SOME COMMON METHODS AND MATERIALS USED IN PLANT HISTOLOGY.

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Histology, in its broadest sense, is the study of the structure, chemical nature and functions of healthy and diseased tissue. The idea which is often prevalent that histology consists merely in the study of micro-anatomy seems to be somewhat too narrow. The subjects anatomy, physiology and histology are so interrelated that distinct boundary lines are impossible. All tissues are composed of cells and cell derivatives and their study, whether along physical, chemical, or morphological lines, must involve more or less of histological methods.

Owing to the similarity of the refractive indices of cell constituents, the study of living cells is very difficult and often unprofitable. The problem then confronts the student of killing and preserving the cell without altering its chemical nature, its minute detail, or sometimes even its gross structure. The killing, fixing and staining of cells offer their separate problems, which vary with the kind of tissue under consideration and frequently involve most complex physical and chemical factors.

The purpose of this paper is to describe some of the more common materials and methods used in histological research. Part of these have been used by the writer. The remainder are added for the sake of completeness.

Since tissue examined under the ordinary compound microscope is viewed by transmitted light, delicate instruments are necessary for the cutting of thin sections of the tissue. It is often desirable to obtain sections one cell in thickness to avoid the confusion due to superimposed cells. This necessitates still more accurate instruments.

Apparatus.

The following is a list of the apparatus used in the present study. It includes the necessities for any general histological work: a microscope magnifying about 1200 diameters; a razor; a hone; a hand microtome; a rotary microtome with knife; a paraffin bath with thermal regulator; a scalpel; a pair of dissecting needles; a pair of scissors; a pair of curved forceps; a turn table; petri dishes; test tubes; stender dishes; minots; a water basin; a graduate (50 cc.); pipettes; slides; round covers; square covers; long covers; cover clamps; bottles.

The microscope should have two eyepieces, coarse and fine adjustments, a double nosepiece, two objectives, 4 mm and 16 mm., and an iris diaphragm and a condenser.

An old style flat sided razor may be used for hand sectioning. A better blad is hollow ground on one side and flat on the other. The ordinary razor hollow ground on both sides could not be used satisfactorily.

One of the first advances beyong the simple blade was made by Valentin who used two blades placed close to each other. When this knife was drawn through a piece of tissue a thin section remained between the blades. Consective sections however were impossible with this instrument.

According to Mann, in 1861, A. B. Stirling at Edinburgh constructed the first device for cutting consective sections of approximately the same thickness. This instrument consists of a brass well with a brass plate flush with its upper surface. The tissue is placed in this well on another plate which can be raised by means of a screw from beneath. The idea is commonly used in present day hand microtomes. A gauge was added by Rutherford in

1871 for determining the thickness of sections. Lewis later introduced a glass plate for the razor to slide upon while making the cut.

Numerous sliding and rotary microtomes have arisin since those beginnings were made. In these machines both the knife and tissue are held mechanically. The sliding machine may make either an oblique or a square cut while the rotary can make only a square cut. The latter instrument gives the most convenient series of sections from tissue imbedded in paraffin.

The most convenient of the many types of paraffin baths consists essantially of a water jacket, wells for the melted paraffin and imbedding tubes, thermometer, thermal regulator (when gas is available). There should be two wells for paraffin of different melting points.

The remaining miscellaneous articles require no description and will allow considerable variation to suit individual tastes and conditions.

Reagents.

It is useless to attempt in this paper anything like a complete enumeration of the agents in use for killing, fixing, and staining plant tissue. Only those which are in constant use in histological work will be considered. To give an idea of the relative amounts of agents desired, the quantities necessary for an average student during a three months' course are given. (Chamberlain).

Killing and Fixing Agents.

The formulae for the preparation of solutions from the following materials will be given later.

Absolute alcohol, 300 cc.; 95% alcohol, 2 liters; ether, 50 cc.; chloroform, 50 cc.; formaldehyde, 200 cc.; corrosive sublimate, 10 g.; chromic acid, 10 g.; picric acid, 5 g.; glacial

acetic acid, 25.cc.; hydrochloric acid, 50 cc.; osmic acid, 1% aqueous solution, 25 cc.; nitric acid, 100 cc.

