COLLOIDAL GOLD COMPLEX PROBES TO DETERMINE USUAL CHITIN OCCURRENCE OR ITS ALTERATIONS IN WALLS OF SOME FUNGAL CELLS AND MEANS THESE HAVE TO OVERCOME SUCH ALTERATIONS.

Guillemond Ouellette, Hélène Chamberland, Louis Bernier, Pierre M. Charest*

*3413 rue de Sarnia, Québec (QC), Can G1X 2K5;

519 rue Givot, Québec (QC) Can G1B 3A1;

CEF, Université Laval, Québec (QC) Can G1K 7P4;

Dept. Phytologie, FSAA, Université Laval, Québec (QC) Can G1K 7P4

Abstract

Results of ultrastructural studies of fungal plant wilt pathogens primarily concerning their chitin cell wall components, using colloidal gold complex probes, are reported. Formation of irregular growth forms and of thin hyphae (microhyphae), in *vitro* as well as in *vivo*, were related to fungal wall alterations. Endocells were formed as a reaction to fungal wall disruptions. The endocell and mother cell walls did not label as for typical cells, indicating that these were probably modified. Fungal cells in close contact with host walls and even of other fungal cells had thin or imperceptible walls that generally also did not label for chitin. In contrast, in cases of pathogen development in resistant plants or in those showing mild external symptoms, the lucent chitin-containing fungal wall layer was much thickened, coincidental with alteration of the outer opaque wall layer. These fungal wall aberrations need to be considered to properly understand pathogenesis of the diseases under study.

Introduction

As known from observations of *in vitro* grown fungi, these are characterized as apically growing cylindrical hyphae or branches and their cell walls usually display an inner lucent chitin-containing layer and an opaque external layer. In previous studies we have indicated that in their host plants some fungi may show diverse irregular forms paralleled by aberrant cell walls [1-4]. By growing *Ophiostoma novo-ulmi* Brasier on Millipore membranes of low porosity, the elements penetrating them were small and distorted and delimited by disorganized wall layers that generally did not label for chitin and irregularly also for cellulose [4], which also occurs naturally in cell walls of this pathogen. Hypha, often less than 0.2 µm in diameter and delimited by thin walls, or even not displaying a distinct wall, were also observed pervading host cell walls in plants or pre-sterilized substrates [2-5]. The development of similar microhyphae in soft-rot fungi, as first observed by Liese *et al.* [see 6], was followed by time-lapse photography by Hale and Eaton [7]. These authors confirmed the initial lack of a wall for these elements, showing that their channel of ingress was first delimited by opaque bands extending into the wood cell walls. We have also frequently observed that similar bands were continuous with the outer layer of fungal elements [2]. These types of fungal growth, as well as the other means fungal elements, may have to overcome alterations of their cell walls may be of critical importance in infecting host plants. This paper presents an overview of these features. The extracellular matter produced by fungi may also be

determinant for properly understanding pathogenesis of some plant diseases. This topic is presented in the companion chapter of these proceedings.

Material and Methods

The plant pathogens and hosts studied were: *Ophiostoma novo-ulmi* Brasier – elm trees; *Fusarium oxysporum* f. sp. *dianthi* (Prill. & Delacr.) Snyder & Hans. race 2 – susceptible carnation; *F. oxysporum* f. sp. *radicis-lycopersici* Jarvis & Shoemaker – tomato; *F. oxysporum* f.sp. *callistephi* (Beach.) W.C. Snyder & H.N. Hans. race 3 Armstrong & Armstrong – staghorn sumac; *Verticilium dahliae* Kleb. – eggplant. Plant inoculations were made by cutting with a sterilized scalpel across a drop of inoculum (appr. 1 x 106 conidia) obtained from the fungus cultured on an agar medium placed at the stem or branch bases that were previously surface-sterilized with 70 % ethyl alcohol. Controls were H2O-injected or non inoculated plants. In the case of *O. novo-ulmi*, samples from inoculated non hosts or pre-sterilized substrates (Millipore membranes of low porosity and elm wood sections sterilized with propylene oxide) were also used.
Samples, obtained at regular intervals after inoculation, and in the case of plants, at increasing distances from the inoculation point were double fixed (glutaraldehyde and osmium tetroxide) or, in some cases, by high pressure freezing (4). Embedding was with Epon.

