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Vertical spread of *Hymenoscyphus fraxineus* propagules

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Abstract

Currently, the ash dieback causal agent *Hymenoscyphus fraxineus* is an established invasive pathogen in most European countries. Its potential to spread quickly among invaded forests is based on its propagules: airborne inoculum composed mainly of ascospores originated in apothecia growing on leaf litter infected during the previous vegetation season. The spread of the inoculum by air masses to distant areas is probable and depends on the availability of the ascospores in higher levels of air. Our study aimed to detect the inoculum in an infected area at heights of more than 20 meters. Our study was conducted in a municipal locality (Boršov nad Vltavou) with tens of infected ash trees (*Fraxinus excelsior*) in South Bohemia (SW Czechia). The infected trees surround an agricultural silo where five rotating arm spore traps (rotorods) were mounted for ten consequent 48h samplings during the peak of the sporulating season (17th July to 6th August 2020). The spore traps were mounted 48, 37, 25, 14 and 0,3 meters above ground. Samples were quantified by qPCR. Results clearly proved the ability of the spores to reach a height of 48 meters. Furthermore, *H. fraxineus* DNA was detected from all five spore traps during all ten samplings. Mostly, the amount of detected spores showed a decreasing trend with height, and varied a lot. During some of the samplings, higher spore concetrations were achieved at the top than at the lower traps, which can be explained by horizontal air transfer of the inoculum from other infected areas. Based on GLM analyses, higher spore concentrations were achieved during days without rain, lower air temperatures, after cloudy, humid and rainy weather without strong winds. A combination of rotorod ROTTRAP 52 with qPCR quantification proved to be an efficient technology for a study focused on the vertical spread of *H. fraxineus* propagules.

Keywords

airborne inoculum, ash dieback, riseability, rotorod, ROTTRAP, spore trap

Introduction

Hymenoscyphus fraxineus (T. Kowalski) Baral, Queloz, and Hosoya is the causal agent of the ash dieback (Kowalski 2006). It is an invasive pathogen introduced to Europe from Eastern Asia (Zhao et al. 2012) probably during the 1990's (Przybył 2002), first reported in Poland (Kowalski 2006). Its current distribution apart from Eastern Asia covers the whole of Europe apart from Portugal, Greece, Albania, Macedonia, Bulgaria and Moldova (EPPO 2022). *H. fraxineus* severely attacks native European ash species *Fraxinus excelsior* and *F. angustifolia* (Kowalski and Holdenrieder 2009) causing a serious threat to mixed deciduous forest habitats, especially riparian forests where other deciduous tree species are endangered by invasive pathogens (Jankovský and Holdenrieder 2009).

The symptoms of the ash dieback and mechanism of infection of the host trees have been well described in numerous publications (Kowalski 2006; Kowalski and Holdenrieder 2009; Gross et al. 2012, 2014; Husson et al. 2012; Cleary et al. 2013; Kräutler et al. 2015; Chandelier et al. 2016; Fones et al. 2016; Haňáčková et al. 2017)

The predominant way to spread this pathogen is via the airborne ascospores (Gross et al. 2012), although the conidia of the imperfect stage *Chalara fraxinea* play an important role (Fones et al. 2016). The ascospores are typically released from apothecia growing on shredded infected leaf rachises during the following growing season, mainly from June to September (Timmermann et al. 2011; Gross et al. 2012; Hietala et al. 2013; Chandelier et al. 2014; Dvořák et al. 2016). The ascospores have been shown to be able to span long distances and in France they have been observed to even reach 50 to 100 km (Grosdidier et al. 2018b).

The probability of the long distance spread of the ascospores depends on the height they can reach to be blown with the air masses (Chandelier et al. 2014; Oteros et al. 2015; Aguayo et al. 2021). Existing studies proved that the inoculum of *H. fraxineus* rises from the ground where it is actively ejected from the apothecia (Timmermann et al. 2011) and it is reliably detectable in aerobiological samples collected by the RNSA (French Network of Aerobiology) at heights of between 10 to 20 meters (Aguayo et al. 2021). According to these authors, ascospores present at this height prove the presence of the pathogen in the landscape scale in the range of tens of kilometres.

The aim of our study was i) to test a methodological approach for describing the vertical spore dispersal pattern of *H. fraxineus*, and ii) to prove the presence of the inoculum as high as possible, at such a height where the spread of the aerosols is more likely affected by horizontal movement of air masses rather than by convection from the ground surface.

Methods

Sampling in ADB infected locality

The sampling point was located in an agricultural silo in Boršov nad Vltavou (South Bohemia; SW Czech Republic); GPS: 48.9244°N, 14.4414°E; 412 m a. s. l. It is an

industrial area of a village adjacent to Moldava River. It is located on the SE edge of a large plain called České Budějovice Basin (Českobudějovická pánev), where foothills of Šumava mountains called Blanský les start to rise. Due to the surrounding landscape being characterised by plains (especially in NW, N and E direction), the 52 m high silo of Boršov is probably exposed to wind currents blowing from areas that are at least tens of kilometres away. Trees in this area are mostly represented by mixtures of deciduous middle-European species formed at the riverbanks, gardens and parks, or alleys along railways and roads. Most of the groups of trees comprise a significant portion of ashes (*Fraxinus excelsior*) with typical symptoms of ash dieback (Fig. 1A); in a few cases they are even completely dead trees. The closest source of inoculum = rachises with apothecia was found 60 m from the silo, where a lowest spore trap (R1) was installed. This closest point is an edge of mostly untreated vegetation with many infected ashes following adjacent railway and roads, partly visible in Fig. 1B at the right side of the horizon. This forest-like suburban vegetation is in the northern direction altered with recently recovered park with heavily infested trees; one of them, 170 m distanced, is well visible in the foreground of the Fig. 1A. The sampling was carried out during the peak of the ascospore production season in Czechia (Dvořák et al. 2016) continuously from 17th July to 6th August, 2020. The presence of apothecia was checked in the litter of ash trees adjacent to the sampling point.

