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Fluctuación estacional del inóculo de *Hymenoscyphus fraxineus* en la República Checa

(Seasonal fluctuations of airborne inoculum of *Hymenoscyphus fraxineus* in Czech Republic)

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☐ Copia para el tutor/a

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1. ABSTRACT

Ash dieback caused by the ascomycete fungus *Hymenoscyphus fraxineus* (anamorph: *Chalara fraxinea*) is a serious disease that has emerged during the past twenty years. It was first observed in Poland and has rapidly expanded over most of the distribution area of European ash (*Fraxinus excelsior*) in Europe. *Hymenoscyphus fraxineus* is a lethal invasive pathogen from East Asia that attacks living shoots, leaves and, later, stems, collars, and roots of ash trees of all ages, and later lives as a saprotroph decomposing fallen petioles. Airborne ascospores seem to be the only infectious propagules and outset of new infections on green leaves. They are released by apothecia on decomposing, pseudosclerotial leaf petioles and rachises found in the leaf litter mainly in summer. Knowledge on the spore content of the air is important to research into the epidemiology of plant diseases, and therefore, to find sustainable control methods which may be helpful to manage the disease.

The objectives of this study were (*i*) to describe the seasonal pattern of *Hymenoscyphus fraxineus* airborne inoculum in a forest stand infected by ash dieback in the South West of the Czech Republic and (*ii*) to determine the influence of meteorological conditions on its biology. For this purpose, a moderate infected mixed forest in the SW Czech Republic was chosen. A seven-day automatic volumetric spore trap (SAVST) and a weather station were installed to continuously sample the pathogen inoculum from mid-May to the end of October 2015.

DNA was extracted from all the air samples from the spore trap SAVST and, subsequently, was quantified by real-time PCR. The qPCR analysis was carried out with the dual-labeled fluorogenic TaqMan probe, TaqMan Universal PCR Master Mix (dNTPs, a suitable buffer solution, a thermo-stable DNA polymerase and water) and species-specific primers for *Hymenoscyphus fraxineus*. Simultaneously with the molecular technique, Saturday air samples were observed using optical microscopy allowing the detection of the ascospores of *H. fraxineus*. Results show occurrence of inoculum of *H. fraxineus* throughout the entire sampling period with peak levels in August, which was confirmed through the identification of its ascospores by microscopy. According to the relation with meteorological variables, minimal air humidity showed a significantly influenced in the amount of *H. fraxineus* DNA. In particular, an inverse correlation was found, differing from former studies. This different pattern may be caused by the high levels of relative humidity present in our study area over the sampling period, which may somehow inhibit the release of *H. fraxineus* ascospores. There is a need to find sustainable control methods to ash dieback, biological studies focused on the occurrence of the *H. fraxineus* air-inoculum and its relation to meteorological conditions, are essential for a more precise disease management.

2. INTRODUCTION

2.1. OVERVIEW AND BACKGROUND OF THE ASH DIEBACK

European forests are characterized by relatively low tree species diversity (Svenning and Skov, 2007). Under the current scenario of global change, the introduction of exotic pathogens constitutes a major risk for European forest ecosystems (Moslonka-Lefebvre *et al.*, 2011). Significant number of new tree diseases caused by alien invasive forest pathogens has been reported in Europe over the last decades (Santini *et al.*, 2013), such as Dutch elm disease caused by *Ophiostoma novo-ulmi* Brasier, Chestnut blight produced by the fungus *Cryphonectria parasitica* (Murrill) M.E. Barr, the serious disease, Pine pitch canker caused by *Fusarium circinatum* Nirenberg and O'Donnell or *Phytophthora alni* Brasier and S.A. Kirk an oomycete which has become an important cause of death of alders in many European countries.

The ascomycete *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, and Hosoya (=*Hymenoscyphus pseudoalbidus* Queloz *et al.*, anamorph *Chalara fraxinea* T. Kowalski) causes a lethal disease known as ash dieback in Europe. The disease is severe and it produces high mortality rates of *Fraxinus excelsior* and *Fraxinus angustifolia* over large parts of the continent. The disease also affects many ecologically ash dependent organisms, and unique forest ecosystems for which it is a keystone tree species. The death of vast numbers of common ash trees is likely to have major ecological consequences for European biodiversity and forest ecosystems (including tree biomass and, thus, carbon sequestration), as observed in other common tree species' declines (Pautasso *et al.*, 2013).

Common ash (*Fraxinus excelsior*) has been suffering from a large-scale decline in Europe for nearly 20 years. Before the current epidemic, dieback symptoms on mature ash trees had been occasionally noted in Europe, but they were interpreted as abiotic damage caused by frost or drought (Gross *et al.*, 2014), as a consequence of excessive fructification, maturation feeding of bark beetles (*Leperisinus varius* and *Hylesinus* spp.) or combinations of abiotic and biotic factors (Hull and Gibbs, 1991; Boudier, 1994).

The phenomenon was initially observed in Lithuania and Poland during the 1990s (Kowalski and Łukomska, 2005). Firstly, the causing agent was discovered and described in its anamorphic stage *Chalara fraxinea*, based on conidia and conidiophore morphology of the vegetative stage, since no sexual stage of the fungus was known at the time (Kowalski, 2006). Kowalski and Holdenrieder (2009) suggested that *C. fraxinea* is the anamorph of *Hymenoscyphus albidus*, a widespread non-pathogenic endophyte of ash petioles involved in the decomposition of ash leaves after autumn shedding. Subsequently, two morphologically almost identical *Hymenoscyphus* species on *F. excelsior* were distinguished and the ash dieback pathogen was described as a novel taxon, *H. pseudoalbidus* (=*H. fraxineus*). Significant differences were found between the species in the loci calmodulin, translation elongation factor 1-α and the internal transcribed spacers (ITS) of the rDNA genes, another strong differentiation was obtained with inter-simple sequence repeat (ISSR) markers (Queloz *et al.,* 2011). This standpoint has been confirmed based on collections in France, describing differences in ITS rDNA and three single copy nuclear genes in *H. albidus* and *H. pseudoalbidus* (Husson *et al.,* 2011). The second species is likely to be the causal agent of ash dieback because it was only associated with diseased ash trees (pathogenicity demonstrated by artificial

inoculation tests), whereas *H. albidus* seems to be non-pathogenic and was the only species found in healthy ash stands (Husson *et al.,* 2011; Queloz *et al.,* 2011).

Hosoya *et al.,* (1993) reported *Lambertella albida* (synonym of *H. albidus*) from petioles of decaying leaves on *Fraxinus mandshurica* var. *japonica*, as a new species in Japan. Then, Zhao *et al.* (2013) showed that *L. albida* from Japan is actually comparable with *H. pseudoalbidus*, they are conspecific, and that the sequence diversity among Japanese specimens is considerably higher than among European ones. A subsequent study incorporating large Japanese and European *H. fraxineus* populations confirmed the finding of a greater genetic diversity in Japan (Gross *et al*., 2012a). This led to the conclusion that the fungus has been introduced to Europe from eastern Asia.

At the times when the asexual morph name *Chalara fraxinea*, and the sexual morph name *Hymenoscyphus pseudoalbidus*, were described, separate scientific names for the different morphs were allowed. However, with the change in the rules of nomenclature pertaining to fungi, the oldest species epithet had to be placed in the correct genus. According to the suggestion of the International Code of Nomenclature for algae, fungi, and plants (Art. 59, ICN Melbourne, McNeill *et al.,* 2012), the name *Hymenoscyphus pseudoalbidus* needed to be changed, because the specific epithet of *C. fraxinea* T. Kowalski 2006 is older than that of *H. pseudoalbidus* Queloz *et al.* 2011, while the generic name *Hymenoscyphus* Gray 1821 predates that of *Chalara* (Corda) Rabenh 1844. Gams *et al.* (2012) recommended to generally accept the epithet which is in the prioritized genus: 'When a binomial in a prioritized genus has a younger epithet than the corresponding name in the suppressed genus, only priority of extant names in the prioritized genus should count.' However, this proposal was later not accepted. Since molecular data on *H. albidus* and *H. pseudoalbidus* supported that these species are closely related to the type species of *Hymenoscyphus* (Queloz *et al.,* 2011), and because *"fraxinea"* yielded in online search engines about 5 times as many hits as *"pseudoalbidus"* (52.200 vs. 10.100, Google Web Search, 22.V.2014), Baral *et al.* (2014) proposed the new combination *Hymenoscyphus fraxineus*.

The disease seemed to have spread from eastern to Western Europe (Husson *at al.*, 2011). The presence of the disease was observed in Lithuania (Lygis *et al.*, 2005) and confirmed in Germany in 2007 (Schumacher *et al.*, 2007). Then, *C. fraxinea* was isolated for first time in symptomatic young trees in two localities in Austria (Halmschlager and Kirisits, 2008). Ash dieback fungus was also identified in the Scandinavian peninsula in subsequent years, Sweden (Bakys *et al.*, 2009b), Denmark (Thomsen *et al.*, 2007), Norway (Talgø *et al.*, 2009), and Finland, Latvia and Estonia a little later (Rytkönen *et al.*, 2011). This event was also repeated in Czech Republic (Jankovsky *et al.*, 2008), Hungary (Szabó, 2008) and Slovenia (Ogris *et al.,* 2009). The results of Husson *et al.*, (2011) confirmed that *C. fraxinea* (*Hymenoscyphus fraxineus*) is also a recent invader in France. In 2012, symptoms caused by *H. fraxineus* were reported in Great Britain for the first time (Forestry Commission, 2013). In eastern Ukraine, first symptoms of dieback were observed on common ash (*F. excelsior*) in 2010 (Davydenko *et al.*, 2013) and likewise in Russia, near St. Petersburg (Selikhovkin *et al.,* 2013). Nowadays, Spain remains free of *Hymenoscyphus fraxineus*, but the rapid advance through France and the large plant trade network due to globalization, it can be expected its introduction in the Iberian Peninsula.

In the Czech Republic, *H. fraxineus* was initially isolated in 2007 from *Fraxinus excelsior* cv. pendula in the Krtiny Arboretum (South Moravia). Nevertheless, the first infections had likely occurred already by the end of the 1990s (Jankovsky and Holdenrieder, 2009), and at present, ash dieback can be found throughout the Czech

Republic. The level of infection, however, differs among the vegetation types, where humidity seems to be the limiting factor for the pathogen (Havrdová and Černý, 2013).

