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ORIGINAL ARTICLE

Individual resistance of *Fraxinus angustifolia* and *F. excelsior* clones to *Hymenoscyphus fraxineus*

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Abstract

Ash dieback, caused by *Hymenoscyphus fraxineus*, is a serious disease of common and narrow-leaved ash in Europe. The resistance of individual trees seems to be important for the maintenance of ash in European forests. In this in situ wound inoculation study, the susceptibility and differences in resistance to *H. fraxineus* between *Fraxinus excelsior* and *F. angustifolia* clones were assessed. Neither of the tested clones revealed total resistance to ash dieback; variety between the tested clones was observed. Differences in necroses lengths were significant between clones and between two ash species. Longer necroses were formed in *F. angustifolia* than in *F. excelsior*. Some clones exhibiting some resistance to the pathogen were identified.

Keywords: ash dieback, *Chalara fraxinea*, in situ, pathogenicity tests, susceptibility

Introduction

In Europe, ash dieback was first observed in the middle of the 1990s on plants in forest nurseries and in young plantations in northern Poland and Lithuania. During the next two decades this disease gradually emerged in other European countries (Husson *et al.* 2011; Timmermann *et al.* 2011). The first symptom of the disease in young ash trees was dieback of the terminal shoot demonstrated by marked dark coloration of shoot bark. Since 2004, the mentioned symptoms of ash dieback have also been recorded in Slovakia (Kunca 2006).

The ash dieback, wherever it occurs causes immense damage to common ash (*Fraxinus excelsior* L.) and in some parts of Europe also to narrow-leaved ash (*Fraxinus angustifolia* Vahl) (Kowalski and Łukomska 2005; Kirisits *et al.* 2010; Schumacher *et al.* 2010; Schumacher 2011). In addition, ash dieback has also been reported on a few ash species not native to Europe (Drenkhan and Hanso 2010). This is an emerging disease, which results in massive tree mortality, threatening the existence of *Fraxinus* over the continent.

The disease-causing agent in Europe was identified in 2006 as a new fungal species *Chalara fraxinea* T. Kowalski, the asexual stage of the ascomycete fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya with taxonomic synonym *H. pseudoalbidus* Queloz, Grünig, Berndt, T. Kowalski, T.N. Sieber & Holdenr. (Kowalski 2006; Kowalski and Holdenrieder 2009a, b; Queloz *et al.* 2011; Baral *et al.* 2014). In 2013, the fungus *Ch. fraxinea* was isolated from damaged symptomatic tissues of *F. excelsior* from eight localities in Slovakia. The fungus was identified based on amplification of internal transcribed spacer (ITS) region and the retrieved sequences were 100% identical to the fungus *H. fraxineus* (Adamčíková *et al.* 2015). In Slovakia in 2014 the pathogen was confirmed and identified on *F. angustifolia* as well (Kádasi-Horáková *et al.* 2017).

Fraxinus L. is an important genus among the deciduous tree species. Although it is not the main forest species in Slovakia, it is commonly planted in forests and parks and is used for urban greenery. Ash trees form small forest plantations (usually less than 1 ha), which represent 1.5–2% of the forest land in Slovakia corresponding to about 30 000 ha. Ash trees used for genetic purposes and as a genetic resource, grow on

122.81 ha in Slovakia. There are 616.40 ha of approved ash stands for seed collection, 206 plus trees and three seed orchards covering an area of 1.80 ha (Longauerová *et al.* 2013).

Currently, limited options are available to control the disease (Hauptman *et al.* 2013, 2015; Dal Maso *et al.* 2014; Gross *et al.* 2014). Moreover, the resistance of individual trees seems to be important for the maintenance of ash in European forests. A considerable intraspecific variability in symptoms and intensity of dieback has been observed in *F. excelsior* infected by the fungus *H. fraxineus*. Their susceptibility to infection by *H. fraxineus* is primarily hereditary. Tolerant individuals able to tolerate the infection and successfully reproduce make up only 1 to 5% of ash populations. Conservation of the larger number of tolerant individuals is crucial because they are able to provide for new generations that will not be weaker than the present one (McKinney *et al.* 2014).

