Blue-stain fungi in xylem of lodgepole pine: a light-microscope study on extent of hyphal distribution[1]

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In midsummer mountain pine beetles emerge from lodgepole pine trees and fly to unattacked trees. While chewing vertical egg galleries in the inner bark of the tree, they inoculate into it a blue-stain fungus complex. Initially, the fungi are confined to the beetle frass of the egg gallery, but they soon grow into the sapwood. The fungi spread radially via the parenchyma of the xylem rays. Once established in the xylem rays, fungal hyphae move into the tracheids of the axial water-conducting system. Here they occlude bordered-pit pairs and occasionally the entire lumen of the cell. Fungal hyphae also attack and destroy resin-duct epithelial cells. This may result in release of resin into surrounding tissues. Destruction of storage and water-conducting tissues in the tree trunk is detrimental to renewed shoot tip expansion the following spring.

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Les dendrotocnes du pin ponderosa émergent de pins lodgepole au milieu de l'été et volent vers des arbres sains. En creusant des galeries de ponte verticales dans l'écorce intérieure de l'arbre, ils inoculent un complexe fongique produisant une coloration bleue. Les champignons sont d'abord confinés aux déchets de l'insecte dans les galeries de ponte, mais ils croissent rapidement vers l'aubier. Ils se répandent radialement par le parenchyme des rayons du xylème. Une fois établis dans les rayons du xylème, les hyphes envahissent les trachéides du système conducteur axial, où ils bouchent les paires de ponctuations aréolées et occasionnellement toute la lumière de la cellule. De plus, les hyphes fongiques attaquent et détruisent les cellules épithéliales des canaux à résine, ce qui peut provoquer la libération de la résine dans les tissus environnants. La destruction des tissus de stockage et des tissus conducteurs de l'eau dans le tronc de l'arbre est nuisible à l'expansion de l'extrémité des pousses le printemps suivant.

[Traduit par le journal]

Introduction

In the western regions of Canada and the United States, mountain pine beetle (*Dendroctonus ponderosae* Hopk.) infestations are devastating lodgepole pine (*Pinus contorta* var. *latifolia* Engelm.) forests. Mountain pine beetles in their feeding attack on host trees inoculate, via specialized "maxillary mycangia" (Whitney and Farris 1970), a blue-stain fungus complex (BSF) into the tree. The "complex" consists of several species of *Ceratocystis* and *Europhium* as well as several other mycelial fungi and yeasts (Robinson 1962; Robinson-Jeffrey and Grinchenko 1964; Robinson-Jeffrey and Davidson 1968; Whitney 1971). The complexity of the fungal species infecting the wood suggests that a microbial succession takes place. Though controversy exists (Hetrick 1949; Barras 1970; Whitney 1971; Yearian *et al.* 1972), studies on southern pine beetle (Barras 1973) have suggested a symbiotic beetle–fungus association to the extent that each partner is able to complete its life cycle more successfully in the presence of the other. Excellent reviews on this complex symbiotic partnership between beetle and fungi and their host trees are presented by Graham (1967), Franke-Grossmann (1964), Berryman (1972), Birch (1978), and Coulson (1979).

Although it is acknowledged that fungi benefit from transport to a food source and new reproductive potential, it is not fully understood what benefit is derived by beetles in this relationship or what role the fungus complex plays in the death of the host tree. Several questions concerning beetle–fungus interaction have been addressed in regard to possible nutritional relation,

