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Ultrastructure of the alveolar network and its relation to coating on vessel walls in elms infected by *Ophiostoma novo-ulmi* and in other plants affected with similar wilt diseases

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Abstract

In elms infected with Dutch elm disease, alveolar networks, demarcated by filamentous-like bands and confluent with similar matter (the coating) accumulating on vessel walls, occurred regularly in vessel elements. Similar material lined vessel walls in inoculated, sterilized, thin elm wood sections fixed by high pressure freezing. The coating was observed to connect with fungal cells and occasionally contained small opaque particles, the size of ribosomes, membranous and vesicular structures, and, following incubation of wood chips taken from diseased samples incubated on an agar medium, it still displayed similar matter. Coating and alveolar bands increased in thickness by confluence of other bands or membranous structures. Similar matter and structures also occurred in other plants affected by similar fungal wilt diseases. In all systems, the compact coating did not label for chitin, cellulose and pectin. In staghorn sumac, the probe for DNA attached to the coating. Altogether, in the light of these data, it appears that the coating and alveolar networks are not inert components, a fact which indicates their primordial probable pathogen origin. It is proposed that these elements might be important not only in the initial infection stages but also in older or recurrent infections at a time when host resistance mechanisms are ineffective.

Key words: *Fusarium oxysporum*, overwintered fungal cells, wilt diseases.

Resumen

Ultraestructura de la red alveolar y su relación con el recubrimiento de las paredes vasculares en olmos infectados con *Ophiostoma novo-ulmi* y en otras plantas infectadas con enfermedades similares de marchitamiento

En olmos afectados por la grafiosis, la red alveolar, demarcada por bandas filamentosas, y confluente con acumulaciones de la misma sustancia (cubrición) presentes en las paredes de los vasos, aparece regularmente en los elementos conductores. Sustancias similares tapizan las paredes de los vasos en secciones de madera delgada de olmo inoculada y esterilizada, y posteriormente criofijadas a altas presiones. Se observó que la cubrición se conecta con las células del micelio y que ocasionalmente contenía pequeñas partículas opacas del tamaño de los ribosomas, estructuras membranosas y vesiculares, así como que, tras la incubación de astillas leñosas cogidas de muestras enfermas incubadas en agar, aún se presentaba una sustancia similar. La cubrición y las bandas alveolares aumentaron su espesor en la confluencia con otras bandas o estructuras membranosas. Estructuras y sustancias similares aparecieron también en otras plantas afectadas por enfermedades similares originadas por hongos que producen marchitamiento. En todos los sistemas, la cubrición compacta no pudo ser marcada como quitina, celulosa ni pectina. En zumaque (*Rhus typhina*), la sonda de ADN se pegó a la cubrición. En resumen, a la vista de estos datos, parece ser que la cubrición y la red alveolar no están formados por componentes inertes, un hecho que indica su probable origen patogénico. Se sugiere que esos elementos podrían ser importantes no sólo en las fases iniciales de la infección, sino también, en infecciones más desarrolladas o recurrentes, en el momento en que los mecanismos de resistencia del hospedante no son efectivos.

Palabras clave: *Fusarium oxysporum*, células de hongo, enfermedades vasculares.

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Introduction

One main issue concerning vascular plant wilt diseases is related to the accumulation and possible effect of extraneous material in vessel elements. The concepts regarding this aspect have first been formulated mostly on the basis of light microscope (LM) observations. For example, gums and gels were mentioned to contain pectin, postulated to originate from host breakdown products and phenolics and advocated to be the major vessel occluding components involved in the wilt syndrome (Beckman, 1987). The formation of tyloses was also reported to be important as vessel occluding structures, but in these cases they were considered by some workers as being an expression of defence as well. For an overview and discussion of these aspects, one is referred to our previous publications on these diseases, particularly Dutch elm disease (DED) (see citations in Ouellette *et al.*, 2004).

Observations at the transmission electron microscope (TEM) level, matched by histochemical tests, have permitted to complement and in some cases to enlighten some of the problems related to the origin and nature of some of the vessel occluding components. Thus, tests with phloroglucinol-HCl detected phenolic compounds in vessel elements and increased lignification of host walls, in carnation as well as in elms in paratracheal cells and in compartmentalization tissue (Baayen *et al.*, 1996; Rioux and Ouellette, 1991a, 1991b). These modifications could also be well differentiated from other types of wall modifications which occurred in these hosts (Rioux and Ouellette, 1991b). By using gold-complexed probes to detect pectic components in elms and non-host trees inoculated with *O. novo-ulmi*, it was shown that these compounds occurred as fine, branched fibrils which were secreted from parenchyma cells, including tyloses (Rioux *et al.*, 1995, 1998). These pectic substrates occurred singly in some vessel elements or intermixed with other material, but were not of general occurrence and as abundant as originally thought, with some variation according to the disease stage. The results of labelling for pectin were also similar for carnation affected with fusarium wilt (Ouellette *et al.*, 1999a). Indications were obtained that much of the extraneous material in vessel lumina of DED infected elms could also be of pathogen origin (Ouellette *et al.*, 1995, 1999b). This aspect was illustrated further in Ouellette *et al.* (2004).

Concerning tyloses, their structure, particularly of their walls, was well characterized, including often thick, inner suberized walls (Ouellette, 1980; Rioux *et al.*, 1995). In Ouellette (1980), it was mentioned that other structures, later designated as an alveolar network (Ouellette and Rioux, 1992), regularly occurred in vessel lumina, which at first sight, particularly in LM observations, might have been confused with tyloses. Observations in LM at low magnifications may be open to this type of misidentification (see for examples Figs. 38 and 50, in Stipes and Campana, 1981). However, to substantiate this point of view, it seems essential to satisfactorily characterize the layers delimiting these networks and to determine their origin and nature in order to better evaluate their role and importance in the DED pathogenesis. It is the purpose of this paper.

This undertaking appears to be all the more important since the alveolar networks were observed to be closely related to the material accumulating on vessel walls, and generally referred to as coating. As we estimate that the origin and nature of coating has never been really determined, it is relevant that this question be concomitantly considered, particularly under the premise that coating is also of paramount importance in several other wilt diseases, for example fusarium and verticillium wilt diseases in a number of plants (Charest *et al.*, 1984; Street *et al.*, 1986; Nicole *et al.*, 1994; Gold and Robb, 1995; amongst others). The present work thus reports on numerous observations, often based on cytochemical tests and obtained from a variety of situations, concerning the alveolar networks and associated coating during the development of DED and in other wilt diseases.

Material and Methods

Nursery-grown, 1-2 meter high plants of *Ulmus americana*, of *U. glabra*, and of *U. pumila* were inoculated with isolates of *Ophiostoma novo-ulmi* (confirmed by either Dr. C. Brasier, Alice Holt Lodge, Farnham, U.K, or Dr. Louis Bernier, Université Laval) or isolates from the Netherlands (also thought to be more aggressive than the usual *O. ulmi* isolates). *U. pumila* trees were also inoculated with an isolate obtained from a naturally infected hybrid of this species, growing in Québec City, Canada. Several inoculations of *U. americana* were performed from 1971 to 1991, and two inoculation experiments for each of the other species were made. Sampling was made at various intervals after inoculation, including of recurrent infections

in some cases. Samples from naturally infected elms were also examined. Non-hosts to *O. novo-ulmi* growing in natural stands were also inoculated with the same isolates. Some samples also came from taller, planted *U. americana* trees injected with a glucose solution prior to inoculation, as pre-treatment with this sugar was reported to reduce symptom expression in elm (Feldman *et al.*, 1950).

Other samples were obtained from cut twigs of *U. americana* plunged into a cerato-ulmin (CU) suspension (Takai and Richards, 1978) or from seedlings with roots still attached but a few severed ones bathing in the CU suspension. Sampling was made 1-4 days afterwards.