Air samplers

To sample the air inoculum rotating arm spore traps (rotorods) ROTTRAP 52 (Miloň Dvořák, Boršov nad Vltavou, Czech Republic) were employed (Fig. 1C). Our air-sampler was a further developed rotorod of Chandelier et al. (2014), based on the description of Perkins and Leighton (1957) and improved by McCartney et al. (1997). A 10 cm-long aluminium arm whirled 2067 rotations per minute with vertical squared brass rods mounted on both ends. The impaction side of the rod (0.8 × 50 mm) was covered by double-sided non-woven tape (Tesa SE, Norderstadt, Germany), which was renewed every 48 hours; exposed stored in sterile 2-ml microtubes at -20 °C before further processing. The rotorods were powered from an electric network via adaptor 220V AC/9V DC. Such an arrangement of rotorod samples 52 litres of air per minute, with a theoretical sampling efficiency close to 100% for particles with a diameter bigger than 10.94 μm (Noll 1970; Dhingra and Sinclair 2017). Further methodological details are described in Dvořák (2022).

Sampling points

The rotorods were installed at five different heights (Fig. 1B): 0.3 m (further mentioned as „*R1*“), 13.84 m („*R2*“), 25.09 m („*R3*“), 36.57 m („*R4*“) and 48.06 m („*R5*“) above ground. The *R1* was positioned close to the ground of the nearest group (60 m apart from the silo) of infected ash trees to monitor the source of inoculum. The *R2* – *R5* were mounted on windows in regular heights on the NNW side of the silo. This

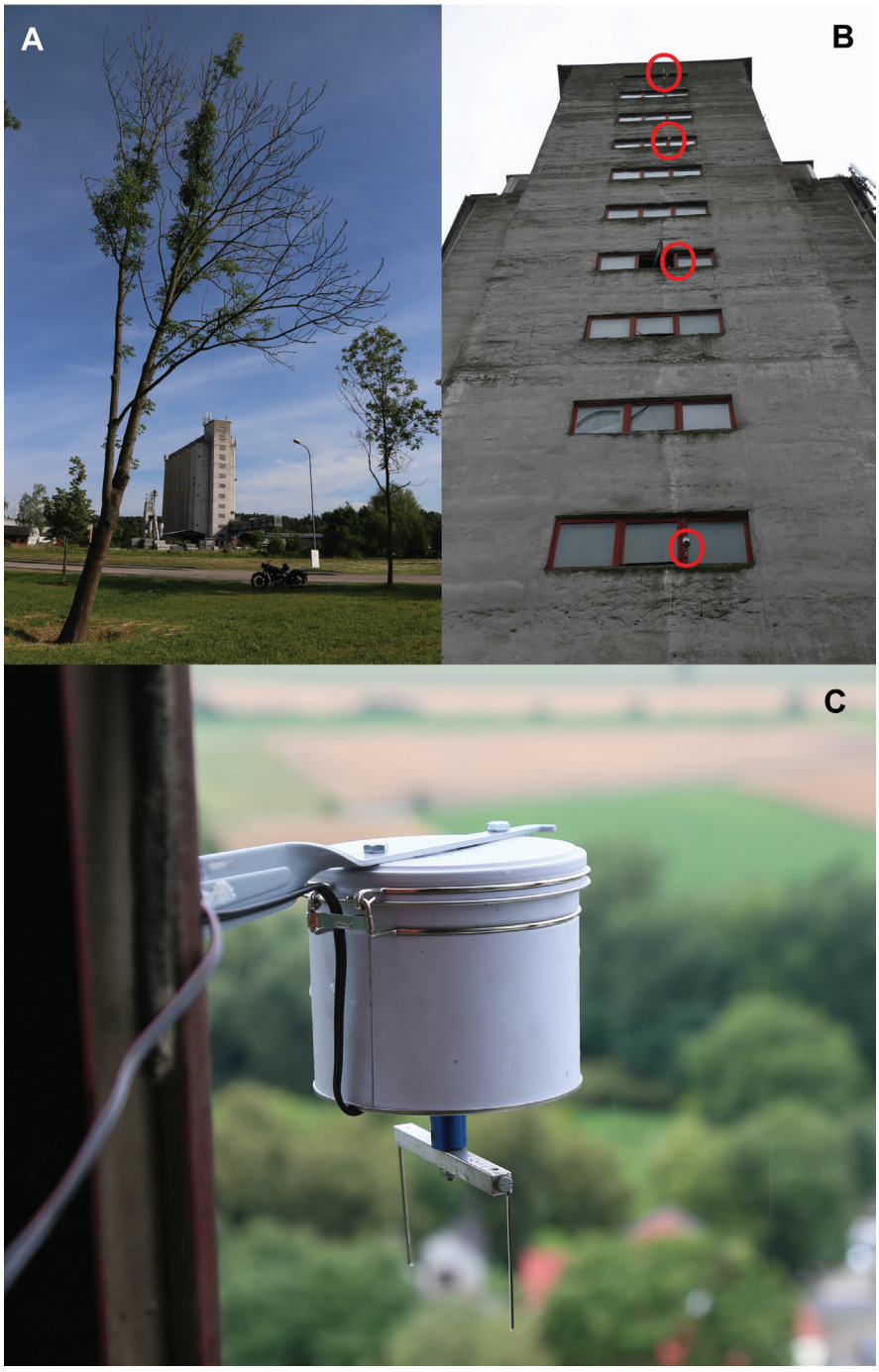

Figure 1. Sampling locality and sampling tools **A** the sampling point in the focus of ash dieback. Agricultural silo is surrounded by ashes infected with *H. fraxineus* **B** sampling spots in the windows of the silo. ROTTRAPs 52 are installed in different heights *R2* - *R5* in the windows (red circles) of the silo on its NNW side **C** rotating arm spore trap ROTTRAP 52 installed at *R5*.

side was chosen for its non-exposure to sun (to avoid additional thermic air currents), and due to the prevailing wind direction. It is exposed to the most common wind (NW), which is supposedly bringing the airborne inoculum from the infection sources present in that direction, including the closest inoculum source where *R1* was installed.

