EXPERIENCES WITH THE INCLUSION OF BOTANICAL MATERIAL IN PEG

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INTRODUCTION

Within amateur microscopy, perhaps the field least practiced by hobbyists is to make histological preparations. It is logical for several reasons. On the one hand we find the particular difficulty of the histological process, alongside which it is necessary to make operations such as fixation of the material, inclusion, hydrations and dehydrations, microtomy, stains and mounting. On the other hand, there is the difficulty in obtaining, outside of professional, academic or research environments, the necessary laboratory material and the large number of reagents used. Complicated, but also fascinating and attractive trying to adapt this process to the domestic environment.

In the domestic field we can, however, get some fixing substances, alcohol and dyes, such as those used for food. We can also get by with imagination and ingenuity and make a lot of laboratory material from household utensils. But within these histological methods the microtomy is perhaps the toughest and complicated to perform at our amateur level. Obtaining fine cuts of tissues, both animals and plants, is an arduous task within the work of histology that requires a laborious process of inclusion, usually in paraffin, and highly specialized and expensive apparatus such as microtomes. However, this same aspect can serve the amateur as a personal challenge, raising and developing home-like procedures to obtain blocks of inclusion and subsequent cuts.

MOTIVATION

Personally, since this affair appeared in me, even as a child, one of the things that fascinated me most was precisely that fine cuts of less than 15 microns could be obtained, unimaginable for a mind of 13 years. That is why for many years I have dedicated myself to gather information and practice different methods within the school and amateur environment to get those cuts. The process has been logical. Of course, first I tried to make microtomes with screws and nuts. The discovery of craft knives changed my life. I started with stems because they are everywhere and they are cylindrical, which fit well in the hollows of the nuts. I encased them with candle wax, even without knowing that I had to dehydrate the material ... All disastrous. And finally I saved to buy a hand microtome with its corresponding knife, which, incidentally, is the type of equipment I still use to obtain my cuts.

At first I tried supporting in carrot and elder pith. Good cuts are obtained but the support seems to me deficient. On the one hand, carving the grooves where the sample is placed is complicated. The placing of the pieces leaves to be desired, although less with the elder pith because it can be soaked in water and better adapted to the material. In addition, when making cuts, on the table there are many carrots or pith remains that are messing up the workplace. Finally, coupling the block well into the microtome clamp is also

a difficult process. I had to try inclusion, but in paraffin it seemed complicated to do it at home. Fortunately, I discovered some articles that described the inclusion in a water-soluble polymer and that hardly needed 50º C to melt. All this led me to try the inclusion in PEG, a process that I found attractive, totally safe and with which I could experiment. Practicing and improving this inclusion I have achieved the best results in terms of quality and thickness of cut using a simple hand microtome with its histological knife. And of course, I got a lot of hours of pleasant distraction. The advantages are obvious: soluble in water (we save dehydration and hydration), non-toxic, melts at low temperatures and not very expensive. The most complicated thing is to get it. The one I got was PEG 1500, (the number indicates the average molecular weight of the polymer) but the 2000 or 2500 can be used.

My microtomy equipment.

Yes, I sharpen my knives with wet sandpaper.

OBJECTIVE

It must be said that the process does not have to become a perfect inclusion. The aim is to get a good support of the material and that it was minimally embedded to achieve an acceptable cut. Of course, in the scientific literature, variations of the procedure are proposed that are carried out with different types of PEG and mixtures of these as well as different periods of impregnation and inclusion. The process that I describe is based on these studies, making variations and many tests with different species of plants, until I achieve my objective that was not other than getting cuts of about 30 microns of good quality, in a comfortable and clean way.

At first, I have concentrated the work on botanical histology for obvious reasons: the material is very easy to obtain and the cuts do not need to be as fine as those of animal tissue. Likewise, it is aimed at obtaining transverse sections of stems, but the method I suppose will be applicable to all types of organs. The material can be fresh, preserved in alcohol or fixed in a fixing mix that we have on hand. In any case, before starting the process, the stems should be washed thorougly with distilled water.