Field-grown staghorn sumac (*Rhus typhina* L.) plants were inoculated (two experiments) with either of two isolates of *Fusarium oxysporum* f.sp. *callistephi* and also sampled at various intervals after inoculation (present illustrations are from 2 dpi). Greenhouse grown eggplants (*Solanum melongena* L.) were inoculated (two experiments) with an isolate of *Verticillium albo-atrum*, and sampled at 1-14 dpi.

Double-fixation of samples was usually made with glutaraldehyde and osmium tetroxide and embedding was with Epon 812. Elm (*U. laevis*) sterilized wood sections inoculated with *O. novo-ulmi* and fixed by high pressure freezing were also examined.

Cytochemical tests were carried out for cellulose, with a gold-complexed exoglucanase, for chitin with gold-complexed wheat germ agglutinin, and for DNA with a gold-complexed monoclonal antibody to this substrate. For further details concerning methodology, one is referred to Ouellette *et al.* (2004, and citations therein).

Results

Characteristics of alveolar networks

The basis for denoting the structures under discussion as alveolar was because of their intricate, small or larger, tortuous or circular shapes that were delimited by membranous-like layers. Contrary to tylosis walls, these layers did not label for either pectin or cellulose, even when they were closely intermixed with these compounds (Fig. 1a). As already mentioned, the tylosis walls, even when they were very thin, were clearly distinguishable from the alveolar layers (Fig. 1b). The following descriptions give further details of these structures which occurred at all stages of the infection process in the hosts in which they have been observed.

In *U. americana* and *U. glabra*, the alveolar layers had various configurations, delimiting large interconnected circles of diverse dimensions, confluent with the coating and frequently traversing vessel lumina (Fig. 1c-e). The layers were particularly intertwining when they were compressed between tyloses, or between tyloses and vessel walls (Fig. 1d). The alveolar bodies were devoid of content or encompassed fibrillar matter, globose bodies and opaque matter (Fig.1a, d-e). Similar structures and configurations were as abundant in seedlings treated with the cerato-ulmin (CU) suspension (Fig. 2a) as in inoculated elms.

The thinnest alveolar-delimiting layers were membranous-like having lucent cores and being bordered by opaque bands (Fig. 1a), whereas the thicker layers were formed by the juxtaposition of the thinner layers, between which the opaque bands were still distinguishable (Fig. 1c). Whereas the arrays of thin alveolar layers were mostly but exclusively present in early infection, the networks with thicker layers were only observed in advanced or recurrent infections. When the networks were compressed, the individual layers were still indicated by opaque bands and these similarly encompassed fine matter, but it was not clear if this matter was circumscribed by the layers or included between them (Fig. 1d). The layers were not always tightly confluent and were either separated by void spaces (Fig. 1c) or they encompassed or merged with other types of matter, at places appearing as a swelling of the bands (Fig. 1e). In other instances, what appeared to be void spaces corresponded in fact to a middle, less opaque stratum (Fig. 2b), compared to the limiting bands, and which, upon bifurcation of the latter, became the external demarcating band of the layer. The alveolar layers also surrounded fungal cells and converged with their extracellular opaque layer (Fig. 2b).

Stacked or contorted networks were also prominent in *U. glabra* (Fig. 2c), and as in elms (Fig. 2d), the layers had a membranous appearance and were delimited or separated by similar opaque bands. Similar structures occurred in vessel elements invaded during the year of the initial infection in the case of recurrent infections (Fig. 2e). Overwintering fungal cells in these vessels were also linked to similar structures and intermixed with opaque matter, or were circumscribed by these structures (Fig. 2f, g). In *U. pumila*, the occurrence of alveolar networks was exceptional.

