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EFFECTIVE MICROTOMY TECHNIQUE FOR WOODY PLANT SPECIES

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ABSTRACT
Microtomy is the technique where any tissue is processed and then sectioned (thickness upto micrometer level), using a microtome. It can be described as microscopic anatomy. Histology is the broad subject, which includes microtomy and study of the sectioned tissue sample. It is an essential tool of biology. Histopathology, the microscopic study of diseased tissue, is an important tool of anatomical pathology since accurate diagnosis of cancer and other diseases usually requires histopathological examination of samples. The trained scientists who perform the preparation of histological sections are Histotechnicians, Medical Scientists, and Medical Laboratory Technicians. Their field of study is called histotechnology.

INTRODUCTION
Plant anatomy is an exciting subject, which informs us about the internal organization of the plant tissues. In fact, structure and function of any plant is directly related to its internal tissue organization. And any disturbance in these aspects can be studied via studying the associated anatomical changes. Besides there are several characteristics, which a plant tissue possesses internally but is not, manifested externally, just like presence of internal vestigial organs in animal. History of microtome is more than 135 years. Purkinje was the first to use the microtome (to slice thin tissue sections) in something around 1850's using glacial acetic acid, potassium bichromate and Canada balsam in the preparation of tissue samples for microscopic examination. In 1872, the precision engineer Rudolf Jung and the pathologist Rudolf Thoma developed the first microtome in series production. This marked the beginning of a new era in histology (Artschwager, 1926; Bailey, 1956). Remarkable work in the field of plant anatomy using was done by Johansen and Cutter (Johansen, 1940; Cutter, 1971).
For the first time it was possible to cut tissue samples in reproducible thin sections for microscopic evaluation - an extraordinary precision for this time period. Bausch and Lomb's Company in 1890 - began production of microtomes.
Softer tissue of herbs with non woody stem can be cut easily in most cases even with hand blade. But for woody herbs, shrubs and trees' portions, microtomy with precision protocol is needed to get fine results.
The tissue is cut in the microtome at thickness of micrometer level. The tissue can be mounted on a slide, stained and examined using a light microscope. If the material is very soft or very hard such thickness can't be achieved through normal blade and through hand sectioning process. Serial transverse sectioning helps in studying every minor change taking place in a tissue or organ during any developmental stages (Negi *et al.,* 2011).

MATERIALS AND METHODS
Materials
Microtome

A microtome is a mechanical instrument used to cut biological specimens into very thin segments for microscopic examination. Most microtomes use a steel blade and are used to prepare sections of animal or plant tissues for histology. Besides rotary microtome which is used for sectioning animal and plant histological microscopic study, now there is a long list of specialized microtomes used in varying cases.

Principle: When the external wheel is rotated manually, a dog pushes (to a fixed distance depending upon the micron to which we have set the microtome) another wheel inside the microtome (internal wheel). This internal wheel is in turn attached with a block holder, which move forwardly upward and downward. During this forward movement the material present in the paraffin block is cut as fine ribbon (See Fig. 1, 2)

Operation: First with the help of a knob, set the width of the section to be cut, about 5-15 micrometer. Attach broad 3-4 mm thick base of the paraffin block to the microtome wooden peg using warm scalpel. Insert or clamp the microtome peg into the microtome. Move the wheel of the rotary microtome rhythmically for obtaining continuous, straight ribbon of paraffin sections.

Uses: Using microtome, the tissue is cut at thickness varying from 1 to 20 micrometer. The tissue can be mounted on a microscope slide, stained and examined using a light microscope. If the material is very soft or very hard such thickness can't be achieved through normal blade and though hand sectioning. Through serial transverse sectioning it helps in studying every minor change taking place in a tissue or organ during any developmental stages.

Glass wares And Accessories:

Slides, coverslips, sharp steel blade, scalpel, wooden peg/block, dropper, needle, forceps, hot plate, filter paper, staining jars, oven and measuring cylinders, funnel, spirit lamp reagent bottles.

General Solutions:

Fixatives: The tissues are mechanically and biochemically stabilized in a fixative. Here tissues are killed rapidly by placing them in special chemical called fixative. Most common fixatives include- Formalin-Aceto-Alcohol (FAA) and Cornoy's fluid.

