THE ANATOMICAL STRUCTURE OF THE RHIZOME OF TWO GENERA IN THE FAMILY OPHIOGLOSSACEAE

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THE ANATOMICAL STRUCTURE OF THE RHIZOME OF TWO GENERA IN THE FAMILY OPHIOGLOSSACEAE

A Research Paper

Presented to

the Graduate Council of

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

James Stewart Johnson

April, 1971

Austin P

To the Graduate Council:

I am submitting herewith a Research Paper written by James Stewart Johnson entitled "The Anatomical Structure of Two Genera in the Family Ophioglossaceae." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

Floyd L. Brown
Major Professor

Accepted for the Council:

Wayne E. Stamp
Dean of the Graduate School

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The author wishes to thank his wife for helping in every way possible during the study.

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Section I

INTRODUCTION

Systematic studies of an anatomical nature have proven to be extremely valuable in the understanding of the various cell types and cell arrangements from a phylogenetic point of view and have assisted greatly in adding more naturalness to current systems of classification. For example, in the family Ophioglossaceae, two genera, Botrychium and Ophioglossum, appear completely different morphologically, but their anatomical structure is very similar.

Many studies involving anatomical descriptions have been concerned with relationships within a particular species or between related species. Each investigation has at sometime been concerned with the preparation of both temporary and permanent glass slides which would enable study over an extended period of time. In either case, the techniques employed must avoid damaging the delicate plant material as this would allow more accurate and detailed descriptions to be made.

This author's study has been concerned with the development of a technique for permanent slide preparation especially tailored for the rhizome of two mature ferns, Botrychium virginianum and Ophioglossum vulgatum, which occur in the Northern Middle Tennessee area.

Hopefully the photographs of several representative slides, prepared during the course of the study will present a clearer understanding of the extent of the study, and will also act as a foundation for future investigations of this nature.

Section II

LITERATURE REVIEW

In a literature survey conducted during the course of this study, it was noted that frequently the descriptions of cell types and their arrangements were vague. Bierhorst (1960) indicated that whenever terms such as "sclariform", "reticulate", and even "helical", "annular" and "pitted" are used without accurate descriptions and illustrations, the meaning of the author is in doubt. One possible factor leading to the misuse of terminology without accurate illustrations could have been the use of improper techniques in slide preparation, thereby, distorting the delicate plant tissue.

Throughout this study, it was noted that numerous methods and techniques for permanent slide preparation existed (Simpson, 1929; Steere, 1931; Wilson and Shutts, 1957). No one method or procedure suffices for all needs or purposes. Feder and O'Brien (1968) found that frequently the most common procedures destroy or distort the cell contents. Johansen (1940) stated that one purpose for the preparation of his book, Plant Microtechnique, was the necessity for a sifting and synthesis of the hundreds of methods and procedures that have been proposed during the past years. It is apparent that each method or technique must be adapted to fit the needs and purposes of the investigation being conducted.

Section III

METHODS AND MATERIALS

Plant material for this study was collected on two different occasions in separate areas. Botrychium virginianum, commonly called Rattlesnake fern, was collected May 4, 1970, on a semi-moist hillside about fifty yards west of the Ashland City Road (Tennessee 12) and 200 yards south of Brush Creek.

The material collected was in a mature stage with a fertile spike and sterile leaf blades. The average size of the plants collected was 18 centimeters in height with a leaf spread of about 9 to 13 centimeters. The rhizomes of the three plants collected were from 3 to 5 centimeters in length and averaged 2 millimeters in diameter. Each rhizome consisted of from 7 to 10 branches extending laterally just beneath the surface of the soil. The lateral rhizomal projections were for the most part unbranched and free of hair. The dark brown rhizomes were greatly contorted.

The second source of material was made available through the assistance of Mrs. Barbara Turner, Department of Biology, Vanderbilt University. Ophioglossum vulgatum, commonly called Adder's-tongue fern, was collected June 20, 1970, in the area of the junction of Couchville Pike and Anderson Road, near Percy Priest Reservoir.

Each plant, approximately 8 centimeters in height, possessed a fertile spike and a sterile leaf blade. The rhizomes of these ferns collected were smaller than those of the Rattlesnake fern in both length and diameter. The rhizomes of Ophioglossum vulgatum were 2 to 3 centimeters in length and averaged 2 millimeters in diameter. The brownish rhizome consisted of 5 to 7 lateral branches originating in the hypocotyl region. The lateral portions were unbranched and entirely free of hair.