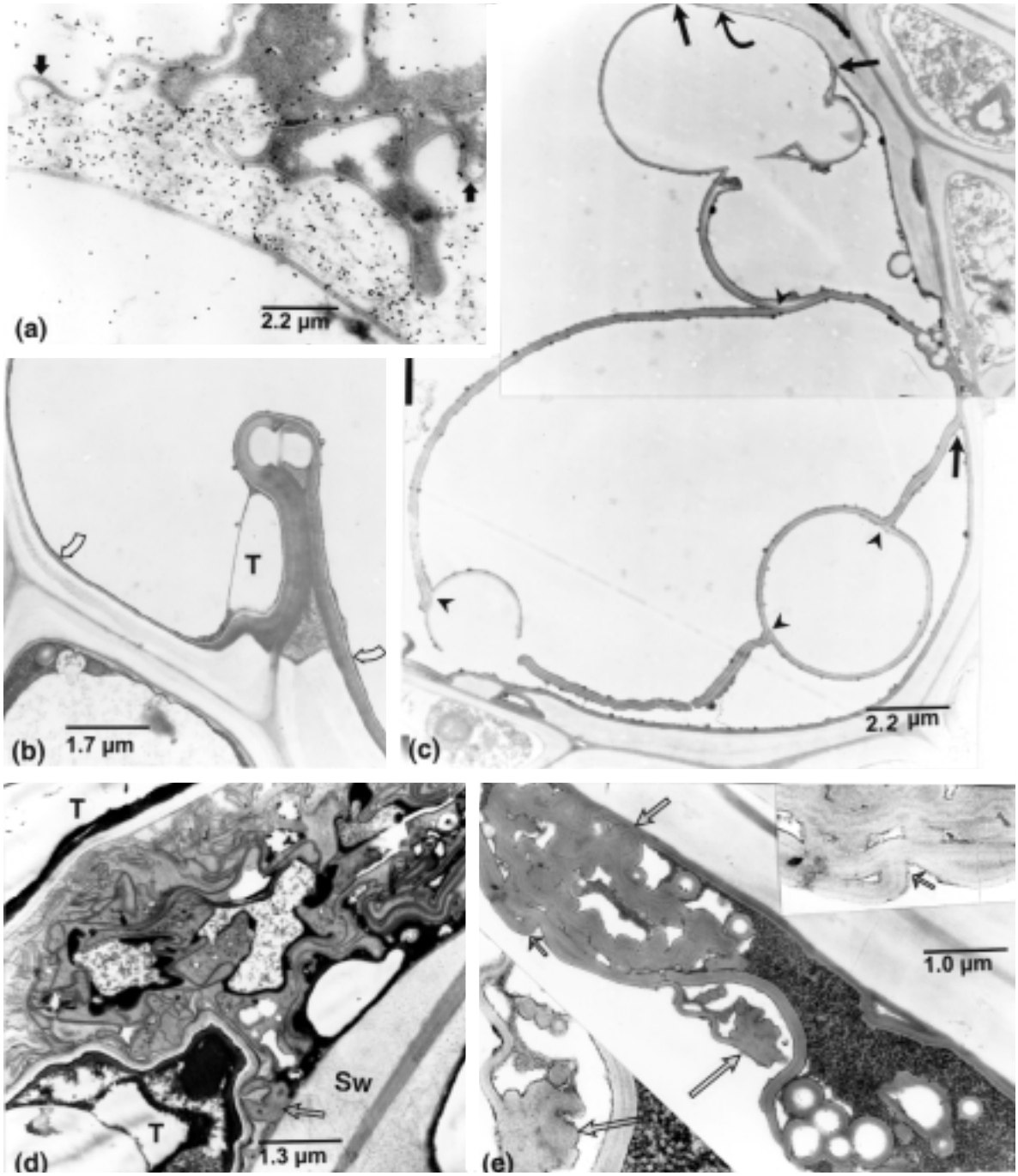

Figure 1. Sw, vessel secondary wall; T, tylosis. **(a)** Labelling for pectin with JIM 5. *Prunus pensylvanica*, 21 dpi. Unlabelled layers (arrows) circumscribe labelled fibrillar material and unlabelled more opaque and compact matter. **(b)** Staghorn sumac inoculated with *F. oxysporum*, 21 dpi. A tylosis with an inner lucent layer (arrows) is firmly appressed to a vessel wall. Arrows point to an inner lucent layer in the tylosis, which, in the left hand portion, is separated from the vessel wall by only a thin, opaque layer. **(c, d)** *U. americana*, from recurrent infections, the third year after inoculation. Alveolar layers, arranged in circles, merge or diverge from the coating (straight arrows), which occurs only as a thin opaque band in between (curved arrow). Arrowheads indicate the point of juncture of two layers, separated by opaque bands. Similar bands border the layers. **(d)** Numerous, contorted, alveolar layers, of various thicknesses, and also delimited by opaque bands, are compressed between the tylosis and vessel walls. Confluence of coating and network layers is apparent (arrow). **(e)** *U. americana*, 1-year-old infection. Shows similar but thicker alveolar layers linked to the coating (short, large arrow). The longer arrows indicate an apparent expansion of the layers limited by opaque bands, confluent with that of the layer (enlarged portion, inset, lower left). The thin arrows point to a stratified alveolar layer (enlarged portion, upper right).

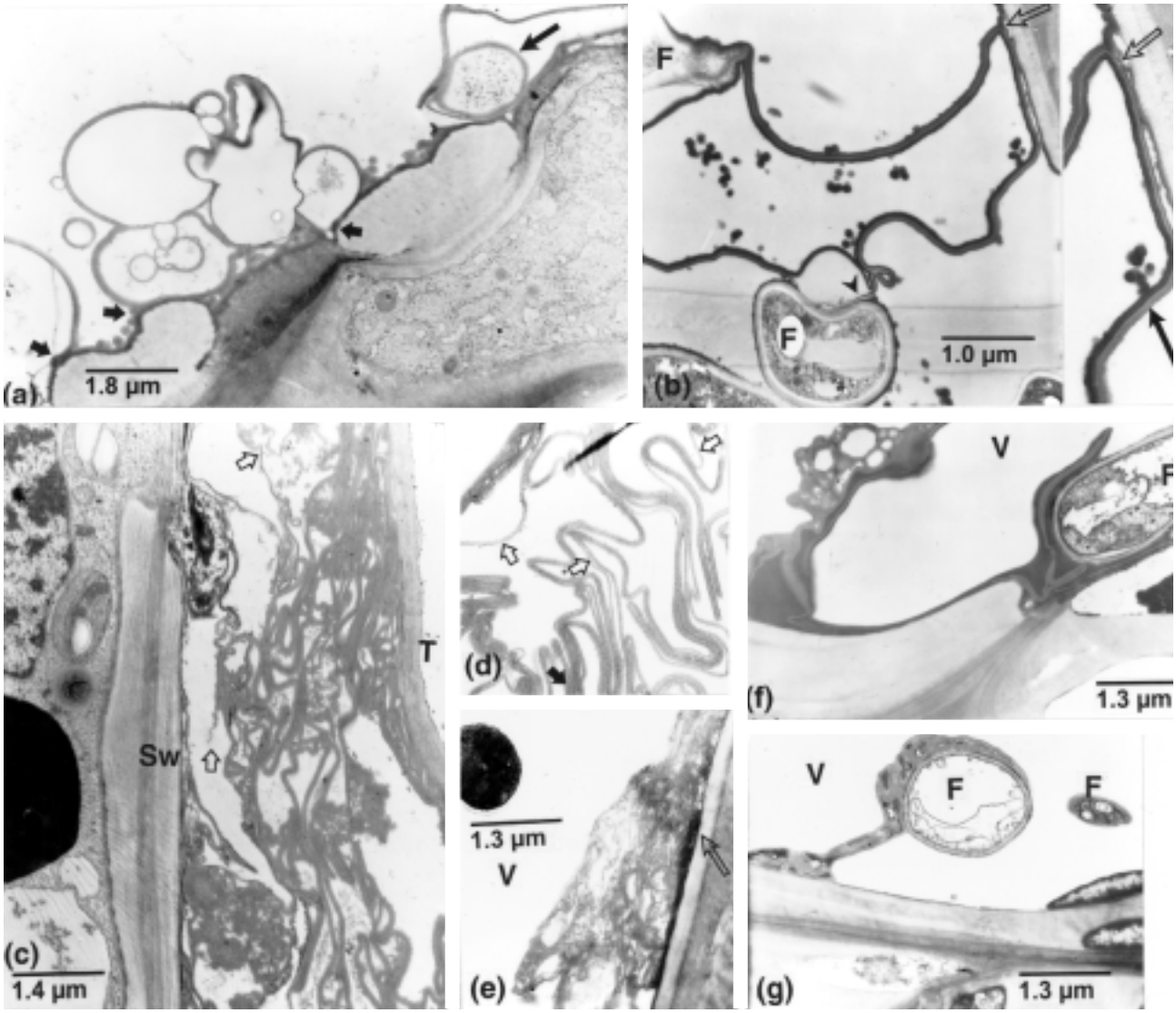

Figure 2. F, fungal cell; Sw, vessel secondary wall; T, tylosis; V, vessel element. **(a)** *U. americana*, injected with cerato-ulmin, 5 dpi. Alveolar layers of varying thickness, linked to the coating (short arrows) and circumscribing fibrillar or globoid bodies (long arrow). Bodies with a paler contour also occur along or are fused with the coating. **(b)** Recurrent infection, third year after inoculation. Alveolar layers linked to or encompassing fungal cells. The thin, more lucent middle portion of the layer, connected to the upper fungal cell, extends along the vessel wall (empty arrow; the corresponding area is enlarged in the right hand portion). The inner layer becomes the external, thicker layer (full arrow) along the vessel wall, but is thin again where it reaches the other fungal cell (arrowhead). **(c)** *U. glabra*, 45 dpi. Contorted, thin layers present between the tylosis and the vessel walls. Globoid bodies are also noticeable encompassed by the layers (arrows). **(d)** *U. americana*, 30 dpi. Networks of layers (opaque arrow); the thinnest ones are delimited by opaque bands (empty arrows), which remain distinct between the juxtaposed layers. **(e-g)** *U. americana*, 1-year-old infection. **(e)** Crowded arrays of thin layers similar to those of the alveolar networks are still distinguishable and confluent with the coating (arrow). **(f)** A fungal cell is partly circumscribed by alveolar layers embedded in opaque matter. **(g)** A fungal cell is «hooked» by the alveolar network.