Formalin-Aceto-Alcohol (FAA)

Formalin-Aceto-Alcohol (FAA) is prepared by mixing formalin, acetic acid and 70% ethanol in a proportion of 1: 1: 18. Similarily Cornoy's fluid is prepared by mixing absolute ethanol, chloroform and glacial acetic acid in a proportion of 6:3:1.

Principle: Fixatives increase optical contrast between structures, this occurs due to irreversible changes in the colloidal system of the protoplasm. The changes in the optical differentiations are due to the destruction of the colloidal system of the protoplasm. This destruction of unwanted structures actually increases the optical density of the protoplasm.

Use: By this method original shape and structure of the tissues are maintained in life- like conditions, and tissues harden so that the tissues can be cut. It makes the cellular invisible or barely visible structures easily observable.

Chemicals and Other Requirements: For preparing 100ml FAA, we need Formalin- 90ml, acetic acid- 5ml and 70% ethanol- 5ml. For 100ml cornoy's fluid we require- absolute ethanol, chloroform and glacial acetic acid in a proportion of 60ml, 30ml, 10ml respectively. Other requirements include measuring cylinder, funnel, and reagent bottle 100ml.

FAA preparation:

Measure formalin- 90ml, acetic acid- 5ml and 70% ethanol- 5ml, with the help of a 100 ml measuring cylinder. Pour them in a 100ml reagent bottle. Mix thoroughly. Dehydrating reagents: Reagents, which possess hygroscopic properties, are those most commonly used for dehydrating tissues. The perfect dehydrating fluid is one which mixes well with water, ethyl alcohol, DPX/balsam and paraffin and which doesn't produce desiccation of the tissues. Most commonly used dehydrating fluid is tertiary butyl alcohol (TBA).

Methods: A series of increasing percentage of dehydrating reagents is prepared by mixing TBA with absolute alcohol and water as given in the table ahead:

Table 1: Dehydrating series used in Plant Microtomy

S. No.	Dehydration series (containing approx. total % of alcohol)----	50%	70%	85%	95%	100%
1.	Distilled Water (ml)	50	30	15	-	-
2.	95% Ethyl alcohol (ml)	40	50	50	45	-
3.	TBA (ml)	10	20	35	55	75
4.	100% Ethyl alcohol (ml)	-	-	-	-	25

Principle: The dehydrating fluid is mixable in water as well as alcohol. So when any tissue is placed in the fluid the fluid enters inside the cell/tissue as the protoplasm consists of water. Since the concentration of alcohol is negligible inside the cell the alcohol from the fluid will replace the water inside the cell, as per the principle of osmosis. As we go on increasing the percentage of alcohol in the dehydrating (see table, from 50% to 100%) fluid the amount of alcohol will increase inside the cell and after the last step of dehydration water inside the cell or tissues will be completely replaced by the alcohol.
Use: dehydrating fluid, like alcohol, completely removes water from the tissue with itself. This alcohol is in turn soluble in liquid paraffin and the liquid paraffin in turn is again soluble in solid paraffin. So dehydration reagent helps in infiltration of wax into the tissue or cell, which in turn help hardening of the tissue and its smooth cutting through microtome.
Chemicals and Other Requirements: Distilled Water, TBA- 200ml, Absolute ethyl alcohol
Steps*:*
For preparing series of dehydration reagent i.e. 50%TBA series, mix the chemicals (serial no. 1,2,3 and 4) mentioned under the column 50% in a reagent bottle one by one.
Mix the chemicals thoroughly.
Similarly follow the above process for each and every remaining series (i.e. 70%, 85%, 95% and 100%).
Adhesive: they are the fluids or substances used for affixing paraffin sections to slides. Most common adhesives used in botanical microtechnique are Haupt's adhesives.
Haupt's adhesives:
Methods: for preparing Haupt's adhesive, one requires 1gm gelatin powder, 100ml distilled water, 2gm phenol crystal and 15cc glycerin.
Principle: its adhesive property is due to gelatin, which easily and stiffly adheres to the slide as well as with cell surface/ cell wall material on drying.
Use: It is used for affixing paraffin sections to slides, so that during dewaxing process sections remain stuck to the slide.
Chemicals and Other Requirements: for preparing Haupt's adhesive, one requires 1gm gelatin powder, 100ml distilled water, 2gm phenol crystal and 15ml glycerin.
Steps:
Dissolve 1gm gelatin powder in 100ml-distilled water at 30^0C.
Add 2gm phenol crystal and 15 ml glycerin. Stir well and then filter.
Embedding media: The most common technique is wax embedding. The samples are immersed in multiple baths of progressively more concentrated ethanol to dehydrate the tissue, followed by a clearing agent such as xylene and finally hot molten paraffin wax (impregnation). During this process, paraffin wax will replace the liquid paraffin: soft, moist tissues are turned into a hard paraffin block, which is then placed in a mould containing more molten wax (embedded) and allowed to cool and harden.
Methods: Pure bee wax can be procured from the market
Principle: Wax is soluble in liquid paraffin so when completely dehydrated tissue is kept in hot molten paraffin wax, the melted wax impregnates the tissue. During this process of impregnation, wax will replace the liquid paraffin inside the cell and soft, moist tissues are turned into a hard paraffin block, which is then placed in a mould containing more molten wax and allowed to cool and harden.
Use: It is hardening material, which supports the soft tissue, and help in smooth cutting of the material.

