

Research Papers

## Ultrastructural modifications in Common ash tissues colonised by *Chalara fraxinea*

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**Summary.** *Chalara fraxinea*, the cause of ash dieback, is present in many European countries and constitutes a severe threat to *Fraxinus* spp. in forests, urban areas and nurseries. This study investigated tissue colonisation strategies of the parasite in Common ash at ultrastructural level and demonstrated that *C. fraxinea* develops intracellularly, moving through the cells and easily colonising the phloem, paratracheal parenchyma and parenchymatic rays. However, damage to either the plasmalemma or cell walls was not observed under our experimental conditions. Furthermore, the presence of intrahyphal hyphae was frequently detected, which are likely associated with a regeneration mechanism.

**Key words:** ash dieback, *Fraxinus excelsior*, *Hymenoscyphus pseudoalbidus*, ultrastructure, TEM.

### Introduction

Ash dieback, caused by *Chalara fraxinea* Kowalski (teleomorph *Hymenoscyphus pseudoalbidus*) is a serious emerging disease in Europe (Kowalski 2006; Kowalski and Holdenrieder, 2009; Queloz *et al.*, 2010).

The disease was first observed on *Fraxinus excelsior* L. in Poland in the 1990s (Przybyl, 2002), but *C. fraxinea* was only identified as the primary causal agent in 2009 (Kowalski, 2006). In the next few years, the symptoms were observed in both European (*F. angustifolia* Vahl. and, only under artificial conditions, *F. ornus* L.) and introduced ash species (*F. nigra* Marsh., *F. pennsylvanica* Marsh., *F. americana* L. and *F. manschurica* Rupr.) too (Drenkhan and Hanso, 2010). The disease spread rapidly from Poland (Kowalski, 2006) to (in alphabetical order): Austria, Belarus, Belgium, Croatia, the Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, Lithuania,

the Netherlands, Norway, Romania, Russia, Slovakia, Slovenia, Sweden and Switzerland, United Kingdom (EPPO, 2010; Timmermann *et al.*, 2011; EPPO, 2012). According to the available information, dieback is present in 60-90% of forest stands in Denmark, in 51% of ash stands in Pomerania (Germany), and in over 30,000 ha of ash stands in Lithuania (Thomsen, 2010; Schumacher, 2011; Vasaitis and Lygis, 2008). Due to the severity of the disease, in 2007, the fungus was added to the EPPO (European and Mediterranean Plant Protection Organization, 2007) Alert List and the NAPPO (North American Plant Protection Organization) Phytosanitary Alert List (2009).

Symptoms of the disease include necrotic, lens-shaped spots on the leaf rachises and subsequently on the bark of young shoots, evolving into stretched, perennial cankers, wilting and premature shedding of leaves and top and shoot dieback (Bakys *et al.*, 2009; Skovsgaard *et al.*, 2010, Pautasso *et al.*, 2012). Below the bark, the necrotic lesions frequently extend to the xylem, especially in the axial and paratracheal ray tissue (Schumacher *et al.*, 2009). In young stands, dieback causes major problems for establishment, whereas older trees show a slower, chronic process

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(Keßler *et al.*, 2012). According to recent studies, it is likely that high-moisture conditions in the air and on the ground (i.e., plants growing in excessive density or close to streams) mitigate the spread of the fungus (Cech, 2008; Ogris *et al.*, 2008). There is little histological information available, however, on the development of infection in host tissues. Kirisits and Cech (2010) suggested that the fungus could penetrate through the leaves, petioles, buds and young twigs. Once the fungus penetrates, it grows rapidly in the pith, vessels of the paratracheal parenchyma, and wood ray parenchyma, whereas the cambium and phloem are colonised at slower rates (Schumacher *et al.*, 2009). According to Andersson *et al.* (2010), the spread of the fungus in tissues could be associated with the phytotoxic effect of one or more toxins.

To provide further information on the tissue colonisation strategies of *C. fraxinea*, we performed an ultrastructural analysis using artificially infected Common ash plants.

## Materials and methods

### Artificial infections

Colonies of the pathogenic *C. fraxinea* strain FS8 (Ogris *et al.*, 2009) were grown on malt extract agar (MEA) plus streptomycin (0.5% w:v) for 60 days at 21±1°C in the dark.

