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Extended Abstract

The ash dieback pathogen *Hymenoscyphus pseudoalbidus* is associated with leaf symptoms on ash species (*Fraxinus* spp.)

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Ash dieback caused by the ascomycete fungus *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*) is characterized by a wide range of symptoms. Leaf symptoms have previously been related to this emerging infectious disease. In fungal isolations from necrotic lesions on leaf petioles and rachises as well as leaflet veins of *Fraxinus excelsior*, *H. pseudoalbidus* was consistently obtained at high frequencies. When inoculated onto leaf rachises of *F. excelsior*, the ash dieback pathogen induced symptoms identical to those seen after natural infections (necrotic lesions, wilting and leaf dropping). The fungus also caused symptoms on leaves of *Fraxinus angustifolia* and *Fraxinus ornus* in artificial inoculations and was re-isolated at considerably high frequencies from all three *Fraxinus* spp. Kochs postulates were thus fulfilled to conclude that *H. pseudoalbidus* is associated with leaf symptoms on *F. excelsior*. On *F. angustifolia* leaf damage resulting from natural infections still remains to be detected. In contrast, *F. ornus* may not be a natural host of *H. pseudoalbidus*, although it proved to be somewhat susceptible in the leaf inoculation experiments.

Key words: *Chalara fraxinea*, *Fraxinus excelsior*, *Fraxinus angustifolia*, *Fraxinus ornus*, emerging forest disease.

INTRODUCTION

Ash dieback, an emerging infectious disease of common ash (*Fraxinus excelsior*) and other *Fraxinus* species in Europe, is caused by the ascomycete fungus *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*; Kowalski, 2006; Bakys et al., 2009; Kowalski and Holdenrieder, 2009; Kirisits et al., 2009, 2010a; Drenkhan and Hanso, 2010; Queloz et al., 2011). The disease is characterized by a wide range of symptoms (Bakys et al., 2009; Kirisits et al., 2009; Kirisits and Cech, 2010). Besides necrotic lesions in the bark, cambium and phloem as well wood discoloration leading to dieback of shoots, leaf symptoms have been related to ash dieback (Figure 1). These include necrotic lesions on leaf petioles and rachises (Figure 1A and B) as well as leaflet veins (Figure 1C), often followed by wilting (Figure 1A) and early leaf shedding (Bakys et al., 2009; Kirisits et al., 2009, 2010b; Kirisits and Cech, 2010).

Besides damaging ash trees in their own right, leaf infections incited by the pathogen's ascospores have been suggested to be the primary path enabling *H. pseudoalbidus* to grow into shoots and twigs of its host trees (Kirisits and Cech, 2009; Kirisits et al., 2009, 2010b; Timmermann et al., 2011). This view is based on the temporal sequence of appearance of symptoms and on the observation that small, localized necrotic lesions, representing early stages of disease, frequently occur around leaf scars (Figure 2; Kirisits et al., 2009, 2010b; Timmermann et al., 2011). So far it has, however, not been definitely established that *H. pseudoalbidus* is associated with leaf symptoms on *Fraxinus* spp. and the role of leaf infections in the disease cycle of ash dieback remains to be proven.

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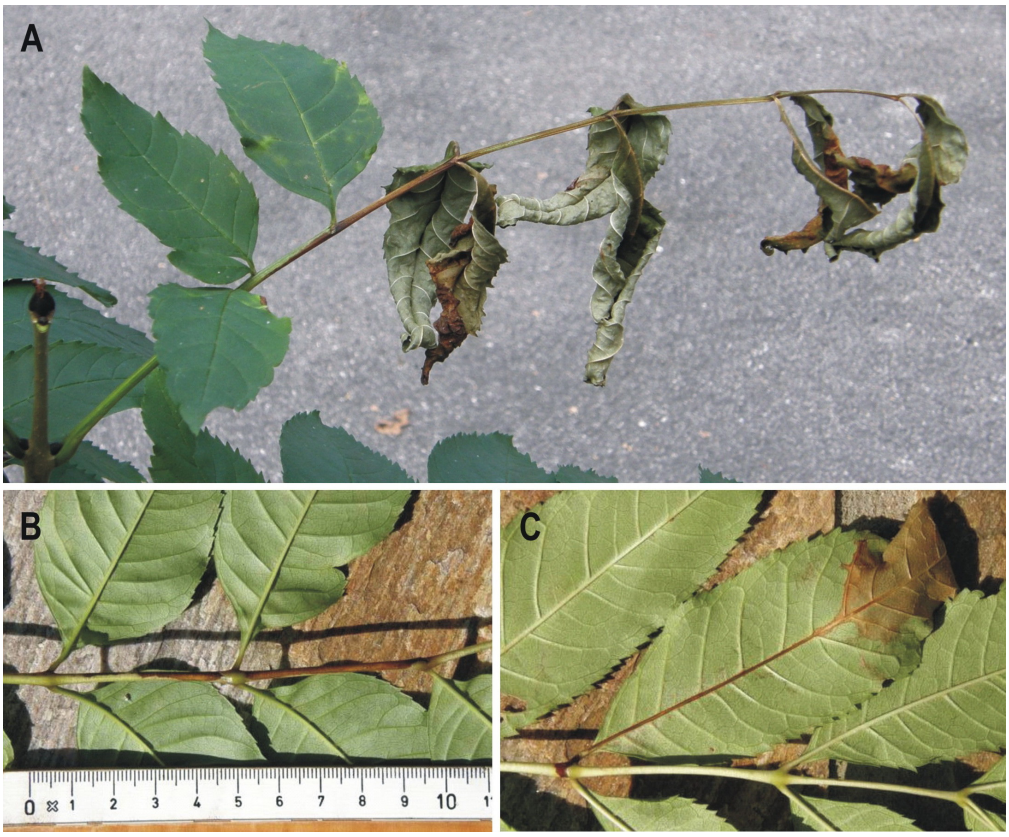