For cytochemical tests, the following colloidal gold complex probes (with 15 nm size particles) were used: for chitin, wheat germ agglutinin (WGA, ordinarily with ovomucoid as a second step) and chitinase; for cellulose, an exoglucanase; for galactose, a Ricinus lectin; and for pectin, a monoclonal antibody (JIM 5). Controls were by treating beforehand the complex with correspondingly: tri acetyl-glucosamine, glucan from barley, galactose, citrus pectin.

Result and Discussion

Fungal growth forms, e.g. not regularly cylindrical and delimited by typical wall layers, are presently illustrated. Thus, chitin-labelling revealed various conditions of fungal elements correlated to disturbances in their wall layers or even of their outer opaque one. This was particularly evident regarding *O. novo-ulmi* growing through Millipore membranes (Fig. 1A, B), except for one of the isolates tested whose elements having penetrated only slightly the membrane displayed typical chitin-labelled wall layers (not illustrated). Frequently, very thin cell walls or their suspected absence was often indicated by a close contact of the cell plasma membrane or outer wall layer with host components (Fig. 1C, D) where the close boundary between both was often indicated by gold particles of the probes labelling these components (Fig. 2A). In so-called normal situations, the lucent layer labelled for chitin but not the outer opaque layer (Fig. 2B). However, where the former was altered, numerous gold particles overlaid the latter and opaque matter related to it, which appeared to locally impinge on the host cell wall (Fig. 2C). Fungal walls in close contact with host walls or content or spanning appreciable distances intramurally as microhyphae, or even juxtaposed to other fungal cells, were thin and frequently unlabelled for chitin (Fig. 2A). In the same and similarly treated samples, fungal cell walls distant from host walls were strongly labelled. Whereas the host cell primary walls did not label for chitin, the vessel secondary walls were strongly labelled (Fig. 2B, 3A). This chitin-labelling was general in elm, carnation, eggplant, and tomato, but not in staghorn sumac.
When fungal cells were not in direct contact with host cell walls or content, they were frequently delimited by distinct, albeit often thin walls, and displayed bursts or ruptures. However, indications were obtained that these alterations were not always conducive to complete degradation of cell content, but part of which could be released into the surrounding medium. As a reaction to these alterations, adventitious wall layers were formed in the affected cells, delimiting apparently still intact content and sometimes also patches of more opaque seemingly amorphous matter (Fig. 3B, C). The formation of these types of endocells or intrahyphal hyphae (Fig. 3B), of frequent occurrence in all the pathogens studied, generally did not result from proliferation of a living into a

dead cell, as can often be the case. Whereas the walls of the enclosing cell was of normal appearance and labelled normally for chitin (Fig. 3D), it was often very thin, only partially labelled or unlabelled, contrasting with the strongly labelled and even thickened wall of the endocell (Fig. 3D). It can be seen in the latter that fungal cell walls are not only thin when in contact with host cell walls but even also with other fungal cells, and nearly free of labelling. In opposition, in many other cases, the wall of endocells frequently did not labelled for chitin, or only erratically, compared with the strongly wall of the mother cell (Fig. 3D).

In many cases, the only indication that a fungal cell had given rise to an endocell was its being delimited by a double chitin-labelled layer (Fig. 4A). As shown here, and representative of general occurrence, the endocell had proliferated and grown through host tissue, only partly delimited by a distinct wall. Thus, the possible delay in fungal cell development occasioned by these wall alterations may be only temporary or even not be of consequence, as the cell content released following the wall disruptions may lead to host cell content breakdown.

Finally, as a possible reaction to the sole alteration of the outer opaque layer, a thickening of the inner lucent layer, labelling for chitin, was noticeable in many fungal cells (Fig. 4B). This reaction was noteworthy in resistant plants, such as in carnation [5], or in plants showing milder symptoms as in staghorn sumac or non host trees inoculated with *O. novo-ulmi* [8].

