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Phytoprotection

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Volume 85, Number 3, décembre 2004

URI: https://id.erudit.org/iderudit/010905ar
DOI: https://doi.org/10.7202/010905ar

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Publisher(s)

Société de protection des plantes du Québec (SPPQ)

ISSN

0031-9511 (print)
1710-1603 (digital)

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Cite this article

B. Ouellette, G., P. Baayen, R., Rioux, D. & Simard, M. (2004). Peculiar ultrastructural characteristics of fungal cells and of other elements apposed to and in vessel walls in plants of a susceptible carnation cultivar, infected with *Fusarium oxysporum* f.sp. *dianthi* race 2. *Phytoprotection*, *85*(3), 121–138. https://doi.org/10.7202/010905ar

Article abstract

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Peculiar ultrastructural characteristics of fungal cells and of other elements apposed to and in vessel walls in plants of a susceptible carnation cultivar, infected with *Fusarium oxysporum* f.sp. *dianthi* race 2

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Received 2005-02-10; accepted 2005-04-08

PHYTOPROTECTION 85 : 121-138

Ultrastructural characteristics and cytochemical reactions of unusual, irregular elements (IE) in vessel elements in susceptible carnation plants infected with *Fusarium oxysporum* are reported. As revealed by labelling for chitin, fungal cells in contact with host cell walls or content had altered or defective lucent layers, and labelling was frequently associated with their outer, opaque layer or matter located outside the cells. Coating matter on vessel walls occurred at all stages of infection, and IEs only in later stages. IEs were delineated by opaque, often folded bands, some contouring pit borders, and contained membranous and vesicular structures mixed with other fine components. Only then, IEs were strongly but not uniformly labelled for chitin. Coating, IE-delineating bands, and the opaque outer layer of typical fungal cells were texturally similar, not labelled for chitin or cellulose, except where they impinged upon host walls. Both probes for chitin and cellulose strongly attached to vessel secondary walls. IEs were often confluent with coating, and with fungal cells connected to them by means of microfilamentous structures. Similar microfilamentous structures and opaque bands connected to IEs, the coating, and the microhyphae, or protruding from fungal cells reached into host walls, associated with alterations of these walls. The possible malleable IEs might be a counterpart of the coating, and although they do not occur in the initial stages of the disease, they could play an important role in the final stages of tissue degradation.

[Caractéristiques particulières des cellules fongiques et d'autres éléments accolés aux parois de vaisseaux, ou présents dans celles-ci, de plantes d'un cv. d'œillet sensible à l'infection par le *Fusarium oxysporum* f.sp. *dianthi* race 2]

Ce travail concerne les caractéristiques ultrastructurales et cytochimiques d'éléments, de formes et de structures irrégulières (IEs), présents dans les vaisseaux de plants d'œillet sensibles à l'infection par le *Fusarium oxysporum* f.sp. *dianthi.* Avec les sondes utilisées pour détecter la chitine, les cellules fongiques étant en contact avec les cellules hôtes ou leur contenu ont montré des parois altérées ou défectueuses; ainsi, le marquage pour ce substrat était fréquemment relié à de la matière opaque constituante de la couche externe des cellules ou entourant celles-ci. Un tapissement compact couvrant les parois de vaisseaux se trouvait à tous les stades d'infection, mais les IEs ont été observés seulement dans les stades plus avancés. Les IEs étaient délimités par des lamelles opaques et souvent tortueuses, contournant les parois secondaires des ponctuations. Ces IEs ont réagi à divers degrés pour la chitine, dans les cas où ils étaient pourvus de structures membranaires ou vésiculaires et d'une fine substance homogène. Le tapissement, les lamelles limitantes des IEs et des cellules fongiques avaient la même apparence, non marqués pour la chitine ou la cellulose, sauf lorsque ces structures avaient empiété sur les parois cellulaires de l'hôte; celles-ci se sont marquées pour la chitine comme pour la cellulose. Les IEs se rattachaient au tapissement ou aux cellules du champignon, souvent via de fines structures filamenteuses. Des structures semblables et des bandes de matière opaque, contiguës aux IEs, au tapissement ou à des microhyphes, ou provenant de cellules fongiques, atteignaient les parois des cellules hôtes, ainsi altérées. Vu les liens possibles entre le tapissement et les IEs multiformes, ces éléments dans les plants sensibles pourraient alors jouer un rôle important dans la dégradation ultime des tissus.

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INTRODUCTION

Of the material extrinsic to mature vessel elements in many plants affected by fungal wilt diseases, opaque matter (the coating) covering vessel walls is found in most of these diseases (Bishop and Cooper 1983, 1984; Pegg *et al.* 1976; Robb *et al.* 1975, 1979, 1987; Shi *et al.* 1992), and considered by many to be compounds of host origin and often as an expression of resistance. Investigations of wilt of carnation (*Dianthus caryophyllus* L.) caused by *Fusarium oxysporum* Schlechtend.:Fr. f.sp. *dianthi* (Prill. & Delacr.) Snyder & Hans. have revealed marked differences in tissue invasion and reactions between the resistant and the susceptible plants (Ouellette and Baayen 2000; Ouellette *et al.* 1999a, 2002, 2004a). The coating in both types of plants was associated with fine filamentous-like structures (Nicole *et al.* 1994; Ouellette *et al.* 1999a, 2002), related to vessel wall alterations, and did not appear *per se* to be a factor of resistance. The lack of or at least the less extensive wall modifications in the susceptible plants compared with the resistant plants, was congruent with profuse host wall colonization, generally by means of microhyphae (Ouellette *et al.* 1999a). This led to tissue collapse and leaf withering (Baayen and Elgersma 1985; Baayen *et al.* 1988).

In elm trees infected with *Ophiostoma novo-ulmi* Brasier, the coating is generally present at all stages of infection and associated with alterations of vessel walls (Ouellette *et al.* 2004b). Prominent networks (denoted alveolar) of layers, delimited by opaque bands, are also closely linked to the coating (*Ibid).* To our knowledge, the question concerning the origin and nature of coating in this and other wilt-affected plants has not yet been resolved adequately, despite the fact that some authors have stated that coatings may contain compounds, assigned a host origin, such as suberin, pectin, phenolics, and so on (Bishop and Cooper 1983, 1984; Gold and Robb 1995; Robb *et al.* 1978; Shi *et al.* 1992; Street *et al.* 1986). However, with gold-complexed probes, pectin, cellulose and chitin residues were found to be of exceptional occurrence in the coating in the diseases we have studied (Ouellette *et al.* 1999a, 2002, 2004a, 2004b; Rioux *et al.* 1998). Most histopathological studies of fungal wilt diseases concerned annual plants; generally, these provided little information about the advanced infection stages, during which coating material in particular could have become modified, and host walls, including those of vessels, increasingly altered. In this respect, the possible primary contribution of the pathogen in coating formation has been overlooked in the models proposed.

It is thus of paramount importance to discriminate between compounds of pathogen vs. host origin and to determine the various possible modes of pathogen development and to which extent its elements may withstand modifications in their walls without losing all their inherent metabolic activities. For example, *O. novo-ulmi* growing in its hosts as well as on various substrates, including growth through Millipore membranes (Ouellette *et al.* 1995), may produce various irregular growth forms and subsequently revert to so-called typical growth forms. Similar irregular growth forms were reported for *Fusarium* infection in carnation and tomato (Charest *et al.* 2004; Ouellette *et al.* 2001).

The present work concerns the occurrence and characteristics of various unreported elements on vessel walls in susceptible carnation infected with *Fusarium*, mostly in advanced stages of infection (early stages of infection have been described in the references referred to above). The association of these elements with vessel wall alterations and their links with more typical fungal forms have been noticed. Likewise, attention has been given to fungal wall modifications occurring in these cells. Many of the observations are based on cytochemical tests for cellulose and chitin. Other peculiar structures related to vessel wall alterations are reported in Ouellette *et al.* (2004c).

MATERIALS AND METHODS

Material

Rooted cuttings of susceptible carnation plants (cv. Early Sam) were greenhouse-grown for 3-4 wk before inoculation with an isolate of *Fusarium oxysporum* f.sp. *dianthi* race 2, as previously described (Baayen *et al.* 1996). Briefly, inoculation was performed by placing a droplet of a conidial suspension (10^6 to 10^7 conidia mL^{-1}) in the axil of one of the lower leaves of the plants (which were then from 25-35 cm in height) and an incision was made across the droplet (Baayen and Schrama 1990). Inoculum was prepared from slant cultures of isolate WCS816. Four inoculated, two water-injected, and two intact plants were sampled (past the first cm above the incision), and sampled from 2 to 37 d after inoculation, with the last samples being taken when the plants showed external disease symptoms.

TEM observations

Samples (2 mm x 2 mm) were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon 812, as described previously (Ouellette *et al.* 1999a). Ultrathin sections (straw color), cut with a Reichert Ultracut II were contrasted with uranyl acetate and lead citrate before examination at 80 kV with a Philips 300 electron microscope. At least two samples from each sampling date and ordinarily five sections from each sample were examined.

Cytochemical tests

Some of the sections were processed for cytochemical labelling (Ouellette *et al.* 1995). Cellulose was detected using an exoglucanase (EC 3.2.1.91), having affinity for ß-(1-4)-D-glucans; the enzyme directly conjugated to colloidal gold, as previously described (Ouellette *et al.* 1995), using a colloidal gold particle size of 15 nm (Frens 1973). Briefly, ultrathin sections were floated on a drop of phosphate buffer saline (PBS)-PEG (20 000, 0.02%) at pH 6.0 and incubated for 30 min on a drop of the enzyme complex. Specificity of the labelling was verified either by incubating sections over a drop of the exoglucanase-gold complex that was previously adsorbed with ß-(1-4)-D-glucans from barley (Sigma

Chemical Co., St. Louis, MO, U.S.A.), or over the non-complexed enzyme followed by incubation with the complexed enzyme.

Chitin was detected using a gold-complexed wheat germ agglutinin (WGA) as described by Ouellette *et al.* (1999a). Sections on nickel grids were incubated on 25 μg of WGA per mL of PBS followed by exposure to an ovomucoid-gold complex (pH 4.95 in PBS-PEG). Control tests included omitting the lectin in the procedure or neutralizing it with N-acetylchitotriose. Both compounds were from Sigma Chemical Co.

Some illustrations are from samples tested with gold-complexed monoclonal antibodies to pectin, giving only sparse labelling. These microphotographs have thus been used here to illustrate other features.

