15 Microwave Paraffin Techniques for Botanical Tissues

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Introduction

The popularity of *in situ* hybridization of nucleic acids and immunolocalization of proteins has caused a resurgence of interest in paraffin microtechnique by the plant biology community. However, the amount of time required for proper anatomical preservation of plant tissues results in degradation of nucleic acids and proteins in the sample (Jackson 1991). There are protocols that may be used to reduce degradation, but they often result in poor anatomical preservation and target (nucleic acid, protein) conservation (Jackson 1991; Kouchi and Hata 1993; Ruzin 1999).

The authors became interested in developing a protocol for microwave paraffin embedding of plant tissue after reviewing the benefits that microwave ovens have brought to the field of electron microscopy (Kok and Boon 1989; Login and Dvorak 1994; Giberson *et al.* 1997). The authors have developed a protocol that in five hours yields embedded plant tissue in paraffin for *in situ* hybridization, immunolocalization, and standard anatomical observation (Schichnes, Nemson and Ruzin 1999). With this technique, the quality of tissue preservation for *in situ* hybridization and immunolocalization is superior to traditional procedures, which usually require 7 days to complete. The tissue preservation for anatomical study is equivalent to the traditional protocol (Johansen 1940; Berlyn and Miksche 1976; Ruzin 1999), which requires a minimum of 9 days to complete. In addition, we have developed a microwave protocol to mount paraffin ribbons to gelatin coated slides (Haupt 1930; Sass 1958) and Fisher Probe-on Plus® slides (Fisher Scientific, Pittsburgh, PA). The microwave method requires 30 min, as opposed to the standard protocol which requires a minimum of 6 hr at 42°C. Finally, we have adapted a classic plant microtechnique staining protocol, Johansen's Safranin O and Fast Green FCF protocol (Johansen 1940) for the microwave oven. This adaptation yields staining equivalent to the traditional procedure in 40 min, rather than 2 days.

The authors protocol was originally developed using *Zea mays* shoot apices. This tissue is difficult to embed in paraffin due to variance in tissue densities. Additionally, the developing leaf primordia often trap air which impedes the infiltration of solutions (Freeling and Lane 1994). The authors have successfully applied the microwave protocol to many other plant tissues, ranging from grasses to gymnosperms, and even *Quercus suber* (cork oak), with some modifications for particularly delicate plant tissues, such as *Arabidopsis thaliana* and friable callus. The benefits of this microwave protocol have been threefold for our laboratory, the Biological Imaging Facility, a core facility for the UC Berkeley campus.

The authors have achieved an increased quality of tissue for *in situ* hybridization and immunolocalization studies, and the ability to process tissue much more rapidly.

The authors are able to embed delicate tissue in paraffin that was previously only usable if embedded in resin, saving an enormous expense in time and chemical cost.

The combination of microwave fixation, paraffin embedding, ribbon mounting, and staining protocols have proved an invaluable teaching tool, allowing us to condense

our plant microtechnique class from one semester into a one-week workshop, and cover more material.

Materials and Methods

Materials

1) Pelco 3440 MAX laboratory microwave oven with variable wattage control (Ted Pella Inc., Redding, CA)
2) PolyTemp Polysciences load cooling waterbath (Polysciences Inc., Warrington, PA)
3) Static water load: 400 ml Tri-corner beaker (Fisher Scientific, Pittsburgh, PA) #2-593-50D with 400 ml water
4) Glass scintillation vial, 15 ml size (Fisher Scientific, Pittsburgh, PA) #3-338-E
5) Sample water bath, plastic, measuring 8.5×12×5 cm
6) Fixative FAA - from Ruzin (1999), 50 ml 100% ethanol, 5 ml acetic acid, 10 ml 37% formalin, 35 ml water
7) 10×PBS - Phosphate Buffered Saline, from Ruzin (1999), 1.3 M NaCl, 0.07 M Na_2HPO_4, 0.03 M NaH_2PO_4, pH to 7.0 using NaOH
8) Fixative PFA - from Ruzin (1999), 100 ml 1×PBS, pH to 11 with NaOH, heat to 60°C, add 4 g paraformaldehyde, stir until solution is clear, pH to 7.5, cool on ice
9) 10 mM Sørensen's buffer, pH 7.2 (Sørensen 1909), 5.6 ml 0.2 M NaH_2PO_4, 14.4 ml 0.2 M Na_2HPO_4, 380 ml deionized H_2O
10) Paraplast Extra paraffin (Fisher Scientific, Pittsburgh, PA) #12-646-113
11) Modified Haupt's gelatin (Ruzin 1999)
12) Glass slides
13) Probe-on Plus® slides (Fisher Scientific, Pittsburgh, PA)
14) Johansen's Safranin O and Fast Green FCF staining procedure (Johansen 1940)

