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AN OVERVIEW OF BOTANICAL CLEARING TECHNIQUE

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Abstract Clearing techniques are outlined with reference to their action on the chemical constituents of plant tissue. The most general technique would include pretreatment with solvents, dissolution of protoplasm, dissolution of other substances, bleaching, infiltration with a dense fluid, and staining. Extensive chemical changes go on during these steps and may prevent satisfactory clearing, an important example being the discoloration of phenolic compounds. Rational design of clearing methods for the chemically distinct cell types and tissues seems a likely future development.

For the last twenty-five years or so, clearing techniques have enjoyed a quiet popularity with morphologists and taxonomists and a considerable number of different methods and schedules have been offered (see the brief reviews by Bersier and Bocquet 1960, Bohm and Kisser 1961, and the annotated bibliography of Lersten 1967). A consideration of the chemistry of major plant constituents leads to fuller understanding of the clearing process and enables rational schedules to be drawn up for particular purposes.

As a definition we may say that a piece of plant material is cleared when some of its components are made visible at the expense of others while the form of the material remains more or less unaltered. Although the term "clearing agent" is used in microtechnique for solvents of embedding media and fluids of high refractive index (clove oil, xylene, *etc.*) this term will be applied more loosely below to include fluids which have a clearing effect by selectively dissolving tissue components. Such usage is fairly general (witness the phrase "clearing with caustic soda"), seems unlikely to lead to confusion, and will serve to emphasize that some clearing agents have their effect both through their high refractive index and through their ability to dissolve protoplasm (see later).

In a cleared preparation then, one to several cell or tissue types will stand out because of their special chemical or physical properties. The dense, resistant nature of the lignified elements generally ensures their persistence, and most clearing studies have been to demonstrate such features as vascular anatomy and sclereid distribution. However, by using an appropriate technique many other interesting plant structures can be shown—crystals, silica cells, tanniferous idioblasts, trichomes, laticifers, stomata, and even nuclei (in embryos). It is convenient to describe all these clearing techniques piecewise under the section headings below so that the chemistry of each method can be made apparent.

Pretreatment

It is usually possible to clear a piece of tissue regardless of whether it is fresh, fixed, or dried, but a different technique may be needed in each of these cases and the rate of clearing will also vary. Dried material, often used in

comparative studies, may clear more quickly than fresh material, presumably because of the disruption of protoplasts in drying, but this tendency can be offset by the production of hard-to-remove dark pigments resulting from air and enzymic oxidation of phenolic compounds. (Plant phenols will recur in this discussion—generally, they cause most of the browning and discoloration in cleared tissue.)

Fixing of material, particularly in 70% ethanol or either of the Carnoy's solutions, can accelerate clearing slightly by extracting various constituents. However, fixing with any formaldehyde-containing fluid is detrimental to the clearing process. Firstly, the strong cross-linking of protein achieved by formaldehyde enables such tissue to resist the usual subsequent treatment with alkali (Walker 1944, Baker 1958) and the whole specimen may disintegrate through alkali attack on the cellulose before it clears. Secondly, formaldehyde reacts with many plant phenols, especially the condensed tannins (leucoanthocyanins and catechins), to form dark polymers which can only be destroyed by strong bleaching, which may also destroy the tissue. A corollary of this is that formaldehyde fixation should be restricted to clearing studies where preservation of tannins is needed, e.g. in tanniniferous idioblasts. When clearing heavily tanniniferous material it is useful to fix in warm, slightly acidified 70% ethanol for 1–5 days, using several changes, to extract as much of the tannin as possible. This step may be essential if the material is too delicate for later bleaching.

Preliminary dissection and marking of the specimen to be cleared may greatly assist later study in cases where natural "landmarks" tend to be difficult to see when clearing is complete. For example, study of leaf traces near the stem apex is made easier by trimming each petiole off so that it does not overtop the leaves closer to the apex and obscure the phyllotactic spiral. Judicious cutting of a flower can enable the orientation of the vascular traces to be determined even when the external parts of the flower fall off during clearing or when the outline of the locules can not be seen, as in very transparent specimens.

Hairs and other epidermal appendages generally resist clearing and should be removed from material in which they are not of interest. Hairs may be removed from stems, leaves, or even robust flowers by the method of Gardner (1974) but remain a problem with delicate clearings and have to be removed with a brush during the final dissection.

The number of procedures applied in the pretreatment stage can be expected to increase as clearing techniques become more ambitious, with certain tissue constituents being stabilised against the actions of the clearing agents to be applied, and others extracted or rendered inert.

Dissolution of Protoplasm

The first step in many clearing techniques is to soak the tissue in a caustic alkali solution (Foster 1950, 1955; Arnott 1959). Loss of protoplasm through the ensuing hydrolysis and oxidation can be fairly complete after about 3 days at 40 C using a 5% NaOH solution (Gardner, unpublished), so longer

treatment is unnecessary. A weak solution of NaOH or KOH (2%–5% w/v) at a moderate temperature (30 C–50 C) is recommended to avoid alkaline degradation of cellulose in the tissue.