RESULTS

Irregularities in fungal walls

Fungal cells that closely contacted host walls or were included therein often had aberrant walls, as shown by marked differences in labelling for chitin. Whereas labelling was associated with lucent layers of septa or of walls facing vessel lumina, it was absent or linked to the outside opaque layer touching the host wall (Fig. 1a, b). In fact, some fungal cells were wholly or in part solely delimited by an opaque layer (Fig. 1c, d) and the host wall-fungal cell interface in other instances was marked by only a membrane-like structure (Fig. 1b). Marked differences in wall structure even occurred in the same cell (Fig. 1a, e); some walls also showed abrupt discontinuities in, or transformation of, both their wall layers mostly again when these contacted host cell walls or content (Fig. 1c, f, g). In these cases, labelling for chitin was mainly associated with opaque matter, and walls of adjoining fungal cells also often appeared to be fused or separated by outside opaque layers (Figs. 1g, 2a). The plasma membrane in many of these cells was seemingly also indistinct in the parts showing altered walls but was distinct in the other parts delimited by more typical walls (Figs. 1a-c, g, 2a). Points of membrane discontinuities in these cases were marked by loops (Figs. 1g, 2a).

Intramural cells were generally well confined by host wall structures and connected to small elements that were delimited by solely a membrane-like structure (Fig. 2b). Arrays of fine, parallel-oriented filamentous-like structures frequently occurred perpendicularly across walls of these fungal cells, reaching into host walls and in continuity with those surrounding the elements (Fig. 2c). A similar association of filamentous structures, often in large masses, and fungal cell walls also occurred in cells present in vessel elements or parenchyma cells (Fig. 3a-c) that likewise pervaded the host walls they contacted. Similar fine structures pervading vessel walls also occurred in the compact coating, appearing to be structurally similar to those other masses (Fig. 3d); paired opaque bands associated with these structures also occurred in this coating.

Elements apposed to vessel walls

Compact coating of varying thickness occurred in most vessel elements at all stages of infection, albeit predominantly in early infection (Figs. 3d, 4a). In samples collected 15 d after inoculation and later (however, sampling between d 8 and 14 was lacking), when host walls were profusely invaded and degraded by the fungus, other types of thin elements equally occurred for appreciable distances over vessel walls or extended into the vessel lumen, intermixed or not with other types of components (Fig. 4b-i). These elements were delimited by paired, opaque bands that were frequently of varying thickness and unequally spaced, the thinnest ones appearing as simple opaque lines (Fig. 4b, d-g). These bands, also associated with fine opaque material of filamentous appearance, were often contorted (Fig. 4b, f, i), with the band apposed to the vessel wall contouring pit borders (Fig. 4d, g). The elements had sparse content in the form of opaque or vesicular-like bodies and fine matter (Fig. 4b, g). Similar structures were connected to fungal cells (Fig. 4h) whereas the element delineating bands and fungal walls were of the same opacity and texture (Fig. 4d-f). For convenience, the peculiar elements just described will be referred to hereafter as IEs.

Labelling for cellulose and chitin

In labelling tests for cellulose, fungal walls, IEs, and the compact opaque coating were not labelled except when these elements impinged upon host walls (Fig. 4c, h; see also Ouellette *et al.* 2004a). The compact, opaque coating, even in intimate contact with labelled fungal cells, was also free of labelling for chitin, except over areas that contained lucent matter (not illustrated), or where it likewise possibly impinged upon the labelled vessel secondary walls.

The IEs, in comparison, generally labelled strongly and exclusively for chitin over their whole width (Fig. 5a, b), which was considered most meaningful when these IEs bordered unlabelled pit membranes or primary walls or the generally slightly labelled secondary walls and thickenings of small vessel elements (see below). Even when they were affixed to strongly labelled vessel secondary walls, the IEs were separated from them by their generally unlabelled delimiting bands (Fig. 5a). The opaque coating, not delimited by bands, did not label, unless it had encompassed labelled vessel wall material (Fig. 5b). Moreover, IEs were labelled only when they contained membranous or vesicular-like structures (Figs. 5a, b, 6a, b). Generally, this labelling was not clearly associated with a distinct lucent layer, as for typical fungal cells, but with more diffuse lucent or even opaque matter (Fig. 6b). Occasionally, labelling was restricted to a lucent band present across the IE (Fig. 6a). The apparently empty, tiny elements (as those illustrated in figure 4) were also labelled (Fig. 6b), but they were elsewhere connected with larger portions with visible content (Fig. 6a). Thin intramural microhyphae and larger but irregular cells in vessel lumina, which displayed only thin and apparently discontinuous lucent layers, labelled in the same manner as the IEs (Fig. 6b).

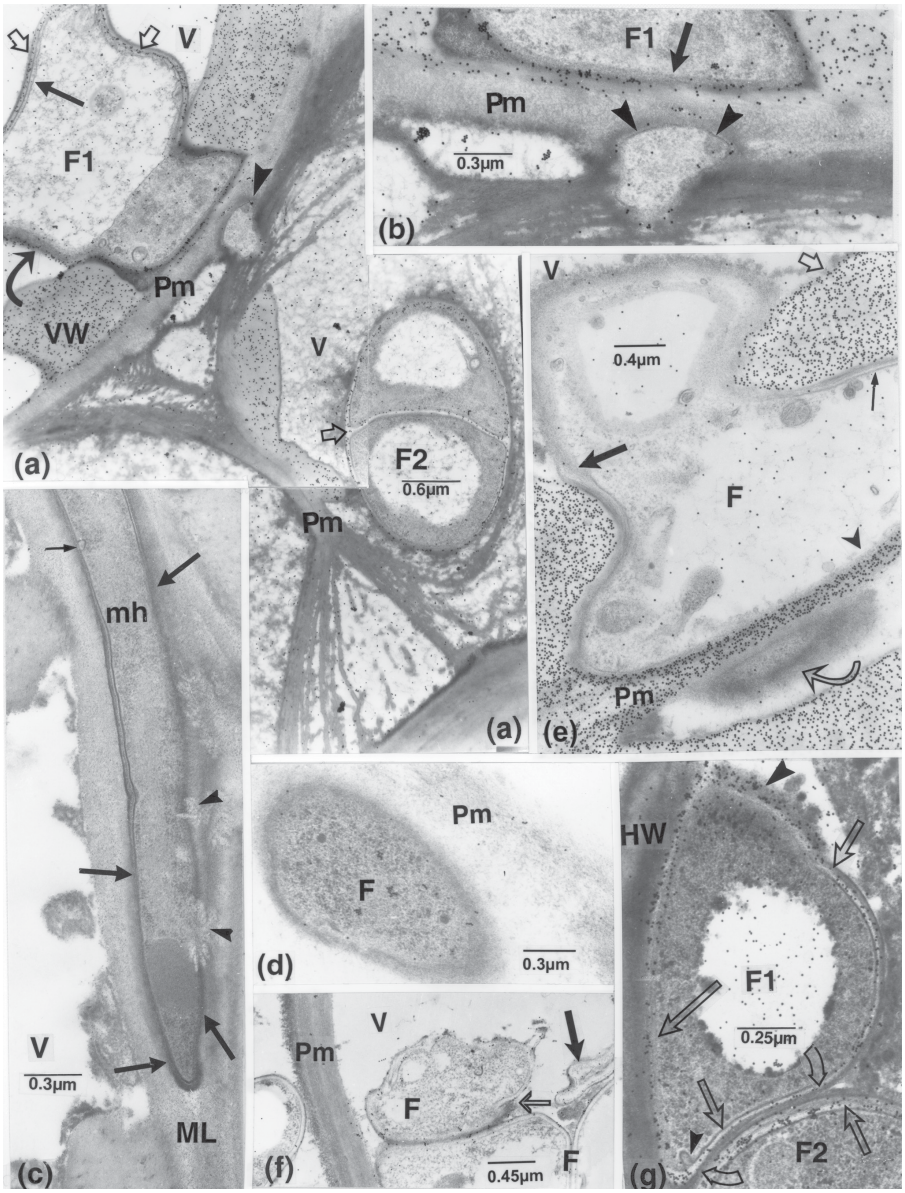

Figure 1. F, fungal cell; HW, host wall; mh, microhypha-like element; ML, middle lamella; Pm, pit membrane; V, vessel element; VW, vessel wall. **(a** and, enlarged portion, **b):** labelling for chitin is associated with: 1) the fungal lucent wall layers (light arrows), in cell F1, not to portions contacting the host walls. An inner double layer (straight, dark arrow) is present in the cell; 2) vessel secondary walls; 3) traces of fibrillo-granular matter in their lumina, and fungal cell vacuoles; and 4) the outer opaque layer (cell F1, curved arrow) (with its cytoplasm concentrated in the part filling the pit chamber) in portions close to or juxtaposed to the pit membrane. The inner fungal wall has a loosened appearance (arrow in **b**). The peg (arrowheads), in the pit chamber, is not delimited by a distinct wall, as indicated by its lack of labelling. The pit membranes are dislocated as shreds. **(c):** an aseptate intramural microhyphal element is seemingly locally delimited by only opaque bands (between thick arrows); these also appear to be discontinuous with signs of cell content invasion into the host wall (arrowheads). An incurving of the plasma membrane is discernible (thin arrow). **(d):** a lucent layer is not discernible in the intraparietal fungal cell (present in an altered pit membrane), demarcated by a layer of filamentous appearance. **(e):** labelling for cellulose. A fungal cell contouring a pit chamber shows a distinct, lucent layer in one portion (thin arrow); a thin and loosened layer along the labelled pit membrane (arrowhead); and a thicker layer seemingly becoming confluent with a more distended layer pervaded by opaque material (dark arrow); the latter is confluent with the coating (light, straight arrow). In the pit chamber of an adjoining vessel, an element (curved arrow), delimited by thin bands, and somewhat similar to that in **d,** is surrounded by a layer of fine filamentous matter. **(f):** a fungal cell wall is discontinuous or apparently overlaps at one point (light arrow). A collapsed hypha occurs nearby (thick arrow). **(g):** labelling for chitin is associated with fungal cell inner lucent layers (short, straight arrows). The region of cell F1 contacting the host wall has an imprecise lucent layer, with labelling mostly associated with outer opaque matter (long arrow) including a larger mass in a void space (large arrowhead) surrounding the cell; this irregular labelling is matched throughout by a non discernible plasma membrane, contrary to its clear presence elsewhere (between the short, light arrows). Bands of opaque matter and other fine components occur in the outer cell cytoplasm (in line with the large arrowhead), and a kink (small arrowhead) is visible in the plasma membrane. The outside opaque layers of cells F1 and F2 appear confluent at places (curved arrows).

