

Full Length Research Paper

Impact of *Hymenoscyphus pseudoalbidus* on *Fraxinus excelsior* Seed Plantations in Austria

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Ash dieback, an emerging fungal disease incited by *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*), causes immense damage to *Fraxinus excelsior* in many parts of Europe. There is hope that some individuals of this tree species display high levels of resistance to the disease. In 2009 and 2010, the intensity of ash dieback was investigated in an ash seed plantation in Upper Austria, consisting of 51 clones of local provenance. A specific rating system for visual inspections was developed to assess shoot, twig and branch dieback as well as leaf shedding. Considering all 187 evaluated trees, mean ash dieback intensity was 18.1% in July 2009 and 17.6% in July 2010. Disease intensity varied greatly between clones, ranging in both years from almost no dieback to more than 80% dieback in the most severely affected clone. Likewise, levels of leaf shedding in July and September differed considerably between clones. However, no clear relationship between leaf shedding and dieback intensity was observed. The results indicate that *F. excelsior* clones in the seed plantation may indeed differ substantially in their resistance to *H. pseudoalbidus*.

Key words: *Chalara fraxinea*, common ash, disease assessments, emerging fungal disease, resistance.

INTRODUCTION

Dieback of common ash (*Fraxinus excelsior*), narrow-leaved ash (*Fraxinus angustifolia*) and other *Fraxinus* species is caused by the recently described ascomycete fungus *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*; Kowalski, 2006; Bakys et al., 2009; Kowalski and Holdenrieder, 2009a, b; Kirisits et al., 2009, 2010a; Drenkhan and Hanso, 2010; Schumacher et al., 2010; Husson et al., 2011; Queloz et al., 2011). This emerging tree disease was first observed around 1992 in Poland and has by 2011 been recorded in at least 25 European countries (Timmermann et al., 2011; Kirisits, unpublished). Although its origin is still enigmatic, the high intensity and the gradual appearance of ash dieback in Europe, related to the successive spatial spread of the causative pathogen, may suggest that *H. pseudoalbidus*

or an aggressive mutated strain of this species is behaving as an alien invasive organism (Husson et al., 2011; Queloz et al., 2011; Timmermann et al., 2011).

Ash dieback is characterized by a remarkably wide range of symptoms (Kirisits et al., 2009; Kowalski et al., 2010). The most conspicuous ones are necrotic lesions and cankers in the bark, combined with wood discoloration, leading to dieback of shoots, twigs, branches and smaller stems. Likewise, *H. pseudoalbidus* has been suggested to be associated with leaf symptoms on *F. excelsior* and other ash species (Bakys et al., 2009; Kirisits et al., 2009, 2010b; Ogris et al., 2009; Drenkhan and Hanso, 2010; Kowalski et al., 2010; Kräutler and Kirisits, 2012). Leaf infections are also thought to play a key role in the disease cycle of ash dieback, as they have been postulated as a major path for the fungus to grow into shoots and twigs of their host trees (Kirisits and Cech, 2009; Kirisits et al., 2009, 2010b; Schumacher, 2011; Kräutler and Kirisits, 2012).

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Ash dieback occurs on *F. excelsior* trees of all ages (Kowalski and Łukomska, 2005; Kirisits et al., 2009, 2011; Kowalski et al., 2010; Schumacher et al., 2010). It is often a lethal disease of seedlings and natural regeneration, but thicket- and pole- sized trees are also frequently killed. Older ash trees are severely damaged as well, but can endure the disease for a longer time and often succumb due to secondary damaging factors such as *Armillaria* spp. (Kowalski et al., 2010; Kirisits et al., 2009; Bakys et al., 2011). The disease is questioning the future of *F. excelsior* as economically valuable and for ecological reasons appreciated noble hardwood species (Kowalski et al., 2010; Kirisits et al., 2009, 2011).

