General discussion with emphasis on the occurrence of the tissue-invading opaque matter

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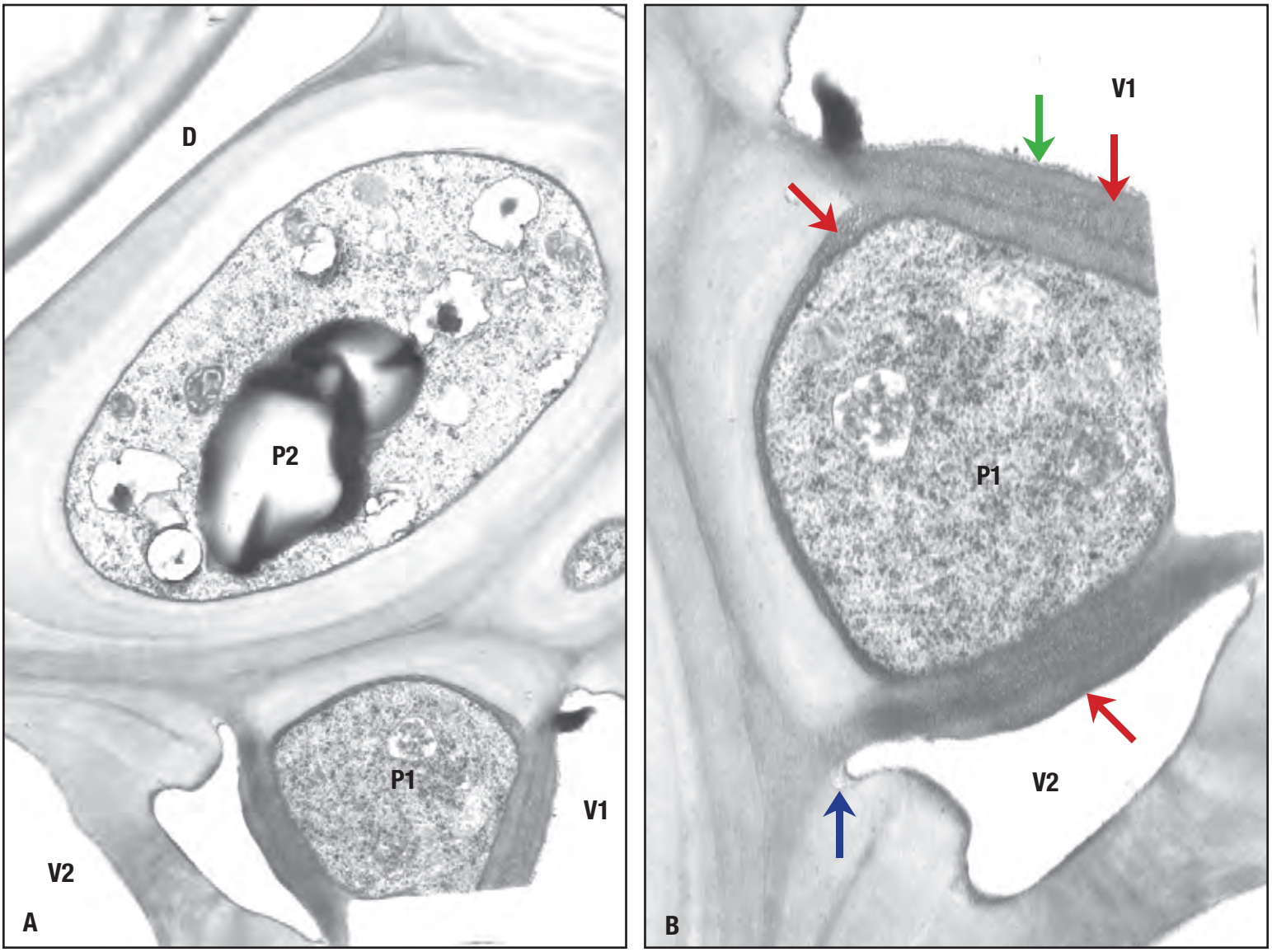
"*What might have been is an abstraction*
Remaining a perpetual possibility
Only in a world of speculation" (T.S. Eliot, from Four Quarters)

It is likely a truism mentioning that fungal wilt diseases are some of the most intricate ones to comprehend, in the sense that demarcating between the fundamental and secondary causes and between the primary and secondary effect is complicated by the fact that the initial determinant course of infection is located in vessel elements. In the models proposed concerning the mechanics of fungal wilt diseases, it is for the most part difficult to sort out the indubitable evidence from the circumstantial one. A main reason for this situation, as we see it, is that the exact role of the pathogens in their respective hosts has not been properly studied or when they were investigated at a certain depth, many of the resulting observations were not properly recognized because they were seemingly marginal or too unusual. Indeed, comments received regarding our own observations, amongst all kinds of dubious reasons, were that they were "over-interpreted" or too "speculative". One has to realize, however, that extensive speculation has been at the root of the classical models to explain mechanisms of symptom expression and of possible resistance regarding these diseases. In this sense, the well written paper by Talboys (1972) may be cited as a good example. One is probably not mistaken saying that the concepts presented in it have been perpetuated one way or another in many of the subsequent publications concerning these diseases, most often from observations restricted to LM studies and at low magnifications. It is perhaps for this reason that observations susceptible to challenge some of these views are awaiting for accreditation and a more decisive adhesion. This author does not feel reluctant, then, to broaden the speculation on the subject, which we nevertheless consider to be based on sound observations, with the hope that a more realistic comprehension of these diseases will emerge.

