Host Phenological Stage Potentially Affects Dieback Severity after *Hymenoscyphus fraxineus* Infection in *Fraxinus excelsior* Seedlings

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Abstract

Ash dieback is a serious forest health problem throughout Europe attributed to the emerging, infectious pathogen *Hymenoscyphus fraxineus*. Preceding studies have revealed large genetic variation among *Fraxinus excelsior* trees in disease susceptibility, but the underlying mode of tolerance is still unknown. Previous research has revealed a genetic correlation between susceptibility and phenology, where the more tolerant genotypes were characterized by early flushing in the spring and early senescence (leaf yellowing) in the autumn. The main objective of the present study was to explore the influence of host phenological stage on symptom development after controlled inoculation with *H. fraxineus*. We induced early budburst in a set of seedlings, and compared the symptom development after artificial inoculation, with a control group where flushing had not been induced prior to infection. We observed that severe dieback symptoms were more frequent in seedlings infected prior to budburst compared to seedling inoculated after budburst. We speculate that resistance mechanisms may be more effective in the ash trees while in their growing seasons, and this can contribute to the observed genetic correlation between early flushing and reduced susceptibility.

Keywords: *Fraxinus excelsior*, *Hymenoscyphus fraxineus*, Inoculation, Phenology, Susceptibility, Tolerance

Introduction

Common ash, *Fraxinus excelsior* L., is currently under threat by the invasive pathogen *Hymenoscyphus fraxineus* (Bakys et al. 2009; Kowalski and Holdenrieder 2009). The pathogen has spread rapidly across Europe since it was first observed in Poland and the Baltic region in the 1990s, and ash forests in several countries are currently severely affected by the disease (Pautasso et al. 2013; McKinney et al. 2014). Several studies have revealed significant genetic variation in disease susceptibility among ash trees exposed to natural infections (McKinney et al. 2011; Kirisits and Freinschlag 2012; Stener 2012; Lobo et al. 2014; Enderle et al. 2015). The partial resistance is inherited from parents to their offspring (Lobo et al. 2015; Pliura et al. 2011; Kjær et al. 2012) giving hope for the future conservation and breeding of ash.

The genetic mechanisms behind disease tolerance among ash clones are not yet understood. Danish and Swedish studies have found significant genetic correlations between phenological traits and crown damage due to *H. fraxineus* infection (McKinney et al. 2011; Stener 2012). The genetic correlation between spring phenology (bud burst) and health of clones was significant but moderate in the Danish study, and showed that earlier flushing clones on average were less susceptible to the disease. Stronger genetic correlation was observed both in Denmark and Sweden for leaf senescence (measured as autumn colouring of leaves) and crown damage where less-affected clones showed earlier leaf yellowing interpreted as indicative of early senescence (McKinney et al. 2011; Stener 2012). As *H. fraxineus* is commonly believed mainly to infect the trees through their leaves during late summer (Gross et al. 2012) it has been speculated that an asynchrony between the phenology of the host (i.e. early senescence) and the

pathogen life cycle could assist the early-senescing trees to escape the disease. However, a controlled inoculation study showed that trees with a high level of resistance towards natural infections also developed shorter lesions when the leaf infection pathway was circumvented by controlled inoculation directly into the cambium with infected wood plugs (McKinney et al. 2012). The lesions continuously increased in size both while trees were foliated from late spring to early autumn and while trees were without leaves from autumn to spring (McKinney et al. 2012), but the lesions were shorter in the tolerant clones. This result points towards the presence of an active defence mechanism that can suppress the development of disease symptoms once the pathogen has invaded the branches, and that the observed partial resistance therefore cannot solely be attributed to phenological disease escape.

The few tested clones that performed well in the controlled inoculation study from 2012 (McKinney et al. 2012) were also among those that performed well under natural infection i.e. those with early bud burst and early leaf yellowing (McKinney et al. 2011). We therefore speculate that the phenological stage of the trees plays a role in an active host defence towards the pathogen. In the present paper we test whether ash seedlings are less affected by ash dieback if infected during their active growing season compared to being infected before flushing.