After collections were made, each rhizomal stock was cut into short cylindrical pieces approximately .5 to 1 centimeter in length and placed in a solution of Formalin Aceto-Alcohol (more commonly known as FAA). These rhizomal stock portions were allowed to remain in this killing and fixing solution for at least 36 hours. The material was then rinsed several times with cold distilled water just prior to the dehydration procedure.

Each method or technique for permenent slide preparation has one common objective--the sections of plant material must resemble as closely as possible living tissue. It is virtually impossible to avoid tissue damage without first having some type of pre-treatment. Johansen (1940) and others have found that if the plant material, through preparation, can be made to have uniform hardness, then the friction of cutting and the resulting damage can be avoided. In recent years, paraffin, with a low melting point, has offered good results.

Replacement of the moisture in the various plant tissues with paraffin is facilitated through several steps. A dehydration series composed of several solutions of alcohol and water, followed by a second series of alcohol and xylol, will remove moisture, harden the plant tissue, and facilitate the infiltration of paraffin.

The methods and techniques used in the preparation of permanent slides generally follow those recommended by Johansen (1940). However, certain modifications in the procedure were established. For example, Johansen (1940) recommended the use of tertiary butyl alcohol as a dehydration agent but this author substituted ethyl alcohol. Another slight modification which allowed for a more rapid dehydration was the increase in concentration of alcohol in the dehydrating solutions. Johansen (1940) suggested a 10% change between steps, whereas, this author used a 20% change. Both modifications enabled a more rapid preparation of the material for study without any apparent damage of the tissue.

To facilitate the handling of the delicate plant material while in the dehydration phase of preparation and to prevent damage to the tissue, a one-half inch diameter glass tube cut into 2 inch cylinders was heated on one end and flaired slightly. A piece of nylon mesh was then placed over this end, draped down the side and tied securely with string. Five to ten rhizomal pieces could then be placed into the tube and the tube immersed

in the first of the ten separate dehydrating solutions thus allowing the dehydration solution to flow freely over and around the material. At the same time, it aided in the handling of the rhizomal material in bulk rather than individually. The rhizomal tissue was allowed to remain at each dehydration step for 24 hours before being moved to the next solution.

At the conclusion of the 10 day dehydration period, the rhizomal pieces, in a 100% xylol solution, were removed from the glass tubes and placed in several 20 milliliter vials, each containing approximately 15 milliliters of filtered 100% xylol. To each vial, approximately 5 milliliters of liquified paraplast (melting point, 56.5^{o}) were added. The vials were then allowed to remain at room temperature for 24 hours. For one week, at 24 hour intervals, a small portion of the solution in each vial was removed and replaced with fresh clean liquified paraplast. By the seventh day, the rhizomal pieces were in a pure solution of paraplast. The vials were then placed in an oven at 60^{o}, which allowed any remaining xylol to be removed by evaporation while ensuring complete infiltration of the plant tissues.

Small petri dishes, about 1 and 1/2 inches in diameter and 3/8 inches deep, coated with glycerine and warmed slightly, were filled with liquified paraplast. Each rhizomal piece was then carefully and quickly immersed in the paraplast filled embedding dish and oriented into rows

of three or four. A small strip of paper identifying the contents was placed on the edge of each dish. The material was allowed to solidify in ice and the cylindrical discs were removed.

When a large number of the discs had been prepared, each was cut into several smaller cubical portions, each containing only one rhizomal piece. Each cube was then mounted on a small wooden block, oriented in the proper position for the section to be made (C.S. or L.S.), and trimmed until perfectly square with a razor blade. This step was very crucial in later obtaining straight uniform ribbons during sectioning.

Sections ranging in thickness from 10 to 15 microns were made with the aid of an American Optical 820 rotary microtome. No microtome blade was available at the time of sectioning and an adapter fitted with a Gillette stainless steel razor blade acted as a rather poor substitute.

The ribbons obtained during sectioning, ranging from 6 to 8 inches in length, were cut into shorter strips. This allowed approximately 20 to 30 sections to be placed on a single glass slide in serial form.

Glass slides, cleaned in concentrated nitric acid for four or five days, were thoroughly rinsed in distilled water and allowed to air dry just prior to use. A small portion of Haupt's adhesive was spread uniformly over the slide surface and permitted to dry before being placed on

a thermostatically controlled slide warmer with a temperature level just slightly below the melting point of paraplast (50°). The smaller ribbons, containing multiple sections, were floated in distilled water, allowed to expand over the warmed surface, and align in straight rows before becoming attached to the slide surface. After a short period, the slides were removed, labelled and set aside in a cool place to await staining procedures.

