Leaf-inhabiting endophytic fungi in the canopy of the Leipzig floodplain forest

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Contents

List of abbreviations

ANOVA	-	Analysis of variance
BLAST	-	Basic Local Alignment Search Tool (Program for searching the databases GenBank, EMBL, DDBJ, and PDB)
bp	-	Base pair
CI	-	Consistency index
CNI	-	Close-Neighbor-Interchange (algorithm to find the Maximum Parsimony tree)
CTAB	-	Cetyltrimethylammonium bromide (20 g/l CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM TRIS/HCl, pH 8,0)
D1/D2	-	Large subunit ribosomal DNA domain
DBH	-	Diameter breast height
DCA	-	Detrended correspondence analysis
DDBJ	-	DNA Data Bank of Japan
ddH_2O	-	Double-distilled water
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxyribonucleotide
DSE	-	Dark septate endophytes
DWD	-	Deutscher Wetterdienst (National Meteorological Service of Germany)
EDTA	-	Ethylenediaminetetraacetic acid
EMBL	-	European Molecular Biology Laboratory
EtOH	-	Ethanol
HPLC	-	High-performance liquid chromatography
INSD / INSDC	-	International Nucleotide Sequence Database Collaboration (comprises GenBank, EMBL, and DDBJ)
ITS	-	Internal transcribed spacer (nrDNA region of the LSU)
LAK	-	Leipziger Auwaldkran (Leipzig Canopy Crane)
LSU	-	Large subunit (of the ribosom)
MEA / MEA+T	-	Maltextract agar / Maltextract agar with tetracycline added (see appendix A)
MT	-	Morphotype; the abbreviation is followed by a series of digits including the year and a consecutive number
nrDNA	-	Nuclear ribosomal DNA
OA	-	Oatmeal agar
PCA	-	Potato carrot agar
PCR	-	Polymerase chain reaction
PDA	-	Potato dextrose agar
PDB	-	Protein Data Bank
RCI	-	Rescaled consistency index
RI	-	Retention index
SD	-	Standard deviation
SSU	-	Small subunit (of the ribosom)
TRIS	-	Tris(hydroxymethyl)aminomethane

Tween20	-	Polyoxyethylene (20) sorbitan monolaurate (a polysorbate surfactant)
UV	-	Ultraviolet
V8	-	Vegetable juice agar

1 Introduction

Endophytic fungi are a highly diverse fungal community mostly consisting of Ascomycota which are able to symptomlessly occupy all kinds of apparently healthy plant tissues. Since endophytes exist without causing any immediate, overt negative effects their host, their existence has widely been ignored in the past. However, numerous fungal studies have shown that endophytes can be found in various habitats, including tropical, temperate, and boreal forests (e.g. Halmschlager et al., 1993; Fisher et al., 1994; Pehl & Butin, 1994; Lodge et al., 1996a; Fröhlich & Hyde, 1999; Helander et al., 2006; Arnold & Lutzoni, 2007; Higgins et al., 2007). They occurred in all plant groups examined to date, not only in gymnosperms and angiosperms, but also in algae (e.g. Hawksworth, 1988), mosses and hepatics (e.g. Read et al., 2000), and ferns (e.g. Fisher, 1996). Due to the broad systematic range and diverse life strategies, there are many different kinds of interactions between the endophyte and its host, the hosts herbivores, and other endophytic and pathogenic microorganisms. Therefore, the effects of endophytes on their host can be diverse and their influence on forest ecosystems depends on the species composition and diversity of the endophytic community. To make reliable assessments about the effect of leaf-inhabiting endophytes on forest ecosystems, it is necessary to have a wide knowledge about the endophytic community in all forest layers all over the vegetation period. The leaf-inhabiting endophytic community in turn is influenced by the successful colonisation of the leaves. This can occur in several different ways and might therefore be influenced by a variety of factors like microclimate, herbivory, previous infection of other plant tissues, and nearby spore sources.

1.1 A short definition of endophytic fungi

The term "endophyte" was used for the first time by de Bary (1866) and has been used frequently in literature since then. However, the opinions about its meaning differed widely. At first, all organisms that live inter- or intracellularly in plants were given the denomination of "endophytes". Since this definition comprises parasitic, pathogenic, and non pathogenic life-forms, used in this way the term was vague and

not very useful (Wennström, 1994).

A considerably more precise and widely accepted definition is that of Wilson (1995). It will be applied in this thesis. Accordingly, endophytes are "(...) fungi or bacteria which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but cause no symptoms of disease" (Wilson, 1995). Here, endophytes are determined ecologically by their way of life, and not by their taxonomical identity. This definition comprises almost all kinds of interactions in which fungi and plants participate, from mutualism and commensalism, via neutral relations, through to parasitism. There are pathogenic fungi covered by this definition, which spend some time latently within the host tissue before the outbreak of the disease, as well as fungi, which are known as pathogens, but do not cause symptoms, for example, because of a mutation. The transitions to other life-forms like parasitism with obvious damage or pathogenic stages are continuous. Mycorrhizal fungi are excluded because of their nutritional particularities.

Although numerous, and in all likelihood ecologically relevant, endophytic bacteria will not be regarded in the present thesis.

1.2 Aims and structure of this thesis

The importance of endophytic fungi in forest canopies has, up until now, rarely been examined. Studies about the interaction of endophytes with their host trees and the hosts herbivores have given a first insight into the species richness and ecological importance of endophytic fungi (reviewed amongst others by Saikkonen et al., 1998; Stone et al., 2004; Schulz & Boyle, 2005; Arnold, 2007; Rodriguez et al., 2009), but they do not take into account the specific characteristics of microhabitats in the tree crowns (e.g. Lodge & Cantrell, 1995; Lodge et al., 1996b; Parker & Brown, 2000; Unterseher & Tal, 2006).

To date, no investigation has looked in detail at different endophytic communities in different canopy layers during the vegetation period, or compared the colonisation of the higher crown regions of full-grown trees with saplings in the understorey at the same site. Several studies have been done about endophytic fungi in European trees, but none of them dealt with endophytes of the European Ash (*Fraxinus excelsior*). The European Ash, a common European tree species, came to the fore during recent years because of a dieback which is spreading in Europe. It is caused by the pathogenic fungus *Chalara fraxinea* (e.g. Kowalski, 2006; Kowalski & Holdenrieder, 2009a).

This thesis will therefore explore the dynamics of spatial and temporal endophyte colonisation of European Ash (*Fraxinus excelsior*) over an entire vegetation period (May to October 2008). The endophyte colonisation of the light and the shade crowns of four host tree species will also be compared. For this purpose, the Leipzig Canopy Crane offers ideal conditions to reach all parts of the canopy.

Several hints that rain is important for the dispersal of propagules of endophytic fungi and the success of the leaf infection have been found (e.g. WILSON, 1996; ARNOLD & HERRE, 2003; DEVARAJAN & SURYANARAYANAN, 2006). However, none of these studies look in detail at the influence of precipitation on the infection rate or the species composition of endophytes in relation to other ways in which leaf infections can occur.

The present thesis focuses on different mechanisms of endophyte infection of trees with a special attention to the role of precipitation.

Within this thesis, several stand-alone investigations will deal with the leaf colonisation of broad-leaved trees in a deciduous temperate floodplain forest by endophytic fungi, based on the following overarching hypotheses:

- There is a specific endophyte composition for leaves of different host trees. It is dominated by host-specific species, which occur alongside to ubiquitous endophytes.
- Considerable differences exist for the species composition, the abundance, and the diversity of different forest layers.
- The colonisation density and species richness will grow during the vegetation period.
- Precipitation, herbivory, and root lesions promote infection of the leaves by endophytic fungi.

The thesis is divided into three major parts. Chapter two describes and discusses the general methods that have been used for all studies. Exploration of the diversity of endophytic fungi at the Leipzig Canopy Crane (LAK) research facility follows in chapter three. This chapter includes an investigation of the diversity of endophytic fungi in different hosts - of which the data has already been published in UNTERSEHER et al. (2007) - and a detailed investigation of spatial and temporal patterns of endophytic fungi in *Fraxinus excelsior*. In chapter four, experimental approaches are described, aimed at studying infection pathways on which endophytes can intrude into the leaves, where the roles of precipitation, herbivore feeding, and root lesions are explored more closely.

2 Methods applied to all studies

Five studies were carried out on the one hand to investigate the diversity of foliar endophytic fungi in the crowns of deciduous trees at the Leipzig Canopy Crane research facility, and on the other hand to explore different possible ways of endophyte infection. All these studies relied on a culture-based approach. The cultures obtained were separated into morphotypes with the same colony and mycelium morphology, pigmentation, and spore characteristics (if developed). These morphotypes were determined according to morphological characters and nrDNA sequence analyses. The following chapters will describe and discuss these methods in detail.

2.1 General methods

2.1.1 Isolation and cultivation of endophytic fungi

Entire leaves or leaflets without any obvious symptoms of fungal infection were collected from the host trees, stored in paper bags, kept cool and processed at the same day.

In accordance with Gamboa et al. (2002), a surface-sterilisation procedure was used to kill all viable fungi including spores that were epiphytic on the leaves. First of all, the leaf surfaces were washed with 0.01% Tween20, a surfactant which is used to rid the leaves of coarse particles of dirt. Then the leaves were immersed in 75% ethanol for one minute. Ethanol serves as a wetting agent that reduces the leaf surface tension and has a considerable antibiotic activity. For the basic sterilisation the leaves were treated with a dilution of 0.5% sodium hypochlorite (NaClO), which is a strong oxidant, for three minutes. Subsequently the leaves were rinsed with 75% ethanol for 30 seconds to remove the sterilant.

After drying the leaf surfaces under sterile conditions, a fragment of 1 x 2 cm was cut out along the midrib and divided into four fragments of 1 x 0.5 cm (Fig. 2.1). Each leaf fragment was placed into a petri dish of 90 mm diameter containing 2% malt extract agar and 0.1% tetracyclin (MEA+T, for recipe see appendix A).

To confirm the success of the surface sterilisation, the upper and lower surfaces of randomly chosen sterilised leaves were pressed onto culture plates for a few seconds.

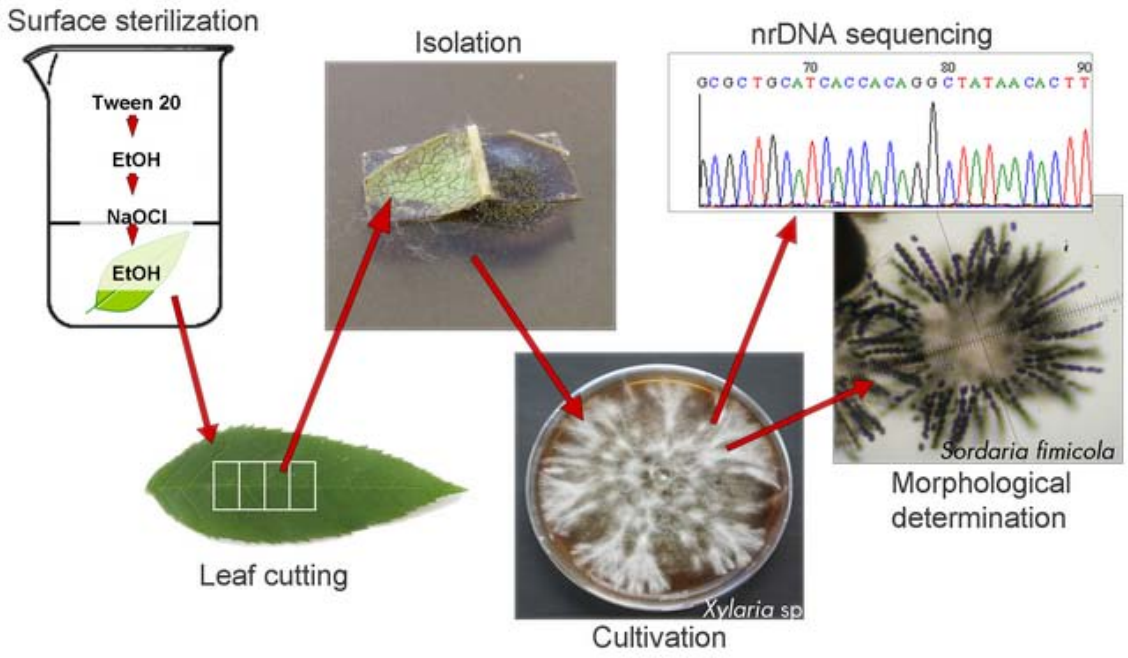

Figure 2.1: Overview of the procedure of isolation, cultivation, and identification of endophytic fungi from the collected leaves

If the sterilisation was successful, no mycelium should develop on the plates after culturing.

The leaf fragments were incubated at room temperature and under natural diffuse light conditions for eight weeks. Every 2-3 days they were investigated for growth of new fungal colonies. Leaf fragments which remained sterile for the entire incubation period were regarded as not infected. All colonies were marked on the back of the plate, and about five square millimetres of each were separated onto fresh culture plates with MEA. The original culture plates with the leaf fragments were supplementarily inspected for spore production.

The axenic fungal isolates were cultivated under the same conditions. Isolates which did not sporulate after several weeks were inoculated on oat agar (OA), potato dextrose agar (PDA), potato carrot agar (PCA), vegetable juice agar (V8) and MEA again (for all recipes see appendix A) and cultivated in an incubator under controlled conditions, with a day/night regime of 13/11 h and additional UV radiation (350-400 nm) at 22°C, to stimulate spore production.

2.1.2 Formation of morphotypes and morphological identification

At first, all cultures were grouped into morphotypes in accordance with Lacap et al. (2003). They were distinguished by means of their colony morphology and pigmentation, hyphal morphology, and reproductive structures as far as could be observed by light microscopy. The isolates which produced reproductive structures were identified morphologically, as far as possible. For this purpose, identification literature mainly from the European region was used (Table 2.1). The nomenclature of the taxa followed the Dictionary of the Fungi (Kirk et al., 2001) and Index Fungorum (http://www.indexfungorum.org).

Table 2.1: Used literature and Internet resources for the morphological identification of endophytic fungi

Authors	**Title**	**Taxa**
Barnett & Hunter (1998)	Illustrated genera of imperfect fungi	Fungi imperfecti
Boerema et al. (2004)	*Phoma* identification manual	*Phoma*
Braun (1995)	A monograph of *Cercosporella*, *Ramularia* and allied genera (phytopathogenic hyphomycetes)	selected genera of hyphomycetes
Crous et al. (2004)	MycoBank: an online initiative to launch mycology into the 21st century. www.mycobank.org	all fungal taxa
Domsch et al. (1980)	Compendium of soil fungi	selected Ascomycota, Basidiomycota, Oomycota, Zygomycota, and Fungi imperfecti
Ellis (1971)	Dematiaceous hyphomycetes	dematiaceous hyphomycetes
Ellis (1976)	More dematiaceous hyphomycetes	dematiaceous hyphomycetes
Gerlach & Nirenberg (1982)	The Genus *Fusarium* - a Pictorial Atlas	*Fusarium*
Hennebert (1973)	*Botrytis* and *Botrytis*-like genera	*Botrytis* and *Botrytis*-like genera
Ho (1999)	*Cladosporium* and *Cladophialophora* in culture: Descriptions and an expanded key	*Cladosporium*, *Cladophialophora*
de Hoog (1972)	The genera *Beauveria*, *Isaria*, *Tritirachium*, and *Acrodontium* gen. nov.	*Beauveria*, *Isaria*, *Tritirachium*, *Acrodontium*

Table 2.1: (continued)

Authors	Title	Taxa
de Hoog (1974)	The genera *Blastobotrys*, *Sporothrix*, *Calcarisporium*, and *Calcarisporiella* gen. nov.	*Blastobotrys*, *Sporothrix*, *Calcarisporium*, *Calcarisporiella*
de Hoog et al. (2000)	Atlas of clinical fungi	selected Ascomycota, Basidiomycota, Oomycota, Zygomycota, and Fungi imperfecti
Leslie (2006)	The *Fusarium* Laboratory Manual	*Fusarium*
Lundqvist (1972)	Nordic Sordariaceae s. lat.	Sordariaceae
Rao & de Hoog (1986)	New or critical hyphomycetes from India	selected genera of hyphomycetes
Schubert (2005)	Morphotaxonomic revision of foliicolous *Cladosporium* species (hyphomycetes)	*Cladosporium*
Sogonov et al. (2008)	Leaf-inhabiting genera of the Gnomoniaceae, Diaporthales	Gnomoniaceae
Sutton (1980)	The Coelomycetes - Fungi Imperfecti with Pycnidia, Acervuli and Stromata	Coelomycetes

2.1.3 DNA sequencing and taxonomical placement of the sequences

The multicopy nuclear ribosomal DNA region (nrDNA) includes the 18S nrDNA of the small ribosomal subunit (SSU), the internal transcribed spacers (ITS1 & ITS2), and the 5.8S and 28S nrDNA of the large ribosomal subunit (LSU; Fig. 2.2). It is the most popular locus for DNA-based mycological studies (e.g. Horton & Bruns, 2001). After isolating the genomic DNA of the fungal cultures, the target area on the nrDNA was amplified with specific primers, and following this, sequenced. The resulting sequences could be compared to reference sequences in databases. Phylogenetic analyses showed and ensured the taxonomical placement of the cultures. The methods used for these analyses will be described in detail in the following sections.

DNA isolation

The fungal DNA was isolated using a modified protocol of the CTAB method of Doyle & Doyle (1987). CTAB is used to separate interfering polysaccharides and proteins by complex forming. A small amount of a freshly grown mycelium (about

1 cm^2) was pestled with 100 μl of CTAB and filled up with 400 μl of the same reagent. A thermic decomposition followed for 30 min at 60°C.

The following purification with phenol and chloroform-isoamyl alcohol produces two phases. The upper aqueous phase contains the DNA, the lower chloroform phase contains degraded proteins, lipids, and secondary compounds. The interface between these two phases contains most of the cell debris and degraded proteins. Therefore 250 μl of phenol and 250 μl of chloroform-isoamyl alcohol (24:1) were added to the solution and mixed gently and thoroughly. The mixture was centrifuged with 1 600 g at 4°C for 5 min. The upper aqueous phase was transferred into a new tube, 500 μl of chloroform-isoamyl alcohol were added and the solution was centrifuged under the same conditions.

Once again the upper phase was transferred and 500 μl of isopropanol (4°C) were added to precipitate the DNA. While centrifuging for 15 min at 13 000 g the DNA accumulated into a pellet. The supernatant was removed, the pellet was washed with 200 μl of ethanol and centrifuged again for 10 min at 13 000 g. The supernatant was removed again and the pellet was dried for 15 min at 37°C. Finally 100 μl of distilled water were added to the pellet and the tubes were stored at -20°C.

PCR amplification of the D1/D2 region

The D1/D2 region is a variable region at the 5' end of the 28S nrDNA. It was amplified with the primers NL1 and NL4 (O'Donnell, 1993, Table 2.2). NL1 and NL4 comprise a region of about 600 bp (Fig. 2.2).

primer	**sequence**
NL1	5'-GCATATCAATAAGCGGAGGAAAAG-3'
NL4	5'-GGTCCGTGTTTCAAGACGG-3'
ITS5	5'-GGAAGTAAAAGTCGTAACAAGG-3'
ITS4	5'-TCCTCCGCTTATTGATATGC-3'

Table 2.2: Primers, that were used for amplification of the D1/D2 (NL1, NL4) and the ITS (ITS5, ITS4) region

The amplification was performed with 49.2 μl of a mixture consisting of:

- 5 μl of 10x reaction buffer (Mg free; Promega, Mannheim, Germany),
- 4 μl of $MgCl_2$ (25 mM; Promega),
- 4 μl of dNTP Mix (MBI Fermentas, St. Leon-Rot, Germany),
- 34 μl of ddH_2O,
- 1 μl of each of the primers NL1 and NL4 (5 μM; MWG-Biotech AG, Ebersberg, Germany), and
- 0,2 μl of *Taq* DNA polymerase (Promega),

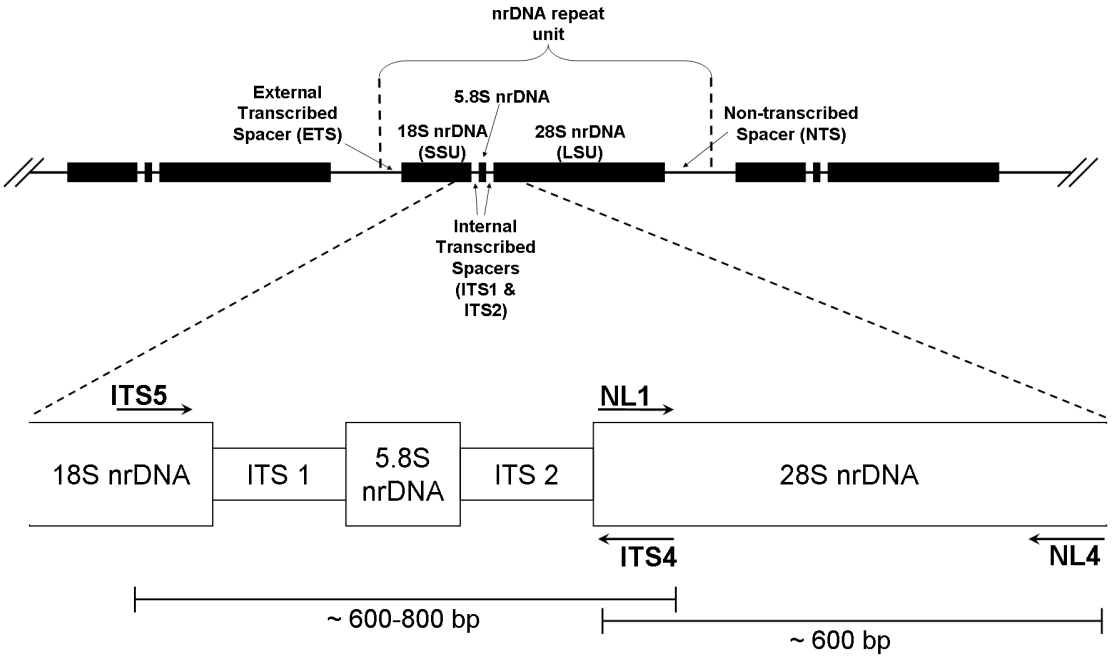

Figure 2.2: Location of the PCR primers ITS5, ITS4, NL1, and NL4 on the nuclear rDNA.

to which 1 μl of the isolated DNA was added. A negative control using water instead of DNA was included in every run to assure that no contamination was amplified. The amplification was run in an automated thermal cycler (PTC-200^{TM}, MJ Research) under the following conditions:

- initial denaturation for 3 min at 94°C,
- followed by 35 cycles with:
  - 30 sec denaturation at 94°C,
  - 45 sec annealing at 54°C, and
  - 1 min elongation at 72°C,
- and a final elongation for 7 min at 72°C.

An aliquot of 4 μl of each PCR product was dispersed by electrophoresis in a 1% (w/v) agarose gel with an addition of 5 μl of ethidium bromide per 100 ml, and subsequently visualised under UV light to check the success of the PCR. PCR products were purified by using the NucleoSpin Extract II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instruction.

Both DNA strands were sequenced. For this purpose 3 μl of the purified DNA were amplified with a mixture of:

- 6 μl of ddH_2O,
- 3.5 μl of 5x buffer (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Warrington, UK),

- 1 μl of Terminator mix (BigDye Terminator v3.1 Cycle Sequencing Kit), and
- 1 μl of either Primer NL1 or NL4 (only one primer per reaction).

In accordance with the BigDye manufacturer's manual, 24 cycles in the thermal cycler followed, with:

- 10 sec denaturation at 96°C,
- 5 sec annealing at 50°C, and
- 4 min elongation at 60°C.

For the purification, the products were mixed gently with 16 μl of HPLC-H_2O and 64 μl of 96% ethanol, left at room temperature for 20 min, and centrifuged for 20 min at 12 000 *g*. The supernatant was removed, and the pellet was washed with 250 μl of 70% ethanol and centrifuged again for 15 min at 12 000 *g*. Again the supernatant was removed and the pellet was dried for 30 min at room temperature in the dark. The pellet was dissolved in formamide and sequenced in an automated sequencer (ABI PRISM 3100 Genetic Analyser, Applied Biosystems).

PCR amplification of the ITS region

The internal transcribed spacer region (ITS) of the nrDNA consists of the variable ITS1 and ITS2 regions and the strongly conserved 5.8S region. It was amplified with the primers ITS5 and ITS4 (White et al., 1990, Table 2.2). ITS5 and ITS4 comprise a region of about 600-800 bp (Fig. 2.2).

The amplification was performed with 50.2 μl of a mixture consisting of:

- 5 μl of 10x reaction buffer (Mg free; Promega, Mannheim, Germany),
- 4 μl of $MgCl_2$ (25 mM; Promega),
- 4 μl of dNTP Mix (MBI Fermentas, St. Leon-Rot, Germany),
- 35 μl of ddH_2O,
- 1 μl of each of the primers ITS5 and ITS4 (5 μM; MWG-Biotech AG, Ebersberg, Germany), and
- 0,2 μl of *Taq* DNA polymerase (Promega)

to which 1 μl of the isolated DNA was added. A negative control using water instead of DNA was included in every run to assure that no contamination was amplified. The amplification was run in an automated thermal cycler (Eppendorf Mastercycler, Hamburg, Germany) under the following conditions:

- initial denaturation for 3 min at 94°C,
- followed by 32 cycles with:
  - 40 sec denaturation at 94°C,
  - 30 sec annealing at 54°C, and
  - 40 sec elongation at 72°C,
- followed by a final elongation of 10 min at 72°C.

An aliquot of 4 μl of each PCR product was dispersed by electrophoresis in a 1% (w/v) agarose gel with an addition of 5 μl of ethidium bromide per 100 ml, and subsequently visualised under UV light to check the success of the PCR.

The PCR products were purified by adding 34.5 μl of isopropanol to 46 μl of PCR product, incubating at room temperature for 20 min and subsequently spinning for 20 min at 2 550 *g*. The supernatant was removed and the pellet was washed with 75 μl of 80% ethanol and centrifuged again for 10 min. Once more the supernatant was removed and the pellet was dried at 35°C for 30 min. HPLC-H_2O was added to attain a final DNA concentration of 10 ng/μl, but at least 10μl of H_2O were added. The success of purification was controlled again by electrophoresis in a 1% agarose gel (conditions see above).

Both DNA strands were sequenced. For this purpose 2 μl of purified DNA were amplified with a mixture of:

- 6 μl of ddH_2O,
- 3.5 μl of 5x buffer (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Warrington, UK),
- 1 μl of Terminator mix (BigDye Terminator v3.1 Cycle Sequencing Kit), and
- 1 μl of either Primer ITS5 or ITS4 (only one primer per reaction).

In accordance with the BigDye manufacturer's manual, 24 cycles in the thermal cycler followed, with:

- 10 sec denaturation at 96°C,
- 5 sec annealing at 50°C, and
- 4 min elongation at 60°C.

For the purification, the products were mixed gently with 16 μl of HPLC-H_2O and 64 μl of 96% ethanol, left at room temperature for 20 min, and then centrifuged for 20 min at 12 000 *g*. The supernatant was removed, the pellet was washed with 250 μl of 70% ethanol and spun again 15 min at 12 000 *g*. Once again the supernatant

was removed and the pellet was dried for 30 min at room temperature in the dark. The pellet was dissolved in formamide and sequenced in an automated sequencer (ABI PRISM 3100 Genetic Analyser, Applied Biosystems).

Molecular identification and phylogenetic analyses

The D1/D2 or ITS1/5.8S/ITS2 sequences were aligned using the ClustalW option of *MEGA* version 4.0.2 (Tamura et al., 2007). The resulting alignments were adjusted manually if necessary. Maximum parsimony analyses were performed with *MEGA*, including only parsimony informative characters. Due to the high computational effort, a branch-and-bound search was only performed for alignments up to 20 sequences, whereas for alignments of more than 20 sequences, a heuristic search with CNI branch swapping was used. To confirm the results, bootstrap analyses with 1000 replicates were enclosed. The fungi were classified in the taxonomical system (orders and subclasses) by their sequences. For morphotypes which had not been determined morphologically, the first best hit in BLAST (Altschul et al., 1990), which was determined at least to genus level, was taken to assess the order and subclass, but only if there was not more than 5% difference from the query sequence.

All sequences were submitted to GenBank. The accession numbers and a list of the best hits of the BLAST search and their coverage with the query can be found in appendix B.

2.1.4 Computing *rarefaction* curves, richness estimators, and diversity indices

Rarefaction curves were estimated to compare the species-richness of different data sets using the program EstimateS version 8.2.0 with default settings, if not recommended otherwise by the software (Colwell, 2006). The x-axis of all curves were rescaled to "individuals", according to the suggestion of Gotelli & Colwell (2001). As an "individual" one isolate of a certain morphotype per leaf fragment, or per leaf for the study with different host tree species (chapter 3.4), was counted. The problem of identifying fungal individuals in the leaves and separating individual colonies from each other will be discussed in chapter 2.2.4. To estimate the total species richness of endophytic fungi that could be found in a given microhabitat or host tissue with the used methods, the nonparametric estimator *Chao2* (Chao, 1987) was used. Furthermore EstimateS computes the Shannon, the Fisher's α, and the Simpson diversity index.

2.2 Discussion of the used methods and their restrictions

2.2.1 Isolation and cultivation

The applied technique of surface sterilisation has been examined amongst others by Schulz et al. (1993), Bissegger & Sieber (1994), and Gamboa et al. (2002) and proved itself to be satisfactory in many studies. The additional tests of the efficiency of surface sterilisation, which were performed according to Schulz et al. (1998) by imprinting sterilised leaves on MEA, have been successful. All control plates remained sterile, whereas imprints of non-sterilised leaves left numerous colonies of fungi.

The amount of endophytic fungi that can be isolated from a defined leaf area depends on the number and the size of leaf fragments into which it is divided. An approach with more and smaller leaf fragments would for sure have increased the amount of isolated fungi per leaf (Gamboa et al., 2002). This is because there is a larger extent of cut surfaces where endophytes can grow out easily and a lower chance of overgrowing since the number of infections per leaf fragment is smaller. The size and the number of leaf fragments that were investigated for this thesis have been restricted by the amount of work that had to be carried out. The chosen leaf area of 2 cm^2, which was divided into only four parts, combined with a rather high amount of sampled leaves, seemed to be good for the accomplished diversity survey. Correspondingly Lodge et al. (1996a) concluded that sampling would be more efficient if the number of isolations per leaf is reduced and the number of sampled trees is increased instead.

75 % of all fungal isolates grew out of the leaf fragments between the third and the seventh day after collecting. Especially in the autumn, when up to five different morphotypes could be isolated from one fragment, slowly growing fungi were sometimes overgrown by fast growing ones. Such slow-growing colonies could be observed repeatedly, but could not be isolated. As a consequence, slowly growing fungi could not be recorded, and the morphotype might be underrepresented, especially at the end of the vegetation period.

MEA is widely used as primary isolation medium (Stone et al., 2004), because most culturable fungi can grow well on it. The studies of the present thesis relied solely on this complex medium, because of the much higher amount of work when using different isolation media.

To deal with the problems of fast-growing fungi which overgrow slow-growing ones,

media with selective growth inhibitors can be used (e.g. Bills & Polishook, 1991; Stone et al., 2004). Following these suggestions, tests with an addition of 0.4 g cycloheximide per liter MEA, to slow down the growth rate of filamentous fungi, were performed. These did not lead to a satisfying result because the fungi that grew out of the leaf fragments on this medium neither grew well nor sporulated.

Another possibility to solve the problem of overgrowing is the dilution to extinction approach (Collado et al., 2007), which Unterseher & Schnittler (2009) have adapted for the first time for endophytic fungi. They homogenized the surface-sterilised leaves and filtered particles of 100 to 200 μm size from the homogenate. These particles were resuspended, and a dilution was pipetted into multiwell-plates with MEA so that ideally only one fungal colony grew in one well. Unterseher & Schnittler (2009) yielded an entirely different species composition compared to the traditional culture based approach, so that this method might be an appropriate complement for future studies.

