AN ABSTRACT OF THE THESIS OF

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(Name) (Degree)

in BOTANY presented on August 7, 1962
(Major) (Date)

Title: Anatomical Studies of Pinus ponderosa Laws. Infested by Elytroderma deformans (Weir) Darker

Abstract approved: [redacted]
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Pine needle blight of Pinus ponderosa Laws. caused by Elytroderma deformans (Weir) Darker is recognized by changes in the needles, in the bark and in the development of the branches. Anatomical studies of the host-parasite relation have been made previously on young pine tissue up to four years of age but not on older tissues. Neither has the pattern of invasion been worked out in individual plants.

Permanent microsections using standard methods of microtechnique were made on material collected from Round Mountain and from the Pringle Falls Experimental Forest near Bend, Oregon. Normal bark development was studied in noninfected trees from age one through 25. Comparisons made with infected stems revealed:

1. Whereas deep periderms do not occur in uninfected stems up to 25 years old they do occur in infected stems anytime

after the second year. The cork cambiums which arise within parenchyma cells of the primary and the secondary phloem in association with hyphal invasion of sieve cells ultimately produce pathological resin canals. Hyphal degeneration results and tissues external to the wound periderms become necrotic. These reactions are generalized responses such as any tissue makes to a foreign agent. However, there is no straight-line relationship between the intensity of tissue response and the severity of the invasion.

2. In stems, hyphae are confined entirely to the sieve cells of the phloem. They may spread vertically within a specified phloem layer and radially from one growth increment to the next.
3. When hyphae invade secondary phloem sieve cells close to the vascular cambium, the vascular cambium is stimulated to produce abnormal parenchyma cells.
4. Phloem horizontal resin canals are more abundant in infected than in noninfected tissue.
5. Sclereids occur in larger groups and more abundantly in both the cortex and the pith of infected stems.
6. Mycelia and microscopic anatomical changes may be present within stems which are macroscopically asymptomatic and bear green needles.

The distribution of hyphae within infected trees was determined by free-hand sections of suppressed ponderosa pine saplings up to 30 years old. Hyphae were found in stem tissue up to 20-years-old. Evidence is conclusive for hyphal growth from the trunk along branches toward growing tips and is suggestive of growth from apical meristems toward the trunk.

Anatomical Studies of *Pinus ponderosa* Laws. Infested by *Elytroderma deformans* (Weir) Darker

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

August 1967

APPROVED:

[redacted]

Professor of Botany
in charge of major

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Date thesis is presented August 7, 1969

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ACKNOWLEDGEMENTS

To Dr. Frank H. Smith, Professor, Department of Botany, Oregon State University, who directed this thesis, go my warmest thanks. I am especially indebted to him for his consistent advice on the anatomical aspects of the problem, for his critical review of the original draft, for his patience, and for his valuable suggestions concerning the final preparation. I am also grateful to him for the use of equipment and laboratory space.

The personal interest and encouragement of Dr. Lewis F. Roth, Professor, Department of Botany, Oregon State University, are most gratefully acknowledged. He provided the basic elements of the problem and counseled in mycological questions which arose.

More than any other single person my husband, Dr. John H. Wirtz, deserves my deepest appreciation. His assistance with photographic techniques and his persistence in the belief of this final accomplishment were endless.

To my colleagues, who sustained me with their humor in trying moments, wherever they may be now, I send my thanks.

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CHAPTER I

INTRODUCTION

The present study attempts to clarify certain anatomical features of *Pinus ponderosa* Laws. which accompany infestation by *Elytroderma deformans* (Weir) Darker. The disease caused by this fungus is commonly known as pine needle blight. Seven species of two- and three-needled pines reportedly are attacked but the effects are most extensive on ponderosa pine.

Resin cyst formation in the bark has been reported to be a symptom and the fungus has been identified in the phloem of needles, buds and young twigs up to four years of age. However, no detailed anatomical study has been made of older tissues despite the fact that distinct visible changes are present. Neither has the pattern of invasion been worked out in an individual host plant although the erratic distribution of necrotic phloem has caused speculation regarding the direction and manner of fungus growth.

Pine needle blight is widespread throughout most of the ponderosa pine forest, causing epiphytotic injury at sporadic intervals. Control is limited to timely salvage of infected merchantable

trees and spread of the disease remains unpredictable. A more complete understanding of the reactions of the host and of the persistence and direction of invasion of the fungus within the host, as presented in this study, may suggest solutions to these field problems.

CHAPTER II

REVIEW OF LITERATURE

Elytroderma deformans (Weir) Darker is an ascomycete which causes very serious needle blight of P. ponderosa Laws. (Western Yellow Pine). Other pines attacked, apparently in lesser degree, include P. banksiana Lamb. (Jack pine), P. contorta Dougl. (Lodgepole pine), P. echinata Mill. (Shortleaf pine), P. edulis Engelm. (Piñon Nut pine), P. jeffreyi Murr. (Jeffrey pine) (12; 25) and P. attenuata Lemm. (Knobcone pine) (7).

According to Darker (12) E. deformans is found only in North America. It extends southward from British Columbia to California and eastward from the Pacific Ocean to Montana, Idaho and Colorado with two isolated stations in Georgia and northern Ontario. Ellis (14) reported it from three national forests in Arizona while an unpublished memorandum by Gill to Lightle (25) placed it in New Mexico. In Oregon it is particularly prevalent in the eastern and central sections (20), especially in the Ochoco and Wallowa-Whitman National Forests (34) and also in the Deschutes National Forest (27) (Table I).

The disease on P. ponderosa has been serious at least as far back as 1913 when Weir's attention was first drawn to it (40). He described the species as a parasite on living needles of P. ponderosa from Sumpter in the Whitman National Forest, Baker Co., Oregon

Table I

Reported Locations of Elytroderma deformans (12; 14; 25; 27; 40)

State or Province	Specific Location	Collector	Pine Species
Arizona	Coconino NF	D. Ellis	ponderosa
	Kaibab NF	D. Ellis	ponderosa
	Prescott NF	D. Ellis	edulis
British Columbia	Thompson R.	J. Weir	ponderosa
		G. Darker	contorta
California	Lake Tahoe	E. Meinecke	jeffreyi
	Lassen NP	W. Wagener	ponderosa
	Latour State F	W. Wagener	ponderosa
	McCloud	Bynum & Miller	attenuata
Colorado	Mancos	Bethel & Payson	edulis
Georgia	Elijay, Gilpin Co.	G. Hedgecock	echinata
Idaho	Boise NF	J. Weir	ponderosa
	Clearwater NF	J. Weir	
	Coeur D'Alene NF	J. Weir	
	Kanisku NF	J. Weir	
	Nez Perce NF	J. Weir	
	Payette NF	W. Wagener	
	Pend Oreille NF	J. Weir	
	Salmon NF	W. Wagener	
	St. Joe NF	J. Weir	
	Selway NF	J. Weir	
Montana	Bitteroot NF	J. Weir; Hedgecock	ponderosa
	Cabinet NF	J. Weir	
	Deerlodge NF	J. Weir	
	Flathead NF	J. Weir	
	Helena NF	J. Weir	
	Jefferson NF	J. Weir	
	Kootenai NF	J. Weir	
	Lolo NF	J. Weir	
	Missoula NF	J. Weir; Waters	
	Sioux NF	J. Weir	
New Mexico	common	L. Gill	?
Ontario	Lake Tamagami	J.H. Faull	banksiana
Oregon	Crater NF	J. Pernot	ponderosa
	Deschutes NF	J. Pernot; L. Roth	ponderosa
	Grant Co.	G. Darker	contorta; ponderosa
	Hood River Co.	J. Boyce	contorta
	Malheur NF	J. Pernot	ponderosa
	Ochoco NF	J. Weir; P. Lightle	ponderosa
	Umatilla NF	Hunt & Childs	ponderosa
	Wallowa-Whitman NF	J. Weir	ponderosa
Washington	Colville	J. Pernot	ponderosa
	Okanogan NF		
	Wenatchee	J. Pernot	ponderosa

in 1916 under the name Hypoderma deformans. Childs (10) reported outbreaks in 1945 to 1955 from Oregon, Washington, Idaho and Montana. Wagener et al. (34, p. 195) also reported that in 1946, 1947, and 1948 "E. deformans developed in severe form on P. ponderosa in many localities east of the Cascades especially Ochoco and Whitman National Forests." In 1947 and 1948, it was almost as severe in the Boise and Payette National Forests of southern Idaho. During this time, heavy infections also developed in the Salmon National Forest in Idaho, and in Lassen Volcanic National Park and Latour State Forest in northern California. In 1957, Waters (39) expressed concern over a definite, rapid increase in the spread of the disease in western Montana. Field studies by Lightle (25) in California and Oregon during 1949 and 1950 and those of Waters reported in 1957 (39) showed that the fungus attacks trees of all sizes, from seedlings six inches high to mature trees. Both Wagener et al. (34) and Lightle (25) remarked that the attacks are usually heaviest toward the upper altitudinal limits for ponderosa pine. Actual economic loss has not been determined but it appears that without salvage cutting it would be extensive (25). Sikorowski (30) estimated that up to 50% of the saplings infected on the Deschutes National Forest are killed. Wagener et al. (34) reported a 90% mortality in parts of the Ochoco National Forest. Probably E. deformans damage to ponderosa pine is secondary in extent only to

that caused by dwarf mistletoes and root pathogens (37).

Weir (40) not only described and identified E. deformans but also performed spore germination and inoculation tests with reported success. He likewise described certain macroscopic symptoms, especially needle changes and witches'-broom formation. From the latter he deduced the perennating nature of the fungus in the tissues of the shoot but reported no further supporting evidence. The next major work was a taxonomic one by Darker (12) in 1932 in which about 50 species of needle cast fungi, including the newly transferred Elytroderma deformans, were discussed. Darker also mentioned the presence of hyphae in the branch tissue but presented no proof. Except for several field descriptions of infected stands by Wagener et al. (34), and Hunt and Childs (20), no further work was reported until 1954 when Lightle provided a description of host pathology and performed controlled experiments to clarify the life history of Elytroderma. His anatomical studies included a description of the formation of "pathological resin cysts" from parenchyma cells surrounding what he interpreted as degenerating phloem cells (25, p. 567). No suggestion of the role of these resin cysts was made. He postulated that degeneration of the phloem cells was caused by a phytotoxin produced by hyphae within the needles, and emphatically disclaimed the presence of fungal mycelium in the shoot. The studies of Waters (35 - 39), which were published in 1957, 1958,

1959, and 1962, showed the progressive development of his belief in the perennating nature of the fungus. In the earliest report (39) he agreed with Lightle regarding disintegration of phloem elements. In 1958, Waters (38) made an announcement before the Montana Academy of Sciences which was then published as an abstract (35). He said, "Hyphae have been found associated with all cases of disorganized phloem and it is now believed the primary phloem is the medium through which the pathogen travels within the host." However, no illustrations were published. Further experiments (36) using enzymes, specific stains, and tissue transfer techniques, convinced Waters that hyphae are actually present in primary phloem cells, not, however, until Roth (27) had experimentally established the perennial nature of the fungus. In 1962, in a paper published after his death, Waters (37) reported the presence of hyphae in one-year-old needles, in the axes of terminal buds and in the developing needles contained therein. He reported, then, that the fungus grows distally into new shoots from a previously infected bud.

Concurrent with Waters' work Sikorowski and Roth (31) reported the perennial infection in the bud and published photomicrographs of the mycelium in needles and buds. Both Sikorowski and Roth have contributed evidence pointing to the survival of the mycelium in the bark of young shoots and of stems at least four years old. Roth (27) suggested that the mycelium may extend back into much

older tissue. He considered that only the presence of a perennial infection could explain the erratic symptoms observed in the field.

Very little information is available regarding the normal structure and development of bark and phloem constituents in ponderosa pine. Chang (8; 9) in 1954, and Srivastava (32) in 1963, presented such studies for North American conifers in general, including the genus Pinus. Chang only briefly mentioned P. ponderosa. Srivastava gave more detail on this species but based his remarks on a single sample.

CHAPTER III

MATERIALS AND METHODS

Area of Study

Material used in this study included trees growing in the Deschutes National Forest. Asymptomatic as well as visibly infected saplings were sampled from the Pringle Falls Experimental Forest near Bend, Oregon. This is the same area from which Sikorowski's collections came (30; 31). Two asymptomatic trees were collected from Round Mountain, five miles northwest of Pringle Falls in an area free from any evidence of infection. Both of these areas are at an altitude of approximately 4700 feet.

Sampling Technique

The trees sampled varied in age and size as well as in symptom expression. Sampling was done in 1963 and 1964 during June, July, August and October. Samples included branches of asymptomatic trees, healthy appearing branches of infected trees, symptomatic branches of infected trees, junctions of asymptomatic-infected branches and junctions of infected-infected branches.

In order to study normal bark development in asymptomatic trees samples were taken at intervals from the bud through stems twenty-five years old. Each age level was represented by samples from five different trees. Samples of infected branches were taken at intervals from the bud through stems twelve years old. The number

of samples ranged from two to fourteen for each of these ages.

The degree of spread of the mycelium was determined by free-hand sections of each of the last 25 annual increments. One infected tree, potted in 1957, was sectioned from leader through roots.

Prior to each sampling, a free-hand sketch was made of the tree in question, and the locations of symptomatic branches and needles were indicated. Notations were made regarding the types of macroscopic lesions present, stem ages, and sampling points.