Present observations may help to explain the often reported scarce occurrence of typical fungal cells in plants infected with plant wilt pathogens, particularly in earlier studies of the Dutch elm disease [9] and in the invasion front. Other aspects are presented in a companion chapter of these proceedings. A point to highlight is that the pathogens involved may develop into varied forms and in all cases display abnormal, altered or even defective walls, as shown to occur by the use of probes for chitin and other components. Obviously, surface or points of host attacks may be greatly increased by these types of fungal cell development. As mentioned here and in other publications (referred to above), the fungal chitin-containing layer may be modified and thus be protected from the plant chitinase action. A thigmotactic effect may also be involved in these wall modifications, as they also occurred in contact with other fungal cells or in host cells in pre-sterilized substrates. Also, the outer opaque fungal cell wall layer impinging on host walls and important in tissue invasion often permitted to trace tiny fungal elements in the hosts. This consideration may be inline with findings that proteins of fungal origin may counteract the action of plant endo-β1-,3-glucanases and chitinases [12]. The strong labelling of host cell secondary walls for chitin may appear to complicate matter, not so much in the ability of determining whether chitin-labelled fungal walls are due to fungal or host components, as possible in some cases, but because they might falsify results of chitin content analyses of infected plants to evaluate the occurrence therein of the fungal biomass. To circumvent such a possible shortcoming, PCR techniques have been used to evaluate the fungal DNA content in colonized tissue [10], attempting to take into account the possible recycling of fungal cell content. Indeed, increases in fungal chitinases have been noticed in some systems and implicated in this type of recycling [11].

Although present considerations may not apply to all systems, they nevertheless indicate that conducting ultrastructural and cytochemical studies of infected hosts to determine all growth potentialities of the pathogens involved and their extent of tissue invasion is germane to fully understand the diseases they cause.

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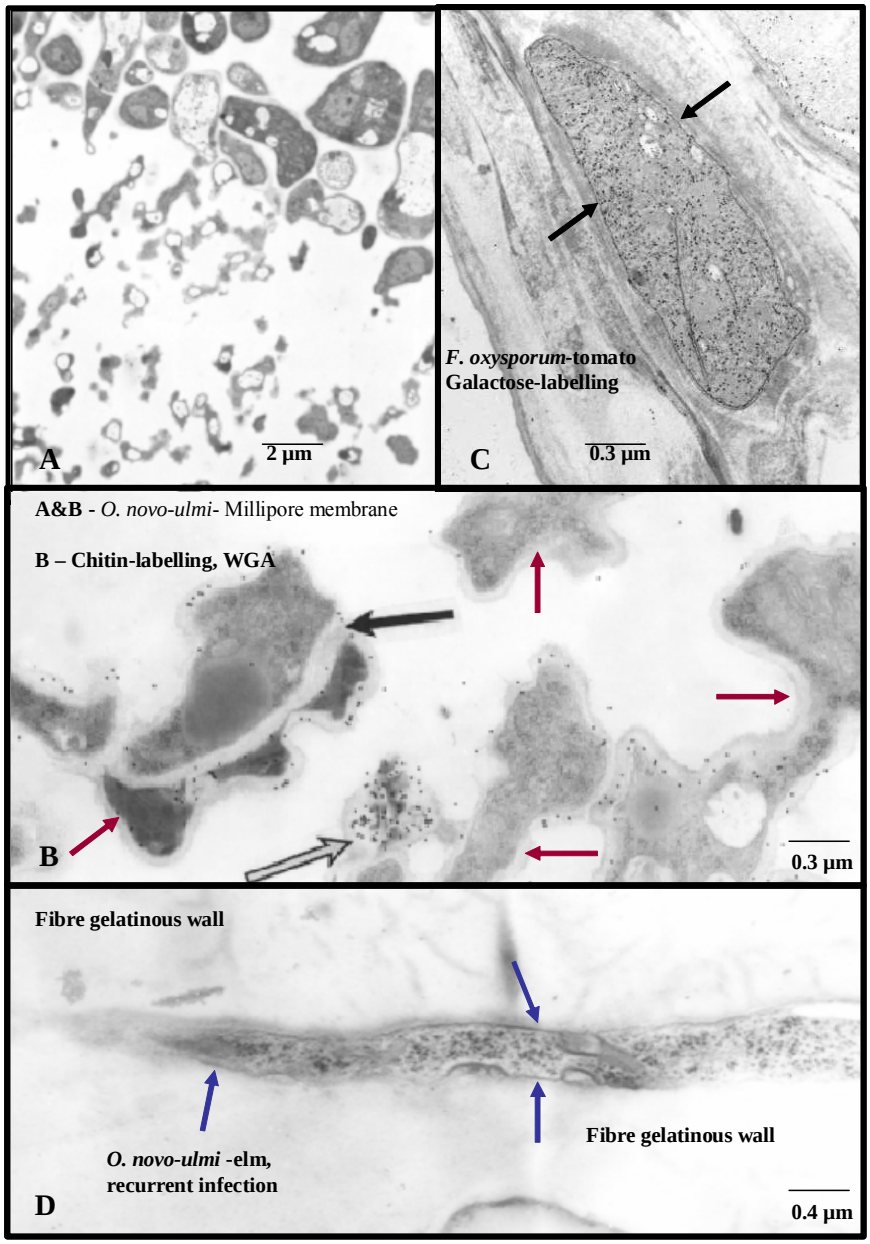