Two ash seed orchards are registered in Slovakia. One seed orchard is located in the western part of Slovakia, in Trstice and the second one is in central Slovakia in Zbojská. Nevertheless, only the seed orchard in Trstice is used for real seed production. The trees in the seed orchard in Zbojská have not blossomed yet since the orchard was established in an unsuitable place. The presence of ash dieback and infection caused by *H. fraxineus* has been confirmed in both seed orchards and also in an abandoned seed orchard in Pusté Pole (east Slovakia) (Longauerová *et al.* 2015).

The aim of this study was to identify the susceptibility of selected ash clones planted in the seed orchard in Trstice against the pathogen *H. fraxineus*, to determine any differences in resistance to *H. fraxineus* between *F. excelsior* and *F. angustifolia* clones used as aseed source and subsequently for seedling production.

Materials and Methods

Site and plant material

Field work was performed in the seed orchard in Trstice, which is situated in the Galanta region, Slovakia (48°01'03"N, 17°47'37"E). The orchard was established in 2009 using 49 different clones of *F. excelsior* and *F. angustifolia*. A total of 566 grafts were planted, spaced 6 × 6 m, in 12 replicates per clone distributed randomly in the plot.

Clones for inoculation trials were selected based on the results of visual damage assessment, i.e. the intensity of tree defoliation (Longauerová *et al.* 2015). Three clones (visually assessed as being the healthiest, 028 and 088, of average health, 043 and 083, and the most damaged, 020 and 011 clones) were selected for each ash species. Each test was replicated five times on different individuals of the same clone.

Fungal isolates

Four different strains of *H. fraxineus* (Č 2e, D 27, J 1b2, SA NI6B) originating from different localities (Černík, Duchonka, Jarok, Svätý Anton) in Slovakia were used for inoculation trials (Table 1). The species identity of the strains used was previously confirmed by the species specific primers of Husson *et al.* (2011) and/or by sequencing of ITS region (Adamčíková *et al.* 2015; Kádasi-Horáková *et al.* unpublished). For production of inoculum, *F. excelsior* sapwood sticks (6 × 5 × 3 mm in size) were sterilized (20 min, 120°C) and colonized with the four fungal strains on 2% ash leaf malt extract agar (AMEA, Kirisits *et al.* 2013) for approximately 1 month (Fig. 1). For control inoculations, sterile wood sticks of similar size were used.

Inoculation trials

Inoculations in the Trstice seed orchard were performed on March 28, 2017 as described by Kowalski and Holdenrieder (2009a) and Gross and Sieber (2016). For inoculations healthy looking branches of each clone were selected. The inoculum (sapwood stick colonized with mycelium) was placed on the xylem surface in the bark incisions (approx. 1 cm long, Fig. 1) without removing the bark and wrapped with stretched Parafilm M (Bemis Company Inc., Neenah, WI, USA) to keep the inoculum from drying (Fig. 1). Control inoculations were made in the same way with sterile sapwood sticks.

All inoculated branches were cut of 106 days after inoculation (July 12, 2017), transported to the laboratory and were evaluated the next day. The presence of typical symptoms of infection was recorded and lengths of discoloured necroses on the outer bark distally and

Table 1. Characteristics of *Hymenoscyphus fraxineus* isolates used in the inoculation trials

Code of isolate	Locality	Year of sampling	Host tree	Coordinates
Č 2e	Černík	2014	*F. excelsior*	48°09'05"N, 18°12'54"E
D 27	Duchonka	2015	*F. excelsior*	48°42'53"N, 18°02'33"E
J 1b2	Jarok	2013	*F. excelsior*	48°16'39"N, 17°58'33"E
SA NI6B	Svätý Anton	2013	*F. excelsior*	48°24'30"N, 18°53'19"E

Fig. 1. Inoculation trial: (A) sapwood sticks colonized with the mycelium of *Hymenoscyphus fraxineus* strains on ash leaf malt extract agar, (B) the bark incisions without removing the bark, (C) sapwood stick colonized with mycelium placed on the xylem surface into the bark incision, (D) incision wrapped with stretched Parafilm

proximally from the inoculation point were measured. Afterwards, the bark was removed, and the length of wood necrosis was measured in same way. The total length of necroses was calculated as the sum of both measured values.