2.2. *Hymenoscyphus fraxineus* (T. KOWALSKI) BARAL, QUELOZ, AND HOSOYA

The genus *Hymenoscyphus* belongs to the family *Helotiaceae*, it is a worldwide genus that contains more than 150 species, most of them considered saprotrophic decomposers of plant material. Before the emergence of ash dieback none of them had been found to cause plant diseases (Wang *et al.*, 2006). However, some forest pathogens within the *Helotiaceae* are known, e.i. *Gremmeniella abietina*, causes Brunchorstia disease in conifers, *Cenangium ferruginosum* causes Cenangium dieback of pines and *Crumenulopsis sororia* causes bark cankers on pines. Important tree pathogens are also found in other helotialean families, e.i. *Lachnellula willkommii* (*Hyaloscyphaceae*), which causes cankers on European larch (*Larix decidua*), and *Phacidium infestans* (*Phacidiaceae*), a snow mould on Swiss stone pine (*Pinus cembra*). In addition, many species within the Helotiales are endophytic and tend to shift to a pathogenic lifestyle if the host is under stress (Sieber, 2007).

KINGDOM	*Fungi*
PHYLUM	*Ascomycota*
SUBPHYLUM	*Pezizomycotina*
CLASS	*Leotiomycetes*
SUBCLASS	*Leotiomycetidae*
ORDER	*Helotiales*
FAMILY	*Helotiaceae*
GENUS	*Hymenoscyphus*

Hymenoscyphus fraxineus (T. Kowalski) Baral, Queloz, and Hosoya (anamorph *Chalara fraxinea* T. Kowalski) forms numerous white-stalked apothecia on decomposing petioles and leaflet veins from the previous year in the leaf litter (Kowalski and Holdenrieder, 2009). It is a discomycetous fungus. The hymenium forms hyaline single-celled ascospores (13–21 × 3.5–5.0 µm). During germination, the ascospores become melanized and single septate in most cases, and appressoria and/or germ tubes are formed. The asexual stage (*C. fraxinea*) is characterized by brown phialides (16–24 × 4–5 µm), which form short cylindrical hyaline single-celled conidia (3.2–4.0 × 2.0–2.5 µm; Kowalski, 2006). The substrate colonized by *H. fraxineus* (leaf debris in the litter, occasionally also small stems) becomes blackened by a conspicuous pseudosclerotial layer on which the apothecia develop during the summer (Gross and Holdenrieder, 2013).

Population studies of *H. fraxineus* in Europe have shown high genotypic diversity (Rytkönen *et al.*, 2011; Kraj *et al.*, 2012) suggesting an outbreeding mating system (Gross *et al*., 2012c) and long-range dispersal via ascospores, and reported low allelic richness and low differentiation among European populations of *H. fraxineus* (Bengtsson *et al.*, 2012). Collectively the different studies conducted throughout Europe suggest no population structure, and that the pathogen must have gone through a strong genetic bottleneck in the European populations (Rytkönen *et al.*, 2011), with the exception of some differentiation between the Polish highland and lowland populations (Kraj *et al.*, 2012). The absence of clonal reproduction together with other features of the

pathosystem *H. fraxineus–F. excelsior*, such as the aerial dispersal over long distances (Timmermann *et al.*, 2011; Gross *et al.*, 2014), the more or less continuous distribution of the host (FRAXIGEN, 2005) and the huge carrying capacity of the host (up to eight different genotypes discovered on a single petiole; Gross *et al.*, 2012b), might have prevented the formation of a distinguished population structure.

The entire life cycle of *H. fraxineus* is completed on *Fraxinus* leaves. Ascospores are produced in the leaf litter by apothecia formed during summer on fallen leaf petioles of the previous year (Queloz *et al.*, 2011; Timmermann *et al*., 2011). Most apothecia are formed on petioles and rachises. The main sporulation period is from June to early September, but, under favourable conditions, sporulation can start earlier and last until October (Kirisits and Cech, 2009; Hietala *et al*., 2013). The wind-dispersed ascospores (Kowalski and Holdenrieder, 2009; Timmermann *et al*., 2011) adhere to the healthy leaf surface via a secreted mucilage, which is more or less hyaline around the spore and conspicuously melanized around appressoria formed on cellophane on ash leaf agar. Ascospores penetrate the ash leaf cuticle via appressoria (Cleary *et al*., 2013) and spread along the leaf veins and rachis developing necrotic lesions in a proximal direction. Occasionally, the pathogen crossed the junction between the petiole and stem, thereby initiating a necrotic lesion on the stem (Kirisits *et al*., 2009). Stem infection is normally a dead-end in the life cycle of the pathogen, because fructification occurs only very rarely on dead stems. The hypothetical life cycle of the pathogen *Hymenoscyphus fraxineus* is represented in the figure 1.

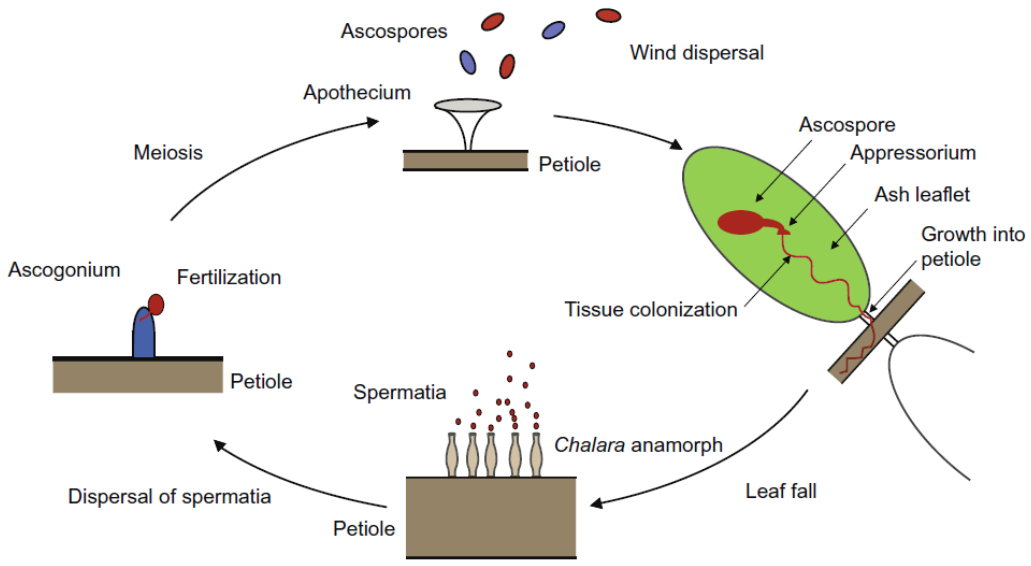

Figure 1. *Schematic representation of the hypothetical life cycle of H. fraxineus: red and blue colors of spores and mycelia represent the two mating types of H. fraxineus.* ***Source****: Gross et al., 2012c.*

Ash petioles in the litter are epidemiologically very important. On this substrate, there *H. fraxineus* develops a characteristic black pseudosclerotial plate on the surface of the petiole and overwinters inside (Kowalski and Holdenrieder, 2009). The asexual stage occurs during the autumn and winter at low temperatures, preferentially near developing pseudosclerotial plates (Kowalski and Bartnik, 2010). Fertilization is mediated through conidia, which acts as spermatia. In the summer of the next vegetation period, new apothecia develop and start a new infection cycle. Because the conidia of the ash dieback fungus did not germinate in laboratory experiments, it has been suggested that they do not play a role in the infection process and that the ascospores are the primary source initiating host infections (Kirisits and Cech, 2009) and responsible

for the rapid recent spread of *H. fraxineus* in Europe. As is established from disease monitoring programs, the disease front can move up to 75 km per year (Gross *et al.*, 2014), implicating a high migration potential. Moreover, seeds of *Fraxinus* may contribute to dispersal: in 8.3% of the investigated seeds DNA of *H. fraxineus* could be detected (Cleary *et al.*, 2012).

The first visible symptoms on the ash tree can be described as brown to blackish necrotic lesions on leaf petioles, rachises and leaflet veins. These characteristics are followed by wilting of leaves distal to the necrotic lesions and premature leaf shedding (Bakys *et al.*, 2009a). Necroses of leaves, buds, leaf stalks and bark are formed; top and shoot dieback, and cankers on shoots, branches and stems can also be observed (Jankovsky and Holdenrieder, 2009). Necrotic lesions usually start to grow in the bark at the junction with a diseased twig or around a leaf wound. The expansion of the lesions produces a girdle in the cambium and phloem (Kirisits *et al.*, 2009). Bark necroses may be temporarily suppressed by host reactions. Kirisits *et al.* (2009) states that phloem girdling and sapwood obstruction occurring outside the vegetative period prevent shoots from flushing in spring and lead to dieback. A brownish discoloration is frequently associated with axial direction of the xylem infection that spreads preferentially within the central tissues (Gross *et al.*, 2014). Long-term severely affected trees may recover their crowns by the formation of epicormic shoots (Kowalski and Holdenrieder, 2008).

The pathogen can also colonize parts of the root system (Kowalski and Łukomska, 2005; Schumacher *et al*., 2010). In addition, the roots of severely infected trees are susceptible to be attacked by opportunistic fungi (e.g. *Armillaria* spp.), which enhance the death of the tree (Bakys *et al*., 2011; Husson *et al*., 2012). On young individuals, the disease frequently leads to tree death within a few years after infection. Whereas on older trees, ash dieback often becomes chronic, resulting in their weakening and predisposing them to other damaging factors. Stand mortality of up to several hectares has been recorded (Zachara *et al.*, 2007).

Trees of various ages in natural, commercial and ornamental situations are affected by this disease (Kowalski and Łukomska, 2005). However, young trees, dense stands and water deficiency have been shown to promote the development of the disease (Cech and Hoyer-Tomiczek, 2007). Different *Fraxinus* species have been shown to develop disease symptoms: the European *Fraxinus excelsior* (Kowalski, 2006) and *F. angustifolia* (Kirisits *et al.*, 2010); the North American *F. nigra, F. pennsylvanica* and *F. Americana*; and the Asian *F. mandshurica* (Drenkhan and Hanso, 2010) and *F. sogdiana* (Drenkhan *et al.*, 2015). *Fraxinus nigra*, *F. excelsior* and *F. angustifolia* are the most susceptible species while for the introduced *F. pennsylvanica*, field observations of natural infections in several European countries indicated that this is less susceptible to ash dieback (Drenkhan and Hanso, 2010). This was corroborated by stem inoculations in a phytotron chamber (Gross and Sieber, 2016). The flowering ash, *Fraxinus ornus*, apparently does not develop the disease under field conditions (Gross *et al.*, 2014). The original hosts of *H. fraxineus* seem to be *Fraxinus mandshurica* and *Fraxinus chinensis*, hosts reported from Asia. The fungus is not considered a pathogen in Japan (Zhao *et al.*, 2013).

2.3. GENERAL FEATURES OF THE MAIN HOST SPECIES *Fraxinus excelsior* L.

The family Oleaceae, pertaining to the order Lamiales, is composed of 24 extant genera that comprise about 600 species (Wallander and Albert, 2000). They occur in all continents except the Antarctic, from northern temperate to southern subtropical regions and from low to high elevations. Well-known genera in this family are *Jasminum, Forsythia*, *Ligustrum*, *Syringa*, *Olea* and *Fraxinus*. The members of the family are trees, shrubs, or woody climbers with opposite, simple, or compound leaves.

