Penetration and Degradation of Cell Walls in Oaks Infected with Ceratocystis fagacearum

I. B. Sachs, V. M. G. Nair, and J. E. Kuntz

Research Technologist, Forest Products Laboratory, Forest Service, USDA, Madison, Wisconsin 53705; Assistant Professor, College of Environmental Sciences, University of Wisconsin, Green Bay 54301; and Professor, Department of Plant Pathology, University of Wisconsin, Madison 53706, respectively.

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ABSTRACT

Ceratocystis fagacearum initially invades the xylem vessels of the outer sapwood in infected oaks. Later, hyphae penetrate into the adjacent xylem parenchyma through the pits. Thereafter, hyphae grow intercellularly, intracellularly, and within the cell wall itself. Thus, the fungus also degrades the walls of infected sapwood cells, including the middle lamella. Lysis progresses from the lumen outward, much like the thinning action of white rot or stain fungi. Both the cellulose and lignin are affected. Hyphal penetration within the cell wall leads to cavities of varying size, shape, and direction. Electron-dense material forms within invaded and adjacent parenchyma cells. Fine structure of conidia and hyphae is described. Phytopathology 60:1399-1404.

Ceratocystis fagacearum (Bretz) Hunt invades the vascular tissue of susceptible oak species and induces plugging of the water-conducting vessels. Foliage wilt and premature defoliation follow. Red and black oaks die soon after infection, but infected white oaks may survive for several years. A considerable literature exists on various aspects of the oak wilt disease and the causal organism. Still lacking, however, is information concerning changes induced in the xylem elements of the infected host and the pathogen's fine structure as it relates to different stages of the fungus within the infected tissues.

This paper describes the penetration and degradation of cell walls in oak sapwood of diseased trees infected by the oak wilt fungus (17). Also reported are observations of the ultrastructure of the fungal cells of *C. fagacearum*, both in conidia and mycelium.

MATERIALS AND METHODS.—Sapwood chips, ca. 1 × 2 × 2.5 mm, were collected from several heights in the trunks, 15-20 cm diam at breast height (137 cm), of bur oaks (*Quercus macrocarpa* Michx.) and northern pin oaks (*Q. ellipsoidalis* E. J. Hill) previously inoculated in a single basal trunk wound and now showing various stages of foliage wilt. Each chip was divided radially; one-half was fixed immediately in the field and the other half was plated on acidified Nutramigen agar medium to determine the presence of *C. fagacearum*. The samples for histological study were fixed for 4 hr in 4% gluteraldehyde in 0.1 M cacodylate buffer with 0.2 M sucrose, and adjusted to pH 7.4. The material was then postfixed for 1 hr in 2% OsO_4 in buffer (2). After washing in distilled water, the specimens were dehydrated gradually in an alcohol series, then in propylene oxide, and finally embedded in epoxy resin.

The fungus was grown for 2 weeks in pure cultures on a modified Nutramigen agar (13). Mycelia and conidia from the advancing margin of the actively growing colonies were fixed and embedded as described above. Fixation with min disturbance to the fungus was best accomplished by pouring the fixatives directly into the culture dishes. Portions of the fixed cultures were removed for dehydration and embedment.

Sections of all embedded material were cut with diamond knives on a Porter-Blum I ultramicrotome. The sections were stained in uranyl acetate for 1.5 hr, in lead citrate for 1.5 min, and examined with an RCA EMU3D electron microscope.

RESULTS.—*Ceratocystis fagacearum* was recovered from most sapwood chips.

Histology of infected xylem.—The most obvious alteration in host xylem elements was the formation of tyloses and gums, but marked changes also occurred in the cell wall layers. Geary & Kuntz (6) reported that in addition to a cellulase, *C. fagacearum* elaborates a pectinase. Oxidizing enzymes capable of attacking or at least modifying lignin also must be produced by the fungus (17), as its hyphae passed easily through and readily degraded the compound middle lamella (Fig. 1, 2).

Hyphae frequently were found in the lumen of various xylem elements, and lysis of wall layers of such cells occurred. Hyphae also occupied bore holes both parallel and perpendicular to the cell wall axis (Fig. 2, 3). Cavitation and degradation of the cell wall layers were distinct. The conspicuous wall cavities, some with ragged edges and others with smooth edges, were without definite size, shape, or direction. Usually the cavities with ragged edges were close to areas of extensive wall dissolution, where thinning had progressed from the invaded lumen outward. In many sections of the tissue, the S_3 layer was etched and corroded. In others, etched zones apparently due to fungal enzymes also occurred in the S_2 and S_1 layers (Fig. 2, 3, 4). In addition, hyphae penetrated between host cells decomposing areas in the compound middle lamella (Fig. 1). Thus, lysis of the middle lamella often accompanied intercellular penetration.