Figure 1: **A.** Distorted fungal (F) elements in membrane (bottom part). **B.** Walls of F-elements mostly free of labelling (←) including a dividing wall (←). In comparison, masses (light arrow) connected to two other elements are strongly labelled. **C.** At most places, the F-cell plasma membrane is in close contact with host components (arrows). **D.** A lucent F-wall not discernible; a band corresponding to the outer wall layer (arrows) or to the plasma membrane is in close contact with host wall.

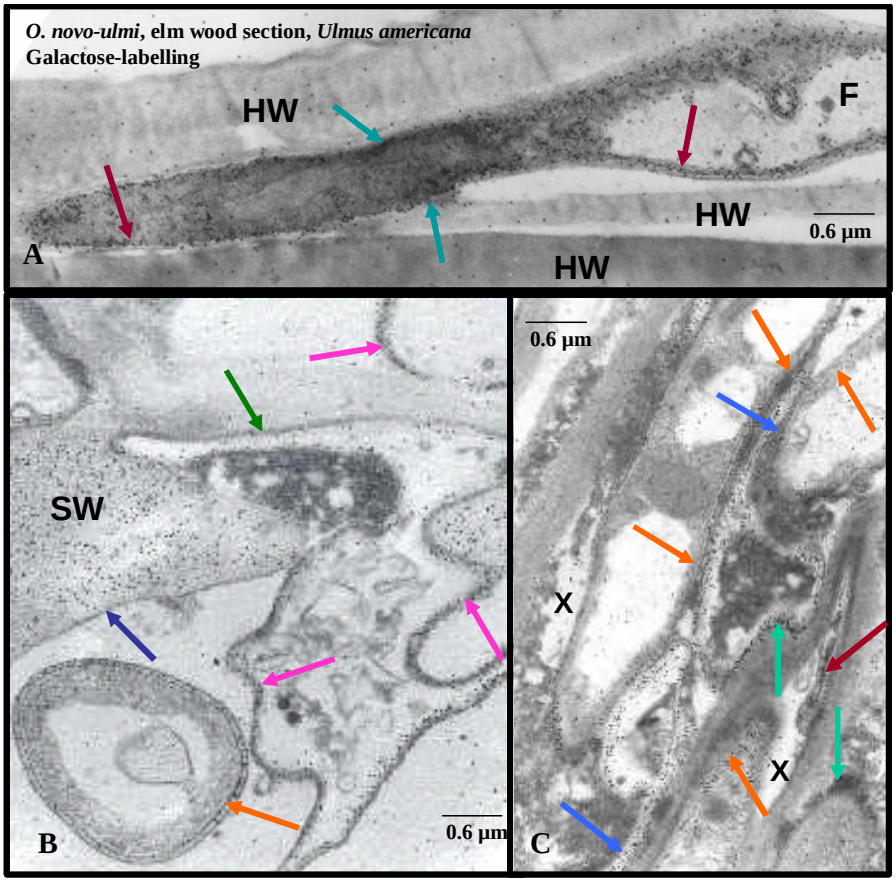

Figure 2: Rows of Au-particles in close contact with wood cell wall (⟵) indicates absence of a discernible F-wall; elsewhere, labelling is mostly associated with cell content and plasma membrane (⟵). B & C.⟶ = inner, labelled wall layers in F-cells; ⟶ = F-cell outer opaque layers overlaid with gold particles in areas of alteration of the inner layer; ⟶= unlabelled F-wall layers in contact with primary unlabelled host wall; ⟶ = the outer wall layer in intact cells and extra-cellular matter also overlaid with Au-particles; ⟶ = numerous gold Au-particles over tiny elements; ⟶ = a thin element delimited by opaque bands; ⟶ = thin, unlabelled bands apposed to strongly labelled V-secondary walls; X = numerous Au-particles in void spaces may correspond to degraded or released wall components.

