Article

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Extracellular sheath formation by *Sphaeropsis hypodermia* and association with its infection in elm trees

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Sphaeropsis hypodermia, isolated from a cankered American elm branch, was grown on agar medium (PDA), on autoclaved wiping paper (Kimwipes), and American elm (*Ulmus americana*) wood chips, or inoculated into greenhouse-grown American elm saplings. Samples from each treatment were double-fixed with glutaraldehyde and osmium tetroxide and examined with the light and the transmission electron microscopes. Ultrastructurally, the hyphae on PDA and inert substrates appeared surrounded by large extracellular sheaths which were delimited by rigid opaque bands of various thicknesses. The sheaths extended appreciable distances from the fungal cells, as evidenced by their adherence to rigid substrates. Individual or aggregated opaque bodies, even as large masses on elm wood chips, were the main components of the sheath. This opaque material was often associated with penetration and ruptures of the wood cells. Inoculated into elm trees, the fungus rapidly caused pronounced alterations of cambial tissues and colonized the adjoining bark and xylem cells. The prominent penetration and breakdown of the inner and outer bark cells by the fungus were associated with opaque material, particularly in cortical fibres. The material was structurally similar to the sheath formed on the rigid sterilized substrates. In the xylem, only the walls of the recently deposited cells were visibly altered, and although mature fibres were generally colonized, the passage of the fungus from one fibre to another was rarely observed, contrary to the passage from vessel and ray cells to adjoining cells. In that instance, only bands of opaque material present in the walls of fibres were connected with fungal cells in their lumen. In the inner bark and cambial regions, cell hypertrophy and hyperplasia occurred next to host walls that were altered and contained similar opaque material. The extracellular sheath of *S. hypodermia* under *in vitro* conditions and the opaque material associated with host wall alterations *in vivo* are considered to be analogous.

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[Formation de couches extracellulaires par le *Sphaeropsis hypodermia* et lien avec l'invasion de celui-ci dans l'orme]

Nous avons observé le mode de développement de *Sphaeropsis hypodermia* (isolé d'une branche d'orme (*Ulmus americana*) atteinte d'un chancre) dans les milieux suivants : un milieu gélosé (PDA), un tissu essuie-tout (Kimwipes), des blocs de bois d'orme stérilisés à l'autoclave, et des ormes d'Amérique inoculés en serre. Des échantillons de chaque substrat ont été fixés pour les observations en microscopie photonique et en microscopie électronique avec du glutaraldéhyde et du tétroxyde d'osmium. Observés en microscopie électronique, les hyphes accolés au substrat étaient entourés d'une épaisse couche extracellulaire devenant circonscrite par des bandes rigides et contenant des corps opaques simples ou agrégés, ou des masses de matière opaque plus volumineuses dans le cas des blocs de bois d'orme. Sur celui-ci et sur le tissu Kimwipes, la couche extracellulaire s'étendait sur une distance appréciable, loin des cellules fongiques, et avait également pénétré les parois de cellules de parenchyme et des fibres, selon le cas. Dans les ormes inoculés, le champignon a rapidement produit des dommages notables dans les tissus du cambium et colonisé abondamment les régions de l'écorce et du xylème avoisinantes. La pénétration et la dégradation des parois cellulaires de l'écorce étaient marquées, en relation également avec de la matière opaque entourant les cellules du champignon. Cette matière ressemblait à celle liée aux cellules fongiques sur les milieux stérilisés. Bien que les cellules du xylème étaient généralement colonisées, des altérations pariétales n'étaient apparentes que dans les cellules récemment formées. En outre, le passage du champignon d'une cellule à l'autre à travers les parois n'a été observé que dans le cas des éléments de vaisseaux et des cellules de rayon. Concernant les fibres, seule y était visible une bande de matière filamenteuse dans les parois et liant les cellules fongiques présentes dans la lumière de ces fibres. En réponse à la dégradation de parois cellulaires liée à de la matière opaque, l'hypertrophie et l'hyperplasie des cellules du cambium et de l'écorce interne ont été observées, liées possiblement à la formation d'une barrière de protection. On discute du rôle possible de la couche extracellulaire des cellules fongiques *in vivo* et *in vitro*.

INTRODUCTION

In 1996, an approximately 10-m tall American elm tree (*Ulmus americana* L.), growing on the campus at Université Laval (Sainte-Foy, Quebec, Canada), showed a withering of branches that might have been at first sight attributable to a wilt disease. Upon sampling, small cankers were observed on these branches, from which a fungus, which produced large pycnidial conidia in culture, was isolated. The fungus was keyed to a *Botryodiplodia* sp. (Von Arx 1974), thus as one of the possible canker-causing pathogens on elms (Riffle 1981; Sinclair *et al.* 1987). *Botryodiplodia hypodermia* (Sacc.) Petrak & Sydow is known to cause cankers on several elm species in the United States but most importantly on *Ulmus pumila* L. (Krupinsky 1981; Riffle 1978; Sinclair *et al.* 1987), and to be more damaging on this species than other canker-causing fungal pathogens, such as *Tubercularia ulmea* J.C. Carter and *Cytospora* sp. (Krupinsky 1981). A slight difference in the size of conidia in our isolate (see below) from those given by others (Petrak and Sydow 1926; Riffle 1981) has nevertheless been observed, but in want of further collections of the fungus in this part of the country to confirm the persistence of such differences, we have

identified our isolate as *Sphaeropsis hypodermia* (Sacc.) Höhnel, which is now considered to have precedence over *B. hypodermia* (W. Sinclair, Cornell University, Ithaca, NY, personal communication).

As far as known, this pathogen has been reported only twice (under *Botryodiplodia* sp.) in Canada (Ginns 1986), but its presence in Quebec is now reported for the first time. As its occurrence may be more prevalent on small branches of American elm trees than hitherto suspected, it should be taken into consideration in control programs for Dutch elm disease. Also, this pathogen may occur frequently as a cause of branch mortality on *U. pumila* in Quebec as in the Great Plains in the United States (Krupinsky 1981; Riffle 1978). Up to now, branch dying in *U. pumila* in Quebec might have been attributed to infections by *Tubercularia* sp. As to our knowledge, no published studies on the histopathology of the elm canker caused by *S. hypodermia* exist, it seemed relevant to undertake such studies, particularly with a view to determining whether the thick extracellular sheath that was observed to form around cells of the fungus in culture (see below) was also produced in the host and involved in the infection process. The present work reports on results of such studies.

MATERIALS AND METHODS

Pathogen isolate

The pathogen was isolated in 1996 from a 10-m tall American elm tree (planted 2 yr earlier on the campus of Université Laval (Sainte-Foy, Quebec, Canada) and kept since then on a 2% potato dextrose agar (PDA) medium at 4°C. A transfer from that culture, grown on PDA at room temperature and on a laboratory bench, was used as inoculum. Pycnidia formed on this medium as well as on the other substrates mentioned below, including cankered elm branches. The pycnidia contained large, hyaline, thick-walled conidia, which were of consistent sizes on the substrates on which the fungus was grown (see below).