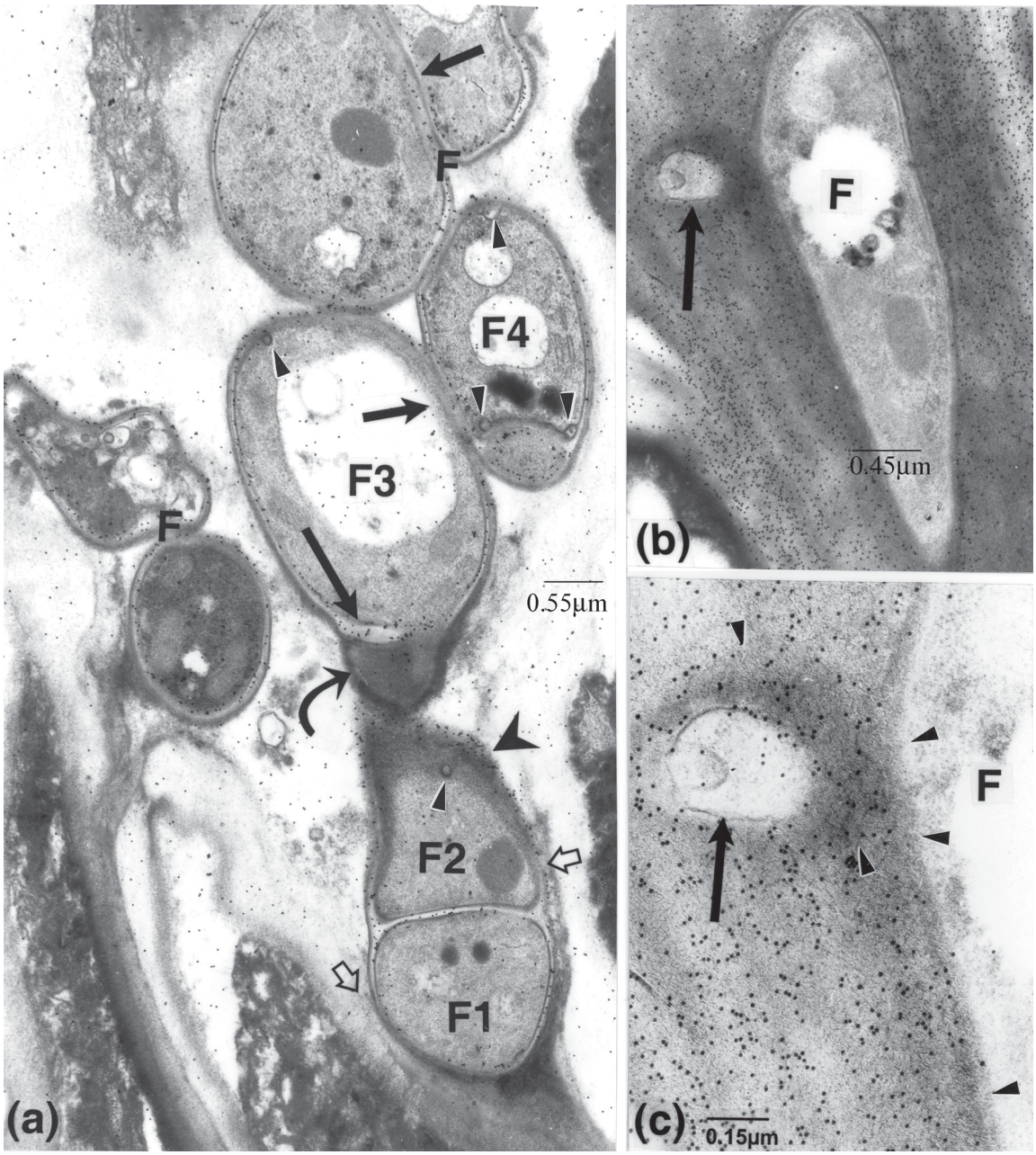

Figure 2. F, fungal cell. **(a):** labelling for chitin. Fungal cell walls are not uniformly or not labelled in regions of contact between them (short, dark arrows) or with host wall debris (light arrows). Gold particles overlay opaque matter (large arrowhead) surrounding cell F2. Cell F3 is separated from a cone of opaque matter (dark, curved arrow) similar to that in cell F2, by a seemingly discontinuous thickened septum (long, dark arrow); a more lucent band in this cell reaches from the cell content into the cone. Circular structures (small arrowheads) occur at apparent discontinuities or kinks in plasma membranes in cells F2-F4, in cell F4 at the juncture with an oval cap; a plasma membrane is not discernible in this cap, marked by a row of gold particles. **(b):** a small element (arrow) neighboring an intramural fungal cell. **(c):** an enlarged portion of **b**. Fine filamentous structures (arrowheads) extend through the fungal wall into the host wall, encircling the small element (arrow), delimited by only a membranous structure.

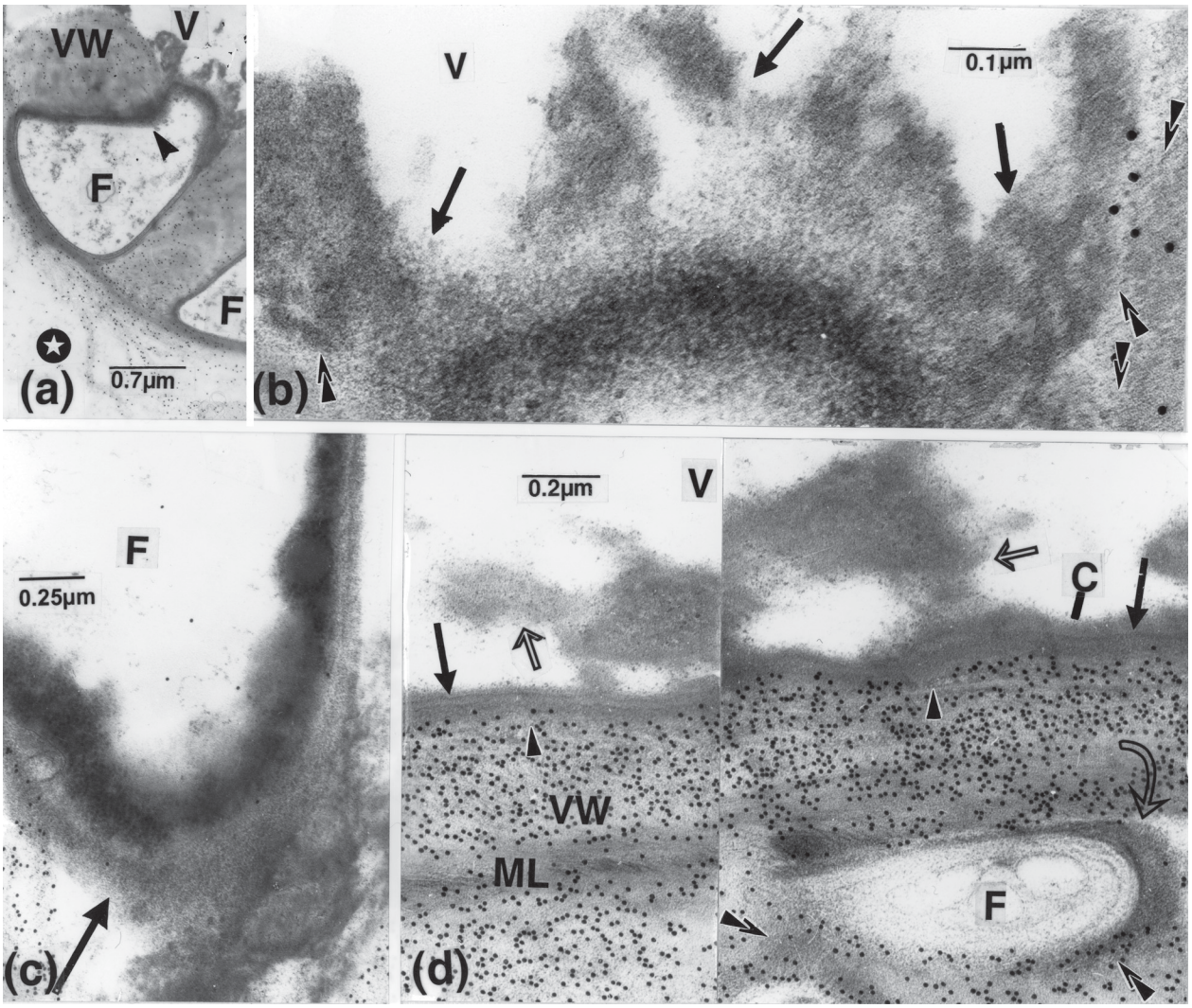

Figure 3. C, coating; F, fungal cell; ML, middle lamella; V, vessel element; VW, vessel wall. **(a):** labelling for cellulose. Fungal cells contour borders of opaque secondary thickenings. The pit membranes and adjacent host wall are altered (star). **(b):** enlarged portions from **a** (above the arrowhead). Filamentous structures stemming across the fungal wall and into the vessel lumen (arrows) extend into the vessel wall (superimposed arrowheads). **(c):** late stages of infection. Filamentous structures reach from the opaque matter present in an intracellular fungal cell to the matter surrounding it (arrow). **(d):** the compact coating, the mass of opaque matter connected to it (light arrows), and the extracellular material of the intraparietal fungal cell (curved arrow) contains fine filamentous structures extending into the host wall (superimposed arrowheads). The vessel wall displays similar structures (arrowheads) extending from the coating, containing paired bands (dark arrows).

