Ecological and histological investigation of species complex *Diaporthe eres* on *Fraxinus excelsior* seed from Montenegro

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Abstract

Investigation of different seed diseases of common ash has great significance due to occurrence of ash dieback across Europe caused by *Hymenoscyphus fraxineus* Baral, Queloz and Hosoya (T.Kowalski) and still not completely defined strategies about regeneration of this species. Species complex *Diaporthe eres* are familiar pathogens of common ash. During the monitoring of health condition of common ash in Montenegro *Diaporthe eres* was frequently isolated from common ash seeds. The aim of this research was to investigate temperature conditions that contribute to the growth of *Diaporthe eres* strains from Montenegro in infected seeds and histological characteristics of *Diaporthe eres* spread in seeds. Growth of *Diaporthe eres* pure cultures on tested temperatures showed the fastest growth on 25°C and the absence of growth on 35°C. Mechanical damage did not significantly contribute to occurrence of infection. Microscopic examination of heavy infected seeds showed that hyphae of *D.eres* were visible on outer parts of seed spreading inside the core. In this research we investigated some basic bio ecological characteristics of *D. eres* associated with decline of *F. excelsior* seed in Montenegro.

Keywords

Diaporthe eres; *Fraxinus excelsior*; Seed; Bio ecology

Contents

1 Introduction

Decline of common ash (*Fraxinus excelsior*) across Europe raised concerns about adaptive potential of this species to fast recover due to invasive fungal disease-causing ash dieback (Pautasso et al. 2013). Also, a change in ground flora diversity due to loss of ash trees is one of the consequences of ash dieback (Mitchell et al. 2016). Restoration of ash forests should include preserving trees with low level of damage (Skovsgaard et al. 2017). However, a large number of the other fungi were recorded on common ash trees (Bakys et al. 2009; Kowalski et al. 2016) that can lead to concatenation of already significant damage).

Recent investigations of fungi associated with common ash (*Fraxinus excelsior*) seed showed the presence of 30-46 fungal taxa (Cleary et al. 2013; Hayatgheibe 2013). Fungus *Diaporthe eres* Nitschke was one of rare fully identified taxa on common ash seed (Cleary et al. 2013). Also, *Diaporthe eres* aff. is weak pathogen to older ash seedlings (Kowalski et al. 2017), while on younger seedlings it can cause more damages (Vemić et al. 2019; Linaldeddu et al. 2020).

Association between infections of different plant organs of common ash should be investigated more due to prevention of further infections and better restoration of ash forests. During the investigation of the fungal diversity of common ash, *Diaporthe eres*. species complex was frequently isolated from common ash seed in Montenegro (Vemić 2020). Prediction models for development of this pathogen on the fruit of the common hazel (*Corylus avellana* L.) based on its ecological characteristics were developed (Arciuolo et al. 2021). The aim of this research was to investigate bio ecological characteristics of *Diaporthe eres* aff. from Montenegro including the influence of temperature on growth of pure cultures and development of mycelium in seed. These results will serve as basis for developing prediction model of the occurrence of *Diaporthe eres* on common ash fruit in this part of its areal.

2 Material and method

2.1 Isolation and identification of fungus

Isolation of fungus was performed from undamaged and mechanically damaged seed with 20 seeds within each damage status. Also, seed that looked visually healthy and diseased was used for isolation with 30 seeds within each health category. Isolation of fungus was performed from collected seed using basic procedure of fungal isolation described in Muntanola Cvetković (1990). Seed was surface disinfected with 96% alcohol and placed on 3% MEA medium.

Identification of fungus was by comparing morphological characteristics of cultures obtained from seed with morphological characteristics of cultures obtained from one-year-old seedlings that were molecularly identified using ITS1/ITS4 and EF728F/EF986R primers (Vemić 2020). All cultures belonged to the same morphotype (Figure 1).

2.2 Influence of temperature on mycelium growth

Cultures of one strain were grown in standard petri dishes on temperatures 10, 15, 20, 25, 27.5, 30, 32.5 and 35°C. On temperatures 10, 27.5, 30, 32.5 and 35°C there were 40 repetitions within each temperature. There were 20 repetitions at 15°C and 39 repetitions at 25°C. Experiment was finished after one week. Average growth within each temperature was calculated.

