

## Light and scanning electron microscopy studies of the early infection stages of *Hymenoscyphus pseudoalbidus* on *Fraxinus excelsior*

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The ontogeny and morphology of infection structures associated with the early stages of infection of *Hymenoscyphus pseudoalbidus* on common ash (*Fraxinus excelsior*) leaves and leaf petioles were investigated using light and scanning electron microscopy. Ascospores were produced in mature ascocarps and infections on ash leaves were first observed 2 weeks later. Ascospores developed germ tubes, followed by appressorium formation and penetration of epidermal cells on ash leaves and petioles. *Chalara fraxinea* spores, the anamorph of *H. pseudoalbidus*, appeared and were arranged in chains, surrounded by a membranous sheath, and varied considerably in size and shape. Host invasion and colonization of all cell types of leaves and petioles were observed using light microscopy. The role of leaves and petioles as sites of infection in the life cycle of *H. pseudoalbidus* and the disease cycle of ash dieback is discussed.

**Keywords:** appressorium, ash dieback, *Chalara fraxinea*

### Introduction

Ash dieback, caused by the ascomycete fungus *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*), is a serious disease of European or common ash (*Fraxinus excelsior*) and other native and non-native *Fraxinus* species (Kirisits *et al.*, 2009; Drenkhan & Hanso, 2010) in many parts of Europe (Kowalski, 2006; Bakys *et al.*, 2009a; Queloz *et al.*, 2011). It was first observed in Poland in the early 1990s (Przybył, 2002; Kowalski, 2006) and has since emerged rapidly throughout the natural range of ash in central and northern Europe (Timmermann *et al.*, 2011). To date, more than 25 countries have reported the occurrence of ash dieback and it is expected that *H. pseudoalbidus* will successively spread into currently non-affected areas, threatening common ash in large parts of its natural distribution range.

Symptoms include dieback of shoots, twigs and branches and wilting of the foliage as a result of shoot girdling, necrotic lesions and cankers in the bark, and discoloration of sapwood. Trees of all ages on various site types in forests, urban and nursery settings are affected (Timmermann *et al.*, 2011). The earliest visible symptoms of ash dieback are suggested as brown to blackish necrotic lesions on leaf petioles and rachises (herein referred to as petioles) and leaflet veins, followed by wilting of leaves distal to the necrotic lesions and premature leaf shedding

(Bakys *et al.*, 2009b; Kirisits *et al.*, 2009; Kräutler & Kirisits, 2012). Necrotic lesions in the bark usually form at the junction with a diseased twig or around a leaf scar and often expand and girdle the cambium and phloem (Kirisits *et al.*, 2009). Lesion development is affected by season (Bengtsson, 2013) and bark necroses may be temporarily repressed by host reactions. Internally, brown to grey discoloration of the sapwood extends beyond the visible necrotic bark lesion (Kirisits *et al.*, 2009). Epicormic shoots are often formed as a stress response on affected trees (Johansson *et al.*, 2009; Kirisits *et al.*, 2009). Phloem girdling and sapwood occlusion occurring outside the vegetation period prevent shoots from flushing in spring and lead to dieback (Kirisits *et al.*, 2009). Early wilting of leaves and withering of shoots soon after flushing in spring may occur due to girdling related to the progression of the fungus, already established in shoots in the previous vegetation period, during winter months (Kirisits *et al.*, 2012). Similarly, wood discoloration can extend into the roots and affect coppice sprouts.

Apothecia of *H. pseudoalbidus* are predominantly formed on decomposing petioles and leaflet veins from the previous year in the leaf litter (Kowalski & Holdener, 2009b; Timmermann *et al.*, 2011). The native *H. albidus* is morphologically similar to, and occupies the same ecological niche as, *H. pseudoalbidus*, although strong genetic differentiation exists between the two species (Bengtsson *et al.*, 2012). Moreover, it has become increasingly apparent that competitive exclusion by *H. pseudoalbidus* has resulted in *H. albidus* becoming locally extinct in some areas (McKinney *et al.*, 2012). Infections through leaves of *F. excelsior* incited by

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ascospores of *H. pseudoalbidus* have been suggested to be the primary infection court for establishing new infections on trees (Timmermann *et al.*, 2011). However, the exact infection court and mode of entry has not been described. The objective of this study was therefore to elucidate the infection process of *H. pseudoalbidus*, by examining the early sequences of pathogenesis on leaves and petioles exposed to ascospores using light- and scanning electron microscopy.