At first branches were cut from trees in the field, wrapped in wet paper, and transported, under refrigeration, to the laboratory for sectioning. Later, vials of water were taken into the field where branch segments were cut and dropped directly into the water to be transferred to fixative at the laboratory. Ultimately, entire saplings were cut off at the base and transported to the laboratory to provide material for either fixation or free-hand sectioning.

Microtechnique

Each sample of stem was cut into discs one-half inch long for longitudinal sections and about one-fourth inch long for transverse sections. The specimens were killed and fixed for twenty-four hours in Randolph's modification of Navashin's solution, prepared according to Johansen (21). Air was evacuated from all samples while in killing solution. The specimens were washed in running tap water for three hours, dehydrated by passing them through the tertiary butyl alcohol-paraffin oil series of Johansen (21) and embedded in

56-58° C., Fisher's Tissuemat. Embedded samples were soaked in a solution of glycerin and Dreft at approximately 37° C. (2). The soaking time varied from 48 hours to as long as two months for the older material. These were either sectioned immediately or stored in water under refrigeration until needed.

The softened tissue was cooled with carbon dioxide and sectioned at from eight to ten microns on the rotary microtome. Transverse, radial and tangential sections were made as needed. Transverse and radial sections usually included a small amount of xylem together with the bark. Sections of young tissues up to six years old included the pith. Haupt's adhesive was used for mounting the sections on the slides.

The progressive safranin-hematoxylin and the chlorazol black E-safranin techniques were used on permanent mounts. The details of the staining procedures followed Johansen (21) with slight modifications. Free-hand sections were stained with lactophenol-aniline blue (26), heated, and allowed to stand overnight before reading.

To illustrate the essential parts of bark structure as well as macroscopic and microscopic changes associated with the diseased condition, photographic and photomicrographic plates were prepared.

CHAPTER IV

RESULTS

Certain symptoms are usually associated with branches of P. ponderosa infected by E. deformans. In branch segments which are over two years brown necrotic streaking occurs in the inner bark (Fig. 1). Lightle (25) illustrated what he considered to be large resin cysts in the phloem zone which are conspicuous in transverse sections of an infected branch (Fig. 2). Often there is an abnormal branch rigidity and abnormal bark thickness (Roth, personal communication). Witches brooms, stimulation of lateral bud formation and dwarfing of branches have been described by several authors (4; 11; 25; 27; 34; 40). These same authors also stated that needle symptoms include reddening in the spring, premature dropping and the presence of fruiting bodies, either pycnidia in early spring or hysterothecia later in the season.

In my initial collections, a number of apparently uninfected branches were collected either from completely asymptomatic trees or from trees bearing the infection in branches at a distance from the symptomatic one. It soon became evident upon microscopic examination that a tree or a branch could be visibly asymptomatic yet have E. deformans hyphae and microscopic anatomical changes. It is thus not always possible to detect the presence or to trace the

extent and distribution of the mycelium from a macroscopic inspection alone.

The recent work of Srivastava (32) included in the introduction a summary of work done on the structure of phloem of gymnosperms. His paper and those of Chang (8; 9) provide the most specific information on the microscopic anatomy of the bark of Pinus. According to morphological characters, the genus Pinus has long been subdivided into two subgenera, Haploxylon or soft pines, and Diploxylon, hard pines or pitch pines (28). All the pines attacked by E. deformans except P. edulis belong to the subgenus Diploxylon. Chang described the bark structure of four species of this subgenus. A detailed description was given of P. echinata Mill. (9), P. banksiana Lamb., P. contorta Dougl. and P. elliottii Engelm. (Slash pine) (8). P. jeffreyi Murr. and P. ponderosa were mentioned briefly (8) and one illustration of ponderosa pine, a radial section of rhytidome, was presented (8). Other members of Diploxylon were in the specimen list but were not discussed in the text. P. edulis (Haploxylon) was listed among the specimens studied (8) but no further mention was made of it.

Srivastava (32) included P. ponderosa, P. jeffreyi, P. attenuata Lemm., P. muricata D. Don (Bishop pine), P. radiata D. Don (Monterey pine), P. sabiniana Dougl. (Digger pine), P. pinea L. (Italian stone pine) and P. murrayana Grev. ex Balf. (Lodge-pole

pine) of Diploxylon in his descriptions and illustrations. P. edulis was not studied. Although he obtained serial transverse, radial and tangential sections for each species, the species in each case was represented by only a single sample so that variations among individuals of a species were not allowed for. He mentioned that "the age of the samples varied considerably but was rarely less than twenty years" (32, p. 2). Thus he made no developmental study of bark of various ages.

On the other hand, Chang mentioned bark samples from two-to-five-year old twigs and other samples taken of "mature bark cut at breast height of average growth trees" (8, p. 5). Since Chang had collections from at least three trees in each species, and took both mature and young bark samples from the same species, the developmental history of the phloem in hard pines in general seems to be adequately covered. Specific information of P. ponderosa based on several representative individuals, however, is lacking. Such information may be of value in interpreting lesions presumably caused by E. deformans in ponderosa pine.

Structural Changes in the Bark of Uninfected Trees

Initial Periderm

One-year-old stems have a collenchymatous epidermis

consisting of a single layer of cells with a distinct cutin over the free surface. The initial phellogen arises subepidermally in the parenchyma cells of the outer cortex within the first year of tree growth. This is consistent with Esau's (15) observations for most gymnosperms. A single row of thick-walled cork cells is present immediately under the epidermis. The remaining cork usually consists of two to three layers of thin-walled, suberized cells.

By the second year the initial phellogen is continuous. Localized regions of two to three rows of thick-walled, woody, cork cells forming one or two tangential bands alternate with the suberized cork cells (Fig. 3). The number of bands of thick-and thin-walled cells is not predictable beyond the third year, sometimes being but one, at other times being as many as five in the same age stem. The number of thin-walled phellem cells in young stems is greater than that of thick-walled ones but as the stems become older this ratio becomes equal (at about nine to ten years) and ultimately thick-walled cells predominate as the thin-walled cells become crushed. The epidermis is usually sloughed by the fifth year.

The general description of the phellem of P. ponderosa agrees with that of Chang (9) for various species of hard pines, and with that of Srivastava (32) for the Pinaceae in general. Chang (8) considered the thick-walled cells to be transformed phelloderm although Srivastava (32) did not agree nor did Grillos and Smith (19) who

considered them to be merely lignified phellem cells. Srivastava (32) pointed out that the alternating bands of phellem cells were described by Moeller in 1882 under the designations Schwammkork (spongy cork) and Steinkork (stony cork).

The phelloderm of the superficial periderm at first consists of one or two rows of thin-walled cells. In stems eight years old, three rows are present occasionally. In stems up to 25 years old the phelloderm is still generally only two to four layers deep (Fig. 4). Chang (8) found that in P. banksiana the normal phelloderm was four to seven cells deep. The continuous superficial cork cambium persists up to 25 years as it does in Douglas Fir (19).

Cortex and Deep Periderms

In uninfected stems up to 25 years old, plates of deep periderm do not occur. Occasional localized deep cortical pockets of cork occur beginning in stems two years old but these are associated with superficial wounds or with larval infestation.

The cortical region consists mostly of parenchyma cells many of them filled with resins and tannins. Interspersed is an occasional solitary sclereid and sometimes small groups of sclereids even in the bud and one-year-old samples. These cells generally are brachysclereids with distinctly lamellated, thick walls but they may be short astrosclereids. The lumen is usually obliterated. Within

the cortex are numerous vertical resin canals. These canals have wide lumens bounded by three layers of cells, a unicellular layer of thin secretory cells encircled by a double layer of heavier-walled epithelial cells as described by Lightle (25). Cells of the outermost of the three layers are often filled with secretion droplets. Starting about the fourth year, there is slight proliferation and dilatation of the secretory lining cells of an occasional resin canal. Occasionally, also by the fourth year, the lumen of a resin canal is filled with degenerating thin-and-thick-walled phellem cells. No phelloderm is present around these structures.

Phloem

The primary sieve cells collapse during the first year as adjacent parenchyma cells enlarge (Fig. 5).

The secondary phloem shows the basic pattern of arrangement of sieve cells, vertical parenchyma cells, ray parenchyma and ray albuminous cells which has been described for the phloem of Pinaceae (8; 9; 32). Sieve areas on the radial walls of the sieve cells are in a single, vertical row but are irregularly spaced. They may be extremely crowded or widely separated. Vertical parenchyma cells in the newly formed secondary phloem may be scattered but often are arranged in short radial rows. These strand parenchyma cells often are filled with a homogeneous, light brown

secretion even when very near the cambium. Occasionally a fusiform parenchyma cell is seen close to the cambium. Various authors (1; 9; 19; 32) report that phloem parenchyma cells may contain tannins, oil droplets, starch, resinous material or styloid crystals. Styloid crystals were observed by the present writer in the lumens of phloem parenchyma of P. ponderosa as early as in the buds and one-year-old stems. These were often in the form of crystal strands and thus agree with Srivastava (32, p. 11) who said they were "frequently seen in young phloem (e. g. P. ponderosa)." Chang (9) considered that the presence and shape of these crystals are of sufficient diagnostic importance to be included in his Key to Genera of N. A. Conifers separating Haploxylon from Diploxylon. He stated, also, that "Ohara used their presence as a basis for separating Japanese hard and soft pines" (9, p. 37). The fact that they occur in the bud and in the very early years, as well as in older stems, might be construed as a corroboration of their importance. The vertical parenchyma cells of the secondary phloem begin to enlarge by the end of the first year and to become round in transverse section. This is in agreement with the findings of Grillos and Smith (19) for Pseudotsuga menziesii and of Srivastava (32) for Larix decidua. The cells are slightly enlarged by the third year but not enough to crush the sieve cells between them. By the third year the parenchyma cells form somewhat irregular tangential bands

with one to three cells arranged in short radial rows separating radial tiers of four to eight sieve cells. Although the vertical phloem parenchyma is sometimes markedly scattered throughout an annual increment of the first few years' growth as Srivastava (32) mentions, older stems often show more uniform tangential bands so that there are definite annual rings of crushed sieve cells bounded by phloem parenchyma. In seven-and-eight-year old stems all the annual increments can be readily counted and it is possible to count the last nine phloem increments in the samples of 13-year-old stems studied (Fig. 6). In one sample, 14 of 19 annual increments were distinct enough to be counted. In other instances, however, the phloem parenchyma was scattered so that even four to five annual increments were difficult to identify in older stems. Thus it would seem that seasonal growth cannot be determined reliably by a count of annual increments in the phloem.

The ray parenchyma cells of the first year stems are mainly uniseriate but fusiform rays with resin canals are also present. By the second year rays consist of both erect and procumbent cells and are from one to four cells high. The outermost cells of some rays are enlarged and rounded but by the third year this is only moderate so that the basic orderly arrangement of the sieve cells is not disrupted. In older stems the rays may be up to nine cells high. It is not until about the seventh to the ninth year that the expanded ray

cells and the numerous moderately enlarged vertical parenchyma cells cause any disturbance of the basic sieve cell pattern. Even in stems 25 years of age this distortion occurs only to a moderate degree and only in the outer phloem.

Horizontal, but no vertical, resin canals are present in the secondary phloem. This is in agreement with Chang's (9) observations of the Pinaceae. The horizontal resin canals enlarge into bulbs or cysts similar to those observed in Picea (33) and may appear on transverse section as unconnected resin sacs oriented parallel to the periphery of the stem (9; 32). These sac-like structures are constant features in P. ponderosa phloem (Fig. 7) and are found in different phloem increments. In one 12-year-old stem, for example, one or two large structures of this type are present in annual increments of age 12 and again of age ten. According to Srivastava (32) the resin canals of old phloem of P. jeffreyi and P. murrayana may have periderm formation around them and he illustrates tyloids in resin canals of P. flexilis bark. Resin canal periderm was also noted in Picea by Thomson and Sifton in 1925 (33). Tylose-like cells are described by Gerry (17) in both horizontal and vertical resin canals in the xylem and phloem of Pinus. He differentiated between tyloses and tylose-like structures in the resin canals, the latter being "a growth or expansion of the whole cell." Bloch (3) in 1941 disregarded this distinction and considered that all cells which

extended into any cavity are tyloses. In the samples I examined up to age 25, periderm and tylosoid formation was the exception rather than the rule. One sample of P. ponderosa which seemed completely normal showed epithelial cell proliferation of the horizontal resin canals. Other samples showed strong periderm formation but this material was from a series showing generalized strong wound reaction probably to the presence of an insect larva.

It should be noted also that Chang (8; 9) considered that the absence of sclerenchyma in the secondary phloem is worthy of taxonomic importance at the generic level in separating Pinus from other genera of the subfamily Abietoideae, family Pinaceae. In two slides, one of a five-year-old stem, the other of a stem 26 years old, an occasional sclereid was noted in the cortex very close to the outer phloem. However, no sclereids were observed in the secondary phloem in any stem of normal P. ponderosa. Srivastava (32) reported a few sclereids in older secondary phloem of P. jeffreyi and P. sabiniana. Shimakura (29, p. 210) flatly stated, "The Pinoideae, in whatever condition, completely lack sclerenchyma" in in the secondary phloem.

Pith

Parenchyma cells of the pith have a moderate amount of storage material. Occasional to many thick-walled cells are

formed, most of them either still containing the nucleus or with large lumens filled with resins in one-year-old stems. These sclereids are about the size and shape of the parenchyma cells and are similar in general structure to cortical sclereids.

