Assessment of Seasonal Patterns in Tissue-specific Occurrence of *Hymenoscyphus fraxineus* in Stems of *Fraxinus excelsior*

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Abstract

Dieback of European ash (*Fraxinus excelsior* L.), a disease caused by the ascomycete *Hymenoscyphus fraxineus* (previously referred to as *H. pseudoalbidus* or *Chalara fraxinea*), was first observed in Poland in the early 1990ies, and is currently present almost throughout the entire distribution area of European ash. The characteristic symptoms of the disease include dead shoots with necrotic lesions in the bark and discoloration of xylem and pith but the seasonal dynamics of pathogen spread in shoot tissues remain poorly understood. To investigate whether the internal spread of the fungus involves season-specific patterns, saplings with necrotic bark lesions in 1-2 -year-old stem regions were collected during 2014-2015 at time intervals in spring, summer, autumn and winter at several localities in western Ukraine and at two localities in south-eastern Norway. Tissue-specific presence of *H. fraxineus* was determined by a highly sensitive quantitative real-time PCR assay that is specific to DNA of *H. fraxineus*. The relatively high proportion of bark samples positive for *H. fraxineus* in the saplings collected during spring provides support to a model that *H. fraxineus* can be a primary causative agent of bark lesions and that other fungi may eventually replace it in old infection areas.

Key words: ash dieback, *Hymenoscyphus fraxineus*, bark, phloem, pith, qPCR (Real-Time PCR).

Introduction

The virulent fungal pathogen, *Hymenoscyphus fraxineus*, causing 'chalara ash dieback,' disease of ash trees in northern and central Europe has been reported from countries across Europe: Czech Republic, Denmark, Finland, Germany, Slovakia, Austria, Hungary, Poland, Romania, Slovenia, Switzerland, France, Estonia, United Kingdom, Ukraine and Norway (Zubrik and Kunca 2007, Halmschlager and Kirisits 2008, Jankovsky and Holdenrieder 2009, Szabo 2009, Drenkhan and Hanso 2009, Engesser et al. 2009, Ioos et al. 2009, Kowalski and Holdenrieder 2009, Davydenko et al. 2011, Matsiakh and Kramarets 2014, Timmermann et al. 2014).

According to the current model, shoot infection by *H. fraxineus* is caused by fungal mycelium that originates from ascospores germinating on the leaf surfaces. Following the infection of leaf tissues, the mycelium spreads through the petiole into shoots and twigs, and causes characteristic bark lesions and crown dieback (Gross et al. 2014, list some other references also). Within the stem, it is proposed that further colonization and infection occurs by an axial and radial expansion through the pith and the sapwood (Schumacher et al. 2010). The authors proposed that this leads to eventual necrotization of cambium and bark and this in turn triggers the development of many secondary fungi that are commonly associated with necrotic bark lesions of ash. In our recent study, we combined histologi-

cal observations with a highly sensitive qPCR assay specific to *H. fraxineus* DNA, and profiled stem tissues associated with regions with necrotic bark lesions and adjacent asymptomatic tissues in naturally infected saplings of *F. excelsior*: outside bark lesion areas pathogen DNA was detected in pith and sapwood, but not in the radially corresponding bark, and pathogen growth fronts were characterized by abundant presence of fungal mycelium in the starch-rich perimedullary zone of the pith during the summer season (Matsiakh et al. 2016).

The present work was designed to increase our understanding of seasonal growth dynamics of the fungus in shoot tissues. For this purpose, *H. fraxineus* specific qPCR assay was used to profile the presence of pathogen DNA in pith, xylem, phloem and outer bark in naturally infected stems of ash saplings that were collected from Ukraine and Norway at different seasons.

Materials and Methods

Experimental material

The experimental material originated from forest stands in continental climate in Ukraine and Norway and was collected at time intervals during 2014-2015. Stems of living 5-10 -year-old saplings of European ash with necrotic lesions in 1-2 -year-old stem regions were collected at vil. Nyvytsi (Lviv region) and vil. Ishkivtsi (Ternipol region) in western Ukraine and at Norderås, Ås (Akershus) and Årungen, Ås (Akershus) in south-eastern Norway.

