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Ultrastructural and cytochemical studies of host and pathogens in some fungal wilt diseases: retro- and introspection towards a better understanding of DED

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Abstract

This report presents a survey of previous and more recent ultrastructural and cytochemical investigations of disease development in elm, caused by *Ophiostoma novo-ulmi,* with results of some comparative studies of other wilt diseases caused by f.spp., of *Fusarium oxysporum* and of *Verticillium* sp. For cytochemical studies, probes complexed to colloidal gold to detect cellulose, pectin, chitin, and DNA were used. Thus, the formation of tyloses, pit membrane alterations, and the disease effect on parenchyma cells in mature as well as in young tissue were characterized. Vessel lumina in these plants, in diverse situations, contained heterogeneous matter, among which occurred masses of opaque matter and in certain instances pectin fibrils secreted by parenchyma cells and tyloses. Numerous globoid, opaque bodies of regular sizes, which have been mostly overlooked previously, abounded in vessel elements of *U. americana* and *U. glabra,* including trees injected with glucose solutions prior to inoculation. Coarser fibrillar material was also noted in vessel lumina, but mostly shortly after inoculation. Other peculiar forms of *O. novo-ulmi* are also described. The possible role of the components under study is discussed, and a model for DED is proposed in which hindrance to vessel invasion, including downward spread of the pathogen, and reactions of parenchyma cells are complementary and considered to be conducive to defence mechanisms, including compartmentalization of the invaded xylem.

Key words: *Ophiostoma novo-ulmi, Ulmus,* cytology.

Resumen

Estudios ultraestructurales y citoquímicos de huéspedes y patógenos en algunas enfermedades de marchitamiento fúngicas: una retro e introspeción dirigida al mejor entendimiento de DED

En este informe se presenta una revisión de los más recientes estudios sobre la ultraestructura y la citoquímica del desarrollo infectivo causado por *Ophiostoma novo-ulmi* en el olmo, y su comparación con otras enfermedades vasculares causada por hongos como *Fusarium oxysporum* y *Verticillium* sp. En los estudios citoquímicos se usaron inmunolocalizadores de oro coloidal para la detección de celulosa, pectina, quitina y ADN. De esta manera se caracterizó la formación de tilosas, las alteraciones en las punteaduras, y los efectos de la infección en las células parenquimáticas, tanto en tejidos adultos como jóvenes. En estas plantas, el interior de los elementos conductores contiene, en diversas ocasiones, sustancias heterogéneas en las que aparecen masas de sustancias opacas, así como, en ciertas ocasiones, fibrillas de pectina segregadas por células parenquimáticas y por tilosas. Numerosos cuerpos opacos globulares de tamaño medio, que previamente habían pasado en gran medida inadvertidos, eran abundantes en los elementos conductores de *U. americana* y *U. glabra,* incluyendo árboles a los que se inyectó soluciones de glucosa antes de la inoculación. También se detectó la presencia, en el interior de los vasos, de material fibrilar de mayor diámetro, sobre todo al poco de la inoculación. También se describen otras formas peculiares de *O. novo-ulmi*. Se discute el posible papel de los componentes en estudio, y se propone un modelo para la grafiosis en el cual los obstáculos para la invasión del vaso, incluidas la transmisión corriente abajo del patógeno y la reacción de células parenquimáticas, son complementarios, considerándose que son conducentes a la aparición de mecanismos de defensa, incluida la compartimentación del xilema invadido.

Palabras clave: *Ophiostoma novo-ulmi, Ulmus,* citología.

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Introduction

Someone mentioned in the early 1960s that the most voluminous body of literature existing on any single plant disease was that concerning Dutch elm disease (DED). One may rightly observe that this trend is persistent. By consulting newspaper headlines concerning the spread of, and losses caused by, this disease over the years, it may be seen that news of control measures against it alternate with news of their precariousness, and of the necessity to find the ideal control. These types of headlines were well exemplified by Jorgensen (1982) at one of the symposia held on this disease.

This literature has been accumulating since the 1920s when the disease was noticed in France and was initially attributed to the unfavourable environmental factors that prevailed at that time and to heavy injuries that were inflicted on trees during the war. We know that it took more than 6 years thereafter to discover the real cause of the disease. There may have been, however, earlier reports of problems with elms whose descriptions suggested it might have corresponded to the DED syndrome. One such report was published by De La Peirouse (1788), of a study undertaken to determine the cause of extensive mortality of elm trees in Toulouse, France. Leaf browning and wilting were initially ascribed to severe drought conditions. The author concluded that drought was not the main cause, as young trees not exposed to drought were also seriously affected. As it was presumed, then, that leaves were necessary for «humidity absorbtion» in plants, the large infestations of elm leaf beetle occurring at the time were indicated as the cause of tree mortality, together with the presence of bark beetles (Scolytidae). Considering these cases retrospectively, one realizes how long it may take to correct assumptions and preconceived ideas and to fill gaps in our knowledge in order to properly perceive problems and solve them. Today, can we really state that, with greater knowledge and technical advances, our efforts to comprehend the mechanisms of wilting in plants, and of their resistance to those diseases, are exempt of similar drawbacks? It seems indeed that it may often take as long, if not longer, to reach final answers, and that frequently, the new data acquired may lead to new unexpected problems for the recognition of which a comparatively still longer period may elapse.

When the first author undertook research on DED (Ouellette 1962a, 1962b), one objective was to discover the mode of development and action of the pathogen and, by inference, the cause of wilting. Indeed, existing notions, based then on light microscope observations, were that pathogen cells occurred sparsely in vessel elements, that they did not but exceptionally invade paratracheal parenchyma cells in the early infection process, and that, on account of this scarcity of pathogen cells, but which was seemingly attributable to a loss of fungal elements during handling of the specimens, the occurrence and action of host wall degrading enzymes was not considered important (see discussion in Ouellette 1978b, 1978c, 1978d). Consequently, the theories first proposed to explain the wilt syndrome were based on notions that vessel blockage by gums and gels might be responsible for detrimental interference to sap flow, thus mostly the result of a physical effect. The production of toxins by the pathogen was subsequently considered as being related to disease development (for an overview of the question see Sheffer 1984; Richards 1994). With the development of new methods of fixing and embedding tissues, a greater number of fungal cells were detected in vessels in light microscopy as well as in transmission electron microscopy (Pomerleau, 1970; Ouellette, 1978d), but it was often uncertain whether these were representative of early or later invasions. As external symptom expression occurred only when the pathogen reached the shoots (Pomerleau, 1968; Ouellette and Rioux, 1992, 1993), observations of inoculations of shoots and leaf petioles were carried out and compared with branch inoculations. Thus, extensive invasion of these younger tissues was noted, and invading fungal cells were more adequately characterized (see some illustrations in Ouellette *et al.*, 1995, 1999b). This type of information appeared fundamental. Nonetheless, the questions as to how the fungus develops in branches following beetle inoculation in lower parts of the tree, and how it spreads downwards, remained open.

Despite the wealth of information obtained on this disease since the 1970s that could foster progress towards controlling the disease, it may not yet be safely stated, unfortunately, that all problems with host-pathogen relationships have been solved. In this respect, hopefully, at least some results of our investigations on DED and related diseases may have helped to perfect knowledge of the pathogens as well as of the affected hosts. As a corollary, these studies may have unravelled new fundamental problems which, in fact, may be more challenging to resolve than those envisaged at the onset of the investigations. The purpose of this

paper is to summarize the various facets of our investigations which, hopefully, might contribute to finding the best means of controlling the disease.

Study material

Present ultrastructural observations concern studies started in 1970 on *O. ulmi* in elms and, subsequently, on other plant wilt diseases and their pathogens, i.e. *Ophiostoma novo-ulmi* Brasier; *Fusarium oxysporum* f.spp. in carnation (*Dianthus caryophyllus* L.), staghorn sumac (*Rhus typhina* L.) and tomato (*Lycopersicon esculentum* Miller); and *Verticillium albo-atrum* Reinke & Berth. in eggplant (*Solanum melongena* L.). Concerning elms and staghorn sumac, naturally infected as well as inoculated materials were used. A number of *O. novo-ulmi* isolates from the Quebec City, Montreal and New Brunswick regions, in Canada, as well as isolates 14 K and 16 K from Barnes, Holland, were employed. The latter isolates were considered to be more virulent in *U. americana*, but were originally identified as *O. ulmi*. The isolates of *F. oxysporum* f.sp. *callistephi* Arm & Arm. were from the Quebec City region, and those of *V. albo-atrum* were from Guelph, Ontario (1969). The isolate of *F. radicis-lycopersici* Jarvis & Shoemaker in tomato was referred to in Charest *et al.* (1984).

Inoculated samples from elm (*Ulmus americana* L., *U. pumila* L., *U. glabra* Huds.) and non-host trees were collected at various intervals after inoculation, sometimes after 1 to 3 years, and, in some cases, samples were collected from trees that showed recurring infection. Similar samples from natural infection, in larger trees, were also examined. Samples from inoculated *U. americana* were obtained from yearly inoculations from 1971 to 1983 (sometimes more than one), and samples from the other species were obtained from 1980 inoculations of field-grown saplings. One- to 4-year-old seedlings of *U. americana*, grown in open soil in a greenhouse or in growth chambers under controlled conditions were used in two experiments or in tests with cerato-ulmin (CU). This compound was absorbed through cut branches or sectioned roots of seedlings, with the others left intact, by immersion in the solution for various intervals. Some samples also originated from seedlings fertilized with different regimes or treated with test fungicides, one being Arbotect®. Field-grown trees were similarly treated with formulas of this fungicide and with glucose solutions, as sugars were suspected of reducing elm disease symptoms (Caroselli and Feldman, 1951; Feldman and Caroselli, 1951).

Some samples were from pieces of diseased xylem placed on an agar medium or a nutritive solution for various intervals before fixing, to localize therein the types of fungal elements that would regenerate into typical fungal cells and stand out from other inert components in the sample. Similarly, to characterize fungal growth in wood tissue in the absence of host reactions, the pathogen was grown on sterilized wood sections in a number of tests. Observations of pathogen growth through Millipore membranes of 0.1, 0.22, 0.4, and 0.8 µm were also made.

Fixation was generally performed with glutaraldehyde and osmium tetroxide, occasionally reduced with ferric chloride, and, in some instances, with a mixture of paraformaldehyde and glutaraldehyde before fixation with osmium. In some cases, it was also performed by high pressure freezing of sterilized wood sections of *U. laevis* L. and of cultures of the pathogen on a solid medium.

Following the advent of labelling with the colloidal gold technique, a number of tests using gold-complexed probes were conducted, particularly for chitin, pectin, peptidorhamnomannan, β-1, 3-glucan and β-1, 4-glucan.

Light microscope (LM) observations were made with various microscopes, but from 1970 on with a Leitz Orthoplan microscope or a Polyvar Reichert-Yung microscope equipped with ultraviolet observations facilities. Transmission electron microscope (TEM) observations were made with either one of the following: Philips 200, 300, 420 or JEOL 1200.

For greater details of these experiments, see Casagrande and Ouellette, 1971; Ouellette, 1978d; Charest *et al.*, 1984; Benhamou *et al.*, 1985; Chamberland *et al.*, 1985; Ouellette and Rioux, 1992, 1993; Rioux *et al.*, 1995; Ouellette *et al.*, 1995, 1999a, 1999b, 2001, 2002.

Observations regarding the host

The following observations are not precisely ordered according to the possible chronological stages of the Dutch elm disease development in elm, but rather according to the overall events occurring in the different types of tissues and treatments, first in the vessel elements, and then in the mature xylem and the newly deposited and/or cambial tissues.

Mature xylem

Tylosis formation. Outgrowth of parenchyma cells into vessel lumina occurred mostly in the late stages of the disease in *U. americana* and in *U. glabra* (Fig. 1a), but more generally in *U. pumila* (Ouellette, 1980). Tyloses also formed in some non-host trees (Rioux and Ouellette, 1989, 1991b). Novel observations concerning tyloses were made by using probes to localize pectin and cellulose (Rioux *et al.*, 1995). Thus, the first limiting wall layer of tyloses, confluent with the inner, protective-like layer of parenchyma cells (Fig. 1b), labelled similarly for pectin, as did the fibrils that extended from this layer into the surrounding medium. The next wall layer (Fig. 1c) labelled for cellulose, and when other layers were laid down, they were unlabelled and corresponded to suberin layers (Fig. 1a, c). These eventually lamellate layers (Fig. 1d),

