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Hymenoscyphus fraxineus can directly infect intact current-year shoots of *Fraxinus excelsior* and artificially exposed leaf scars

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Summary

Hymenoscyphus fraxineus, the causal agent of ash dieback, was inoculated onto intact, unwounded current-year shoots and leaf scars of 4-year-old, potted *Fraxinus excelsior* seedlings. Pieces of ash wood colonized by the fungus were used as inoculum. Three of 25 (12%) of the inoculated intact shoots and nine of 25 (36%) of the inoculated leaf scars were infected by *H. fraxineus* and developed typical symptoms of ash dieback, including necrotic lesions on the shoot surface and wood discoloration as well as shoot and leaf wilting distal to the inoculation site. No symptoms occurred on control seedlings, which had been inoculated in the same way but with sterile wood pieces. Visible necrotic lesions on shoots and wood discoloration were statistically significantly longer in proximal than in distal direction from the inoculation site, a pattern which resembles symptoms after natural infection. The ash dieback pathogen was re-isolated from nine of 12 (75%) of the symptomatic seedlings. These results provide indirect supportive evidence that the fungus infects shoots via leaves and shows that it is able, under experimental conditions using a massive mycelial inoculum, to directly infect intact, unwounded current-year shoots of its main host in Europe.

1 Introduction

European or common ash (*Fraxinus excelsior* L.) is presently endangered within its natural distribution range by the ascomycete *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (synonyms: *Hymenoscyphus pseudoalbidus* Queloz, Grünig, Berndt, T. Kowalski, T. N. Sieber & Holdenr and *Chalara fraxinea* T. Kowalski [asexual stage]), the causal agent of ash dieback (Kowalski 2006; Baral et al. 2014; Gross et al. 2014). During the last two decades, a drastic increase in diseased ash populations has been observed, as the pathogen has successively spread across most of Europe (Timmermann et al. 2011; McKinney et al. 2014). *H. fraxineus* is native to *F. mandshurica* Rupr. in Asia and behaves as an aggressive, invasive, alien pathogen on its main host in Europe, *F. excelsior*, most individuals of which are severely and often lethally damaged (Zhao et al. 2012; Gross et al. 2014; McKinney et al. 2014).

Symptoms of ash dieback are manifold and occur on a wide range of tree organs (leaves, shoots, twigs, branches, stems, root collars and even roots) and tissue types (Bakys et al. 2009a; Kirisits et al. 2009; Schumacher et al. 2010; Husson et al. 2012; Kräutler and Kirisits 2012; Cleary et al. 2013; Gross et al. 2014). Crown symptoms include dieback of shoots, twigs and branches and wilting of leaves due to girdling, as well as necrotic lesions on leaf petioles, rachises and leaflet veins followed by wilting and premature shedding of single leaflets and entire leaves. The stem, root collar region and aerial roots are damaged by necrotic lesions in the bark accompanied by discoloration of the sapwood. Ash trees gradually decline due to repeated infections, over the course of several years, and many eventually succumb to the disease (McKinney et al. 2014).

According to current understanding, wind-dispersed ascospores, produced mainly during summer in apothecia on decomposing, pseudosclerotial leaf petioles and rachises in the leaf litter, are the only infectious propagules of the ash dieback pathogen, while its asexual spores may function as spermatia in the course of sexual reproduction, but most likely do not play any role in infecting the host (Timmermann et al. 2011; Zhao et al. 2012; Chandelier et al. 2014; Gross et al. 2014). *H. fraxineus* is primarily a leaf pathogen, and ash leaves are considered to be the main target for ascospore infections (Kräutler and Kirisits 2012; Cleary et al. 2013). Observational evidence suggests that *H. fraxineus* can grow from infected leaves into shoots of *F. excelsior* and that leaves are the main entrance court for the pathogen into woody parts of its main host (Kirisits and Cech 2009; Schumacher 2011; Kräutler and Kirisits 2012; Cleary et al. 2013). The fungus may also be able to infect shoots directly (Kirisits and Cech 2009; Kirisits et al. 2009; Bengtsson et al. 2014), but this has not yet been proven. Husson et al. (2012) presented evidence that necrotic lesions and wood discoloration on the stem base, root collar and on aerial roots of *F. excelsior*, which are commonly observed in areas affected by ash dieback, are primarily caused by *H. fraxineus*. The infection processes leading to these symptoms are presently unknown, but as they were not associated with leaf scars, side twigs or sprouts, Husson et al. (2012) suggested that *H. fraxineus* ascospores can infect intact bark via lenticels. These findings emphasize that there must be an infection court other than leaves enabling the ash dieback pathogen to enter woody parts of common ash.

