EXPERIENCES WITH THE INCLUSION OF BOTANICAL MATERIAL IN PEG. PART II

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INTRODUCTION

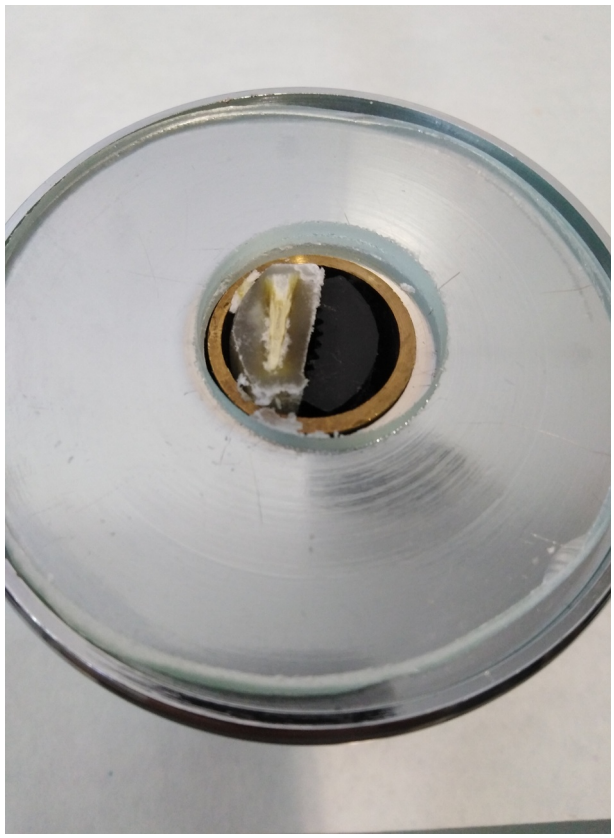
In a previous article[1] I explained a possible way to adapt the technique of inclusion in PEG to a domestic field to obtain histological sections of plant material for its study under optical microscopy. Specifically, in said article, the followed process was described to obtain inclusion blocks from which to obtain transverse sections of stems and roots (cylindrical material in general) using a simple hand microtome. In the present article I intend to expand the information, telling my experience in applying the method to obtain longitudinal sections of stems and transverse sections of leaves. Also, I will comment on some improvement of the general process and some observations made.

MOLDS FOR LONGITUDINAL CUTS

The process followed to make inclusion blocks to obtain this type of sections is practically the same as the one explained in the article reviewed except for the mold used. The main problem was to obtain the correct orientation of the pieces of material for this type of cuts, so once the block was placed in the hand microtome, the longitudinal sections could be obtained. Obviously, PVC cylinders were not appropriate for size or waste of much of the mold. The first solution was to make small aluminum foil boxes. Later, I used some plugs (made of plastic material) from containers and bottles. With both types of molds I found some difficulties such as sealing, the large amount of PEG used, the unmolding of the plugs and the carving of the block to introduce it into the microtome hole. Finally, I found a blister of certain medications with a size that fitted very well to the microtome hole and that could contain several pieces of stems oriented so that placing the block in the microtome would be horizontal and thus be able to perform the intended longitudinal cuts. As shown in the figure, the preparation of the block is comfortable because it is done horizontally and several pieces can be included, orienting them in a convenient way. It is advisable to keep an eye on and control the solidification process in case it is necessary to correct the placement of the material (which is done with a needle). In addition, if the blister has a metal cover on the outside, a considerable improvement is obtained since the material may have some more time on the hot plate with the pure melted PEG, improving the infiltration and subsequent inclusion.

The unmolding of the block is very easy, obtaining a tablet with a curved area that adapts well to the outer edge of the microtome hole and another flatter surface that faces the clamp, offering a perfect hold. Before placing the block in the microtome it is advisable to separate with a knife some PEG from both sides of the block and even from the edge you are going to cut. The excess polymer previously cutted can be reused in another inclusion process. It is also possible to reuse the blister several times by returning it to its original shape with your hands.

Placement of the material inside the blister with the PEG completely melted on the heating plate (glass ceramic).



Placement of the block in the microtome, showing the advance of the cuts and how the orientation of the material for the longitudinal cut is achieved.