Study material

Wood chips of approximately 2 mm were collected from American elm branches at the beginning of the dormant season in 1998, autoclaved, placed next to plugs of PDA colonized by the fungus in a small Petri dish, and left to be invaded by it. The chips were bark free following autoclaving. Pieces of autoclaved 2 mm x 2 mm Kimwipes paper strips (Type 900-L, Kimberly-Clark Corporation, Toronto, Ontario, Canada) were similarly placed on inoculated PDA medium. As controls, non-inoculated, sterilized, wood chips and pieces of the Kimwipes paper were used. Incubation was at room temperature with alternating light and dark periods. Sampling was done 5 and 14 d after inoculation.

Two inoculation experiments were conducted with greenhouse-grown 3-yr-old American elm seedlings of the same seed source, in March and April 1999. These were grown in a 2:1 mixture of peat moss and vermiculite, under a 16-h illumination period with 400 W high-pressure sodium lamps, and at 24°C daytime and 18°C nighttime. Trees were fertilized weekly with 1 g of 20-20-20 fertilizer. In the first experiment, typical cankers had girdled the inoculated branches within 7 d, over more than 10 cm from the inoculation point, and leaves were wilting. The cankers in the second experiment developed less rapidly, but similar symptoms were pronounced from 10-18 d post inoculation. Seedlings were inoculated by placing a disk of the PDA-grown pathogen in a 5 mm hole made with a sterilized borer into a surface-sterilized portion of 1-yr-old branches. Controls consisted of plugs of non-inoculated agar. Both inoculated and control wounds were covered with pieces of waxed paper to prevent them from drying. Samples obtained from the second test 3, 7, and 18 d after inoculation, with one plant for each interval, were fixed for microscopic observations. Similar inoculations were also made at the base of 2-yr-old stems and branches. Larger branches, from dormant trees, were cut into lengths of about 20 cm and similarly inoculated to verify the effectiveness of the inoculation method. They were also placed in the greenhouse

under the conditions mentioned above. In these, as well as in inoculated samples, pycnidia with large conidia developed through the invaded region of the bark tissues. A transfer of these conidia on PDA yielded cultures similar to the original culture.

Fixation procedures

The following samples were fixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde, postfixed in reduced osmium (Tamaki and Yamashina 1994), and dehydrated in a series of ethanol:

1. 2 mm x 2 mm strips of the Kimwipes paper;
2. approximately 2 mm x 2 mm pieces from the agar medium and the inoculated elm wood chips; and
3. 2 mm pieces from inoculated branches, some with adhering bark tissues, and taken 1 cm from the inoculation wound up to the visible tip of advancing infection.

A portion of the samples was embedded in Epon 812 and another one in LR White medium. As embedding in Epon was generally better than with LR White medium, present observations concern mainly the first samples. From 3 to 7 blocks from the 1-yr-old branches from each sampling date and for both embedding media were prepared.

Microscopy

For light microscopy, sections (1 µm thick) were stained with Toluidine blue O and Safranin O as previously described (Rioux and Ouellette 1989) and examined with a Polyvar microscope. Ultrathin sections (90 nm) mounted on copper or nickel grids were contrasted with uranyl acetate and lead citrate according to standard procedures. Examinations with transmission electron microscope were done with a Philips 300. At least four sections from two samples from each sampling date were examined in light and electron microscopy.

RESULTS

The pathogen

When inoculated on PDA or on the other sterilized substrates, large, hyaline guttation droplets accumulated on aerial and prostrate hyphae, as an apparent initial step in the formation of pycnidia in a manner similar to that described by McPhee and Colotelo (1977) for fruiting bodies in cultures of other fungi. Large pycnidial conidia formed within 7 d of culture incubation. Measurements of the four sets of 50 conidia from the various substrates gave sizes of 32-36 µm x 12-16 µm (mean 34 µm x 16 µm). The mode of pycnidial formation also presented peculiarities that will be described elsewhere.

Growth on sterilized substrates

Ultrastructural observations

Extracellular sheaths, which encompassed individual as well as groups of fungal cells, were regularly observed in samples from PDA as well as from wood chips and Kimwipes paper. The extracellular sheaths had various configurations and contents of diverse appearances. Some features of the sheaths are presented below as a point of reference for the observations to be presented hereafter.

As seen in Figure 1, the sheaths were generally thick and limited exteriorly by opaque bands and were similar whether they surrounded cells with intact or altered contents. Sheaths were at times localized as a kind of mound on one portion of the fungal cell, close to the fungal wall, or over a compact, opaque layer apposed to it. Membranous-like layers extended free in the surrounding medium and often fused with the sheath- or mound-delineating bands, which accounted for the localized thicker regions of the bands.

The sheaths contained opaque bodies that were increasingly greater in size the further they were from the fungal wall (Fig. 1). The denser areas in the sheath likely resulted from a compaction of such bodies. Arrays of fibrillar material (not illustrated in detail here) also occurred in the sheath, but were

generally the main components present in the vicinity of the fungal wall.

On Kimwipes paper, compact, opaque bands, similar to the sheath-delimiting bands described above, extended for a noticeable distance affixed to fibre contours as well as along the exposed side of the sheath (Figs. 2, 3). Bands of opaque matter adjacent to the sheath or linked to fungal cells having grown in the fibres also extended through the fibre walls (Figs. 2, 3). The sheath material itself was ordinarily more dispersed than on the agar medium (Fig. 2). Fibres of non-inoculated paper were completely free of any opaque material, as were those located away from the fungal cells (not illustrated).

Large masses of material likewise occurred around individual or groups of superimposed hyphae present in the vicinity of developing pycnidia on the surface of the elm wood chips, and likewise extended some distance from fungal cells. Opaque components were a major part of this material, which often extended from the surface of the wood chips to between fibres or other types of cells sectioned crosswise (Figs. 4, 5). Secondary walls of the outermost cells of the elm wood sample, and more rarely of some of the inner cells, were cut across by bands or strands of the opaque material (Fig. 5).