Fraxinus comprises 48 tree and shrub species indigenous to the temperate and subtropical regions of the Northern Hemisphere. The genus comprises species from America (i.e. *F. nigra*), Europe and East Asia (i.e. *F. mandshurica*). In Europe, *F. excelsior* L. is the most widespread native species, while *F. angustifolia* Vahl and *F. ornus* L. thrive in Southern Europe. *Fraxinus angustifolia*, narrowed-leaved ash, is a medium-sized tree with a wide range, which covers central-southern Europe and northwest Africa, up to the Caucasus. The northern part of its distribution overlaps with that of common ash (*Fraxinus excelsior*), with which it can naturally hybridise (Fernandez-Manjarres *et al.*, 2006; Heuertz *et al.*, 2006).

F. excelsior is one of the most important tree species in Europe (Dobrowolska *et al.*, 2008). It is the second or third most frequent deciduous tree species after European beech (*Fagus sylvatica*) in central European countries (Kirisits and Cech, 2009). Common ash is a medium-sized deciduous tree, usually growing to 20-35 m and only occasionally reaching 45 m (Ellenberg, 1988). The crown is domed and open with ascending branches. The trees develop a smooth, pale grey bark that thickens and develops fissures with age. Its leaves are compound, with 9-13 leaflets, odd pinnate, serrated, and stalkless. The individual leaflets measure 3-12 cm by 0.8-3 cm, composing leaves of 20-35 cm. The flowers open before the leaves unfold, which occurs relatively late in spring compared with other trees. The flowers develop in bunches of 100 to 400, without petals, exposing the pale green styles and filaments and the dark purple stigmas and anthers. This ash species is termed as polygamous, because plants can develop only male or female flowers, or unisexual inflorescences with only male and female flowers carried separately, or even hermaphrodite flowers. Ash pollen as well as ash seeds are dispersed by wind (Dobrowolska *et al.*, 2011). The seeds ripen individually in oval-shaped samaras, flattened, 2-5 cm long, that by the end of summer hang in bunches from the branches. Seeds usually lie dormant for two years before germinating.

Common ash is naturally found throughout the European temperate zone, from the Atlantic coast to the Volga River (figure 2). It is absent from the centre and South of the Iberian Peninsula, south of Italian and Balkan peninsulas, northern Fennoscandia and Iceland. Its regional distribution limits appear to be set by the energy requirements to complete its annual life cycle in the north, minimum temperatures in the east, and moisture availability in the South and South-East (Dobrowolska *et al.*, 2011).

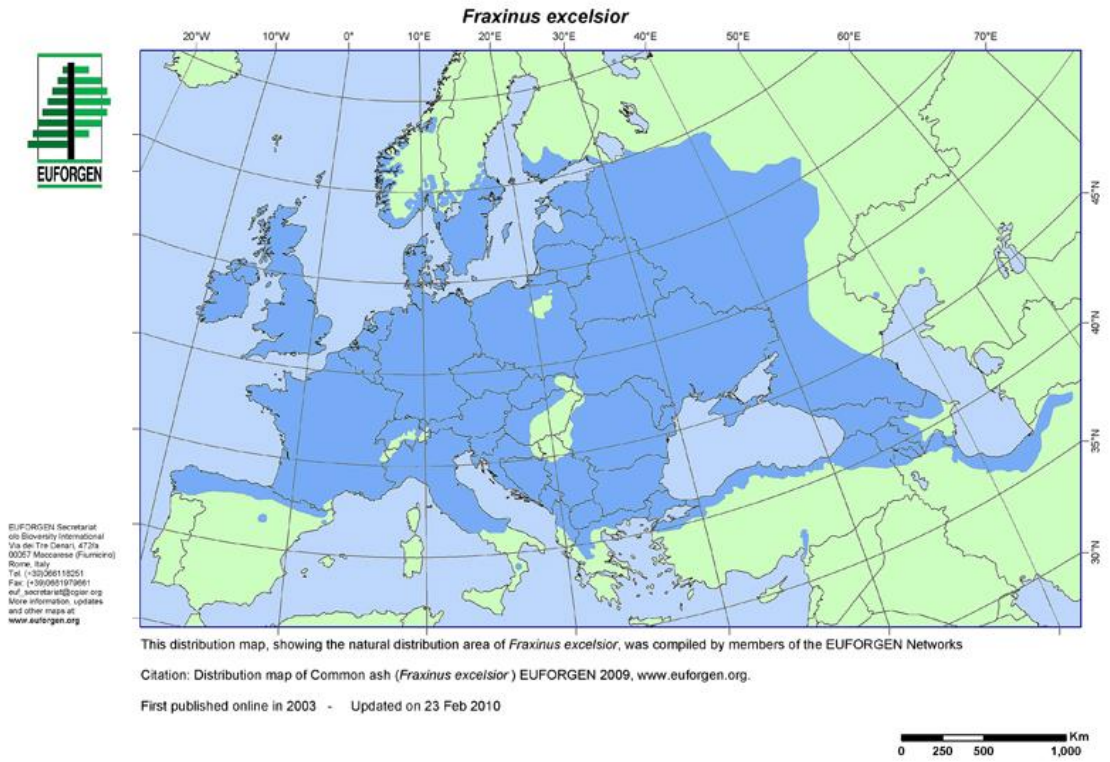

Figure 2. *Distribution map that shows the natural distribution area of Common ash (Fraxinus excelsior).* ***Source****: EUFORGEN, 2009.*

F. excelsior can grow in pure stands, but it is a typical tree species of mixed forests (Dobrowolska *et al.*, 2008). Soils which are fertile, pH-neutral, deep, moist and freely draining create optimal conditions for the growth of ash. Only optimal site types provide the fast growth rates which are needed to produce high quality ash timber (Dobrowolska *et al.*, 2008). Currently, in Czech Republic, there are differentiated alluvial, mountain and lime ecotypes (Střeštík and Šamonil, 2006).

Ash requires shadow in younger states but then, it becomes increasingly light demanding with increased age (Dobrowolska *et al.*, 2011). Ash is sensitive to climatic fluctuations, such as extreme high temperatures or severe winter and late spring frosts. Under harsh conditions of snow and temperature its stems may break. Young trees are susceptible to late spring frost which may harm the foliage (Dobrowolska *et al.*, 2008).

Like many other valuable broadleaved species, ash is widely used in European forestry and in the timber industry. Its timber is in high demand because of its physical properties (elastic, hard, resistant to pressure), which make it very valuable for the production of furniture, veneer, flooring, tool handles, and sports equipment, such as hockey sticks, oars and hurdles (Pliûra and Heuertz, 2003). In central Europe it has been widely used as an ornamental tree along roads and city streets.

2.4. DETECTION AND QUANTIFICATION OF AIRBORNE INOCULUM USING REAL-TIME PCR ASSAYS

Apparently, airborne ascospores are the first and only source of infection on healthy leaves (Kowalski and Holenrieder, 2009; Timmermann *et al.*, 2011). Knowledge on the spore content of the air is important to research into the epidemiology of plant diseases. The methods for monitoring airborne inoculum involve two steps: spore sampling and spore detection. A wide variety of methods is available for collecting bioaerosol samples. However, samplers such as Hirst-type spore traps (Hirst, 1952) and rotating-arm samplers (Perkins, 1957), that collect particles by impaction onto surfaces are used extensively in aerobiological studies (figure 3), particularly for spore and pollen sampling and in plant disease epidemiology (McCartney *et al.,* 1997). The seven-day automatic volumetric spore trap is often used for the continuous sampling to follow ascospore occurrence. This type of spore trap is a suction-type trap which works through a small orifice. The use of this type of traps increases the efficiency of 'impaction' by artificially accelerating a narrow air stream towards a sticky surface. Movement of the sticky surface at a constant rate past the orifice through which this air-stream flows, producing a trace showing changes of atmospheric spore content with time. Comparison of this trace with recording meteorological instruments would greatly increase the accuracy of its interpretation (Hirst, 1952; Dhingra and Sinclair, 1985).

Figure 3. *Left: installation of two rotating arm spore traps (centre) and Hirst-type spore trap (left) in SE Czech Republic (Dvořák et al., 2016). Right: detail of the rotating-arm spore trap (Chandelier et al., 2014), that is made of two square-section rods covered with double-sided tape.*

The seven-day automatic volumetric spore trap has been constructed in which a sticky 336 × 40 mm slide is moved at 2 mm·h^{-1} past an orifice 14 × 2 mm, to which suction at the rate of 10 L·min^{-1} is applied. A continuous trace of the day's spores is deposited on an area 48 × 14 mm. A wind-vane on the mounting directs the orifice into the wind. The sticky surface must permit a high efficiency of impaction; this requires a soft, almost wet surface, which is not hygroscopic or subject to dry during the period of exposure. It must have good optical properties, allowing the swelling of shrunken spores, so they can be identified. Of the substances, Vaseline seems to be the best one (Hirst, 1952). Glycerine jelly was rejected because it is very hygroscopic and not easy to mount without some movement of the spores. Silicone grease gives high impaction efficiency and has good optical properties, but the spores are rapidly wetted by the grease and any shrinkage of the spores is irreversible (Hirst, 1952).

Real-time polymerase chain reaction (qPCR) method of DNA amplification is one of the most sensitive and reliably technique with broad applications in fields such as spore detection by molecular biology. A qPCR has three phases, exponential phase, linear phase and plateau phase as shown in figure 4. The exponential phase is the earliest segment in the PCR, in which product increases exponentially since the reagents are not limited. The linear phase is characterized by a linear increase in product as PCR reagents become limited. The PCR will eventually reach the plateau phase during later cycles and the amount of product will not change because some reagents become depleted. Real-time PCR exploits the fact that the quantity of PCR products in exponential phase is in proportion to the quantity of initial template under ideal conditions.