In previous artificial stem inoculation studies with *H. fraxineus*, aimed at confirming its pathogenicity to *Fraxinus* spp., fulfilling Koch's postulates or testing the susceptibility of *F. excelsior* genotypes, the inoculation procedures involved wounding of the host (Bakys et al. 2009a,b; Kirisits et al. 2009, 2010; Kowalski and Holdenrieder 2009; Ogris et al. 2009,

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2010; Szabó 2009; Husson et al. 2011; McKinney et al. 2012). Here, we report on an inoculation experiment in which wood pieces colonized by *H. fraxineus* served as inoculum and were experimentally attached to intact, unwounded current-year shoots and to artificially exposed leaf scars of *F. excelsior* seedlings. Both methods of inoculation resulted in successful infection of a portion of the test plants by the ash dieback pathogen. The implications for the infection biology of *H. fraxineus* are discussed.

2 Materials and methods

The inoculation experiment was carried out with 4-year-old seedlings of *F. excelsior* (from a forest nursery in Ottenstein, Lower Austria; provenance: forest ecoregion 5.2, 300–600 m a.s.l., according to Kilian et al. 1994), which were purchased and transplanted into 5.5-l plastic pots filled with a ready mix potting soil for woody and perennial plants (Terra Vita Pflanzsubstrat, T6-Gehölze/Stauden; Franz KRANZINGER GmbH, Straßwalchen, Austria) in April 2009. The potted seedlings were kept in the garden of the institute and watered as required. At the time of inoculation, the plants were visually free of ash dieback symptoms. The seedlings (n = 60) were 39–91 cm high (mean = 57.1) with stem diameters of 2.6–7.6 mm (mean = 4.1 mm) at the height of the inoculation site. At the beginning of the experiment in June 2010, their shoots were fully expanded and their leaves completely unfolded.

Five isolates of *H. fraxineus*, collected between 2007 and 2009 from symptomatic trees of *F. excelsior* and *Fraxinus angustifolia* Vahl at different localities in Austria, were used in the experiment (Table 1). All isolates were identified as *H. fraxineus* by sequencing the ITS rDNA region (see GenBank accession numbers in Table 1) and are maintained in the culture collection of IFFF-BOKU. Four of them have also been deposited at the CBS Fungal Biodiversity Centre (CBS), Utrecht, the Netherlands (Table 1).

For preparation of fungal inoculum, cultures of the selected isolates were grown on malt extract agar [MEA; 20 g DiaMalt malt extract (Hefe Schweiz AG, Stettfurt, Switzerland), 16 g Becoagar agar (W. Behrens & Co, Hamburg, Germany), 1000 ml tap water, 100 mg streptomycin sulphate (Calbiochem; Merck KGaA, Darmstadt, Germany), added after autoclaving] and incubated at 20°C in the dark. After 35 days, autoclaved, rectangular pieces of ash wood, approximately 10 mm in length, 4 mm in width and 2 mm in height, were placed on the surface of the freshly grown cultures, where they remained for 20 additional days under the same incubation conditions prior to their use as inoculum in the experiment. For the control variant, autoclaved wood pieces were placed on sterile MEA.