## Materials and methods

### Plant material

The experiment was conducted with 19 2-year-old bare-root seedlings of *F. excelsior* (30–50 cm in height) obtained from a commercial nursery near Helsingborg, Sweden in March 2011. The seed originated from Billingen, near Skövde, Sweden. The seedlings were grown in 20 cm diameter plastic pots filled with potting media consisting of 60% light peat sieved, 25% black peat and 15% sand (Hasselfors Garden) in a greenhouse with a 16 h photoperiod of 20/15°C (day/night). Seedlings were allowed to grow for 3 months before they were individually transferred to separate moist chambers for fungal inoculation. Moist chambers consisted of cylindrical flexible plastic tubes (c. 30 cm in diameter and 100 cm in height) with mesh screens (10 × 10 cm) mid-height to facilitate aeration and misting.

### Exposure of plants to ascospores of *H. pseudoalbidus*

Leaf petioles from the previous year covered by black pseudo-sclerotial plates of *H. pseudoalbidus* were collected on 30 May 2011 from the leaf litter in a *F. excelsior* seed orchard located at Trolleholm (55°57' N, 13°12' E) in southern Sweden. Most petioles carried immature apothecia but a few petioles partially covered by litter already had fully developed apothecia. In the greenhouse, a minimum of 25 petioles were placed on moist filter paper and a plastic mesh screen fitted to the base of each potted seedling. The petioles and seedlings were maintained in a moist chamber with manual daily light misting through the mesh screen fitted to the chamber in the afternoon over several weeks to allow apothecia to mature. The daily misting also resulted in water accumulation on leaves that would protect spores against desiccation. For control purposes, three comparable seedlings were treated in the same way, but leaf petioles were not added to the moist chambers. The seedlings were regularly inspected for the occurrence of external symptoms during the period between late June and early October 2011. Leaves from plants with symptoms and healthy control plants were collected bi-weekly throughout the experiment for light- and scanning electron microscopy (SEM). Dead petioles bearing apothecia of *H. pseudoalbidus* were also sampled for SEM. At the end of the experiment, abscised leaves from trees with symptoms and uninfected control trees were collected to confirm the presence of the fungus by PCR amplification with *H. pseudoalbidus*-specific primers (Johansson *et al.*, 2010). Samples were kept at –70°C until DNA extraction could be performed.

### Scanning electron microscopy (SEM)

Leaf tissue samples (1 × 1 cm), collected from seedlings, were pre-fixed in 3% glutaraldehyde plus 2% paraformaldehyde in a

0.1 M sodium cacodylate (CAC) buffer (pH 7.2) for 2 h at room temperature and then stored at 4°C until further processing. Samples were washed twice in the CAC buffer for 30 min and then post-fixed in 0.1% w/v osmium tetroxide in the same buffer for 2–3 h at room temperature. After fixation, samples were washed (2 × 30 min) with distilled water and dehydrated with a graded ethanol series (20, 40, 60, 70, 80, 90, 95 and 99.5%) consisting of 10 min steps for each ethanol concentration followed by a graded ethanol–acetone series with 30 min steps. Specimens were subsequently dried in a critical-point drier (Agar Scientific Ltd) with liquid CO<sub>2</sub> as the transition fluid, mounted on SEM stubs with double-sided sticky tape and coated with gold (40 nm thickness) using an Emitech K550X sputter coater (Quorum Technologies). Observations of plant and fungal structures were made using a Philips XL30 ESEM scanning electron microscope operated at 10 kV and equipped with a camera for digital micrographs.

### Light microscopy

To examine fungal growth inside tissues, leaf and petiole samples were fixed in FAA (70% ethanol, 37% formaldehyde, glacial acetic acid, glycerol; 85:5:5:5 by volume) for 3 days and then stored in 70% ethanol until further processing. Samples were embedded with Technovit 8100 (Kulzer) according to the manufacturer's instructions with some modification. Samples were dehydrated in a graded ethanol series as described above followed by a pre-infiltration with ethanol and the base-liquid under vacuum. Tissue samples were treated with the infiltration solution for 12 h at 4°C. Samples were then placed in moulds with the embedding solution and allowed to polymerize for 24 h. From the embedded samples, 6 µm serial sections were cut on a Leica RM2265 microtome, stained with 0.5% toluidine blue for visualization of fungal hyphae, and mounted on fresh glycerol on glass slides. Sections were examined using a Leica DM5500B microscope equipped with digital camera for photomicrographs.

