PLANT MICRO-TECHNIQUE

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BY

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Isolated cells from macerated herbaceous stem of *Pelargonium zonale*. Stained with safranin, dehydrated with hygrobutol, and infiltrated with balsam.

PREFACE

Four considerations prompted the preparation of the present text: (1) the acute necessity for a sifting and synthesis of the hundreds of methods and procedures that have been proposed during the past ten years of rapid development in microtechnique; (2) the need for a manual of modern botanical technique methods by botanists lacking special training in that field but who must prepare slides as part of their work in other fields; (3) to bring together the accumulated and mostly unpublished results of some sixteen years of extensive personal experience in collecting materials and preparing slides of plants from over the entire range of the plant kingdom; and (4) to provide an answer to the increasingly numerous enquiries received by the author concerning the methods he employs in routine and research work.

The main purpose of the book is to acquaint the user with the principles and procedures of all phases of botanical microtechnique. The specific aim is to enable elementary and advanced students, instructors and research investigators to prepare their own microscope slides of plant materials.

The text is in no sense an encyclopedia of botanical microtechnique methods. Many proposed fixing fluids, staining and dehydration methods, etc., have been omitted because it was concluded after thorough trial that they had not demonstrated their superiority over accepted procedures. With but few exceptions, every procedure cited has been carefully tested by the author or by students under his immediate supervision. Methods which are questionable under certain circumstances are so indicated. Such minor changes as might be required to adapt older schedules to modern conditions have been incorporated. In general, if a procedure or formula has not been credited to a specified person, the author may be held responsible.

The text has been divided, for reasons of convenience and experience, into two sections.

The first section describes the apparatus, reagents, dyes, etc., and the general methods universally employed by botanical technicians. Partiality in the selection of methods for presentation has been avoided, but it must be confessed that experience has engendered a preference for the paraffin over the celloidin method. Each method, however, has been described in sufficient detail to permit mastery in all its various phases. Inessentials have been eliminated and procedures made as concise and explicit as possible. The chapters on Whole-mount Methods, Smear Methods, and Cytological Methods constitute features that have never before been included as such in any text.

The second section takes up all plant phyla in phylogenetic order and gives as detailed directions or suggestions as are available for the treatment of specific groups in each phylum. The plants described are mainly those occurring naturally in the United States and Canada. The chapters on the algal phyla are particularly comprehensive. At the beginning of each chapter are given both general and specific suggestions regarding the collection, preservation, cultivation and manipulation of each phylum as an entirety, following which the orders and families are taken up in succession and more detailed directions are cited for genera and species. Innumerable procedures are described for the first time. Whenever references to other sources are not mentioned, the technical treatment recommended for a particular plant or group of plants is the one which has been found preferable from the author's personal experience.

References to the literature are intended primarily for the guidance of those who may wish to pursue a topic further, and secondarily to indicate the sources of statements or for more detailed descriptions of procedures. Only articles and books cited in the text, therefore, are included in the bibliography at the end of the second section.

A chapter on photomicrography has not been included because of the large number of excellent texts on the subject now available. Paleobotanical methods are omitted since suitable material is not available to most botanists and because the author has had insufficient personal experience in this field.

The figures have in general been selected to illustrate the results of a given technical treatment on the material concerned. Except as otherwise noted, all slides from which the photomicrographs have been made were prepared by the author. The photographs are practically all the work of a former student, Mr. John D. Poindexter.

For assistance in solving difficult technique problems and for a critical reading of portions of the first section, the author is deeply indebted to Miss Enid A. Larson. Portions of the second section have been read, and numerous suggestions made, by Dr. G. M. Smith. The author must also acknowledge the advice and assistance, particularly in connection with the commercial aspects of microtechnique, rendered by Dr. George H. Conant over a period of many years.

Botanical microtechnique is a science in which such rapid progress is being made at present that it is difficult for a person, without access to a large botanical library which receives all current journals, to keep abreast with proposed new procedures and changes in older methods. The author, therefore, would appreciate it if users of the text would favor him with reprints of their articles, or would otherwise keep him informed concerning new or improved methods.

STANFORD UNIVERSITY, CALIF.,
May, 1939.

DONALD A. JOHANSEN.

