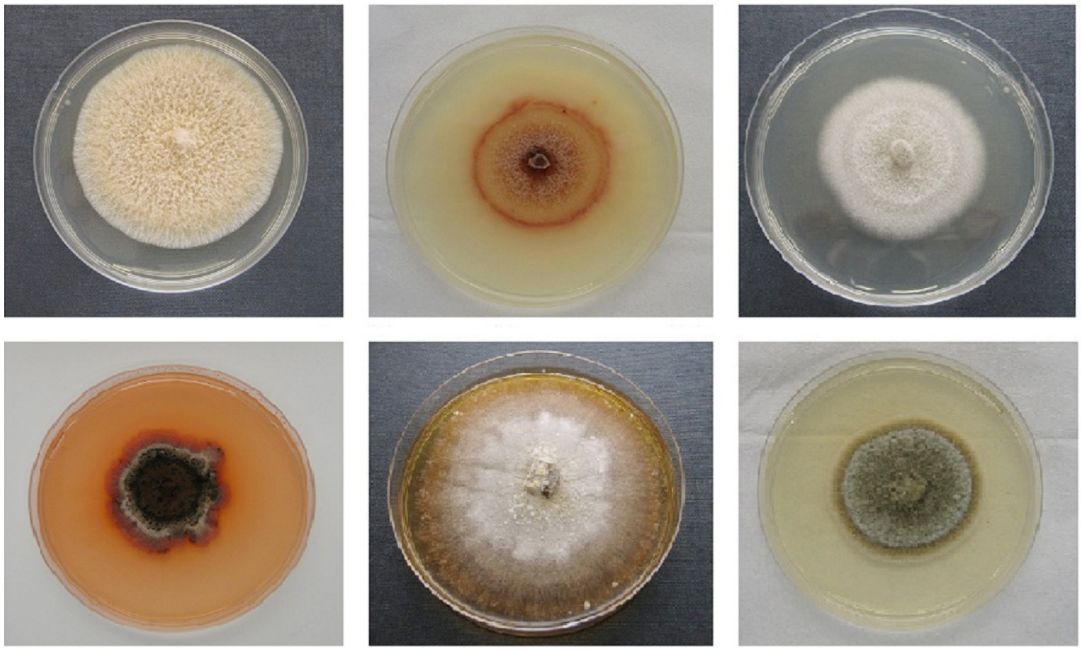
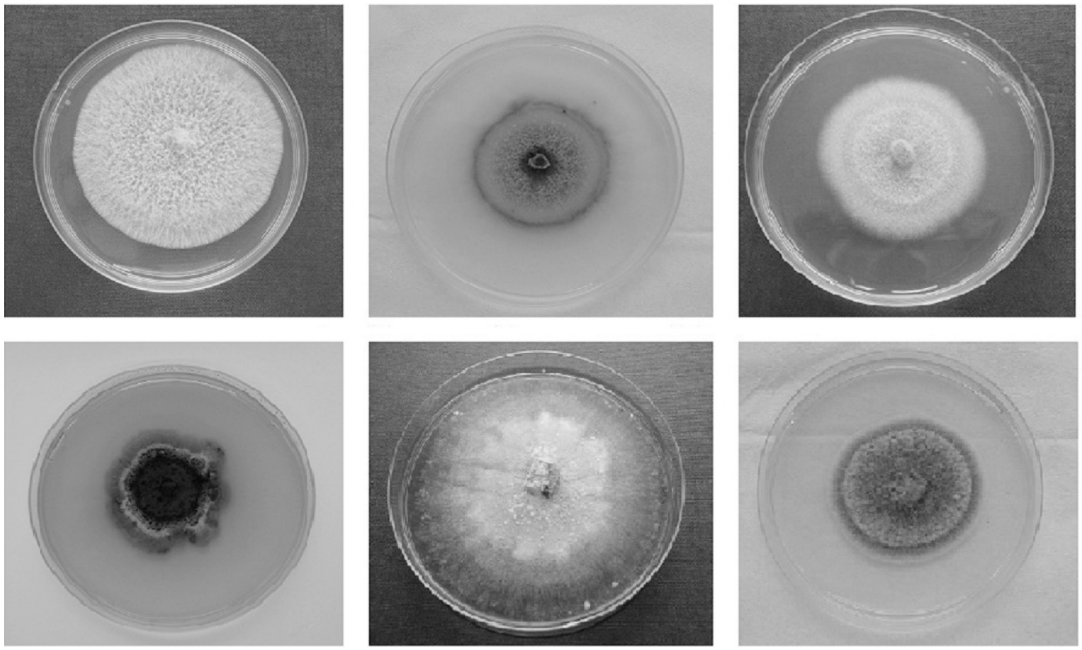
Variation in fungal endophyte communities of pedunculate oak (*Quercus robus* L.): spatial, temporal and environmental aspects



Agostinelli Marta
Supervisors: Johanna Witzell and Kathrin Blumenstein

Swedish University of Agricultural Sciences
Master Thesis no. 195
Southern Swedish Forest Research Centre
Alnarp 2012

Variation in fungal endophyte communities of pedunculate oak (*Quercus robus* L.): spatial, temporal and environmental aspects



Agostinelli Marta
Supervisors: Johanna Witzell and Kathrin Blumenstein
Examiner: Jörg Brunet

Swedish University of Agricultural Sciences
Master Thesis no. 195
Southern Swedish Forest Research Centre
Alnarp 2012

Master Thesis in Biology, Euroforester MSc Program SLU and
SUFONAMA MSc Program 30 ECTS Advanced Level, SLU course code EX 0279

CONTENTS

List of figures

List of tables

ABSTRACT

Fungal endophytes are a taxonomically and ecologically heterogeneous group of ubiquitous intercellular fungi that live part of their life within the tissues of the plants without causing apparent harm to the host. They may contribute to the stress tolerance and resistance of the plants, and endophytes may also be involved in decomposition processes. Thus, these fungi are an interesting part of biodiversity e.g. in forest ecosystems. Little is still known about the temporal and spatial patterns in endophyte community structures in trees. Moreover, while it is known that endophytes spread to trees from the environment (horizontally) it is not clear at which scales their spreading occurs, and if e.g. neighbouring vegetation determines endophyte communities in trees. In addition, it is not known if the general vitality of the trees affects the frequency and diversity of endophyte infections in trees. Using culture-dependent method (isolations), endophyte communities were compared in young pedunculate oak (*Quercus robur* L.) trees that were surrounded by herbs and shrubs in different combinations and that showed different vitality status. The isolations were done from current and previous year's twigs (xylem and bark) in early and late summer to study the temporal and within-tree spatial patterns. A total of 384 samples were plated with bark and xylem pieces. Altogether 172 of them produced a total number of 285 isolates, which could be grouped in 22 morphological groups (morphotypes). The colonisation and isolation rate values showed the succession of endophyte communities during the season and inside the twigs but there were no significant effects of tree vitality on these rates. However, in June the declining trees had higher morphotype evenness, indicating that declining trees were compromised in their ability to defend themselves against fungal infections, which may have reduced the competition between the different invading fungi. The Jaccard's similarity index and Shannon's diversity index showed that there was no clear pattern of infection from the closest surrounding vegetation, suggesting that the spreading of these fungi occurs at a larger scale.

Keywords: Pedunculate oak, Quercus robur, endophytes, fungal communities, neighboring effect.

Acknowledgment

I express my gratitude to my supervisor associate professor Johanna Witzell for her valuable advices and support!

I extend special thank to Kathrin Blumenstein for the help in the lab.

I wish to thank Anna Jensen for allowing me to collect my samples in her field plots, and professor Jörg Brunet for the constructive comments on my work.

Grazie alla mia famiglia, per il supporto incondizionato mostrato in questi anni, per avermi spronato ad aprire i miei orizzonti e inseguire i miei sogni.

Alla mia nonnissima, perchè è la nonna più incredibile che ci sia.

Un ringraziamento speciale a Marco per tutto quello che ha fatto, per essere stato presente nonostante le migliaia di chilometri e per le ore spese in viaggio.

1. INTRODUCTION

Endophytic fungi

Fungi are a group of heterotrophic organisms that can be found everywhere. They have a fundamental role in nature as decomposers of organic material. Some fungi have established close interactions with plants: they can interact as mutualists, symbionts or pathogens (Arnold et al., 2000; Arnold et al., 2003; Helander et al., 2006; Helander et al, 2007; Herre et al., 2007; Gennaro et al., 2003). Certain microscopic fungi live at least part of their life cycle within the tissues of the plants without causing visible symptoms: these fungi are called fungal endophytes (Petrini 1991).

Fungal endophytes are a taxonomically and ecologically heterogeneous group of ubiquitous intercellular fungi belonging to the Ascomycotina, Deuteromycotina (Petrini et al., 1992), Basidiomycotina and Oomycetes (Saikkonen et al., 1998) that seem to make up a large fraction of the fungal biodiversity (Arnold et al, 2000 and Arnold et al, 2001). They have been found in every studied plant species (algae, mosses, ferns, conifers, grasses, palms, shrubs and monocotyledonous and dicotyledonous angiosperms) from agricultural land to natural forests (Arnold, 2007; Hyde and Soytong, 2008). Even though some species have been studied for several years (Zabalgogeazcoa, 2008), there is still a lack of knowledge about their roles and interactions with their host plants (Petrini, 1996), moreover, there are still many plants whose endophytes have not been studied yet (Arnold et al, 2000 and Arnold et al, 2001).

Depending on the colonized host the endophytes are classified as clavicipitalean (grass-inhabiting) and non-clavicipitalean (non grass-inhabiting) (Sieber, 2007; Hyde and Soytong, 2008). Within the clavicipitalean group, the endophytes are transmitted *vertically* from original plants to the seeds and offspring (Fig. 1). Instead, the non-clavicipitalean endophytes, such as endophytes of forest trees, seem to be transmitted mainly *horizontally* (Arnold et al, 2003; Devarajan and Suryanarayanan, 2006), i.e. from the surrounding environment, via asexual spores (Saikkonen et al., 1998) (Fig. 1). The dispersion of fungal endophytes can be favoured also by other biotic factors such as insects: according to Devarajan and Suryanarayanan (2006), folivory and phytophagous insects can facilitate the spreading of endophytes in tropical regions.

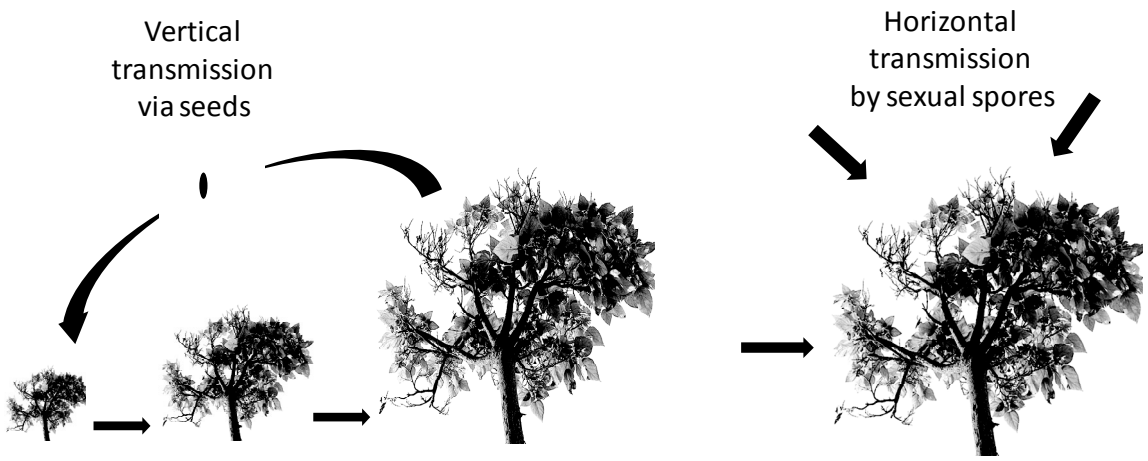


Figure 1 Vertically and horizontally transmission of endophytes spores within the plant tissues (modified from: http://www.sciencedirect.com/science/article/pii/S1360138504001025. Photo: Microsoft Clip art 2012)

Variation of endophytic communities and host interaction

The distribution of endophytes within a tree may be highly variable: some species seem to be almost ubiquitous and are found on hosts belonging to different families and in diverse site conditions, other species seem to be more specific and have the tendency of establishing organ and host specific communities (Sieber, 1989). It is known that within one tissue type, few endophytic species usually dominate. However, the number of species and their frequency and distribution within a host vary greatly according to the period, the site and weather conditions and the host's characteristics (Petrini et al., 1992; Sieber, 2007). According to Lorenzi et al. (2006) also the height and the density of the crown, the crown's homogeneity, the age of the tree, the altitude, the humidity, the exposure to winds, the rainfalls and the temperature influence the formation of endophytic communities. The surrounding vegetation is likely to be an important source of endophytic infections that determines the endophytic communities in the host (Rodriguez et al. 2008).