Hyphae developed at certain points in the xylem vessels, and from these loci invaded the surrounding xylem parenchyma, especially the uniseriate and multiseriate ray cells which abut the xylem vessels. Hyphae

penetrated through the pits from xylem vessels to xylem parenchyma, but from xylem parenchyma to xylem parenchyma cells they penetrated both through pits and through cell walls (Fig. 3, 6) (8, 16). Hyphae frequently penetrated primary pit fields containing plasmodesmata (Fig. 7). Moreover, appressoria with penetration pegs often developed at the point of penetration of the thickened walls of ray and xylem parenchyma (15). The organism readily degraded the walls of invaded fiber, xylem parenchyma, and ray parenchyma cells. The appearance of the corroded host cell walls indicates that enzymes necessary for the depolymerization of cell wall constituents may be secreted not only at the tip of the hypha but also along its entire length (Fig. 2) as reported for other fungal species (10, 11). Lysis of wall layers of invaded host cells was greatest at the hyphal tip. Infected oak roots showed similar cell wall degradation.

Fine structure of C. fagacearum.—Transverse and longitudinal sections of young hyphae exhibited a fine grain cytoplasm bound by a definite cell wall approximately 0.06 μ thick (Fig. 8). The hyphal cell wall was evident in most sections; however, in some instances the wall was indistinct and the hypha appeared autolyzed. Many longitudinal and transverse sections exhibited areas with reduced cytoplasm and without nuclei, mitochondria, or other cytoplasmic organelles.

The cell wall appeared rather dense and uniform, usually with a distinct plasmalemma closely appressed over most of its inner surface. In many hyphal cells the plasmalemma was wavy, with occasional finger-like projections into the cell lumen and invaginations into the cell wall (Fig. 9). Hawker (7) reported that the undulating-type plasmalemma with invaginations is found in old fungal cells. But in this study the undulating type occurred in young hyphal cells of actively growing fungus colonies. In transverse sections, these invaginations appeared as vesiclelike structures. They measured from 150 to 250 Å in diam (Fig. 9). In addition, circuitous structures 30 to 80 Å in diam and channellike structures 25 to 85 Å in diam were found in the cell wall (Fig. 10). These may be portions of a branching system of channels distributed through the wall similar to those reported in *Pythium debaryanum* by Manocha & Colvin (12). Sections through the outer edge of the cell wall revealed minute circular areas that may be openings and a part of the above-mentioned channel system.

Nuclei measured approximately 1.0 to 2.0 μ in diam, and in cross section occupied a major portion of the cell (Fig. 12). The nuclear membrane contained pores, as has been reported for nuclei of other fungi (1). The nucleolus measured 0.3 μ in diam. Mitochondria frequently were seen in transverse and longitudinal sections of the hyphae. Double membranes surrounded the mitochondria; cristae projected from the inner membrane. Complex concentric membranes, ribosomes, vesicles, and lipid bodies were distributed throughout the cytoplasm. Occasionally a large osmiophilic body was associated with a vacuole and in contact with the smooth endoplasmic reticulum. Frequently, lomasomes also were found in contact with the plasma membrane (Fig. 11) or free in the cell lumen.

The septa between hyphal cells were of the Ascomycete type with a single simple pore (14). The pores were simple, not swollen or cupped, and measured approximately 0.2 μ in diam. Occasionally a septal plug occupied the center of a pore. The membrane lining the pore appeared as a continuation of the plasma membrane of adjacent cells (Fig. 13). In some sections, cytoplasmic organelles were found within the septal pore itself, suggesting that the material was in the process of migration at the time of fixation.

The fine structure of conidia appeared similar to that of the hyphal cells in respect to most of the cytoplasmic organelles, but the cytoplasm was more dense and the nucleus did not occupy a large part of the cell. Most conidial nuclei measured approximately 0.5 μ in diam.

In longitudinal and transverse sections of conidia, large voids and lomasomes were not observed. Electron-dense bodies which may be glycogen were present. Only rough endoplasmic reticulum has been observed; Golgi apparatus was present.