Meteorological data

Meteorological data were partly measured by automatic meteorological station Signalizátor (AMET, Velké Bílovice, Czech Republic) and partly received from the archive of the Czech Hydrometeorological Institute (CHMI), station České Budějovice – Rožnov. From the sampling point the CHMI meteorological station is positioned 3.6 km to NE. Data taken from Signalizátor were daily means of relative air humidity (further in the text only air humidity). Data received from CHMI were: i) daily mean of air temperature (air temperature); ii) daily duration of sunshine (sunshine); iii) daily mean of air pressure (air pressure); iv) daily amount of precipitation (precipitation) and v) daily mean of wind speed (wind speed).

DNA extraction

The genomic DNA from samples was extracted with a DNeasy plant minikit (Düsseldorf, Germany). Each microtube with exposed tape was supplemented with one 3-mm sterile tungsten bead and 20 pcs 2-mm glass beads, 400 µl of AP1 buffer and 4 µl of RNase. This mix was ground twice for 60 seconds using a high speed homogenizer Millmix 20 (Domel, d.o.o., Železniki, Slovenia) set at 30 Hz and incubated for 10 minutes at 65 °C. The microtubes were inverted three times during the incubation. Further steps were following the manufacturer's instructions; however, the last step (elution) was not repeated to obtain higher concentration of DNA. DNA samples were eluted in 100 µl and stored at -20 °C before further processing.

Real-time quantitative PCR conditions

Direct specific qPCR was performed using a QuantStudio 6 Flex Real-Time PCR System (Life Technologies Holdings Pte. Ltd., Singapore), Light Cycler 480 Probes master (Roche Diagnostics Nederland BV, Almere, the Netherlands) and primers and probes specific to *H. fraxineus* (Chandelier et al. 2010). The cycling conditions followed the master mix manufacturer's instructions and the setting optimised by (Chandelier et al. 2010): preincubation: 10 min, 95 °C followed by 45 cycles of denaturation: 10 sec, 95 °C; annealing: 30 s, 60 °C; extension: 1 s, 72 °C. Composition of the reaction mixture was following: 0,2 µl of each primer (final concentration 400 nM), 0,2 µl of TaqMan probe (200 nM), 5 µl of TaqMan Universal PCR Master Mix, 1,4 µl of sterile deionized water and 3 µl of template DNA. Every reaction was performed in two technical repetitions together with a negative control containing the master mix without template DNA.

Absolute quantification

The concentrations of *H. fraxineus* DNA in the samples were expressed as numbers of copies of the target sequence in 1 µl of template DNA (further only *CN*). These values were obtained using a standard curve generated from reactions with different *CN*s (2.5×10^{2} to 2.5×10^{-2}) of plasmid pCR 2.1 TOPO TA vector (Invitrogen, Carlsbad, California, USA) by QuantStudioTM Real-Time PCR System Version 1.3 (Thermo Fisher Scientific). Plasmids contained species - specific insert (PCR products amplified with Cf-F and Cf-S primers). DNA was extracted from pure cultures of *H. fraxineus* (collection of Mendel University in Brno).

To express the absolute amount of ascospores in every analysed sample, an absolute quantification of ascospore suspension was performed. For that purpose ten apothecia were collected from ash leave rachises and immersed in 1 ml of distilled water in a 2-ml microtube. The following day, the clear liquid upper part without apothecia and other debris was transferred into a clean 2 ml microtube. Vortexed ascospore suspension was quantified in Bürker chamber by microscope. Ten-fold serial dilutions from 18750 to 1.875 ascospores in 100 µl of distilled water were transferred into clean 2 ml microtubes and DNA was extracted with the same protocol as for the spore trap samples. Extracted DNA samples were used as standards for a qPCR absolute quantification of the plasmids previously used for the quantification of the environmental samples. The lowest detectable concentration which turned positive in all three technical repetitions of the sample was 18.75 ascospores per sample (*Ct* = 36.328, SD = 0.862).

Statistical analysis

To describe the influence of meteorological factors on the ability of the inoculum to spread vertically, meteorological variables were averaged for three particular days of every sampling. Furthermore, the factor of riseability (*FR*) has been defined. It is expressed as the ratio of the ascospore concentration recorded at the highest sampler (*R5*) to the concentration at the lowest sampler (*R1*). It takes values lower than 1.0 in case the *R5* ascospore concentration is lower than *R1* concentration.

In order to describe the relationship between *CN* and the character of the weather described by explanatory variables (air temperature, precipitation, sunshine, air humidity, wind speed and air pressure), generalised linear regression models were constructed with explanatory variables measured on the same day as the dependent variable *CN*, or with explanatory variables recorded during previous four samplings (i.e. one sampling lag = period of preceding two days before the sampling started, two samplings lag = two to four days before, three samplings lag = four to six days before and four samplings lag = six to eight days before sampling) to simulate the lag of the pathogen's reaction on the changing weather. Due to the nature of the dependent variable (a strictly positive variable showing a positive skew for *R1*, *R5* and *FR*), we used the gamma distribution with a logarithmic link function when