In the study of Helander et al. (2007) it was shown how a fragmented environment can influence the frequency of the endophytes within the birch leaves' tissue. Moreover, the authors emphasize that the occurrence and the species composition of the endophytic fungi of birch leaves vary due to abiotic and biotic conditions because of the fact that the leaves are re-infected at every new season. In a tree, this could be seen also as a difference in endophyte communities in woody tissues, for example different aged twigs: while the older parts of a twig should present an established community, there should be a more dynamic, establishing population in the younger parts of the same twig.

The colonisation of the host may be different according to the kind of endophytes and the reaction of the hosts. Several types of colonisations have been recorded: intracellular and limited to a singular cell, intercellular and localized, systemic and both inter- and intracellular, limited to specific organs of the host (i.e. roots, leaves/needles) (Schulz and Boyle, 2005). The penetration of the host tissues occurs with help of appressoria and haustoria, through the stomata and substomatal chamber or through the cell wall (Schulz and Boyle 2005) but the infection strategies of most endophytes are still poorly understood (Wilson, 1996).

Ecological roles of endophytes

The endophytic life style has evolved during millions of years and these fungi may have versatile ecological roles in plants. These roles are suggested to be linked in particular to growth, tolerance and defence of the plants. Furthermore, endophytes may significantly contribute to the decomposition processes e.g. in forest ecosystems (Korkama-Rajala et al. 2008).

There are different opinions about the influence of endophytes on the growth of the host plants. According to Arnold (2002) there is no clear evidence that endophytes cause changes in the growth and biomass accumulation of the host. On the other hand, Wilson (2000) states that the presence of endophytes could enhance the ability of the leaves of absorbing nutrients through their surface. Herre et al. (2007), however, affirm that there is some evidence that endophytes can cause a reduction in the host´s growth and they take as a possible motivation the fact that endophytes are heterotrophic organisms which consume some of the substances produced by the plants. The question whether the endophytes could influence the growth of the host trees is clearly a difficult one and is complicated by the fact that it is difficult, if not impossible, to have a control tree completely free from endophytes to be used as a comparative object.

While the influence of endophytes on plant's growth and physiology is still unclear, there are some studies on forest trees that support the hypothesis of a defensive mutualism role against pathogen, insects and allelochemicals (Arnold et al., 2003, Saikkonen et al., 1998, Lorenzi et al., 2006). For example, Webber (1981) showed that an endophytic fungus living in the inner bark of elm trees was an antagonist to a bark beetle spreading the Dutch elm disease. Moreover, some endophytic fungi seem to be capable of killing the gall-forming insects

within the galls: an example of this is the fungus *Deadelea quercina*, which invades the galls of a cynipid insect and kills the wasp inside them (Wilson, 1995).

While the endophytes may have several positive effects on plant's survival, there are also reports of endophytic fungi that become pathogens when the genotypic condition of the plant favours the development of the disease (Helander et al., 2007; Moricca and Ragazzi, 2007; Sieber, 2007) or when the host suffers from a biotic or abiotic stress (Arnold et al., 2007, Ragazzi et al., 2003; Ragazzi 2004). Some fungi start their life cycle as endophytes in the healthy trees and when the tree is under stress, they become pathogens that contribute to the death of the tree. This kind of shift has been recorded for *Apiognomonia quercina* and its anamorph *Discula quercina*, *Diplodia mutila*, *Biscogniauxia mediterranea* and *Phomopsis quercina.* (Lorenzi et al., 2006). Thus, while the endophyte community of a tree can promote the tree's vitality by protecting it from enemies (Arnold et al., 2003, Saikkonen et al., 1998, Lorenzi et al., 2006) and stress (Tan and Zou, 2001 cited by Schulz and Boyle, 2005), it can also be affected and modulated by the tree's condition. Schulz and Boyle (2005) call this *balanced* interaction.

According to Schulz and Boyle (2005), the interaction between endophyte and host is balanced because the two partners live together with unapparent signs of antagonism but this interaction may be momentary and there could be a shift, due to biotic or abiotic factors, that change the endophytic life style to a pathogenic one. When and why this shift happens is still uncertain, and little is known about life-history strategies of the endophytes within the host. Moreover, how the balanced interaction between the two partners is maintained is still unknown. Several factors are likely to be involved (i.e. the host defence responses, the virulence of the fungus, the changing of abiotic factors) to regulation of the balance, and to the possible pathogenicity of an endophyte (Schulz and Boyle, 2005).

The defense mechanisms developed by the trees against pathogens should also influence the overall community of endophytes and also, on the contrary, the fungal endophytes will influence the defense mechanisms of the plants. According to Schulz and Boyle (2005) the host may benefit from the interaction with the endophytes e.g. through the induction of defence metabolites potentially active against pathogens, the production of phytohormones, the provision of nutrients from the rhizosphere and the increase of the metabolic activities. While it is generally well documented that the clavicipitalean endophytes play a role against browsing and in favour of plant growth (e.g. Clay 1988), the case is less clear for the non-

clavicipitalean endophytes: experimental demonstration has been mostly inconclusive (Sieber, 2007). However, some studies on mutualistic interactions of endophytes with above-ground plant organs show protection against insect herbivory (Schulz and Boyle, 2005, and refs. therein).

A possible advantage for the plant after being infected by endophytes is production and secretion of mycotoxins by the endophytes into plant tissues, which may enhance the resistance against other pathogens (Bultman and Murphy, 2000 cited by Schulz and Boyle, 2005). Endophytes may also produce, or stimulate the plant's production of antioxidants that protect plants from the oxidative stress associated with plant diseases, droughts, heavy metals and other oxidative stressors (White and Torres 2010).

Given all this, it seems likely that vital trees, which are likely to have stronger resistance mechanisms, host a different endophytic community than less vital trees. Different outcomes are possible. It could be that a vital tree supports certain kinds of, or highly diverse, communities of endophytic fungi that keep the tree healthy and suppress infections caused by pathogens and saprophytic fungi. On the other hand, it could be that a less vital tree might have many endophytes because it is generally weaker against any fungal infections due to its poorer defensive system. However, it has rarely been tested whether the phenotypic differences in tree vitality could be reflected in tissue-specific patterns of endophyte communities, or whether the environmental influence (at small or large scale) on endophyte communities overrides the inherent controls in plant tissues. Moreover, while it is clear that the horizontal spreading of endophytes occurs from surrounding vegetation and soil, it is not known at what spatial scale the surrounding environment is forming the endophyte communities: are the neighbouring plants close by the crucial factor (small scale variation in vegetation), or does the environmental regulation act mainly at a larger scale.

Aim of the study

Through their different roles in trees, the fungal endophytes may contribute to various ecosystem services. Therefore, to support informed forest management decisions, it is important to understand how endophyte diversity varies in trees at temporal and spatial scales. The object of this study was to analyse and compare the variation in endophyte communities within the woody tissues of a deciduous tree species, pedunculate oak (*Quercus robur L.*). To investigate the dependencies between tree vitality and endophytes, twigs were collected from

trees showing high or low vitality. The trees were growing in plots where the surrounding vegetation had been systematically manipulated in different ways which allowed studying of the effect of neighbouring vegetation on endophyte flora within oaks. Samples were collected at two time points in order to study the temporal patterns in endophyte communities.

The following questions were studied:

1. Do the endophyte communities in the trees that show a high vitality differ from those in trees that show poor vitality?
2. Are there age-and tissue-specific quantitative and qualitative differences among the endophyte populations?
3. Does the neighbouring vegetation have an important role as a determinant of the quantitative and qualitative variation and distribution of the endophytes?
4. How does the endophyte community change during the growth season in different-aged tissues?

2. MATERIALS AND METHODS

2.1 Study area

The samples were collected in Alnarp, in Skåne, Southern Sweden at a field-experiment area ("Trädgårdslaboratorium") (55°39'40''N, 13°05'04''E). The meteorological data on average precipitation and temperature for the studied area are shown in Fig. 2.

In the year 2007 an experiment was established for studying the influence of herbal and shrub vegetation on the growth of oak seedlings (Jensen 2011). The oaks planted for this experiment were used to carry out this work.

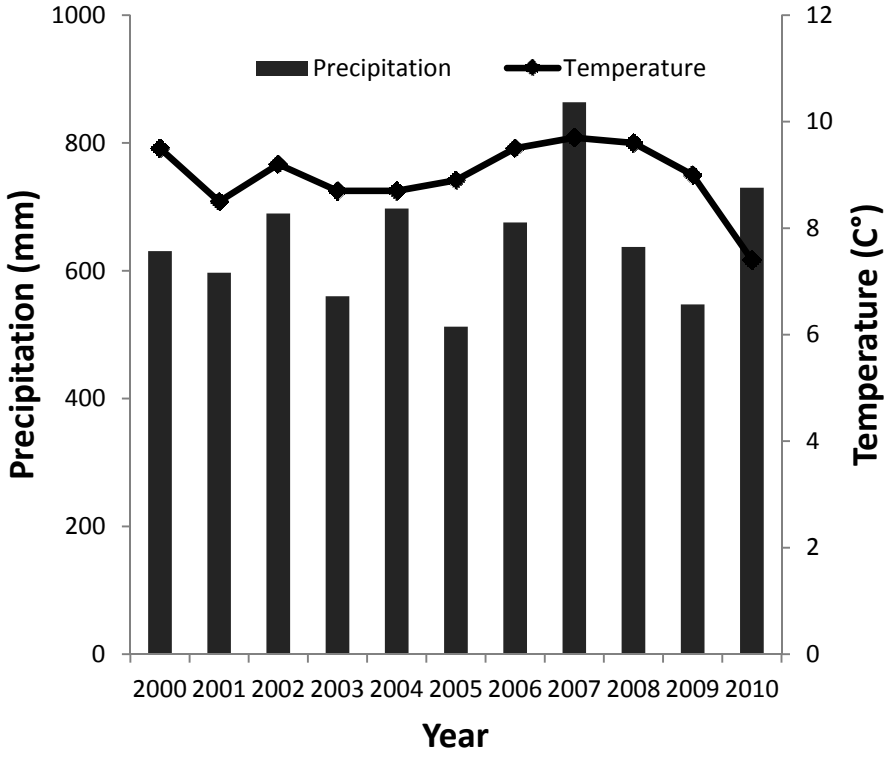


Figure 2 Mean annual precipitation and temperature in Malmö for the years 2000–2010. Data source: SMHI (Swedish Meteorological and Hydrological Institute) from the meteorological station 5235, Malmö A, coordinates X 6163610 and Y 1327530.

2.2 Pedunculate oak (*Quercus robur* L.)

The genus *Quercus* belongs to the *Fagaceae* family and has about 300-350 species in the northern hemisphere. Those can be arboreal or shrub, deciduous, evergreen or semi evergreen species (Gellini and Grossoni, 1997). The pedunculate oak (*Quercus robur* L.) is native to the most of Europe and to the area going from Anatolia to Caucasus. In the Scandinavian area it has a natural distribution in the southern regions of Sweden, on the southern coastal area of

Norway, on the coastal area in the South-Southwest of Finland and in all Denmark. In Sweden it is possible to find this species up till Stockholm and Uppsala's region (Fig. 3).

Pedunculate oak grows on a wide range of soil conditions in forests, wooded pastures, and the agricultural landscape (Mossberg and Stenberg, 2003). It usually reaches 30-35 meters height and it is a very long-lived tree (Gellini and Grossoni, 1997). It has a grey, fissured bark and standing alone it can reach a crown diameter of 10 m. The buds are rounded. The petiole is usually not exceeding 10 mm. The leaves blade is 7-20 cm with a dark green colour and not hairy on the above side, pale green and sparsely haired on the lower side (Mossberg and Stenberg, 2003).