In May 2010, artificial inoculations were performed on 10 three-year-old container-grown asymptomatic *F. excelsior* plants obtained from a single seed stock collected in an official ash provenance (provenance "Feltre" (BL), 46°00'55" N; 11°58'30" E, seed lot 08PA0081).

At a position of ten centimetres above the collar, the plants were superficially brushed with 5% hydrogen peroxide (v:v) and gently rinsed with sterile water. The disinfected portion was then wounded with a sterile, 6 mm diameter cork borer, inoculated with a mycelial plug of the same diameter, and protected with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA). The plants were grown in a greenhouse at 23±3°C, 80% R.H. and 12 h per day of natural light (Ogris *et al.*, 2010). As controls, ten plants were treated in a similar manner but using sterile MEA and streptomycin plugs.

After 80 days, when all of the inoculated plants exhibited at least a 5 mm-long bark necrosis arising from the wound, the maximum length and depth of

the lesions were measured, and the damaged portion was cut 10 mm above and below the bark necrosis. The control plants were similarly treated.

To verify the presence and vitality of the fungus, four equidistant 3 mm<sup>3</sup> samples were collected along each necrotic edge and plated as described above, after removing the periderm.

To perform the ultrastructural observations, 5 inoculated plants with a lesion length as close as possible to the average length of the 10 measured necroses were selected, and one stem portion including the lesion was collected. Along the necrotic edge, 4 equidistant 1 mm<sup>3</sup> samples including wood and cortex were gently removed along the necrotic edge. The control plants were similarly treated.

### Sample preparation for transmission electron microscopy

To observe the main features of the strain used for the inoculations (diameter, cell wall thickness and composition, septa structure, number and location of the Woronin bodies, fine structure characteristics), four 1 mm<sup>3</sup> portions of *C. fraxinea* were collected from the edge of a pure FS8 colony and prepared for TEM observations according to D'Ambra and Mutto (1975, 1977), without the addition of propylene oxide (Dykstra, 1993): samples were placed in 4% Gluteraldehyde in Na Cacodylate Buffer 0.1 M for 2 h at 4°C and post-fixed in buffered 1% Osmium Tetroxide for the same time and at the same temperature; the samples were rinsed with buffer, dehydrated by immersion in a graded acetone series (30-50-70-90-100%) and embedded in Fluka Durcupan ACM (Sigma-Aldrich, MO, USA). The previously collected woody and cortical samples of both the infected and control plants were treated in the same way.

For each sample, 20 semithin sections (10 tangential, 10 transversal) 1µm thick were then cut using a XTL-2400 Ultracut microtome (Reichert-Jung Optische, Vienna, A), stained with AZUR II on a hot plate for 10–60 seconds according to Hunter (1984), and observed using a DMRB microscope (Leica Microsystems, Wetzlar, D) up to 1000×. For each sample, 20 ultrathin sections 120 nm thick were obtained using the same Ultracut microtome, supported on formvar-covered grids (Electron Microscopy Sciences, Hatfield, PA, USA), stained with lead citrate for 2 minutes (Reynolds, 1963; Hunter, 1984) and observed using a Philips EM 308 TEM.

## Results

### Presence of *C. fraxinea* in the inoculated samples

After 80 days, all the inoculated plants showed at least 5 mm long and 3 mm deep bark lesions (ave. 8 mm, ranging from 5 to 21 mm, and ave. 4 mm, ranging 3 to 5 mm, respectively). In the control plants, all wounds were closed, and necroses were visible only along the wound edge. *C. fraxinea* was consistently reisolated from the inoculated seedlings but never from controls.

### *C. fraxinea* mycelium

Under TEM, the hyphae of the pure FS8 culture were 0.84–1.5  $\mu\text{m}$  in diameter, with cell walls 0.14–0.26  $\mu\text{m}$  thick, composed of layers that changed in density, ending in an outer layer that was electron-dense and amorphous. As expected, the fine structure displayed mitochondria, vacuoles, nuclei, endoplasmic reticulum, microbodies and lipid droplets. Ribosomes, with a maximum diameter of 22 nm, were often distributed in the cytoplasm of the growing hyphae (Figure 1). The hyphal septa showed a single central pore (ave. 0.11  $\mu\text{m}$  diameter; Figure 2) and a wafer-like structure: a central layer, which started from elongated triangular shapes, appeared to be less electron-dense than the adjoining layers (Figure 3). Groups of 1–3 Woronin bodies (ave. 0.23  $\mu\text{m}$  diameter) were often present alongside the septum or plugging the pore (Figure 2).