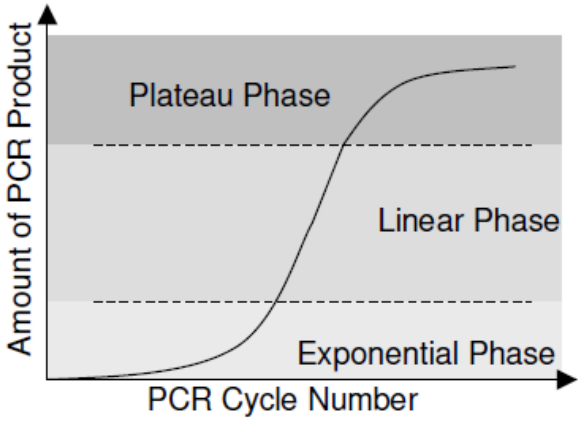

Figure 4. *Theoretical plot of PCR cycle number against PCR product amount is depicted. Three phases can be observed for PCRs: exponential phase, linear phase and plateau phase.* ***Source:*** *Yuan et al., 2006.*

Mixed with DNA template, the major reaction components involved in real-time PCR experiments are dNTPs, at least one pair of specific primers (strands of short nucleic acid sequences that serves as a starting point for DNA synthesis), a suitable buffer solution, a thermo-stable DNA polymerase (the enzyme that synthesize DNA molecules) and water. Quantification of amplified product is obtained using fluorescent probes or fluorescent DNA-binding dyes and real-time PCR instruments that measure fluorescence while performing the thermal cycling needed for the PCR reaction. Once the device starts to run, denaturation begins. In this step the separation of the double-stranded DNA into single strands occurs at high temperature. Subsequently, during the annealing step of the qPCR, both fluorescent probe and primers anneal to the DNA target. Finally, the enzyme polymerase begins to amplify in a stage called extension. The series is repeated in each cycle.

Amplification products are measured as they are produced using the fluorescent label. During amplification, a fluorescent dye binds via a labeled hybridizing probe, to the accumulating DNA molecules, and fluorescence values are recorded during each cycle of the amplification process. Since PCR is a geometric amplification, ideally doubling every cycle, a linear plot of the data should show a classic exponential amplification, as described before (figure 4). During a typical qPCR experiment, the initial concentration of template is extremely low; therefore the resulting product-related fluorescence is too low to be detected. After the yield has reached the detection threshold, the reaction course can be followed through the exponential phase. Using a standard curve for the

target of interest, copy number values can be determined for any unknown sample. Coupled with volumetric air sampling, the specificity, sensitivity and high-throughput nature of real-time PCR assays should make the employed approach optimal for profiling the occurrence of air-borne fungi (Hietala *et al.*, 2013).

3. OBJETIVES

The main aim of this Master Thesis is to shed light on the biology of the invasive fungal species causing the ash dieback, *Hymenoscyphus fraxineus*.

In order to achieve this aim, the following partial objectives are proposed:

1.- To describe the seasonal pattern of *H. fraxineus* airborne inoculum dispersal in a forest stand infected by ash dieback (*Hymenoscyphus fraxineus*) in the South West of the Czech Republic.

2.- To determine the influence of meteorological conditions on the biology of *Hymenoscyphus fraxineus.*

4. MATERIALS AND METHODS

4.1. SAMPLING LOCALITY

The experiment was carried out in the Novohradské Mountains that belongs to Český Krumlov District located in Southern Bohemia (South West of the Czech Republic; figure 5), with the Austrian border nearby. The coordinates of the experiment location are showed in table 1. The forest stand is called "Jelení Hora" and is located in a mixed broadleaved forest composed by spruce (*Picea abies*) and beech (*Fagus sylvatica*), where there is a small group of *ca.* 60 years old European ashes (*Fraxinus excelsior*) admixed (figure 6). The typology of the stand is *Piceeto-Fagetum fraxinosum humidum*, according to the Czech forest ecosystem classification. The whole group of ashes showed more or less moderate infection of ash dieback (*Hymenoscyphus fraxineus*; figure 7, 8 and 9).

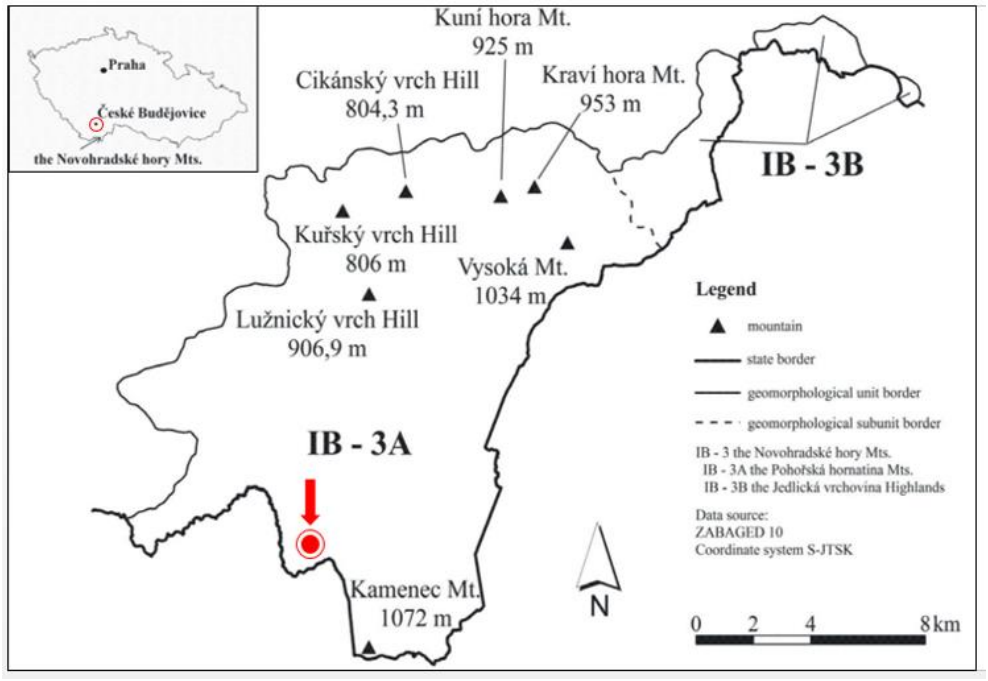

Figure 5. *The location map of the Novohradské hory Mts. within the Czech Republic and their basic geomorphological division.*

Table 1. *Forest stand GPS coordinates of the experiment.*

COORDINATES	
LATITUDE	48° 36' 38.935'' N
LONGITUDE	14° 38' 11.528'' E
ALTITUDE	883 meters above sea level

Figure 6. *Mixed broadleaved forest in Jelení Hora (Czech Republic) composed by spruce (Picea abies) with beech (Fagus sylvatica) and a small group of European ashes (Fraxinus excelsior), where the assay was done.*

Figure 7. *The group of ashes in the assay location (Jelení Hora, Czech Republic) showed more or less moderate infection of ash dieback (Hymenoscyphus fraxineus).*

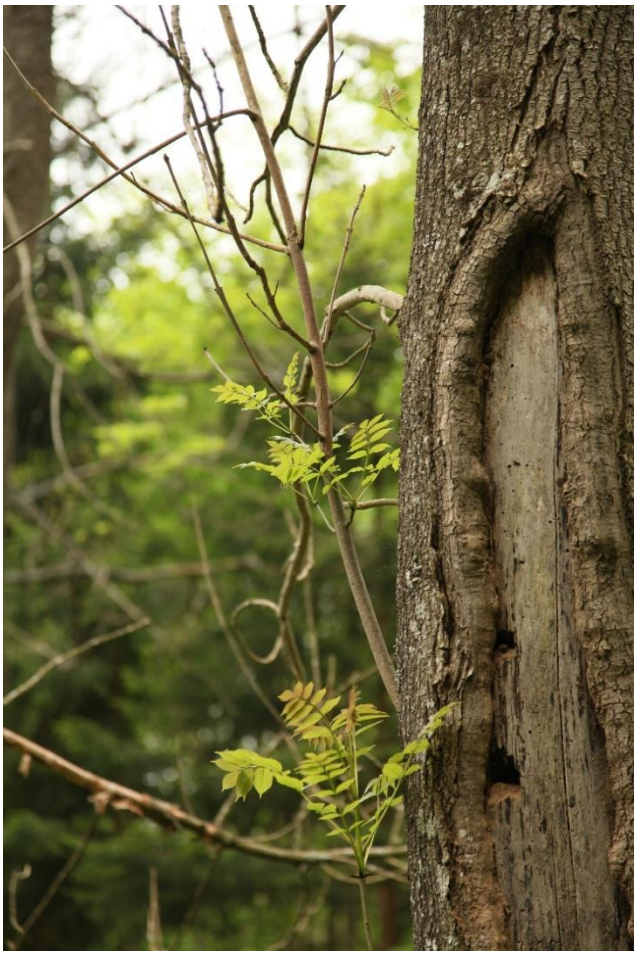

Figure 8. *Brown necrotic lesions on leaves of European ash (Fraxinus excelsior) in Jelení Hora (Czech Republic).*

Figure 9. *Ash leaf litter on the ground with numerous white-stalked apothecia of Hymenoscyphus fraxineus in Jelení Hora (Czech Republic).*

In order to estimate the influence of meteorological conditions on the ocurrence of the inoculum of *Hymenoscyphus fraxineus*, an automatic weather station Signalizátor (Amet, Velké Bílovice, Czech Republic) was used. Air temperature, relative humidity, and leaf wetness were measured every hour through the whole air-sampling season. A weather station was placed on the ground to measure the microclimatic conditions of the source of inoculum—ash leaf litter, which was checked weakly for presence/absence of apothecia of *Hymenoscyphus fraxineus* (figure 10).

Figure 10. *Automatic weather station Signalizátor (Amet, Velké Bílovice, Czech Republic) placed in the assay location.*

4.2. AIR SAMPLERS

The technique for measuring the occurrence of the inoculum of *Hymenoscyphus fraxineus* in our assay was the spore capture by the seven-day automatic volumetric spore trap (SAVST) made by AMET (Velké Bílovice, Czech Republic; figure 11) which is based on the description of Hirst, 1952. This trap allowed a continuous sampling to follow ascospore occurrence in real-time. It consists essentially of three units: the impactor unit with an adhesive-coated transparent tape placed on a drum rotating once a week, the wind-vane mounting and the motor housing. A vane tail keeps the cylindrical housing facing the wind. The motor housing contains a vacuum pump.

Figure 11. *Seven-day automatic volumetric spore trap (SAVST) made by AMET (Velké Bílovice, Czech Republic).*

Figure 12. *The seven-day automatic volumetric spore trap was placed under the group of moderated-infected ashes in Jelení Hora (Czech Republic), with its orifice 30 cm above soil surface.*

In the weekly sampler, the Melinex tape is mounted on the drum. The tape is 336 mm long and corresponds with the 7 days of the week. Once the Melinex tape is on the drum, the petroleum jelly must be applied by slightly and continuously turning the drum without stopping, ensuring that a very fine and uniform layer is obtained (Carvalho *et al.*, 2008). This substance works as a trapping medium. The operating mode of the spore trap consists in fixing the revolving drum in the drum carrier, facing an orifice through which 10 L of sampled air were sucked every minute. This device was installed to sample the air inoculum continuously from 15 May to 30 October 2015 and was placed under the group of moderated-infected ashes having its orifice 30 cm above soil surface (figure 12).