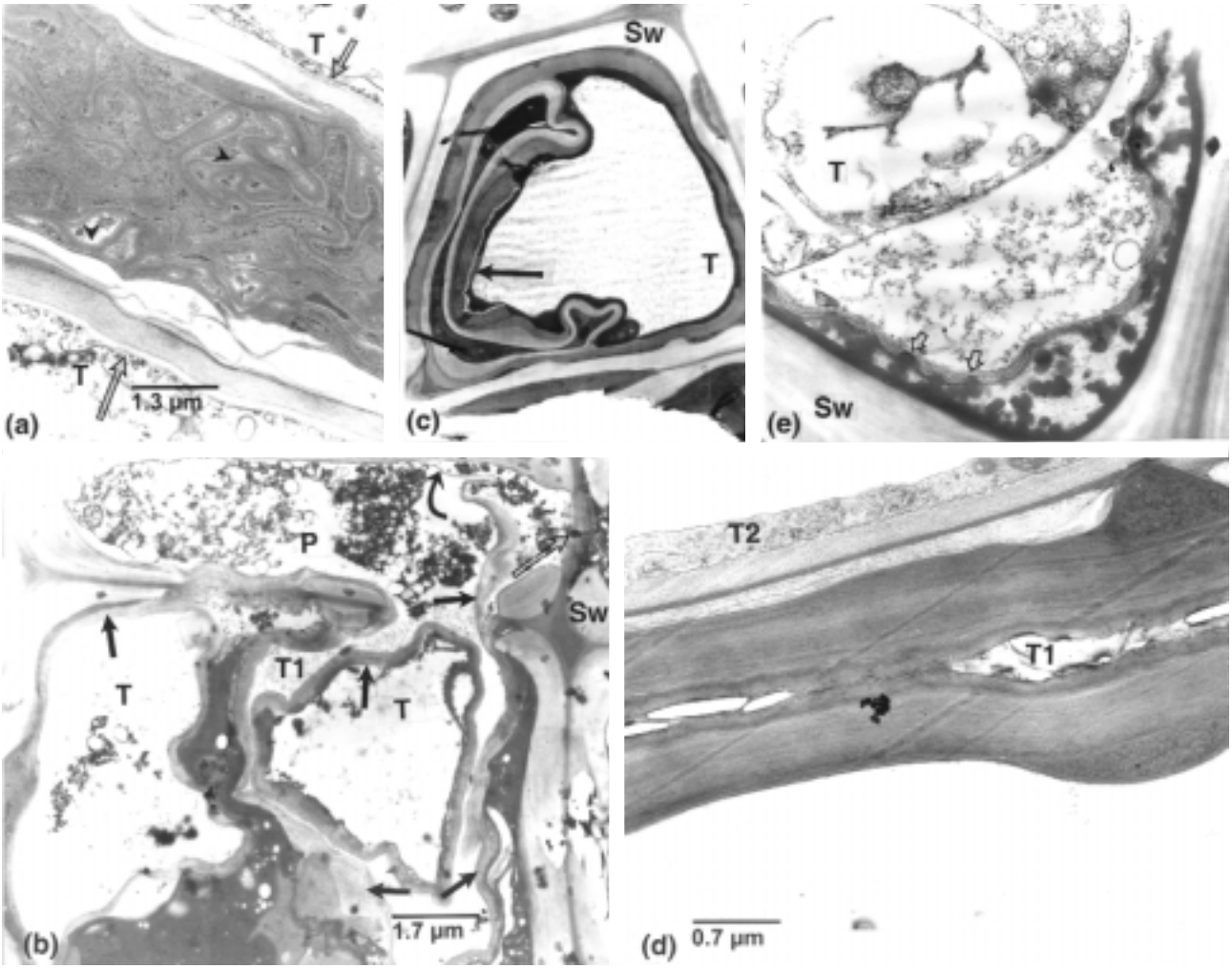

Figure 1. P, parenchyma cell; Sw, vessel secondary wall; T, tylosis. **(a)** *Ulmus glabra*, 15 months after inoculation. Two tyloses having suberin layers (arrows) circumscribing alveolar networks, the thick meandering bands of which (arrowheads) are encompassing masses of other mostly fibrillar matter. **(b)** *U. americana*, 38 days after inoculation (dpi). Tangles of tylosis walls (opaque arrows) of varying thickness encircling other types of matter. Walls of T1 are continuous with the inner, protective layer (curved arrow) of the parenchyma cell. This thin layer is retracted on the opposite side of the cell, next to the pit membrane which is pervaded by an opaque band (empty arrow). **(c)** *U. pumila*, natural infection, the first year of symptom expression, in July. In the tylosis, a type of cross wall has been laid down, itself covered by a disrupted opaque one lined by a thin lucent layer (long arrow). **(d)** *U. pumila*, 9 months after inoculation. Two juxtaposed tyloses, one with thick opaque walls and a suberized layer (T1), and the other (T2), with a thinner wall and a band of normal cytoplasm. **(e)** *U. americana*, 30 dpi. Before sampling, the short inoculated twig was perfused with 10 mL of a 1% osmium tetroxide. The tylosis wall, which encompasses masses of opaque matter at sites (arrows) has a speckled appearance.

often alternating with other labelled layers in non-hosts, occurred in the three elm species studied, but were often discontinuous (Fig. 1c) and frequently lacking in *U. americana*. In these instances, tyloses had altered walls and content (Fig. 1b, e), resulting in their abortion or collapse. Physical barriers from other components or tyloses might have interfered with tylosis formation. The mode of tylosis wall alterations was analogous to that of pit membranes of half-bordered pits (Ouellette, 1978c; see below). The tylosis walls were observed to be directly apposed to, and often seemingly confluent with, the vessel walls, in the same or in contiguous vessel elements, but tyloses were generally separated from the latter by opaque or other type of material (Fig. 1b), some of it even appearing to be circumscribed or included in the tylosis wall (Fig. 1e). Several tyloses, with their walls tangled and intermixed with other components, seemingly had formed at different intervals in the same vessel element, some with thick and suberized walls and others with thinner walls and normal cytoplasm (Fig. 1d). Tylosis walls always formed as an extension of the protective layer of the paratracheal cell, a feature which is well illustrated in Fig. 1b, where this layer has retracted or developed following damage to the pit membrane of the adjacent vessel element. Cross walls also formed in some tyloses (Fig. 1c).

Tyloses were clearly shown to be different from the networks of alveolar-like structures (Fig. 1a; Ouellette, 1980; Rioux and Ouellette, 1989; Ouellette and Rioux, 1992, 1993) which, at the light microscope level, can easily be mistaken for tyloses. However, in Ouellette's first report (1962b) on these structures, it was mentioned that this network might have corresponded to remnants of fungal cells. Detailed descriptions of these networks and of their links with the coating forming on vessel walls are presented in Ouellette *et al.* (2004b).

Other extraneous matter in vessel lumina. As described above, tyloses and alveolar networks were shown to be part of the components occurring in vessel elements. Tyloses, however, were not generally present in the earlier stages of infection, contrary to the alveolar networks and other heterogeneous matter which frequently completely occluded some vessel elements in the types of samples studied, particularly in shoots, twigs and small branches. Some of the occluding components were fine fibrils, which labelled for pectin (Rioux *et al.*, 1998), other were coarser fibrillar components and masses of more opaque matter. Some of that matter appeared to correspond to the material currently identified as «gums», particularly when it occurred as distinct humps in pit chambers of half-bordered pits, and thus as possible secretion products from paratracheal parenchyma cells (Fig. 2a). Other dense matter, however, did not seem to have the same origin.

Shortly after inoculation, opaque matter accumulated on vessel walls, filling the pit membranes of both the bordered and half-bordered pits, or even extending into the host walls (Fig. 2b). Similar matter often bridged the material affixed to vessel walls and fungal elements (Fig. 2c) and, as observed at the TEM level, masses of this matter surrounding fungal cells often showed connections with the cell content through the fungal walls (Fig. 2d). Similar fungal cell-opaque matter connections were less frequent in more advanced infections, as in fact also were typical fungal cells, but the occurrence of analogous material was noticeable and linked to host cell disturbances (see below).

Besides the above-mentioned components, vessel lumina frequently contained large numbers of round opaque bodies of regular sizes (Fig. 2e-g). They were of general occurrence in early (Fig. 2f) as well as late infection (Fig. 2e) in the three elm species studied. The peculiarities and common presence of these bodies have previously been overlooked, particularly their being ornamented with fine fibril-like structures which were still distinguishable when the bodies coalesced (Fig. 2g, h). The bodies were also confluent with similar opaque matter pervading deeply the pit membranes (Fig. 2g). A possible indication of the origin of these bodies was obtained by occasionally noticing that they were linked to, and even included in, fungal cell walls in vessel lumina (Fig. 2i).

Similar bodies also occurred in samples from trees having been given various treatments, including fungicide injections (Fig. 3a). These bodies were again confluent with opaque matter forming the coating on vessel walls or pervading pit membranes of half-bordered pits; they were likewise similar to bodies present in periplasmic areas of these cells. In more advanced infection (1 month and over after inoculation), the bodies which could have corresponded in number, shape and sizes were less opaque and surrounded by a lucent halo (Fig. 3b); however, they were noticeable only when they were circumscribed by compact bands or tylosis walls, or intermixed with other compact matter. These bands were formed of an inner opaque layer and an outside paler layer, analogous to that surround-

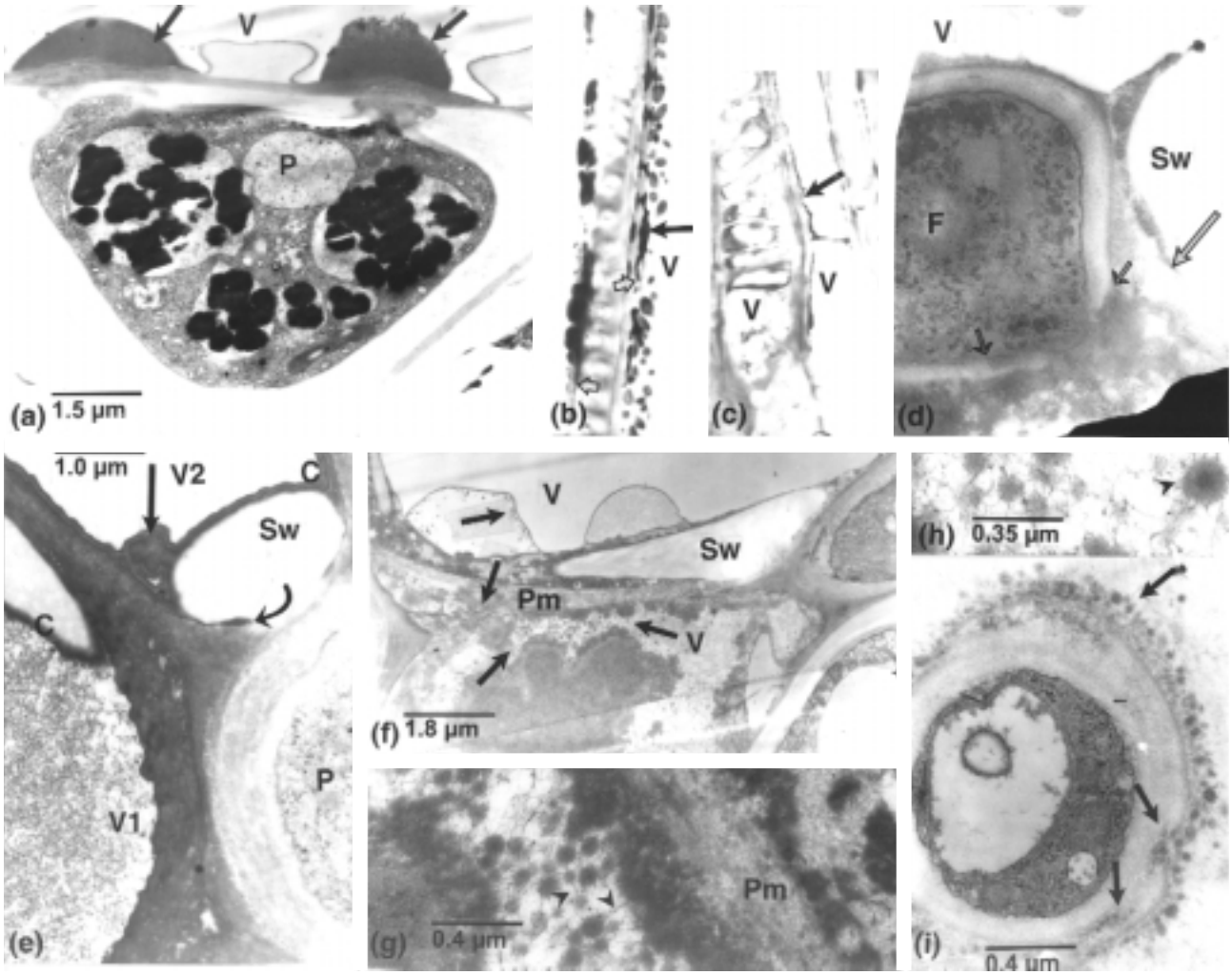

Figure 2. F, fungal cell; C, coating; P, parenchyma cell; Pm, pit membrane; Sw, vessel secondary wall. *U. americana*. **(a)** 1 year after inoculation. Humps of matter (arrows) cover the central portion of the pit membranes in line with the pit aperture in the adjacent parenchyma cell. **(b, c)** *U. americana,* LM observations. **(b)** 1 dpi. Masses of opaque matter (as that indicated by the long arrow) occur along vessel walls intermixed with fungal cells (likely of the inoculum), and fill pit chambers. Small arrows point to similar matter cutting across the pit borders. **(c)** 2 dpi, at the tip of an advancing streak in a shoot. In a small vessel element, opaque matter lining its wall (arrow) is linked to similar material traversing the lumen and confluent there with a fungal-like element. **(d)** From a seedling grown in a controlled chamber. Petiole inoculation. Large amounts of opaque matter (bottom part) surrounding a fungal cell have links with the cell content through the fungal wall (between short arrows), and with that apposed to the wall of the adjacent border (long arrow). **(e)** 3 dpi. Vessel V1 contains numerous opaque bodies of equal size. Walls of V1 and V2 are covered by coating material, which has a stratified appearance in V2, due to the presence of equidistant opaque bands and enclosing (arrow) a mass of opaque matter in the vessel lumen. Some of the opaque matter occurs in the secondary wall thickening (curved arrow). **(f)** 2 dpi, (with inoculum grown in presence of tritidiated thymidine). Occurrence of numerous opaque bodies (arrows) in protoxylem vessel, some of which are included in the pit membrane or confined by the alveolar bands, in the upper vessel. **(g)** From a section contiguous to that in f. Fibrillar-like structures (arrowheads) are linked to the globoid bodies, even to those which have coalesced. In the bottom left portion, arrays of filamentous-like structures abut on and intermingle with the bodies. **(h)** A slightly underexposed print, showing the close link of the fribrillar structures with the bodies shown in g. Some of the structures appear to reach into the bodies. **(i)** 2 dpi. Globoid bodies (arrows) are linked to or included in the wall of a pathogen cell.

ing the bodies (Fig. 3b). Thinner bands, in these cases, enclosed other types of matter. Attempts to further characterize these bodies were not exhaustive, but in samples placed in a nutrient solution before fixing, opaque bodies of regular dimensions and still linked to filamentous-like structures abounded in vessel lumina, whereas other components had been lost, except for empty networks of nearly lucent bands (Fig. 3c).