In the trees injected with glucose solutions prior to inoculation, only networks of thin bands occurred and their thinness was obvious even when merged (Fig. 3a). As already stated (Ouellette *et al.*, 2004b), the content of paratracheal cells in these samples was dense and opaque and their pit membranes facing vessel lumina were almost free of covering matter, except for small vesicular bodies that were seemingly linked to fine bands included in the outside portion of the membrane (Fig. 3b). Similar bodies were linked to the bands present in vessel lumina (Fig. 3c). Configurations demarcated by similar thin bands, confluent with, or bi-

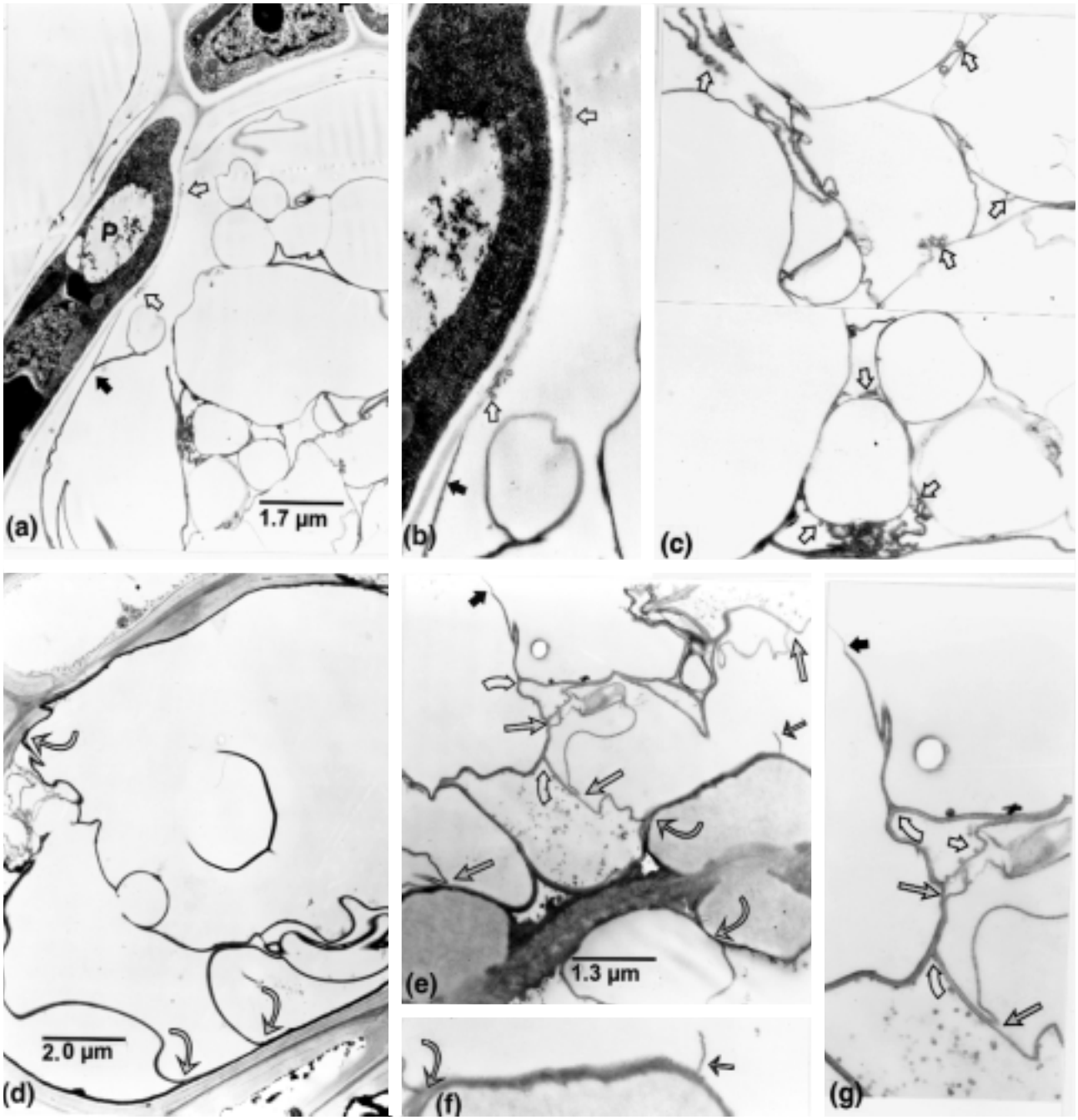

Figure 3. Alveolar networks demarcated by thin membranous-like layers. **(a-c)** Injected with a glucose solution, 30 dpi. **(a)** The content of parenchyma cells (P) is dense and opaque and the pit membrane of the half-bordered pit is intact. The area between empty arrows is enlarged in b. **(b)** Vesicular-like bodies linked or proximate to a thin opaque band on the vessel wall (opaque arrow, corresponding to that in a) encroaches on the pit membrane. **(c)** Another enlarged portion of **a**. The arrows point to vesicular-like bodies linked to the membranous layers, some bulging from these and others seemingly detached from them (lower part). **(d)** Recurrent infection, the third year after inoculation. Thin alveolar layers linked to or bifurcating from the coating on both sides of the vessel element (curved arrows) and crossing its lumen. **(e-g)** Injected with cerato-ulmin and collected 5 days later. Enlarged portions in **f** and **g**, with the same type of arrows corresponding to the same features. Thin bands (long, straight arrows) merge producing larger layers (or bifurcate from these) or are confluent with the coating, some short ones even at right angles from it (thin arrows). The opaque arrows indicate an abrupt narrowing of a layer, the curved short arrows point to some kind of roundabouts formed by apparent extensions of membranous layers, and the long curved arrows point to a merging with or extensions of thin bands from the coating.

furcating from, the coating at right angles from it and often spanning across the vessel lumen, were observed in recurrent, seemingly milder infections in inoculated elms (Fig. 3d). These observations raised the question as to whether the configurations just illustrated (Fig. 3a-d) were related or not to the alveolar networks circumscribed by thicker layers.

In this connection, a glimpse of thinner membranous structures bridging thicker layers has already been illustrated (Fig. 2a). The thinner and opaque bands were also observed as resulting from a possible thinning out of larger bands or as bifurcations of the outside bands of the alveolar layers (Fig. 3e, g); looking at it differently, the thin bands could also have merged to produce various configurations, including some kinds of roundabouts. Similar bands, once more, merged with or emerged from the coating (Fig. 3e, f). Globoid bodies were likewise circumscribed or linked to the network (Figs. 3g and 4a).

Indications that the thin opaque bands could spread in a somewhat autonomous manner were the following. The paired bands of a layer were dislocated and wound individually in diverging directions (Fig. 4a); they appeared as single bands bifurcating from a layer element, extending over wall thickenings and pit membranes and impinging on it (Fig. 4b) or being linked to their erosion or disruption (Fig. 4c, d). These bands could be traced back not only as possible bifurcations from larger layers, but also seemingly as reuniting, following noticeable meandering and some increase in thickness, into larger layers (Fig. 4e, f).

Networks in other plants

Alveolar networks abundantly occurred in *Prunus pensylvanica* inoculated with *O. novo-ulmi*. They did not occur in other non-hosts, but the coating layers at times extended into vessel lumina or bulged over pit membranes (Fig. 5a).

In eggplants inoculated with *Verticillium*, networks delimited by thick layers were not observed in vessel lumina, but the coating often bulged out into these or into pit chambers, and similar matter often bridged them (Fig. 5b). Fungal cells were connected between themselves or to the coating by similar layers (not illustrated) that were bordered by more opaque bands (Fig. 5b). Networks of thinner filamentous-like structures, similarly bound as in elms, occasionally occurred in vessel elements along vessel walls or vessel rims (Fig. 5c). Fungal cells were also surrounded by similar structures. Occasionally, thinner bands linked to the coating extended into vessel lumina (Fig. 5d). As in elm trees, the coating was distinguishable in some places as opaque bands and extended into adjacent vessel elements across the pit membrane. In tomato infected by *Fusarium oxysporum* f.sp. *radicis-lycopersici*, the alveolar network was pronounced and analogous to that present in elm (not illustrated).

Other peculiarities of coating in elms and other plants

Vessel elements did not always contain alveolar networks, but the material on their walls was similar to that associated with the networks, as shown in the following illustrations. Of analogous texture, it was often delimited by a more lucent film (Fig. 6a) and it likewise displayed more opaque bands (Fig. 6b). The material also permeated pit membranes and extended into vessel secondary walls (Fig. 6a). Similar coating material occurred on walls of vessels in inoculated sterilized elm sections fixed by high pressure freezing (Fig. 6c).