Inside the hyphae, other hyphae having both a wall and cytoplasmic contents were observed to develop outward (Figure 4). Often, additional hyphae, with electron-dense cytoplasmic contents, were observed inside the intrahyphal hyphae (Figure 4, arrow).

### Woody tissues

The fungus was never found in the tissues of control plants, based on the semithin sections and TEM observations.

In all the woody tissues observed from the inoculated plants, the hyphal features were comparable to those observed in pure cultures (1.1–1.7  $\mu\text{m}$  in diameter, with cell walls 0.14–0.24  $\mu\text{m}$  thick and a single central pore of ave. 0.12  $\mu\text{m}$  diameter). Many of the hyphae appeared to be degenerated, showing a larger size and an electron-transparent cytoplasm

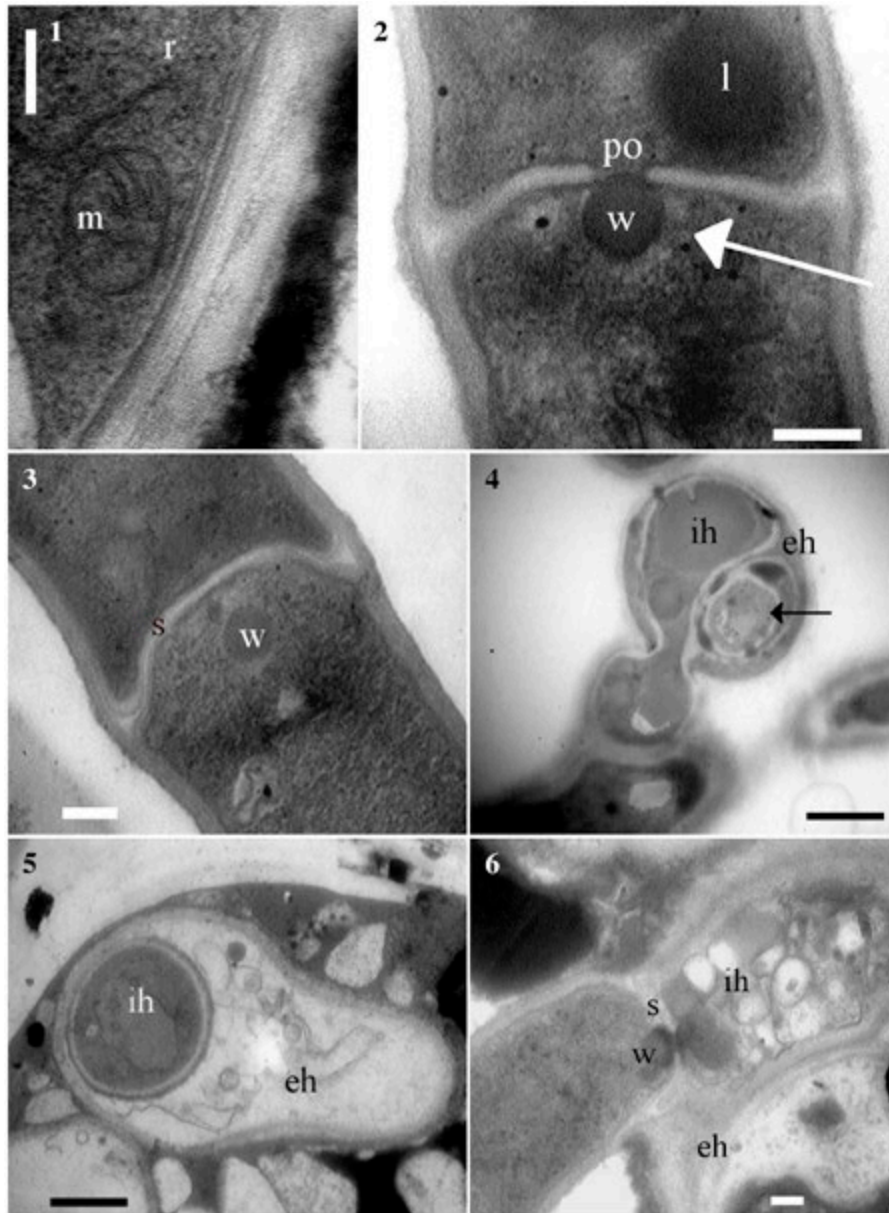
without cellular organelles. Inside these, further fungal structures with a cell wall, condensed cytoplasm and without septa were frequently present (Figure 5) and able to develop outward, directly crossing the outer hypha, or arising through a septal pore. Upon perforation, a septum was observed between the outer hypha and an electron-dense plug occluding the pore and comparable to a Woronin body (Figure 6). The entrance of one hypha into another was also observed (Figure 7).