### DNA extraction and PCR

A PCR test with *H. pseudoalbidus*-specific primers (Johansson *et al.*, 2010) was performed to detect the presence of the pathogen in leaves, including leaf veins and petioles, collected from trees with symptoms and uninfected control trees. Leaf tissues were homogenized in 2 mL screw cap tubes, using a screw nut and a screw in a fast prep Precellys control device. Extraction CTAB buffer (3% cetyltrimethylammoniumbromide, 2 mM EDTA, 2.6 M NaCl, 0.15 M Tris-HCl; pH 8.0) was added to remove membrane lipids followed by incubation at 65°C for 1 h. After centrifugation, the supernatant was transferred to new 1.5 mL Eppendorf tubes and then mixed with an equal volume of chloroform. After centrifugation for 7 min at 6708 g the supernatant was precipitated with 1.5 volumes isopropanol, washed with 70% ethanol and dissolved in 50 µL milli-Q water. DNA concentration was measured using a NanoDrop spectrometer (Thermo Scientific). Amplifications with the *H. pseudoalbidus* primer pairs were performed in 10 µL reaction volumes. The PCR cycling conditions included an initial denaturation step at 95°C for 5 min followed by 35 amplification cycles of denaturation at 94°C for 30 s, annealing at 62°C for 1 min and extension at 72°C for 30 s. The reaction was finished by an extension step at 72°C for 7 min. PCR products were visualized by gel electrophoresis on a 1% agarose gel in SB buffer.

