Botanical Microtechnique

'***Microtechnique***' is the various methods by means of which, sections can be prepared for microscopic examination.

It aims, in general, to study the structure and functioning of cells and tissues.

Botanical microtechnique includes

- ***Preparation*** of botanical sections
- ***Staining*** of these sections

I- Paraffin Technique

'Paraffin technique' was regarded to be the general procedure called for the preparation of sections, while other methods are of special applications only.

A. Preparation of paraffin sections

There are nine essential steps in preparation of paraffin sections before staining has begun, namely

1-Selection of material
2-Fixation
3-Washing
4-Dehydration
5-Clearing
6-Infiltration (Impregnation)
7- Embedding
8-Sectioning (Cutting)
9- Affixing of sections on the slides.

1- Selection of material

For the study of ***normal*** structure, ***healthy*** and ***representative*** materials must be used, cut in suitable pieces (if necessitated).

For the study of mitosis, root tips can be picked either from germinating seeds, sprouting shoot or bulbs, or from a sandy soil-potted plant but **never from** a plant growing in the field to avoid the damage of delicate tissues by pulling up the plant.

Procedure

a. Fully ripen seeds were selected for uniformity of size and shape then soaked in tap water for 24 hr. ***Soaking water must be aerated or changed***, at least, every 12 hr to avoid quantitative and qualitative harmful effects of stagnant water on mitotic division (***Anoxia***).

b. Seeds were sown, vertically, in loose sand, with the ***hilum downwards*** to allow roots germinate straightly, for 3 days.

c. Seedlings with healthy, straight roots having almost the same length (about 4 cm) were selected. Root tips, 1 cm length, were ***severed***, washed with distilled water and ***immediately immersed*** in the fixing solution. **Never use** dry or wilted material.

2- Fixation

It is the process dealing with the treatment of the fresh materials with certain agents to ***stabilize*** and ***preserve*** their chemical and structural components in as nearly as the natural condition.

a. Fixed tissues…..Why?

Fixed tissues are preferably stained than living tissues. This is because

i- ***Fixation protects tissues*** from damaging factors as drying, autolysis and microbial attack (**putrefaction**).

ii- Fixation enables tissues to be **sectioned and stained more easily** than the living tissues through:

* ***Hardening of tissues*** by coagulating their proteins that constitute the framework of their cells.

* Producing ***optical differentiation*** by altering the refractive indices of constituents so that they may be better distinguished from each other.

* Acting as a mordant for certain stains whereas ***mordanting*** means allowing the color of the stain to be more fixed to the tissues.

iii- Fixed tissues can be made in ***permanent preparations*** in contrast to temporary preparations of the unfixed tissues.

b. Types of Fixation

i. Physical Fixation

This does not involve chemical fixatives. For example, **Desiccation** and **Heating** can be satisfactorily applied for some smear preparations as bacterial films. Then fixed films can be directly stained and permanently mounted as normal.

ii. Chemical Fixation

As the cell contains a large number of chemical constituents, so there is **no single substance** has been found to derive the optimum fixation for a given tissue. Chemical fixatives are usually consisted of several ingredients in such proportions that fulfill the successful chemical and structural fixation. Generally, it is better to use **more than one fixative** when initiating studies on unfamiliar tissues.

c. Some Precautions for Laboratory Fixation of a Material

i- ***Immediate Fixation*** is essential to avoid postmortem degenerations.

ii- ***Size of the specimen*** should be very small to be rapidly killed by its specific fixatives even they were of low penetration rate.

iii- ***Amount of the fixative*** should be, at least, 10-30 times the size of the specimen.

iv- ***Time of Fixation*** may range from few minutes to several days depending upon the characters of both the fixative and specimen.

Procedure

a. Severed roots were fixed for 24 hr, in freshly prepared ***Farmer's solution*** composed of 3:1 v/v mixture of absolute ethanol and glacial acetic acid.

b. Fixed roots were then kept in a refrigerator in 70% ethanol till use.

3- Washing

This is applied ***to remove the excess of the fixative*** used before proceeding with the next steps. This is because fixative may inhibit proper staining or leave precipitates (**artifacts**).