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initiation, or confusion of host defense mechanisms against attacking beetles and predisposition of tree tissues for brood development and survival (Nelson and Beal 1929; Nelson 1934; Mathre 1964; Reid *et al.* 1967; Shrimpton and Whitney 1968; Berryman 1972; Shrimpton 1973, 1978; Wong and Berryman 1977; Webb and Franklin 1978). However, few detailed developmental or anatomical studies of the fungi within the host have been attempted. For example, as far as is known, only one study with a single illustrative micrograph showing fungal development in rays is available for lodgepole pine (Rumbold 1941). Shepherd and Watson (1959) reported on the presence of fungi in blue-stained wood in lodgepole pine, but no micrographs were included in this report. A few early reports (Nelson 1934; Nelson and Beal 1929) are available concerning fungal development in southern pines. They also present a few light micrographs showing hyphal establishment in ray parenchyma and some tracheids. Hubert (1931 (after von Schrenk 1903)) shows micrographs of fungi in xylem of northern white cedar and has a drawing showing BSF in bordered pits in Sitka spruce. Boyce (1938) recorded hyphae in radial views of southern yellow pine wood. Liese and Schmid (1961) studied pines and spruce with blue stain and showed passage of *Ophiostoma* sp. hyphae through bordered pits, mechanical destruction of pit membranes, and presence of hyphae in ray cells. This study seems to be the most comprehensive to date on hyphal development in blue-stained wood. Wong and Berryman (1977) described, in *Abies grandis*, the spread of *Trichosporium* sp. radially via sapwood rays, vertically via axial tracheids, and circumferentially via bordered pits of tracheids.

This preliminary report examines, at the light-microscope level, distribution of blue-stain fungi in the secondary xylem and phloem of *P. contorta* at the time of expression of tree death. It is recognized that fungal symbionts of the mountain pine beetle are numerous and complex, but it was not within the scope of this preliminary study to isolate, identify, and reinoculate with each, but rather to describe the extent of BSF colonization in lodgepole pine sapwood and inner bark at this stage of the beetle life cycle as occurs in nature.

Materials and methods

Study sites were located near the head of Logan Canyon in Cache County, UT (elevation 2300 m). Fifteen lodgepole pine trees ranging in size from 30 to 60 cm diameter at breast height were baited in mid-July 1978 and again in mid-July 1979 for beetle attack. Bait consisted of pheromone-impregnated tygon tubing tied around tree trunks 1–2 m aboveground. In the weeks after baiting, overwintering beetles emerged from trees attacked the previous year and successfully attacked 12 experimental and nearby unbaited trees. Sampling was conducted on two trees in the spring of the year following beetle attack (spring 1979 and 1980) until foliage of these trees faded from green to brown. As controls, bark and sapwood of nearby unattacked trees were sampled periodically throughout the growing season.

To determine microscopically the nature and extent of BSF hyphal development, diseased tissue was processed for 3 h in a sodium-cacodylate - buffered, full-strength Karnovsky's (Karnovsky 1965) fixative and postfixed for 2 h in 1% (aqueous) osmium tetroxide. Tissue samples were dehydrated in ethyl alcohol and infiltrated and embedded in Spurr's low-viscosity medium (Spurr 1969). Sections, 1–2 μm thick, were taken with glass knives on a Sorval MT-2 Porter-Blum ultramicrotome, stained with toluidine blue O, and viewed and photographed with a Zeiss WL research microscope.

Results

In northeastern Utah at an elevation of 2300 m young beetles emerge from their host, lodgepole pine, near the end of July. Usually, female beetles fly to large, unattacked trees, where they bore into bark to establish nuptial chambers. After mating the female bores a vertical egg gallery through the inner bark, cambium, and outer sapwood tissues (Figs. 1 and 2). It is through this activity that BSF becomes inoculated into host tissue.

Several weeks before beetle attack, cambial activity has renewed in lodgepole pines. Cambial growth in this species appears to proceed much the same as in other pines (Alfieri and Evert 1968), except for differences related to the higher elevations at which they grow. In mid-August or soon thereafter, after beetle attack has begun, resin ducts of late wood have differentiated as distinct, if not yet functional, entities (Fig. 1). During this period the female has constructed her egg gallery (Fig. 2, at the right margin). With the hatching of eggs and subsequent feeding activity of larvae, new routes of lateral growth are opened to fungi in the bark region. Important avenues for radial fungal development are the numerous uniseriate rays of the sapwood and phloem. The fungi appear to grow through and destroy the living ray parenchyma cells (Figs. 4, 5, 6, and 9), a feature which has been observed in other studies (Nelson and Beal 1929; Nelson 1934; Rumbold 1941; Liese and Schmid 1961). The ray parenchyma is a tissue involved with food storage and radial translocation of sugars and water. This would thus be the most favorable path of growth for the fungus. As a consequence, fungal development within rays is extensive before other nonliving portions are colonized. In accordance with this, hyphae would have little difficulty moving into axial tracheids via the relatively large pinoid, half-bordered pit pairs (Figs. 4 and 5). Should fungal contact be made with the less numerous horizontal resin ducts (Fig. 3), radial development is much the

same. These ducts, lined by epithelial cells also provide an "easy" nutrient-filled pathway for fungi into sapwood because the horizontal resin ducts of the phloem are continuous with those of the xylem. Here, growth is through the epithelial cells and the duct lumen itself. Destruction of resin ducts could conceivably result in release of resin into surrounding tissues.