In preparing slides of macerated material, a solution of potassium dichromate and sulfuric acid acted very well in removing the pectin from between cells, thus permitting good separation of the material. Small pieces of the rhizomal stock remained in this solution for 24 hours. The macerated material was then washed several times with cold distilled water, centrifuged at a low speed and the precipitated material collected. In order to speed the permanent slide preparation of the macerated material, this author devised a process which worked rather effectively. Haupt's adhesive and distilled water in a one to one ratio was first spread uniformly on a glass slide and allowed to dry. The macerated material was then spread over the adhesive and left to evaporate to dryness on the thermostatically controlled slide warmer at 50°. This material was then labelled and set aside for staining procedures.

Two coal-tar dyes which have proven to be dependable on sections of almost every type of plant material were selected for their ease of preparation and application. Safranin, which

selectively stains the lignin portion of the cell wall, and fast green, which stains the cellulose portion of the cell wall, together give a clear differentiation within the section. These coal-tar dyes enabled separation at a glance of the once living and non-living portions of the section.

Preliminary to staining, both the macerated and sectioned material require "hydration" which is a reverse of the dehydration process described earlier. Ten to twelve glass slides were placed in small staining dishes filled with the hydrating solutions and were permitted to remain 5 to 10 minutes in them before being moved. Those sections embedded in paraplast ribbons had to first be passed through the xylol-alcohol series while the macerated material could begin directly on the alcohol-water series.

When both the sectioned and macerated slides reached the 50% alcohol-water "hydrating" solution, they were immersed in a concentrated solution of safranin for three hours. The slides were then quickly rinsed in distilled water and dehydrated once again with only one minute intervals between solutions. Fast green in clove oil was administered after the slides reached the last of the xylol-alcohol series (100% xylol). The stain was briefly washed over the slide surface for about 10 seconds and the excess stain was poured off. The slides were quickly immersed in 100% xylol. Fast green in clove oil and the 100% xylol rinse perform two functions each. The fast green in clove oil will remove

any excess residual safranin while selectively staining the cellulose portion of the cell wall. The 100% xylol will remove any excess fast green while leaving enough of its solvent properties behind to aid in the attachment of the glass cover slide, the next step.

Once the slides were removed from the xylol rinse, they were immediately drained of the excess solution. A drop or two of Canada-balsam, the permanent mounting media, was placed on the slide surface and the glass cover slip was carefully placed over the tissue sections. The slides were oven dried for 4 to 6 days at 70°. The solidified excess Canada-balsam could then be easily removed with a razor blade and the finished slides were cleaned and labelled.

Approximately 200 multiple section permanent slides were prepared during this study. The instrumentation used in the preparation of the slides is pictured in Figure 1. From start to finish, the entire process encompassed approximately four weeks.

Since photographs often prove to be a valuable asset in any study of an anatomical nature and are also required for publication, several attempts to photograph the sectioned and macerated rhizomal tissue were undertaken. To insure good photographic quality, several combinations between film of different speeds and lens filters of varying capacities were investigated. Photomicrographs and exposures of a projected image were attempted.

The best results were obtained by first projecting the tissue image on a white semi-glossy surface and then taking several exposures of the reflected image adjusting the shutter speed and aperture of the camera diaphragm. By varying the distance between the camera lens and reflecting screen surface, and by changing the angle of incidence, a good quality of photographs were obtained. To insure good contrast and clarity within the photographs, various lens filters designed to elminate gray hues were also tried.

An Asahi Pentex Spotmatic camera, Kodak Tri-X 400 film and a Tiffen Photar Filter 15G (orange) proved to be the most successful combination. Approximately 100 exposures were made during the course of study and of those, 35% offered good contrast and clarity.

Section IV

OBSERVATIONS AND DISCUSSION

Paraffin sections, studied under a compound microscope, revealed that little tissue damage occurred as a result of the techniques applied to both Botrychium and Ophioglossum. The stains selected for use gave a clear differentiation and contrast with little residual debris. The sections studied revealed that the rhizome of both species were very fleshy with the cortex being composed primarily of starch-filled parenchyma cells. The stelar structure, differing somewhat in arrangement, occupied approximately 15% of the rhizomal tissue. The xylem, tetrach in arrangement in Botrychium and triarch in Ophioglossum, was easily differentiated from the surrounding tissue (Figures 2 and 4). Bower (1926) referred to the arrangement of the stele as being of a "quite usual construction with either a triarch or terarch arrangement of the vascular tissue." In other studies, the stele varied from a triarch arrangement (Boddle, 1901) to a pentarch arrangement (Baas-Becking, 1921). Wright (1920) referred to the rhizome of Ophioglossum vulgatum as consisting of a large, starch-filled cortex surrounding a siphonostele of endarch bundles of primary wood.