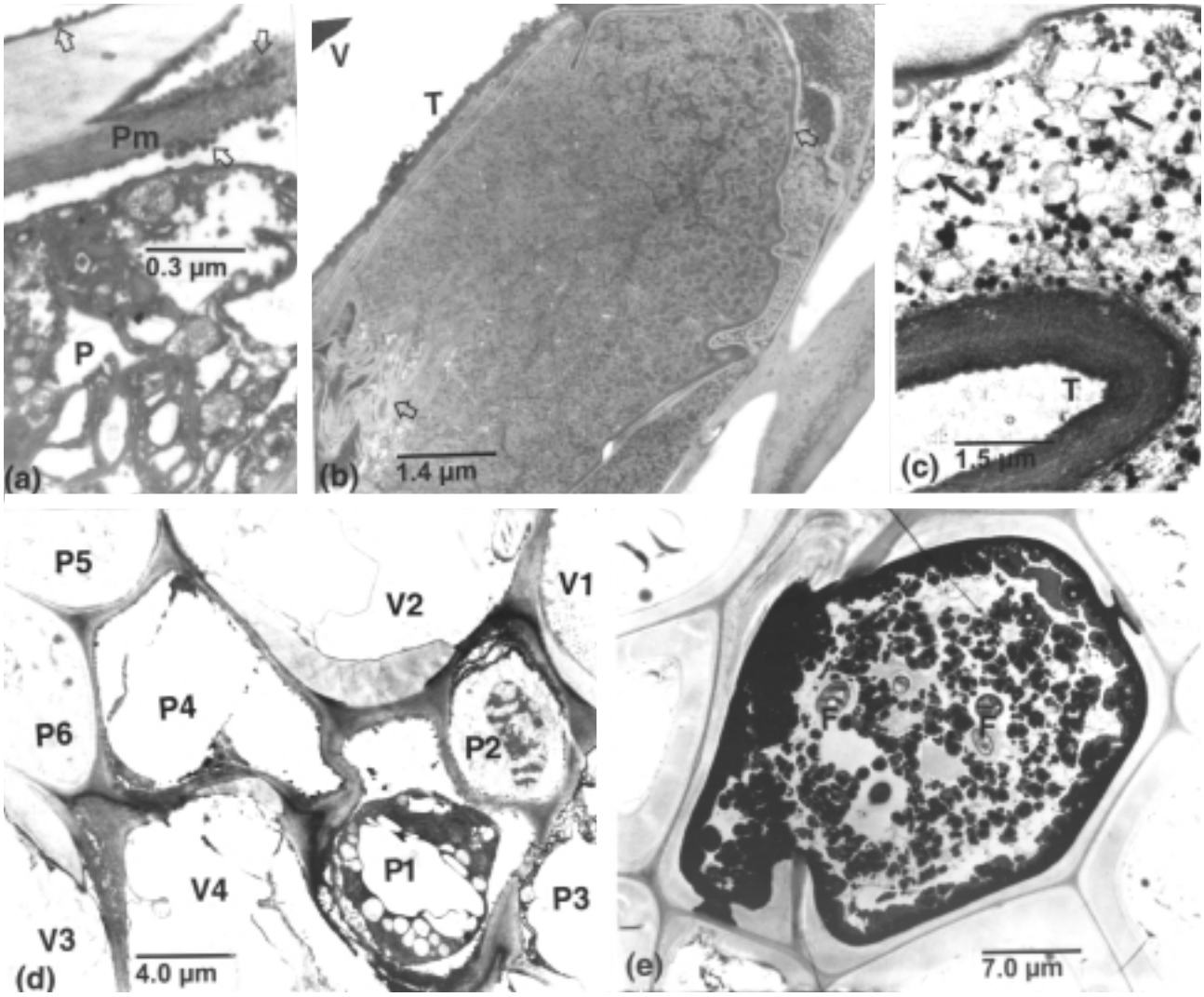

Figure 3. F, fungal cell; P, parenchyma cell; Pm, pit membrane; T, tylosis; V, vessel element. *U. americana*, except c, *U. glabra*. **(a)** Fungicide treatment prior to inoculation, 20 dpi. Globoid, opaque bodies (arrows) of equal size occur in the altered pit membrane, the cell periplasm, and as part of the coating. **(b)** 40 dpi. Masses of globoid bodies, with lighter margins, some coalescing, are enclosed between bands of an alveolar network (arrows) and a tylosis wall. Fibrillar material is similarly circumscribed close to the vessel wall. **(c)** 4 months pi. Specimens placed in a nutritive solution 18 h before fixing. Numerous globoid bodies of regular size are present in a vessel element, intermixed with remnants of the alveolar network (arrow). **(d, e)** Absorption of a cerato-ulmin suspension through cuttings (**d,** 112 h) or seedlings (**e**, 48 h). **(d)** Altered (P1-P4) and more intact (P5, P6) parenchyma cells next to vessel elements containing traces of alveolar networks and coating (V2-V4).

The coating material present on vessel walls, associated with these structures, became thicker and somewhat stratified as infection progressed (Fig. 2e; see also Ouellette *et al.*, 2004b). Although pit membranes, including those of bordered pits, were covered by the coating and were even altered or impregnated, they nevertheless remained well demarcated and their disintegration products appeared to stay localized (Figs. 2e, f, 3b).

Mentions of material occurring in vessel lumina following injections of compounds which have been isolated from the pathogen, some as possible toxins and others as elicitors of defence reactions (Strobel *et al.*, 1978; Scheffer, 1984; Richards, 1994; Yang *et al.*, 1994), seem warranted [for discussions concerning the possible role of toxins in DED pathogenesis, one is referred to Strobel *et al*. (1978); Scheffer (1984); Richards (1994)]. When the monoclonal antibody (mab) produced against the saccharide moiety of the peptidorhamnomannan [isolated by Strobel *et al*. (1978)] was used in immunocytochemical tests, this mab at-

tached to the pit membranes and the protective layer of paratracheal parenchyma cells (Benhamou *et al.*, 1985). Although these mabs might have been used to better comprehend DED disease development, they were not used in further work due to subsequent results showing that they attached to the middle lamellae of some of the non-inoculated trees, including non-hosts, and particularly *Prunus pensylvanica* (Rioux, D., unpublished observations).

A few samples tested with CU suspensions absorbed by cut twigs, as described by Takai and Richards (1978), or by seedlings bathing in the toxin solution with some of their roots sectioned and the others kept intact, have been examined. The injected twigs showed accumulation in some vessel elements of the alveolar networks and of globose bodies similar to those described above (see Ouellette *et al.*, 2004b). Pit membrane alterations and noticeable parenchyma cell reactions were pronounced in young tissue (Fig. 3d) (in fact, secondary xylem was present only at the base of the seedlings). However, the pathogen was isolated from the injected plants in both tests and visually detected in occluded vessels (Fig. 3e). Furthermore, the fungus was isolated directly from the toxin samples. As isolation of the toxin was performed by filtration through Millipore membranes, a number of filtration tests were conducted with membranes of low porosity, and the trees injected with the filtrates became diseased; similarly, samples of filtrates plated on an agar medium yielded the fungus, occasionally in filtrates from the 0.1 µm membrane and in up to 35% of trials (n = 20) with the 0.22 and 0.4 µm membranes (unpublished observations). By growing *O. novo-ulmi* on these membranes and on an impoverished medium, fungal cells were found to traverse the membrane in various irregular forms (Ouellette *et al.*, 1995; Ouellette *et al.*, 2001) with clear indications that cell wall organization, particularly that of chitin deposition but also that of cellulose (see below), was strongly disturbed; the smaller the porosity of the membrane was, the more pronounced these disturbances were (see also Ouellette *et al.*, 1995). Much extracellular material also surrounded the fungal elements in or on the membrane. It may be recalled that cellulose is a normal wall component of this pathogen.

Parenchyma cell and pit membrane alterations. Although parenchyma cells in mature xylem were rarely colonized, they showed various types of alterations and reactions. Some of these have already been reported (Ouellette, 1978c, 1978d, 1982; Ouellette and Rioux, 1992, 1993). The severity of parenchyma cell alterations was found to be linked to the number of altered or pervaded pit membranes communicating with the same or with other adjoining vessel elements (Fig. 4a, b). This feature can only be approximated, as numerous contiguous sections should have been examined to adequately evaluate this relationship. As a counterpart, however, it can be stated that when paratracheal cells adjoined less altered pit membranes or membranes that contained little pervading matter, their content was visibly less altered (Fig. 4c, d). Similarly, slight alterations of parenchyma cells paralleled slight alterations of pit membranes even in 1-year-old or recurrent infections (Fig. 4d). Pit membranes of half-bordered pits were similarly less affected in inoculated *U. pumila*, but they were more altered as well as their external symptoms in naturally infected trees of this species.

Whether they were concomitant or not with pit membrane alterations, masses of opaque matter, texturally similar to that found in vessel lumina, occurred in periplasmic areas below the membranes and around the cell and, in pronounced cases, extended into the cell content (Fig. 4b; see also Ouellette and Rioux, 1992, 1993). Often, such matter was the only remaining component at the periphery of cells having otherwise almost completely disintegrated content (see below).

Whereas distensions of pit membranes of half-bordered pits consistently occurred, as shown by labelling for cellulose, and in association with included opaque matter (Fig. 4f) (Ouellette *et al.*, 1978b, 1978c), pit membranes of bordered pits were rarely disrupted but they regularly appeared swollen, but mostly due to their being covered by matter which was continuous with the coating and similarly not labelling for cellulose (Fig. 4g).

Reactions of parenchyma cells. Various types of reactions occurred in xylem paratracheal cells (Ouellette, 1981, 1982; Ouellette and Rioux, 1992, 1993). Cell modifications, which are considered to be most expressive of defence reactions, are further illustrated here. Cell content was obviously much modified in paratracheal cells, even sometimes when the adjoining pit membrane was heavily pervaded by opaque matter (Figs. 4d, e, h, 5a). These modifications were characterized by dense and opaque cytoplasm, nuclei having dense content, the presence of opaque or fine matter in organelles, pronounced vesiculation of the endoplasmic reticulum, the presence of large vacuoles containing opaque and more lucent compounds, and so on.

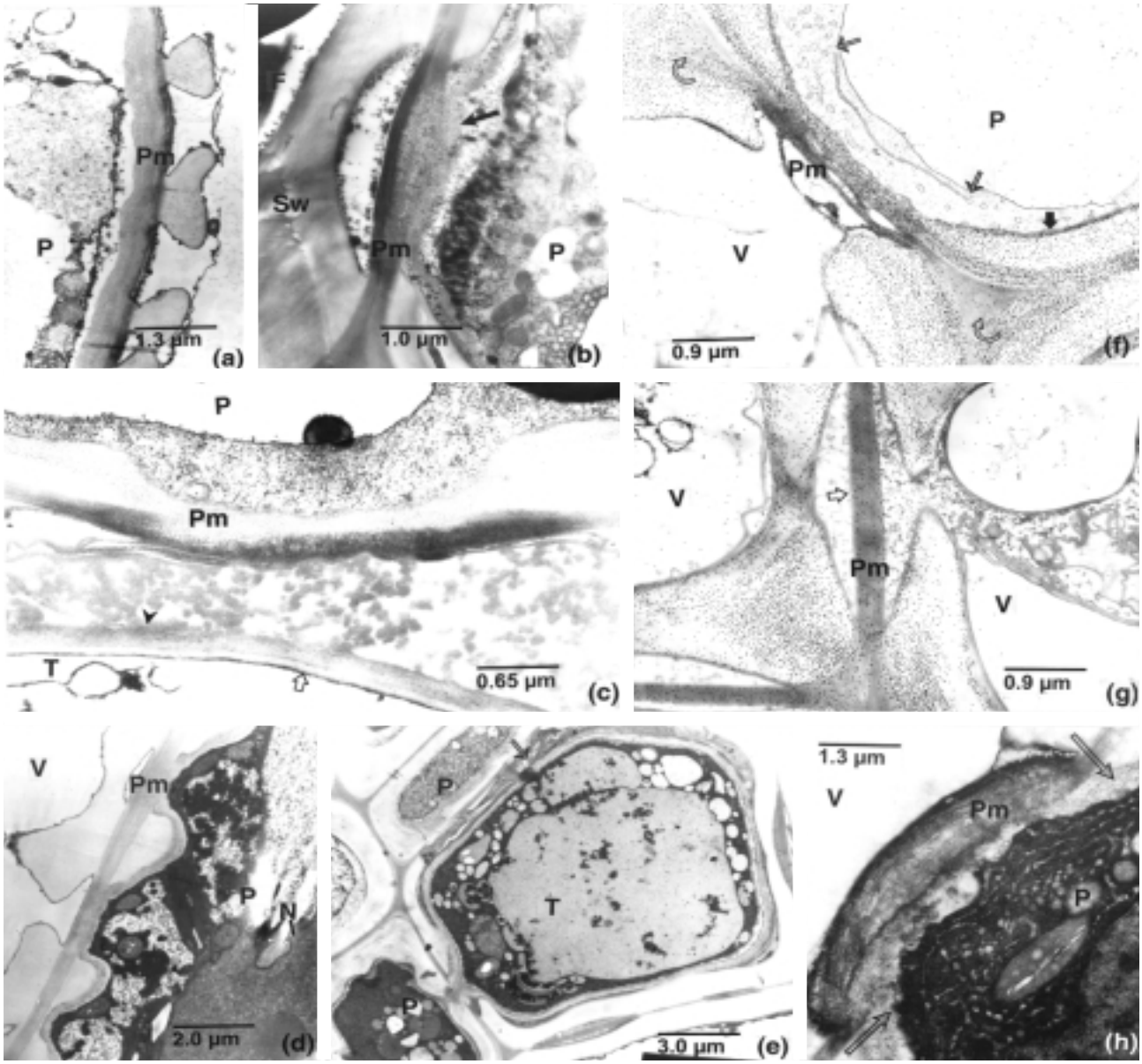

Figure 4. N, nucleus; P, parenchyma cell; Pm, pit membrane; Sw, vessel secondary wall; T, tylosis; V, vessel. *U. americana*. **(a)** 18 dpi. Four pit membranes of half bordered pits are covered with an opaque layer which has impinged on the membranes. The content of the subtending parenchyma cells is much altered. **(b)** 15 dpi. The pit membrane is impregnated with opaque matter (arrow), similar to that present in the periplasm of the subtending parenchyma cell and extending into the altered cell content. **(c)** 15 dpi. The pit membrane is only partially pervaded by opaque matter and content of the parenchyma cell is nearly normal. The tylosis has an inner suberin layer (empty arrow), but its external layer is pervaded by matter (arrowhead) confluent with that enclosed between the tylosis and pit membrane. **(d)** Same type of sample as for a. In comparison, very little matter covers pit membranes which are seemingly intact. The reacting parenchyma cell has dense and opaque content including a dense nucleus. **(e)** 16 months pi. Various reactions are noticeable in cells adjoining a vessel element with a tylosis, having content similar to that of the parenchyma cell with which it is connected. The spaces between the tylosis and vessel walls are filled with material in which meander bands (arrow) of the alveolar network. **(f)** 18 dpi. Labelling for cellulose. The probe has attached to the inner opaque layer in the parenchyma cell (black arrow), to the dislocated pit membrane, and to the native host walls of vessels and parenchyma cell. The intercellular areas (curved arrows) are almost not labelled. Dilated, smooth endoplasmic reticulum (empty, straight arrows) is noticeable in the cell. **(g)** 15 dpi. Vessel elements contain alveolar networks and opaque bodies. Labelling for cellulose, strong and uniform, except in areas of the pit borders, is restricted to the middle lamella, the pit membranes, and the vessel secondary walls. **(h)** 15 dpi. The plasma membrane in the parenchyma cell, next to the altered and pervaded pit membrane, is not perceptible at locations (between arrows). The nucleus and plastids are modified and the more lucent areas probably correspond to altered endoplasmic reticulum.

The layer of distinct cytoplasm was often very thin, not thicker than the plasma membrane itself (Fig. 5a). Transformation of this membrane was also noted as being thicker around one side of or around the entire cell (Fig. 5b), and displaying the presence of many circular structures (Fig. 5c) which likely corresponded to cross sections of filamentous components. Also, although the cell plasma membrane was in some instances noticeably altered beneath the pit membrane, it was still well demarcated elsewhere around the cell and linked to an apparent deposition of opaque matter on the cell wall (Fig. 5d).