Once the fungus complex has established itself in what formerly were living cells of the sapwood (Figs. 5, 6, and 9) and has moved into the axial tracheids (Fig. 5), the lumen of these tracheids may become heavily colonized with hyphae (Fig. 7, tracheids). The fungi move from tracheid to tracheid via bordered-pit pairs (Figs. 8 and 9A) and thus eventually fill the lumen of a number of these cells in what appears to be a significant proportion of the water-conducting system. Carlquist (1975) states that conifer tracheids are of such great length and wood rays are so numerous that tracheid contact with at least one ray by any given tracheid is inevitable. The consequence of this close association between rays and tracheids ensures eventual colonization of tracheids by these fungi, even if growth away from rays is not extensive.

It should be emphasized that the evidence presented in Figs. 4–9 was taken from xylem roughly 8–11 months after beetle attack. Further, it is important to stress the extensiveness of successional fungal development prior to needle browining. Tree death is herein defined by needle browning (necrosis) which follows beetle attack by aprpoximately 10 months. The living portions of the trunk, (i.e., sapwood rays, cambium, and phloem) have all been killed prior to final needle browning.

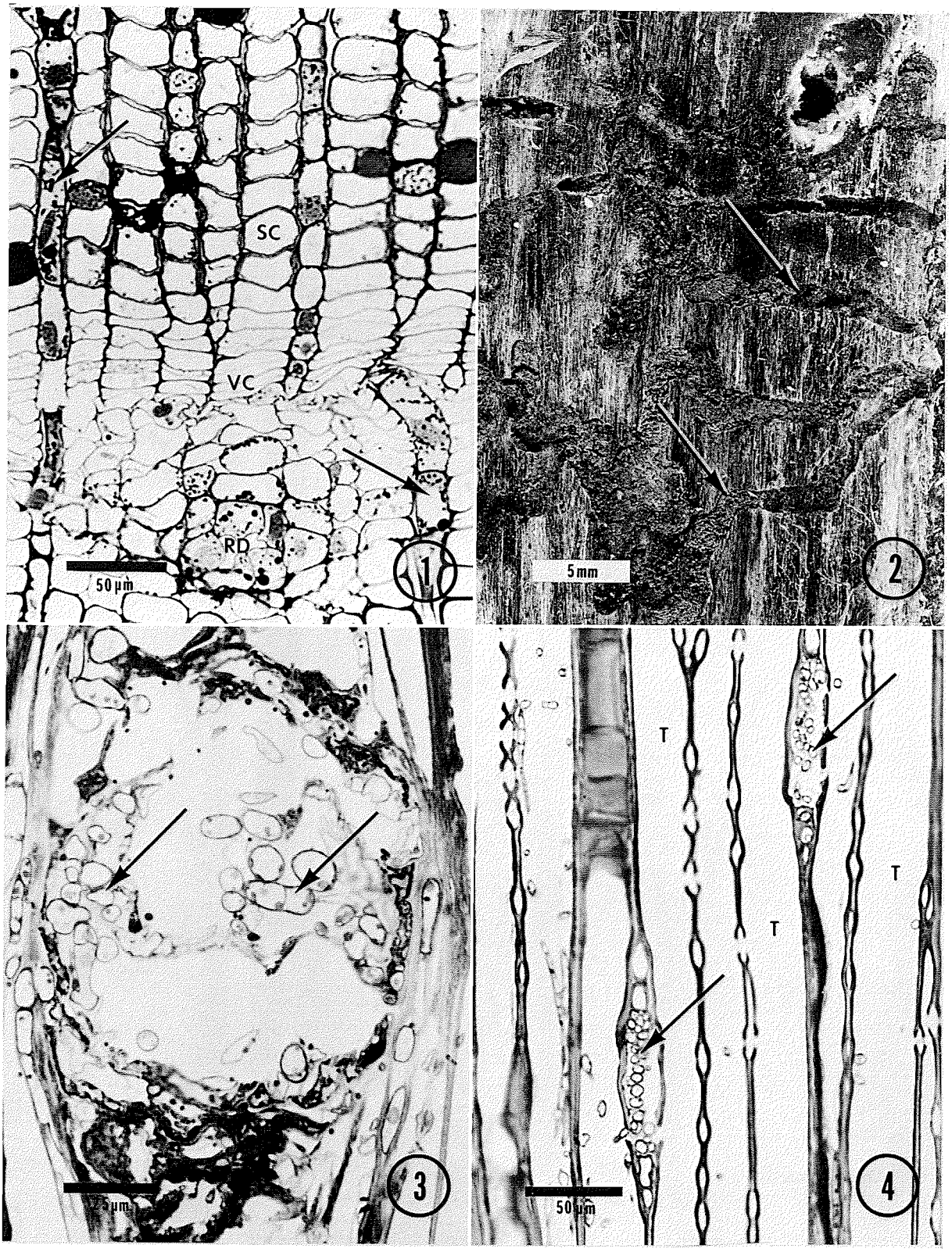
Discussion

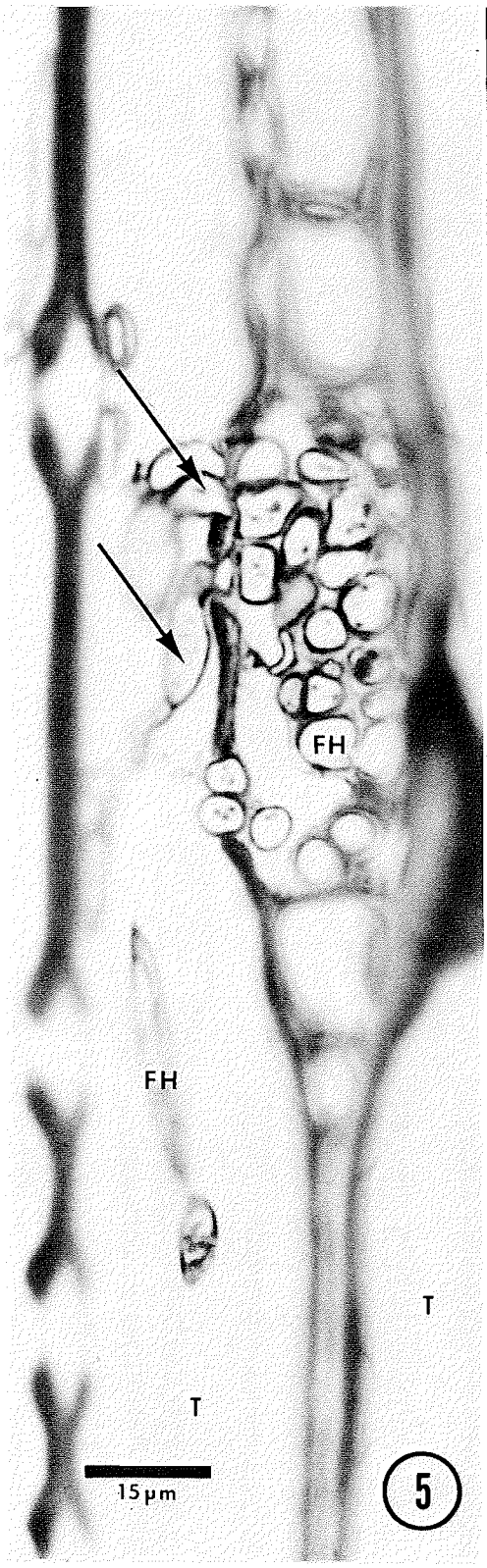
Nelson (1934), Caird (1935), and Bramble and Holst (1940) studied the effect of *Ceratocystis minor* on water conduction in southern pines and Mathre (1964) conducted similar investigations with Ponderosa pine. They all found that in trees, where inoculum was sufficient, water conduction up the tree was severely affected. Water was conducted around, but not through, infected regions of sapwood. The transpiration stream was also shown by Mathre (1964) to be disrupted between 0 and 6 mm in advance of the stained wood. Our preliminary water potential studies show beginings of water stress about 8 weeks after initial beetle attack (R. G. Ballard, unpublished observations). In the trees studied, water stress was always associated with development of blue stain in the sapwood. All studies have shown that when the entire circumference of the sapwood was stained blue, complete disruption of the transpiration stream ensued. Nelson (1934) suggested aspirated bordered pits were responsible for transpiration stream disruption. Mathre (1964) suggested that embolism in tracheids caused water flow disruption. Fares *et al.* (1980) have suggested a bimodal system of disruption. First, embolism would occur in tracheids subsequent to penetration by fungal hypahe. Second, with destruction of the resin-duct epithelium, resin leaks into surrounding tissues; the resin subsequently crystallizes