Unlike control plants (Figure 8), strong hyphal colonisation was observed in the phloem, axial paratracheal parenchyma and parenchymatic rays (Figures 9 and 10). In the rays, the amount of starch grains was visibly less than in the control samples, and the degeneration deeply affected the cytoplasmic constituents (Figure 10). The plasmalemma and cell walls remained undamaged, whereas the plastid membranes were more degraded and electron-transparent than the controls, and the dimensions of the starch granules were notably reduced. The vacuoles were convoluted, with a decondensed and indistinct tonoplast. More rarely, only electron-dense amorphous cytoplasmic residues were visible, with a collapsed nucleus toward the walls and a cytoplasm rich in electron-dense material (Figure 11). In the xylem the vessels and tracheids were colonised by mycelium but to a lesser degree than the parenchymatic cells. In all observations, the mycelia developed only within the cell lumen, and the cell walls never showed degradation. The hyphae crossing the simple pits (Figure 12) showed a notably reduced diameter, which became a normal size thereafter, and the fungal wall partially tailed off (Figures 12 and 13). Perforation involved only a small part of the pit's membrane, without distorting it, and electron-opaque materials were detectable all around (Figure 14). When the intrahyphal hyphae described above passed through the pits within an outer hypha, no further damage was observed to the middle lamella (Figure 15).

## Discussion

In this study, we investigated the colonisation strategies of *C. fraxinea* in the bark and woody tissues of Common ash at ultrastructural level.