Stains.

The following are some of the most reliable and widely known stains:

Haematoxylin-Delafield's: To 100 cc. of saturated solution of ammonia alum add drop by drop a solution of 1 gram of haematoxylin dissolved in 6 cc. of absolute alcohol. Expose to air and light for 1 week then filter. Allow to stand until sufficiently dark then filter and place in a tightly stoppered bottle. The solution should go through a ripening which consists essentially in the oxidation of haematoxylin into haematin. This requires about two months' time naturally, but may be accomplished in a few minutes by the use of hydrogen peroxide.

Eosin. 1 gram dissolved in 100 cc. of water or 95% alcohol.

Safranin. Dissolve 1 gram of safranin in 100 cc. distilled water and 1 gram in 100 cc. 95% alcohol, then pour the two together.

Orange Gentian. One gram dissolved in 100 cc. of distilled water.

Cyanin, Erythrosin, Gentian Violet. Make a 3% solution of analin oil in distilled water; shake thoroughly and frequently for a day; add alcohol to make the solution 20% alcohol. Add 1 gram of the dye to 100 cc. of the solution.

Pianeze's Solution (IIIb) for Differential Staining of Fungus and Host Tissue. Malachite green, .5 gram; acid fuchsin, .1 gram; martius gelb, .01 gram; distilled water, 150 cc.; 95% alcohol, 50 cc. The latter combined stain is not in general use but in tests made by the writer it promised well as a rapid, practical differential stain.

Alcohols.

Alcohol of any desired strength can be obtained from a more concentrated alcohol in the following way: Subtract the percent of the required alcohol from the percent of the alcohol to be diluted. The difference is the number of cubic centimeters of water to be added to a quantity of the higher alcohol equivalent to the lower percent. Example: Given 95% alcohol and required 15% alcohol. 95 - 15 = 80 = cc. of water to be added to 15 cc. of 95% alcohol to make 95 cc. of 15% alcohol. When the dilution has once been made the bottle can be marked to measure 15 cc. and 95 cc. Then the alcohol and water can be poured in to fill up to their respective marks.

Clearing Agents.

Xylol is the most common and widely known clearing agent. Other useful ones are carbric acid, turpentine, clove oil, cedar oil, and bergamot oil.

Methods.

While most of the technique involved in histological work pertains to permanent mounts, simple temporary mounts may often be used to almost if not quite as good advantage. For finding fungus mycelium in plant tissue a pair of dissecting needles may be used to tease up the tissue in a drop of water on a slide. When a cover is added to this the observing eye can often note more details than are possible in a series of carefully prepared sections. Softer materials may simply be placed without any treatment in such a drop and will often thus appear to as good advantage as any other way.

A convenient method for preserving fresh tissue such as the epidermis of a leaf is the glycerine method.

A ring of balsam is put on the slide by means of the turning table and 10% glycerine in water is put inside the ring. The tissue is put into the solution and the glycerine allowed to concentrate. When it has become quite thick it should just fill the ring. A cover is then added. An additional ring may be put around the cover to insure sealing.

Usually, however, for one reason or another, it is desirable to preserve sections of tissue under consideration in a still more permanent form. To do this requires a more or less elaborate procedure. This will be followed through in some detail.

Killing and Fixing.

Although it is customary to use the same reagent for both these processes they are notwithstanding separate and distinct processes. The purpose of the killing agent is to bring the life processes to as sudden a termination as possible, while fixation comprises those processes, physical or chemical, by which we preserve the tissue in a state approaching that of the living tissue. Material to be fixed should be cut into small cubes or strips so that the agent will not have to penetrate more than one-sixteenth to one-eighth of an inch. The solution should be greatly in excess; from ten to fifty times the volume of the tissue. The time required for the action varies with the tissue and agent used. In general, twenty four to forty eight hours will be necessary.

A few of the principal agents in use for killing and fixing should be noted here.

