Notes RBG Edinb. 43(2): 299–301 (1986)

A SIMPLIFIED TECHNIQUE FOR CLEARING AND STAINING THE VASCULAR SKELETON OF WHOLE PLANT MATERIAL

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ABSTRACT. A technique for clearing and staining plant vasculature for morphological or anatomical study is described. It employs basic fuchsin in KOH, resulting in the differential staining of lignified tissue, producing a deeply-stained, three-dimensional vascular skeleton of the plant *in situ* within a transparent whole plant body.

INTRODUCTION

Several methods for clearing and staining whole plant material to supplement microtome sections have evolved in the past (e.g. Foster, 1949, 1953 & 1955; Morley, 1949; Brady, Wemple & Lersten, 1965). Their techniques involved clearing with NaOH and staining with safranin or with a safranin and fast-green combination. The method described here involves the use of fuchsin in KOH. It was adapted primarily from that of Barratt (1920) but also from Fuchs (1963) by one of us (C.N.P.) for the study of *Equisetum* vasculature (Page, 1972), which is otherwise difficult to stain. It has also been found useful for displaying the taxonomically significant venation patterns of fern pinnae, and the technique was later found successful by one of us (K.T.) for use with flowering plant material. Essentially the process stains the xylem *in situ* within a transparent whole plant body. By this method the vascular structure from its main arteries to its finest ramifications can be displayed in a three-dimensional setting, suitable for teaching, illustration or photography.

PREPARATION OF THE STAIN

The following procedure prepares 500ml of 1% leuco-basic fuchsin stain:

i. Weigh out 5 gm basic fuchsin.

ii. Dissolve as much as possible in 500ml boiling water. Do not filter at this stage. The solution is now dark red.

iii. Take off heat. Continuously stirring, add 30gm KOH pellets (*one* at a time!). This weight can be varied to give solutions of KOH between 3–10% according to the hardness or blackness of the tissue to be treated.

Note: Great caution is essential at this stage in allowing the reaction to die down following the addition of each pellet of KOH, before adding another—addition of KOH at too rapid a rate causes super-heating of the liquid, which can take place explosively! The colour of the solution should now have disappeared, with the deposition of a dark or nearly black precipitate.

iv. Cool slightly. Filter with filter pump, or centrifuge using litre vessels. Discard precipitate. Retain the filtrate, which should be a clear, slightly straw-coloured liquid.

v. Bottle filtrate for immediate use or store in cool dark place (a dark bottle in the refrigerator is ideal). So stored, the stain can last up to 3 months, but to obtain best results, freshly made solutions are recommended.

METHOD OF USE

The following schedule is recommended for staining plant material.

1. Immerse plant material in 70% alcohol (ethanol) until decolorized of chlorophyll and other alcohol-soluble pigments. Transfer to water.
2. If heavily pigmented tissues remain (such as those coloured by tannins), bleach by immersion in Eau de Javelle solution until material is colourless, or until it shows no further change in colour. (Starch-containing tissues are also beneficially cleared at this stage.) To avoid destruction of more delicate material immersion time in Eau de Javelle should be kept to the minimum necessary to achieve bleaching. Wash well in running water to remove remaining bleach.
3. Immerse plant material in leuco-basic fuchsin in KOH (prepared as above). Raise to 60°C in an oven and maintain at this temperature in a covered container for approximately 6–12 hours, or until any remaining colour has disappeared from the specimens, which should now be a pale straw yellow throughout.
4. Remove from oven. Specimens are usually now soft, colourless, and particularly delicate. Onward procedures should now be carried out by removing and replacing liquids using a safety pipette.
5. Remove and discard excess stain. Wash specimen gently with water, then replace by 50% alcohol and stand for 15 minutes.
6. Discard 50% alcohol and replace with 70% alcohol, previously acidified with a few drops of conc HCl (e.g. five drops in 500ml). Purple-coloured veins now begin to appear in the specimen. Cover and allow to stand in the solution until all venation is distinct, and there is no further change.
7. Rinse and allow to differentiate in 70% alcohol, until any excess stain which has appeared in tissue, other than the vasculature, has been removed.
8. Dehydrate through a graded series of alcohols to absolute alcohol.
9. Pass through 1:1 absolute ethanol-xylene solution and then clear in two changes of xylene. Remove any bubbles which have accumulated within the tissue in a vacuum-desiccator. The resulting material should now be totally clear and transparent, except for its vasculature which is stained deep red, magenta or purple. The specimen is now ready for study and can be mounted in any xylene-soluble mounting medium (e.g. Euparal), or may be left permanently in xylene in sealed glass tubes.

DISCUSSION

The reaction of the stain following the acidification stage is probably based on mild hydrolysis of deoxyribofuranose (the carbonate component of DNA) to release aldehyde groups which then unite with fuchsin to give

a purple- or magenta-coloured product. Thus, in addition to lignified walls, all tissues containing some DNA such as anthers, ovaries and pteridophyte sporangia also stain in shades of reddish-purple.

The depth of stain achieved within the vasculature, as well as with any other tissues, varies directly with the time of treatment (stage 3) as well as with the stainability of the material itself. Even with material which has xylem that is difficult to stain (such as *Equisetum*), overnight immersion has usually proved adequate. Excess time however, can result in difficulty in later differentiation.

The shelf-life of specimens successfully treated varies considerably with different material. Many fade within four months if exposed to normal daylight. Prolonged storage in xylene also causes some material to become rather brittle. Study of the material whilst freshly prepared is hence desirable.

We have, however, found this method applicable to a range of both pteridophytes and flowering plants, including relatively massive stem as well as more delicate leaf and floral material. Successful results have been achieved irrespective of whether the original material was fresh, fixed, or obtained from a previously dried herbarium specimen (providing that it could first be adequately reconstituted). The results give an excellent overall three-dimensional picture of the complete vascular arrangement of the plant specimen and its organ relationships. It also provides an excellent comparison for achieving orientation and placement of microtome sections whenever more detailed cross-sectional study is required.

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