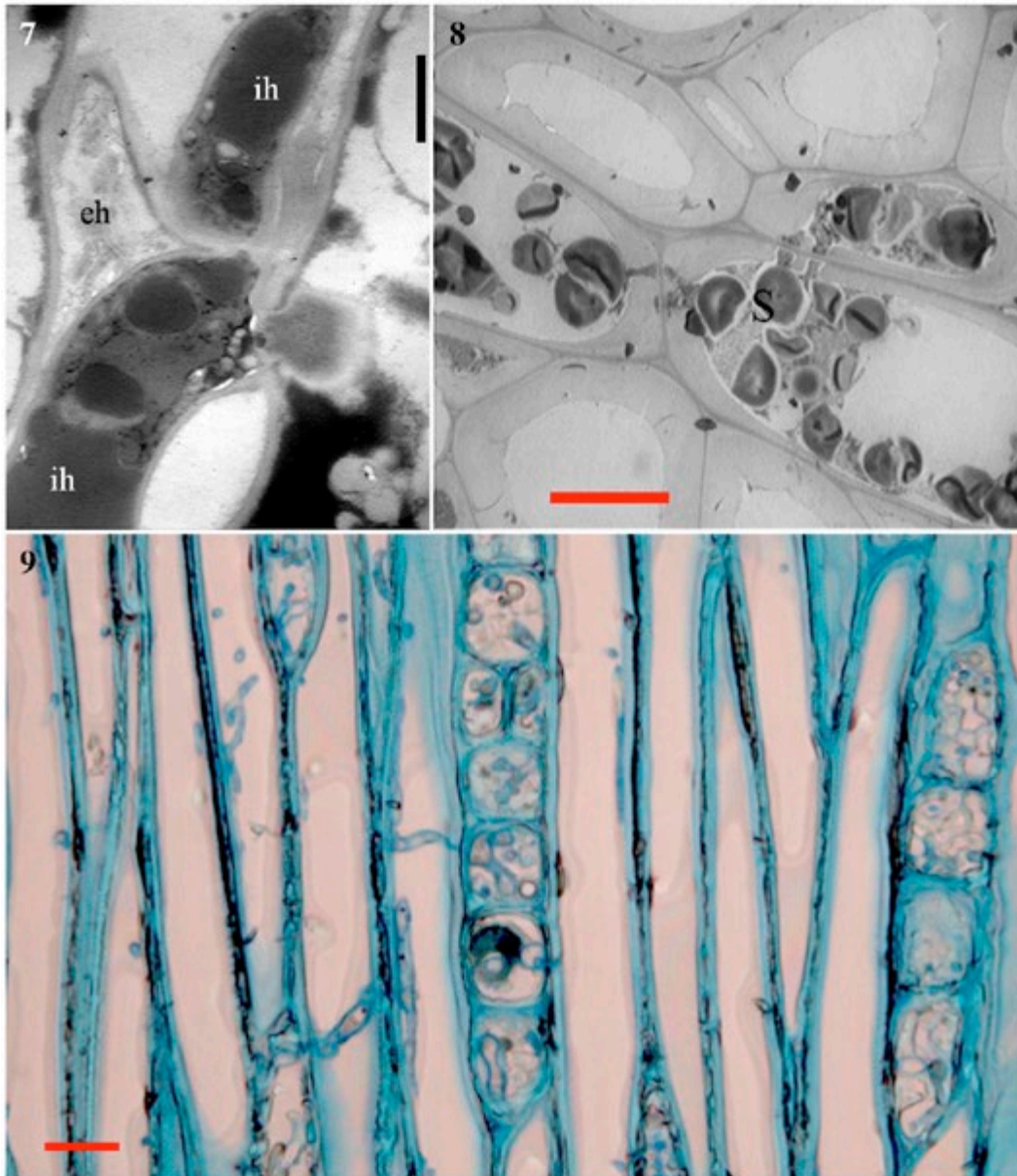
As revealed by electron microscopy, the same fungal features of the pathogenic strain used for the inoculations were observed in both the pure



**Plate A. Figure 1.** Hypha. Cell wall composed of several layers; inner layers ending in an amorphous outer layer. TEM. **Figure 2.** Hypha. Septum with a single pore in the middle; a Woronin body seems to migrate and plug the pore. TEM. **Figure 3.** Septum with a wafer-like structure. Two electron-dense layers enclose a central layer starting from elongated triangular shapes. Woronin bodies are present. TEM. **Figure 4.** Intrahyphal structure. Cytoplasmic contents and wall. A third internal walled structure (arrow) and a hypha exiting the outer hypha are visible. TEM. **Figure 5.** Hyphae inside a parenchymatic cell. The hyphal cellular contents are degenerated, and an inner fungal structure is present. **Figure 6.** An intrahyphal hypha is perforating and departing from the outer hypha. At the interface with the outer hypha, a new septum develops and a hyphal plug occludes the pore. (fungal structures: eh, external hypha; ih, intrahyphal hypha; l, lipid droplet; m, mitochondrion; po, pore of septum; r, ribosomes; s, septum; w, Woronin body; white bar, 200 nm; black bar, 1  $\mu$ m).

culture and wounded tissues. In particular, a outer electron-dense amorphous fungal layer, similar to