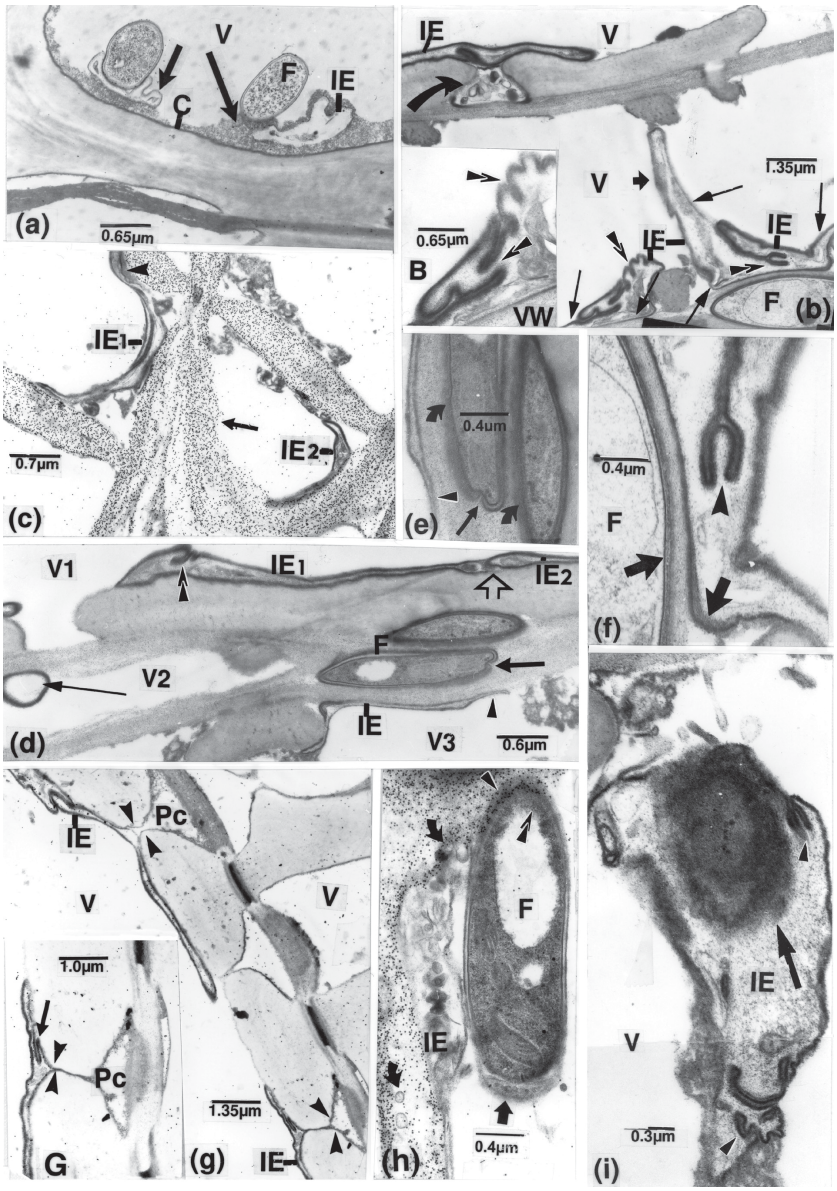

Figure. 4. C, coating; F, fungal cell; IE, irregular element; Pc, pit chamber; V, vessel element; VW, vessel wall. Intermediate to later stages of infection. Cross sections, except in **b** and **d**, longitudinal. **(a):** a vessel element contains typical fungal cells, a thin apparently collapsed hypha (short arrow), and an irregular element included in fine matter (long arrow), bordering a thin coating. **(b-i):** a variety of IEs. **(b):** IEs, delineated by mostly thin, opaque bands (long arrows) extend along vessel walls or across a vessel lumen (short arrow), enclosing either vesicular-like bodies and/or traces of fine matter. Similar bands circumscribe dark bodies in the pit chamber (curved arrow). Infolds in the bands are noticeable (superimposed arrowheads), whose interstices are of lighter opacity (enlarged in inset, B). **(c):** labelling for cellulose. IE1 is confluent with opaque material and other bands. One band (arrowhead) is included in matter similar to that of the IE. The flaring extremity of IE2 impinges upon the locally eroded (arrow) vessel wall. **(d,** and enlarged portion, **e):** in V1, an IE with an infolded margin (superimposed arrowheads) abuts on another one (open arrow). The circular body (long, thin arrow) in V2 (likely a fiber tracheid) may correspond to a cross-sectioned IE. A thin IE attenuates into a filament-like structure (arrowhead) in V3. Small intramural fungal cells are delimited by thin, discontinuous walls (thick arrow, **d**, and arrow, **e)**, or by a single opaque band elsewhere (curved arrows, **e;** note the similarity of this wall layer with that of IE (arrowhead) on the vessel wall). **(f):** an enlarged portion from **b,** including the horseshoe-like folding (arrowhead) of the IE bands; these are bordered by fine structures. The thin, outer opaque layer of the intramural fungal cell is analogous to the band of the adjoining IE (arrows). **(g):** thin IEs extending along a vessel wall, with the band affixed to it contouring the pit borders (arrowheads), and incurved at one point (arrow, inset G, enlarged portion). Note the absence of membranous structures in the IE and the near absence of gold particles of the chitin over it and elsewhere on the section. **(h):** labelling for cellulose. Vesicular-like bodies occur in the largest part of the IE; similar ones are seemingly unbound in the altered vessel wall (curved arrows) and in a mound capping the fungal cell (short arrow). The fungal wall is distinctly labelled where it encroaches on the host wall (arrowhead). Fine matter occurring in the adjoining fungal cell extremity (superimposed arrowheads) seemingly pervades its wall. **(i):** an IE, also delimited by bands infolded at locations (arrowheads), spans across a vessel lumen. The lower IE portion is confluent with a mass of fine matter, and the upper part includes a body (arrow) surrounded by opaque material containing arrays of fine filamentous-like structures.

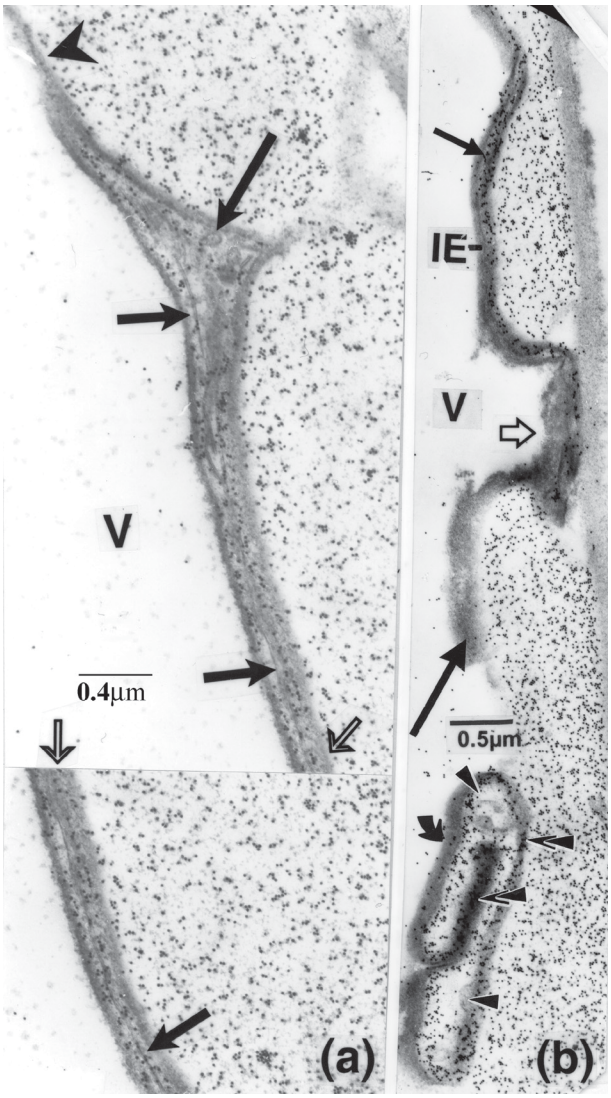

Figure 5. IE, irregular element; V, vessel wall. Labelling for chitin. **(a):** vesicular bodies (long arrow) and membranous structures (short arrows) are present in matching portions (light arrows) of a labelled IE, which spanned a long distance over the labelled vessel secondary wall. Note: gold particles are nil over the IE-delimiting bands, including that affixed to the vessel wall and extending (arrowhead) past the IE. **(b):** one part of an IE (short arrow), apposed to a labelled vessel secondary wall and bordered by opaque bands, is overladen with numerous gold particles, as is its extension (light arrow) over the pit membrane; the contiguous part (long arrow) is not labelled. A patch of strongly labelled material (except in the presence of fine components, arrowheads) is encircled by an opaque band (curved arrow); strong labelling of the band is limited to the portion encompassing the vessel wall (superimposed arrowheads).