Statistical analysis

The data were analysed by factorial analysis of variance (ANOVA). The response variable 'necrosis length' (measurement at wood level) was $\log_e$-transformed to achieve normally distributed residuals (which was checked by visual inspection of residual plots). Statistical analyses were performed using the statistics software STATISTICA (StatSoft, Inc. 2011), version 10 (www.statsoft.com).

Re-isolation of pathogens from ash tissues

Re-isolations were carried out from all inoculated branches showing visible wood necroses larger than 2–3 mm. From the margin of necrosis, i.e. from the boundary between infected and healthy parts, small wood tissue was taken, superficially sterilized according to Adamčíková *et al.* (2015) and transferred to AMEA (Kirisits *et al.* 2013) supplemented with antibiotics (100 mg streptomycin/1 l media).

Results and Discussion

The presence of ash dieback based on the typical symptoms was confirmed in the seed orchard in Trstice. The symptoms were noticed on both ash species: *F. excelsior* and *F. angustifolia.* Clones 028, 043, 020 (*F. excelsior*) and 088, 083, 011 (*F. angustifolia*) were selected for inoculation trials based on their existing health status according to the results of average defoliation (Longauerová *et al.* 2015).

Sterile inoculum (control) did not cause necroses; all were bound with callus around the inoculation hole.

From a total of 120 inoculations, typical ash dieback lesions were observed on 65 (54%). No necroses were formed on 55 inoculations on *F. excelsior* and *F. angustifolia* in all, 31 and 24, respectively. The lengths of discoloured necroses on the outer bark and of wood were not statistically different (F = 1.1590, p = 0.422), but generally necroses length in the outer bark was shorter than the length of wood necroses. Also, the results showed a strong correlation between outer bark and wood necrosis; necrosis lengths in the outer bark increased with the length of wood necroses (n = 120, r = 0.86, p < 0.001, Fig. 2). Statistically significant differences were recorded for the necroses direction. Differences were observed in necroses length in both distal and proximal directions of development (F = 1.9496, p = 0.000317). The necroses in the proximal direction were longer (n = 120, average length of necrosis 13.7 mm, maximal necrosis length 95.2 mm, Fig. 3A) than in the distal direction (n =120, average necrosis length 8.3 mm, maximal necrosis length 103.6 mm, Fig. 3A). No correlation was determined between the diameter of branches and length of necrosis (r = 0.15, p = 0.085).

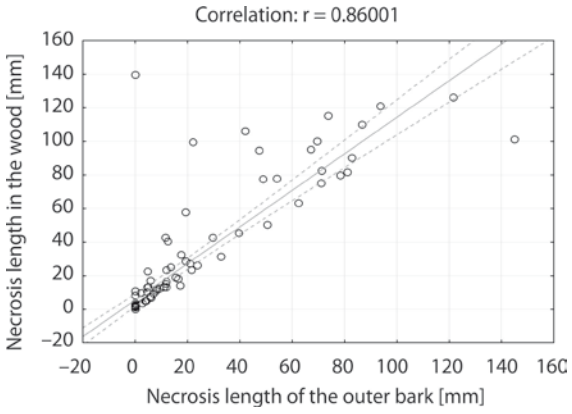

Fig. 2. Correlation between the necrosis length on the outer bark and in the wood