In order to get our samples, the drum was removed from the drum carrier by holding the top and avoiding the contact with the surface of the Melinex tape. Subsequently, the tape was extracted from the drum and cut in half lengthwise. We remained one of the halves for our assay. As the drum rotates at a 2 mm per hour, and the Melinex tape was 336 mm long, seven 48 mm pieces (each one corresponds to one day) were cut with the aid of a transparent ruler in the laboratory. The processing of the samples is described in the figure 13. Each segment was cut into two equal sections, one of them corresponding to the first part of the day (0:00 am - 12:00 pm) and the other one to the second part (12:00 pm – 23:59 pm). The two sections were stored in 2 mL microtubes at -20ºC before further processing. Therefore, from each day we had two microtubes called A and B (for example: 15.09.15A and 15.09.15B).

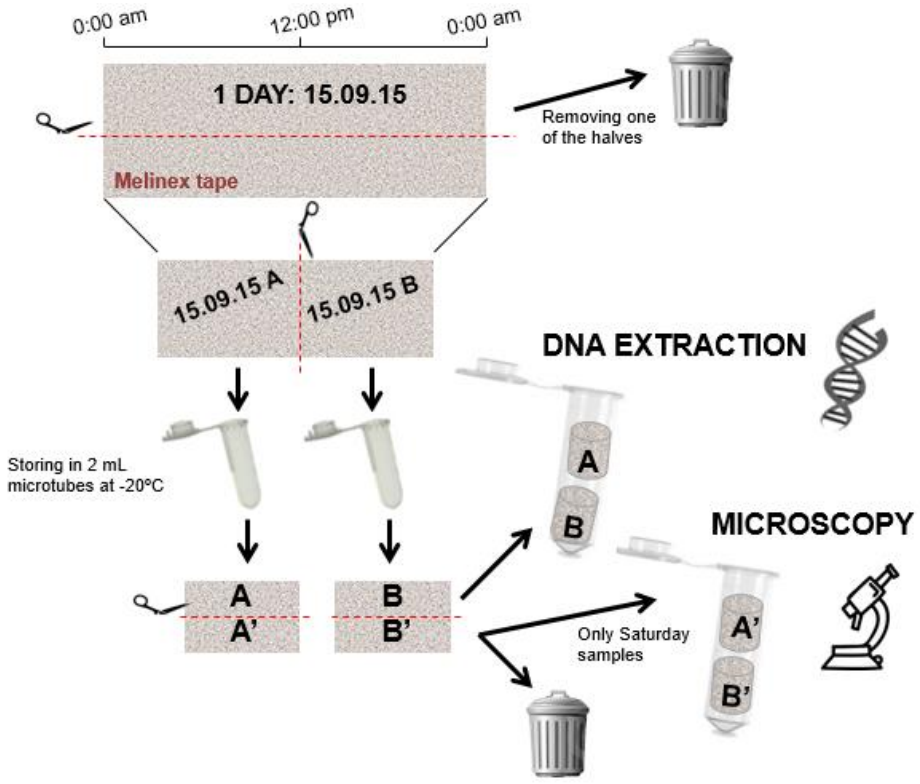

Figure 13. *Schema of the processing of the samples and final destination.*

For the part of the DNA extraction, we used from each day only one half of the segment A and another half of the B, removing the rest except those days which we were going to use it in the microscope part. Therefore, in the final microtube of the sample we had the representation of the two parts of the day, both for the DNA extraction as for microscopy technique.

4.3. DNA EXTRACTION

The DNA extraction was carried out in the laboratories of the Department of Forest Protection and Wildlife Management in Mendel University of Brno (Czech Republic). DNA was extracted from all the samples together with an empty microtube as a negative control of extraction. Firstly, the spores' disruption was performed similarly to Hospodsky *et al.* (2010), so 0.4 g of 0.1 mm balotina beads were added to the microtubes with the samples together with 250 µL of 0.1% Nonidet P40. They were disrupted in the Mixer Mill MM400 (Retsch, Haan, Germany) for 10 min by 30 Hz.

For further processing, the protocol for isolation of DNA from plant tissue with the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) was followed. The first step consisted in the lyses of the cells. For this purpose, 400 µL of lysis buffer and 4 µL of RNase A (supplied with the kit) are added in each microtube and, after vortex 5 s, the samples are incubated during 60 min at 65ºC in a heating bath. Then, in order to precipitate detergent, proteins, and polysaccharides, 130 µL of P3 buffer must be added previously incubated for 5 min on ice. The next step consists in centrifuge the lysate for 5 min at full speed. After that, the supernatant of the lysate is poured to the QIAshredder spin column sitting in a 2 mL collection tube and centrifuged for 2 min. QIAshredder removes most precipitates and cell debris. Subsequently, 1.5 volumes of AW1 buffer are added to the cleared lysate, mixed by pipetting and the result is passed to the DNeasy mini spin column sitting in a 2 mL collection tube for DNA binding. Centrifugation for 1 min (discarding the flow-through). Now, we can start the washing phase with 500 µL of AW2 buffer applied twice. In the last step, DNeasy column is transferred to a 1.5 mL microcentrifuge tube and 100 µL of preheated (65°C) elution buffer is pipetted. Once incubated for 10 min at room temperature, the DNA extraction must be centrifuged for 1 min to eluate. Therefore, in the end of this process, 100 µL of DNA will have been achieved for the quantification by real-time PCR.

4.4. qPCR CONDITIONS

Extracted DNA from the samples was quantified by qPCR. Direct specific qPCR was performed using a PCRmax Eco48 which amplifies DNA exponentially, doubling the number of target molecules with each amplification cycle. The number of cycles and the amount of PCR end-product is used to calculate the initial quantity of genetic material by comparison with a known standard. In our case, the fluorescent reporter used was a probe that hybridizes with PCR product during amplification.

The qPCR analysis was carried out with the dual-labeled fluorogenic TaqMan probe, TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and species-specific primers. Probe and primers were designed and their specificity was proved by Chandelier *et al.* (2010) for *Hymenoscyphus fraxineus.* Their nucleotide sequences and names are mentioned in table 2. The procedure was followed according

to the manufacturer's instructions. Composition of the reaction mixture was following: 0.4 µL of each primer (final concentration 400 nM), 0.2 µL of TaqMan probe (200 nM), 5 µL of TaqMan Universal PCR Master Mix, 1.1 µL of sterile deionized water and 3 µL of template DNA. Every reaction was performed in two repetitions together with a positive control, a negative control from the DNA extraction and a negative control containing the master mix without template DNA (NTC) (figure 14).

Each cycle of the amplification process was programmed. The pre-incubation previous to the cycles lasted 10 min at 95ºC. After that, it started 45 cycles of 15 s at 95ºC followed by 1 min at 60ºC (figure 15).

Table 2. *Specific primers and probe used for the real-time PCR for the amplification to Hymenoscyphus fraxineus.*

Nucleotide designation	Sequence (5' - 3')	Source
Cf-F	CCCTTGTGTATATTATATTGTTGCTTTAGC	Chandelier *et al.* (2010)
Cf-R	GGGTCCTCTAGCAGGCACAGT	Chandelier *et al.* (2010)
Cf-S	6FAM-TCTGGGCGTCGGCCTCGG-BHQ1	Chandelier *et al.* (2010)

Figure 14. *Plate layout from one of the amplification process by PCRmax Eco48 (well designation: U: Unknown; +: positive control; -: negative control from DNA extraction; N: NTC). EcoStudy Software v5.0.*

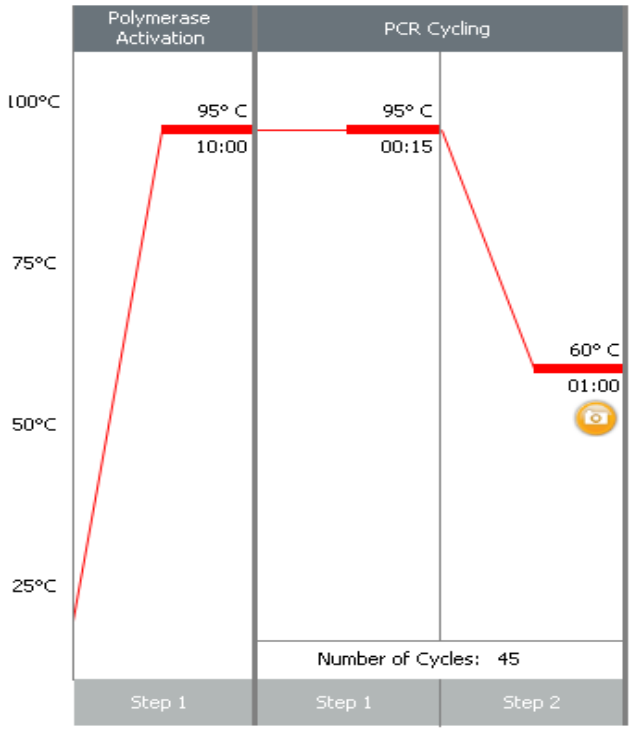

Figure 15. *Thermal profile for each amplification process by PCRmax Eco48.*

4.5. ABSOLUTE QUANTIFICATION

The method that uses a dual-labeled fluorogenic probe (TaqMan Probe) is based on the application of the 5' nuclease assay first described by Holland *et al.* (1991), which uses fluorescent Taqman methodology which requires a hybridization probe labeled with two different fluorescent dyes (Lee *et al.*, 1993). One dye is a reporter dye [FAM (i.e., 6-carboxyfluorescein)], the other is a quenching dye [TAMRA (i.e., 6-carboxy-tetramethylrhodamine)]. When the probe is intact, fluorescent energy transfer occurs and the reporter dye fluorescent emission is absorbed by the quenching dye TAMRA. During the extension phase of the PCR cycle, the fluorescent hybridization probe is cleaved by the 5'-3' nucleolytic activity of the DNA polymerase. On cleavage of the probe, the reporter dye emission is no longer transferred efficiently to the quenching dye, resulting in an increase in peak fluorescent emission spectra, becoming detectable. The instrument uses a charge-coupled device for measuring this spectrum. The fluorescence signal is directly proportional to DNA concentration over a broad range, and the linear correlation between PCR end-product and fluorescence intensity is used to calculate the amount of template present at the beginning of the reaction. In the case of our study, using a standard curve performed by PCN assay, copy number values could be determined for the entire samples in real-time.

A plasmid copy number (PCN) assay is a widely used method in quantitative genetic stability analysis (Fujimura *et al.*, 1996). This method consists in create a standard curve generated by reactions with different amounts of plasmid with species-specific inserts (from the target DNA) using the qPCR method. Absolute quantification determines the exact copy concentration of target DNA by relating the CT value to the standard curve generated with the plasmids.

The concentration 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 numbers of copies). These numbers of copies were quantified using a PCN with plasmid pCR™2.1-TOPOr TA vector (Invitrogen, Carlsbad, CA, USA). Plasmids contained species-specific inserts (PCR products amplified with primers described in table 2). DNA was extracted from pure cultures of *H. fraxineus* (collection of Mendel University in Brno).