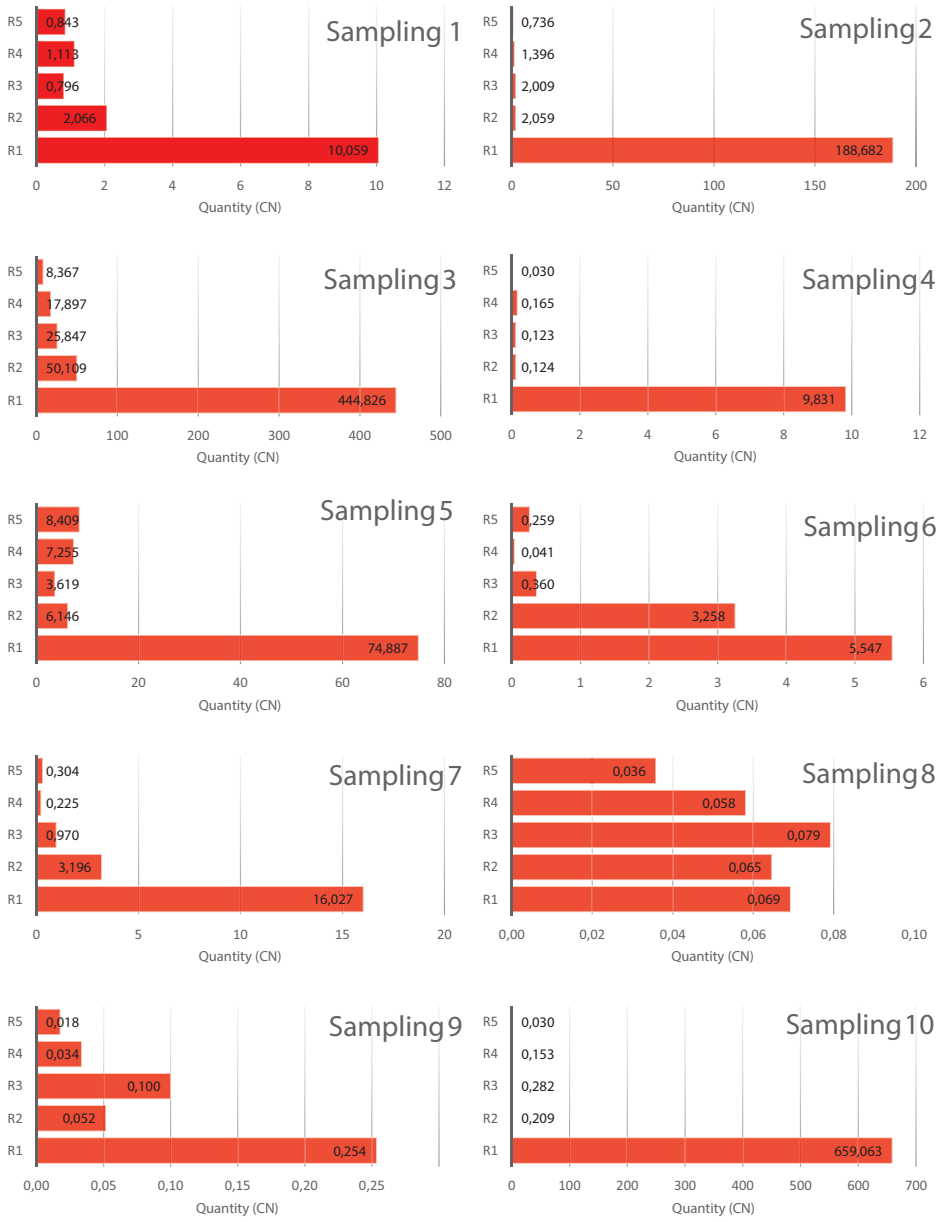

Figure 2. Vertical profiles of spore concentrations recorded during the ten samplings.

fitting the regression models. To select variables for individual models, we used the procedure described in Morgan and Tatar (1972) implemented in the "bestglm" library for the R software (R Core Team 2018). This approach allowed the selection of the best subset of explanatory variables for particular GLMs. However, because of the low number of observations, in some cases the models did not converge

numerically, and it was not possible to obtain the optimal regression model. The selection of suitable models was made with regard to the AIC values achieved (the smallest the best).

At the same time, we tried to model the relationship between *CN* and the height of the sampler. Due to convergence problems with an exponential model based on a differential equation, we used a somewhat simpler empirical exponential model with the following analytical form:

$$CN = \beta_0 \cdot \exp(-\beta_1 \cdot h)$$

where β_0 and β_1 are the estimated regression coefficients, and h is the height in meters. All numerical calculations were performed using the R 4.2.0 programming environment (R Core Team 2018).

Results

Fresh apothecia were observed on infected rachises at the sampling locality from the beginning of July until the end of the sampling (6th August, 2020). Consequently, all ten 48-h samplings showed presence of *H. fraxineus* in the air (Fig. 2).

Every sampler positively detected inoculum during every sampling. The lowest positively detected spore concentration was detected in samples from the highest sampler *R5* from the sampling started on 4th August (*CN* = 0.018, *Ct* = 37.428). This least concentrated sample contained 1.89 ascospores; however, this amount was calculated extrapolating the ascospore suspension standard curve, hence the exact amount cannot be taken in consideration. The highest concentration was recorded at *R1* during the following sampling started on 6th August (*CN* = 659.063, *Ct* = 27.181). Taking in account the sampling rate 52 l/min and sampling period of 48 h, we detected average spore concentration in a range from 0.013 to 462.12 spores per m^3.

Most of the detected spore concentrations showed a clear decreasing trend following the height of the sampling point. The resulting nonlinear regression model (Fig. 3; residual SE = 99.85, DF = 96), corresponding to the aforementioned parameterization, describing the generally decreasing trend, was estimated as follows:

$$CN = 150.3243 \cdot \exp(-0.2155 \cdot h)$$

GLM analyses resulted in 10 significant models to estimate *CN*s from meteorological variables measured during the sampling and lagged of 2–4, 4–6 and 6–8 days. Their parameters can be found in supplementary file "Parameters of GLM models". The calculated *p* and positive or negative meaning of each parameter are displayed in Table 1.