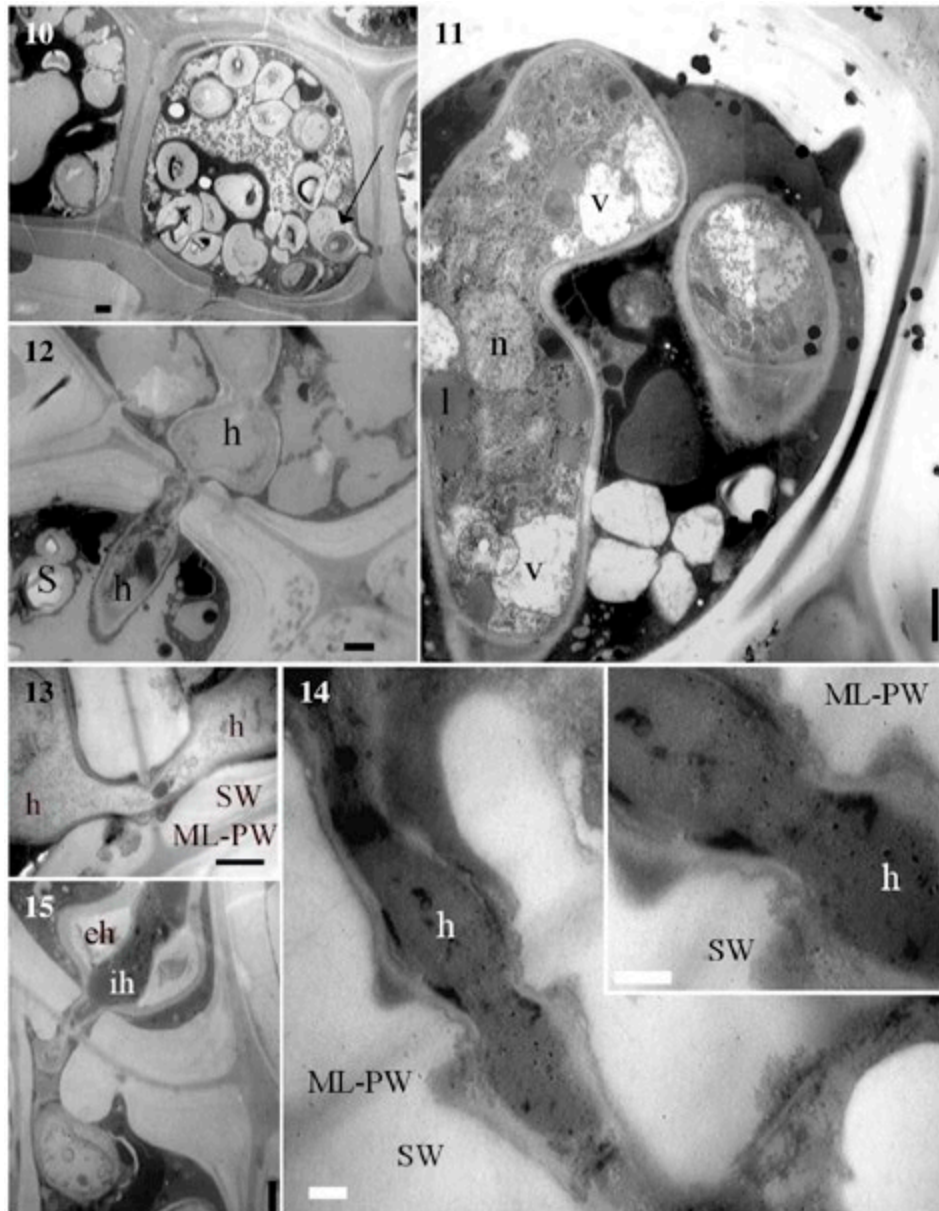
the extrahyphal matrix described by Deacon (2006), was observed in the hyphae grown in both the artifi-



**Plate B. Figure 7.** Hypha in woody tissues with degenerated cytoplasm and septa. Another hypha enters the outer hypha, and develops inside. **Figure 8.** Control woody sample. The ray's parenchymatic cells with deposition of a secondary wall and cytoplasm rich in starch grains. **Figure 9.** Xylematic zone. The ray's parenchymatic cells and fibres are colonised by hyphae. Light microscopy. (fungal structures: eh, external hypha; ih, intra-hyphal hypha; plant structures: S, starch grains; black bar, 1  $\mu\text{m}$ ; red bar, 5  $\mu\text{m}$ ).

cial medium and woody tissues, demonstrating that this layer is a characteristic of the fungus *per se*. The presence of a triangular electron-dense thickening at the junction between the cross wall and lateral wall,

previously reported for other fungi (Pock-Steen and Kobayasi, 1970; Deacon, 2006), could be associated with the protrusion of the inner layers of the hyphal walls, but a definitive explanation is not possible.