There are numerous references of fungi being unable to grow in culture (e.g. Duong et al., 2006; Seena et al., 2008), because they are for instance obligatorily biotrophic and therefore dependent on living hosts. A cultivation-based approach would exclude this group of fungi, and reveals limits in this respect. Cultivation-independent approaches have been rarely applied to endophytic fungi so far (Gao et al., 2005; Arnold et al., 2007). They would provide a possibility to include endophytes that can not be cultivated successfully, as obligate biotrophic, as well as slow-growing species, and would avoid the effort of cultivating the isolates on different media for a long time, to induce spore production. To determine the detected fungi, it is necessary that there are reliable sequences in the databases which is by far not the case for all endophytic fungus species (see chapter 2.2.3). However, these cultivation independent approaches remain problematic because there has been no evidence so far that the DNA of epiphytic mycelia and adhering spores will be effectively destroyed by traditional sterilisation methods and will thus not be included into the subsequent DNA isolation. The necessity to destroy fungal DNA from epiphytic fungi exists also for modern metagenomic analyses such as 454 pyrosequencing. However, they have been used successfully for a survey of the complete community of leaf associated fungi (e.g. Jumpponen & Jones, 2009, 2010).

2.2.2 Morphotype formation and morphological identification

The morphological identification of endophytic fungi is often difficult, because many isolates do not sporulate in culture, and the appearance of characteristcs that are relevant for determination may differ on different media. The use of further culturing media besides MEA increased the number of morphologically identifiable fungi. A long culture period of up to one year also led to spore production of some fungi, which was perhaps caused by the slowly drying or the decreasing nutrient availability of the culture medium.

According to previous studies, about 15 to 25% of the isolated endophytes do not sporulate in culture (Lacap et al., 2003) but these values may increase to more than 50% (Fisher et al., 1994; Guo et al., 1998). To handle these cultures not merely as one large group of mycelia sterilia, it is common practice to classify them into morphotypes. Many morphotypes can be distinguished from each other by typical characters like pigmentation, anastomoses of hyphae, or the diffusion of pigments into the medium. However, the appearance of different morphotypes can vary on different media and is further dependent on the cultivation conditions, and the age of the culture. Lacap et al. (2003) verified the reliability of the morphotype concept by sequencing the isolates. They have reasoned that the species number is rather underestimated, because several of their studied morphotypes might even comprise more than one species. Nevertheless, the data by Lacap et al. (2003) mainly supports the conventionally used morphotype concept. Hence, for the purpose of this study, it is sufficient even if not every single species is detected.

Despite all efforts to divide the isolates into morphotypes, there have been some isolates left which neither satisfactorily differed from a particular morphotype nor definitely belonged to it. That is why some isolates are treated as undetermined, and not as a particular morphotype, and therefore have not been integrated in the further analyses. They might be singletons of a separate and unregarded morphotype, as well as poorly grown or invisibly contaminated members of an identified and more frequent morphotype.

Even sporulating cultures were not always able to be determined to species level. Sometimes, ultra-structural characters like spore ornamentation have to be determined, sometimes, comprehensive keys of the family or genus were missing and there are still many fungal groups which are lacking accepted species and genus definitions, such as the genus *Phomopsis*. These morphotypes were determined as far as possible and the determination was combined with the results of the BLAST search and the phylogenetic examination.

2.2.3 Phylogenetic analyses and taxonomical placement of the sequences

The phylogenetic analysis has been used to integrate the non-sporulating morphotypes into the taxonomical system and to confirm the morphological determinations. The method reaches its limits at different points. First, there must be a sufficiently determined reference sequence in the databases. This is often not the case, as ascertained for publicly available fungal ITS sequences in the International Nucleotide Sequence Database (INSD) by Nilsson et al. (2006).

To illustrate the problem of determination by sequences, one example of the present study is given: The best hit for a sequence of the ITS region of the morphotype MT0843 was an undetermined fungus with 99% sequence similarity, the first determined one *Tumularia aquatica* with only 92% sequence similarity. For a reliable determination of a sterile mycelium, this BLAST result is of no use, considering that the intraspecific ITS variability of the Ascomycota on average is only 1.96% (SD 3.73; Nilsson et al., 2008). Another problem is the inclusion of the highly conserved 5.8S and variable amounts of the 18S and the 28S nrDNA. They will inevitably add percentages to the best hits. A solution to this problem was recently published by Nilsson et al. (2010). Their software tool, however, was not yet available for the present thesis.

Furthermore, the determination has to be correct and reliable. According to Bridge et al. (2003), up to 20% of the fungal sequences that are submitted to the European Molecular Biology Laboratory (EMBL) seem to be misidentified or dubious. Even if there has been a vehement protest against this particular study by Hawksworth (2004) and Holst-Jensen et al. (2004), the problem of misidentified, poorly documented, or not accurately produced sequences in the databases can not be denied. As a direct consequence, all database entries which were used in this study have been checked for their reliability, as far as possible.

At least for some groups the phylogenetic examination using the D1/D2 or the ITS1/5.8S/ITS2 region by means of maximum parsimony analyses has been problematic, because the intraspecific variability is too high. For example, in the present study, there have been difficulties with the genus *Xylaria* with the D1/D2 as well as the ITS region. Several reference sequences from the order Xylariales and sequences of the found *Xylaria*-like morphotypes clustered all in one clade without further separating. This can be explained by a very high intraspecific DNA variability. Nilsson et al. (2008) found an ITS variability of 24.2% for *Xylaria hypoxylon*, whereas it has not been higher than 3% for other surveyed Ascomycota. Thus, these nrDNA re-

gions, which can be sufficiently used for a broad range of fungi, reach their limits at least with some taxa of the Xylariales.

2.2.4 *Rarefaction*, species richness estimation, and species abundance distribution

The number of isolates was used to compute *rarefaction* curves, species richness estimations, and species abundance distributions, instead of the number of individuals, as described in chapter 2.1.4. It is a severe problem to distinguish single infections from each other, and to determine the size of an endophytic mycelium within the host (Stone et al., 2004). Usually, infections of endophytic fungi in woody plants are spatially restricted (e.g. Lodge et al., 1996a; Rodriguez et al., 2009). However, it is impossible to ascertain whether colonies of a fungal species, growing out of adjacent leaf fragments or several times out of one fragment, belong to one mycelium from a single spore which has been divided by leaf cutting, or whether they belong to different mycelia. Our approach counted every species only once per leaf fragment or leaf respectively, regardless of the number of its colonies. This provided a useful opportunity to quantify the infection-rate per sample point.

For hyperdiverse groups of organisms, to which endophytic fungi belong (Arnold et al., 2000), it is nearly impossible to do a complete inventory of all species in an investigation area (Colwell & Coddington, 1994). To determine how far the sampling is from being exhaustive, sample-based *rarefaction* curves have been computed.

Rarefaction curves, in contrast to species accumulation curves, have the advantage that they deduce what the species richness of an assemblage would be, if the sampling effort had been reduced by a specified amount. This allows a direct comparison amongst communities of different size on the basis of the number of individuals in the smallest sample (Magurran, 2004). Furthermore, sample-based *rarefaction* curves are preferable to individual-based ones to account for natural levels of sample heterogeneity in the data (Gotelli & Colwell, 2001). To compare the species richness instead of the species density of different host trees, forest layers, or sample dates, the sample-based curves were rescaled to isolates as recommended by Gotelli & Colwell (2001).

EstimateS computes a number of species richness estimators, which try to predict how many species could be found in the examined site with the used methods, if the sampling had been exhaustive. While species accumulation curves are well established in mycological diversity studies, the use of species richness estimators

seems to be less common (UNTERSEHER et al., 2008). However, there is no one-size-fits-all solution. The *Chao2* richness estimator has been used for the present study, because UNTERSEHER et al. (2008) showed that it performs best with mycological data, even at smaller sample sizes, and own test with other estimators confirmed these findings. Since *Chao2* is a non-parametric estimator, the estimations might be too small, as O'HARA (2005) shows by means of computing several estimators for simulated data. O'HARA (2005) states that at present it is nearly impossible to decide whether any of the estimation methods will give a realistic estimate, as not enough is known about the true numbers of fungal species or other microbes in natural communities.

It has to be kept in mind that the applied estimators try to compute the total species richness of a mainly homogenous area, which could be found with the methods applied. No forecast can be made about how many endophytes would be found additionally, by using other isolation media, cultivation independent approaches, or extending the investigation area to other habitats, for example.

3 Exploring the diversity of endophytic fungi at the Leipzig Canopy Crane research facility

There are numerous studies about endophytic fungi in woody plants. On the one hand the composition and the ecology of root associated fungi, so-called dark septate endophytes (DSE) or Class 4 endophytes (Rodriguez et al., 2009), are intensively investigated (e.g. Jumpponen & Trappe, 1998; Jumpponen, 2001). DSE seem to be strictly localised in the root system, with no overlap to above ground parts of the plant body. On the other hand, in above ground tissues, the study of foliar endophytes comprises the largest amount of published studies (e.g. Halmschlager et al., 1993; Fisher et al., 1994; Pehl & Butin, 1994; Lodge et al., 1996a; Fröhlich & Hyde, 1999; Helander et al., 2006; Arnold & Lutzoni, 2007; Higgins et al., 2007). Only very few studies look up to the canopy (e.g. Halmschlager et al., 1993; Bahnweg et al., 2005), even though most of the leaves of full-grown trees in a forest can be found in a height of more than 15 m (Eermak, 1998). This is probably due to two facts: The canopies of full-grown trees are difficult to reach, and most economically important plants are small and grow in the understorey like *Theobroma cacao* (Arnold & Herre, 2003). The Leipzig Canopy Crane offered ideal conditions to reach large parts of the canopy and to study spatial and temporal patterns, as well as host-specificity of leaf-inhabiting endophytic fungi.

3.1 An introduction to endophyte diversity in deciduous trees

Endophytic fungi inhabiting leaves of woody plants typically belong to the Class 3 endophytes. They have been described by Rodriguez et al. (2009) as a highly diverse group of horizontally transmitted endophytes that are restricted to aboveground tissues and form localised infections. The successful establishment of these infections may be affected by a patchy distribution of habitat characteristics, such as solar radiation and microclimate, or, alternatively, by dispersal limitation (i.e. a lack of nearby spore sources). This leads to spatial and temporal patterns in the composition of the endophytic community. Moreover, differences in the tissue quality and in the chemistry of the host species e.g. in secondary metabolites might lead to a colonisation by host-specific fungi and thus to host-specific endophyte communities. Since the interactions of foliar endophytes with their hosts are manifold, these patterns might influence the effect of the endophytic community on the forest ecosystem.

3.1.1 Spatial patterns

The few studies which have been carried out on exploring spatial patterns of the endophyte infection of woody plants so far, arrive at contradictory conclusions. Helander et al. (2007) found a heterogenous distribution in the endophytic colonisation between mainland and island forest fragments, and between different forest management systems (Helander et al., 2006). In contrast, comparing tree stands in different habitat types, Arnold & Herre (2003) found strong differences in the number of air-borne fungal propagules, but no significant differences in the infection rates. Even less is known about vertical patterns in tree canopies, and the few existing studies are limited to single host - endophyte combinations. For example Bahnweg et al. (2005) found differences in the colonisation of the light and the shade crown of *Fagus sylvatica* by *Apiognomonia errabunda*, likewise Wilson et al. (1997) for *Ophiognomonia cryptica* in *Quercus emoryi*, and Halmschlager et al. (1993) for different endophytic fungi in *Quercus petraea*. While direct observations of vertical gradients in endophyte frequency and diversity are rare, there is evidence that endophytic fungi are sensitive to environmental conditions (Wilson, 1996; Bahnweg et al., 2005). As these are known to change drastically within the forest canopy (e.g. Parker & Brown, 2000; Unterseher & Tal, 2006; Horchler, 2007), vertical patterns of the endophyte community mirroring the environmental gradients can be expected. This thesis, therefore, compared the endophyte commu-

nity of light and shade crowns of full-grown trees (see chapter 3.4 and 3.5) as well as the understorey (chapter 3.5).

Even within leaves, spatial patterns can be found. Amongst others Sieber & Hugentobler (1987), Halmschlager et al. (1993), and Wilson & Carroll (1994) report a higher infection density along the midrib, especially at the leaf base, than in the marginal lamina. This is probably due to differences in spore deposition and retention, which are affected by leaf surface structures and elevated water availability along the midrib, and lead to increased germination rates (Allen et al., 1991). Variability within leaves was not regarded in this thesis, but leaf fragments were always taken from the middle part of the leaf.

3.1.2 Temporal patterns

More frequently examined than spatial patterns are temporal changes in the endophytic community. Directly after the spring leaf flush, leaves are usually free of endophytes. Gradual spore deposition and germination lead to an increasing infection density and species number as the leaves get older (e.g. Sieber & Hugentobler, 1987; Halmschlager et al., 1993; Pehl & Butin, 1994; Faeth & Hammon, 1997; Wilson et al., 1997). At least a few species of endophytic fungi act as primary decomposers and utilize fallen leaves as a habitat for sexual reproduction and sporulation (Frankland, 1998; Osono, 2006). Infections of these fungi increase in space short before and even after leaf fall. Although the total number of species increases with leaf age, it is possible that the infection density of single species decreases after some time, which might, for example, be due to high summer temperatures (Bahnweg et al., 2005; Hashizume et al., 2010). This could lead, for example, to the fact that Hyphomycetes are dominating the species community in young leaves and Coelomycetes are more frequently found in older leaves (Suryanarayanan & Thennarasan, 2004). Since the microclimatic conditions develop differently in the forest layers, it can be assumed, that changes in the endophytic colonisation also run differently. For this thesis, the endophytic community of light and shade crowns was compared for full-grown Ashes and Ash saplings in the understorey, throughout an entire vegetation period (see chapter 3.5).

3.1.3 Host tree specificity

Host specificity of endophytic fungi in the leaves of deciduous trees is discussed controversially. On the one hand they seem not to be very specific to single host species (Rodriguez et al., 2009). This probably applies particularly to the relatively large

portion of endophytes sporulating on fallen leaves, which usually are cosmopolitan saprobionts (Frankland, 1998). On the other hand Petrini (1991) states that the host specificity of endophytic fungi is probably much narrower than often thought, and that even widespread fungal endophytes form host-specific strains. Furthermore, host preferences can be observed at higher taxonomical levels, as fungi dominating the endophytic communities of angiosperm trees usually belong to the Diaporthales whereas dominant endophytes of gymnosperms belong to the Helotiales (Sieber, 2007).

The endophytic fungal colonisation has been studied for several deciduous host tree species in Europe, e.g. for *Acer pseudoplatanus* (Pehl & Butin, 1994; Gange, 1996), *Betula pendula* and *B. pubescens* (Helander et al., 2006, 2007), *Fagus sylvatica* (Sieber & Hugentobler, 1987; Pehl & Butin, 1994; Bahnweg et al., 2005; Unterseher & Schnittler, 2010), *Quercus petraea* (Halmschlager et al., 1993), *Quercus robur* (Pehl & Butin, 1994; Gonthier et al., 2006), and *Tilia cordata* (Pehl & Butin, 1994). But only (Helander et al., 2007) compared the endophytic communities of different hosts at the same site. Since the composition of the endophytic community depends on the composition of the aerial inoculum (Helander et al., 2007), differences in the endophyte composition of various hosts at different sites can not necessarily be traced back to host specificity. Therefore the endophytic diversity of four different host species from the same site was compared for this thesis (see chapter 3.4)

3.2 Canopy research

For a long time forest canopies were beyond the regard of environmental scientists. This was for one thing due to the limited technical possibilities. At the beginning of canopy research the crowns could be reached only by time-consuming and strength-sapping climbing techniques, for which the equipment was available only to the army (Mitchell, 1986). The sampling was thereby confined to the surrounding of sturdy branches close to the tree trunk, while the leafy regions of the tree crowns were out of reach, if not shot down with firearms. Thus, the areas of the highest metabolic activity, where for example leaf flush, flowering, fruit development, and the maximum level of photosynthesis take place, could not seriously be examined. Furthermore, the scientists did not expect to find something new and spectacular, because their ecological ideas about forest canopies were based on observations made on the forest floor. Only since the 1970s, have increased activities to an exploration of canopies been undertaken. New techniques like fogging of the tree crowns with insecticides and subsequent collecting of the insects (Erwin, 1982) showed that many ecological and biological secrets were still to discover.

It is now known that the canopy plays an important role in the maintenance of forest biodiversity (Basset et al., 2003). This is underlined by the fact that 22 of 25 global biodiversity hotspots embrace forest habitats. Approximately 20 to 25% of the invertebrates are proposed to be unique to the canopy and 10% of all vascular plant species are epiphytic canopy dwellers (Ozanne et al., 2003). Particularly the external canopy layers, which have not been accessible before, seem to possess a great biological activity and a high species richness (Basset et al., 2003).

The comprehensive exploration of forest canopies has started in the early 1980s (Lowman, 2009). At that time the techniques had developed so far, that ascending the trees became considerably simpler and intervened in the ecosystem with fewer disturbances. Use of permanently installed systems like canopy walkways, towers, and cranes is spreading as well as the use of balloons (Hallé et al., 2000; Nadkarni et al., 2004; Pennisi, 2005; Lowman, 2009).

The first construction crane for observation purposes was set up in 1992 by Alan Smith to study forest canopies in Panama (Parker et al., 1992). The advantage of cranes compared to walkways is the greater flexibility to reach positions in all crown layers, and they are considerably less weather-dependent than balloons. The outer canopy regions can be reached particularly easily by a gondola attached to the crane's jib, and exactly the same locality can be investigated repeatedly.

The use of cranes allows study of trees in their natural environment and exploration of biodiversity, nutrient cycles, energy transfer, and various other interac-

tions. The investigation of abiotic influences, just like the flow of water vapour, CO_2 (KÖRNER et al., 2005), and light, facilitates an advanced appreciation of ecosystem processes. Conversely, the forest canopies influence abiotic factors. An intact canopy, for example, increases the evapotranspiration, the water vapour of the atmosphere and contributes to cloud formation. On the other hand, up to 25% of the precipitation is held back by the canopies (OZANNE et al., 2003), instead of running off quickly and contributing to soil erosion. Such findings show the necessity of canopy research for the appreciation of complex biological and ecological processes in forests.

3.3 The study site at the Leipzig Canopy Crane research facility (LAK)

The study site is located in the area of the city of Leipzig (Germany) at an altitude of 102 m, with a mean annual precipitation of 512 mm and a mean temperature of 8.8°C. It is at the margin of a mixed deciduous floodplain forest stand as it is typical for the upper alluvial zone (Querco-Ulmetum minoris Issler). Due to absence of flooding and a falling groundwater level in the last decades, the species composition has changed. Species that are less tolerant towards flooding, as maple (*Acer* spp.), became more numerous and the population of the previously dominant English oak (*Quercus robur*) declined (Schöne & Jentsch, 2007). Altogether, 15 woody species (with > 5 cm diameter at breast height - DBH) can be found at the crane site (Fig. 3.1).

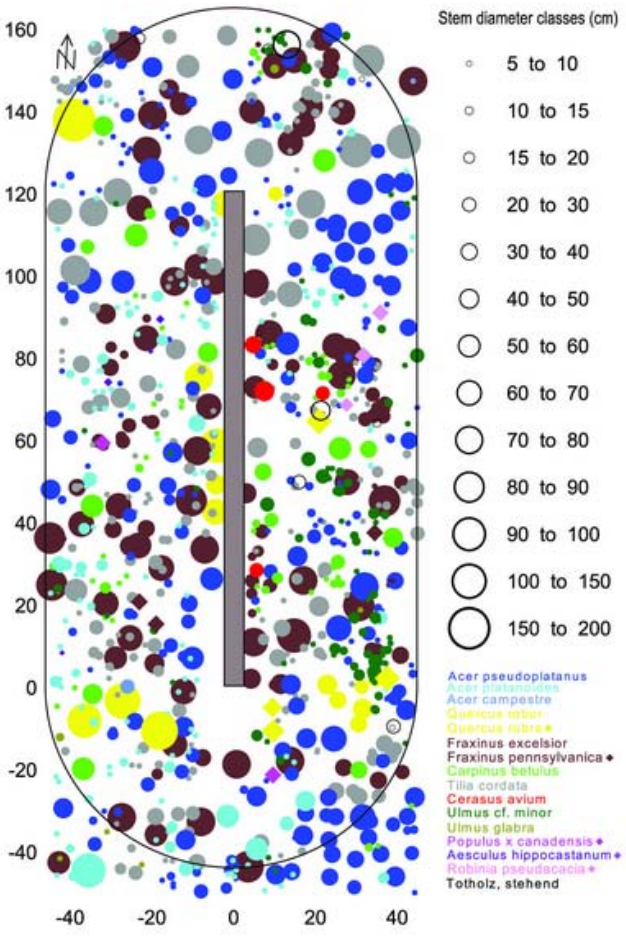

Figure 3.1: Species composition and position of trees with more than 5 cm diameter at breast height at the Leipzig Canopy Crane research facility (source: Morawetz & Horchler, 2003)

By means of a tower crane (Liebherr 71EC) with a height of 40 m, a jib length of 45 m, and a maximum sampling height of about 35 m, which is movable on a track of 120 m length, all layers of the canopy can be reached within an area of 1.6 ha (Fig. 3.1 and 3.2).

The study site shows a great heterogeneity with its comparatively high tree species number and its mixture of old and young individuals. This is reflected in a highly fragmented canopy surface. The canopy is currently mainly formed by *Quercus robur* (up to 250 years, 7% of the canopy cover), *Fraxinus excelsior*, *Acer* spp., and *Tilia cordata* (all younger than 130 years; 49, 21, and 10% of the canopy cover, Rohrschneider, 2007).

Figure 3.2: The Leipzig Canopy Crane in spring (source: LAK project)

3.4 Investigating the diversity of endophytic fungi in different hosts

3.4.1 Methods

In September 2005, about four weeks before the end of the vegetation period, leaves (or leaflets in the case of *Fraxinus*) were collected from *Acer pseudoplatanus* L., *Quercus robur* L., *Fraxinus excelsior* L., and *Tilia cordata* Mill.. Two individuals of each tree species were selected randomly considering that (1) the individuals had a height of at least 25 m, (2) the distance of the two individuals of each species was at least 20 m, and (3) the tree crowns were accessible with the gondola from different sides. From each tree, samples were taken at four randomly selected points of the tree crown at heights of 15 to 35 m, noting that two of the points were located in the light crown and two in the shade crown. At each sample point, three leaves were collected randomly within a cubic space with an edge length of 0.5 m. In the case of *F. excelsior* one middle leaflet of the pinnate leaf was taken (Table 3.1).

The subsequent handling of the samples followed the general methods of isolation, cultivation, grouping of morphotypes, and morphological determination (see chapters 2.1.1 and 2.1.2). Deviating from this, for this study, a leaf fragment of 2 x 2 cm was cut out of the middle part of the leaves or leaflets, and divided into 16 fragments of 0.5 x 0.5 cm.

The sequence of the D1/D2 nrDNA region was determined for at least one isolate per morphotype, as described in chapter 2.1.3. A maximum parsimony analysis was performed for all 36 obtained sequences, including a sequence of *Glomus mosseae* (GenBank accession number DQ469131) as the outgroup, to get an overview of how the morphotypes arrange in the taxonomical system of the fungi.

Each morphotype was counted only once per leaf, regardless of the real number of isolates (see chapter 2.1.4). Thus, every morphotype could have a frequency between 0 and 3 at each sample point.

Based on these quantitative data, sample-based *rarefaction* curves were computed for the whole collection of 32 sample points, as well as for the different host species and crown layers to estimate and compare the diversity of different endophytic communities. The Fisher's α, Shannon, and Simpson diversity indices have likewise been computed with EstimateS for this purpose (see chapter 2.1.4).

Table 3.1: Sampling design for the light and the shade crowns of four tree species at the LAK site in 2005

Host species	Individual	Crown layer	Sample points	Leaves	Fragments
Acer pseudoplatanus	A	light crown	2	6	96
	A	shade crown	2	6	96
	B	light crown	2	6	96
	B	shade crown	2	6	96
Fraxinus excelsior	A	light crown	2	6	96
	A	shade crown	2	6	96
	B	light crown	2	6	96
	B	shade crown	2	6	96
Quercus robur	A	light crown	2	6	96
	A	shade crown	2	6	96
	B	light crown	2	6	96
	B	shade crown	2	6	96
Tilia cordata	A	light crown	2	6	96
	A	shade crown	2	6	96
	B	light crown	2	6	96
	B	shade crown	2	6	96
Total	8		32	96	1536

3.4.2 Results

Morphological identification

Provided that every morphotype has been isolated only once per leaf altogether 343 fungal cultures were isolated, out of which 298 were divided into 40 morphotypes. The remaining 45 isolates could not be distinguished unambiguously from the others and were treated subsequently as a non-assignable group of mycelia sterilia. They were not included in further calculations, because nothing is known about their taxonomical status. 21 morphotypes could be determined by morphological characters to genus or species level (Table 3.2).

Systematical grouping of sequenced isolates by maximum parsimony analysis

For *Aspergillus niger*, *Nigrospora sphaerica*, and the morphotypes MT0526 and MT0537, no sequence of the D1/D2 region was obtained. The alignment of the other 36 morphotypes and *Glomus mossae* as the outgroup comprised 179 parsimony informative sites that were included in the further analysis.

Table 3.2: Morphologically identified species or genera of endophytic fungi from four host tree species (*Acer pseudoplatanus*, *Fraxinus excelsior*, *Quercus robur*, and *Tilia cordata*) and their appearance in culture as anamorph (A) or teleomorph (T)

Fungal species	**appearance in culture**
Alternaria alternata (Fr.) Keissl.	A
Aspergillus niger var. *niger* Tiegh.	A
Aureobasidium pullulans (de Bary) G. Arnaud	A
Cladosporium cladosporioides (Fresen.) G.A. de Vries	A
Colletotrichum gloeosporioides (Penz.) Sacc.	A
Diplodina acerina (Pass.) B. Sutton (T: *Apiognomonia hystrix* [Tode] Sogonov)	A
Discula umbrinella (Berk. & Broome) M. Morelet (T: *Apiognomonia errabunda* [Roberge ex Desm.] Höhn.)	A
Epicoccum nigrum Link	A
Fusarium sp.	A
Fusicladium fraxini Aderh. (T: *Venturia fraxini* Aderh.)	A
Nigrospora sphaerica (Sacc.) E.W. Mason	A
Periconia cookei E.W. Mason & M.B. Ellis	A
Phoma sp. 1	A
Phoma sp. 2	A
Phomopsis sp.	A
Anamorphic state of *Pleospora herbarum* (Pers.) Rabenh. (T)	A
Pyrenophora erythrospila A.R. Paul	T
Sordaria fimicola (Roberge ex Desm.) Ces. & De Not.	T
Sordaria humana (Fuckel) G. Winter	T
Sporormiella minima (Auersw.) S.I. Ahmed & Cain	T
Xylaria sp.	A

The maximum parsimony analysis led to a 50% majority rule consensus tree that is shown in Fig. 3.3. All 36 morphotypes group into two classes of Ascomycota: Dothideomycetes and Sordariomycetes. From the unsequenced morphotypes, *N. sphaerica* also belongs to the Sordariomycetes, whereas *A. niger* belongs to the Eurotiomycetes.

A list of the best hits of the BLAST search for all sequences can be found in appendix B.

Species richness of endophytic fungi

56.6% of the isolates belonged to the class Dothideomycetes and 29.4% to the Sordariomycetes. Only 0.3% (one isolate) belonged to Eurotiomycetes and 13.7% were

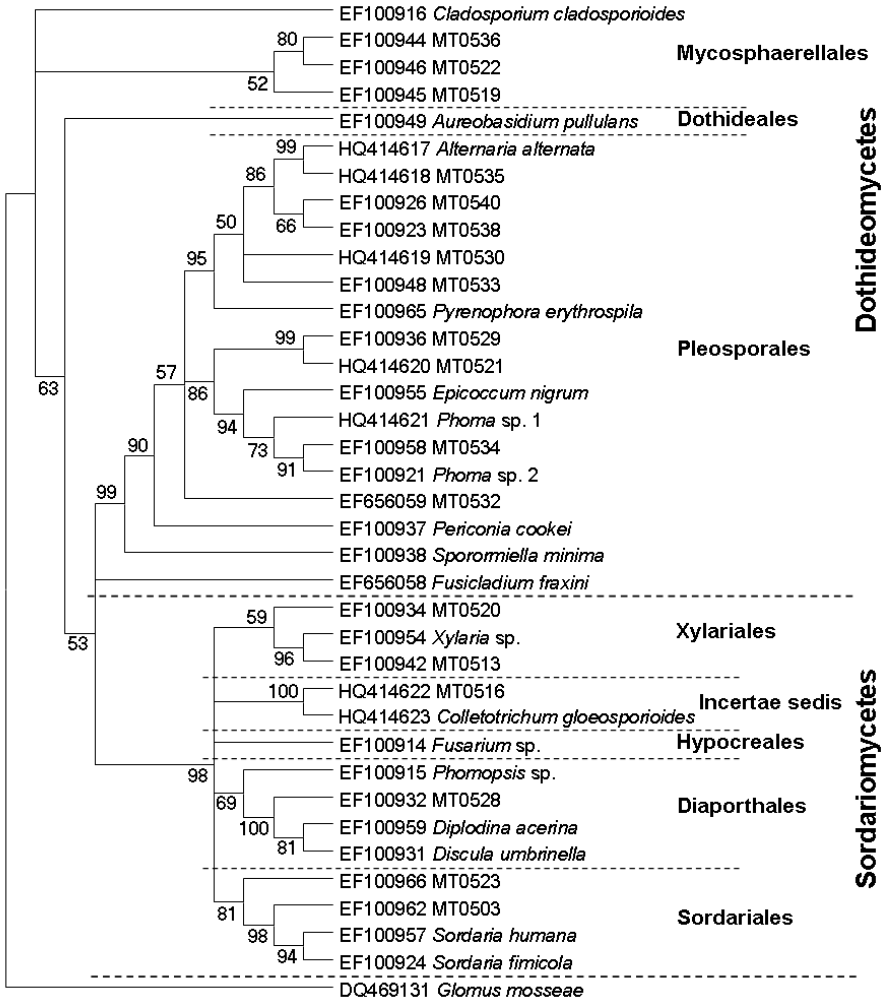

Figure 3.3: A 50% majority rule consensus tree of maximum parsimony analysis based on D1/D2 region (LSU) nrDNA sequences of 36 morphotypes, rooted with a sequence of *Glomus mosseae* (DQ469131) wich was selected from GenBank (tree length 658, parsimony informative characters 179, consistency index (CI) 0.451, retention index (RI) 0.761, rescaled consistency index (RCI) 0.344). The sequences are labelled with the GenBank accession number, and the species or genus name for morphologically determined morphotypes or the shortcut for undetermined morphotypes. The given orders and classes were deduced from the best hits of the BLAST search (see appendix B), if the morphotype had not been determined. Bootstrap values of ≥50% from 1000 replicates are shown on the branches.

ungrouped mycelia sterilia or morphotypes which could not be determined on class level. With respect to the grouped isolates, 65% were Dothideomycetes, 34% were Sordariomycetes, and 1% were Eurotiomycetes and unidentified morphotypes.

Three of the 40 isolated morphotypes were abundant in the sampled trees: *Alternaria alternata*, *Cladosporium cladosporioides*, and *Aureobasidium pullulans* were found in more than 30 of the 96 examined leaves (Fig. 3.4). 13 other morphotypes were isolated frequently (from 5 to 17 leaves), and the remaining 24 morphotypes (60%) were rare or very rare (from 1 to 4 leaves).