In staghorn sumac infected by *F. oxysporum* f.sp., alveolar networks were not detected but the coating was of general occurrence, formed early after inoculation, appeared as membranous-like structures delimited by bands (Fig. 6d) similar to those observed in elms and in carnation (not illustrated). Some coating also had a fuzzy appearance, concealing or not opaque bands, and connected with similar matter reaching into the vessel lumen (Fig. 6e). A surprising result with the DNA probe was that this type of coating strongly labelled (Fig. 6e, f), except in areas containing the distinct opaque bands (Fig. 6e). The slightly or more altered cell content in these cells and the host walls was free of gold particles of the probe (Fig. 6e) or they were sparse in periplasmic areas and host organelles (Fig. 6f).

Another intriguing peculiarity in elm was the occurrence of numerous distinct opaque particles, the approximate size of ribosomes, intermingled with fine opaque matter along vessel walls and associated with their alterations (Fig. 7a-e). The layer bearing these particles was bound or seemingly unbound, particularly on the vessel wall side (Fig. 7a). This layer also encompassed membranous and vesicular-like structures, and fibrillo-granular material similar to the mate-

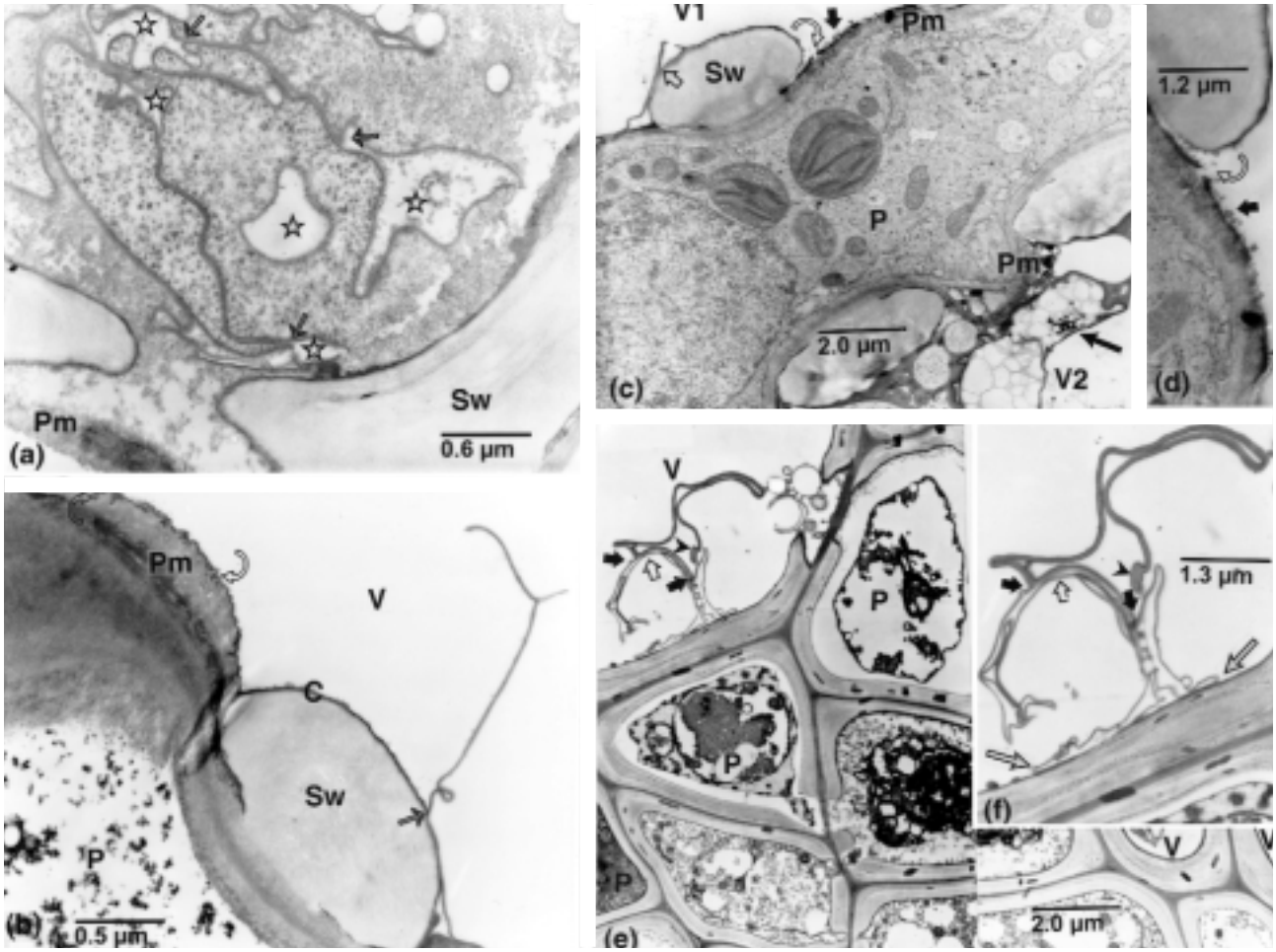

Figure 4. C, coating; P, parenchyma cell; Pm, pit membrane; Sw, vessel secondary wall; V, vessel element. *U. americana.* Thin opaque bands viewed as basal units of the alveolar layers in vessel lumina. **(a)** One-month-old infection. Arrows point to separations of the bands, and stars indicate lacunar areas demarcated by these bands. Globoid bodies occur, circumscribed by the layers. **(b-d)** Injected with cerato-ulmin and collected 5 days later. **(b)** The opaque band of a membranous layer crossing the vessel lumen separates from it (arrow) at the coating level and running appressed to the vessel wall (also penetrated by opaque matter at its base) and the adjacent pit membrane on which it has impinged (curved arrows). The content of the parenchyma cell is degraded but new wall material has been deposited in the cell. **(c,** with enlarged portion in **d**, the corresponding parts indicated by the same type of arrows). Pit membranes in V1 and in V2, on both sides of a parenchyma cell, are altered, distended in V2 and ruptured in V1 (curved arrows). In the latter, vesicular bodies are linked to the opaque band (short, opaque arrows), and a thin opaque band covers the wall thickening, bifurcating over an apparent eroded part of it (empty short arrow), and expanding over the adjacent pit membrane. Vessel V2 contains a thick coating delimited by bands extending as alveolar layers (long, opaque arrow) which encompass frothy-like structures. **(e, f)** One-year-old infection, in an area of re-invasion near the cambium. The vessel element (top left) displays an alveolar network, enlarged in **f**. The opaque bands delimiting the membranous layer bifurcate from it (opaque arrows), extend into convolutions and reunite again (empty arrow). The cells closest to this vessel have altered content, compared to those more remote from it. Fungal cells occurred in this vessel (cropped portion) and in one of the other five contiguous vessel elements, all displaying coating.

rial found in the vessel lumen (Fig. 7a, b). Paired thin bands encompassed a similar type of material and expanded around pit borders and along the pit membranes (Fig. 7c). Opaque particles were likewise linked to fine matter and filamentous structures winding across vessel lumina or apposed to their walls, even more than 1 year after inoculation (Fig. 7f-h). These masses of particles were intermixed with matter which was texturally similar to that of the alveolar layers, in overwintered trees that showed only mild symptoms during the year of inoculation. In these trees, numerous fungal cells originated from the layers present on vessel walls, shortly after incubation of the samples on an agar medium before fixing.