Structural Changes in the Bark of Infected Trees

Cortex

The subepidermal origin and development of the superficial periderm in infected stems is identical to that found in noninfected stems through the first three years of growth. Some stems of ages six to nine, and one of 12, years show a superficial phellogen which continues to be active with approximately the same degree of orderliness as in uninfected stems of the same age. As many as seven bands of thick-walled woody cork cells alternating with thin-walled suberized ones form the superficial phellem. Although the phelloderm rarely becomes more than four cell layers deep even in 22-year-old, noninfected stems, in infected stems the phelloderm may be six cell layers wide in nine-to-12-year-old stems.

Although deep periderm is not present in uninfected stems up to 25 years of age, such periderms occur in infected stems anytime after the second year. They arise within primary and secondary phloem parenchyma cells in association with hyphal invasion of sieve

cells. The distribution of these periderms is thus dependent upon the distribution of hyphae which, in severely infected stems, ultimately circumscribe the stem. In two samples of four-year-old stems studied, the necrosis involved about three-fourths of the total circumference. The necrotic bark in trees infected by the fungus thus includes phloic as well as cortical elements. The phelloderm of such periderms may be over 20 cell layers deep (Fig. 8).

In regions not destroyed by necrosis the cortex of infected stems is essentially similar to that of noninfected stems. The number of resin canals present is approximately equal in both and they are similar in structure. In both infected and noninfected stems slight proliferation of the inner lining cells of the canals occurs beginning at ages three to four years. These do not increase with increasing stem age which is consistent with the opinion of Kirsch (23) who stated that tylosal growths do not depend upon the age of the tissues. Noninfected stems four years old and older occasionally have a resin canal whose lumen is filled with degenerating thin- and thick-walled phellem cells. This condition occurs more frequently in infected stems, especially those with wide cortical necrosis. No phelloderm is formed around cortical resin canals in either the infected or noninfected stems. There are no Elytroderma hyphae within the cortical canals of any stem of all ages studied.

The cortex of both noninfected and infected stems contains sclereids which are similar in shape and size. In infected stems sclereids form larger groups and are much more abundant (Fig. 9). The number of sclereids in the cortex vary. Some infected one-year-old stems show only an occasional sclereid, but others have numerous, scattered, solitary sclereids as well as groups of as many as eight to ten. There is no correlation between the number of hyphae present within the stem and the number of sclereids in the cortex.

Phloem

Elytroderma deformans hyphae occur in sieve cells of the primary phloem at the base of buds (Fig. 10), with diminishing number toward the tips, and in one-year-old stems. This agrees with the observations of Waters (38), and the description and illustrations of Sikorowski and Roth (31). The gelatinous sheath, the well defined septa and the branching habit leave no doubt that these structures are hyphae (Fig. 11) and not degenerated cell walls as was stated by Lightle (25, p. 566) who said, "No trace of them (hyphae) were found in current year's buds." The first hyphae to penetrate the phloem in the early stages of the infection are narrow (Fig. 12). Very quickly they become thickened and penetrate walls of adjacent sieve cells. Waters in 1959 (36) demonstrated by his iodine potassium

iodide test that "the action upon walls of the sieve cells appears to be hydrolytic in nature." The hyphae thus come to lie freely in the resultant cavity (Fig. 13).

Parenchyma cells surrounding the invaded primary phloem sieve cells show marked division and enlargement. These enlarged cells tend to push the primary sieve cells away from the secondary (Fig. 14). The distorted, hyphal-filled cells might then be misconstrued as cortical rather than phloic. Although some of the parenchyma cells undergo nuclear distortion and many become filled with secretion droplets, none contain hyphae. By the second year of invasion some of the phloem parenchyma cells in the vicinity of the infected zone initiate a cork cambium which encloses a zone of sieve cells. The cork cambium produces concentric rings of phellem and phelloderm cells. The phelloderm consists of up to five layers of cells filled with secretion droplets. Phellem cells are laid down toward the center of this structure (Fig. 15). Hyphal strands are present in the center of each of these structures (Fig. 16).

The lining cells of these newly formed periderms then secrete resin into the hyphal-laden cavity (Fig. 17). The resultant structures can be seen on macroscopic inspection of cut surfaces and were referred to by Lightle (25) as "pathological resin cysts." Since they traverse the stem vertically they may more properly be called "pathological resin canals" (Fig. 18). Frequently, normal

horizontal resin canals are present immediately adjacent to these pathological canals without themselves being involved (Fig. 19).

The cambiums first form around isolated hyphal groups (Fig. 20). Later, adjacent isolated cambiums may connect (Fig. 21). In one sample of a three-year-old stem the periderm forms a continuous ring around the circumference of the stem.

Hyphae within the resin-filled cavities gradually degenerate. Tissues external to the deep periderms undergo necrosis (Fig. 22).

Cork cambiums are not always initiated immediately by hyphal presence. In a particular stem some portions of the primary phloem may show marked reactions whereas other portions are relatively unaffected in the presence of equal amounts of invading hyphae.

Even during the early stages of invasion hyphae spread in a radial direction so that sieve cells of the secondary phloem nearest the primary phloem of one-year-old stems sometimes contain small hyphae (Fig. 23). Vertical parenchyma cells in the vicinity of the hyphal-filled cells often show extremely elongated nuclei as seen in longitudinal section. Many secondary sieve cells immediately adjacent to enlarged primary phloem parenchyma cells are crushed. Both vertical and ray parenchyma cells of the secondary phloem enlarge (Fig. 24). The basic phloem pattern is often severely disrupted in young stems with an invasion time of one to two years. This is markedly different from the completely orderly arrangement

of phloem cells in noninfected two-year-old stems. However, the disruption is usually localized rather than involving the entire circumference of the stem. There may be only slight crushing of sieve cells and minimal parenchyma cell dilation in some regions but marked distortion in others. The E. deformans mycelium found within the secondary phloem sieve cells may involve cells immediately adjacent to a horizontal resin canal but, again, are found only within sieve cells, never within the resin canal or epithelial cells. Neither do the hyphae invade either ray or vertical parenchyma cells.

In stems invaded for at least three years hyphae may be found in any secondary phloem growth layer although not in every growth layer of every stem studied. Hyphae may occur in scattered cells throughout one annual increment as well as in cells of several different annual increments (Fig. 25). Lightle (25) illustrated hyphae in several phloem growth layers but made no further mention of this condition in his text. Usually where hyphae of the primary phloem are either unenclosed by periderm or are partially circumscribed by discrete periderms secondary sieve cells show invasion. Where the periderms of the primary phloem are widespread and confluent, hyphal-bearing secondary sieve cells seldom occur. On the other hand, hyphae may be present in younger secondary phloem sieve cells, as was seen in the second-and-third-year increments in a six-year-old stem, without being present in any of the primary, or

the older secondary, phloem cells. Thus, hyphae may spread vertically solely within the secondary phloem in older stems.

Cork cambiums arising in both vertical and ray parenchyma cells are produced around infection loci of the secondary phloem. Ultimately, pathological resin canals identical to those seen in the primary phloem develop. These may be present within various growth increments of definitely secondary phloem in association with hyphal invasion. Sikorowski and Roth (31, p. 334) mentioned finding "phloem cavities scattered throughout the secondary phloem."

As in the primary phloem, not all hyphae are surrounded by periderm nor are the tissue reactions necessarily proportionate to the amount of invasion. In the same region of the stem hyphae may be unenclosed by periderm in older phloem but be markedly cut off by periderm production in younger phloem cells. In one six-year-old stem deep periderm arises from cells of the secondary phloem in the second annual growth ring where sieve cells are markedly invaded by hyphae. It encompasses about one fourth of the circumference of the stem resulting in massive necrosis. Older secondary phloem sieve cells are crushed, some contain hyphae and a few are surrounded by highly dilated parenchyma cells. Conversely, however, in another region of the same stem infected cells of older phloem are surrounded by periderm while younger cells, although invaded by hyphae, are without periderm.

Wherever the pathological resin canals occur they have certain features in common. They always surround hyphae, the phellem cells are always laid down toward the lumen and secrete resins into it, and the phelloderm always consists of many cell layers, often nine or more tiers in the older structures. Bramble (6) described wound periderm such as this in his study of Chestnut bark. He was dealing with a fungus which attacks the outer bark and described the rows of phelloderm forming toward the uninfected bark while the thin-walled phellem forming four or more layers lay adjacent to the infected tissue. The development of these pathological canals, whether they occur in the primary or the secondary phloem, may be summarized as follows:

1. Dilation of a hyphal-bearing sieve cell
2. Lysis of adjacent sieve cell walls resulting in the formation of a cavity
3. Crushing of surrounding sieve cells coincident with proliferation of nearby parenchyma cells, both ray and vertical, which form an irregular cell mass
4. Development of a cork cambium
5. Periderm formation circumscribing hyphal-laden sieve cells
6. Resin production
7. Degeneration of enclosed sieve cells and mycelia with resultant necrosis

8. Confluence of adjacent circumscribing periderms

9. Ultimate necrosis of all cells external to periderms

There is some correlation between the presence of phloic pathological resin canals and the age of the infection. In stems infected no longer than two years the resin canals occur in not more than 25% of the samples. Sixty-five to 75% of those infected for three to six years had such structures and 85 to 90% of the stems infected for seven to nine years are involved. In stems infected for over eight years pathological resin canals are present in all of the stems examined.

There is also a tendency toward a complete enclosure of hyphae by periderm. In some stems in each of the ages studied, except year one, the hyphae were completely enclosed by pathological resin canals and the hyphae were necrotic. With increasing age of the infection the pathological resin canals gradually encompass the entire circumference of the stem. Tissues external to the periderm die, sometimes leaving several phloem layers free of hyphae, sometimes leaving infected younger phloem. Thus, there is a gradual walling off of the progress of invasion. When it is not complete the fungus spreads radially and vertically. Figure 29 is a diagram of a tree which had only two symptomatic branches. Sections were taken from the distal branch, labeled B, showing minimal macroscopic symptoms at the base. Although the seven-year-old tissue has much

periderm formation, numerous unenclosed hyphae are present in phloem increments one, two, three and four. A sample taken of six-year-old tissue shows some pathological resin canals enclosing hyphae but also unenclosed hyphae in the sieve cells of the one-year-old phloem increment. The five-year-old stem beyond this discloses a moderate amount of hyphal mass in the two last formed phloem increments but no phloic pathological resin canals. The four-year-old stem shows minimal hyphal invasion in the last increment formed and no pathological resin canals. Sections of stems distal to this indicated that the fungus, which was growing in a distal direction through the youngest growth layer of phloem, had not progressed to the tip of the asymptomatic branch.

This situation was also seen in much older tissue. Figure 30 depicts an infected branch from a very large tree whose total age was undetermined. This 19-year-old branch is the only part of the entire tree manifesting infection. On microscopic examination this branch shows hyphae within sieve cells scattered in several different age layers of phloem as well as hyphae enclosed within one to two rows of pathological resin canals. Hyphae have invaded two of the side twigs of the branch. The fungus is also found both above and below this branch junction in main axis tissue 19 and 20 years old. Hyphae in the main axis are mostly surrounded by pathological resin canals in two and three parallel zones of different age levels of

phloem. A few remain unenclosed in newly-formed phloem of the 19-year-old portion of the stem.

There is a tendency toward an increase in the number of horizontal resin canals present. Usually the nonpathological resin canals show neither increased tylosoids nor periderm formation which is also true of noninfected stems.

An intensive search was made for sclereids in the secondary phloem tissue of both infected and noninfected trees but none were found.

Vascular Cambium and Xylem

In the majority of the stems studied the cells of the cambial zone and xylem are not affected by the presence of the hyphae. However, in four stems of different ages, taken from different trees, the vascular cambium has produced cells in various isolated regions which have remained as expanded, irregular, thin-walled parenchyma cells rather than differentiating into regular rows of phloem sieve cells and xylem tracheids. In two of the stems, one three-years-old, the other seven-years-old, these abnormal cells are found in the last year's growth (Fig. 26). In another, a six-year-old stem, such cells are present, in localized regions, in the last two xylem increments. Adjoining sieve cells are crushed. Elsewhere in the stems the cambium has produced cells which differentiated into the usual rows of

xylem and phloem elements. In a four-year-old stem, for approximately one-fourth of its circumference, the irregular parenchyma cells are present as a band within the xylem half-way through the third year's growth (Fig. 27). The vascular cambium at this location laid down distorted rows of tracheids the next year. In the phloem region external to these tracheids only a few crushed sieve cells are present. The majority of cells are the phelloderm of a deep periderm which is continuous into the adjoining region where only three and a half years growth of normal xylem is present. Elsewhere throughout the stem circumference four annual increments of xylem tracheids are present.

In these stems hyphae have invaded secondary phloem sieve cells close to the vascular cambium (Fig. 28). Large pathological resin canals have formed around invaded sieve cells in the older phloem.

Pith

Sclereids form earlier and in greater numbers in the pith of infected shoots at all ages. Apparently Lightle (25, p. 564) found many sclereids in his samples of infected tissues because he mentioned that twigs of P. ponderosa were "heavily armored with stone cells." Whether these were in the cortex or the pith was not stated. The increase in pith sclereids occurred in stems with increased

cortical sclereids but could not be correlated with the severity of the hyphal invasion.