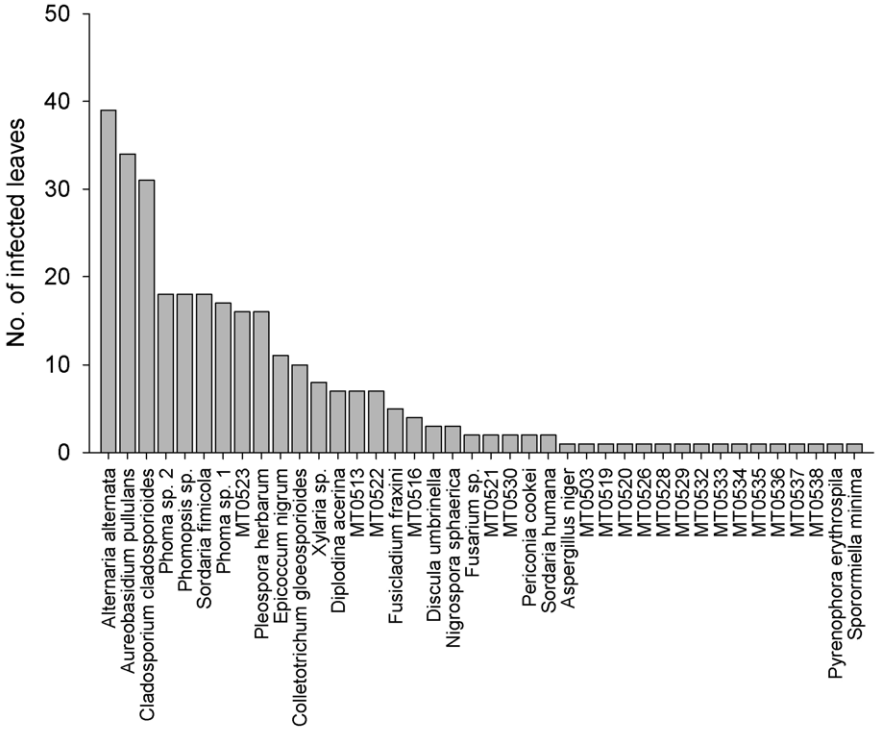

Figure 3.4: Rank-abundance plot of the endophytic fungal morphotypes isolated from four host tree species (*Acer pseudoplatanus*, *Fraxinus excelsior*, *Quercus robur*, and *Tilia cordata*) sorted by the number of infected leaves

The large number of rarely isolated morphotypes led to a *rarefaction* curve that continuously rises almost linearly (Fig. 3.5). Accordingly, the *Chao2* estimator computed far larger values (61 estimated vs. 40 observed morphotypes).

Influence of host tree species and light regime on the occurence of endophytic fungi

The number of endophytic taxa hardly differed between the host tree species. 22 endophytic morphotypes were found in *Acer* foliage, followed by *Fraxinus* and *Tilia*

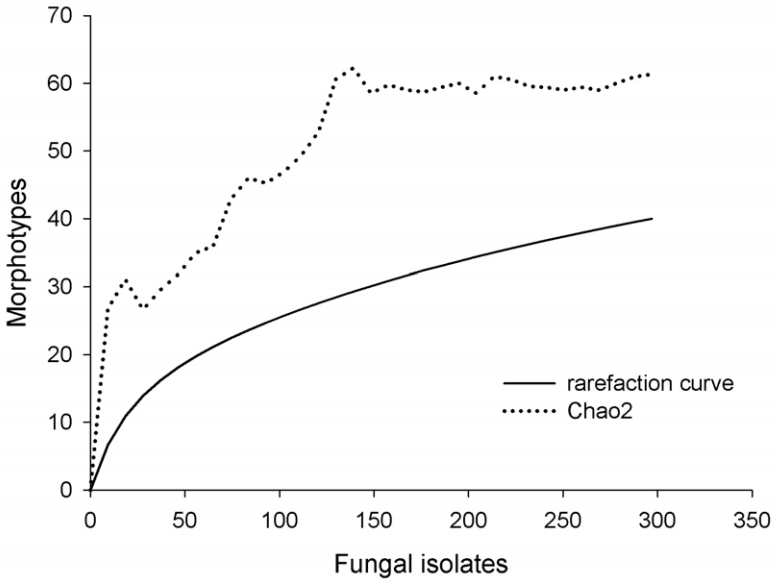

Figure 3.5: Sample-based *rarefaction* curve, rescaled to isolates, of the whole endophytic community isolated from four different host species, and the species richness estimator *Chao2*

containing 21, and *Quercus* containing 20 morphotypes. The different diversity indices identified different most endophyte-rich host tree species. The Shannon diversity index is highest for *Acer*, followed by *Quercus*, *Tilia*, and *Fraxinus*. The Fisher's α index in contrast is highest for *Quercus*, followed by *Acer*, *Fraxinus*, and *Tilia*. The Simpson index is also highest for *Quercus*, followed by *Acer*, but then by *Tilia*, and *Fraxinus* (Table 3.3).

Table 3.3: Diversity indices of the complete sampling and different subsamples of endophytic fungi from the light and the shade crowns of four host tree species

	Fisher's α	**Shannon**	**Simpson**
Total	12.45	3.02	15.6
Light crown total	9.99	2.73	12.09
shade crown total	11.53	2.99	17.20
***Acer* total**	10.20	2.81	16.23
***Fraxinus* total**	9.27	2.56	10.19
***Quercus* total**	11.69	2.75	16.60
***Tilia* total**	8.86	2.70	13.79
***Acer* light crown**	9.88	2.55	14.72
***Acer* shade crown**	15.12	2.75	19.53
***Fraxinus* light crown**	6.17	2.07	6.87
***Fraxinus* shade crown**	8.18	2.48	12.72
***Quercus* light crown**	10.03	2.22	11.88
***Quercus* shade crown**	16.21	2.74	22.00
***Tilia* light crown**	6.44	2.31	10.25
***Tilia* shade crown**	9.05	2.57	14.55

Whilst especially the most common morphotypes *A. alternata*, *A. pullulans*, and *C. cladosporioides*, were distributed almost evenly among all host tree species, some less frequent morphotypes exclusively appeared in a single host tree species, as *Diplodina acerina* in *Acer*, and *Discula umbrinella* in *Quercus* (see appendix C.1).

For all tree species, the endophytic species richness (Fig. 3.6) and species density (not shown) were higher in the shade crown than in the light crown, though the differences were not significant (based on the lower and upper 95% confidence interval of the particular curve). All *rarefaction* curves did not approach an asymptote. The diversity indices in the light crown were lower than in the shade crown for each single tree species as well as the complete sampling (Table 3.3).

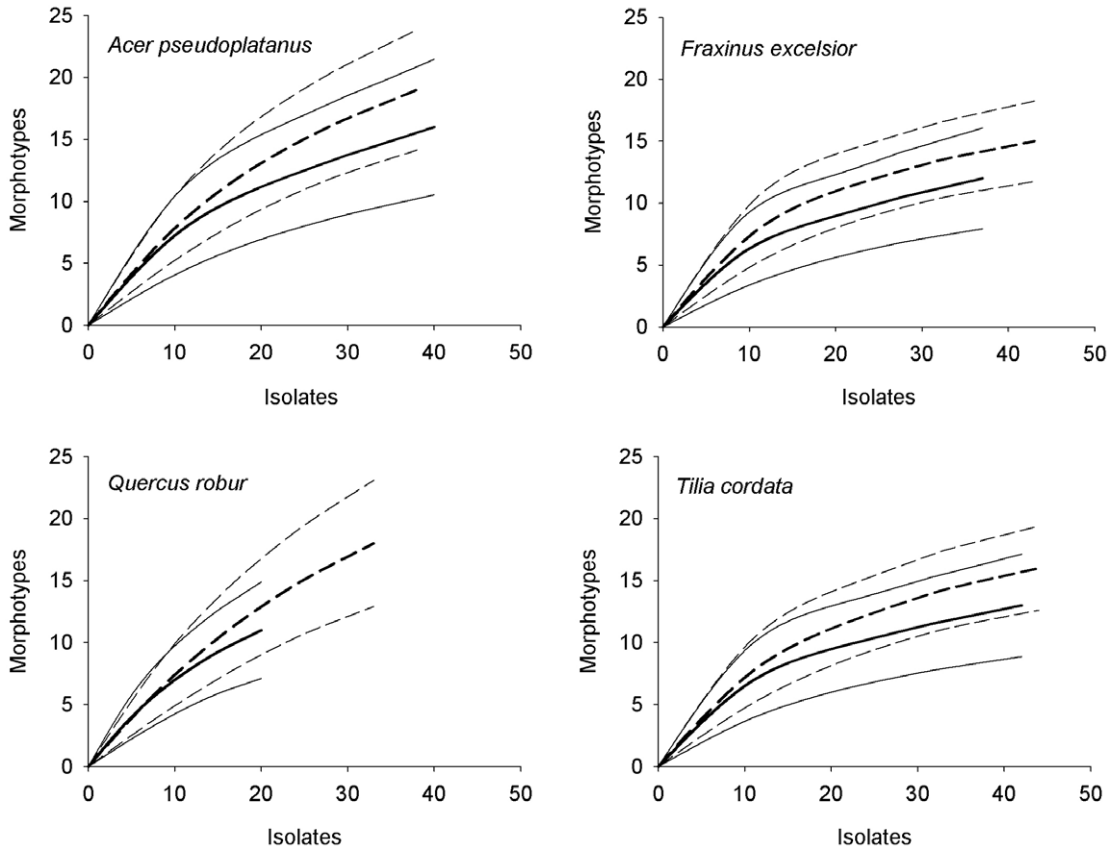

Figure 3.6: *Rarefaction* curves of endophytic fungi communities of *Acer pseudoplatanus*, *Fraxinus excelsior*, *Quercus robur* and *Tilia cordata* in the light crown (thick solid lines) and in the shade crown (thick dashed lines) and their 95% confidence intervals (thin lines)

A closer look at the most common morphotypes (Fig. 3.7) showed that the distribution between light crown and shade crown differed between the morphotypes. While some morphotypes followed the general pattern and were isolated mostly or exclusively from the shade crown (e.g. *Colletotrichum gloeosporioides*, *Fusicladium*

fraxini, and MT0513) others inverted that trend and were most common in the light crown (e.g. *Epicoccum nigrum*, *Sordaria fimicola*, and *Alternaria alternata*).

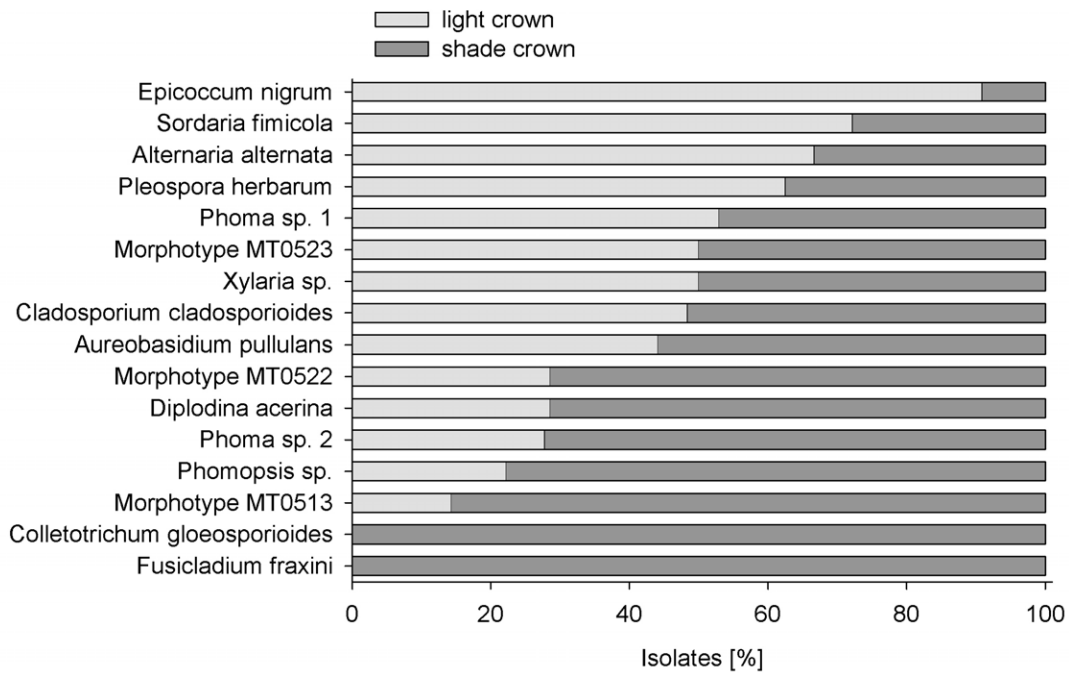

Figure 3.7: Distribution of the most common species of endophytic fungi in the light and the shade crowns of four host tree species

3.4.3 Discussion

The study revealed higher rates of endophytic infection in the shade crowns than in the light crowns of all four examined host tree species. Only slight differences were found comparing the colonisation of the different hosts.

Sampling design

In contrast to other studies that investigated the fungal endophytic community of only one or two host tree species (e.g. Gange, 1996; Bahnweg et al., 2005; Gonthier et al., 2006; Helander et al., 2007), four host tree species were investigated for this study at the same time.

Since studies of endophytic communities in different canopy layers are rare (see chapter 3.1.1), the present work can be viewed as a novelty in endophytology and may allow comprehensive assessment of diversity patterns of temperate forest endophytes. The higher number of host tree species however led to a smaller number of samples per host species. On the other hand, the sampling design at hand allowed identification of trends in colonisation variability.

The four host tree species (*A. pseudoplatanus*, *F. excelsior*, *Q. robur* and *T. cordata*) were chosen, because they are ecologically dominating the investigation area (Seele, 2007), thy are predominant in the canopy (Rohrschneider, 2007), and they are widespread in Europe, and therefore typical for European temperate forests. The canopies of the same host tree individuals were investigated for wood-decaying fungi and eumycetozoans before (Unterseher et al., 2005; Schnittler et al., 2006; Unterseher & Tal, 2006) and will enable more detailed analysis of the fungal diversity in the forest canopy.

Morphological and molecular identification of the morphotypes

About 70% of all cultures developed either conidia or ascospores during the cultivation period. These cultures belonged to the 53% of morphotypes, that could be determined morphologically at least to the genus level. The amount of mycelia sterilia was comparable to other studies on endophytic fungi and depends, to a certain degree, on the extent of efforts to promote sporulation with different media and cultivation techniques (Guo et al., 2000). Nevertheless, there will always be sterile mycelia remaining, which can only be classified with molecular methods.

For these mycelia sterilia a molecular determination at species level is not always possible with the used D1/D2 region of 28S nrDNA (e.g. Abliz et al., 2004). Whilst the D1/D2 sequences in many cases distinguished between species from the same genus, this region of the conserved 28S nrDNA showed no or only slight differences for other genera (e.g. *Cladosporium*), or, in contrast, showed a high intraspecific variability, as in *Xylaria*. Multigene approaches would solve this problem, but were not applicable due to time and money constraints. However, the D1/D2 region is beneficial for the molecular identification of a broad range of fungal taxa, and the aim of this study was not phylogenetic so the methods chosen seem to be reasonable.

Species richness and diversity of endophytic fungi

The observed endophytic community was dominated by two classes of Ascomycota: Dothideomycetes (65%) and Sordariomycetes (34%). According to Arnold & Lutzoni (2007), this proportion was between the proportion found for boreal forests (only about 1/6 Sordariomycetes) and that of temperate forests (more than 50% Sordariomycetes). It was remarkable that only 1% of the isolates belonged neither to Sordariomycetes nor to Dothideomycetes, as Arnold & Lutzoni (2007) found that at least 5% of the isolates belonged to other classes. This might be due to the fact that the boreal forest, as well as the tropical forest and the temperate forest researched in the study by Arnold & Lutzoni (2007), are at least semi-deciduous

or even indeciduous, whereas the Leipzig floodplain forest is deciduous. The present study indicates that leaf age and basic character (deciduous, coniferous) influence the ratio of higher taxonomic levels of foliar endophytes. The amount of Sordariomycetes is higher in leaves that only reach an age of several months. Also, the diversity at the LAK site (Fisher's $\alpha = 12.45$) was between that of a boreal forest (Fisher's $\alpha = 9.2$) and a temperate semi-deciduous forest (Fisher's $\alpha = 25.7$; Arnold & Lutzoni, 2007). It seems that climate and leaf age also influence the species composition and diversity.

The rank-abundance curve showed few frequent species at the left and a long right-hand tail of rarely isolated species. It is, as well as the almost linearly rising *rarefaction* curve of the total sampling, typical for hyperdiverse groups (Hughes et al., 2001), into which endophytic fungi can be counted (Arnold et al., 2000), and may indicate an undersampling of the underlying parent communities (Ulrich et al., 2010). For hyperdiverse groups it is usually not feasible to carry out a complete survey for an investigation site with traditional techniques, but new approaches such as 454 pyrosequencing might successfully supplement the efforts (Jumpponen & Jones, 2009; Tedersoo et al., 2010).

However, statistical approaches to describe the species richness and estimate the total number of species could greatly increase the explanatory power of any fungal diversity assessment (Arnold et al., 2000; Hughes et al., 2001; Unterseher et al., 2008). The computed richness estimator *Chao2* predicts a species richness of barely more than 60 morphotypes which could be isolated from the investigated sample points, with the methods applied. *Chao2* reached an asymptote, as it is required to make a reliable predication. This corroborates the results of Unterseher et al. (2008), who recommend the *Chao2* richness estimator as one that computes reliable figures for mycological data. Furthermore, it is to be considered that strictly biotrophic endophytes cannot be isolated, and endophytes that colonise only single cells were most likely underrepresented in the data, because they were not traceable with the used methods. Therefore, the calculated diversity should be considered as a truly conservative estimate.

The observed species composition is typical for endophytic communities in European broadleaf trees of the host tree genera *Acer*, *Quercus*, and *Tilia* (e.g. Halmschlager et al., 1993; Pehl & Butin, 1994; Gonthier et al., 2006). As yet nothing is known about endophytic communities of *Fraxinus* leaves, but at least some of the isolated endophytic genera occur as wood-inhabiting endophytes in *Fraxinus* branch bases (Kowalski & Kehr, 1992).

The most frequent species *Alternaria alternata*, *Aureobasidium pullulans*, and

Cladosporium cladosporioides are cosmopolitan saprobionts (Ellis, 1971). Wilson (1993) supposes that endophytic fungi are involved in the senescence and the degradation of leaves. The high frequency of fungi known as ubiquitous saprobionts in autumn corroborates this assumption. Later, these species may act as primary colonisers and decompose the leaf litter until they are displaced by Basidiomycota and soil fungi (Frankland, 1998). The number of 40 morphotypes in total and 20 to 22 per host species is comparatively low. For instance Sieber & Hugentobler (1987) found 64 species in leaves of *Fagus sylvatica* and Halmschlager et al. (1993) 78 species in leaves of *Quercus petraea*. This difference is probably due to the relatively small sample size per host species, which is owed to the fact that four different hosts were investigated simultaneously in this study.

The influence of the host tree species and the light regime

Even though the sampling has been far from being exhaustive, differences in the endophytic density and diversity between the light crown and the shade crown could be observed for all host tree species. Differences in the abundance of single endophyte species between the light crown and the shade crown have already been reported earlier (Halmschlager et al., 1993; Wilson & Faeth, 2001; Bahnweg et al., 2005) but no publication could be found which extensively compares the total species richness and the diversity of different tree crown layers.

Numerous explanations are imaginable for the fact that more species can be found in shade leaves than in leaves of the light crown and that the infection density is higher in the shade crown. The shade leaves provide a less fluctuating habitat, while only a few specialists can develop well in the light crown leaves which are exposed to a higher UV radiation and a stronger variation in temperature and humidity. These extreme conditions in the light crown might reduce the successful infection of leaves by endophytic fungi. A similar effect has been found by Sumampong et al. (2008) for *Phoma argillacea*, a common pathogen on *Rubus spectabilis*, which showed a considerable decrease of colony growth and conidia germination for temperatures above 25°C. The high light intensity also leads to thicker cuticles and scleromorphic cell structures as well as enhanced hairiness (e.g. for sun leaves of *Tilia cordata*) which hinder a successful infection of the leaves (Allen et al., 1991). Another important point, which will be discussed in greater detail in chapter 4.2, is the limited availability of water on the leaf surface. The leaves in the light crown are more exposed to the sun and the wind, so that the leaf surfaces dry faster after precipitation (Unterseher & Tal, 2006).

The rather high frequency of fungi known as ubiquitous saprobionts in the light

crown (especially *Epicoccum nigrum*, and *Alternaria alternata*) could well be due to the fact that these primary colonisers of leaf litter can already benefit from unspecific compounds (as carbohydrates) that are released in the leaves during senescence processes, which start considerably earlier in the light crown than in the shade crown.

The endophytic fungi showed less host specificity than wood-decaying fungi from the same host tree species and the same study site (Unterseher et al., 2005; Unterseher & Tal, 2006). Most endophytic species seem to be broad in host range as Rodriguez et al. (2009) supposed. However at least *Diplodina acerina* for *Acer* and *Discula umbrinella* for *Quercus* were found in only one host species. *Discula umbrinella* (teleomorph: *Apiognomonia errabunda*) has been reported to occur in a wide variety of host plants, amongst others also in *Fraxinus*, *Acer*, and *Tilia*. This might be due to the suggestion of Petrini (1991) that endophytes can form host-specific strains and populations. Haemmerli et al. (1992) actually could satisfactorily show such host specificity for *Apiognomonia errabunda* in different host tree species. The species might be minor competitive in the other hosts and therefore be overgrown by other endophyte species. However, multi-species interactions and the influence of substratum type and quality are difficult to assess (Lopez-Llorca et al., 1999) and were beyond the scope of the present thesis.

Furthermore, host-specific endophytes might be underestimated in this study, because with common media, such as malt extract agar, it is not possible to cultivate obligatory biotrophs which are expected to be more host-specific. Besides, host-specific species could be slow-growing on artificial media, and they would thus be inferior in the competition with fast-growing ubiquitous ones (see chapter 2.2.1).

3.5 A detailed investigation of spatial and temporal patterns of endophytic fungi in *Fraxinus excelsior*

The study of the endophytic composition of different host species (chapter 3.4) has revealed differences in the endophyte colonisation of the light and the shade crown for all four examined host tree species. None of the hosts in this study appeared to be investigated sufficiently in respect of species richness and species composition, which is recognizable by the *rarefaction* curves that did not reach an asymptote. Furthermore, new questions appeared: If there is a difference between the light crown and the shade crown, will there be also a difference between the shade crown and the understorey as Espinosa-Garcia & Langenheim (1990) found for mature trees and basal sprouts of coastal redwood? And how does the endophytic community in the different forest layers change in the course of the vegetation period (see chapter 3.1.2)?

Because no exhaustive sampling could be done for all four previously investigated host tree species, *Fraxinus excelsior* was chosen for further research. The European Ash is frequent and ecologically important, not only in the study site, but also in different forest types of Europe. There also seems to be as yet no published study about the endophyte community of *Fraxinus excelsior*. This is currently of particular interest, as *Chalara fraxinea*, first observed in Poland about 10 years ago (Kowalski, 2006), has spread over Europe (e.g. Germany: Schumacher et al. (2007), Austria: Halmschlager & Kirisits (2008), Hungary: Szabo (2009), Norway: Talgo et al. (2009), Slovenia: Ogris et al. (2009), Italy: Ogris et al. (2010)). This fungus has been found to be the cause of a new dieback of ash (Kowalski, 2006) and to be the anamorph of the ascomycete *Hymenoscyphus albidus* which occurs on *Fraxinus* petioles in the leaf litter and might even live endophytically before. The teleomorph was described already in 1850, and has been widespread in Europe without causing any disease thus far (Kowalski & Holdenrieder, 2009b).

3.5.1 The host tree *Fraxinus excelsior* at the LAK research facility

The European Ash (*Fraxinus excelsior* L.) is native to most of Europe with exception of southern Spain, northern Scandinavia, and the northern and eastern parts of the European Russia. It is a typical deciduous tree found in hardwood floodplain vegetation, preferring calcareous and nutrient rich soils and tolerant of occasional

flooding. Since it is sensitive to late freezing, leaves of full-grown trees do not sprout till the end of April at the LAK-site and drop off with the first night frosts at the end of October. Saplings in the understorey that rise more protectedly against low night temperatures, already sprout at the beginning till middle of April (personal observation). Ashes can grow up to a height of 40 m and can reach a diameter at breast height (DBH) of more than one metre. The oldest trees reach an age of about 300 years (Roloff & Pietzarka, 2006).

Fraxinus excelsior is together with *Acer pseudoplatanus* and *Tilia cordata* the most ecologically dominant tree species in the study site (Seele, 2007). It does not have the highest number of individuals, but the highest number of full-grown trees and provides almost 50% of the canopy cover (Rohrschneider, 2007). The population structure of *Fraxinus excelsior* in the study site has been described in detail by Seele (2007): On the area of 1.6 ha which can be reached by the crane, 111 individuals of *Fraxinus excelsior* with a DBH of more than five centimetres were recorded in 2004. Tree populations that are rejuvenating themselves naturally usually show a unimodal distribution of DBH-classes. In contrast to this, the ash population at the LAK facility shows a bimodal DBH-class distribution with peaks at 15-30 cm and 60-75 cm DBH. There are only very few young ashes, so that natural regeneration can only take place at a limited rate. This is evidently due to the fact that very young ashes can tolerate shade, but as they grow older they need plenty of light (Roloff & Pietzarka, 2006). On the crane trail, which builds a gap in the forest, the successful settling of many young ashes could be observed during the last years. Presumably, the high number of trees in the dominant DBH-classes can be traced back to the fact that since the 19th century ashes have been planted in several phases in the area. Previously ashes did not naturally occur in the Leipzig floodplain forest (Seele, 2007).

3.5.2 Methods

Sampling at the research facility

Leaflets of *Fraxinus excelsior* showing no symptoms of disease, were collected in three different forest layers, once a month from the end of May until the end of October 2008, at the same sample points each time (Table 3.4). The leaflets were collected from the light crown of full-grown trees between about 25 to 35 m height (Fig. 3.8 A), from the shade crown of the same trees between about 15 to 20 m height (Fig. 3.8 B), and from about 10 years old saplings in the understorey (Fig. 3.8 C). At four sample points in every canopy layer, within a cubic space with an edge length

of 0.5 m, one middle leaflet of each of three randomly chosen pinnate leaves was collected.

Table 3.4: Sampling design for the light crown, the shade crown, and the understorey of *Fraxinus excelsior* at the LAK site during the vegetation period 2008

Date	**Number of sample points/leaflets/ leaf fragments**			**Total**
	Light crown	shade crown	Understorey	
May (26.05.08)	4/12/48	4/12/48	4/12/48	12/36/144
June (23.06.08)	4/12/48	4/12/48	4/12/48	12/36/144
July (21.07.08)	4/12/48	4/12/48	4/12/48	12/36/144
August (25.08.08)	4/12/48	4/12/48	4/12/48	12/36/144
September (22.09.08)	4/12/48	4/12/48	4/12/48	12/36/144
October (20.10.08)	3/9/36	4/12/48	4/12/48	11/33/132
Total	23/69/276	24/72/288	24/72/288	71/213/852

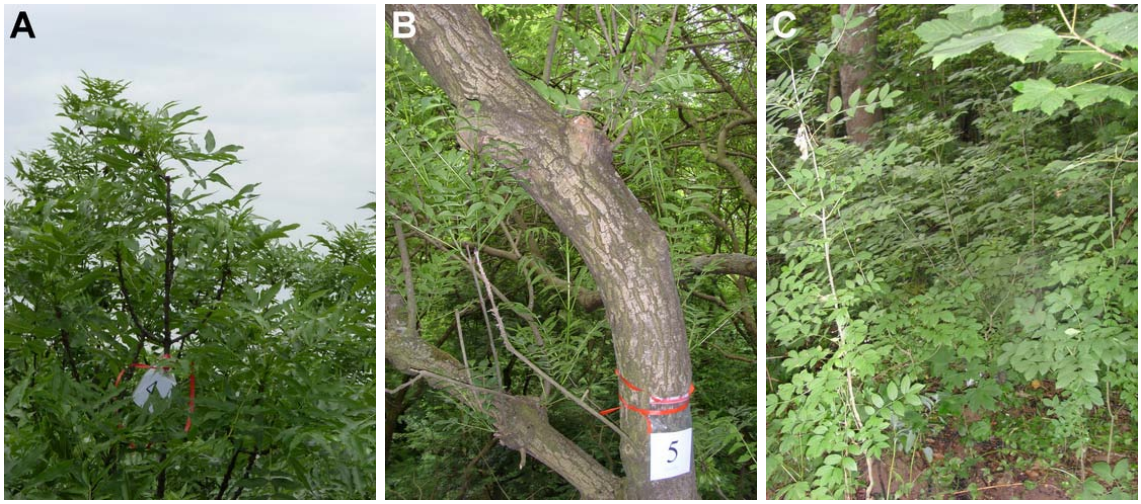

Figure 3.8: The pictures show one sample point each in the light crown (A), the shade crown (B), and the understorey (C) of *Fraxinus excelsior*

The structure and size of the leaflets from the light crown, the shade crown, and the understorey differed noticeably. The leaflets from the light crown were leathery-thick and had a thick, shiny cuticle (Fig. 3.9 A). In contrast to that the leaflets from the shade crown were thin and had a thin cuticle which was not shiny (Fig. 3.9 B). In the understorey, the leaflets from the saplings were frail, considerably smaller than the leaflets from both crown layers, the cuticle was thin, and the shape was not lanceolate but rather elliptical (Fig. 3.9 C).

In the light crown in October, a part of the leaves or leaflets had already dropped off. Therefore leaflets could not be collected randomly from all leaves of the sample

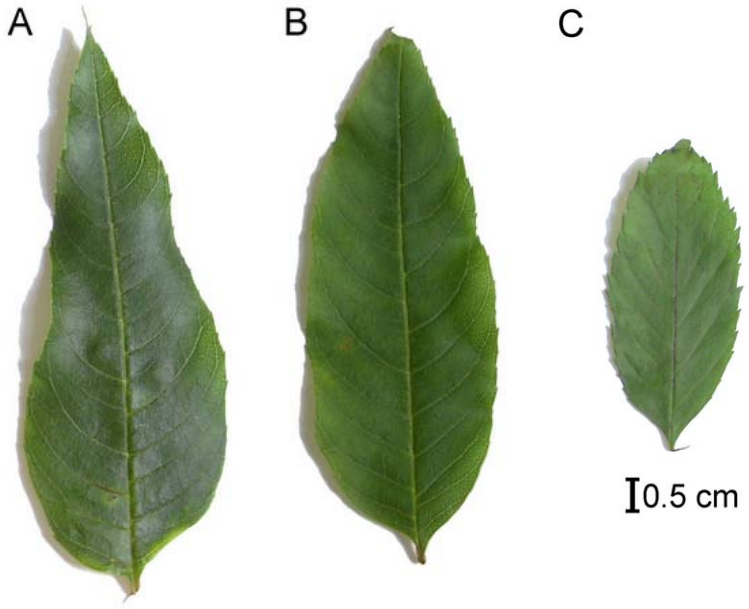

Figure 3.9: The pictures show a typical leaflet of *Fraxinus excelsior* from the light crown (A), the shade crown (B), and the understorey (C)

points. Only leaflets that had been more persistent than the others were left. At one sample point, all leaves had already dropped off so that it could not be sampled in October. These problems were taken into account for the interpretation of the results, and will be discussed in chapter 3.5.4.

Sample processing

The subsequent processing of the samples followed the general methods of isolation, cultivation, grouping of the morphotypes, and morphological determination (see chapters 2.1.1 and 2.1.2).

Sequences of the ITS1, ITS2, and 5.8S nrDNA region were determined for at least one isolate of selected morphotypes as described in chapter 2.1.3. A maximum parsimony analysis was performed according to chapter 2.1.3. The 29 obtained sequences from own sequencing and 15 additional sequences from GenBank representing morphologically-identified endophytic species were included into the maximum parsimony analysis to get an overview of how the morphotypes arrange in the taxonomical system of fungi.

Data analysis

To deal with the problem of defining endophytic individuals (see chapter 2.1.4) each morphotype was counted once per leaf fragment, regardless of the number of colonies it produced. According to the 12 leaf fragments from three leaves that were examined per sample, a morphotype could reach an amount of 0 to 12 for each sample.

Based on these quantitative data *rarefaction* curves were estimated to compare the species-richness of different data sets using the program EstimateS as described in chapter 2.1.4.