Figures 2 and 4 displayed a layer of cells, the pericycle, which separates the stele from the cortex. In Botrychium, it consisted of parenchyma tissue, two to three cells in thickness, surrounded by an endodermis two to four cells

in thickness. In Ophioglossum, the pericycle, essentially two cell layers in depth, was not surrounded by an endodermis, according to Wright (1920).

An attempt to photograph the vascular elements of the species under study presented some difficulty. Magnification and lens resolution had to be coordinated perfectly for the best results. The tracheary elements (Figures 3 and 5) were not clearly visible but those glass slides studied revealed the presence of spiral thickenings in both species.

The phloem sieve cell members (Figures 3 and 5) appeared in many instances densely protoplasmic with conspicuous nuclei. This would indicate that the methods used in the preparation of the rhizomal tissue may be satisfactory for intracellular examination. Further experimentation and modification will have to be attempted before conclusive evidence of the reliability of this method can be determined.

Section V

SUMMARY

It would appear that those methods developed and applied during this study were satisfactory for the rhizomal tissue of both Ophioglossum vulgatum and Botrychium virginianum as tissue suffered little damage. Those stains selected and used during the study gave a clear and distinct differentiation between the tissue types present, while leaving only a minimum amount of residue on the slide surface. While minute cellular details were not boldly apparent, the dense protoplasm and conspicuous nuclei of the phloem tissue indicated the possible adaptation of the methods used for intracellular study.

It is hoped that those methods and techniques developed during the course of the study will aid others in the anatomical study of delicate plant tissue. Even though each method of slide preparation essentially must be tailored to fit the plant material in question, perhaps, this work will act as a starting point for further modifications and investigations.