Inoculations were carried out on 24 June 2010. In the experiment, two inoculation methods, according to the position of the inoculum, were tested on the common ash seedlings: (i) intact, unwounded internodes of shoots (Fig. 1a,c) and (ii) leaf scars exposed by artificially inflicted leaf abscissions (Fig. 1b,d). For inoculation of the unwounded shoots, only current-year main shoots were chosen, and fungal inoculum was placed with forceps onto the intact epidermis and fixed to the shoot with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA). Inoculation of leaf scars was performed by removing a healthy, mature leaf, which created a fresh abscission and exposed a leaf scar, onto which a wood piece colonized by *H. fraxineus* was placed and fixed to the seedling shoot with Parafilm. Very short current-year main shoots were avoided; instead, leaf scars on older sections of the main stem were chosen for inoculation. When placing a fungal inoculum onto a

Table 1. Collection data, CBS numbers, rDNA ITS GenBank accession numbers and infection rates of isolates of *Hymenoscyphus fraxineus* for the two inoculation methods.

IFFF[1] code	Date of isolation, collection locality in Austria and collector[2]	Host and substrate	CBS[3] no.	GenBank accession no.	Infection rate[4] by inoculation method, n (%)	
					Intact shoot (n = 5)	Leaf scar (n = 5)
N1/3 Holz	20-Jun-2007, Upper Austria, Edt bei Lambach, EH & SMK	*F. excelsior*, discoloured wood	122191	KJ197294	1 (20)	3 (60)
N/5/4/A	20-Jun-2007, Styria, Altaussee, EH & SMK	*F. excelsior*, shoot	122192	KC529352	1 (20)	1 (20)
HO/II/6/1	06-Feb-2008, Lower Austria, Hohenau an der March, TK & SMK	*F. angustifolia*, shoot	123140	KJ197295	0	1 (20)
SFB/II/7/2	22-Apr-2008, Vienna, Schafberg, TK & SMK	*F. excelsior*, shoot	123365	KJ197292	0	2 (40)
WER 2/17	03-Apr-2009, Salzburg, Werfen, TK & MM	*F. excelsior*, shoot		KJ197300	1 (20)	2 (40)

[1]Culture collection of the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), University of Natural Resources and Life Sciences, Vienna (BOKU), Vienna.
[2]Abbreviations of the names of the collectors: EH = Erhard Halmschlager, TK = Thomas Kirisits, MM = Michaela Matlakova, SMK = Susanne Mottinger-Kroupa.
[3]Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, the Netherlands.
[4]Based on the presence of an externally visible necrotic lesion on the shoot.

seedling, the side of the wood piece that lay on the surface of a culture was directed towards a shoot or leaf scar. For the controls, sterile wood pieces were applied similarly, according to the two inoculation methods. Before each inoculation, forceps were flame sterilized using 96% ethanol and a Bunsen burner. Each seedling received one inoculation, either on a shoot or an exposed leaf scar, and for each of the two inoculation positions, there were five replicate plants per isolate and the control variant (total n = 30 seedlings for each of the two methods of inoculation; Table 1).

Plants were regularly inspected for necrotic lesions on the shoots and wilting of leaves above the inoculation site until 4 October 2010 and again on 17 March and 8 June 2011. Plants exhibiting symptoms by October 2010 were processed 8–33 days after the first symptoms had been observed. Plants with necrotic lesions in 2011 were processed on 23 March 2011. All symptomatic plants were cut at ground level and transported to the laboratory. On each seedling, any evidence of wilt above the inoculation site due to girdling and/or sapwood occlusion was recorded, and the length of the externally visible necrotic lesion on the shoot surface was measured. Thereafter, the shoot of a seedling was split longitudinally, and the length of the discoloration in the wood was measured. All measurements were made from the middle of the inoculation site to both the top (distal) and the bottom (proximal) of the lesion or the wood discoloration, respectively, and total lengths were later calculated from the two respective records. Plants which were asymptomatic by 8 June 2011, both those inoculated with *H. fraxineus* and the control seedlings, were not further analysed and remained intact in the garden of the institute.