Formalin. Although less widely known, formalin is rapidly becoming one of the few reliable agents. It is slightly acid in reaction and should be neutralized to get best results. When this is done, formalin produces a comparatively smooth, homogeneous mixture of the cell protein. The action of formalin is slower than that of corrosive sublimate, hence it should be allowed

to act at least three days. For preparations to be sectioned at once a 70% solution is recommended. When it is desired to preserve soft tissue for some time it may be placed in a solution of 2% to 4% formalin in water. The formalin may be washed out with water when the tissue is to be used.

Picric Acid. This acid may be used as a saturated solution in water or 70% alcohol. One gram of the acid will saturate 75 cc. of water or alcohol. Picric acid 1% and $HgCl_2$ 2.5% make a powerful combination. The acid increases the already vigorous action of the sublimate. This combination precipitates practically all the cell solutes but in coarse form. When it is necessary to obtain finer details formalin may be added. When formalin is used fixing should not extend over more than 48 hours. Otherwise the picric acid overcomes the formalin or the formalin breaks up the coagulum. Picric acid alone should act 1 to 24 hours if cold, or if hot only a few minutes will be necessary. Alcohol should be used in removing the agent.

Chromic Acid. Chromic and its combinations are universally used in botanical work. While effective in a 1% aqueous solution it is generally used to best advantage with other agents, as sublimate, acetic acid, osmic acid. The proportions and time of action of this combination should be varied to suit individual cases. A standard solution is 1/2% each of chromic and acetic acids in water. It is believed that the most delicate tissue should be fixed for at least 24 hours and that in most cases 2 or 3 days will give better results. This agent tends to harden tissue and should be replaced by picric acid or other agents which tend to soften material when more obstinate tissue is to be sectioned.

The following formulae for Flemming's Fluid give good results in most cases:

A	1% Chromic Acid,	25 cc.
	1% Acetic Acid,	10 cc.
	Water,	55 cc.
B	1% Osmic acid,	10 cc.

Mix A and B just before using.

Ethyl alcohol. This alcohol in varying concentrations has been long and widely used as a fixative. Like formol it kills, fixes and preserves at once, which triple action makes its use simple and convenient. A 40% or 50% solution will probably give best results. The tissue may then be run up to the higher alcohols. If weaker alcohol is used, coagulation will not be uniform while on the other hand if strong alcohol is used coarse flocculi are formed and the protoplasm shrinks more or less, invariably. Absolute alcohol, however, does not cause serious shrinkage and when speed is desired this agent gives satisfaction.

The following mixture, known as Carnoy's Fluid has excellent power of penetration and requires only a few hours for fixing. The material may be washed for two hours in absolute alcohol and imbedded without delay:

Absolute alcohol,	6 parts.
Chloroform,	3 "
Glacial acetic acid,	1 "

Corrosive Sublimate. This material may be used alone in a 2 to 5 percent solution in water or 70% alcohol. One to ten hours will usually be found sufficient for complete fixation. In watery solution the agent is dissociated into HgO and

2HCL. When chlorides are present the demand for chlorine ions will be satisfied and the $HgCl_2$ will not be dissociated and hence the mercury cannot bombine with the albumin, which action is necessary for fixation. If NaCl be to $HgCl_2$ as 3:7 the formation of albumates is prevented. When tissue is fixed too long in sublimate the $HgCl_2$ is hydrolysed to form Hcl which may cause the precipitate of albumin to be dissolved. Material fixed in sublimate and removed to alcohol soon becomes brittle and hence should be imbedded promptly. The coagulating power of subhimate is increased by the addition of 1% of glacial acetic acid. The following combined formula has been used by the writer almost exclusively:

Gilson's Alcoholic-mercuro-nitric Fixing Fluid.

Corrosive sublimate,	5 gm.
Nitric acid, (80% approx.)	4 cc.
Glacial acetic acid,	1 cc.
Alcohol (70%),	25 cc.
Water, (distilled)	220 cc.

Care should be taken not to undo the work accomplished by the fixative ~~in working.~~ Aqueous fixing solutions with sublimate or chromic acid as their principal ingredient may be washed in water. Sublimate may be removed in alcohol. Solutions containing alcohols or picric acid must be removed by alcohol.

Dehydrating.

The next important step after washing is dehydrating. This is accomplished almost exclusively by alcohol which at the same time causes a hardening that is generally desirable and often necessary.