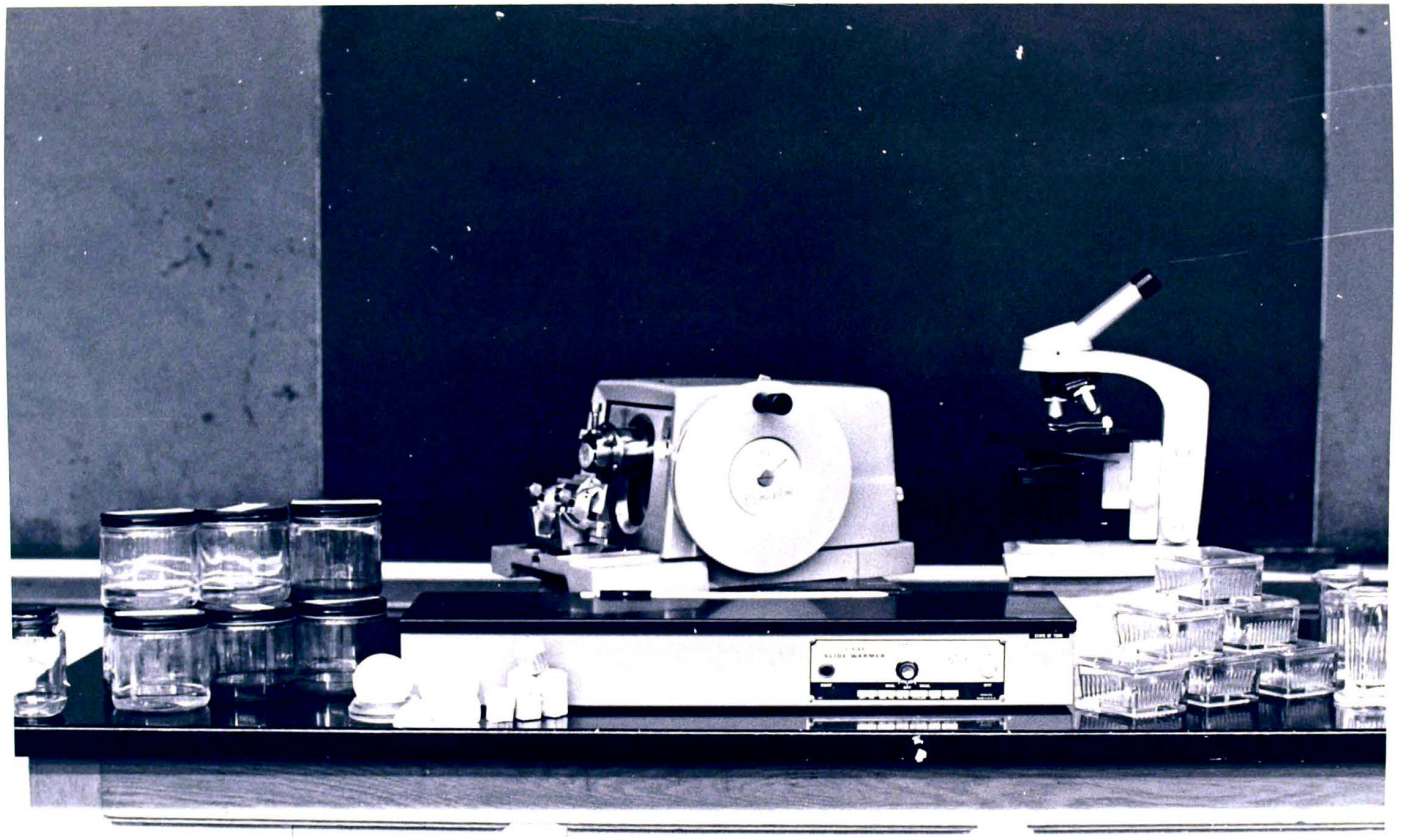

Figure 1. The instrumentation used during the course of study. From left to right: Killing and fixing solution; Dehydration series; embedding dish; paraplast cylinders; mounting blocks; slide warmer; rotary microtome; compound microscope and staining dishes.