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CHAPTER I

INTRODUCTION

Immense strides have been made during the past decade in all aspects of microscopical technique, and even greater progress has been made in revealing the life histories of plants and their phylogenetic relationships. There has thus been rendered imperative an attempt to correlate the information provided by these two sources and to digest it in such a manner that it may be of the optimum service to workers in all the various botanical sciences. The present text constitutes an attempt at such a correlation.

Modern synthetic chemistry has made available innumerable reagents with which both botanical and zoological technicians have recently begun to experiment. Many of the attempts turned out to have been founded upon overoptimism: there seems to be currently prevalent a delusion that there exists such a thing as a single foolproof cure-all for every one of the difficulties with which technicians are continually confronted. Dioxan may be cited as an example: expectations at first were very high, but this reagent soon turned out to have only a limited, though valuable, application. Experiments with other reagents are continuing, and every encouragement should be extended to such efforts. However, statements with regard to the applicability of a new reagent should always be made with due caution: it would be well continually to bear in mind that the innately complex structural differences among plants will always prevent any one reagent or method from being of universal application.

Although the older methods and ideas in vogue among botanical technicians often verged on superstition, there are nevertheless many sound conceptions which have been evolved and have survived all attempts at obliteration. The more prominent of the older superstitions were that absolute ethyl alcohol must always be used to ensure complete dehydration and that a clearing agent must be used to render the tissues perfectly transparent before they were in a fit condition to be infiltrated with paraffin. On the other hand, methods immediately became more refined when botanical technicians got rid of a procedure, apparently borrowed from clinical laboratories, in which time is the essence of any method, and began to use a graduated series of reagents for both dehydration and infiltration. None of the newer reagents provided by modern

chemistry has demonstrated its ability to work successfully at a given single unitary concentration, and it is extremely doubtful if any ever will, for the nature of plants cannot be changed any more than can that of human beings.

Each species of plants must be treated as individualistically as are specimens of *Homo sapiens*. The days when both plants and people were treated en masse have passed. To deal successfully with a given species, the technician must know something of the life history, the manner through which each stage is passed, the physical structure and chemical properties of the various tissues composing the organism and each of its parts, and the probable reactions of the latter to the reagents which it is proposed to use upon them. To this must be combined an essential knowledge of the physical and chemical characteristics of each reagent and of dyes and stains, their interactions with one another, and their general effects upon plant tissues.

The amazing manual skill of the older technicians has been supplanted by procedures which are partly physical but mainly chemical. In place of learning how to wield a razor and how to keep it sharp, the present-day technician must learn how to handle chemicals. Probably no other profession so abounds with "tricks" peculiar to it, as does microtechnique. A technician becomes successful only to the extent that he masters these strategems, in addition to inventing a few more of his own. It is not possible to describe all the artifices. After a thorough mastery of methods, one instinctively learns about them and recognizes the moment when it becomes necessary to apply one of them. Some small or relatively insignificant matter very frequently determines the success or failure of a procedure. Accidents and near accidents occur all the time: the mark of the technician is his ability to avoid them and to plan detours around obstacles placed in the path by the plants themselves. Experience showed that there was more than one way of killing a cat; likewise, there are different methods of dealing with obdurate plant tissues. The only trouble is that the other and correct method is not always immediately plain; more or less experimentation may be required, but this occasionally gives one the chance to devise a new method which would be of great service to other technicians. As a matter of fact, very many methods have resulted because some one could not get the customary procedure to work as it supposedly should.

The beginning technician and those who are proceeding for the first time should always follow directions exactly as given. It is highly presumptuous to imagine that one knows more about the procedure, even before attempting to use it, than did the originator. One is not at liberty to proceed differently until and unless one is convinced that the nature of the material demands a digression. The formulae of most

reagents have been determined by carefully controlled experiments; the schedules for all the principal staining methods generally represent the combined experience of many competent workers over long periods and with all sorts of plant tissues under a variety of conditions. The iron-acetocarmin method, for example, as devised by Belling, has been standardized in its basic principles, and one should first learn to obtain exactly the same results as do other workers before one starts in to make modifications. Others are prevented from understanding and interpreting one's results if undesirable or unnecessary modifications are made in familiar procedures.