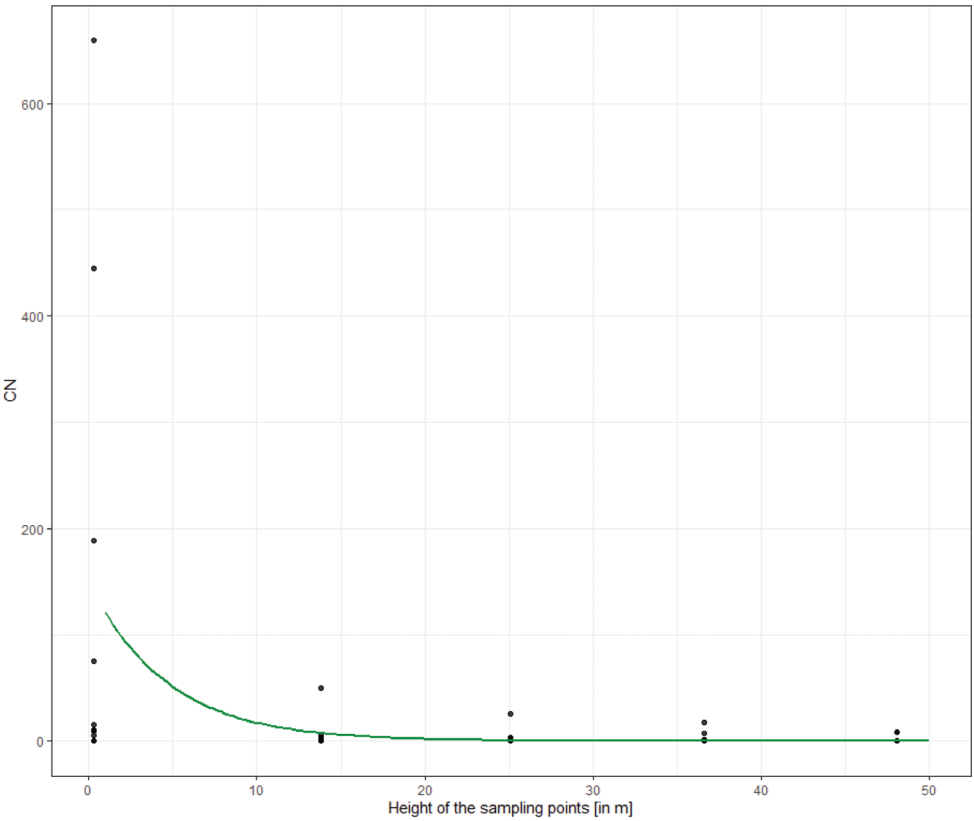

Figure 3. Model of the vertical spread of the inoculum. The nonlinear regression model described the decreasing trend of spore concentrations with increasing height of sampling.

Discussion

Our results prove for the first time, that propagules of *H. fraxineus* are reliably detectable at almost 50 meters above ground, where they have their main source in infected rachises (Gross et al. 2012). Repeated sampling revealed *H. fraxineus* DNA's presence to be more than twice as higher as in an aerobiological study, where air was sampled by 7-day volumetric spore traps (Hirst 1952) of RNSA (Aguayo et al. 2021).

The results of this study also confirm statements of other authors (Chandelier et al. 2014) who described decreasing trend of ascospore concentrations up to three meters, regardless of the site and time of the sampling. This was a general trend among our samplings. The ground sampling spot *R1* always showed higher *CN* than the top spot *R5*. In one case even more than 20,000 times.

However, not in every sampling did the *CN* values descend with increasing height. The sampling spot *R4* showed lower values, than *R5* for samplings 5, 6 and 7. This was probably due to overloading the sampling rods with dust, which is a critical handi-

Table 1. Meteorological variables as parameters of GLM models and their *p* during sampling and 0–8 days before sampling (d.b.s.).

R1	**6–8 d.b.s.**	**4–6 d.b.s.**	**2–4 d.b.s.**	**0–2 d.b.s.**	**sampling**
Air temperature				.014	.026
Sunshine				.009	.034
Precipitation			.032		.002
Air humidity			.032	.045	
Wind speed		.01	.031		
Air pressure			.012		
R5	**6–8 d.b.s.**	**4–6 d.b.s.**	**2–4 d.b.s.**	**0–2 d.b.s.**	**sampling**
Air temperature		.043		.040	.018
Sunshine				.001	
Precipitation		.001		.001	.002
Air humidity		.006	.004		
Wind speed					
Air pressure					
FR	**6–8 d.b.s.**	**4–6 d.b.s.**	**2–4 d.b.s.**	**0–2 d.b.s.**	**sampling**
Air temperature			.016		
Sunshine		.009	.029		
Precipitation	.034	.018		.009	.048
Air humidity				.011	
Wind speed			.016	.015	
Air pressure					
Legend:		Influence			
		positive	negative		
Significant parameter ($p < .05$)		*p*	*P*		
Highly significant parameter ($p < .01$)		*p*	*P*		

cap of rotorods (Lacey and West 2006; Chandelier et al. 2014; Dhingra and Sinclair 2017). The dust apparently came from outlets of ventilators of the silo adjacent to *R4* while depositing the harvested grain.

Furthermore, the sampling site *R3* gave higher *CN*s than it could be expected to follow the descendent trend during samplings 8 and 9. Similarly, *CN*s at *R5* from samplings 1, 5 and 6 were also not lower than *CN* of lower sampling points. An explanation for this anomaly could be the effect of horizontal air currents which could bring a higher amount of inoculum from more distant localities, influencing the results of sampling only in these cases of low local concentrations. Generally, abnormalities in the trend of decreasing *CN* with the height of sampling can be interpreted as a consequence of the fact that inoculum detected in heights above 10 m might be representative for areas within a perimeter of at least tens of kilometres (Aguayo et al. 2021). Study focused on long distance spread of pollen grains found correlation of pollen data from the sampler (elevated 10–20 m) and land use even 200 km apart (Oteros et al. 2015, 2017). In the case of our experiment, samplers *R2*, *R3*, *R4* and *R5* were mounted higher than 10 meters; *R5* even in more than 48 m. Taking into account the previously mentioned studies (Oteros et al. 2015, 2017,