The effects of forest layer (light crown, shade crown, understorey) and season (May, June, July, August, September, October) on the number of isolates and the number of morphotypes were analysed with Two Way Repeated Measurement ANOVAs using SigmaPlot version 11.0 (Systat Software, Inc.). Before the analyses, the data was square-root transformed to remove the mean/variance relationship of data which had the character of Poisson data (count data). The Repeated Measurement ANOVA includes the computation of an error-term to accommodate the repeated sampling of the sample points. Pairwise comparisons were calculated using the Tukey test.

A detrended correspondence analysis (DCA) was performed with the programme PC-ORD Version 4.25 (McCune & Mefford, 1999). Since the data, due to the low colonisation density, was sparse for the first months and analyses of the complete data set failed, the analysis was done exemplarily with a reduced data set of the autumn months (August, September, and October). The analysis was performed with quantitative data (0 to 12 isolates per sample and species) and with default settings (rescale axes = on, rescaling threshold = 0, number of segments = 26). Outlier analyses with Euclidean distance measure were done previously to the ordination, but no species or sample had to be excluded.

Environmental parameters

Temperature and humidity were measured with Hobo® Pro Series data loggers, at one sample point each, in the light crown and the shade crown every hour. It was necessary to fix a rain shield above the loggers, because the humidity sensors can be damaged by stagnant moisture. Thus, it was impossible to fix a data logger directly in the highest part of the tree crown, because the twigs were to thin there. It was unfortunately not possible to fix a data logger in the understorey, because the study site region is intensively used as a public recreation area, so the risk of instrument loss was too high.

3.5.3 Results

Isolation frequency and morphological identification

From altogether 852 leaf fragments, 845 fungal cultures were isolated. 72% of them grew out of the leaves within the first week after sampling, another 13% within the second week, the remaining 15% within the following six weeks.

809 of these isolates were divided into 50 morphotypes. The remaining 36 isolates could not be distinguished unambiguously from the others and are treated subsequently as a remaining group of non-assignable mycelia sterilia. They were not included into further calculations. 25 morphotypes were identified by morphological characters to genus or species rank (Table 3.5). According to the morphotype concept, morphotypes identified to genus rank usually contain only one species but might also comprise more than one species.

Alternaria infectoria and *Alternaria alternata*, the two most frequent taxa, were isolated from 148 and 127 of the 852 examined leaf fragments. On the other hand, 50% of the morphotypes were isolated from only one or two leaf fragments (Fig. 3.10). The resulting rescaled *rarefaction* curve of the whole sampling rises almost linearly, and the richness estimator *Chao2* computed 104 species. This are more than twice as much species as could actually be isolated by the methods applied (Fig. 3.11).

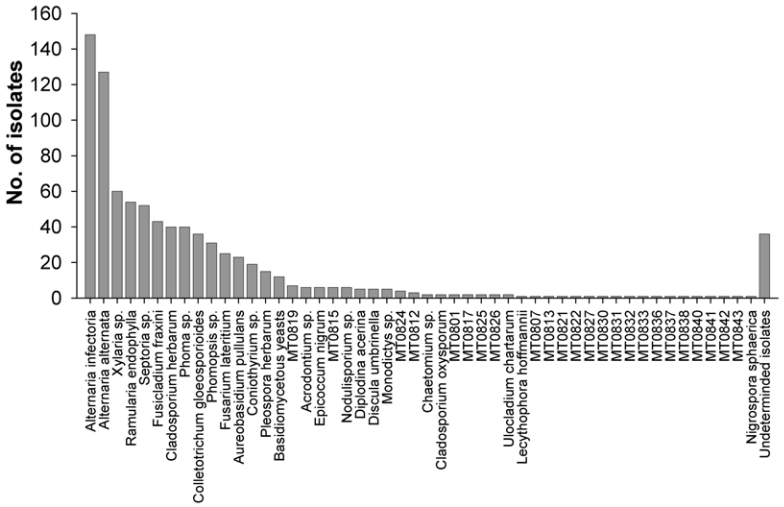

Figure 3.10: Rank abundance curve of the complete sampling of endophytic fungi from *Fraxinus excelsior* in three different forest layers during the vegetation period 2008

Taxonomical identification by sequencing and systematical grouping of the morphotypes

For six morphotypes (MT0801, MT0821, MT0822, MT0825, MT0826, MT0832) no sequence of the ITS region was obtained. The alignment of the other 44 morphotypes comprised 267 parsimony informative sites that were included in further analysis. In

Table 3.5: Morphologically identified species or genera of endophytic fungi from *Fraxinus excelsior* and their appearance in culture as anamorph (A) or teleomorph (T)

Fungal species	**appearance in culture**
Acrodontium sp.	A
Alternaria alternata (Fr.) Keissl.	A
Alternaria infectoria E.G. Simmons (T: *Lewia infectoria* [Fuckel] M.E. Barr & E.G. Simmons)	A
Aureobasidium pullulans (de Bary) G. Arnaud	A
Basidiomycetous yeasts	A
Chaetomium sp.	T
Cladosporium herbarum (Fresen.) G.A. de Vries (T: *Mycosphaerella tassiana* [De Not.] Johanson)	A
Cladosporium oxysporum Berk. & M.A. Curtis	A
Colletotrichum gloeosporioides (Penz.) Sacc.	A
Coniothyrium sp.	A
Diplodina acerina (Pass.) B. Sutton (T: *Apiognomonia hystrix* [Tode] Sogonov)	A
Discula umbrinella (Berk. & Broome) M. Morelet (T: *Apiognomonia errabunda* [Roberge ex Desm.] Höhn.)	A
Epicoccum nigrum Link	A
Fusarium lateritium Nees (T: *Gibberella baccata* [Wallr.] Sacc.)	A
Fusicladium fraxini Aderh. (T: *Venturia fraxini* Aderh.)	A
Lecythophora hoffmannii (J.F.H. Beyma) W. Gams & McGinnis	A
Monodictys sp.	A
Nigrospora sphaerica (Sacc.) E.W. Mason	A
Nodulisporium sp.	A
Phoma sp.	A
Phomopsis sp.	A
Anamorphic state of *Pleospora herbarum* (Pers.) Rabenh. (T)	A
Ramularia endophylla Verkley & U. Braun (T: *Mycosphaerella punctiformis* [Pers.] Starbäck)	A
Septoria sp.	A
Ulocladium chartarum (Preuss) E.G. Simmons	A
Xylaria sp.	A

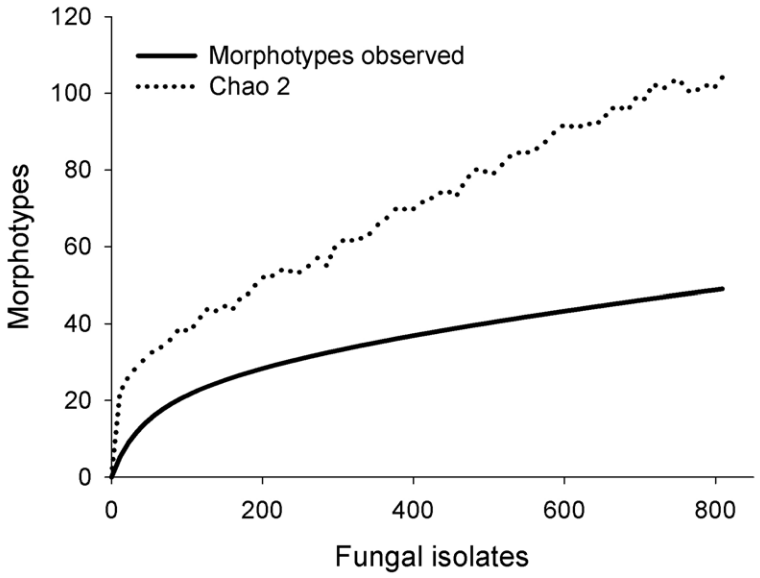

Figure 3.11: Sample-based *rarefaction* curve, rescaled to isolates, of the whole endophytic community isolated from *Fraxinus excelsior*, and the species richness estimator *Chao2*

the maximum parsimony analysis of these 44 morphotypes, 43 morphotypes grouped into two classes of Ascomycota: Dothideomycetes and Sordariomycetes (Fig. 3.12).

The 44th, a basidiomycetous yeast, had been previously defined as the outgroup. The taxonomical placement of the unidentified morphotypes, which is given in figure 3.12, refers to the taxonomical placement of the best hits in the BLAST search (see Appendix B). For MT0843 there was no identified best hit with at least 95% sequence identity. For this reason, the order of this morphotype is indicated with question marks.

Diversity of endophytes in dependence of forest layer and season

The number of infected leaves and leaf fragments increased considerably during the vegetation period (Fig. 3.13). The slight decrease in October was due to a decrease only in the light crowns. Up to five different morphotypes could be isolated from one single leaf fragment. Partially there were even more than five morphotypes, but in this case, the isolation of slowly growing fungi was impossible.

The rescaled *rarefaction* curves of the single months show that the number of morphotypes related to a determined number of isolates was higher in spring than in the autumn (Fig. 3.14 A), even though the differences were not significant. In contrast to this, the Shannon diversity indices increase over season (Fig. 3.14 B).

For the different forest layers, the species richness and the number of morphotypes related to a determined number of isolates was highest in the understorey, followed by the shade crown, and lowest in the light crown (Fig. 3.15 A), as was the number of isolates. Likewise, the Shannon diversity index was high for the understorey and the shade crown and considerably, but not significantly, lower in the light crown (Fig. 3.15 B).

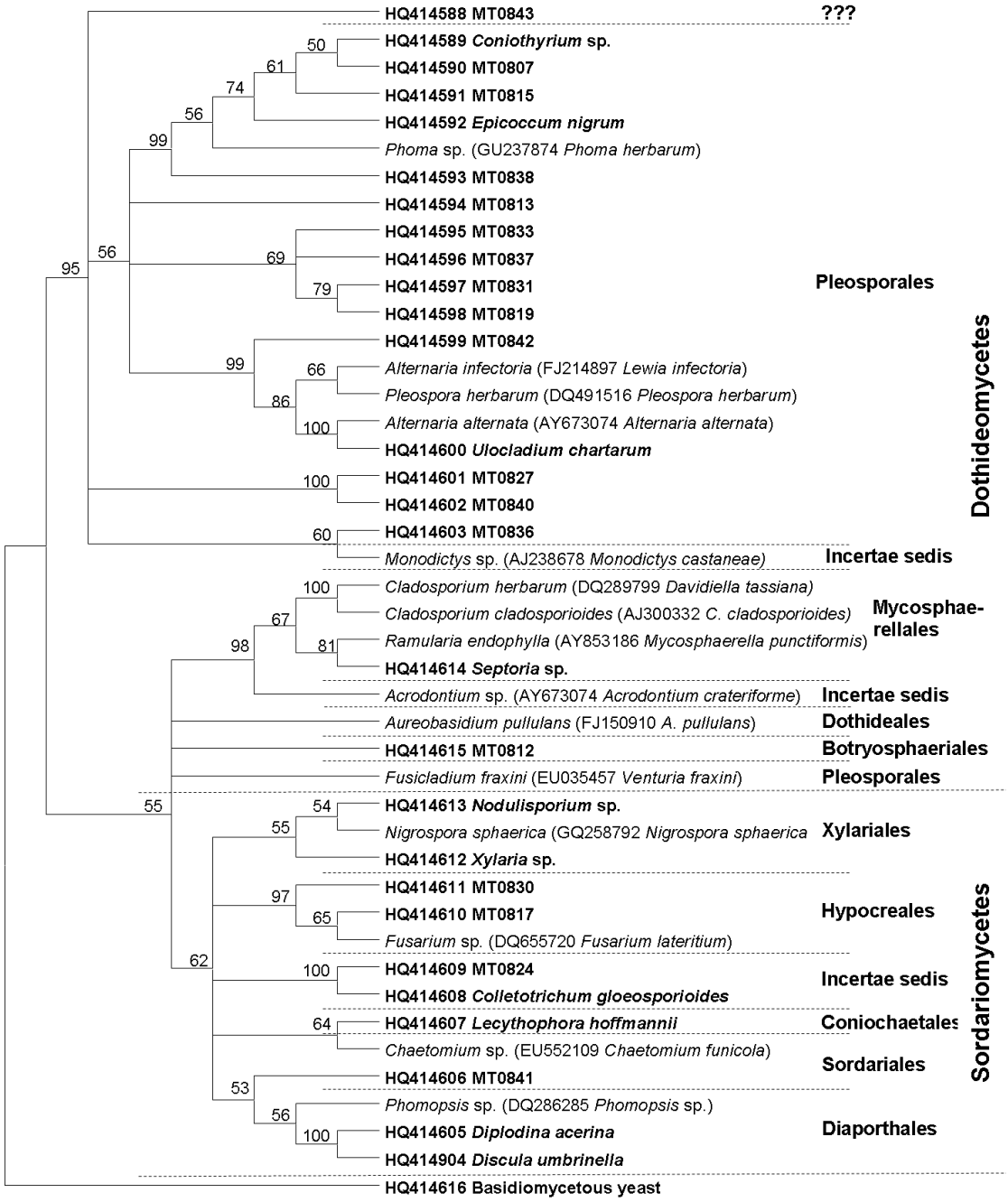

Figure 3.12: A 50% majority rule consensus tree of maximum parsimony analysis based on ITS1/5.8S/ITS2 nrDNA sequences of 44 morphotypes, rooted with a "basidiomycetous yeast" (tree length 1804, parsimony informative characters 267, consistency index (CI): 0.345, retention index (RI): 0.631, rescaled consistency index (RCI): 0.218). Sequences printed in bold were determined in the current study. The other sequences, which represent morphotypes that were not sequenced, were selected from GenBank. The accession number and the name under which the sequence can be found there are given in brackets. The given orders and classes were deduced from the best hits of the BLAST search (see appendix B), if the morphotype had not been determined. Bootstrap values of $\geq$50% from 1000 replicates are shown on the branches.

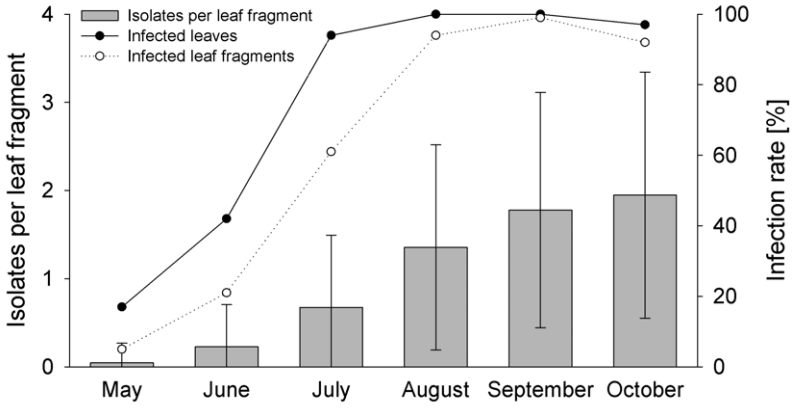

Figure 3.13: The endophyte infection rates of leaflets and leaf fragments of *Fraxinus excelsior*, and the number of isolates per leaf fragment during the vegetation period 2008

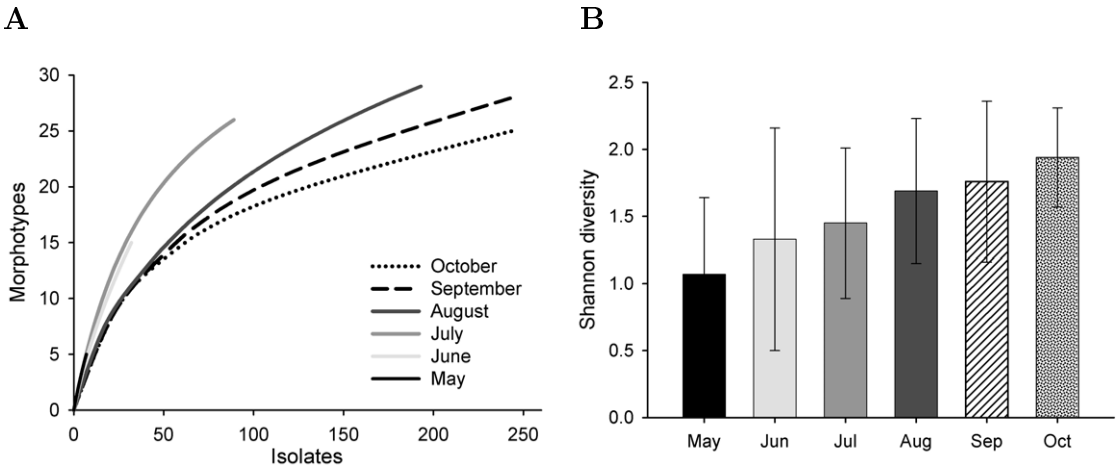

Figure 3.14: Sample-based *rarefaction* curves, rescaled to isolates, (A) and Shannon diversity indices per sample (B) of endophytes of *Fraxinus excelsior* for the single months (36 leaflets / 144 leaf fragments per month).

The DCA of the data from the months August to October confirms these differences between the forest layers (Fig. 3.16). The left-hand shift of the August shade crown samples in this figure can be explained by overlaying the sample matrix with single species (not depicted). This shows that the high number of *Xylaria* sp. isolates which appeared only in these samples (see Fig. 3.20 F) could be causal for the shift.

In all forest layers, the number of isolates increased from May to October, except for a slight decrease in the light crown and the understorey in October (Fig. 3.17 b, c, d). However, this decrease was not significant (Fig. 3.17 b & d). The highest numbers of endophytes were isolated in all months from the understorey, followed by the shade crown and fewest were isolated from the light crown (Fig. 3.17 a).

For the number of morphotypes, a similar picture as for the number of isolates became apparent (Fig. 3.18 a). However, in October, the number of morphotypes in the understorey was slightly lower than in the shade crown. In the shade crown the number of morphotypes decreased in August and then increased again until October, while the number of isolates increased continuously. Similarly, in the light

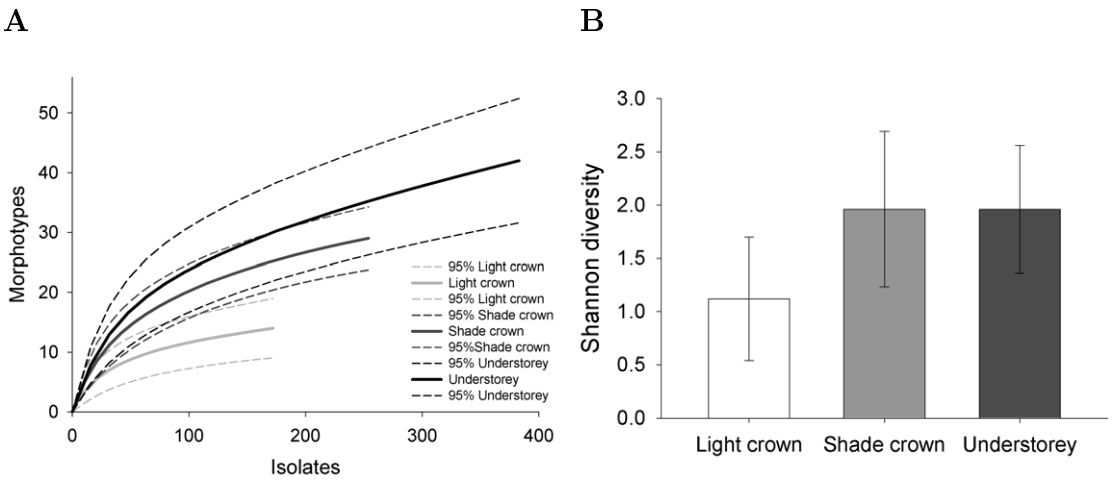

Figure 3.15: Sample-based *rarefaction* curves, rescaled to isolates, (A) and Shannon diversity indices per sample (B) of endophytes of *Fraxinus excelsior* for the light crown, the shade crown, and the understorey (69 leaflets / 276 leaf fragments for light crown, 72 leaflets / 288 leaf fragments for shade crown and understorey, respectively).

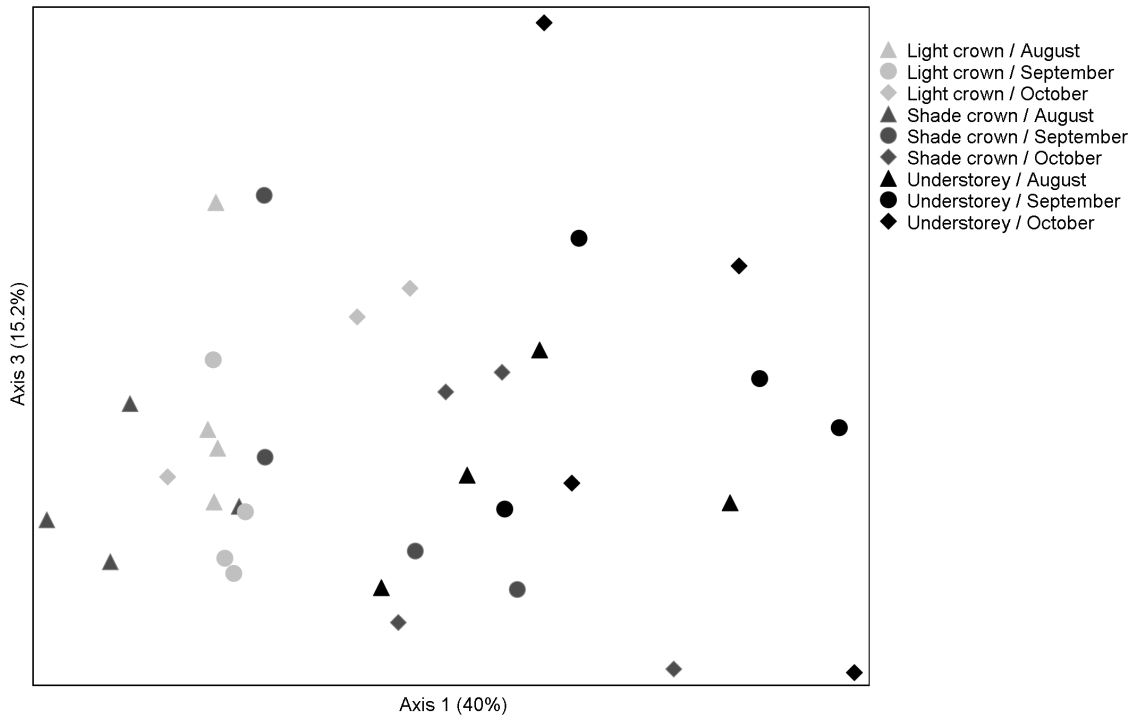

Figure 3.16: Shift of the endophyte community of *Fraxinus excelsior* from the light crown to the shade crown to the understorey, illustrated by a detrended correspondence analysis of samples from August, September, and October. The percentages at the axes provide information about their explanatory value.

crown the number of morphotypes decreased in September and increased again in October, while the number of isolates increased continuously until September and decreased in October (Fig. 3.18 b, c, d).

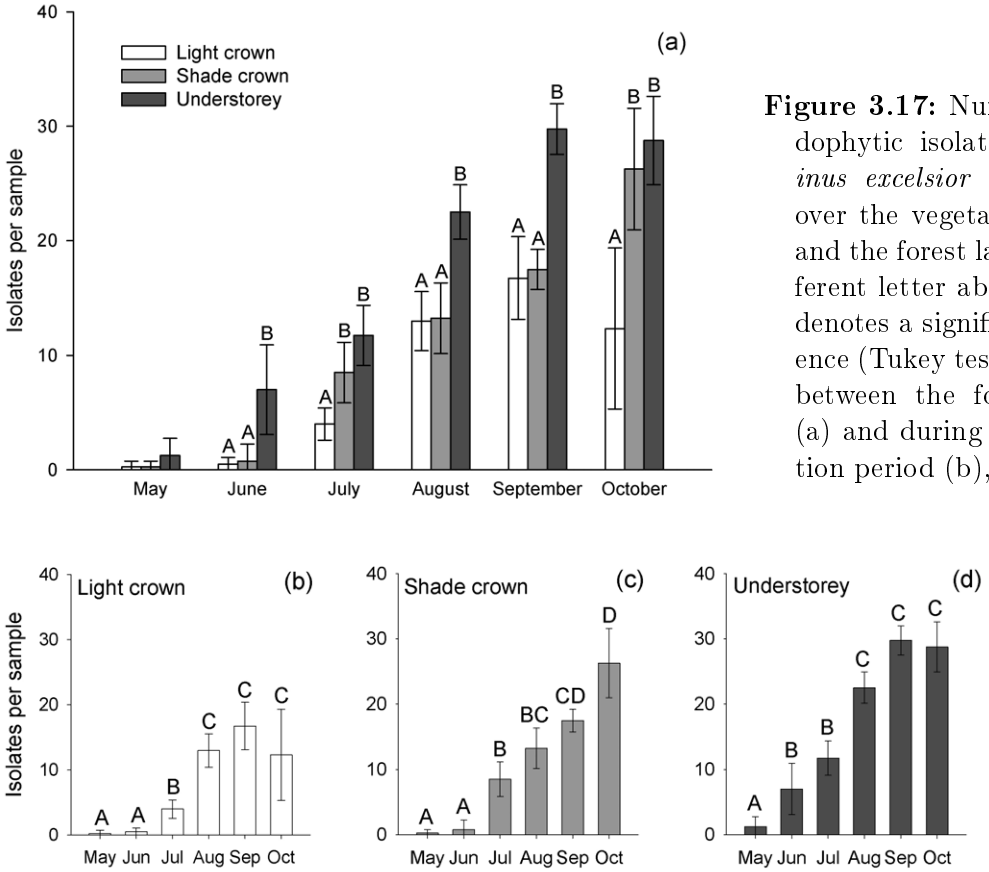

Figure 3.17: Number of endophytic isolates of *Fraxinus excelsior* per sample over the vegetation period and the forest layers. A different letter above the bar denotes a significant difference (Tukey test, $p < 0.05$) between the forest layers (a) and during the vegetation period (b),(c),(d).

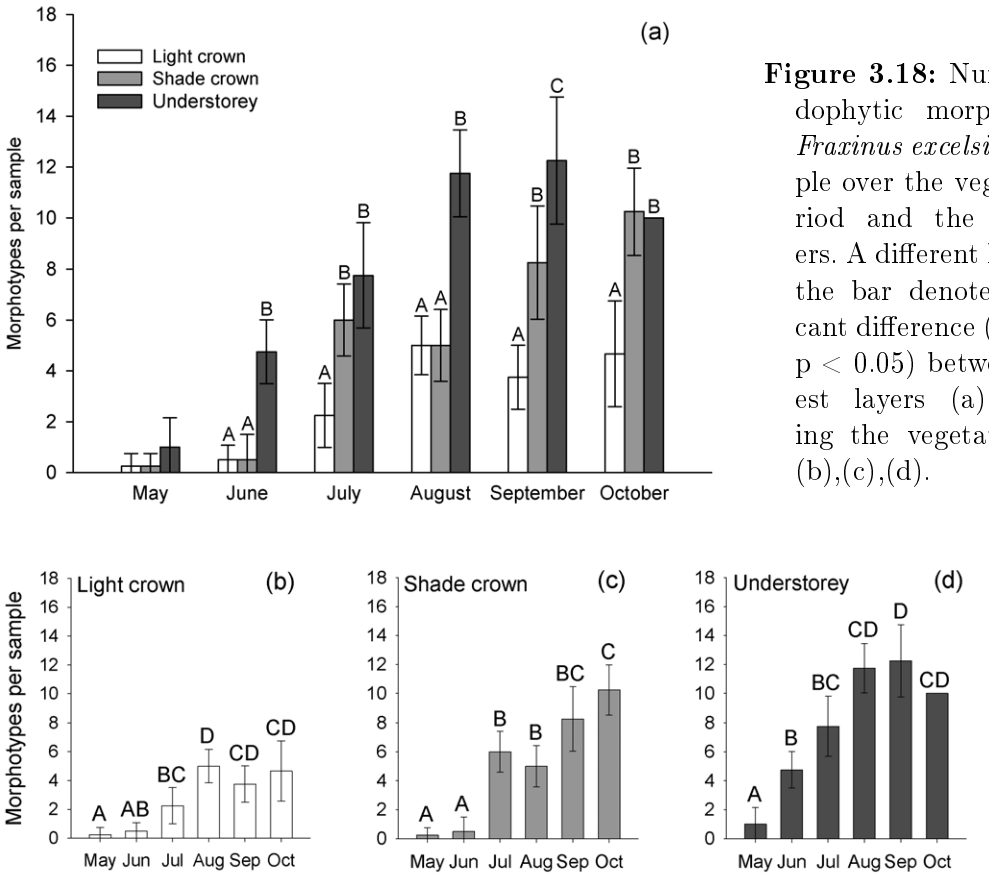

Figure 3.18: Number of endophytic morphotypes of *Fraxinus excelsior* per sample over the vegetation period and the forest layers. A different letter above the bar denotes a significant difference (Tukey test, $p < 0.05$) between the forest layers (a) and during the vegetation period (b),(c),(d).

The Shannon diversity indices exhibited a similar pattern as the number of morphotypes, except that the differences between the shade crown and the understorey in July and September were smaller (Fig. 3.19).

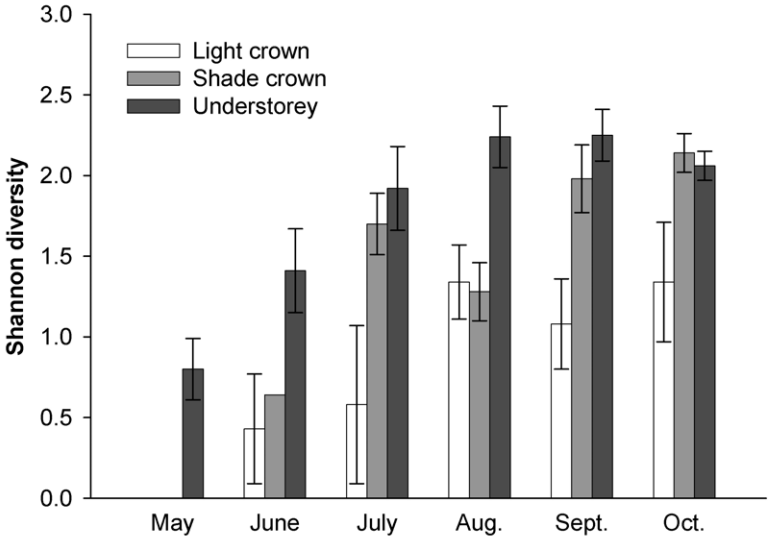

Figure 3.19: Average of Shannon diversity index of endophytes of *Fraxinus excelsior* per sample in the light crown, the shade crown and the understorey during the vegetation period

Infection patterns of selected endophyte species

A closer look at the individual morphotypes showed that most of them like *Ramularia endophylla*, *Septoria* sp., *Colletotrichum gloeosporioides*, *Phoma* sp., *Phomopsis* sp., and *Coniothyrium* sp. followed the aforementioned patterns with high infection rates in the autumn and in the understorey (i.e. Fig. 3.20 A & B, for complete data see Table C.2 in the appendix). Some of them like *Diplodina acerina*, *Discula umbrinella*, and *Fusarium lateritium* even occurred almost exclusively in the understorey. Other species showed the directly opposed pattern. Especially the two *Alternaria* species were isolated most frequently from the light crown (Fig. 3.20 C & D). Finally, some species showed an irregular pattern of infection. For example, *Fusicladium fraxini* showed a shift of frequency with respect to the season and forest layers (Fig. 3.20 E) and *Xylaria* sp. was very frequent only in August and there especially in the shade crown (Fig. 3.20 F). However, most species were found too rarely for assessment of the infection patterns.