Sampling

Up to 1-m-long stem segments enclosing regions with necrotic bark lesions, and up to 10 cm healthy bark on both sides of the lesions, were cut from a total of 21 saplings sampled throughout the year and transported to laboratory. The vertical orientation of the stem piece was marked. In laboratory, the stem lesions were split longitudinally with a knife and the split lesion halves were scanned with a photocopy machine to obtain an image of tissue discoloration. A total of 55 five-mm-long stem segments were sampled in areas with necrotic bark and a total of 131 such stem segments were sampled in adjacent areas with healthy bark. The position of each stem segment sampled was marked in the scanned image. These stem segments were further divided into radially corresponding tissue samples and outer bark, inner bark (i.e. phloem), xylem and pith samples were separately subjected to DNA isolation.

DNA extraction and real-time PCR

All tissue samples were weighed (up to 30 mg fresh weight) and placed in a 2 mL Eppendorf tube with a lid reinforced with glue. The samples were then pulverised with the aid of liquid Nitrogen and steel beads in a Retsch mill (max speed, 1.5 min). DNA isolation was performed with DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The real-time PCR detection of *H. fraxineus* DNA was performed using Takyon Low Rox Probe MasterMix dTTP Blue w/o UNG for probe Assay Low ROX (Eurogentec, Seraing, Belgium), and the forward primer Cfrax-F 5′-ATTATATTGTTGCTTTAGCAGGTC-3, reverse primer Cfrax-R 5′-TCCTCTAGCAGGCACAGTC-3′ and probe C-frax-P5′-FAM- CTCTGGGCGTCGGCCTCG-BHQ1-3′ designed and tested for species specificity by Ioos et al. (2009). The primer and probe concentrations were 300 nM and 100 nM, respectively (Ioos et al. 2009).

Pathogen DNA amount standard curves with known DNA concentrations were prepared from fungal pure cultures (Hietala et al. 2013). For both the experimental and standard curve samples, 3 µl of the DNA solution was used as the template for each 20-µl PCR reaction. Each single-plex reaction was repeated twice. PCR cycling parameters were 95°C for 3 min, followed by 40 cycles of 95°C for 3 s and 65°C for 25 s (target DNA) or 60°C for 60 s (reference DNA). Fluorescence emissions were detected with an Applied Biosystems ViiA^{TM} 7Systems (Applied Biosystems, Foster City, CA, USA). Standard curves were constructed based on the relationship of cycle threshold (Ct) values and known DNA concentrations: the Ct values were plotted against log-transformed DNA amounts, and linear regression equations were calculated for the quantification of DNA pools by interpolation in unknown samples.

Statistical analyses

Sampling time and tissue type –specific differences in the presence of *H. fraxineus* DNA were tested by ANOVA and Tukey HSD post hoc tests in SPSS 22.0 (IBM Inc., Armonk, NY, USA), and considered statistically significant at $P < 0.05$.

Results

No significant differences were observed in the occurrence of *H. fraxineus* between the Norwegian and Ukrainian shoot material in the different tissue types, neither within a bark lesion area nor outside this region. Therefore, the material was pooled together for subsequent statistical analyses. Of the stem segments analysed from regions with necrotic bark lesions, 27, 80, 89 and 84% were positive for *H. fraxineus* DNA in the outer bark, phloem, sapwood and pith, respectively (Table 1). Regarding stem segments analysed from regions from adjacent regions with healthy bark, 22, 31, 43 and 48% were positive for *H. fraxineus* DNA in the outer bark, phloem, sapwood and pith, respectively (Table 1). Except for outer bark (*p* 0.35), the differences in the tissue-type specific occurrence of *H.fraxineus* DNA between regions with necrotic bark and regions with healthy bark were statistically highly signifi-

Table 1. Presence of *Hymenoscyphus fraxineus* DNA in different stem tissues of naturally infected *Fraxinus excelsior* saplings during the seasons (specified as n, number of trees or stems with positive detections of the pathogen and quantified as percentage % of total trees analysed; ns, not sampled). Samples were taken either in a stem region with a necrotic bark lesion or in adjacent regions with healthy bark. Samples collected in spring are from the study of Matsiakh et al. (2016)