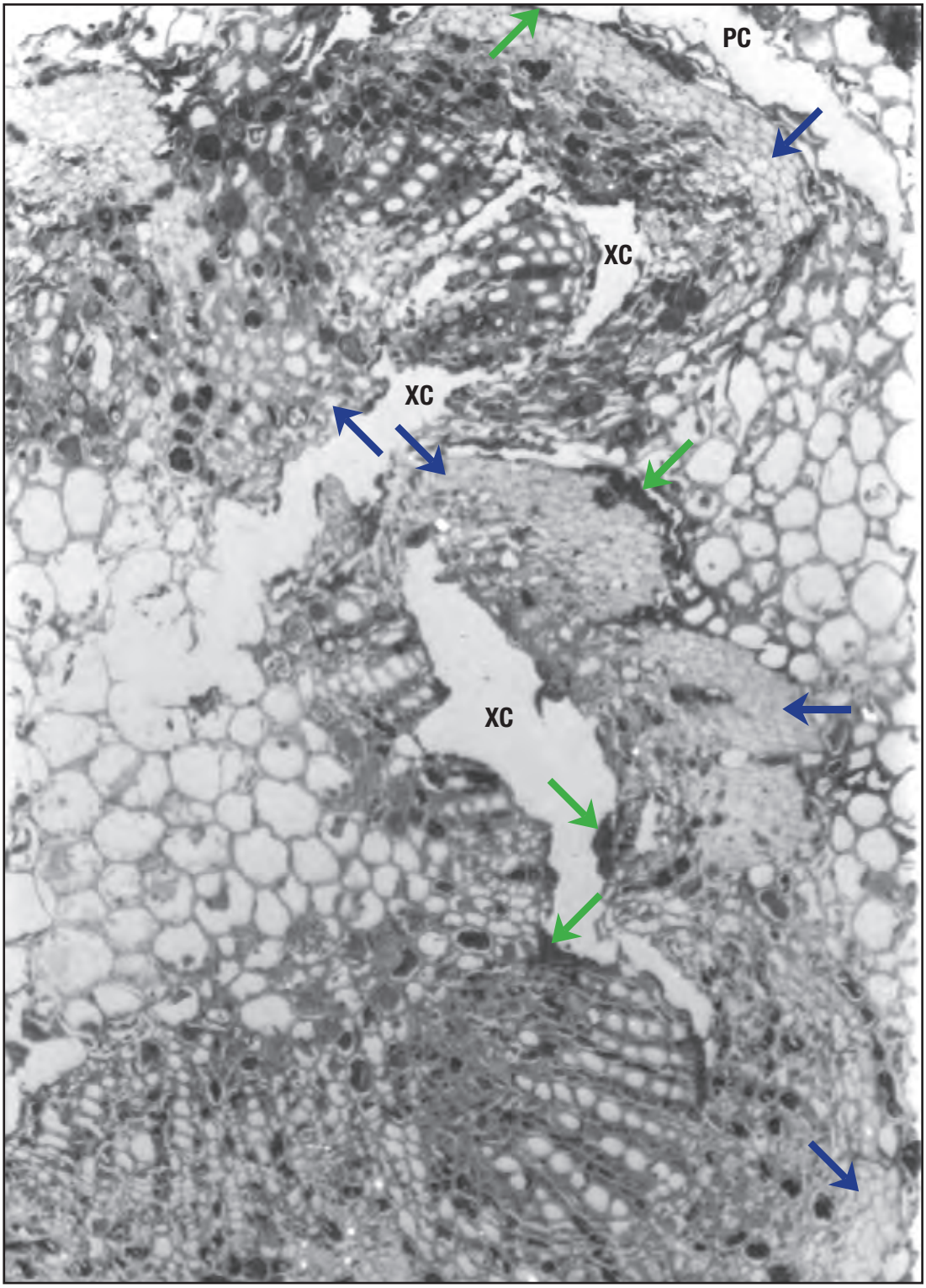
In our investigations, a search was made of features that could enhance this comprehension. At this stage, despite the doubts it can cast, we propose that the primary cause of the disease is not attributable to the classical forms of fungal elements but to unusual ones and, in many cases, to components released from these and with a potential of host invasiveness. One main component, in this respect, appears to be the structures that we have called P-elements. Some insight into these possibilities has been provided by the many illustrations and interpretations presently given. We may add that related elements have also been detected in many other published works on fungi and plant diseases that we have consulted. For example, the author feels confident of having detected similar elements in the extracellular layer separating a fungal cell wall from a host cell, and in a proximate body in the latter in Fig. 17 in O'Connell et al. (1996); similar structures can be distinguished in Figs. 4 & 5 in Gianinazzi-Pearson et al. (1996); and so on. It is interesting to note that the illustration in the former was from material fixed by high pressured freezing. Also, many reports exist showing that compounds released by cells of fungal pathogens in a number of species were related to host cell attacks (for example, Cole et al. 1998; Healy et al. 2004; Mercure et al. 1995; Melchinger et al. 1980; O'Connell et al. 1996; Sugui et al. 1998; Tenberge et al. 1996). Key evidence in this scheme was the pronounced alterations and reactions at all stages of infection occurring in vasicentric cells, particularly of the metaxylem and recently differentiated tissue, following invasion of middle lamellae and of adjoining primary cell walls and reaching into the cell content. It would even seem