PROCESS

MOLD CONSTRUCTION

Initially, I made molds of aluminum foil, winding strips of this material to a cylindrical object (a 1.5 v battery) and fixing them with adhesive tape. The dimensions of the strips

were about 3 cm wide by about 6 cm long. One end was flattened to make a kind of background. Subsequently, the cylinder stuck to the base flattened to a coin by some Loctite type glue. The molds thus manufactured worked well but some failed in the sealing, leaving the PEG by the base or by the board stuck with the adhesive tape. In addition, at the time of demolding the paper was broken, which made the process very difficult.

A considerable improvement was to manufacture the molds with flexible PVC tube between 1-1.5 cm in diameter. Simply cut small 2.5 cm high cylinders with a knife and stick to coins. The problem of sealing is solved and to unmold the coin (which is reused) is detached and the tube is cut longitudinally, so that the demolding is faster and the resulting cylinder is handled less.

Materials for making the molds.

Some molds ready to be filled.

IMPREGNATION / INFILTRATION

For the whole process it would be desirable to have a heating plate, but it can be done perfectly on the smallest heater of a ceramic hob (as long as it is available in the kitchen). This heater will adjust to the minimum. The impregnation can be done in a 50 ml beaker or in a small glass that can withstand the heat, although it will not be necessary to heat to more than 60 ºC.

There are many variations in the literature on the percentages of the dilutions to be used and the times during which the material to be impregnated must remain in each one. For example, start with a dilution of PEG in water at 20 % and increase from twenty in twenty to reach pure PEG, keeping the material for one hour in each bath. After many tests, I observed that the most comfortable and that simultaneously gave me very good results was to start from a 50 % dilution in weight of PEG in water, which with the passage of time, as the water heated and evaporated, the solution is concentrated until it almost reached pure PEG. So you do not have to prepare different dilutions and go from one to another. In this way, even the process is even more gradual.

Definitely, my way of proceeding is the following. I prepare in a beaker 10 ml of water and 10 g of PEG, I set the heater to 1-2 and let it melt and dissolve the PEG. When the mixture is perfectly liquid add about five or six pieces of stem about 2.5-3 cm and I leave everything about 3-5 hours. From time to time I shake and check that the temperature is not excessive. It would perfect if you had a laboratory thermometer, if not

simply the glass should not burn and could be touched by hand. It should not exceed 50 ºC. Little by little, the water evaporates and the mixture is enriched in PEG. In some texts it is found that the addition of a few drops of glycerin improves the cuttability. So far, my experience has been that the plant species in question is more determinant than the addition of glycerin. Another aspect that everyone can experience.

Actually, in this process a perfect inclusion does not occur like the one that could be obtained with paraffin or carrying out the process more professionally. The infiltration would coincide with the last period of time of impregnation, when almost all the water has evaporated leaving the polymer almost pure. During this last period, it should be noted that the stems shrink to a greater or lesser extent depending on the species and its original diameter. At first, I thought that the process spoiled the samples, but after cutting and leaving it in water to dissolve the PEG, the stems rehydrate and acquire their original size.
The theory says that the infiltration must get all the cavities of the material filled to harden it, but we must not forget that what we want to achieve is a good and efficient filling that helps us improve the thickness which doesn't have to be perfect so perfect. All this process could be delayed in time, but for our amateur purposes to do it between 3-5 hours is enough.

MOLDED

Placing the stems well oriented is essential to make good cuts. To do this you can cross perpendicularly the stems with a needle at one end and drop the set on the edge of the PVC mold. The final step would be to fill the mold with pure melted PEG and place the stem in the exposed manner, allowing the block to cool and solidify at room temperature. They should not be cooled in the refrigerator because it would absorb moisture.