Process

1) Preparation of tissue. Dissect tissue into glass scintillation vial with 10 ml of fixative at 4°C and keep on ice until ready to begin MW protocol. Use PFA as a fixative for immunolocalizations, use SØrensen's buffer with no chemical fixative for delicate tissues, and FAA for all other tissues.
2) Determining sample location. Time: ~ 2 min. Using a neon bulb array, determine sample and water load placements. Locations are marked on a sheet of paper taped to the oven floor.
3) MW fixation. Time: ~ 45 min.
    i) Place glass vials in plastic water bath. Fill water bath until water level is equal to fixative level.
    ii) Set the microwave variable wattage to 650 watts for most samples, 450 watts for delicate tissue. Place temperature sensor in a vial with the samples.
    iii) MW for 15 min with temperature limit set to 37°C. Replace with fresh fixative and cool on ice to 12°C. Repeat twice.
    iv) Change the static water load after each cycle.
4) Alcohol dehydration. Time: ~ 10 min.

i) MW sample at 67°C for 1 min, 15 s each step in the ethanol dehydration series. 1×50%, 1×70%, 1×70% with Safranin O (0.1%), 2×100%.
ii) Change static water load.
iii) MW sample at 77°C for 1 min, 30 s each step in the isopropanol dehydration series. 1×50% EtOH:50% Isopropanol, 1×100% Isopropanol.
iv) Change static water load.

5) Infiltration. Time: ~ 3 hrs.
i) MW sample in 50% Isopropanol:50% molten paraffin for 10 min at 77°C.
ii) MW sample in 100% molten paraffin for 10 min at 67°C.
iii) MW sample in 100% molten paraffin for 30 min at 67°C. Repeat 4 times.
iv) Change water load between each cycle.
v) Embed sample in aluminum or paper boats and cool to room temperature.

6) Mounting Paraffin Ribbons. Time: 30 min.
i) Do not put slides directly on MW floor. Place them on cardboard or stack in a glass staining dish placed on its side.
ii) Place temperature sensor in a 100 μl drop of water on an adjacent slide. Use a hydrophobic slide (Fisher ProbeOn) to retain the water drop.
iii) MW slides for 30 min at 43°C.

7) Staining Sections. Time: ~ 1.5 hrs.
i) Deparaffinize slides in xylene 2×10 min. Hydrate sections to 70% EtOH.
ii) Place slides in staining dish and cover with Johansen's Safranin O solution. Place staining dish in a water bath. Loosely cover staining dish with plastic wrap to prevent spattering. Insert temperature probe through the plastic wrap into the staining dish. MW at 60°C for 40 min.
iii) Dehydrate slides for 5 s in 95% EtOH with 0.5% picric acid.
iv) Wash slides for 5 s in 95% EtOH with ammonium hyroxide (4 drops per 100 ml EtOH).
v) Dehydrate slides for 5 s in 100% EtOH.
vi) Counterstain for 10–15 s in Fast Green FCF staining solution.
vii) Wash for 5 s in Used Fast Green Clearing Solution. Wash 5 s in Fast Green Clearing Solution.
viii) Clear in xylene 2× for 10 min, keep slides in xylene until mounting coverslips.

Results

Typical examples of microwave and traditionally processed *Zea mays* shoot apices are shown for comparison in Fig. 1. Microwave fixation and embedding required five hours, while the traditional protocol took 9 days. The overall quality of the tissue prepared using the microwave is comparable to the tissue prepared through the traditional protocol. The overall morphology of the sample is preserved, as well as the internal anatomy. There are no indicators of poor fixation and infiltration, such as holes, rips or tears in the samples. However, when looking at the highly magnified region of the meristem in Figs. 1B and 1D, the difference in quality becomes apparent. The meristem is a delicate, densely cytoplasmic structure that is easily damaged, and therefore a good indicator of the quality of tissue preservation. The two outer layers of the microwave-prepared specimen (Fig. 1B) are plump and intact.

