

PERMANENT PRESTAINING IN BOTANICAL MICROTECHNIC¹

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One of the many problems in botanical microtechnic is the retainment of safranin at the proper intensity and brilliance when counterstaining with fast green FCF or any of the commonly used counterstains. Especially is it difficult when attempting to stain plant tissues previously treated with hydrofluoric acid.

The term "permanent" is used here for the lack of a better word and to distinguish this type of staining from ordinary procedures. The most common prestaining is a process of staining bulk material so that it can be easily seen in the paraffin block or ribbon. In permanent prestaining, the bulk material is stained once and the stain used in prestaining becomes the primary stain of the finished preparation.

In this instance a specific method was sought, which would be suitable for the staining of bluegrass leaves in transverse section. By using this type of prestaining, several days may be added to the dehydration process; however, time is saved from imbedding to the finished preparation.

The preparation of bluegrass leaves by this technic is relatively simple but certain procedures must be adhered to if the desired results are to be obtained. First, the leaves are cut transversely with a sharp razor blade into one-eighth inch lengths. If they are cut any longer uneven penetration of the dye is unavoidable. Also, siliceous compounds will not be uniformly removed when treated with hydrofluoric acid.

OUTLINE OF PROCEDURE

1. Place material in formal-acetic-alcohol (using 70 per cent ethyl alcohol), or Bouin's Fluid, and by means of a vacuum pump or an apparatus similar to Wittlake's (2) vacuum apparatus, pump at 600 mm. mercury for 12 hours.
2. Rinse quickly in distilled water and transfer to commercial hydrofluoric acid (48 per cent)², 120 hours.
3. Remove material from the hydrofluoric acid and wash in running tap water for one hour.
4. Transfer material to Johansen's (1) 50 per cent Tertiary Butyl Alcohol. Pump one hour at 600 mm. mercury.
5. Stain in Safranin Y³ made up according to Johansen's (1) procedure of preparing Safranin O with methocellosolve. Stain from 96 to 120 hours.
6. Dehydrate by Johansen's (1) Tertiary Butyl Alcohol method up to 100 per cent at intervals of two hours.
7. Transfer to 10, 25, 50, 70, 85, 95, 100 per cent toluene series made up in tertiary butyl alcohol at two-hour intervals.
8. Transfer to equal parts of xylene and toluene, and finally to 100 per cent xylene. Change at two-hour intervals.
9. Place specimen bottle in the imbedding oven for one-half hour, allowing the xylene to warm up. Infiltrate in a good grade of rubber paraffin by adding paraffin to the xylene at several three-hour intervals and pouring off half the volume of the mixture

¹Papers from the Department of Botany, The Ohio State University, No. 463.

²The hydrofluoric acid used is obtainable from J. T. Baker Chemical Co., Phillipsburg, New Jersey, in $\frac{1}{4}$ -lb. (113.4g) paraffin container at a concentration of 48 per cent.

³The Safranin Y used was manufactured by the Coleman and Bell Co., Norwood, Ohio, certification No. C S-7.

each time until no odor of xylene can be detected. If a vacuum is used in the infiltrating process only four or five changes will be necessary before all the xylene is removed.

10. Section material at 10 microns and remove paraffin from slide with xylene, which will occur within about 5 minutes.
11. Transfer to equal parts of xylene and absolute alcohol for 45 seconds, agitating the slide constantly.
12. Transfer to absolute ethyl alcohol for 45 seconds and continue agitation.
13. Counterstain with Johansen's Fast Green FCF³ 30 to 45 seconds and agitate.
14. Transfer slides to Johansen's (1) Fast Green Rinse for one minute with vigorous agitation. (This is made up of one part xylene, one part clove oil and one part 100 per cent ethyl alcohol.)
15. Clear in synthetic methyl salicylate two minutes.
16. Complete in two changes of xylene at two-minute intervals.
17. Mount in xylene balsam or clarite.