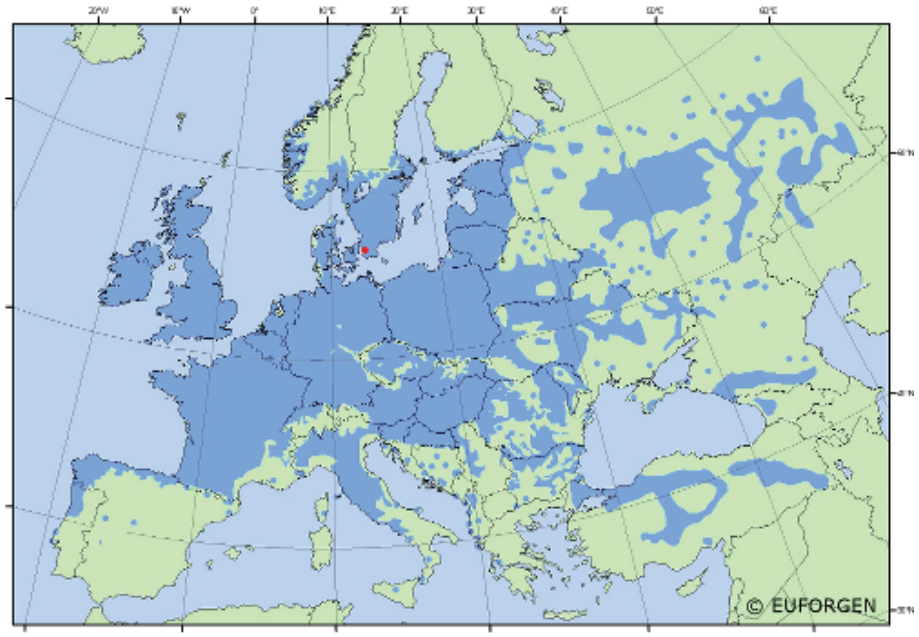


*Figure 3 Natural distribution of pedunculate oak (*Quercus robur L.*) in the Scandinavian region. (Source: http://ieg.ebd.csic.es/arndthampe/Quercus)*

2.3 Experiment setup

The oak plants studied in this experiment originated from nurseries in southern Sweden (Ramlösa Plantskola) or in Denmark (Hede-Danmark) and were two years old when planted in the field at the SLU campus in Alnarp in 2007. Therefore, they were seven years old when the samples were collected in summer 2011.

The experiment's design has 4 repetitions (blocks A, B, C, D) and each block has 6 subplots (1, 2, 3, 4, 5, 6) (Figs 4 and 5) with different combinations of neighbouring species (herbs and shrubs). Each treatment has a code composed by the letters H and S. The plus or minus signs

following the letter mean respectively if the competition by herbs (H) or shrubs (S) is present or absent. In treatment 1 (H-S-) there is no herbs or shrubs competition. Treatment 2 (H+S-) has competition of herbs but not competition from shrubs. Treatment 3 (H-S+) has competition only from shrubs. Treatment 4 (H+S+) has competition both from herbs and shrubs. Treatment 5 (H-AS+) has shrubs competition from above-ground canopy (A), but root-zone competition was restricted by plastic barriers. Treatment 6 (H-BS+) has shrubs competition from below (B), but the above-ground competition was restricted by bending the shrub canopy away from oak shoots with metal wires. The shrub species planted around the oaks were *Rubus idaeus* (35%), *Betula pendula* (25%), *Corylus avellana* (20%), *Fraxinus excelsior* (10%) and *Populus tremula* (10%), which are commonly found in regeneration areas in Southern Sweden (Jensen 2011).

Per each subplot 25 bare-rooted oak seedlings were planted. From each subplot, two trees were selected and marked with a red ribbon. Totally, 48 trees were chosen for this study in the Trädgårdslaboratorium field experiments.

Figure 4 Aerial photo of the experiment in the Trädgårdslaboratorium in Alnarp. The division between blocks and subplots is clear also from an aerial photo (Digital Globe, GeoEye, Map data, 2012, Google).

A		B		C		D	
H-S-	H+S-	H+S+	H-BS+	H-AS+	H+S-	H-BS+	H+S-
H-S+	H+S+	H-S+	H+S-	H-S-	H-S+	H-S-	H-AS+
H-AS+	H-BS+	H-S-	H-AS+	H-BS+	H+S+	H+S+	H-S+

Figure 5 Disposition of the six vegetation competition treatments in each block repetition of the experiment.1: no competition, control of shrub and herb vegetation (H-S-). 2: competition from herb vegetation, control of shrub vegetation (H+S-). 3: competition from shrub vegetation, control of herb vegetation (H-S+). 4: competition from herb and shrub vegetation (H+S+). 5: above ground competition from shrub vegetation, control of herb vegetation (H-AS+). 6: below ground competition from shrub vegetation, control of herb vegetation (H-BS+).

2.4 Data collection

The samples were harvested during two different periods in summer 2011. The first collection was carried out between the middle and the end of June, the second one a month and a half later between the end of July and the middle of August 2011. The harvesting of the samples material at two different time points was done to analyse the possible quantitative and qualitative variation in the composition of the endophyte community in the trees during the growth period.

The selection of the sample trees was based on visual estimate of the health status. Apical shoot condition, length of the twig of the current and previous years, spacing between the whorls and condition of leaves (colour, general appearance) were the criteria used for choosing the sample trees. Trees looking clearly sick were not chosen to avoid bias towards saprophytic fungi among isolates.

A pair of highly vital and a clearly less vital tree was chosen in each plot and marked with a red ribbon so that they could be recognized easily during the second session of harvesting. Per each tree, one branch (appr. 15 cm long) was collected at cardinal point north of the compass and taken to the laboratory in plastic bags within a couple of hours. Once in the laboratory, the branches were processed as follows:

1) The leaves were removed.
2) From each branch, the last and the current year's growth were separated, resulting in two samples, hereafter referred to as old (last year) and young (current year) part.
3) From each part, a 5 cm long piece was cut from the middle area and surface sterilized.

2.4.1 Detection of endophytic fungi

The endophyte community was analysed in a total of 384 samples detached from the processed branches. Only culturable fungi were studied, i.e. no molecular detection based on fungal DNA was used in this study. The fungal communities were captured using the most common, 3-step technique for detecting fungal endophytes in plants (Guo et al. 1998):

I. Surface sterilization of the plant tissue;
II. Incubation of discs cut from plant tissue on agar and isolation of endophytes which grow out;
III. Identification of the sporulating cultures by traditional methods, mainly microscopy.

The surface sterilization was done following the procedure used by Helander et al. (2007) with some modifications (Blumenstein, 2010):

- Cut approximately 3 cm long piece from each branch;
- Dip the samples into 75% ethanol for 30 seconds;
- Dip the samples in 4% Na-hypochlorite solution for 5 minutes;
- Dip the samples in 75% ethanol for 15 seconds;
- Air drying of the samples for 5 minutes.

The procedure was carried out under a laminar hood in aseptic condition.

Afterwards, the sterilized material was cut in smaller pieces. Each old and young part of the branch was cut in a smaller piece of approximately 5 x 5 mm. From each piece, xylem and bark were separated and plated in 5 cm (diam.) Petri dishes with a 2% Malt Extract Agar (MEA, Appendix 1) which favoured the growth of diverse fungi (Arnold et al., 2003). The dishes were sealed with Parafilm and incubated at room temperature in a dark shelf. The classification of the samples is schematized in Fig. 6.

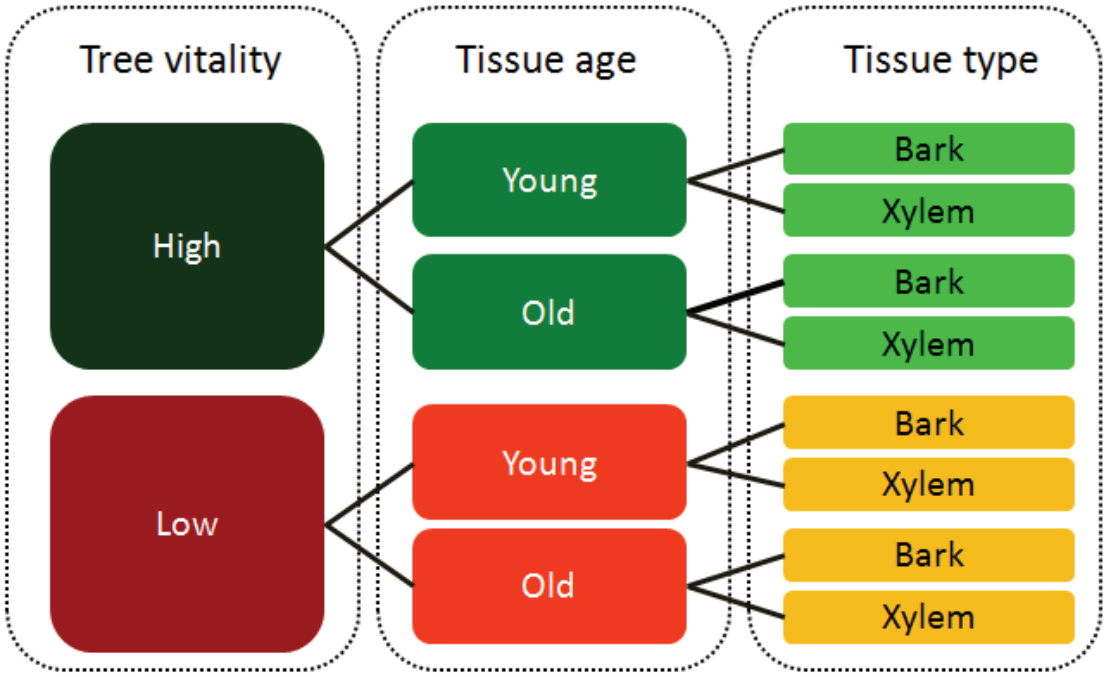


Figure 6 Schematic presentation of the types of samples collected from each tree. The sampling resulted in eight different combinations for each plot sampled: High vitality, old bark; High vitality, old xylem; High vitality, young bark; High vitality, young xylem; Low vitality, old bark; Low vitality, old xylem; Low vitality, young bark and Low vitality, young xylem.

The Petri dishes were checked every day to control if some hyphae were emerging. Each time a new colony was emerging from xylem or bark segments, it was sub-cultured on new 2% MEA plates in order to obtain pure cultures. Some samples were infected by bacteria. In these cases the fungi were cultured on Petri dishes with MEA amended with the antibiotic chloramphenicol (App. A).

2.4.2 Classification of the endophytes

The recovered isolates were classified using a morphospecies approach, i.e. the isolates were grouped to *recognizable taxonomic units* according to their macromorphological traits (Krell, 2004). In diversity analyses, morphotypes were used instead of true taxonomic species. This method is commonly used for grouping those fungi that do not sporulate in culture (Arnold et al., 2000). The method used for morphotyping the fungal endophytes was slightly modified from Arnold et al (2000) and Guo et al (2000), (J. Witzell and K. Blumenstein, pers. comm.).

2.4.3 Morphotype (MT)

The following seven morphological characteristics were used as criteria to divide and group all the fungi (App. B). Each fungus was analysed according to the chosen characteristics and

assigned to a specific morphological group. To be part of the same group, a fungus had to have the same 4 characteristics fixed for the group.

- Colour. The colour of the colony is the first element that was used to discriminate the fungi. If a fungal colony had more than one distinct colour, the description was done from the middle towards the outer part.
- Agar colour**.** Some fungi produce substances that can change the colour of the agar where the colony is growing.
- Liquid drops. The presence and the colour of the liquid drops that some fungi produced on their surface were recorded.
- Colony shapes. Each colony grows in a certain way forming different shapes (Fig. 7). A total number of 8 shapes were distinct: crenate, entire edge, erose or dentate, fimbriate, lobate, radially striate with lobate edge, undulate, with rhizoids (Crous et al., 2009).

