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A Refinement of the Staining Techniques for Hevea *Latex Vessels*

SAMSIDAR HAMZAH*, J.B. GOMEZ* AND L.H. HO*

A refined method to enhance the staining of Hevea *latex vessels is described. Tissues were prepared following procedures for electron microscopy. Semi-thin sections (1-2 μm thick) were double-stained with aqueous safranin and 1% malachite green. The contents of latex vessels were stained cyclamen (purple) and their cell walls capri blue. The contents of tannin cells stained dark blue. These temporary preparations were good for colour photography for a few days. Comparison is made with the conventional staining of fixed or resin-embedded material.*

When *Hevea* bark tissues are prepared for light microscopy in the usual manner, *i.e.* using paraffin wax as the embedding medium and staining the sections with safranin and fast green, the latex vessels are not clearly differentiated. This is due to the incompatibility of infiltration procedures[1] used, which leads to loss of the latex vessel contents. Modifications to the tissue preparation have been carried out to prevent this dissolution. One method is to brominate the tissues after the formalin-acetic-alcohol (FAA) fixation[2] which renders the rubber insoluble. Wimalaratna[3] added a few drops of oleic acid to the molten wax to retain the coagulated latex during embedding. A mixture consisting of a latex coagulant and a solvent for paraffin was also used during the de-waxing prior to staining. Recently, using fresh samples *i.e.* not fixed and embedded, Jayabalan and Shah[4] found oil red O and iodine-potassium iodide to be the best stain for rubber in guayule.

Tissues prepared for electron microscopy using osmium tetroxide fixative give good preservation of the latex vessel contents but the stains commonly used do not differentiate the latex vessels well. We now describe a staining technique that can be used successfully on semi-thin sections to distinguish both walls and contents of latex vessels from those of other cells.