Re-isolations were made from three sections of a symptomatic shoot: (i) from the inoculation site, (ii) from the lower end of the necrotic lesion and (iii) from the midpoint between inoculation site and lower end of the lesion. After surface sterilization (1 min in 96% ethanol, 3 min in 4% NaClO, 30 seconds in 96% ethanol) of a shoot sample, the epidermis or bark was carefully peeled off with a sterile scalpel and discs with an approximate thickness of 2 mm, containing xylem and phloem tissues, were cut with sterile pruning shears. From each of the three sections, three discs were sampled, which were placed on one MEA plate, resulting in a total of nine discs and three Petri dishes per symptomatic seedling. The Petri dishes were sealed with Parafilm and incubated at 6–10°C in the dark. From the developing mycelial colonies *H. fraxineus* was identified based on morphological characteristics of its asexual stage (phialophores, phialids and spores; Kowalski 2006). Fungal species other than the ash dieback pathogen were not further examined. A symptomatic seedling was scored positive for *H. fraxineus* if the fungus was re-isolated from at least one of the nine shoot discs, irrespective of re-isolation section. Similarly, the fungus was considered as positively re-isolated from a shoot section if it was recovered from at least one of the three sampled pieces.

Total counts and percentages of successful infection of the shoots, indicated by the presence of externally visible necrotic lesions, were calculated separately for each isolate and inoculation method (Table 1). Due to the low number of replicates per isolate, measurements of lesions on the shoot and wood discoloration were pooled according to inoculation method (Table 2). To examine whether or not there were differences in the extension of necrotic symptoms (externally visible