Phenolic oxidation is almost universal at this stage; dark pigments are produced by at least three mechanisms (Singleton 1972):

(a) Oxidative phenol-phenol coupling to give quinones and related compounds.

(b) Phenol-aldehyde condensation to give very stable polymers whose constitution depends on reaction conditions. Naturally-occurring aldehydes or those produced in protoplasm breakdown, e.g. furfural from carbohydrates, may be involved.

(c) Phenol-amine coupling, involving quinonoid phenol derivatives and amine-containing substances, e.g. peptides.

The affinity many of these polymeric phenols have for protein (cf. the true tannins) seems to result in a kind of protection for the tissue, and the rate of clearing in NaOH slows down. They may remain in the tissue as insoluble precipitates or diffuse through the alkali solution to be absorbed onto the cell walls of the tissue. Some improvement in clearing heavily-pigmented material is found if 50% ethanol is used in making up the alkali solution, and this solution should be changed several times over the 2–5 days needed for complete action of the alkali. Generally, tissue that blackens during alkali treatment will need to be bleached.

Acid has been used in place of alkali to dissolve protoplasm but is usually too vigorous in its action. Papain, a naturally-occurring protease, has been recommended by Rodin and Davis (1967) for dissolving NaOH-resistant protein. This gentle method seems worthy of wider attention, although Schmid (1972) found papain unsatisfactory in clearing highly tanniniferous flowers of *Syzygium* (Myrtaceae).

Postalkali Treatment

The phenolic pigments produced during alkali treatment will prevent an increase in specimen transparency even though loss of protoplasm has occurred. Repeated washing with a succession of warm water, warm 70% ethanol, and warm 95% ethanol can remove some of these pigments, and, if done thoroughly, may make unnecessary the strong bleaching usually required for decolorisation.

Another reason for poor transparency at this stage is the presence of crystals, commonly of calcium oxalate. If crystals are not the objects of the study, they may now be removed by bathing the tissue in warm dilute HCl for as short a time as possible (10% HCl for 2 hours at 40 C is usually sufficient). Removing crystals before alkali treatment is not recommended because the acid then needs longer to penetrate to the crystals and the specimen may become badly weakened by acid hydrolysis.

Slow attack on the crystals is carried out during alkali treatment and is accelerated by the presence of ethanol (Gardner, unpublished). Therefore,

studies of crystal distribution in cleared material must avoid prolonged alkali treatment. Artefacts of crystal distribution may be caused by differing rates of penetration of the alkali into different parts of the specimen. In this connection the replacement of the alkali by a protease, as discussed in the previous section, would be indicated.

Bleaches

Many oxidative bleaches have been recommended for removing the dark pigments that may be formed at any of the earlier clearing stages: hydrogen peroxide (Stebbins 1938, Sporne 1948), oxygen (Morley 1968), chromium trioxide, as in Stockwell's solution (Schmid 1972), hypochlorite (Debenham 1939), chlorine (Janes 1962), acidified sodium chlorite (Barghoorn 1948). These are certainly all effective but most of them can dissolve cellulose and lignin, and must therefore be used cautiously on fragile tissue. This is especially true of the bleaches which operate in, or produce, acidic conditions. A frequent problem is to ensure that a bleach penetrates rapidly enough to destroy the innermost pigments before the exterior of the tissue crumbles under the strong oxidation it suffers. A more uniform reaction can often be obtained by soaking the specimen with the appropriate solution for some time before allowing the bleaching action to begin, *e.g.* by soaking with dilute H_2O_2 in the cold and then allowing the reaction to proceed at room temperature under alkaline conditions. Of the bleaches, 20 volume H_2O_2 applied with an overnight soaking can be recommended. Most lightly-pigmented tissues are bleached overnight, and darker specimens need only a short additional time at room temperature. If chromium trioxide is preferred, it must be thoroughly washed from the tissue after bleaching to avoid green chromic ion formation *in situ* (Baker 1958).

Reductive bleaches do not appear to have been used in clearing techniques. A strong solution of sodium dithionite, NaS_2O_4, has proved satisfactory for tissues which are not deeply discolored (Gardner unpublished), and has the advantage of neutral operating conditions so that hydrolysis of cellulose is negligible. However, sodium dithionite will not destroy all pigments and heavily tanniniferous tissues tend to bleach to a brown color and remain more or less opaque.

The bleach to be used must not attack crystals if these are the objects of the study. Hydrogen peroxide or sodium dithionite are then the appropriate reagents, the acid conditions of chromium trioxide or the chlorine group of bleaches giving rapid crystal dissolution.