Options for disease management are very limited (Kirisits et al., 2009; Kowalski et al., 2010). The observation of healthy and only slightly affected trees in severely diseased ash stands may, however, indicate that there are considerable differences in resistance between individuals of *F. excelsior* to the ash dieback pathogen (Kirisits et al., 2009, 2010b; McKinney et al., 2011). Investigations in seed plantations in Denmark revealed that damage levels of ash dieback vary greatly amongst 39 clones, indeed suggesting the presence of natural genetic resistance in *F. excelsior* to *H. pseudoalbidus* (McKinney et al., 2011). Moreover, the length of necrotic lesions in the bark following artificial inoculation of *H. pseudolabidus* differed amongst clones and showed a strong and significant correlation with phenotypic damage levels resulting from natural infections (McKinney et al., 2012). *F. excelsior* individuals with putative high resistance to ash dieback may form the basis for the maintenance of the species in the future. These genotypes are expected to be favoured by natural selection and *ex-situ* genetic conservation and breeding programs could be initiated, providing prospects that the overall resistance levels of populations of *F. excelsior* will increase over time (McKinney et al., 2011).

In Austria, there are three seed plantations of *F. excelsior*, one in the province of Upper Austria and two in the province of Styria. These plantations consist of clones of local provenance which have been selected based on traits desirable for timber production. The original aim of the plantations was to yield genetically diverse, site-adapted forest reproductive material of high quality. With the occurrence of ash dieback, seed orchards and clone collections have become a valuable resource for investigations on the resistance of *F. excelsior* to this emerging disease. Hence, from 2009 onwards, the intensity of ash dieback has been monitored in the three *F. excelsior* seed plantations in Austria. The objectives of these investigations were: (i) to document differences in the intensity of ash dieback between clones; (ii) to use the seed plantations as general monitoring plots to follow disease development seasonally within a year and between years; and (iii) to increase the knowledge on the symptomatology and the disease cycle of ash dieback. In this report, preliminary results from one of the seed plantations are presented.

MATERIAL AND METHODS

The investigations were carried out in a seed plantation of *F. excelsior* located in Feldkirchen an der Donau (14.02 E, 48.20 N, 264 m asl., flat terrain) in the Austrian province of Upper Austria. In this 1.36-hectare-large plantation, initiated in 1993 on former agricultural land, 51 different ash clones of local provenance (forest ecoregion 7.1, northern foothills of the Alps, western part, submontane altitudinal zone; Kilian et al., 1994) are represented. Originally four grafted ramets per clone and thus 204 ash trees were planted at a spacing of 7.50 m (distance between rows) and 8.66 m (distance of trees within rows). The trees were arranged in a randomized pattern in the plantation. Twelve trees died after planting. Further five trees were trimmed in order to maintain a telephone line crossing the plantation and they were therefore not considered in the assessments. The total number of investigated trees was thus 187; 35 clones were represented with four ramets, 15 clones with three ramets and one clone with two ramets.

Ash dieback was for the first time recognized in the plantation in 2008, but it is likely that it caused damage even earlier there, as the first unambiguous observations of the disease in Upper Austria were made in 2005 (Cech, 2006). On 30 April 2009 dead shoots and twigs or those with localized necrotic phloem lesions, representing early stages of ash dieback were collected from selected *F. excelsior* ramets. In the laboratory, about 4 to 6 cm long segments, containing the transition between necrotic and healthy phloem tissues and/or discoloured and healthy xylem were cut from the samples. After surface sterilization (1 min in 96% ethanol, 3 min in 4% NaOCl, 30 s in 96% ethanol), the outer bark was carefully peeled off and 3 to 10 mm wide discs containing wood and phloem tissues were cut under aseptic conditions 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 *H. pseudoalbidus* and to give it competitive advantage over other fungi, thereby increasing the likelihood to detect the ash dieback pathogen (Kirisits et al., 2009). *H. pseudoalbidus* was identified based on morphological characteristics of its *C. fraxinea* stage (colony morphology, phialophores and spores). Other fungi were not determined. Besides fungal isolation, leaf petioles and rachises on the ground were in both years examined for the occurrence of black pseudosclerotial layers and apothecia of *H. pseudoalbidus*. Observations were made at the dates of the ash dieback severity assessments (see below) and during visits in the plantation in mid-June 2009 and early June 2010.