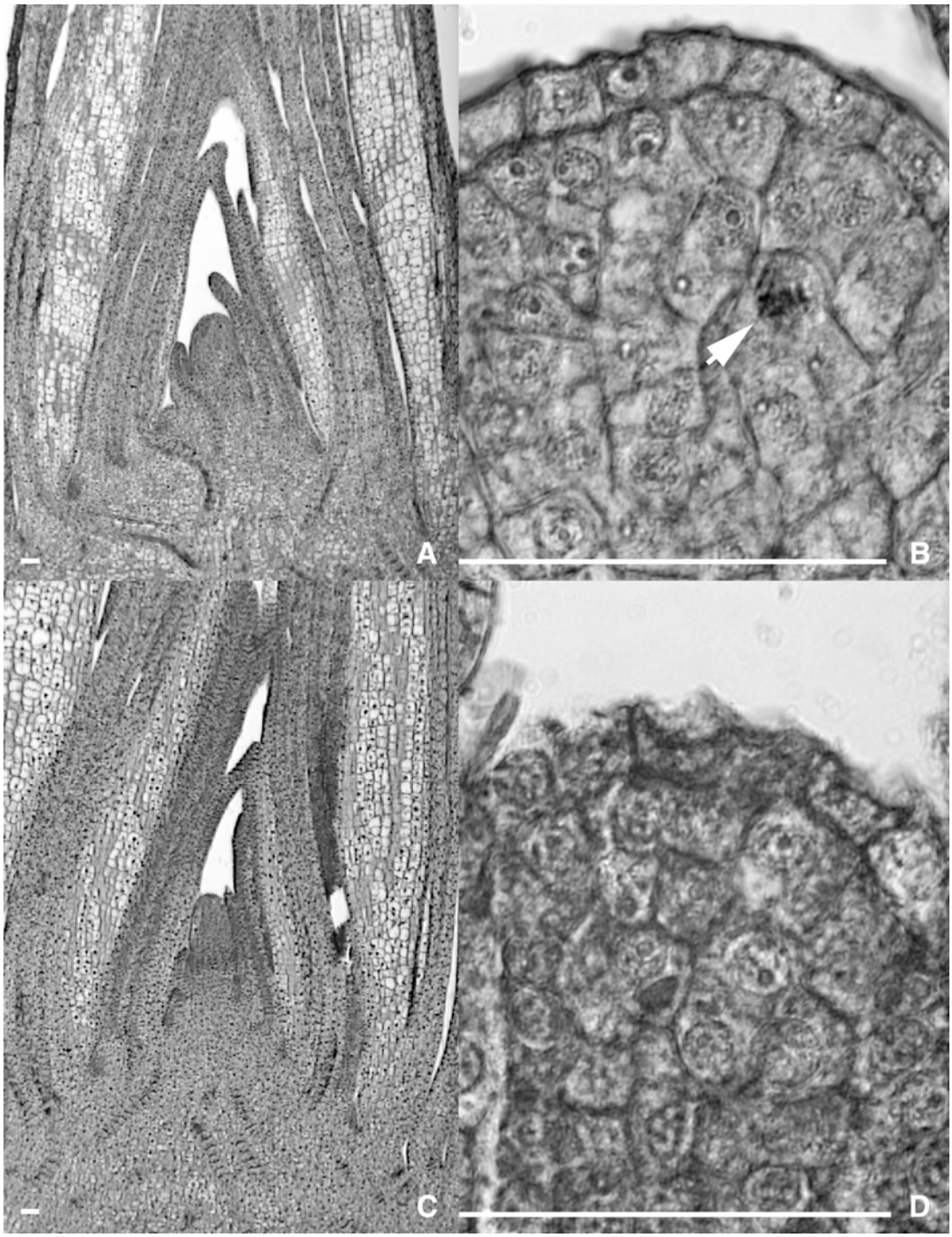

Figure 1. Examples of *Zea mays* shoot apical meristem and surrounding leaf primordia in longitudinal section. The sample shown in 1A was fixed, dehydrated, and embedded using our microwave protocol. A detailed view of the same sample is shown in 1B. The arrowhead points to a mitotic figure. The sample shown in 1C was processed using traditional methods. A detailed view of the same sample is shown in 1D. All samples were stained using Johansen's Safranin O and Fast Green FCF protocol. Sample A was stained using the microwave protocol outlined in this chapter, sample B was stained conventionally. Bar equals 50 μm.