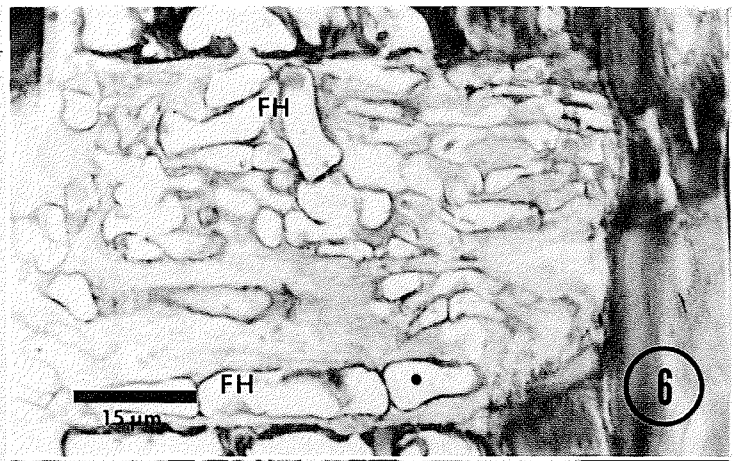
FIG. 1. Transverse section of *P. contorta* showing inner bark, vascular cambium (VC), and young sapwood. Sample was taken August 21, 1978. Axial resin ducts (RD) of the late wood have differentiated. SC, sieve cell. FIG. 2. Close-up view of egg (vertical) and larval (arrows) galleries constructed by *Dendroctonus ponderosae* as viewed from cambium looking outward. Beetles tunnel their way through bark and in doing so inoculate it with blue-stain fungi. Fungi grow vertically or tangentially into living phloem and radially into xylem through the living ray system. Note beetle exit hole in upper right. Sample taken the end of July about 1 year after beetle attack. FIG. 3. Tangential view of phloem ray possessing horizontal resin duct. Epithelial cells of duct have been destroyed by fungi (arrows). Resin ducts appear to represent a very efficient pathway for fungal entry into sapwood. Sample taken September 29, 1978, about 2 months after beetle attack. FIG. 4. Tangential view of sapwood showing extent of hyphal development (arrows) through living parenchyma cells of rays. Some axial tracheids (T) also exhibit portions of hyphae. Sample taken July 8, 1979, the year following inoculation (about 11 months after beetle attack). Tree is in final stages of browning.