For the assessments of the severity of ash dieback on ramets of the various clones, a specific rating system was developed (Figure 1). For the ratings, the crown of each individual tree was divided into thirds. Thereafter, each crown third was assigned to one out of seven ash dieback severity classes. Using class means (0, 2.5, 12.5, 35, 65, 90 and 100), the values of the three crown thirds were averaged, to obtain an ash dieback severity rating in percent for each ramet (Figure 1). The rating scheme of dieback of shoots, twigs and branches was tested on 30 April 2009, prior to the main surveys. For this, about 15 trees were evaluated independently by both authors and the results were compared. Subsequently, the second author did all assessments alone. Leaf shedding was evaluated in the same way as dieback of shoots, twigs and branches (Figure 1). Assessments were done at three dates in 2009 and 2010, at mid-May, at the end of July and in early to mid-September.

RESULTS

Fungal isolation and occurrence of apothecia of *H. pseudoalbidus*

H. pseudoalbidus was isolated from 17 out of 21 (81%)

Ash dieback rating system

Step 1: Divide crown into thirds.

Step 2: Rate each third separately. Each third is given a rating from 0 to 6, as described below.

0	No dieback	Class mean 0%
1	< 5%	Class mean 2.5%
2	> 5% to 20%	Class mean 12.5%
3	> 20% to 50%	Class mean 35%
4	> 50% to 80%	Class mean 65%
5	> 80 to 100%	Class mean 90%
6	100%	Class mean 100%

Step 3: Add ratings of crown thirds to obtain a total rating for a tree; the tree on the right will receive a rating of $4 + 5 + 5 = 14$.

Step 4: Calculate an average percentage value of crown dieback for a tree, using class means (see above); the tree on the right will receive a value of $(65 + 90 + 90) / 3 = 81.7\%$.

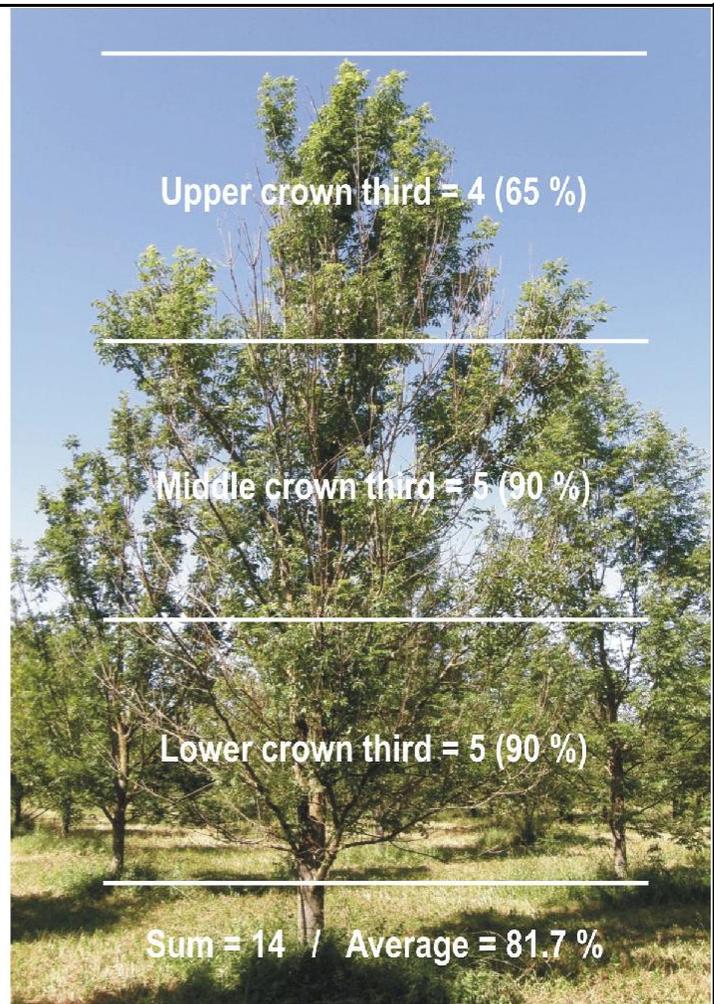


Figure 1. Rating system developed for the assessments of ash dieback severity in seed plantations in Austria.

symptomatic shoots and twigs collected from 20 ramets in the seed plantation. From 43% of the samples, it was obtained in pure culture, 38% of the isolations yielded *H. pseudoalbidus* in mixed culture with other fungi and from the remaining 19% of the samples other fungi, but not the ash dieback pathogen were isolated. Overall, *H. pseudoalbidus* was the most frequently isolated fungal species.