Figure 1. *Diaporthe eres* aff. cultures.

2.3 Histological examination of diseased seed

The infected seed was examined under a microscope at 400 x magnification. Fresh histological sections for microscopic examination were stained by cotton blue stain.

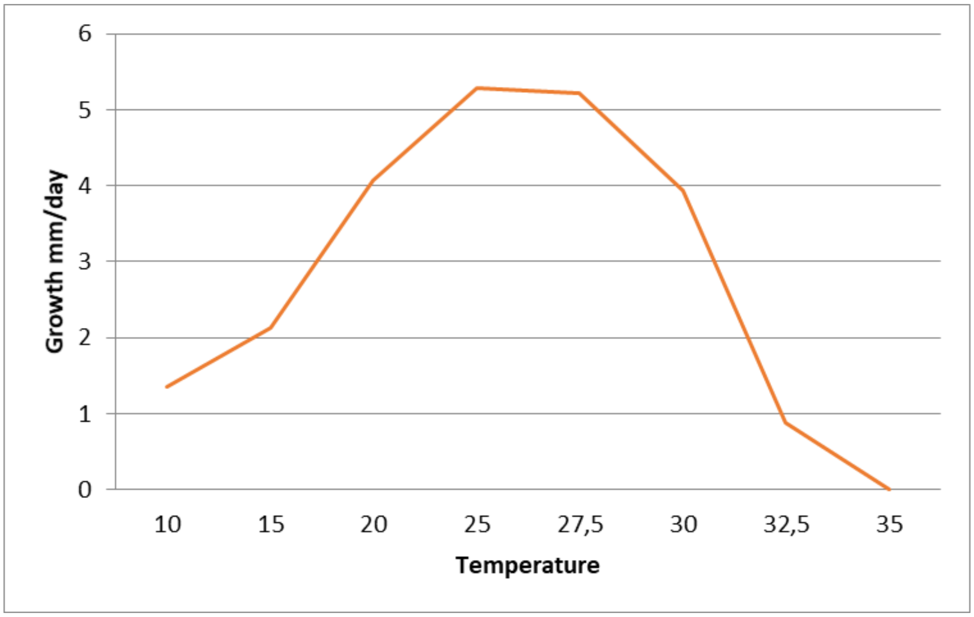
2.4 Statistical methods

Kruskal Wallis test was used to test difference of mycelium growth between different temperatures. Mann Whitney U test was used to test difference of mycelium growth between different pairs of temperatures. Chi square test was used to test dependence between mechanical damage of seed and occurrence of infection. Descriptive statistics was used to show average growth of mycelium within different temperatures. All analyses were performed in SPSS 21 and Microsoft Office Excel 2010.

3 Results

3.1 Influence of temperature on mycelium growth

There was statistically significant difference in mycelium growth on different temperatures ($H = 282.769$, $p < 0.000001$). The fastest growth was recorded on 25°C, the lowest was on 32°C and absence of growth on 35°C (Graph 1, Table 1). Temperature of 35°C caused the mortality of most cultures. There was significant difference in growth rate between different temperatures (Table 1).



Graph 1. Average growth of cultures according to temperature.

Table 1. Average growth of cultures according to temperature.

Temperature	Mean*	Std. deviation	N
10	1.3575a	0.18931	40
15	2.135b	0.14609	20
20	4.0775c	0.3393	40
25	5.282051d	0.49411	39
27.5	5.2125d	0.24929	40
30	3.9375c	0.68301	40
32.5	0.875e	0.12558	40
35	0	0	40

*Statistically significant differences in rows are labeled with different letters.

3.2 Development of mycelium in seed

Fungus was isolated from both visually healthy and diseased seed. Visually diseased seed was light infected and heavy infected. There was not significant dependence between mechanical damage of seed and occurrence of fungus (χ^2 = 1.905; $p = 0.168$). Hyphae of fungus were recorded on various parts of heavy infected seed including seed coat and core.

Light infected seed didn't have any damages in core and damages of seed coat were slightly (Figure 2). In light infected seed hyphae were visible only in outer part of seed.