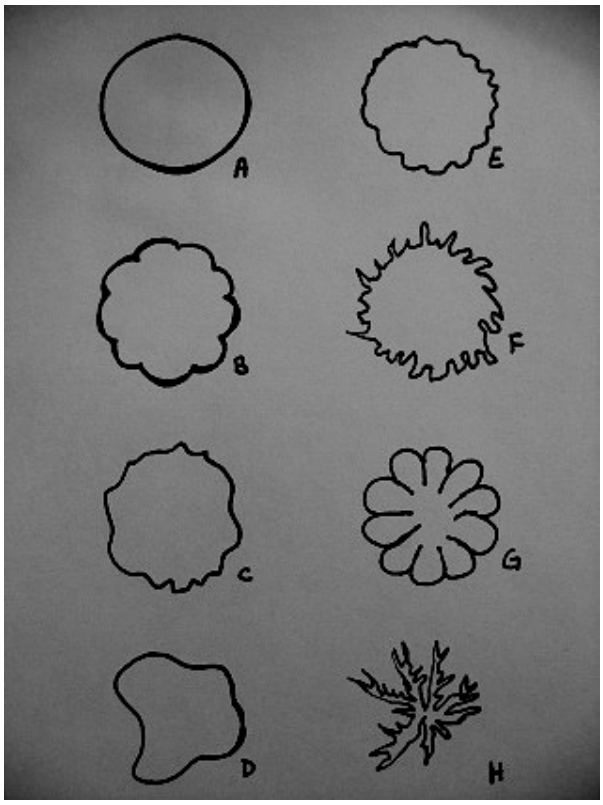


Figure 7 Terminology of the colony morphologies. A: entire edge. B: lobate. C:erose or dentate. D: undulate. E: crenate. F: fimbriate. G: radially striate with lobate edge. H: with rhizoids. (From Crous et al, 2009).

- Colony texture. Seven fixed textures were distinct: woolly, velvety, hilly, spongy, dry, furry and creamy (App. C).

- Spore accumulation. It was taken note if the fungi developed a clear accumulation of spores on their surfaces and the colour of the spores was registered.
- Growth rate. The growth rate was studied as an additional characteristic in order to increase the common characteristic within a group. For carrying out the growth rate measurement, three fungi for each group were randomly selected with the random selection function available in the Excel software. For each selected fungus, three replicates were sub-cultured in small Petri dishes with 2% MEA. To uniform the starting point, a cork borer was used to cut a circular mycelial plug (4 mm in diameter) from the leading edge of 3 to 4 months old colony. Then, the plug was placed in the middle of the small Petri dish. The middle point of each plate was marked in advance to guide the placement of the plug and define two perpendicular axes, α and β, where the measurements were taken (Fig. 8). The measurements of the colony expansion along these axes were done using a ruler with an accuracy of 1 mm daily (on working days) during a 14-day period. For the missing measurements (weekends) an interpolated data was calculated. An average was calculated for the replicates (n=3) and a rating system was determined in relation to the fastest growing group that covered the entire Petri dish in 8 days (the groups were defined comparing their speed of growth with the fastest group). The values go from 1 to 5 where 1 represents the slowest and 5 the fastest (data growth rate App. D).

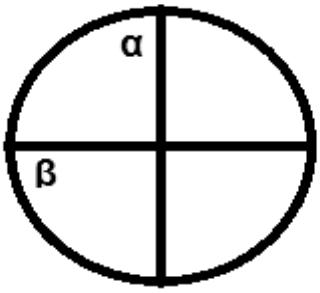


Figure 8 Schematic presentation of the axes α and β used for the measurement of the colony growth rate on Petri dishes

The amount of morphotypes yielded by a single Petri dish was recorded. The positive samples produced at least one fungus up to a maximum of six. In Fig. 9 the schematization of yielded fungi is shown.

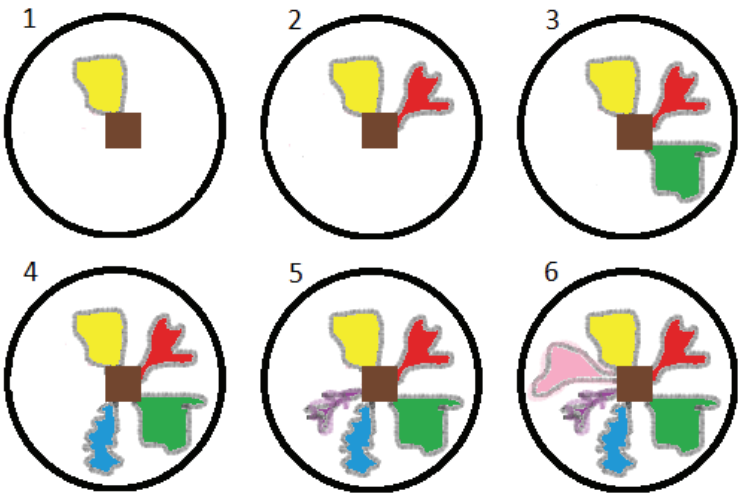


Figure 9 Scheme of the numbers of yielded fungi/morphotypes from plated samples. 1 produced only one morphotype; 2 produced two morphotypes; 3 produced three morphotypes; 4 produced four morphotypes; 5 produced five morphotypes: 6 produced six morphotypes.

2.5 Data analysis

The purpose of statistical analysis was to investigate eventual differences in diversity of endophyte communities between two different time periods and among different origins of the samples (vitality, age, tissue type). The following descriptors were calculated on basis of the isolation data.

2.5.1 Colonisation and isolation rate

The colonisation rate and the isolation rate were calculated as the mean of the four replicates (blocks) in order to study the qualitative and quantitative differences in communities as described by Kumar and Hyde (2004).

For comparison of endophyte infection frequency in general, the **colonisation rate** was calculated as the total number of replicates in a sample yielding one or more isolates, divided by the total number of sections in that sample:

$$\text{Colonisation rate} = \frac{\text{}}{\text{}}$$

For comparison of the morphotype frequency, the **isolation rate** was calculated by the total number of morphotypes yielded by a given sample divided by the total number of replicates in that sample.

$$\text{Isolation rate} = \frac{\text{}}{\text{}}$$

Because of many zero-values, the data did not fill the assumption for normal distribution, nor could the samples be regarded independent. Therefore, the non-parametric Wilcoxon signed-rank test was used for pair wise comparisons (vitality, age, branch part, collection period) and the Friedman test was applied to study the colonization and isolation rates data collected from trees in different vegetation treatments.

2.5.2 Diversity and similarity indices

The morphotype data was studied using indices. The diversity and similarity indices are two different types of indices exploring the community structure (Washington, 1984). They are used to facilitate the interpretation of large amount of data (Pontasch, 1988) and can be useful in the case of samples with a limited number or intensity, on the other hand, it is important to keep in mind that they are usually sensitive to the sample size (Chao et al., 2005).

The **diversity indices** try to combine the data or the abundance within a certain species belonging to a community into a single number that expresses the state of the community with a single number (Washington, 1984).

The **similarity indices** are used to compare two samples or two areas' population where one of the two is usually the control site (Washington, 1984; Danilov and Ekelund, 1999). They are also used in plant ecology even if they are mostly used for the evaluation of aquatic areas after cases of pollution (Washington, 1984).

In this study the **Jaccard similarity index** and **Shannon diversity index** were calculated to compare the endophyte community structures in the different vegetation manipulation treatments and in the two different periods of collection.

2.5.2.1 Jaccard's similarity index

The Jaccard index is the oldest and simplest similarity index expressing the percentage of species shared in common within two samples (Washington, 1984). It is based on the presence/absence of a species within a certain population giving a qualitative comparison of the samples (Pontasch, 1988; Chao et al., 2005). The formula is:

$$J = \frac{C}{A+B-C}$$

where A is the number of species in sample 1, B the number of species present in sample 2 and C is the sum of the species in common between A and B. The value of Jaccard index

varies between 0 and 1, where 0 means there is no similarity at all between the two analyzed population and 1 a complete similarity between the two samples.

In this study, Jaccard index was used to compare the similarity of the morphotypes population in the six vegetation treatments between the collection done in June and August.

2.5.2.2 Shannon's diversity index

This index allows knowing the number of species and how the abundance of the species is distributed among all the species in the community.

$$H' = -\sum_{i=l}^{s}(Pi * lnPi)$$

Where Pi is the fraction of the entire population made up of a certain morphotype, S is the number of morphotypes encountered, Σ is the sum from morphotype 1 to morphotype S. The higher the value of H' is, the more the two analyzed samples differ in their endophyte diversity. When a sample has only one species, it would have an H' value equal to 0.

Sometime the diversity index values are not comparable to each other and the evenness is used to allow a fair comparison among the data. It ranges between 0 and 1 and it is measured as the division between the H' values and H'_{max}

$$E = \frac{H'}{H'max}$$

when the values are equal to 1 it means that all the species of the samples are equally distributed; on the contrary, when the value is closer to 0 it means that the species are not equally distributed within the population (Jost, 2006).

To better compare the results from Shannon´s index, the diversity needs to be calculated in order to find the true diversity of the population. The true diversity is calculated as

$$D = exp^{(H')}$$

The values of the diversity show how many individuals for species are equally distributed for the highest value of H' (Jost, 2006).

2.5.3. Statistical tests

The data was organized in Microsoft Office Excel 2007 and analysed using Minitab 16 and SPSS version 17 softwares. The colonization and isolation rate data were not normally distributed and therefore pair wise comparisons (time points, vitality groups, tissue type and age) of these values were done with the nonparametric Wilcoxon signed rank test and the Friedman test was used for multiple comparisons of data from the six treatments. Because initial analysis showed significant differences between the data from the two sampling dates, the early summer and late summer data were treated separately. For the normally distributed index values (Jaccard and Shannon indices, true diversity), no significant treatment effect was found in initial analyses (ANOVA, GLM). Therefore, the index data from all treatments were pooled to compare the data from the collection dates and vitality groups with paired samples *t*-test.

3. RESULTS

3.1 Temporal and spatial variation in endophyte isolation frequency

The number of positive isolations (i.e., at least one fungus recovered) and the colonization density, measured as percentage of segments infected by at least one fungus from the total number of segments in each tissue are shown in table 1. The data for colonization and isolation rates, divided to tree vitality groups and sampling times are shown in Fig. 10.

*Table 1 Number (N) and percentage (%) of positive and sterile endophyte isolations from the xylem and bark of the current (2011) and previous (2010) year's growth (young, "Y", and old, "O", respectively) in twigs of young oak (*Quercus robur L.*) trees showing high or low vitality in early and late season.*

Early season (June)								**Late season (August)**							
Isolation result	***N (%)***	***Tissue***	***N (%)***	***Vitality***	***N (%)***	***Age***	***N (%)***	**Isolation result**	***N (%)***	***Tissue***	***N (%)***	***Vitality***	***N (%)***	***Age***	***N (%)***
Positive	72 *(37.5)*	***Xylem***	7 *(9.7)*	***High***	5 *(71.4)*	**Y**	2 (28.6)	**Positive**	100 *(52.1)*	***Xylem***	10 *(10)*	***High***	6 *(60)*	**Y**	2 (20)
						O	3 (42.9)							**O**	4 (40)
				Low	2 *(28.6)*	**Y**	0 (0)					***Low***	4 *(40)*	**Y**	1 (10)
						O	2 (28.6)							**O**	3 (30)
		Bark	65 *(90.3)*	***High***	*34 (52.3)*	**Y**	10 (15.4)			***Bark***	90 *(90)*	***High***	*47 (52.2)*	**Y**	23 (25.6)
						O	24 (36.9)							**O**	24 (26.7)
				Low	*31 (47.9)*	**Y**	7 (10.7)					***Low***	*43 (47.9)*	**Y**	19 (21.1)
						O	24 (36.9)							**O**	24 (26.7)
Negative	120 *(62.5)*	***Xylem***	89 *(74.2)*	***High***	43 *(48.3)*	**Y**	22 (24.7)	**Negative**	92 *(47.9)*	***Xylem***	86 *(93.5)*	***High***	42 *(48.8)*	**Y**	22 (24.7)
						O	21 (23.6)							**O**	20 (22.5)
				Low	46 *(51.7)*	**Y**	24 (27.0)					***Low***	44 *(51.2)*	**Y**	23 (25.8)
						O	22 (24.7)							**O**	21 (23.6)
		Bark	31 *(25.8)*	***High***	14 *(45.2)*	**Y**	14 (45.2)			***Bark***	6 *(6.5)*	***High***	1 *(16.7)*	**Y**	1 (16.7)
						O	0 (0)							**O**	0 (0)
				Low	17 *(54.8)*	**Y**	17 (54.8)					***Low***	5 *(83.3)*	**Y**	5 (83.3)
						O	0 (0)							**O**	0 (0)-