Inoculated elm trees

Observations with the light microscope

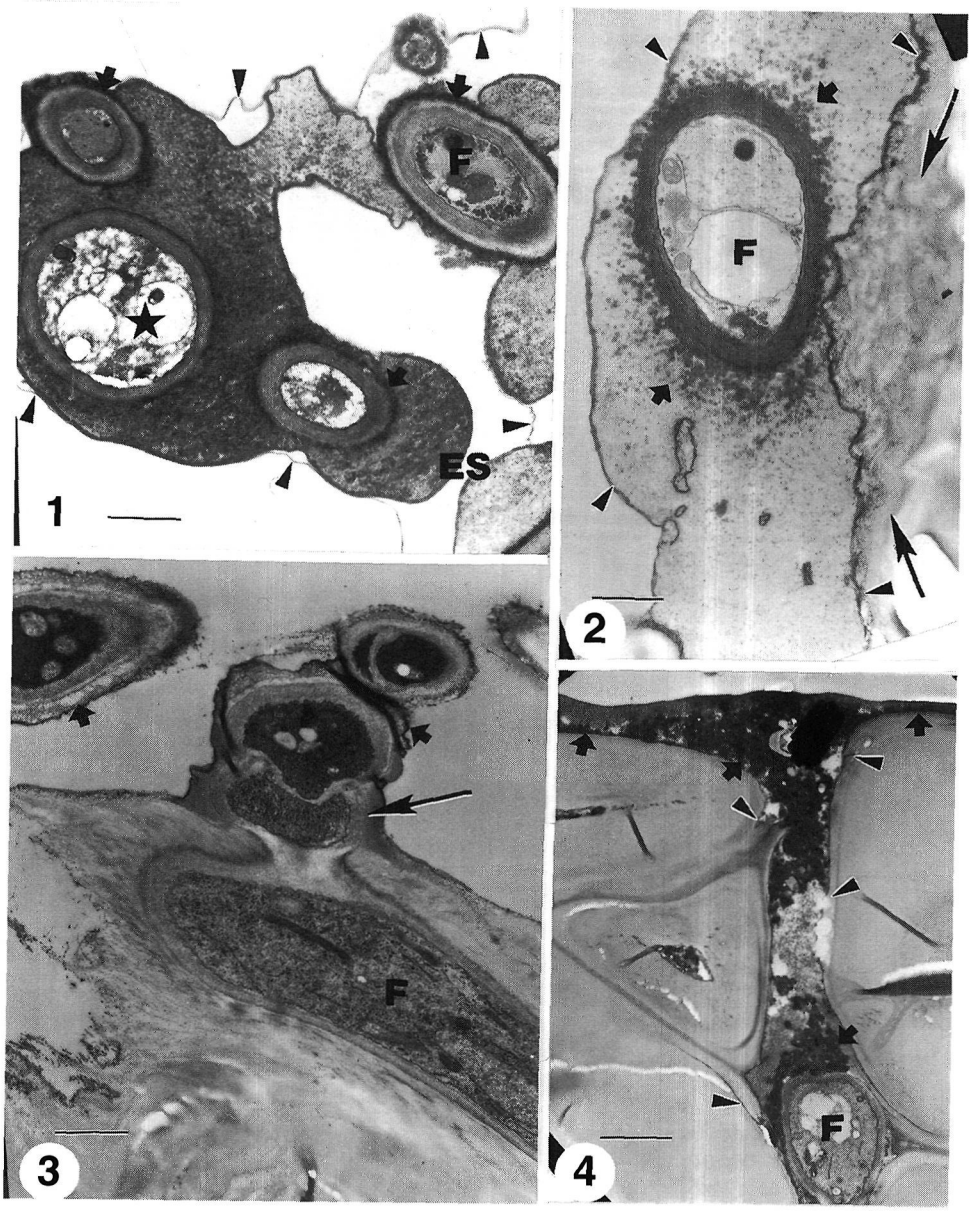
The cambial tissues in control samples were generally intact, as were the adjoining bark and xylem (Fig. 6). The inner bark had rows of spherical cells with dense and opaque contents and intercalary rows of tissues that contained bundles of fibres with gelatinous walls and other thin-walled cells of various shapes. Some gelatinous-walled fibres also occurred in the xylem. Xylem ray cells rarely had dense contents compared with the contiguous ray cells that extended into the bark. In a few cases, some cell dislocation and cytoplasm liberation were observed close to the control wounds, but the adjacent bark and xylem tissues were visibly intact.

In samples from 3 or 7 d after inoculation, the pathogen had invaded cambial tissues (Figs. 7, 8) some 5 cm or more above and below the inoculation point. The cambial cells and some of the adjoining bark cells were collapsed and distorted (Fig. 8). Many of the bark cells had contents that were denser than in controls, and some had enlarged and expanded into the void spaces made by the path of pathogen invasion (Figs. 8-10). These paths extended between the xylem and the cambium and crossed the cambial cell layers up to within the outer bark and even the phellem layers (Figs. 7, 9). The regions of invasion displayed many fungal cells, particularly on d 7, intermixed with masses of slightly stained material (Figs. 7, 9, 10). The host cells in these regions had altered walls and were much dislocated, as were even the bundles of fibres (Fig. 7). From the regions of invasion, which also occurred between the layers of bark tissues (Fig. 7), the pathogen apparently also spread inwards from invaded outer bark towards unaffected inner bark (Figs. 9, 10).

On d 18, most of the affected bark tissues appeared as opaque, irregular bands or masses that surrounded or included collapsed cells (Fig. 11); even the cortical fibres were heavily colonized and pycnidia initials occurred in the outermost bark tissues. Although fungal cells were not easily detectable in the opaque regions, ultrastructural observations showed their general occurrence therein (see below).

Colonization of xylem cells, which was evident on d 3 in the first rows of cells adjoining the cambium, was general by d 7 (Fig. 12) and extended down even to the preceding growth ring. Direct passage of pathogen cells through host walls was mostly evident from vessel elements and ray cells to adjoining cells. The fungal passage from fibre to fibre, rarely observed, was apparently by means of small and only lightly stained bands that connected with fungal cells in the host cell lumina (Fig. 12).

Some cell reactions occurred that were possibly indicative of defence mechanisms. One of these was the apparent formation of a lateral barrier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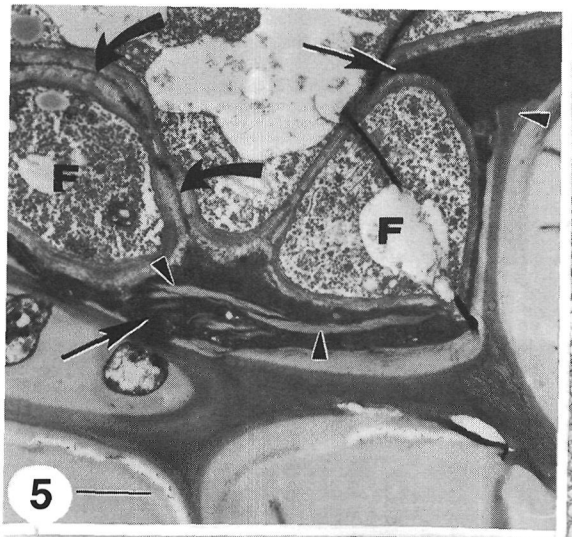
Figures 1-4. Ultrastructural observations. **Figure 1.** A thick extracellular sheath (ES) surrounds a group of fungal cells (star) or occurs as mounds over one of these (F). The sheath contains opaque particles that are bigger at increasing distances from the cells. These particles are aggregated here in compact masses. Closer to the cells, it is composed of fibrillar-like structures. An opaque layer (short arrows) separates the sheath from the fungal cells. Thin, membranous-like bands (arrowheads) that extend into the surrounding medium merge at several sites with the firm layer delimiting the sheath. From a 7-d-old culture on PDA. Bar = 1.38 μm. **Figures 2-3.** Samples from Kimwipes paper. Bar = 0.75 μm. **Figure 2.** The opaque particles in the extracellular sheath (short arrows) are here bigger near the fungal cell (F) than more distally from it. The compact, opaque bands (arrowheads) that delimit the sheath exteriorly and the portion affixed to the fibre extend an appreciable distance from the fungal cell. The fibre in contact with the sheath band is pervaded by opaque strands (long arrows). **Figure 3.** A fungal cell (F), present in a fibre, is linked to other cells located outside it. A thin sheath (short arrows) surrounds the other cells. The contents of the connecting cell (long arrow) appear to be radially oriented. **Figure 4.** From autoclaved elm wood chips, 5 d after inoculation. A mass of material with compact and opaque components (short arrows) extends over the wood chip surface and between the fibres. A fungal cell (F) is also part of this material, as were others (not shown) that connected with the layer of material on the wood surface. Arrowheads point to altered portions of cell walls associated with the opaque components. Traces of opaque matter occur in the lumen of fibres, as was also the case in control samples. Bar = 0.92 μm.

zone in the cambial region, and in the inner bark adjoining the paths of invasion (Figs. 9, 10). Cell hypertrophy and hyperplasia were apparent in these regions, which seemingly yielded numerous, large and globoid cells, many of which were rich in opaque contents. Whether these callus-like tissues would have generated normal bark and xylem was not established, as the cankers almost completely encircled the inoculated branches sampled on d 18. In one of the samples taken near the extremity of the canker, a thin layer of xylem tissue had developed in which fungal cells were almost restricted to the newly deposited vessel elements. However, pronounced alterations of the cambial and bark tissues were visible, which likely resulted from subsequent invasion (not illustrated). Inoculations on 2-yr-old branches and stems of larger diameter kept in a greenhouse for over a month eventually formed visible callus at the margins of the cankers, but no histological observations were made of them.