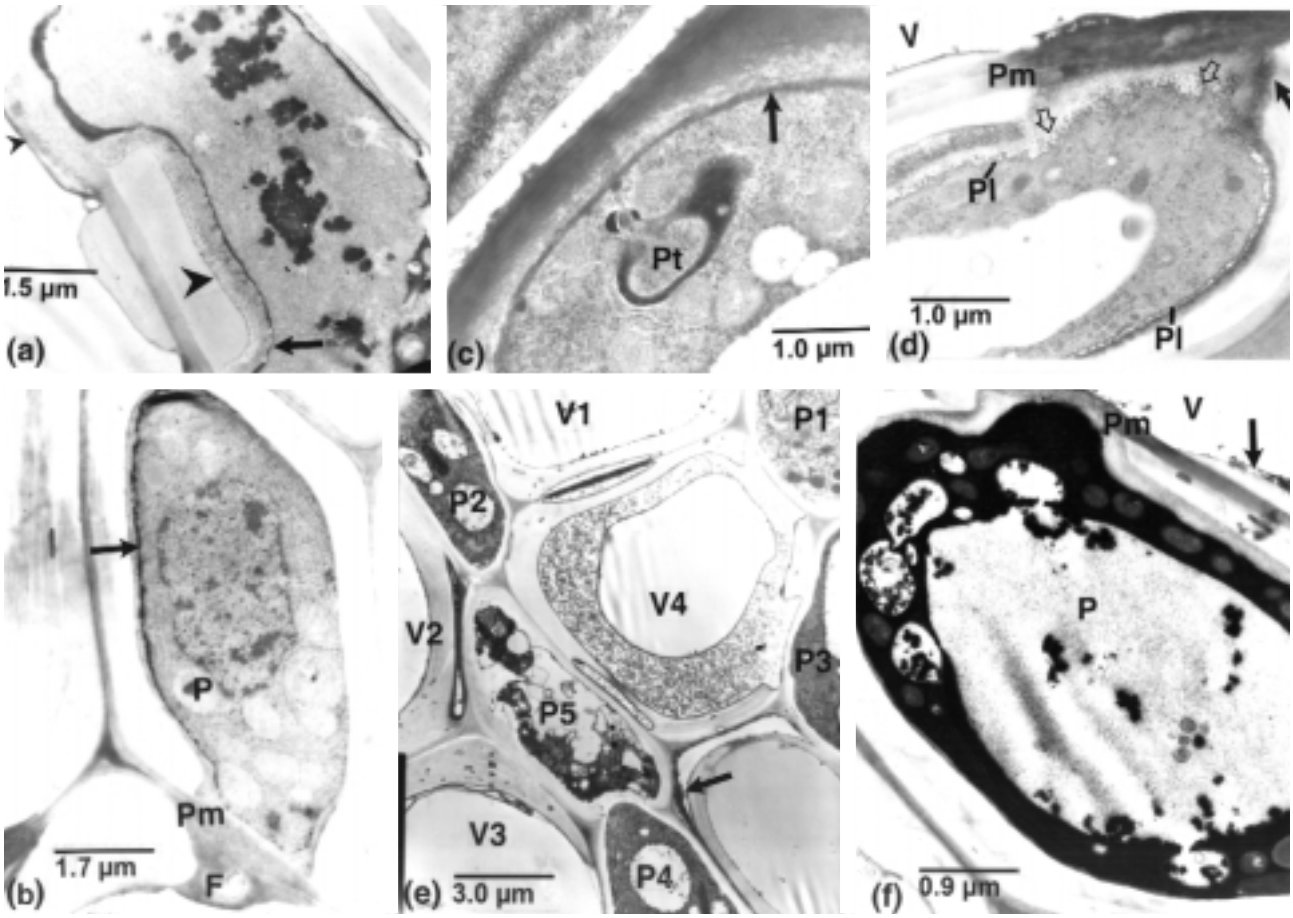

Figure 5. F, fungal cell; P, parenchyma cell; Pl, plasma membrane; Pm, pit membrane; Pt, plastid; V, vessel element. *U. americana*. **(a)** 3 dpi. A parenchyma cell has a thin layer of cytoplasm, no thicker at locations than the plasma membrane (arrow), surrounding a large vacuole filled with fibrillo-granular and opaque matter. The inner wall in the cell is fibrillar, thicker on the vessel side, but loosened at places (large arrowhead). The pit membranes are covered by a thin coating and slightly pervaded by opaque, filamentous-like material connecting with it (small arrowhead). **(b)** 30 dpi, greenhouse-grown seedling, fertilized with a 5-10% NPK solution 2 days before inoculation. The band corresponding to the plasma membrane on one side of the parenchyma cell is thick and opaque (arrow). The adjacent pit membrane is impregnated and the pit chamber is occluded with fine material, surrounding an element identified as a fungal cell. **(c)** A deformed plastid in a parenchyma cell whose content is delimited by a thick layer (arrow) containing structures with a lucent core (enlarged area, top left portion) and fine opaque matter. **(d)** 14 dpi. The plasma membrane, below the heavily pervaded and coated pit membrane, is much altered in association with opaque matter extending into the cytoplasm (empty arrows), and is distinct but irregular elsewhere around the cell. Similar matter, present in the inner cell wall layer, is confluent with that in the pit membrane (opaque arrow). **(e)** 30 dpi, from a tree (ca. 12 cm in diameter) injected with 5 L of a 5% glucose solution, prior to inoculation. Four of the parenchyma cells (P1-P4) are intact having content typical of reacting cells, whereas cell P5, which has pits (obliquely sectioned) connecting with three vessel elements, has altered content. Thin coating layers line vessel walls (V1-V4) and pit membranes are little permeated by opaque matter. Vessel V4 also contains numerous opaque bodies of equal size. A mass of opaque matter (arrow) is included in the intercellular area and middle lamella next to another, empty cell. **(f)** 4 dpi, greenhouse-grown, 2-year-old seedlings, planted directly in soil. Ca treatment (50 g in 500 mL H_2O) 4 days prior to inoculation. Dense and opaque cytoplasm in a paratracheal cell, communicating with a half-bordered pit having a slightly permeated membrane. Little matter (arrow) lines the vessel wall.

In American elm trees submitted to particular treatments prior to pathogen inoculation, reactions of parenchyma cells appeared to be enhanced in some cases (Fig. 5e, f). Thus, of the various fertilization treatments with NPK and Ca in greenhouse-grown elm seedlings, parenchyma cell reactions were mostly noticeable in the Ca treatments (Fig. 5f), coincident with a lessening of external symptom expression, compared to the other treatments. Following injection of a glucose solution, pit membranes of paratracheal cells were rarely altered (Fig. 5e) compared to untreated trees, and most of the cells had modified cell contents similar to those described above. In agreement with the results obtained following glucose as first reported by Feldman *et al.* (1950), the intensity of symptom expression was also lessened in our similar tests. Other differences related to glucose treatments are mentioned in Ouellette *et al.* (2004b). Some apparent differences in cell reactions linked to fungicide treatments were also observed but were more difficult to evaluate, as examinations were limited to samples collected the second year after treatment.

Deposition of new wall layers was also a marked reaction of parenchyma cells and fibres, a feature which was common in cells related to barrier zone formation (Rioux and Ouellette, 1991a, 1991b). Thick layers of fibrillar material (Fig. 6a, b), shown to label for pectin (Rioux *et al.*, 1998), were deposited in many cells. The plasma membrane was frequently not discernible or was impregnated in these cells, particularly in parts facing vessel elements (Fig. 6a, b). An inner opaque layer occurred in other cells, which clearly labelled for cellulose, as did tylosis walls (Fig. 6c). On the contrary, some of the usual labelling of secondary walls was lost in some of these cells (Figs. 4e, 6c). Additional secondary wall layers alternating with more opaque ones occurred in some cells, a feature which was common in *U. pumila* trees having recovered from infection (Fig. 6d) (see also Ouellette, 1978a; Ouellette and Rioux, 1992). However, similar new wall layers were also occasionally observed in some non-inoculated trees (not illustrated). The presence of a suberin-like layer in parenchyma cells, including tyloses as mentioned above and also fibres, was frequent in *U. pumila*, occasional in *U. glabra*, and exceptional in *U. americana* trees, except in barrier zones. Cell reactions, similar to the ones just mentioned, were commonly observed in reacting cells of the susceptible *Fusarium*-infected carnation plants (Ouellette *et al.*, 2002), and in which compartmentalization of invaded xylem was also well marked (Baayen *et al.*, 1996). A gelatinous-like layer occurred in many fibres in elms displaying adjacent wall alterations (Fig. 6e) or in more intact cells as also part of the compartmentalization tissue (Ouellette 1981; Rioux and Ouellette, 1991b). Outstanding formation of similar tissue in barrier zones was also observed in *Fusarium*-inoculated sumac plants (Fig. 6f).

Young and cambial tissue

Besides assigning the usual proto- and metaxylem to this category of tissue, the other criteria for denoting tissues as being newly deposited were either based on their location close to the cambium or on the cell wall characteristics (thin and/or not displaying a secondary wall).

Cell content and wall modifications. Outstanding features in the less altered cells in both situations were the deformation or alteration of plant organelles. A few of these modifications are illustrated (Fig. 7a-e) as a complement to previous mentions (Ouellette and Rioux, 1992, 1993). Thus, pronounced proliferation or cupping of mitochondria or of plastids, some containing paracrystalloids or numerous vesicular structures, was frequent in these cells (Fig. 7a-c, e). A peculiar thickening of the endoplasmic reticulum, bordered by rows of ribosomes and bridged by a strand of smooth reticulum, was noticeable (Fig. 7d).

Alterations of pit membranes of the proto- and metaxylem were often outstanding with their residues appearing mostly as opaque bands intermixed with other types of opaque matter (Fig. 8a). Yet their breakdown products remained seemingly confined by thin or thick layers of compact material and were not liberated into vessel lumina. However, occasionally altered cell content, including vesicular structures, were seemingly extruded through pronounced distensions of pit membranes in these and other tissues (Ouellette, 1978c).

During the first year of infection or in recurrent infections, the pathogen had often reached the cambial area and invaded the last deposited vessel elements, next to which the cambium cells were damaged to various degrees (Fig. 8b). The most severe cases were characterized by distorted and collapsed cells having necrotic content, through which cells with more normal content had intruded. The vessel elements in these areas were much flattened or twisted, their presence being indicated by bands of secondary-like walls,

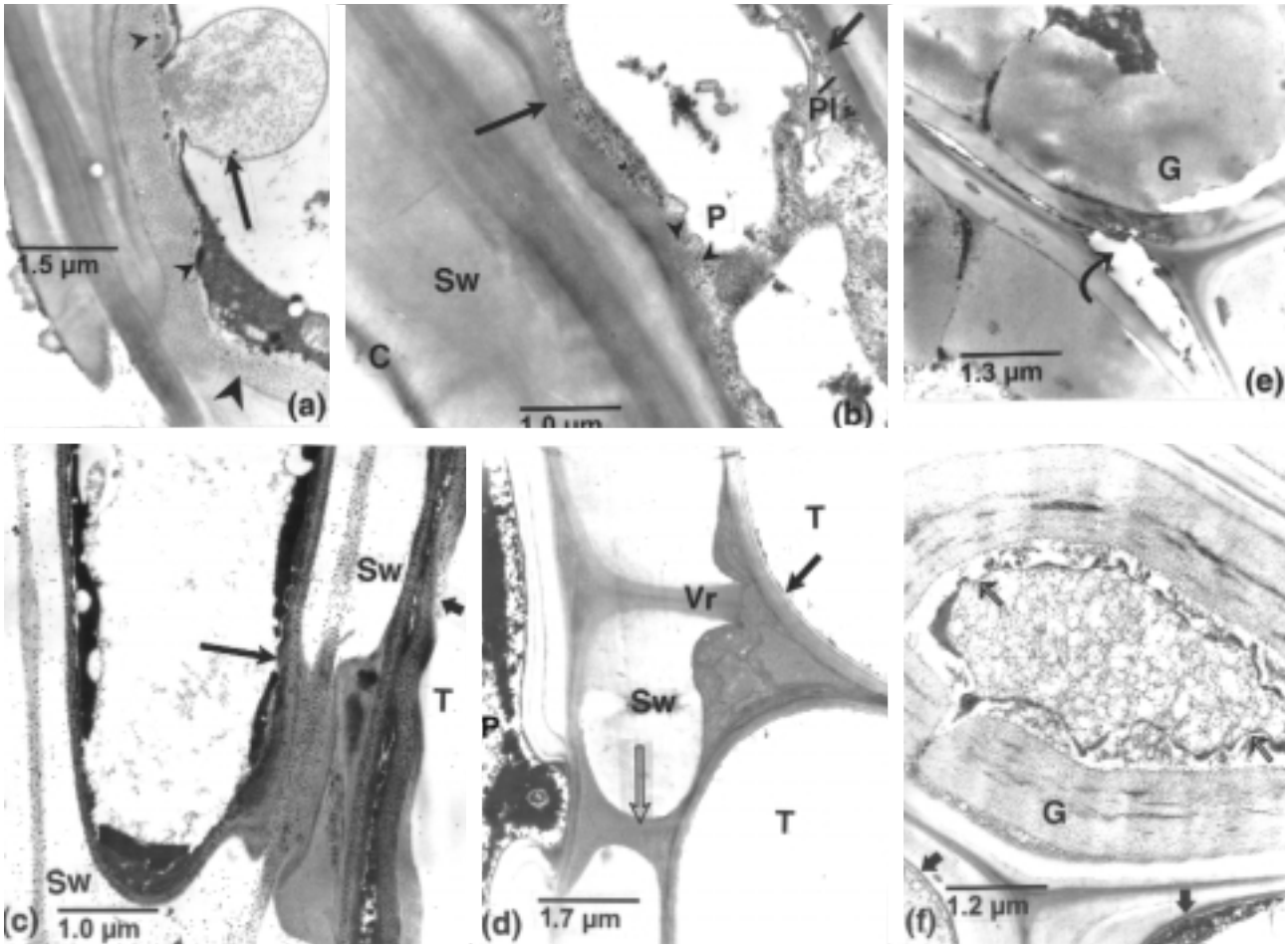

Figure 6. C, coating; G, gelatinous layer; P, parenchyma cell; Pl, plasma membrane; Sw, vessel secondary wall; T, tylosis; Vr, vessel rim. *U. americana*, except where otherwise stated. Deposition of new wall layers in reacting cells. **(a)** 34 dpi. A thick fibrillar layer (thick arrowhead) is apposed to the native wall in a parenchyma cell, and an invaginated portion of the plasma membrane (long arrow) contains fibrillo-granular. Opaque matter (thin arrowheads) is present in the fibrillar layer, the cell periplasm as in the outer cytoplasm. **(b)** 24 dpi. In a parenchyma cell, the new wall layer (long arrow) on the side facing the vessel, with its wall lined with a coating, is irregular and loosely textured, matched by a barely perceptible plasma membrane, compared with the more compact and distinct one (short arrow) on the opposite side. Cortical microtubules (arrowheads) are abutting on the irregular wall. **(c)** 60 dpi. The middle lamellae, the pit membrane, and the inner new wall layer in the parenchyma cell (long arrow) are strongly labelled, but not the matter enclosed between the tylosis and the vessel walls. A loss of labelling of the secondary walls is noticeable, whereas elsewhere in the same section they were more normally labelled. Short arrow: a more lucent patch in the tylosis wall. **(d)** *U. pumila*. Tylosis walls, with an inner suberin layer (arrow), are separated from the vessel secondary wall by only a thin layer of matter, except near the vessel rim by masses of fibrillar matter, confluent with that filling the adjacent pit chamber (empty arrow). Two additional secondary wall-like layers, separated by opaque layers, are present in the adjoining parenchyma cell. **(e)** 48 dpi. Thick gelatinous layers have been deposited in fibres, next to altered portions (curved arrow) of the middle lamella and the other two outside layers, in association with opaque matter. **(f)** Staghorn sumac inoculated with *F. oxysporum*, 15 dpi. Reactions of cells, in part of a barrier zone. A thick, gelatinous-like layer is present in a fibre next to other cells having an inner thin opaque layer (black arrows). Note: the thin layer of cytoplasm (empty arrows) enclosing a large amount of material similar to that in the cell periplasm and in the invaginated area in a.