I saw no evidence to substantiate Waters' statement (39, p. 43) that there was an "abnormal development of pith helping to cause thickening or enlargement of the twig in cases of trees which are lightly infected." The increased number of sclereids is the only difference noted between piths of noninfected and infected trees. In this regard a tree with just one fully symptomatic branch, which might thus be assumed to be only lightly infected, showed, on microscopic examination, a pith packed with sclereids. However, another lightly infected tree had few to no sclereids, while still another had a moderate number. Highly symptomatic trees showed varying numbers of sclereids in different sections of the same tree.

Correlation of Macroscopic Symptoms and Microscopic Anatomy

External signs of infection are always accompanied by specific microscopic structures. The rusty red needles of spring always show the presence of E. deformans hyphae and this is also true of shoots with premature needle drop. This agrees with the findings of Sikorowski and Roth (31).

The deep brown streaking noted in the inner bark is correlated directly with the pathological resin canals of the phloem. These are

a direct response of the phloem tissue to the hyphae since they are formed only in the presence of hyphal-laden sieve cells.

The areas of bark necrosis seen macroscopically are directly correlated microscopically with large necrotic regions involving the phloem and the cortex and sometimes part of the xylem which result from the formation of deep periderms. Sometimes such necrotic areas are localized, at other times they involve almost the entire circumference of the bark. The visible distribution of the necrosis is sporadic along infected branches. When the necrotic regions are relatively small and localized, macroscopic identification is sometimes questionable since there may be dark streaks within the outer bark but not visible necrosis. Whenever any abnormal bark can be detected macroscopically it is accompanied by some degree of microscopically visible necrosis.

In three samples of noninfected trees studied, the bark became rough between the ages of 22-26 years. Microscopically, at these points, there was a large amount of phellem but the phelloderm was only two to four layers deep. Two other noninfected trees had rough bark at age 11 but both of these showed evidence of external wounding. In Elytroderma infected trees there was a range of ages from eight to 25 in which rough bark began. The average was about year 15 to 16. In sections through these stems concentric phloic periderms which included nine and more rows of phelloderm cells were present.

Frequently mycelia and even pathological resin canals are present within branches showing no visible lesions and bearing macroscopically normal appearing green needles several years of age. Environmental factors of moisture and temperature affect the rate of growth of the fungus (18). During a period of slow hyphal growth within a tree, the tree may appear macroscopically symptom-free. It thus may be considered to be "disease enduring" (41) rather than disease resistant.

The Distribution of Hyphae

In order to determine the degree of vertical distribution of the fungus in infected ponderosa pine, free-hand sections were made of each internode from the tip down through the varying age levels to 25 years. Trees with only one or two symptomatic branches seemed most likely to reveal the direction and extent of spread.

In one tree which had one slightly infected branch bearing four years' growth of rusty-red needles no hyphae are found in tissue older than five years. The branch forked at this point and each fork showed evidence of being diseased. Figure 31 is of a tree also with only one mildly infected branch. Although the needles of the current (1964) year of this branch are macroscopically asymptomatic nevertheless hyphae are present within them. Only a few of the 1963 needles had the rusty red color of blight and needles were retained

for four years. The fungus, however, is present in the last 15 years' growth of the branch. No fungus is found in the 16-year-old branch tissue nor in the main trunk.

The tree illustrated by Fig. 32 had two diseased branches that were widely separated. The upper of the two, labeled M on the drawing, was ten years old. It bore macroscopically asymptomatic 1964 needles but infected 1963 needles. On microscopic examination the 1964 needles are found to be free of hyphae but hyphae are present in the 1964 stem and throughout the ten-year branch growth. The main trunk has hyphae through years 11 and 12 where the hyphae are enclosed by pathological resin canals, and through tissue of ages ten to six where hyphae are also present enclosed by pathological resin canals. The canals are very numerous in infected younger axial tissue above branch M but only a few are present in the 12 year old stem below branch M. From tissue six years old to the current bud of the trunk and all its branches above this point, no hyphae or lesions are present either microscopically or macroscopically.

The lower of the two infected branches, labeled Y in Fig. 32, was 22 years old. The only macroscopic evidence of infection is the rusty-red, 1963 needles of three and the stems of two of the side-shoots of this branch. Needles, persisting for three years, of the axis of the branch are asymptomatic. Microscopic examination revealed hyphae in the following locations:

1. 1964 needles of branch Y
2. 1963 needles of each of the three side shoots of branch Y
3. the axes of the side shoots
4. the main axis of branch Y in the terminal three years' growth

From year four to its junction with the first side shoot the branch axis is without hyphae. Hyphae again are present from this junction at year eight to the point of emergence of the third of the infected side shoots at year 12. From years 13 to 22, where branch Y joins the main trunk, the axis of branch Y is without hyphae.

The trunk, both the year above and the year below the Y branch junction, is likewise free of fungus.

Another tree, depicted by Fig. 33, has an infected leader and one nine-year-old infected branch separated by an asymptomatic branch. The main axis below the infected branch shows hyphae and one ring of pathological resin canals in the tenth year. No hyphae are present in older axial tissue but a few pathological resin canals are seen unilaterally in 11-year-old tissue and only three such canals are seen throughout the entire circumference of 12-year-old axial tissue.

The separation of symptomatic branches by asymptomatic ones is a common phenomenon. In one example almost every other branch is asymptomatic. The oldest symptomatic branch has fungus present

from its junction at the main stem halfway to the tip (Fig. 34). The last six years' growth are hyphae-free. The trunk below this branch has hyphae through five years' growth and has pathological resin canals through a distance of seven years. The total involvement of the main trunk by the fungus is 22 years.

CHAPTER V

DISCUSSION

From the comparison of the structure and development of normal against infected ponderosa pine bark, pathological responses of the host to the parasite can be recognized and explanations of them inferred. The most significant changes of the infected host occur within the phloem. Only here, in infected stems, is the fungus present. Parenchyma cells in the immediate vicinity of hyphal-laden sieve cells, both in the primary and secondary phloem, undergo hypertrophy and repeated divisions to form cork cambiums. While Lightle (25) described these divisions as associated with attacks of Elytroderma it is more likely a general rather than a specific response of the tissue as a type of wound reaction which would occur wherever and whenever a foreign agent is present. Bloch (3, p. 121), in reviewing wound healing, mentions that "a traumatic stimulus produces a number of conspicuous tissue changes near the wound surface indicating a resumption or stimulation of metabolic activities." Haberlandt in 1902, and Petri in 1929, working with Echeveria and potato, suggested that wound hormones, perhaps the oxidation products of substances normally present in the cells, might cause cell divisions of those normal cells neighboring on necrotic cells (3).

Wilhelm in 1930 used various substances such as tissue sap,

sugar solution and horse serum to study divisions of pith parenchyma cells in Vicia faba, and concluded that cells could be unspecifically irritated to produce a division hormone (3). Esau (16) mentioned that some viruses of Solanaceae cause hypertrophy and hyperplasia in the vicinity of first developing sieve tubes producing sieve tube elements of abnormal shape.

The wound cambiums produce concentric rings of phellem and phelloderm cells surrounding groups of hyphal-laden sieve cells as early as the second year. Such occurrences were described by Bloch (3) in discussing the planes of cell divisions of wound meristems. He mentions the formation of concentric sheaths parallel to the centers of necrosis through all cells capable of rejuvenation which thus separated the necrotic part of the tissue from the healthy portion. He stated that this arrangement was found not only beneath external surface wounds, but also around inner centers of necrosis. Bramble (6) in his work on Chestnut bark blight, mentioned that wound cork might bar progress of a fungus infection, thus separating infected tissue from normal tissue. In older ponderosa pine stems, the increased phelloderm and phellem are macroscopically observable as a roughening of the bark.

By the fourth year resin is secreted and the hyphae now enclosed by these vertical, pathological resin canals degenerate. These resin-filled canals result in the dark brown streaking noted

in the inner bark of E. deformans infected stems in the field. Such pathological resin canals are not specific to ponderosa pines. In 1964, Bynum and Miller (7) noted such lesions in macroscopic transverse sections of "older broomed branches of knobcone pine" infected with E. deformans. The age was not specified but the illustrations seemed to be of a tree at least five years old. These lesions were described as being located in the outer phloem of a diseased tree and were reported as being absent in healthy knobcone twigs and branches. It would be interesting in this regard to know the pathological anatomy of P. sylvestris L. stems infected by Lophodermium pinastri (Schrad. ex Fries) Chev. which also exists within phloem tissue (22). Lightle (25, p. 567) remarked on the obliteration of "diseased phloem cells" by the influx of resin, not realizing that it was actually the obliteration of hyphae which was occurring. Dufrenoy (13), working with potato to establish the relation of cellular immunity to the presence of phenolic compounds, found that the progress of fungus penetration was checked by the toxic effect of phenolic compounds in wound tissue. Such a suggestion is probably applicable here.

Deep periderm formation in the phloem results in cortical and phloic necrosis which, when severe, is visible externally.

The only other change of the infected host is an increase in the number of sclereids of cortex and pith. Again, this is a nonspecific

reaction to a foreign agent. An increase in sclereids also occurred in samples of ponderosa pine infected by insect larvae. Various cells can redifferentiate as thick-walled sclereids in response to wounding as reported by Bloch (3). In air roots of Philodendron glaziovii wound meristems redifferentiate in this manner and in many members of the Commelinaceae internal bundle sheaths which are normally thin-walled become sclerified after wounding.

The second major aim of this study is to determine the pattern of spread of the mycelium within the host tissue. Although the mode of entrance of the fungus into the tree has not actually been demonstrated it seems, from numerous reports, to invade through the stomates of the leaves. Waters (37) felt that the invading pathway might be similar to that reported by Jones (22) for Lophodermium pinastri (Schrad. ex Fries) Chev. Lophodermium, closely related to E. deformans, is a parasitic blight fungus on P. sylvestris L. and was proven to penetrate through the stomata. Gordon and Laurent (18), studying juvenile ponderosa pine needles, reported that the first evidence of E. deformans hyphae there occurred beneath the stomata. Nothing in the distribution pattern as found in this study tends to dispute these suggestions.

There is no longer any question of the perennial character of the mycelium once it has invaded the trees. Weir (40) stated that the mycelium of E. deformans perennated in the tissues of the shoot of

ponderosa pine, but, unfortunately, gave no evidence to support this statement. Darker (12, p. 108) also mentioned that E. deformans "invades branch tissues and stimulates them to produce witches' brooms." He pointed out that such invasion has also been claimed for related species by Fron, 1911, for Hypoderma dezmazierii and Tubeuf, 1901, for Lophodermium pinastri. In 1954 Lightle (25) remarked that hyphae of E. deformans were found in the needles but not in the stem, and in his early work of 1957, Waters (39) concurred. In both cases this opinion resulted from failure to recognize the mycelium as it occurred in the sieve cells of the phloem even though Waters (39, p. 44) described the "twisted mass of continuous, swollen, and tortuous strands," resembling "a long section of coiled intestine" which he saw on longitudinal microscopic sections. By 1958 the true nature of the mycelium was recognized by Waters (38) and reported in the phloem of one-year-old stems but no illustrations of the hyphae within the phloem accompanied this report. In 1959, Waters presented a paper to the Seventh Western International Forest Disease Conference at Pullman, Washington, describing various supportive evidence of the presence of this fungus within phloem cells. He presented the following: 1. pictorial evidence obtained through use of a diastase to expose the hyphae and subsequent staining with lactophenol-aniline blue; 2. growth of hyphae from dissected phloem tissue placed in a medium; 3. growth of

sclerotia-like hyphal cells in a needle decoction to which phloem had been transferred. However, publication was delayed until March, 1962 and was posthumous (37). Meanwhile, Sikorowski (30), while working on his M. S. degree during 1958-59, also arrived independently at a recognition of E. deformans mycelium within the phloem of needle, bud and two-year-old ponderosa stem. In his thesis he supplied both pictorial and descriptive evidence of the organism. Publication of his findings was made in April, 1962 under joint authorship with Roth (31).

Childs (11, p. 1) suggested that "the fungus is perennial in bark of infected twigs and small branches and invades most or all of the new needles soon after they appear." Waters (37; 38) agreed with this when he reported that hyphae moved from the terminal bud axis into developing dwarf shoots and needle fascicles "in an unbroken chain of infection." During pruning studies of blighted ponderosa pine trees performed at the Pringle Falls Experimental Forest near Bend, Oregon in 1956 and 1957, Roth (27) suggested not only that the infection can be perennial but also that it grows into mature woody branches. In 1963, however, Gordon and Laurent (18) denied that hyphae occur in older host tissues and stated that the "growth of hyphae after reaching the phloem of the stem was always toward the apical meristem." I tried to determine whether anatomical support

of hyphal penetration occurring proximally as well as distally could be found.

The mycelium was found in the current year's shoot even though needles of this shoot showed no symptoms while needles of the previous season and sometimes of two or three seasons were symptomatic. When young buds were studied abundant hyphae were found in the primary phloem at the base of the bud, in diminishing number toward the tip and in decreasing amounts out the new leaf traces. This much was in agreement with Gordon and Laurent (18). Hyphae were also present in the mature tissue of trunks both above and below visibly infected branches. Here they travel within sieve cells of the secondary phloem at various annual increments.

Growth of hyphae within mature tissue, but toward the apical meristem, was evident in a tree with many infected branches, one of which (Fig. 34G) was invaded by hyphae from its junction with the trunk through eight years' growth. The last six years' growth were hyphal free. The tree depicted in Fig. 29 also shows such spread along branch B in tissue seven, six, and five years old.