Aguayo et al. 2021) there is a theoretical presumption that the detected inoculum can be related to sources at a distance of up to hundreds of kilometres. Also fungal spores' long distance proofs are known. Based on spatial air sampling and modelling it was revealed that tobacco blue mould outbreak was caused by *Peronospora tabacina* spores blown from sources several hundred km away (Aylor et al. 1982). Another example of long distance transport of spores was studied by Vasaitis and Enderle (2017) considering a possible way of ash dieback introduction to Great Britain. Agricultural silo used for this study is located very beneficially from this point. Its height is almost three times bigger than the canopy layer of surrounding vegetation, which acts as a natural barrier for the vertical spread of the inoculum (Aylor 1999). Taking into account the geomorphology of surrounding landscape and predominant wind direction (NW), it is highly likely exposed to air masses from an adjacent plane area called "Českobudějovická pánev" (České Budějovice Basin), which is a tectonic ditch oriented NW – SE, elevated 380–410 m a. s. l., almost 70 km long and 10–12 km wide. From personal experiences we know that infected ashes are very frequently present there, especially in alleys along roads, riverside plantations and municipal green spaces. We assume that inoculum detected in *R2* – *R5* may originate in this area, not only from the sources adjacent to the sampling point. Transfer of spores by air masses up to 100 km has been already proven (Grosdidier et al. 2018b), which is enabling this hypothesis.

Long distance transfers of *H. fraxineus* by air masses have always been an important issue. It was considered that *H. fraxineus* had been introduced into Great Britain between 2008 and 2011 via long distance transfer of the air inoculum from mainland Europe. This statement was strongly supported with a model (Vasaitis and Enderle 2017). Recently, *H. fraxineus* was found for the first time in Spain (Asturias, NW Spain) on matured trees and its surrounding regeneration (Stroheker et al. 2021). The fact that it has not yet been observed in NE Spain, which is adjacent to the closest distribution of the pathogen, implies the possibility of long-distance transport of the inoculum from France via the Bay of Biscay. However, the introduction of the pathogen through plant material import cannot be overlooked.

Results of the GLM modelling of the determination of the detected spore concentrations by meteorological factors discovered numerous relations. Although the low number of repetitions decreases the reliability of the results' interpretation, we would like to highlight some of them:

Air temperature proved to have significantly positive effect two days preceding the sampling at both *R1* and *R5*. This confirms previous observations of Chandelier et al. (2014), who found a positive influence of higher temperatures on spore concentrations. On the contrary, sooner than two days before and directly during the sampling, the effect of air temperature was significantly negative. This pattern indicates, and confirms, that for the ascospore development and release the high air temperature is not favourable (Grosdidier et al. 2018a). Since the entire sampling period can be characterized like very warm with maximal temperatures exceeding 30 °C, this biological limitation of the pathogen was more apparent within our experiment.

The daily amount of precipitation showed different effects at different sampling heights. *R1* was significantly positively affected by precipitations two to four days before sampling. Through the enhanced humidity of the ground surface, rain proved to be essential for successful ascospore maturation and release (Timmermann et al. 2011; Hietala et al. 2013; Dvořák et al. 2016) and the consequent progress of ash dieback (Chumanová et al. 2019). On the other hand, concentrations at *R5* and riseability were significantly negatively affected by rain during the days before the sampling, and even highly significantly during the sampling. Two explanations for this phenomenon are probable: i) ascospores currently present in the air are washed out by rain from the aerosol which is a known factor (Aylor 1999), and ii) trapped ascospores are partly splashed from the sticky sampling surface. For the significantly positive effect of precipitation on riseability during sampling we do not have any reasonable explanation; however, the p for this parameter is approaching the limit of significance (p = .048) hence its importance is ambiguous.

Daily duration of sunshine showed highly significantly negative effect on spore concentration at *R1* and *R5* two days before the sampling and on riseability during the preceding four to six days. However, the sunshine was significantly positive during the sampling at *R1* and *R5* and during the preceding two to four days it was significantly positively affecting the riseability. This partly confirms and partly neglects results of Burns et al. (2022) who found a clear determination of *H. fraxineus* spore release after five-day-average net radiation and leaf moisture.

Air humidity was found to be an important factor through our sampling. At *R5* the humid air was significantly determining the inoculum load after two to six days; similarly, at *R1* there was a significantly positive effect on inoculum concentrations lagged by two to four days. The promoting effect of air humidity on the disease spread and establishment has already been emphasised many times (Timmermann et al. 2011; Hietala et al. 2013; Dvořák et al. 2016; Čermáková et al. 2017; Chumanová et al. 2019; Volke et al. 2019). However, up to two days before the sampling, the air humidity had an opposite effect at *R1* and negatively affected also the riseability. This ambiguity of the air humidity effect for sporulation emphasizes the necessity for more detailed study of the ascospore discharge mechanism such as in Ingold (1999).

Wind speed proved to have a significantly negative influence on ascospore concentrations at *R1* after two to six days. This effect is probably due to the desiccation of leaf rachises and growing apothecia which are not able to mature and sporulate under such conditions (Timmermann et al. 2011; Hietala et al. 2013). The significantly negative effect of wind speed was also found to affect the sampling directly. Since the wind speed is measured in horizontal direction, it is logical that strong wind cannot be beneficial for vertical spore transfer. Horizontally, moving air masses are supposedly continuously removing the local inoculum, which would otherwise reach higher air levels by convection (Garratt 1994; Lacey and West 2006).

Air pressure did not show much importance in our experiment. It was a significant parameter with negative influence on spore concentrations at *R1* after two to four days. At the same period and sampling height two other factors had positive influence on the

spore concentrations: precipitations and air humidity. Naturally, rainy and humid weather is characterized by low air pressure, which we assume to be a reason for this result.

From a methodological point of view, rotating arm spore trap ROTTRAP 52 proved to be a reliable tool for the detection of *H. fraxineus* inoculum. All samplers successfully completed all ten 48h samplings at five sampling spots without any blackout even in hot or rainy weather. This reliability has been improved compared to previous experiments (Dvořák et al. 2016, 2017; Čermáková et al. 2017). Rotorods combined with quantitative real-time PCR detection and quantification of *H. fraxineus* in the air continuously produce robust and valuable data (Chandelier et al. 2014, Dvořák et al. 2016; Čermáková et al. 2017). Similarly, in our study all samplings yielded positive results. On the other hand, the possibility of overloading by other, more concentrated particles (in this study's case it was dust) must be always taken into consideration in experiment planning and interpretation of the results.