even included in the region of other moribund cells lacking secondary walls (Fig. 8b, c). Rows or groups of hypertrophied cells having thin walls or containing only a narrow band of secondary wall bordered these vessels, and sometimes located at an appreciable distance from the dead cells (Fig. 8c) (see also Ouellette and Rioux, 1992, 1993). Cells with these types of reactions often abutted on a zone of compartmentalized xylem during the year of inoculation (Rioux and Ouellette, 1991a, 1991b). In some of the distorted, reac-

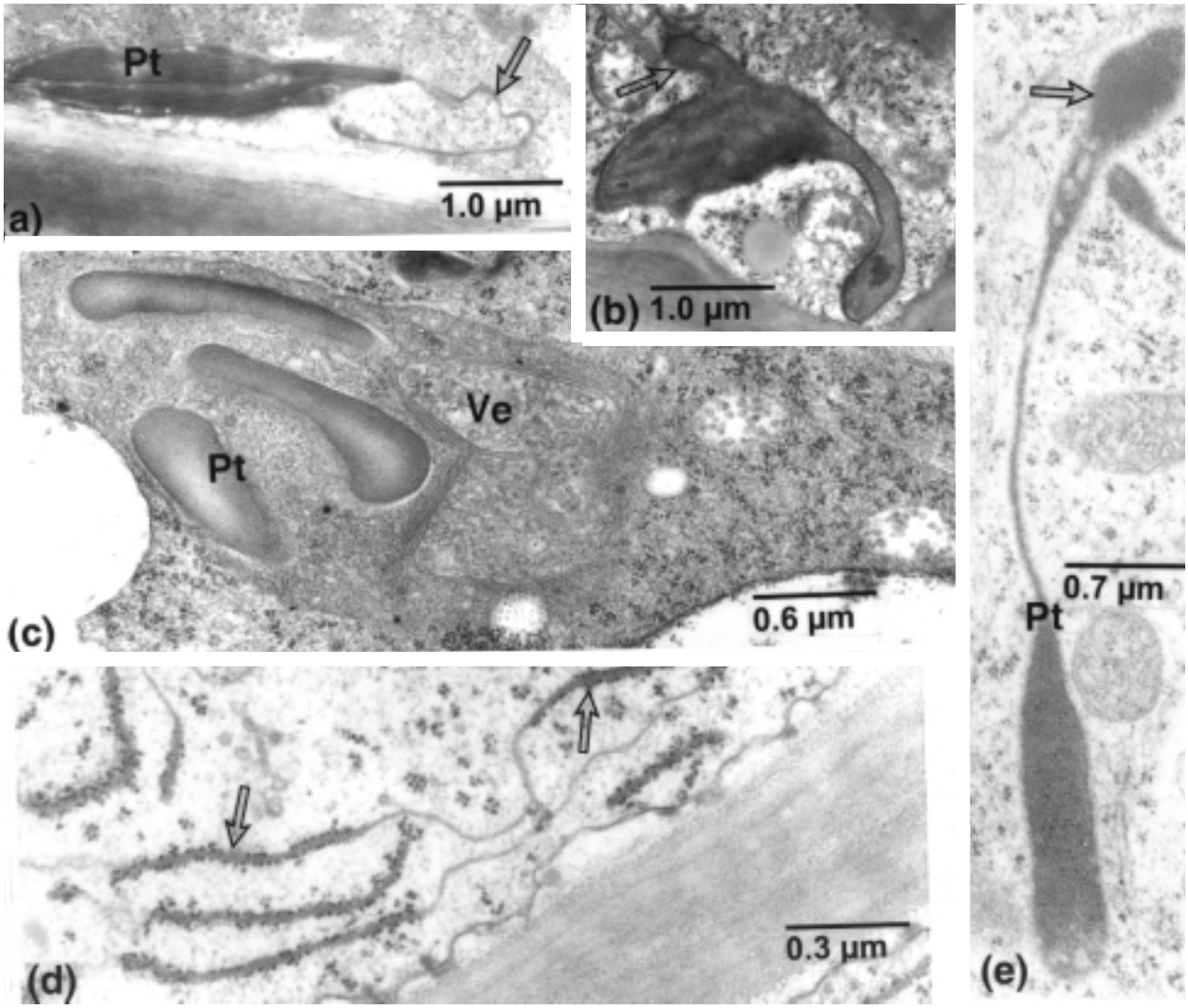

Figure 7. Pt, plastid; Ve, vesicular structures. From diverse inoculation and sampling times, *U. americana*. **(a-c)** Modified plastids: one with an elongated hook-like cupping (a, arrow); one also proliferated and with a lateral outgrowth (b, arrow); one containing numerous vesicular structures in its inflated portion (in c). **(d)** Rows of ribosomes are linked to bands of fine opaque matter (arrows), separated by membranous structures of various lengths. **(e)** A much elongated plastid contains a paracrystalloid (arrow) in one of its inflated ends.

ting cells, irregular patches or tangles of wall material were apposed to or extending from the native wall into the cell, next to other cells having patches of secondary wall (Fig. 8d).

A characteristic feature linked to anomalies in the newly deposited xylem cells was the general and abundant occurrence of bands and masses of homogeneous opaque matter in their walls and in the cell periplasm (Fig. 8e-h). Some of it also infringed on the cell content (Fig. 8f) and the cell plasma membrane was seemingly discontinuous or absent in these cells or frequently impregnated by opaque matter (Fig. 8f-i). As shown in Fig. 8e (and also below), the opaque matter appeared as arrays of filamentous matter. Many cells had gigantic nuclei and nucleoli, whose borders often contacted the cell walls (Fig. 8h, i). These cells were delimited by irregular walls, with portions being secondary-like but abutting on more opaque portions in some places, next to much distended primary walls and/or middle lamellae (Fig. 8g, h). Other cells with more normal content frequently neighboured the altered cells (Fig. 8g, i). Examples of increasing severity of cell disturbances from larger vessels over several rows of cells up to the cambium were noted, in which

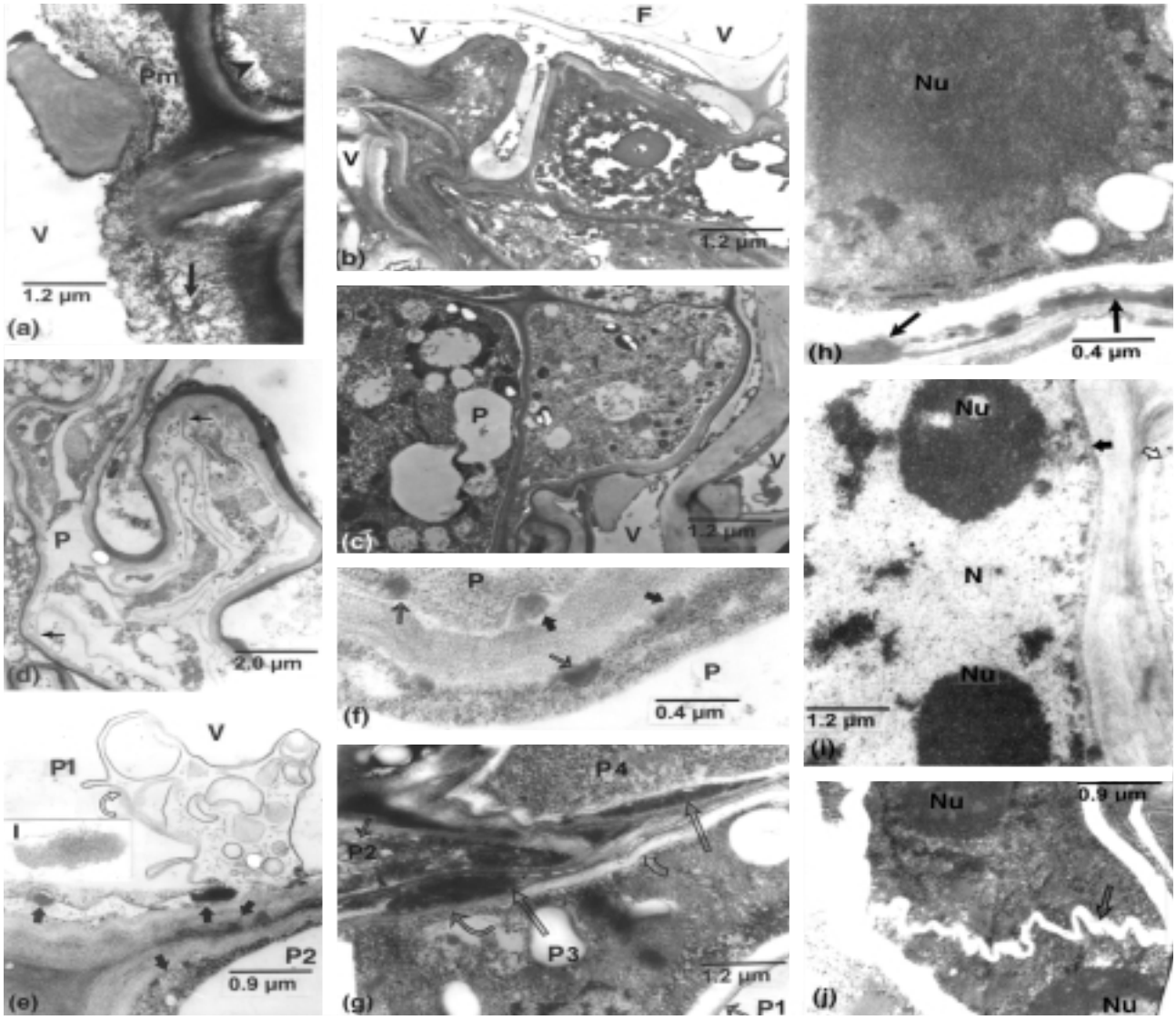

Figure 8. F, fungal cell; N, nucleus; Nu, nucleolus; P, parenchyma cell; Pm, pit membrane; V, vessel. Young tissue, alterations and reactions. **(a)** 54 hpi. Pit membrane alterations in proto- and metaxylem tissue. Breakdown products of the membrane appear as opaque bands intermixed with opaque matter (arrow), some covering the membrane. The cytoplasm in an adjacent parenchyma cell is retracted (arrowhead). **(b, c)** Natural infection, sampled late May 1979, the year following the first appearance of external symptoms. The vessel elements are elongated and collapsed, next to or among necrotic cells (in b), and by hyperplastic and hypertrophied ones (in c). Similar vessels also occurred more distally in this type of tissue. **(d)** Wall-like divisions stemming from the wall (arrows) of and meandering in a long, hypertrophied and distorted parenchyma cell with altered content. Similar cells adjoin this cell. **(e)** Masses of opaque matter (straight arrows) occur in the periplasm of cells P1 and P2 and in the middle lamella. In cell P2, the plasma membrane is locally altered in contact or close to the opaque matter, also in association with opaque matter. The inset (I) is an enlargement (in a shorter exposed print) of the mass at the base of the invaginated plasma membrane enclosing vesicular structures and other matter (curved arrow). **(f)** A plasma membrane is not discernible in these parenchyma cells, except where it surrounds masses of opaque matter (opaque arrows); other similar matter impinges (lucent arrows) on the cell content. **(g)** 38 dpi. Masses of opaque matter (long arrows) are included in the middle lamella and/or primary wall of large cells. A plasma membrane is visible (short, straight arrows; partly cropped one in P1) in altered cells P1 and P2; in cell P3 and P4, it is indistinct or appressed to the cell wall (short, curved arrow). An irregular secondary wall-like layer has been deposited in these cells but is thinner and not lucent (long, curved arrow) bordering the opaque mass, in cell P3. **(h, i)** 24 dpi. **(h)** A large nucleolus is present in a hypertrophied cell with a secondary-like wall layer, bordered by masses of opaque matter (arrows) included in the primary wall. **(i)** The cell contains two nucleoli in a gigantic nucleus having its margin reaching the cell wall (dense arrow). Content of the adjacent cell is also altered (empty arrow). **(j)** A tortuous wall division (arrow) is separating two not well demarcated nuclei in a ray cell, in a region where several rows of cells were increasingly more disturbed from an invaded vessel element towards the cambium.

most of the cells having also gigantic nuclei and other altered organelles included in dense cell content corresponded to ray cells (Fig. 8j). Chaotic secondary wall formation, without a perceivable presence of a primary wall, was noticeable in these cells, including the sinuous layer dividing the cells (Fig. 8j).

Characteristics of the opaque matter

Intriguing observations concerned the wide occurrence in elm of opaque matter spanning mostly intercellularly over appreciable distances as masses bifurcating into bands and fine strands in seemingly unruptured cell walls (Fig. 9a), and linked to cell alterations, particularly in shoots and twigs (Ouellette, 1978d; Ouellette and Rioux, 1992, 1993; Nicole *et al.*, 1994). This matter, in which intact or altered organelles were not detected, displayed fine, opaque homogeneous matter intermixed with more lucent areas. In enlarged and underexposed prints, the more lucent portions contained arrays of filamentous-like components, intermixed with more opaque bodies (Fig. 9b, c). In many instances, similar matter was linked to chipping of vessel secondary walls (Fig. 9d). Following injection of tritidiated thymidine shortly after inoculation, masses of similar matter were highly labelled, as shown in autoradiography (Ouellette, 1978d; Ouellette and Rioux, 1992, 1993).

In light of these observations, it appeared that this matter could not be ascribed a primary host origin, although sometimes some components present in ruptures of host walls could be identified as being of host origin; but in these cases, they displayed discernible remnants of host cell organelles. In order to further verify our assumption, experiments were conducted by inoculating sterilized elm wood sections in which host cell reactions would be obviated and cell content would generally be degraded. The formation of irregular growth forms by *O. ulmi*, particularly microhyphae, was highlighted using this method (Casagrande and Ouellette, 1971). Although the occurrence of masses of opaque matter was then also observed in cell walls, linked to fungal elements, those masses were not examined in TEM. Present TEM observations showed that opaque matter, similar to that just described, widely occurred as thick and bifurcating smaller bands in middle lamellae and secondary cell walls (Fig. 9e). This matter was in close contact with wall components, eliminating the possibility that they occupied the path of pre-existing ruptures, had these existed before inoculation. Other striking examples of opaque matter occurrence in this type of samples, treated with a labelled probe for detecting cellulose, was its presence along and within vessel walls (Fig. 9f). When remnants of host cell cytoplasm occurred, it could be recognized as such (Fig. 9f).

The occurrence of similar opaque matter was also observed in other plants affected by wilt diseases, particularly in staghorn sumac inoculated with *F. oxysporum* f.sp. (Fig. 9g-i). This matter also spanned long distances into intact middle lamellae, bound by thin bands and also often terminating into fine filament-like structures (Fig. 9h); in some tests, it was found to extend from the invaded vessel elements, radially up to the cambium area (Fig. 9i) associated thereabout to pronounced cell alterations and reactions, as in elms. Deposition of new wall layers in adjoining fibres was also noticeable (Fig. 9i). By testing these samples with gold-complexed monoclonal antibodies specific to double- and single-stranded DNA, this matter labelled strongly, as did the one present in periplasmic areas of cells adjoining invaded vessels (Fig. 9g), even more so than the normal host DNA-containing organelles. It is to be noted here that the plastid with which is associated appreciable labelling shows signs of rupture and proliferation.