A word of encouragement is in order. Many occasions will arise when one experiences a feeling of helplessness when confronted with the task of dealing with an unfamiliar plant. Even experienced technicians often find themselves in such situations. Their procedure is to employ a killing and fixing fluid with whose reactions they are familiar, then to use dehydration, infiltration and staining methods with which they have had successful previous experience, and finally to observe the results in the completed preparation. A little study will quickly reveal where improvements should be made when the rest of the material is to be worked up. This is only a sort of trial-and-error method, of course, but if each step is carried out after due consideration, the result is generally fairly satisfactory, and only minor changes need to be made afterward. The beginner should follow the same procedure. If the plant concerned is not specifically described in the text, then the methods recommended for the most closely related forms should be used for guidance.

Persons who happen to be afflicted with color blindness are under a handicap in microtechnique since the ability to judge variations in stain reactions is one of the prerequisites for successful work.

CHAPTER II

LABORATORY RULES

These rules are the self-imposed regulations of all competent technicians; by following them, the beginning technician will avoid considerable trouble.

1. First and foremost: Keep everything clean.

2. Know what you are doing. If in doubt, stop at once, and orient yourself before proceeding further. Do not try to rush things: there is no more certain way of courting grief. There will, of course, be frequent occasions for becoming exasperated with the proverbial innate perversity of inanimate things, but one should never permit such irritations to exhaust his patience.

3. Keep your desk or table in order. Have a definite place for every object. Label all chemicals, reagents, and solutions; do not trust your memory or senses to recognize them.

4. Use only clean glass vessels in preparing reagents, except in such rare cases as when special containers might be indicated. Always clean the glassware while still damp, or place it temporarily in a dishpan with running water.

5. Keep your hands clean and dry, especially when mounting paraffin ribbons on slides. Be careful not to leave traces of poisonous substances (*e.g.*, mercuric chloride and phenol) on your hands or clothes. It would be wise to wear a rubber apron in order to protect the clothing.

6. Use acids with great caution. The fumes of most acids are extremely irritating. Always pour acids into water, never water into acids. If heat is evolved, add small quantities at a time and cool between additions.

7. Keep containers holding anhydrous solutions tightly corked or stoppered. Do not use the dregs, as of absolute alcohol, for completing dehydration, for such remnants are no longer anhydrous. Use vaseline or petrolatum on the covers and edges of all stenders and coplins containing volatile or hygroscopic fluids, if they are to be left standing for more than a day or two.

8. Keep card or other simplified records, as accurately as possible, of all materials. Record on each card all data concerning the manipulation of the material. Do not leave anything of consequence to memory.

9. In weighing solid chemicals, take care to avoid contamination. Protect the pans of scales or balances with paper.

10. Do not throw solids or celloidin solutions into the sink. Flush the sink with water when pouring acids or stains into it.

11. Do not use pipettes indiscriminately. Have one for each type of alcohol, one for xylol, one for stains, another for acids, etc.

12. Take extreme care to avoid contamination of osmic acid solutions: they are very expensive. Never breathe the fumes of osmic acid, nor permit them to come near the eyes.

13. Keep balsam containers out of the light. The balsam might become acid and is then ruinous to stains.

14. In collecting material, remember that changes in the cells occur rapidly after removal from the plant, from water, etc. Shorten the time elapsing between removal and placing into the killing fluid as much as possible. It should not exceed more than a few seconds. Avoid crushing. Remove all superfluous tissue and cut into as small pieces as practicable.

15. Before starting to kill and fix tissues, be sure you have selected what you consider to be the proper solution. Better still, use several different fluids at the first trial, and later select for future collections the one giving the best fixation. Make up the solutions and have ready for use before starting work on the plants.

16. Study staining schedules carefully before starting to use one with which you are unfamiliar. Make certain that all the reagents called for are at hand.

17. Budget your time both a day and several days ahead. Plan future operations far enough ahead to ensure that the most may be made of the available time. Experienced technicians frequently have as many as a dozen operations proceeding simultaneously.

18. Remember not to leave tissues too long in killing solutions, in the dehydrating fluids, and in the paraffin oven.

19. Examine all your preparations critically and also, whenever possible, obtain the opinions of competent specialists. Never be satisfied with mediocre results. Judge on the basis of killing and fixing, infiltration, microtoming, staining, and mounting. Be honest in your judgment, even if you feel that poor results were the fault of the material or the schedule followed. You will be a better technician if you blame yourself first and your materials and schedules afterward.