The evidence for hyphal growth from apical meristems toward the trunk is suggestive but not conclusive. A few examples (Fig. 32M; Fig. 33) show more severe pathological resin canal formation in trunk tissue closest to an infected branch with decreasing severity down the trunk in older tissue. If hyphal spread were always toward

the apical meristem the older tissue would, of necessity, be infected longest and would show the greatest response to the invader. This has been found to be not always true.

Evidence of multiple infection of the tips of two branches far apart on the tree without hyphal connection between them was demonstrated (Fig. 32). Contiguous branches may likewise be separately infected with no fungus found in the section of the trunk between them.

Hyphal invasion of branches is sporadic and branch and needle symptoms need not appear simultaneously with invasion. Frequently a latent period between infestation and symptom production occurs so that Sikorowski's (31, p. 334) statement that "asymptomatic branches evidently were not infected" is not necessarily true. The length of this latency varies and may be due to ecologic factors governing hyphal growth within the host. Since E. deformans does perennate throughout the tree it becomes clear why control efforts so far have been unsuccessful. The attempts have been by means of sprays, both eradicants and protectants (24), which may attack ascospores or conidiospores but not affect the mycelium proper.

The free growth of the mycelium within new as well as mature tissues, the possibility of multiple sporic infections, the varying intensities of reaction of host tissue to the invader, and the asymptomatic presence of the fungus can easily account for the frequently

observed "random scattering of diseased branches among healthy branches" mentioned by Roth (27).

CHAPTER VI

PROBLEMS FOR FURTHER STUDY

As is usual in the course of research, questions have arisen whose solution is beyond the scope of this study. For example, existing data indicate that members of the subgenus Diploxylon are more prone to infection by E. deformans than those of the Haploxylon. All species reported as affected belong to the hard pines except P. edulis for which two separate identifications, one from Colorado (12) and the other from Arizona (14) are available. Is this simply a question of insufficient field data regarding host infection or is there some intrinsic cause for greater susceptibility of pines in this subgenus? Shaw (28) indicated that, in general, hard pine needles are more stomatiferous, especially on the dorsal surface, than are the soft pines. This could be significant if the mode of entrance of E. deformans is truly through the stomata. There may also be a correlation between the ability of these two subgenera of pines to produce resin structures and their susceptibility to invasion. According to Chang (8), soft pines show a distinct tendency toward more and larger horizontal resin canals although Shaw (28) listed the wood of Haploxylon as having little resin.

Many puzzling features of this disease still remain unsolved. One of the most intriguing is why certain branches, as fully infected

as others, do not show macroscopic pathological lesions. Controlled field experiments should be done to establish a particular time of infection of one particular tree with a view to determining the possible time lapse between infection and onset of symptoms. Several investigators, among them Gordon and Laurent (18), Roth (27), and Waters (39), have been unable to duplicate the positive inoculation results reported by Weir (40), a necessary prerequisite to the proposed experiments, although successful infection experiments have been reported for closely related species such as Hypoderma desmazierii (12), Hypoderma lethale Dear. (5), and Hypodermella laricis, concolor and nervata (12). One might speculate that the needles of ponderosa pine do perhaps actually become infected but that the mycelium of E. deformans may remain dormant for some period of time. Microscopic analyses of inoculated trees should be done at yearly intervals in an attempt to show the mycelium within the host even when it may remain asymptomatic. Of course, the reasons why such a latent period would occur present another aspect of the problem. Ecologic studies of factors governing hyphal growth should be correlated with anatomical and morphological studies.

Another question revolves around the possibly greater susceptibility of a previously infected tree to subsequent invasion than one which has never been infected. In his inoculation studies Roth (personal communication) pruned a diseased tree and after it had

remained asymptomatic for five years, inoculated the terminal branches. Within two years most of the inoculated branches became symptomatic whereas other trees not previously infected did not significantly respond to repeated inoculations (27). Did the fungus remain completely latent for five years only to be stimulated somehow by the attempted inoculations, or did new inoculations actually take place, or was it simply coincidence that the symptoms reappeared at a time when they could conceivably be correlated with the inoculation procedure?

Other questions revolve around the tissues involved by fungal invasion with regard to the leaf and the stem. Although I found hyphae only within the phloem sieve cells of the stems, many workers report invasion of a variety of cells in the leaves. Waters (35), Gordon and Laurent (18), and Sikorowski (30) variously reported the mycelium within the leaf penetrating the hypodermis, mesophyll, endodermis and transfusion tissue to ultimately invade the phloem. Sikorowski (30) described the mycelium in the mesophyll as both inter- and intracellular. Hyphae apparently travel through the vascular rays of the xylem in the leaf (18) but not in the stem. Perhaps the difference reported (18; 25; 30; 35) in the morphology of the hyphae occurring in sieve cells compared to the occurrence in any other cell is responsible. Also, some workers (25; 30) describe E. deformans mycelium in lacunae of resin canals of the leaf but it

is not present within resin canals of the stem, either vertical or horizontal. Why?

Finally, further anatomical work should be done on the tissue responses of other pines infected by E. deformans and of other pines infected by other phloem invading fungi, for example, Hypoderma lethale Dear. on southern hard pines (5).

Fig. 1. Necrotic areas in inner bark and abnormally rough bark due to infection

Fig. 2. Transverse section of a pine stem four to five years old showing pathological resin "cysts" in the phloem (4X)

Fig. 3. Two layers of woody cork in a three-year-old, noninfected stem (890X)

Fig. 4. Normal superficial periderm in a 22-year-old stem with phelloderm cells only two to four layers deep (330X)

Fig. 5. Groups of enlarged primary phloem parenchyma cells and collapsed sieve cells in uninfected two-year-old tissue (890X)

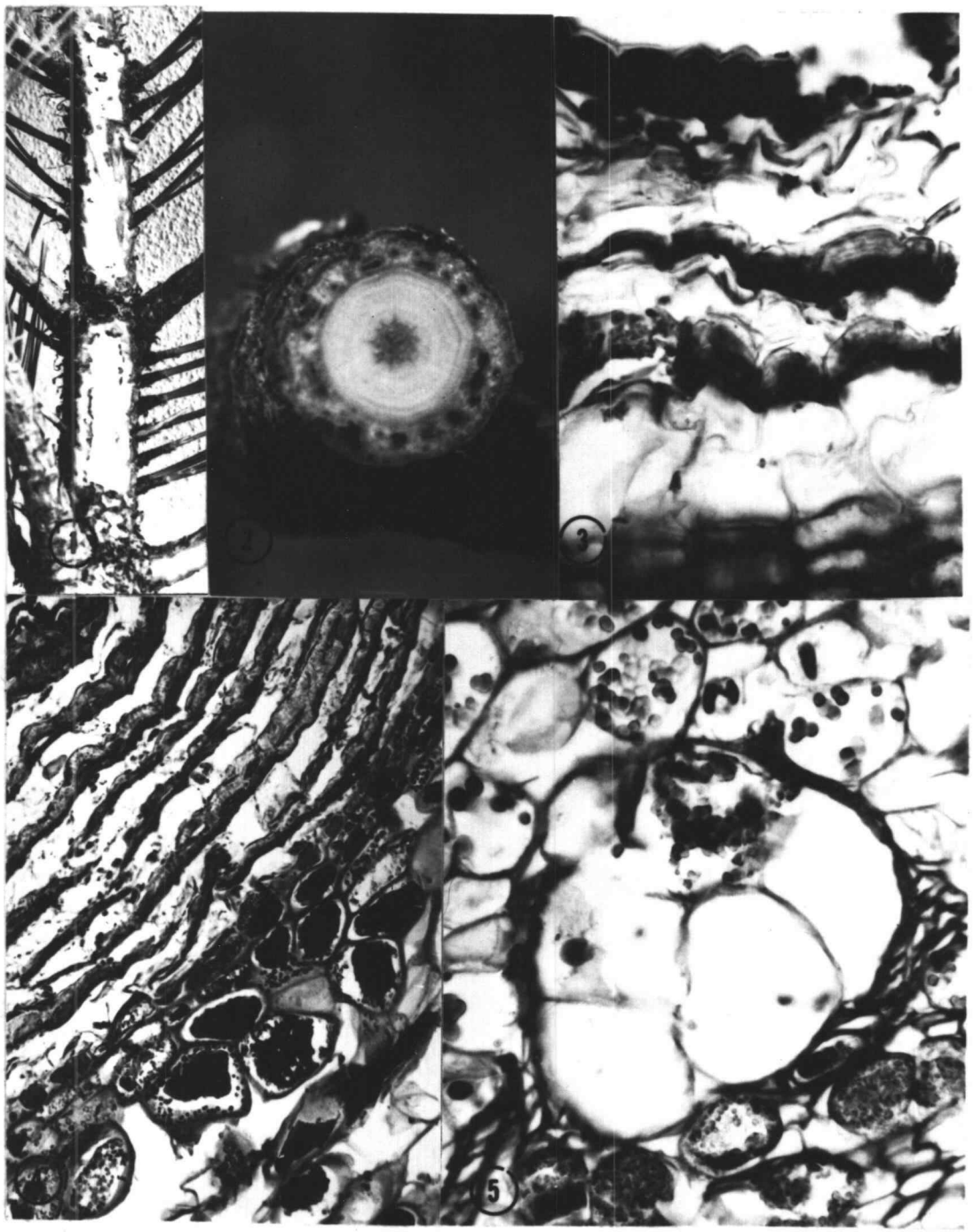
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Fig. 6. Annual phloem increments demarcated by tangential bands of parenchyma cells are distinguishable for nine years in a 13-year-old stem of healthy ponderosa pine (330X)

Fig. 7. A normal horizontal resin canal expanded into a cyst within the phloem of an 11-year-old stem (200X)

Fig. 8. Deep periderm with numerous layers of phelloderm cells in an infected six-year-old stem (200X)

Fig. 9. Numerous sclereids in the cortex of an infected one-year-old stem (33X)

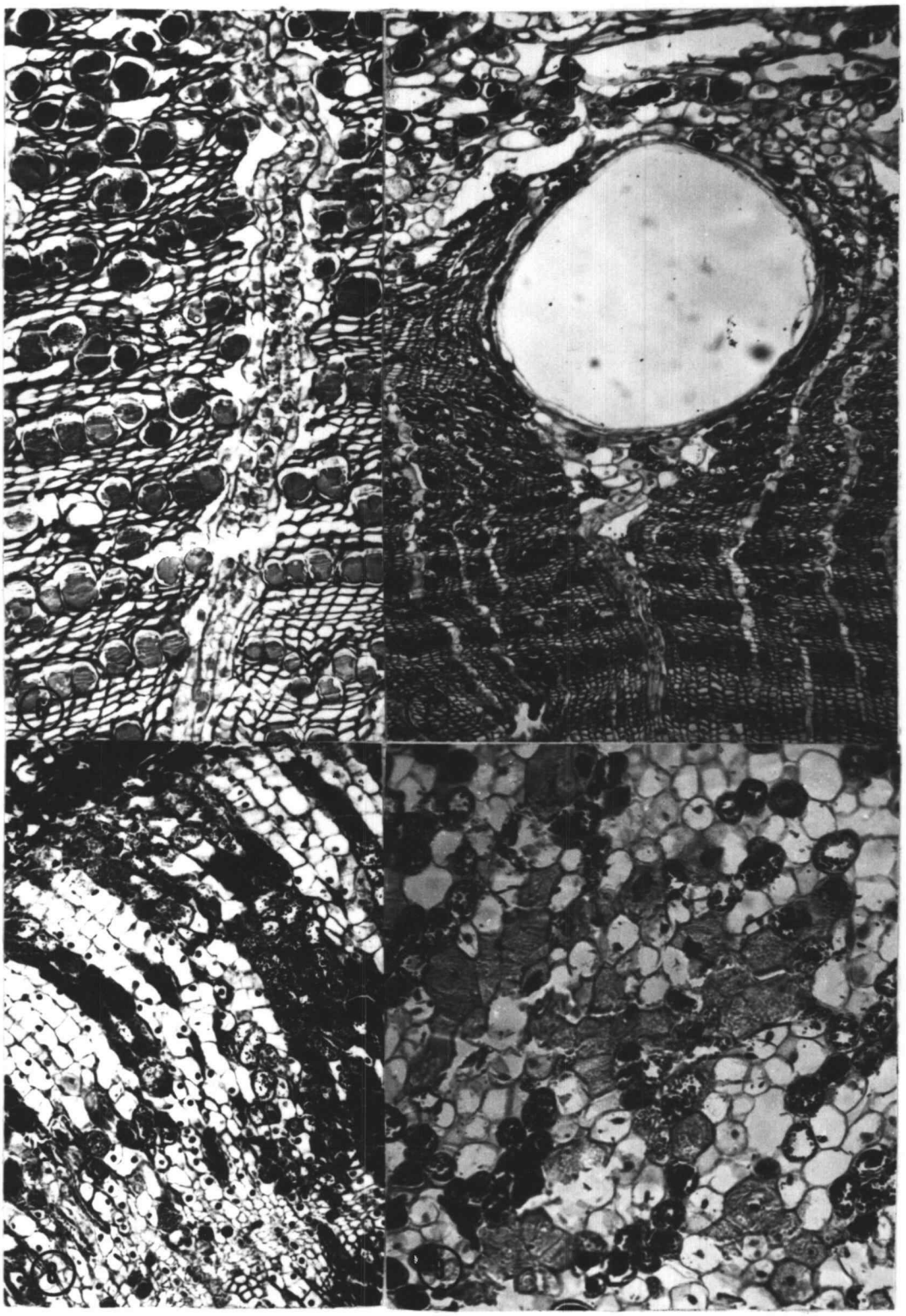

Fig. 10. Hyphae invading the base of a bud (890X)