UNMOLDED AND STORAGE

Once the block is solidified, the coin is peeled off and a cut is made along the cylinder with a knife, trying not to damage the PEG a lot. The PVC layer is separated and quickly the block is wrapped in film. Several of these blocks are stored in a bag and can be kept in the refrigerator for months. It is advisable to put some silica gel in these bags, but if it is well wrapped in film it is not necessary. What you have to avoid as much as possible is the contact with moisture, which would cause the dissolution of the polymer. It is advisable to keep the blocks overnight before being cut, nevertheless it is possible to cut them recently unmolded.

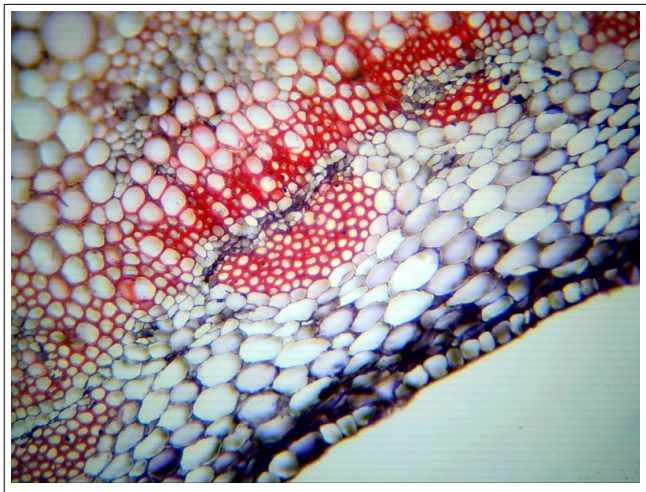
RESULTS

I made inclusions of different species such as *Schefflera arboricola, Lavandula spica, Petroselinum crispum, Malva sylvestris* and *Bellis perennis,* all of them a Dicotyledons type.

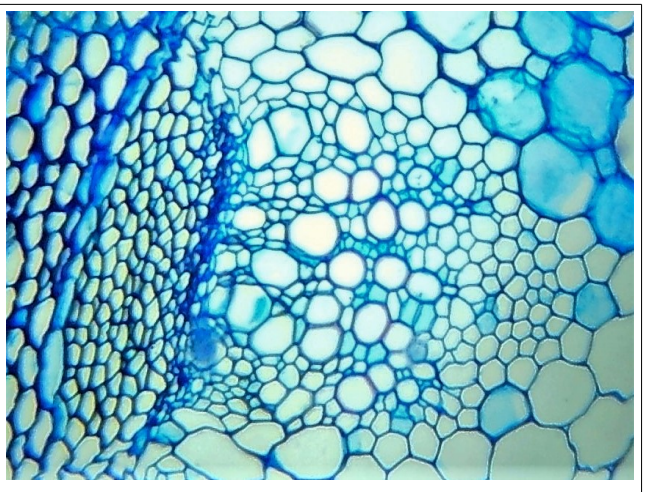
For each species, every several days, I took a block and obtained a large number of cuts. The rest of the unused block returned to wrap in film and kept it for some time, even months, to see how it evolved. The cuts were easily obtained thanks to the good fit of the block in the microtome, to which it remained perfectly fixed. The cutting technique was the usual one of passing the knife all along its edge and with an angle no higher than 5 degrees. In this case do not go moistening the blade, much less the block. The best cuts are obtained when the block is already somewhat advanced, because at the beginning of it you can find some cavities around the stem due to an irregular solidification speed of the PEG. As the knife is moved the cut is rolled on it, so that the more curly it appears the thinner is the cut.

The cuts are put in a Petri dish with water to dissolve all the polymer and the stem acquires all its size. Later I proceeded to make different routine stains within plant microtechnique, especially combinations of Safranin (SA) with other colorants like Fast Green (FG), Gentian violet (GV) or Delafield´s Hematoxylin (DH) and Astra Blue (AA) with Basic fuchsin (BF) or a simple stain with Toluidine blue (TBO). Later they were dehydrated, clearing and mounted in Balsam or DPX.