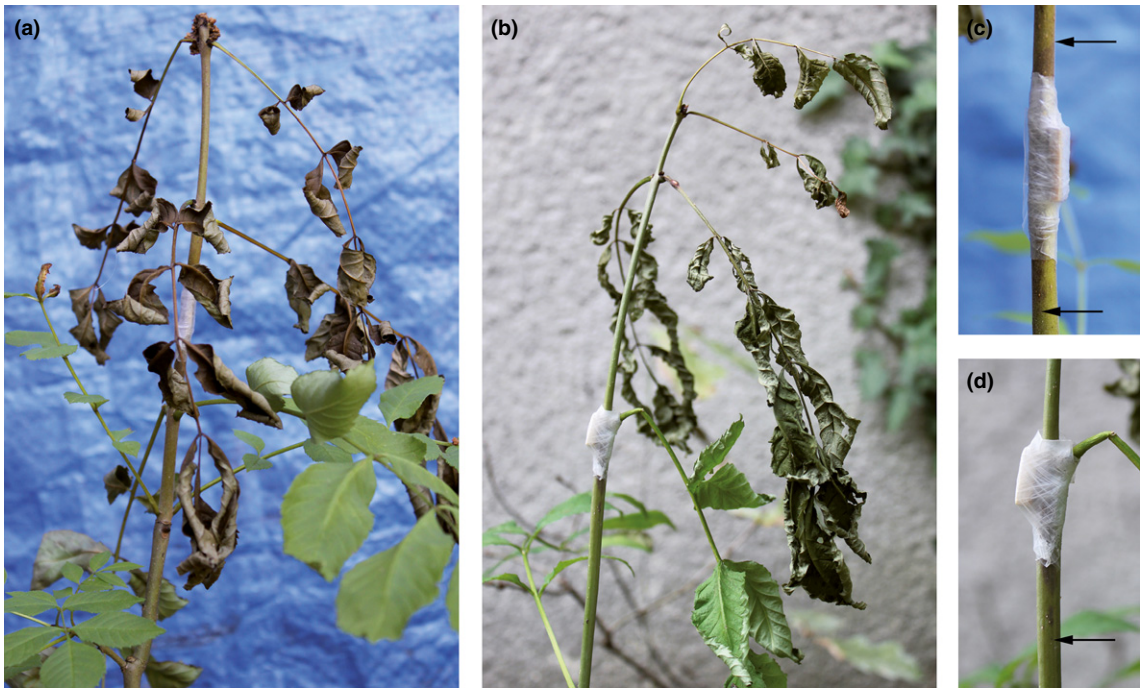

Fig. 1. Symptoms after inoculation of *Fraxinus excelsior* shoots with wood pieces colonized by *Hymenoscyphus fraxineus*. Wilting symptoms on seedlings following both methods of inoculation: (a) on an intact, unwounded current-year shoot, (b) on a shoot where fungal inoculum was placed onto an artificially exposed leaf scar. Corresponding necrotic lesions on the same seedlings: (c) following inoculation of an intact shoot, (d) following inoculation of a leaf scar. Black arrows (c, d) indicate the edges of the visible lesions on the shoot surface.

Table 2. Lengths of necrotic lesions on the shoot surface and wood discoloration for the two inoculation methods.

			Lengths in mm: mean ± SD[2] (min–max)	
Inoculation method	N[1]		Visible necrotic lesion	Wood discoloration
Intact shoot inoculation	2	Distal	53.0 ± 18.4 (40–66)	68.0 ± 31.1 (46–90)
		Proximal	140.5 ± 0.7 (140–141)	155.5 ± 7.8 (150–161)
		Total	193.5 ± 17.7 (181–206)	223.5 ± 23.3 (207–240)
Leaf scar inoculation	8	Distal	20.4 ± 22.3 (0–64)	15.6 ± 11.6 (0–32)
		Proximal	74.9 ± 48.8 (16–161)	82.4 ± 56.8 (0–176)
		Total	95.3 ± 48.5 (27–161)	98.0 ± 52.6 (24–167)

[1]Number of shoots on which necrotic lesion and wood discoloration lengths were measurable both in proximal and distal direction from the inoculation site.
[2]Standard deviation.

lesion on the shoot surface and wood discoloration) in proximal and distal direction from the inoculation site and to compare the total lengths of the externally visible lesion and the wood discoloration, the Wilcoxon signed-rank test for paired samples was applied to symptomatic plants that had been inoculated onto the leaf scars. Statistical analyses were performed with the program IBM SPSS STATISTICS, version 20 (SPSS IBM, New York, NY, USA).

3 Results

Both inoculation methods resulted in necrotic lesions on the shoot and in wilting symptoms on a portion of the common ash seedlings that had been inoculated with *H. fraxineus* (Table 1; Fig. 1). Disease symptoms were identical to those seen on naturally infected plants. In contrast, seedlings inoculated with sterile wood pieces did not show any shoot symptoms and were therefore not processed further.

By 4 October 2010, 102 days after application of *H. fraxineus*, two seedlings following shoot inoculation and four seedlings following leaf scar inoculation showed necrotic lesions on the shoot and wilting of shoots and leaves. The first symptomatic seedling was observed 47 days after inoculation in the intact shoot variant and 57 days after inoculation in the leaf scar variant. By March 2011, another necrotic lesion had developed on a seedling from the unwounded shoot inoculation, and necrotic lesions were observed on five additional seedlings from the leaf abscission inoculation. These plants were processed in March 2011, prior to sprouting, and the occurrence of wilting symptoms could therefore not be assessed. It is likely that at least a portion of the plants would have shown shoot dieback above the inoculation site if processing had been delayed. No further symptoms were found on any seedling by the beginning of June 2011, when the observations were terminated. Considering the symptom assessments both in 2010 and 2011, the overall infection rate of *H. fraxineus* was 12% (three of 25 plants) following shoot inoculation and 36% (nine of 25 plants) for seedlings that had received the leaf scar inoculation (Table 1). Summarizing both inoculation methods, all five *H. fraxineus* isolates caused symptoms on at least one inoculated seedling, and the number of symptomatic seedlings per isolate ranged from 1 to 4 of 10 of the inoculated seedlings (Table 1).