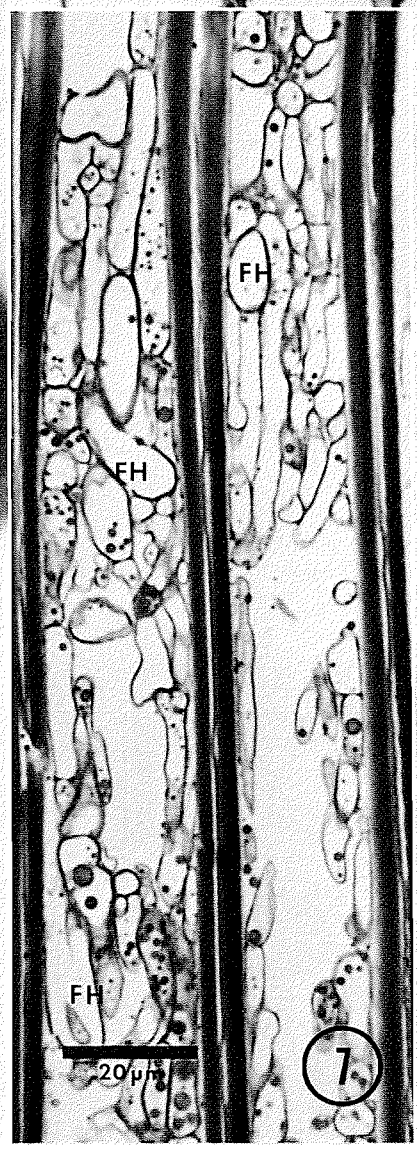
FIG. 5. Tangential view of xylem ray showing growth of fungal hyphae (FH) through pinoid, half-bordered-pit pairs (arrows) into lumen of tracheids (T). Sample taken July 8, 1979, the year following inocualtion. FIG. 6. Radial view of portion of xylem ray showing extensive development of fungal hyphae (FH) in region of former ray parenchyma cells. Sample taken July 8, 1979, the year following inoculation. FIG. 7. Radial view of xylem showing portions of two axial tracheids densely occluded by fungal hyphae (FH). Sample taken July 8, 1979, the year following inoculation. FIG. 8. Radial view of xylem showing face view of bordered-pit pairs of axial tracheids. Arrows point to bordered-pit paris where fungal hyphae traverse the walls. Compare this view with that shown in Fig. 9. Sample taken June 16, 1980, the year after inocualtion (about 10 months after beetle attack). The tree was still green.

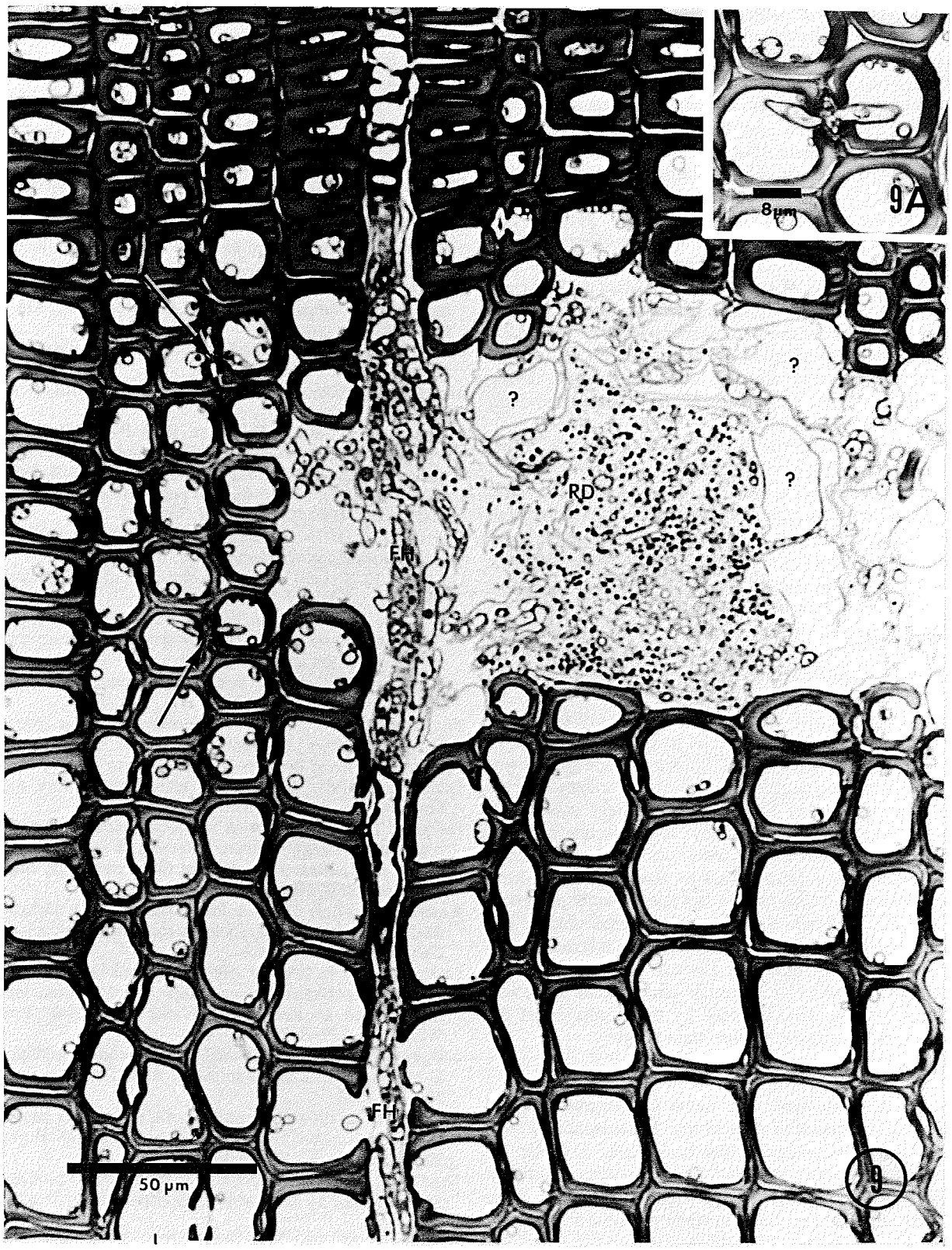
FIG. 9. Transverse section at lower magnification of xylem showing portions of early wood and late wood infected with blue-stain fungal hyphae (FH). Arrows point to hyphae traversing axial tracheid walls at bordered-pit pairs which are shown again at higher magnification in inset (Fig. 9A). A resin duct (RD) contains much evidence of fungal growth and destruction. Small black dots are probably either yeast cells or microconidia. Regions labelled (?) are probably remains of epithelial cell walls. Note the extensiveness of hyphae in the single ray and the cross-sectional views of hyphae in the single ray and the cross-sectional views of hyphae in virtually every axial tracheid. Sample taken July 8, 1979, 1 year after inoculation.