A 10-fold serial dilution series of the plasmid, ranging from 1×10^4 to 1×10^8 copies/µL (figure 16), was used to construct the standard curve (figure 17). The qPCR conditions were equal to the natural samples, described in the previous paragraph. Each level of dilution was performed in three technical repetitions together with a positive control, a negative control from the DNA extraction and a negative control containing the master mix without template DNA (NTC).

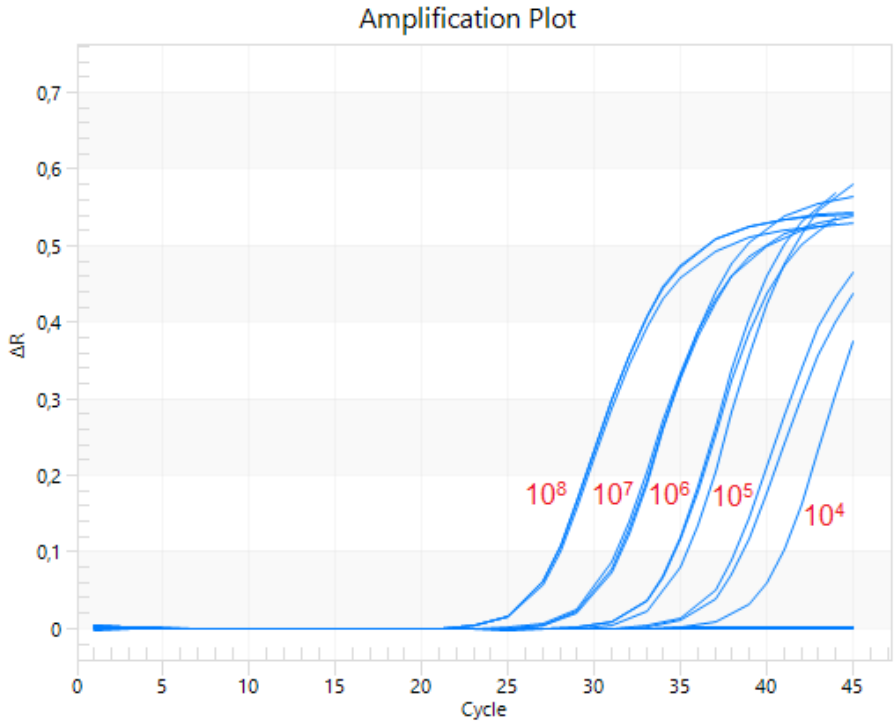

Figure 16. *Amplification plot of the 10-fold serial dilution of the plasmid, which shows the different levels of amplification of the series. EcoStudy Software v5.0.*

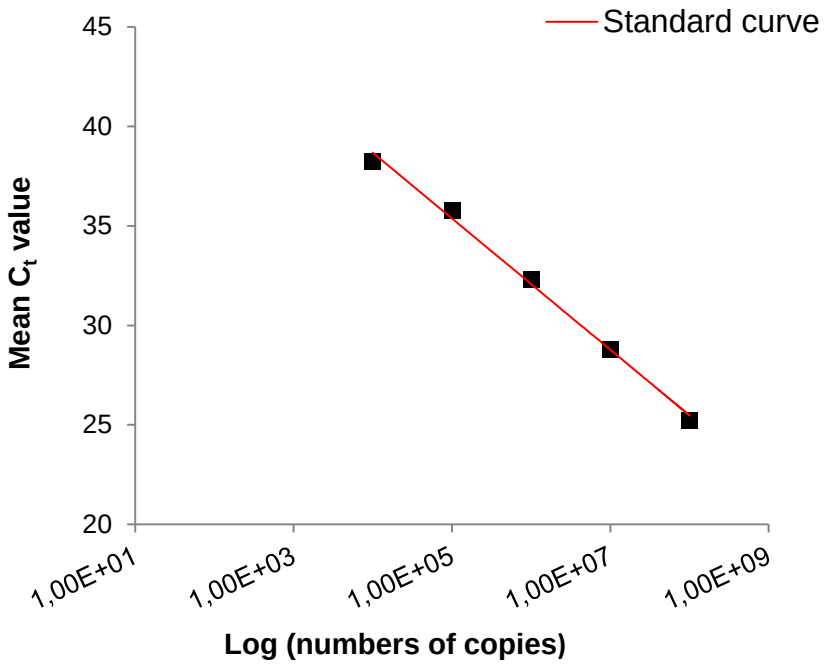

Figure 17. *Standard curve of the mean cycle threshold (Ct) values versus the log 10-fold serial dilution of the plasmid (mean Ct values calculated from three series of data).*

The DNA concentration standard curve for *H. fraxineus*, based on the relationship of the log 10-fold serial dilution (x) of the plasmid and their corresponding Ct values (y), were y = -1,434·ln(x) + 51,882 (R^2 = 0.9961).

4.6. MICROSCOPY ANALYSIS

Simultaneously with the molecular technique, optical microscopy technique under visible light was performed. This technique consists in direct microscopic observation of the adhesive tape used in the spore trap. Microscopic technique allowed the morphological identification of the spores of *Hymenoscyphus fraxineus*.

The samples were collected on Saturdays within the period of the study and their processing is described in the paragraph 3.2 and figure 13. Each fragment of Melinex tape (two per day) was attached to a microscope slide with a drop of adhesive mixture and a cover glass was mounted. The slides were analyzed at 40x magnification along one horizontal line of 24 mm (=12h) using a light microscope (figure 18). The number of ascospores of *H. fraxineus* (fusiforme-elliptical, broadly rounded apex, narrow base and presence of large oil globules: Kowalski and Holdenrieder, 2009) (figure 19) was then recorded. In order to estimate the number of ascospores of the whole fragment, a simple formula was used:

$$Number\ of\ ascospores = \left(\frac{Sample\ area}{Analyzed\ area}\right) \cdot N$$

where N is the number of ascospores recorded in the analyzed area. Two N number was obtained for each day, thus these two numbers should be summed.

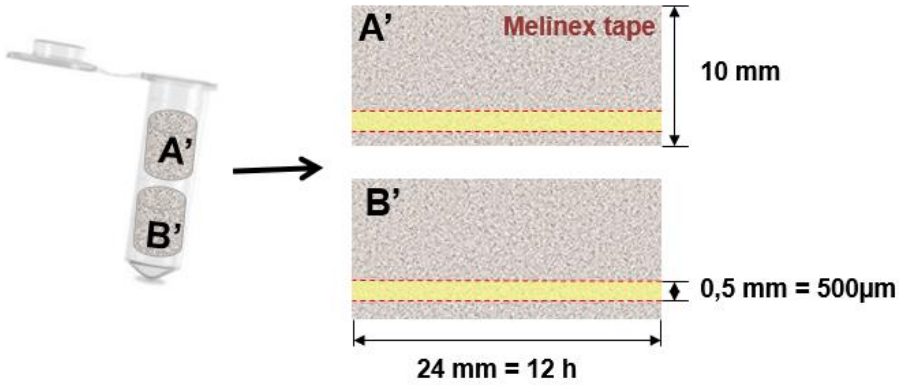

Figure 18. *Each fragment of Melinex tape (two per day) was analyzed along one horizontal line of 24 mm (=12h) using a light microscope.*

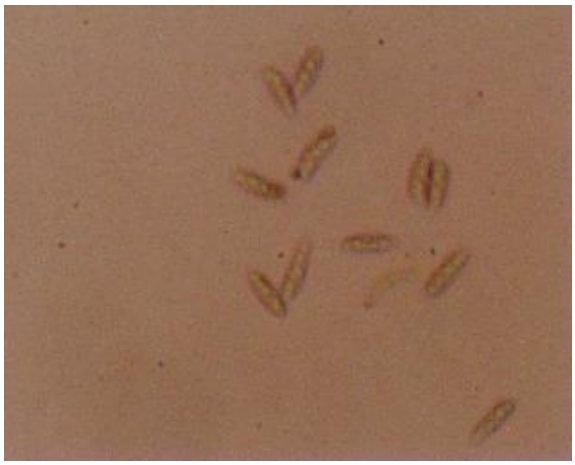

Figure 19. *Hymenoscyphus fraxineus ascospores through light microscope.*

4.7. STATISTICAL ANALYSIS

For the purpose of determining the influence of meteorological conditions on the biology of *Hymenoscyphus fraxineus*, a statistical analysis was carried out. A high number of zeros in the periods of less occurrence of the pathogen, mainly at the beginning and at the end of the season, took place. The most likely reason could be the level of precision of the qPCR device. Actually, zero values could be values between 0 and the detection threshold (around 10^3 copies·μL^{-1}). Hence, these data were excluded from statistical analyses, working on the period from 27 July to 17 September, with samplings each 10 days. The parameters selected for the meteorological analysis were mean temperature, mean relative humidity and mean leaf wetness. In addition, minimal relative humidity and minimal leaf wetness were included in the analysis since these meteorological variables

were the most influential in the production of air-inoculum in the previous study done by Dvořák *et al.* (2016).

A three-step approach was used to evaluate the relative influence of quantity of copies in 1 µL of template DNA and meteorological conditions (estimated as a set of variables: mean temperature, mean relative humidity, minimal relative humidity, mean leaf wetness and minimal leaf wetness). First, principal component analysis (PCAs) was applied to the quantity of copies in 1 µL of template DNA to avoid the use of highly correlated variables in subsequent steps. This PCA was carried out in R (R Core Team, 2014), with the ‘‘Vegan’’ package (Oksanen *et al.*, 2015). Second, the most closely correlated variable of each of the retained axes were selected. Significant axes were also selected by applying the broken-stick method with the ‘‘BiodiversityR’’ package (Kindt, 2014). The PCA axes with higher percentages of variance than broken-stick variances are significant. Third, a linear regression was performed using the variable selected in the previous step. In addition, the normality hypothesis of the regression model was tested by Shapiro-Wilk normality test and the homoscedasticity by the Breusch-Pagan test using the “Car” package (Fox and Weisberg, 2011).

5. RESULTS

DNA of the target fungi was detected in the samples from the beginning of the experiment (May 16) until almost the end (29 October) (figure 20). The amounts of numbers of copies of the target sequence were ranging between 0 and 18,204,975.5 per 1 µL of template DNA of air sampled by SAVST. The period of permanent occurrence of air-inoculum can be pointed from mid- July until mid- September with extremely variable values (e.g. 15 August, 20.118,6 and 22 August, 18.204.975,5 copies per 1 µL of air sampled by SAVST). The long-term occurrence of air-inoculum was also observed during other months. From the beginning of the study to early July (9 July) the values remain between 0 and $4{,}5 \times 10^4$, with a large number of zeros which could be due to the level of detection of the qPCR device (no less than 10^3 copies/µL). Following the sample period, it start to show values higher than 10^4, until reaching numbers of copies upper to 10^7 in the 31 of July and 10 and 22 of August. The values decrease after mid- September. In this last period there are again a large number of zeros and the number of copies don't exceed 5×10^4, except a little upturn of *ca.* 7,1 and $8{,}2 \times 10^4$ in 23 and 25 of October respectively.