## Results

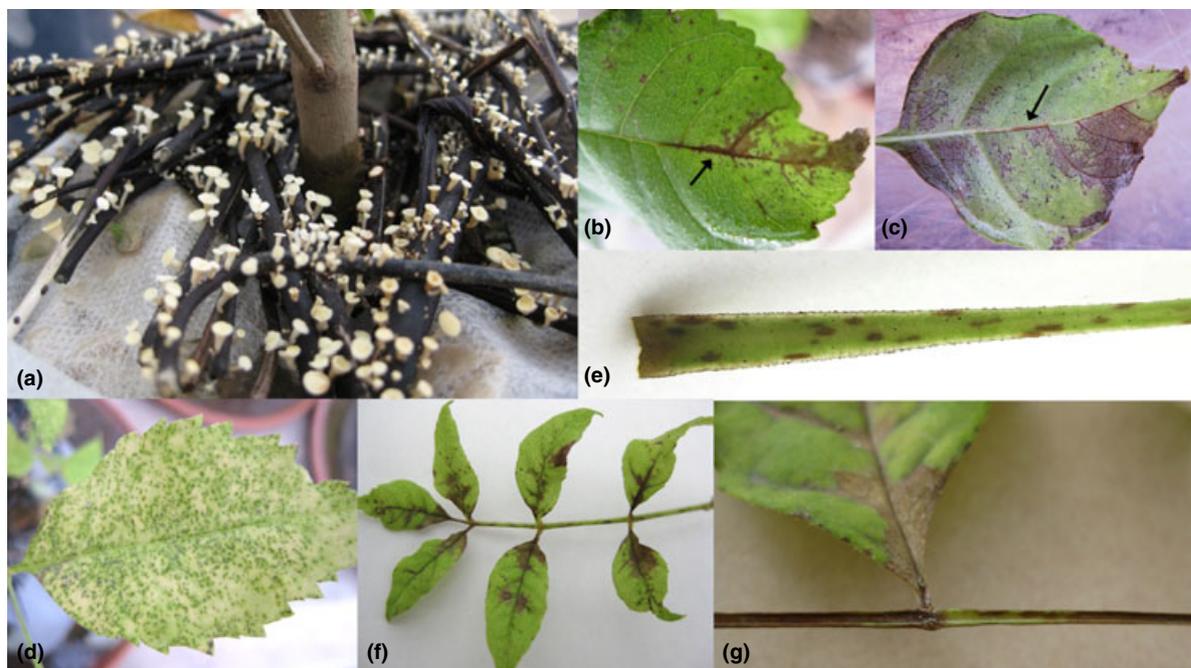
### Symptoms

Symptoms on leaves were noted during early July, *c.* 2 weeks after the first mature ascocarps had occurred (e.g. Fig. 1a), although most apothecia matured during a period of 2 months between July and August. All inoculated plants showed symptoms similar to those previously reported on naturally infected trees (e.g. Bakys *et al.*, 2009b; Kirisits *et al.*, 2009) although the appearance and severity of symptoms varied from small necrotic lesions on leaves and leaflet veins (Fig. 1b,c) to mottling and chlorosis of leaflets (Fig. 1d), which then turned brown and abscised. Leaf petioles showed numerous localized and small necrotic lesions (Fig. 1e) which amalgamated over time into elongated lesions. Necrotic symptoms on leaflets usually occurred prior to lesion development on petioles (Fig. 1f,g), but in a few cases, only necrotic lesions on the petioles were evident and caused abscission of single symptomless leaflets. At the end of the experiment in October, most leaflets and petioles had abscised. No lesions were observed on the main shoots. The test with *H. pseudoalbidus*-specific primers resulted in successful amplification for the leaflet and petiole samples obtained from trees with symptoms. In the uninfected control trees, no symptoms developed and no amplification was obtained in the PCR test.

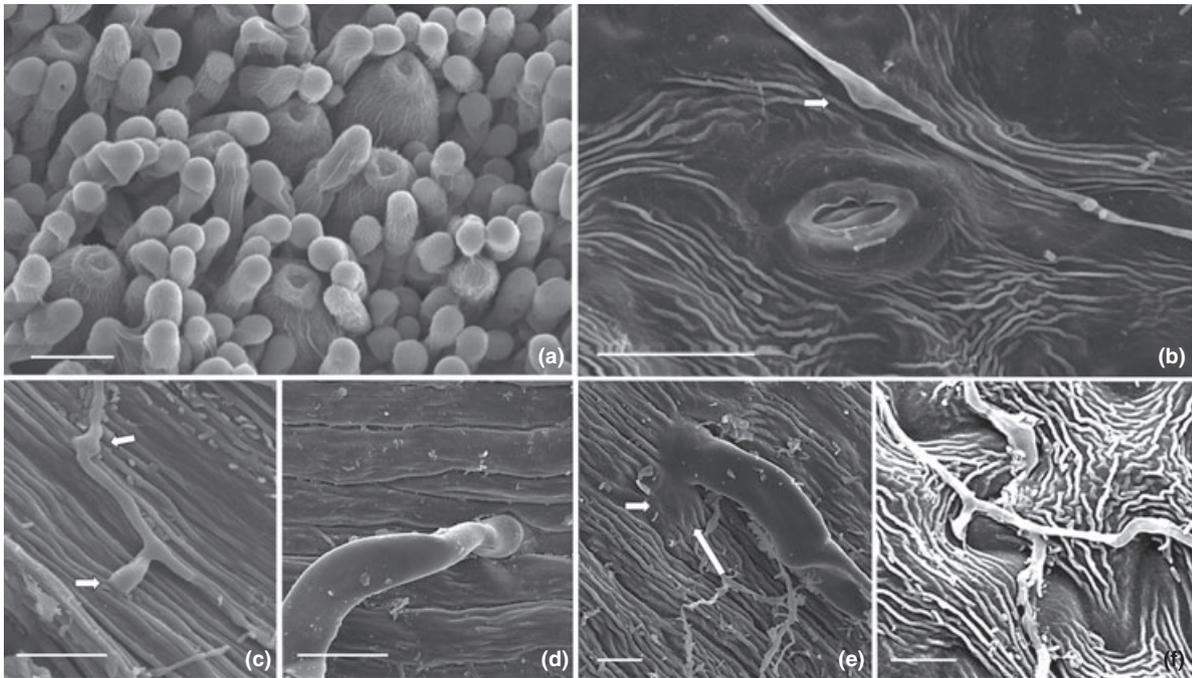
### Infection sequence of *H. pseudoalbidus*

Scanning electron microscopy revealed that the hymenium of ascomata of *H. pseudoalbidus* consisted of mature asci containing ascospores, interspersed with cylindrical, septate paraphyses of approximately the same length as the asci (Fig. 2a). On leaf and petiole surfaces, firm attachment of ascospores due to an amorphous mucilage matrix was observed. Ascospores germinated by giving rise to one short germ tube, whose tip formed a swollen adhesive disc that adhered firmly to the leaf surface. Subsequently, appressoria formation occurred (Fig. 2b). The extracellular mucilage apparently formed by the appressorium also appeared to play a role in adhesion to the host surface. Parts of the cuticle exposed to the mucilage around the fungal structure appeared to be structurally modified. No such alterations in wax platelets were observed in control leaves. In more advanced differentiation, the appressoria appeared subglobose with irregular and variable margins (Fig. 2b,c,d) and caused partial dissolution of the cuticle (Fig. 2e). Following differentiation of appressoria, penetration of epidermal cells occurred.