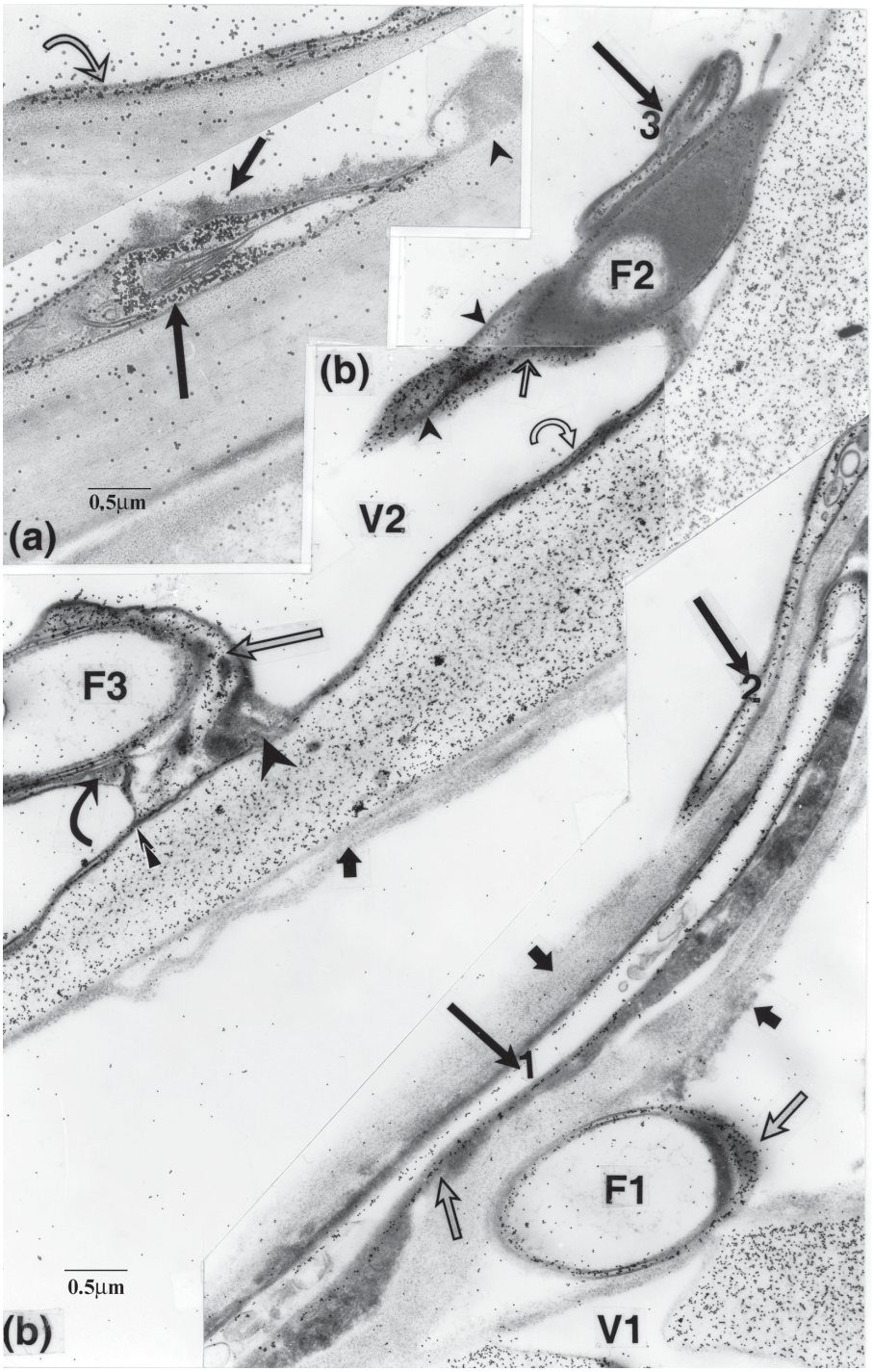

Figure 6. F, fungal cell; V, vessel element. Labelling for chitin. **(a):** numerous gold particles ornament the lucent matter crossing the IE (long arrow) and circumscribing an array of membranous structures, one ending (arrowhead) in a mass of fine matter confluent with that surrounding the IE (short arrow). Gold particles in the other part of the IE have attached to a lucent layer for a distance but cover wholly the other, thin end of the IE (curved arrow). **(b):** microhyphal elements (long arrows 1 and 2, the element indicated by arrow 2, being somewhat similar to the IEs illustrated above) in the unlabelled, dislocated host walls (short, opaque arrows) mostly do not display a lucent wall and are irregularly labelled, with some particles occurring over the opaque matter (light, short arrows) bordering the element, as over that capping cell F1. In V2, cell F2 with thin, poorly labelled walls is encased at one end by labelled homogeneous matter (thin, light arrow), containing thin bands (small arrowheads); this matter is confluent with the layer, present as paired bands (curved, light arrow) on the vessel wall, reaching cell F3. An IE (long arrow 3), forked at one end (compare with Fig. 4f), is juxtaposed to this element. Cell F3 is surrounded by labelled matter, at one end as a lucent mass (long, light arrow) and on the opposite side, as opaque material (curved, opaque arrow), both being bound by opaque bands; one of these merges with opaque matter (large arrowhead) on the vessel wall and the other with the single band on the wall (superimposed arrowheads). The vessel walls are strongly labelled.

Peculiarities of labelling for chitin

Although few gold particles of the probe occurred in control tests (comparable, for example, to those present on walls of some small vessel elements, as in Fig. 7e), the number of particles in some vessel lumina was relatively high. This situation mainly occurred next to strongly labelled IEs that occurred on or in unlabelled primary walls and pit membranes (Fig. 7a-g). Single or superimposed IEs opened up at places on the vessel lumen (Fig. 7a, b; compare with Fig. 6a). Gold particles did not attach to free opaque matter in these cases, except when it was closely associated with IEs, membranous structures, or lucent bands (Fig. 7e, A inset). High numbers of particles were also noticeable in elements extending across vessel lumina, and, once more, the element-limiting, firm bands, and their extensions along or contouring vessel walls were free of labelling (Fig. 7d, f, g). Networks of meandering opaque bands, parts of or connected to the IE or connected to their limiting bands, were also free of labelling (Fig. 7d, g). The typical labelling of the lucent fungal wall layer in Fig. 7d, the absence of gold particles over the cell content, except over traces of material in the vacuoles (compare with Fig. 1a), and the buckling of the plasma membrane in this cell are to be noted.

In other situations, numerous gold particles, bound to traces of dense material, occurred in the vicinity of opaque, paired or single bands and membranous structures which had seemingly pervaded unlabelled vessel walls (Fig. 8a, b). Sparse gold particles occurred elsewhere over the section, except in areas corresponding to fungal elements. The presence of these particles in vessel lumina could, at first sight, also have been associated with chipped portions or remnants of vessel secondary walls (Fig. 8c, d); however, in figure 8c, due to the occurrence of intermixed opaque bands and vesicular bodies and traces of ruptured, unlabelled middle lamella in conjunction with these, it was difficult to visualize what corresponded to the original cell walls. When vessel secondary walls were eroded, this alteration still appeared to be associated with opaque bands and more diffuse matter related to IEs (Fig. 8d; compare with the material confluent with cell F2 in Fig. 6b); it is to be noted here that the labelled band contacting the unlabelled pit membrane is texturally similar to a fungal cell wall (Fig. 8d). The only content in these cells was texturally similar to extracellular material, and in turn to coating that, in figure 8d, covers a pit membrane of increased opacity.

Links of fungal cells with IEs and the opaque matter

The IE-delimiting bands often appeared to be confluent with the outer fungal wall layer (Fig. 9a). In some cases, IEs' bands seemed to encapsulate larger fungal elements (Fig. 9b, c; see also Fig. 6b), as indicated by the chitin-labelled content of IEs that were continuous with the fungal cell walls (Fig. 9b). Similarly, the IE-limiting band appeared to coalesce or to be continuous with material (analogous to coating) apposed to vessel walls of the same or adjoining vessel element (Fig. 9a, c). IEs' bands also merged with fungal cell walls, making them appear as if they were double-layered at some locations (Fig. 9d, e). Typical fungal cells and IEs were also connected to one another by means of crowded arrays of fine filamentous-like structures, often grouped in large masses (Fig. 10a, b).

The content of many fungal cells was nearly homogeneous, not showing typical organelles and internal membranes, except for a few particles of ribosomal appearance bordering an area of more lucent filamentous structures (Figs. 9b, d, 10b). In contrast, the plasma membrane was regular, except for some small localized kinks (Fig. 9d, e). In some cases, similar ingrowths were the only visible membrane-like structures in the cell, whereas other juxtaposed cells, in the same hypha, had a distinct plasma membrane and a different type of content (Fig. 10a).

Figure 11a may be considered to summarize the main aspects of tissue invasion by the pathogen, characterized by tiny intramural hyphae similar to the IEs, by large masses of fine matter connected to these IEs and associated with larger fungal cells, and by similar tiny elements in vessel lumina. Connections of similar matter with fungal cells by means of filamentous structures and with coating material also occurred in vessel lumina (Fig. 11b).

DISCUSSION

The crucial problem in this work was to discover the possible origin of the IEs and to obtain some insight into their nature. It must first be emphasized that using current terminology, based mainly on observations of fungal growth *in vitro*, was often problematic in attempting to describe these novel elements. For example, elements are described as filaments or layers that might have been part of a larger frondose body. Hence, these could cover a large surface or be part of ramifying elements and be difficult to visualize in their entirety, an undertaking, however, which could undoubtedly provide interesting information. One may raise questions as to whether the tiny elements could correspond to collapsed or degenerative hyphae. First, collapsed hyphae would not be expected to have one of their limiting structures closely contour vessel rims and pit borders, whereas others become deeply infolded or recurvate, or have bridged fungal cells (see Fig. 6b). Also, these IEs in vessel elements were quite similar to elements that were well confined within host walls. The same types of considerations would apply to the question of degenerative hyphae, and one would expect to find in this case various intermediate stages of fungal wall degradation; but this was not the case.

In any event, these IEs and associated elements and structures need to be considered in a new perspective. If they were of pathogen origin, it means that they could maintain a potential for infection, as shown here and in other works for more typical larger fungal cells with defective walls, particularly in microhyphae spanning, non septate, long distances in host cell walls (Charest *et al.* 2004; Ouellette *et al.* 2001, 2002, 2004a, for example). Alterations or absence of fungal walls in hyphae penetrating host walls have