On two seedlings of the intact shoot inoculation and on eight seedlings of the leaf scar inoculation method, the length of necrotic lesions and length of the wood discoloration were measurable both in proximal and distal direction from the inoculation site, and total values could be calculated from the two measurements. Lesions and the wood discoloration resulting from inoculation of the intact shoots were generally larger than those originating from inoculation of the leaf scars (Table 2). Due to an advanced stage of necrosis and dieback of the shoot distally to the inoculation site, total values for the two remaining symptomatic seedlings could not be calculated.

Taking into account all 10 seedlings of both inoculation methods for which lesions were fully measurable, the extension of necrotic lesions and the wood discoloration was greater in proximal than in distal direction from the inoculation site. Mean values of the two intact shoot-inoculated seedlings for proximal lesion length on the shoot surface and the extension of the wood discoloration were 87.5 mm longer than the respective mean distal measurement values (Table 2). Likewise, following leaf scar inoculation, six of eight externally visible shoot lesions and seven of eight wood discoloration measurements were longer in proximal than in distal direction. Mean proximal values were 54.5 mm (shoot lesions) or 66.8 mm (wood discoloration) longer than the respective distal measurements (Table 2). The differences in the proximal and distal extension of symptoms were statistically significant for both measurement categories (Wilcoxon signed-rank tests for paired samples, shoot lesion: $Z = -2.100$, $p = 0.036$, wood discoloration: $Z = -2.240$, $p = 0.025$). Referring to the same eight plants, no statistically significant difference was detected between the total lengths of the externally visible shoot lesion and wood discoloration (Wilcoxon signed-rank tests for paired samples, $p > 0.05$), although there was a tendency to a slight increase in the extension of necrosis from the shoot surface to the wood (Table 2). For the two seedlings following shoot inoculation, the extension of the wood discoloration was also greater than that of the lesion on the shoot surface (Table 2).

Hymenoscyphus fraxineus was re-isolated in pure culture or together with other fungi from all three (100%) symptomatic shoots which had been directly inoculated and from six of the nine (67%) diseased shoots which were

inoculated onto leaf scars, resulting in a total re-isolation rate of 75% for both methods. There were no obvious differences in the re-isolation rates among shoot sections. The overall re-isolation rate for shoot sections was higher in autumn 2010 (83%, n = 18) than in spring 2011 (22%, n = 18), and pure cultures of *H. fraxineus* were obtained three times more frequently at the earlier processing date in autumn (56%) compared to the later processing in spring (17%).

4 Discussion

Using mycelial inoculum, *H. fraxineus* was able to infect a fraction of the inoculated *F. excelsior* seedlings via the intact epidermis of current-year shoots and via artificially exposed leaf scars, which confirms, in agreement with previous wound inoculation experiments (Bakys et al. 2009a,b; Kirisits et al. 2009; Kowalski and Holdenrieder 2009; Ogris et al. 2009, 2010; Szabó 2009; Husson et al. 2011), its pathogenicity to shoots and stems of its main host in Europe. In previous pathogenicity trials with *H. fraxineus*, in which the fungus was wound-inoculated onto stems of ash plants, high infection rates up to 100% were observed (Bakys et al. 2009a; Kowalski and Holdenrieder 2009; Kirisits et al. 2010; Ogris et al. 2010; Husson et al. 2011; McKinney et al. 2012), although Bakys et al. (2009b) recorded an infection rate of only 50%. In contrast, infection rates in the present study were lower: 12% following inoculation of intact shoots and 36% following inoculation of leaf scars. This is likely due to the different inoculum procedures which, in the present study, did not involve any wounding of the stem and exposure of the phloem or xylem. With wound inoculation, constitutive protection of the host by the epidermis or bark is completely circumvented, in contrast to the shoot inoculation in this study in which the epidermis was not injured. Likewise, injury of the host by artificial leaf abscission was less than in previous wound inoculation experiments.