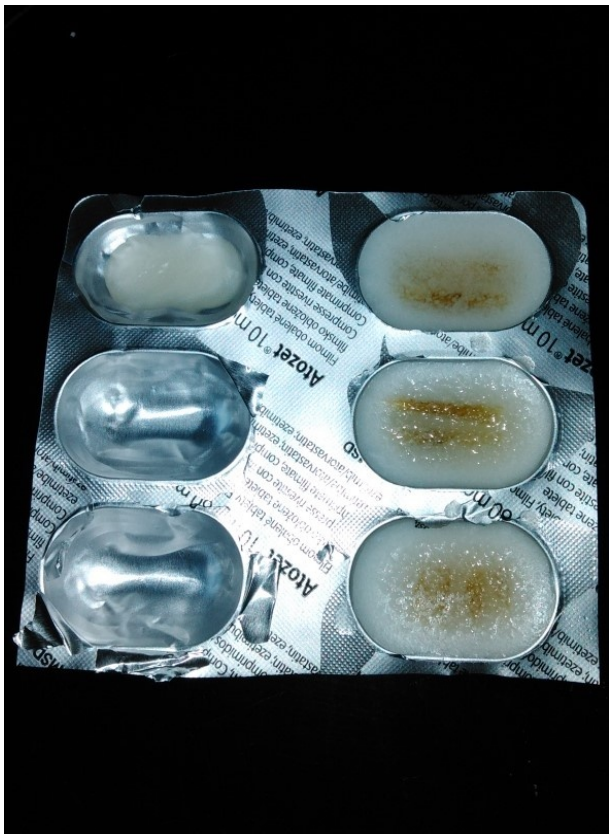
The most complete cuts are usually obtained at the beginning and end of each piece of stem, because if the medular area is reabsorbed, the section can be divided by the center in two parts. This is not a problem for the histological study given the cylindrical symmetry of the stem. It is also true that as you go deeper, each cut is different and can show different configuration of the vascular and tissue bundles, contrary to what happens in the cross section where all cuts follow the same pattern. This is another reason to try to make this type of cuts.

INCLUSION OF LEAVES

The blister was also shown as an ideal mold to fit pieces of leaves to obtain its transverse section, as shown in the previous figure. The leaves are impregnated in the same way, placed at the bottom of the blister and covered with PEG, taking care the material does not float. If this happens, it is necessary to take the material to the bottom with a needle while the polymer is a little bit molten. For this reason, it is necessary to be aware of solidification and the process need to be controlled. When the block it is going to be used, again a little PEG is removed from the sides of the block to fit well in the microtome.

PROCESS IMPROVEMENTS

The first is about the mold. After checking the suitability of the blisters as molds for the longitudinal cut, it occurred to me to place two pieces of stem inside one of them so that when using a block I could obtain two cuts in a single pass, where the number of effective cuts increase considerably.



Molds with two pieces of stems placed in each, with the PEG almost completely solidified.

Block mounted on the microtome showing two stems included simultaneously.

Secondly, when the blister has a metal cover, it can be placed directly on the heating plate, so it is possible to melt the PEG in the same mold (with the consequent polymer saving as it melts just the necessary amount) and the pieces of material can be maintained longer in pure PEG melted, therefore the impregnation improves notably. However, the use of the blister has an obvious drawback that is not other than the diameter of the stem included is determined by the depth of the blister. (If we use the PVC mold in a cylindrical shape, we can always look for a tube of the right diameter).

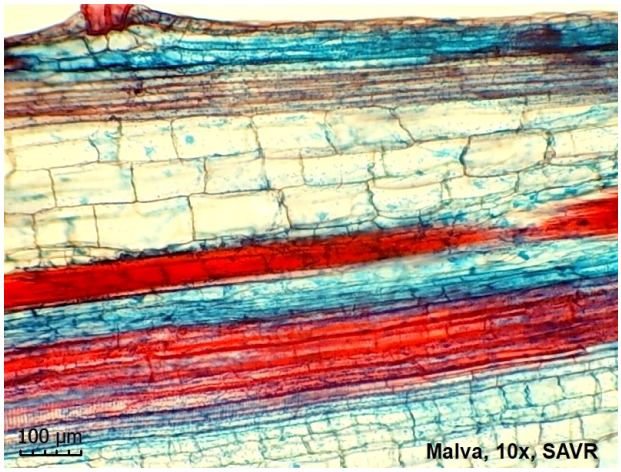
Another important fact is that the times I tried to add some glycerin in the impregnation; later, the blocks softened and presented some moisture, which made cutting difficult or even impossible. Actually, the ease of cutting is determined mainly by the hardness of the material itself and the fluid used for the fixation, since this reagent can harden the tissues too much. I find that the most comfortable, fastest and best result I have obtained is by fixing the material with Farmer's mixture (absolute ethyl alcohol and glacial acetic acid in 3:1 ratio) and not adding glycerin in any time of the process. Definitely the addition of glycerin is not decisive and can be dispensed with, without any inconvenience.

An important precaution is to control the temperature well. For PEG 1500, it must not exceed 50º C since there is a risk of cooking the stems. It is also important to control time. Although three hours give good results, it is best to do it in five hours, including about 30 final minutes with the pieces already in the blister with pure PEG melted before removing from the fire.