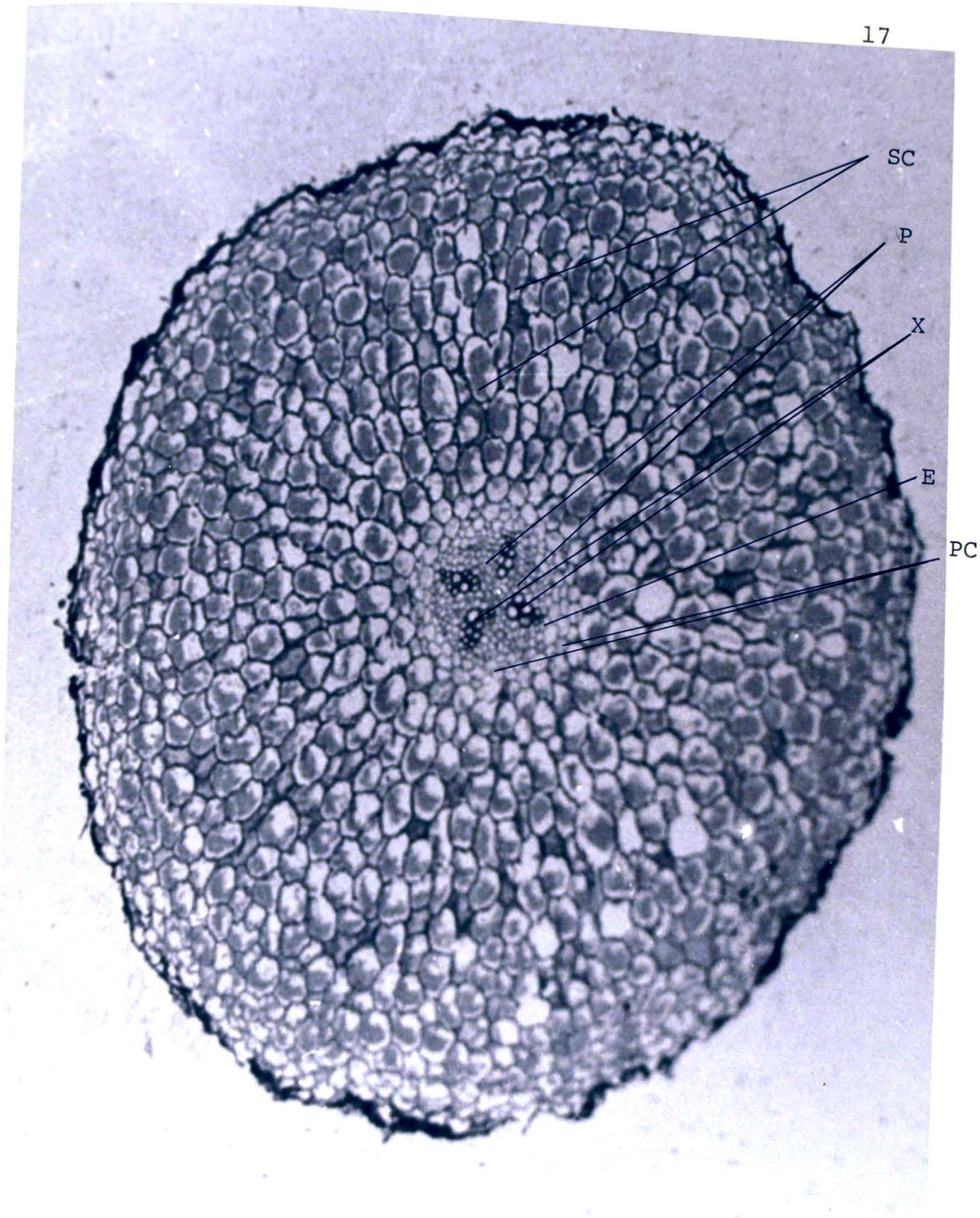

Figure 2. Cross section of Botrychium virginianum showing xylem (X); phloem (P); pericycle (PC); endodermis (E) and starch-filled cortex (SC).