Acceptable finished preparations, from tissues fixed in formal-acetic-alcohol, were obtained, showing very little plasmolysis and both the primary stain and the counterstain were up to standard as to brilliance and intensity. However, Bouin's Fluid proved to be the preferred fixative for this particular leaf tissue. When using either of these fixatives, fixation was accomplished in a twelve-hour interval under vacuum at 600 mm. mercury.

Since it is almost impossible to cut satisfactorily the leaves of many grasses without first removing the silica, a commercial grade of hydrofluoric acid was used so that the material could be sectioned easily in paraffin. Of course, all containers in which this acid is used should be first paraffined on the inside, as well as the cork used for the container. A standard 50 cc. wide-mouthed bottle is sufficient for most material. Care should be taken not to breathe the fumes of this acid. When the material becomes translucent to opaque in appearance, it is ready to be removed from the acid. This is usually from 96 to 120 hours. All material in this experiment was treated for 120 hours.

In this staining method, the proper staining time of the bulk material was determined by selecting the material which showed after prestaining, a minimum of diffusion of the dye into the mesophyl of the leaf from the vascular strands. The bulk material selected in this way turned out to be the same material that was acceptable when carried through the above schedule to the finished preparation. A decrease or increase in the timing of the counterstain altered the acceptability of the finished slide. When a counterstain time was secured with bulk material of these characteristics and showed the proper balance between the safranin and the fast green in the finished preparation, this counterstain time was used as the standard time. When bulk material was selected which showed a great amount of diffusion of the dye into the mesophyl, it was impossible to vary the counterstain time sufficiently to produce a satisfactory finished preparation. The same thing was true in the case of bulk material that was understained.

In the safranin-fast green combination, the balance between the two stains changes even with the particular tissue being stained; hence alterations in the timing of stains in most of the general staining schedules is necessary. A safranin-fast green combination which is not up to standard can be detected by definite gradations of red to purple and blue to green from parts of tissue which should stain a brilliant red to parts of tissue which stain a brilliant green color. In other words, a well differentiated preparation should show definite areas of green and red.

The writer has found that the range in time intervals between strictly alcoholic

³The Fast Green FCF used was manufactured by the National Aniline and Chemical Co., Inc., New York, New York, certification No. NG f-4, dye content 91 per cent.

safranin and alcoholic fast green is much longer than the staining time interval between Johansen's (1) Metho-cellosolve safranin and Johansen's Metho-cellosolve fast green. However, when the proper time interval is obtained with Johansen's stains, they yield excellent results. Although, these time intervals between Johansen's safranin and fast green are more difficult to balance properly without obtaining blue and purple gradations of color, it would seem that a beginner would have more success with alcoholic safranin-fast green schedules.

In this particular procedure the writer has used only a part of Johansen's Tertiary Butyl Alcohol Dehydration Method. When 100 per cent tertiary butyl alcohol was reached, the material was transferred to 10 per cent toluene made up in 100 per cent tertiary butyl alcohol. This was done to avoid the use of paraffin oil in Johansen's schedule. Paraffin oil is extremely hard to extract from plant tissues once it gains entrance. The author, as well as several of his associates, have tried Johansen's Tertiary Butyl Alcohol Dehydration Method with no success. When ready to cut infiltrated material where there is a trace of paraffin oil left in the tissues, it is almost impossible to get a satisfactory ribbon.

Toluene was used with 100 per cent tertiary butyl alcohol and after extended use of this particular combination of liquids, it was found that a minimum of hardening of tissues resulted. The lack of brittleness was very apparent in contrast to material run through a long series of xylenes and ethyl alcohols.

Although this staining procedure was worked out specifically for bluegrass leaves it has been successfully used on other material. Many other tissues not treated with hydrofluoric acid were also used with the result that safranin was retained easily and to a very high intensity. Another observation is that material permanently prestained by this method can be left in the dehydration alcohols much longer than in regular staining schedules.

REFERENCES

- (1) Johansen, D. A. 1940. Plant Microtechnique. McGraw-Hill, New York, N. Y.
 - (2) Wittlake, E. B. 1942. An efficient vacuum apparatus for microtechnic. Ohio Journal of Science, 42: 65-69.
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