Ultrastructural observations

As described above, the initial points of pathogen invasion in the inoculated trees were through the cambial tissues. Cell walls and contents of the cambial cells in regions invaded by the pathogen cells were drastically altered (Fig. 13). In the vicinity of such zones, cambial cells also had much altered contents and somewhat bleached but intact walls (Fig. 14); masses of opaque matter occurred in the periplasm of most of these cells. More remotely from the visibly colonized area, cambial cells likewise displayed altered contents, but rare traces of opaque material in the periplasm, and walls that were of a more uniform opacity (not illustrated). In regions where the cambial and inner bark regions appeared as opaque layers (Fig. 8), cells had dense and opaque contents, and were delimited by distorted walls of unequal thicknesses, next to some void spaces (Fig. 15).

On d 7, pathogen cells, surrounded for the most part by an opaque layer, were present throughout the severely affected inner bark regions. Towards the periderm, fungal cells had likewise penetrated host walls and were abundant in intercellular areas. Often, the colonizing cells were very irregular in outline and somewhat camouflaged by opaque host walls and cell contents (Fig. 16). These fungal cells were closely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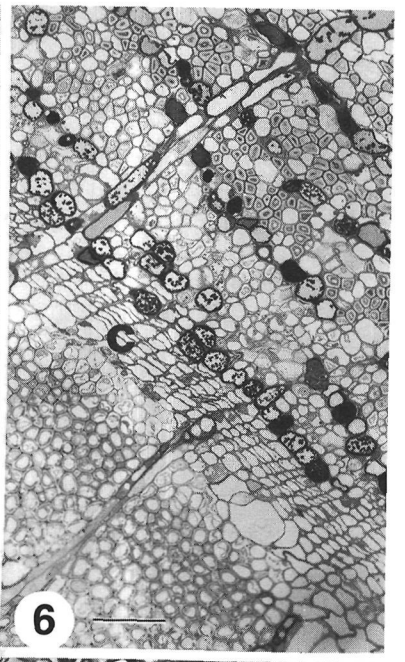
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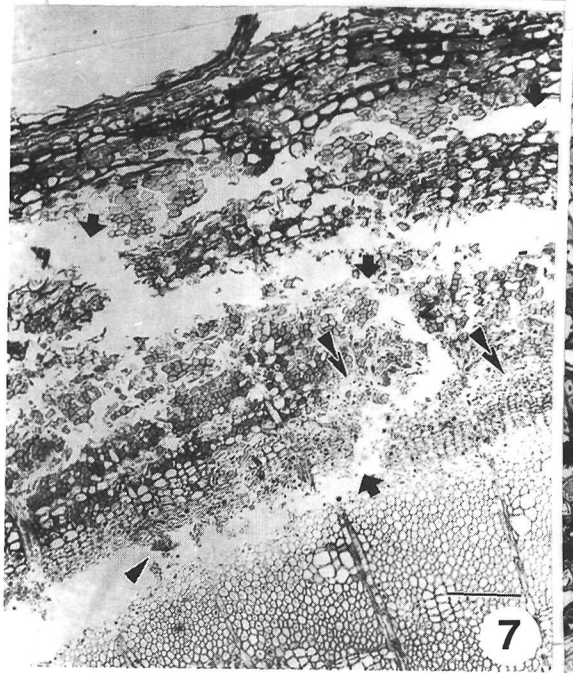
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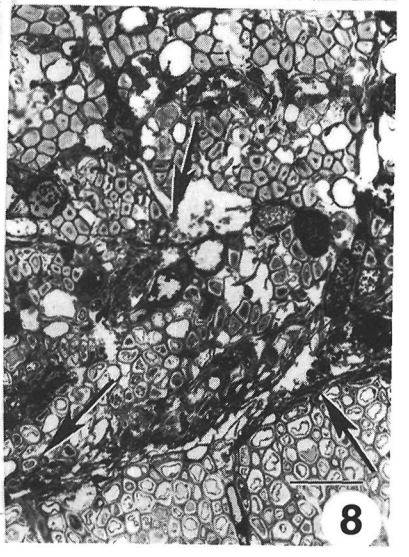
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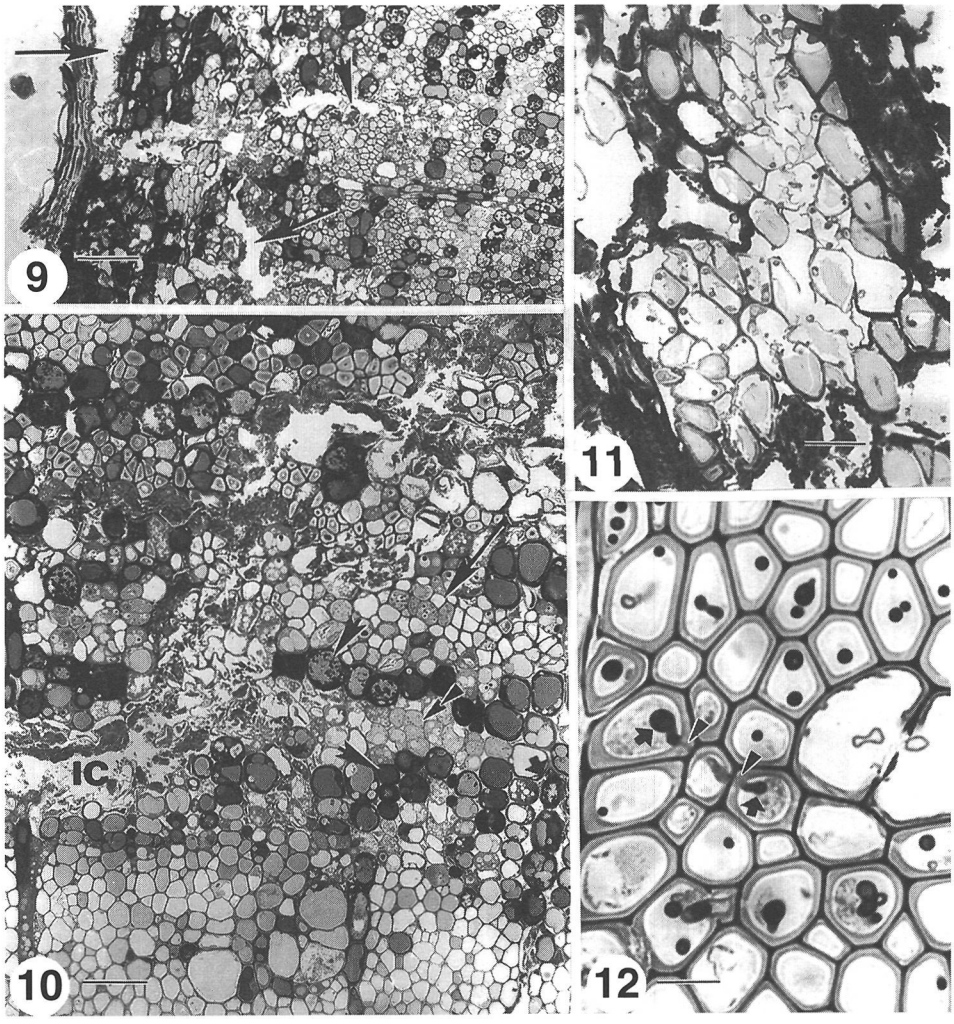
Figure 5. Sample from wood chips as in Fig. 4. Large masses of opaque matter (long arrows) are present between a row of fungal cells (F, part of a 3 to 5 cell-thick layer), and the wood surface these cells cover. Arrowheads point to portions of the secondary walls of fibres, which are included in or cut across in association with the opaque matter. Curved arrows indicate the continuity of the opaque matter present between the fungal cells with that of the larger mass. Bar = 0.92 μm. **Figures 6-8.** Light microscope observations. Samples from 1-yr-old elm branches. Bar = 38 μm Figs. 6 and 8, and 91 μm Fig. 7. **Figure 6.** Wound control sample. Xylem (lower part), cambial (C), and bark tissues (upper part) are intact. Rows of rounded cells with opaque contents alternate with layers of tissues containing thick-walled fibres and thin-walled cells of various sizes. **Figure 7.** 7 d after inoculation. Extensive damage to cambial tissues is evident either on the xylem (single arrowhead) or the bark side (double arrowheads) in an area that contains numerous fungal cells (visible as black dots at this magnification). A path of invasion (short arrows) extends from the cambium into the outside bark, bordered by many altered and disrupted cells, including fibres. **Figure 8.** 3 d after inoculation. The cambial and adjacent inner bark cells are dislocated and collapsed in opaque bands and masses (long arrows) in an area that bordered colonized cambial cells.