Visually healthy seed i.e. without the presence of pathogenic fungi didn't have any damages (Figure 3A). However, advanced stages of infection caused damage in seed embryo (Figure 3B).

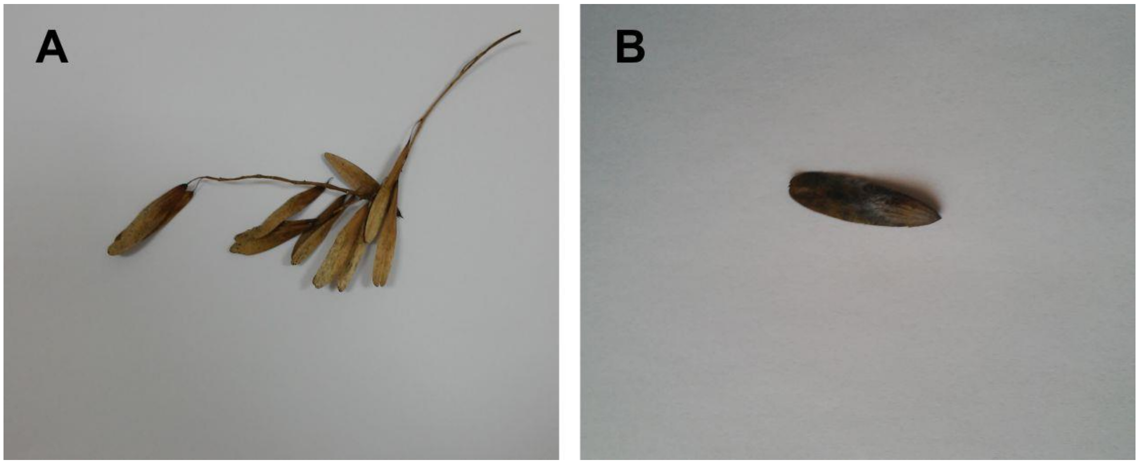


Figure 2. Common ash seed affected with *Diaporthe eres* aff. A-B light infection with slightly damaged seed coat.

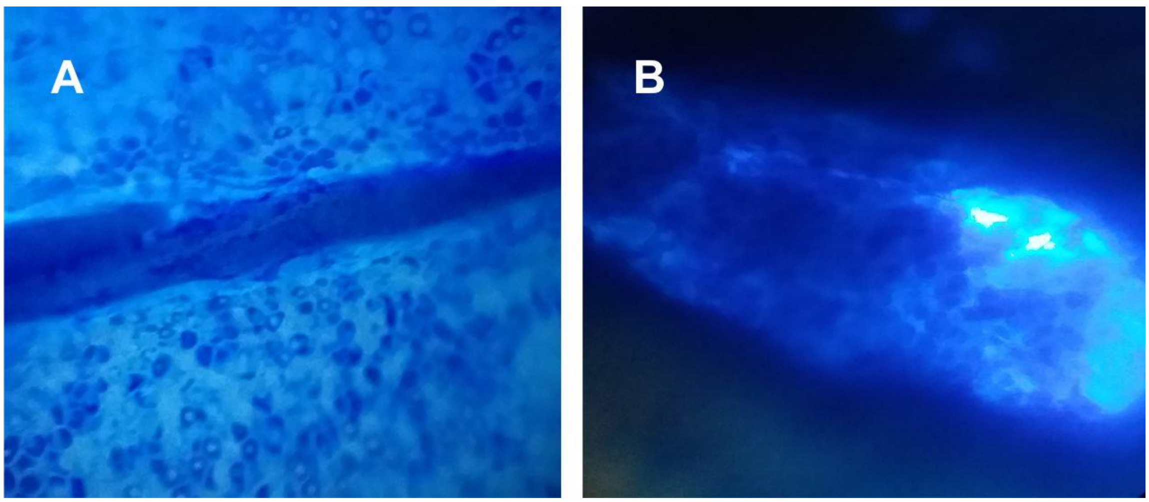


Figure 3. Histological section of common ash seed: A-Healthy embryo with compact dark blue color, B-Damaged embryo with light blue color showed damaged area.