Chemicals and Other Requirements: Pure solid paraffin.
Stains:
To see the tissue under a microscope, the sections are stained (coloured) with one or more pigments. This is done to give contrast to the tissue being examined, as without staining it is very difficult to see differences in cell morphology. Safranin, light green, fast green, Hematoxylin and eosin are the most commonly used stains in histology and histopathology. Hematoxylin colors nuclei blue, eosin colors the cytoplasm pink. There are hundreds of various other techniques, which have been used to selectively stain cells and cellular components. Histochemistry refers to the science of using chemical reactions between laboratory chemicals and components within tissue.
Methods: Method of preparation varies for different types of stains, discussed under the subheading, steps.
Principle: Stains react specifically with the chemical constituents of the cell and give colour, which may be different from the colour of the stain itself. Light green stains the cytoplasm and also extensively used to stain cellulose cell wall. Similarly stain like safranin is used to stain lignified, cutinized, suberized and chitinized structures.
Use: Stains help to locate the subcellular structures by specifically binding with these structures and giving good contrast when observed under the microscope.
Chemicals and Other Requirements: Safranin powder, light green powder, distilled water, ethanol, reagent bottle, measuring cylinder, funnel.
Steps:
Steps for preparing various stains varies for different types of stains.
Safranin:
Dissolve 4gm of the dye thoroughly in 100ml 25% alcohol.
Light Green:
Dissolve 0.2gm of the dye thoroughly in 100ml 95% alcohol.
Ehrlich's Acid Haematoxylin:
Distilled water-100ml, absolute alcohol-100ml, Glycerine-100ml, Glacial acetic acid-10ml, Haematoxylin-2g and Aluminium ammonium sulphate-20g is taken.
Eosin Y:
It is prepared by taking Eosin Y-0.2g and 95% ethanol-100ml.

Procedure:
Get ready the Microtome, Slides, Long coverslips, 5-10ml glass vial, sharp steel blade, scalpel, oven, wooden peg, dropper, needle, forceps, hot plate, filter paper, reagent bottles, glycerin and small petriplate, 5%, paraffin processing and staining series, formalin solution, dehydrating series, staining series, DPX, small petriplate, spirit lamp, matchbox, glass rod, staining jars, haupt's adhesive.

Steps:

1. Fix the plant material in fixatives like FAA for 24hrs.
2. Transfer the material in 70% alcohol for long-term preservation.
3. Transfer the required number of plant material pieces in the TBA Series in increasing order of percentage of alcohol i.e. 50%, 70% upto 100%. Keep the material in each series for 8-12 hours.
4. Transfer the above-dehydrated material into 50% liquid paraffin (by mixing 50ml liquid paraffin and 50ml absolute alcohol) and then in pure liquid paraffin.
5. Fill molten wax in a clean 10ml vial and allow it to solidify slightly.
6. Place the plant material over the top of this solidified wax.
7. Keep the vial in an oven preheated at 60° C, for 12 hrs or overnight.
8. Next day morning again take out the tissue from the vial and place them in another vial having solidified wax, and keep it also for 8hrs.