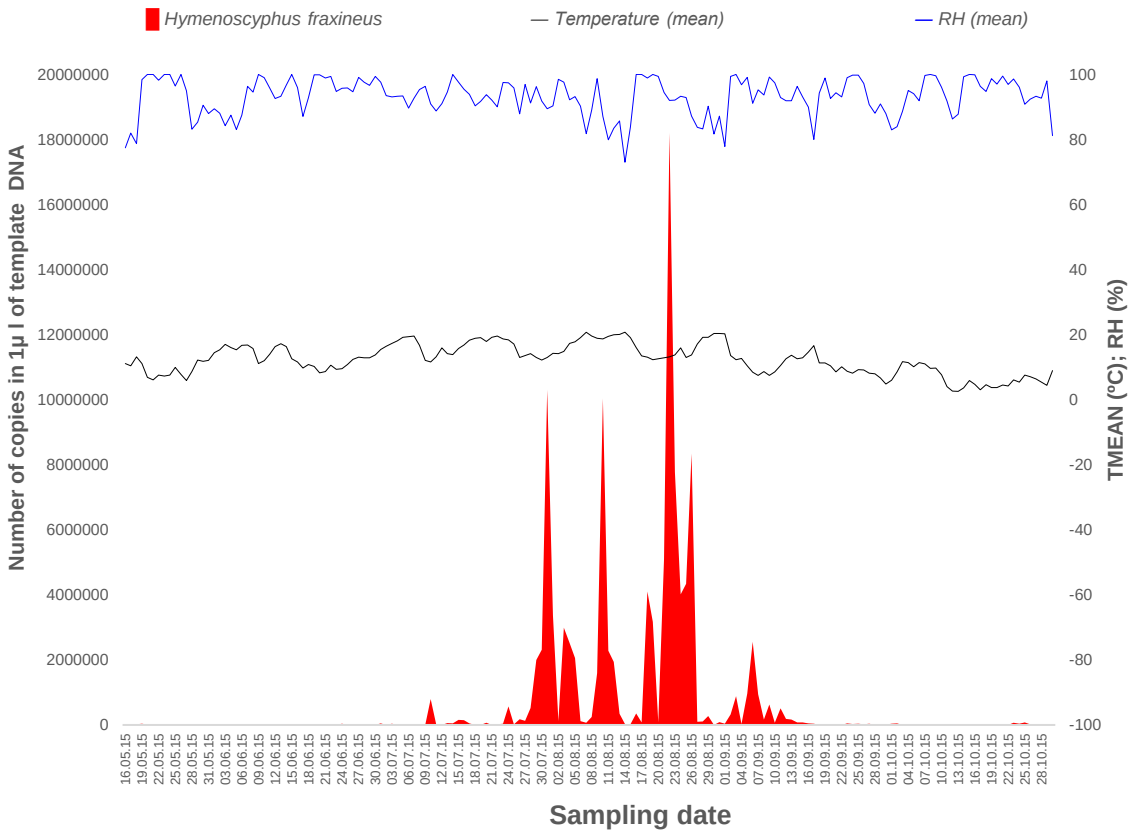

Figure 20. *Seasonal air-inoculum dispersal pattern of H. fraxineus (numbers of copies of the target sequence in 1 µL of template DNA in particular days of sampling by SAVST), accompanied by curve of temperature mean (TMEAN ºC) and air relative humidity mean (RH %). The graphic represent the whole period of study.*

The technique of microscopic observation of the Saturday samples confirms the presence of *H. fraxineus* ascospores during the period of study. The number of ascospores identificated in each sample is showed in the table 3. The higher values appear in early September.

Table 3. *Number of Hymenoscyphus fraxineus ascospores detected by microscopy in each sample date of the period of the study.*

Sample date (Saturdays)	Number of ascospores detected
16.05.15	180
23.05.15	160
30.05.15	400
06.06.15	160
13.06.15	360
21.06.15	200
27.06.15	180
04.07.15	280
11.07.15	100
18.07.15	160
25.07.15	200
01.08.15	400
08.08.15	100
15.08.15	560
22.08.15	240
29.08.15	540
05.09.15	920
12.09.15	620
19.09.15	320
26.09.15	140
03.10.15	20
10.10.15	240
17.10.15	40
24.10.15	60

As explained in material and methods part, we focused our statistical analysis in the period of highest and most sustainable amount of copies in order to avoid the large oscillations in the number of copies. This period was determined from 27 July to 17 September. Principal component analysis (PCA) was performed to select the variables for use in the linear regression. In particular, the broken-stick method only retained the first axe in the PCA for meteorological variables (percentage of variance, *ca.* 56%) (table 4). The variable selected from the axe retained in the PCA was minimal relative humidity, being the most influential (table 5). This RHmin variable show a clear inverse correlation with the number of copies in 1 µL of template DNA (cor = -0.8926).

Table 4. *Selection of significant axes from principal component analyses by the broken-stick method. Note: PCA axes with larger percentages of variance than broken-stick variances are significant.*

	Axes			
	1	2	3	4
Meteorological variables				
Eigenvalue	2,797	1,298	0,788	0,117
Percentage of variance	**55,942**	25,961	15,766	2,331
Broken-stick percentage	**52,083**	27,083	14,583	6,25

Table 5. *Coefficients of correlation between meteorological variables and the significant axe of the principal component analysis. Note: The highest correlation is shown in bold type.*

	PC1
Mean temperature	0,6946
Mean HR	-0,8219
Minimal HR	**-0,8926**
Mean leaf wetness	-0,6968
Minimal leaf wetness	0,2480

Finally, with the chosen variable, in our case minimal relative humidity, a simple linear regression was performed. The results revealed significant differences between the number of copies in 1 µL of template DNA and minimal relative humidity (F-statistic = 22,47; P-value = 0,0178). The values of number of copies of DNA tend to increase when minimal relative humidity decrease. The simple linear regression model is represented in the figure 21. The normality of the model was tested by Shapiro-Wilk test (W = 0.976; p-value = 0.91) and the Breusch–Pagan chi-square test was conducted to check the homoscedasticity (Chi-square = 0.174; df = 1; p-value = 0.677). Both hypotheses were fulfilled.

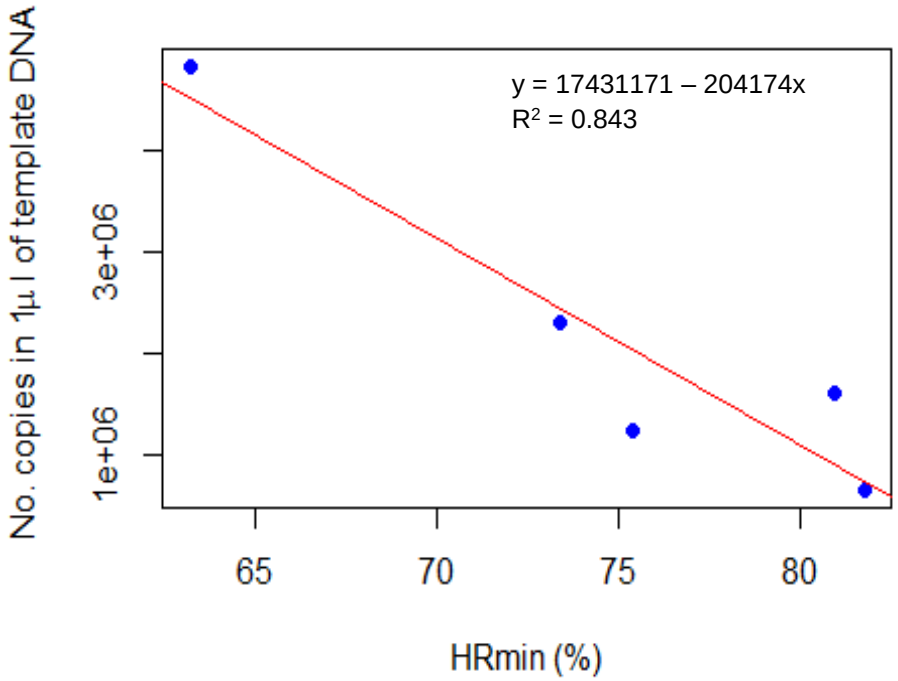

Figure 21. *Linear regression model for the number of copies in 1 µL of template DNA and minimal RH.*

6. DISCUSSION

During the past two decades, dieback of European or common ash (*Fraxinus excelsior*) has become a major threat to this tree species in Europe. Airborne ascospores seem to be the only infectious propagules and outset of new infections on green leaves (Kowalski and Holenrieder, 2009; Timmermann *et al.*, 2011). Knowledge on the spore content of the air is important to research into the epidemiology of plant diseases. Our results demonstrate that *H. fraxineus* is present in the air of the infected forest Jelení Hora since mid- May until the end of October (figure 20), which means some time before and after the ascospores are known to be released and wind-dispersed in nature. These results are supported by the air sampling technique seven-day automatic volumetric spore trap.

Several authors have examined the spore dispersal of *H. fraxineus*. Timmermann *et al.* (2011) and Hietala *et al.* (2013) employed seven-day automatic volumetric spore trap based on the description of Hirst (1952) to investigate the diurnal and seasonal pattern of *H. fraxineus* in Southern Norway from the beginning of July until the end of September. Chandelier *et al.* 2014 applied rotating arm spore traps based on principles of Perkins (1957) and McCartney *et al.* (1997). Their results described the spatial (horizontal and vertical) spore dispersal together with the seasonal pattern of the occurrence of *H. fraxineus* ascospores, sampled extensively in different years at different localities from April to November. According to Dvořák *et al.* (2016), SAVST (seven-day automatic volumetric spore trap) and RAST (rotating arm spore trap) for air-sampling of *H. fraxineus* provide similar results. However, rotating arm spore traps have certain disadvantages. They are not suitable to sample very small particles and after a relatively short time they may become overloaded (Dhingra and Sinclair, 1985). Basically, they are not applicable for continuous sampling to follow ascospore occurrence in real-time. Conversely, compared to seven-day volumetric spore traps, they normally work with higher sampling rate, even over 100 L·min-1 (McCartney et al., 1997; Dhingra and Sinclair, 1985), which is 10-times higher in a volume of air than SAVST. On the other hand, this difference in sampled volume is balanced by the efficiency of sampling. According to Lacey and West (2006), the efficiency of the Burkard seven-day volumetric spore trap (similar construction to SAVST used in this study) is almost indifferent to the size of sampled particles, if the particles are bigger than 2 µm. This limit cannot affect the sampling of ascospores of *H. fraxineus*, whose size is 13–21 x 3.5–5 µm (Kowalski and Holdenrieder, 2009). Advantages of rotating arm spore traps are their lower price and weight (McCartney *et al.*, 1997; Dhingra and Sinclair, 1985; Chandelier *et al.*, 2014). Nevertheless, the most suitable device in order to carry out a continuous sampling of the *H. fraxineus* occurrence in real-time, the goal of the study, was the SAVST.

