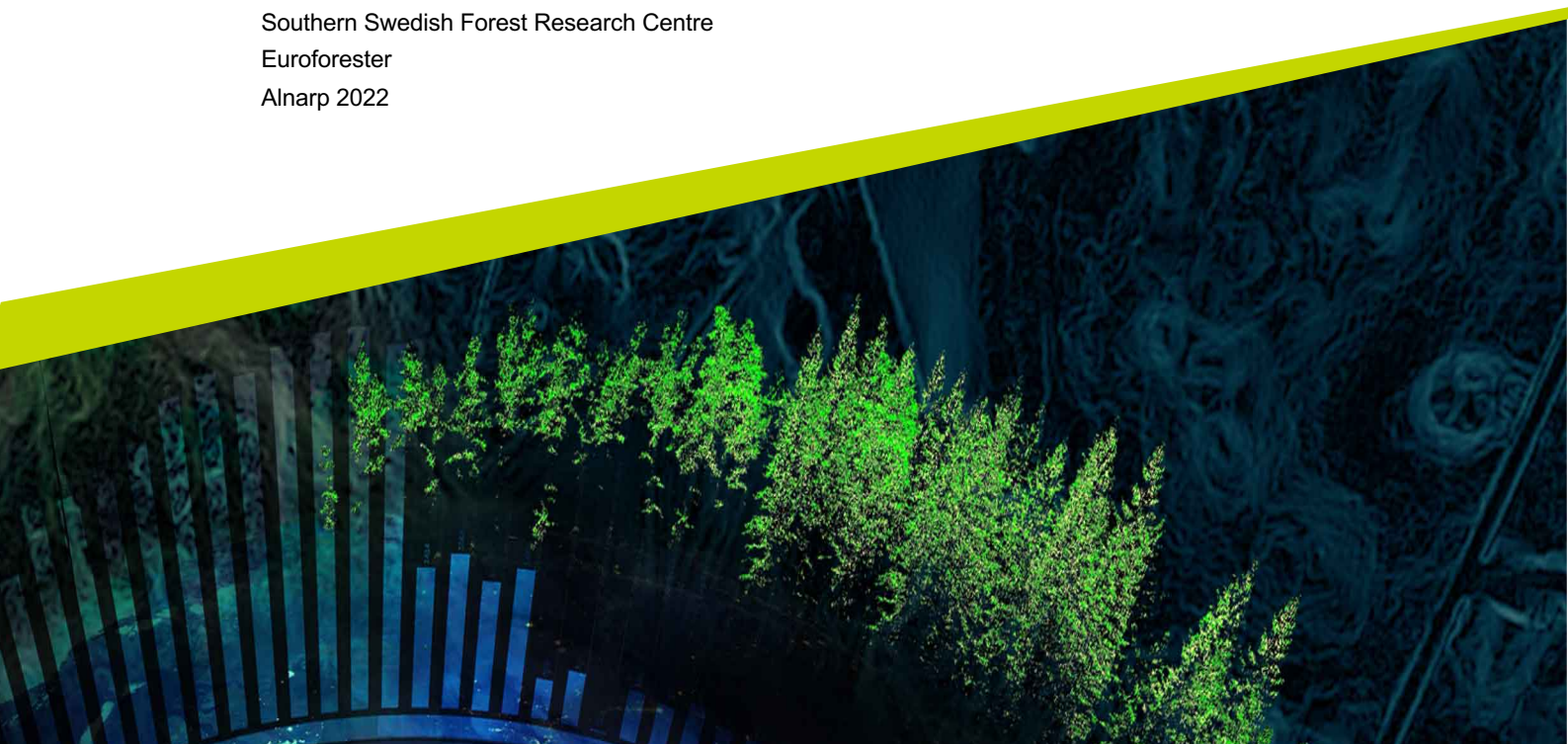
The effect of various nutrient media on the captured diversity of culturable seed endophytes in European beech (*Fagus sylvatica* L.)

Kinga Stolarek

Master's thesis • (30 credits)
Swedish University of Agricultural Sciences, SLU
Southern Swedish Forest Research Centre
Euroforester
Alnarp 2022



The effect of various nutrient media on the captured diversity of culturable seed endophytes in European beech (Fagus sylvatica L.)

Author's name: Kinga Stolarek

Supervisor:	**Iva Franić, Swedish University of Agricultural Sciences, Southern Swedish Forest Research Centre**
Assistant supervisor:	Michelle Cleary, Swedish University of Agricultural Sciences, Southern Swedish Forest Research Centre
Examiner:	Jorge Aldea, Swedish University of Agricultural Sciences Southern Swedish Forest Research Centre

Credits:	30 credits
Level:	A2E
Course title:	Master's thesis in Forest Science
Course code:	EX0984
Programme/education:	Euroforester - Master´s Programme
Course coordinating dept:	Southern Swedish Forest Research Centre
Place of publication:	Alnarp
Year of publication:	2022
Keywords:	fungal endophytes, seeds-associated fungi, *Fagus sylvatica* L, nutrient media, tree pathogens

Swedish University of Agricultural Sciences

Faculty of Forest Sciences

Southern Swedish Forest Research Centre

Publishing and archiving

Approved students' theses at SLU are published electronically. As a student, you have the copyright to your own work and need to approve the electronic publishing. If you check the box for **YES**, the full text (pdf file) and metadata will be visible and searchable online. If you check the box for **NO**, only the metadata and the abstract will be visible and searchable online. Nevertheless, when the document is uploaded it will still be archived as a digital file. If you are more than one author, the checked box will be applied to all authors. Read about SLU's publishing agreement here:

- https://www.slu.se/en/subweb/library/publish-and-analyse/register-and-publish/agreement-for-publishing/.

☒ YES, I/we hereby give permission to publish the present thesis in accordance with the SLU agreement regarding the transfer of the right to publish a work.

☐ NO, I/we do not give permission to publish the present work. The work will still be archived and its metadata and abstract will be visible and searchable.

Abstract

The associations of fungal endophytes with trees are poorly understood, especially within tree seeds. Recent molecular studies show that tree seeds harbour highly diverse fungal communities, including potential plant pathogens that could be introduced to new areas when seeds are exchanged. However, only a small fraction of these fungi can be detected when traditional culturing is used. Traditional culturing is widely used for assessing fungal diversity in plant tissues because it allows the detection of viable fungi and provides pure isolates for future experiments. However, various nutrient media have been used for fungal culturing and it is unknown if the choice of nutrient media affects the captured diversity. This study assesses the overall diversity of endophytes in seeds of European beech (*Fagus sylvatica* L.) and tests the effect of media and seed origins on endophyte diversity by growing fungi from seeds from six locations on three commonly used types of media that differ in carbon sources (Malt Extract Agar, Potato Dextrose Agar and Water Agar). The results show that almost all tested seeds of European beech yielded fungi, and reveal high diversity of fungal endophytes, including plant pathogens. Captured diversity and community composition stayed unchanged irrespective of the medium type, but it differed across locations of seed origin. The results show that similar communities are obtained using three most commonly used media which indicates that most of the fungi are able to utilise simple and complex carbohydrates and allows comparison of diversity assessed by those media. The results also indicate that dangerous fungal pathogens could be moved within the seeds exchanged for tree reforestation purposes which calls for the use of local seeds for reforestation.

Keywords: fungal endophytes, seeds-associated fungi, *Fagus sylvatica* L, nutrient media, tree pathogens

Preface

I would like to express my deepest appreciation to my Supervisor, Iva Franić. Thank you for your invaluable contribution, suggestions and advice. Thank you for your profound belief in my work and the intention to teach me as many skills as possible.

I am also extremely grateful to my co-supervisor, Michelle Cleary, and to the all community of Forest Pathology Lab in Alnarp for constructive advice and guidance during my laboratory work. I would like to thank Beatrice Tolio for her help with photos and general support.

I would like to extend my sincere thanks to my family, especially to my parents and siblings. Particularly supportive to me during this time was Edžus Romāns. I am also grateful to my friends including the Euroforester program 2020/21.

Table of contents

List of tables

List of figures

Abbreviations

DNA	Deoxyribonucleic acid
HTS	High-throughput sequencing
MEA	Malt Extract Agar
PDA	Potato Dextrose Agar
WA	Water Agar

Introduction

1.1 Importance of European beech (*Fagus sylvatica* L.) in Europe

European beech (*Fagus sylvatica* L.) is an important and widespread broadleaf tree species in Europe (Durrant et al. 2016). Together with Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) H.Karst), it contributes to more than a half of all forest reproductive material (i.e., tree parts that can be used for its regeneration e.g., seeds, seedlings) in Europe (SoEF, 2020; Konnert et al. 2015). It is one out of five economically most important tree species in Europe, mainly due to its high-quality wood (SoEF, 2020). This species is also important for increasing biodiversity, especially for the presence of lichens' and bryophytes' communities in old-growth beech stands (Brunet et al. 2010). The natural range of European beech extends from Bergen in southern Norway in the north to Sicily in southern Italy in the south, and from the Cantabrian Mountains in Spain in the west to the Balkan and Carpathian Mountains to the east (Fig. 1; Durrant et al. 2016). This tree species needs at least 140 days of vegetation period (i.e., part of a year with appropriate temperature and humidity for plant growth) which currently limits its presence in Scandinavia (Durrant et al. 2016, Koncewicz and Lewak 2007). However, future forecasts suggest that the distributional range of European beech will decrease in eastern and southern Europe due to climate change while expanding towards northern Europe (Kramer et al. 2010). European beech may thus become an increasingly important tree species for northern Europe.

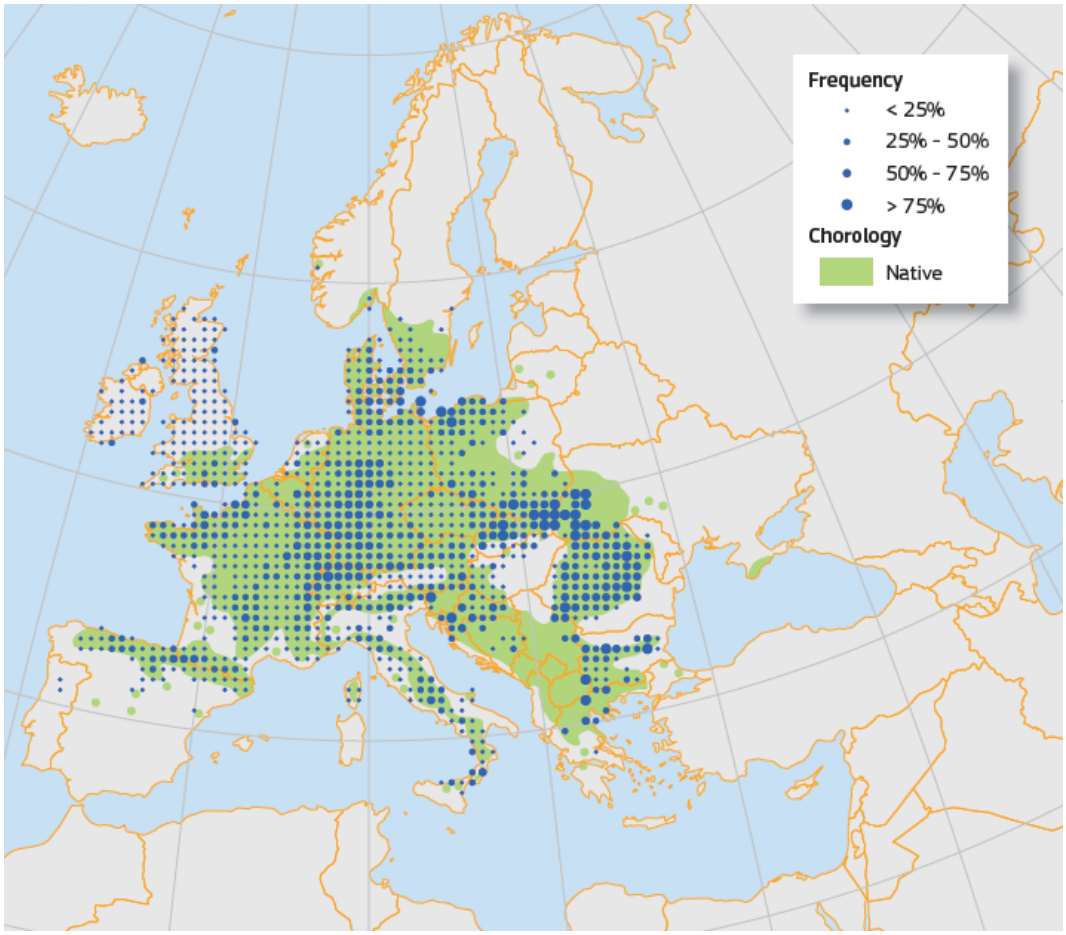


Figure 1. Distribution and occurrence frequency of European beech in Europe (Durrant et al. 2016)