Hyphae were observed closely appressed to the leaf cuticle (Fig. 2f) and extended over and into natural leaf openings (e.g. stomata; Fig. 3a,b). In general, fungal growth appeared to lack direction on the leaf surface, although linear orientation of hyphae was sometimes observed, following the contours of epidermal cells (Fig. 3c). Fungal-induced necrosis of epidermal cells appeared as sharply



**Figure 1** (a) Mature apothecia of *Hymenoscyphus pseudoalbidus* on dead leaf petioles at the base of an ash seedling in a moist chamber system; (b,c) necrosis on leaflet blades and leaflet veins (arrows); (d) mottling and chlorosis of a leaflet blade; (e) leaf petiole showing numerous, distinct and necrotic lesions; (f,g) necrosis extending from leaflets to leaf petioles.



**Figure 2** Samples examined with scanning electron microscopy. (a) Hymenial layer of an apothecium of *Hymenoscypha pseudoalbidus* showing asci surrounded by paraphyses, bar = 5  $\mu\text{m}$ ; (b) formation of an appressorium (arrow) on an ash leaf, bar = 20  $\mu\text{m}$ ; (c) appressoria formation (arrows) on an ash petiole, bar = 10  $\mu\text{m}$ ; (d) penetration of epidermal surface, bar = 20  $\mu\text{m}$ ; (e) alteration of wax platelets surrounding appressorium (arrows) suggesting enzymatic degradation at point of penetration, bar = 20  $\mu\text{m}$ ; (f) hyphal growth over cuticular surface of a leaf, bar = 10  $\mu\text{m}$ .

delineated lesions, in which cell walls had lost turgidity and shape (Fig. 3d). Necrosis expanding along leaflet veins resulted in softening and local dissolution of the cuticle and the epidermal cell walls. Large infection cushions consisting of an aggregation of branched hyphae, probably originating from one or more ascospores, resulted in extensive mycelial development and penetration points (Fig. 3e). At the surface of host cells confronted by fungal hyphae, abundant extracellular material was observed.

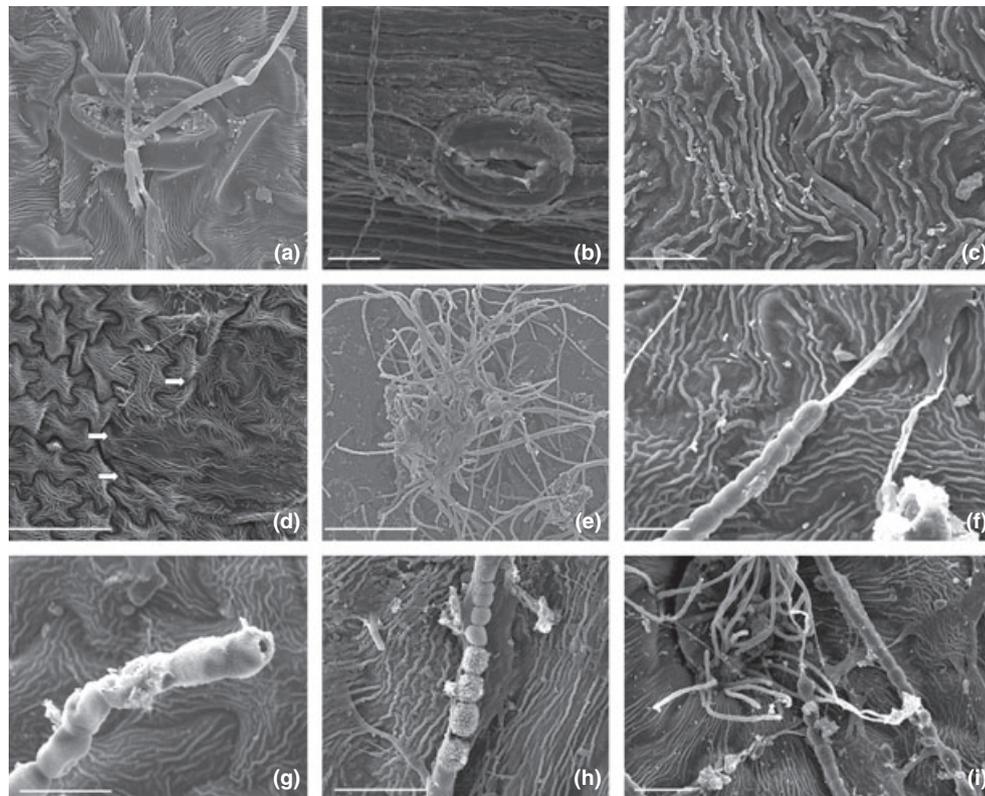
Putative conidia of *C. fraxinea* emerged from terminal hyphae and were extruded in long, persistent chains (Fig. 3f,g). Spores varied considerably in size and shape and were commonly enclosed within a membranous sheath (Fig. 3h), extending from the top of the phialides to the apical conidium of the chain. Extensive mycelia developed from infection cushions (Fig. 3i) and growth and penetration was observed on the abaxial and adaxial surfaces of leaflets as well as petioles.