that the invasive elements could utilize some of the basic host cell components for their build up. The occurrence of fungal cells in still well delimited nuclei, as illustrated here, in cells otherwise containing completely degraded content, and the stretches of P-element-containing matter spanning long distances through the still partly organized cell content are considered to support this assertion. In the secondary xylem, the P-elements were shown to reach the paratracheal cell content through pit membranes, and the intensity of reactions in these cells appeared to be related to the degree of that invasion, with exceptions as shown below.

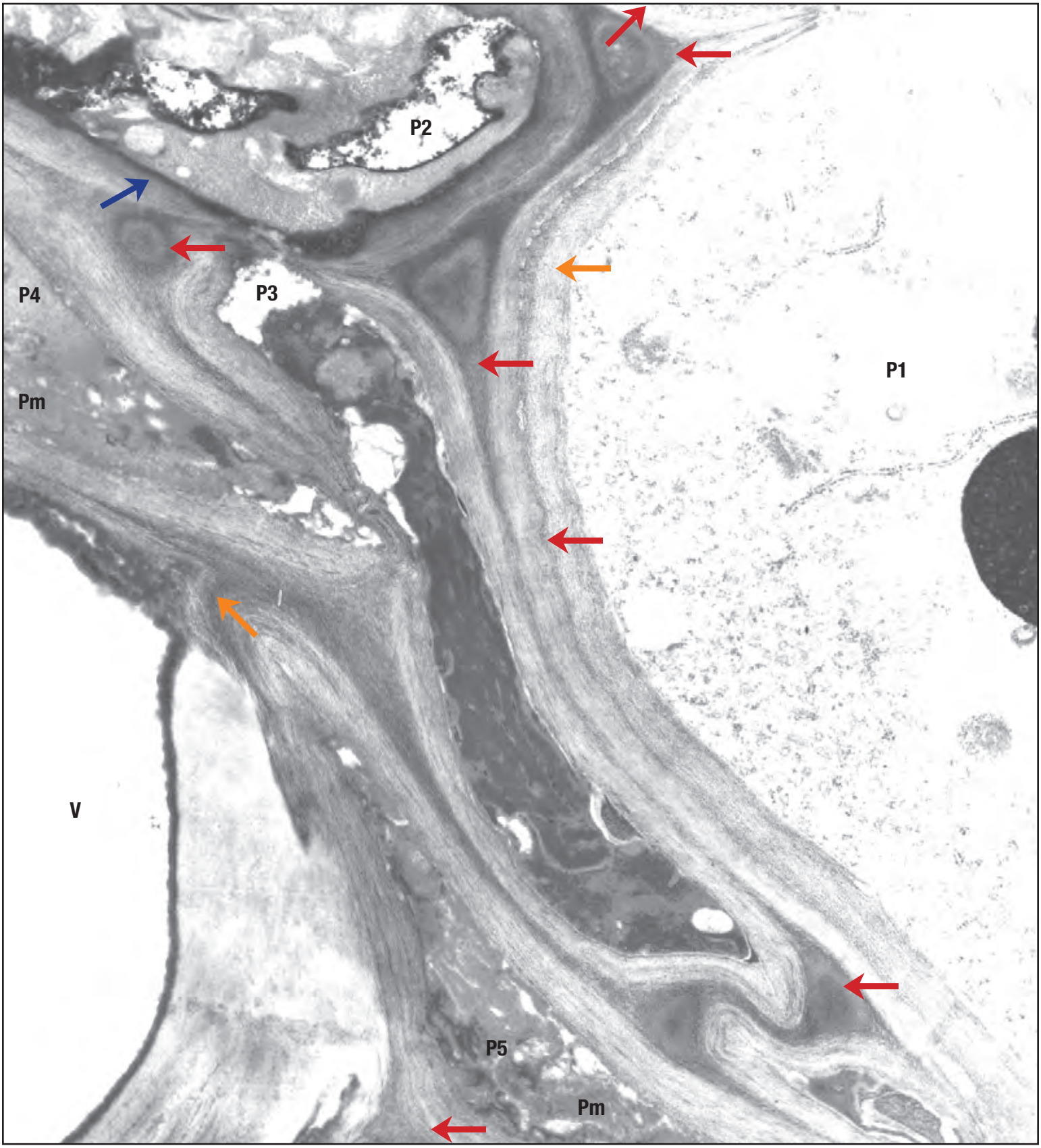


A paratracheal cell (P1) (A) and adjoining one (P2) are separated from adjacent cells by a dislocated (D) middle lamella. P-elements (→), as indicated in enlarged cell P1 (B), are distinguishable throughout the pit membranes and the P-cell wall, including the protective layer. These elements are seemingly absent at this level in adjoining intercellular spaces and confluent middle lamella, except near an eroded portion of the vessel wall (→). Small particles (→) are associated with the lining in V1.