1.2 Risk associated with the exchange of European beech reproductive material

European beech currently does not encounter any major pests (i.e., any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products; FAO, 2007) in Europe (SoEF, 2020), but climate change and international trade might cause the emergence of new pests in the future (Durrant et al. 2016; Santini et al. 2013). The historical example of a devastating disease caused by a non-native pest is chestnut blight caused by *Cryphonectria parasitica* (Murrill) M.E. Barr on American chestnut (*Castanea dentata* (Marsh.) Borkh.) and European chestnut (*Castanea sativa* Mill.). This pathogen was introduced in the

19th century from Asia to North America where it caused especially huge dieback of the American chestnut with serious ecological and economic consequences (Prospero and Cleary 2017). Another more recent, non- native fungal pest, *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya has been threatening European forests and causing dieback of European ash (*Fraxinus excelsior* L.) (Baral et al. 2014) with unpredictable consequences for forest ecosystems. The number of non-native fungal pathogens has been increasing significantly during last decades (Santini et al. 2013) mainly due to the exchange of plant material. The elimination of already established non-native forest pathogens is difficult and sometimes even impossible (Prospero and Cleary 2017). Therefore, it is crucial that potential pests are detected before or early upon their introduction in order to reduce the damage and protect the forests.

Although natural regeneration is the most common way of regeneration of European beech (Övergaard, 2010), beech stands are sometimes established from seeds and seedlings obtained from international trade (Jansen et al. 2019). For example, during 2004–2014 Sweden imported around 45,000 kg of beech seeds annually from seven different European countries, predominantly from Hungary. Similarly, around 163,000 seedlings were imported to Sweden during that period, mainly from Germany and Denmark (Jansen et al. 2019). Phytosanitary measures are in place to reduce the risk of the introduction of non-native pests via plant trade. In Europe, those measures target trade of tree species and commodities which are likely to serve as a pathway for regulated pests (Eschen, 2015). Most of the tree seeds are considered as non-significant pathways for non-native pests, and their trade is thus not regulated. Nowadays, only the exchange of seeds of Douglas-Fir (*Pseudotsuga menziesii* (Mirb.) Franco) and seeds of *Pinus* spp. are regulated for international or national movement (Vettraino et al. 2018). However, recent studies have shown that tree seeds carry highly diverse fungal communities including known and unknown pathogens (Franic et al 2019, Cleary et al 2019). Furthermore, these pathogens are often asymptomatic and because of their microscopic size, almost impossible to detect during phytosanitary inspections (Brasier 2008). The large amounts of traded plant material, including seeds, and variety of origins increase the potential of spreading known and unknown fungal pathogens of trees. It is thus important to increase our knowledge about pathogens hidden in tree seeds to improve biosecurity of seed exchange.

1.3 Fungal endophytes associated with European beech

Endophytes are organisms (e.g., bacteria or fungi) occupying plant tissues for at least some part of their life cycle showing no visible signs of presence (Petrini, 1991). Thereby, they do not cause any disease symptoms. Endophytes can be dormant saprotrophs, mutualists, or even latent pathogens (Rodriguez et al. 2009). Moreover, they can change the character of their relationship with the host during the inhabiting time. For example, the activation of latent pathogens may depend on the genotype of the host or the appearance of stress conditions (Bacon et al. 2008, Khare et al. 2018). For instance, the *Ramularia collo-cygni* B. Sutton & Waller is the endophyte which can become a pathogen during the vegetative season (Walters et al. 2008, Khare et al. 2018). Also, endophytes of woody plants of the Botryosphaeriaceae family may become active pathogens when a tree is under stress (Slippers and Wingfield, 2007). Characterization of endophytes associated with European beech seeds would help to better understand current and emerging phytosanitary risks associated with seed exchange.

Endophytes are a widespread and highly diverse group of organisms. They were detected in all terrestrial ecological systems from polar regions to the tropics (Rashmi et al. 2019; Zhang et al. 2013). They are known to live in association with about 300, 000 of different plant hosts around the world. Their diversity is currently probably underestimated considering that they were characterized from only 1-2% of plant species (Strobel and Daisy 2003, Khare et al. 2018). Abiotic and biotic conditions (e.g., climate, surrounding plant community composition and host tree species) can influence endophyte diversity (Franic et al. 2020). Endophytes can occupy various parts of plants such as roots, leaves and seeds (Rana et. al 2019). Studies on beech' endophytes explored several tree tissues such as xylem, whole stems (Petrini and Fisher 1988), leaves (Unterseher and Schnittler 2010), buds and twigs (Toti et al.1993). Previous studies recorded high diversity of fungal taxa associated with European beech (Sieber and Hugentobler 1987, Pehl and Butin 1994, Unterseher and Schnittler 2010). The phylogenetic diversity analysis of endophytes' beech shows that the majority of detected fungi belong to non-parasitic Pezizomycotina lineages (Unterseher and Schnittler 2010). On the other hand, among endophytes associated with buds and twigs of beech, almost

half (49%) were assigned to species *Apiognomonia errabunda* (Rob.) von Hohnel, which causes leaf anthracnose of beech (Toti et al.1993).

Seed-borne fungi have been studied for years using traditional culturing methods (Cappelli and Covarelli 2005). However, most of the studies have been targeting foliar fungal endophytes, and lower diversity of seed-borne fungi in comparison with foliar endophytes might have been because of the research bias. Recent molecular studies revealed that true diversity of seed-endophytes might be much higher than previously anticipated (Franic et al. 2019, Franic et al. 2020, Cleary et al. 2019). Recent study recorded a high diversity of fungi in 12 seed lots of European beech – 38 fungal genera, 11 of which are known as potential plant pathogens were characterised from those seeds (Franic et al 2020). The other study identified two potentially pathogenic genera among endophytes of beech seeds: *Fusarium* sp. and *Phomopsis* sp. (Manka et al. 2012). A large diversity of fungi detected in seeds and limited understanding of their distribution, host preferences, and potential pathogenicity stresses the need for more research.

Endophytes, like all fungi, can be characterized by oligotrophic or copiotrophic life strategy depending on their ability to utilise available nutrient resources. Oligotrophs grow slower and they are more successful in utilization of complex carbon sources than copiotrophs. On the other hand, copiotrophic fungi prefer simple carbohydrates as carbon sources. Some copiotrophic fungi can also change their life strategy and start to expose more oligotrophic traits. In the majority, the kingdom of fungi is dominated by oligotrophs. Life strategies determine the prevalence of microorganisms in the environment (Ho et al.2017). The life strategy of fungi may also have implications for endophyte diversity studies. Based on information about the life strategy of a particular fungus, researchers can select the most proper methods and materials for their detection

1.4 Traditional plating as a method for the diversity assessment of fungal endophytes

One of the most common methods for characterizing fungi in plant tissues is plating. This method allows for obtaining fungal cultures from plant tissues by putting fragments of plants on a particular nutrient medium. Therefore, a type of a

medium is a very important component of experiments using traditional plating. Among other features, media vary in nutrient concentration. According to this, we can distinguish between nutrient-poor and nutrient-rich media. Nutrient-poor media pose the base for preparing all other media (Kwasna 2014). However, they can also be used as a substrate for fungal growth. For example, Water Agar (WA) is commonly used as a nutrient-poor medium (Ajah and Mohammed, 2015). The main ingredient of this medium, besides water, is only agar (Ritchie et al. 2009, Ajah and Mohammed, 2015). Agar is an extract of a sticky glycan from the cell walls of red algae (Solomon et al. 2014). The main carbon source of this medium which is available for microorganisms is agarose and agaropectin, both being complex polysaccharides. Examples of nutrient-rich media are commonly used Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA). PDA is a nutrient-rich medium consisting of agar, dextrose and potato extract (Ravimannan et al. 2014, Ritchie et al. 2009). Fungi growing on PDA can utilize monosaccharide dextrose molecules as a “simple” carbon source and potato extract as a nitrogen source. MEA is also a nutrient-rich medium (Nunez et al. 2007). Generally, it consists of agar, malt extract, dextrin and gelatin peptone (Ajah and Mohammed, 2015). Present maltose is a disaccharide carbon source, vegetable peptone is the source of nitrogen and dextrin is an additional component. All three mentioned types of nutrient media (WA, PDA and MEA) are commonly used for fungal assessment (Black 2020; Choi et al. 1999). Furthermore, we can differentiate between universal, semi selective and selective types of media. Universal media allow for the detection of various microorganisms groups. Semiselective media contain the addition of antibiotics, which give an opportunity of testing the diversity of fungi while excluding bacteria. The purpose of using selective media is to detect a specific group of organisms or even particular species. It can be also used for obtaining selective taxon of fungi by adding chemicals that inhibit or support the growth of specific fungi or create a favourable environment for a narrow range of fungi (Tsao 1970; Kwasna 2014).

In general, previous studies do not present a detailed summary of differences in fungal diversity between nutrient-poor and nutrient-rich media. However, significant differences in fungal diversity and community composition are possible because copiotrophic and oligotrophic fungi might grow differently depending on a nutrient medium. Fungi growing on nutrient-rich media can grow faster and

produce more abundant antibiotics, which inhibit other fungi (Bu'lock 1975, Whipps 1987). Whereas nutrient-poor media promote slow-growing fungi, and the same they minimize the risk of overgrowing one fungus by other. Also, testing of low- nutrient media can exhibit the fungi diversity in conditions of lack of some nutrients, which effect can be similar to the occurrence of stress factors (Whipps 1987). The majority of publications use only one type of medium for culturing of microbes which do not allow the comparison of fungal communities obtained on different media (Oita et al. 2021). Therefore, it is crucial to explore the more detailed effect of various nutrient media on endophytes diversity.

