once the PEG had hardened, it was affixed to metal specimen discs (Electron Microscopy Sciences; catalogue no. 62355) whose surface had been heated by immersion in boiling water for 20–30 s and then dried with a paper towel. The heated disc was placed flat side down on the embedded material, which was still in the embedding cups, and allowed to cool for at least 10 min. The mounted blocks were then trimmed and sectioned. We also tested

the use of a Bunsen burner to heat the metal specimen disc, but this method produced uneven heating and resulted in unstable attachment to the PEG. The larger pieces of tissue, embedded in aluminium weighing dishes, were mounted onto wooden blocks instead of metal specimen discs. The embedded tissue was first cut out of the weighing dishes and trimmed into rectangular blocks with a single edge razor blade. The bottoms of the trimmed blocks were coated with melted PEG and then mounted onto wooden blocks to be sectioned.

Mounted blocks were sectioned on a Spencer Lens Company (Buffalo, New York, USA) rotary microtome using the same procedures as for paraffin sectioning (Berlyn & Miksche, 1976). We were able to obtain ribbons at section thicknesses of 8–15 μm. The thicker sections were easier to obtain. The ribbons could be stored flat, at room temperature, for up to 2 years with no detrimental effects. If the blocks became too dry to section due to prolonged exposure to air, the sections would crumble and ribbons could not be formed. In this case, water or 50% EtOH was used to paint the face of the block before sectioning (Table 4). The additional moisture allowed sectioning to continue until the block dried out again, when additional water was painted on the surface. Note that the addition of water to the face of the block worked only if the PEG was sectioned within 5 days of embedding. After that, the blocks had lost or gained too much moisture and were unable to be sectioned.

Good PEG sections were milky white or clear, and easily formed ribbons. If the blocks lacked moisture, the sections were opaque and powdery or brittle, and would not form ribbons (see Table 4). Good sections had no curling, breakage or folding, although some compression of the sections was evident.

Mounting of sections

Bissing's modification of Haupt's adhesive was used to adhere the sections to standard microscope slides (Bissing, 1974). First, 1.5 g of gelatin and 100 mL of ultrapure water were

Table 4. Troubleshooting guide for polyethylene glycol (PEG) embedding

Problem	Possible cause or solution
Block has a rough or cracked exterior	• The PEG was left in the oven too long during infiltration • The PEG cooled too fast after embedding • There was too much PEG 4000 in the mixture
Sections crack or fragment	The block lost too much moisture during storage
The block is too soft to section	• The block gained too much moisture during storage • There is too much PEG 1450 in the embedding mixture
Sections curl	The block is too dry − apply a small amount of H_2O or 50% EtOH to the block face before sectioning
Sections do not adhere to the slide	Allow the section to fully dry on the slide
PEG still present on the slide after staining	Allow the slide to remain in 1% Triton X-100 for longer before dehydration

heated at 30°C and stirred slowly for approximately 20 min until the gelatin had dissolved. Then, 2 g of phenol crystals and 15 mL of glycerin were added to produce the raw adhesive. The raw adhesive was filtered and allowed to return to room temperature. A working solution was prepared using one part raw adhesive to 140 parts 3% formalin. If pieces of undissolved gelatin remained in the working adhesive after it had cooled, it was filtered again. The working adhesive could be stored at room temperature for at least 3 months.

    Several drops of the working adhesive were applied to a clean microscope slide as a flotant for the PEG sections, which were placed on the slide individually or in groups of two or three. Sections were picked up with forceps and manually transferred to the adhesive solution on the slide. The slide was then placed on a warming tray at 35°C. The mounting solution allowed the sections to expand and relieved the compression caused by sectioning. The slide was maintained on the warming tray for c.5 min or until the adhesive dried down and the sections had adhered to the glass (see Table 4). If the sections showed signs of compression, a few drops of ultrapure water were added on top of them; this did not affect the adherence of the sections to the slide.

    Once the liquid had dried, the slide was removed from the warming tray and placed in a Coplin jar containing 1% Triton X-100 to remove the PEG (Smithson *et al.*, 1983). Mild intermittent agitation (four or five times) was used for 2 min (see Table 4). After the slide had been removed from the Triton X-100, the excess solution was drained by holding the slide at an angle and touching one end to a paper towel. Staining could begin immediately.

    Because of the solubility of PEG in water, we were unable to mount ribbons of serial sections. As soon as a ribbon was placed on a slide, the PEG would dissolve and the ribbon would come apart. This allowed the sections to float away from their original locations. The sequential order of the sections could not be preserved unless they were placed on the slide one or two at a time. This method was time consuming and unnecessary for our work, and therefore it was not pursued further. The break up of the sections on contact with water also prevented the embedding of flowers with separate parts. Once sectioned, these parts were held together only by the PEG and came apart when the sections were floated on water.