9 Repeat step number 8 for another two times.
10 Apply glycerin over the internal surface of a petriplate and then add pure molten wax in the petriplate.
11 When the wax is solidified at the bottom to the petriplate, with the help of a preheated forceps, place the tissue in the molten wax.
12 Leave the petriplate to solidify.
13 Put the petriplate in a trough full of cold water.
14 The wax will float in the water after some time.
15 Trim the block and attach it to the wooden peg.
16 Attach the wooden peg to the microtome.
17 Set the angle of the blade properly.
18 Set the micron to 10 and move the wheel of the microtome.
19 Collect the paraffin ribbon on a filter paper.
20 Cut the paraffin ribbon into one inch long pieces with the help of a blade.
21 Take a microscopic slide, first apply one drop of haupt's adhesive to it uniformly with a glass rod, then add to it 4-5 drops of 5% formalin solution.
22 Now place the 2-3 paraffin ribbon pieces over the formalin solution.
23 Slightly warm the slide so that the ribbon may get straighten.
24 Discard the remaining formalin solution over a filter paper.
25 Again warm the slide so as to vaporize the remaining formalin solution present over the slide surface.
26 Keep the slide in any dust free areas for two three days for drying.
27 After drying process is over pass the slide to the paraffin processing vis-à-vis staining solutions in the following order (the entire processes will be performed in the staining jars and keep the slide at every step for five minutes except the first two steps which should be of 30 minutes each):-
28 Pure xylene
29 50% xylene (50ml xylene+50ml pure ethanol)
30 100% alcohol
31 75% alcohol50% alcohol
32 50% alcohol
33 25% alcohol
34 2% safranin (keep the slides for 30 minutes)
35 25% alcohol
36 50% alcohol
37 75% alcohol
38 Light green (only for 30 seconds)
39 100% alcohol
40 50% xylene (50ml xylene+50ml pure ethanol)
41 100% xylene (keep the slides for 30 minutes)
42 Take a slide from the staining jar (full of pure xylene), place it over the filter paper.
43 Apply immediately 5-6 drops of mountant like- DPX over the slide.
44 Cover the slide with long coverslip.
45 Store the slide in horizontal position at room temperature for at least 24 hrs.
46 Now the slide is ready for microscopic observation

Flow Chart/Diagram:

Plant material fixation 24hrs
↓
70% alcohol (preservation)
↓
Transfer in TBA Series in increasing order of percentage of alcohol i.e. 50%, 70% upto 100%. (Each step 8-12 hours)
↓
50% liquid paraffin
↓
Pure liquid paraffin.
↓
Fill molten wax in a clean 10ml vial
↓
Place the plant material over the top of this solidified wax.
↓
Keep the vial in an oven at 60° C (12 hrs or overnight)
↓
Change the wax similarly for two days
↓
Apply glycerin in petriplate and add pure molten wax
↓
Place the tissue in the wax.
↓
Leave the petriplate to solidify.
↓
Put the petriplate in a trough full of cold water.
↓
Attach block to the wooden peg
↓
Attach the wooden peg to the microtome.
↓
Set the angle of the blade properly.
↓
Set the micron to 10
↓
Move the wheel of the microtome.
↓
Collect the paraffin ribbon on a filter paper.
↓
Cut the paraffin ribbon into one inch long pieces
↓
Apply haupt's adhesive to the slide then add to it 4-5 drops of 5% formalin solution.
↓
Now place the 2-3 paraffin ribbon pieces over the formalin solution.
↓
Slightly warm the slide so that the ribbon may get straighten.
↓
Discard the remaining formalin solution over a filter paper.