MATERIALS AND METHODS

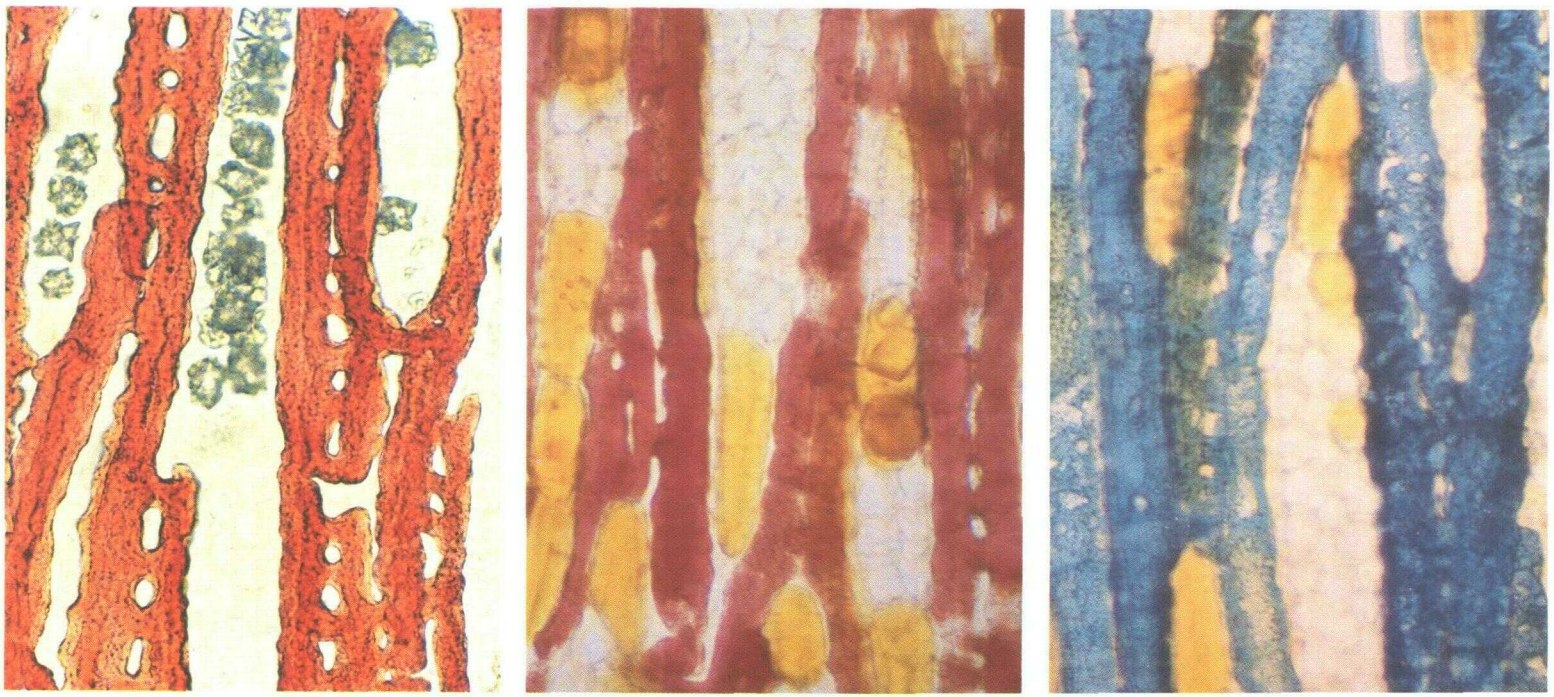
Refined Technique

Bark samples of *Hevea* were prepared for electron microscopy. These were double fixed with 6% glutaraldehyde and 1% osmium tetroxide in 0.1M phosphate buffer (pH 7). Dehydration in graded series of ethanol was followed by embedding in araldite-polybed mixture. Polybed is a substitute of Epon 812 in Mollenhauer's mixture[5].

Some of the staining procedures described below follow the well established method of Johansen[1] with some slight modifications. Semi-thin sections of 1-2 μm thickness were floated on a drop of water on a glass slide and were heated on a hot plate (60°C-70°C) until dry. Prior to staining the slides were soaked in toluene for 1 h followed by a rinse in absolute alcohol. This was carried out with the intention of dissolving the embedding medium, but it did not. Nevertheless, it was found that this routine helped to stain the sections better.

A drop of undiluted aqueous safranin (BDH 580270) was added to the sections and allowed to dry on a hot plate. Excess stain was removed with water followed by differentiation for about 10 s in a solution of 95% alcohol containing a few drops of saturated picric acid. The

*Rubber Research Institute of Malaysia, P.O. Box 10150, 50908 Kuala Lumpur, Malaysia



Sudan III *Oil red O* *Sudan blue*

Figure 1. Tangential longitudinal sections of FAA-fixed bark stained with Sudan III, oil red O and Sudan blue. Note that the section stained with Sudan III was sampled from a different type of bark to those stained with oil red O and Sudan blue as shown by the presence of numerous calcium oxalate crystal inclusions in the parenchyma cells. (Magnification 240×).

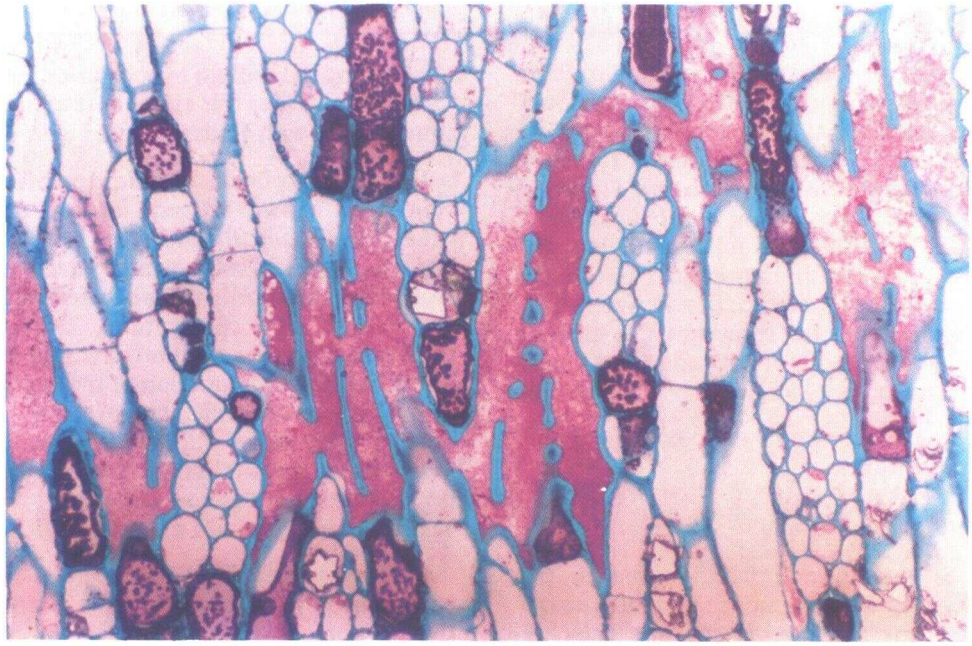


Figure 2. Tangential longitudinal section of bark stained with safranin and malachite green. The latex vessels are distinguished by the cyclamen (purple) contents and the tannin cells by the dark blue colouration. Many tannin cells have grainy contents in this picture. (Magnification 225×)