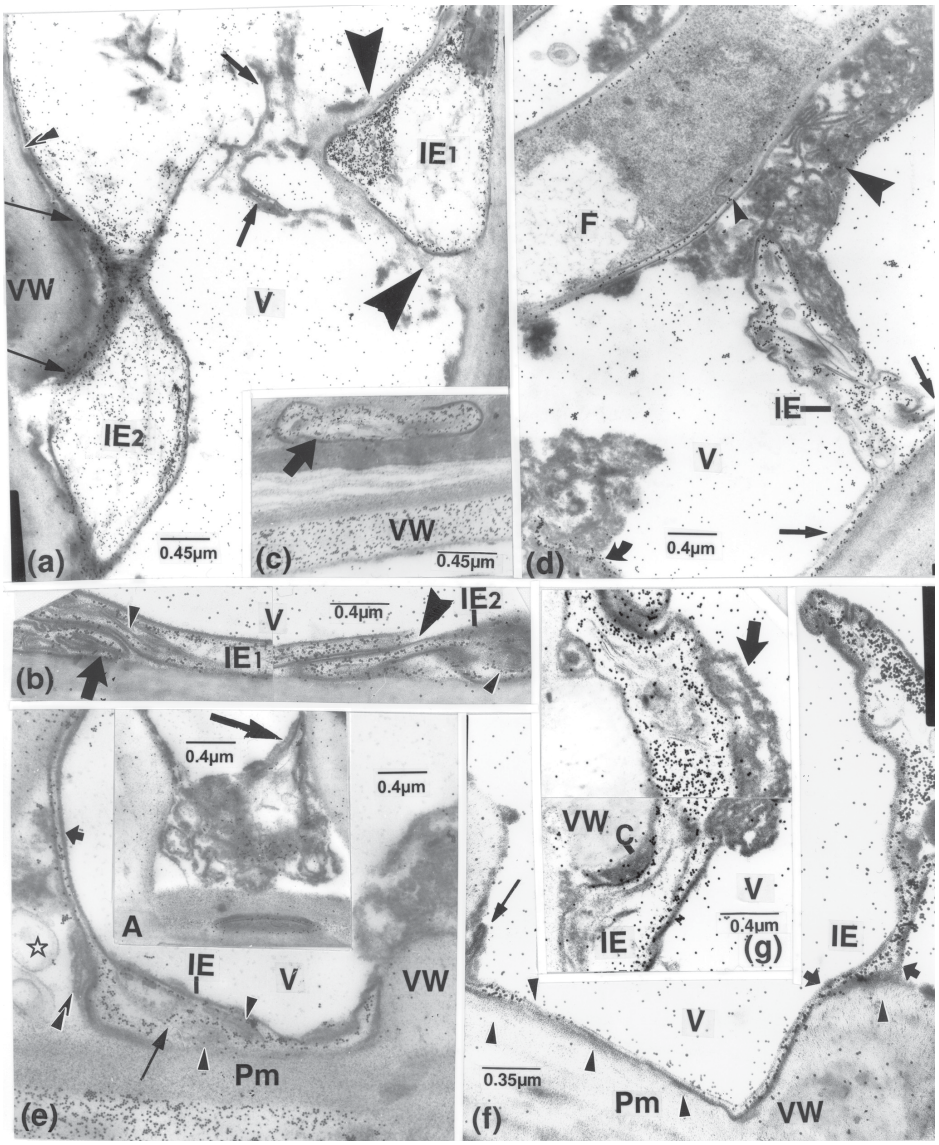

Figure 7. C, coating; F, fungus; IE, irregular element; Pm, pit membrane; V, vessel element; VW, vessel wall. Labelling for chitin. **(a):** numerous gold particles have attached to large IEs which are delimited solely by opaque bands. IE1 is surrounded by material (between large arrowheads) similar to and confluent with the vessel wall. The IE2 limiting layer facing the vessel lumen is somehow interrupted next to intertwining bands (short, thick arrows); on the vessel wall, this layer is generally thin, but parts next to it, possibly due to their being obliquely sectioned, are thicker and then appear highly labelled (between long, thin arrows). Another opaque band (superimposed arrowheads) is included in the vessel wall, bordering, lower down, a void space. **(b):** two superimposed strongly labelled IEs (the print portion of IE1 was reduced by 2 cm), containing membranous structures and fine matter (small arrowheads). One extremity of element IE1 (part of which was cropped out) opens up (large arrowhead) onto the surrounding medium, and the lower limiting band at the other extremity (arrow) overlaps a network of other meandering bands. Gold particles occur over opaque matter being part of IE2 or surrounding it. **(c):** a labelled IE-like element (arrow) occurs in an altered, unlabelled middle lamella, next to a strongly labelled vessel wall. **(d):** concentrations of gold particles occur in conjunction with: 1) lucent layers of fungal cells; 2) the IE (containing membranous structures) and its flaring portions on the vessel wall (straight arrows); and 3) the lucent band (curved arrow) extending through unlabelled material in the vessel lumen. Particles are sparse over the host primary wall, the fungal cytoplasm, and the network of opaque bands (large arrowhead), confluent with the IE. The numbers of particles in the vessel lumen and the fungal cell vacuole, containing traces of fibrillar components, are similar. An apparent inflection of the plasma membrane (small arrowhead) occurs in the fungal cell. **(e):** a thin IE on the vessel wall (short arrow) is confluent with a larger portion overlaid by several gold particles, next to unlabelled pit membrane and vessel wall thickening. The IE-limiting band, at one point, appears thicker and tortuous (superimposed arrowheads), and the labelled material (long, thin arrow) contours blobs of unlabelled matter (arrowheads). Vesicular-like bodies (star) are present between the IE and the vessel wall. The inset (A), from a contiguous part of the section, shows a labelled mass of opaque matter which adjoins vesicular-like bodies circumscribed by thin opaque bands (arrow). **(f, g):** strongly labelled IEs; in **f**, it flares out into a thin element over the unlabelled vessel wall (short arrows) and pit membrane, ending up in paired bands (thin arrow), adjoining a coating-like layer, and in **g** the band affixed to the unlabelled vessel wall coalesces with opaque matter similar to coating material. In **f**, filamentous-like structures extend from the IE band into the vessel wall (arrowheads). Gold particles are absent over the IE-limiting bands and connected networks in **g** (thick arrow), and their numbers over the vessel wall and lumen are obviously many times fewer than over the IEs.

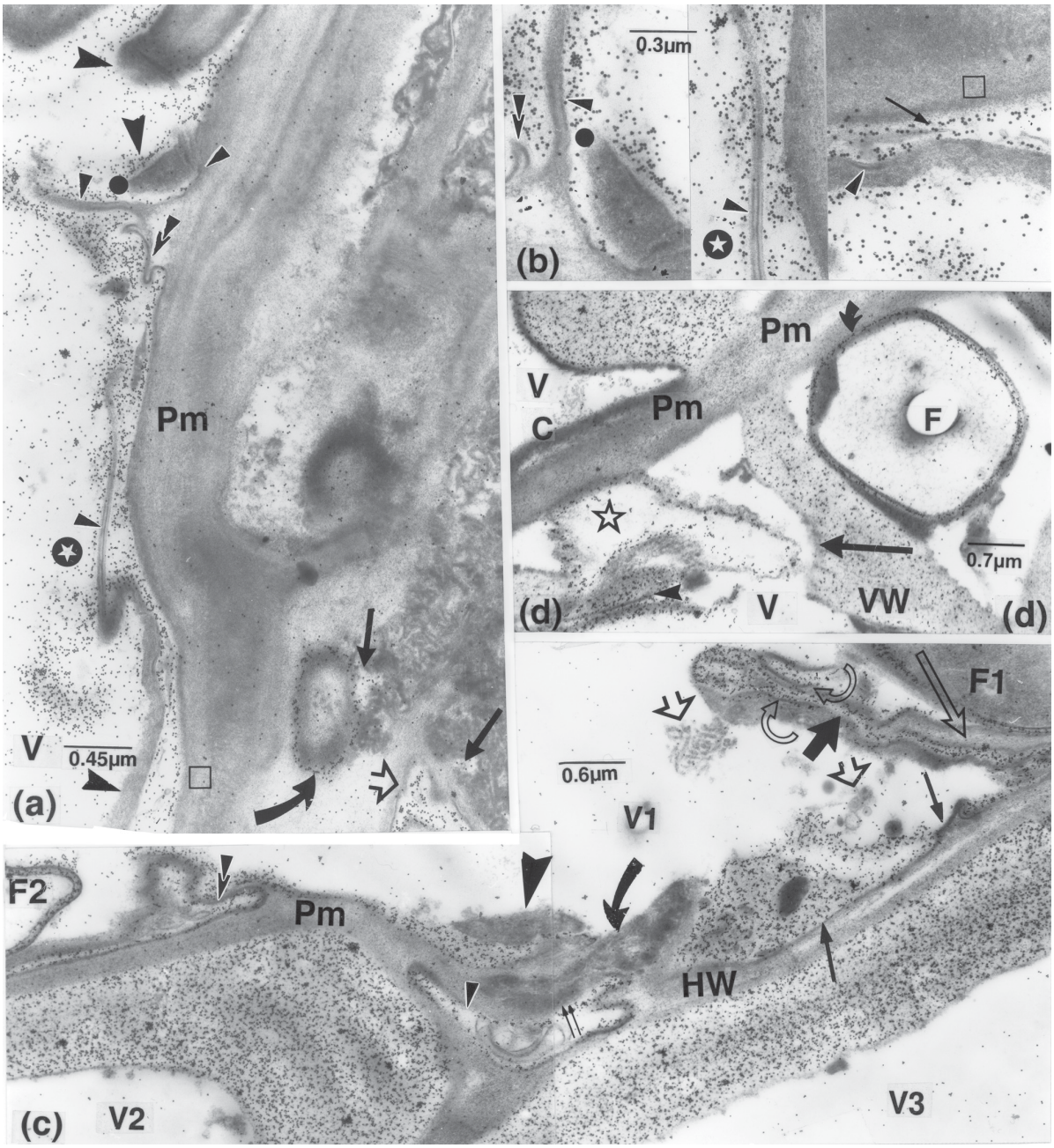

Figure 8. C, coating; F, fungus; HW, host wall; Pm, pit membrane; V, vessel element; VW, vessel wall. Labelling for chitin. Of the host cell walls, only the vessel secondary walls are labelled. **(a)**: numerous gold particles occur near or between altered portions (large arrowheads) of unlabelled host wall; in the adjacent cells, they cover a ring of opaque material surrounding a fungal element (curved arrow) and islands of lucent matter (small arrows), one delimited by thin opaque bands (open arrow). The dark circle, star, and square indicate areas that are presented as enlargements in **b. (b)**: host wall alterations are associated with opaque bands, single or paired (arrowheads), or locally bent (superimposed arrowheads), or with membranous structures (thin arrow). Gold particles in these regions have attached to traces of opaque matter. **(c):** in V1, a fungal cell (F1) is next to an unlabelled stretch of material (long, light arrow) which is structurally similar to the also unlabelled host wall; this material is bordered by a column of labelled material (thick arrow) extending into the vessel lumen, delimited at places by opaque bands (curved, light arrows) which are similar to that present along the distended wall (thin arrows). A bulge of the intramural labelled element into the vessel lumen (superimposed arrowheads), near labelled cell F2, is surrounded by numerous gold particles; another element (small arrowhead), in the intercellular area, has its upper limiting band (double small arrows) confluent with opaque matter (curved, dark arrow) associated with a rupture in the primary host wall. An unlabelled piece of wall-like matter (large arrowhead) overlays another stretch of labelled substrate. Open arrows point to fine structures and vesicular-like bodies. **(d)**: gold particles overlay: vessel wall thickenings; the opaque material bordering a straight band (arrowhead) next to an altered thickening; limiting bands of an IE (star), matching a notch (long arrow) in the thickening; and a fungal cell lucent layer and its confluent opaque part adjoining an unlabelled pit membrane (curved arrow). Note: similarity in the IE layer and fungal wall on both pit membranes, the more opaque one being covered by a coating in the adjacent vessel.