Leaf petioles and rachises with black pseudoclerotial plates were commonly observed in the litter during all visits in the seed plantation. Apothecia of *H. pseudoalbidus* occurred abundantly on leaf petioles and rachises at the end of July 2009 and 2010. Fruiting bodies were not seen at the inspections in May and were rare during the visits in September. In 2009, apothecia were observed on 17 June and in 2010 already on 9 June. Based on the irregular inspections in both years, the main period of ascocarp formation of *H. pseudoalbidus* in the ash seed plantation likely extends from the middle or the end of June until late August or early September.

Dieback of shoots, twigs and branches

When testing the ash dieback rating system (Figure 1) in April 2009, the ratings of individual trees by the two authors were always identical. It was therefore decided to use this scheme for subsequent investigations. Considering all 187 evaluated trees, mean ash dieback intensity was 14.6% in May 2009, 18.1% in July 2009, 14.2% in May 2010 and 17.6% in July 2010. Due to early leaf shedding, evaluation of dieback severity proved to be difficult in September and the data of these assessments are therefore not presented.

Disease intensity varied considerably between clones, ranging in both years from almost no dieback to more than 80% dieback in the most severely affected clone (Figure 2). In May 2010 mean dieback intensity was below 10% in 28 out of the 51 clones (55%), between 10.1 and 20% in 13 clones (25%), between 20.1 and 30% in 5 clones (12%) and between 30.1 and 40% in one clone (2%) (Figure 2). Only 4 clones (8%) had average