The schedule of dehydrating usually employed by the

writer is as follows:

15% alcohol		2 hours.
35% "		2 - 4 "
50% "		6 -12 "
70%, 85%, 95%, 100%,		24 " each.
3 parts alcohol:	1 part xylol,	24 hours.
2 " " :	2 " "	24 "
1 part " :	3 " "	24 "
Pure xylol,		24 "

The xylol is added primarily to remove the alcohol but it acts also to make the tissue transparent. Hence it is called a clearing agent. Benzene may be used in the same way. The agent must be a solvent of both alcohol and paraffin. (In the celloidin method the process differs after the absolute alcohol).

Imbedding in Paraffin.

Paraffin should be added very gradually. A small block may be added to the tissue while still in the clearing agent. This will dissolve slowly and make the infiltration gradual. When the clearing agent has been almost saturated with added paraffin the solution should be poured off and pure melted paraffin should be added. This is kept on a paraffin bath at a temperature about 1°C. above the melting point of the paraffin. Changing of the paraffin may be necessary to remove all the clearer. Failure to do this may result in granular paraffin difficult to cut. It is best to have one grade of paraffin melting at about 45° C. for the first half of infiltration and another melting from 50° to 54° C. for the second half. Opinions vary widely as to the time necessary for infiltration. The minimum will probably be one hour. The writer has used 24 hours for each grade of paraffin with good results. The tissue may be imbedded in any container which allows of rapid cooling.

Small paper trays are very good. A little paraffin should be poured in and allowed to begin hardening. When it is firm enough to support the tissue it is added with just enough paraffin to completely cover it. The tray should be placed in a basin of water and immersed completely as soon as the paraffin is sufficiently firm to allow it. This rapid cooling is necessary to prevent crystallization of the paraffin.

For imbedding in collodion the process is the same through the absolute alcohol as that for the paraffin method. The tissue is then placed through the following:

12 to 24 hours in equal parts of alcohol and sulphurous ether.
12 to 24 hours in sulphurous ether.
24 hours in dilute collodion (1%).
24 to - hours in thick collodion (5%).

The material is then imbedded in thick collodion and as soon as the surface glazes it is transferred to chloroform vapor or liquid chloroform for 12 to 24 hours. It is then transferred to equal parts of glycerine and alcohol where it is kept until needed. Colloidion is pure gun cotton made by a special process. Collodion is a solution of gun cotton in a mixture of alcohol and ether.

The collodion method is of value because material can be sectioned which could not be cut in paraffin. It has many objections, however. A series of sections cannot be obtained from collodion imbedding. The process is slower, and the sections are difficult to fix to the slide. Sections in celloidin cannot be stained in basic dyes since the celloidin being acid stains deeply. Paraffin on the other hand causes hardening of some tissue and may cause shrinkage when heated above 45°C.

Cutting Sections.

The oldest and simplest method of obtaining sections consists in grasping the object to be sectioned between the thumb and forefinger and drawing the razor freehand across the fore finger. Gradually materials such as pith, wax, and carrots were brought into use to hold unstable bits of tissue in position to be sectioned. Various microtomes were devised for holding the tissue and operating the knife. The ordinary hand microtome, although one of the simplest and oldest, is still one of the most useful. Where a little more care and time are to be put into the operation the sliding microtome, in which both tissue and blade are held mechanically, gives excellent results. The complex rotary microtome gives best results when very delicate paraffin sections are desired. The principal disadvantage of this machine is that an oblique cut cannot be made and hence only very soft tissue can be sectioned in this way. The paraffin is cut into blocks with at least two sides parallel. The other two are best trimmed in slight wedge shape. The base of the wedge and the supporting block are then warmed and the two brought quickly together. The joint can be strengthened by running a hot needle around the base of the paraffin block. The sections will come in serial order from the knife in a very delicate ribbon. The knife is hollow ground on one side and should be honed on that side only.

Cleaning Slides and Covers.

Before using slides and covers they should be passed through 10% hydrochloric acid and 95% alcohol for several hours each, rinsing between the solutions. Just before using they should be removed and dried with a linen cloth free from lint.

Fixing Sections to the Slide.