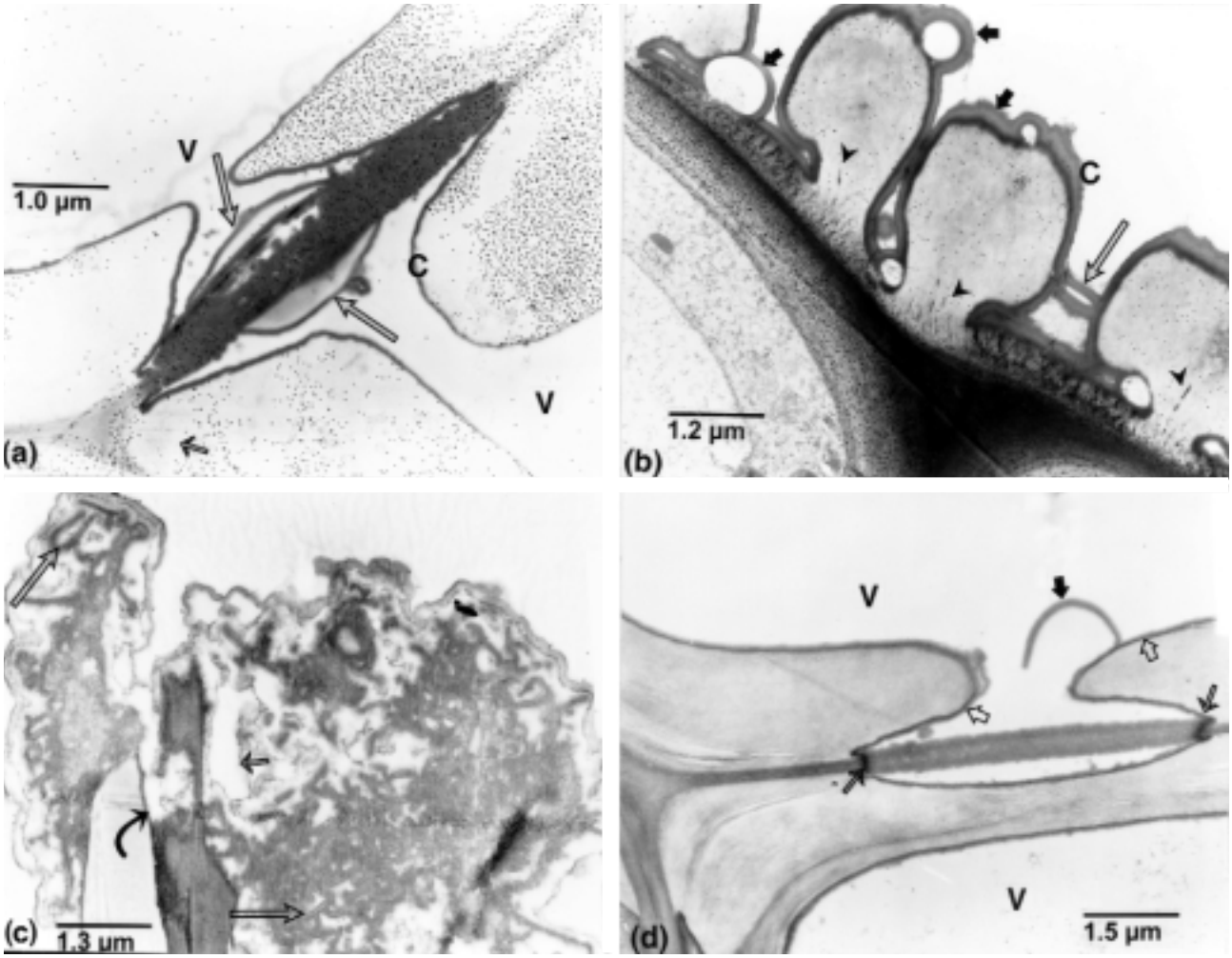

Figure 5. C, coating; V, vessel element. Coating and alveolar networks in hosts other than elm. **(a)** *Sorbus americana* inoculated with *O. novo-ulmi*. Labelling for cellulose. Vessel walls are covered with coating, and the one over the pit membrane has expanded (long arrows) into the pit chambers. The pit membrane is altered and ruptured at one end. Labelling is absent or dispersed over the vessel secondary walls, particularly in the more opaque areas (short arrow). **(b-d)** Eggplant inoculated with *Verticillium*. 15 dpi. **(b)** Labelling for cellulose. The thick coating, displaying a pale outside layer (short arrows), bulges at locations. Bands similar to the coating bridge it across the pit chamber (long arrow). The vessel secondary thickenings are unequally labelled, compared to the strongly labelled, altered pit membranes and adjacent parenchyma cell walls. Opaque bands present in the secondary thickenings (arrowheads) also occurred in controls. **(c)** A network of membranous structures (long arrows) similar to those occurring in elm. The middle lamella and portions of the secondary wall of a vessel rim are altered (curved arrow and small straight arrow, respectively). **(d)** A hooking of the coating (opaque arrow) into the vessel lumen. Opaque bands are discernible in the coating (empty, thick arrows), and opaque matter, continuous with the coating, traverse the pit membrane (thin arrows); it is confluent with the coating in the adjacent vessel element.

Discussion

Present observations indicate that the coating material and the alveolar network formed under various conditions in elm vessel lumina in host (*U. americana* and *U. glabra*) and non-host trees inoculated with *O. novo-ulmi*, and equally in other plants affected by wilts diseases, including fusarium of wilt of carnation (Nicole *et al.*, 1994; Ouellette *et al.*, 1999a) and root and crown rot of tomato (Charest *et al.*, 1984), are comparable ultrastructurally and texturally. These observations are also complementary to those expressed and discussed in Ouellette *et al.* (2004). It stands out that the numerous thin membrane-like layers are structurally analogous to the larger layers, particularly by their being delimited by opaque bands, bifurcating in the same manner, similarly merging with one another and with the coating, or extending from it. The globular