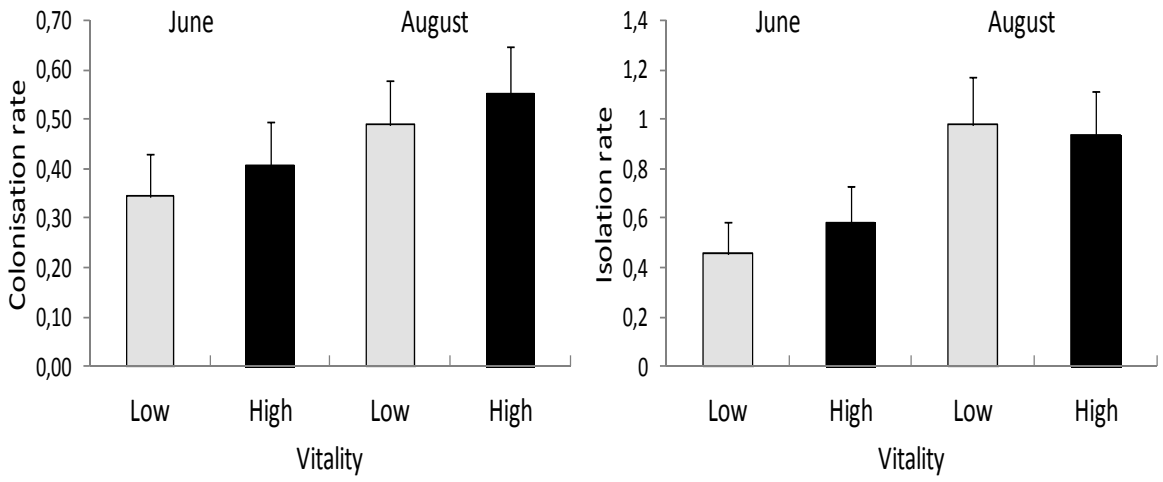


*Figure 10 Mean values of colonisation and isolation rate by endophytes from current and last year xylem and bark in oak (*Quercus robur L.*) twigs (vertical bars represent standard error = 4).*

Within-seasonal differences

The number of productive isolations was higher in the late summer (August) than in early summer (June) (Table 1). This temporal difference in colonisation rate was significant both in declining trees (Wilcoxon Signed Rank test p=0.0017) and in the vital trees (p= 0.002).

Differences between asymptomatic and declining trees

In most cases, a higher number of positive isolations were obtained from trees with a high vitality, as compared to declining trees (Table 1).

In June, the colonisation rate (Figure 10) differed nearly significantly between the trees showing different degree of vitality (Wilcoxon Signed Rank test p=0.059). In August, the difference in colonisation rate was not significant (p=0.116).

Differences between younger and older branch parts

In all cases the previous year's growth ("old") tissues yielded more positive isolations than the current years' ("young") growth. Both in June and August the effects of age on colonisation rate was significant (p=0.001 and p=0.045, respectively)

Tissue-specific differences

The majority of the positive isolations were obtained from bark, while only a few positive isolations were obtained from xylem. Both in June and August the results showed significant

difference among the two tissues (Table 1; Wilcoxon Signed Rank test for tissue type, p= 0.000).

Among the xylem samples, there was a negligible increase in the number of positive samples during the season. In the bark samples, a different pattern was observed: the old bark produced 24 positive samples out of 24 already in June (regardless of the vitality), while the numbers of positive samples in young bark tissue samples was more than doubled during the summer (Table 1).

3.2 Temporal and spatial variation in morphotype frequency

On basis of the macromorphological characters, the fungal isolates were classified to 22 different morphotype groups (1 to 12, 15 to 17, 19, 21, 22, 24 and 26 to 28) (App. 5). The total number of the morphotypes tended to be somewhat higher in vital trees as compared to declining trees. Of the individual morphotypes, morphotype 4 was the most abundantly isolated one. It was found as 62 isolates (21.8% of all isolates; Fig. 11 and 12) and from nearly all types of samples types (App. E). Other abundant morphotypes were 22 (isolated from bark in early summer, and from all tissues except current-year xylem later in the season); 9 (isolated from older bark throughout the season and also from younger bark in late season); 1 (isolated particularly from trees with high vitality); 24 (with irregular pattern of occurrence among the sample types); 2 (with similar occurrence pattern than morphotype 9) and 3 (isolated particularly often from bark samples). The other 16 morphotype groups were represented by 33.6 % (App. E; Fig. 11).

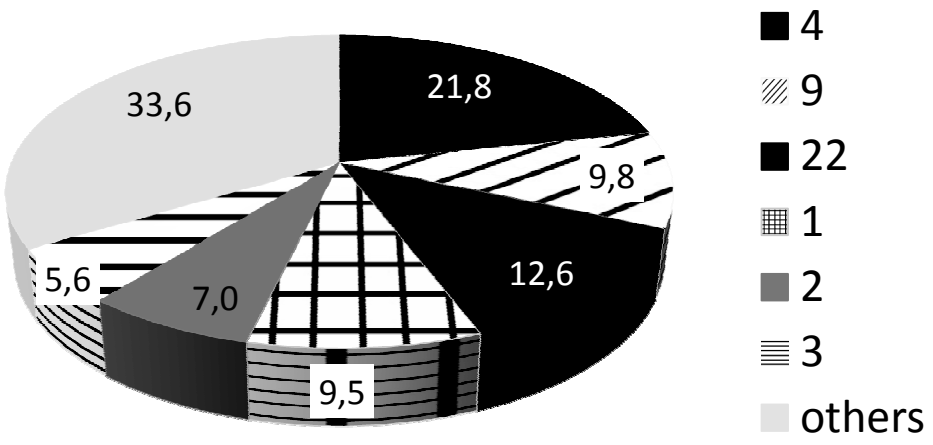


Figure 11 Abundance of morphotypes in oak twigs (xylem and bark). The six most abundant morphotypes were groups 4, 22, 9, 1, 2 and 3, in this order, and the others were groups 5, 6, 7, 8, 10, 11, 12, 15, 16, 17, 21, 24, 26, 27, 28

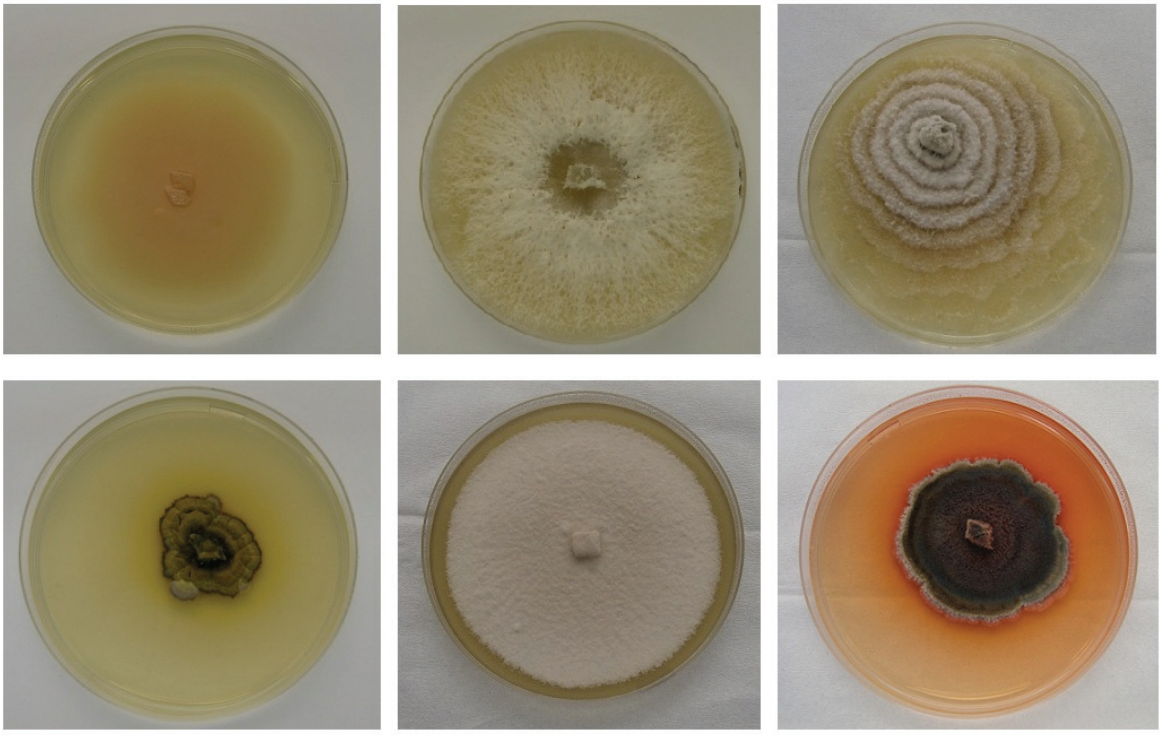


Figure 12 Pictures of representative isolates belonging to the most abundant morphotypes. From the left to the right respectively in the first row morphotype groups number 1, 2, 3 and in the second row number 4, 9 and 22

Within-seasonal differences

The isolation rate differed significantly between the trees with different vitality in June (Wilcoxon Signed Rank test p=0.002), and also in August (p=0.049).

Eight out of 22 morphotype groups showed a close relation with the season of collection. Morphotypes belonging to groups 19 and 27 were found only in twigs collected during the early summer, whereas those belonging to groups 5, 7, 11, 16, 17 and 26 were found in twigs harvested in the late summer (App. E).

Differences between asymptomatic and declining trees

In June, the isolation rate was slightly higher in vital trees, while the contrary pattern was found in August (Fig. 10). However, the difference in isolation rate between the trees showing different vitality status was not significant in June (p=0.065) nor in August (p=0.944).

Only few morphotype groups showed a clear relation with the health status of the tree: groups 7 and 11 were formed only by fungi recovered from twigs of vital trees, and groups 17, 27 and 28 from less vital trees (App. E).

Differences between younger and older branch parts

Significantly higher numbers of morphotypes were recovered from older twig parts in June (p=<0.0001) but not in August (p=0.344) (App. E). Most of the groups (thirteen) contained fungi that were found either from the younger or older branch parts, while the morphotypes 15 and 17 were exclusively found in the younger branch part and 6, 7, 8, 11, 16, 19 and 27 in the older branch part (App. E).

The variety of morphotypes present in the old and young tissues differed mainly in the first period of collection. The young twig parts collected during the early summer produced few positive samples and consequently there were less morphotype groups. The harvesting done in the late summer revealed a quite uniform distribution of morphotypes among old and young bark samples, while the samples belonging to the xylem part still resulted in a smaller number of positive samples.

Tissue-specific differences

Significantly higher numbers of morphotypes were recovered from bark as compared to xylem for both time periods (Wilcoxon Signed Rank test for isolation rate in xylem and bark p=0.000)

Fourteen of the morphotypes contained only fungi that were recovered from bark tissue, but there were no morphotypes specific to xylem; the remaining eight groups were formed by fungi from either the bark or the xylem (App. E).

3.3 Effect of surrounding vegetation on twigs endophyte communities

The difference in surrounding vegetation had no significant effect on the colonisation rate in June (Friedman test $\chi2 = 5.86$, p = 0. 329) nor in August (Friedman test $\chi2 = 7.02$, p = 0. 219). Similarly, no significant effect of the vegetation on the isolation rate was detected in June (Friedman test $\chi2 = 8.64$, p = 0. 124) nor in August (Friedman test $\chi2 = 5.86$, p = 0. 320).

In most treatments, the samples collected in the early summer produced only one morphotype (Fig. 13). In the late summer, the probability of finding three or more morphotypes per sample was higher than in the early summer (Fig. 13). At this time point, the samples belonging to declining trees seemed to have a higher tendency in producing more than three morphotypes, as compared with those from vital trees.