A statistically significant difference was recorded in necroses length between the two ash species (F = 4.2563, p = 0.0415). Longer necroses were formed in *F. angustifolia* (n = 60, average necrosis length 31.4 mm, maximal necrosis length 139.8 mm, Fig. 3B) than in *F. excelsior* (n = 60, average necrosis length 15.1 mm, maximal necrosis length 100 mm, Fig. 3B). The average development rate of necrosis in a proximal direction was 0.07 mm in *F. excelsior* and 0.18 mm in *F. angustifolia*. The average necroses growth rate for inoculations with *H. fraxineus* on *F. excelsior* varied between 0.26 to 0.86 mm per day according to Gross and Sieber (2016) and Bakys *et al.* (2009). For *F. angustifolia* the mean necrosis length in inoculation trials varied between 0.75 to 1.23 mm per day according to Hauptman *et al.* (2016), which was higher than for *F. excelsior*. The results of the current study yielded lower values. Development of necroses varied in relation to several factors, with the time of inoculation having the most significant effect on this variation (Bolvanský *et al.* 2014). Lesions that developed during a dormant period were two times smaller than those developed during a vegetative period. Similar results were also obtained by Guérin and Robin (2003) after inoculation of 4-year-old *Castanea sativa* sprouts with *Cryphonectria parasitica in situ* and *in vitro* on stem segments. In both trials, necroses length was lower in a dormant period than in a vegetative season (Guérin and Robin 2003; Bolvanský *et al.* 2014).

Significant differences were also noted between tested clones of *F. angustifolia* (F = 13.905, p = 0.000017) due to markedly longer necroses in clone 083 (n = 20, average necrosis length 64.34 mm, maximal necrosis length 126.3 mm, Fig. 4A). The shortest average necroses were measured in clone 011 (n = 20, average

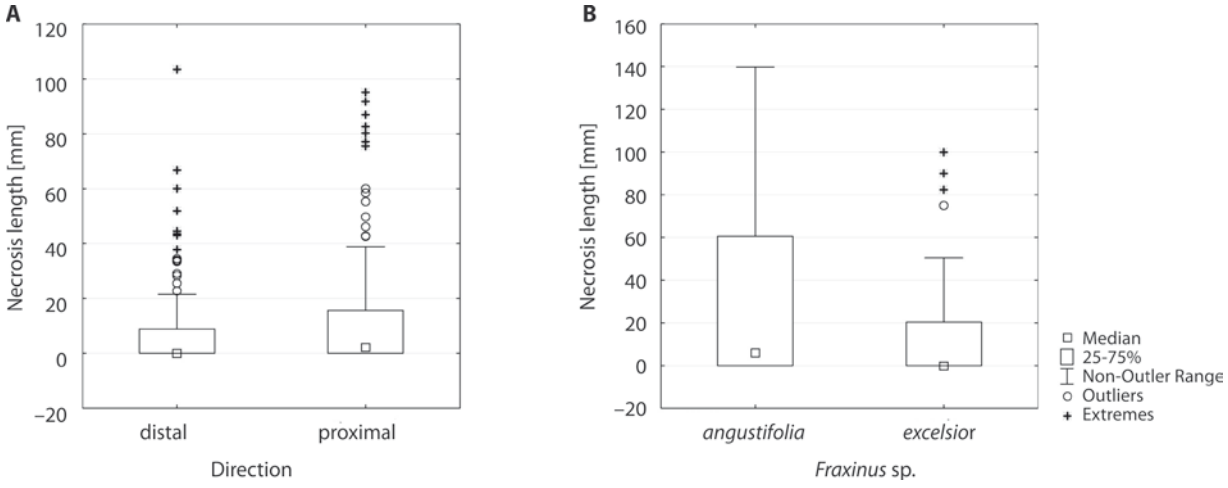

Fig. 3. (A) Comparison of necrosis length in distal and proximal directions, (B) differences in necrosis length between two ash species (*Fraxinus excelsior*, *F. angustifolia*)