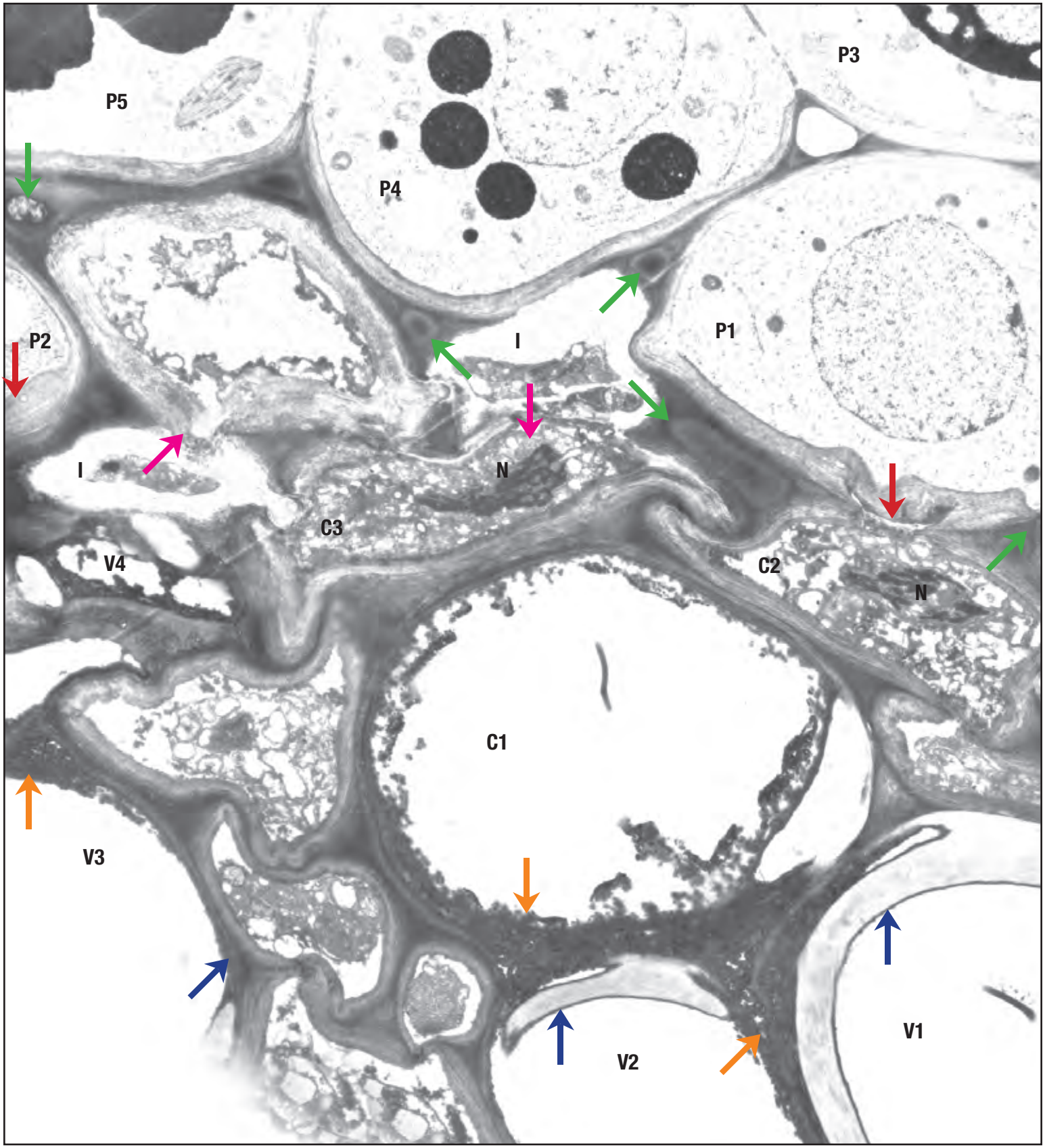
Therefore, to our mind, there lies the primary cause of infection. One may argue that, as we are dealing with vascular wilt diseases, the primary cause needs to be sought in vessel elements. That these are avenues for infection to progress, as "primary or secondary determinant" (Talboys 1972) is not denied, as, indeed, fungal propagules and/or unwalled elements need to be introduced therein as a means to establish tissue ingress. The proposed effect of disease development on vasicentric cells, as mentioned by Talboys and others has been considered as an indirect one related to secretion of compounds either favouring further development or inhibition of fungal elements. A direct detrimental effect from early alterations of vasicentric cells has rarely been considered to be involved. However, we feel that without this type of cell attacks in relation with the structures that we have mentioned, perhaps there would not be a disease at all. In this line of thought, the role of vessel occlusion would need to be re-assessed, considering that much of the occluding matter may have a pathogen origin. Here again, the main effect of the material produced and released by fungal cells may be on vasicentric cells. Mostly unbound material, containing P-elements and small particles the size of ribosomes, amongst other components, was regularly apposed to vessel walls (the lining) and associated with the type of invasion just mentioned. As this lining was observed to precede the visible presence of fungal cells (as observed at the tips of advancing streaks), thus past the stage of inoculation (in DED disease, we have observed that the pathogen first developed in feeding wounds of the European Scolytus beetle from which it reached vessel elements, Ouellette 1960), the determinant considered as primary may in fact be secondary but producing a primary effect, leading to a cascade of reactions, as proposed by other researchers for other systems (Graham & Graham 1991) and as we have discussed in other writings. Hence, the presence of fungal cells in any large amounts in vessel lumina may not be indicative of a primary attack front. We have already mentioned that in a functional vessel element any extraneous element present therein would be directed towards the vessel wall, the heavier components first and then the lighter ones, due to a theoretically more rapid sap movement in the center of the column. In the same line of thought, the released components of fungal cells might become part of the invasion front, unless their movement is hindered by vessel occlusions. These have been considered by some as leading to wilting and by others to reflect a mechanism of resistance. This question has been discussed in preceding chapters and in other published work (particularly in Ouellette et al. 2004a, 2004b). Whether the presence of tyloses and gels were directly involved in producing symptoms was questioned in these works, but the observed dying of twigs and shoots were considered to be of paramount importance in both the acute and chronic symptoms of the disease. The following LM and TEM photomicrographs are presented as a recapitulation.