Procedure

Fixed roots were then washed 3 times with distilled water to ***get rid*** the traces of the ***excess fixative***.

4- Dehydration

This means the ***removal of any water traces*** from the fixed tissues to make them firm, hard and ready for the infiltration process. Furthermore, dehydration increases the visibility of the specimens.

Importance

The principle of the paraffin technique is to substitute paraffin wax for the water initially present in the tissue. Since water is not miscible with the paraffin wax, so; all the water must be eliminated firstly. This can be achieved by a process of gradual dehydration through increasing strengths of alcohol to absolute alcohol.

Procedure

Tips were dehydrated in increasing ethanol concentrations for their corresponding times.

Conc.	50 %	70 %	80 %	96 %	absolute (3 changes)
Time	3 h	overnight	3 h	3 h	overnight / each

N.B.

* Ethanol represents the most common dehydrating agent in class work. This is because other dehydrating agents, for example, may be ***toxic*** (e.g. **methanol**) or ***volatile*** (e.g. **acetone**).

* In research studies, 2,2-dimethoxypropane **(DMP)** may be used as a ***rapid dehydrating agent*** (1-2 min) as it instantly converts water in the tissue samples to **methanol** and **acetone**.

5- Clearing

It deals with the replacement of alcohol in the dehydrated specimen by a suitable agent (clearing agent) to be ready for the following infiltration process.

As alcohol is not miscible with wax, clearing agent (e.g. chloroform, xylol) is an intermediate having the property of **mixing with** the absolute alcohol (***dehydrating agent***) in one side **and with** paraffin wax (***infiltrating agent***) in the other side.

Since clearing agents render the tissues **quite translucent**, the process is, accordingly, known as '***clearing***'. It is worth noting that translucency of tissues is a suitable condition for their study by transmitted light.

Procedure

a. Dehydrated tips were cleared by a series of chloroform-alcohol at ratios of (1:3), (1:1) and (3:1), respectively for 2 h / each step.

b. Tips were finally placed in pure chloroform in stoppered bottles together with some pieces of paraffin wax (m.p. 56-58 ˚C), which was used for infiltration. Chloroform amount must be just enough to cover tips, so that it will not need prolonged evaporation later on.

6- Infiltration (Impregnation)

It is a process applied to ***support tissues internally by paraffin wax*** which infiltrated using a suitable solvent.

Procedure

a. Bottles were left overnight in the oven at 40 ˚C for the preliminary infiltration. This gradual infiltration will allow wax to penetrate to each cell of the material before the evaporation of chloroform.

b. Stoppers were then removed and more wax was added for 3 h.

c. Contents were then poured in a watch glass with enough molten wax at 60 ˚C.

d. Molten wax was changed many times till the complete evaporation of chloroform (as no odor could be detected) and the material become well infiltrated and ready for embedding.

Note that traces of chloroform would cause crystallization of wax and damage of plant cells.

7- Embedding

Root tips were embedded in paraffin matrix that serves to

a. ***support*** tissues externally against the impact of the knife.

b. ***stick*** the cut sections together in a ribbon.

Procedure

a. A tray (***boat***) made of stiff glazed paper (or with ***L-shaped brass pieces***) was placed on a hot plate (or warmed Petri-dish) then molten wax was poured.

b. Infiltrated tips were placed and arranged parallel rows (5 mm apart) using two warm needles.

c. Tray was left to float on the surface of cold water in a large container till the wax surface was solidified then the tray was ***rapidly***, and ***gently dipped*** to ***avoid the crystallization of wax***.

N.B.

Cast blocks should be ***stored in refrigerator*** to minimize or ***prevent bending*** of block, ***white areas*** or ***bubbles*** that may develop if blocks were kept at room temperature.

8- *Sectioning* (Cutting)

Material embedded in paraffin was almost cut using the rotary microtome at a thickness of **10-12 µ**.