Fig. 11. Morphology of E. deformans hyphae in a one-year-old stem (890X)

Fig. 12. Narrow hyphae at an early stage of infection. One-year-old stem (890X)

Fig. 13. Elytroderma hyphae lying free in a cavity formed by lysis of sieve cell walls of the primary phloem. One-year-old stem (890X)

Fig. 14. Enlarged parenchyma cells surrounding hyphae in the primary phloem of an infected two-year-old stem (890X)

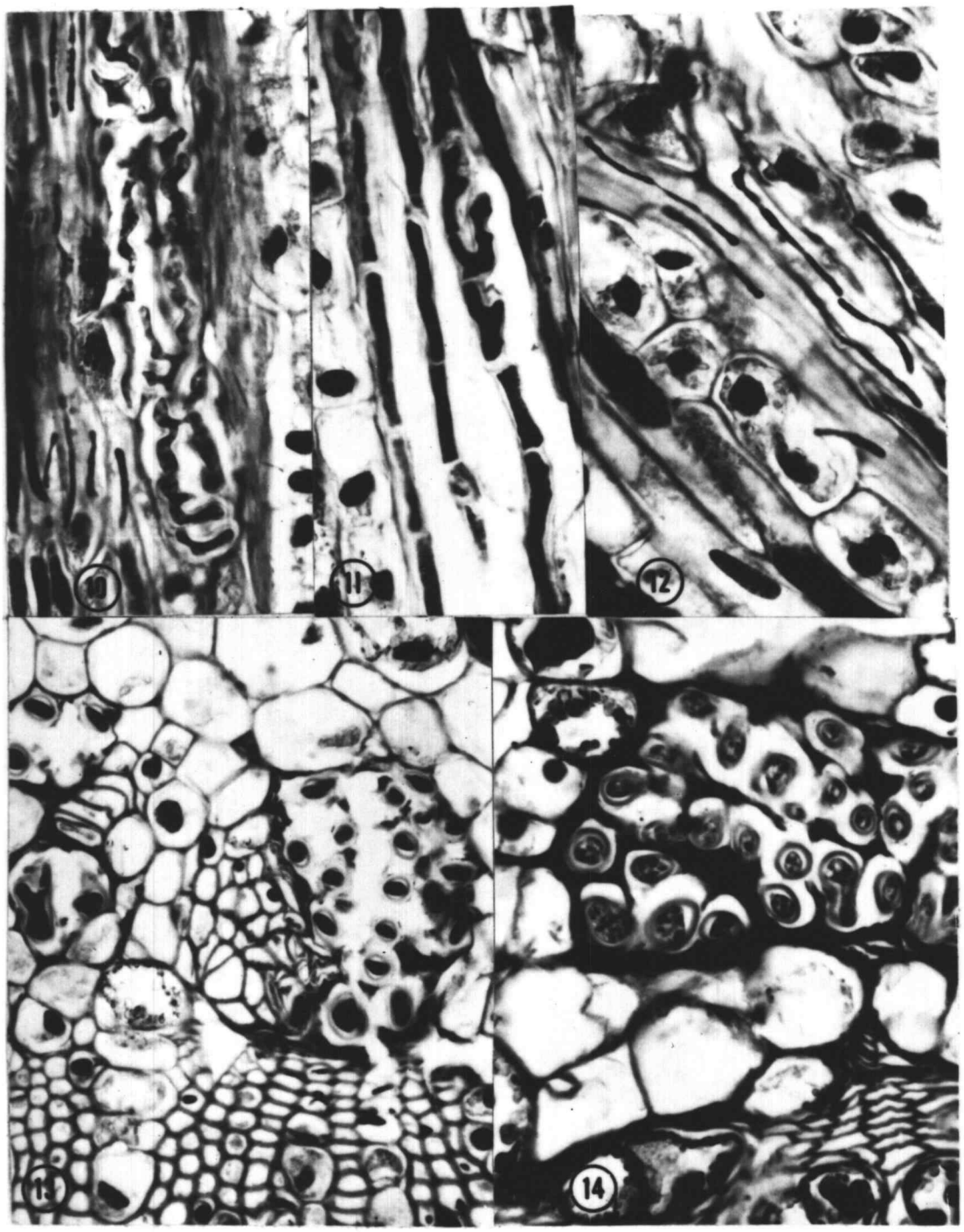
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Fig. 15. Longitudinal section showing cork cambium and early phellem formation around hyphae in primary phloem cells (890X)

Fig. 16. Concentric rings of phelloderm and phellem produced by a cork cambium surrounding hyphal strands in the primary phloem of a three-year-old stem (440X)

Fig. 17. Resin secretion by phellem cells of the cork cambium. Hyphae at tip of pointer (100X)

Fig. 18. A longitudinal section showing a pathological resin canal traversing the stem vertically (20X)

Fig. 19. Transverse section of normal horizontal resin canal cysts adjacent to a pathological resin canal of the primary phloem. Five-year-old stem (200X)

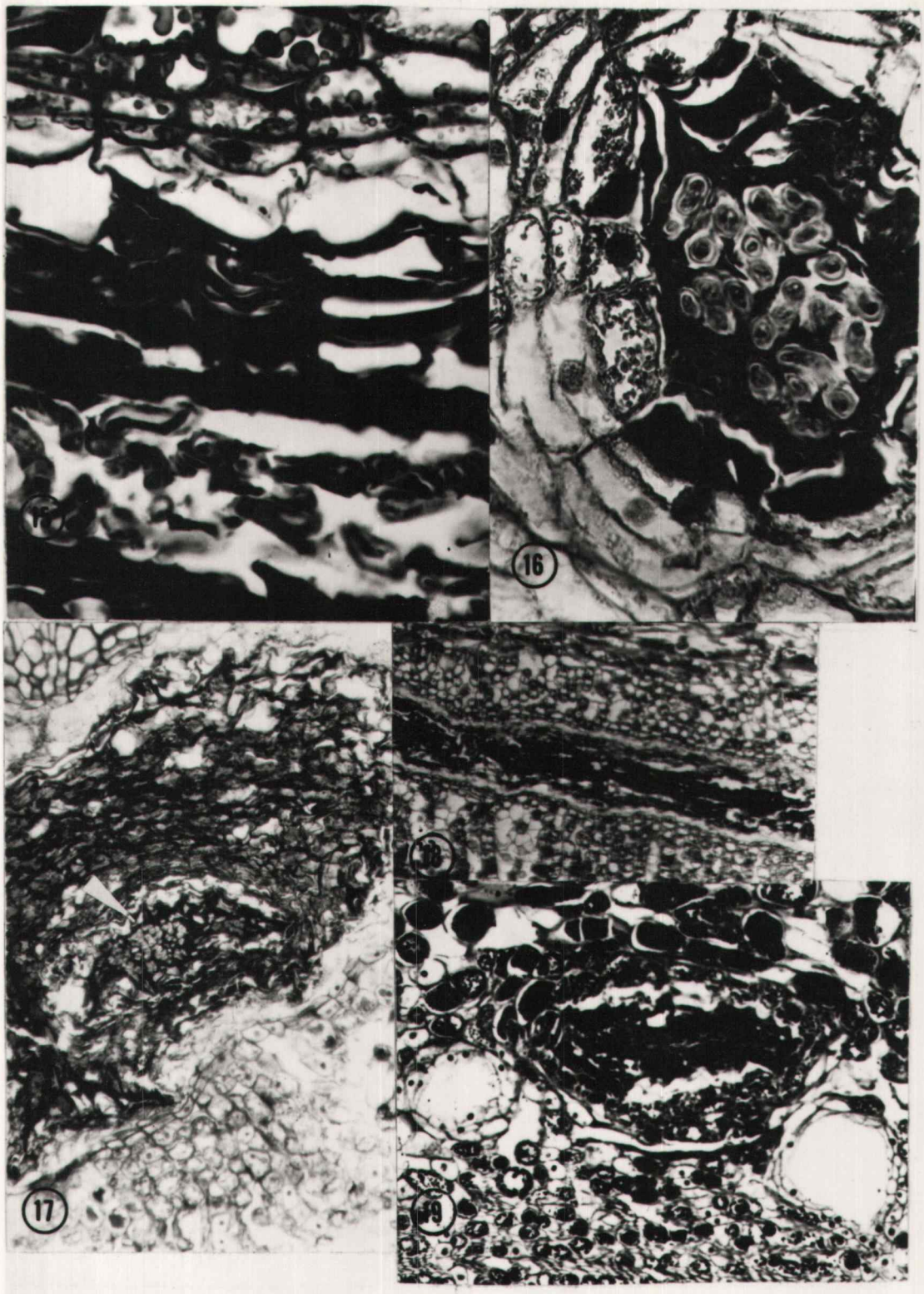
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Fig. 20. Cork cambiums forming separately around contiguous strands of hyphae (100X)

Fig. 21. Transverse section showing the union of cambiums which formed separately around infection loci (33X)

Fig. 22. Necrosis of the primary phloem and cortex external to a deep periderm (33X)

Fig. 23. Mycelium is present in both the primary and the secondary phloem sieve cells in this infected one-year-old stem (890X)

Fig. 24. Enlarged parenchyma cells in the secondary phloem of an infected two-year-old stem. Hyphae at tip of pointer (100X)

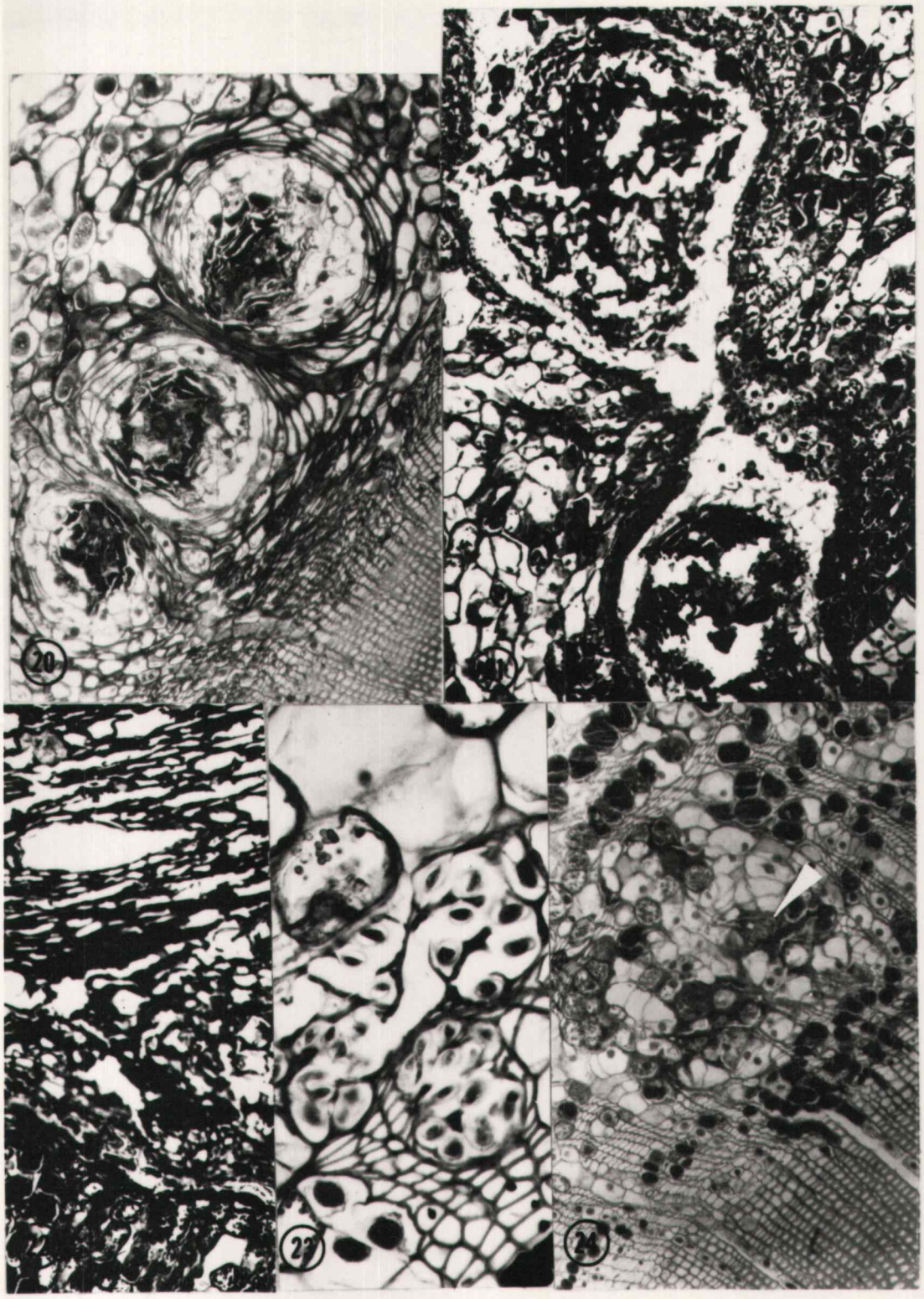
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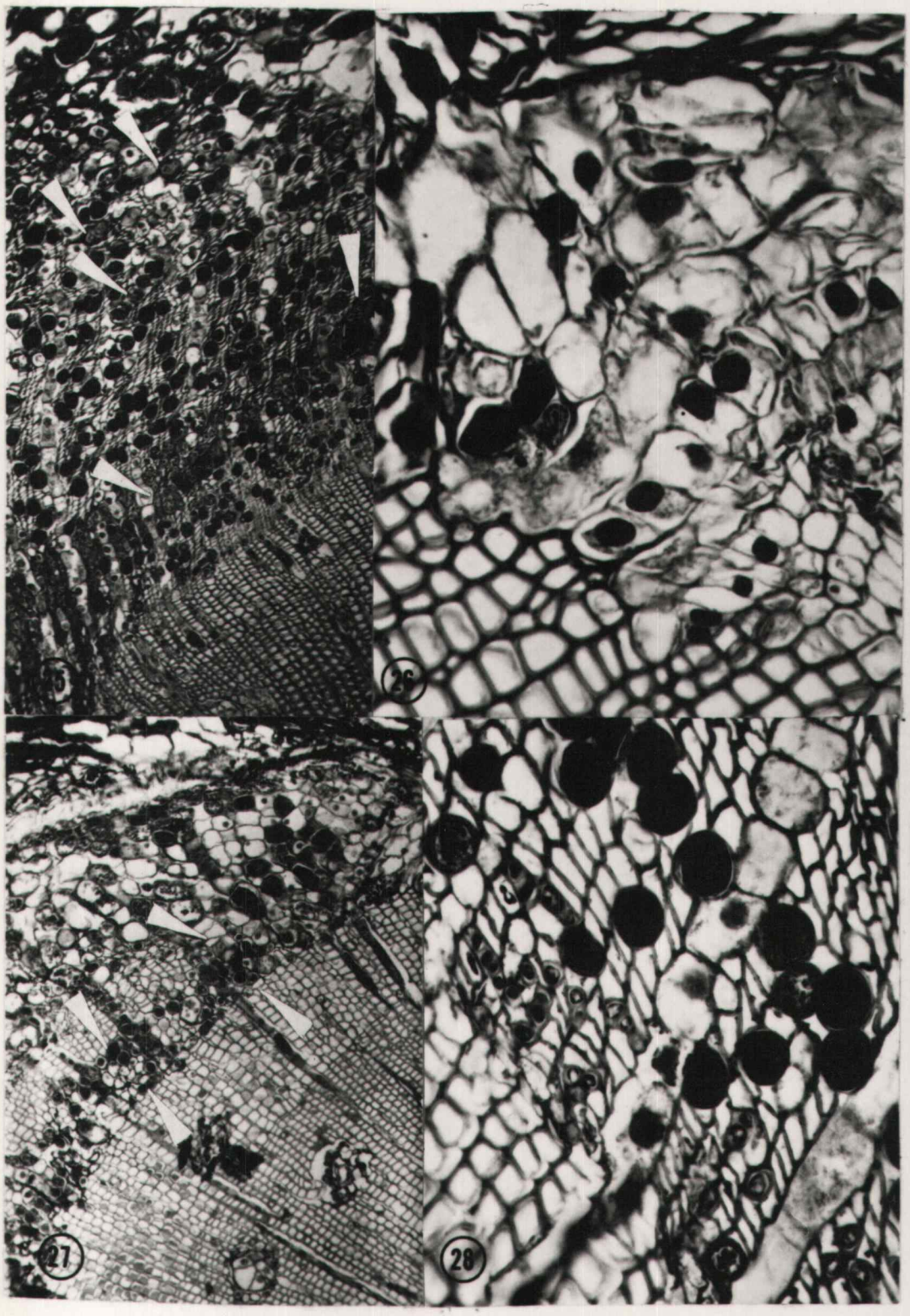
Fig. 25. Fungal hyphae are present in several annual phloem increments in a six-year-old stem. Pointers mark the hyphae (100X)