Conclusion

Our study revealed the permanent presence of the *H. fraxineus* inoculum up to 48 meters above the ground during the whole sampling period. Its concentration is continuously changing depending on previous and current weather, and decreases with height. It poses a persistent threat to ash trees, either at local or landscape scale. This finding supports a sceptical outlook for the future of ashes in European forests, but also confirms the important role of high height air sampling of the propagules of this invasive alien pathogen to ensure its reliable monitoring.

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References

Aguayo J, Husson C, Chancerel E, Fabreguettes O, Chandelier A, Fourrier-Jeandel C, Dupuy N, Dutech C, Ioos R, Robin C, Thibaudon M, Marçais B, Desprez-Loustau ML (2021) Combining permanent aerobiological networks and molecular analyses for large-scale surveillance of forest fungal pathogens: A proof-of-concept. Plant Pathology 70(1): 181–194. https://doi.org/10.1111/ppa.13265

Aylor DE (1999) Biophysical scaling and the passive dispersal of fungus spores: Relationship to integrated pest management strategies. Agricultural and Forest Meteorology 97(4): 275–292. https://doi.org/10.1016/S0168-1923(99)00072-6

Aylor DE, Taylor GS, Raynor GS (1982) Long-range transport of tobacco blue mold spores. Agricultural Meteorology 27(3–4): 217–232. https://doi.org/10.1016/0002-1571(82)90007-3

Burns P, Timmermann V, Yearsley JM (2022) Meteorological factors associated with the timing and abundance of *Hymenoscyphus fraxineus* spore release. International Journal of Biometeorology 66(3): 493–506. https://doi.org/10.1007/s00484-021-02211-z

Čermáková V, Kudláček T, Rotková G, Rozsypálek J, Botella L (2017) *Hymenoscyphus fraxineus* mitovirus 1 naturally disperses through the airborne inoculum of its host, *Hymenoscyphus fraxineus*, in the Czech Republic. Biocontrol Science and Technology 27(8): 992–1008. https://doi.org/10.1080/09583157.2017.1368455

Chandelier A, André F, Laurent F (2010) Detection of *Chalara fraxinea* in common ash (*Fraxinus excelsior*) using real time PCR. Forest Pathology 40(2): 87–95. https://doi.org/10.1111/j.1439-0329.2009.00610.x

Chandelier A, Helson M, Dvorak M, Gischer F (2014) Detection and quantification of airborne inoculum of *Hymenoscyphus pseudoalbidus* using real-time PCR assays. Plant Pathology 63(6): 1296–1305. https://doi.org/10.1111/ppa.12218

Chandelier A, Gerarts F, San Martin G, Herman M, Delahaye L (2016) Temporal evolution of collar lesions associated with ash dieback and the occurrence of *Armillaria* in Belgian forests. Forest Pathology 46(4): 289–297. https://doi.org/10.1111/efp.12258

Chumanová E, Romportl D, Havrdová L, Zahradník D, Pešková V, Černý K (2019) Predicting ash dieback severity and environmental suitability for the disease in forest stands. Scandinavian Journal of Forest Research 34(4): 254–266. https://doi.org/10.1080/02827581.2019.1584638

Cleary MR, Daniel G, Stenlid J (2013) Light and scanning electron microscopy studies of the early infection stages of *Hymenoscyphus pseudoalbidus* on *Fraxinus excelsior*. Plant Pathology 62(6): 1294–1301. https://doi.org/10.1111/ppa.12048

Dhingra OD, Sinclair JB (2017) Basic plant pathology methods. CRC Press, 434 pp. https://doi.org/10.1201/9781315138138

Dvořák M (2022) Detection of Airborne Inoculum of *Hymenoscyphus fraxineus*: The Causal Agent of Ash Dieback. In: Luchi N (Ed.) Plant Pathology. Methods in Molecular Biology, vol 2536. Humana, New York, 119–137. https://doi.org/10.1007/978-1-0716-2517-0_8

Dvořák M, Rotková G, Botella L (2016) Detection of airborne inoculum of *Hymenoscyphus fraxineus* and *H. albidus* during seasonal fluctuations associated with absence of apothecia. Forests 7(12): 1–13. https://doi.org/10.3390/f7010001

Dvořák M, Janoš P, Botella L, Rotková G, Zas R (2017) Spore dispersal patterns of *Fusarium circinatum* on an infested monterey pine forest in North-Western Spain. Forests 8(11): 432. https://doi.org/10.3390/f8110432

EPPO (2022) *Hymenoscyphus fraxineus*: Distribution. EPPO Global Database. https://gd.eppo.int/taxon/CHAAFR/distribution [July 14, 2022]

Fones HN, Mardon C, Gurr SJ (2016) A role for the asexual spores in infection of *Fraxinus excelsior* by the ash-dieback fungus *Hymenoscyphus fraxineus*. Scientific Reports 6(1): 1–10. https://doi.org/10.1038/srep34638

Garratt JR (1994) Review: The atmospheric boundary layer. Earth-Science Reviews 37(1–2): 89–134. https://doi.org/10.1016/0012-8252(94)90026-4

Grosdidier M, Ioos R, Marçais B (2018a) Do higher summer temperatures restrict the dissemination of *Hymenoscyphus fraxineus* in France? Forest Pathology 48(4): 1–8. https://doi.org/10.1111/efp.12426

Grosdidier M, Ioos R, Husson C, Cael O, Scordia T, Marçais B (2018b) Tracking the invasion: Dispersal of *Hymenoscyphus fraxineus* airborne inoculum at different scales. FEMS Microbiology Ecology 94(5): fiy049. https://doi.org/10.1093/femsec/fiy049

Gross A, Zaffarano PL, Duo A, Grünig CR (2012) Reproductive mode and life cycle of the ash dieback pathogen *Hymenoscyphus pseudoalbidus*. Fungal Genetics and Biology 49(12): 977–986. https://doi.org/10.1016/j.fgb.2012.08.008