Disrupted shoot xylem tissue, in inoculated elm. Often mechanical ruptures resulting from sample collection was invoked to account for such xylem cavitation (XC) (thus, causual) or that it occurred as a wilting effect. The mounds of neoplastic tissue (→) that were formed past these dislocations indicate they were present at the time of sampling or were deposited when the twig was still living. Masses and bands of opaque matter (→) border regions of tissue dislocations, including similar ones (PC) in parts corresponding to the outer bark.



A second attack in the cambial area following a partially recovered recurrent infection in elm. Collapsed vasicentric cells (P2-P5) with variously altered content next to invaded vessel (V) elements. The intercalary middle lamellae and intercellular spaces are altered, displaying P-elements (→) at many places, including parts adjoining the pervaded pit membranes (Pm). A new wall layer (→) separated from the native wall by a fibrillo-granular layer (also showing P-elements) has been deposited in the hypertrophied cells (P1, and other adjacent cells) having mostly intact content. P-elements can also be distinguished in the opaque material present in the periplasm of cell P2 (→), and seemingly associated with pronounced degradation of its content.



Hyperplastic cells (P1-P5) adjoin rows of severely altered cells, next to contiguous, invaded vessel elements (V1-V3), having their walls covered with a lining. This illustration may be considered to summarize a few of the features related to infection of young tissue in elm elm in this case, following a second attack at the cambial level. Thus: the cell (C1) with most degraded content and altered wall, and probably the first one to have been affected, are bordered by two altered pit membranes (→); other adjacent cells have thicker but wavy primary walls, some locally ruptured (→); intercellular spaces are visibly altered, displaying areas delimited by a lucent or opaque layer (→) (as in the preceding Fig.). Cells P1 and P2 show localized thickened walls, seemingly in association with traces of opaque matter (→).

As many authors claim that the lining (coating) on vessel walls and the similar material lining fungal cells is of host origin, adding a few considerations on the subject may be relevant. The question raised by Pegg et al. (1976), concerning infections by *Verticillium* spp. and still kept open by Pegg & Brady (2002) whether this relation really exists, at least in some cases, remains pertinent. Evidently, as the lining may be primarily of pathogen origin, the question needs to be evaluated on a completely different angle. We believe having shown convincingly that release of material from fugal elements contributed to gradual build up of the lining, its first layer being regularly shown to be associated with host wall and cell attack, and displaying in many cases cytoplasmic-like content, and, at least in carnation, to label for chitin (Ouellette et al. 2004c). If these linings solely originated from paratracheal cells, sealing off their pit membranes (as pointed out by Pegg et al. 1976; Pegg & Brady 2002), then one would still have to explain how these same cells could continue to secrete material leading to that seemingly continuous increase in thickness of the linings. Also, a downward movement of lining material leading to a similar build up cannot be easily envisaged. Furthermore, the presence of that material should always be detectable first in the paratracheal cells. We have provided evidence that the occurrence of material covering pit membranes preceded the presence of any comparable material in the periplasmic areas of paratracheal cells in each of the diseases studied, including the present addition of fungal infection in *Tillia*. We stress that these results cannot be compared with LM observations alone (i.e. not paralleled by TEM observations of contiguous samples), which also in many cases have been the ground for mentions that the material accumulating in vessel elements, such as in *Verticillium*-infected tomato plants (Newcomb & Robb (1989), originated from similar matter in middle lamellae and intercellular spaces (also Robb et al. 1991; Gold & Robb 1995).