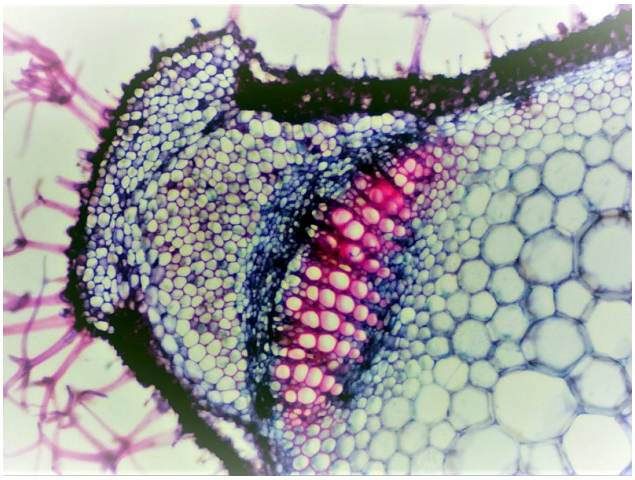
The photos were obtained through a Nikon Labophot microscope using 10 and 40 magnification planacromatic objectives and a 5 Mpx digital eyepiece camera. The photos were not treated with any program except to give them the appropriate format and size to the text with the free program AVS Image Converter.