DISCUSSION.—*C. fagacearum* can "decay" invaded wood cells. The pattern or character of wood decay often indicates the type of causal organism (3). So-called "soft rot" of sapwood commonly is induced by Ascomycetes (4, 5) that invade the outer sapwood. Elongated cavities are formed in the secondary cell

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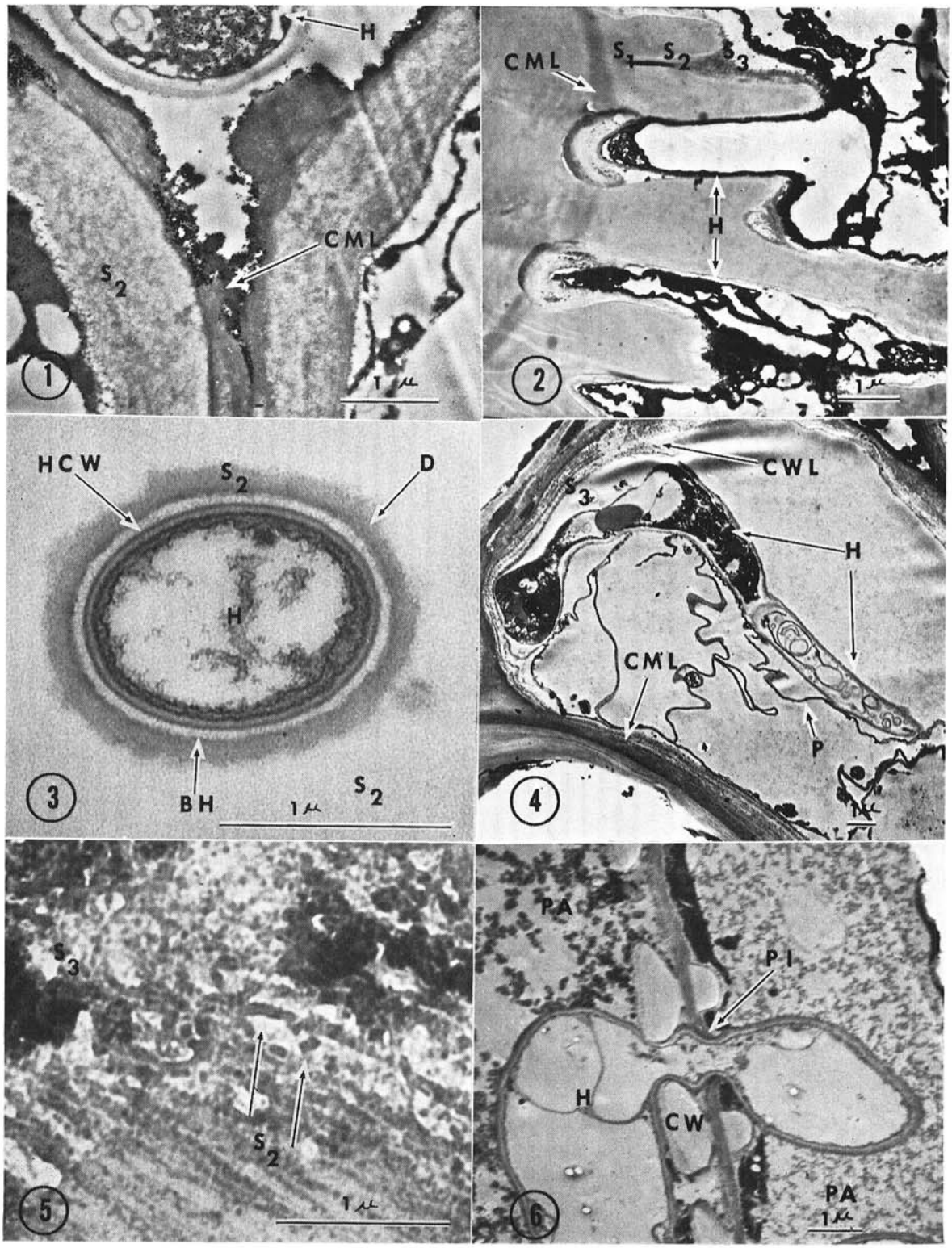
Fig. 1-6. **1)** Transverse section of xylem parenchyma cells of infected bur oak and of a hypha fixed in OsO_4. Lysis of the cell wall is quite clear. Compound middle lamella (CML), secondary wall (S_2), and normal hypha (H). (ca. ×16,000) **2)** Branching hyphae penetrating cell wall from the lumen outward. Enzymes appear to be secreted not only at the tip of the hypha but also along its entire length. The organism was fixed during penetration of the middle lamella. Note swelling of the constituents of the cell wall and middle lamella. The organism has undergone autolysis. Compound middle lamella (CML), secondary wall ($S_{1,2,3}$), and autolyzed hyphae (H). (×10,000) **3)** Transverse section of hypha-occupied bore hole in the secondary wall. Dark area (D) may be due to hyphal enzymatic action on chemical constituents of the secondary wall (S_2). Hypha (H), hyphal cell wall (HCW), bore hole (BH). (×37,000) **4)** Transverse section of a springwood parenchyma cell infected with a hypha (H) of the pathogen *Ceratocystis fagacearum*. Cell wall lysis (CWL) appears to have proceeded from the S_3 outward. Compound middle lamella (CML), plasmalemma (P) of host cell. (×4,500) **5)** Lysis of cell wall, a consequence of enzymatic action by *C. fagacearum*. As the cellulose is digested, the remaining suppositive lignin appears to match the cellulosic fibrils before complete separation takes place. In some areas, it appears that there is an unwinding or uncoiling before the separation is complete (arrows). Secondary wall ($S_{2,3}$). (×28,000) **6)** Hypha (H) passing through pit (PI) of cell wall (CW) between ray parenchyma cells (PA). (×8,200)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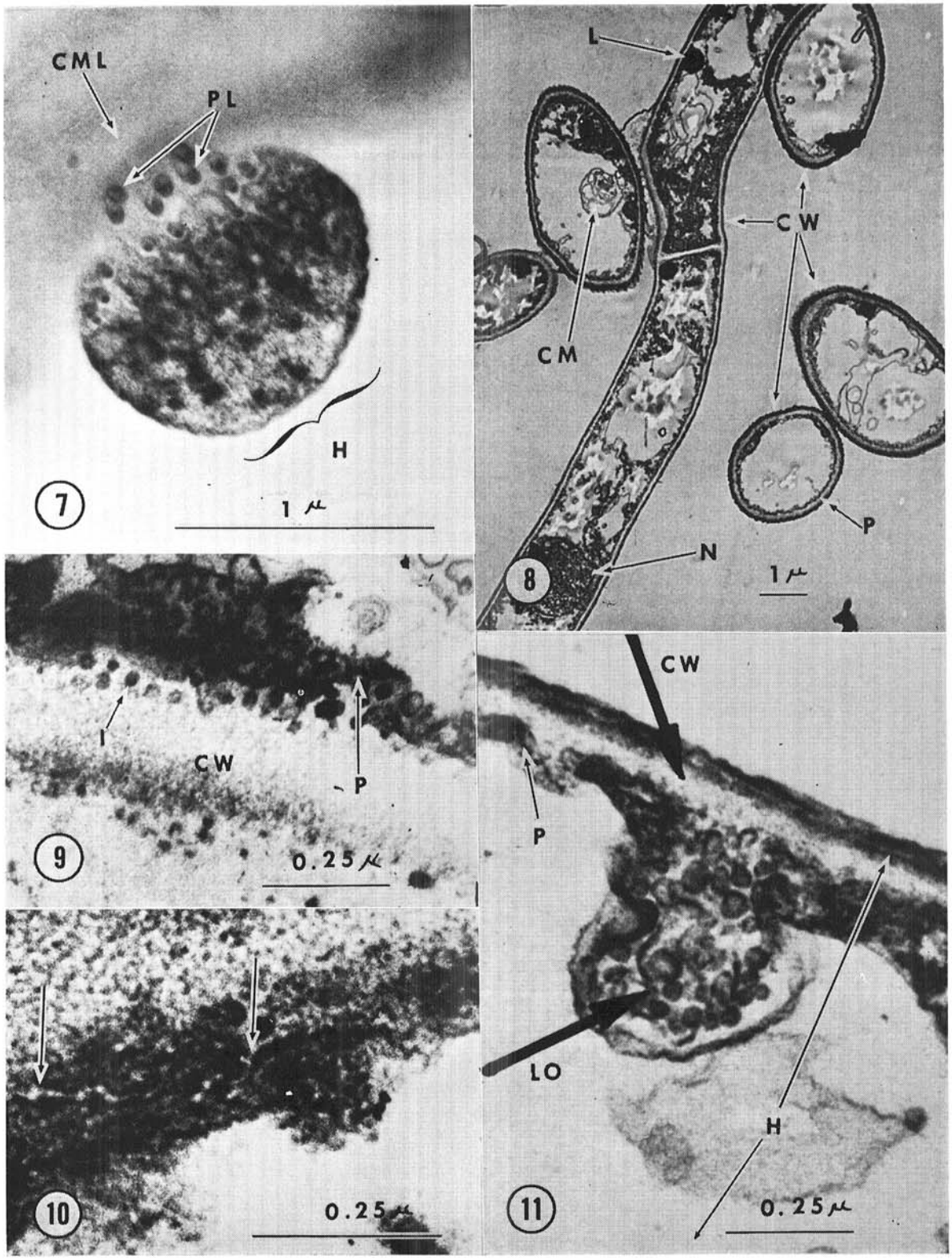