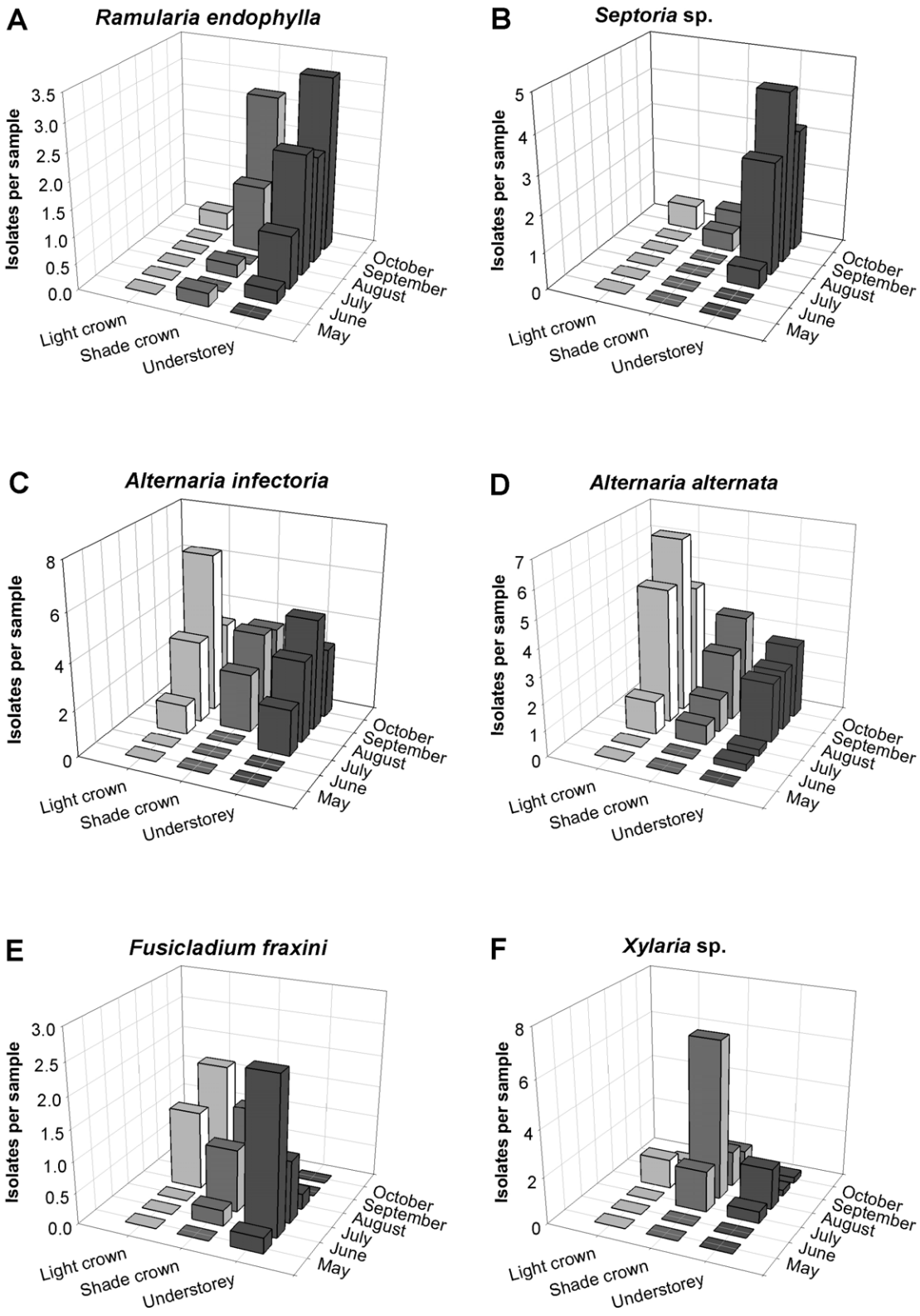

Figure 3.20: Occurrence of selected endophyte species (A *Ramularia endophylla*, B *Septoria sp.*, C *Alternaria infectoria*, D *Alternaria alternata*, E *Fusicladium fraxini*, F *Xylaria sp.*) of *Fraxinus excelsior* in different forest layers during the vegetation period. The means of all four samples of a certain forest layer and month are shown.

Environmental parameters

In average the temperature in the light crown was 0.48°C (SD 0.66°C) higher than in the shade crown, the relative humidity was 5.9% lower (SD 5.4%) (Fig. 3.21). The highest measured temperature difference was 4.24°C warmer in the light crown than in the shade crown, and the highest difference in humidity was 30.6% more in the shade crown than in the light crown.

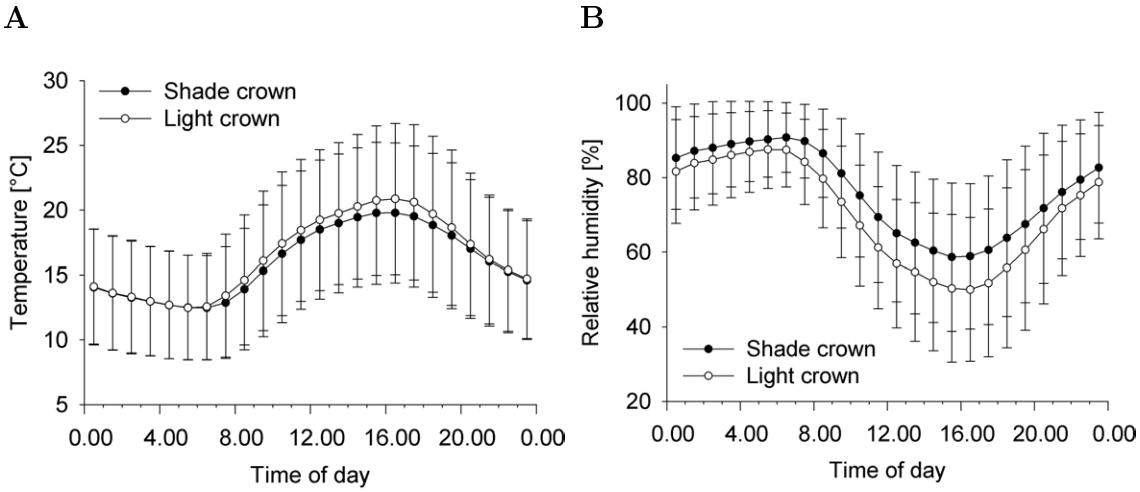

Figure 3.21: The course of a day of temperature (A) and humidity (B) in the light crown and the shade crown of *Fraxinus excelsior*. Plotted are the means of all days during the sampling period.

The precipitation was distributed almost equally over the vegetation period in 2008 (source: DWD, station 10469 Leipzig/Halle). Solely in May, it was very low (Fig. 3.22). In contrast to that, the increase of isolates was low in spring, rose up until August and than decreased again. No correlation could be found between the amount of precipitation in the month before a sampling and the increase of isolates compared to the previous sampling.

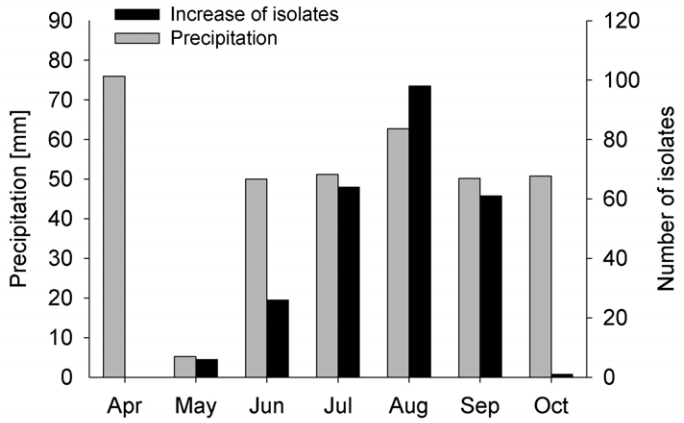

Figure 3.22: Precipitation during the vegetation period 2008, and the increase of the number of endophytic isolates of *Fraxinus excelsior* compared with the previous sampling

3.5.4 Discussion

This study revealed changes in the endophyte community of *Fraxinus excelsior* in three different forest layers during the vegetation period. For the first time, it was shown that the species richness and diversity of endophytes in leaves exposed to the light from the top of full-grown trees is considerably lower than in shade leaves and in the understorey. However, the two by far most frequent endophyte species were isolated particularly from the light crown.

Sampling design

To allow the extensive sampling at six sampling dates and three forest layers, the sampling in the current study has been restricted to one host species. Attention was paid to collect only obviously undamaged leaflets. However, in September and October it was partially not possible to find leaflets without sap-sucking whiteflies in the understorey. Thus leaflets with as few animals as possible were chosen.

To get a detailed picture of the colonisation of single sample points by a particular endophytic morphotype, morphotypes have been counted not only once per leaflet, but once per leaf fragment. Because of this, more differentiated statements about the progression of the endophyte colonisation could be made, compared with the former study (chapter 3.4).

Morphological and molecular identification

About 90% of all cultures developed conidia during the cultivation period. These cultures belonged to the 26 morphotypes (52%) that could be determined morphologically, at least to genus level. The remaining number of mycelia sterilia is comparable to other studies and depends, to a certain degree, on the efforts of researchers to promote sporulation with different media and cultivation techniques (Guo et al., 2000). Nevertheless, there will always be sterile mycelia remaining, which can only be classified with molecular methods.

As for the D1/D2 region, a molecular examination has not always been successful with the used ITS1/5,8S/ITS2 region. Especially for the Xylariales the intraspecific variability of the ITS region is too high for a reliable classification (Nilsson et al., 2008). The ITS region therefore did not perform better than the D1/D2 region. Even the availability of reference sequences was similar for both regions.

Species composition and diversity of the complete sampling

The taxonomical range (73% Dothideomycetes, 21% Sordariomycetes, 6% others) and the diversity (Fisher's $\alpha = 11.48$) were comparable to former studies at the same site (see chapter 3.4), and at other sites (Arnold & Lutzoni, 2007). The number of Dothideomycetes was, at the expense of the Sordariomycetes, slightly higher than in the former study. This is probably due to the different methods of assessing the frequency of the morphotypes. In the current study, every morphotype was counted once per leaf fragment. Species which colonise the leaf with few, but large, mycelia create the same signal as species that form many small-scaled mycelia, and both are assessed as being more frequent than species that form only scattered mycelia of a minor extent. In contrast to this, counting each morphotype only once per leaf, as done in the former study (see chapter 3.4), ignores the dominance of individual species within a single leaf, and all species detected within one leaf are weighted equally. Many of the very frequent species as *Alternaria alternata* and *A. infectoria* belong to the Dothideomycetes, which were for that reason more dominant than in the former study.

The species composition is typical for endophytic communities in the leaves of European broad-leaved trees, as many of them don't seem to be host-specific, and appear also in other host trees in Europe (compare, amongst others, with Halmschlager et al., 1993; Pehl & Butin, 1994; Gonthier et al., 2006). Some of them can also be found in branch bases (Kowalski & Kehr, 1992) or in necrotic or declining parts of ash (Przybyl, 2002; Lygis et al., 2005). The most frequent species *Alternaria alternata* and *A. infectoria* are known as cosmopolitan saprobionts. This is not surprising as endophytic fungi often colonise different plant parts as primary decomposers (Frankland, 1998). At least some species, particularly *Fusicladium fraxini*, might be host-specific or at least form host-specific strains. *Diplodina acerina*, which has been isolated only from *Quercus* in the study with four host species, could be isolated from *Fraxinus* in the current study, too. Thus, it is not host-specific at the study site, or at least forms only host-specific strains. This is according to Sogonov et al. (2008) who also found it to occur on *Acer pseudoplatanus* and various other hardwoods.

The new fungal pathogen causing the ash dieback, *Chalara fraxinea*, was not found endophytically in the examined ashes, although it is able to grow on MEA (Kowalski, 2006). Since no case of ash dieback has been reported from the surrounding of Leipzig, it can be assumed that it has not yet reached the area until now.

Species richness estimation

Compared to the former study (see chapter 3.4), more than twice as many morphotypes were isolated from *Fraxinus excelsior*. This is obvious because it has been shown that the sampling of *Fraxinus excelsior* was not exhaustive in the first study and the total species number could only be estimated very vaguely (see chapter 3.4.3). However, even in this second study the rescaled *rarefaction* curve of the total sampling does not reach an asymptote, which means that despite the more extensive sampling, the exploration was still far from being exhaustive.

To get an idea of the species number which can be expected with the used methods, the species richness was estimated. The species richness estimator *Chao2* was chosen because UNTERSEHER et al. (2008) recommended it for mycological data, and because it worked well in the former study (see chapter 3.4).

It converges to an asymptote at 104 morphotypes for the one host species *Fraxinus excelsior*. Hence, it is way above the richness estimation in the former study (see chapter 3.4), which computed about 60 morphotypes for four different host tree species. This shows that the quality of the richness estimation strongly depends on the amount of the underlying data (MAGURRAN, 2004). The estimation did not reach the asymptote as early as in the study with different host trees. This might be due to rare species being able to be detected more often, due to the greater sample size, or to there being more differences caused by changes during the vegetation period. Even in the estimation of 104 morphotypes fungi which are strictly biotrophic, slowly growing, or restricted to only a few cells are underrepresented or not included.

Species richness and diversity in dependence of forest layer and season

The first sample in May was taken about 4 weeks after bud burst in the light and the shade crown, and about 6 weeks after bud burst in the understorey. Since only 5% of the leaf fragments carried endophytes at this time, the leaves were probably endophyte-free, directly after the bud burst in the spring, as also reported by TOTI et al. (1993) for *Fagus sylvatica*. Apparently, endophytic fungi neither penetrate the bud scales, nor do they grow from infections in the twigs into the very young leaves of the bud. However, infections of older leaves by primarily twig-inhabiting fungi can not be excluded, since SIEBER & HUGENTOBLER (1987) and HALMSCHLAGER et al. (1993), for example, have found numerous identical endophyte species in both twigs and leaves of *Fagus sylvatica* or *Quercus robur*.

During the vegetation period, fungal propagules can accumulate on the leaf surface. It has been assumed that the effectiveness of the subsequent leaf infection depends on the amount of precipitation (e.g. FRIAS et al., 1991; WILSON & CARROLL,

1994; Wilson et al., 1997; Devarajan & Suryanarayanan, 2006). Therefore no evidence could be found in this study. However, the precipitation was rather evenly distributed over the vegetation period, and the decrease of new infections at the end of the vegetation period might be due to the already high infection rates. For that reason, this correlation will be analysed in detail in chapter 4.2. Furthermore, feeding and sucking herbivores, especially white flies, might have acted as vectors for spores and hyphae (e.g. Petrini, 1991; Feldman et al., 2008), and possibly facilitated the penetration of hyphae into the leaves (Faeth & Hammon, 1997). A detailed discussion of this fact will follow in chapter 4.3.

The number of infected leaves and leaf fragments increased up until September and than decreased again slightly in October. This decrease was due to a decrease in the light crown samples. Since the sampling was restricted there, because the leaves had already partially dropped off, the following two explanations for this lower infection density are conceivable: (1) The leaflets which have a low infection density with endophytic fungi remain longer on the tree than leaflets with a high infection rate or (2) The infection density in the light crown decreases as a whole, shortly before leaf fall. The first scenario is more probable. Amongst others, Wilson (1993) supposes that endophytes could play a role in leaf senescence, and lead to a premature abscission of leaves. This would as well explain the high variability in the infection rate in the light crown in October compared to the previous months. At sample points where only the leaflets with the fewest endophytes have been left, the infection rate was very low, whereas at sample points where most of the leaflets still have been there, the infection rate was still higher. With data at hand, a retreat of fungi from senescing leaves cannot be excluded, although it seems unlikely given the saprotrophic potential of many endophytes. This warrants further study particularly focusing on freshly fallen leaves.

While the infection density, the number of morphotypes, and the overall diversity increased during the vegetation period, the species richness (number of morphotypes per isolates) decreased. One explanation is that especially rare species could be isolated less often in the autumn, because either they could not compete successfully in the leaves and have been displaced by highly competitive species, or they just could not compete on the isolation medium and thus have been overgrown and could not be detected (see chapter 2.2.1).

Comparison of the rescaled *rarefaction* curves for different forest layers shows that the species richness in the light crown was significantly lower than in the shade crown and the understorey. This is confirmed by the fact that the confidence intervals of their *rarefaction* curves do not overlap (Colwell et al., 2004). This is in agreement

with the earlier study comparing different host tree species (see chapter 3.4), and with studies of other authors (e.g. HALMSCHLAGER et al., 1993). Whilst single species can be more frequent in the light crown, the species richness tends to be higher in the shade crown and the understorey. As already explained in chapter 3.4, This might be traced back to different causes, like different leaf structures, water availability on the leaf surfaces, and microclimate. The measurements of the microclimate showed higher temperatures and lower humidity in the light crown than in the shade crown especially during the day. Since the datalogger in the light crown could not be fixed in the outermost crown regions, and the rain shield partly shielded the solar radiation, the actual differences are expected to be higher than the measured ones.

No difference in species richness emerged between the understorey and the shade crown, whereas ESPINOSA-GARCIA & LANGENHEIM (1990) found a higher species richness in the needles of saplings than in the needles of shade crown branches of full-grown trees of coastal redwood. However, in the current study, the number of isolates has been significantly higher in the understorey than in the shade crown at almost any time during the vegetation period, as HELANDER et al. (2006) found it for saplings of *Betula pendula* compared to full-grown trees of managed, as well as natural, forest stands of the same host. This is probably due to the close proximity to the leaf litter, since the composite of the aerial inoculum strongly depends on the amount and quality of spore sources in the close neighborhood, and many endophytes live as primary saprotrophs and sporulate on dead leaves (e.g. FRANKLAND, 1998; TOKUMASU, 1994; OSONO, 2006). Some of them which were frequently isolated in the understorey, such as *Ramularia endophylla*, might form many slowly growing infections, so that the infection density would be higher whilst the species number would be on the same level as in the shade crown.

A closer look to the colonisation patterns of single months shows that the endophyte density and species richness in the first months of the vegetation period is highest in the understorey. This can most likely be explained by the earlier bud burst in the understorey in spring. The leaves were about two weeks older than those in the crowns, and therefore could be invaded by endophytes earlier. Another important point is the structure of the leaves, since the ability of fungi to infect leaves depends on the structure of the leaf surface (ALLEN et al., 1991). It is conceivable that endophytes can more easily penetrate the thin tissues of understorey leaves.

Likewise, feeding and sap-sucking insects, which possibly act as vectors for endophytic fungi (see chapter 4.3) might prefer these tender understorey leaves. Whiteflies could be commonly observed, especially in the later vegetation period, in the

understorey more often than in the other forest layers. It is possible that they transfer fungal propagules and open infection gates. But it could also be the other way round: Whiteflies might prefer those leaves that are already more strongly infected by endophytes, as GANGE (1996) has reasoned for the correlation between the pathogenic fungus *Rhytisma acerinum* and the appearance of aphids.

Infection patterns of selected endophyte species

The DCA depicted a shift in the species composition from the light crown to the shade crown to the understorey, additionally to differences in the number of isolates and morphotypes. This is due to differences in the distribution of single endophyte species. While most species followed the spatial and temporal patterns discussed above, there were some notable exceptions: The two most frequently isolated species, *Alternaria infectoria* and *A. alternata*, were found most often in the light crown, and seem to be well adapted to the harsh conditions there. Particularly, the comparatively fast drying of the leaf surfaces can hamper the survival of spores and the successful penetration of leaves by the most endophyte species. The considerable decrease in infection rate in the light crown in October suggests that leaves intensely colonised by the *Alternaria* species drop off earlier than others (see above). It may be assumed that the high infection rates of *Alternaria* lead to a premature leaf abscission as WILSON & FAETH (2001) found it for *Ophiognomonia cryptica*. The evidence of this hypothesis has to be provided in further studies, since there could be other parameters that lead to a premature leaf senescence and *Alternaria* and other early decomposers could benefit from this decay. Since Alternaria is known to sporulate on dead leaves, the high infection rates in the light crown are in contrast to the hypothesis of a limitation of infection by the proximity of spore sources.

Several other species show distribution patterns which cannot be explained easily. For instance *Xylaria* seems to have better growing conditions in the shade crown than in the light crown and the understorey. The highest infection rates for all forest layers can be observed in August. The abundant *Xylaria* species of Central Europe, like *X. hypoxylon*, *X. polymorpha*, and *X. longipes*, of which especially the latter is frequent at the study site, start to produce large quantities of conidia in the summer months. This would explain the high infection rates in August. Obviously especially in the shade crown conditions such as microclimate and leaf quality are good for an infection. Since these *Xylaria* species grow and sporulate on lying deadwood and stumps they are presumably not very competitive in leaves. If this is the case, a displacement by more competitive species like *Alternaria* spp. would explain the decline in September and October.

The remarkable distribution pattern of *F. fraxini* can be explained by its growing rate during the isolation. The colonies grow very slowly on MEA and therefore *Fusicladium* could only be isolated, if there were no fast-growing fungi nearby which competed with it and overgrew it. This has been the case for example in the spring and the early summer when the frequency of many other fungi is still low, or in the light crown in October when leaves with a strong *Alternaria* colonisation had already dropped off and the remaining leaves therefore had lower amounts of endophytic fungi.

4 Experimental approaches to find factors that influence leaf infection by endophytic fungi

Patterns in the endophytic colonisation of leaves in different forest layers and host tree species were revealed in chapter 3. It was hypothesised that they were mainly caused by differences in the microclimate, the composition of the aerial inoculum, the leaf structure, and the herbivore activity. Changes in these factors therefore might alter the endophytic community. Since the endophytic community in turn diversely influences the host tree, it is necessary to know which of these factors are most important. Based on this knowledge, it might be possible to assess how altering endophytic communities, caused for example by climatic change or changes in the forest management, can affect forest ecosystems. Several factors which might influence the leaf infection by endophytic fungi were examined in the present thesis, based on experimental approaches. These will be discussed in the following chapters.

4.1 Introduction to infection modes

The colonisation of leaves by endophytic fungi can be divided into two general stages: First, fungal propagules need to be transferred to the leaf; second, they have to intrude it. Both stages might be influenced diversely. Propagules can be deposited on the leaf surface in various ways, for example by wind, rain, or herbivores (e.g. Petrini, 1991; Devarajan & Suryanarayanan, 2006; Arnold, 2008). Once deposited on the leaf, they need adequate preconditions for a successful intrusion. As well as an appropriate host, abiotic factors like the availability of humidity on the leaf surface, the temperature or the amount of UV-irradiation might affect the survival of the propagule and the success of the intrusion into the leaf (e.g. Wilson, 1996; Bahnweg et al., 2005). This intrusion can proceed directly through the intact epidermis, by means of appressoria, or through stromata of an intact leaf. It might be facilitated by lesions of the plant tissues, caused by a mechanical damage or herbivore

feeding. A direct inward transfer by feeding, sap-sucking, or boring herbivores is also conceivable (Vega et al., 2008). Furthermore, small spores that have entered the plant through damaged vessels, or mycelial fragments that detached from an established infection might be transported to the leaf with the transpiration stream.

4.1.1 The role of precipitation

The availability of water plays an important role for the infection of host plants with endophytic fungi. Petrini (1991) supposed that there might be a correlation between moisture and the infection of tree leaves by endophytes. Arnold & Herre (2003) observed that the duration of moisture on the leaf surface differed between seedlings in a clearing and in a forest. Seedlings in the clearing dried rather rapidly after rainfall, whereas the forest-grown seedlings maintained water on the leaf surface for several hours. Arnold & Herre (2003) supposed that this might have an influence on spore germination and survival on the leaf surfaces, as it has already been proved for pathogenic fungi (e.g. Frias et al., 1991; Huber & Gillespie, 1992). Wilson & Carroll (1994) and Wilson et al. (1997) showed a correlation between the cumulative precipitation and the infection of *Quercus garryana* with *Discula quercina* and *Ophiognomonia cryptica*. Likewise Devarajan & Suryanarayanan (2006) assumed that there is a correlation between the precipitation and the infection rate of endophytic species. However, these studies did not investigate the number of new infections in correlation with the current amount of precipitation but only the total infection rate which is influenced by growth and also by decrease of earlier infections. Furthermore, they did not distinguish between infections that are due to precipitation and others which might be due to other causes, for example, growing out of vascular tissue or being channeled in by herbivores.

A better hint on this topic was given by Wilson (1996) who demonstrated that plastic bags which exclude rain from branches have a negative effect on infection of *Q. garryana* leaves for several endophytic fungi. The bags, however, might at least partially interfere the spore deposition and are sure to change the microclimate, so that the comparability to leaves without bags must be doubted.

This thesis therefore studied the role of precipitation for the endophyte colonisation of leaves in an experimental approach, taking into consideration that the microclimatic conditions were as similar as possible and the leaves were shielded from precipitation, but not from spore fall (see chapter 4.2).

4.1.2 Dispersal of endophytic fungi by herbivores

The influence of systemic grass endophytes on herbivores has been examined extensively: In these systems, endophytes often enhance the resistance of the host to herbivore feeding (Clay, 1988), but also to other pests like nematodes (Kimmons et al., 1990), and pathogenic fungi (Clay et al., 1989) by producing secondary metabolites (e.g. Findlay et al., 2003; Sumarah et al., 2010). For the nonsystemic endophytes in woody plants the interactions are by far more diverse and difficult to assess. The whole range of effects, from positive (Gange, 1996), via neutral (Faeth & Hammon, 1997), through to negative (Vega et al., 2008; Albrectsen et al., 2010), has been reported.

But how do herbivores affect the colonisation of leaves by endophytic fungi? Does herbivore feeding open infection opportunities to the endophytes, and which role do herbivores play in the dispersal of fungal propagules?

There are indications that endophytes can be dispersed by herbivores. For instance, entomopathogenic endophytes such as *Beauveria bassiana* (Vega et al., 2008) could be transferred, when the infected herbivore migrates to new feeding places. Accordingly, Monk & Samuels (1990) found that spores and hyphae of several saprobic and plant-pathogenic fungi can be isolated from faecal pellets of grasshoppers living in Indo-Malayan rainforests. Subsequently, Devarajan & Suryanarayanan (2006) have shown that even endophytic fungi can indeed pass the gut of grasshoppers without being destroyed, and can be dispersed in this way. It is almost certain that other herbivorous species also have the potential to act as dispersal vectors for endophytes, as Feldman et al. (2008) have demonstrated it for moths. In this cases, herbivory might increase the colonisation frequency of horizontally transmitted endophytes.

Besides, endophytes which are once deposited on the leaf surface might easily infect the leaf through openings that are caused by herbivores. Up until the present, answers to this question are contradictory. For many phytopathogenic fungi it is known that the infection is enhanced by previous damage of the leaf, caused by insect feeding or oviposition in the plant tissue. The insects are vectors of the fungi and they are able to place fungi in the wound site (e.g. Hatcher, 1995). Furthermore, Simon & Hilker (2003) found herbivory to induce the systemic susceptibility of *Salix cuspidata* towards rust fungi, not only on the leaves which had been damaged by herbivores. In contrast to this, Hatcher & Paul (2000) demonstrated that beetle grazing can significantly reduce the infection of *Rumex obtusifolius* by several plant pathogenic fungi. Little is known about to what extent this result can be transfered to endophytic fungi. Faeth & Wilson (1996) concluded that plant damages, caused

by herbivores, increase the colonisation of horizontally transmitted endophytes of woody plants. Accordingly, ARNOLD (2008) found that parts of leaves of *Gustavia superba* which were damaged by hesperiid larvae had significantly higher rates of endophyte infection, higher endophyte species richness, and a different community of endophytes, relative to undamaged areas, of the same leaves. As a consequence, horizontally transmitted endophytes might tolerate or even promote herbivory, in contrast to vertically transmitted endophytes of grasses for which a negative effect on herbivores has often been reported (CLAY, 1988). However, GANGE (1996) detected a positive correlation between sycamore aphids and the infection with *Rhytisma acerinum* but found no evidence that the aphid stylets act as pathways of entry. SAIKKONEN et al. (1996) found no correlation between the frequency of endophytes of two tree species and the density of five species of herbivorous insects.

To approach the question of whether herbivore feeding facilitates the infection of leaves by endophytic fungi and its importance in the development of colonisation patterns, infection rates of intact leaves and leaves severely damaged by herbivores have been compared in this thesis (see chapter 4.3).

4.1.3 Additional infection modes

There might be additional ways for endophytic fungi to enter the leaves. At least some fungal genera and species that occur in branch bases (KOWALSKI & KEHR, 1992) can be found endophytic in leaves, too. They might be able to infect leaves as well as branches, or grow from the branches into the leaves, and vice versa.

They might furthermore be introduced into plants through lesions in stems, and twigs (SIEBER et al., 1995), or even roots. Small hyphal parts or spores might then be transported with the transpiration stream from root lesions to the leaves. To verify this hypothesis, an experiment that exposes fresh root lesions to a suspension with fungal spores has been carried out (see chapter 4.4).

4.2 Experiment to find the role of precipitation for endophyte infection

The availability of water on the leaf surface seems to play an important role in the successful infection of leaves by endophytic fungi (see chapter 4.1.1). Since precipitation, i.e. rainfall, is the most important source for water on the leaves, the effect of rain on the endophyte infection of ash saplings was tested with a rain exclusion experiment.

4.2.1 Methods

Experimental design and sampling

Thirty saplings of *Fraxinus excelsior* growing in the LAK site were dug out carefully in October 2008. The saplings were between 0.5 and 1 m tall and about 5 years old. They were potted into a mixture of 2/3 original forest soil and 1/3 sterile garden mould and left in the open-air area of the Botanical Garden Leipzig over winter. At the end of March 2009, about two weeks prior to the beginning of foliation, 25 of the saplings were placed in a greenhouse construction with two open sides. This allowed circulation of air and entry of fungal propagules but protected the plants effectively from rain and splash water. The glass roof was covered on sunny days allowing diffuse light transmission. The remaining five plants were left outdoors as control group at a half-shaded place. From this control group, leaf samples were taken every four weeks (see Table 4.1).

From the middle of May, every four weeks, five randomly chosen saplings from the greenhouse were placed outdoors. On the date they were put outside, as well as after two, four, and eight weeks, leaf samples were taken. The last group remained a control group in the greenhouse, until the end of the experiment.

One leaflet of the middle of three different leaves was collected at each sampling date from every tree sampled at this date. It was taken care that leaflets of the first foliation period were collected, so that all leaflets were of the same age.

The leaf samples were processed as described in chapter 2.1.1 and 2.1.2. Additionally, randomly collected leaflets were pressed onto culture medium dishes (MEA+T) without previous surface sterilisation, to examine if fungal propagules had accumulated on the leaf surfaces in the greenhouse and outdoors. It appeared to be sufficient to estimate the amount of mycelia and not to invest time counting them.

The plants in the greenhouse were watered extensively twice a week without moistening the leaves. The plants outdoors were watered as necessary. The precipitation

Table 4.1: Overview over the treatment of saplings of *Fraxinus excelsior* and leaf sampling design

tree number (group)	**date**	**action**	**number of leaflets**
01-05 (group 1)	19.05.	placed outdoors	
	19.05.	1st sampling	15
	02.06.	2nd sampling	15
	16.06.	3rd sampling	15
	14.07.	4th sampling	15
06-10 (group 2)	16.06.	placed outdoors	
	16.06.	1st sampling	15
	30.06.	2nd sampling	15
	14.07.	3rd sampling	15
	11.08.	4th sampling	15
11-15 (group 3)	14.07.	placed outdoors	
	14.07.	1st sampling	15
	28.07.	2nd sampling	15
	11.08.	3rd sampling	15
	14.09.	4th sampling	15
16-20 (group 4)	11.08.	placed outdoors	
	11.08.	1st sampling	15
	25.08.	2nd sampling	15
	14.09.	3rd sampling	15
21-25 (group 5)	14.09.	1st sampling	15
26-30 (control outdoors)	19.05.	1st sampling	15
	16.06.	2nd sampling	15
	14.07.	3rd sampling	15
	11.08.	4th sampling	15
	14.09.	5th sampling	15
		total	315

was measured daily at the outdoor location during the whole experimental period from the middle of April until the middle of September, by means of a rain gauge, according to Hellmann.

Temperature and humidity were measured outdoors as well as in the greenhouse by Hobo® Pro v2 dataloggers every hour, to monitor the conditions in the greenhouse and compare them to those outdoors.

The density of the aerial inoculum was randomly determined on two sunny, calm days, between which there was an interval of two months. Two culture dishes (90 mm diameter, 2% MEA+T) for each were placed amongst the greenhouse and the outdoor plants for 30 minutes, between 11 and 12 a.m. The dishes were sealed

subsequently after exposure and incubated at room temperature for three days, and the number of growing fungal colonies was counted subsequently.