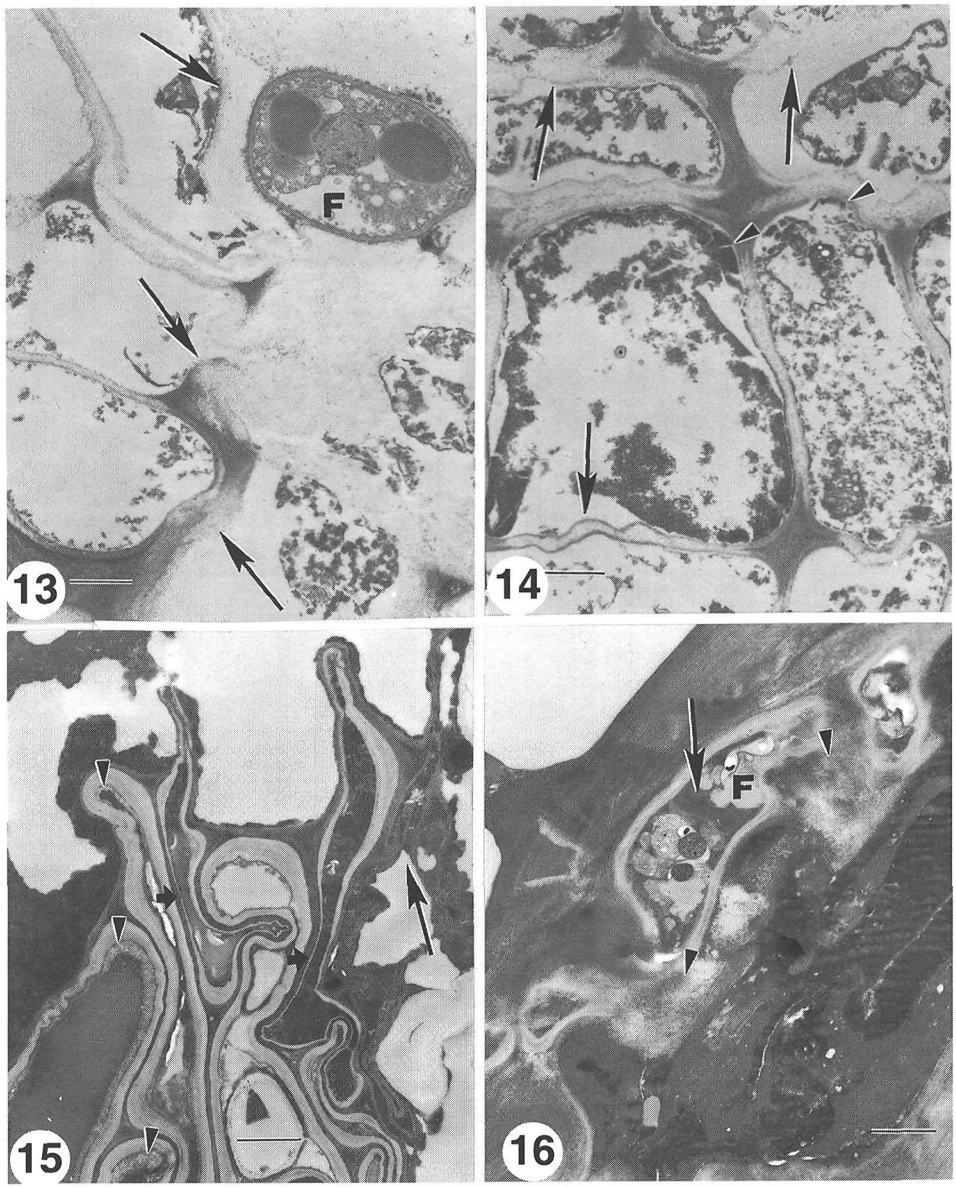
linked to masses of fine, filamentous-like material. Other larger fungal cells with thick, lucent walls and contents analogous to those of pycnidial conidia (not shown) were present in bark tissues.

In the samples from 18 d after inoculation, most of the inner and outer bark cells were collapsed, entangled in layers of opaque appearance, whereas the peridermal layers and some bundles of fibres were still distinct. However, the gelatinous-like wall layer in many colonized fibres was extensively degraded in apparent association with opaque material that contacted most fungal cells (Fig. 17). This material, which was localized between the gelatinous and the inner wall layers over the whole perimeter of the fibre, also impinged on the inner secondary-like layer (Fig. 17). Other structures, suggestive of collapsed fungal elements, visible as wall-like material surrounded by opaque matter, were present between dislocated fibres (Fig. 17). Such elements were likewise bordered by fine fibrillar material delimited by thin opaque bands, an association which recalled that observed in relation to the fungus in culture, as described above. Non-invaded fibres in infected trees had, as in non-inoculated trees, intact, thick gelatinous layers and were free of opaque material in the interwall regions, contrary to the invaded fibres. The fibre cell lumen, which is characteristically narrow in normal gelatinous fibres, rarely contained remnants of cytoplasm.