Infiltration With A Fluid Of High Refractive Index

The cellulose walls now forming the bulk of the specimen become transparent when infiltrated with a fluid of about the same refractive index and the denser lignified structures, the crystals which cannot be infiltrated, the dark

deposits of tannin *etc.*, all become apparent. The refractive index of cellulose varies according to origin and treatment but does not critically determine the infiltrating fluid to be used, most dense noncorrosive fluids being satisfactory. Specimens may be dehydrated and taken to one of the traditional clearing agents of microtechnique or infiltrated immediately with reagents such as lactic acid, strong solutions of phenol and chloral hydrate, or mixtures of these substances. The popularity of the latter group of clearing fluids probably stems from their ability to give a one-step clearing procedure, tissue simply being left in the fluid until it becomes transparent, a procedure which may take several months at room temperature. Sections of material treated in this way show that the protoplasts have shrivelled up and disintegrated, probably through osmosis and acid hydrolysis. Destruction of the protoplasts is more or less complete if acid is added to the clearing fluid as is sometimes advocated (Bisalputra 1961). It is normal in one-step procedures to warm or even boil the clearing fluid to hasten clearing; this frequently produces specimen discoloration, probably due to phenolic compounds again. This problem is accentuated if phenol itself is in the clearing fluid. Continued heating will not remove these pigments and the specimen must be washed, bleached, and infiltrated again. In this connection it is imperative to wash formaldehyde-containing fixatives out of tissues completely if clearing with solutions containing phenol. Generally, one-step procedures are only satisfactory for slender nontanniniferous objects, *e.g.* herbaceous stems.

Herr (1971) describes a one-step clearing and squash procedure for the study of ovule development, using a mixture of lactic acid, chloral hydrate, phenol, clove oil, and xylene; the frequent occurrence of phenols in the integuments of ovules from certain families, *e.g.* Escalloniaceae, and the cutinized, lignified or suberized walls of the integumentary tapetum and hypostase, may restrict this method somewhat, and as Herr (1971) suggests, necessitate pretreatment.

It has been claimed (Morley 1968, Schmid 1972) that strong bleaching, with or without NaOH treatment, is of little value in clearing studies because pigments are bleached *in situ* and tissue becomes snow-white and more or less opaque. However, examination of leaf discs that had been bleached with potassium chlorate/HCl and infiltrated with a variety of clearing fluids showed that much of the opacity was due to minute bubbles throughout the tissue; these could be removed by gentle heating to give greatly increased transparency (Gardner unpublished).

Some practical considerations guide the choice of the clearing fluid. It should not be so dense as to show prominent viscosity currents which obscure the specimen during dissection, nor should it give off an unpleasant or harmful vapor which might prevent prolonged observation. There are also chemical considerations relating to the possibility of testing for various substances in the cleared specimen (see next section).

Staining

Because of the variety of chemical changes they have undergone, cleared tissues may not exhibit their normal staining reactions. The most frequent problem here is to obtain a good differentiation between lignin, and cellulose which has become weakly acidic from NaOH treatment and bleaching. Morley (1949) discusses some schedules and Lersten (1967) gives others. Staining is usually done after clearing is judged satisfactory, but Gourley (1930) and Fuchs (1963) give methods for staining before clearing. Neither of these authors have a bleaching step in their procedures. As a general oversight stain and for vascular tissue, alcoholic safranin applied for 24–48 hours followed by a lengthy period of differentiation in 95% ethanol can be recommended. Specimens stained for lignin are usually dehydrated and examined in xylene; the hardening effect that this has on the tissue enables even the most delicate objects to be dissected—in this respect xylene is distinctly superior to lactic acid, phenol and chloral hydrate.

Instead of using basic dyes to demonstrate lignin, one or other of the lignin color reactions can be applied, this technique being most effective if the reaction can be carried out in a fluid which maintains the transparency of the specimen. Galaxazi (1965) and Bohm and Kisser (1961) demonstrate lignin in cleared tissues with phloroglucinol/HCl applied in acidic methyl benzoate, and in acidic chloral hydrate-phenol, respectively. The thiobarbituric acid color reaction with lignin (Berheim *et al.* 1953) can be applied in lactic acid or xylene-ethanol but is generally too faint to be of much utility. Since both the phloroglucinol and the thiobarbituric acid reactions depend on aldehyde groups in the lignin (Brauns and Brauns 1960), color development is fainter in NaOH-cleared tissue and is prevented completely by previous oxidative bleaching. If a chlorine bleach has been used, the specimen can then be soaked in a saturated solution of sodium sulphite when the lignin becomes pink-red (Jensen 1962) and this color is preserved to some degree if an alkaline infiltrating fluid is chosen, *e.g.* sodium lactate.

Conclusions

It is hoped that this attempt to bring together information on clearing techniques will give some basis for choosing between different methods when one has some elementary knowledge concerning the object under study (presence of tannin, crystals, *etc.*) .

Not all plant parts can be demonstrated by clearing techniques known at present. Phloem tissue is a major problem—it may be shown by polarisation microscopy (Bisalputra and Esau 1964), aniline blue staining (LaMotte and Jacobs 1962) , or aniline blue staining and fluorescent microscopy (Peterson and Fletcher 1973) but these methods are not satisfactory for bulky tissue. Other substances which are often well-localized and may be able to be shown in clearings include essential oils, proteinaceous latex, gums and mucilages,

and lipids. Development of clearing methods here would no doubt benefit comparative studies considerably.

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