The most common detection methods for fungi in plant tissue are plating and more recently high-throughput sequencing (HTS). HTS is the technology of DNA and RNA sequencing in a relatively short time (Szefler et al. 2017). It reveals much higher fungal diversity than traditional plating (Franic et al. 2019) and is cheaper when large numbers of samples are included (Eschen et al. 2019). However, HTS mostly uses DNA genes as barcodes for fungal detection and identification, and thus does not discriminate between DNA coming from dead or living organisms (Franic et al. 2019, Gullino et al. 2020). Culturing in contrast to HTS reveals only living cultures, which is essential for the evidence of the presence of a viable pathogen, for identification of unknown species, and experiments that need living pure cultures. It is thus necessary to develop and improve the traditional plating method for fungal diversity assessments.

1.5 Research aims and hypothesis

The objectives of this study were to: 1) assess the overall diversity of fungal endophytes of European beech seeds from several locations in Europe, including plant pathogens, 2) assess the differences in diversity and community composition of obtained fungi among different media and seed origins (i.e., location of collection). For this purpose, fungi were grown for seeds of European beech obtained from six locations within Europe on three types of nutrient media differing in nutrient sources. The media chosen for this experiment are Water Agar (WA;

nutrient-poor medium), Potato Dextrose Agar (PDA; nutrient-rich medium) and Malt Extract Agar (MEA; nutrient-rich medium).

In relation to research objective 2) I hypothesize that:

i. endophyte diversity will be significantly different between three types of nutrient media - the diversity on WA will be higher than on PDA and MEA. This is because fungi might grow slower and produce less antagonistic compounds on WA than on PDA and MEA, which could increase the chances of capturing higher diversity on WA than on nutrient-rich media.
ii. community composition of fungal endophytes will differ among three types of media because of the differences in fungal life strategies, i.e., copiotrophic fungi are adapted to nutrient-rich environments while oligotrophic fungi thrive in nutrient-poor environment
iii. diversity of endophytes will differ between locations.

The results of this study are contributing to the knowledge about fungal endophytes associated with European beech seeds from different locations within Europe and will help to assess the phytosanitary risks associated with the movement of beech seeds. Furthermore, the results will show the efficacy of various nutrient media commonly used for the assessment of tree endophytes for revealing the fungal diversity and will help to determine and help to choose the best media for detecting fungi in plant tissue during traditional culturing.

2. Materials and methods

2.1 Study materials

Study seeds of European beech were obtained from commercial seed suppliers from six locations within Europe: Denmark, Germany, Switzerland, Poland, Slovakia and Austria.

2.2 Methods

2.2.1 Surface-sterilization of seeds

Seeds were first surface sterilized to reduce environmental contamination. In total, about 200 seeds from each seed lot (i.e., seeds of European beech from a location) were surface-sterilized by subsequent immersion in a solution of 70% ethanol (1 minute), 1% sodium hypochlorite (5 min), 70% ethanol (30 sec), and sterile water (30 sec) (Fig.2; Gamboa et al. 2003). After sterilization, seeds were left to dry in a Biological safety cabinet (i.e., enabling sterile conditions for laboratory work) and stored at 3-5 °C until processing.

Figure 2. Surface-sterilization of seeds using 70% ethanol, 1% sodium hypochlorite and sterile water.

2.2.2 Cultivation of seed fungi

Total of 90 seeds per seed lot was used for fungal assessment. Seeds were crushed with a sterile pestle to allow the emergence of fungi from the inside of the seeds. Crushed seeds were placed on three types of media, 30 seeds per seed lot and medium. I used: 1.5 % water agar (WA, Agar bacteriological, 15 g/L; VWR Chemicals, Solon, Ohio, USA), Potato Dextrose Agar (PDA, 39g/L; Merck KGaA, Darmstadt, Germany) and Malt Yeast Extract Agar (MEA, 30g/L; Duchefa Biochemie, Haarlem, The Netherlands, WA, Agar bacteriological, 15 g/L; VWR Chemicals, Solon, Ohio, USA). Each medium type was supplemented with streptomycin (0.05 mg/ml) to prevent bacterial growth. Media were selected to allow the emergence of fungi that utilize different carbon sources. Selected media varied in nutrient content including different carbon and nitrogen sources. Water agar (WA) was used as a nutrient-poor medium that contains two complex polysaccharides as a carbon source, agarose and agaropectin. The main carbon source in Malt Yeast Extract Agar (MEA) is disaccharide maltose, and in Potato Dextrose Agar (PDA) it is monosaccharide dextrose. MEA and PDA belong to

nutrient-rich media and they both contain nitrogen sources. Two seeds were placed on each plate with media. Plates were stored in the laboratory room in boxes. Boxes were sealed with tape with vaseline to protect plates from mites.

2.2.3 The isolation of pure fungal cultures and grouping into morphotypes

Plates were checked for occurring endophyte colonies at equal intervals for 30 days, every five days. Each observed fungus was transferred to a small plate with Potato Dextrose Agar (PDA: 39 g/L; Fig. 3). PDA was used for cultivation of pure cultures because on PDA fungi develop species specific morphologies which is not the case when WA is used. Clean fungal cultures were grouped according to macro-morphological characteristics such as colour, texture, form, margin (i.e., the appearance of their edge) and the elevation (Microbiology, 2014; Fig.4).

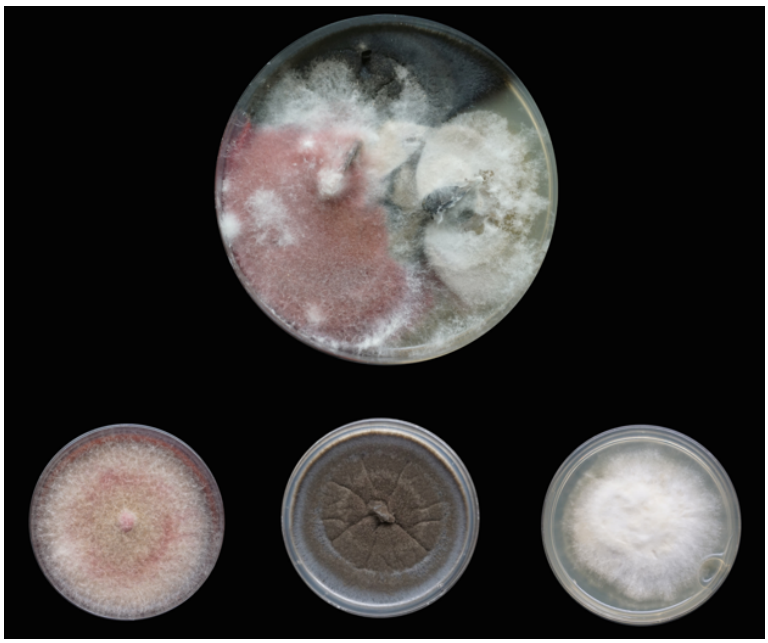


Figure 3. Three pure fungal cultures (small PDA plates at the bottom of the photo) were isolated from one big original PDA plate with two seeds (upper part of the photo). The taxonomy of mentioned pure fungal cultures: *Fusarium* sp., *Discosia artocreas* (Tode) Fr., *Fusarium* sp. (respectively, from the left side of the photo).

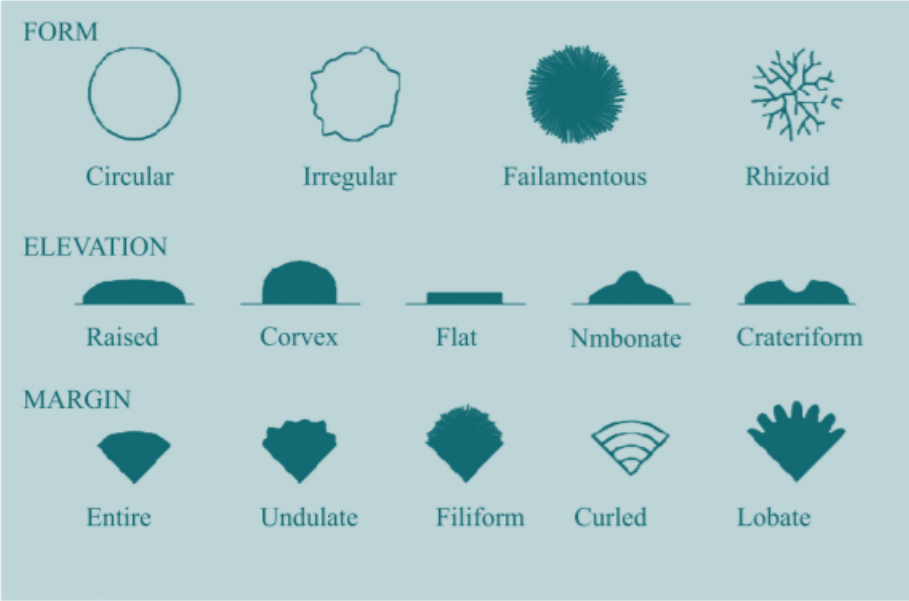


Figure 4. The examples of morphological features of fungal isolates growing on nutrient media (Microbiology, 2014).

2.2.4 Identification of fungi

2.2.4.1 DNA extraction

One representative culture of each group of morphotypes was chosen for DNA extraction. Fungal mycelia was scraped off and placed in 2 ml centrifuge tubes and freeze-dried overnight. The process of freeze-drying preserves samples by removing water from fungal mycelia so samples can be stored at room temperature until further processing (Wolkers and Oldenhof 2015).

Extraction of DNA was carried out using E.Z.N.A.® SP Plant DNA Kit (Omega Bio-Tek, Norcross, Georgia, USA), following the manufacturer's instructions. This kit allows DNA extraction from fungal mycelia and consists of lysis of fungal tissues and DNA purification from proteins and cellular fluid.

2.2.4.2 Amplification of rDNA ITS region and Sanger sequencing

DNA concentration in each sample was measured using a DS-11 UV-Vis Spectrophotometer (DeNovix), which provides qualitative and quantitative DNA analysis, and DNA was then diluted to 5 ng/ul. The dilution step reduces the

concentration of inhibitors which allows the successful amplification of marker genes during Polymerase Chain Reaction (PCR; Castle et al. 2018). Samples with DNA concentration below 5 ng/ul were not diluted. The ribosomal internal transcribed spacer (ITS) region, which provides the most successful identification for fungi, was amplified in PCR (Schoch et al. 2012). ITS region was amplified in the volume of 25 µl in PCR, including 2 µl of DNA, 8.5 µl of H2O, 12.5 µl of DreamTaqPCR Master Mix (2X), ITS1 (forward) and ITS4 (reverse) primers in the quantity of 1 µl each (Tedersoo et al. 2014). PCRs were carried out using Eppendorf Mastercycler following the conditions: 2 min at 95°C, 35 cycles of 1 min at 95°C, 45 s at 55°C, 1 min 30 s at 72°C, and strand completion by 5 min at 72°C. The presence of fungal DNA in samples was confirmed with electrophoresis in which DNA fragments of different lengths migrate under the influence of an electric field. Subsequently, they can be properly distinguished in comparison with the known length of DNA fragments from the DNA marker (Lee et al. 2012). Afterwards, PCR products were sent to Macrogen (Amsterdam, Netherlands) for purification and sequencing using chain termination method (i.e., Sanger sequencing) with the same primers that were used in PCRs.

2.2.4.3 Sequence analysis and identification

The Bioedit program (Hall, 1999) was used to trim the low quality ends and to align forward and reverse DNA sequences into consensus sequences. The Basic Local Alignment Search Tool (BLAST) was used for comparing obtained sequences with sequences in The National Centre for Biotechnology Information (NCBI) database (Geer et al. 2010). Fungal sequences were assigned to species if the similarity showed less than 1% divergence from the reference sequence. If a specimen was matched to multiple taxa with the same similarity rank, the assignment was ranked down to the next taxonomic level (i.e., genus, family, suborder, order, class and subphylum level). The sequence was not identified when the similarity was lower than 99%. Finally, I used the Fungal Databases, U.S. National Fungus Collections, ARS, USDA (Farr and Rossman 2019) for collecting data about potential pathogenicity, host associations and distribution of identified fungal species.