Season	Sapling	Within bark lesion area					Outside bark lesion area				
		Total	Outer bark	Inner bark	Xylem	Pith	Total	Outer bark	Inner bark	Xylem	Pith
Spring	NOR1	3	ns	2	3	3	5	Ns	0	0	1
	NOR2	2	ns	2	2	2	2	Ns	2	2	2
	NOR3	2	ns	2	2	2	2	Ns	1	0	0
	NOR4	4	ns	4	4	4	3	Ns	1	0	1
	NOR5	0	ns	ns	ns	ns	8	Ns	1	4	5
	NOR6	3	ns	3	3	3	13	Ns	6	6	8
	UKR1	4	ns	4	3	4	8	Ns	2	2	4
	UKR2	4	ns	3	4	3	6	Ns	2	2	1
	UKR3	2	ns	2	2	2	8	ns	3	2	3
	UKR4	4	ns	4	4	4	4	ns	0	2	1
	Sum (n)	28		26	27	27	59		18	20	26
	Sum (%)	100		93	96	96	100		31	34	44
Summer	NOR7	2	2	2	2	2	7	2	2	2	5
	NOR8	1	1	1	1	1	9	8	2	6	3
	UKR5	4	2	1	2	4	3	0	1	1	2
	UKR6	6	2	3	6	3	4	0	1	0	1
	UKR7	2	0	1	1	1	6	3	1	3	2
	UKR8	1	1	1	1	0	9	4	3	8	6
	Sum (n)	16	8	9	13	11	38	17	10	20	19
	Sum (%)	100	50	56	81	69	100	45	26	53	50
Autumn	NOR9	3	1	3	2	2	5	0	1	3	2
	UKR9	0					9	6	6	6	6
	UKR10	4	4	4	4	4	4	2	4	3	3
	Sum (n)	7	5	7	6	6	18	8	11	12	11
	Sum (%)	100	71	100	86	86	100	44	61	67	61
Winter	NOR10	2	1	1	2	2	9	2	0	5	5
	UKR11	2	1	1	1	0	7	2	1	0	2
	Sum (n)	4	2	2	3	2	16	4	1	5	7
	Sum (%)	100	50	50	75	50	100	25	6	31	44
All seasons	Sum (n)	55	15	44	49	46	131	29	40	57	63
	Sum (%)		27	80	89	84		22	31	44	48

cant ($p < 0.01$). To obtain a sufficient number of replicates for considering the significance of seasonal differences, the samples collected from autumn and winter were pooled together. No tissue type showed significant seasonal changes in the presence of *H. fraxineus* DNA, neither within a bark lesion area nor outside this region (Fig. 1). Within a bark lesion area, the greatest differences in the occurrence of *H. fraxineus* DNA were observed between spring and summer for inner bark (p 0.09) and pith (p 0.08).

Discussion

To explore seasonal growth dynamics of *Hymenoscyphus fraxineus* in shoots of European ash, we used *H. fraxineus* DNA – specific qPCR to assess tissue type - specific presence of the pathogen in stems of naturally infected saplings of European ash that had been collected from Ukraine and Norway during spring, summer, autumn and winter. According to the current model that is based on tissue microscopy and pathogen DNA profiling, *H. fraxineus* spreads axially in shoot tissues by growing in the starch-rich cells in the perimedullary pith and in xylem, while the necrotic bark lesions arise in association with radial spread of pathogen mycelia to phloem through the rays that bridge pith and xylem with inner bark (Schumacher et al. 2010, Matsiakh et al. 2016). The now recorded generally higher occurrence of *H. fraxineus* in pith
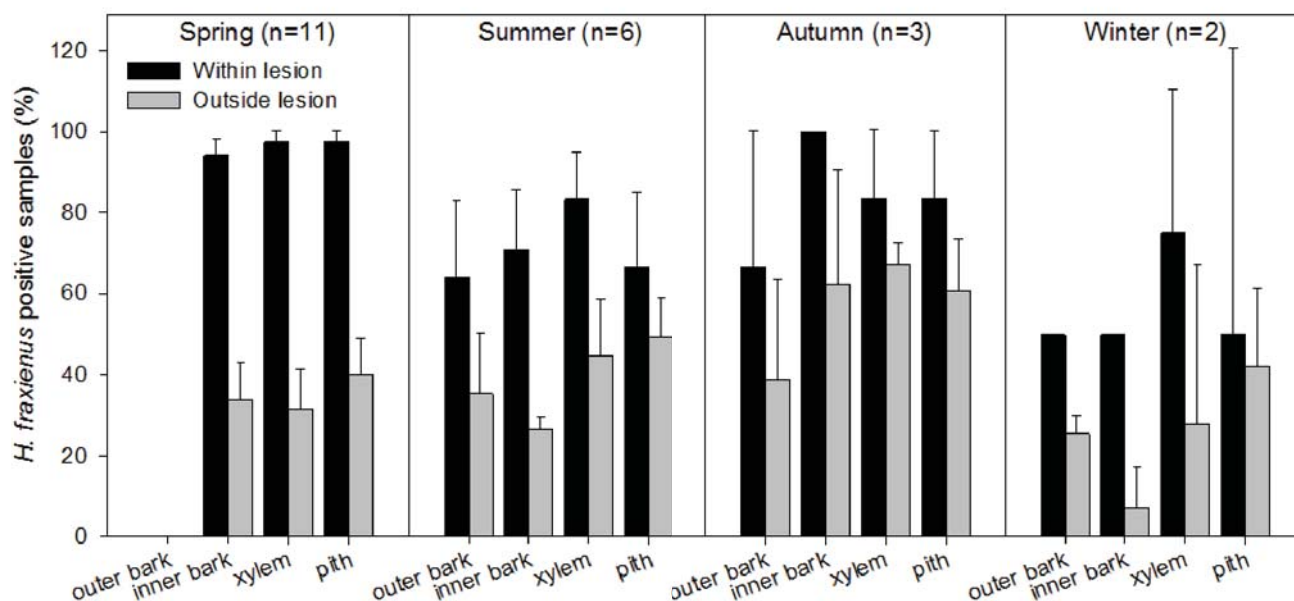
Figure 1. Occurrence frequency of *Hymenoscyphus fraxineus* DNA (% mean ± *SE*) in different stem tissues of naturally infected *Fraxinus excelsior* saplings collected in Ukraine and Norway during the seasons in 2014-15. The analysed samples were collected either from a region showing a necrotic bark lesion ("Within lesion") or from adjacent regions with healthy bark ("Outside lesion"). No samples were taken from the outer bark in the samples collected in spring that are derived from our previous study (Matsiakh et al. 2016).