Therefore, as an overall consideration, a search for disease resistance should be aimed at discovering reactions in vasicentric cells as well as in vessel elements susceptible to hinder tissue invasion. In the diseases studied, only in a resistant carnation cv. do really effective means of resistance occur. Concerning elms, the elm progenies showing resistance that were obtained or are being developed by some workers in Europe (Gil and co-workers, Santini, see New approaches to elm Conservation, INIA, Madrid, Spain) as well as in the United States (Wisconsin University by late Dr. Smalley, and workers at USDA Forest Service, Delaware Ohio, in particular) may contain this type of combined resistance. As an example may be mentioned the work of Et-Touil et al. (2005) who have illustrated the histological changes occurring in inoculated elm strains produced by Prof. Smalley and co-workers. In American elms, reactions that can be considered as of mechanisms of resistance may take place, but they are apparently not general or persistent enough to hinder disease development or occurrence.

Even in the presence of a supposedly effective occlusion of vessel elements, invasion could progress via the middle lamellae by means of microhyphae in some cases (particularly in carnation and recurrent infection in elm) or by unbound opaque matter (particularly in elm, eggplant, and staghorn sumac), highlighting again that this mode of invasion might correspond to a primary cause. The immediate damage to host cell walls would be a primary effect of little consequence if compounds of host origin neutralized it and prevented its repercussion on adjoining living cells (an indication of defence mechanism) but much more damaging if the invasive agent reached content of adjoining cells. This last condition would generally apply to the hosts studied, with variations in their cell reactions.

Hence, in this proposed model the presence of so-called typical fungal cells in vessel lumina would be to perpetuate the "primary cause", also assuring means of disease recurrence and production of the next inoculum. Just how these cells can be generated is, however, a moot question. We have shown by incubating samples of diseased samples on an agar medium and fixing after short to longer time intervals thereafter that fungal cells issued from the vessel wall lining. Other evidence was that when liquid cultures of *O. novo-ulmi* were filtered through Millipore membranes of either 0.22 µm or 0.45 µm porosity, it could be recovered from the filtrates, seemingly developing from tiny particles present therein. In the hosts studied, also, unbound masses of matter containing particles of ribosomal appearance contained or were closely associated with fungal cells and, following incubation of diseased samples, as mentioned above, some of these masses appeared as if becoming delimited by membranous structures and wall layers.

Considering the irregularities in forms of fungal elements and of their particular modes of development in their hosts it would become unreliable to evaluate the fungal biomass in infected plants in terms of degree of infection on the base of quantifying chitin, particularly in host plants containing chitin analogs in their secondary walls, as shown by labelling and other means (Benhamou & Asselin 1989). This was the case for the hosts studied except staghorn sumac, presenting another kind of control for the test. Similarly, the extent and intensity of tissue invasion extrapolated from the number of fungal propagules expressed in colony forming units, obtained from exudates of diseased plants (Brandt et al. 1984, as an example) may not be meaningful to measure the colonizing potential of the pathogen (Pegg 1985). Anyhow, a close relationship of results obtained with these methods and the intensity of disease development or resistance to it

was not evident (see Pegg & Brady 2002). Even by using PCR techniques (Hu et al. 1993; Heinz et al. 1998, as examples), the fungal DNA content that could thus be estimated may not be accurate, as, in the case of microhyphae, their content could be translocated to their growing tips. Also, developing cells were often connected to empty cells by means of fine filamentous structures, not corresponding to true budding, indicating that the DNA content in the latter cell had either been transferred to the growing cell or become degraded.

The author having promoted all along (Ouellette 1978b; Ouellette & Baayen 2000; Ouellette & Chamberland 2006; Ouellette et al. 2004c, 2005b, 2006) that unbound opaque matter attributed to the pathogens could extensively invade host walls and cells, the final words of this discussion will concern this issue. Clearly associated with host tissue alterations, this OM may be considered as having a pivotal role in the pathogenesis of the diseases studied. Many observations in support of this view, which we consider not to be mere speculation, were discussed in Ouellette et al. (2004b, 2005a, 2007) and other cited publications. Due to the perceived germane importance of the subject, may it be stressed once more that ascribing components of this matter, particularly the basic uniform opaque particles, to the host rather than to the pathogen would not be less challenging. Indeed, how could one easily explain the grouping of these particles, of ribosomal size and appearance, in the periplasm of host cells that otherwise contain degraded content. Also, the content of some fungal cells was observed to be structurally analogous to some of the present matter. Considering that the opaque matter can be foreign to host cell content does not exclude, however, that some host components may become included in components of pathogen origin particularly in case of close contact between both.