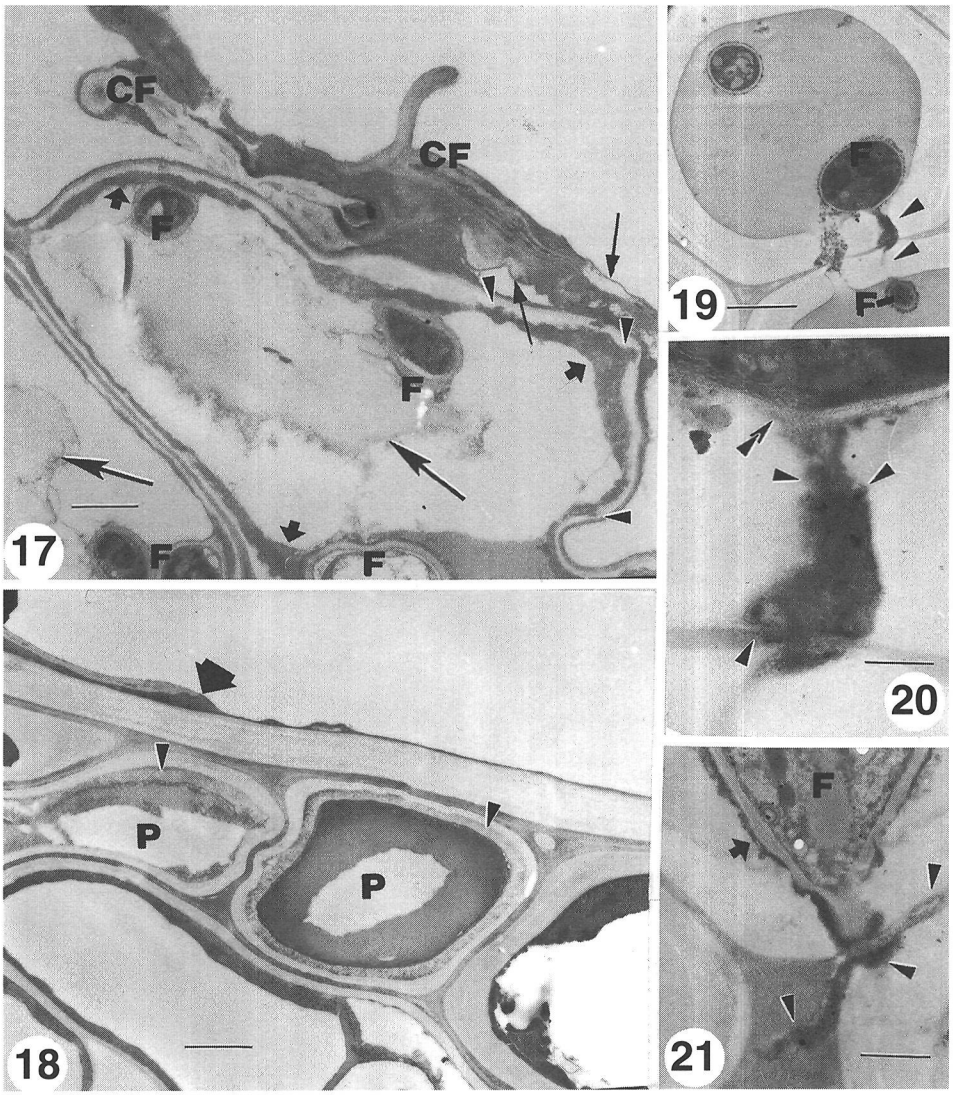
In the newly formed xylem, pathogen cells were present only in vessel elements, even though the adjacent cambial and bark tissues were quite altered. Vessel walls of invaded vessels were also coated with material (Fig. 18) similar to that which extended over the cells in sterilized wood chips (compare with Fig. 4). In contrast, the pathogen had invaded almost every fibre in the last two growth rings of the inoculated branches (see Fig. 12). Observations with the electron microscope did not reveal the fibre to fibre passage of the pathogen. The only components present in fibre walls that connected with fungal cells in the fibre lumina appeared as bands of nearly homogeneous fibrillar, opaque material (which could have corresponded to that shown in Fig. 12) that infringed on the fibre secondary walls (Figs. 19, 20). Similar material, also in continuity with the extracellular material of pathogen cells, extended apparently unbound into fibre secondary walls and middle lamellae (Fig. 21). However, opaque material was mostly absent around fungal cells in ray cells (not illustrated). These fungal cells were generally irregular, with dense contents, lacked septae, and contained many



Figures 9-12. Light microscope observations. Inoculated 1-yr-old elm branches. Bar = 91 μm Fig. 9, 38 μm Fig. 10, 31 μm Fig. 11, and 18 μm Fig. 12. **Figures 9-10.** From a region located laterally from a heavily colonized cambial region. **Figure 9.** A path of invasion (arrows), which originated in the cambial region (see Fig. 10, IC), reaches the periderm layer, and another invasion path appears to have spread downwards (arrowhead) towards a region of tissue reaction (see enlarged portion of Fig. 10) at the cambial level. **Figure 10.** Laterally to an invaded cambial region (IC), a kind of barrier zone has formed, where in place of normal cambial cells occur only slightly stained, meristematic-like cells (double arrowhead) and large globoid cells (arrowheads) with very dense contents. Some of these cells are aligned with xylem ray cells (short arrow, right : hand side). Meristematic-like cells also occur in the inner bark (long arrow). **Figure 11.** 18 d after inoculation. Fungal cells (round opaque bodies) are present in or between each of the cortical fibres in the bundle illustrated. In most fibres, the internal gelatinous-like layer has been much degraded. **Figure 12.** 7 d after inoculation. Bands of medium opacity present in walls of xylem fibres (arrowheads), connected to fungal cells of greater opacity (short arrows) in the fibre lumina, in regions where fungal cells occurred in almost every host cell.



Figures 13-16. Ultrastructural observations. 7 d after inoculation, 1-yr-old elm branches. Bar = 1.2 μm Figs. 13-15, and 0.92 μm Fig. 16. **Figure 13.** Walls of cambial cells are disrupted and bleached (arrows), close to a fungal cell (F). **Figure 14.** Cambial cells in a non-colonized region, but located close to a colonized one (as in Fig. 13), have altered contents with masses of opaque material in the periplasm (arrowheads), and partly bleached but intact walls (arrows). **Figure 15.** Cambial cells with dense contents border collapsed cells (long arrow), and a large gap next to these. The secondary walls in these cells are distorted and of unequal thickness (short arrows), and a gelatinous-like, also irregular layer (arrowheads) is present in some of the cells. **Figure 16.** Fungal cells (F) with irregular contours are tightly caged by opaque walls or contents of altered host cells in the outer bark region. Masses of less opaque material (arrowheads) (which at a higher magnification appeared filamentous-like in nature) are linked to the fungal cells. The contents of these cells are fragmented, but display well-delimited bodies, in one of which occur islands of opaque matter (arrow).