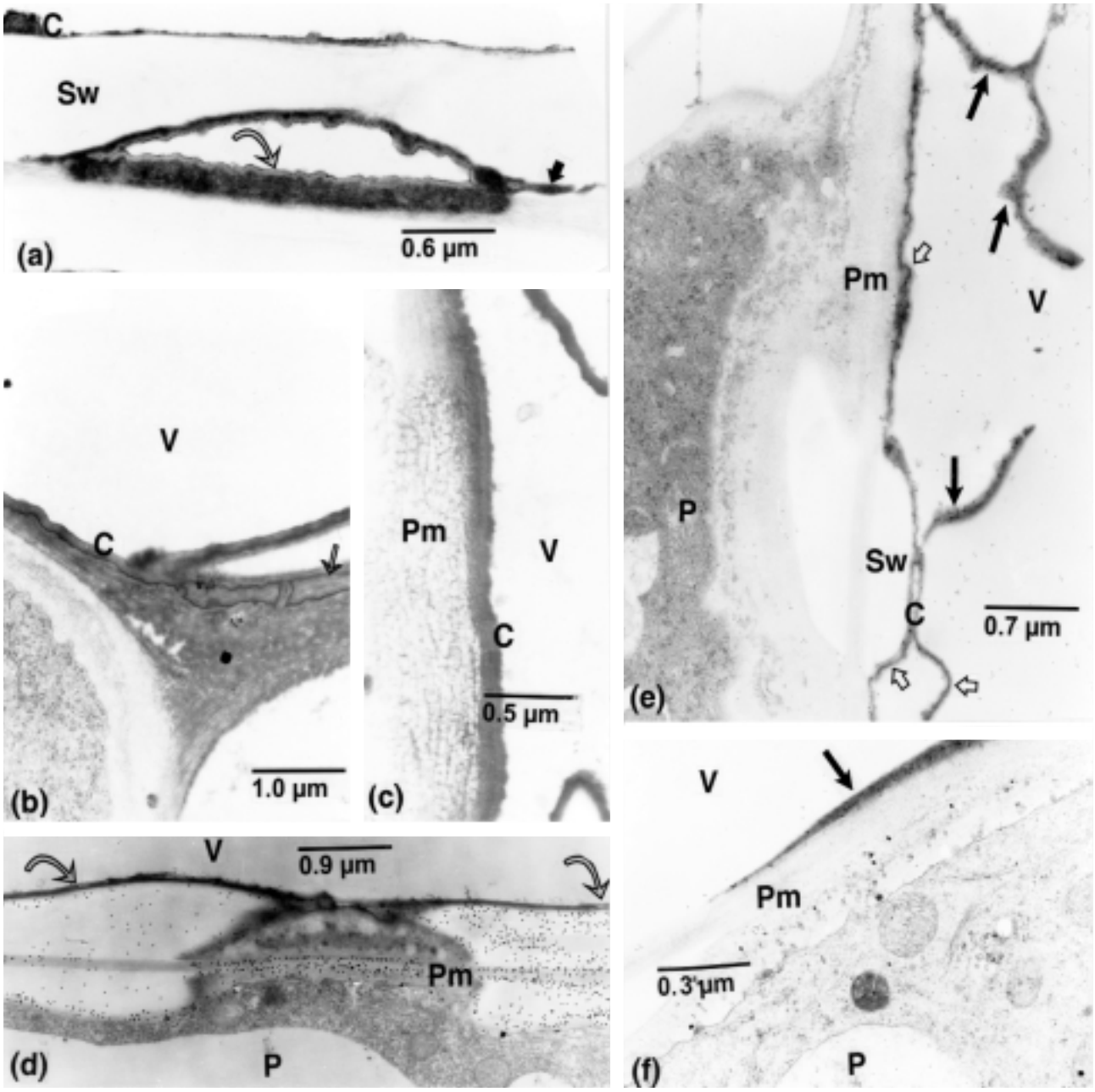

Figure 6. C, coating; P, parenchyma cell; Pm, pit membrane; Sw, vessel secondary wall; V, vessel element. **(a, b)** *U. americana*, 17 dpi. **(a)** The coating extending over the pit border and pit membrane is covered by a paler film (curved arrow). The opaque matter permeates the pit membrane and extends into the vessel secondary wall (arrow). **(b)** Opaque bands, paired at one location and circumscribing another type of matter, are present in the coating (arrows). **(c)** From an inoculated, sterilized section of *U. laevis*, fixed by high pressure freezing. A layer, similar to coating material, extends over the vessel secondary wall and pit membrane. **(d-f)** Staghorn sumac inoculated with *Fusarium*, 2 dpi. **(d)** The coating is delimited by thin bands (curved arrows), and the vessel and parenchyma cell walls are unequally labelled for cellulose, particularly in the more opaque areas. **(e, f)** Labelling for DNA with a gold-complexed monoclonal antibody to single- and double-stranded DNA. **(e)** The coating present on the pit membrane and adjacent pit borders extends into vessel lumina. Concentrations of gold particles occur over the coating (long arrows), but the paler film delimiting it (empty arrows) and its extensions, and the compact bands (short arrows) are nearly free of gold particles. They are sparse over the host cell content and the host walls, except close to the coating, but a few more are dispersed in vessel lumina. **(f)** A concentration of gold particles occurs over the material (arrow) covering part of a pit membrane of a half-bordered pit. Few particles are linked to traces of opaque matter in the parenchyma cell periplasm and to vesicular-like bodies in the cell content; others are dispersed elsewhere over the cell.

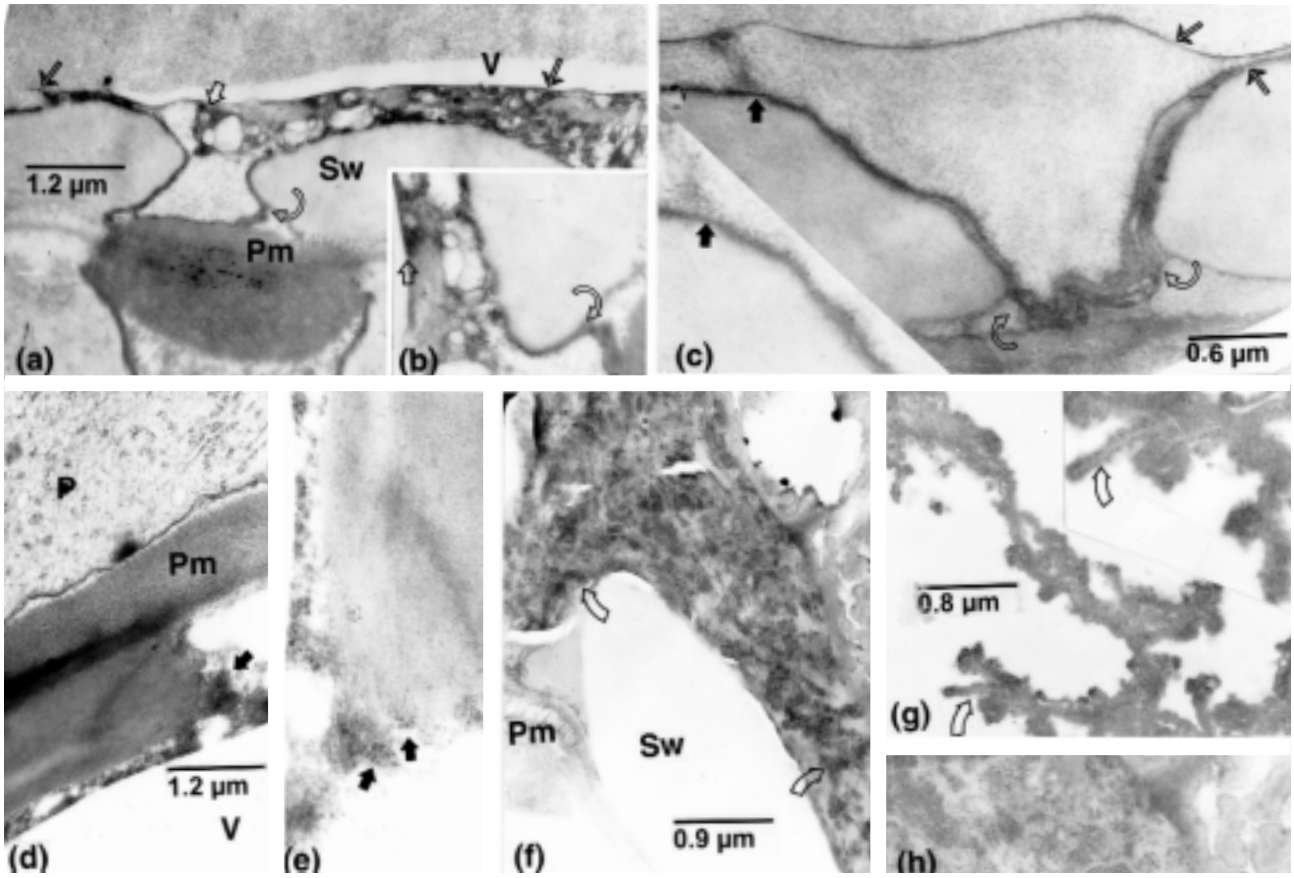

Figure 7. P, parenchyma cell; Pm, pit membrane; Sw, vessel secondary wall; V, vessel element. Inoculations, *U. americana*. **(a,** enlarged portion in **b**) Opaque particles (thick arrow), intermixed with other types of components (some membranous), occur in a thick layer along the vessel wall. A thin band (thin arrows) delimits the layer on the vessel lumen side, but is mostly not perceptible on the opposite side. The limiting bands, present around pit borders (curved arrows) and over the pit membrane, cut across fibrillo-granular material similar to that found in the vessel lumen. **(c)** Paired, thin bands (thin arrows) (found as a prolongation of the layer in **a**) encompass fibrillo-granular material similar to that present in the vessel lumen. The bands contour the pit borders and span across the pit chamber (curved arrows). The inset (left portion), an enlarged area along the portion indicated by the opaque arrows, shows links of fine components with the band. **(d, e)** An unbound layer on the vessel wall contains opaque particles the size of ribosomes (arrows, enlarged in **e**). The particles, along the pit border, are associated with, and apparently even included in, the altered vessel wall (arrows). **(f)** One year after inoculation. Masses of opaque particles (arrows) are present in vessel lumina, intermixed with islands of paler components. **(g)** Four months after inoculation. Opaque particles are linked to filamentous-like structures (enlarged portion in the inset, arrows indicating the corresponding parts) or included throughout in fine, paler matter, some resembling that in **f** (an enlarged portion of which is shown in **h**).