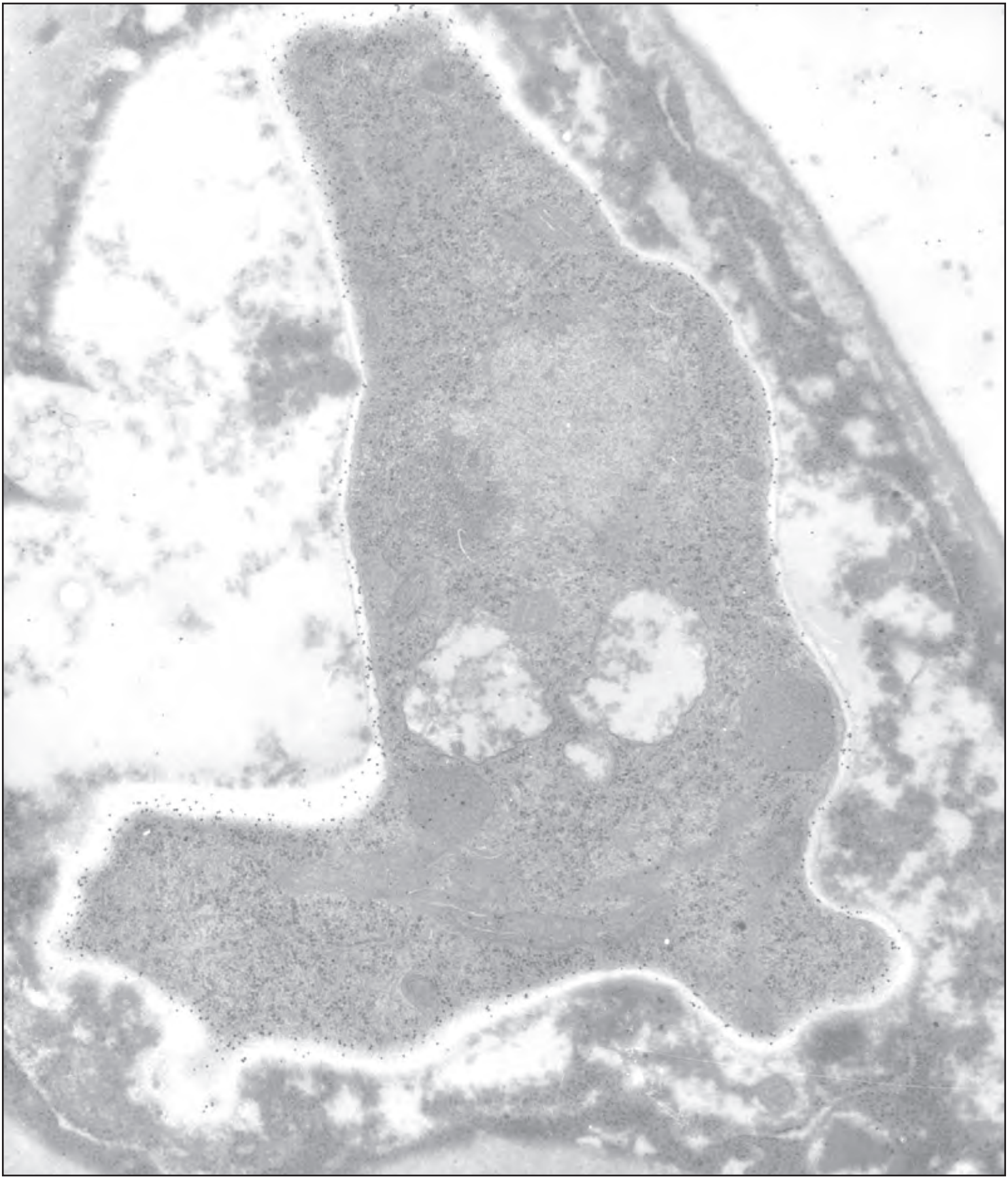
Obviously, one condition here would be to account for the mode of formation and/or preservation of this matter. Considering that fungal elements may not always be bound by a wall, as shown in this and other works (Charest et al. 2004; Ouellette et al. 1999a, 2004a, 2004c, 2005a, and citations therein), any one section from embedded samples would show only a portion of the whole possible ramifications of this malleable matter and hundreds of contiguous sections, if not more, would be necessary to visualize it in its entirety in order to detect all its components. Also, high magnifications in properly exposed prints of photomicrographs, as adopted for the present documents, would be necessary to adequately detect details of these components, a requisite, which unfortunately was not always satisfied in most works. Yet, a more controversial aspect would be to explain the presence of nucleic acids in the OM which was observed spanning long distances in host walls. Considerations that can be invoked to make this condition less improbable are the following. Nuclei in developing cells were regularly observed not to be limited by a classical envelope and thus bound to develop into irregular forms and thin extensions; many of our observations point to this possibility (Chamberland & Ouellette 1977; Ouellette et al. 1995, and some present observations). Thus, tiny filaments or small isthmuses bridging larger masses of matter that labelled for DNA could be avenues of DNA transport and thus become part of fungal elements of various shapes and sizes. Reasonable evidence has been obtained that labelling for this compound was reliable (Ouellette & Chamberland 2006; Ouellette et al. 1999b, 2004a, 2005b). Extracellular material was also observed to label for RNA that extended into the surrounding medium. To know then what would be the minimal size of these extensions or part thereof of being able to regenerate the organism in its fullness is a question to investigate. An insight into possible answers to the question was provided by the observed formation of conidia at the tip of long small filaments and by obtaining regeneration of *O. novo-ulmi* cells from elements passing through Millipore membranes, as mentioned above. Thus, much of the opaque matter present over long distances in host walls (Ouellette 1978; Ouellette & Chamberland 2006; Ouellette et al. 2004a, 2005b, as main examples) might be correlated to the matter containing particles of ribosomal appearance. As already considered, nuclei in some other groups of organisms were shown to transform into masses of opaque matter (Carothers 1973; Renzaglia et al. 2002, for example). In the case of the opaque matter in question in infected plants, its opacity could also be partly due to some host compounds, such as adsorbed phenols. However, this could not be the situation regarding the nuclear areas in fungal elements of *O. novo-ulmi* growing on or penetrating Millipore membranes (Ouellette et al. 1995, 1999b). In this scheme also, accounting for the presence of DNA in the opaque matter in question, particularly in its long stretches, would be facilitated if synthesis of this acid could occur outside a well delimited nucleus, granted that the necessary basic components are present. Considering that DNA components can now be synthesized in vitro, thanks to the PCR techniques, this eventuality might not be that improbable.