Large numbers of copies of *H. fraxineus* DNA were recorded, with the maximum number of them occurring from the end of July to the end of September. This fact coincides with the presence of apothecia in ash leaf litter. Timmermann *et al.* (2011) and Hietala *et al.* (2013) observed that the higher number of ascospores, resembling those of *H. fraxineus*, occur from the end of July to mid- August with the highest release levels early in the morning. In the case of Chandelier *et al.* (2014), the period was between the end of July until the beginning of September. A similar study was realized in the South East of Czech Republic by Dvořák *et al.* (2016), whose results were in agreement with our findings. Although in this last case, the number of copies of target sequence in 1 L of template DNA was much higher, with the maximum around 10^{13} compared to 10^{7} in the present study. This could be explained for the different stand characteristics and different level of disease. While the location of Dvořák *et al.* (2016) had a high number of severely infected *F. excelsior*, our location belonged to a mixed broadleaved forest with a small group of European ashes showing moderate infection and nearby twice as

altitude. Investigations along landscape gradients are needed for *H. fraxineus* to assess environmental variation in ash dieback severity. Altogether, it seems that the highest levels of *H. fraxineus* DNA appear in the warmest summer period. Hence, the quantification of airborne inoculum is a prerequisite to evaluate the influence of meteorological conditions and stand characteristics on the spread of the disease.

Minimal air humidity was the most influential meteorological variable in the production of air-inoculum in our study. This correlation was inverse, contrary to former studies where air humidity has been a crucial factor for *H. fraxineus* ascospore release (Dvořák *et al.*, 2016; Gross *et al.*, 2012c). In fact, probably the ascospore maduration takes place primary during the night when air humidity is high and the discharge during the period with morning dew may protect the ascospores from desiccation during the subsequent infection process and may stimulate their germination (Timmermann *et al.*, 2011). It is suggested that air humidity is essential for the growth and maturation of apothecia (Ogris, 2010). Our results could be explained because the levels of minimal air humidity ranges between 63 to 81%, which are very high being minimal values. Compared with Dvořák *et al.* (2016) where the minimal air humidity was at around 30-40%, much lower values. Therefore, we can't affirm that occurrence of *H. fraxineus* is conditioned by low air humidity. Perhaps, it is possible that too high air humidity values could somehow inhibit the release of *H. fraxineus* ascospores.

Even though the highest amount of *H. fraxineus* DNA was from the end of July to the end of September, also is present in the air of the infected forest Jelení Hora the rest of the sampling period. But this contrast with that, based on the hypothetical life cycle of *H. fraxineus* depicted by Gross *et al.* (2012c), ascospores are produced in the leaf litter by apothecia formed during summer. Apparently, this means that *H. fraxineus* DNA detected in unexpected periods, when fresh fruiting bodies are not present, could be from ascospores that remain in the air or other parts of the pathogen. The origin of the inoculum sampled in the periods without apothecia is discussed by Dvořák *et al.* (2016). The hypothesis posed is that it might be (*i*) conidia (spermatia), (*ii*) ascospores from apothecia appearing in pseudoscplerotial petioles with postponed fructification and/or (*iii*) pseudosclerotia of disintegrated petioles which may become airborne particles. The hypothesis of long distance transport of inoculum by air masses was rejected because authors throughout Europe limited the ascospore release season equally (Gross *et al.,* 2012c; Chandelier *et al.,* 2014; Timmermann *et al.*, 2011; Kowalski and Bartnik, 2010). Therefore, there is no reason to await long distance-transported inoculum apart from the main season of ascospore production (Dvořák *et al.*, 2016).

Our simultaneous microscopy analysis allows demonstrating the presence of *H. fraxineus* ascospores in the absence of apothecia. However, we can't confirm entirely that all ascospores detected by microscopy belong to *H. fraxineus*. This is because it is easy to confuse the species of the spores, especially when common *Hymenoscyphus* species occur simultaneously. It is the case of *H. albidus*, with which was wrongly identified by 2009. *H. fraxineus* is said to have slightly longer ascospores (Queloz *et al.*, 2011), but variation and overlap in this feature forbid recognition of the two species on *Fraxinus* from spore length alone. *H. fraxineus* ascospores can be distinguished from *H. albidus* by the presence of croziers (Baral and Bemmann, 2014), but sometimes this feature could be unclear as well and requires considerably skill (Zhao *et al.*, 2013). The presence of *H. fraxineus* in Europe is replacing *H. albidus* (McKinney *et al.*, 2012), the overlapping niches result in presumed competition with *H. fraxineus* being strongly favoured. It seems that *H. fraxineus*, during invasion of Switzerland, hardly surpass an altitude of 850 m while *H. albidus* appears at higher altitudes (Queloz *et al.*, 2011). Our study forest is located at that elevation limit (883 m), then the possibility of coexistence

of both species exists, and consequently, the susceptibility to identification mistake. Additionally, *Cyathicula fraxinophila*, an helotialean species commonly colonizing and specific to decaying ash petioles, could be confused with *H. fraxineus*. Specifically, *C. fraxinophila* has usually been found later in the season, mostly in September and October (Koukol *et al.*, 2016). This could correspond to the ascospores detected in the end of the sample period. Another closely related, *Cyathicula coronata*, is present on ash petioles and together with *C. fraxinophila* do not appear to be critically affected and displaced by the invasion of *H. fraxineus* (Koukol *et al.*, 2016).

The methodology of DNA extraction used in our survey, in which the disruption of the sample is performed with balotina beads combined with two different lysis buffers together with qPCR measurement is the most sensitive technique for detection of *H. fraxineus* in nature (Dvořák *et al.*, 2016). The detection and quantification of spores on the sticky surfaces of volumetric spore traps is usually carried out using microscopy, but this method is time-consuming and, as described previously, occasionally is ambiguous. Molecular methods with specific primers offer several advantages over microscopy (West *et al.*, 2008), especially in the case of real-time quantitative PCR (qPCR) analytical methods, which are (*i*) adapted to quantification, (*ii*) able to detect low amounts of DNA, and (*iii*) species-specific (Duvivier *et al.*, 2013) in environmental samples. PCR have permitted the analysis of minimal starting quantities of nucleic acid. This has made possible many experiments that could not have been performed with traditional methods. In addition, the method of dual-labeled fluorogenic probe (TaqMan Probe) is sensitive and specific, and provides very accurate and reproducible quantification of gene copies (Heid *et al.*, 1996).

As the ash dieback has become a serious threat to forests and nurseries, the European Plant Protection Organization (EPPO) Secretariat decided to include *H. fraxineus* in the EPPO Alert List in 2007 (EPPO, 2010). The control of established ash dieback in the forest is practically impossible and silvicultural recommendations are limited to avoid the loss of the value of mature ash stands. Only when the pathogen has recently been introduced through infected nursery stock and there are no infections in the surrounding ecosystems, the removal of infected plants is recommended (DEFRA, 2013). There is a need to find sustainable control methods, which may inhibit the development of the disease. In order to design these control methods to ash dieback, biological studies focused on the occurrence of the *H. fraxineus* air-inoculum and its relation to meteorological conditions, are essential for a more precise disease management (Chandelier *et al.*, 2014).

Chemical control might be possible for small plants. Knowing the sporulation period, can help in the design of using these chemicals. Treatment of infected ash leaf debris to prevent sporulation of *H. fraxineus* seems to be one of the most effective control measures. It must be taken into account that these products could be counterproductive, as it makes no sense to preserve susceptible plants, which would enhance the epidemic after outplanting. In addition, sublethal fungicide treatments might prevent symptom development and thereby inhibit long-distance dispersal with latently infected plants (Gross *et al.*, 2014). Although fungicides have proved immensely successful in controlling many diseases, their use is increasingly threatened by the development of fungicide-resistant strains, health hazards and the risks to the environment (Pearson, 2009). According to Hauptman *et al.* (2015), infection potential of the pathogen could be significantly reduced with the use of urea. Urea acts directly on the fungus, but also stimulates the activity of antagonistic microorganisms and occurrence and reproduction of saprophytic fungi accelerating the degradation of the treated leaf debris. Therefore,

the application of urea for treatment of infected ash leaf debris could be even more effective than the use of fungicides and also more environmentally acceptable.

In recent decades, many parts of the world have experienced the devastating results of epidemics caused by introduced pathogens and insect parasites, which have impacted trees of great economic and/or ecological importance. Ash dieback will thus dramatically reduce the size of its host populations in Europe, thereby threatening not just ash, but also the organisms depending on *F. excelsior* (Pautasso *et al.*, 2013). The aerial distribution of fungal inoculum over tens of kilometres, the long potential sporulation period spanning May to November (Timmermann *et al.*, 2011; Chandelier *et al.*, 2014), and the rather high density of ash in central European countries (Pautasso *et al.*, 2013; Gross *et al.*, 2014a) all contribute to the difficulty in eradication or mitigation this disease. Hence, protection and prevention methods are currently one of the most discussed topics in this field.

To date, more than 25 countries have reported the occurrence of the disease and it is expected that *H. fraxineus* will successively spread into currently non-affected areas and threat ashes in large parts of its natural distribution range (Cleary *et al.*, 2013). The big threat of the ash dieback disease is in the gates of the Iberian Peninsula. The speed of spreading of *H. fraxineus* has allowed reached to the South of Europe in a short period of time. It would be necessary to start the prevention methods for avoiding the threatening situation, such as establishment of sentinel areas with monitoring methods coupled with spore traps in ash forest of Spanish Pyrenees. Surveys, similar to the present study, ensure a fast and reliable detection of the *H. fraxineus* which could be used for a rapid identification of the pathogen.

7. CONCLUSIONS

1. *Hymenoscyphus fraxineus* is present in the air of the infected forest Jelení Hora (South West of the Czech Republic) since mid- May until the end of October, with peaked values from mid- July until mid- September. As expected, the higher amount of *H. fraxineus* DNA appeared in the warmest summer period. This is supported by the air sampling technique seven-day automatic volumetric spore trap and the real-time quantitative PCR assay (qPCR).

2. Minimal air humidity was the most influential meteorological variable in the production of air-inoculum in the study location, with an inverse correlation. This different pattern may be caused by the high levels of relative humidity present in our study area over the sampling period, which may somehow inhibit the release of *H. fraxineus* ascospores.

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