2.2.5 Statistical analyses of data

All statistical tests were conducted in R (R Core Team 2018). Statistical tests were considered significant at $P < 0.05$.

2.2.5.1. Differences in fungal diversity among media and locations

Three measures of fungal diversity: endophyte richness (i.e., number of fungal morphotypes per seed lot), Shannon diversity index (H') and Simpson's index (1-D) were considered in this study. Endophyte richness is based on presence/absence data, whereas the Shannon diversity index and Simpson's index take into account the abundance data (diversity function from vegan package, Oksanen et al. 2020). Shannon diversity index increases with biodiversity and depends on the number of species in a sample and the abundances of those species, i.e., it is a measure of "evenness" which tells us how similar the abundances of different species are in a sample. Simpson's index is a dominance index because it gives more weight to common or dominant species. In this case, a few rare species with only a few representatives will not affect the diversity. This approach allowed to assess the effects of study factors on the overall fungal diversity, and diversity of dominant species.

The effects of medium type and location on three measures of fungal diversity Endophyte richness, Shannon diversity index and Simpson's index were tested according to the following mathematical equation of the model: Y ~ country + media, in which Y represents the response variable and "country" and "media" represent the independent variables. Generalized Linear Model (GLM) was used to test the effect of medium type and location on endophyte richness. For this, the glm function from the stats package (R Core Team, 2021) with a Poisson distribution, which is usually used for modelling count data (Venables and Ripley, 2002; Sellers and Shmueli, 2010) was used. The effects of medium type and location on the Shannon diversity index (H') and the Simpson's Index (1-D) were analysed using log-transformed data with linear models (lm function from stats package; R Core Team, 2021). The significance of the fitted models and individual factors was analysed using the Anova function from the car package (Fox, J. 2016). The Tukey's post hoc tests were run using the emmeans function (package 'emmeans'; Lenth et al. 2020) for the factors showing significant differences. This

allowed me to estimate the marginal mean for each variable and determine which groups within a factor are statistically different. Plots of fungal diversity measures were constructed using the ggplot function (package ggplot2; Wickham 2009).

2.2.5.2 Differences in fungal community composition among media and locations

The effects of medium type and location on three measures of the differences in community composition (i.e., beta diversity): Sørensen's dissimilarity index, Horn index and Morisita–Horn index were also assessed. The Sørensen's dissimilarity index is calculated from presence-absence data, in contrast to Horn index and Morisita–Horn index which are based on the abundance data. All these indices were calculated using vegdist function from vegan package (Oksanen et al. 2020). These three measures of differences in community composition were used because of their different sensitivity to dominant species, similar as for fungal diversity. The Sørensen's dissimilarity index weights species equally because of its insensitivity to species abundance, Horn index considers abundance, but weights common and rare species equally, and the Morisita–Horn index gives more weight to abundant than rare species (Libório and Tanaka, 2016; Jost 2007).

Differences in fungal community composition across samples belonging to different media types and to different locations were assessed with permutational multivariate analysis of variance [PERMANOVA (Anderson 2001); adonis2 function from the vegan package (Oksanen et al. 2020)], based on the mathematical equation of the model – Y ~ country + media, in which Y represents the response variable and "country" and "media" represent the independent variables. The significance of each variable was analysed using marginal tests available in adonis2, which assesses the unique impact of each variable when added after all others. Corresponding R2 values that indicate the size of the effect of each variable were also calculated. To visualize the differences in fungal community composition among media and locations I used Nonmetric Multidimensional Scaling (NMDS, function metaMDS, vegan package; Oksanen et al. 2020) with plotting 95% confidence interval ellipses (Ordiellipse; vegan package; Oksanen et al. 2020).

Moreover, I used the Venn Diagram tool (Venn function; package ‘limma’ Ritchie et al. 2015) for showing the number of fungal morphotypes that are unique and shared between different media.

3. Results

3.1 Overall fungal taxonomy

Fungal endophytes were cultured from about 99.4 % of all seeds (537 seeds out of 540). A total number of 1,436 fungal cultures were isolated from those seeds. Obtained cultures were assigned to 145 morphotypes. More than 80% of morphotypes (121 out of total 145) were represented by more than 1 isolate, whereas 24 morphotypes were singletons (i.e., represented by only 1 isolate). The most abundant morphotypes (n = 26) represent 62% of total 1,436 isolates (Tab.1; Fig.5).

DNA was successfully extracted from around 92% of representative cultures (133 out of 145). From all DNA samples ITS region was successfully amplified in PCR and sequenced. About 91% (121 out of 133) of fungal sequences provided good quality data for the identification of fungal taxonomy. Around 18% of obtained sequences were assigned to species, 36.2% to genus, 10.5% to family, 1.5% to suborder, 2.3% to order, 6% to class and 16.5% to subphylum level. Around 9% of sequences could not be assigned to any taxonomy rank. The majority of identified fungal sequences belonged to phylum Ascomycota (99.2%) and only one individual to Basidiomycota (0.8%). Identified sequences were assigned to four main classes of fungi. Within Ascomycota, fungi were assigned to three classes: Sordariomycetes (39.7%), Dothideomycetes (37.2%) and Eurotiomycetes (4.1%). Within Basidiomycota, the identified species belonged to class Agaricomycetes (0.8%). Remaining fungal sequences (18.2%) were assigned to higher taxonomy rank of subphylum.

Potential tree pathogens were present in 42% of seeds (229 seeds out of 540). They posed about 21% all isolates (302 out of 1,436) and about 10% (27 out 261)

of obtained DNA sequences. All belong to two classes within phylum Ascomycota: Sordariomycetes (83.3%) and Dothideomycetes (16.7%). Plant pathogenic fungi were represented by three species: *Apiognomonia errabunda* (Rob.) v. Höhn., *Apiognomonia veneta* (Sacc. & Speg.) Höhn., *Diaporthe rudis* (Fr.) Nitschke) an four genera: *Diaporthe* sp., *Fusarium* sp., *Phoma* sp., *Xylaria* sp.

Table 1. The relative abundance of the 26 most abundant morphotypes and their taxonomy rank

Morphotype	Abundance [%]	Taxon
M1	11.9	*Alternaria* sp.
M35	6.8	*Apiognomonia veneta*
M2	4.4	*Phoma* sp.
M58	4.3	Pleosporaceae
M27	4.1	Chaetosphaeriaceae
M8	2.3	*Fusarium* sp.
M16	2.0	unidentified
M38	2.0	Pezizomycotina
M25	1.8	Dothideomycetes
M4	1.8	*Fusarium sporotrichioides*
M36	1.8	Pezizomycotina
M39	1.8	Pezizomycotina
M30	1.5	*Diaporthe* sp.
M23	1.5	*Discosia pseudoartocreas*
M40	1.5	Pezizomycotina
M29	1.3	*Diaporthe rudis*
M13	1.2	*Alternaria alternata*
M26	1.2	Didymellaceae
M31	1.2	Pezizomycotina
M24	1.2	Dothideomycetes
M18	1.1	*Discosia* sp.
M28	1.1	Chaetosphaeriaceae
M60	1.1	Pleosporaceae
M123	1.0	*Cladosporium* sp.
M61	1.0	*Fusarium* sp.
M9	1.0	*Fusarium* sp.

Figure 5. Photos of fungal colonies of the twelve most common morphotypes (from the top left to the bottom right corner: M1; M2; M27; M8; M16; M38; M25; M4; M30; M23; M40; M13).

3.2 Differences in fungal diversity among media and locations

No significant differences in endophyte richness, Shannon diversity or Simpson's index were observed between three types of media (**Endophyte richness**: $\chi 2 = 0.89$, df = 2, P = 0.641; overall mean ± standard error = 33.8 ± 2.3; **Shannon index**: $\chi 2 = 0.004$, df = 2, P = 0.492; overall mean ± standard error = 3.19 ± 0.088; **Simpson's index**: $\chi 2 = 0.0008$, df = 2, P = 0.236; mean ± SE = 0.94 ± 0.0076; Fig 6. A, B and C, respectively, Supplementary Table 1).

Endophyte richness was not significantly influenced by location ($\chi 2 = 10.5$, df = 5, P = 0.0623; mean ± standard error = 33.9 ± 2.3; Fig 7. A), unlike the Shannon and Simpson's index (Supplementary Table 1). Samples from Poland had significantly lower Shannon index than samples from Switzerland ($\chi 2 = 0.05$, df = 5, P = 0.033; 2.96 ± 0.090 vs 3.44 ± 0.098, respectively; Fig. 7.B). Similarly, Polish samples had significantly lower values of Simpson's index in comparison with samples from Switzerland and Germany ($\chi 2 = 0.005$, df = 5, P = 0.025; 0.92 ± 0.013 vs 0.96 ± 0.0043 and 0.96 ± 0.0032, respectively; Fig. 7. C).

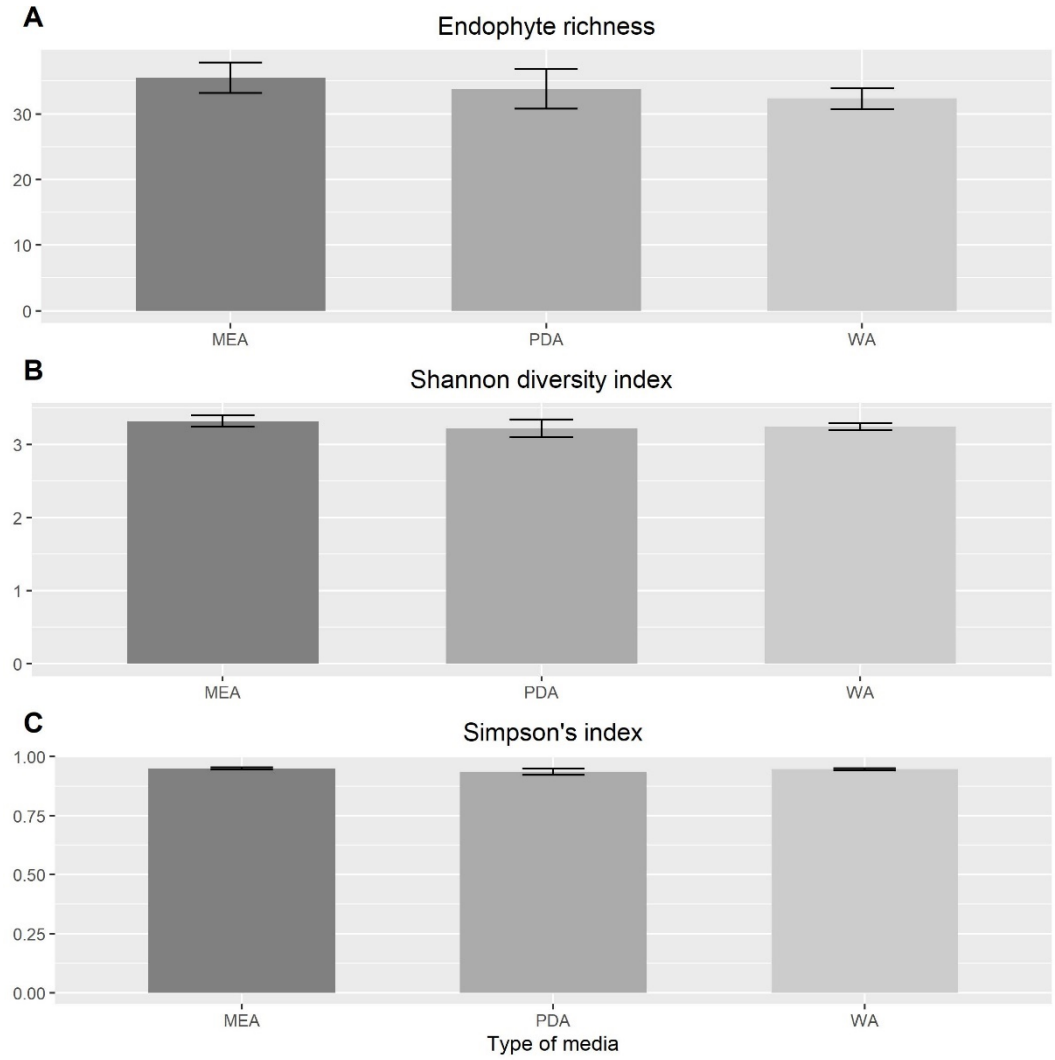


Figure 6. Fungal diversity across three types of media: Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Water Agar (WA). Fungal diversity is represented by three measures; Endophyte richness (A), Shannon diversity index (B) and Simpson's index (C). Values are mean and standard error.