Gross A, Holdenrieder O, Pautasso M, Queloz V, Sieber TN (2014) *Hymenoscyphus pseudoalbidus*, the causal agent of European ash dieback. Molecular Plant Pathology 15(1): 5–21. https://doi.org/10.1111/mpp.12073

Haňáčková Z, Koukol O, Čmoková A, Zahradník D, Havrdová L (2017) Direct evidence of *Hymenoscyphus fraxineus* infection pathway through the petiole-shoot junction. Forest Pathology 47(6): 1–6. https://doi.org/10.1111/efp.12370

Hietala AM, Timmermann V, BØrja I, Solheim H (2013) The invasive ash dieback pathogen *Hymenoscyphus pseudoalbidus* exerts maximal infection pressure prior to the onset of host leaf senescence. Fungal Ecology 6(4): 302–308. https://doi.org/10.1016/j.funeco.2013.03.008

Hirst JM (1952) An automatic volumetric spore trap. Annals of Applied Biology 39(2): 257–265. https://doi.org/10.1111/j.1744-7348.1952.tb00904.x

Husson C, Caël O, Grandjean JP, Nageleisen LM, Marçais B (2012) Occurrence of *Hymenoscyphus pseudoalbidus* on infected ash logs. Plant Pathology 61(5): 889–895. https://doi.org/10.1111/j.1365-3059.2011.02578.x

Ingold CT (1999) Active liberation of reproductive units in terrestrial fungi. The Mycologist 13(3): 113–116. https://doi.org/10.1016/S0269-915X(99)80040-8

Jankovský L, Holdenrieder O (2009) Short communication *Chalara fraxinea* - Ash dieback in the Czech Republic. Plant Protection Science 45(2): 74–78. https://doi.org/10.17221/45/2008-PPS

Kowalski T (2006) *Chalara fraxinea* sp. nov. associated with dieback of ash (*Fraxinus excelsior*) in Poland. Forest Pathology 36(4): 264–270. https://doi.org/10.1111/j.1439-0329.2006.00453.x

Kowalski T, Holdenrieder O (2009) Pathogenicity of *Chalara fraxinea*. Forest Pathology 39(1): 1–7. https://doi.org/10.1111/j.1439-0329.2008.00565.x

Kräutler K, Treitler R, Kirisits T (2015) *Hymenoscyphus fraxineus* can directly infect intact current-year shoots of *Fraxinus excelsior* and artificially exposed leaf scars. Forest Pathology 45(4): 274–280. https://doi.org/10.1111/efp.12168

Lacey ME, West JS (2006) The Air Spora – A Manual for Catching and Identifying Airborne Biological Particles. Springer, 156 pp. https://doi.org/10.1007/978-0-387-30253-9

McCartney HA, Fitt BDL, Schmechel D (1997) Sampling bioaerosols in plant pathology. Journal of Aerosol Science 28(3): 349–364. https://doi.org/10.1016/S0021-8502(96)00438-7

Morgan JA, Tatar JF (1972) Calculation of the Residual Sum of Squares for all Possible Regressions. Technometrics 14(2): 317–325. https://doi.org/10.1080/00401706.1972.10488918

Noll KE (1970) A rotary inertial impactor for sampling giant particles in the atmosphere. Atmospheric Environment (1967) 4: 9–19.

Oteros J, García-Mozo H, Alcázar P, Belmonte J, Bermejo D, Boi M, Cariñanos P, Díaz de la Guardia C, Fernández-González D, González-Minero F, Gutiérrez-Bustillo AM, Moreno-Grau S, Pérez-Badía R, Rodríguez-Rajo FJ, Ruíz-Valenzuela L, Suárez-Pérez J, Trigo MM, Domínguez-Vilches E, Galán C (2015) A new method for determining the sources of airborne particles. Journal of Environmental Management 155: 212–218. https://doi.org/10.1016/j.jenvman.2015.03.037

Oteros J, Valencia RM, del Río S, Vega AM, García-Mozo H, Galán C, Gutiérrez P, Mandrioli P, Fernández-González D (2017) Science of the Total Environment Concentric Ring Method for generating pollen maps. Quercus as case study. The Science of the Total Environment 576: 637–645. https://doi.org/10.1016/j.scitotenv.2016.10.121

Perkins WA, Leighton PA (1957) The rotorod sampler. Second Semi-Annual Report No. CML 186, Aerosol Laboratory.

Przybył K (2002) Fungi associated with necrotic apical parts of *Fraxinus excelsior* shoots. Forest Pathology 32(6): 387–394. https://doi.org/10.1046/j.1439-0329.2002.00301.x

R Core Team (2018) R: A language and environment for statistical computing. https://www.r-project.org/

Stroheker S, Queloz V, Nemesio-Gorriz M (2021) First report of *Hymenoscyphus fraxineus* causing ash dieback in Spain. New Disease Reports 44(2): 10–12. https://doi.org/10.1002/ndr2.12054

Timmermann V, Børja I, Hietala AM, Kirisits T, Solheim H (2011) Ash dieback: Pathogen spread and diurnal patterns of ascospore dispersal, with special emphasis on Norway. Bulletin OEPP. EPPO Bulletin. European and Mediterranean Plant Protection Organisation 41(1): 14–20. https://doi.org/10.1111/j.1365-2338.2010.02429.x

Vasaitis R, Enderle R (2017) Dieback of European Ash (*Fraxinus* spp.) -Consequences and Guidelines for Sustainable Management The Report on European Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, 301 pp.

Volke V, Knapp S, Roloff A (2019) Survey of *Hymenoscyphus fraxineus* in a central European urban area and exploration of its possible environmental drivers. Urban Forestry & Urban Greening 40: 165–173. https://doi.org/10.1016/j.ufug.2018.05.013

Zhao YJ, Hosoya T, Baral HO, Hosaka K, Kakishima M (2012) *Hymenoscyphus pseudoalbidus*, the correct name for Lambertella albida reported from Japan. Mycotaxon 122(1): 25–41. https://doi.org/10.5248/122.25