Fig. 26. Expanded, irregular parenchyma cells produced by the vascular cambium in an infected three-year-old stem. Edge of unaffected xylem at lower left (890X)

Fig. 27. Irregular parenchyma present within the xylem (demarcated by pointers) in a four-year-old, infected stem (100X)

Fig. 28. Hyphae have invaded the sieve cells of the last phloem increment almost to the vascular cambium. Xylem at lower left, phloem at upper right (890X)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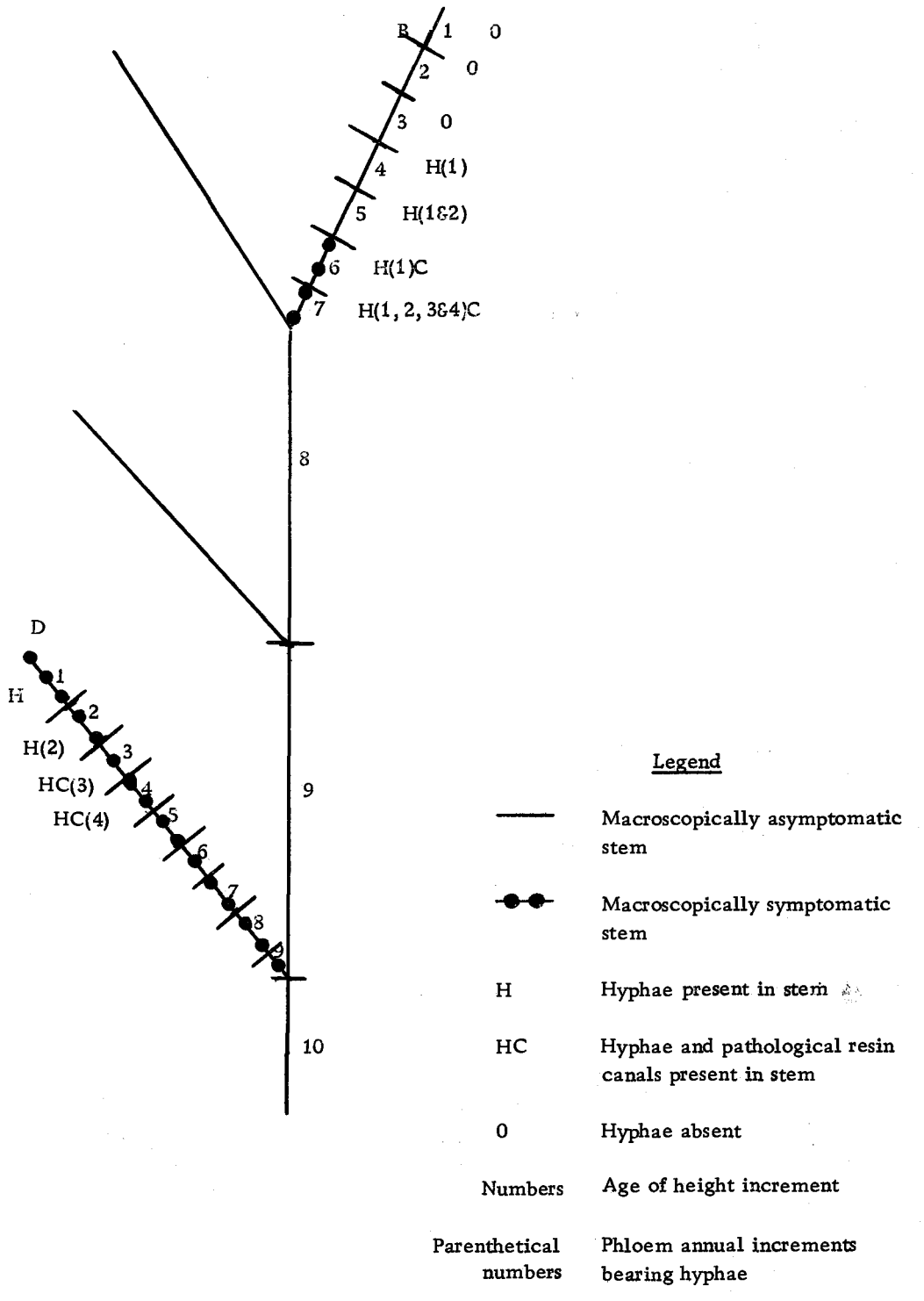

Figure 29. Distribution of hyphae and pathological resin canals within young stem tissue.

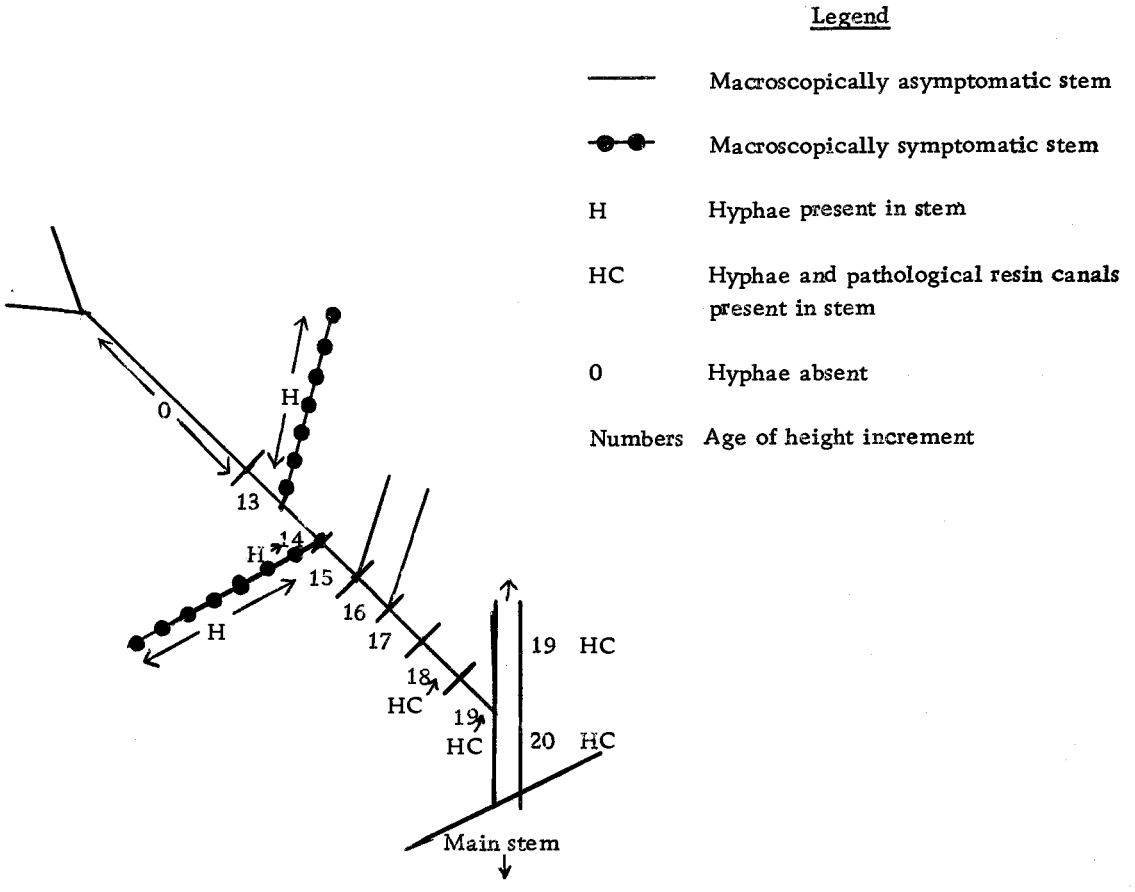

Figure 30. Distribution of hyphae and pathological resin canals within older stem tissue as determined by free-hand sections.

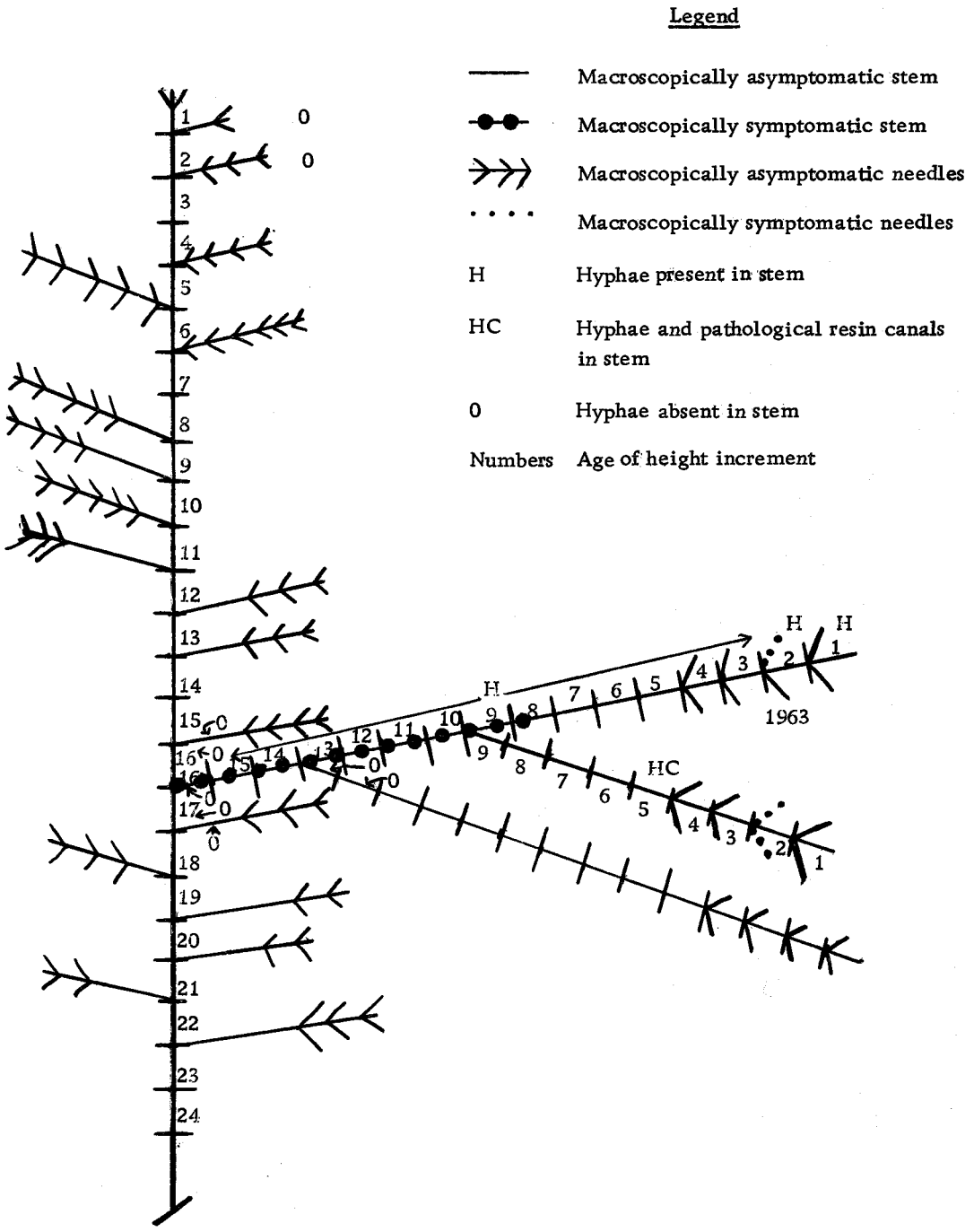

Figure 31. Distribution of hyphae along a single infected branch.