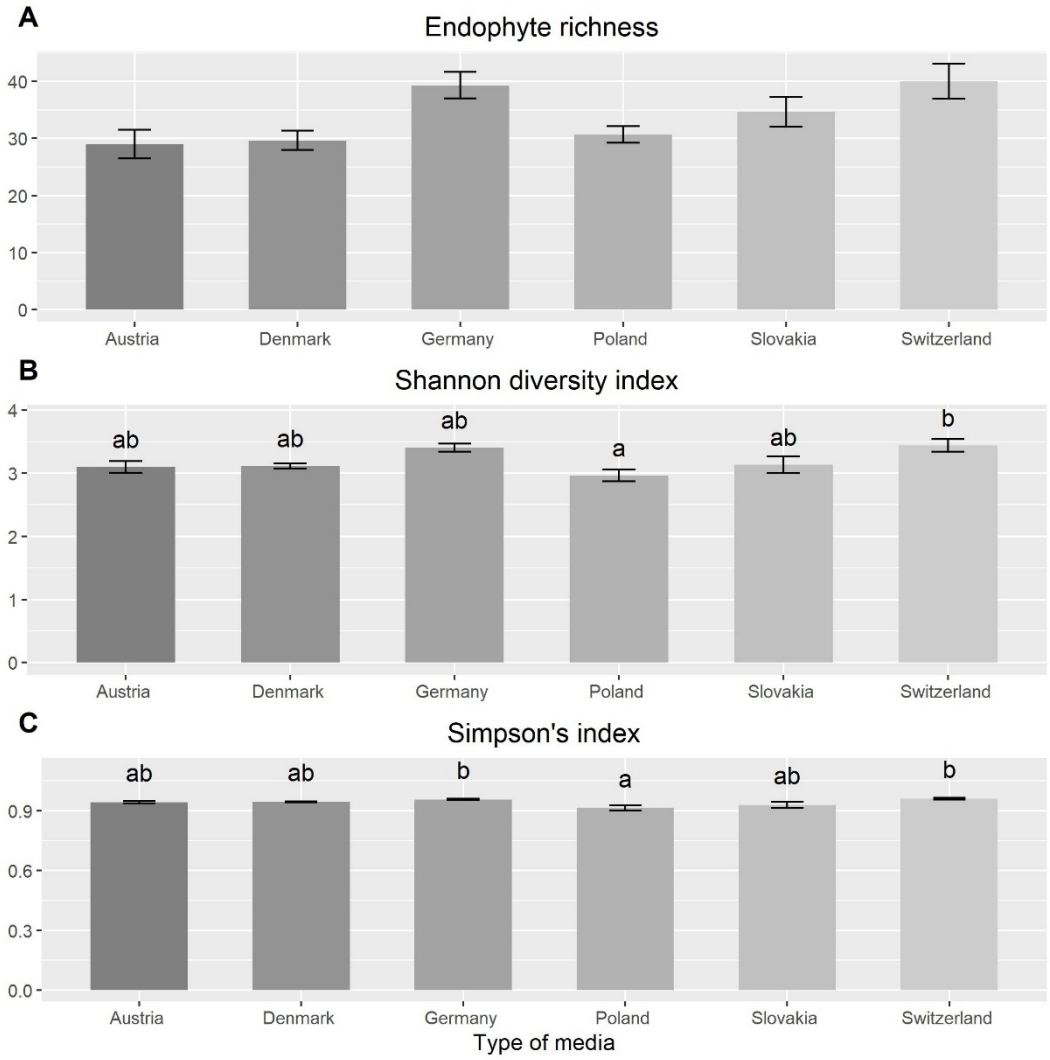


Figure 7. Fungal diversity across study locations. Fungal diversity is represented by three measures; Endophyte richness (A), Shannon diversity index (B) and Simpson's index (C). Different letters above the bars indicate significantly different values ($P < 0.05$). Values are mean and standard error.

3.3 Differences in fungal community composition among media and locations

The results show no significant differences in fungal community composition among three media, irrespective of the dissimilarity measure (Supplementary Table 2). Sørensen's dissimilarity index did not differ between three types of media (r2 = 0.091, df = 2, P = 0.190; Fig. 8A). Total of 62 out of 145 morphotypes (43%) appeared on all three types of media (Fig. 8B) and 44 out of 145 morphotypes (30%) have been found on two media. Around 27% (n = 39) of morphotypes were unique for a specific medium. The results were similar when the Horn's index was considered (r2 = 0.059, df = 2, P = 0.141; Fig. 8C). However, the analyses indicate significant differences in Morisita-Horn index among different media (r2 = 0.045, df = 2, P = 0.002; Fig. 8D).

Unlike the medium, location was an important factor driving the differences in endophyte communities (Supplementary Table 2). The Sørensen's dissimilarity index (r2 = 0.534, df = 5, P = 0.001; Fig. 9A), the Horn's index (r2 = 0.754, df = 5, P = 0.001; Fig. 9B), and the Morisita-Horn index (r2 = 0.926, df = 5, P = 0.001; Fig. 9C) all show significant differences between locations.

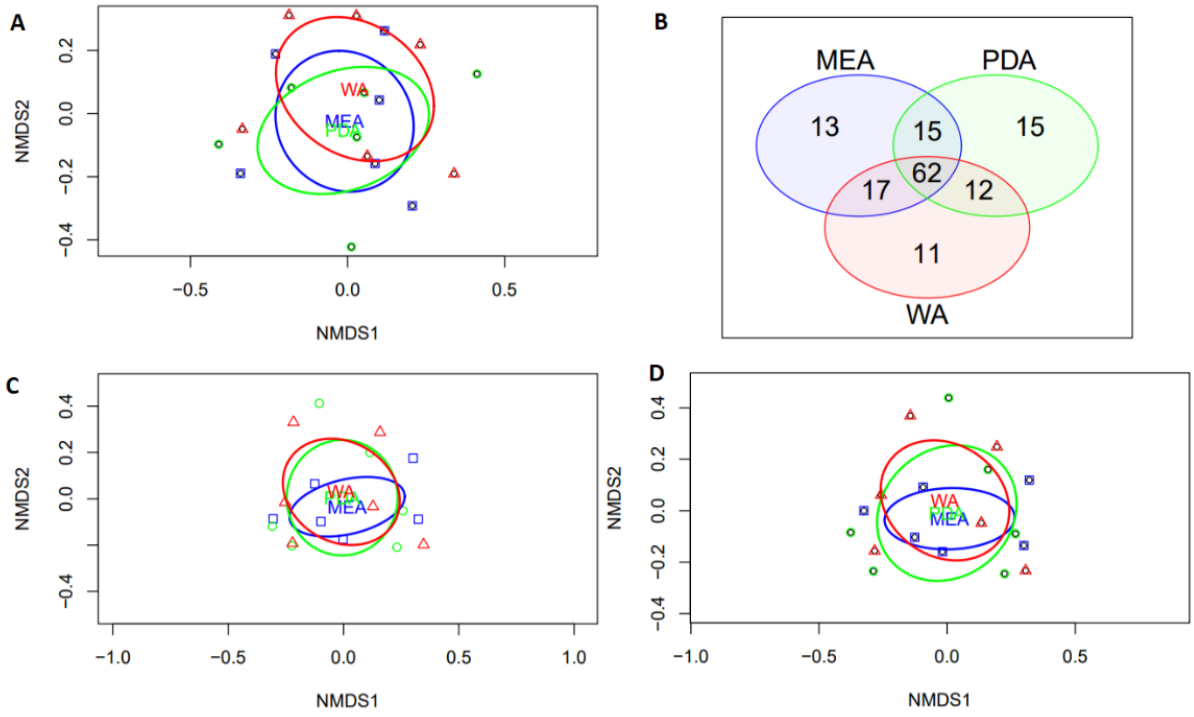


Figure 8. Non-metric multidimensional scaling (NMDS) plot presenting differences of endophyte communities on three types of media. Each point represents a sample (i.e., a sample is 30 seeds from a location per medium). Samples belonging to different media are distinguished by blue squares (MEA), green circles (PDA) and red triangles (WA). Plots are based on different dissimilarity measures: Sørensen's dissimilarity index (A), Horn's index (C) and Morisita- Horn index (D). The Venn diagram shows numbers of fungal morphotypes that are unique and shared between different media (B).