Figure 1. Leaf symptoms on *F. excelsior* commonly seen from late summer onwards in connection with ash dieback: (A) Necrotic lesion on a leaf rachis leading to wilting in the part of the leaf distal to the lesion, (B) Detailed view of a necrotic lesion on a leaf rachis, (C) Necrotic lesion on a leaflet vein extending to the leaf rachis.

FUNGAL ISOLATION FROM SYMPTOMATIC *F. EXCELSIOR* LEAVES

In early September 2008 and early October 2009 symptomatic leaves (126 in total for both years) were collected from young *F. excelsior* trees at one site in Vienna (Schafberg) and fungi were isolated from necrotic lesions on leaf petioles and rachises (Figure 1A and B; subsequently referred to as 'leaf rachises' only). After surface sterilization (1 min in 96% ethanol, 1 to 3 min in 4% NaOCl, 30 s in 96% ethanol), the epidermis was carefully peeled off and small fragments of necrotic tissue were cut and placed on malt extract agar (MEA; 20 g/L malt extract, 16 g/L agar, 100 mg/L streptomycin sulphate). Isolation plates were incubated at cool temperatures (between 4 to 10°C) in the dark. This was done in order to stimulate anamorph production of the ash dieback pathogen and to give it competitive advantage over other fungi, thereby increasing the likelihood to detect the fungus (Kirisits et al., 2009). *H. pseudoalbidus* was identified based on morphological characteristics of its *Chalara fraxinea* stage (colony morphology, phialophores and spores).

In both years *H. pseudoalbidus* was isolated at high frequencies from necrotic leaf rachises and it was the most frequently isolated fungus. The overall isolation frequency of the ash dieback pathogen was 89.7%. From 52.4% of the rachises the fungus was obtained in pure culture. Various other fungi were also isolated, especially in October 2009, but they were not determined. None of the other fungal species reached the high frequency of *H. pseudoalbidus*. In other isolation series from necrotic leaf rachises, using leaves collected in late summer and early autumn 2008 at five localities in Lower Austria and in August 2011 at one site in Upper Austria, the ash dieback pathogen was also recovered abundantly, with isolation frequencies ranging from 80 to 100% at the various sites. Moreover, at the site Vienna-Schafberg *H. pseudoalbidus* was obtained from 26 out of 35 (74.3%) necrotic leaflet veins (Figure 1C), from which isolation had been attempted.

The high isolation frequencies of the ash dieback pathogen from symptomatic leaves in the present study agree well with the investigations by Bakys et al. (2009). Using fungal isolation on various media and molecular detection methods directly from plant tissues, these authors detected *H. pseudoalbidus* commonly in ash leaf rachises and leaf blades. Ogris et al. (2009) also obtain-

Figure 2. Small, localized necrotic lesions on *F. excelsior* shoots, representing early stages of ash dieback (Vienna, Penzing, March 2010). The position of the lesions adjacent to leaf scars may indicate that shoot infections occurred via leaves.

ed the fungus from necrotic leaf petioles of *F. excelsior.* Moreover, Drenkhan and Hanso (2010) isolated *H. pseudoalbidus* from petioles of four *Fraxinus* species not native to Europe (*F. americana*, *F. mandshurica*, *F. nigra* and *F. pennsylvanica*).

LEAF INOCULATION EXPERIMENT

At the end of June 2010 leaf rachises of potted *F. excelsior*, *F. angustifolia* and *F. ornus* seedlings were wound-inoculated with five *H. pseudoalbidus* isolates. Inoculum consisted of autoclaved wood fragments (approximately 5 mm long and 2 mm in diameter) obtained from *F. excelsior* shoots that had been placed for 24 days on the various *H. pseudoalbidus* cultures on MEA. Sterile wood fragments of similar size were used as control treatment. Inoculation was done by initiating an approximately 1 cm long superficial wound on the upper surface of a leaf rachis, placing inoculum on the wound and fixing it with parafilm. For *F. excelsior* and *F. angustifolia* 20 plants per treatment were inoculated, while for *F. ornus* 10 seedlings per treatment were used. After inoculation the seedlings were regularly inspected for leaf symptoms until early November, when the remaining inoculated rachises were harvested. Upon dropping of an inoculated leaf from a seedling or at the termination of the experiment, re-isolation of *H. pseudoalbidus* from the leaf rachis onto MEA was attempted as described above. The positions of the inoculated leaves on the shoots were marked and during autumn, winter and spring 2010/2011 shoots were examined at irregular intervals for the occurrence of necrotic lesions.