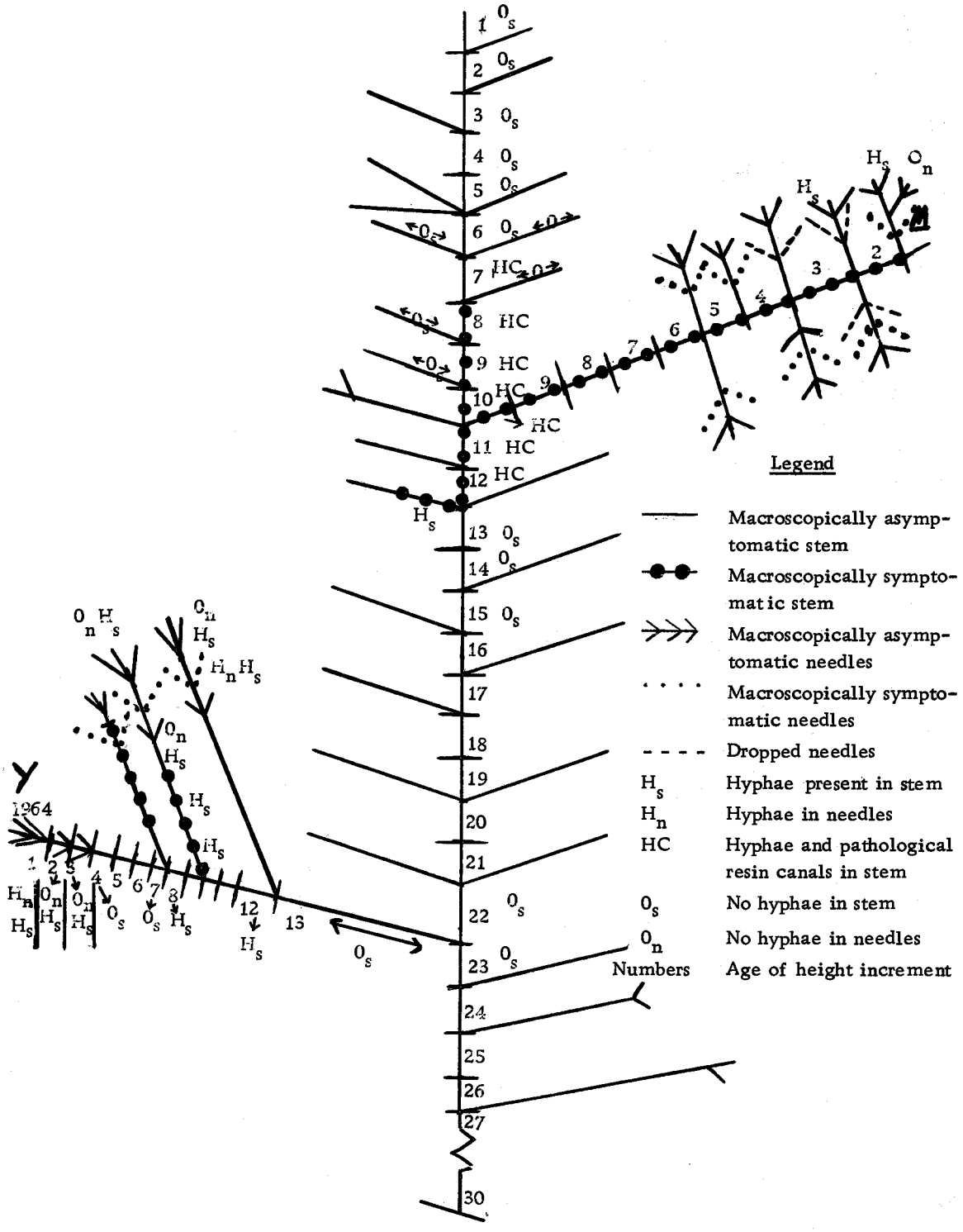

Figure 32. Distribution of hyphae within a tree with two widely separated, infected branches.

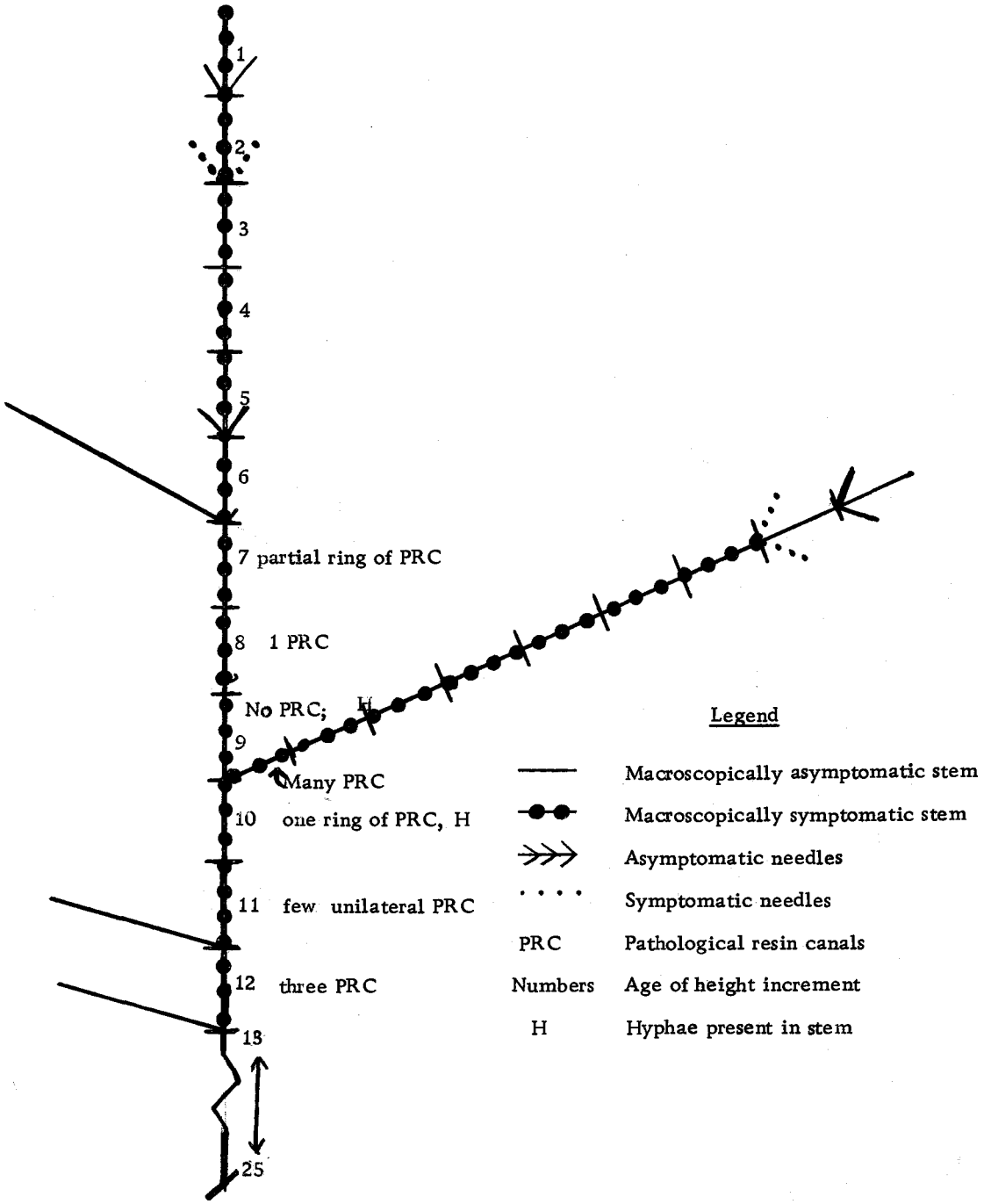

Figure 33. Distribution of hyphae within a tree bearing an infected leader and one infected branch.

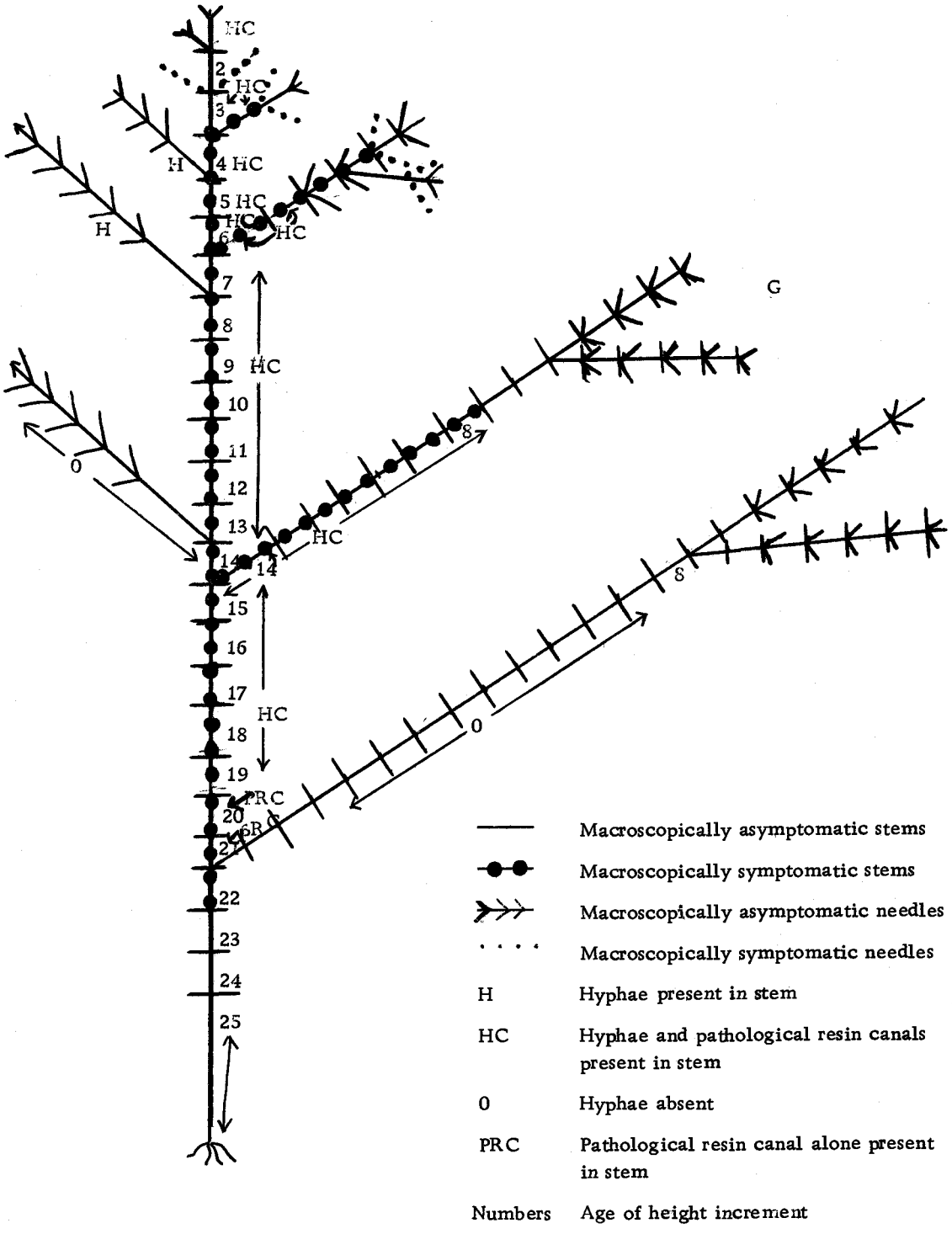

Figure 34. The distribution of hyphae within mature tissue.

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