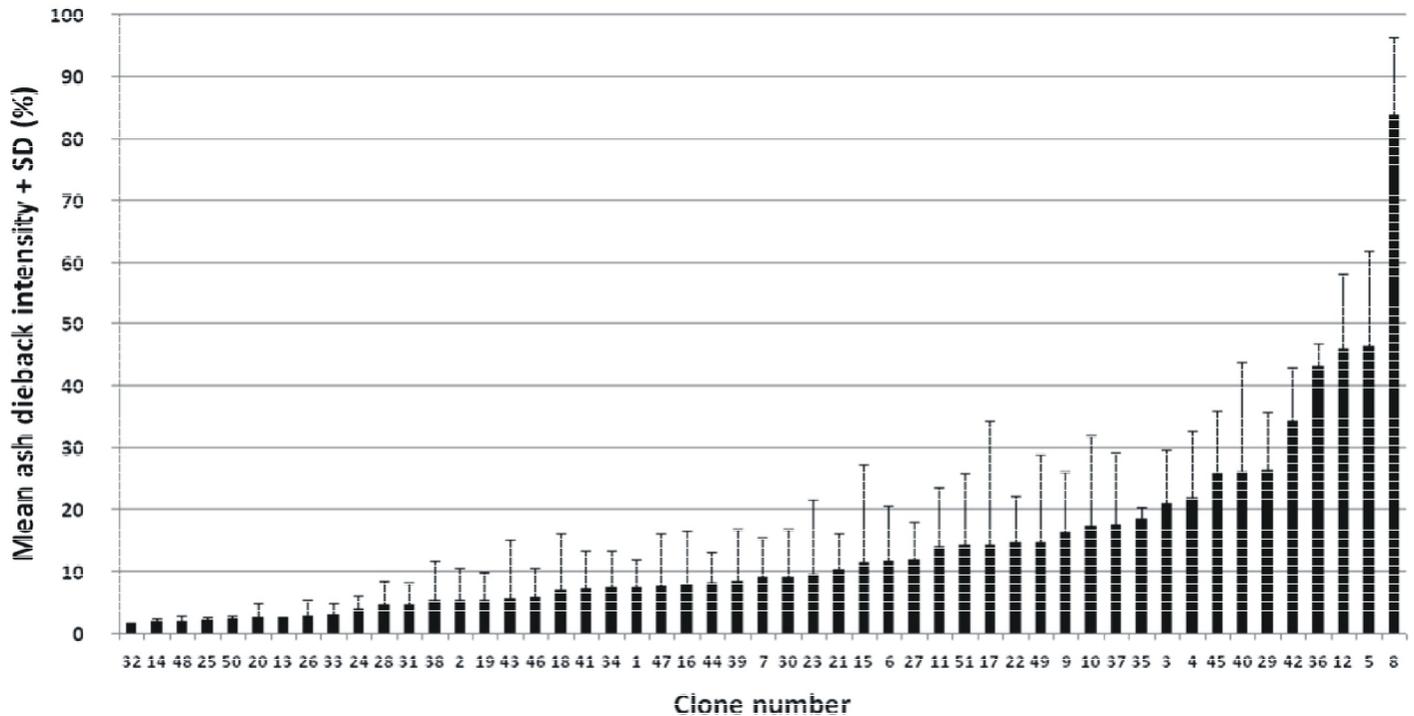


Figure 2. Mean ash dieback intensity (+ standard deviation, SD) in percent by *F. excelsior* clone on 13 May 2010 in the seed plantation in Feldkirchen an der Donau (Upper Austria). The overall sample size was 187 trees: 35 clones were represented with 4 ramets, 15 clones with 3 ramets and one clone with 2 ramets.

values higher than 40% (Figure 2). Ramets of two extreme clones (number 8, the most severely damaged clone and number 14, one of the least damaged clones) are shown in Figure 3. A comparison of the assessments in May 2009 and May 2010 showed that the mean ash dieback ratings remained unchanged for two clones, those of 28 clones had improved, while those of 22 clones got worse.

Leaf shedding

The overall patterns of leaf shedding were consistent in 2009 and 2010. In both years, the mean percentage of shed leaves was low at the end of July (8% in 2009, 1% in 2010), but high in late summer (67% in mid-September 2009, 46% in early September 2010). As it was observed for dieback, the intensity of leaf shedding varied considerably between clones, both in the July and September assessments (data not shown). While many clones showed high levels of defoliation by September, others were to a lesser degree affected by this symptom and a few remained densely foliated until late summer or even early autumn (for example clone number 14, Figure 3B). However, no obvious positive or negative relationships between the intensity of leaf shedding in September 2009 and dieback intensity in May 2010 were detected (data not shown). This is exemplified by clones numbers 8 and

14 (Figures 2 and 3), both of which are not so much affected by early leaf shedding, but differ substantially in the levels of dieback.

DISCUSSION

H. pseudoalbidus was isolated at high frequencies and often in pure culture from shoots and twigs displaying early symptom of ash dieback on selected ramets of *F. excelsior* clones in the seed plantation in Feldkirchen an der Donau. The massive occurrence of pseudosclerotial plates and apothecia on leaf petioles and rachises in the litter also confirmed the presence and abundant occurrence of the fungus. The results therefore clearly suggest that *H. pseudoalbidus* is associated with dieback of shoots, twigs and branches in the *F. excelsior* seed plantation.

The rating system developed in the present study is in our opinion convenient to monitor ash dieback over time and it may be useful for other studies evaluating the severity of this emerging disease. Assessments of disease severity based on visual inspections need to fulfil several criteria. They should be sufficiently accurate to reflect disease levels, easy to apply and assessments of the same tree by different observers should lead to the same result or at least be fairly comparable. Doing the assessments separately for crown thirds, which was



Figure 3. Differences in the intensity of ash dieback between *F. excelsior* clones in the seed plantation in Feldkirchen an der Donau (17 August 2011): A. Ramet of the most severely damaged clone (number 8), B. Ramet of one of the least damaged clones (number 14) with virtually no dieback symptoms. In both 2009 and 2010 ramets of this clone were also hardly affected by early leaf shedding and remained densely foliated until early autumn.

inspired by the 6-class dwarf mistletoe rating system (Hawksworth et al., 1996), and designation of the particular seven classes used for the ratings (Figure 2) contribute in our opinion to meet the three criteria mentioned previously (accuracy, easy application and comparability). All ratings in the seed plantation were done by the same person (C. Freinschlag), which should have a positive influence on data quality and guarantee comparability of assessments at various dates.

