Methyl Green-Pyronin Staining in Germinated Fruit Tree Pollen: Methods to Enhance Stainability of the Nuclei and Chromosomes¹

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Fruit tree pollen is a unique cell which cannot be studied by standard cytochemical methods, but only by the use of special techniques. DNA and RNA and their mutual relationship are commonly investigated by the methyl green-pyronin technique of Unna (1902). However though this procedure differentiates RNA from DNA it does not conclusively define the chemical nature of the stained material. Not all the cellular material which stains red is RNA and Brachet (1940) improved chemical identification of the cell by the addition of RNase. However, the staining procedure proved difficult to standardize and lack of success with the original method led to many modifications (Taft 1951 a, b, Kurnick 1952, Tepper and Gifford 1962, Elias 1969).

The standard Brachet (1940, 1942) procedure was successfully applied to the pollen of *Tulipa* (Jona and Coppa 1970), with modifications based on the characteristics of the object to be stained.

However, this procedure was not suitable for fruit tree pollen, because after treatment with RNase at 37°C and parallel control treatment with lukewarm distilled water, methyl green failed to stain the small sized nuclei and chromosomes. To overcome this difficulty an alternate method was devised based on results of our investigation of the cause of lack of methyl green binding.

Materials and methods

The pollen is grown on Millipore membrane filters (Jona 1967) placed on an aqueous medium of sucrose (cane; 0.5-0.8 M depending on the species), agar (0.5-1 % depending on the species) and boric acid (0.01 %).

After an appropriate time interval the pollen is fixed by transferring the membrane filters to a filter paper soaked with a modified Navashin (Jensen 1962) mixture.

Solution A: glacial acetic acid	ml	22.5
chromium trioxide	gm	1.0
distilled water	ml	51.5
Solution B: commercial formalin	ml	40.0
(formaldehyde 40% in water)		

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distilled water	ml 35.0
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Solutions A and B are mixed just before use. After 3 hrs contact with the fixing fluid the membrane is thoroughly rinsed overnight transfer to a filter paper held in a frame on the surface of a gentle stream of running water. The pollen is then bleached by leaving the membrane for 3–6 hrs on a filter paper soaked with several changes of a mixture of equal parts saturated aqueous ammonium oxalate and 3% hydrogen peroxide followed by rinsing with several changes of distilled water.

The pollen is squashed onto a slide as described elsewhere (Jona and Coppa 1970).

RNA is removed by incubation at 37°C for 2 hrs with RNase (pancreatic; Merck cat. No. 7602) 0.5 mg/ml in an aqueous solution of cysteine hydrochloride 0.05 M (to retain its activity) at a pH adjusted to 6.8 (Tepper and Gifford 1962). The control slides are treated in the same way, but distilled water is substituted for the enzyme.

At the end of these reactions the nuclei barely stain with methyl green. In order to achieve a satisfactory degree of staining, nuclei and chromosomes of fruit trees, after careful dehydration, should undergo preliminary treatment with pure acetic anhydride for 18 hrs at room temperature. To remove acetic anhydride the slides are thoroughly rinsed in absolute alcohol. Satisfactory results can also be obtained with glacial acetic acid instead of acetic anhydride.

After rehydration the slides are stained in methyl green-pyronin solution for 2 hrs at 37°C.

The methyl green-pyronin solution used was Kurnick's (1955) modified mixture.

Methyl green (British Drug House, C. I. 42585) 2% (w/v) and pyronin Y (Merck, C. I. 45005) 2% w/v, are separately purified by shaking with equal volumes of chloroform until the layer which separates is colorless for the methyl green and pale pink for the pyronin solution. Once purified the following mixture is prepared:

2% Methyl green solution	ml 15
2% Pyronin solution	ml 12.5
Buffer (acetic acid-sodium acetate pH 4.7; 0.1 M)	ml 72.5

Before use the mixture must be filtered on a Buchner funnel through a pad of three No. 589 Schleicher & Schüll paper filters in order to remove impurities from the pyronin. The solution may be reused several times provided that it is filtered each time. In order to achieve more balanced staining the mixture should be stored at 0°–4°C in a dark closed bottle.

For best results 10 days aging is advisable, but after 40 days the mixture will no longer stain properly and should be discarded.

The stained cells can be differentiated by dipping the slides in ice cold water (Elias 1969) and immediately transferring to two changes of t-butyl alcohol for 5–40 min depending on the intensity of the stain. The differentiation should be followed by microscopic observation and the slides mounted in Euparal without further clearing.

Results and discussion

Because of the greater sensitivity to damage of fruit tree pollen the alcoholic

fixation procedure devised for *Tulipa* (Jona and Coppa 1970) would have led to serious shrinkage. On the other hand, the chrome-osmic fixative which was successfully employed prior to the Feulgen reaction (Jona 1967) either prevented the staining of the cytoplasm or inhibited the RNase activity.