Materials and methods

Plant material

A total of 58 seedlings (offspring from a clonal seed orchard FP202 (Nielsen et al. 2009)) were used in the study. On January 20th, 2011, 28 two years old seedlings were placed in a greenhouse with the following light and temperature settings: 16 hours of daylight (from 06:00) at 19ºC (suitable for *H. fraxineus* (Kowalski and Bartnik 2010; Bengtsson et al. 2014)), 8 hours of darkness at 17ºC. During the testing period (March 21st to May 5-6th) the average midday temperature was around 20ºC in the greenhouse except for one week (21/4-30/4) where the temperate was between 24º and 27ºC. The remaining 30 seedlings (controls) were kept under semi outdoor conditions in a cold green house where temperatures followed outdoor conditions, although without frost.

Inoculation

Two isolates of *H. fraxineus* (3.2.1/1 and 3.5.1/2) were tested on the seedlings. The isolates were obtained from single spore cultures derived from two apothecia collected in August 2010 from a forest stand, Nørreskov, close to the city Aabenraa (GPS coordinates 55º03.531, 009º 24.825). The inoculum was grown on malt extract agar at room temperature and two weeks prior to inoculation sterile ash wood plugs (~1cm long) were added to the medium allowing the hyphae to invade the wood plugs. The stem of the seedlings were inoculated on March 21st, 2011 by cutting into the bark (1.5 cm in length) and placing an infected wood plug into the wound. Parafilm was wrapped around the inoculated stem. From the heated greenhouse 14 seedlings were inoculated with isolate 3.2.1/1 (Isolate 1) and another 14 were infected with isolate 3.5.1/2 (Isolate 2), while from the cold house 15 plants were inoculated with the two isolates, respectively. Of the 28 plants from the heated greenhouse all except one had flushed at the time of inoculation while the 30 plants from the cold greenhouse were all without signs of budburst.

Assessment

Plants were assessed on 5-6/5 2011, approximately 6 weeks after inoculation. The longitudinal spread of the necrosis was assessed by measuring the sum of the upwards and downwards visible length of the necrosis (*i.e.* discoloration of the outer bark). Crown damage of the plants was scored in categories where 0 was no visible symptoms, 1: few visible symptoms, 2: clear visible symptoms with main shoot beginning to wilt 3: shoot above inoculation spot dying or dead, 4: entire seedling dead. Three plants never flushed (1 from the warm house and 2 from the cold house), hence, we could not score crown damage of these plants.

Data analysis

Analysis of variance (ANOVA) was performed for necrosis length with stage (before flushing versus after flushing) and isolate (3.2.1/1 versus 3.5.1/2) as treatments. Necrosis length was square root transformed before analysis to meet the assumptions behind the analysis of variance model. For crown damage, significance of differences between stage and isolate were analysed by Fishers exact test of the 2 x 4 frequency tables.

Results

Around 6 weeks after inoculation the lesions were approximately 4 cm long for the non-flushed cold house seedlings (mean ± SE for isolate 3.2.1/1: 5.23 ± 0.75 cm and isolate 3.5.1/2: 3.65 cm ± 0.99 cm) while around 5 cm for the flushed seedlings inoculated in the heated greenhouse (isolate 3.2.1/1: 5.36 cm ± 0.54 cm and isolate 3.5.1/2: 5.56 cm ± 1.45 cm. These differences were neither significant between the stage of the plant when inoculated ($P < 0.42$) or between the two isolates ($P < 0.34$). However, the distribution of crown dieback symptoms was significantly different between seedlings inoculated before and after flushing, ($P = 0.04$; based on Fishers exact test, Table 1). The frequency of seedlings with severe symptoms (class 3) was thus 25% (7/28) in seedlings inoculated prior to flushing compared to 4% (1/27) in seedlings inoculated

after flushing (Table 1). Differences between isolates were not significant ($P = 0.27$) based on Fishers exact test.