Externally asymptomatic shoots were not examined for necrosis in the phloem and wood discoloration, and no re-isolations were made from them at the end of the experiment. This would have definitely clarified whether or not these shoots had been infected by *H. fraxineus*. However, it is most likely that the absence of wilt and visible necrotic lesions by 8 June 2011 (more than 11 months after inoculation) in shoots that had been inoculated with *H. fraxineus* reflected in all cases failed infections rather than an asymptomatic, endophytic persistence of the fungus. Likewise, in previous experiments (Bakys et al. 2009a; Kowalski and Holdenrieder 2009; Ogris et al. 2009, 2010; Szabó 2009), including own ones (Kirisits et al. 2009, 2010; K. Kräutler and T. Kirisits, unpublished data), control inoculations never resulted in symptoms or infection by or isolation of the ash dieback pathogen. It is also considered as extremely unlikely that any of the shoots were already latently infected by *H. fraxineus* prior to inoculation in June 2010 and that this could have influenced the results of the experiment. This is because necrotic lesions on the shoots were always unambiguously associated with the positions onto which the fungal inoculum had been placed.

Leaves have been suggested to be the most important entrance court of *H. fraxineus* into woody parts of *F. excelsior* (Kirisits and Cech 2009; Kirisits et al. 2009; Schumacher 2011; Timmermann et al. 2011; Kräutler and Kirisits 2012; Cleary et al. 2013; Gross et al. 2014). However, the sequence of infection from leaves to shoots has not yet been reproduced experimentally (Kräutler and Kirisits 2012; Cleary et al. 2013). The ability of *H. fraxineus* to infect shoots through leaf scars exposed by leaf abscissions in the present study can be seen as indirect supportive evidence that the fungus is indeed able to enter shoots via leaves, a process which also involves passing the leaf scar region. In addition, symptoms following inoculation of artificially inflicted leaf scars were very similar to those occurring after natural infection. Examination of infected shoots in the present study revealed that necrotic lesions on the shoot or stem surface as well as the wood discoloration were significantly longer towards the base of the shoot (proximally) than towards the tip of the shoot (distally). A similar pattern of lesion and wood discoloration extension was observed in naturally infected shoots, twigs and small stems of *F. excelsior* at early stages of disease, when lesions extended on average much more in proximal than in distal direction from a leaf scar, which was the location where *H. fraxineus* was suspected to have grown into woody parts (see Fig. 2 in Kräutler and Kirisits 2012; T. Kirisits and K. Kräutler, unpublished data). The substantially greater size of lesions and of the wood discoloration in proximal than in distal direction both after natural infection and artificial inoculation may be due to the anatomy of the shoot in the area of the leaf scar and, more specifically, due to the pattern of connection of vascular bundles of leaves with vascular bundles of shoots, favouring the downward spread of *H. fraxineus* at early stages of infection of woody parts. In contrast to the present study, Kowalski and Holdenrieder (2009), who wound-inoculated *F. excelsior* plants by exposing the xylem and inserting inoculum of *H. fraxineus*, did not find differences in the proximal and distal sizes of necrotic lesions.

To our knowledge, this is the first report that *H. fraxineus* can directly infect unwounded, intact current-year shoots of *F. excelsior*. Gross et al. (2014) also conducted an experiment in which fungus-colonized wood blocks were attached to unwounded current-year shoots of *F. excelsior*, but this method of inoculation did not, contrary to the present study, lead to shoot infections by *H. fraxineus*. Cleary et al. (2013), who exposed common ash seedlings to ascospores of the ash dieback pathogen in moist chambers, induced symptoms on leaves, but did not observe infections directly on shoots. In the present study, current-year shoots were inoculated before the periderm had replaced the epidermis. The infection process of *H. fraxineus* into shoots was not studied in detail, and it is therefore unknown whether the fungus directly penetrated the unsuberized epidermal cells or grew through natural openings. A recent histological investigation of leaf tissues at early stages of infection by *H. fraxineus* showed that after ascospore germination and formation of appressoria, infectious hyphae are able to penetrate the cuticle layer and the epidermal cell walls of leaves but also that infection occurs through stomata (Cleary et al. 2013).