forms of the networks were seemingly attributable to the expansion of their layers when they were not compressed between walls of tyloses and vessels. However, intertwined, overlapping and/or spliced layers were also meandering uncompressed in vessel lumina. The confluence or disruption of layers may thus account for their apparently reduced numbers in the later stages of development. Also, fewer layers may be formed in some cases. Increases in layer thickness may also occur following the addition of new material in their middle portion. Some figures (Figs. 1c, e and 2e) well illustrate this aspect.

In this respect, the thin opaque bands delimiting the alveolar layers may not simply be a confining structure whose opacity might not have been factual, but corresponded to a real structure. Indeed, it occurred not only freely as such in vessel lumina of vessels or over their walls, but it was also linked to their pervasion and alterations. In fact, as already mentioned, the diversity in thickness of layers was due to the juxtaposition of the bands. The thinner bands were particularly obvious in elm trees injected with a glucose solution prior to inoculation. It may be recalled that pre-treatments with this monosaccharide were reported

to reduce DED symptoms in elms (Feldman *et al.*, 1950). Similar configurations of thin bands were also observed in elm seedlings treated with CU. Caution concerning treatment with this compound was expressed in Ouellette *et al.* (2004), considering that the pathogen was isolated from treated trees.

It cannot easily be ascertained whether the alveolar networks and associated coating have any particular effect on disease development or whether they are an expression of defence mechanisms (for example see Street *et al.*, 1986; Gold and Robb, 1995). In an attempt to assess the situation, one would need to consider the following: 1) the ultrastructural similarities between the membranous structures, the thick alveolar layers, and the layers of medium thickness suggest that they all have a common origin and nature; 2) the alveolar layers are closely linked to the coating which is often associated with vessel secondary wall and pit membrane alterations; and 3) these structures cannot be considered simply as inert components. Some people may argue that the various configurations of the networks have formed during sample processing and fixation procedures. An answer to this type of question would be that the alveolar layers, be they thin or thicker, show the same type of organisation and seem to increase in thickness as infection progresses. Also, the layers, compressed and filling completely the gaps between tylosis and vessel walls are similar to those wandering in vessel lumina. Finally, one would not expect that the thick alveolar layers were made to merge by specimen handling solely in samples from advanced infection. Anyhow, if the procedure was to be questioned, it always produced the same effect on the same type of structures. In this respect, the alveolar networks in overwintering diseased trees, or in recurrent infections more than one year after inoculation, were similar to those occurring during the first year of infection. Their relationships with fungal cells might therefore be informative as to the conditions of pathogen survival and regeneration. Moreover, as the coating did not label for either pectin or cellulose, it does not seem to correspond to host wall breakdown products. Even in cases where the vessel secondary walls labelled for chitin, particularly in carnation (Ouellette *et al.*, 1999a, 2002) and elm, the coating proper and the networks did not label.

The large amounts of the networks and associated coating in vessel lumina could interfere with sap movement, but they may not necessarily be the primary and sole factors inherent to the wilt syndrome, as these components were estimated to have generally formed concomitantly with or subsequently to other types of disorders, e.g. alterations of pit membranes. Nevertheless, considering that these structures may be somehow active, they could contribute to maintain tissue alterations and be important in disease recurrence. It may be warranted to speculate that such an activity is related to the opaque bands, which, when they appeared as single units bifurcating from the membranous structures or merging with them (depending on how one looks at it), spanned long distances over vessel walls and pit membranes into which they dipped in many forms. These bands were also similar to those that extended through masses of fibrillo-granular matter in vessel lumina or those present in the coating and that cut across the thicker bands. These thin bands, therefore, may be considered as the basal elements of the membranous structures.

Reference was made to the links between the alveolar networks and the coating. There are peculiarities in the coating that deserve attention. Indeed, the extensive material often closely apposed to vessel walls and not always clearly delimited by bands (see also Ouellette and Rioux, 1992) occasionally contained numerous particles that were the size of ribosomes and that were linked to vessel wall erosion, as was also the case in susceptible carnation plants infected by *F. oxysporum* (Ouellette and Baayen, 2000; and unpublished observations). When material from overwintered infected elm trees was incubated on an agar medium before fixing, large numbers of similar bodies were observed in matter present in lumina of vessels or apposed to their walls. Whatever the origin and nature of these structures, one would not expect such distinct, unbound bodies to have been preserved from disintegration over long periods, such as during cold winters, unless the bodies were somewhat structured. Following similar sample incubation for various intervals before fixing, fungal cells generally originated from the material apposed to the vessel walls, but no clear indications were obtained that they formed directly from the matter in question. It is noteworthy, however, that the particles were intermixed with matter similar to that of the alveolar bands (see also Ouellette *et al.*, 2004).

When considering results in this and other work (Ouellette *et al.*, 2004), the following may be recapitulated: the coating under study is similar to the opaque matter, free or linked to fungal cells, as well as to the matter pervading host cell walls or accumulating in parenchyma cell periplasmic areas, in at least three

systems. Both the coating and the opaque matter present in the periplasm of parenchyma cells in sumac were found to label for DNA (Ouellette *et al.*, 2004). In autoradiographic tests, the opaque material in host walls in elm also labelled with ^{3}H thymidine (Ouellette, 1978; Ouellette and Rioux, 1992, 1993). Regarding this issue, it needs to be considered that fungal elements such as microhyphae (which also occur on vessel walls in carnation; unpublished observations) and larger cells have often defective walls, and that this condition does not appear to hinder their invasion process (Charest *et al.*, 2004; Ouellette *et al.*, 1999a; Ouellette *et al.*, 2001). If one correlates these observations in the sense that among a number of features, when one is analogous to a second and, in turn, to a third, it can be said that the features are analogous between themselves; therefore, it would not be mere speculation to mention that these interrelated structures cannot be primarily ascribed a host origin. This does not exclude, however, that other types of compounds may be adsorbed or intermixed with the structures under study.

As a concluding remark, may it be mentioned that in all our observations few indications were obtained that the alveolar networks and coating could be linked to host degradation products. Even when vessel occluding matter was assigned to swollen and degraded pit membranes (see Vander Molen, 1978), the location of the membrane was still distinct and the matter overlaying it was of a compact and different structure. Some of this matter even appeared to cross pit membranes as we have shown in the present study. Whatever the situation is, it is interesting to note that the material ascribed to breakdown products is first surrounded by a fine, fuzzy layer, comparable to that bridging fungal cells (compare Figs. 6 and 13 in Vander Molen), then by a pale layer surrounding a more opaque core. Moreover, this fuzzy material appears to be analogous to the extracellular matter linked to cells of *O. novo-ulmi* (Ouellette *et al.*, 1995, 1999b) and other fungal pathogens (see Discussion in Charest *et al.*, 2004). It is our contention that to understand the key aspects of disease development in DED and other similar diseases, one needs to concentrate on the properties of this type of opaque matter which was shown to contain filamentous structures.

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