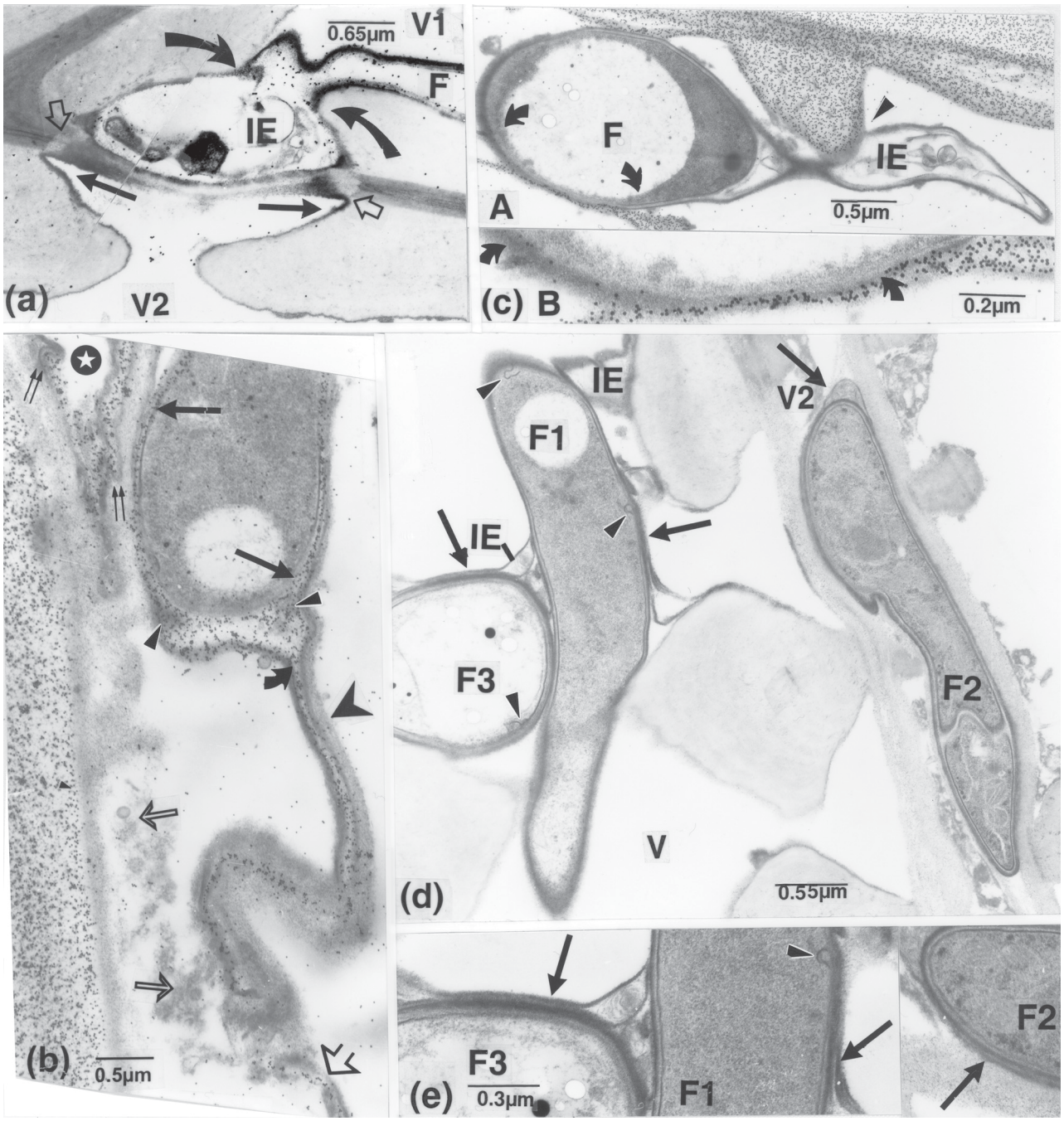

Figure 9. F, fungus; IE, irregular element; V, vessel element. **(a, b):** labelling for chitin. **(a):** the opaque layers contouring a pit chamber (curved arrows) are continuous with the outer, opaque layer of a long hypha and are connected to the coating in V2 (straight, opaque arrows) and across the paler pit membrane portions (light arrows). **(b):** gold particles occur over: 1) lucent wall layers in the fungal cell (arrows) confluent at its extremity with a mound of similar substrate, itself continuous with the labelled IE (curved arrow); 2) some opaque matter at the base of the fungal cell and of the IE (small arrowheads); 3) a layer, also bordered by a compact band (thick, light arrow), coming at a right angle with the E; and 4) an irregular, microhyphal-like element (star) also precisely delimited by bands (double small arrows) in the unlabelled, altered host wall (next to the labelled vessel wall). An unlabelled layer, encompassing both the IE and the fungal cell (large arrowhead), may correspond to detached vessel wall components. Vesicular-like structures (thin, light arrows) occur next to the IE and in the region of the altered host wall (compare with Fig. 4h). **(c):** labelling for cellulose. IE-limiting bands (in A) are continuous with the outer opaque layer of a fungal cell. One IE layer is seemingly interrupted, meeting with a similar layer (arrowhead) contouring the labelled vessel wall thickening. An opaque layer (enlarged portion, between curved arrows, in B) surrounds the fungal wall which impinges upon the host wall (compare with Fig. 4h). The layer displays filamentous structures, connected to similar matter in the fungal cell. **(d):** bands, similar to those limiting IEs coalesce (arrows) with walls of cells F1 and F3 and with the intramural cell F2. Thin structures (arrowheads) extend from the cell margin inwards. **(e):** enlarged portion of **d.** At points of contact with IEs, the fungal walls appear as if they were double layered (arrows). The content of cell F1 is nearly homogeneous, with its central part being imperceptibly delimited.

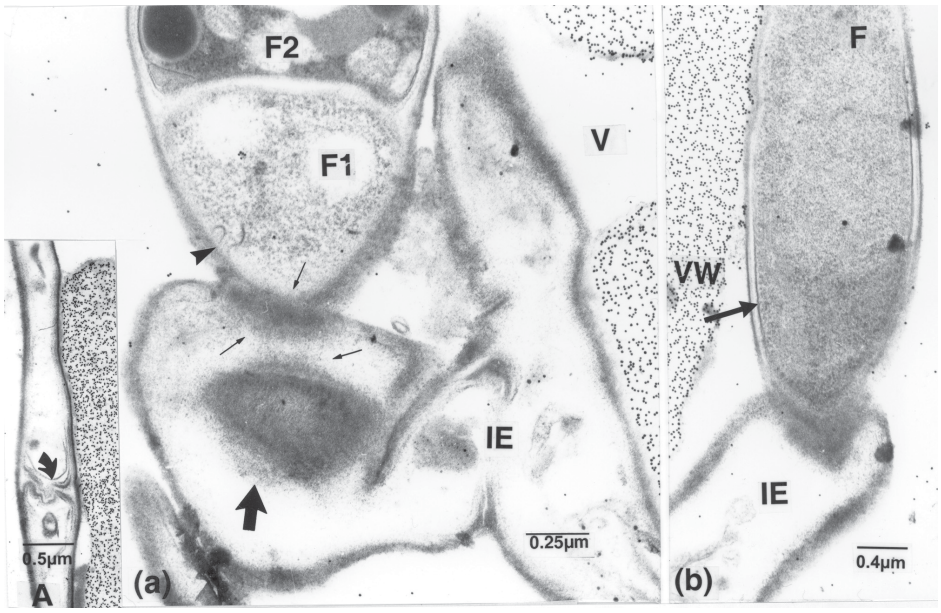

Figure 10. F, fungus; IE, irregular element; V, vessel element; VW, vessel wall. Labelling for cellulose. **(a):** filamentous structures (small thin arrows), connected to cell F1 and to an IE, reach a body in IE (large arrow) which is surrounded by similar structures (compare with Fig. 4i). F1 and F2 content differs; in F1 it does not display a plasma membrane and two structures (arrowhead) extending from the cell periphery inwards. The inset (A) is from a lower portion of the IE, showing a septum-like division (curved arrow). **(b):** similar links of a fungal cell with an IE. The cell content is nearly homogeneous with a lighter central zone displaying filamentous structures extending throughout the more opaque area next to the IE. Except for a distinct plasma membrane (arrow), no other membranes are discernible in the cell.

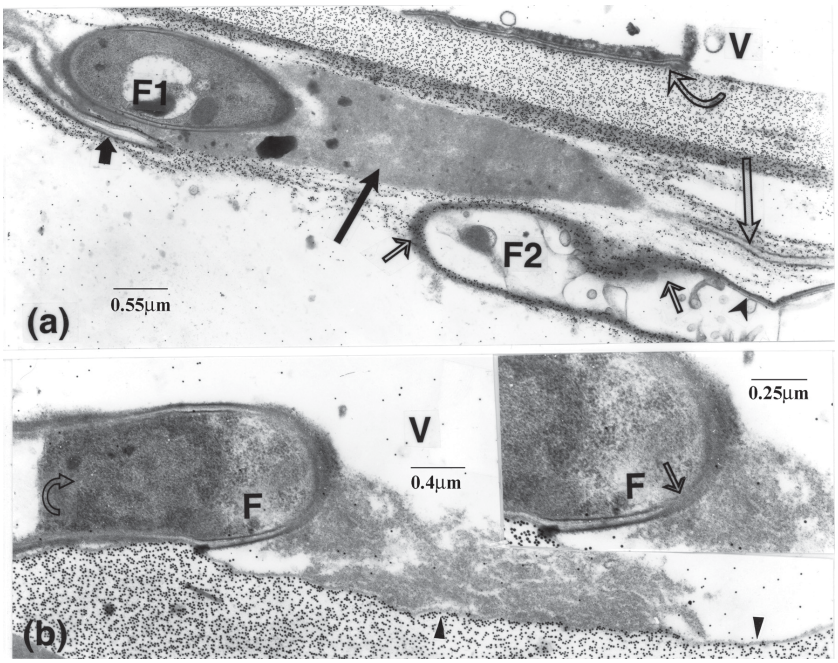

Figure 11. F, fungal element; V, vessel element. **(a, b):** labelling for cellulose. **(a):** next to a vessel element, an intramural mass of fine matter (long, dark arrow) borders cell F1 and abuts below (long, light arrow) on a tiny element, delineated by opaque bands. Cell F2 is delimited by an opaque layer overlaid by many gold particles (thin, light arrows), except the portions containing a lucent layer (arrowhead). A similar layer occurs in cell F1 and at the tip of the adjacent microhyphal element (short, opaque arrow). A thin IE is included in opaque matter apposed to the vessel wall (curved arrow). **(b):** a mass of homogeneous matter, connected to a fungal cell by means of filamentous-like matter (arrow, inset), is separated from the vessel wall by a band of similar opacity (arrowheads). The area corresponding to the cell nucleus (curved arrow) is discernible but not clearly demarcated, whereas the plasma membrane nearby is distinct.

now also been well illustrated in other systems using immunocytochemical means (Charest *et al.* 2004; O'Connell and Ride 1990; Tenberge *et al.* 1996). Moreover, some fungi may grow as protoplasts in culture as well as in nature (Tyrrell and Macleod 1972). Granted that the general or localized absence of typical walls would not be fatal to fungal development *in vivo*, then the ability of the fungus to develop into innumerable forms and sizes, as presently perceivable, can be more easily understood.