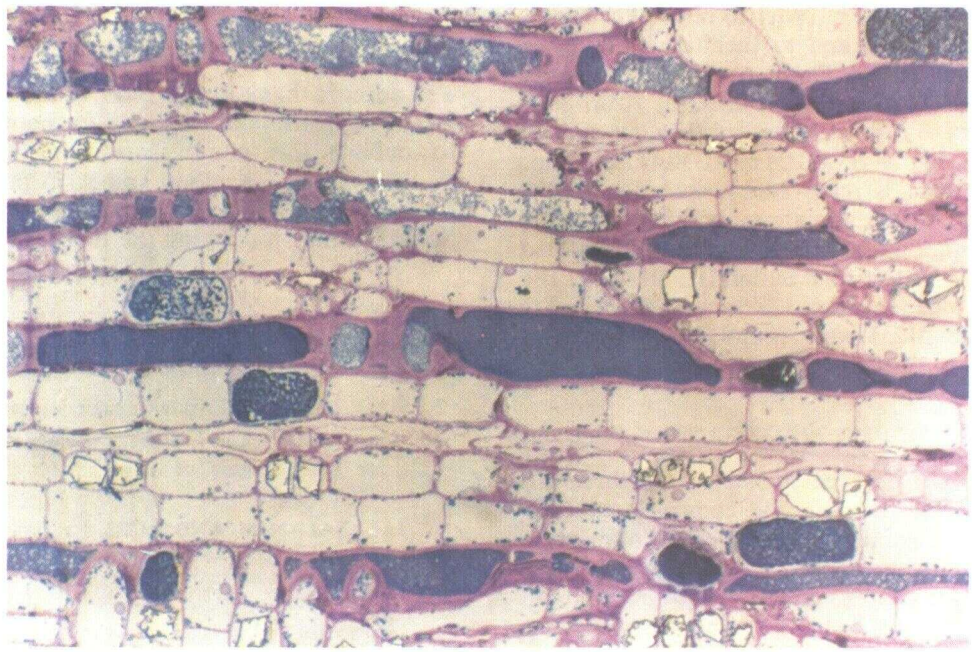


Figure 3. Tangential longitudinal section of bark stained with toluidine blue O. No colour differentiation is seen between latex vessels and tannin cells. (Magnification 225×)

sections were next rinsed in a solution of 95% alcohol containing a few drops of ammonia solution (commercial 22%) in order to stop the action of acid. A final wash in water followed and the sections were allowed to dry. The dried sections were counter-stained with 1% malachite green (BDH 1867720) in coplin jars, overnight if possible, or for a minimum of 2 h. Similar rinsing procedures followed: first in water, then in ammoniacal alcohol solution (95% alcohol containing a few drops of ammonia) and finally in water again.

The stained sections were examined and micrographs taken.

Conventional Technique

This is the normal method used in the laboratory for routine counting of latex vessel numbers, density of latex vessels, *etc.* The bark samples are first fixed in formalin-acetic-alcohol [3:3:94 (50% alcohol)] for a minimum of 24 h. Sections of 50–70 μm thick are obtained by using a bench microtome and are collected in 50% alcohol. The sections are washed and then kept in sodium hypochlorite (commercial grade) overnight in order to render them partially colourless and to facilitate staining of the latex vessels. These are washed in water followed by dehydration in 50%, 70%, 80% alcohol (10 min each) before they are stained with 1% Sudan III (BDH 6980330A) for 3 min. Excess stain is removed by washing in 50% alcohol before these are mounted in glycerine jelly. Other oil-based dyes are sometimes used instead of the normal Sudan III. Of these, 1% Sudan blue (Harleco 3788) and 1% oil red O (Harleco 3125) were found to be the most suitable.

RESULTS AND DISCUSSION

The only difference shown by the three oil-based stains used in the conventional non-embedded samples is the different colouration taken up by the latex vessels *(Figure 1)*. Sudan III stains the latex vessels burnt orange; Sudan blue stains them Indian blue and oil red O stains them red. Tannin cells are stained yellow by Sudan blue and oil red O when only partially bleached by the sodium hypochlorite. Excessive bleaching removes this effect as seen in the section stained with Sudan III in *Figure 1*.

With the refined technique, differential staining is shown between latex vessels and other cells *(Figure 2)*. The latex vessel walls are stained capri blue, its contents cyclamen purple while the tannin cells can be differentiated by the darker blue colouration. This refined technique combines good preservation of tissue as well as the differentiation of the latex vessels and is a considerable improvement on the normal practice of staining resin-embedded sections with 0.5% toluidine blue O, which does not show distinct colour differences between cells containing tannin and latices *(Figure 3)*.

Permanency by the refined method can only be obtained through photographic records as all the mountants tried like Canada Balsam, Euparol, glycerine and glycerine jelly proved to fade the colouration on storage after a few weeks. In the non-embedded sections however, with the right kind of mountant e.g. glycerine jelly, the colour of the oil-based stains is preserved for a couple of years.

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