The *F. excelsior* ramets rated in the present study were relatively tall, justifying separate assessments for crown thirds. For smaller trees, dividing the crown into thirds is probably not necessary and it is preferable to evaluate the entire crown. In two other seed plantations in Austria, which consist of younger *F. excelsior* ramets, tall trees were rated separately for crown thirds, while for small trees a single value was assigned to the undivided crown. Using class means and averaging crown thirds, ash dieback severity can be calculated in percent, which can

loosely be treated as a continuous variable in statistical analyses. These calculations also allow averaging disease severity data of trees which have been assessed separately for crown thirds and those for which ratings have been done for the entire crown.

Overall disease intensity changed very little during the entire observation period and in 2010 it was slightly lower than in 2009. This stagnation as well as the short-term changes in the performance of individual clones should, however, not be overestimated. Heavily diseased trees often respond intensively with the formation of auxiliary and epicormic shoots (Figure 3A), in order to compensate the loss of killed shoots and twigs. This often leads to a decrease in disease severity, but it is questionable, whether this reflects a long-term tendency. Likewise, killed twigs and branches become decayed, subsequently fall down and can therefore not be considered in future ratings. Moreover, it is possible that climatic factors contributed to the slight decrease in disease intensity from

2009 to 2010. In agreement with the assessments in the seed plantation, only a slight overall increase of ash dieback intensity was observed from 2008 to 2010 on monitoring plots in Lower Austria (Keßler et al., 2012) as well as from 2009 to 2010 in surveys in Norway (Solheim et al., 2011).

The assessments in the ash seed plantation provide added evidence that leaf symptoms are associated with ash dieback. In agreement with earlier reports (Bakys et al., 2009; Kirisits et al., 2009, 2010b; Kowalski et al., 2010; Kräutler and Kirisits, 2012) necrotic lesions on leaf petioles and rachises as well as leaflet veins, followed by wilting and early leaf shedding were commonly observed on *F. excelsior* ramets in the plantation. Symptom observations, fungal isolations and inoculation studies suggest that *H. pseudoalbidus* is associated with these leaf symptoms and that leaf infections are indeed the cause of early leaf shedding (Bakys et al., 2009; Kirisits et al., 2009, 2010b; Ogris et al., 2009; Kräutler and Kirisits, 2012). The phenology of early leaf shedding in the seed plantation, approximately starting in July and subsequently increasing in intensity, until many trees are to a large extent or even totally defoliated by September, corresponds well with the suggested period of ascocarp formation of *H. pseudoalbidus* from mid- /end of June to late August/early September. Given that ascospore production starts already in June and leaf symptoms first appear approximately by the middle of July, an incubation period (time from inoculation to the appearance of visible symptoms) of several weeks can be assumed for *H. pseudoalbidus*. The temporal pattern of leaf shedding also agrees well with the proposed disease cycle of ash dieback (Kirisits and Cech, 2009; Kirisits et al., 2009; Schumacher, 2011).

The intensity of shoot, twig and branch dieback and the levels of leaf shedding varied greatly amongst *F. excelsior* clones in the seed plantation. It is most likely that this variation reflects genetically determined differences in the resistance of ash genotypes to *H. pseudoalbidus*. No relationship between the intensity of leaf shedding and dieback intensity was detected. This is in contrast to the study in seed plantations in Denmark where a strong correlation between dieback intensity and late leaf shedding / leaf senescence was observed. Thus, clones retaining their leaves longer in autumn displayed higher levels of dieback (McKinney et al., 2011). Moreover, early leaf shedding was supposed as a means to partially escape disease, leading to lower dieback intensity (McKinney et al., 2011).