Some pivotal points of convergence for the pathogen origin of IEs and some problems inherent to this situation have been noted thus: as structural analogies between the outer opaque layer of typical fungal cells and the IE-delimiting bands and networks of associated networks; as an association of these elements or even of their individual limiting bands with host wall penetration and alteration; and the inferred analogy in structure and content of the IEs and associated matter lining vessel walls with the invasive microhyphae (see also Ouellette and Baayen 2000; Ouellette *et al.* 1999a, 2001). Similar microhyphae, which were demonstrated in sterilized wood sections and in elm trees (Casagrande and Ouellette 1971; Ouellette *et al.* 2004d), have also been well characterized by Hale and Eaton (1985a, 1985b). Hence, these types of elements are not to be ranked as inert components. However, IEs occurring in vessel elements would be less confined by host walls than generally are the elements first described as microhyphae. In this perspective, the following is theoretically perceivable: if the content of a fungal cell of about 3 μm in diam became flattened out possibly into a 0.1 μm thick plate-like IE (as some of these were estimated to be), the host wall surface then covered by the same fungal mass would be many times greater; similarly, if this mass divided into tiny filaments, the possible points of attack would be that much greater. Also, this type of development, in the absence of rigid limiting structures, could translate into a greater efficacy of fungal components apt to be translocated more rapidly towards the pathogen invasion front. Previous observations on the intense physical interaction of microhyphae with middle lamellae and primary walls of vessels in carnation (Ouellette *et al.* 1999a) indicated that surface interaction may be critical to infection by the pathogen. Indeed, close contact of fungal elements, particularly with thin or defective walls, might promote the efficacy of pectic and other cell wall degrading enzymes (Baayen *et al.* 1997; Charest *et al.* 2004; Tenberge *et al.* 1996). The possible role of these enzymes, as discussed by the last authors, may not necessarily be properly evaluated according to their amounts obtainable from cultures of the pathogen (Cooper *et al.* 1981, as an example).

Striking transitions were observed between IEs and typical fungal cells. Clearly, in assuming that IEs were of fungal origin, they must initially have formed from typical fungal cells, as an extension of microhyphal-like elements or otherwise. Connections between these elements and fungal cells were observed. However, most IEs extended freely for long distances along or even inside vessel walls (Fig. 5a, for example; see also Ouellette and Baayen 2000), or across vessel lumina (Fig. 4b, i, for example), without displaying evident links with typical fungal cells. Moreover, as shown by the types of connections between IEs and hyphae, it appeared that some larger hyphal cells might even have formed as enlargements from the former (Fig. 9b-d). Fungal cells and IEs were also interconnected by means of microfilamentous-like structures, whereas arrays of similar structures present in host walls were traceable to fungal cells, IEs, the coating and similar opaque matter. These observations add to previous ones from other studies (Ouellette *et al.* 1995, 1999a, 2002, 2004a, 2004d) showing the possible general occurrence of these structures in fungal infection and the need to unravel their exact nature.

The strong labelling of IEs for chitin can be considered meaningful in cases where they contacted unlabelled vessel walls, another feature substantiating that IEs were not primarily of host origin. When labelled IEs were in contact with labelled secondary vessel walls, they either generally contained membranous structures or were separated from them by an unlabelled opaque band, except where they impinged upon these vessel walls, as did the compact coating. The strong labelling of vessel walls for chitin is in agreement with reports that chitin analogues occur in vessel walls in other plants (Benhamou and Asselin 1989). In regular fungal cells, the probe specifically attached to the inner, lucent wall layers that are known as the chitin backbone of the fungal wall (Ruiz-Herrera 1992; Wessels 1994).

The abundance of gold particles of the chitin probe in vessel lumina, compared with the few elsewhere in void spaces and controls, could have been related to substrates released from the vessel wall. In this instance, the presence of the cellulose probe particles should have been alike in similarly colonized vessel lumina (as verified in some contiguous sections), but those were found to be almost nil therein. In comparison, breakdown products of host walls surrounding intramural fungal cells were strongly labelled (see also Ouellette and Baayen 2000; Ouellette *et al.* 2004a). As numerous gold particles occurred next to strongly chitin-labelled IEs apposed to unlabelled host walls for an appreciable distance (e.g. young vessels with only sparse thickenings), chitin-labelled compounds in vessel lumina could then have mainly come from IEs. The fact that chitin or chitosan moieties can be released from fungal cells has previously been demonstrated in some *Fusarium*-infected plants (Hadwiger *et al.* 1981). In staghorn sumac, vessel secondary walls did not label with the WGA probe and gold particles were present in vessel lumina only again in proximity of labelled fungal cells (unpublished). In similar and simultaneous tests with samples of the resistant carnation plants (Ouellette *et al.* 1999a, 2002), gold particles were few over vessel lumina and the uncommon coating except where it also encroached on vessel walls (see also Fig. 12C in Nicole *et al.* 1994); moreover, IEs were lacking in the resistant cv. At any rate, when vessel secondary walls were eroded, this action was associated with IEs and other matter (see Ouellette *et al.* 2004c), a feature which would be most striking in figure 8c, if the largest labelled mass in vessel V1 was really part of the

vessel wall. In any event, the features illustrated in this figure and others indicate that host wall alterations occurred in a non-classical manner.

If the occurrence of gold particles in IEs have bound to possibly dispersed chitin moieties, the occurrence of this substrate therein would still need to be explained. As one possiblility, their labelling patterns could primarily have been related to autolysis of chitin or to its alteration as an effect of extraneous enzymes, with these being apt to permeate the often thick, element-delimiting opaque bands. This possibility can at least be questioned. If, in opposition, the profuse chitin occurrence in IEs corresponded to native chitin, then its particular location would seem to deviate from the main model proposed for chitin synthesis and deposition, as established for fungi growing in culture (Ruiz-Herrera 1992; Sietsma *et al.* 1995; Stewart and Rogers 1983; Wessels 1994). However, exceptions to this have been noted that were attributed to stress or other unfavorable factors (see Gooday and Schofield 1995). Hence, it would seem that an interaction of several chitin synthase genes, some existing in "latent" forms with boosted activities in some instances, might be involved and active at the plasmalemma level. Examples are the high chitin synthase activities in protoplasts of *Aspergillus* sp. (Archer 1977; Hardy and Gooday 1983) and the chitin-rich septa in "morphologically aberrant hyphae in *Neurospora* due to a disruption of a chitin synthase gene" (Yarden and Yanofsky 1991, citation on p. 115 in Gooday and Schofield 1995). In this perspective, it is noteworthy that irregularities in chitin labelling of typical fungal cells in the present and other cases (unpublished observations) were associated with apparent anomalies of the plasmalemma. In any event, labelling for chitin in IEs or at their margins was noticeable only when they contained cytoplasmic-like structures or were confluent with portions containing these structures.

Contrasting with the presence of opaque coating and other associated matter which were present at all stages of infection in both the susceptible carnation plants, IEs were not detected in early infection in the susceptible plants nor in the resistant plants at all stages of infection. However, some components similar to those of the IEs also occurred in the coating, including homogeneous opaque matter often of filamentous appearance, and paired bands. Small filament-like structures delimited by similar bands also occur in vessel lumina in elms and non-hosts infected with *Ophiostoma novo-ulmi* (Ouellette *et al.* 2004b), and in staghorn sumac infected with *Fusarium oxysporum* f.sp. *callistephi* (unpublished observations). In support to this aspect, the following cases may be referred to: a pure, cellulose synthesizing enzyme, similarly fixed for TEM observations, was illustrated as having a filamentous appearance (Colvin 1980), and movement proteins located on tubules have been observed to extend freely outside virus–infected host cells (Ritzenhaler *et al.* 1995). Likewise, numerous fungi are known to produce fimbriae (Poon and Day 1975), now shown to contain RNA and collagen as some main components (Celerin *et al.* 1994, 1996), that extend freely into the surrounding medium. Present observations, therefore, might be of germane importance not only in the study of plant diseases but also of other systems.

If the opaque coatings were somehow related to IEs and microhyphae, one may assume that host defence reactions might contribute to hinder further action or development of coatings. Hence, the fungitoxic and wall-enforcing compounds formed in resistant cv. Novada (Baayen *et al.* 1996; Ouellette *et al.* 2002) might also be active in fixing the coating material. In this scheme, a pathogen origin of the coating would not necessarily preclude the subsequent or simultaneous occurrence of other components of host origin. It seems appropriate to re-emphasize that the coating and IEs did not label for pectin and cellulose in the infected plants studied (Ouellette *et al.* 1999a, 2002, 2004b, 2004d, references cited therein, and unpublished observations).

In summary, reliable evidence has been presented that the IE elements were related to host tissue and cell alterations, despite their not displaying a so-called normal organization, and hence were most probably of pathogen origin. Fine matter, often of a filamentous appearance, associated with IEs, fungal cells and coating also seemed to be involved in host attack. This matter was not only connected to fungal cells (as also shown in Ouellette *et al.* 2004c) but was similar to the observed, nearly homogeneous and dense content of some fungal cells, including a large seemingly unbound portion corresponding to the nucleus region, conditions which can be considered to be atypical of eukaryotes. Similar observations have been made concerning *O. novo-ulmi* growing in elm trees as well as on other substrates (Chamberland and Ouellette 1977; Ouellette *et al.* 1995, 1999b) and *Fusarium* in sumac (not illustrated). Evidence for the production and extrusion of constituents from fungal cells, of this and other pathogens that are apparently able to invade in an unrestricted manner host tissue is increasing (see discussions in Ouellette and Baayen 2000; Ouellette *et al.* 1999a, 1999b, 2000, 2002, 2004c, 2004d). Material in the form of tiny filaments has also been observed to be associated with fungal cells in staghorn sumac and eggplant infected respectively with *F. oxysporum* and *Verticillium dahliae* Kleb., and likewise with cell disturbances (unpublished observations). However, it is not so much the extrusion of such matter that may cause concern, but its spread and activity once outside the cell.

Finally, granted that IEs are active elements, and hence most probably of pathogen origin, they could play an important role in final intensive tissue invasion, as in other wilt diseases (Bishop and Cooper 1983). Alternatively, IEs could be involved in disease recurrence in perennial plants, as for Dutch elm disease (Ouellette and Rioux 1993), even though only a limited number of typical fungal cells might be directly regenerated at one point or another from these elements, or from their fusions. From these few cells, other cells might proliferate to invade more fully the moribund tissues and then to produce the next source of inoculum.

ACKNOWLEDGMENTS

We express our gratitude to all the people from LFC who have contributed to this work, in particular: Ms. Nicole Lecours, for her assistance in conducting the TEM observations, to Ms. Pamela Cheers, Ms. Isabelle Lamarre, and Mr. Benoît Arsenault for their technical editing of the manuscript, to people in the Informatics Services, to Dr. Ariane Plourde and the management of this Centre who have provided the facilities for completing this work. This research was supported in part by grants from NSERC, Canada, to the senior author.

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