Fig. 7-11. **7)** Oblique section of hypha entering the primary pit field. Compound middle lamella (CML), plasmodesmids (PL), hypha (H). (×42,000) **8)** Longitudinal and transverse sections of *Ceratocystis fagacearum* vegetative hyphae illustrating some cytoplasmic details. Prominent cell wall (CW), plasmalemma (P), nucleus (N), lipid bodies (L), complex concentric membrane (CM). (×7,800) **9)** An oblique longitudinal cut through a hypha exposing a transverse view of plasmalemma invaginations (I). Plasmalemma (P), cell wall (CW). (×81,000) **10)** Longitudinal view of the branching channel system (arrows) throughout the cell wall. (×130,000) **11)** Longitudinal section of hypha (H). Lomasome (LO) in close contact with the plasmalemma (P). Cell wall (CW). (×90,000)

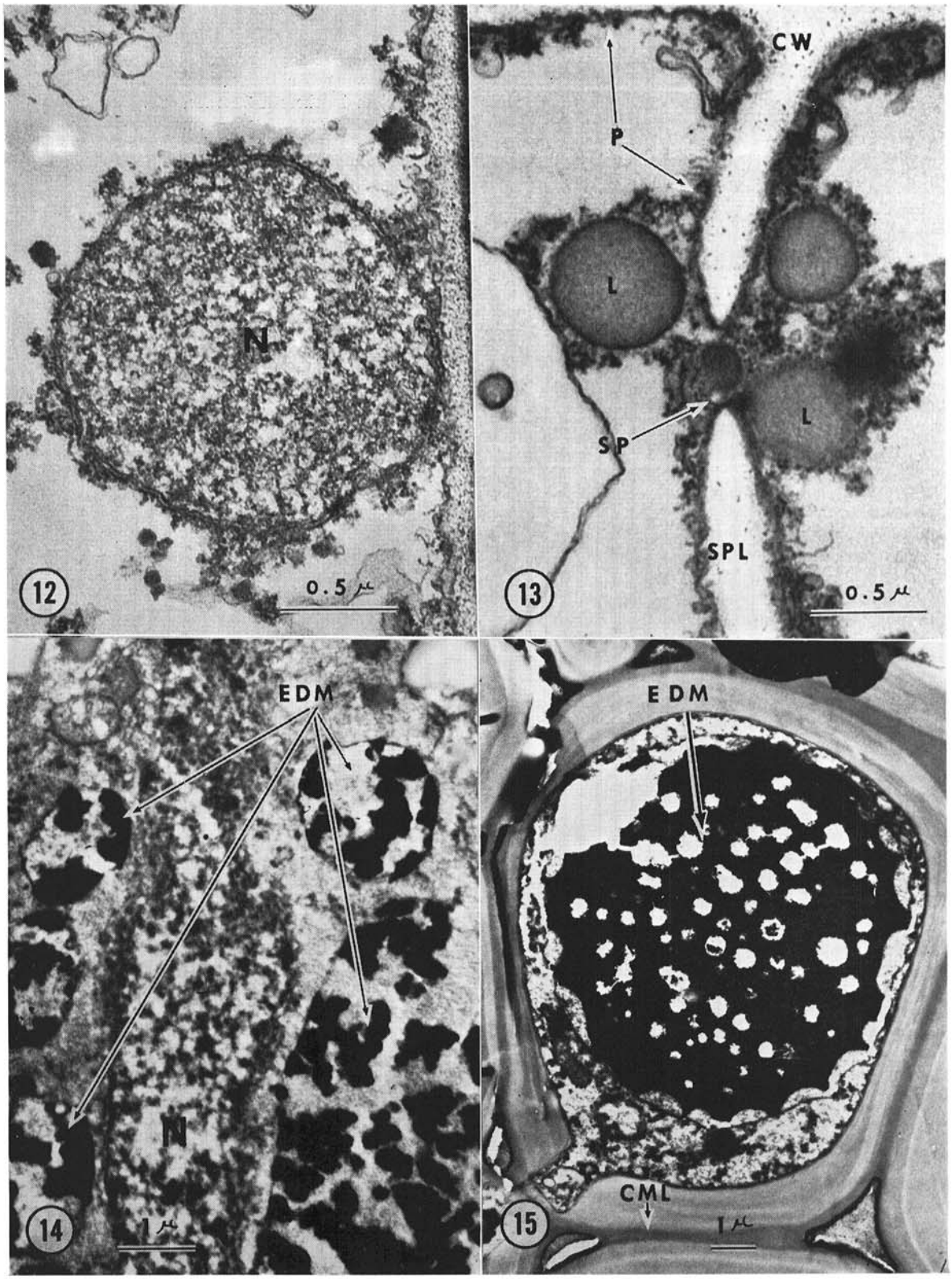

Fig. 12-15. 12) Transverse section of a hypha. Nucleus (N) is surrounded by a perforated double membrane. (×37,200) **13)** Hyphal section through septum showing pore and associated structures. Lipid body (L), cell wall (CW), plasmalemma (P), septal pore (SP), septal plate (SPL). (×37,200) **14)** Transverse section of ray parenchyma cell showing small islands of electron-dense material (EDM) building up in the cells. Nucleus (N). (×12,300) **15)** Transverse section of ray parenchyma cell showing electron-dense material (EDM) that formed from coalesced islands seen in Fig. 14. Compound middle lamella (CML), secondary wall (S). (×7,000)

walls as cellulose is destroyed. Duncan (4) described such soft rot of wood invaded by *Chaetomium globosum*. Cellulose of the secondary wall was dissolved enzymatically to form cavities that were oriented either helically around or parallel to the long axis of the cell. Such attack suggested hydrolysis along planes determined by the structural orientation of the cellulose. The cavities were diamond-shaped with pointed ends.

Ascomycetous fungi of the genus *Ceratocystis* also cause wood stain. Hyphae develop sparingly in vessels and tracheids, but abundantly in xylem and ray parenchyma. These fungi live mainly on substances within the cell, but may attack the cell walls to a limited extent (18). Their hyphae often pass directly through walls by means of bore holes, but usually through simple or bordered pits. Hyphae sometimes occur in the middle lamella. Walls between infected ray cells may be conspicuously thinned or even decomposed completely.