Figs. 1-6. Pollen of *Malus* (cv. Golden Delicious) cultured for 1 day on agar-sucrose medium on Millipore membranes and stained with methyl green pyronine. The material not submitted to the RNase extraction was extracted, as control, with warm water under identical conditions. In order to enhance the nuclear stainability the material was submitted to the following treatments before staining: 1, no pretreatment, warm water. $\times 640$. 2, no pretreatment, warm RNase. $\times 530$. 3, acetic anhydride pretreatment, warm water. $\times 480$. 4, acetic anhydride pretreatment, warm RNase. $\times 450$.

Navashin's mixture (Jensen 1962) was, therefore, applied but with a more than twofold increase in concentration of acetic acid to remove some interfering mitochondrial structures.

Furthermore the composition of the dying mixture is critical, as pointed out by many researchers (Brachet 1940, Kurnick 1955, Elias 1969). We found that staining of fruit tree pollen requires a more concentrated solution of methyl green and a higher purity of pyronin than other cells (Jona and Coppa 1970). Stability of the pH is also very important and all solutions should be carefully buffered. Differentiation after staining is another critical step which has been approached in various ways: Kurnick (1955) modified previously used methods of differentiation (Cowdry 1948, Lee 1950, Conn 1946) by substituting n-butyl alcohol followed by clearing agents for water followed by dehydration in acetone. Elias (1969) achieved satis-

Figs. 5-6. 5, nitrous deaminating pretreatment, warm water. $\times 480$. 6, nitrous deaminating pretreatment, warm RNase. $\times 450$.

factory results, as we did, by using ice cold water followed by t-butanol in further modification of the techniques of Kurnick (1955) and Taft (1951).

All these authors cleared the slides either by xylene alone or by xylene and cedar oil; but these treatments caused complete fading of the stain with our material. The procedure of Elias (1969) was therefore adopted with the omission of the clearing step.

Another problem which had to be overcome was the decrease in DNA uptake of the methyl green stain after the obligatory pretreatment with lukewarm RNase and the parallel control treatment with distilled water. Because of the need for high specificity which can only be obtained with RNase we did not attempt replacement of the enzyme with other reagents which, though they extract RNA, also unspecifically react with other molecules.

The use of perchloric acid, for example, did not seem advisable, since some DNA

may be extracted during DNA removal (Barka and Dallner 1959, Di Stefano 1952, Woods 1957) and alterations in the dye-tissue interrelationship may result from DNA denaturation (Swift 1966).

According to Deitch (1966) lowering of methyl green binding by DNA after heating was observed by several researchers, but its cause remained unexplained.

According to Alfert (1952) the heat cleaves peptide bonds in the basic nuclear proteins which, in the presence of free reactive amino groups, interfere with the DNA staining process.

In contrast Pollister and Leuchtenberger (1949) postulated that the effect of heat on DNA depolymerisation accounted for the decrease methyl green staining.

To gain more information in support of one of the two hypotheses we treated the pollen tubes, with acetic anhydride, as recommended by Alfert (1952); this treatment resulted in good staining of the nuclei. In order to decide whether selective binding of methyl green by repolymerized DNA or blocking of the amino groups was responsible for this success, we subjected the slides to acid extraction with 1N or 2N HCl for 5 to 10 min at room temperature, also improving the nuclear features. It appears most likely that some proteins, whose amino groups were in competition with the DNA, were extracted and washed off, leaving the DNA available for reaction with methyl green.

This postulated mechanism of reaction was confirmed by deamination with 4N aqueous NaNO_2 and 4N acetic acid 1:1 for 3 hrs, at room temperature, (Beccari e Mazzi 1966) which also led to an improvement in the appearance of the stained nuclei.

Summary

Brachet's method for the identification of tissue DNA and RNA is very specific because the nature of the stained material is confirmed by the enzymatic extraction. However, the small sized nuclei of pollen of fruit trees (*Malus*, *Persica*, *Corylus*) lose their affinity for methyl green after heating for 2 hours at 37°C with RNase or distilled water (as control). RNA cannot be extracted with cold perchloric acid, because of the low specificity of this extraction.

In contrast, treatment with glacial acetic acid or acetic anhydride for 18 hrs, following proper alcoholic dehydration, enhances the nuclear stainability. The recommended fixative is Navashin's, with increased concentration of acetic acid up to 30% (to remove mitochondria), for 3 hrs. After thorough washing and bleaching with mixture of equal parts saturated aqueous ammonium oxalate and 3% H_2O_2 the slides are incubated with either enzyme or distilled water at 37°C for 2 hrs and treated with glacial acetic acid or acetic anhydride. Staining with methyl green (B.D.H.) 0.3% (w/v) and pyronin (Merck) 0.25% (w/v) in acetate buffer at pH 4.7, is performed at 37°C for 2 hrs. Differentiation is achieved by dipping in ice cold distilled water followed by a 5–40 min rinse in two changes of t-butyl alcohol.

The slides are mounted in Euparal. The acetic anhydride treatment was shown to block the amino groups of basic proteins which, otherwise, would interfere with the DNA staining by methyl green.

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