Leaf symptoms (necrotic lesions on the rachises, wilting and leaf dropping) appeared on all three ash species tested. Some inoculated leaves showed typical symptoms

already two weeks after inoculation. The temporal patters of leaf shedding varied considerably between the three ash species. For example, five weeks after inoculation only one fungus-inoculated *F. ornus* leaf had dropped, whereas the proportions of shed leaves were more than 50% in *F. excelsior* and 11% in *F. angustifolia*. Necrotic lesions on the leaf rachis developed on 91% of the *F. excelsior* seedlings, 56% of the *F. angustifolia* seedlings and 53% of the *F. ornus* seedlings inoculated with *H. pseudoalbidus*. The values for the occurrence of leaf wilting were 74% (*F. excelsior*), 29% (*F. angustifolia*) and 30% (*F. ornus*). Besides a few natural infections, no symptoms occurred on the leaves that had received the control treatment.

H. pseudoalbidus was re-isolated from all three *Fraxinus* spp., often in pure culture, but results varied between species. Re-isolation rates from fungus-inoculated seedlings were 64% for *F. excelsior*, 53% for *F. angustifolia* and 27% for *F. ornus*. Until the end of May 2011 necrotic lesions in the bark of shoots and/or the main stem had developed on a small portion of the test seedlings. All the lesions resulted from natural infections and none of them could unambiguously be related to the leaves that had been inoculated with *H. pseudoalbidus* in the previous summer.

DISCUSSION AND CONCLUSIONS

Kochs postulates were fulfilled to conclude that *H. pseudoalbidus* is indeed associated with leaf symptoms on *F. excelsior*. Likewise, in the inoculation experiment the ash dieback pathogen caused leaf damage on *F. angustifolia* and also on *F. ornus*. On these two ash species, leaf symptoms resulting from natural infections have so far not been reported in connection with ash dieback. It is, however, most likely that they occur also on *F. angustifolia* which is clearly affected by the disease (Kirisits et al., 2009, 2010a). In contrast, ash dieback has till now not been confirmed to occur on *F. ornus*. This ash species may therefore be immune or highly resistant to *H. pseudoalbidus*, although it proved to be somewhat susceptible in stem (Kirisits et al., 2009) and leaf inoculation experiments (this study). In the leaf inoculation trial reported here, it appeared to be the least susceptible species amongst the three *Fraxinus* spp. tested.

The ash dieback pathogen predominantly forms its apothecia on previous year's ash leaf petioles and rachises in the forest litter (Kowalski and Holdenrieder, 2009; Kirisits and Cech, 2009). This emphasises the great importance of leaves as primary habitat of *H. pseudoalbidus* and for the entire life cycle of the fungus. Observations and circumstantial evidence suggest that green leaves are infected by ascospores and colonized by the fungus during the vegetation period, while further development of *H. pseudoalbidus* on leaf petioles and rachises as well as leaflet veins, particularly formation of black pseudosclerotial layers, takes place during the following autumn, winter and spring (Kowalski and Holdenrieder, 2009; Kirisits and Cech, 2009; Kirisits et al., 2009, 2010b; Timmermann et al., 2011). Depending on latitude, altitude and varying climatic conditions apothecia first appear at the end of May, June or early July in the year following infection (Kowalski and Holdenrieder, 2009; Kirisits and Cech, 2009; Kirisits et al., 2009; Queloz et al., 2011; Timmermann et al., 2011).

Although we were not able to induce shoot infections in the leaf inoculation experiment, we still suppose that *H. pseudoalbidus* can grow from infected leaves into phloem and xylem tissues of ash to cause necrotic phloem lesions and wood discoloration (Kirisits and Cech, 2009; Kirisits et al., 2009, 2010b). Field observations suggest that only a relatively low portion of leaf infections may lead to shoot infections, because in many cases leaves are shed before the ash dieback pathogen has reached phloem and/or xylem tissues (Kirisits et al., 2010b). This phenomenon likely also occurred in our leaf inoculation experiment. Thus, due to methodological problems it may be difficult to fully mimic natural infections in inoculation trials with the ash dieback pathogen. Further studies are required to fully elucidate the infection biology of *H. pseudoalbidus* and particularly to definitely determine the organs and tissues of the hosts which are subjected to infections. Such studies include especially also experiments using ascospores as source of inoculum.

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