20. Finally, under no circumstances become discouraged if your first efforts culminate miserably, but *try again*. Seek for the cause or causes of the failure. As Bolles-Lee, one of the greatest of zoological technicians, has truly said, even the most experienced technicians often turn out perfectly atrocious preparations on their preliminary trials.

CHAPTER III

APPARATUS

It is presumed that a person commencing the study of plant microtechnique intends to familiarize himself, to some extent at least, with the majority of the different methods. On this basis, each student should provide himself with the supplies noted in the following summarized list. Some of the items will be discussed at greater length.

One or more boxes of slides of the standard size, 25 × 75 mm. (1 × 3 inches).
Coverslips: A medium thickness, generally known as No. 1, is preferable. As a start, a half ounce each of the following sizes will serve: 22 mm. squares, 18 or 22 mm. circles, and 22 × 40 mm. rectangles.
100-cc. graduate (also 50-cc. and 500-cc. sizes if desired).
A dozen ordinary pipettes.
Giant pipette.
Large all-steel scalpel, or sharp pocketknife.
Scalpel with ebony handle and long, thin, straight blade; intended primarily for trimming paraffin blocks for microtoming.
Several needles, in holders.
2 or more camel's-hair brushes, assorted sizes.
2 pair of scissors, small and large.
Forceps: a strong one for handling slides, and a narrow-pointed one for use with coverslips.
Waterproof India ink and fine-pointed pen.
12 or more solid (Syracuse) watch glasses.
10 or more Coplin jars.
8 or more Stender dishes.
Several flat staining dishes.
8 or more bottles of 100-cc. capacity.
Several bottles of 500-cc. capacity.
Alcohol lamp: (Use only clean ethyl alcohol; traces of xylol, etc., will cause coverslips to become smudged.)
Balsam bottle, with glass-rod dropper.
6 or more square Petri dishes (for smears).
Suction pump for water faucet.
Grease or china-marking pencil (red).

Microscopes.—A microscope is, of course, necessary, but all that is required in microtechnique is an inexpensive one with a low-power objective and a single ocular. A microscope intended for research purposes emphatically should not be employed, as the chances are that it will soon be somewhat damaged by chemicals and minor accidents. The stage of the microscope should be protected by a glass plate; a large

lantern-slide cover serves the purpose admirably. It should not be fastened to the stage, as it is frequently easier to move the glass than a wet slide placed upon the glass. Also one might wish to examine a completed preparation without getting it wet, for which purpose it is merely necessary to withdraw the glass plate.

If the student has not had previous experience in the manipulation of the microscope, he should first inform himself fully upon the subject. The optical companies usually provide small handbooks to accompany their own instruments. One can pursue with great profit Simon H. Gage's "The Microscope" (Comstock Publishing Company, Ithaca, N. Y., 15th ed., 1932).

Microtomes.—Although not required at the beginning, a microtome soon becomes an absolute necessity. The modern microtomes are, on the whole, very efficient precision machines. They are of two general types, the sliding and the rotary. Technicians are not in agreement as to which type is the best; the choice of either type apparently depends upon the possession of "mechanical ability." Those who have a feeling for mechanical skill will do their best work with the sliding microtome; beginners, and even those with little or no aptitude in the use of machines, should place their main reliance upon the rotary microtome.

The rotary microtome should be used only for sectioning material embedded in paraffin; in all other methods requiring the cutting of sections, a sliding microtome is indicated.

Many students find it desirable to begin with a simple form of the sliding microtome, and to progress to a larger and somewhat more complicated as well as more accurate machine as skill increases.

There are two types of rotary microtomes. In the older type, as exemplified by the Minot microtome manufactured by the Bausch and Lomb Optical Company, the forward movement is directly related to the up-and-down movement. For optimum results, the two movements should be separated, but satisfactory sections can be cut on the Minot models if due care is exercised. In the new Spencer No. 820 microtome, the horizontal and vertical movements are wholly independent, giving greater stability and precision. The universal joint clamp for holding the object in this microtome, though of simple and rigid construction, is a little difficult for beginners to manage.

Knives.—Microtome knives are easily available, not too expensive, and those intended for rotary machines are interchangeable. Each student will find it more convenient to purchase his own knife, rather than to depend upon a laboratory knife which is liable to have been handled by careless students. Some sliding microtome knives have special two-pronged handles for clamping into the sliding block. The personal possession of such a knife is at the student's option; if he pre-