The use of wood pieces colonized by mycelium of *H. fraxineus* as inoculum in this study is not representative of inoculation under natural conditions, which occurs by ascospores. Shoots were inoculated at the end of June, at a time when *H. fraxineus* already produces apothecia and ascospores in central and western Europe (Kirisits and Cech 2009; Chandelier et al. 2014). The susceptibility of fully formed, unwounded shoots in this experiment, at a time when inoculum of *H. fraxineus* is available, suggests that direct infection of shoots by ascospores can also occur naturally. The possibility of direct infection of shoots and woody parts in general by ascospores is circumstantially supported by previous investigations. Husson et al. (2012) postulated that necrotic lesions and wood discoloration on the stem base, root collar and on aerial roots of *F. excelsior*, which were not found to be associated with leaves, side twigs, low sprouts or wounds, originate from ascospores penetrating the intact bark through lenticels, a mode of infection which may occur particularly frequently on moist sites, promoting massive sporulation and increasing the inoculum pressure of *H. fraxineus.* Moreover, investigating naturally infected *F. excelsior* trees, Bengtsson et al. (2014) observed a considerable portion of newly emerging necrotic lesions in the bark that were not connected to a leaf scar or damage, and these authors suggested that such lesions may originate from direct infections of primary shoots or from infections via lenticels.

In stems and branches of *F. excelsior* trees naturally infected by *H. fraxineus*, the length of the wood discoloration was significantly greater than that of the corresponding externally visible necrotic lesions in the bark, which suggests that the fungus spreads more rapidly in longitudinal direction in the wood than in the cambium and phloem (Schumacher et al. 2010; Bengtsson et al. 2014). Likewise, Kowalski and Holdenrieder (2009) noted that on a portion of inoculations, necrosis extended from the externally visible lesion margin up to 8 cm further into the inner bark and cambium. In the present study, the length of the wood discoloration was on an average slightly greater than the externally visible lesion. Differences were, however, not statistically different, which may be due to the low number of artificial infections available for analysis. Nevertheless, the appearance of necrotic lesions and the wood discoloration in shoots and stems of naturally infected and artificially inoculated common ash plants appears to be very similar.

Previous research has indicated a long incubation period in the disease cycle of ash dieback (Schumacher 2011; Kirisits et al. 2012; Gross et al. 2014), and this was confirmed in the present experiment. By 4 October 2010, 102 days after inoculation, disease symptoms had emerged on six inoculated seedlings. During the evaluation in March 2011, necrotic lesions were found on six additional plants, which were scored as asymptomatic at the latest assessment in the previous year, but had most likely already been latently infected. These findings emphasize the risk posed in planting ash, where *H. fraxineus* could be introduced into new areas by externally disease-free ash plants that have already been infected (Timmermann et al. 2011; Kirisits et al. 2012).

Hymenoscyphus fraxineus was re-isolated frequently from symptomatic seedlings in this study, which confirmed its association with the symptoms occurring after artificial inoculation. The lower re-isolation rate from shoot sections at the later processing in March 2011 compared to that in September and October 2010 indicates that a prolonged time from inoculation to re-isolation decreases the likelihood to detect *H. fraxineus* and, more specifically, to recover it in pure culture. This interpretation is in agreement with the study by Kowalski and Holdenrieder (2009), who recorded in three independent experiments re-isolation rates of *H. fraxineus* (based on the investigation of several tissue samples per inoculated ash plant) of 40, 19 and 9%, 2, 3 and 12 months after inoculation, respectively.

In conclusion, this study demonstrated that the ash dieback pathogen can, at least under experimental conditions employing a massive mycelium inoculum, infect through the intact, epidermis of current-year shoots of common ash. This finding reinforces the view that there is another important path of infection other than that via leaves. Mycelia of *H. fraxineus* were also able to penetrate through leaf scars, which provides additional evidence for the supposed main infection path from leaves to woody parts.

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