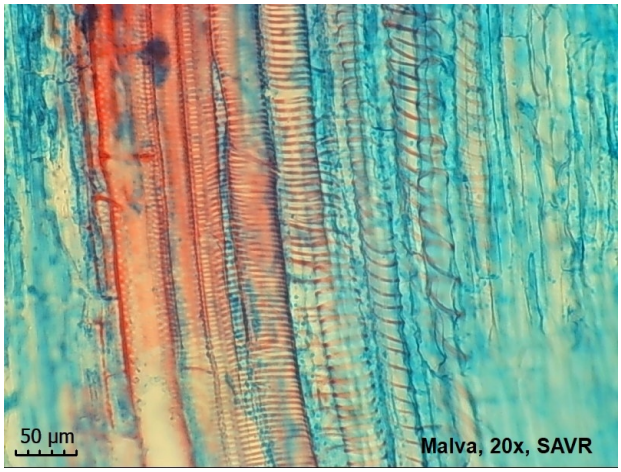
RESULTS

For the practice of the longitudinal cuts, *Malva sylvestri* stem was used, fixed in a Farmer or FAA mixture and included in PEG as explained. The cuts obtained by means of the hand microtome are put in a Petri dish with water to dissolve all the polymer and the stem or leaf re-hydrates, recovering its full size. Subsequently, I proceeded to perform different routine stains within the botanical microtechnique following the well-known authors Johansen, Sass, D'Ambrogio[2] and Roeser[3], especially combinations of SA (Safranin) with AN (Aniline Blue) since this dye is able to stain the callose, revealing certain elements of the Floema (cribosas plates). Other combinations were SA with VR (Fast Green) and AAFB (Blue Astra and Basic Fuchsin). Later they were mounted in resinous medium with Balsam of Canada or DPX or in aqueous medium in Karo to 75% or mixture type von Apathy (Glycerin, Karo, Gum Arabic, what I call GliKaGom), following the instructions on safe mounting media for amateurs of Walter Dioni[4]. Finally proceeded to observe the greater or lesser quality of the cut in terms of thickness, deterioration of the fabrics when making the cut and ease to make this.

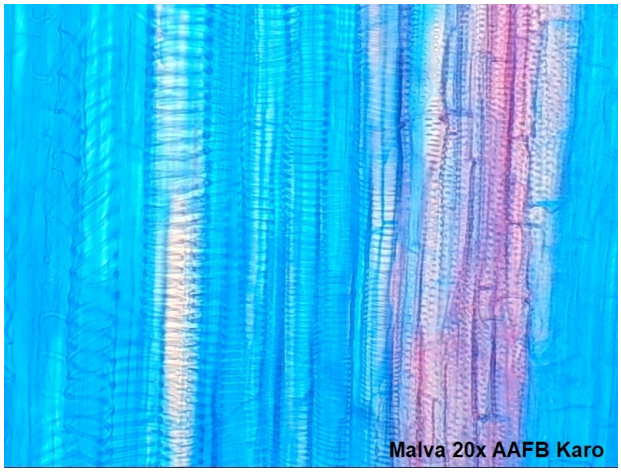
The photos were obtained through an Olympus CX31 microscope using 10 and 40 magnification planoacromatic objectives and a 5 Mpx digital microscope. The photos were registered and treated with the MiCam 2.4 program for exposure correction, insertion of legend and to give them the format and size appropriate to the text.