Table 1. Distribution of seedlings to ash dieback damage classes six weeks after controlled inoculation. Inoculations were done March 21 2011. Approximately half of the seedlings had been forced to flush in a heated greenhouse prior to inoculation (inoculated after flushing) while the other half was kept in a cold house and had not flushed (inoculated before flushing)

Inoculation	Class 0 No symptoms	Class 1 Few symptoms	Class 2 Severe symptoms	Class 3 Very severe
Before flushing	17	2	2	7
After flushing	23	3	0	1

Discussion and Conclusions

Our results suggested that the phenological stage of the tested ash trees (seedlings) influenced the symptom severity when challenged by *H. fraxineus* through controlled stem infection. The growth (lesion lengths) of the pathogen was slightly higher in plants inoculated in the heated greenhouse, probably because the temperature was more suitable for the growth of *H. fraxineus* (Bengtsson et al. 2014; Kowalski and Bartnik 2010). Similar to field observations by Bengtsson et al. (2014) lesions did also develop under cold conditions and the difference in lesion length was not significant, and fairly fast necrosis development was observed in some seedlings kept in the cold greenhouse. The significantly stronger crown dieback symptoms in seedlings from the cold house compared to seedlings that had already flushed before inoculation may indicate a less effective defence mechanism in the seedlings while partly in winter dormancy. The presented dataset is small and additional studies are required to verify this hypothesis.

A relation between the severity of infections and host tree phenology has been observed in other tree pathosystems. Sudden oak death threatens the coast live oak (*Quercus agrifolia*) in central and northern California, where the pathogen (*Phytophthora ramorum*) is known to sporulate from December to May (Davidson et al. 2005). An annual cycle of inoculation experiments showed that the lesion size after infection was highly correlated with the cambial activity measured (Dodd et al. 2008). Trees with an early onset of cambial activity would therefore be more susceptible to infections because the activity coincides with the sporulation of the fungus. Similarly, in elm, it has been shown that budburst and susceptibility to Dutch elm disease was highly correlated in *Ulmus minor*, suggesting that early flushing clones were less affected by inoculations (Santini et al. 2005). The time of maximum susceptibility to *Ophiostoma novo-ulmi* happens simultaneously with the maximum growth rate and formation of large size vessels, indicating that infection is highly dependent on host cycle and phenology (Solla et al. 2005).

In the above cases, the timing of host susceptibility must match with a limited window for infection and proliferation of the fungus. Asynchrony of these events may therefore contribute to lower disease susceptibility in these species in the form of disease escape. It has been questioned if the strong correlation between crown damage and senescence in *F. excelsior* (early senescing ash clones being the most healthy ones) could likewise reflect disease escape (McKinney et al. 2011). It is known from Norway that pathogen sporulation peaks from mid-July to mid-August with corresponding fungal DNA concentrations in living ash leaves reaching a high plateau around mid-August, several weeks before leaf shed (Hietala et al. 2013). These results contradict the explanation of lower susceptibility by disease escape, but pre-dormancy changes (biochemical or physiological) taking place already in the early autumn could perhaps dis-favour the spread of *H. fraxineus* from infected leaves into the stem.

Our suggestion, that host phenology at the time point for infection plays a role in disease development, supplements earlier results showing that crown damage correlates negatively at the genetic level to early spring flushing (McKinney et al. 2011). If an active defence response depends on the host being in its active growing season, this could give the early flushing ash trees an advantage in supressing early infections before the temperatures become more suitable for *H. fraxineus* during late spring and summer.

The physiological, anatomical and/or chemical mechanisms involved in the observed correlation between phenology and partial disease resistance in *F. excelsior* remain unknown. Recent studies have revealed a strong association between disease susceptibility and MADS box transcription factors (Harper et al. 2016; Sollars et al. 2016) that may function as regulators of plant developmental processes (Pařenicová et al. 2003). These MADS box genes may be involved in the observed patterns.

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