Figures 17-21. Ultrastructural observations. Figs. 17 and 18, 18 d after inoculation, and Figs. 19-21, 7 d after inoculation. **Figure. 17.** Opaque material (short arrows) in contact at sites with fungal cells (F), extends between the inner, strongly degraded, gelatinous-wall layer (long arrows) and the outer secondary-like layer, in cortical fibres. The opaque material also impinges on the outer layer (arrowheads). Opaque bands (small arrows) that circumscribe fine, homogeneous material, and collapsed fungal cells (CF) also occur in the altered intercellular area. Bar = 0.92 μm. **Figure 18.** The slightly distorted paratracheal cells (P), next to a vessel element with its wall covered with coating material (thick arrow, compared with Fig. 4) contain a thin cytoplasm layer, large vacuoles filled with opaque matter, and a gelatinous-like, inner wall layer (arrowheads). Bar = 1.2 μm. **Figure 19.** Some opaque material (arrowheads), present across the walls of adjacent xylem fibres, is linked to fungal cells (F) in the lumen of the fibres. Opaque material also surrounds the fungal cells. Bar = 1.7 μm. **Figure 20.** Enlarged portion of Fig. 19, showing the link of the opaque material with the fungal cell contents, through its wall (double arrowhead). This material, of a fibrillar appearance and intermixed with discrete particles, impinges on the fibre wall (arrowheads). Bar = 0.6 μm. **Figure 21.** Opaque material, confluent with that surrounding the fungal cell (F, short arrow), extends seemingly unbound into the wall of a xylem fibre and the adjoining middle lamella and intercellular area (arrowheads). Bar = 1.2 μm.

opaque bodies and profiles of mitochondria.

Host cell reactions

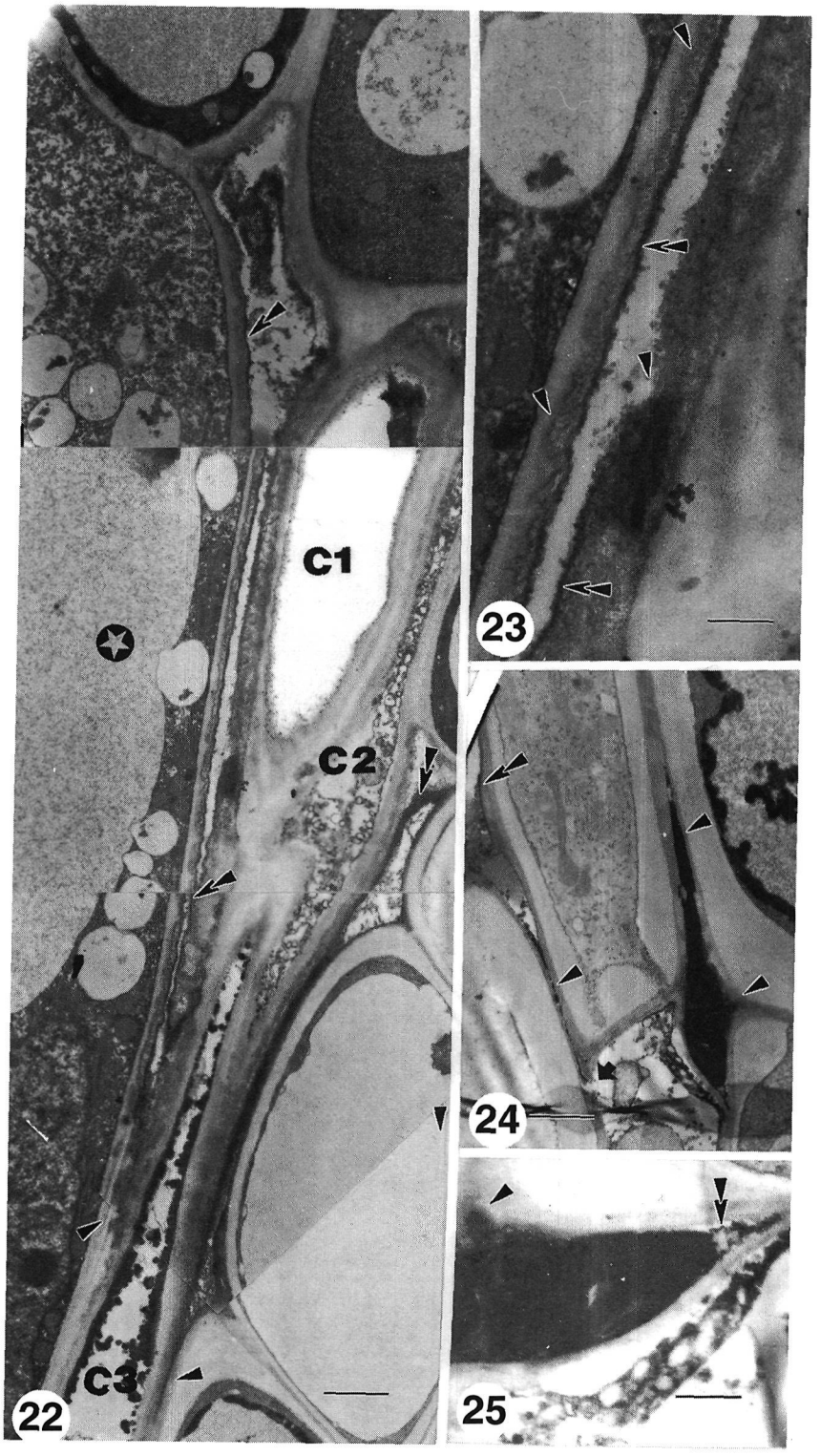
Various reactions, seemingly of a similar nature, occurred in cells that bordered collapsed cambial cells in early infection (Fig. 15) or in paratracheal cells that were deposited subsequently to the time of inoculation (Fig. 18). In both cases, reacting cells had dense contents and had laid down a new, somewhat gelatinous wall layer. In the altered cambial region, however, such reactions were not uniform and appeared to have been halted in some cells. Another type of reaction is illustrated in Figures 9 and 10. The corresponding tissues observed in transmission electron microscopy showed that the large intercellular areas between the rounded cells were either empty or contained structures that were identifiable as remnants of host cytoplasm. The marked modifications of contents of hyperplastic cells, such as indicated by a much granulated cytosol, nuclei with dense contents and irregular contours, and large vacuoles filled with fibrillo-granular components were also evident in the long ray cells (Figs. 22, 23) that were included in the region of cell hyperplasia and hypertrophy. These reacting ray cells bordered other collapsed ray cells, and the intercellular eroded areas were demarcated by opaque bands and masses (Figs. 22, 23). The collapsed ray cells contained less opaque vacuolated cytoplasm, except in the periplasm of which occurred opaque components.

Distally from the markedly altered cambial tissues, bands and masses of opaque material were likewise present intercellularly, partly between apparently still intact cells, and partly between altered cells (Fig. 24). In the latter instance, some of the intercellular opaque material was associated with a wall rupture and distension of a cell. The opaque material which impinged on the host cell walls, as also shown above, was delimited at locations by thin opaque bands (Fig. 25).

DISCUSSION

This is the first histological study of infection of elm trees by *Sphaeropsis hypodermia,* and one of the few published ultrastructural studies of tree bark infections by fungi (Biggs 1992). *S. hypodermia* invaded extensively and caused the rapid breakdown of the cambial and adjoining bark tissues. The majority of xylem cells, in the immediate vicinity of affected cambium as in the preceding growth rings, were also colonized. The observed transformation of the cambial cells, next to the region of tissue alterations, was seemingly indicative of an initial barrier zone formation, as observed in host and non-host trees inoculated with *Ophiostoma novo-ulmi* Brasier (Rioux and Ouellette 1989, 1991), and representative of wall 4 of the CODIT model (Shigo 1984). The wall 4 formation is characterized by the deposition of new types of tissue at the cambium level, but in the present case, it represents a lateral as well as a tangential reaction. Many of the present cell reactions might approach some of the events described in the cascade of reactions related to hypersensitivity (Goodman and Novacky 1994). In response to obvious cell death and collapse in the cambium and inner bark regions, cell hypertrophy and hyperplasia were evident. Many distorted cells with very dense and opaque contents, however, would have been denoted as dead with a light microscope. Nevertheless, at the ultrastructural level, new additional wall layers were observable in such cells. The hyperplastic or hypertrophied cells, some with pronounced altered walls, also contained opaque, modified cell contents and lobed and elongated dense nuclei. Nuclei with irregular contours have also been observed in diseased plants or plants exposed to adverse conditions (Fink 1999). It is worth noting that Jones *et al.* (1975a, b) observed similarly configured nuclei in cells located next to cells considered to have undergone a hypersensitive response.