For convenience in staining it is necessary to fix the sections firmly to the slide. A convenient and effective agent for this purpose is prepared from 50 cc. each of glycerine and white of egg. The glycerine serves only to prevent too rapid drying. One gram of salycilate of soda is added as a preservative. A small drop of this mixture is added to the slide and spread very thinly. The slide is then flooded with water and the sections are floated on the water. The water is heated gently until the paraffin becomes smooth. The water is then drained off. There is danger of coagulating the albumen if the preparation is heated sufficiently to melt the paraffin. The writer has obtained good results with a 1% solution of gum arabic which will stand higher heating. In this case a very weak solution of potassium bichromate is used to flood the slide. It is not necessary to spread the drop of fixative.

Staining.

The paraffin is removed from sections by dissolving in xylol. This process is accelerated by gently warming the slide before passing to xylol. The xylol in return is removed by 95% alcohol. The preparation is then ready for the stain if alcoholic stain is to be used. If aqueous stains are used it will be desirable with thick sections to run the slide down through the alcohols to the 35% strength.

All the colored substances made from coal tar are derivatives of benzene. The principal compounds involved in dyeing, besides benzene, are its homologues toluene, xylene, quinone, naphthalene, anthracene. Color is produced by substituting a radical called a chromophone for a hydrogen of the benzene ring. Chromophores may be basic due to the presence of nitrogen, or acid when they contain oxygen compounds or compounds derived from them by substituting divalent sulphur or trivalent nitrogen. The entrance of a stain into tissue

may be physical only or it may react chemically with the tissue. A possible explanation of differential staining is that stains diffuse into different parts of tissue at different rates, possibly due to physical structure.

No specification, however general, can be laid down as to the time required for a stain to act. Each individual case must be left to the judgment of the worker. The stain can be intensified by dipping in water containing a few drops of ammonia. If it is too intense it may be reduced by dipping in 95% alcohol containing 3% of hydrochloric acid. The stain is removed in 95% alcohol as a rule. This is followed by clove oil, carbol turpentine or some other clearing agent. Xylol is then added to remove the clearer and to prepare for the balsam. The final clearer must be a solvent of balsam. Xylol may be used alone as the clearer. A drop of balsam is then added and a cover quickly placed over it. The sections should not be allowed to dry after the paraffin has been dissolved out.

A few of the commonest staining procedures should be mentioned.

Eosin. For staining fungus mycelium in woody tissue no stain or combination of stains has given better results for the writer than plain eosin in 1% alcoholic solution.

Fleming's Triple. This is a widely used differential stain. Remove the paraffin in xylol and wash in 95% alcohol; stain over night in safranin; reduce in alcohol if necessary and rinse with water; stain in gentian violet 3 to 15 minutes; rinse quickly in water; stain in orange gentian 1/2 to 1 minute; run through 95% alcohol and xylol and mount in balsam.

In a modification of this method the material is passed through safranin 30 seconds, gentian violet 1 minute, and orange gen-

tian 10 seconds or less. This permits a great saving of time and gives good results.

Haematoxylin and Eosin Special (Durand). Stain deeply in haematoxylin; wash in 95% alcohol and counterstain with eosin 5 to 10 minutes; remove the surplus stain with a cloth and clear in carbol turpentine; wash in xylol and mount in balsam.

This is an excellent combination for intercellular mycelium. The haematoxylin stains the cell wall, cytoplasm and nuclei. The eosin stains the fungus mycelium. The preparation should not be washed between the eosin and clearer. The clearer is composed of 3 parts of turpentine and 2 parts of melted carbolic acid crystals.

Planeze's III b. Wash in water and stain in this solution 15 to 45 minutes; wash in water and decolorize in acid alcohol; clear in carbol turpentine and mount in balsam.

This is a good differential stain for fungus and host tissue and has to recommend it that all the stains are combined in a balanced mixture, thus simplifying the application. This is a comparatively new stain and the extent of its use remains to be determined.

Labeling.

All labels should be placed in the same position on the slide both for the sake of order and for convenience in reading. The label should contain the following data: Name of tissue; plane of the section as transverse, tangential, or radial; stain used; date; name of worker.

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