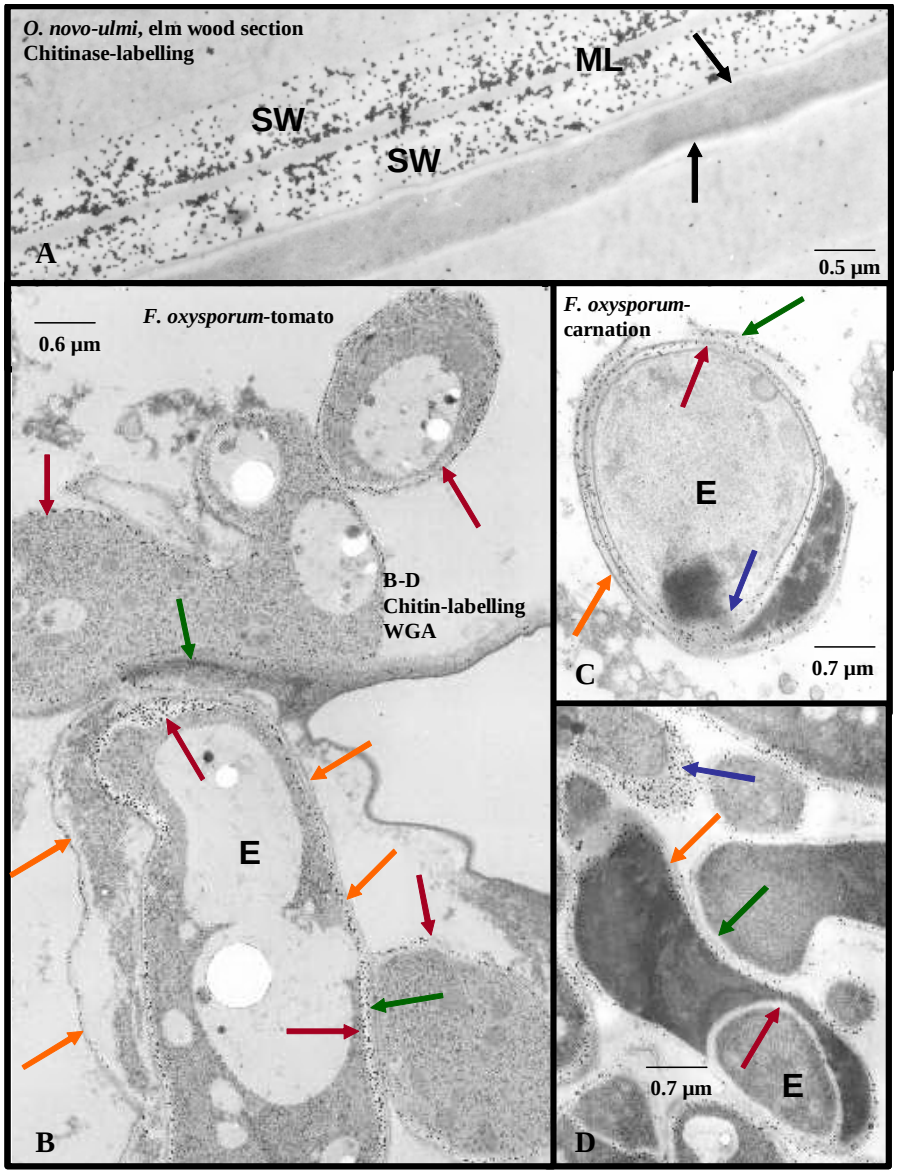

Figure 3: A. Microhypha wall (arrows) and host cell middle lamella (ML) unlabelled; secondary walls (SW), labelled. B-D. Endocells (E) or intrahyphal hyphae. → = lucent E-wall; → = mother (M) cell wall. B. E-wall, with thickened portions, strongly labelled. M-wall, left hand side is mostly unlabelled and thin, and on opposite side is thicker and unlabelled. F-wall adjoining host walls or other F-cells (→) is thin and almost free of Au-particles. Legends for C & D, on next page.