Data analysis

To deal with the problem of defining endophytic individuals (see chapter 2.1.4), each morphotype was counted once per leaf fragment, regardless of the number of colonies it produced. According to the 12 leaf fragments from three leaves that were examined, a morphotype could reach an amount of 0 to 12 per tree.

The effects of the treatments (0, 4, 8 weeks outdoors, control outdoors) on the number of isolates were analysed with a One Way ANOVA using SigmaPlot version 11.0 (Systat Software, Inc.). Before the analysis, square root transformation was applied to the data to remove the mean/variance relationship of the data, which had the character of Poisson data (count data). Pairwise comparisons were calculated using the Tukey test. The data from the aerial inoculum test failed the equal variance test, and were therefore compared with Mann-Whitney Rank Sum Tests.

4.2.2 Results

Environmental conditions

On average, the temperature in the greenhouse was 1°C (SD 0.97°C) higher than outdoors, the relative humidity was 3.3% lower (SD 4.1%; Fig. 4.1).

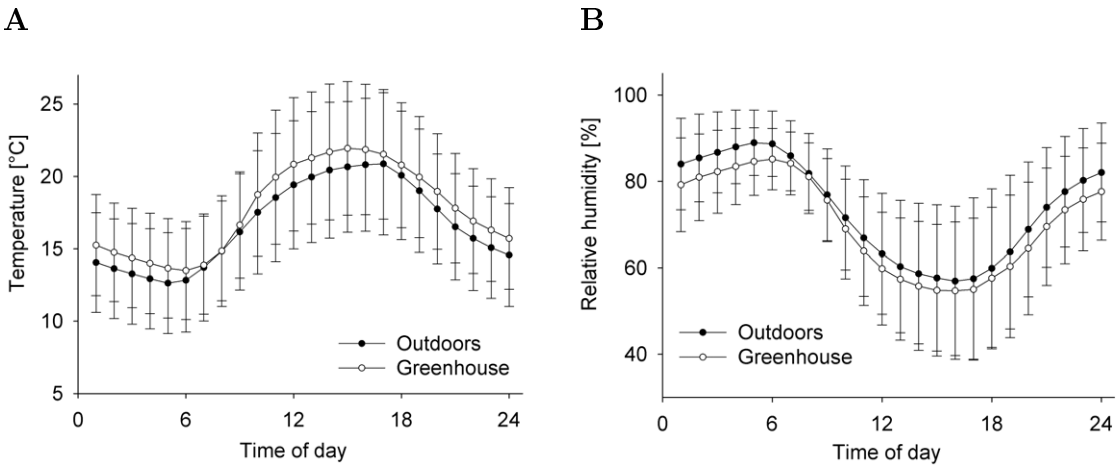

Figure 4.1: Temperature (A) and humidity (B) in the course of a day, in the open greenhouse and outdoors. Plotted are means of all days during the sampling period.

The precipitation was distributed relatively equally over the sampling period (Fig. 4.2). Drought periods did not last longer than a few days. Summed up to months

the amount of precipitation differed only marginally from the long-term mean (1961-1990).

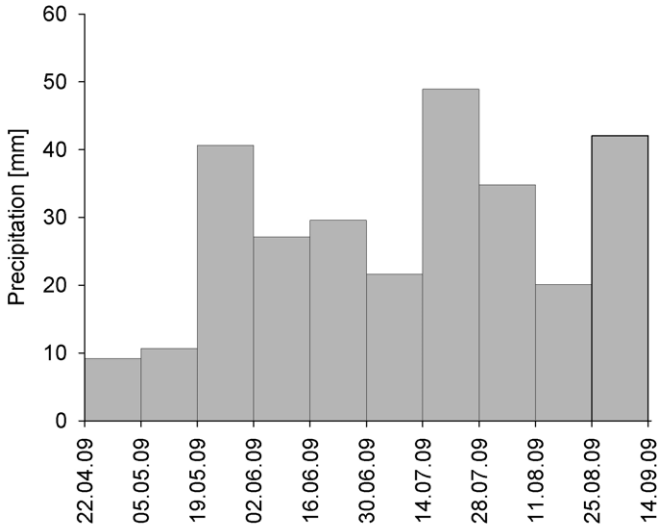

Figure 4.2: Precipitation during sampling period from middle of April until middle of September 2009

On the two sunny and calm days, no significant difference of the aerial inoculum density was observed between the location outdoors (1.9 $\pm$0.24 deposited propagules*$cm^{-2}h^{-1}$) and in the open greenhouse (1.62 $\pm$1 deposited propagules*$cm^{-2}h^{-1}$, Fig. 4.3).

Figure 4.3: Number of fungal propagules of the aerial inoculum that were deposited by the natural sporefall in the open greenhouse and outdoors

The incubated leaf imprints showed a large amount of viable propagules both from the greenhouse and from outdoors. The number of propagules was in both cases considerably higher on the upper side of the leaflets than on the lower side (Fig. 4.4).

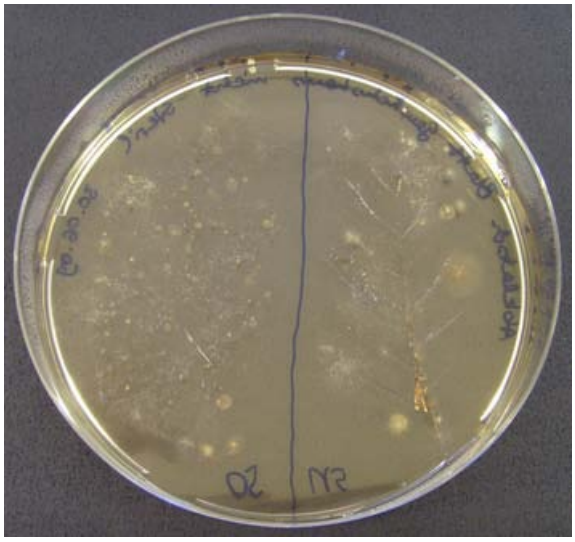

Figure 4.4: Growth of fungal propagules from the imprint of a leaflet from the open greenhouse on malt extract agar (left: upper side, right: lower side)

Colonisation of leaves by endophytic fungi

A total of 546 fungal cultures were isolated. The mean infection rate of the outdoor control plants increased continuously during the study, from 2.6 (±1.82) isolates per tree to 17.2 (±3.77) isolates. The leaflets of plants in the greenhouse remained almost free of endophytes until August (0.2 [±0.45] to 0.4 [±0.55] isolates per tree, groups 1 to 4 first sampling). The infection rate increased considerably as late as September (4.4 [±2.3] isolates, group 5). After placing these plants outside, the infection rate increased continuously during the eight weeks of observation (Fig. 4.5).

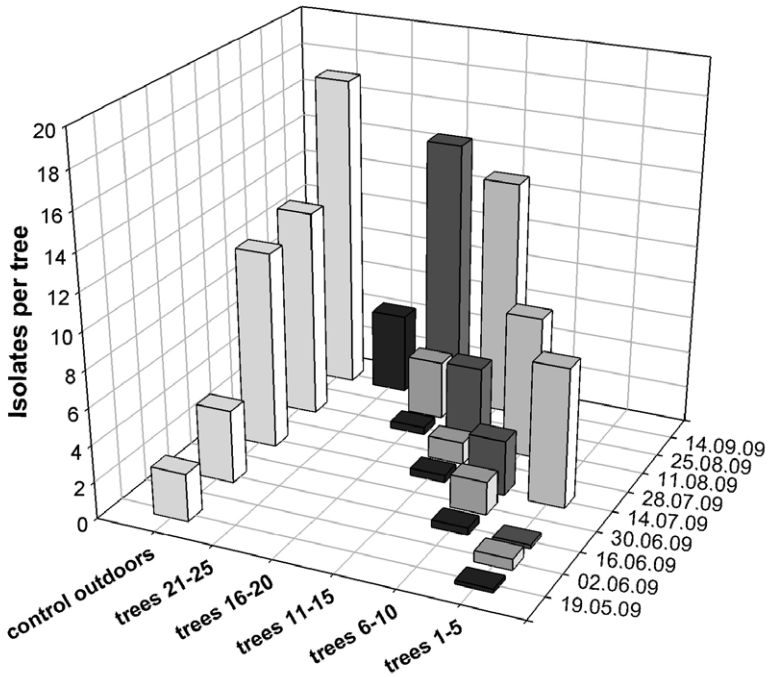

Figure 4.5: Number of isolates of endophytic fungi per tree over time for different treatments of rain exclusion (error bars not shown)

Significant differences in the number of fungal isolates per tree were found between the control group outdoors and the trees in the greenhouse during the whole sampling period (Fig. 4.6). The infection rates of trees that had been outdoors for four weeks were still significantly lower compared with the outdoor control from June till August, whereas in September there was no longer a significant difference. Trees that had been outdoors for eight weeks showed no significant difference in the infection rate compared with the control outdoors.

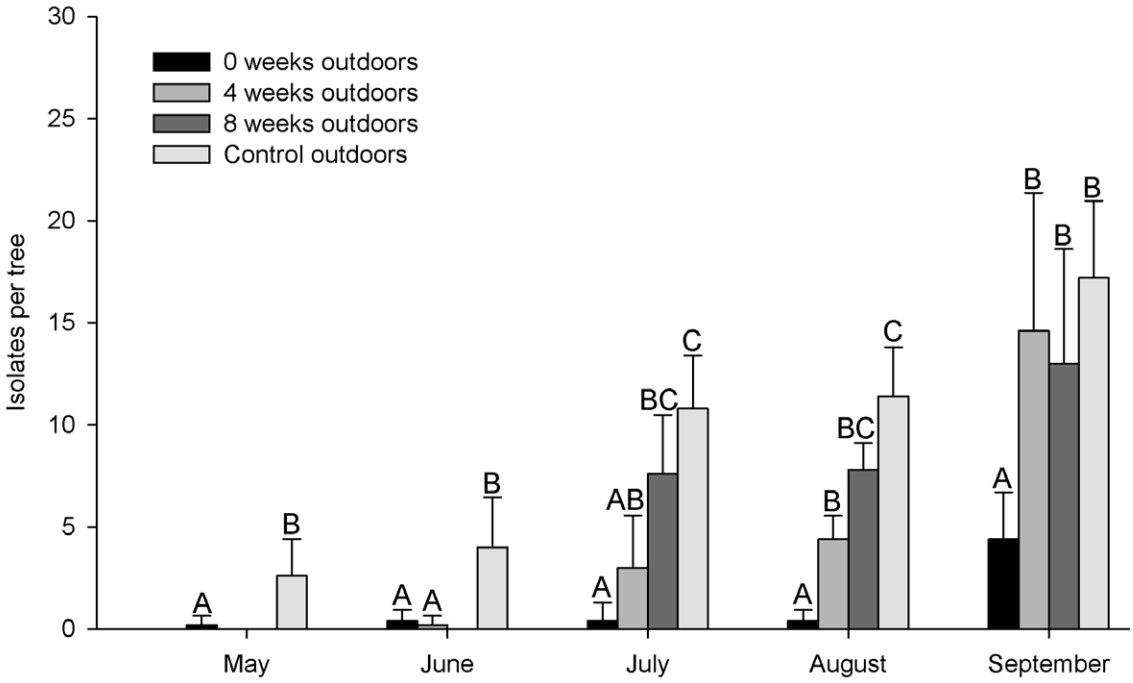

Figure 4.6: Isolates of endophytic fungi per tree from May until September 2009 for the different treatments (0, 4, and 8 weeks outdoors, control outdoors). A different letter above the bar denotes a significant difference between the treatments within a single month (Tukey test, $p < 0.05$).

The 546 isolates were grouped into 46 morphotypes. 20 morphotypes could be identified by morphological characters to genus or species level (Table 4.2). According to the morphotype concept, morphotypes determined to genus rank usually contain only one species but might also comprise more than one species.

Only 19 morphotypes were isolated from at least three leaf fragments. The other 27 (59%) appeared only once or twice (Fig. 4.7).

Trees stored for some time in the greenhouse and those of the control group outdoors differed in species composition (Fig. 4.8, Table C.3 in the appendix). Particularly, the percentage of *Fusicladium fraxini* and morphotype MT0901 was higher in trees that were outdoors all the time than in those standing in the greenhouse for some time. In contrast, *Septoria* sp. was more frequent in trees standing in the greenhouse for some time.

Table 4.2: Morphologically identified species or genera of endophytic fungi of *Fraxinus excelsior* in the rain exclusion experiment and their appearance in culture as anamorph (A) or teleomorph (T)

Fungal species	**appearance in culture**
Acrodontium sp.	A
Alternaria alternata (Fr.) Keissl.	A
Alternaria infectoria E.G. Simmons (T: *Lewia infectoria* [Fuckel] M.E. Barr & E.G. Simmons)	A
Aureobasidium pullulans (de Bary) G. Arnaud	A
Basidiomycetous yeasts	A
Chaetomium sp.	T
Cladosporium sp.	A
Colletotrichum gloeosporioides (Penz.) Sacc.	A
Epicoccum nigrum Link	A
Fusicladium fraxini Aderh. (T: *Venturia fraxini* Aderh.)	A
Lecythophora hoffmannii (J.F.H. Beyma) W. Gams & McGinnis	A
Nigrospora sphaerica (Sacc.) E.W. Mason	A
Nodulisporium sp.	A
Phialophora sp.	A
Phoma sp.	A
Phomopsis sp.	A
Pleospora herbarum (Pers.) Rabenh.	A
Ramularia endophylla Verkley & U. Braun (T: *Mycosphaerella punctiformis* [Pers.] Starbäck)	A
Septoria sp.	A
Sordaria fimicola (Roberge ex Desm.) Ces. & De Not.	T
Xylaria sp.	A

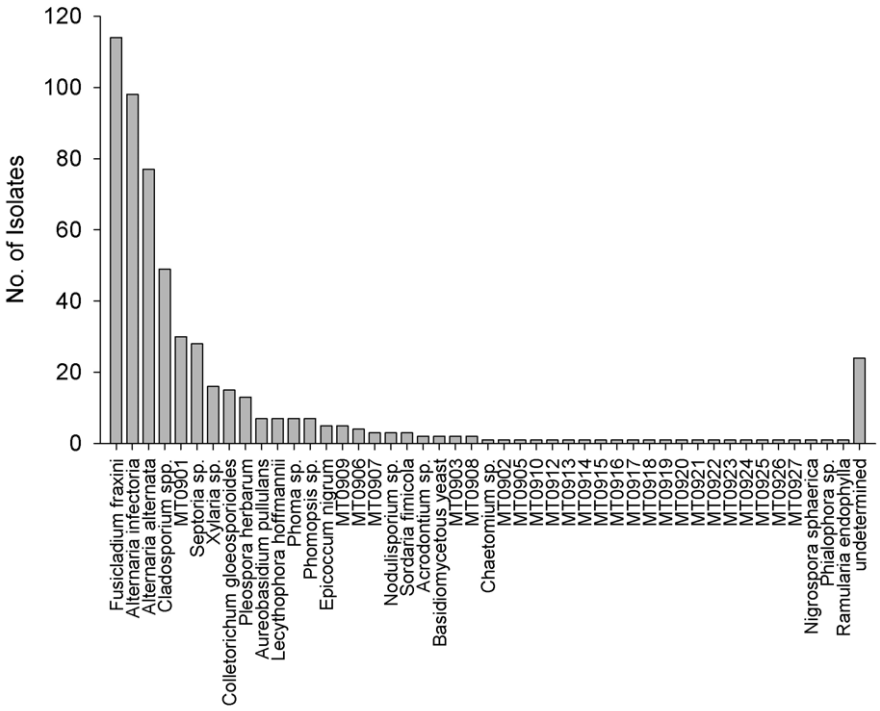

Figure 4.7: Rank abundance curve of the complete sampling of endophytic fungi of *Fraxinus excelsior* in the rain exclusion experiment

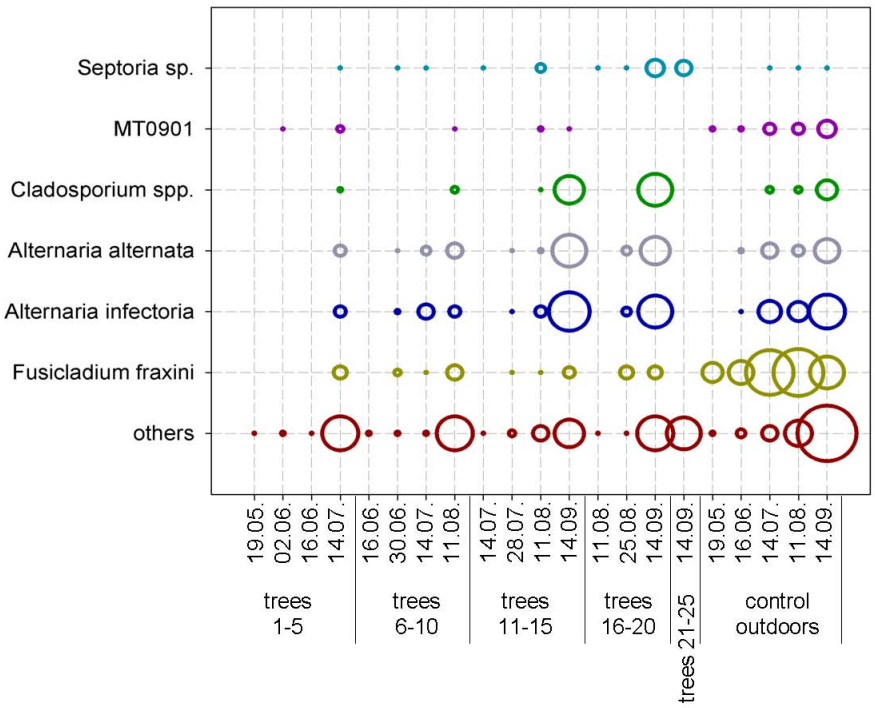

Figure 4.8: Isolation rate of the most common endophyte species of *Fraxinus excelsior* in the rain exclusion experiment for all treatments at all sampling dates. The circle diameters are correlated with the isolation frequency.

4.2.3 Discussion

The study revealed a significant increase of colonisation rates of *Fraxinus excelsior* leaflets by endophytic fungi for trees which were exposed to rain. In contrast, trees that were excluded from rain remained almost endophyte free, although no significant difference was observed in the number of viable propagules between greenhouse and outdoors, in either the aerial inoculum or deposited propagules on the leaf surface.

Experimental design

As water stress might influence the infection rates and composition of the endophyte flora (Arnold & Herre, 2003; Gonthier et al., 2006), all plants were extensively watered if necessary, but without causing waterlogging.

An increase in the temperature due to shielding the trees against rain was unavoidable. However, the difference to outdoors was successfully kept to a minimum. Only in times of weather changes, especially of sudden cooling in the occurrence of a thundery front, the difference between outdoors and the greenhouse increased for a short time, because cooling in the greenhouse followed with a delay of about one hour. The difference in the relative humidity is the direct consequence of the temperature difference. Particularly divergences of the relative humidity, and therefore of the dew point, could influence endophyte infections. If a relative humidity of a certain magnitude is sufficient to promote the infection, it would be reached more easily outdoors. But if a film of available water is necessary for fungal growth and establishment, the relative humidity has to achieve 100% to begin dew formation. This was not the case during the sample period, at least not at the height of the leaves where the dataloggers had been fixed.

The homogenous distribution of the precipitation over the study period provided ideal conditions for the experiment. Since the duration of wetness periods and their interruptions considerably influences the success of leaf colonisation by fungal pathogens (Huber & Gillespie, 1992), it may also influence the colonisation by endophytes. Therefore, long drought periods might have led to reduced infection rates of the plants outdoors and would have complicated assessment of the influence of precipitation on the endophyte colonisation.

The density of the aerial inoculum was low compared to the tropics, where Arnold & Herre (2003) found up to 15 deposited viable propagules $cm^{-2}h^{-1}$. However, since no significant difference could be found between the open greenhouse and outdoors, the infection rates should not have been influenced by the amount of viable fungal propagules in the aerial inoculum.

Another indicator for differences in the number of fungal propagules due to the microclimate and the air circulation is the amount of viable propagules on the leaf surface. It was checked by imprinting unsterilised leaflets on agar plates. This procedure showed high amounts of fungal propagules on both leaflets of the greenhouse and outdoors. In general the amount of living fungal propagules should have been high enough in all treatments, that the observed low infection rates of the greenhouse plants were not caused by a lack of fungal propagules.

Species composition

Only slight differences in the fungal species composition were observed between the trees in the open greenhouse and those outdoors. The differences observed e.g. for *Fusicladium fraxini*, morphotype MT0901, and *Septoria* sp. might be due to differences in the composition of the aerial inoculum (Helander et al., 2006) and therefore on the leaf surface. Since only the amount of viable propagules both in the aerial inoculum and deposited on the leaf surface was observed, differences in the composition due to nearby spore sources, for instance, cannot be excluded. The accumulation of spores on the leaf surface depends on the physical characteristics of the leaf, like hairs, that affect deposition and retention of fungal propagules (Allen et al., 1991). Furthermore, spores are able to modify the leaf surface within minutes after contact, to increase the adhesion (Nicholson & Epstein, 1991). This explains why spores are not just washed of the surface after the plants were placed outside and the species composition is different for plants that have been in the greenhouse for some time, even though they were later placed outdoors at the same location as the outdoor control. To confirm these assumptions, it would be useful to study the composition of the aerial inoculum and the viable propagules deposited on the leaf surfaces, however, this is very time-consuming. An influence on the isolation frequency of *F. fraxini* from overgrowth by other species, as was expected for the previous study at the LAK site (see chapter 3.5.4), can almost be excluded for this study, since the total infection rates were comparatively low over the whole sampling period and not more than three isolates were growing out of a single leaf fragment.

The number of morphotypes, as well as the species composition, was comparable to former studies (see chapters 3.4 and 3.5) and the rank abundance plot was again typically for hyperdiverse groups (Hughes et al., 2001). However, it was conspicuous that the fungal morphotype MT0901, which was relatively frequent, especially in the trees outdoors, did not occur in previous studies at the LAK site. Since the experimental site was about 9 km away from the LAK site amidst the city of Leipzig, this could be due to differences in habitat structure. The greater distance and there-

fore isolation of tree patches from each other in the city may control the foliar fungal communities by influencing the aerial inoculum (Helander et al., 2007). Watering and potting the trees into a mixture of soil from the former habitat and garden mould, which is more nutrient rich than the substrate at the LAK site, may also lead to an altered resistance against some phyllosphere fungi, and thus might influence the species composition (Jumpponen & Jones, 2010). Finally, these differences between the LAK and the experimental site should be mentioned but should have no great influence on the issue of effects the precipitation might have on the endophyte colonisation.

The influence of precipitation

Very low infection rates of leaves which were excluded from precipitation were observed. However the spores did also accumulate on these leaves. As soon as the plants were placed outdoors and the rain applied water to the leaves, the infection started, and almost gained the extent of the control trees within eight weeks. Later in the year, the infection rates reached those of the control trees within four weeks. This might be due to the fact that more viable propagules had accumulated by then on the leaf surface.

The observed increase of endophyte infections in the greenhouse in September is presumably due to an infestation of whiteflies. A relatively high number of the observed infections belong to *Septoria* sp. which has also been found frequently in the understorey trees at the LAK site between August and October. In this case, a high infestation with whiteflies has also been observed (see chapter 3.5). Possibly, *Septoria* sp. is transferred by these sap-suckers and introduced into the leaves directly, by leaf penetration caused by the whiteflies stylets. This assumption might be supported by the findings of Walling (2000), who reported that some some piercing and sucking insects induce the defense-signaling pathways most commonly activated by bacterial, fungal, and viral pathogens. This is possibly a preventive reaction to defend the leaf tissue against microorganisms invading through the sap-suckers' stylets. This could be examined by researching into fungi on the leaf surface, compared to those in the stylets and the digestive system of the insects.

In contrast to the assumption of Arnold & Herre (2003), in the current study, high relative humidity of the air was evidently not enough to allow the infection of leaves: there also has to be a water film on the surface. This would also conclusively explain the lower infection rates of the light crown of full-grown trees (see chapter 3.5), since stronger exposure to wind and sun leads to the leaves drying more rapidly after rainfall. The time during which a water film is available might be too short for

a successful penetration of the leaf (SUMAMPONG et al., 2008).

It can be reasoned that climatic change, with modifications in the amount of precipitation, especially during spring and summer, could directly lead to a change in the rates of fungal infections, both of endophytes and pathogens of woody plants, with unforeseeable consequences. On the one hand it is known that higher moisture can promote the colonisation of tree leaves by pathogenic fungi (SALLE et al., 2008). On the other hand a decrease of moisture, e.g. resulting from lower rainfall, can also enhance the pathogen infection of tree species (DESPREZ-LOUSTAU et al., 2006). The effects for endophytic fungi should be similar to those for pathogenic fungi.

4.3 Influence of herbivory on the occurrence of endophytic fungi in *Acer pseudoplatanus*

Noticeable damage to leaves could be observed at the LAK-site already a few weeks after leaf appearance in spring. Since herbivory can influence the infection of leaves with endophytic fungi (see chapter 4.1.2), this study, with its orientational nature, is devoted to the question: Does the endophytic community of *Acer pseudoplatanus* leaves change with a severe herbivore damage? For this reason, the endophyte colonisation of herbivore-damaged and intact understorey leaves of *A. pseudoplatanus* was investigated by means of a culture-based approach. No significant difference could be observed between the two leaf samplings. This might be due to a rapid wound closure, which averted the infection, and also to the low amounts of precipitation before the sampling date, which could overlay the effect of herbivory.

4.3.1 Methods

Leaves of *Acer pseudoplatanus* L. were collected at the LAK site (see chapter 3.3) about eight weeks after the appearance of leaves in spring 2007. Leaves were collected at eight sampling points in the understorey, at heights between 1.5 and 6 m. At each sampling point, three herbivore-damaged and three intact leaves were taken. The eroded leaves were seriously damaged (Fig. 4.9), however neither the damaged nor the intact leaves showed symptoms of disease (e.g. necrotic tissues) or fungal colonisation.

All leaves were stored in a cool and dry place, and processed for the cultivation on the same day, following the general methods (see chapter 2.1.1). The isolates were grouped into morphotypes and determined morphologically according to chapter 2.1.2, and by sequencing of the ITS region (chapter 2.1.3). *Rarefaction* curves were computed as described in chapter 2.1.4.

4.3.2 Results

In total, 192 leaf fragments from 48 leaves were examined. Endophytic fungi were isolated from 54 of the fragments (28%) of 17 leaves (35%). No more than one endophyte per leaf fragment ever appeared during the eight weeks of incubation.

The 54 isolates were grouped into 28 different morphotypes, of which eight were assigned exclusively to intact leaves, a further 11 to herbivore damaged leaves, and 9 grew out of both kinds of leaves. Figure 4.10 shows the rank-abundance plot of all 28 morphotypes.

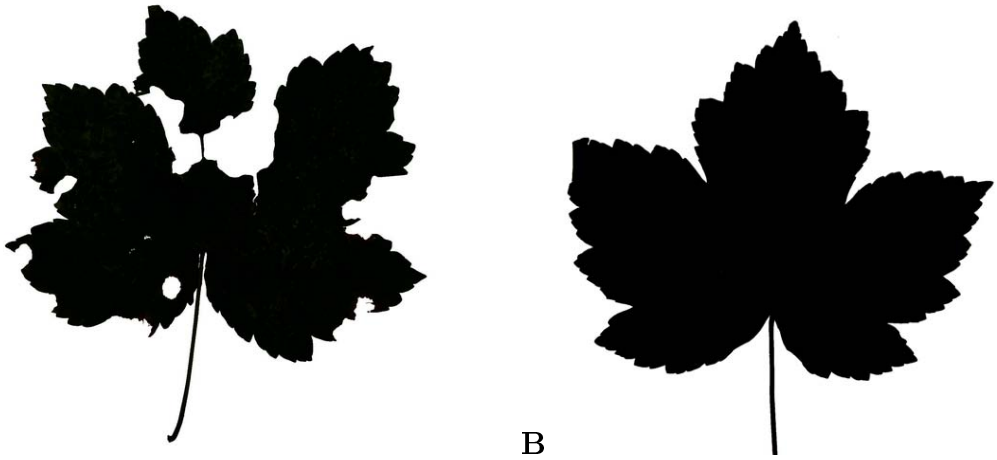

Figure 4.9: Leaves, collected from *Acer pseudoplatanus*, that were seriously damaged by herbivores (A) or completely intact (B)

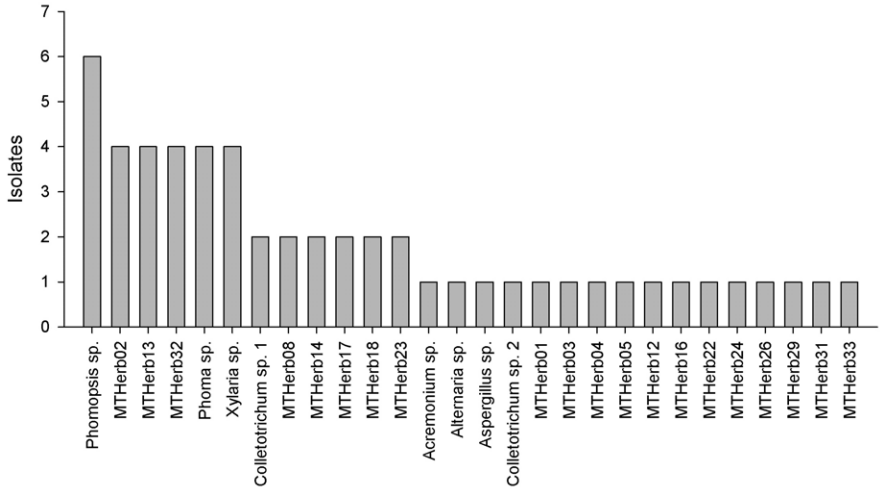

Figure 4.10: Rank-abundance plot of endophytic fungal morphotypes, isolated from intact and herbivore damaged leaves of *Acer pseudoplatanus*, sorted by number of infected leaves

The rescaled *rarefaction* curves, separated into herbivore-damaged and intact leaves, showed no significant difference in the observed species richness (Fig. 4.11). At 25 isolates the herbivore damaged leaves showed a species richness of 18 morphotypes which is nearly identical to that of intact leaves with 17 morphotypes.

The infection density of herbivore damaged leaves was slightly higher than of intact leaves. However, the variance within each group was so high that these differences were not significant (Fig. 4.12).

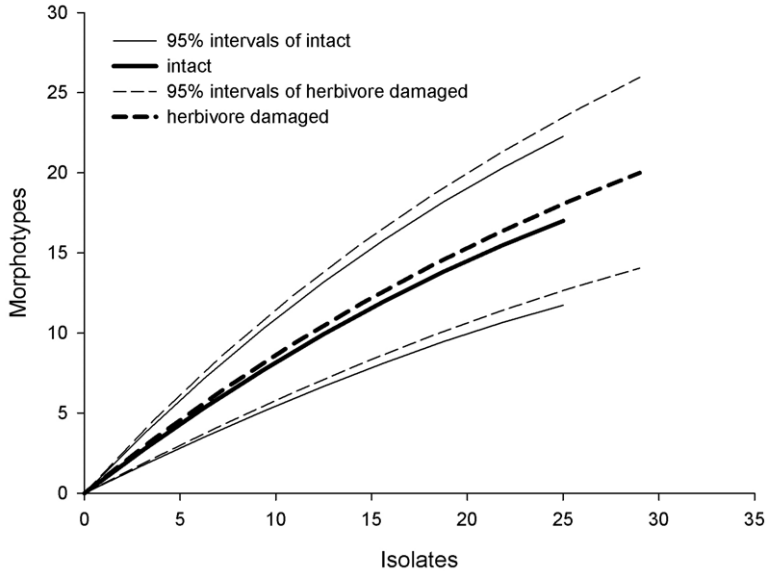

Figure 4.11: *Rarefaction* curves of endophytic fungi isolated from herbivore damaged and intact leaves of *Acer pseudoplatanus* and their 95% confidence intervals

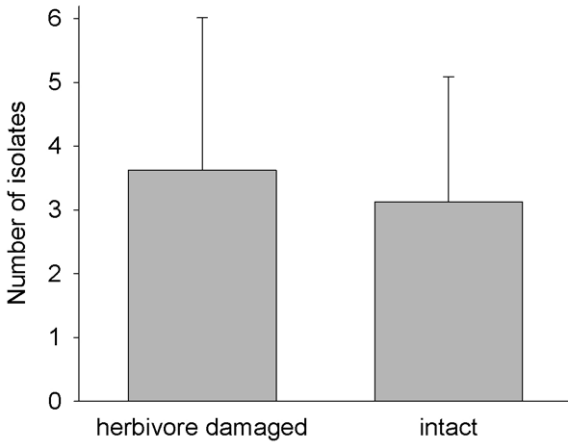

Figure 4.12: Number of isolates of endophytic fungi of herbivore damaged and intact leaves of *Acer pseudoplatanus* per sample point

4.3.3 Discussion

It was noticeable that soon after budding, many leaves were already damaged by herbivores. Some of them were even damaged so heavily that it was not possible to use them for this study, for lack of enough intact leaf area to place on the agar.