Procedure

a. The block with embedded material is fastened in the proper position to the microtome holder using hot scalpel. The paraffin is trimmed rectangular (3 mm from each side), cutting away some of extra paraffin about the material.

b. Cool thoroughly before cutting.

c. Warm the knife slightly, ***don't stop cutting***, successive sections remain attached to each other in the form of ***straight ribbon*** which was received on a stiff paper using two needles.

N.B.

* **Curved ribbon may result because**

- A dull spot on the knife….shift it laterally or replace with a good one.
- Upper and lower edges are not parallel….trim with scalpel.
- Lower edge is not parallel to the knife edge….adjust it.

* **Separation of the specimen from the wax mold** during cutting indicates the improper infiltration; if so, ***repeat the infiltration steps***.

d. Cut the ribbon into suitable lengths considering the size of the cover and further expansion of ribbon warming.

9- ***Affixing***

Median paraffin sections are fastened to a very clean glass slide with an ***adhesive*** prior to staining. ***Gelatin-containing*** adhesives or ***Mayer albumen*** (equal amounts of egg albumen and glycerol, and traces of sodium salicylate) are used.

Procedure

a. Rub evenly the least amount of ***Mayer albumen*** over the entire surface of slide then ***float*** with tap water.

b. Place on it the ribbon pieces, ***warm gently***, ***without melting***, till sections are completely flattened.

c. Allow the ribbon to cool then drain excess water.

d. Leave for 24 h. ***Do not*** stain before complete dryness.

N.B.

Some gelatin containing adhesives (e.g. 1g gelatin + 90 ml distilled water + 10 ml formalin) can be applied for ***floating*** and ***affixing*** of ribbons ***simultaneously***.

B. Staining of paraffin sections

Chromosomes can be stained by 1% aqueous solution of the **gentian** (crystal) **violet** basic stain. As it is bleached easily in higher alcohols that are used for dehydration of sections, it needs **mordanting** with iodine and potassium iodide (1% in 80% alcohol).

Procedure

Place the slides in staining jars and stain as follows

Xylol (10-15 min) → Rinse in 100%-96%-70%-50%-30% ***alcohol*** → ***water*** → ***Gentian violet*** (10-15 min) → ***Water*** (wash) → ***I_2/KI*** (30-45 sec) → ***96% alcohol*** (2 sec) - ***100% alcohol*** (2 sec) → ***Clove oil*** → ***Xylol*** (twice, 15 min/each) → **mount** → **cover** → **Examine** when dry.

N.B.

***Avoid** the dryness of sections between two successive steps.

**** Clove oil*** removes the excess of stain till the proper density of color is attained. This is known as '***differentiation***'. At this step, the slide must be examined microscopically. **If color is bleached**, re-stain the specimen starting with absolute alcohol in step (2)

Mounting

Sections are usually mounted in ***Canada balsam*** as it dries quickly, has a suitable refractive index and prevents fading of staining color.

Covering

Avoiding air bubbles, sections are covered with a suitable cover glass then slides were dried before inspection.

C. Advantages and disadvantages of paraffin technique

1. Advantages

It gives **serial**, **thin** and **easily stained** sections.

2. Disadvantages

It is **not ideal** for **histochemical** studies as:

a. ***Fats*** are ***dissolved by the fixative***.

b. ***Enzymes*** and many chemical components are destroyed by heat.

II- Celloidin Technique

A. Comparison with paraffin technique

In this technique, material is fixed similar to that of paraffin technique but

1. **Dehydration and clearing** are done in absolute alcohol and ether for ***2 days***.
2. **Embedding** is in ***celloidin*** (nitrocellulose) instead of paraffin for ***6 weeks***.
3. **Cutting** is done by a special ***sliding microtome*** into ***thick sections*** (10-25 µ thick).
4. **Staining** is in a watch glass.

B. Advantages and disadvantages of celloidin technique

1. Advantages

a. Normal structures are preserved as **no heating** is used.

b. Techniques is applied for **brittle** and **stiff** (woody) **tissues** for which, paraffin wax is not the ideal supporting material.

2. Disadvantages

a. Technique is **time-consuming**.

b. Sections are **not serial**, **thick** and **difficult to be cut and stained**.