In histological sections from leaflets and petioles with symptoms examined with light microscopy, fungal hyphae were abundant. In leaflets, fungal hyphae occurred in epidermal, palisade and spongy mesophyll cells and in the vascular bundle (Fig. 4a). The cytoplasm and walls of affected host cells appeared mostly disintegrated and cells often appeared collapsed compared to those in samples from healthy control trees (Fig. 4b). Sections of petioles that had stained more intensely contained a large number of hyphae in the xylem vessels, the phloem, fibre and axial parenchyma cells (Fig. 4c,d).

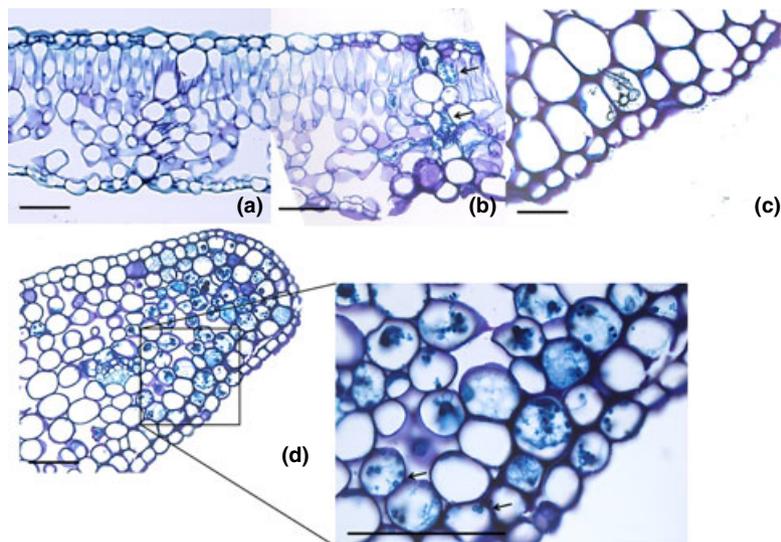
Proliferation of fungal hyphae was primarily through pits. In petioles with more advanced stages of necrosis, hyphae were also found in the pith region.

## Discussion

On *F. excelsior* trees subjected to natural infections, the first visible symptoms of ash dieback are thought to be necrosis of leaflets and leaf petioles, although the association of *H. pseudoalbidus* with leaf symptoms on *F. excelsior* and the role of leaf infections in the disease cycle of ash dieback has not been fully proven (Timmermann *et al.*, 2011; Krautler & Kirisits, 2012). Recently, Krautler & Kirisits (2012) fulfilled Koch's postulates to show that necrotic lesions on leaf petioles resulting in leaf wilting and abscission are associated with *H. pseudoalbidus*, although wound inoculation, and not ascospores, were used. In the present investigation, clear associations were shown between ascospore infection by *H. pseudoalbidus* and subsequent symptom development on leaves. The mottling and chlorotic symptoms on leaflets from just a few trees were atypical compared to that previously reported (e.g. Bakys *et al.*, 2009b; Kirisits *et al.*, 2009; Krautler & Kirisits, 2012), but may have resulted from a higher than normal inoculum pressure using the current method. The multiple, localized necrotic lesions on petioles clearly appeared to reflect individual ascospore infections, not necessarily originating from leaflets.