Links of filamentous-like structures with the opaque matter

Examination of this matter at proper magnifications and print exposures showed that it mostly contained arrays of fine filamentous-like structures. As these structures were related to diverse host wall and cell alterations, it seemed important to illustrate examples of their diversity of occurrence (Figs. 10, 11) in order to unravel the problem their origin and nature may represent.

Thus, large masses of opaque matter in vessel lumina contained almost exclusively these filamentous structures, some of which were linked to fungal cells and extended into the vessel wall (Fig. 10a). Other similar masses occurred in the periplasm of cells with more pronounced alterations of content, had a similar striate appearance and differed from the cell content (Fig. 10b, c). Similarly, filamentous structures could be distinguished in the slightly or more heavily pervaded pit membranes of half-bordered pits, reaching from the coating to the cell periplasm, linked to alterations of the plasma membrane and even pervading it (Figs. 10d, 11a). The material in middle lamellae and primary walls of younger tissue, as illustrated above,

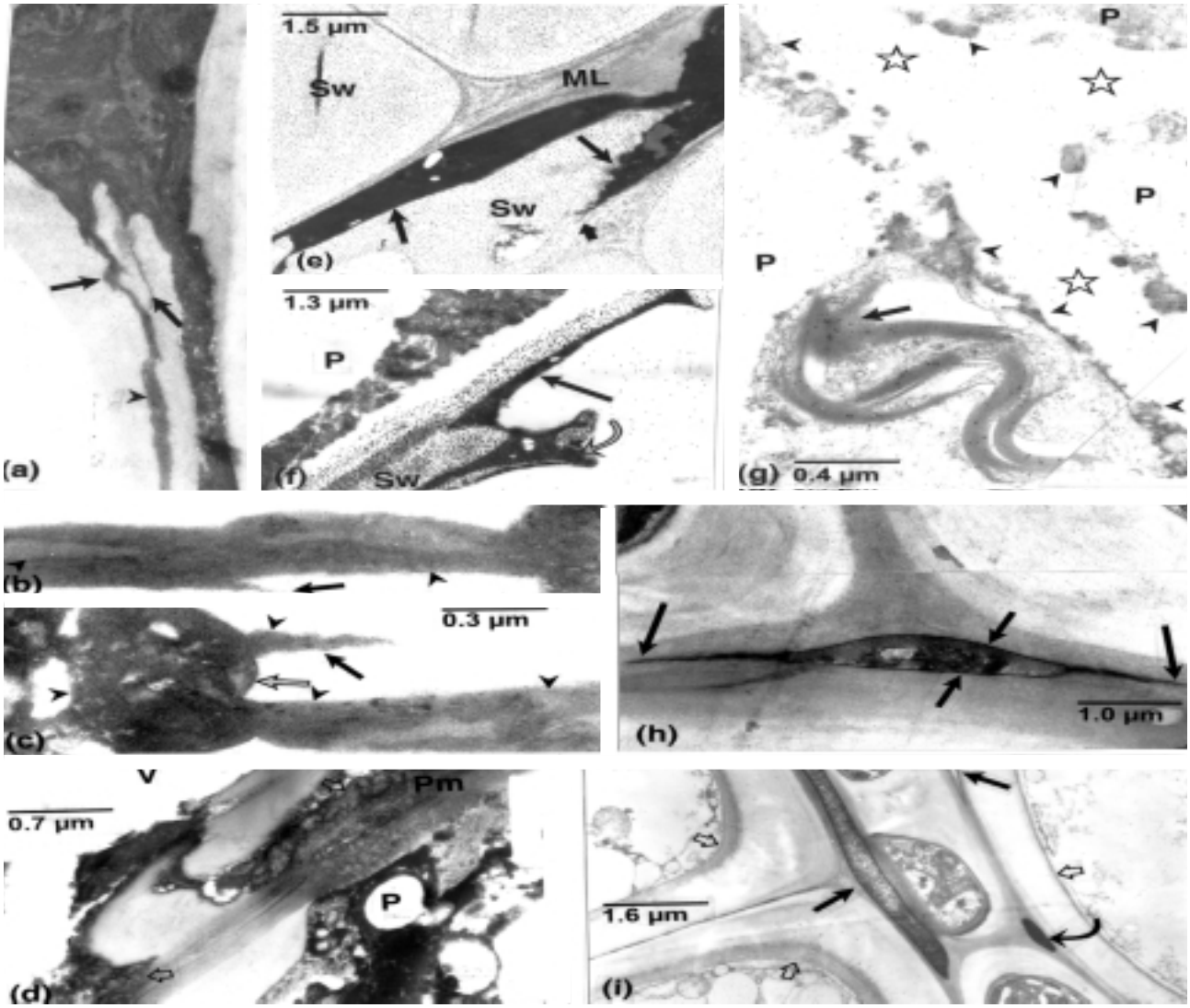

Figure 9. ML, middle lamella; P, parenchyma cell; Pm, pit membrane; Sw, secondary wall. **(a-i)** Characteristics of opaque matter (OM) in host walls and periplasmic areas. **(a-d)** *U. americana*. **(a-c)** 3dpi. **(a)** OM included for a noticeable distance in middle lamella and through an intercellular area, some extending as thinner bands from the larger body (arrows). The OM contains islands of more lucent matter, and displays at locations more opaque particles (arrowhead). **(b, c)** Similar matter viewed at a higher magnification and contrast, showing arrays of filamentous structures (arrowheads) in the more lucent areas and the presence of opaque bodies in the more opaque areas. The long, opaque arrows point to the thinner bands extending from the larger masses, and the empty arrow to a discrete band delimiting the OM. **(d)** 1 year pi. A mass of OM is associated with a chipped and eroded portion of the vessel wall, the extremity of which, if brought in place, would not perfectly fit into the void areas, also with eroded margins. The altered pit membranes display numerous circles of equal size (arrows), delimited by opaque bands, similar to others present in pit chambers and the coating. **(e, f)** Inoculated sterilized wood sections of *U. laevis*. 4-day incubation. Fixed by high-pressure freezing. Labelling for cellulose. **(e)** Large bifurcating masses of OM (long arrows) occur at a noticeable distance in undisrupted middle lamellae and secondary walls, terminating in fine bands (short arrow). **(f)** The apparently bleached pit membrane is covered by a layer of OM (arrow) which is confluent with that included in the dislocated portion of the vessel secondary wall (curved arrow). Note: the difference between this matter and the altered cytoplasm in the parenchyma cell. **(g)** From staghorn sumac inoculated with *F. oxysporum*. 2 dpi, 5 cm from the inoculation point. Labelling for DNA with monoclonal antibody. Short time, print exposure. Concentrations of gold particles are linked to masses of OM (arrowheads) in the periplasm of host cells and to a modified plastid (arrow). A number of particles occur in void spaces next to disintegrated cell content, but are sparse over the host walls (stars) forming the juncture of these three cells; they were absent elsewhere over the cell walls. **(h)** 3 dpi. Masses of OM delimited by compact bands (short arrows) on both interfaces across an intercellular area. **(i)** Staghorn sumac, 8 dpi. OM spans an appreciable distance in intact middle lamellae between ray cells and fibres, bypassing intercellular areas. The curved arrow points to a shorter compact mass in an intercellular area, and the short empty arrows indicate thin opaque layers apposed to the secondary wall layers in the adjoining fibres.

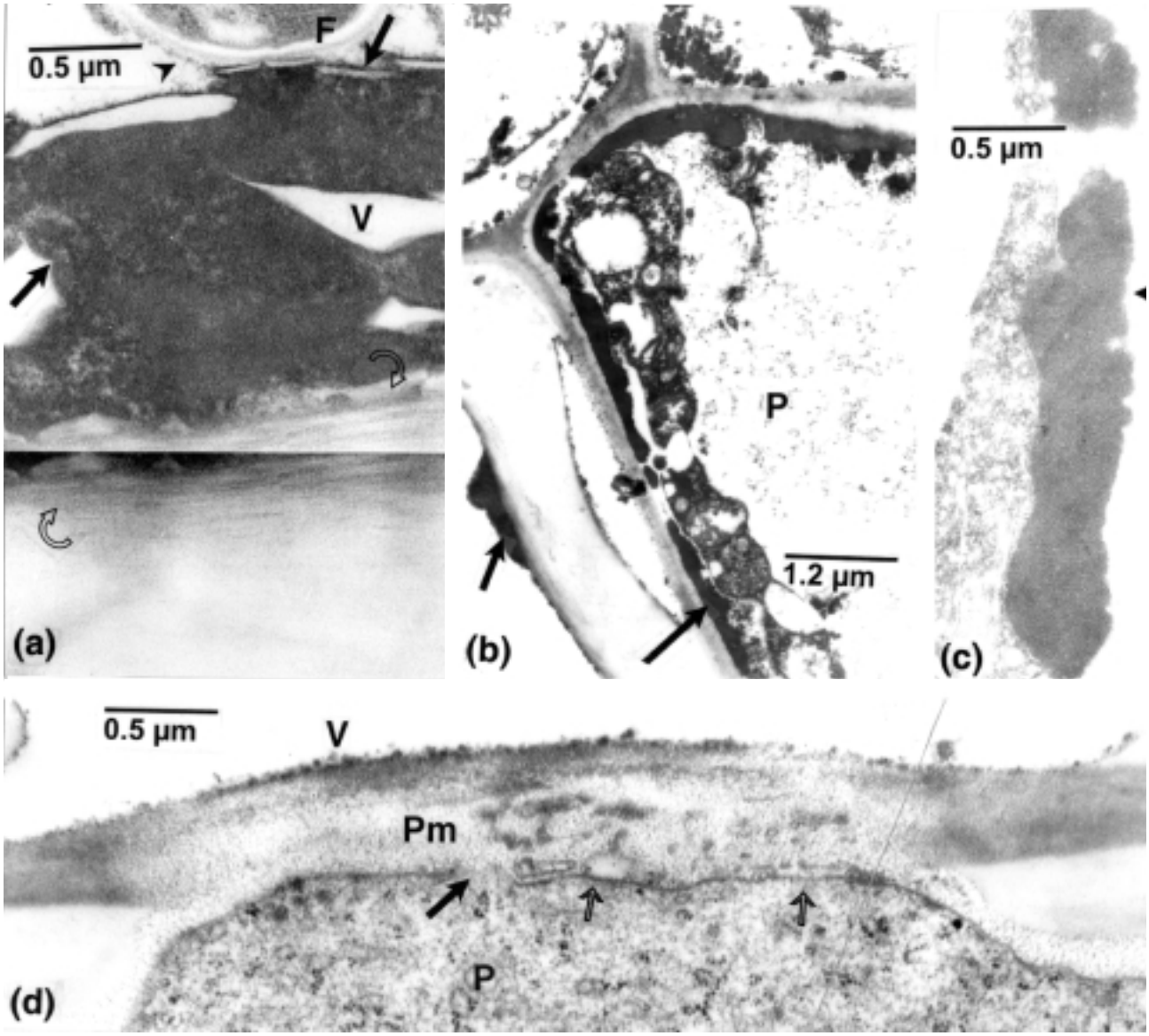

Figure 10. F, fungal cell; P, parenchyma cell; Pm, pit membrane; V, vessel element. **(a-d)** Occurrence of filamentous-like structures (FLS) related to opaque matter (OM). **(a)** 30 hpi. Masses of OM (arrows) displaying arrays of FLS occur next to a vessel wall. Some filaments reach from the former into the latter (curved arrows), associated with its greater opacity. The bottom part of this illustration was confluent with the top part, but was printed at a shorter time exposure to show better the texture of the impregnated vessel wall. Similar matter is also linked to the fungal cell (arrowhead). **(b)** 40 dpi. Masses of OM occur in the parenchyma cell periplasm (long arrow), similar to that along the vessel wall (short arrow), but differing from the altered cell content. **(c)** A similar mass of matter, in a slightly underexposed print and at greater contrast, displays crowded arrays of FLS (arrowhead). **(d)** 4 dpi. A pit membrane is pervaded by FLS across from its margin facing the vessel lumen to the cell periplasm and at locations in the outer cytoplasm through an altered area in the plasma membrane (dense arrow) or linked at places to it (empty arrows).

likewise appeared striated due to the filamentous structures, and the similar structures in the adjacent cell periplasm were continuous with similar ones present in the proximate nuclear envelope (Fig. 11b).

Opaque matter and filamentous structures also occurred in tylosis walls, but they were exceptional in the inner lucent, suberin layer (Fig. 11c). Filamentous structures likewise extended from the coating material into the vessel walls in these vessel elements (Fig. 11c, d). When the sections were tilted at various angles, mounted prints obtained from these various planes showed in stereoscopy that the filamentous structures,