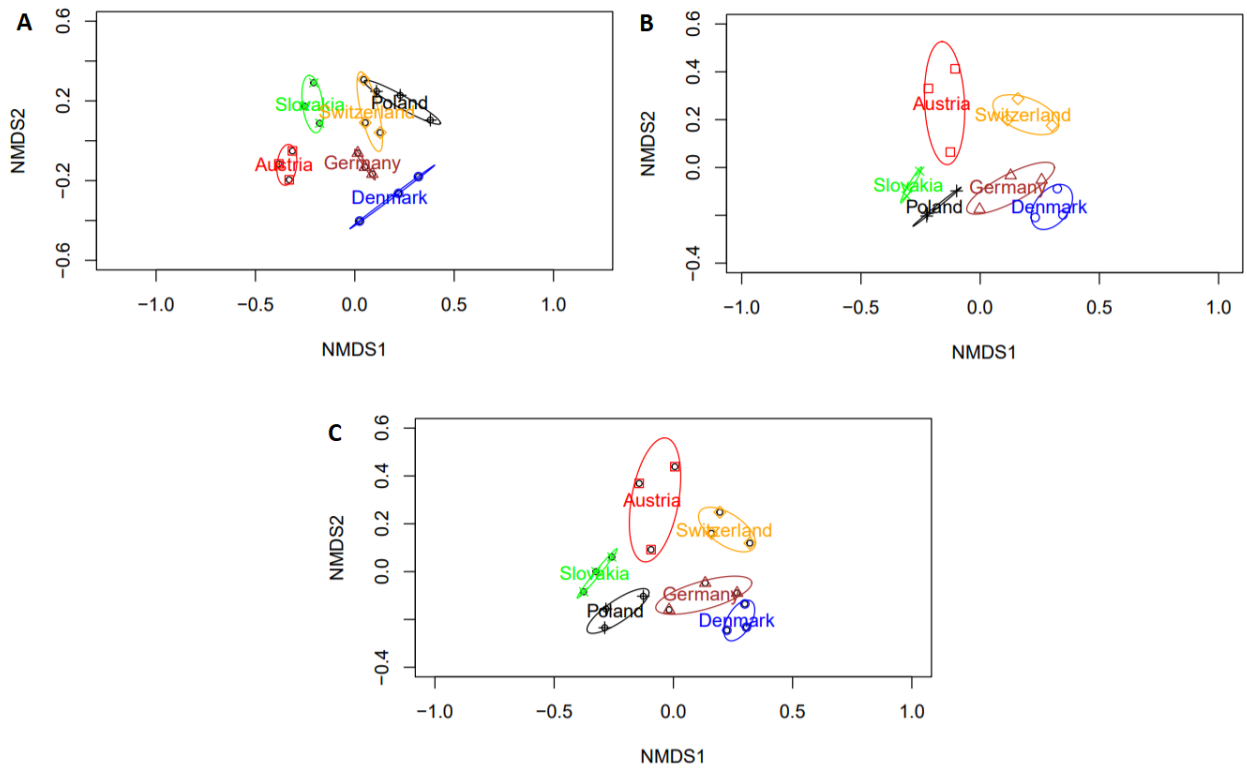


Figure 9. Non-metric multidimensional scaling (NMDS) plot presenting differences of endophyte communities across locations of seed origin. Each point represents a sample (i.e., a sample is 30 seeds from a location per medium). Samples belonging to different countries are distinguished by different colours: red (Austria), blue (Denmark), yellow (Switzerland), brown (Germany), green (Slovakia) and black (Poland). Plots are based on different dissimilarity measures: Sørensen's dissimilarity index (A), Horn's index (B) and Morisita- Horn index (C).

4. Discussion

My results show a high infection level and diversity of fungi in European beech seeds which is similar to previous findings (Franic et al. 2019) and indicates that European beech seeds can serve as an introduction pathway for high diversity of fungi into new environments when foreign seeds are used for reforestation purposes, especially because seeds from different locations carry unique fungi. My results also show that medium type does not influence captured endophyte diversity and community composition. This result is in line with recent findings (Oita et al. 2021; Massimo et al. 2015) and indicates that the results of studies that use common culturing media similar to ones used in this study are in fact comparable and that it is not necessary to use multiple media for diversity studies of seeds.

4.1 Overall fungal diversity

In this study, almost all tested seeds yielded fungi. Also, the overall number of distinguished fungal morphotypes among detected seeds endophytes was high. These results demonstrate a high abundance of diverse fungi in seeds. A similar infestation levels of European beech seeds by fungi were found by Franic et al. 2019 and 2020, in which all or almost all seed lots contained fungi. However, seed-associated fungal endophytes are considered a less frequent and diverse group than foliar endophytes as it was shown in the study of Ganley and Newcombe (2006) who looked at the endophytes associated with needles and seeds of *Pinus monticola* (Douglas ex D. Don). My results tie well with previous studies (Cleary et al. 2019; Franic et al. 2019 and 2020) which showed that seed-associated fungal endophytes should also be considered as a diverse and abundant community.

The majority of identified taxa belong to the phylum Ascomycota. The most abundant classes were Sordariomycetes and Dothideomycetes. A similar

proportion of taxa was noticed in Franic et al. 2020, in which Sordariomycetes and Dothideomycetes classes dominated communities of seed-borne endophytes. The reason for a high prevalence of Sordariomycetes and Dothideomycetes is that both classes comprise of a high number of endophytes able to inhabit a wide range of plant hosts (Maharachchikumbura et al. 2016, Bezerra et al. 2017). The class Sordariomycete is the second most diverse class within Ascomycota and it is characterized by a cosmopolitan distribution, especially among terrestrial hosts (Maharachchikumbura et al. 2016). Also, Dothideomycetes class is described as one of the most diverse and important from Ascomycota phylum (Bezerra et al. 2017).

Although a large number of morphotypes was detected in European beech seeds, obtained fungal community was dominated by only a small number of those morphotypes. A similar diversity of fungal community was detected in leaves of European beech (Unterseher and Schnittler, 2009) – the majority of isolates was assigned to two morphotypes, with around 30% of isolates belonging to each morphotype, and in twigs and buds of European beech in another study which were dominated by *Apiognomonia errabunda* (Toti et al. 1993). The fact that the small number of fungal taxa dominate fungal communities, while the larger number of taxa is represented by a small number of individuals might be due to host specificity of certain fungal endophytes that developed strong association with their host during co-evolution (Franic et al 2020).

My results show that European beech seeds carry potential pathogens in numbers comparable to previous studies, i.e., 20-30% of fungi found in seeds are potential plant pathogens (Cleary et al. 2019; Franic et al. 2019). Four species and three genera belonging to plant pathogens were identified from European beech seeds. In addition to previously mentioned *Apiognomonia errabunda*, I also identified *Apiognomonia veneta* and *Diaporthe rudis*. *Apiognomia errabunda* causes leaf spot and shoot dieback on European beech and is widespread in Europe. *Apiognomonia veneta* causes anthracnose on plane trees for instance on the London plane (*Platanus* × *hispanica* Münchh; Ivanová et al. 2007). The pathogenicity of *Diaporthe* species is increasingly reported but it is still poorly understood regarding forest tree species. However, it is known that *Diaporthe rudis* manifests a pathogenic impact on Maritime pine (*Pinus pinaster* Aiton; Lopes et al.

2021). Moreover, many morphotypes were assigned to several pathogenic genera such as, *Diaporthe* or *Fusarium*. A large number of *Fusarium* sp. found in this study is consistent with what has been found in the previous study by Manka et al. 2012, in which this genus was present almost 10% of all isolated fungi from inside of fresh beech seeds. *Fusarium* sp. are classified as damping-off pathogens and they strongly reduce the production of conifer seedlings for example on Scots pine (Swiecimska et al. 2020) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco; James et al. 1990). Species belonging to identified genera of *Phoma* sp. and *Xylaria* sp. can also show pathogenic activity. For instance, the *Phoma exigua* causes cancer of ash tree seedlings (*Fraxinus* sp.; Schmitz et al. 2006) and *Xylaria* sp. infecting roots of Hedge maple (*Acer campestre*; Tang et al. 2014). In summary, my results provide evidence that European beech seeds carry viable plant pathogens that could be moved to new areas when seeds are being exchanged for reproduction purposes and could affect tree health in nurseries or forests.

4.2 The influence of type of media on endophyte diversity and community composition

The results show no significant differences in endophyte richness among the three types of media used in this study. Abundance-weighted diversity measures, Shannon and Simpson index, also did not differ between different media. These indices increasingly emphasize the importance of abundant species. The absence of the effects of medium type on endophyte richness, Shannon and Simpson index could thus confirm that fungal diversity was also not affected by medium type, irrespective if we considered the number of morphotypes in a sample or also their abundance.

These results of study are consistent with the previous study (Oita et al. 2021) in which the richness of foliar endophytes was also not affected by the type of media containing various carbon sources. Similar to our study, Oita et al. (2021) also used nutrient-rich media (MEA, PDA, Sabouraud's medium and molasses yeast agar) and a more nutrient-scarce medium (CCA consisting of carboxymethyl cellulose and agar). Also, the study of Massimo et al. (2015) does not indicate differences in endophytes isolation frequency (i.e., the percent of tissue segments from which

fungal culture appeared) among two nutrient media (MEA and CMA - BBL cornmeal agar). The lack of differences in species richness among various types of media in my study is surprising because I assumed that endophytes richness may be higher on WA than MEA or PDA. The basis for this assumption is that the carbon source in WA is much more complex (polysaccharide) than in MEA (disaccharide in addition to polysaccharide) and PDA (monosaccharide in addition to polysaccharide). Hence, oligotrophs (slower growth) should be more abundant on WA than copiotrophs (faster growth rate). Slower growth of fungi on WA could affect the precision of isolating by having more time for checking plates and lower risk of overgrowing one fungus by another. This, in turn, could result in catching higher diversity of fungi on water agar than two other tested media. However, additional studies of the life strategies of seed endophytes could determine similar abilities of nutrient utilization among seed endophytes and their ability to utilise all nutrient sources used in this study. Seed fungi might be adapted to utilise both complex carbon sources that are available during seed dormancy, and simple sugars with increasing concentrations during seed germination. However, to fully understand the lack of differences in endophyte richness between three tested media additional studies characterizing life strategies of seed endophytes are needed.

I expected to detect crucial differences in community composition between endophytes obtained from different media. However, two out of three beta diversity measures (i.e., Sørensen's dissimilarity index and Horn index) showed no significant differences between media in our study, which is not surprising because almost half of all morphotypes (43%) appeared on all tested media. Only the Morisita-Horn index, which emphasizes the dominant species, shows significant dissimilarity between communities across media. This result may indicate the relationship between specific groups of the most dominant morphotypes and the particular type of media. However, the PERMANOVA analyses showed a very low R2 value in this case indicating that a very small portion of the variation in community composition as measured by the Morisita Horn index was explained by the media. In general, our results confirm previous findings by Oita et al. (2021) describing no differences in the composition of total and dominant endophyte communities between various types of media. Lack of differences in endophyte community composition was also found in the above-mentioned study by Massimo

et al. (2015) comparing MEA and BBL cornmeal agar (CMA). However, the study of Qi et al. 2012, demonstrated that type of nutrient medium can affect endophyte community composition. In this study authors tested the impact of four media (PDA, Czapek's, WA and Sabouraud's) on differences in composition communities of endophytes associated with *Acer ginnala* (Maxim.). This study showed that using all tested media would give the best result in exploring community composition. However, the disadvantage of this approach is that using multiple media significantly increases the time and costs. Thereupon, researchers analysed which media would give the best recognition of the community composition of endophytes and they pointed to PDA and Czapek's nutrient media. The reason for this can be significant differences in carbon sources between these media. The carbon source in the Czapek's nutrient medium is sucrose, a disaccharide composed of glucose and fructose (Solomon et al. 2014, Ajah and Mohammed 2015). The monosaccharide fructose is an additional carbon source in comparison with PDA nutrient medium which contain only glucose. This differentiation in carbon sources can result in detecting higher fungal diversity and significant differences in community composition. However, specific endophytic communities inhabit different tree organs (Ganely and Newcombe 2006) and their life strategies might be very different, e.g., foliar endophytes might have more copiotrophs than seed fungi due to high concentrations of simple sugars produced in leaves during photosynthesis while seed fungi might consist of more oligotrophs due to low concentration of simple sugars in metabolically inactive seeds. However, as already pointed out, study characterizing life strategies of seed fungi would be necessary to better understand the lack of differences in fungal diversity and community composition among different media.

The great similarity of carbon sources among selected types of media for my study, especially between MEA (maltose) and PDA (glucose), can be the reason for no differences in diversity and community composition of endophytes. The maltose is a disaccharide compound of two glucose monomers bonded together by α- 1,4 bonds of glycogen. All fungi are able to break down bonds of glycogen using extracellular enzymes to obtain simpler carbon molecules (Hobbie et al. 2001). Due to this, fungi could easily obtain glucose molecules by the breakdown of maltose in my study. As shown in the article of Qi et al. (2012) there is still a possibility to obtain significant differences in community composition among

different types of media. My study does not show differences, but a different selection of media could expose some. One of the possibilities is using the nutrient medium with the addition of plant extract, e.g., beech seed extract. Commercially produced media do not mimic conditions in which endophytes live in their environment inside the host. Applying plant extract would allow for exploring endophytes in conditions more similar to their natural environment (Murphy et al. 2014).