Staining

We obtained the best staining with Safranin and Fast Green (Berlyn & Miksche, 1976). After having been rinsed in 1% Triton X-100, the slides were placed in 1% Safranin in 50% EtOH for 30 min to overnight. The Safranin was washed off with a few seconds' wash in 50% EtOH, and the sections were further dehydrated by several seconds in 70% followed by 95% EtOH. The sections remained in each EtOH solution for less than 1 min. The slides were counterstained with 0.5% Fast Green in 95% EtOH for 15–30 s up to a maximum of 45 s. Overstaining in Fast Green resulted in the Safranin being completely removed.

Coverslipping

After staining, dehydration of the sections was necessary before application of an anhydrous mounting medium. The slides were dehydrated in Coplin staining dishes following standard procedures for paraffin sections: two changes of 95% EtOH, one change of 100% EtOH, one change of 1:1 Clearite:100% EtOH, and two or three changes of 100% Clearite. Clearite (Richard-Allan Scientific, Kalamazoo, Michigan, USA) is a less toxic replacement for xylene.

Once the slides were in 100% Clearite, they could be stored for several days until coverslips were added. An anhydrous mounting medium was used (Richard-Allan Scientific Mounting Medium) along with a coverslip. Once the mounting medium had dried, the slides could be stored indefinitely.

Controls

Control sections of *Musa velutina* and *Heliconia psittacorum* were embedded and cut using standard paraffin techniques for comparison with our the PEG-embedded material (Berlyn & Miksche, 1976). A full description of the paraffin methods can be found in Kirchoff (1992).

Comparison of PEG- and paraffin-embedded ovaries

The quality of sections of PEG-embedded ovaries and flowers was the same as or better than that of paraffin-embedded material (Figure). The PEG sections had better tissue differentiation and showed better preservation of crystals, which were more often found *in situ*. Alcohol-soluble, non-polar components should also have been better preserved, but we did not check this in our sections (Jones *et al.*, 1959).

Discussion

Compared with the paraffin method, the PEG protocol was more efficient. Using PEG, material can be embedded directly from water, therefore the dehydration steps needed for paraffin embedding can be omitted. This prevents tannins from hardening as well as keeping intact the cell walls, which are often destroyed during the paraffin method due to the tannins being ripped out of the tissue during sectioning. PEG embedding also decreased the time in the oven. It reduced the embedding time to 3–6 h as opposed to the approximately 2 days needed for paraffin embedding. PEG embedding does not require the paraffin-miscible chemicals, tertiary-butyl alcohol in our case, necessary for paraffin embedding. These chemicals can destroy components of the tissues and cause shrinkage (Riopel & Spurr, 1962). We were also able to achieve lower compression with PEG infiltration.