The rate of herbivore infestation and damage at the LAK-site in 2007 was high compared with other years (personal observation) and might be due to the warm winter and the hot, dry spring. From 24th March till 6th May there were only 3 l precipitation per square metre whereas the long-term average lies between 35 l per month in March and 50 l per month in May (source: Deutscher Wetterdienst [DWD]). This led to optimal conditions for herbivorous activity of insects.

The number of isolates was comparable to the spring samples of the study of endophytic fungi in *F. excelsior* (see chapter 3.5), and low compared with autumn

samples of the same study and autumn samples of *A. pseudoplatanus*, at the canopy of the same site (chapter 3.4). This supported the finding that the infection frequency of young leaves is low, compared with older ones (e.g. FAETH & HAMMON, 1997; WILSON et al., 1997, chapter 3.5). In contrast, with 54 isolates containing 28 morphotypes, the number of morphotypes was relatively high, compared with the endophyte survey of four different tree species in the autumn (see chapter 3.4). At that time, 54 isolates from *A. pseudoplatanus* comprised only 19 morphotypes. These differences approve the findings of chapter 3.5, that the number of morphotypes defined for a determined number of isolates is higher in the spring than in the autumn. Morphotypes that are dominant in the autumn, and probably form extended infections, are still rare in the spring so that other morphotypes get more weight.

Only a slightly but not significantly higher infection density could be observed in herbivore-damaged leaves than in intact leaves. No significant difference in the number of morphotypes was found. It could be that plant responses to herbivore feeding, which are known to occur already within several hours after plant damage (e.g. CARROLL & HOFFMAN, 1980), lead to a mechanical or biochemical resistance, which includes a defense against fungi (WALLING, 2000). Furthermore, availability of water is necessary for spore germination of most endophytic fungi (see chapter 4.2). The hot and dry spring might have increased the herbivore feeding and fungal transference by herbivores on the one hand, but on the other hand it offered adverse conditions for the establishment of the deposited fungal propagules. Only propagules that got in direct contact with the wound, whilst the herbivore was feeding, and thus contacted the tissue water might have benefited from the leaf damage. However, it can not be excluded that herbivore-damage facilitates the endophyte infection considerably, if enough water is available, as FAETH & WILSON (1996) noted, even if plant defences are initiated fast.

4.4 Role of root lesions for endophyte infection

In the preceding studies, it was observed that an endophytic species often grew out of both ends of the midvein crossing a leaf fragment. The issue arises, whether small spores of an endophyte could penetrate through root lesions caused by soil herbivores, and be transported, up to the leaves by way of the transpiration stream. A small study of orientational nature was carried out to test this hypothesis.

4.4.1 Methods

The experiments were carried out at a natural stand of saplings of *Fraxinus excelsior* at the Leipzig Canopy Crane research facility (see chapter 3.3). A spore suspension of *Cladosporium cladosporioides*, in an isotonic sodium chloride solution with a concentration of 5 x 10^4 spores / ml, was used to increase the amount of viable spores in the soil. *Cladosporium cladosporioides*, known as common endophyte of *F. excelsior* was used, because spores of at most 8.8 x 3.5 μm (Ho, 1999) are by far small enough to pass through the vessels of the juvenile ashes. The diameter of early vessel lumina is increasingly 125 to 225 μm in trees of up to 15 years of age (Helinska-Raczkowska & Fabisiak, 1999). Furthermore, *C. cladosporioides* infections are sporulating fast and reliably and can therefore be distinguished easily from other endophyte infections already after a short cultivation period.

From ten randomly chosen individuals one leaflet from the third or fourth leaf pair of the apical shoot was collected, in order to record the endophyte colonisation before the root lesion. Subsequently parts of the root system were exposed, and two major roots were cut. For five trees the cut surface was covered with soil and watered with 100 ml of the spore suspension and one liter of river water. The cut roots of the other five trees were left open and no watering was carried out, in order to observe changes in the endophyte composition which could be induced by effects of water stress, caused by the root cutting. Seven days after the root lesion, one leaflet per tree was collected once again, as described above.

All leaflets were stored in a cool, dry place, and processed for cultivation on the same day, following the general methods in chapter 2.1.1. The number of morphotypes that grew out of the leaf fragments was counted after seven days. Additionally, the leaf fragments were checked for infections with *Cladosporium cladosporioides*.

4.4.2 Results

No significant difference could be observed between the amount of morphotypes before and after the treatment (Fig. 4.13). Likewise, there was no significant difference in the colonisation with *Cladosporium cladosporioides*.

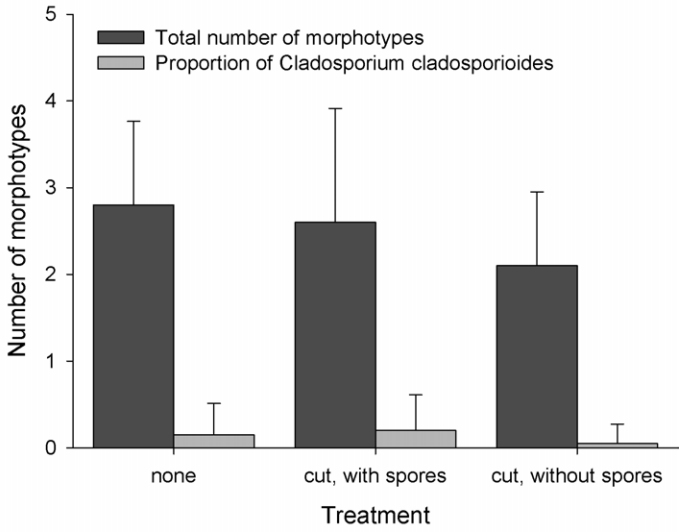

Figure 4.13: Number of morphotypes of endophytic fungi per leaf fragment isolated from saplings of *Fraxinus excelsior* before (none) and after root lesion with (cut, with spores) and without (cut, without spores) following covering with soil and watering with spore suspension

4.4.3 Discussion

As for herbivore-damaged leaves (see chapter 4.3), no increase of the infection rate of endophytic fungi after serious root lesions could be found. For the group of plants where the root cuttings were covered with soil and watered with spore suspension, a transport of *Cladosporium* spores to the leaves in the xylem should, in principle, be possible. The negative result of this study indicates that the following may have happened: the spores were absorbed by soil particles and did not remain free in the water, the root lesions were closed fast enough that spores were not able to invade, or the spores got lost within the xylem on their way to the leaves. Microscopy of the roots after the experiment showed that the vessels at the cut surface were densely filled with starch. This might be the first way for the plant to rapidly close the damaged area and avoid chance of easy entrance for microorganisms. In the near future, a continuation of this study with modified methods and an enlarged number of tree individuals is planned.

5 Conclusions and future prospects

The endophyte flora of the LAK research facility, as well as the potted plants in the botanical garden, was clearly dominated by the two *Alternaria* species *A. alternata* and *A. infectoria*. These two ubiquitous species were very frequent in all explorations of this thesis, especially in the light crowns and in the autumn.

Contrary to the first hypothesis (see chapter 1.2), the endophyte composition of different host tree species was not dominated by host-specific endophytes, but by ubiquitous endophytes. Only a few rare species seemed to be host-specific or at least to form host-specific strains. No statement can be made, in that respect, about the host specificity of obligate biotrophic fungal species, which might be considerably higher, since they could not be detected by the culture-based approach which was carried out.

According to the second hypothesis, considerable differences were observed for the endophytic species composition, infection density, and diversity of different forest layers. Differences could be found for all four host tree species between the light crown and the shade crown in September, as well as for *Fraxinus excelsior*, between all three forest layers and during the whole vegetation period.

The colonisation density grew, as supposed in hypothesis three, during the whole vegetation period, and in all forest layers. Only in the light crown was a decrease observed at the end of the vegetation period, which might be due to a necessary modification in collection at the beginning of leaf fall or to a real decrease of the colonisation density. In contrast to this, the species richness decreased after late summer, due to change in the species composition.

The fourth hypothesis could only be confirmed regarding the influence of the precipitation, as a high amount of precipitation and particularly a long availability of water on the leaf surface obviously promote the infection of leaves by endophytic fungi. Increased infection, due to herbivory and root lesions, could not be observed.

The studies showed that further exploration of the leaf inhabiting endophytic

communities of trees, with regard to different forest layers, is necessary, especially of the ecologically outstanding light crowns. Since climatic changes, particularly hot and dry summers, are predicted for the coming decades in Central Europe, this knowledge will help to make more reliable assessments of the effects that leaf inhabiting endophytic fungi can have on forest ecosystems in the future, for instance by damage of pathogenic species that formerly have been quiescent or by endophyte induced premature leaf abscission (see chapter 3.5).

Further research on endophytic fungi should be done, particularly in respect of changing environmental conditions, e.g. elevated temperatures, as simulated at the canopy crane project of the Tomakomai Experimental Forest (Murakami & Hiura, 2003; Muller et al., 2009), or elevated CO_2, as studied at the Swiss Canopy Crane (Körner & Zotz, 2003; Körner et al., 2005). This might help to assess, whether climatic changes lead to changes in the endophyte composition, as must be concluded from the present thesis.

Further investigations on the role which the *Alternaria* spp. play in premature leaf abscission in the light crowns of *Fraxinus excelsior* will give more evidence about the influence of single endophyte species. Experimental approaches are necessary, to show which factors cause the high *Alternaria* colonisation in the light crowns, particularly regarding limited water availability, increased UV-irradiation, and differences in the aerial inoculum. They might also clarify the question if the strong *Alternaria* colonisation is caused by premature leaf aging, which increases the susceptibility of the leaves or vice versa - that *Alternaria* causes premature leaf abscission.

Summary / Zusammenfassung

Summary

The present thesis deals with the colonisation of leaves by endophytic fungi in different forest layers of a deciduous floodplain forest in Central Europe. The endophytic community of leaves in the light and in the shade crowns of *Acer pseudoplatanus*, *Fraxinus excelsior*, *Quercus robur*, and *Tilia cordata*, four typical tree species at the Leipzig Canopy Crane research facility was compared. Deepening spatial and temporal patterns of the endophyte colonisation of the European ash (*Fraxinus excelsior* L.) were examined in the light and the shade crowns of full-grown trees, as well as in the understorey during the course of a whole vegetation period. The Leipzig Canopy Crane facilitated the repeated access to the same sample points in the canopies of full-grown trees. The investigations of colonisation patterns in the natural habitat were complemented by experiments on the influence of precipitation, herbivory, and root lesions on the endophyte colonisation to find reasons for the different colonisation patterns.

All studies were realised using a culture-based approach. Leaves that did not show any symptoms of disease were collected randomly. The surfaces of the collected leaves were sterilised using natriumhypochlorite and ethanol. Subsequently, leaf fragments were cut out along the midrib under sterile conditions and placed on malt extract agar (MEA, 2%, with 0.1 g/l tetracycline added). The fungal mycelia, that grew out of the leaf fragments within a few days to weeks, were transfered into axenic cultures.

The gained isolates were grouped into morphotypes and identified by means of micromorphological characters and molecular examinations of the D1/D2 respectively the ITS region of the nuclear ribosomal DNA. Endophyte infections of woody plants are, in contrast to the systemic endophytes of grasses, usually locally restricted and comprise partially only a few cells, but may also be more extended. Since it is therefore almost impossible to make sure whether isolates of the same morphotype grew out of the same or of different mycelia within the same leaf, the abundance of a morphotype was determined by counting it only once per leaf or

leaf fragment, respectively. Morphotypes, isolated repeatedly from the same sample, regardless whether they originated from one large or several small scaled mycelia, were assessed as being more frequent than species that were isolated only once.

To compare the endophyte colonisation of different host tree species in September 2005 leaf samples of three leaves per sample point were collected at four sample points each in the light and the shadow crowns of *Acer pseudoplatanus*, *Fraxinus excelsior*, *Quercus robur*, and *Tilia cordata* in heights of 15 to 35 m (96 leaves total). 298 of the 343 obtained isolates were grouped into 40 morphotypes, of which 21 could be identified morphologically to genus or species rank. The remaining 45 isolates could not be typed unambiguously. By means of the molecular examinations all morphotypes could be assigned to two classes of the Ascomycota (i.e. Sordariomycetes and Dothideomycetes). The rank-abundance-plots showed the typical pattern of hyperdiverse groups of organisms with few frequent and many rarely isolated species. Accordingly the *rarefaction* curve did not reach a maximum but increased almost linearly, and the *Chao2* estimator lay far above the detected species number.

Only few endophyte species were isolated from only one host species. The most species, especially the most frequent isolated *Alternaria alternata* (39 isolates), *Aureobasidium pullulans* (34 isolates), and *Cladosporium cladosporioides* (31 isolates), appeared in several hosts and are known as ubiquistic species. For all host species differences in the colonisation of the light and the shade crowns became apparent. The infection density as well as the species richness and the diversity were lower in the light crowns than in the shade crowns of all four host species. The differences were not significant, but proved true in the following extensive exploration of the endophyte colonisation of *Fraxinus excelsior*.

Therefore, leaflets of *Fraxinus excelsior* were collected in different forest layers once a month from end of May until end of October 2008, each time at the same sample points. Three leaflets were collected at four sample points each from the light crown (25 to 35 m height) and the shade crown (15 to 20 m height) of full grown trees, and from about 10 years old saplings in the understorey (213 leaflets total). 809 of altogether 845 fungal isolates were grouped into 50 morphotypes, of which 25 were determined micromorphologically to genus or species rank. The remaining 36 isolates could not be typed unambiguously. Even in this study almost all of the isolates belonged to the classes Sordariomycetes und Dothideomycetes, only 12 isolates belonged to the basidiomycetous yeasts. Again, the *rarefaction* curve and the *Chao2* estimator showed, that the detected species number was considerably lower than to be expected.

The infection density, the species richness and the diversity of all three forest

layers increased significantly during the vegetation period. As late as the end of the vegetation period a stagnation or a slight decrease was partially observed. In contrast, the number of morphotypes related to a defined number of isolates was higher in spring than in the autumn. Therefore, it can be concluded that the composition of the endophyte community shifted from many rarely isolated to fewer but frequently isolated species.

The comparison of the three forest layers showed that the endophyte colonisation was lowest in the light crowns of full-grown trees, followed by the shade crowns, and highest in the understorey, regarding the infection density, as well as the species richness, and the diversity. Most of the morphotypes followed these general distribution patterns with high isolation frequencies in the understorey and in the autumn. However, some species, especially the most frequent ones *Alternaria alternata* and *A. infectoria*, were isolated most frequently from the light crowns.

It is to be assumed that the increasing colonisation of the leaves during the vegetation period can be traced back to an accumulation of fungal propagules on the leaf surfaces, which leads under suitable conditions to an infection of the leaf. The differences in the colonisation of the forest layers were presumably mainly caused by differences in the microclimate, the composition of the aerial inoculum, the leaf structure, and the herbivore activity. Changes of these factors therefore might alter the endophytic community. Since the endophytic community in turn diversely influences the host tree, it is necessary to know which of these factors are most important. Based on this knowledge assessments might be possible, how altering endophytic communities, caused for example by climatic change or changes in the forest management, can affect forest ecosystems.

Therefore, the influence of precipitation, herbivory, and root damages on the endophyte colonisation of leaves was examined. Saplings of *Fraxinus excelsior* were potted and placed into a greenhouse construction with two open sides several weeks prior to the beginning of foliation to shield them from precipitation From end of May until middle of September 2009 every four weeks five of these ashes were placed outdoors. The endophyte colonisation was determined at that time, and two, four and eight weeks later, and compared to a control group that had been outside all the time. Additionally, the precipitation was measured outdoors, and the spore fall from the air, the accumulation of viable fungal spores on the leaves, and temperature and humidity in the greenhouse and outdoors were compared.

While no significant differences were determined in the microclimate and the availability of viable spores, the colonisation of the leaves by endophytic fungi varied significantly. During the whole sampling period the colonisation of trees placed in

the greenhouse by then was significantly lower than that of the outdoor control. The infection rates of plants placed outdoors during the experiment, rapidly increased and did not differ significantly anymore from the outdoor control at the latest after eight weeks. The composition of the endophyte community of plants that were placed in the greenhouse at first partly differed from that of the outdoor control.

The results show that the precipitation plays a decisive role for the colonisation of leaves by endophytic fungi. Although viable spores had accumulated on the leaves, they remained almost free of endophytes as long as the spores had no contact to water. As soon as rain reached the leaves, the colonisation by endophytes also took place. The differences in the endophyte composition suggest that the colonisation of the plants that stood in the greenhouse for some time at least partially originated from spores that had accumulated on the leaves already in the greenhouse.

A comparison of the endophyte colonisation of intact and severely herbivore damaged leaves of *Acer pseudoplatanus* in spring 2007 showed no significant differences. As well, an experiment in September 2008 where roots of saplings of *Fraxinus excelsior* were cut through and exposed to a spore suspension showed no influence on the endophyte colonisation of the leaves. Probably, in both cases the wound closure by the plant proceeded so rapidly that hardly any fungi could intrude into the plant through the wound. It can not be excluded, however, that herbivores influence the composition of the endophytic community as vectors of spores or mycelia.

For the first time the endophyte colonisation of leaves in the uppermost part of a full-grown forest were studied extensively. Considerably greater differences were found in comparison to the shade crown and the understorey than were expected from previous studies about the endophyte colonisation. It could be proved that the availability of water plays an important part in explaining these differences, however influences of other factors like UV-irradiation and temperature can not be excluded. The present thesis showed, that it is necessary to further explore the leaf inhabiting endophytic communities of trees with regard to different forest layers, especially to the light crowns. Since climatic changes, especially hot and dry summers, are predicted for the coming decades in Central Europe, this knowledge will help to make more reliable assessments of the effects that leaf inhabiting endophytic fungi can have on forest ecosystems in the future.

Zusammenfassung

Die vorliegende Arbeit befasst sich mit der Besiedelung von Blättern in verschiedenen Waldschichten eines mitteleuropäischen Hartholz-Auwaldes durch endophytische Pilze. Die Endophytengesellschaften von Blättern in den Licht- und Schattenkronen von *Acer pseudoplatanus*, *Fraxinus excelsior*, *Quercus robur* und *Tilia cordata*, vier typischen Baumarten der Forschungsfläche Leipziger Auwaldkran (LAK), wurden verglichen. Vertiefend wurden zeitliche und räumliche Muster der Endophytenbesiedelung der Gemeinen Esche (*Fraxinus excelsior* L.) in der Licht- und der Schattenkrone ausgewachsener Bäume, sowie im Unterwuchs im Verlauf einer Vegetationsperiode untersucht. Der Leipziger Auwaldkran ermöglichte dabei den wiederholten Zugang zu denselben Sammelpunkten in den Kronen ausgewachsener Bäume. Die Untersuchungen im natürlichen Lebensraum wurden ergänzt durch Experimente zum Einfluss von Niederschlag, Herbivorie und Wurzelverletzungen auf die Endophytenbesiedelung, um Ursachen für die verschiedenen Besiedelungsmuster zu finden.

Für alle Untersuchungen wurde ein kultur-basierter Ansatz gewählt. Es wurden stichprobenartig Blätter gesammelt, die keine sichtbaren Schadsymptome aufwiesen. Die Oberflächen der gesammelten Blätter wurden mit Natriumhypochlorit und Ethanol sterilisiert. Anschließend wurden entlang der Mittelrippe Blattstücke unter sterilen Bedingungen herausgeschnitten und auf Malzextrakt-Agar (MEA, 2%, incl. 0,1 g/l Tetrazyklin) ausgelegt. Die Pilzmyzelien, die innerhalb weniger Tage bis Wochen aus den Blattstücken herauswuchsen, wurden in Reinkulturen überführt.

Die so gewonnenen Isolate wurden in Morphotypen eingeteilt und anhand mikromorphologischer Merkmale sowie molekularer Untersuchungen der D1/D2- bzw. der ITS-Region der nukleären ribosomalen DNA taxonomisch eingeordnet. Endophyteninfektionen in Gehölzen sind, im Gegensatz zu den systemischen Endophyten der Gräser, gewöhnlich lokal begrenzt und umfassen teilweise nur wenige Zellen, können jedoch auch ausgedehnt sein. Da es deswegen nahezu unmöglich ist festzustellen, ob Isolate des gleichen Morphotyps aus demselben oder aus verschiedenen Myzelien innerhalb eines Blattes hervorgegangen sind, wurde zur Ermittlung von Abundanzen jeder Morphotyp einmal pro Blatt bzw. Blattstück gezählt. Morphotypen, die aus mehreren Blättern oder Blattstücken an einem Sammelpunkt isoliert wurden, unabhängig davon, ob sie aus einer ausgedehnten oder mehreren kleinräumigen Infektionen hervorgegangen waren, wurden somit stärker gewichtet, als solche die nur aus einem Blatt bzw. Blattstück isoliert wurden.

Für den Vergleich der Endophytenbesiedelung verschiedener Wirtsbaumarten wur-

den im September 2005 in den Licht- und Schattenkronen von *Acer pseudoplatanus*, *Fraxinus excelsior*, *Quercus robur* und *Tilia cordata* in Höhen von 15 bis 35 m an je vier Sammelpunkten Blattproben im Umfang von drei Blättern je Sammelpunkt entnommen (insgesamt 96 Blätter). Von den 343 daraus gewonnenen Isolaten wurden 298 in 40 Morphotypen eingeteilt, von denen 21 morphologisch bis auf Gattungs- oder Artebene bestimmt werden konnten. Die restlichen 45 Isolate konnten nicht zweifelsfrei typisiert werden. Mit Hilfe der molekularen Untersuchung konnten alle Morphotypen zwei Klassen der Ascomycota (i.e. Sordariomycetes und Dothideomycetes) zugeordnet werden. Die Rang-Abundanz-Plots zeigten das typische Muster hyperdiverser Organismengruppen mit wenigen häufigen Arten und zahlreichen selten isolierten. Dementsprechend erreichte die *Rarefaction*-Kurve keinen Maximalwert, sondern stieg nahezu linear an und der *Chao2* Schätzwert lag deutlich über der gefundenen Artenzahl.

Nur wenige Endophytenarten wurden von nur einer Wirtsbaumart isoliert. Die meisten Arten, vor allem die am häufigsten isolierten *Alternaria alternata* (39 Isolate), *Aureobasidium pullulans* (34 Isolate) und *Cladosporium cladosporioides* (31 Isolate), traten in mehreren Wirten auf und sind als ubiquistische Saprobionten bekannt. Bei allen Wirten zeigten sich Unterschiede in der Besiedelung von Licht- und Schattenkrone. Sowohl die Infektionsdichte, als auch die Artenvielfalt und die Diversität waren in den Lichtkronen aller Wirte niedriger als in den Schattenkronen. Die Unterschiede waren zwar nicht signifikant, bestätigten sich jedoch in der folgenden intensiven Untersuchung der Endophytenbesiedelung von *Fraxinus excelsior*.

Dafür wurden in der Vegetationsperiode 2008 von Ende Mai bis Ende Oktober einmal monatlich jeweils 3 Blättchen an 4 Sammelpunkten in der Lichtkrone (25-35 m Höhe) und in der Schattenkrone (15-20 m Höhe) ausgewachsener Bäume sowie von ca. 10 Jahre alten Schößlingen im Unterwuchs gesammelt (insgesamt 213 Blättchen). 809 von insgesamt 845 Isolaten wurden in 50 Morphotypen eingeteilt, von denen 25 mikromorphologisch bis auf Gattungs- oder Artebene bestimmt werden konnten. Die restlichen 36 Isolate konnten nicht zweifelsfrei typisiert werden. Auch in dieser Untersuchung gehörten nahezu alle Isolate in die Klassen Sordariomycetes und Dothideomycetes, nur 12 Isolate gehörten zu den basidiomycetalen Hefen. Wieder zeigten die *Rarefaction*-Kurve und der *Chao2* Schätzwert, dass die ermittelte Artenzahl bei weitem nicht der zu erwartenden Artenzahl entspricht.

Die Infektionsdichte, die Artenvielfalt und die Diversität stiegen in allen Waldschichten im Lauf der Vegetationsperiode zunächst stark an. Erst gegen Ende der Vegetationsperiode kam es z.T. zu einer Stagnation oder einem leichten Rückgang der Werte. Die Anzahl der Arten bezogen auf eine definierte Anzahl an Isolaten war dagegen im Frühjahr höher als im Herbst, so dass auf eine Verschiebung in der

Zusammensetzung der Endophytengesellschaft von vielen seltenen hin zu wenigen, dafür aber häufig isolierten Arten geschlossen werden kann.

Der Vergleich der drei Waldschichten zeigte die geringste Endophytenbesiedelung in der Lichtkrone ausgewachsener Bäume, gefolgt von deren Schattenkrone. Die höchste Besiedelung fand sich im Unterwuchs, sowohl hinsichtlich der Infektionsdichte, als auch der Artenvielfalt und der Diversität. Die meisten Morphotypen folgten diesen generellen Verteilungsmustern mit hohen Isolatenzahlen im Herbst und im Unterwuchs. Einige Arten, insbesondere die beiden häufigsten *Alternaria alternata* und *A. infectoria* wurden, im Gegensatz dazu, am häufigsten aus Blättern der Lichtkrone isoliert.

Es ist anzunehmen, dass die zunehmende Besiedelung der Blätter im Jahresverlauf auf eine Akkumulation von Sporen und Myzelbruchstücken auf der Blattfläche zurückzuführen ist, die unter geeigneten Bedingungen zu einer Infektion des Blattes führt. Die Unterschiede in der Besiedelung der Waldschichten sind vermutlich vor allem durch Unterschiede im Mikroklima, in der Zusammensetzung des Luftinokulums, in der Blattstruktur und in der Aktivität von Herbivoren bedingt. Veränderungen dieser Faktoren würden dann zu Veränderungen der Endophytengesellschaft führen. Da diese wiederum ihren Wirt auf vielfältige Weise beeinflussen, ist es notwendig zu wissen, welche dieser Faktoren den größten Einfluss ausüben. Basierend auf diesem Wissen wären dann Vorhersagen möglich, wie Veränderungen der Endophytenzusammensetzung, ausgelöst beispielsweise durch den Klimawandel oder durch Veränderungen in der Art der Waldbewirtschaftung, Waldökosysteme beeinflussen können.

Es wurde daher der Einfluss von Niederschlag, Herbivorie und Wurzelverletzungen auf die Endophytenbesiedelung von Blättern untersucht. Um sie vor Niederschlag abzuschirmen wurden Schösslinge von *Fraxinus excelsior* eingetopft und einige Wochen vor dem Blattaustrieb in ein an zwei Seiten offenes Gewächshaus gestellt. Aller vier Wochen von Mitte Mai bis Mitte September 2009 wurden jeweils fünf Eschen ins Freie gebracht und die Endophytenbesiedelung zu diesem Zeitpunkt sowie nach zwei, vier und acht Wochen im Vergleich zu einer Kontrolle im Freiland untersucht. Zusätzlich wurde der Niederschlag im Freiland gemessen und der Sporenniederschlag aus der Luft, die Ansammlung von lebensfähigen Pilzsporen auf den Blättern, sowie Temperatur und Luftfeuchtigkeit im Gewächshaus und im Freiland verglichen.

Während beim Mikroklima sowie bei der Verfügbarkeit lebensfähiger Sporen keine signifikanten Unterschiede festgestellt wurden, unterschied sich die Besiedelung der Blätter mit endophytischen Pilzen deutlich. Während des gesamten Untersuchungszeitraumes war die Besiedelung von Pflanzen, die bis zu diesem Zeitpunkt im Ge-

wächshaus standen, signifikant geringer als die der Freilandkontrolle. Die Infektionsraten der Pflanzen, die im Verlauf des Experimentes ins Freiland gestellt wurden, stiegen rasch an und waren nach spätestens acht Wochen nicht mehr signifikant verschieden von denen der Freilandkontrolle. Die Zusammensetzung der Endophytengesellschaft von Pflanzen, die zunächst im Gewächshaus standen, unterschied sich teilweise von denen der Kontrolle, die die gesamte Zeit im Freiland stand.

Die Ergebnisse zeigen, dass der Niederschlag eine entscheidende Rolle für die Besiedelung von Blättern mit endophytischen Pilzen spielt. Obwohl sich lebensfähige Sporen auf den Blättern ansammelten, blieben sie nahezu endophytenfrei, solange die Sporen keinen Kontakt mit Wasser hatten. Sobald Regen die Blätter erreichte, erfolgte auch die Besiedelung mit Endophyten. Die Unterschiede in der Endophytenzusammensetzung legen nahe, dass die Besiedelung der Pflanzen, die einige Zeit im Gewächshaus standen, zumindest teilweise aus Sporen erfolgte, die sich in dieser Zeit bereits auf den Blättern angesammelt hatten.

Ein Vergleich der Endophytenbesiedelung von intakten mit stark von Herbivoren angefressenen Blättern von *Acer pseudoplatanus* im Frühjahr 2007 zeigte keine signifikanten Unterschiede. Auch ein Experiment im September 2008 bei dem Wurzeln von Schösslingen von *Fraxinus excelsior* durchtrennt und einer Sporensuspension ausgesetzt wurden, zeigte keinen Einfluss auf die Endophytenbesiedelung der Blätter. Vermutlich ging in beiden Fällen der Wundverschluss durch die Pflanze so rasch vonstatten, dass kaum Pilze durch die Verletzung in die Pflanze eindringen konnten. Es ist jedoch nicht ausgeschlossen, dass Herbivoren als Überträger von Sporen oder Myzelstücken trotzdem einen Einfluss auf die Zusammensetzung der Endophytengesellschaft ausüben.

Erstmalig wurden die Endophytenbesiedelung von Blättern im obersten Bereich eines ausgewachsenen Waldes intensiv untersucht. Dabei wurden deutlich stärkere Unterschiede zu Blättern der Schattenkrone und des Unterwuchses gefunden, als aus vorangehenden Untersuchungen zur Endophytenbesiedelung zu erwarten war. Es konnte nachgewiesen werden, dass die Verfügbarkeit von Wasser auf der Blattoberfläche eine bedeutende Rolle bei der Erklärung dieser Unterschiede spielt. Einflüsse von anderen Faktoren wie UV-Strahlung und Temperatur können jedoch nicht ausgeschlossen werden. Die vorliegende Arbeit zeigt, dass es notwendig ist, die Gesellschaften blattbewohnender Endophyten von Bäumen in Zukunft noch umfassender zu untersuchen, insbesondere die der Lichtkronen. Da für die kommenden Jahrzehnte klimatische Veränderungen, insbesondere heiße und trockene Sommer, für Mitteleuropa vorhergesagt sind, würde dieses Wissen helfen die Effekte, die endophytische Pilze auf Waldökosysteme in Zukunft haben können, verlässlicher abzuschätzen.