The results concerning leaf shedding in our study may not be directly comparable with those from Denmark by McKinney et al. (2011). Since the occurrence of ash dieback, the phenology of leaf shedding of *F. excelsior* has been substantially altered in many parts of Austria, due to direct leaf infections by ascospores of *H. pseudoalbidus* (Kirisits et al., 2009, 2010b; Kräutler and Kirisits, 2012). As observed in the course of this study, shedding of

leaves often starts already in late summer and by mid-September a large portion of trees is substantially or totally defoliated (Kirisits et al., 2009, 2010b). McKinney et al. (2011) treated leaf senescence and shedding as a genetically determined phenological trait of *F. excelsior* which is not influenced by *H. pseudoalbidus* infections. We, however, argue that leaf shedding in our study, though varying greatly between clones, is mainly a consequence of leaf infections by the ash dieback pathogen. Because of leaf infections, we suppose that genetic differences in autumn leaf phenology of common ash can no longer be observed precisely in the seed plantation studied here. The inconsistent findings and interpretations in our study and that of McKinney et al. (2011) may be explained by differences in the phenology of the pathogen and the host in different environments, likely resulting in a different temporal development of the disease cycle of ash dieback. For example, formation of *H. pseudoalbidus* apothecia starts slightly later and the period of sporulation is shorter in Northern Europe than in Central Europe (Kirisits and Cech, 2009; Solheim et al., 2011; Timmermann et al., 2011).

Although induced by the pathogen, early leaf shedding could be a resistance mechanism to ash dieback in certain *F. excelsior* genotypes. This is because infected leaves may be shed before *H. pseudoalbidus* can enter bark and wood tissues adjacent to the leaf base (Kirisits et al., 2010b). The lack of a consistent association between dieback symptoms and leaf shedding suggests, however, that leaf shedding as a kind of 'defence reaction' does not operate successfully in all the clones which lose their leaves early. It is worth to note that some clones (for example number 14, Figure 3B) were only slightly affected by both dieback and leaf shedding. This may indicate that these clones possess resistance mechanisms in the leaves and possibly also in the bark and wood that limit infection by and/or spread of *H. pseudoalbidus*. In clones whose leaves are more intensively affected, but show only limited dieback, resistance traits in shoots may, however, be more important. Common ash clones that display limited damage of both leaves and ligneous tissues may in our opinion be most interesting for genetic conservation and breeding programs. Putative traits of leaves and ligneous tissues associated with resistance to ash dieback and the relative importance of resistance mechanisms operating in these two organs will require attention and study in the future, as discussed by McKinney et al. (2011, 2012).

Although this report is clearly preliminary, the assessments in the seed plantation in Upper Austria indicate that *F. excelsior* clones indeed differ substantially in their resistance to *H. pseudoalbidus*, with some clones displaying high putative resistance levels. The results agree well with the findings obtained in the *F. excelsior* seed plantations in Denmark (McKinney et al., 2011, 2012). For the Austrian seed plantations, thorough statistical data analyses are intended in the future,

to support the preliminary trends reported here. Likewise, it is planned to continue the assessments, in order to monitor the future development of damage levels and to check particularly, whether some clones will remain to be affected only to a limited degree by the disease. Moreover, artificial inoculation of *H. pseudoalbidus* onto leaves and branches of various clones displaying contrasting damage levels are desirable, to definitely determine that resistance to this emerging tree pathogen varies in *F. excelsior*.

The results presented in this report and by McKinney et al. (2011, 2012) are of considerable practical importance. They provide substantial evidence that at least a low portion of *F. excelsior* individuals displays high resistance levels to ash dieback. The recommendation to practitioners to maintain and promote disease-free and slightly diseased common ash trees of all ages (for example Kirisits et al., 2009, 2010b, 2011) thus appears to be a reasonable measure for disease management. Clearing ash trees irrespective of their health status bears the risk to eliminate individuals with high resistance levels from the populations.

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