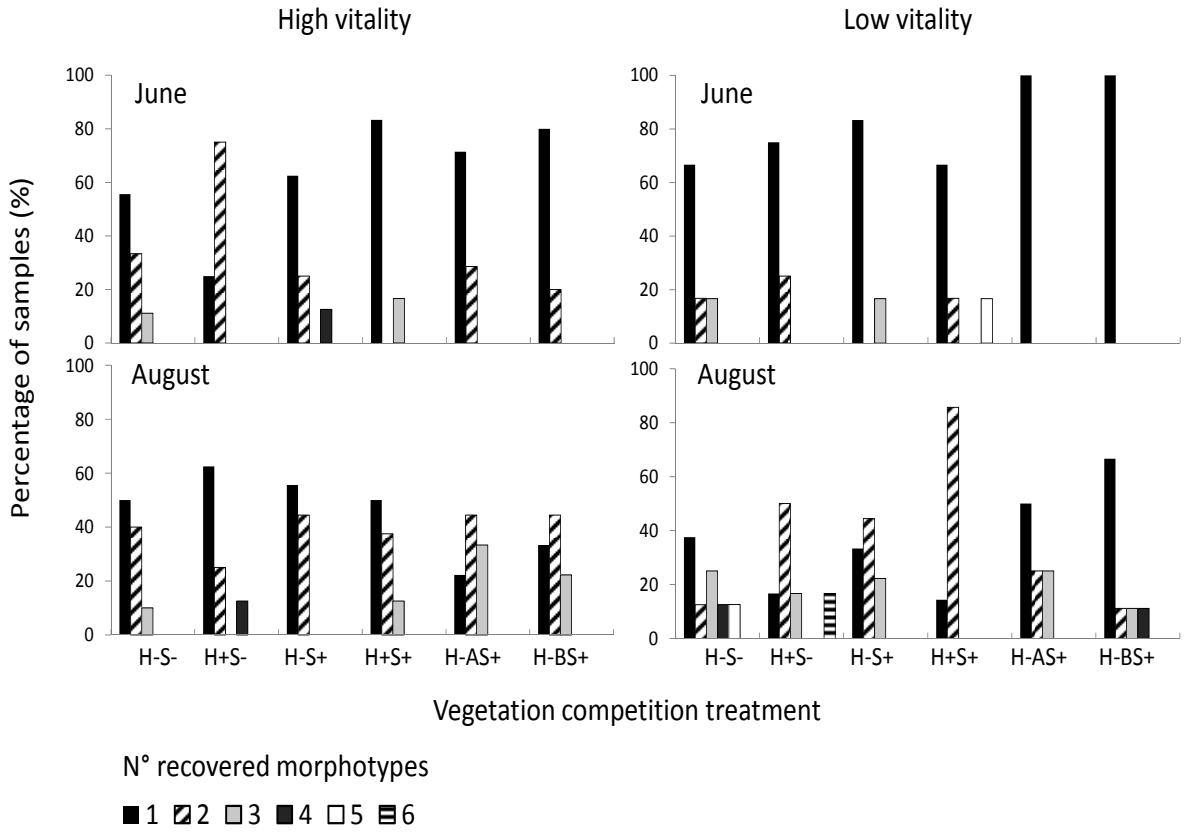


*Figure 13 Percentage of pedunculate oak (*Quercus robur L.*) twig (bark and xylem) samples yielding 1 to 6 morphotypes. The samples were collected at two time points (June and August) from vital or declining young oak trees, growing in six vegetation competition treatments. The percentage is calculated as positive isolations per four tissue pieces (young and old; bark and xylem) per treatment. The colors and patterns of the bars code for the number of different fungal morphotypes recovered. The vegetation competition treatments were H-S- (no competition from herbs or shrubs); H+S- (competition from herbs); H-S+ (competition from shrubs); H+S+ (competition from herbs and shrubs); H-AS+ (aboveground competition from shrubs); and H-BS+ (belowground competition from shrubs).*

Differences between treatments

Morphotype groups 1, 2, 4, 9, 10 and 22 were present in all six different treatments). The groups 3, 5, 6, 8, 12, 15, 24, 26 and 28 were slightly more restricted in their distribution and were found in 3 to 5 treatments. Groups 7, 11, 16, 17, 19, 21 and 27 were most selective and were found only in a couple of treatments.

The isolates belonging to group 5 were found only in treatments 3, 5 and 6 which included competition from shrub vegetation with control of herbal vegetation. The fungi belonging to group 7 were found only in treatments 5 and 6 where there was competition from above and below shrub vegetation with control of herbs.

3.3.1 Comparison of similarity and diversity using indices

3.3.2 Jaccard's similarity index

The values of Jaccard similarity index J were generally lower in the beginning of the summer and higher in August (Table 3 – August values in shaded color). In early summer, the endophyte communities in trees growing without herbs and with below ground competition (and with reduced above-ground competition) had generally the lowest Jaccard index values (Table 3), suggesting that these communities differed most strongly from the others. The highest Jaccard index values (over 0.65) were found mainly, although not exclusively, for treatments with shrubs as component of neighboring vegetation in August.

Table 2. Jaccard's similarity index values for pedunculate oak (Quercus robur L.*) twig endophyte communities at two time points (June and August 2011). The samples were collected from xylem and bark of young trees showing high or low vitality and growing in six vegetation competition treatments. The vegetation competition treatments were H-S- (no competition from herbs or shrubs); H+S- (competition from herbs); H-S+ (competition from shrubs); H+S+ (competition from herbs and shrubs); H-AS+ (aboveground competition from shrubs); and H-BS+ (belowground competition from shrubs).*

Vegetation competition treatment	**H-S-**	**H+S-**	**H-S+**	**H+S+**	**H-AS+**	**H-BS+**
	June					
H-S-		0.55	0.36	0.27	0.40	0.2
H+S-	0.53		0.54	0.43	0.50	0.18
H-S+	0.39	0.67		0.57	0.55	0.25
H+S+	0.60	0.71	0.77		0.31	0.15
H-AS+	0.50	0.50	0.73	0.67		0.25
H-BS+	0.50	0.42	0.44	0.39	0.65	
	August					

The lowest J-values were found between treatment H+S+ and H-BS+ in the early summer, and between H-S- and H-S+, and H+S+ and H-BS+, in the late summer, indicating low similarity of endophyte communities in these pairs. The endophytic populations with the highest J-values and thus highest similarity were found in oaks growing in treatments H-S+ and H+S+ for both time period of collection (Table 3).

3.3.3 Shannon's diversity index, richness, evenness and diversity

Table 4 shows the amount of morphotypes and the values for the Shannon index and their evenness and diversity divided among the treatments, the collection period and the high and low vitality groups.

In most cases the value of richness, Shannon index and diversity, increased significantly during the summer (Table 4; ANOVA results for effect of sampling time: F=21.68, p=0.006 for richness; F=34.32, p=0.002 for Shannon index, and F=59.53, p=0.005 for diversity). There was no significant seasonal trend in evenness values (ANOVA for time effect: F=0.26, p=0.633).

*Table 3 Morphotype richness (number of morphotypes), Shannon's diversity index, evenness and diversity values for pedunculate oak (*Quercus robur L.*) twig endophyte communities. The samples were collected at two time points (June and August 2011) from current and previous year's xylem and bark of young trees showing high (H) or low (L) vitality and growing in six vegetation competition treatments. The vegetation competition treatments were H-S- (no competition from herbs or shrubs); H+S- (competition from herbs); H-S+ (competition from shrubs); H+S+ (competition from herbs and shrubs); H-AS+ (aboveground competition from shrubs); and H-BS+ (belowground competition from shrubs).*

Treatment	Vitality	Richness		Shannon H'		Evenness		Diversity	
		June	August	June	August	June	August	June	August
H-S-	H	6	8	1.38	2.06	0.51	0.76	4.0	7.9
	L	5	12	1.58	2.35	0.72	0.78	4.9	10.4
H+S-	H	4	8	1.55	1.99	0.80	0.78	4.7	7.3
	L	5	10	1.61	2.21	1.00	0.82	5.0	9.1
H-S+	H	6	6	1.61	1.67	0.63	0.65	5.0	5.3
	L	7	9	1.91	1.96	0.92	0.69	6.7	7.1
H+S+	H	5	9	1.55	2.10	0.80	0.82	4.7	8.2
	L	9	7	2.15	1.82	0.89	0.71	8.5	6.2
H-AS+	H	4	11	1.21	2.23	0.55	0.76	3.4	9.3
	L	5	8	1.56	1.87	0.87	0.71	4.8	6.5
H-BS+	H	3	10	0.95	2.12	0.59	0.75	2.6	8.3
	L	3	10	0.95	2.21	0.59	0.82	2.6	9.1

The effect of vitality was significant only for evenness (ANOVA, F=10.02, p=0.025). Pairwise comparisons of values within the two sampling times revealed significant differences between vital and declining trees in Shannon index (T=-285, p= 0.036) and evenness (T=-3.82, p= 0.012), in June when the diversity values showed higher numbers in declining trees, as compared with the high vitality trees. At the same time point, the diversity values of vital and declining trees differed nearly significantly (T=-2.41, p=0.061). The diversity values tended to increase from early summer to late summer.

There was no significant treatment effect on richness ($F=0.21$, $p=0.942$), Shannon index ($F=1.16$, $p=0.437$), evenness ($F=3.33$, $p=0.107$) or diversity ($F=0.39$, $p=0.836$).

4. DISCUSSION

Within-seasonal differences

The endophyte communities within oak twigs showed a clear temporal increase, with more successful isolations and morphotypes in August than in June. The increase in infection's frequency and diversity during the growth season confirms results from several other reports on the succession of fungal communities in plants (Schulz and Boyle, 2005; Sieber, 2007). The succession of the endophyte communities proceeded from more diverse to more similar. For instance, the Jaccard index showed a clear increase in overlap among the treatments in the late summer, showing that the higher time of exposure to the infection leads to a higher similarity between the samples. The Shannon index showed narrower results range in the late summer collection, meaning that the overall species distribution was more equal in August than in June.

Differences between asymptomatic and declining trees

As compared to vital trees, the evenness (distribution of individuals within morphotypes) was higher in declining trees in early summer. Possibly, the declining trees were compromised in their ability to defend themselves against fungal infections. It seems possible that in the absence of strong interference from the habitat (i.e. tree defenses) the interspecific competition between the fungi would have been high, leading to clear dominance by certain species. However, my results indicate that the lower vitality, probably accompanied by low defenses led to reduce the competition between different fungi. Comparable results have been obtained in studies with elms, where the Dutch elm disease resistant trees had restricted endophyte diversity and frequency in xylem (Martín et al., unpublished). If the less vital trees are a more suitable habitat for a larger variety of endophytes as compared to the vital trees, the declining trees have higher value of endophyte diversity. Therefore, the replacement of less vital trees with vital genotypes in forests could lead towards negative effects on the endophyte diversity. Because different endophyte species might be also functionally different, this could affect negatively some ecological functions in forests.

Nevertheless, the lack of temporarily consistent and strong differences in endophyte communities between vital and declining trees in my study suggests that endophyte communities in young oaks do not systematically reflect the health status of the tree. In earlier studies, however, endophyte communities have been found to vary between healthy and

declining trees (Ragazzi et al., 2003; Giordano et al., 2009) or pathogen resistant and susceptible trees (Martín et al., unpublished). It cannot be excluded that a more comprehensive sampling of trees could have revealed more clear differences also in my study. The variation of the endophytes community varies greatly within trees (Saikkonen, 2007) showing organ (i.e. leaves, bark, xylem) and host specificity (Sieber, 1989). Furthermore, the frequency and distribution of endophytes species vary in relation to the season, site, weather condition, host characteristics (Petrini et al., 1992; Sieber, 2007; Helander et al., 2006) and the colonisation of the different tissues might differ according to the endophytes pattern of infection (Schulz and Boyle, 2005).