Both paraffin and PEG blocks can be stored at room temperature; however, the latter will easily dry out or absorb water from the air, depending on the humidity, and can therefore be stored for only short periods. We have successfully sectioned paraffin-embedded material older than 10 years, whereas PEG-embedded material is usable for only about 5 days.
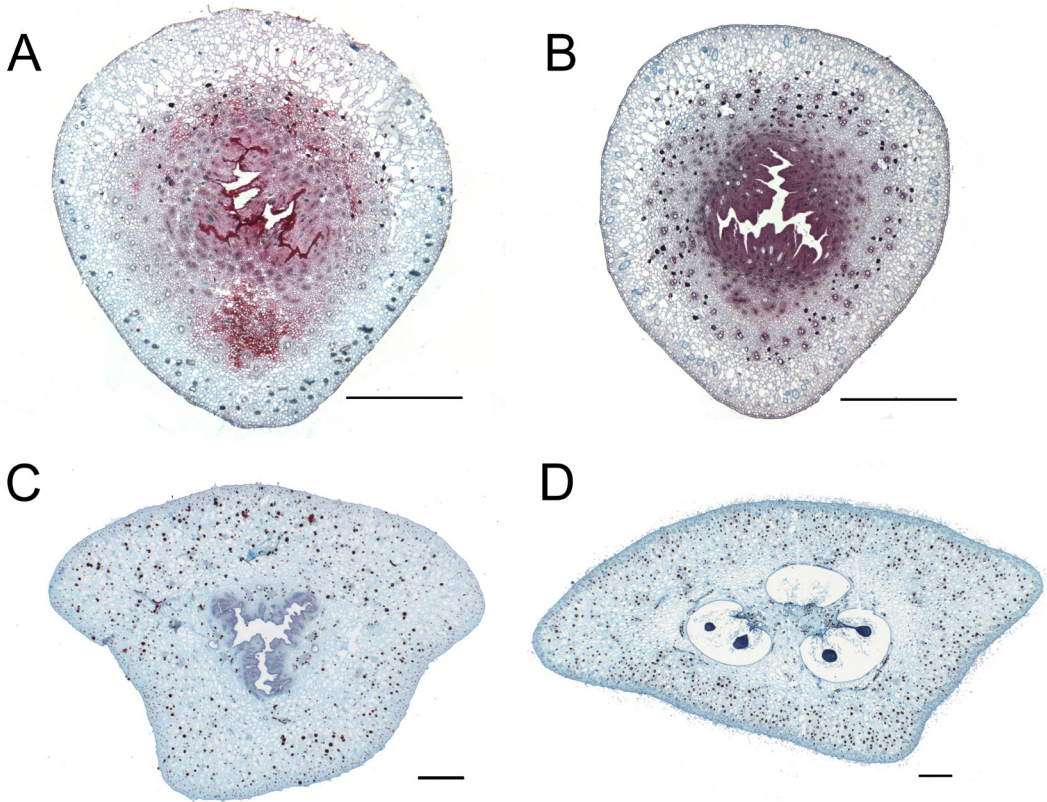
Figure. Cross-sections of ovaries of *Heliconia psittacorum* (A,B) and *Musa velutina* (C,D). Polyethylene glycol–embedded ovaries are on the left (A,C). Paraffin-embedded ovaries are on the right (B,D). Scale bars: 1 mm. Images obtained by Anastasia Romanov (A and C) and Bruce Kirchoff (B and D).

Excess paraffin can also be remelted and reused, but due to its hydrophilic nature, PEG cannot. Only the amount immediately required should be used during embedding. The PEG method can be used for specimens that need to be processed quickly and efficiently in small batches (Holubowicz & Goffinet, 1988; Holtham & Slepecky, 1995).

In the process of developing our protocol we tested several other infiltrating, embedding, mounting and staining techniques. In addition to our recommended procedure we tried four different infiltration protocols, three of which were modified after Smithson *et al.* (1983). However, none of these procedures provided satisfactory infiltration. The procedures that used higher proportions of PEG 4000 to 1450 (1/3 or 1/4) produced blocks that were too dry, whereas those produced with only PEG 1450 absorbed too much water. None of the procedures produced blocks that could be sectioned. With the recommended procedure, the blocks could be sectioned for up to 5 days regardless of humidity level or time of year.

Sriramachari & Ramalingaswami (1952) found that embedding in PEG 1000 during winter produced the best blocks, but that it was necessary to add small amounts of PEG 4000 during the summer. Although we cannot rule out the necessity of altering the embedding mix depending on time of year, we found that our procedure produced consistent results over the range of temperatures and humidities found in a typical air-conditioned laboratory in a building of at least 40 years of age in the southeastern USA.

With the proper modifications, PEG can even be used to section very hard materials, which are otherwise difficult to section. Barbosa *et al.* (2010) used PEG 1500 to embed and section tissue from woody lianas and arboreal palms by pretreating the material with ethylenediamine to soften it (Carlquist, 1982), using slow infiltration, and by stabilising the sections with polystyrene foam solution (polystyrene foam dissolved in butyl acetate) as they were cut. Infiltration was carried out through a graded series of PEG 1500, beginning with 10% PEG 1500 and ending with 100% PEG 1500, over the course of 10 days. The sections were affixed to slides with glycerinate egg albumin (Mayer, 1883). This method was later used to investigate liana anatomy and evolution (Chery *et al.*, 2019, 2020).

In addition to storing the embedded material on the bench top, we tested several other storage locations. During storage in a refrigerator at 4°C the PEG would develop a moist patina and become unsuitable for sectioning. This contradicts the findings of Kauf & Bilan (1977), who chilled the PEG blocks at 0−2°C until just before sectioning. Storage in a desiccator with Drierite (W. A. Hammond Drierite Co. Ltd, Xenia, Ohio, USA) resulted in the PEG becoming dry, powdery and cracked. Ribbons were unattainable because the sections were too friable to form them. In a humidity chamber maintained at 60−70% humidity, the PEG became damp and unsectionable due to the hydration of the block; this was similar to what happened at 4°C.