↓
Vaporize the remaining formalin
↓
Keep the slide in any dust free areas for two three days for drying
↓
Pure xylene
↓
50% xylene (50ml xylene+50ml pure ethanol)
↓
100% alcohol
↓
75% alcohol 50% alcohol
↓
50% alcohol
↓
25% alcohol
↓
2% safranin (30 minutes)
↓
25% alcohol
↓
50% alcohol
↓
75% alcohol
↓
Light green (30 seconds)
↓
100% alcohol
↓
50% xylene
↓
100% xylene (keep the slides for 30 minutes)
↓
Take a slide from the staining jar (full of pure xylene), place it over the filter paper
↓
Apply immediately 5-6 drops of mountant like- DPX over the Slide
↓
Cover the slide with long coverslip.
↓
Store the slide in horizontal position at room temperature for at least 24 hrs.
↓
Now the slide is ready for microscopic observation

Precautions:

Microtome blades are extremely sharp, and should be handled with great care. Safety precautions should be taken in order to avoid any contact with the cutting edge of the blade.

Avoid traces of water in xylene and paraffin solution other wise the purposes will not be achieved.

Always keep the slides in wet condition when you have started de-waxing process till placing coverslip over the slide.
The blade should be sharp otherwise the sections may get brittle.
Crucial Remarks: Time durations mentioned above for dehydration and de-waxing process may vary depending upon the type of plant material. Like if the plant tissue is hard the time duration should be increased. Always take fresh plant material for microtomy and avoid dry or drooped plant parts, and instead use water fed plant material for microtomy.

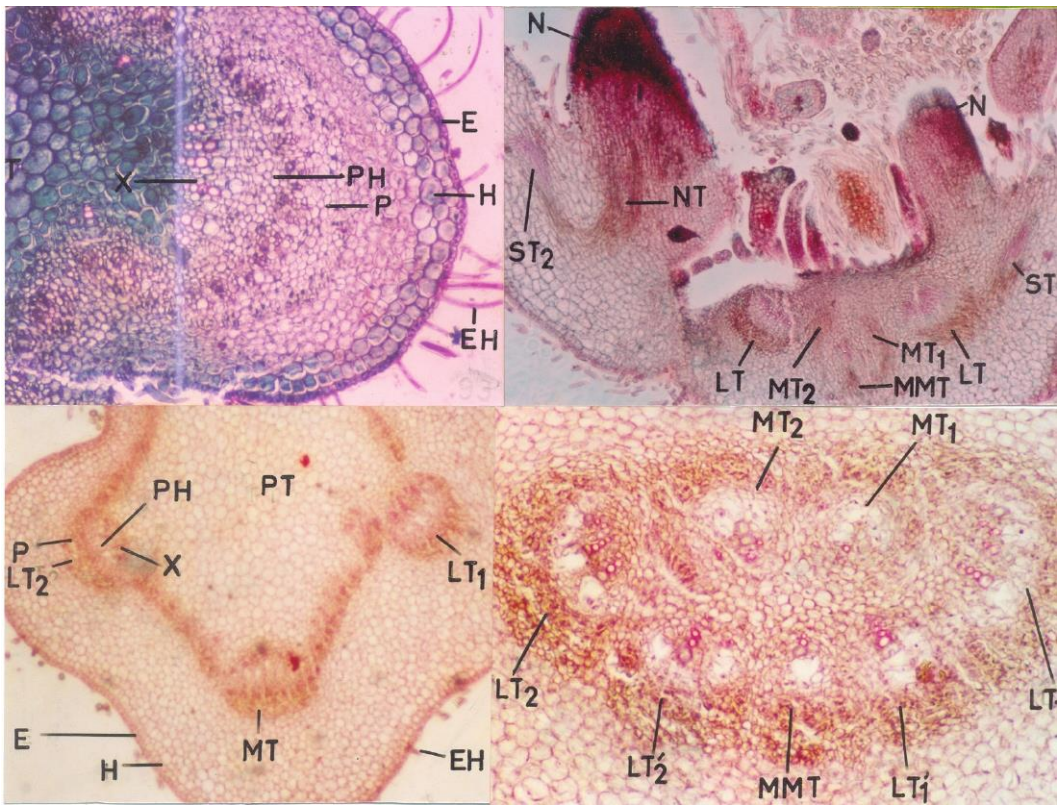

Figure 1: Microphotographs of nodal region in *Cassia auriculalta*

Figure 2: Rotary microtome

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