and xylem in asymptomatic regions adjacent to stem areas with necrotic bark lesions is in line with this model. However, it is noteworthy that healthy outer bark showed the presence of *H. fraxineus* DNA throughout the year. Detection of *H. fraxineus* by fungal isolation and diagnostic PCR assays in collar lesions at ash stem base suggests that the fungus is able to cause direct infection of ash stem (e.g. Chandelier et al. 2016, Marçais et al. 2016). Could some of the now obtained positive signals from outer bark be derived from quiescent endophytic thalli established directly by ascospores, thalli that can resume growth and challenge shoot tissues from outside under certain circumstances? In any case, given the relatively small material now examined from autumn and winter sampling, the generally high occurrence frequency of *H. fraxineus* in phloem in late spring is coherent with the data of Bengtsson et al. (2014), who visually monitored seasonal expansion of bark lesions and observed that they could expand throughout the year but that the high season of lesion expansion initiated in spring. Based on the literature available, Gross et al. (2014) proposed a model according to which shoot bark is initially colonized by *H. fraxineus* but secondary fungi can replace *H. fraxineus* in these lesions and even contribute to lesion enlargement in the tissue. Taken together, the findings of Bengtsson et al. (2014) and the current study may suggest that *H. fraxineus* can indeed be a primary causative agent of bark lesions.

    Does all this point out that *H. fraxineus* is above all a pioneer colonizer that feeds primarily on easily accessible non-structural carbohydrates present in the ash shoot and is eventually replaced by endophytes that are better equipped to feed on cell-wall components? To rigorously consider this scenario, a tissue-specific sampling scheme coupled with fungal community profiling by next-generation sequencing and qPCR verification of tissue colonization level by *H. fraxineus* and fungi competing for this niche would be required.

Conclusions

    1. qPCR assays specific to *Hymenoscyphus fraxineus* DNA provide a sensitive method for monitoring tissue and season-specific growth of this pathogen in ash stems. 2. No significant differences were observed in the occurrence of *H. fraxineus* between the Norwegian and Ukrainian shoot material in the different tissue types, neither within a bark lesion area nor outside this region.

    2. The differences in the tissue-type specific occurrence of *H. fraxineus* DNA between regions with necrotic bark and regions with healthy bark were statistically highly significant.

    3. The relatively high proportion of bark samples positive for *H. fraxineus* in the saplings collected during spring provides support to a model that *H. fraxineus* can be a primary causative agent of bark lesions and that other fungi may eventually replace it in old infection areas.

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