**Plate C. Figure 10.** Infected woody sample. Parenchymatic cells comprising some hyphae (arrow), with degraded starch grains and plastid membranes. The cytoplasm shows residues of degraded organelles; the plasmalemma and cell walls are apparently undamaged. **Figure 11.** Two hyphae in a collapsed parenchymatic cell. The cytoplasm accumulates highly electron-dense amorphous material. TEM. **Figure 12, 13, 14, 15.** Hyphae crossing a pit between two parenchymatic cells. In particular: **(12)** The hyphal size decreases in the passage. The plant cell wall is not altered, and the middle lamella–primary wall complex in the pit is not distorted. TEM. **(13)** Only a small portion of the pit’s membrane is affected by the perforation. The fungal wall is partially decondensed. TEM. **(14)** Hypha crossing a pit and its magnification on the right. A part of the pit’s membrane is degraded, allowing hyphal passage without distortion. Electron-dense material is present all around the passage. The hypha has a wider diameter in the upper part of the passage, indicating the direction of growth. TEM. **(15)** A degenerated hypha contains an osmiophilic fungal structure shrinking and crossing the pit membrane inside the external one, with no further damage to the middle lamella. (fungal structures: eh, external hypha; h, hypha; ih, intrahyphal hypha; l, lipid droplet; n, nucleus; v, vacuole; plant structures: ML-PW, middle lamella–primary wall complex; S, starch grains; SW, secondary wall; white bar, 200 nm; black bar, 1  $\mu$ m).

In woody samples, whole mycelia can pass through the simple pits between the parenchymatic cells and fibres. When the fungus perforates the middle lamella, its hyphal diameter notably decreases, and the fungal walls become thinner. As the pit membrane never warps when this happens, it is difficult to relate such behaviour to a mechanical interaction with the host; conversely, the main hypothesis is a chemical reaction targeted to a limited portion of the middle lamella and part of the primary wall, due to the production of lytic enzymes (Annis and Goodwin, 1997; Wanjiru *et al.*, 2002). However, the available data do not allow the observed degenerations to be related to a biochemical effect of enzymes or to toxic substances such as viridiol (Grad *et al.*, 2009; Andersson *et al.*, 2010).

A particularly interesting observation was the presence of nested hyphae, with both a wall and cytoplasmic contents inside other hyphae; these could be interpreted as hyphae of a different fungal species or intrahyphal hyphae (endohyphae). The first hypothesis seems hardly likely, as the entrance of a young hypha into a degenerated one has never been associated with the easily recognisable structural reactions of hyperparasitised hyphae, such as plasmalemma retraction, cytoplasm aggregation, necrotisation of the hyphae and extrusion of the cell contents (Benhamou *et al.*, 1999). The most probable hypothesis is, therefore, intrahyphal hyphae, the presence of which is known in all major fungal groups (Buller, 1933; Lowry and Sussman, 1966; Kim *et al.*, 2004; Kim and Hyun, 2007). Such hyphae could be associated to a defensive reaction to antifungal compounds or fungistatic environments (Kim *et al.*, 2004) or they could represent a regeneration mechanism to establish the protoplasmic continuity after degeneration or damage to the hyphal cells (Kendrick and Molnar, 1964; Lim *et al.*, 1983). According to this hypothesis, when the outer hypha is partially degraded (i.e. by age, or fungal enzymes such as chitinase or glucanase, or toxic reactions by the host), *C. fraxinea* could regenerate itself by developing a new hypha that can arise from the outer protecting hypha, forming a new septum and occluding it with a Woronin body, as observed, to avoid dehydration (Calonge, 1968).

According to Kowalski (2006), the observations suggest a similarity between *C. fraxinea* and the genus *Ceratocystis*, *C. fraxinea* being able to colonise the wood along the medullary rays moving to piths, tracheids and vessels, to disrupt the parenchymatic cells' organelles, and produce intrahyphal hyphae,

as previously reported in studies performed on *Ceratocystis platani*, *C. fagacearum* and *C. dryocoetidis*, respectively (D'Ambra *et al.*, 1977; Sachs *et al.*, 1970; Kendrick and Molnar, 1964).

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