The cytoplasm has not shrunk away from the cell wall, and mitotic figures (arrowhead) are found frequently. The sample processed traditionally (Fig. 1D) is not

as well preserved. The two outermost layers of cells are shrunken and damaged. The nuclei of these cells are large and not well shaped, and it is difficult to find any

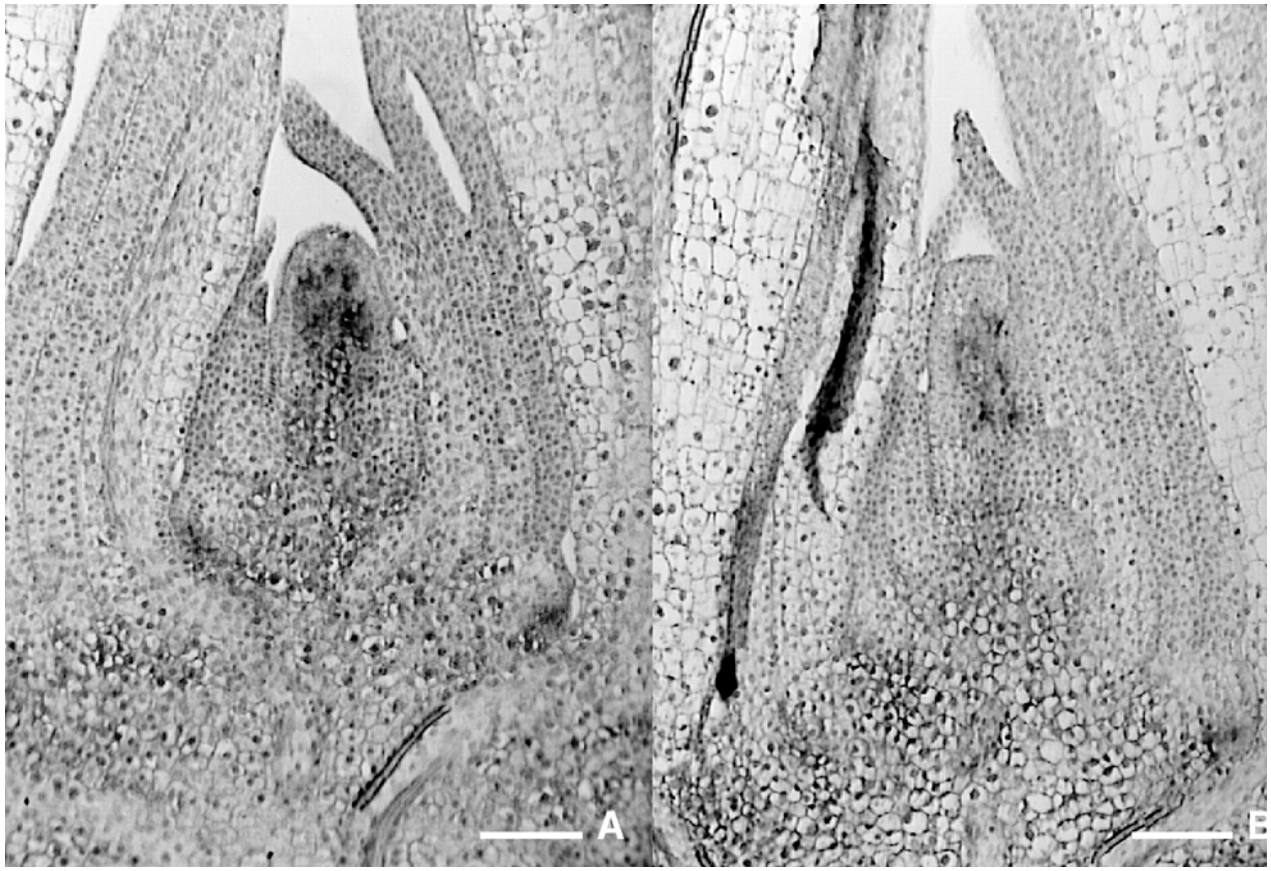

Figure 2. Examples of *Zea mays* shoot apical meristem and surrounding leaf primordia in longitudinal section. *In situ* localization experiments were performed using a DIG-labeled antisense probe which hybridized to *knotted* (Smith and Hake, 1992). Sample shown in 2A was fixed, dehydrated, and embedded using our microwave protocol. Sample B was processed for *in situ* analysis using traditional methods (Jackson, 1991). Bar equals 100 μm.

mitotic figures in this general area.