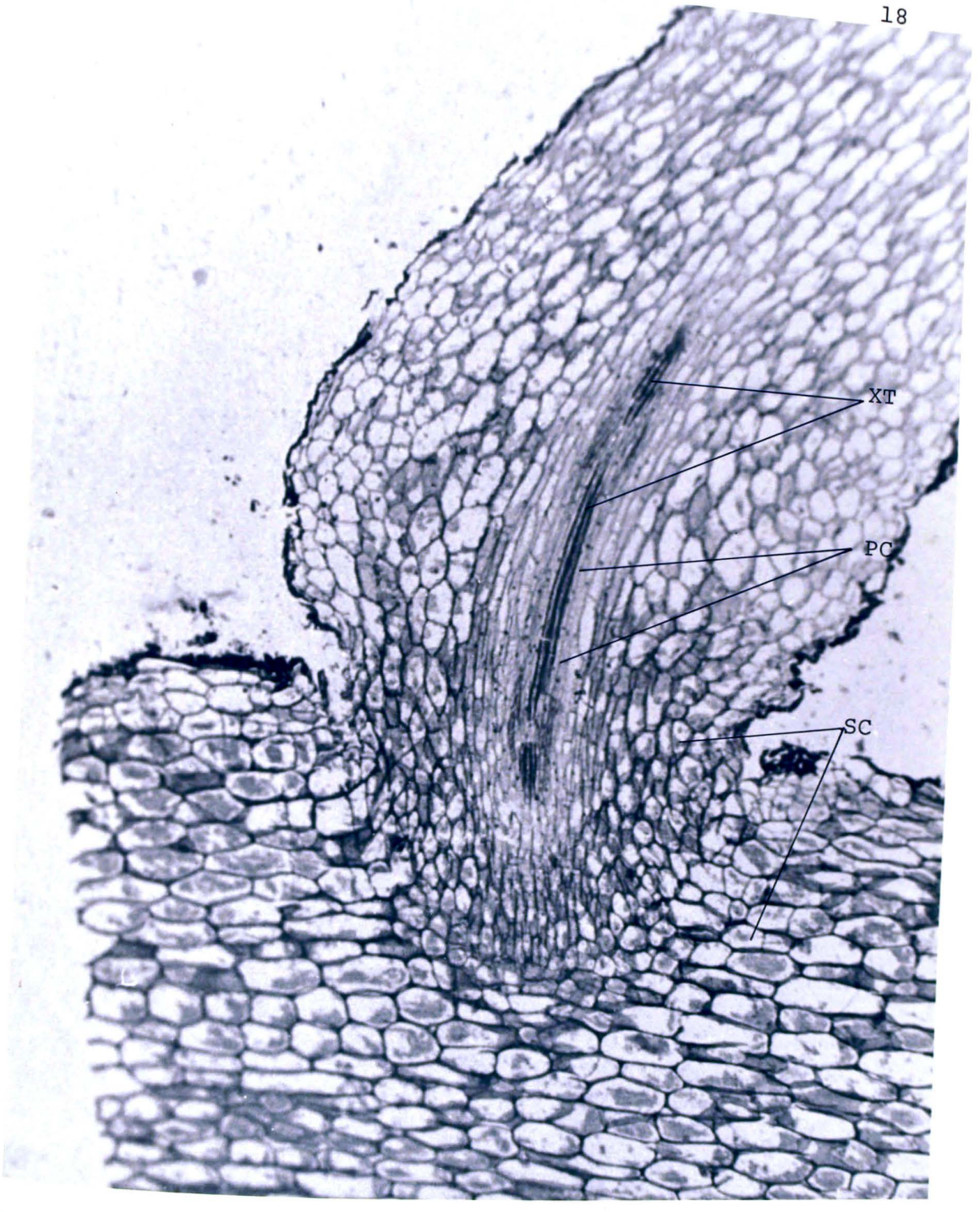

Figure 3. Longitudinal section of Botrychium virginianum showing xylem spiral tracheids (XT); phloem sieve cells (PC) and starch-filled cortex (SC).

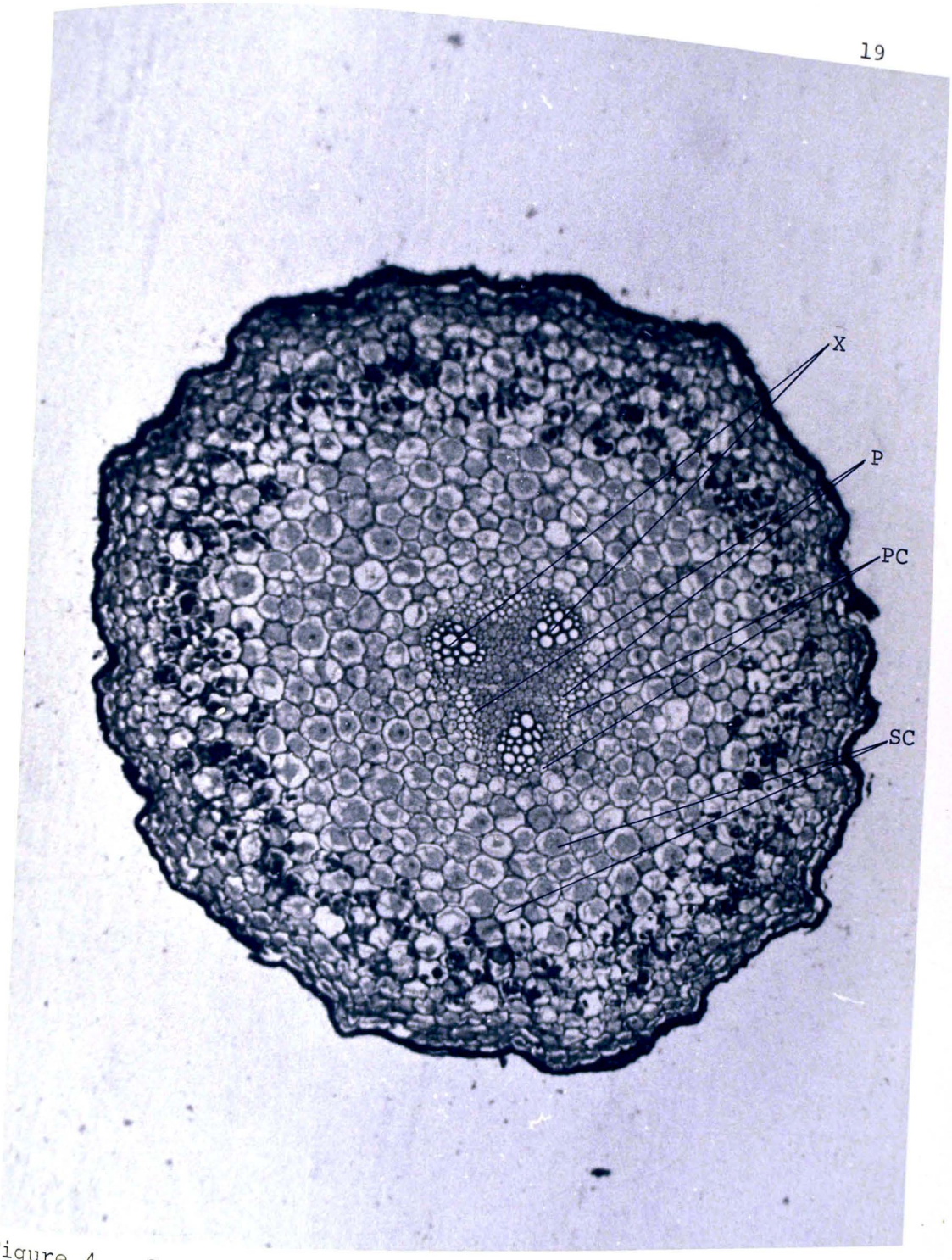

Figure 4. Cross section of Phioglossum vulgatum showing xylem (X); phloem (P); pericycle (PC) and starch-filled cortex (SC).