In this context, some reports of the occurrence of similar matter, surrounding or present in the vicinity of pathogen cells, even in intercellular areas (Lazarovits & Higgins 1976), might be worth looking at in a new perspective. The following references, for example, appear to have a bearing on this issue. Bishop & Cooper (1983) have illustrated stereoscopic images (their Fig. 5c, d) of opaque matter and radiating filamentous-like structures crossing vessel walls, which were clearly connected to similar material lining these walls; those illustrations show analogies with many of the situations that we have observed, for example in elms and eggplant (Ouellette 1978b; Ouellette & Chamberland 2006; Ouellette et al. 2004a, 2005b) and attributed to a pathogen origin. Appreciable amounts of opaque matter extending from fungal cells

and present in host cell walls was also noted in other systems and postulated to be related to pathogen cells (Hickey & Coffey 1977; Seifers & Ammon 1980; Mims & Nickerson 1986; Bauer et al. 1995, 1997; Enkerli et al 1997), but such material was not reported to appreciably extend from these. Similar material surrounding fungal cells in another *Exobasidium* species, observed by Mims & Richardson (2007), was denoted as pertaining to an interaction zone. Fungal cells of *Sphaeropsis hypodermia* (another pathogen of elm) were found to accumulate extracellularly large masses of a similar opaque material; in the host, similar fungal cell-associated material was observed as having permeated cell walls, including secondary walls of fibres (Ouellette et al. 2000). In the accompanying chapter on *Tillia* cultivars, similar material extended long distances into middle lamellae, but in this case, content of adjoining cells was mostly intact, even when the plasma membrane was locally altered. This indicated that the large amounts of opaque matter most likely did not originate from these cells. These observations, added to other similar ones in the other plants studied, showed that localized alterations of the cell plasma membrane do not automatically lead to cell death. In relation with this aspect, considering the occurrence of opaque matter in cell periplasm does not prove that it represents a mechanism of defence (as also discussed in Ouellette & Chamberland chapter I).

In a flashback manner, one may have to return to Eriksson's observations reported at the beginning of the last century, denoting as pertaining to fungal elements non-walled opaque matter pervading host cell walls in some diseased plants (Eriksson 1910; Eriksson & Tischer 1904). Eriksson proposed the term "mycoplasm" for this type of development, a term which was subsequently borrowed, inconsiderately so to our mind, to name another type of organism (Mareschkowsky 1910). It is interesting to note that osmium was used by Eriksson (1904) as one of the fixative for his study material. Investigating further the question might show that Eriksson's observations should not have been so easily rejected, even though those observations might not necessarily have applied in every case to the pathogens referred to. For example, endophytes are now known to be quiescent inhabitants of plants (Stone & Petrini 1997), having been mentioned to occur in some instances as very tiny hyphae or irregular protoplast-like multinucleate bodies (Petrini 1991). Astatt (2003) recently reported having obtained fungus elements from masses or strands of opaque matter present in fungi and other plants, following incubating samples of these on a synthetic culture medium. He proposed the term "mycosome" to accommodate that matter. Whether definition of the term could be extended to include all unbound opaque matter and particles that could be related to development and/or reproduction of the fungi concerned, might be worth looking at.

A closing remark on the subject could be the following: at the opening ceremony of the 1978 International Congress of Plant Pathology held in Münich, DE, Prof. R.K.S. Wood (London University, UK) had a good foresight by saying, in his keynote address, that a possible outcome of plant pathology research at the turn of the century (the last one) would be to show that fungi might infect plants as protoplasts (from memory).



...now, for others to go giant steps further...

(labelling for chitin +Au-complex chitinase)