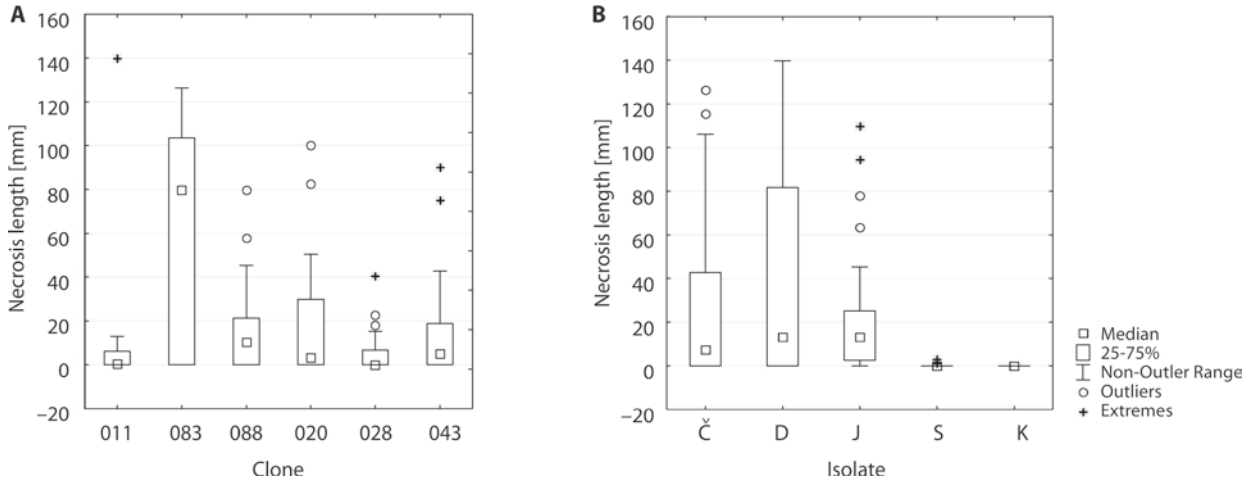

Fig. 4. (A) Comparison of necrosis length after inoculation by *Hymenoscyphus fraxineus* at ash clones level, (B) at fungal strain level (Č – isolate Č 2e, D – isolate D 27, J – isolate J 1b2, S – isolate SA NI6B, K – control)

necrosis length 9.85 mm, maximal necrosis length 139.8 mm, Fig. 4A) and did not differ significantly to necroses in clone 088 (n = 20, average necrosis length 16.15 mm, maximal necrosis length 79.7 mm, p = 0.062, Fig. 4A).

Similarly, significant differences in necroses length were recorded between clones of *F. excelsior* (F = 3.424, p = 0.041), while clone 028 formed markedly shorter necrosis (n = 20, average necrosis length 5.49 mm, maximal necrosis length 40.4 mm, Fig. 4A) than clone 043 (n = 20, average necrosis length 15.95 mm, maximal necrosis length 90.1 mm, Fig. 4A) and 020 (n = 20, average necrosis length 20.42 mm, maximal necrosis length 100 mm, Fig. 4A).

Differences in necroses length were significant between the clones of both ash species (F = 7.48, p = 0.000006). The most susceptible of all tested clones proved to be clone 083 (*F. angustifolia*) and the least susceptible were clones 028 (*F. excelsior*) and 011 (*F. angustifolia*) (Fig. 4A). The significant differences in necroses lengths between the tested clones indicated that some clones more successfully limit fungal growth than others (Hauptman *et al.* 2016).

According to Longauerová *et al.* (2015) all three clones of *F. excelsior* selected for the present study had higher average defoliation expressed in percent (68, 58, and 54%) than clones of *F. angustifolia* (52, 48 and 32%). Clone 020 (*F. excelsior*, 68%) had the highest defoliation and it was considered to be the most susceptible common ash clone in the inoculation test. The results for the other two *F. excelsior* clones (043 and 028) were also in accordance with results of visual evaluation of defoliation according Longauerová *et al.* (2015).