Figure 2 compares *Zea mays* shoot apices processed for *in situ* hybridization studies using the microwave (Fig. 2A) and traditional (Fig. 2B) protocols. The microwave protocol required 5 hr while the traditional protocol required 7 days. The traditional protocol for *in situ* studies is shorter than the protocol for standard anatomical preservation to minimize the time in which mRNA degrades. (Jackson 1991). Due to the short exposure to fixative, the overall anatomical preservation of the tissue is poor. Notice that the shoot apical meristem in Fig. 2B is sunken and not dome-shaped, as it is in its native state. The outer cells layers of the meristem (the l1 and l2 layers) are completely crushed. There is a large tear in the tissue indicative of poor infiltration and embedding. This example is typical of the quality of samples that are embedded according to the traditional protocol. In contrast, the microwave prepared sample (Fig. 2A) is of excellent quality. The apical dome is well preserved and there are no tears in the tissue. The staining is darker, indicating better preservation of the mRNA, and the pattern is more tightly localized than in traditional preparations. The authors are not able to include color figures in this

publication, but refer to Schichnes, Nemson and Ruzin (1999) for a detailed analysis of *in situ* localization results using microwave techniques.

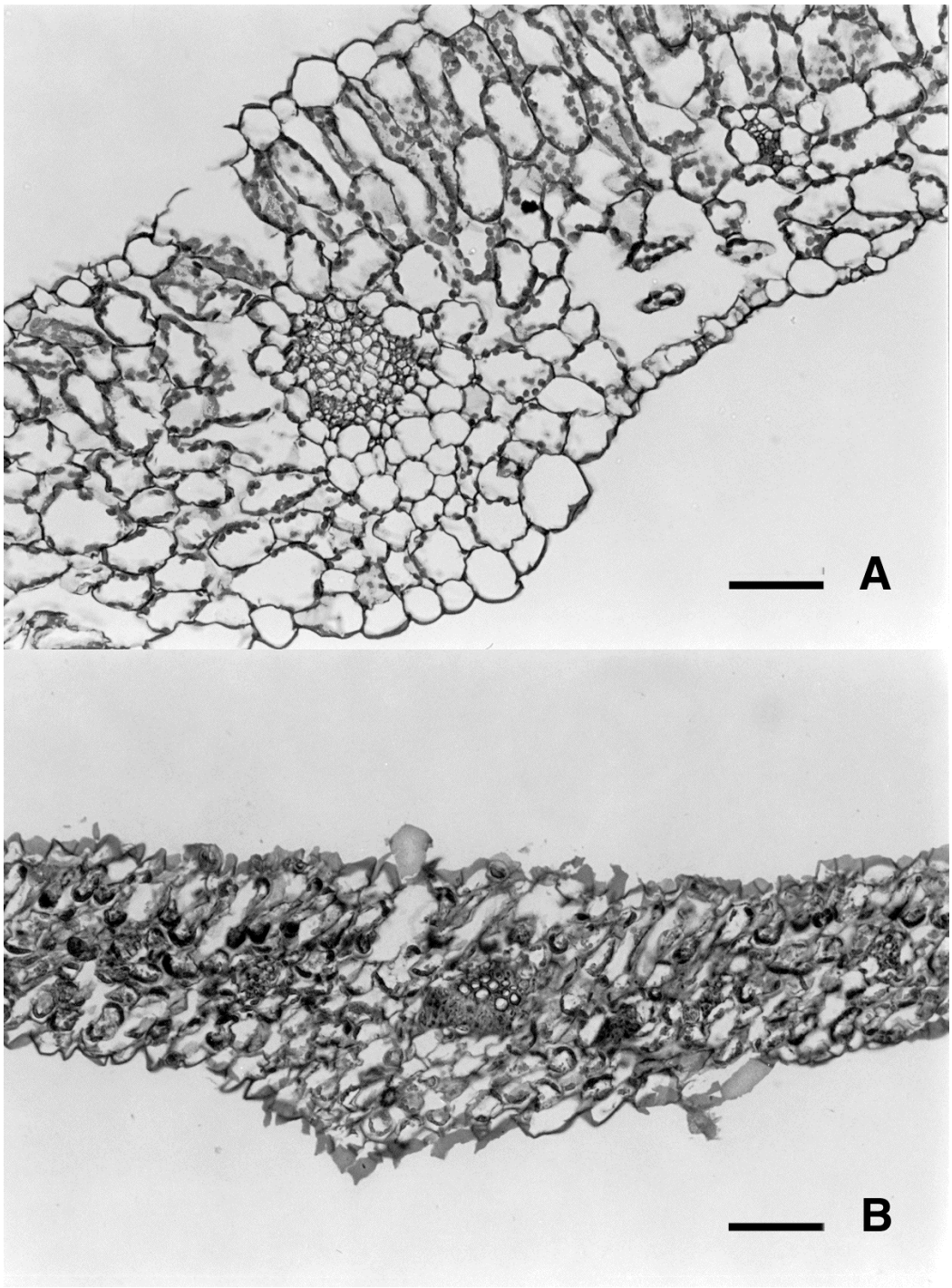

Figure 3. *Arabidopsis thaliana* rosette leaves shown in transverse section. The sample in 3A has been processed according to our microwave protocol, while 3B has been processed according to traditional protocols. The samples were stained using Johansen's Safranin O and Fast Green FCF microwave modified protocol. Bar equals 100 μm.

The final figure, Fig. 3, details results from a variation of our microwave protocol to accommodate delicate tissues such as *Arabidopsis thaliana* and friable callus.

Traditionally these tissues are fixed with a high concentration of glutaraldehyde and embedded in resin or methacrylate for sectioning. Paraffin embedding nearly always yields poor results with these tissue types (Ruzin, 1999). To accommodate delicate tissue we made two major changes to the microwave technique. The authors used 10mM Sørensen's buffer with no chemical fixative and microwaves to stabilize these delicate samples, and we adjusted the power of the oven to 450 watts (normally at 650 watts). Figure 3A shows an *Arabidopsis* leaf in transverse section