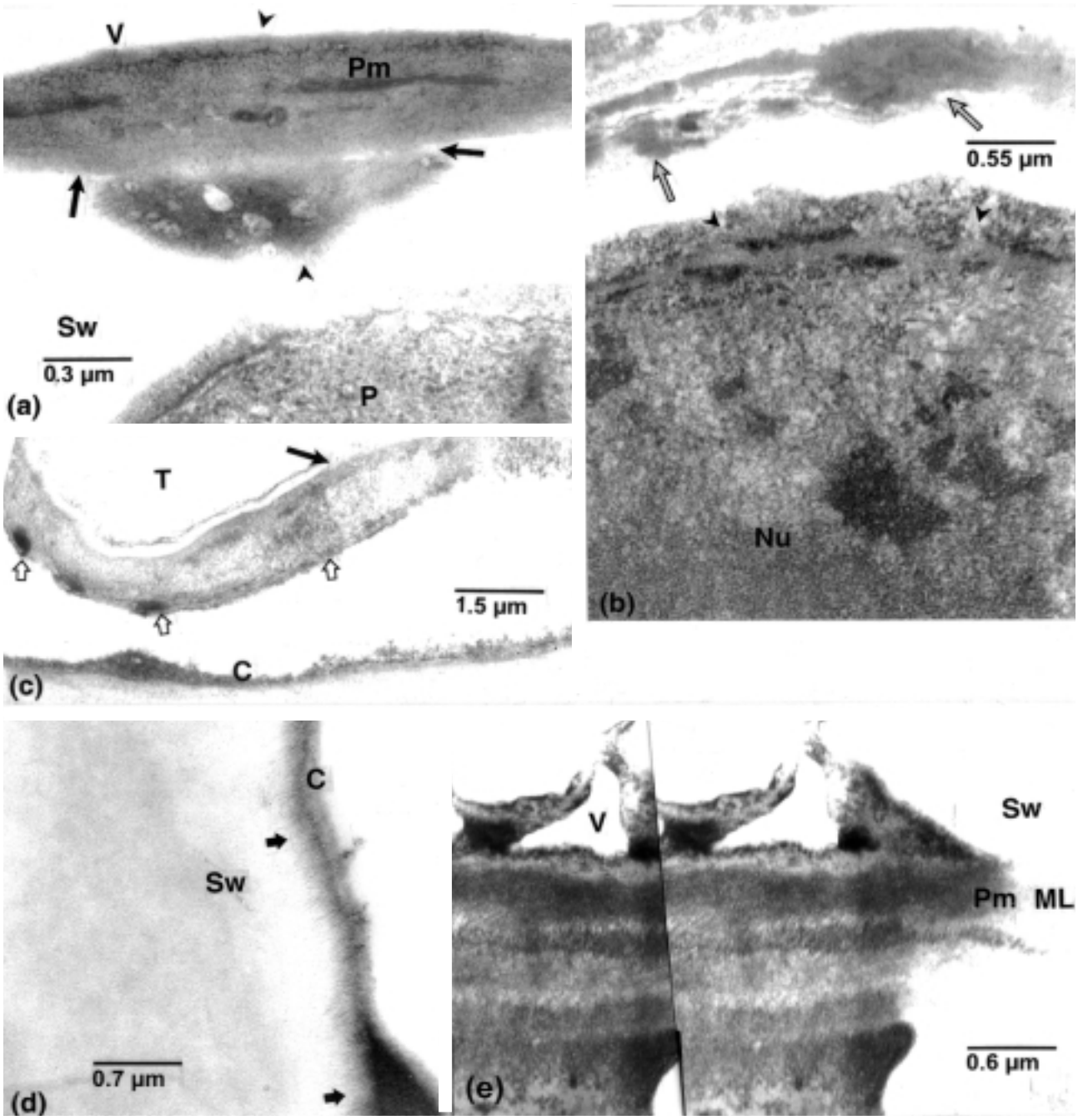

Figure 11. C, coating; ML, middle lamella; Nu, nucleolus; P, parenchyma cell; Pm, pit membrane; T, tylosis; Sw, vessel secondary wall; V, vessel element. **(a-e)** *U. americana*. Occurrence of filamentous-like structures (FLS). **(a)** From a 1 m high seedling, greenhouse-grown in open soil, fertilized with a 10% NPK solution, prior to inoculation. 30 dpi. Oblique section through a vessel secondary wall, pit membrane, and parenchyma cell wall. FLS (arrowheads) extend across (arrows) the pit membrane and its secondary wall borders, reaching the periplasm of the cell, in which the plasma membrane is partially obliterated. **(b)** Enlarged portion of Fig. 8h. Crowded arrays of parallel oriented FLS occur in the OM (empty arrows) in the middle lamella and primary host cell wall. Similar structures extend at sites from the margin of the cell secondary wall into the outer cytoplasm (arrowheads) and across the band corresponding to the nuclear envelope. **(c)** Some OM and fine matter, similar to that of the coating, permeated the outside layer of a tylosis wall (short arrows). The inner suberin-like layer is only slightly pervaded by similar matter (long arrow). **(d)** 34 dpi. Numerous arrays of FLS (arrowheads), present inside the vessel secondary wall, are linked to the coating. **(e)** 1 year pi. Incubated on an agar medium 4 days before fixing. Stereoscopic views showing numerous FLS, some displaying a lucent core, are present parallel and across the pit membrane. They are absent in the secondary wall and also in the middle lamella distal from the membrane.

oriented perpendicularly across the host wall (pit membrane and secondary wall as well) were really present in the section, at all levels (Fig. 11e).

Observations regarding the pathogen

Peculiarities of fungal growth of *O. novo-ulmi* in culture or *in vivo* conditions were noted which might be inherent to a better understanding of pathogen action and host reactions. In conjunction with the presence of the filamentous structures described above, similar structures permeated the fungal cell walls, bridging cells or extending from them into host walls as well as freely into vessel lumina (Fig. 12a, c). In fungal cell portions in close contact with host walls, the fungal wall was often not discernible and the filamentous structures appeared to connect directly with

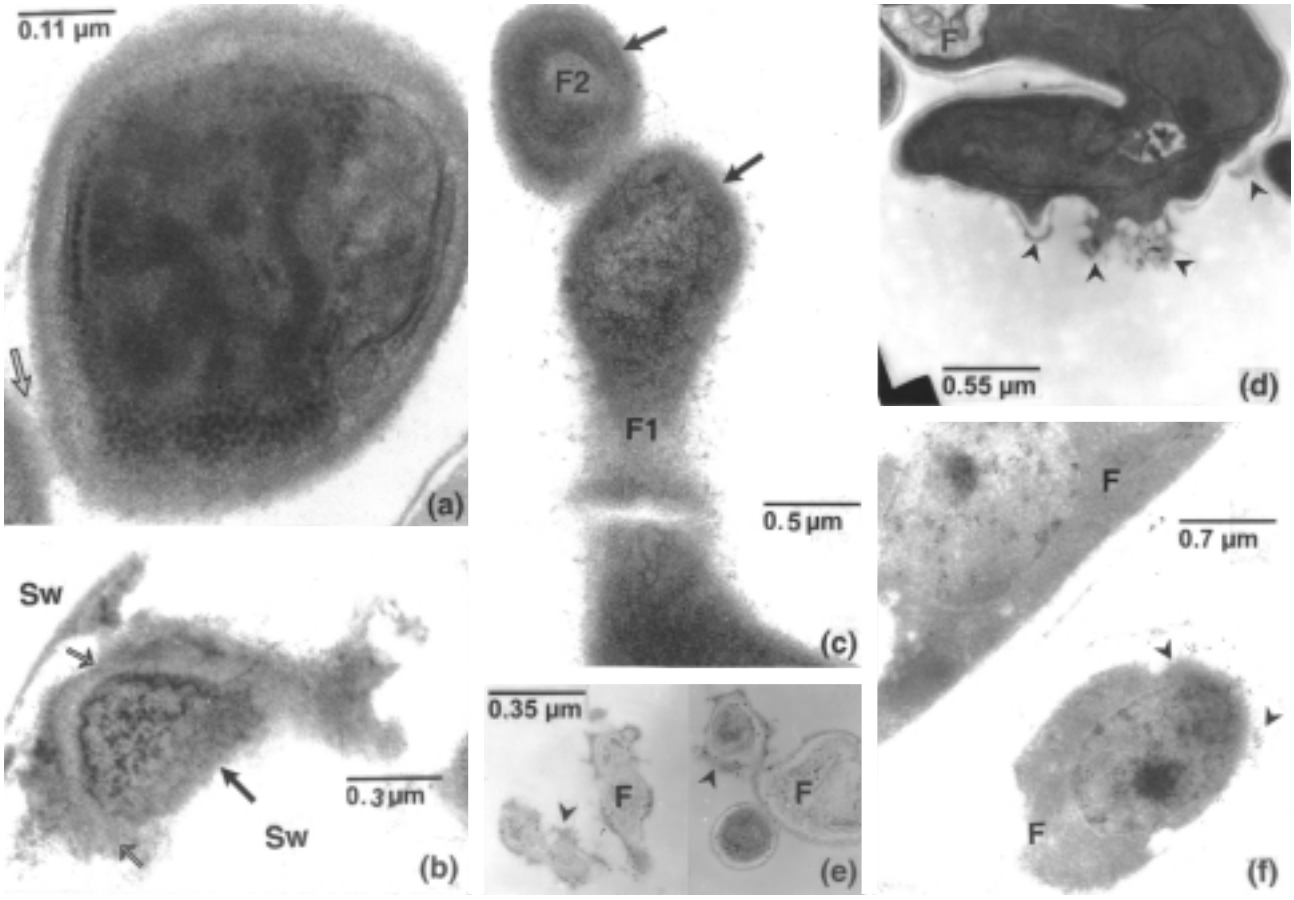

Figure 12. F, fungal cell; Sw, vessel secondary wall. **(a-c)** Features of *O. novo-ulmi* cells in inoculated *U. americana*. **(a)** 4 dpi.Walls of fungal cells are heavily permeated by filamentous-like structures (FLS), reaching the cell content, including a region identifiable as bordering a large nucleus (as indicated by bands of heterochromatin-like matter). Similar filaments bridge this cell with an adjacent one (arrow). **(b)** 40 hpi. A fungal element in which a wall is not discernible in the part appressed to the vessel wall (thick arrow). Numerous FLS, connected to the element, extend through its wall (empty arrows) into the surrounding matter lining a pit border. The element is included in a degraded pit membrane. **(c)** Inoculated in October. 4 dpi. A limiting wall is not apparent in the elements (F1, F2), these being surrounded by a layer of FLS adjoining an opaque membranous structure (arrows). These elements are connected by similar filaments. **(d, e)** Growth of *O. novo-ulmi* on Millipore membranes. **(d)** A cell with dense and opaque content, at the interface with a membrane of 0.4 µm porosity. The «pseudopode-like» structures extending from the cell at the penetration site in the membrane show pronounced wall alterations and are surrounded by fine components (arrowheads). **(e)** Labelling for cellulose. Membrane of 0.1µm porosity. Fungal elements at the membrane penetration site (right hand part) and included in the membrane (left hand portion). These are very irregular and their walls are much altered, surrounded by fine matter (arrowheads). **(f)** Culture. Glutaraldehyde fixation alone. Labelling with monoclonal antibody to DNA. Gold particles occur over nuclei and nucleoli with a concentration over nucleoli and filamentous matter, stretching out from the nucleus (arrowheads). Particles also occur over similar structures and nearby over the fungal wall.

the cell content (Fig. 12b). A similar association was observed concerning fungal cells located in vessel elements of colonized growth rings the year of inoculation but sampled the following year (not illustrated). Similar extracellular matter occurred surrounding cells invading Millipore membranes of even 0.1 µm porosity (Fig. 12d, e), and whose walls, as shown by labelling for cellulose (Fig. 12e) or chitin (Ouellette *et al*., 1995), were strongly altered. Another interesting feature of fungal growth through these membranes was that the content of fungal cells contacting the membrane was generally dense and very opaque (Fig. 12d) compared to the cells growing over the surface of the membrane. This feature might be indicative of a kind of cytoplasm transformation, a possibility that might also apply to the opaque matter referred to above.

Results of labelling with the mab probe to detect DNA in these types of samples showed that the region corresponding to the nucleus was generally not well delimited and frequently bordered the cell wall (Ouellette *et al*., 1995, 1999b). In sterilized elm wood sections (and, in this case, fixed by high pressure freezing), as in culture, the probe attached to nuclei and DNA-containing organelles, but also to masses of opaque bodies in some cells and to strands emerging from the nucleus into the cell wall (Fig. 12 f).

Other aspects of fungal morphology, departing from the so-called normal conditions concerning walls, may be mentioned. For example, some endocell-like elements were observed to form a haphazard protoplasm fragmentation in hyphal cells (Fig. 13a), a means by which fungal cells could withstand some detrimental effect to their walls. Indeed, the presence of endocells was common in overwintered cells in infected trees (not illustrated). Other cell wall modifications were noted. Thus, cells with thin and irregular walls bordered cells with thickened or pluri-layered walls, both having apparently still nearly intact content (Fig. 13b). In fact, cells with thickened walls were more prominent in restricted than in intensive tissue invasion. Aberrant wall divisions in such cells did not label for chitin, compared to the strong labelling of the inner, lucent wall layer with which it merged (Fig. 12c). Some elements apposed to vessel walls or present in walls of host cells colonized during the first year of infection or the following year were thin or they were delimited only by a membranous structure or thin opaque bands (Fig. 13d-f). Cells with normal content, present in overwintered infected trees, and which likely represented the inoculum for re-infection, were included in extracellular material linked to such small fungal elements (Fig. 13g) or to the coating on vessel walls.

Present data are considered as complementary to those already reported concerning Dutch elm disease and its pathogen or other wilt diseases, particularly fusarium wilt of carnation, in which intensive tissue invasion was linked with microhyphae having limited content (Ouellette *et al.*, 1999a, 1999b, 2001). The occurrence of irregular cells with thin and aberrant walls that labelled erratically for chitin, as well as irregular divisions, were also observed in *F. oxysporum* f.sp. *radicis-lycopersici* in tomato (Ouellette *et al.*, 2001; Charest *et al*., 2004).

General considerations

Ultrastructural and cytochemical studies have contributed to clarify problems related to DED syndrome and to other plant wilt diseases. For instance, the mode of formation and the characteristics of tyloses, their differentiation from the alveolar network in elm (Rioux and Ouellette, 1989; Ouellette and Rioux, 1992; Ouellette *et al*., 2004b) and other plants, and their probable role in disease development may be mentioned. First, as tyloses were rarely present in the three elm species studied in early infection, they obviously are not related to infection at this stage. Their greater abundance in later stages, particularly in cases of apparent disease recovery, might be significant in confining xylem invasion. This aspect could be primordial in restricting the downward spread of the pathogen towards the base of the tree. Indeed, this downward invasion, including that of the smallest roots, whose mortality may also be very important, has never been clearly explained. However, the peak of this downward spread possibly occurs during a period of tree dormancy while ambient temperatures may still be high enough to favour pathogen growth in the absence of any strong defence reactions. To verify this possibility, we inoculated trees during winter (Ouellette, 1960) and found that initial invasion of the inoculated branches remained limited, while it continued to progress in the following early spring. Concomitantly, tissue invasion during this period may not be clearly expressed by wood discoloration. In any event, the confining effect of tyloses may be important only inasmuch as they become suberized, as for the parent parenchyma cells, and are thus less subject to their alterations; thus, they may be a complement to cell reactions in the com-