Figure 3, continued. C. Lucent E-and M-cell walls with its ruptured portions neatly labelled. **D.** *O. novo-ulmi*, Millipore membrane. E-wall, unlabelled and M- and of other cell walls, except when they contact other cells (⟵) are strongly labelled, including apparently thickened portions (⟵)

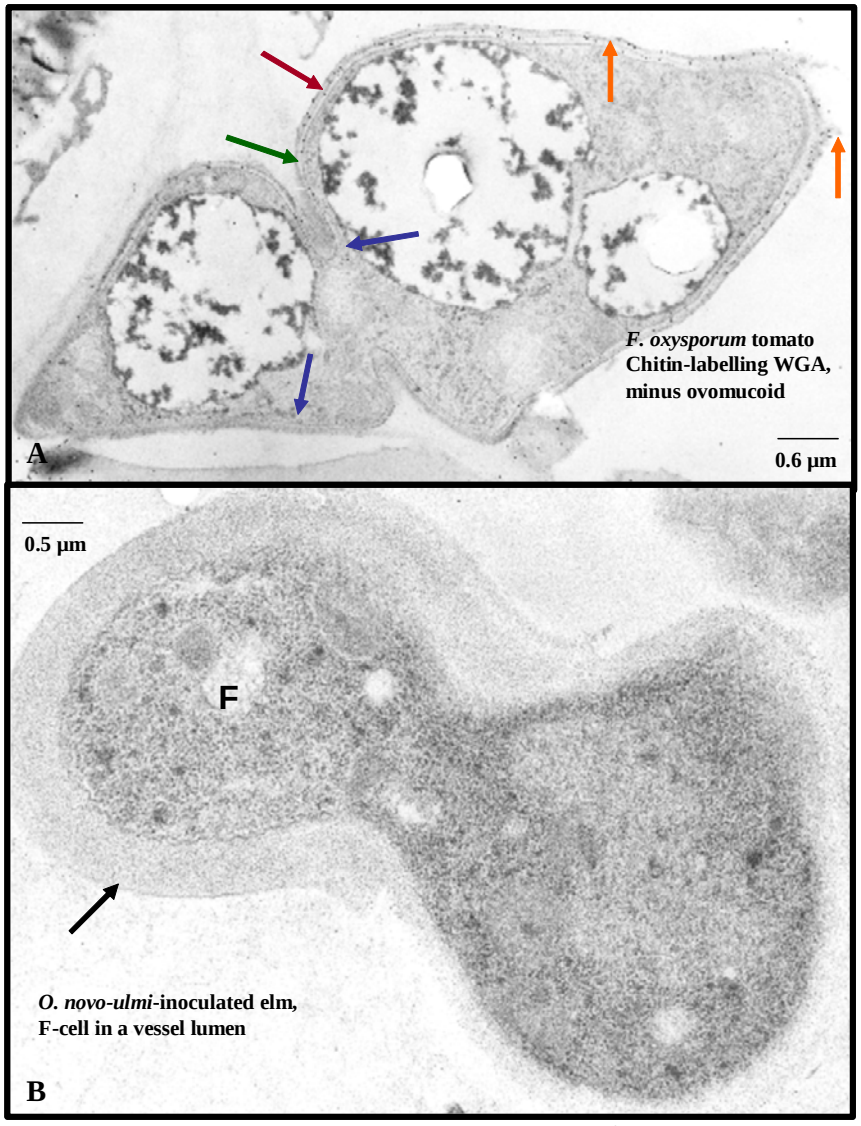

Figure 4: A. A labelled double wall layer (⟵) present on one side of the F-cell, the external one locally ruptured (⟵), or discontinuous below (⟵). This E-like element has outgrown the M-cell and penetrated the contiguous host wall, not displaying a distinct wall, particularly in areas contacting it (⟵). B. *O. novo-ulmi*-inoculated elm. F-cells with much thickened walls (arrows) and almost free of an opaque outer layer.