4.3 The influence of seed origin on endophyte diversity and community composition

Endophytes' richness was not significantly affected by seed origin in our study. Thereby, endophytes richness might not be highly dependent on location specific variables. This result corresponds with previous studies in which the influence of location and environmental variables on fungal richness in seeds (Franic et al. 2020) and other plant tissues (U'Ren et al. 2012; Coince et al. 2014) was tested. I noticed the differences in fungal diversity between different seed origins when abundance-weighted measures were used. Both abundance-weighted indices showed a significant difference in diversity between Poland and Switzerland. The Shannon diversity index shows that diversity based on species richness and level of evenness is significantly higher in Switzerland than in Poland. Then, the Simpson index points out that the fungal communities of Germany and Switzerland are significantly more diverse than the fungal communities of Poland, when rare species are weighted less than the abundant. The detailed explanation of differences in diversity among particular countries requires more studies about endophyte diversity including the recognition of host phylogeny, and environmental variables (Harrison and Griffin, 2020). However, the differences in all three measures of fungal diversity between locations were quite small. Our study thus shows that the fungal diversity of European beech is largely stable across tested locations. The reason for this can be the fact that beech seeds are embedded in the soft-spined husks most of the time (Durrant et al. 2016) and thus protected against the influence of the environment.

Our results show a clear separation of fungal communities based on the seed country of origin, for all three measures of community dissimilarity. The first axis of

NMDS plots divides countries into two groups: 1) Austria and Slovakia and 2) Germany, Switzerland and Denmark. This observation indicates higher similarity in fungal community composition within than among these two groups. This spatial pattern can be referred to as countries' geographic position on the European map. This result corresponds with previous studies, in which location was also an important driver of differences in the composition of fungal communities (Franic et al 2020, Zimmerman and Vitousek 2012; Coince et al. 2014). Endophyte communities associated with a particular host are shaped by abiotic and biotic conditions. Abiotic conditions like climate and environmental variables can favour certain fungi and thus affect community composition of tree-associated fungi directly. Furthermore, climatic and environmental variables can influence fungal community composition of a certain tree species by shaping the species composition of plants in a particular habitat, which are a source of fungal inoculum (i.e., part of fungi that can infest plants such as spores, sclerotia and fragments of mycelium, Agrios (2005)) for other plants in the same environment. It is thus not surprising that significant differences were observed in fungal community composition across locations in Europe.

4.4 Conclusions and recommendations

Results of the present study show that chosen media (PDA, MEA, WA) do not influence endophyte diversity and community composition. Furthermore, I show that European beech seeds carry abundant and highly diverse fungi, including potential plant pathogens that could be harmful to European beech, but also other European tree species. The results thus confirm that new plant pathogens can be brought to new places when seeds are moved which is indicated by differences in community composition between locations. This result emphasizes the need to improve the methods for detection of viable fungi such as traditional plating. Some ideas include: 1) dilution-to-extraction cultivation (Unterseher and Schnittler, 2009) which could help to obtain rare fungi that are difficult to grow by culturing plant fragments and 2) addition of plant extracts to media in order to provide more natural medium for growth. However, the overall assessment of fungal life strategies is needed and will help to improve the methods used for fungal detection.

Appendix

Supplementary Table 1 – Results of generalized linear model (Endophyte richness) and linear models (Shannon diversity index and Simpson's index) for the differences in endophyte diversity among three types of media and six locations. Degrees of freedom (df), chi square (χ2) and P values are shown. Significant P values are bold (P < 0.05).

Model	Response variable	Factor	Df	χ2	P
Endophyte richness ~ Location+Medium	Endophyte richness	Location	5	10.5	0.0623
		Medium	2	0.89	0.641
The Shannon diversity index ~ Location+Medium	The Shannon diversity index	Location	5	0.05	**0.033**
		Medium	2	0.004	0.492
The Simpson's index ~ Location+Medium	The Simpson's index	Location	5	0.005	**0.025**
		Medium	2	0.0008	0.236

Supplementary Table 2 – Results of permutational multivariate analysis of variance using marginal tests available in adonis2 for the differences in endophyte community composition among three types of media and six locations. Degrees of freedom (df), R-squared (r2), and P values are shown. Significant P values are bold (P < 0.05).

Model	Response variable	Factor	Df	r2	P
Sørensen's dissimilarity index ~ Location+Medium	Sørensen's dissimilarity index	Location	5	0.534	**0.001**
		Medium	2	0.091	0.190
Horn's index ~ Location+Medium	Horn's index	Location	5	0.754	**0.001**
		Medium	2	0.059	0.141
Morisita-Horn index ~ Location+Medium	Morisita-Horn index	Location	5	0.926	**0.001**
		Medium	2	0.045	**0.002**

References

Agrios, G. N. (2005). Plant pathology. Elsevier.

Ajah, H. A. and Mohammed, S. T. (2015). Handbook of fungal and parasitic Culture media. Department of Biology-College of Sciences Mustansiriyah University

Anderson MJ. A new method for non-parametric multivariate analysis of variance. Austral Ecol 2001;26:32–46.

Bacon, C. W., Glenn, A. E., and Yates, I. E. (2008). Fusarium verticillioides: managing the endophytic association with maize for reduced fumonisins accumulation. Toxin Rev. 27, 411–446. doi: 10.1080/15569540802497889

Baral, H. O., & Bemmann, M. (2014). Hymenoscyphus fraxineus vs. Hymenoscyphus albidus–A comparative light microscopic study on the causal agent of European ash dieback and related foliicolous, stroma-forming species. Mycology, 5(4), 228-290.

Bezerra, J. D., Oliveira, R. J., Paiva, L. M., Silva, G. A., Groenewald, J. Z., Crous, P. W., & Souza-Motta, C. M. (2017). Bezerromycetales and Wiesneriomycetales ord. nov.(class Dothideomycetes), with two novel genera to accommodate endophytic fungi from Brazilian cactus. Mycological Progress, 16(4), 297-309.

Black, W. D. (2020). A comparison of several media types and basic techniques used to assess outdoor airborne fungi in Melbourne, Australia. PloS one, 15(12), e0238901.

Brasier, C. M. (2008). The biosecurity threat to the UK and global environment from international trade in plants. Plant Pathology, 57(5), 792-808.

Brunet, J., Fritz, Ö., & Richnau, G. (2010). Biodiversity in European beech forests-a review with recommendations for sustainable forest management. Ecological Bulletins, 77-94.

Bu'Lock, J. D. (1975). Secondary metabolism in fungi and its relationships to growth and development. Filamentous fungi.

Cappelli, C., & Covarelli, L. (2005). Methods used in seed pathology and their recent improvements. *Phytopathologia Polonica*, *35*, 11-8.

Castle, S. C., Song, Z., Gohl, D. M., Gutknecht, J. L., Rosen, C. J., Sadowsky, M. J., ... & Kinkel, L. L. (2018). DNA template dilution impacts amplicon sequencing-based estimates of soil fungal diversity. *Phytobiomes*, *2*(2), 100-107.

Choi, Y. W., Hyde, K. D., & Ho, W. H. (1999). Single spore isolation of fungi. *Fungal diversity*.

Cleary, M., Oskay, F., Doğmuş, H. T., Lehtijärvi, A., Woodward, S., & Vettraino, A. M. (2019). Cryptic risks to forest biosecurity associated with the global movement of commercial seed. forests, 10(5), 459.

Coince, A., Cordier, T., Lengellé, J., Defossez, E., & Vacher, C. (2014). Leaf and Root-Associated Fungal Assemblages Do Not Follow Similar Elevational.

Eschen, R., Roques, A., & Santini, A. (2015). Taxonomic dissimilarity in patterns of interception and establishment of alien arthropods, nematodes and pathogens affecting woody plants in E urope. Diversity and Distributions, 21(1), 36-45.

Eschen, R., De Groot, M., Glavendekić, M., Lacković, N., Matosević, D., Morales-Rodriguez, C., ... & Franić, I. (2019). Spotting the pests of tomorrow—Sampling designs for detection of species associations with woody plants. Journal of biogeography, 46(10), 2159-2173.

FAO (2007). International Standards for Phytosanitary Measures

Farr, D. F., & Rossman, A. Y. (2019). Fungal databases, US national fungus collections, ARS, USDA.

Fizjologia roślin. Praca zborowa pod red. J. Koncewicza i S. Lewaka. Wydawnictwo Naukowe PWN Warszawa 2007

FOREST EUROPE, 2020: State of Europe's Forests 2020.

Fox, J. (2016) Applied Regression Analysis and Generalized Linear Models, Third Edition. Sage.

Franic, I., Eschen, R., Allan, E., Hartmann, M., Schneider, S., & Prospero, S. (2020). Drivers of richness and community composition of fungal endophytes of tree seeds. FEMS microbiology ecology, 96(9), fiaa166.

Franic, I., Prospero, S., Hartmann, M., Allan, E., Auger-Rozenberg, M. A., Grünwald, N. J., ... & Eschen, R. (2019). Are traded forest tree seeds a potential source of nonnative pests?. Ecological Applications, 29(7), e01971.

Gamboa, M. A., S. Laureano, and P. Bayman. 2003. Measuring diversity of endophytic fungi in leaf fragments: Does size matter? Mycopathologia 156:41–45.

Ganley, R. J., & Newcombe, G. (2006). Fungal endophytes in seeds and needles of Pinus monticola. Mycological research, 110(3), 318-327.

Geer, L., A. Marchler-Bauer, L. Han, J. He, S. He, L. C. W. Shi, and S. Bryant. 2010. The NCBI BioSystems database. Nucleic Acids Research 38:D492–D496.

Gullino, M. L., Albajes, R., & Nicot, P. C. (Eds.). (2020). Integrated pest and disease management in greenhouse crops (p. 691). Swizerland: Springer.

Hall, T.A. (1999) BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41, 95-98.

Harrison, J. G., & Griffin, E. A. (2020). The diversity and distribution of endophytes across biomes, plant phylogeny and host tissues: how far have we come and where do we go from here?. Environmental microbiology, 22(6), 2107-2123.

Ho, A., Di Lonardo, D. P., & Bodelier, P. L. (2017). Revisiting life strategy concepts in environmental microbial ecology. FEMS microbiology ecology, 93(3).

Hobbie, E. A., Watrud, L. S., Maggard, S., Shiroyama, T., & Rygiewicz, P. T. (2003). Carbohydrate use and assimilation by litter and soil fungi assessed by carbon isotopes and BIOLOG® assays. Soil Biology and Biochemistry, 35(2), 303-311.

Houston Durrant, T., de Rigo, D., Caudullo, G., 2016. Fagus sylvatica and other beeches in Europe: distribution, habitat, usage and threats. In: San-Miguel-Ayanz, J., de Rigo, D., Caudullo, G., Houston Durrant, T., Mauri, A. (Eds.), European Atlas of Forest Tree Species. Publ. Off. EU, Luxembourg, pp. e012b90+

Ivanová, H., Bernadovičová, S., & Pastirčáková, K. (2007). Influence of changed ecological conditions on occurrence of London plane (Platanus× hispanica Münchh.) anthracnose. Folia Oecol, 34(1), 1.