Another relevant aspect to be considered is the influence of the isolation procedure (Guo et al., 2003), the choice of media on which the endophytes were plated and the growth conditions. For detecting the endophyte community, the most common technique for surface sterilization was used (Guo et al., 1998) but even though, it is feasible that a small amount of spores or hyphae pieces present on the surfaces of the twigs survived to the procedure (Petrini, 1986) or penetrated in the host tissues (Petrini, 1986 and refs. therein). Thus, the procedure may have allowed some persistent epiphytic species to be detected along with the true endophytes. On the other hand, a longer sterilization procedure could also negatively affect the endophytes living in the upper parts of the tissues. Isolations were done on malt extract agar because it is considered to be one of the most suitable substrates for the proliferation of endophytes but it could be that some species were not able to grow on this particular medium (Guo et al., 2003). Certain endophytes may be generally uncultivable on agar (Clay, 2006; Moricca and Ragazzi, 2008). Furthermore, fungi show different temperature preferences: the optimal temperature varies among species, causing a possible variation in the growth of the samples of the current study. A further common problem in isolation studies is also that the slowly growing ones could have been overgrown by the fast growth endophytes, and thus escaped detection (Cairney, 2006). Hence, the amount of fungi recorded in the current study and the size of the morphotypes could have been biased or underestimated (Moricca et al., 2004).

The choice of MEA might also have influenced the morphotyping. Indeed, the macromorphological traits and growth rate of colonies can vary according to the type of substrates selected for the experiment (Guo et al., 2003) leading to possible different characteristics and consequently to a different morphotyping. Moreover, although that

parataxonomic method (morphotyping) is an established, rapid and easy method and has a value in preliminary detection of biodiversity patterns (Krell, 2004), more detailed studies utilizing recent molecular methods would be needed to further explore the community structure of endophytes in oak twigs.

Differences between younger and older branch parts

In parallel with the seasonal (temporal) increase in endophyte communities of oak twigs, the succession of horizontally spread endophyte infections was obvious also in different-aged tissues: in the previous year's tissues the endophyte population had an additional year to establish itself within the host and thus a more diverse and abundant endophyte community could be detected in older bark and xylem. This result agrees with the findings of Kowalski and Gajosek (1998) and Kowalski and Kehr (1990) who found tissue age dependent variation in endophytes due to factors such as branch diameter or bark characters.

Tissue-specific differences

The tissue-specific colonization pattern observed in my study (higher colonization of bark and low colonization of xylem) is in agreement with the findings of Kowalski and Gajosek (1998). They reported that less than 4% of the studied birch (*Betula pendula*) xylem samples were colonized by endophytes, whereas over 70% of bark samples had infections. The fact that xylem yielded a lower amount of fungi can be due to the fact that this tissue is found in the inner part of the twig and therefore the endophyte infections that proceed from the surface might need longer time to colonize this type of tissue. Moreover, the specificity of some fungi could have limited the colonization to the bark tissues, which are probably easier to be invaded or a more suitable substrate or habitat to the fungi due to the higher nutrient availability.

Differences between treatments

The results of the current study indicate that the endophyte infections are more strongly dependent on the macro-environment than the immediate micro-environment surrounding the canopy: no significant effect of surrounding vegetation could be detected either on the colonization or isolation frequency of the endophytes, and while there were some morphotypes that seemed to show a relation with the treatments, no strong association of certain morphotypes to either shrubs or herbs could be established.

5. CONCLUSIONS

In conclusion, the results of this study indicate that the endophyte communities in twigs of young oaks did not strongly reflect the overall vitality status of the host trees. However, some results indicate that the declining trees could be suitable to a broader spectrum of species, as compared to the vital trees. A more thorough sampling and analysis of endophyte communities using molecular methods that detect also other fungal species would be needed to confirm this result. The frequency and diversity of endophyte infections was higher in bark than in xylem, and increased during the season in particularly in the bark, as could be expected on the basis of existing literature. These findings underline the temporal and spatial dynamics in endophyte communities due to horizontal spreading, and as a function of a tree's quality as a substrate and habitat for the microfungi. The lack of clear effects of qualitative differences in the neighbouring vegetation around the oaks suggests that the horizontal infections by endophytes spread in the landscape at a larger scale.

REFERENCES

Arnold, A. E., Maynard, Z., Gilbert, G. S., Coley, P. D. and Kursar, T. A. (2000). *Are tropical fungal endophytes hyperdiverse?* Ecology Letters 3: 267-274.

Arnold, A. E., Mejia, L. C., Kyllo, D., Rojas, E. I., Maynard, Z., Robbins, N. and Herre, E.A. (2003). *Fungal endophytes limit pathogen damage in a tropical tree.* PNAS 100: 15649-15654.

Arnold, A. E. (2007). *Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers.* Fungal Biology Reviews 21: 51-66.

Blumenstein, K. (2010). *Characterization of endophytic fungi in the genus Ulmus: putative agents for the biocontrol of Dutch Elm Disease (DED).* Diploma thesis, University of Kassel.

Cairney, J. W. G. (2006). *Mycorrhizal and endophytic fungi of Epacrids (Ericaceae)*. Pages 247-260 in: *Microbial Root Endophytes*. B. J. E. Schulz, C. J. C. Boyle, and T. N. Sieber, eds. Springer-Verlag, Berlin.

Carrol, G. (1988). *Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont*. Ecology: 69, 2-9.

Chao, A., Chazdon, R. L., Colwell, R. K. and Shen, T. -J., (2005). *A new statistical approach for assessing similarity of species composition with incidence and abundance data.* Ecology Letters 8: 148-159.

Clay, K. (2006). *Fungal endophytes of plants: biological and chemical diversity*. Natural Toxins 3: 147-149.

Crous, P. W., Verkley, G. J. M., Groenewald, J. Z. and Samson, R. A. (2009). *Fungal Biodiversity.* CBS Laboratory manual series. CBS-KNAW Fungal Biodiversity Center.

Danilov, R. and Ekelund, N. G. A. (1999). *The efficiency of seven diversity and one similarity indices based on phytoplankton data for assessing the level of eutrophication in lakes in central Sweden.* The Science of the Total Environment 234: 15-23.

Devarajan, P. T. and Suryanarayanan, T. S. (2006). *Evidence for the role of phytophagous insects in dispersal of non-grass fungal endophytes.* Fungal Diversity 23: 111-119.

Gellini, R. and Grossoni, P. (1997). *Botanica forestale II Angiosperme.* CEDAM

Gennaro, M., Gonthier, P. and Nicolotti, G., (2003). *Fungal endophytic communities in helathy and declining Quercus robur L. & Quercus cerris L. trees in northern Italy.* Journal of Phytopathology 151: 529–534

Giordano, L., Gonthier, P., Varese, G. C., Miserere, L. and Nicolotti, G. (2009). *Mycobiota inhabiting sapwood of healthy and declining Scots pine (Pinus sylvestris L.) trees in the Alps*. Fungal Diversity 38: 69-83.

Guo, L. D., Hyde, K. D. and Liew, E. C. Y (1998). *A method to promote sporulation in palm endophytic fungi.* Fungal Diversity 1: 109-113.

Guo, L. D., Hyde, K. D. and Liew, E. C. Y. (2000). *Identification of endophytic fungi from Livistona chinensis based on morphology and rDNA sequences.* New Phytologist 147: 617-630.

Guo, L. D., Huang, G. R., Wang, Y., He, W. H., Zheng, W. H. and Hyde, K. (2003). *Molecular identification of white morphotype strains of endophytic fungi from Pinus tabulaeformis.* Mycological Research 107: 680–688.

Helander, M., Wäli, P., Kuuluvainen, T. and Saikkonen, K. (2006). *Birch leaf endophytes in managed and natural boreal forests.* Canadian Journal of Forest Research 36: 3239–3245.

Helander, M., Ahlholm, J., Sieber, T. N., Hinneri, S. and Saikkonen, K. (2007). *Fragmented environment affects birch leaf endophytes.* New Phytologist 175: 547-553.

Herre, E. A., Mejia, L. C., Kyllo, D. A., Rojas, E., Maynard, Z., Butler, A. and Van Bael, S.A. (2007). *Ecological implications of anti-pathogen effects of tropical fungal endophytes and mycorrhizae.* Ecology 88: 550-558.

Hyde, K. D. and Soytong, K. (2008). *The fungal endophyte dilemma.* Fungal Diversity 33: 163-173.

Jensen, A. (2011). *Effects of facilitation and competition on oak seedlings*. Doctoral Dissertation. Acta Universitatis Agriculturae Sueciae, 2011:58

Jost, L. (2006). *Entropy and diversity.* OIKOS 113: 2.

Korkama- Rajala T., Müller M. M. and Pennanen T. (2008*). Decomposition and Fungi of Needle Litter from Slow- and Fast-growing Norway spruce (Picea abies) Clones.* Microbiotic Ecology 56: 76–89.

Kowalski, T. and Kehr, R. (1992) *Endophytic fungal colonization of branch bases in several forest tree species.* Sydowia 44: 137-168.

Kowalski, T. and Gajosek, M. (1998) *Endophytic mycobiota in stems and branches of Betula pendula to a different degree affected by air pollution*. Österreichische Zeitschrift für Pilzkunde 7: 13–24.

Krell F-T. (2004). *Parataxonomy vs. taxonomy in biodiversity studies –pitfalls and applicability of 'morphospecies' sorting.* Biodiversity and Conservation 13: 795–812.

Kumar, D. S. S and Hyde, K. D. (2004). *Biodiversity and tissue-recurrence of endophytic fungi in Tripterygium wilfordii*. Fungal Diversity 17: 69-90.

Linaldeddu, B. T., Sirca, C., Spano, D. and Franceschini, A. (2011). *Variation of endophytic cork oak-associated fungal communities in relation to plant health and water stress.* Forest Pathology 41: 193–201.

Lorenzi, E., Lorando, E. and Picco, A. M. (2006). *Microfunghi endofitici ed epifitici di Picea abies (L.) Karst. in ambiente naturale ed antropizzato in Lombardia.* Doctoral thesis Dipartimento di Ecologia del Territorio e degli Ambienti Terrestri, sez. Micologia, Università di Pavia.

Moricca, S. and Ragazzi, A. (2007). *Fungal Endophytes in Mediterranean Oak Forests: A Lesson from Discula quercina.* Dipartimento di Biotecnologie agrarie, Sezione di Patologia vegetale, Università di Firenze. Phyto 98: 380-386.

Mossberg, B. and Stenberg, L. (2003). *Den nya nordiska floran.* Wahlström & Widstrand.

Petrini, O. (1986). *Taxonomy of endophytic fungi of aerial plant tissue.* In: N. Fokkema, J. van den Heuvel (Eds.), Microbiology of the Phyllosphere, Cambridge University Press, Cambridge, U.K. (1986), pp. 175–187.

Petrini, O. (1996). *Ecological and physiological aspects of host-specificity in endophytic fungi.* In: Endophytic Fungi in Grasses and Woody Plants: Systematics, Ecology and Evolution. Ed. by Redlin, S. C.; Carris, L. M. St. Paul, MN: APS Press, pp. 87–100.

Petrini, O.**,** Sieber, T. N., Toti, L. and Viret. O. (1992). *Ecology, Metabolite Production, and Substrate Utilization in Endophytic Fungi.* Natural Toxins 1: 185-196.

Pontasch, K. W. and Brusvent, M. A. (1988). *Diversity and community comparison indices: assessing macroinvertebrate recovery following a gasoline spill.* Water Research 22: 619-626.

Pontasch, K. W., Smith, E. P. and Cairns Jr., J. (1989). *Diversity indices, community comparison indices and canonical discriminant analysis: interpreting the results of multispecies toxicity tests.* Water Research 23: 1229-1238.

Ragazzi, A., Moricca, S., Capretti, P., Dellavalle, I. and Turco, E. (2003). *Differences in composition of endophytic mycobiota in twigs and leaves of healthy and declining Quercus species in Italy.* Forest Pathology 33: 31–38.

Ragazzi, A. (2004). Endophytism: knowns and unknowns of an age-old phenomenon. In: "Endophytism in forest trees" (Ragazzi A, Moricca S, Dellavalle I eds). Accademia Italiana di Scienze Forestali, Firenze, pp. 15-32.

Real, R. (1999). *Tables of significant values of Jaccard's index of similarity.* Miscellania Zoologica 22.1 (1999).

Rodriguez, R. J., F. White, F. Jr., Arnold, A. E. and Redman R. S. (2008). *Fungal endophytes: diversity and functional roles*. New Physiologist 182: 314-330.