**Figure 3** Samples examined with scanning electron microscopy. (a,b) Hyphal growth over and into stoma on a leaflet, bar = 20  $\mu\text{m}$ ; (c) orientation of a hypha, following the contour of adjacent epidermal cells, bar = 10  $\mu\text{m}$ ; (d) necrotic lesion on leaf blade surface showing loss of turgidity and alteration of the shape of epidermal cells (arrows), bar = 50  $\mu\text{m}$ ; (e) infection cushions originating from one or more ascospores, bar = 20  $\mu\text{m}$ ; (f) putative spores of *Chalara fraxinea* forming on a terminal hypha, bar = 10  $\mu\text{m}$ ; (g) chain of spores, bar = 20  $\mu\text{m}$ ; (h) membranous sheath enclosing the conidial chain, bar = 20  $\mu\text{m}$ ; (i) extensive development of multiseptate conidia from infection cushions, bar = 20  $\mu\text{m}$ .



**Figure 4** Leaflet and petiole sections prepared for light microscopy. (a) Section through an uninfected ash leaflet from a control plant, showing typical anatomical features (epidermal, palisade and spongy mesophyll cell layers), bar = 25  $\mu\text{m}$ ; (b) section through an infected ash leaflet showing fungal hyphae colonizing epidermal as well as underlying palisade and spongy mesophyll cells (arrows), bar = 25  $\mu\text{m}$ ; (c,d) cross-sections through ash petioles; (c) fungal hyphae in ash petiole parenchyma cells, bar = 15  $\mu\text{m}$ ; (d) infected leaf petiole with fungal hyphae occurring in phloem and xylem tissues, bars = 25  $\mu\text{m}$ .

Successful penetration and colonization of plant tissues by most fungal pathogens requires differentiation of specialized cell types or infection structures (Mendgen & Deising, 1993; O'Connell *et al.*, 1996). In this study, microscope examination of *F. excelsior* leaves exposed to

ascospores of *H. pseudoalbidus* allowed a description of the sequential development of early infection stages associated with the pathogen. Observations suggest that the infection process includes events associated with certain stages of morphogenesis: ascospore germination and

formation of infection structures such as appressoria (hypothesized by Gross *et al.*, 2012), followed by leaf cuticle penetration and subcuticular growth.

Adhesion to the host surface is considered to be an essential prerequisite for fungal infection, as surface substrate recognition and signals are typically required for infection structure formation (Hoch & Staples, 1991; Nicholson, 1996). Essential for the infection process is the release of substances by the ungerminated spore upon its contact with the host. The extracellular matrix probably consists of polysaccharides and glycoproteins released by exocytosis or passively through the bursting of the spore cell wall upon contact with water (Nicholson, 1996). Excreted enzymes in the mucilage may then alter the host epicuticular waxes and the cuticle structure enzymatically, facilitating contact of the pathogen with the host. Appressoria formation will also reinforce contact with the host surface and enzymatically soften the cuticle at the penetration site, allowing infection hyphae to mechanically penetrate the cuticle and the host cell wall. For fungi with necrotrophic behaviour, the site of penetration may be entirely random or occur frequently at the anticlinal junction of epidermal cells (Hoch & Staples, 1991) as was also shown in this study.

Emmett & Parbery (1975) suggested that infection cushions (commonly observed on leaflets in this study) are complex appressoria, which commonly originate from hyphae. Infection cushions serve to increase the host–parasite interface and may enhance the physical contact between fungal toxins or enzymes and the plant (Jamaux *et al.*, 1995). *Hymenoscyphus pseudoalbidus* is known to produce phytotoxins such as viridol (Andersson *et al.*, 2010) and other secondary metabolites (Andersson *et al.*, 2012a,b) which may manipulate the plant physiology to the benefit of the pathogen. The aggressiveness of the fungus (Kowalski & Holdenrieder, 2009a) also suggests that it produces a broad spectrum of hydrolytic enzymes that lyse host cells ahead of colonization and before they can mobilize effective defence, similar to that shown for other fungal pathogens (Hématy *et al.*, 2009).

Once infection was established, mycelia continued to proliferate and colonized neighbouring cells. Schumacher *et al.* (2010) described fungal colonization by *H. pseudoalbidus* in discoloured xylem of 3-year-old nursery ash saplings as abundant hyphae occurring in the axial vessels, paratracheal ray tissues, fibre cells and in the phloem. Similarly, in the current study hyphae were observed in all cell types of leaves and from the pith to the phloem in petioles, with intercellular passage through pits.