James, R. L., Dumroese, R. K., & Wenny, D. L. (1991). Fusarium diseases of conifer seedlings. IUFRO Working Party 82.07-09 (Diseases and Insects in Forest Nurseries), 7, 181.

Jansen, S., Konrad, H., & Geburek, T. (2019). Crossing borders–European forest reproductive material moving in trade. Journal of environmental management, 233, 308-320.

Jost, L. (2007). Partitioning diversity into independent alpha and beta components. *Ecology*, *88*(10), 2427-2439.

Khare, E., Mishra, J., & Arora, N. K. (2018). Multifaceted interactions between endophytes and plant: developments and prospects. Frontiers in microbiology, 9, 2732.

Konnert, M.; Fady, B.; Gömöry, D.; A'Hara, S.; Wolter, F.; Ducci, F.; Koskela, J.; Bozzano, M.; Maaten, T.; Kowalczyk, J. (2015). "Use and transfer of forest reproductive material in Europe in the context of climate change". European Forest Genetic Resources Programme, Bioversity International, Rome, Italy: xvi and 75 pp.

Kramer, K., Degen, B., Buschbom, J., Hickler, T., Thuiller, W., Sykes, M. T., & de Winter, W. (2010). Modelling exploration of the future of European beech (Fagus sylvatica L.) under climate change—range, abundance, genetic diversity and adaptive response. Forest Ecology and Management, 259(11), 2213-2222.

Kwasna, H. (2014). Mikrobiologia rolnicza. Wydawnictwo Uniwersytetu Przyrodniczego.

Lee, P. Y., Costumbrado, J., Hsu, C. Y., & Kim, Y. H. (2012). Agarose gel electrophoresis for the separation of DNA fragments. *JoVE (Journal of Visualized Experiments)*, (62), e3923.

Libório, R. A., & Tanaka, M. O. (2016). Does environmental disturbance also influence within-stream beta diversity of macroinvertebrate assemblages in tropical streams?. *Studies on Neotropical Fauna and Environment*, *51*(3), 206-214.

libRussell V. Lenth. emmeans: Estimated Marginal Means, aka Least-Squares Means, 2020. R package version 1.5.3

Lopes, A. F., Batista, E., Hilário, S., Santos, L., & Alves, A. (2021). Occurrence of Diaporthe species in Eucalyptus globulus, Pinus pinaster and Quercus suber in Portugal. Forest Pathology, 51(2), e12674.

Maharachchikumbura, S. S., Hyde, K. D., Jones, E. B., McKenzie, E. H. C., Bhat, J. D., Dayarathne, M. C., ... & Wijayawardene, N. N. (2016). Families of sordariomycetes. Fungal Diversity, 79(1), 1-317.

Manka, M., Łakomy, P., Cieślak, R., & Szynkiewicz, A. (2012). Fungi inhabiting Fagus sylvatica seeds after harvest and after drying. Phytopathologia, 65, 39-4

Massimo, N. C., Nandi Devan, M. M., Arendt, K. R., Wilch, M. H., Riddle, J. M., Furr, S. H., ... & Arnold, A. E. (2015). Fungal endophytes in aboveground tissues of desert plants: infrequent in culture, but highly diverse and distinctive symbionts. Microbial ecology, 70(1), 61-76.

Microbiology (2014). Morphology and general properties of fungi. https://nios.ac.in/media/documents /dmlt/Microbiology/Lesson-51.pdf

Murphy, B. R., Batke, S. P., Doohan, F. M., & Hodkinson, T. R. (2015). Media manipulations and the culture of beneficial fungal root endophytes. Int J Biol, 7(3), 94-102.

Nunez, F., Westphal, C. D., Bermudez, E., & Asensio, M. A. (2007). Production of secondary metabolites by some terverticillate penicillia on carbohydrate-rich and meat substrates. Journal of food protection, 70(12), 2829-2836.

Oita, S., Carey, J., Kline, I., Ibáñez, A., Yang, N., Hom, E. F., ... & Arnold, A. E. (2021). Methodological approaches frame insights into endophyte richness and community composition. Microbial ecology, 82(1), 21-34.

Oksanen J, Blanchet FG, FriendlyMet al. vegan:Community Ecology Package. R Packag version 25-3 2018. https://cran.r-proje ct.org/web/packages/vegan/vegan.pdf (Accessed January 8, 2020).

Övergaard, R. (2010). Seed production and natural regeneration of beech (Fagus sylvatica L.) in southern Sweden (Vol. 2010, No. 2010: 12).

Pehl, L., & Butin, H. (1994). Endophytische Pilze in Blättern von Laubbäumen und ihre Beziehungen zu Blattgallen (Zoocecidien). Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft

Petrini, O. (1991). Fungal endophytes of tree leaves. In Microbial ecology of leaves (pp. 179-197). Springer, New York, NY.

Petrini, O., & Fisher, P. J. (1988). A comparative study of fungal endophytes in xylem and whole stem of Pinus sylvestris and Fagus sylvatica. Transactions of the British Mycological Society, 91(2), 233-238.

Prospero, S., & Cleary, M. (2017). Effects of host variability on the spread of invasive forest diseases. Forests, 8(3), 80.

Qi, F., Jing, T., & Zhan, Y. (2012). Characterization of endophytic fungi from Acer ginnala Maxim. in an artificial plantation: media effect and tissue-dependent variation.

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rana, K. L., Kour, D., Sheikh, I., Dhiman, A., Yadav, N., Yadav, A. N., ... & Saxena, A. K. (2019). Endophytic fungi: biodiversity, ecological significance, and potential industrial applications. In Recent advancement in white biotechnology through fungi (pp. 1-62). Springer, Cham.

Rashmi, M., Kushveer, J. S., & Sarma, V. V. (2019). A worldwide list of endophytic fungi with notes on ecology and diversity. Mycosphere, 10(1), 798-1079.

Ravimannan, N., Arulanantham, R., Pathmanathan, S., & Niranjan, K. (2014). Alternative culture media for fungal growth using different formulation of protein sources. Annals of Biological Research, 5(1), 36-39.

Ritchie, F., Bain, R. A., & McQuilken, M. P. (2009). Effects of nutrient status, temperature and pH on mycelial growth, sclerotial production and germination of Rhizoctonia solani from potato. Journal of Plant Pathology, 589-596.

Ritchie, M. E., Phipson, B., Wu, D. I., Hu, Y., Law, C. W., Shi, W., & Smyth, G. K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic acids research, 43(7), e47-e47.

Rodriguez, R. J., White Jr, J. F., Arnold, A. E., & Redman, A. R. A. (2009). Fungal endophytes: diversity and functional roles. *New phytologist*, *182*(2), 314-330.

Santini, A., Ghelardini, L., De Pace, C., Desprez-Loustau, M. L., Capretti, P., Chandelier, A., ... & Stenlid, J. (2013). Biogeographical patterns and determinants of invasion by forest pathogens in Europe. New Phytologist, 197(1), 238-250.

Schmitz, S., Zini, J., Etienne, M., Moreau, J. M., Chandelier, A., & Cavelier, M. (2006). Effectiveness of thiophanate-methyl, trifloxystrobin and vinclozolin on canker caused by Phoma exigua Desm. on ash tree seedlings. BASE.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., ... & Fungal Barcoding Consortium. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences*, *109*(16), 6241-6246.

Sellers, K. F., & Shmueli, G. (2010). A flexible regression model for count data. *The Annals of Applied Statistics*, 943-961.

Sieber, V. T., & Hugentobler, C. (1987). Endophytische Pilze in Blättern und Ästen gesunder und geschädigter Buchen (Fagus sylvatica L.). *European Journal of Forest Pathology*, *17*(7), 411-425.

Slippers, B., & Wingfield, M. J. (2007). Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. *Fungal biology reviews*, *21*(2-3), 90-106.

Solomon, E. P., Berg, L. R., Martin, D. W. (2014). Biology, ninth edition.

Strobel, G., and Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. Microbiol. Mol. Biol. Rev. 67, 491–502. doi: 10.1128/MMBR.67.4.491-502.2003

Swiecimska, M., Tulik, M., Šerá, B., Golińska, P., Tomeková, J., Medvecká, V., ... & Šerý, M. (2020). Non-thermal plasma can be used in disinfection of Scots pine (Pinus sylvestris L.) seeds infected with Fusarium oxysporum. Forests, 11(8), 837.

Szefler, S. J., Holguin, F., & Wechsler, M. E. (Eds.). (2017). Personalizing asthma management for the clinician. Elsevier Health Sciences.

Tang, A. M. C., Lam, R. Y. C., & Leung, M. W. K. (2014). Xylaria hongkongensis sp. nov. from an urban tree. Mycotaxon, 128(1), 37-40.

Tedersoo, L., et al. 2014. Global diversity and geography of soil fungi. Science 346:6213.

Toti, L., Viret, O., Horat, G., & Petrini, O. (1993). Detection of the endophyte Discula umbrinella in buds and twigs of Fagus sylvatica. European Journal of Forest Pathology, 23(3), 147-152.

Tsao, P. H. (1970). Selective media for isolation of pathogenic fungi. Annual Review of Phytopathology, 8(1), 157-186.

Unterseher, M., & Schnittler, M. (2009). Dilution-to-extinction cultivation of leaf-inhabiting endophytic fungi in beech (Fagus sylvatica L.)–different cultivation techniques influence fungal biodiversity assessment. Mycological Research, 113(5), 645-654.

Unterseher, M., & Schnittler, M. (2010). Species richness analysis and ITS rDNA phylogeny revealed the majority of cultivable foliar endophytes from beech (Fagus sylvatica). Fungal Ecology, 3(4), 366-378.

U'Ren, J. M., Lutzoni, F., Miadlikowska, J., Laetsch, A. D., & Arnold, A. E. (2012). Host and geographic structure of endophytic and endolichenic fungi at a continental scale. American journal of botany, 99(5), 898-914.

Venables, W. N., & Ripley, B. D. (2002). Random and mixed effects. In Modern applied statistics with S (pp. 271-300). Springer, New York, NY

Vettraino, A. M., Potting, R., & Raposo, R. (2018). EU legislation on forest plant health: An overview with a focus on Fusarium circinatum. Forests, 9(9), 568.

Walters, D. R., Havis, N. D., and Oxley, S. J. P. (2008). Ramularia collo-cygni: the biology of an emerging pathogen of barley. FEMS Microbiol. Lett. 279, 1–7. doi: 10.1111/j.1574-6968.2007.00986.x

Whipps, J. M. (1987). Effect of media on growth and interactions between a range of soil-borne glasshouse pathogens and antagonistic fungi. New Phytologist, 107(1), 127-142.

Wickham, H. ggplot2: Elegant Graphics for Data Analysis; Springer: New York, NY, USA, 2009.

Wolkers, W. F., & Oldenhof, H. (Eds.). (2015). *Cryopreservation and freeze-drying protocols* (pp. 3-25). Humana Press.

Zhang, T., Zhang, Y. Q., Liu, H. Y., Wei, Y. Z., Li, H. L., Su, J., ... & Yu, L. Y. (2013). Diversity and cold adaptation of culturable endophytic fungi from bryophytes in the Fildes Region, King George Island, maritime Antarctica. FEMS microbiology letters, 341(1), 52-61.

Zimmerman, N. B., & Vitousek, P. M. (2012). Fungal endophyte communities reflect environmental structuring across a Hawaiian landscape. Proceedings of the National Academy of Sciences, 109(32), 13022-13027.