Hyphae of *C. fagacearum* developed only sparingly in xylem vessels, at least until the late stages of disease development. Though the hyphae penetrated through pits from infected vessels to the adjacent xylem parenchyma, later they grew intercellularly, intracellularly, and even within cell walls; and developed abundantly in xylem and ray parenchyma. Although chemical analyses have not yet determined which wall constituents are consumed by the fungus, polarized light microscopy has indicated that cellulose had been removed. A residual substance that may be lignin mirrored the shape and arrangement of the cellulose fibrils (Fig. 5). In some areas, the presumed lignin had contracted before fixation, suggesting cleavage of lignin-carbohydrate bonds.

As reported by Levi (9) for other fungi, *C. fagacearum* also degraded most frequently the cell wall from the lumen outward. Multi-layering of the cell wall, orientation of the cell wall microfibrils, and lignin content of the wall appeared to present little or no difficulty to fungus penetration.

In bur oaks, especially, masses of dark, amorphous, electron-dense material frequently developed in parenchyma cells adjacent to infected vessels (Fig. 14). Often, small globules of this material had coalesced and had formed a large mass that completely filled the cell lumen (Fig. 15). This material may be one of the phenolic tannins of oak. Very rarely have hyphae been observed in parenchyma cells containing this material. Whether this substance possesses a fungistatic or fungitoxic effect which restricts hyphal penetration of these cells is unknown. But in bur and white oaks, the fungus often appears restricted to initially invaded sapwood tissues. From these infected tissues, the fungus makes little further growth, either radially or tangentially. Frequently, the tree is able to lay down new wood over the arc of infected wood, and recovers. Thus, these substances may contribute to resistance or recovery mechanisms, particularly in bur and white oaks.

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