Whether the present tissue transformations could have led to an effective barrier could not be determined, prob-


C1
C2
C3
22
23
24
25

Figures 22-23. Ultrastructural observations, the counterpart of Fig. 10. Figure 23 is the enlarged middle portion of Fig. 22. A long ray cell (star) (which extended from the xylem to the inner bark) borders other collapsed cells (C1-C3). The intercalary walls are strongly eroded (Fig. 23, arrowheads) in conjunction with firm bands of compact matter (double arrowheads). The altered contents of cells C2 and C3 do not contain similar matter, except in the periplasm of C3, in regions where the cell walls are fully impregnated with opaque material. The tail ends of the bands of such opaque material (Fig. 23, arrowheads) are present in seemingly still intact host wall. Bar = 1.38 μm Fig. 22, and 0.75 μm Fig. 23. **Figures 24-25.** Also the counterpart of Fig. 10, but from the region located at the other extremity of the visibly altered cambial region. Figure 25 is the enlarged, middle portion of Fig. 24, but tilted counterclockwise. **Figure 24.** Masses and bands of opaque matter are present between ray cells, in apparently intact middle lamellae (arrowheads), and delineated at sites by thin bands (double arrowhead). One band reaches the lower ray cell that has altered contents (short arrow) and one of the ray cells (top right) has accumulated tannin-like material in its vacuole. Bar = 1.2 mm. **Figure 25.** The opaque material impinges on the fibre walls (arrowhead) and is delineated at one location by compact bands (double arrowhead). Bar = 0.4 μm.

ably because of the small size of the inoculated shoots. In this case, a re-infection of the new tissues seemingly ensued from a downward spread of infection in the bark, which in the samples examined, appeared to be very sensitive to infection. However, larger inoculated stems produced cankers that were more restricted than on smaller branches. The present inoculations led to development of the disease that was similar to that on field trees (Riffle 1981), where large cankers formed on still standing trees.

Host wall penetration and breakdown in the inoculated elm branches were associated with bands and masses of opaque material linked to fungal cells. A similar relationship was found in the *in vitro* condition, with regard to fungal development on the sterilized elm wood chips and Kimwipes paper. A remarkable feature on these substrates was that the fungal extracellular components extended noticeable distances from fungal cells, affixed to the substrate. Other plant fungal pathogens grown on sterilized, rigid substrates were likewise shown to produce large amounts of opaque material that covered marked areas of the substrate (e.g. Van Dyke and Mims 1991). Opaque material associated with host wall alterations was likewise observed in plants affected with wilt diseases : elm trees with Dutch elm disease (Nicole *et al.* 1994; Ouellette 1978; Ouellette and Rioux 1992, 1993); susceptible *Fusarium*-infected carnation plants (Ouellette and Baayen 2000; Ouellette *et al.* 1999a); *Fusarium*-infected staghorn sumac (Ouellette, unpublished observations); and *Verticillium*-infected eggplant (Ouellette and Chamberland 1993). The opaque, firm bands that delimit the extracellular sheath in *S. hypodermia* are likewise comparable to those related to the alveolar network formation in vessel lumina in elms affected with Dutch elm disease (Ouellette and Rioux 1993). The opaque material associated with fungal cells of *S. hypodermia* on agar medium and on Kimwipes paper (composed of bleached fibres) cannot be attributed to host degradation or reaction products, as both were inert substrates. In elm wood chips, some opaque material in the non-inoculated samples was noticeable only in some ray and fibre cells, likely as part of coagulated cytoplasm, but was absent over the exposed cell surfaces. In the sterilized, inoculated wood samples, such material occurred abundantly linked to fungal cells, over or between the wood cells, or with host wall chipping. These components were also not attributable to cell reactions products as the cells were dead, nor could it come from noticeable pre-existing material on the chips because it would have been lost during autoclaving.

In sterilized elm wood, alterations of the middle lamellae were particularly pronounced, and besides some chipping, the affected secondary walls were still relatively intact. In the inoculated trees, degradation of the primary walls and middle lamellae and of the gelatinous layer of cortical fibres was outstanding, and secondary wall deterioration was also not pronounced, despite the general colonization of xylem cells of the preceding growth rings. In opposition, the pathogen cells rarely occurred in newly differentiated cells, except vessel elements, a fact which was interpreted as some type of defence had occurred in these cells, but not in the bark tissues, as these were eventually destroyed. Considering the general occurrence of pathogen cells in the xylem cells in the first instance, observations of hyphal passages from one cell to the next would have been expected to be common. This prevision applied only to the spread of the fungus from ray cells and vessel elements to contiguous cells. With regard to fibres, the only links observed between fungal cells in the lumen of adjacent fibres corresponded to bands of the opaque material.

Many reports exist on the formation around fungal cells of large extracellular sheaths that are surrounded or not by an opaque limiting band. In addition to the examples referred to in Ouellette *et al.* (1999b) we may cite : Carver *et al.* 1999; Frederick *et al.* 1999; Hoffert *et al.* 1995. The extracellular components in *S. hypodermia* may be related to those present intercellularly in the sclerotia formed by some fungi (Kohn and Grenville 1989; McLaren *et al.* 1989; Willetts *et al.* 1990). Melanin may also occur in the sheath of *S. hypodermia,* as determined by a few exploratory tests conducted by M. J. Butler (University of Western Ontario, London, Ontario, Canada, personal communication; for a recent comprehensive review of melanin production in fungi, see Butler and Day 1998). The extracellular sheath in *S. hypodermia* probably contains some other active components, as shown by its ability to penetrate freely host walls as well as inert, rigid substrates, and to extend similarly some distance over these. In the case of inert substrates, sap movement, as would occur in the living tree, or any other evident physical driving force cannot be invoked to explain this apparent self-displacement of the opaque material. Cyto- and immunocyto-chemical tests were conducted to determine some of the possible sheath components, but no definitive results have been obtained (unpublished observations).

Some of the hyphal cells present in the bark and ray cells differed structurally from cells with large sheaths. A similar difference in intercellular components, structure, and mode of development occurred between fungal cells of the pycnidial peridium and those located more inwards in the pycnidia (unpublished observations). Analogous differences were likewise noted between the rind and medulla cells in sclerotia (Kohn and Grenville 1989; McLaren *et al.* 1989; Willetts *et al.* 1990).

The production of abundant extracellular material in several fungal pathogens is suspected to be of great importance in pathogenesis in a number of plant diseases, including Dutch elm disease (Ouellette *et al.* 1999b; see citations concerning some of the other cases). Isolating and producing probes for the possible components of the extracellular sheath that forms under various conditions in *S. hypodermia* would undoubtedly contribute to a better understanding of the role of such components in host tissue invasion in this case as in other plant diseases.

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