4 Discussion

This paper study bio ecological characteristics of *Diaporthe eres* aff. on common ash (*Fraxinus excelsior*) seed in Montenegro. Prior to this research there were no these data in domestic literature to our best knowledge. Taxonomic situation of this genus is complicated, with large number of species belonging to species complex *Diaporthe eres* (Gomes et al. 2013; Udayanga et al. 2014). This fungus was recorded on above 60 host species (Wehmeyer 1933). Also, for forest *Diaporthe eres* aff. was recorded on *Abies*, *Acer*, *Aesculus*, *Cedrus*, *Corylus*, *Cupressus*, *Euonymus*, *Fraxinus*, *Hedera*, *Ilex*, *Lingustrum*, *Lonicera*, *Malus*, *Prunus*, *Pyrus*, *Rhododendron*, *Rosa*, *Rubus*, *Salix*, *Sorbus*, *Symphoricarpus*, *Tilia*, *Ulmus* and *Ulex* (Ellis and Ellis 1985). Besides investigation of association between infections of different organs of common ash, association between the infections of different hosts should be studied. Also, there is growing interest in seed sourcing of common ash (*Fraxinus excelsior*) for improving planting and reforestation (FRAXIGEN 2005). Protection of ash seed should play the role in this process especially due to increment of reforestation and restoration needs due to losses caused by ash dieback. *Diaporthe eres* species comples is reported in Serbia on (Petrović et al. 2015) on soybean seed and it would be interested to investigate potential pathways in infection spread between these two species.

It is familiar that factors like temperature can influence development of fungi and limit their development in field conditions or storage conditions. Minimum, optimum and maximum temperatures for storage fungi are 0-5 ºC, 30-33 ºC and 50-55 ºC (Agarwal and Sinclair 1996). Obtained results from tested strain from seed in Montenegro showed that fungus is less likely to develop in proper storage conditions. Also, results are in agreement with Arciuolo et al. (2021) about trend of cultures growth on different temperatures. However, tested strain in this study didn't grow at 35 ºC which implicate that in the case of development of this fungus in storage conditions it could develop only on colder temperatures. Still, this strain of fungus can be active in field conditions making infections to other trees and seeds.

Development of mycelium in seed addressed to different conclusions. Since the fungus was isolated from visibly healthy seed and visually damaged seed it was hard to evaluate if infection speeded from outer to inner parts of seed or vice versa. Histological examination of infected seed showed hyphae were endophytic and ecophytic. Multiple infections by different species of this genus can be present (Mostert et al. 2001) which implicates that results of histological analyses should be carefully interpreted even in this case where only one morphotype was isolated. Also this fungus is morphologically very variable (Castlebury et al. 2002), so different mophotypes should be analysed. One of the reasons could be because strains isolated from the same host species may not be closely related and may represent different taxons (Rehner and Uecker 1994; Farr et al. 2002). For example, morphological characteristics of *Diaporhe eres* aff. cultures isolated from common ash (*Fraxinus excelsior*) fruits in Montenegro differed than cultures isolated from fruits of apple (*Malus* Mill.), pear (*Pyrus* L.), cherry (*Cerasus* Mill.) and plum (*Prunus* L.) in Poland (Abramczyk et al. 2018).

All obtained results serve for development of models of *Diaporthe eres* species complex infection of common ash (*Fraxinus excelsior*) seed in this part of their areal.

5 Conclusion

Results obtained during this research implicated to next conclusions:

- Mycelium grew on temperature range 10-32.5°C. Mycelium growth stopped at 35°C. Also, this temperature affected vitality of most cultures.
- Optimum growth was achieved at 25-27.5°C.
- Fungus was isolated from visibly healthy seed as well as damaged seed. This implicates that in ecological conditions of this part of its areal it can behave as both endophyte, facultative saprophyte or pertophyte parasite.
- Mechanical damages of seed were not primary way of infection due to fungus was isolated from both seed with mechanical damage and without mechanical damage. However, micro damages of seed probably have role in infection process.
- Hyphae were found in seed coat and core. Only in heavy infested seed embryo was damaged. Based on hyphae distribution it was hard to

evaluate if infection speeded from outer to inner parts of seed or vice versa. It is possible that both types of infection pathway were present depending on if infection occurred directly on outer parts seed or spores of pathogen were dormant and started to germinate inside seed.

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