Saikkonen, K., Faeth, S. H., Helander, M. and Sullivan, T. J. (1998). *Fungal endophytes: a continuum of interactions with host plants.* Annual Review of Ecology and Systematics 29: 319-343.

Saikkonen, K. (2007). *Forest structure and fungal endophytes.* Fungal Biology Reviews 21: 67-74

Schulz, B., Boyle, (?) Draeger, C., Rommert A.-K. and Krhon, K. (2002). *Endophytic fungi: a source of novel biologically active secondary metabolites.* Mycological Research 106: 996-1004.

Schulz, B. and Boyle, C. (2005). *Review: The endophytic continuum.* Mycological Research 109: 661–686.

Sieber, T. N. (1989). *Endophytic fungi in twigs of healthy and diseased Norway spruce and white fir.* Mycological Research 92: 322-326.

Sieber, T. N. (2007). *Endophytic fungi in forest trees: are they mutualists?* Fungal Biology Reviews 21: 75-89.

Washington, H. G. (1984). *Review: Diversity, biotic and similarity indices a review with special relevance to aquatic ecosystems.* Water Research 18: 653-694.

White, J. F. and Torres, M. S. (2010). *Is plant endophyte-mediated defensive mutualism the result of oxidative stress protection?* Physiologia Plantarum 138: 440-446.

Wilson, D. (1995). *Fungal endophytes which invade insect galls pathogens, benign saprophytes, or fungal inquilines?* Oecologia 103: 255-260.

Wilson, D. (1996). *Manipulation of infection levels of horizontally transmitted fungal endophytes in the field.* Mycological Research 100: 827-830.

Wilson, D. (2000). *Ecology of wood plant endophytes.* In: *Microbial endophytes* (Bacon, C. W. and White, J. F. Jr.). Marcel Dekker Inc., New York, USA, pp. 389-420.

Webber, J. (1981). *A natural biological control of Dutch elm disease.* Nature 292: 449-451.

Zabalgogeazcoa, I. (2008). *Fungal endophytes and their interaction with plant pathogens.* Spanish Journal of Agricultural Research 6: 138-146.

APPENDICES

Appendix A:

Chemicals

Malt Extract Agar (MEA)

Reagent	**Amount [g/l]**
Malt extract	20
Agar	18
Aqua dest.	1000

Chloramphenicol MEA

Reagent	**Amount [g/l]**
Malt extract	20
Agar	18
Chloramphenicol	100 mg
Aqua dest.	1000

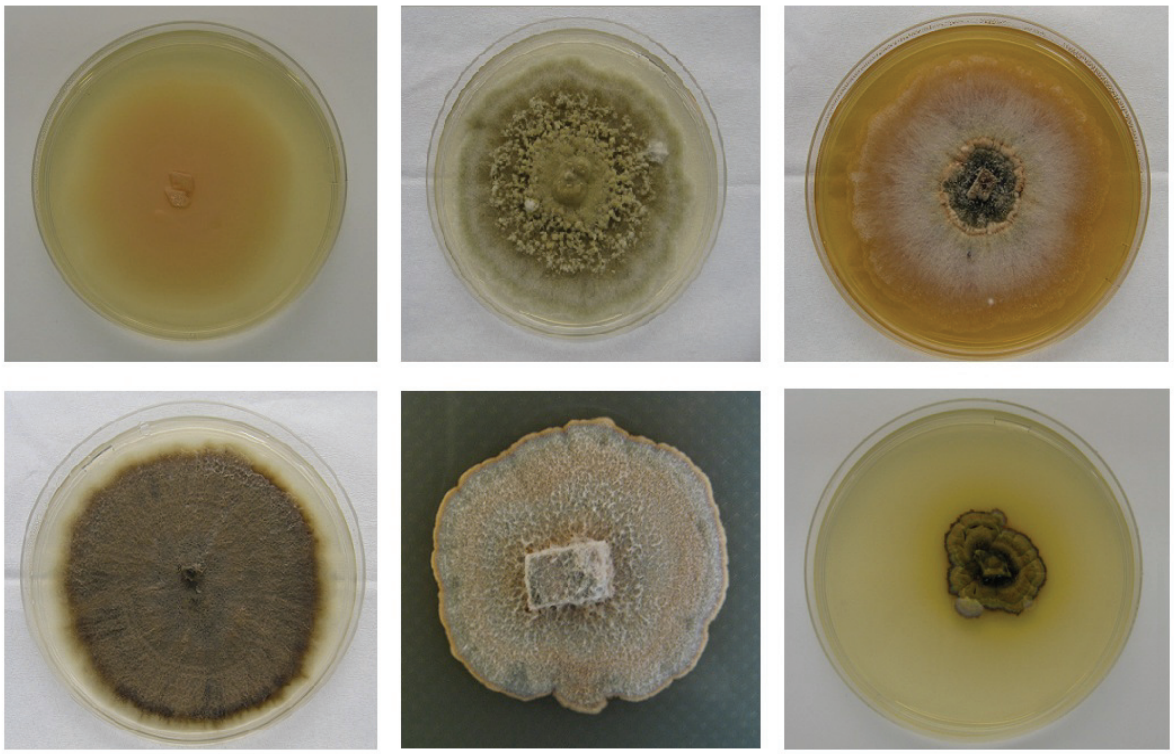
Appendix B

Detailed description of the characteristics used to morphotype the fungi.

MT	Colony pigmentation	Agar pigment	Growth rate	Liquid	Colony shape	Texture	Spore accumulation
1	pink	N	3	N	lobate	creamy	
2	white	N	4	Y	entire edge	woolly	Y
3	white; yellow	N	3	Y	fimbriate	reliefs	
4	yellow; white	brown	1	Y	radially striate with lobate edge	spongy	
5	grey; white	N	3	Y	lobate	velvety	Y
6	beige; white	N	1	N	lobate	woolly	
7	brown; orange; white	orange	2	N	erose or dentate	velvety	
8	brown; white; brown; pink	N	4	N	entire edge	velvety	
9	grey; green	N	4	N	fimbriate	woolly	
10	grey; green; white	N	4	N	fimbriate	woolly	
11	yellow; white	yellow	3	N	erose or dentate	woolly	
12	white; grey	N	5	N	erose or dentate	woolly	Y
15	brown; grey; brown; white	N	2	N	fimbriate	dry	Y
16	grey; white	N	1	Y	undulate	woolly	
17	white; beige; red; white	N	3	N	entire edge	woolly	
19	white	N	2	N	undulate	dry	
21	white; brown; grey	orange	1	N	erose or dentate	furry	
22	pink; brown; white...	orange	2	N	erose or dentate	furry	
26	white	N	4	N	fimbriate	woolly	Y
27	pink; black	N	1	N	erose or dentate	creamy	
28	black	N	3	N	entire edge	velvety	

Appendix C

Colony texture: from the first on the left in the upper part to the third on the right in the lower part (creamy, woolly, velvety, dry, furry and spongy) different types of textures used to describe the endophyte colony.



Appendix D

Table with the measures of the growth rate divided by day

MT	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
1	1,89	4,58	3,97	4,92	3,87	3,87	3,87	3,44
2	0	3,47	8,47	12,19	6,10	6,10	6,10	4,83
3	0	4,11	5,08	6,56	6,00	6,00	6,00	5,22
4	0	0	0	0,39	1,38	1,38	1,38	1,47
5	1,03	5,69	5,44	7,08	4,69	4,69	4,69	3,75
6	0	1,69	0,72	1,69	1,83	1,83	1,83	1,64
7	0	1,03	2,92	3,64	1,87	1,87	1,87	1,97
8	4,28	9,75	7,92	9,58	4,88	4,88	4,88	2,79
9	1,61	5,14	5,78	7,11	5,66	5,66	5,66	4,36
10	1,94	6,92	7,47	7,94	5,48	5,48	5,48	3,96
11	0,00	3,17	4,83	8,11	5,73	5,73	5,73	4,28
12	2,69	13,78	14,81	8,19	7,11	7,11	7,11	4,25
15	0	0,28	2,53	3,75	3,20	3,20	3,20	3,61
16	0	0,25	2,00	1,17	1,50	1,50	1,50	2,29
17	0,83	5,88	6,39	6,38	4,87	4,87	4,87	4,07
19	0	3,19	2,86	3,64	2,73	2,73	2,73	3,61
21	0	0,78	1,28	2,36	1,80	1,80	1,80	3,08
22	0	1,44	1,89	3,11	2,42	2,42	2,42	1,86
26	0	0	0,88	2,50	2,13	2,13	2,13	3,63
27	0	0	0	0,39	1,25	1,25	1,25	1,34
28	0,94	3,89	3,42	4,28	3,30	3,30	3,30	3,53

Table with the morphotypes listed from the slowest (1) to the fastest (5)

MT	growth %	growth rate
27	8,4	1
4	9,2	1
16	15,7	1
6	17,3	1
21	19,8	1
26	20,6	2
7	23,3	2
22	23,9	2
15	30,4	2
19	33	2
28	39,9	2
1	46,8	3
5	57	3
11	57,8	3
17	58,6	3
3	59,9	3
9	63	4
10	68,7	4
2	72,7	4
8	75,3	4
12	100	5

Appendix E

Table with all the percentages of fungi yielded by each morphotype divided in the three categories (vitality, age, tissue) and the total percentage of fungi outgrown in each morphotype (%) per time point

Period																		
MT	June									August								
	High vitality				Low vitality					High vitality				Low vitality				
	Old		Young		Old		Young			Old		Young		Old		Young		
	Bark	Xylem	Bark	Xylem	Bark	Xylem	Bark	Xylem	%	Bark	Xylem	Bark	Xylem	Bark	Xylem	Bark	Xylem	%
1	0.7	0.35	-	0.35	0.35	-	-	-	1.75	1.4	0.35	2.1	-	1.05	-	2.81	-	7.72
2	2.81	-	-	-	0.35	-	-	-	3.16	1.75	-	0.35	-	0.7	-	1.05	-	3.85
3	0.35	0.35	-	-	0.7	-	-	-	1.4	0.7	-	1.05	-	1.75	-	0.7	-	4.21
4	3.16	0.35	3.16	0.35	1.75	0.7	0.7	-	10.17	1.4	-	3.86	-	2.45	0.7	2.81	0.35	11.58
5	-	-	-	-	-	-	-	-	0	-	-	0.35	-	0.35	-	0.7	-	1.4
6	0.7	-	-	-	-	-	-	-	0.7	-	-	-	-	0.7	-	-	-	0.7
7	-	-	-	-	-	-	-	-	0	0.7	-	-	-	-	-	-	-	0.7
8	0.35	-	-	-	-	-	-	-	0.35	1.05	-	-	-	0.7	-	-	-	1.75
9	0.7	-	-	-	2.1	-	-	-	2.81	1.75	-	1.4	-	2.81	-	1.05	-	7.01
10	0.35	-	-	-	1.4	-	-	-	1.75	1.4	-	0.35	-	0.35	-	0.7	-	2.81
11	-	-	-	-	-	-	-	-	0	0.7	-	-	-	-	-	-	-	0.7
12	0.35	-	-	-	0.35	-	-	-	0.7	0.35	-	0.35	-	0.7	-	0.35	-	1.75
15	-	-	-	-	-	-	0.26	-	0.26	-	-	1.75	0.35	-	-	-	-	2.1
16	-	-	-	-	-	-	-	-	0	0.35	-	-	-	0.35	-	-	-	0.7
17	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	1.05	-	1.05
19	0.35	-	-	-	-	0.35	-	-	0.7	-	-	-	-	-	-	-	-	0
21	-	-	0.35	-	0.35	-	-	-	0.7	-	0.35	0.35	-	-	-	-	-	0.7
22	2.1	-	1.05	-	1.4	-	1.4	-	5.96	1.05	0.35	1.75	-	1.75	0.35	1.4	-	6.67
24	1.75	-	-	-	0.7	-	-	-	2.45	1.4	0.35	1.05	0.7	2.1	-	0.7	-	6.31
26	-	-	-	-	-	-	-	-	0	0.35	-	-	-	0.35	-	1.05	-	1.75
27	-	-	-	-	0.7	-	-	-	0.7	-	-	-	-	-	-	-	-	0
28	-	-	-	-	1.75	-	-	-	1.75	-	-	-	-	0.7	-	0.35	-	1.05