Different results were achieved for *F. angustifolia* clones. The most susceptible in inoculation trials was clone 083. While according to the visual evaluation of Longauerová *et al.* (2015), clone 011 was assessed as highly damaged, and had the shortest necroses in inoculation trials. Similar results in inoculation trials on *F. angustifolia* were recorded by Hauptman *et al.* (2016), who compared visual evaluation of crown damage with inoculation trials of two clones. Necroses on the highly damaged clone were shorter than the necroses on the healthier looking clone (Hauptman *et al.* 2016). Thus, the visual health status does not always correspond with the extent of tissue necroses in ash trees or at least not specifically for *F. angustifolia* trees (Hauptman *et al.* 2016).

At the strain level (fungal isolate), all the studied isolates, except one (Svätý Anton) differed significantly from the control (F = 16.44, p < 0.001) and within isolates (F = 6.23, p = 0.0012 and F = 17.69, p < 0.001, Fig. 4B). Significantly shorter necroses were measured in inoculation with isolate SA NI6B on both ash host species (F = 6.23, p = 0.0012 and F = 17.69, p < 0.001, Figs. 4B, 5). Recorded differences in necroses length at the strain level confirmed the previous suppositions on differences in virulence within *H. fraxineus* strains (Bakys *et al.* 2009; Ogris *et al.* 2009; Hauptman *et al.* 2016). It has been suggested that long-term storage might cause a degeneration of strains (Brasier 1991; Kowalski and Holdenrieder 2009a), which could result in decreased virulence (Gross and Sieber 2016). Indeed, in this study isolate D 27 was younger (isolated in 2015) and induced the longest necroses, while inoculations using the oldest isolates (SA NI6B and J 1b2), stored two years longer, induced the smallest necroses. The necrosis length caused by the next three isolates (J 1b2, Č 2e, D 27) did not differ significantly. Similarly, no statistically significant difference was recorded in the growth rate of two similarly aged isolates used in the inoculation trial (Diminić *et al.* 2017).

We successfully isolated *H. fraxineus* only from 1 of 58 inoculated branches, resulting in a very low

Fig. 5. Typical necrosis caused by four *Hymenoscyphus fraxineus* strains used in inoculation trials recorded on the outer bark (left) and in the wood after bark removal (right). Č – isolate Č 2e, D – isolate D 27, J – isolate J 1b2, S – isolate SA NI6B

re-isolation success rate of 1.7%. The only successful re-isolation was gained from *F. angustifolia* clone 011, inoculated by D 27, which was the youngest. The age and long storage of the isolates could be the reason for unsuccessful re-isolations. An other factor that could have an effect on the re-isolation rate was the season, i.e. the time of the year.

The results of this current study indicate differences in resistance to ash dieback. These differences exist not only between individuals of common ash, but also in narrow-leaved ash. Comparing the two ash species, considerably larger necroses were observed in *F. angustifolia* than in *F. excelsior*. For both species, statistically significant differences were recorded in susceptibility to ash dieback and clones with variable susceptibility have been identified. None of the tested clones revealed total resistance to ash dieback; a variable response in tested clones was observed. In inoculation trials clones 011 (*F. angustifolia*) and 028 (*F. excelsior*) were the least susceptible to *H. fraxineus*, and they exhibited some kind of reduced susceptibility to the pathogen that should be tested in future experiments.

The fungus *H. fraxineus* is the main trigger of the ash decline process, and a genetically determined difference in resistance to ash dieback is likely one of the decisive factors contributing to the survival of individual ash trees (Hauptman *et al.* 2016). Resistance of individual ash trees is crucial for the existence of ash in forests of Europe; management in ash stands must be focused on finding resistant ash trees (genotypes) (Hauptman *et al.* 2016; Dimić *et al.* 2017).

The current study presented preliminary data about the susceptibility of ash clones that are the source of new seedlings planted in forests in Slovakia. The results will be helpful for selection of clones to be used for seed collection and subsequent seedling growth. The data obtained could be a good basis for further research focused on host genotypes less susceptible to the pathogen and may provide background for future forestry management.

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