Both Sriramachari & Ramalingaswami (1952) and van Horne & Zopf (1951) suggest storing PEG-embedded material inside glass jars to prevent all contact between the PEG and atmospheric moisture. Sriramachari & Ramalingaswami (1952) recommend wrapping the blocks in cellophane before storage, a technique that was also used by Padilla García (2018, 2019). We tried this method by storing the blocks in the refrigerator, in the freezer, and on the bench top. With cold storage in either the refrigerator or freezer, we found that the blocks became too soft to section after 2−3 days, despite being stored in plastic wrap inside a jar. The blocks that were stored in the jar on the bench top became too dry to section after 5 days.

Zugibe *et al.* (1958) found that the best ribbons were cut when the laboratory humidity was kept below 75% and the temperature below 27°C. Our research was conducted in the central piedmont of North Carolina, USA, mainly during the spring and summer months when humidity and temperature levels varied from day to day, even in an air-conditioned laboratory. Although we were unable to obtain accurate measurements of either temperature or humidity, we were able to obtain good sections throughout these seasons.

We initially had difficulty getting the PEG sections to adhere to slides and tried two other adhesive formulas before we eventually succeeded with Bissing's modified Haupt's adhesive. Both alternative formulas produced very thick layers of adhesive on the slide and resulted in unacceptable levels of background staining. The first recipe was a modification of Haupt's adhesive with a much greater amount of gelatin: 3.75 g of gelatin and 27.5 mL of ultrapure water were heated at 100°C and stirred slowly for approximately 20 min until the gelatin dissolved. One drop of phenol and 15 mL of glycerol were then added to produce the stock adhesive. A working solution was prepared by adding 1 mL of stock adhesive to 15 mL of 3% formalin. This adhesive was wiped on a slide that was placed on a warming tray, and then a drop of water was added. PEG sections were then floated on the water, over the adhesive. As the water evaporated, the sections expanded and adhered to the adhesive. The second adhesive was first suggested by Giovacchini (1958): 7.5 g of gelatin was dissolved in 22.5 mL of water with the addition of 5 mL of glycerol and one drop of phenol. In contrast to these two methods, Bissing's modified Haupt's adhesive, which uses only 1.5 g of gelatin, held the PEG sections in place even through mild agitation (Bissing, 1974). There was little or no background staining.

Although we were unable to successfully mount ribbons, we were successful in mounting sections individually or in small groups by using forceps to carefully place them on a slide (Wade, 1952; Firminger, 1964). Riopel & Spurr (1962) suggest a method of mounting serial sections based on the use of the adhesive suggested by Giovacchini (1958). We did not pursue this method because of the strong background staining, which we were unable to remove. Riopel & Spurr (1962) suggest that it can be removed because gelatin has less affinity for the stain than for the tissues, but they do not specify which stain they used. We did not experiment extensively with the flotation method for mounting sections advocated by Firminger (1950, 1964) and Zugibe *et al.* (1958). We agree with Wade (1952) that this method is unlikely to produce satisfactory results due to the solubility of PEG in water. The positioning of the sections on slides is the only tedious and time-consuming part of the sectioning procedure. Even with this limitation, the overall time spent to obtain good sections with PEG remains shorter than the time needed with the paraffin method. If a new mounting method that does not involve water flotation can be found, it should be possible to mount serial sections because we had no difficulty cutting them.

Besides Fast Green and Safranin (Berlyn & Miksche, 1976), we also conducted numerous tests of various concentrations of Alcian Green with Safranin (Wells, 1973; Joel, 1983). We had hoped to find a mixture of these stains that would produce satisfactory results in combination, as suggested by Joel (1983). Although we occasionally achieved good results, we were unable to find a concentration of Alcian Green that would consistently produce well-stained sections. Even overnight staining did not produce sufficiently dark sections.

Stained slides were dehydrated in a standard dehydration sequence, but we also tested a second dehydration method. The stained slides were immersed in 100% acidified

2,2-dimethoxypropane for 30–45 s in a Coplin staining dish before being transferred to 100% Clearite (Postek & Tucker, 1976). This method was faster but removed some of the Fast Green and Safranin from the sections. It also tended to produce clumping of the stain and was therefore not pursued further. Dehydration with EtOH was easy to carry out and produced good results.

Conclusions

Using PEG, we were able to produce sections of the same quality as with the paraffin method, while reducing the processing time from 2 days to 3–6 h. We also achieved better *in situ* preservation of crystals and tannins. Even though we were unable to mount serial sections, this may be possible in the future if another mounting method can be devised. The PEG method is a beginner-friendly procedure that does not require extensive knowledge of paraffin-embedding techniques. PEG is an efficient and effective embedding medium for larger as well as smaller plant tissues. It is easy and convenient to perform when quick and simple embedding is needed.

Acknowledgements

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