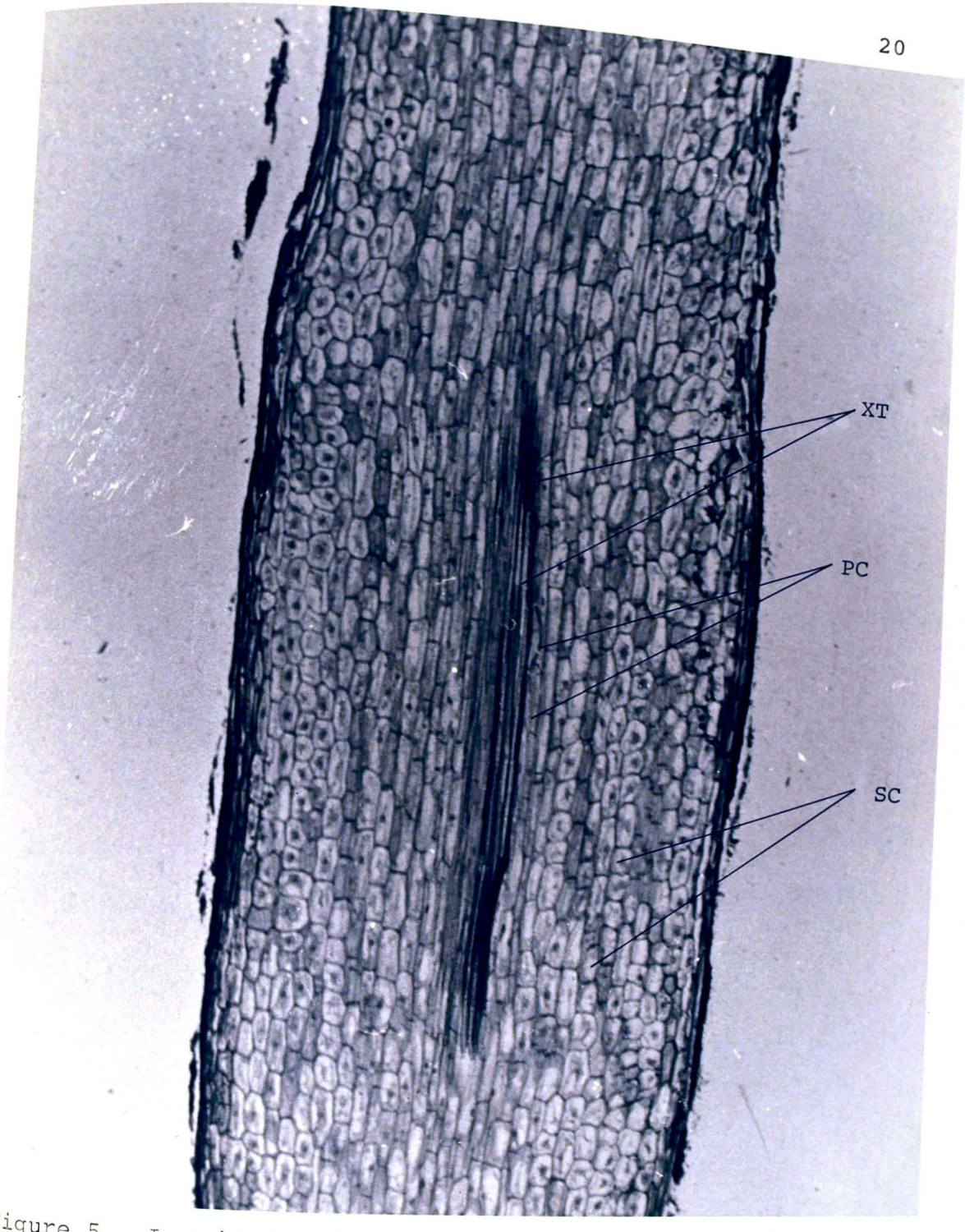

Figure 5. Longitudinal section of Ophioglossum vulgatum showing xylem spiral tracheids (XT); phloem sieve cells (PC) and starch-filled cortex (SC).

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