upon contact with water and lodges against tracheid bordered-pit membranes thus causing increased resistance to axial water flow. These theories have merit, though one possible weakness is that they would require the extensive colonization of host tracheids early in the disease cycle. This may not occur until a later date. More work is being done to elucidate the time frame of these events. In this preliminary report we have attempted to demonstrate the great extent of hyphal growth in tissues of lodgepole pine phloem and xylem. We have shown the complete destruction of the parenchyma system of the sapwood. The destruction occurs by the time of expression of death of the host trees in June of the year following attack. Yet in spite of extensive destruction of tissues in bark by the beetle larvae and colonization of the symplast by fungal hyphae, the trees are still able to maintain living cytoplasm in the extremities of the branching system, most notably the needles.

In early June, at the Logan Canyon study site, of the year following beetle attack, stem water potentials of blue-stained trees exceeded −40 bars (1 bar = 100 kPa) (R. G. Ballard, unpublished observation). Initial expansion of terminal buds in these trees was observed; these buds did not continue to expand normally, as did those of nearby healthy trees (R. G. Ballard, unpublished observation). A possible interpretation of this is that the transpiration stream to the crown of these trees had been disrupted causing a turgor deficit in growing points. Of great significance is the length of time these trees take to die after attack. While southern pines, attacked by *Dendroctonus frontalis* beetles and colonized by *C. minor*, take only a few weeks to succumb, foliage of lodgepole pine takes up to 11 months to fade and brown. This difference suggests the ability of lodgepole pine to minimize water losses in water-stress conditions. Recent studies (Lopushinsky 1969; Running 1976, 1980) have shown that trees suffering water-stress conditions may have effective mechanisms to limit transpirational water loss. When needle water potentials of −16 to −18 bars were reached, stomates closed. The needles still have high water content and it is maintained for a period of time after stomate closure. This may explain the ability of lodgepole pine needles to survive the stress condition imposed by the disruption of the transpiration stream by blue-stain fungi.

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