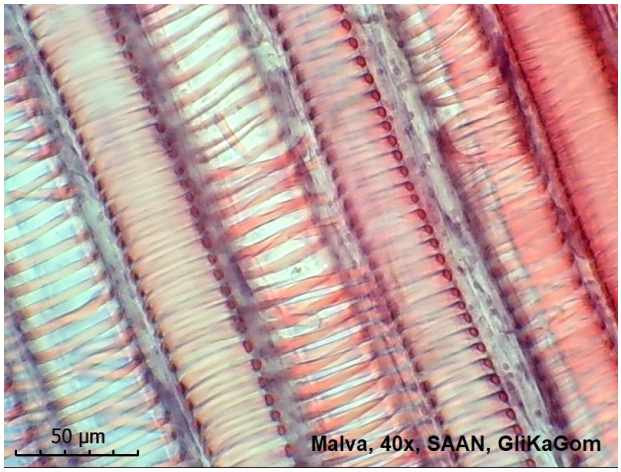
Safranin-Fast Green



Safranin-Fast Green



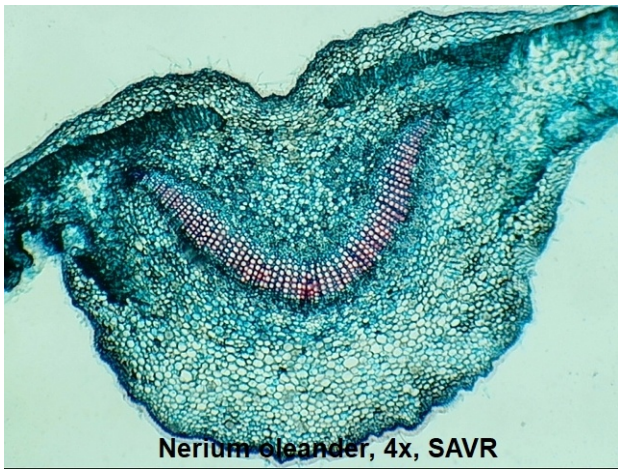
Astra Blue-Basic Fuchsin



Safranin-Aniline Blue

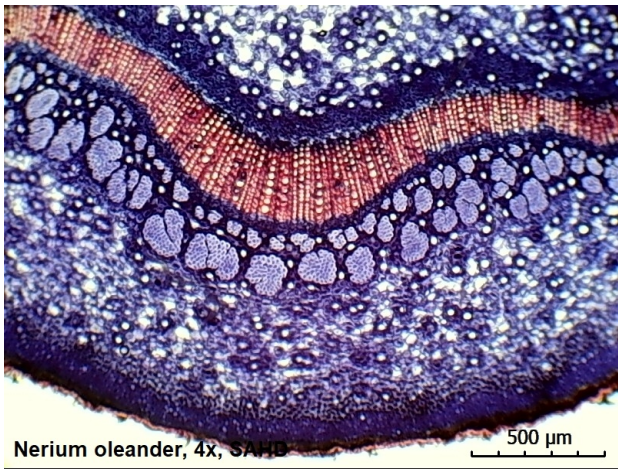
In the photomicrographs, different elements of the conductive vessels (tracheids, fibers, accompanying cells, etc.) and the different tissues that form the different layers (epidermis, marrow, etc.) are observed and are comparable with other photographs found in histology atlases. The thickness of the cuts is quite acceptable for our amateur purposes. These cuts were stained correctly as expected with each stain. Likewise no damage was seen in the sections and the cut was very easy to perform.

For the practice of leaf cutting, *Nerium oleander* was used, which was fixed in a mixture of Farmer or FAA, included as explained and after obtaining the cuts are stained and mounted following the same routine protocols as for the stems.

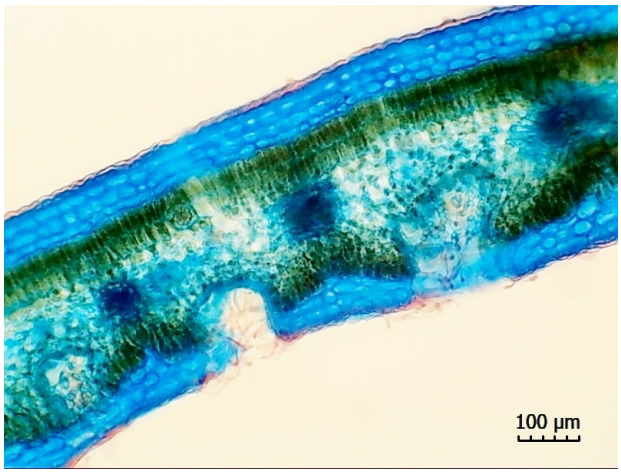


Safranin-Fast Green

Safranin-Fast Green



Safranin-Delafield`s Hematoxylin



Astra Blue-Basic Fuchsin

Again, the photographs show a fairly acceptable thickness and quality of the cuts, being able to observe the different types of tissues dyed as expected. The cutting of leaves does not add any new difficulty, however, the treatment of the cuts offers certain difficulties since these tend to roll up and are more complex to manipulate. However, as the photos show, the inclusion in PEG returns to give fabulous results in both thickness and quality to make sections of leaves within the amateur field.

REFERENCES

1.- Jaime Padilla. *Experience with the inclusion of botanical material in PEG*. MICSCAPE MAGAZINE, December 2018.

2.- Johansen, *Plant Microtechniques*; Sass, *Botanical Microtechnique*; Ana D`Ambrogio, *Manual de técnicas en histología vegetal.*

3.- Rebecca Luque et col, *Astra Blue and Basic Fuchsin double staining of plant materials*. *Biotechnic & Histochemistry, **73** (1998) 5.*

4.- Walter Dioni. *Safe microscopic techniques for amateurs. Part two, solidifying media.* MICSCAPE MAGAZINE, January 2003.

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