after having been processed using the alternative microwave method. The leaf internal structure is well preserved, the vascular bundles are well defined, and the phloem (delicate and easily crushed) is visible. The epidermal cells are expanded and rounded, as in the native state. Stomatal complexes including air spaces in the mesophyll are open and not crushed. Finally, the chloroplasts are present and intact.

Although the leaf in Fig. 3B was the same size and shape as the leaf in Fig. 3A before fixation and paraffin processing, is has shrunk during the procedure. The epidermal layer is crushed and not distinguishable from the other cell layers. It is not possible to distinguish stomatal complexes or mesophyll air spaces, and difficult to find the vascular bundles. Of the large midvein that is discernable, only the xylem elements, which are secondarily supported by lignin and therefore some of the strongest parts of the leaf, are visible.

Discussion

The microwave samples are superior to their traditional counterparts when comparing corresponding indicator regions in the sample, such as the outer cell layers of the meristem. The samples prepared for *in situ* hybridization reflect this as well. Not only are microwave-processed *in situ* samples better preserved, but they show a stronger and more tightly localized signal pattern than their traditionally prepared counterparts. Microwave and traditional mounting and staining are comparable, with less time required for the microwave procedure.

Another important benefit of the microwave technology is the ability to embed delicate botanical tissues, such as *Arabidopsis* and friable callus, in paraffin. Paraffin microtechnique is less expensive, less technically demanding, and generates less toxic waste than resin embedding. Before the variable wattage option on the microwave, it was not possible to adequately prepare these tissues in paraffin, either traditionally or in the microwave. The authors have placed the tissue in an isotonic solution and used microwave energy to stabilize the tissue, which has proved effective for these delicate botanical tissues.

Quick processing times do not mean poor sample quality. Nothing was sacrificed by using the microwave to process botanical tissues. The authors have gained increased tissue quality and the ability to process delicate samples that were previously only available through the time and expense of resin embedding.

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