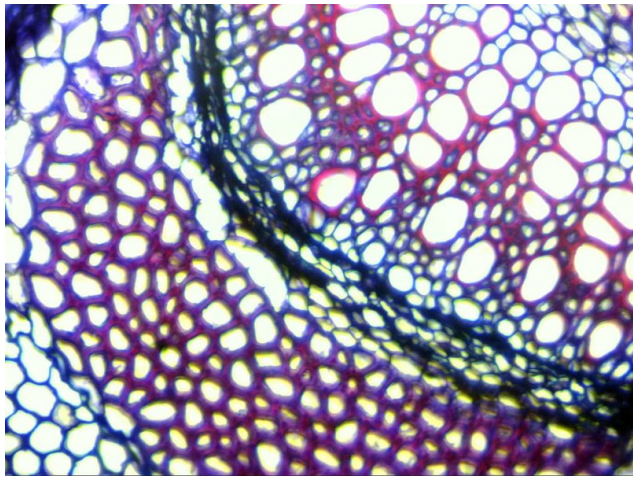
Bellis perennis, SAHD, 10x



Bellis perennis, AABF, 40x



Lavandula spica, SAHD, 10x

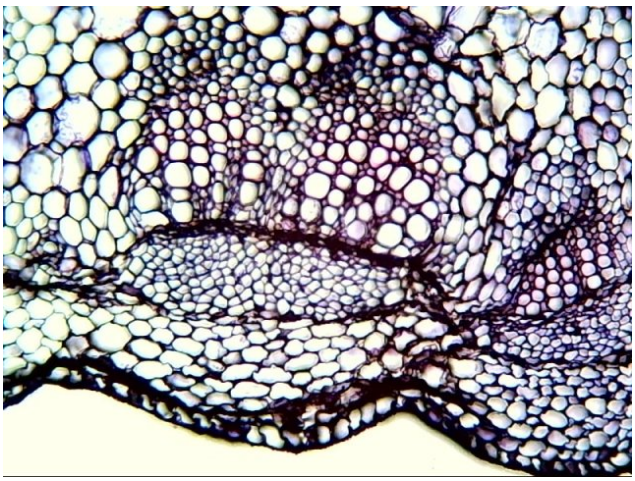


Lavandula spica, SAGV, 40x

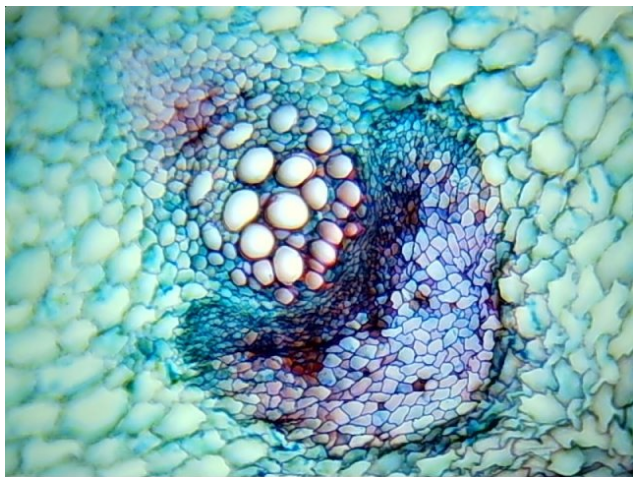
In the photos you can see how the cuts obtained were of a fairly acceptable thickness according to our amateur pretensions. In addition the stems were in very good condition, without tears and with undamaged edges, being able to observe the epithelial cells perfectly.

The different tissues appear correctly stained according to the affinity of these by the dyes. The usual overstaining of Safranin obtained in the free-hand slices was not observed. In addition, the staining times and protocols are much closer to those followed for the slides obtained with paraffin inclusion, especially as regards the reaction times of the reagents.

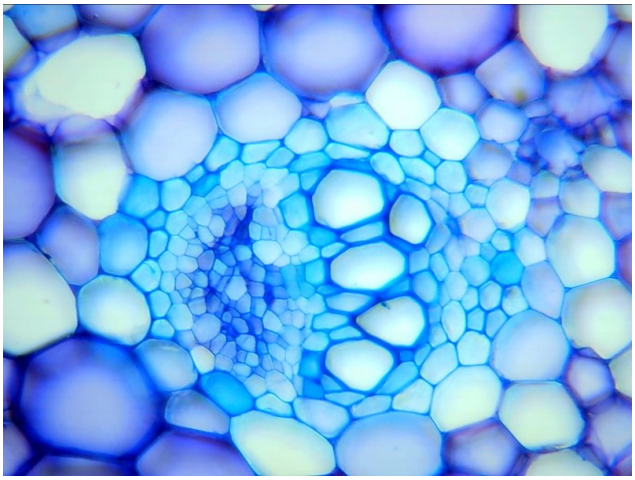
Some cuts were obtained after more than three months of having made the inclusion, keeping the stems perfectly and presenting the same characteristics observed previously.



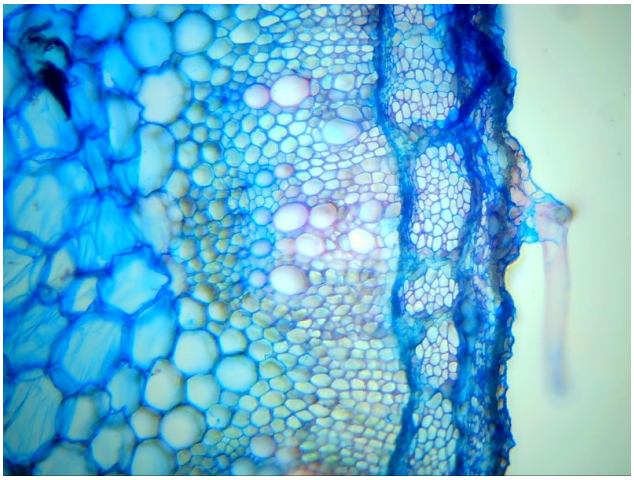
Petroselinum crispum, SAVG, 10x



Petroselimun crispun, SAFG, 40x



Schefflera arboricola, TBO, 40x



Malva sylvestris, AAFB, 40x

CONCLUSIONS

The inclusion in PEG is a very suitable process for the amateur microscopist, who is fond of histology, given its advantages regarding paraffin inclusion. These advantages are: the simplicity and safety of the process since no dehydration and subsequent rehydration are required with the consequent use of toxic substances such as xylene; speed of the process; availability of material and cost. We also have the possibility of preparing several blocks to have them stored and ready for a long period of time to always have samples available. With respect to using elder pith or carrot, better encasing is obtained, (avoiding the process of carving the hole and assembling the "sandwich"), and better fit and grip to the microtome hole, which provides thiner and more homogeneous cuts in an easy and clean way.

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