The spores observed on diseased leaves in this study were morphologically similar to that described by Kowalski (2006), occurring as chains or as long and branched filaments, and similar to descriptions of other *Chalara* species (Hawes & Beckett, 1977a; Kile & Walker, 1987; Carris, 1988; Kowalski & Halmschlagler, 1996; Koukol, 2011). There was though considerable variation in spore size and shape (e.g. Fig. 3f,i). Hawes & Beckett (1977a,b) and Koukol (2011) suggested that

changes in conidial morphology are common, and this is probably also true for the anamorph of *H. pseudoalbidus* observed on leaves in this study. Furthermore, there may be considerable variation in morphology of conidiophores *in vitro* (e.g. Kowalski, 2006) and *in situ* (on natural substrates). The precise role of conidia in the disease cycle is still uncertain. Kirisits *et al.* (2009) reported that inoculations of shoots and leaves of ash seedlings with suspensions of *C. fraxinea* spores resulted in no symptoms. Furthermore, spores of *C. fraxinea* did not germinate on various agar media, on medium supplemented with ash leaf extracts and after *in vitro* inoculation of detached ash leaflets, which led to the suspicion that the spores of *C. fraxinea* are not conidia but rather spermatia that are not capable of inciting infections but play a role in exchanging nuclei as part of sexual reproduction of the ash dieback pathogen (Kirisits *et al.*, 2009). This plausible hypothesis is also supported by Gross *et al.* (2012). In the current study, observations of hyphal structures on leaves in the form of conidial chains resembled those also observed in the phloem and xylem of petioles. It is proposed that the propagation of *C. fraxinea* conidia following initial penetration and infection by ascospores facilitates spread causing staining and dysfunction of host tissues, during and outside the vegetation period.

Accumulating evidence suggests that leaves and leaf petioles are the primary habitat of *H. pseudoalbidus*. The fungus undergoes an extensive saprotrophic stage (overwintering on petioles in the leaf litter) and is later capable of infecting and parasitizing ash leaflets and leaf petioles. Primary inoculum comes from apothecia produced on dead leaf petioles (Kowalski & Holdenrieder, 2009b; Queloz *et al.*, 2011), and occasionally from ligneous tissues and dead shoots of 1- to 3-year-old ash seedlings (Kirisits *et al.*, 2009, 2012; Kowalski & Holdenrieder, 2009b; Kirisits & Freinschlag, 2012) from approximately June to September in the year following infection, with peak periods of spore dispersal probably occurring between July and August (Kirisits *et al.*, 2009; Timmermann *et al.*, 2011). In this study, and as reported by Kirisits *et al.* (2009), ripe apothecia can also appear as early as late May. Thus, it is evident that the sporulation period of *H. pseudoalbidus* is strongly influenced by climatic and microsite conditions.

Kirisits *et al.* (2009) and Timmermann *et al.* (2011) suggested ascospore infections through the leaves of *F. excelsior* are the primary infection court for establishing new infections on trees. In this investigation, both leaves and leaf petioles were confirmed as targets of primary infections by *H. pseudoalbidus*. Furthermore, lesions initiated by ascospores, in turn, produced conidia. Kirisits *et al.* (2009) proposed that direct infection of shoots by ascospores may be possible. Moreover, Husson *et al.* (2012) suggested that collar and aerial root infections on trees may be induced by ascospores infecting via lenticels in the bark. In this study, no such infections initiated on shoots were observed. However, as fungal progression in host tissues and external symptom

development also occur outside the vegetation period, the fungus, given time, may have grown from infected petioles into the phloem and xylem to cause necrotic bark lesions and wood discoloration in the stem.

The observations in this study contribute to the understanding of the role of airborne ascospores of *H. pseudoalbidus* as the primary source initiating host infections, the mode of entry and infection structure formation of the pathogen on host tissues and the temporal spread of the fungus during its early stages of development. The method used in this experiment for inciting natural infection with ascospores has since been repeated successfully in the authors' laboratory in a first screening of resistance in ash genotypes. A moist chamber system appears essential for the development and maturation of apothecia and to condition the host for infection. However, endophytic and opportunistic fungi can also be promoted in such a system and thus pyrosequencing methods should complement future studies to provide a platform for characterizing and quantifying levels of other associated fungi. Further studies are required to fully elucidate the time course for infection structure formation and development of *H. pseudoalbidus* on *F. excelsior* leaves and petioles and also to understand the role of environmental variables in the infection process, especially for ascospore germination and conidial production.

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