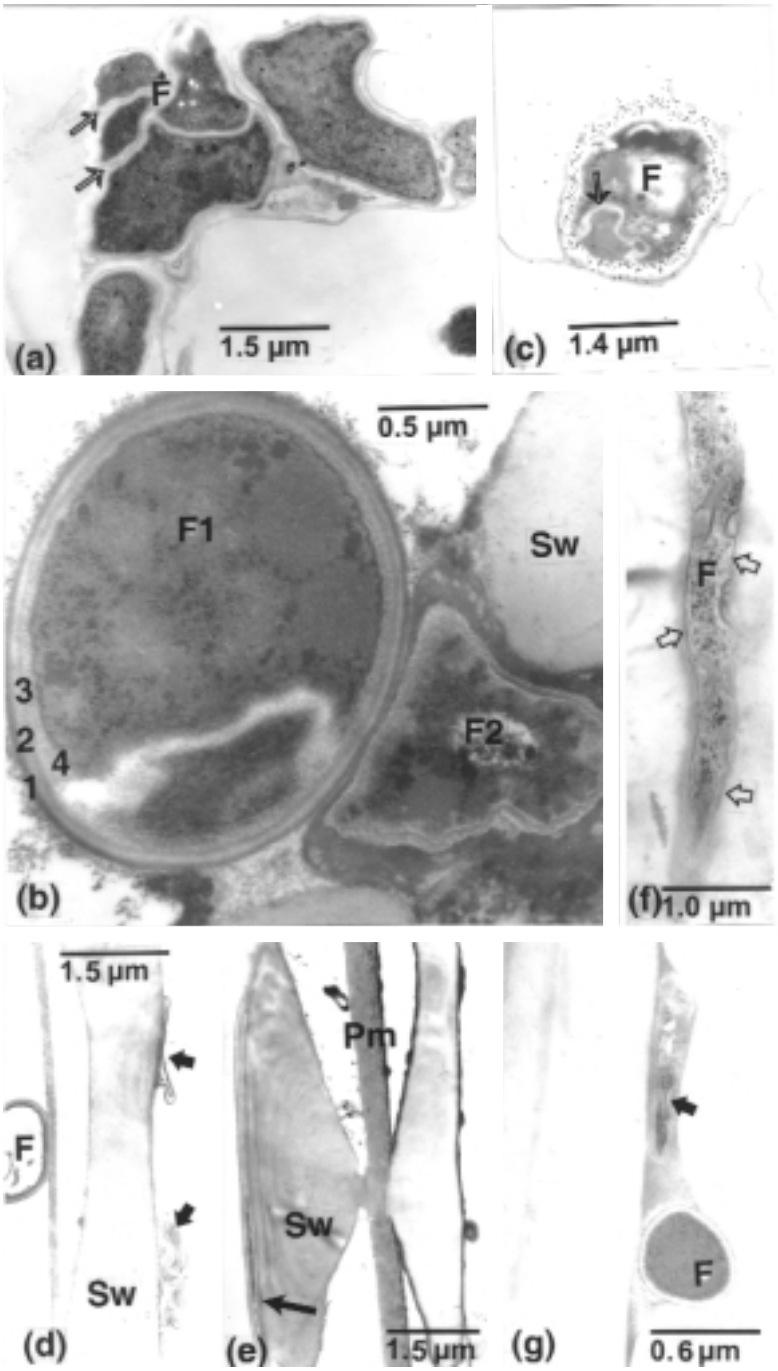

Figure 13. F, fungal elements; Sw, vessel secondary wall. **(a-g)** Peculiarities of *O. novo-ulmi* elements. **(a)** Culture. A hyphal cell has produced adventitious walls (arrows), yielding outgrowing endocell-like elements. **(b)** Greenhouse-grown tree, leaf petiole inoculation, 40 hpi. A fungal cell (F1) displays a four layered wall (1-4) and a sinuous lucent band anchored in layer 4. Another contiguous cell (F2) has a thinner, irregular and notched wall. **(c)** 25 dpi. Labelling for chitin. A fungal cell in a vessel lumen has a thick, lucent labelled wall, but not the wavy band (arrow) stemming from it. **(d-g)** Inoculated *U. americana.* **(d)** 1 year dpi. Thin elements, apposed to vessel walls, are limited by only opaque or contorted bands (arrows). **(e)** 60 dpi. A tiny element (long arrow) affixed to the vessel wall can almost be mistaken as being part of this wall. **(f)** Sampled in early spring, 10 months after inoculation. A hypha, less than 0.5 µm in diameter, growing through a gelatinous layer of a fibre, is seemingly confined by only a membranous structure (arrows). **(g)** Natural infection, sampled from an recurrent infection, in early May the year following appearance of the first external symptoms. A small fungal cell is seemingly emerging from possibly previous coating-like matter, including remnants of a tiny element (arrow, compare with the element in e).

partmentalization process (Rioux and Ouellette, 1991a, 1991b; Rioux and Biggs, 1994; Rioux *et al*., 1995).

Alteration of membranes of half-bordered pits in mature xylem is persistent at all stages of infection, in association with opaque matter. However, the real effect of the concomitant disturbances of parenchyma cells on disease severity may not be easily estimated. It may be significant that pit membranes were less affected in *U. pumila* and in *U. americana* trees that were injected with a glucose solution prior to inoculation than in untreated trees, a treatment which was reported to reduce the severity of external symptoms (Feldman *et al*., 1950). Indeed, in our tests, less pronounced external symptoms were observed, coincidental with reactions in parenchyma cells, which we consider as typical of defence reactions. It would be interesting to determine whether the increased resistance to disease development in elm trees submitted to water stress (Solla and Gil, 2002) might proceed from a similar effect on parenchyma cells. Also, trees with smaller or more dispersed vessel elements may be less susceptible to the disease (Sinclair *et al.*, 1975; Solla and Gil, 2002), possibly not only in restricting fungal movement through translocation, but also on account of fewer pit contacts of paratracheal parenchyma cells with vessel elements. Indeed, we have obtained indications that the severity of parenchyma cell alterations may be paralleled by the number of pit membranes they have with vessel elements, and with their degree of alterations (Ouellette, 1978c).

One possible detrimental effect of parenchyma cell alterations would be to liberate compounds favouring fungal action or to reduce production of fungal inhibitory compounds (Duchesne *et al*., 1985), and perhaps more importantly, to prevent formation of sound tyloses (see Fig. 1b), whereas reactive or less altered parenchyma cells may be involved in reactions leading to barrier formations. Indeed, cells with transformed and opaque content, as exemplified here, surround or are part of barrier zones (Rioux and Ouellette, 1991a, 1991b). These types of cells may be analogous to those denoted as resulting from hypersensitive reactions, preliminary to a cascade of other defensive cell reactions (Graham and Graham, 1991). The cells we have illustrated do not appear, however, to be necrotic cells.

The production of microhyphae, which is of great importance in tissue colonization at all stages of wilt disease development in susceptible carnation (Ouellette *et al.*, 1999a, 2002), appeared important in elm, mostly in the process of pathogen crossing over from one growth ring to the next (Ouellette and Rioux, 1993). Interestingly, the mechanisms of defence in resistant carnation were related to cell wall modifications barring invasion to these small and larger hyphae, and the characteristics of compartmentalization in this plant were quite similar to those observed in elm (Baayen *et al.*, 1996; Ouellette *et al.*, 2002). Even though pronounced reactions may occur in elms at the cambium level in recurrent infections, the quite erratic differentiation of vessel elements in this region may prevent complete tree recovery. These disorders may possibly explain why the numerous trees that showed symptoms for the first time in 1978, in Montreal (Quebec, Canada), died suddenly, as if scorched, in the late spring of 1979.

Another novel addition to the present issue relates to cytochemical localization and characterization of pectin in elm vessel lumina, which was demonstrated in earlier research by others (Gagnon, 1967), but was not precisely localized. Non-crystallized pectin, in our studies, occurred as fine, branching fibrils (Rioux *et al.*, 1998), which were found to be secreted from parenchyma cells and tyloses, but it was not of consistent occurrence in affected vessel elements, as was also the case in *Fusarium*-infected sumac (unpublished observations). In carnation, it occurred mostly in vessel elements bordering recently differentiated cells, in the periplasm and vacuoles of which appreciable amounts of the compound were detected (Ouellette *et al.*, 1999a, 2002).

No clear evidence was obtained that any large amounts of pit membrane products were liberated into vessel lumina in the systems we have studied, except occasionally in metaxylem vessels, and then, these products also labelled for cellulose, as shown in carnation and sumac (unpublished observations). Similarly, if, in elm, the coating and similar material had originated from pit membrane disintegration, as proposed by others for other systems (Beckman, 1987), one expects that at least some of this material would also label for cellulose; this was not the case. Portions of vessel secondary walls in elm were occasionally chipped off (Ouellette, 1978d; Ouellette and Rioux, 1992, 1993), but the detached portion still labelled for cellulose. However, a loss of labelling for cellulose of secondary walls was observed herein, next to labelled middle lamellae and wall depositions in infected elms, indicating that wall modifications in these likely occurred, masking the binding sites for the probe used.

In our studies, noticeable amounts of fibrillar material occurred in elm vessel lumina, surrounding patho-

gen cells (Ouellette *et al*., 1999b). Similar material was observed in large amounts linked to fungal cells in culture, particularly on Millipore membranes (Ouellette *et al*., 2004a). However, this type of material, which differed from pectin fibrils, was observed in abundance only shortly after inoculation, in vessel elements containing cells and hyphae that likely originated directly from the inoculum. Thus, these compounds may only be transient in the host, and may not occur to any great extent following natural infection. This does not imply that these or similar compounds that could be isolated from cultures and injected in quantity into trees are unable to act as elicitors (Yang *et al*., 1989), even though it might not reflect a natural condition.

Another type of matter observed to be linked to fungal cells in appreciable amounts was opaque matter containing arrays of filamentous structures. This association in elm was noted at all stages of infection, even 1 year after inoculation, although, once again, it was more prevalent in early infection. Nevertheless, these structures were consistently and clearly associated with host wall pervasion and alterations. They are discussed in greater detail below.

The presence of appreciable amounts of material traceable to fungal cells in vessel lumina brings about the question of toxins. In this connection, extraneous compounds of possible pathogen origin in vessel lumina have been denoted as either vivoaggressins or vivotoxins (Scheffer, 1984), depending on whether or not they could penetrate pit membranes, as mentioned by van Alfen and Turner (1975). However, as shown in our studies, the fibrillar skeleton of pit membranes and primary walls can be much loosened and thus allow penetration of large molecules. Concerning the possible role of vivoaggressins in DED, if vessel blockage was attributable to any specific compound of pathogen origin, this would represent only a portion of the occluding material, as in cases of severe occlusion, this material was very heterogeneous. In any event, it was noticed that marked vessel occlusions always occurred at appreciable distances below the point of advancing streaks in the xylem, and for this reason we have proposed that the real factors inciting infection should be sought at this level (Ouellette, 1978d; Ouellette and Rioux, 1992, 1993).

Concerning the compound CU, if the various internal symptoms, like tylosis and coating formation, in addition to typical DED external symptom expression (Takai and Hiratsuka, 1984) attributed to its action were confirmed, it could be classified as a vivotoxin. However, in the tests we have conducted with this compound, we have isolated the pathogen from injected trees as well as from the toxin samples. Recently, Del Sorbo *et al*. (2000) have reported that by transferring the CU gene to a non-pathogen of elm, *Ophiostoma quercus*, and that by inoculating it to elm, some symptoms typical of DED were obtained. In this situation, it might be valuable to determine at the TEM level the types of host disturbances that may occur.

Another feature that has not attracted much attention previously is the abundance of opaque bodies of regular sizes at all stages of infection and under different conditions in the three elm species studied. As these bodies were occasionally observed to be connected to fungal cells having typical content and to be associated with pit membrane alterations, and sometimes with the coating on vessel walls as well as with vessel wall alterations, they most likely are of pathogen origin. The same consideration applies to the masses of opaque, mostly homogeneous matter, free of intact or even signs of degraded organelles, and spanning long distances in intact host walls in elm and sumac. The presence of similar matter in sterilized wood sections extending through host walls, including vessel secondary walls, precludes that such matter was related to host cell reactions. Similar matter was occasionally found to be connected to fungal cells, but one is in want, here, of an explanation to account for a mechanism by which it could spread freely at distances from fungal cells. In this issue, it needs to be considered that this matter labelled with a DNA probe which, in intact host and fungal cells in elm and staghorn sumac as in culture, attached only to DNA-containing organelles. Controls for these tests were negative, except for the occurrence of a few gold particles in void spaces, including free embedding medium.

Considering the outstanding reactions occurring in young tissue, including the pronounced deformation of plastids and mitochondria and the gigantism of nuclei and nucleoli, it cannot be visualized that these modifications stemmed from secondary effects, such as wilting for example. Therefore, the search of compounds liable to trigger such drastic effects is worth pursuing. In this respect, the occurrence, in the four wilt diseases studied, of structures that we have tentatively described as filamentous and which permeate host walls, extending from opaque matter and from the coating, might be related to this question. This problem undoubtedly represents one of the main fundamental aspects of the host-pathogen relationship in

these diseases (see discussion in Charest *et al.*, 2004; Ouellette *et al.*, 2002, 2004a, 2004b), and increasing evidence obtained by the use of mabs to extracellular compounds of pathogen cells in other systems indicates the key role these compounds may play in pathogenesis (Hutchison *et al.*, 2002).

An overall impression emerging from these studies is that pathogens may not develop in their hosts as in pure culture, as also hinted to by other authors, and that some compelling imaginative insight and creativity might be indicated to cope with the problem. Thus, fungi may not always develop in perfect cylindrical forms or need a normal wall to invade tissues, as we have illustrated. Furthermore, the extracellular material linked to these is now shown in many systems to be active components. As others, we have regularly assumed that fungal cells occurring in tandem, not as part of a distinct hypha, had resulted from budding. After re-examining numerous examples of juxtaposed fungal cells in vessel lumina of Dutch elm disease-infected trees, of *Fusarium*-infected carnations, tomatoes and staghorn sumacs as well as *Verticillium*-infected eggplant, we have observed that paired or rows of cells were often separated from one another by their external opaque delimiting layers and were bridged by fine filamentous structures (for an example, see Ouellette *et al.*, 2002). To our mind, these problems cannot be ignored or simply attributed to artefacts, in case they could prove to be new key findings concerning these fungi. In this connection, further confirmation that the origin of the opaque matter and associated filamentous structures mentioned above is primarily of pathogen origin is equally paramount, as challenging as it may be to anchored notions on the mode of development and action of plant pathogens. In this respect, confirming that the coating on vessel walls is primarily of pathogen origin would be primordial. In any event, no clear indications were obtained that the large amounts of opaque material in vessel lumina and host walls were secreted from parenchyma cells as, on the contrary, these cells were altered in association with this matter. Thus, its bulk presence in periplasmic areas of cells with degraded content cannot be considered, as reported by other authors (Shi *et al.*, 1992), as indicative of defence reactions. Furthermore, the amount of this matter at any one location increased as infection progressed, raising a problem as to the mode of its synthesis. Above all, it showed a somewhat structured appearance.

In summary, present studies have helped to clarify some problems related to the pathogenesis of DED, but concomitantly revealed other important problems requesting further investigations. Unlike other plant diseases whose development and complete cycle can chronologically be followed, events in DED leading to external symptom expression and the complete colonization of the tree cannot be exactly established. In fact, properly understanding how recurrent infections occur is as urgently needed as the understanding of mechanisms leading to the initial infection. Up to now, many pieces of the puzzle concerning both aspects have been obtained, but others are still lacking to obtain a complete picture. For example, when speaking of early tissue invasion, present data are based mostly on events occurring shortly after inoculation close to the inoculation wound or at the tip of advancing streaks in the xylem. From the inoculation point up to this extremity, examination of samples taken in-between may display many stages of infection, some with interactions, due to the rapid lateral spread of the pathogen outside the initially invaded vessel elements. For this reason, knowledge of the exact mode of tissue invasion following beetle inoculation is wanting, where initial invasion is expected to be more restricted. However, comparing the mode of tissue invasion and reactions near the inoculation wound with that in the infection advancing front, comparable in this respect to that of inoculated twigs and petioles, may provide an insight into the main factors related to infection. In American elm, we have found that external symptoms stand out only when the shoots have been invaded. As the opaque matter we have observed is linked to tissue disorder at these places, this matter would appear to play a key role in this view. A comprehension of all facets related to infection might surely help to develop better control measures for existing native trees, but to produce resistant varieties, one does not have to wait until the complete story is unravelled. Any factor, be it anatomical or chemical, that could satisfactorily prevent tangential as well as vertical spread of the pathogen, in its regular or irregular forms, is worth selecting for; so much the better if this factor is complemented by cell reactions hindering radial spread. In parallel, acceptable tree resistance may be primarily provided by defence reactions at the cellular level, particularly when one considers inoculation by beetles. In a nutshell, the promising outcome concerning DED is to continue working on it in a multidisciplinary fashion.

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