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Appendix

Appendix A - Cultivation media

2% Maltextract Agar (MEA, MEA+T)

- 20 g/l maltextract
- 15 g/l agar
- solve thoroughly in distilled water
- autoclave 16 min at 121°C

- for the isolation 0.1g/l tetracycline were solved in 10 ml sterile distilled water and added after cooling down the agar to 50°C (MEA+T)

Oatmeal agar (OA)

- cook 30 g oatmeal in 500 ml distilled water for 20 min
- pour off through a fine sieve
- fill up to 1 l with distilled water
- add 15 g/l agar
- autoclave 16 min at 121°C

Potato carrot agar (PCA)

- cook 20 g potatoes (peeled and cut up small) in 250 ml distilled water for 60 min and pour off through a fine sieve
- cook 20 g carrots (peeled and cut up small) in 250 ml distilled water for 60 min and pour off through a fine sieve
- mix extracts and fill up to 1 l with distilled water
- add 15 g/l agar
- autoclave 16 min at 121°C

Potato dextrose agar (PDA)

- cook 200 g potatoes (peeled and cut up small) in 250 ml distilled water for 60 min and pour off through a fine sieve
- fill up to 1 l with distilled water
- add 15 g dextrose
- add 15 g/l agar
- autoclave 16 min at 121°C

Vegetable juice agar (V8)

- mix 500 ml vegetable juice (albi GmbH+Co, Bühlenhausen, Germany; mix of tomato, carrot, sauerkraut, red cabbage, beet root, cucumber, pepper, and lettuce) and
- 500 ml distilled water
- add 7.14 g compressed yeast solved in 12 ml distilled water
- add 15 g/l agar
- autoclave 16 min at 121°C

Appendix B - Best hits of the BLAST searches

Table B.1: Best hits of the BLAST searches for sequences of the ITS and the D1/D2 nrDNA region (results from October 2010)

Query		Best hits					
Morphotype	**Accession number**	**No.**	**Description**	**Accession number**	**Max score**	**Query coverage**	**Max ident**
ITS1-5.8S-ITS2 region							
Basidiomycetous yeast	HQ414616	1	Uncultured *Cryptococcus*	EU852364	806	100%	100%
		2	*Cryptococcus dimennae*	AY188365	806	100%	100%
		3-4	*Cryptococcus dimennae*	AF410473, EU266559	748	100%	97%
		5	*Bullera globispora*	FN824496	742	100%	97%
Colletotrichum gloeosporioides	HQ414608	1-2	*Glomerella cingulata*	AJ301952, AJ301925	1052	100%	100%
		3	*Glomerella* sp.	EU622052	1035	98%	99%
		4	*Colletotrichum gloeosporioides*	AB470867	1029	100%	99%
		5-8	*Glomerella acutata*	AF272789, AF489565, EU391655, AM991135	1029	100%	99%
Coniothyrium sp.	HQ414589	1-2	Uncultured fungus clone	GU721701, GU721696	883	100%	100%
		3-6	*Phoma medicaginis*	FJ755264, FJ755260, FJ755259, FJ755258	883	100%	100%
		7	*Marssonia populi*	FJ755256	883	100%	100%
		8	*Phoma medicaginis*	FJ755251	883	100%	100%
Diplodina acerina	HQ414605	1	Uncultured fungus	FJ820749	938	100%	97%
		2-5	*Apiognomonia hystrix*	EU255012, EU255018, EU255032, EU255033	938	91%	100%
		6	*Cryptodiaporthe hystrix*	DQ323531	938	96%	98%
		7-9	*Apiognomonia errabunda*	AJ888475-AJ888477	938	100%	97%
Discula umbrinella	HQ414604	1	Uncultured fungus	FJ820749	956	100%	98%
		2-4	*Apiognomonia errabunda*	AJ888475-AJ888477	956	100%	98%
		5	*Fusicoccum quercus*	AJ293871	946	98%	99%
		6-9	*Fusicoccum quercus*	AJ293872-AJ293875	940	98%	98%
Epicoccum nigrum	HQ414592	1	Fungal sp.	GQ996091	890	100%	99%

Table B.1: (continued)

Query		Best hits					
Morphotype	**Accession number**	**No.**	**Description**	**Accession number**	**Max score**	**Query coverage**	**Max ident**
		2-9	Uncultured fungus	GQ999415, GQ999357, GQ999354, AB520274, AB520262, GU065617, GU065533, GU065522	890	100%	99%
		10-11	*Epicoccum nigrum*	FN868456, FJ914708	890	100%	99%
		12	*Phyllosticta ligustri*	AB470841	890	100%	99%
Lecythophora hoffmannii	HQ414607	1-3	Fungal sp.	FJ235948, HM123356, HM123357	994	100%	100%
		4	Foliar endophyte of *Picea glauca*	AY561213	990	99%	100%
		5	*Coniochaeta ligniaria*	AY198390	981	100%	99%
		6	Fungal sp.	HM123047	977	100%	99%
MT0807	HQ414590	1	Uncultured fungus	GQ999376	888	100%	98%
		2	*Phoma* sp.	GU566295	883	100%	98%
		3-4	*Phoma glomerata*	EU273521, DQ912693	883	100%	98%
		5	Uncultured soil fungus	EU480138	879	100%	98%
MT0812	HQ414615	1-5	*Botryosphaeria stevensii*	AY259093, DQ458886, EU030326, EU856765, EU856766	931	100%	99%
		6	*Botryosphaeria stevensii*	EU856764	925	100%	99%
		7	*Coniothyrium diplodiella*	EU520074	919	100%	98%
		8-9	*Diplodia mutila*	EF445346, FJ481586	917	98%	99%
MT0813	HQ414594	1	Fungal sp.	GQ996179	727	99%	94%
		2	*Ochrocladosporium frigidarii*	FJ755255	721	99%	94%
		3	*Hyalodendriella* sp.	FJ379833	715	99%	94%
		4	Uncultured fungus	GU065525	710	99%	94%
MT0815	HQ414591	1-2	*Peyronellaea pinodella*	AB369504, AB369439	917	100%	99%
		3	*Didymella fabae*	FJ755246	913	99%	99%
		4	Uncultured soil fungus	EU480138	913	100%	98%
		5-8	Uncultured Ascomycota clone	HM239934, HM239891, HM239850, HM239783	906	100%	98%
MT0817	HQ414610	1	Fungal sp.	GQ996094	917	100%	99%
		2	*Fusarium* sp.	GU480953	917	100%	99%

Table B.1: (continued)

Query		Best hits					
Morphotype	**Accession number**	**No.**	**Description**	**Accession number**	**Max score**	**Query coverage**	**Max ident**
		3	*Fusarium tricinctum*	GQ922561	917	100%	99%
		4	Uncultured root-associated fungus	FJ362250	917	100%	99%
MT0819	HQ414598	1	*Neosetophoma samarorum*	FJ427061	950	96%	97%
		2	*Leptosphaeria* sp.	FJ228193	946	95%	98%
		3	Ascomycete sp.	AJ972820	944	100%	96%
		4	Uncultured soil fungus	DQ420932	938	100%	96%
MT0824	HQ414609	1-10	*Glomerella acutata*	AM991131, EF622179, EF622180, EF622182, EF622184, EF622186, EF622203, HM575267-HM575269	979	100%	99%
		11	Fungal endophyte	FJ449924	979	100%	99%
		12-42	*Glomerella acutata*	AB219020, AB219026, AB219031-AB219033, AB219037, AB219041, AB269940, AB269942, AM404275-AM404278, AM404280-AM404289, AY266405, DQ454019, DQ454021, EF175780, EF221831, EF221832, EU301722, EU301723	979	100%	99%
		43	*Colletotrichum acutatum*	AJ749679	979	100%	99%
MT0827	HQ414601	1	Ascomycota sp.	GU566233	1027	100%	100%
		2-15	Fungal sp.	HM122893, HM122955, HM123068, HM123090, HM123169, HM123189, HM123207, HM123243, HM123272, HM123350, HM123362, HM123491, HM123535, HM123559	1013	100%	99%
		16-24	Dothideomycetes sp.	GQ153056, GQ153093, GQ153104, GQ153105, GQ153159, GQ153163, GQ153201, GQ153213, GQ153244	1013	100%	99%

Table B.1: (continued)

Query		Best hits					
Morphotype	**Accession number**	**No.**	**Description**	**Accession number**	**Max score**	**Query coverage**	**Max ident**
		25-27	Dothideomycetes sp.	GQ153209, GQ153218, GQ153226	1008	100%	99%
MT0830	HQ414611	1	*Gibberella pulicaris*	AY188921	946	100%	99%
		2	*Gibberella pulicaris*	AY147370	938	97%	100%
		3	*Gibberella pulicaris*	EU715637	937	97%	100%
		4	*Fusarium* sp.	DQ885387	935	97%	99%
MT0831	HQ414597	1	Uncultured *Coniothyrium*	EU852367	937	99%	98%
		2	Uncultured fungus	GU564969	871	92%	98%
		3	Ascomycete sp.	AJ972820	840	98%	95%
		4	Uncultured soil fungus	DQ420932	837	99%	94%
MT0833	HQ414595	1	Uncultured endophytic fungus	EF505542	875	97%	98%
		2	Uncultured fungus	AB520483	835	100%	96%
		3	Uncultured endophytic fungus	EF505602	829	97%	96%
		4	Uncultured ectomycorrhiza	FJ266734	827	92%	98%
MT0836	HQ414603	1	Leotiomycetes sp.	FR668003	927	100%	99%
		2	Uncultured fungus	FJ820764	921	100%	99%
		3	*Lophiostoma corticola*	HM116751	915	100%	98%
		4	*Lophiostoma corticola*	EU770249	885	96%	98%
MT0837	HQ414596	1	Uncultured fungus clone	FJ820771	1033	100%	100%
		2	*Phaeosphaeria* sp.	EF432300	1033	100%	100%
		3	*Phaeosphaeria avenaria* f. sp. *triticae*	AY196988	1033	100%	100%
		4	Uncultured endophytic fungus	EF505622	1027	100%	99%
MT0838	HQ414593	1-4	Uncultured root-associated fungus	FJ362257, FJ362259, FJ362261, FJ362262	858	100%	98%
		5-6	*Phoma herbarum*	AB470824, AB470886	858	100%	98%
		7-9	Uncultured soil fungus	EU480208, EU480209, EU480210	858	100%	98%
		10	Uncultured root-associated fungus	FJ362260	852	100%	98%

Table B.1: (continued)

Query		Best hits					
Morphotype	**Accession number**	**No.**	**Description**	**Accession number**	**Max score**	**Query coverage**	**Max ident**
MT0840	HQ414602	1-7	Uncultured ascomycete	AY969329, AY969331, AY969332, AY969333, AY969337, AY969457, AY970154	806	66%	96%
		8-9	Uncultured ascomycete	AY969368, AY969991	800	66%	96%
		10	Uncultured ascomycete	AY969402	794	66%	96%
		11	*Paraconiothyrium* sp.	FN868460	777	67%	95%
MT0841	HQ414606	1	*Phialemonium* sp.	DQ286653	798	90%	96%
		2	Ascomycota sp.	HQ157157	702	100%	91%
		3	*Acremonium strictum*	AY138486	690	100%	91%
		4	*Tricladium patulum*	FJ000403	685	100%	91%
MT0842	HQ414599	1-2	*Pyrenophora teres f. maculata*	EF452470, EF452471	963	96%	99%
		3	*Pyrenophora teres*	Y08746	963	96%	99%
		4	*Pyrenophora teres f. maculata*	GU014820	958	95%	99%
		5-6	*Pyrenophora teres f. maculata*	GU014821, GU014822	952	95%	99%
MT0843	HQ414588	1	Fungal sp.	FJ228206	869	94%	99%
		2	Ascomycete sp.	AY568066	756	99%	94%
		3	Uncultured Pezizomycetes clone	AY265337	683	99%	92%
		4	*Tumularia aquatica*	AY568066	675	99%	92%
Nodulisporium sp.	HQ414613	1	*Biscogniauxia nummularia*	GQ428318	1037	100%	100%
		2	*Biscogniauxia nummularia*	EF155488	1031	100%	99%
		3	*Nodulisporium* sp.	AF280630	1021	100%	99%
		4	Fungal endophyte sp.	EU686089	1010	100%	99%
Septoria sp.	HQ414614	1	*Mycosphaerella coacervata*	EU167596	906	100%	100%
		2	*Mycosphaerella linorum*	EU167590	906	100%	100%
		3	*Septoria cucubali*	GU214698	892	100%	99%
		4	*Septoria* sp.	EF394864	883	97%	100%
Ulocladium chartarum	HQ414600	1-2	*Alternaria citri*	EF104220, AY154705	1006	100%	100%
		3	Uncultured endophytic fungus	EF504709	1000	100%	99%
		4	Uncultured endophytic fungus	EF505172	998	99%	99%
		5	*Alternaria* sp.	EU143251	998	100%	99%

Table B.1: (continued)

Query		Best hits					
Morphotype	**Accession number**	**No.**	**Description**	**Accession number**	**Max score**	**Query coverage**	**Max ident**
Xylaria sp.	HQ414612	1	Fungal sp.	GQ996170	981	99%	99%
		2	Sordariomycete sp.	EU680529	981	99%	99%
		3	Fungal endophyte sp.	FJ025307	958	99%	99%
		4	Xylariales	GQ906959	940	99%	98%
D1/D2 region							
Alternaria alternata	HQ414617	1	*Alternaria alternata*	AB566342	1021	100%	100%
		2	*Alternaria maritima*	GU456317	1021	100%	100%
		3	Uncultured fungus	AY464847	1021	100%	100%
		4	*Alternaria arborescens*	AY154706	1021	100%	100%
Aureobasidium pullulans	EF100949	1	Uncultured fungus	FJ040378	952	100%	99%
		2-13	*Aureobasidium pullulans*	FM212450, FJ743629, GQ169729, GQ169730, GQ169732-GQ169734, HM015197, HM060308, FN868849, HM146908, HM627088	952	100%	99%
		14-23	*Aureobasidium pullulans* var. *pullulans*	FJ150947, FJ150948, FJ150950-FJ150957	952	100%	99%
		24	*Kabatiella microsticta*	FJ150945	952	100%	99%
Cladosporium cladosporioides	EF100916	1	*Pseudocercospora* sp.	GU253807	931	100%	99%
		2	*Pseudocercospora fatouae*	GU253747	931	100%	99%
		3	Uncultured Ascomycota clone	HQ006144	931	100%	99%
		4	Uncultured *Cladosporium*	HQ006139	931	100%	99%
Colletotrichum gloeosporioides	HQ414623	1-5	*Glomerella acutata*	DQ286133, EF175780, FJ588238, FR716517, FR716518	1054	100%	100%
		6-7	*Colletotrichum acutatum*	AJ301983, AJ301987	1054	100%	100%
		8	*Colletotrichum gloeosporioides*	AJ301972	1054	100%	100%
		9	*Colletotrichum acutatum*	AJ301971	1054	100%	100%
Diplodina acerina	EF100959	1	*Cryptodiaporthe hystrix*	AF408344.1	938	100%	99%
		2	Uncultured fungus	FJ040375	887	100%	97%
		3	*Diplodina microsperma*	EU754159	887	100%	97%

Table B.1: (continued)

Query		Best hits					
Morphotype	**Accession number**	**No.**	**Description**	**Accession number**	**Max score**	**Query coverage**	**Max ident**
		4-7	*Apiognomonia hystrix*	EU255178, EU255179, EU255181, EU255182	887	93%	100%
Discula umbrinella	EF100931	1	Uncultured fungus	FJ040375	933	100%	99%
		2	*Apiognomonia errabunda*	AF408334	933	100%	99%
		3	*Apiognomonia errabunda*	EF100963	900	97%	99%
		4	*Gnomonia errabunda*	EU255175	881	93%	99%
Epicoccum nigrum	EF100955	1	*Epicoccum* sp.	EU715638	958	100%	100%
		2	*Epicoccum nigrum*	FM991735	958	100%	100%
		3	*Phoma* sp.	FJ430776	958	100%	100%
		4	*Sphaeriothyrium filicinum*	EU552164	958	100%	100%
Fusarium sp.	EF100914	1	*Fusarium lateritium*	AF310980	942	100%	100%
		2-3	*Fusarium lateritium*	EU715672, EU715687	931	100%	99%
		4	*Fusarium flocciferum*	GQ505465	931	100%	99%
		5	*Fusarium* sp.	GQ505451	931	100%	99%
Fusicladium fraxini	EF656058	1	*Venturia fraxini*	EU035457	971	100%	99%
		2	*Gibbera conferta*	GU301814	937	100%	98%
		3	*Venturia maculiformis*	EU035463	931	100%	97%
		4	*Venturia macularis*	EU035462	931	100%	97%
MT0503	EF100962	1	*Sordaria tomento-alba*	AY681161	937	100%	100%
		2	*Asordaria prolifica*	AY681140	927	100%	99%
		3	*Chalara sessilis*	FJ176262	925	100%	99%
		4	*Neurospora tetrasperma*	AY681159	925	100%	99%
MT0513	EF100942	1-2	*Nemania diffusa*	DQ840076, AB376826	892	100%	100%
		3	*Xylaria* sp.	AB512403	881	100%	99%
		4	Xylariaceae sp.	AB376754	881	100%	99%
		5-8	Xylariaceae sp.	AB376758, AB376761, AB376774, AB376788	875	100%	99%
MT0516	HQ414622	1-6	*Glomerella acutata*	DQ286131, DQ286133, EF175780, FJ588238, FR716517, FR716518	1027	100%	100%
		7-9	*Colletotrichum acutatum*	AJ301983, AJ301987, AF275542	1027	100%	100%

Table B.1: (continued)

Query		Best hits					
Morphotype	**Accession number**	**No.**	**Description**	**Accession number**	**Max score**	**Query coverage**	**Max ident**
		10	*Colletotrichum gloeosporioides*	AJ301972	1027	100%	100%
		11	*Colletotrichum acutatum*	AJ301971	1027	100%	100%
MT0519	EF100945	1	*Pseudocercosporella fraxini*	GU214682	904	100%	99%
		2	*Mycosphaerella* sp.	EF619924	904	100%	99%
		3	Fungal sp.	FJ948158	875	96%	99%
		4	*Pseudotaeniolina globosa*	HQ115663	777	100%	95%
MT0520	EF100934	1	Xylariales sp.	GQ906958	908	100%	98%
		2-3	*Thuemenella cubispora*	EF562508, EF562509	873	100%	97%
		4	*Hypoxylon monticulosum*	DQ840067	873	100%	97%
		5	*Annulohypoxylon nitens*	AB376819	862	100%	97%
		6	*Annulohypoxylon moriforme*	DQ840057	862	100%	97%
MT0521	HQ414620	1	*Didymella exitialis*	EU167564	1015	98%	100%
		2	*Ascochyta hordei* var. *hordei*	EU754134	988	98%	99%
		3	Uncultured ascomycete	EU489950	963	98%	98%
		4	Fungal endophyte	EF100936	958	93%	100%
MT0522	EF100946	1	*Ramularia* sp.	EU019285	921	100%	99%
		2	*Venturia hanliniana*	AB100681	921	100%	99%
		3-47	*Mycosphaerella punctiformis*	DQ470968, EU167569, EU343407, EU343412, EU343414-EU343416, EU343418-EU343421, EU343424-EU343432, EU343434-EU343447, EU343450-EU343459, AY490776	904	100%	99%
		48-56	*Mycosphaerella punctiformis*	EU343406, EU343408-EU343411, EU343413, EU343433, EU343448, EU343460	898	100%	98%
MT0523	EF100966	1-17,19-23	*Chaetomium globosum*	GU183111, FN868871, AB449672-AB449684, AB449687, AB449688	919	100%	99%
		18	*Chaetomium caprinum*	GU183107	919	100%	99%

Table B.1: (continued)

Query		Best hits					
Morphotype	**Accession number**	**No.**	**Description**	**Accession number**	**Max score**	**Query coverage**	**Max ident**
		19-23	*Chaetomium globosum*	AF286403, AY545729, AB292591, GQ221865, EU715603	919	100%	99%
		24-25	*Chaetomium globosum*	AB449685, AB449686	913	100%	99%
MT0528	EF100932	1	*Diplodina microsperma*	EU754159	944	100%	100%
		2	*Discula campestris*	AF277140	944	100%	100%
		3	*Cryptodiaporthe salicella*	AF408345	944	100%	100%
		4-5	*Plagiostoma euphorbiae*	AF277131, AF408382	938	100%	99%
MT0529	EF100936	1	*Didymella exitialis*	EU167564	958	100%	100%
		2	*Ascochyta hordei* var. *hordei*	EU754134	935	100%	99%
		3	Uncultured ascomycete	EU489950	906	100%	98%
		4	*Phoma paspali*	GU238125	894	100%	97%
MT0530	HQ414619	1	*Pleospora herbarum* var. *herbarum*	AF382386	1031	99%	100%
		2	*Dendryphiella salina*	EU848587	1025	99%	99%
		3-7	*Bipolaris zeicola*	GQ167205, GQ167206, GQ167210, GQ253957, GQ253958	1019	99%	99%
		8-9	Uncultured ascomycete	EU489968, EU490039	1019	99%	99%
MT0532	EF656059	1	*Pleurophoma cava*	EU754199	1096	97%	100%
		2-3	*Pyrenochaeta cava*	GQ387607, EU754198	1090	97%	99%
		4	*Ochrocladosporium frigidarii*	FJ755255	1052	99%	98%
		5	*Phoma* sp.	AY293785	1052	99%	98%
MT0533	EF100948	1	*Pleospora halophila*	AY849955	958	100%	100%
		2	*Dendryphiella salina*	EU848587	952	100%	99%
		3-4	*Pleospora herbarum*	FN868852, GU238160	952	100%	99%
		5	Pleosporaceae sp.	EF100928	952	100%	99%
MT0534	EF100958	1-2	*Phoma macrostoma* var. *macrostoma*	GU238098, GU238099	952	100%	99%
		3-4	*Phoma macrostoma* var. *incolorata*	GU238096, GU238097	952	100%	99%
		5-6	*Phoma digitalis*	GU238066, GU238067	952	100%	99%
		7	*Didymella fabae*	FJ755246	952	100%	99%

Table B.1: (continued)

Query		Best hits					
Morphotype	**Accession number**	**No.**	**Description**	**Accession number**	**Max score**	**Query coverage**	**Max ident**
MT0535	HQ414618	1	*Ulocladium* sp.	FN868846	1002	99%	100%
		2-8	*Alternaria tenuissima*	FJ755190-FJ755194, FJ755196, FJ755240	1002	99%	100%
		9-10	*Alternaria alternata*	FJ890363, FJ890364	1002	99%	100%
		11-14	Uncultured ascomycete	EU489911, EU489925, EU489956, EU490141	1002	99%	100%
MT0536	EF100944	1	*Mycosphaerella microsora*	EU167599	921	100%	99%
		2	*Passalora* sp.	GU214460	915	100%	99%
		3-4	*Passalora bellynckii*	GU214454, GQ852618	915	100%	99%
		5	*Mycosphaerella keniensis*	DQ246259	910	100%	99%
MT0538	EF100923	1	*Chalastospora gossypii*	FN868853	942	100%	99%
		2	*Alternaria malorum*	GU183130	942	100%	99%
		3	*Chalastospora obclavata*	FJ839651.1	942	100%	99%
		4-10	*Chalastospora gossypii*	FJ839644-FJ839650	942	100%	99%
MT0540	EF100926	1	*Alternaria triticina*	AY154695	958	100%	100%
		2-3	*Lewia infectoria*	AY154690, AY154692	958	100%	100%
		4	*Chalastospora gossypii*	FN868853	946	100%	99%
		5	*Alternaria malorum*	GU183130	946	100%	99%
Periconia cookei	EF100937	1	Uncultured fungus	AY464861	935	100%	99%
		2	*Sporidesmium tengii*	DQ408559	850	100%	96%
		3-4	*Periconia* sp.	HQ130666, HQ130679	819	100%	95%
		5-6	*Periconia* sp.	HQ130681, HQ130683	813	100%	94%
Phoma sp. 1	HQ414621	1	*Peyronellaea lethalis*	GU238010	988	100%	100%
		2-4	*Phoma* sp.	HQ130716, HQ130718, HQ130720	983	100%	99%
		5-7	*Phoma subherbarum*	GU238144-GU238146	983	100%	99%
		8-9	*Phoma pedeiae*	GU238126, GU238127	983	100%	99%
Phoma sp. 2	EF100921	1-2	*Phoma macrostoma* var. *macrostoma*	GU238098, GU238099	958	100%	100%
		3-4	*Phoma macrostoma* var. *incolorata*	GU238096, GU238097	958	100%	100%
		5-6	*Phoma digitalis*	GU238066, GU238067	958	100%	100%
		7	*Didymella fabae*	FJ755246	958	100%	100%

Table B.1: (continued)

Query		Best hits					
Morphotype	**Accession number**	**No.**	**Description**	**Accession number**	**Max score**	**Query coverage**	**Max ident**
Phomopsis sp.	EF100915	1,3,4	*Phomopsis* sp.	HQ130721, AB247162, AB245078	940	100%	100%
		2	*Diaporthe eres*	HQ115664	940	100%	100%
		5	*Diaporthe medusaea*	AB245074	940	100%	100%
		6	*Diaporthe* sp.	DQ377874	940	100%	100%
Pyrenophora erythrospila	EF100965	1	*Drechslera* sp.	FJ236017	958	100%	100%
		2	*Drechslera erythrospila*	EU552124	958	100%	100%
		3	*Pyrenophora tetrarrhenae*	AY849962	937	100%	99%
		4	*Pyrenophora seminiperda*	AY849961	935	100%	99%
Sordaria fimicola	EF100924	1	*Sordaria* cf. *macrospora*	DQ008062	937	100%	100%
		2	*Sordaria fimicola*	AY681160	937	100%	100%
		3	*Sordaria fimicola*	FN868870	931	100%	99%
		4	*Sordaria humana*	EF100957	931	100%	99%
Sordaria humana	EF100957	1	*Sordaria fimicola*	FN868870	937	100%	100%
		2	*Sordaria macrospora*	AY346301	937	100%	100%
		3	*Sordaria fimicola*	AY545728	937	100%	100%
		4	*Asordaria sibutii*	AY681146	937	100%	100%
Sporormiella minima	EF100938	1	*Preussia minima*	GQ203744	948	100%	99%
		2	*Preussia persica*	GQ292752	942	100%	99%
		3	*Preussia* sp.	FJ430777	942	100%	99%
		4-5	Dothideomycetes sp.	GQ153229, GQ153131	937	100%	99%
Xylaria sp.	EF100932	1	Xylariaceae sp.	AB376781	912	100%	98%
		2-6	*Xylaria curta*	EU715621, EU715630, EU715623-EU715625	906	100%	98%
		7-10	Xylariaceae sp.	AB376753, AB376764, FJ425704, AB465203	906	100%	98%
		11	*Xylaria* sp.	AB376724	906	100%	98%

Appendix C - Isolation frequencies

Table C.1: Number of leaves that were infected by a single fungal species in the light and the shade crown of different host tree species in September 2005

	Acer pseudoplatanus		*Fraxinus excelsior*		*Quercus robur*		*Tilia cordata*		
	light	shade	light	shade	light	shade	light	shade	total
Alternaria alternata	4	2	11	8	2	1	9	2	39
Aureobasidium pullulans	4	4	2	4	3	5	6	6	34
Cladosporium cladosporioides	6	5	4	3	2	1	3	7	31
Phoma sp. 2		3		3	1	3	4	4	18
Phomopsis sp.		3	1	6			3	5	18
Sordaria fimicola	5	2	1		5	2	2	1	18
Phoma sp. 1	5	2	4	1		2		3	17
MT0523	1	2		2	2	3	5	1	16
Pleospora herbarum	2	1	8	3				2	16
Epicoccum nigrum	3	1	2				5		11
Colletotrichum gloeosporioides		1		4		2		3	10
Xylaria sp.	2	1		1	1	2	1		8
Diplodina acerina	2	5							7
MT0513	1	1				3		2	7
MT0522	1	1		1			1	3	7
Fusicladium fraxini				4		1			5
MT0516				1				3	4
Discula umbrinella					1	2			3
Nigrospora sphaerica					1	1		1	3
MT0521		1		1					2
MT0530			1				1		2
Periconia cookei	1				1				2
Sordaria humana	1	1							2
Fusarium sp.			1					1	2
Aspergillus niger							1		1
MT0519						1			1
MT0520						1			1
MT0526							1		1
MT0528						1			1
MT0529						1			1
MT0532		1							1
MT0533				1					1
MT0534	1								1
MT0535			1						1

Table C.1: (continued)

	Acer pseudoplatanus		*Fraxinus excelsior*		*Quercus robur*		*Tilia cordata*		
	light	shade	light	shade	light	shade	light	shade	total
MT0536								1	1
MT0537						1			1
MT0538			1						1
Pyrenophora erythrospila	1								1
MT0503		1							1
Sporormiella minima					1				1
undetermined	7	0	8	6	4	1	15	4	45
total	47	38	45	49	24	34	57	49	343

Table C.2: Number of fungal colonies per morphotype that were isolated from leaflets of fraxinus *Fraxinus excelsior* in every single month and forest layer during the vegetation period 2008

	light crown						shade crown						understorey						
	May	Jun	Jul	Aug	Sep	Oct	May	Jun	Jul	Aug	Sep	Oct	May	Jun	Jul	Aug	Sep	Oct	total
Alternaria infectoria			5	14	27	10				10	15	14			8	14	19	12	148
Alternaria alternata			5	20	26	13			3	5	10	14		1	1	9	9	11	127
Xylaria sp.				5					7	27	6	4			2	7	1	1	60
Ramularia endophylla						1	1		1		5	11		1	4	8	8	13	53
Septoria sp.						2					2	3			2	12	18	13	52
Fusicladium fraxini				5	1	5		1	4	2	5	4	1	10	4	1			43
Cladosporium herbarum			1	3	6	1			2		2	13		1	1	3	5	2	40
Phoma sp.						1			1		5	10			3	3	8	9	40
Colletotrichum gloeosporioides											1	7		1	3		10	14	36
Phomopsis sp.									1	1	4	11		2	2	5	3	2	31
Fusarium lateritium	1											1			4	3	10	6	25
Aureobasidium pullulans		1	1		2			2	1		1	3	1		2	3	2	4	23
Coniothyrium sp.											2	1		1	1	5	5	4	19
Pleospora herbarum			2	2	3	2			1	1	2					1	1		15
Basidiomycetuous yeasts									3	1		2				1		5	12
MT0819									1							3	3		7
Acrodontium sp.										1	1			3	1				6
Epicoccum nigrum				1	1							1					2	1	6
MT0815															1		2	3	6
Nodulisporium sp.			1	2		1			2										6
Diplodina acerina														1	1	1	1	1	5
Discula umbrinella													3	2					5

Table C.2: (continued)

	light crown						shade crown						understorey						
	May	Jun	Jul	Aug	Sep	Oct	May	Jun	Jul	Aug	Sep	Oct	May	Jun	Jul	Aug	Sep	Oct	total
Monodictys sp.									1	1					1		1	1	5
MT0824										1	1					1	1		4
MT0812									1			2							3
Chaetomium sp.		1															1		2
Cladosporium oxysporum											1							1	2
MT0801														2					2
MT0817																2			2
MT0825																2			2
MT0826												2							2
Ulocladium chartarum										1							1		2
Lecythophora hoffmannii																1			1
MT0807														1					1
MT0813									1										1
MT0821														1					1
MT0822																	1		1
MT0827															1				1
MT0830																1			1
MT0831																1			1
MT0832															1				1
MT0833															1				1
MT0836																1			1
MT0837																	1		1
MT0838																		1	1
MT0840																	1		1
MT0841																1			1
MT0842											1								1
MT0843										1									1
Nigrospora sphaerica												1							1
undetermined			1		1	1			4	1	6	1		1	3	1	5	11	36
total	1	2	16	52	67	37	1	3	34	53	70	105	5	28	47	90	119	115	845

Table C.3: Number of fungal colonies per morphotype that were isolated from leaflets of *Fraxinus excelsior* per tree over time for different treatments of rain exclusion in 2009

Sampling date	19.05.09		02.06.09	16.06.09			30.06.09	14.07.09				28.07.09	11.08.09				25.08.09	14.09.09				
Trees	1-5	26-30	1-5	1-5	6-10	26-30	6-10	1-5	6-10	11-15	26-30	11-15	6-10	11-15	16-20	26-30	16-20	11-15	16-20	21-25	26-30	total
Fusicladium fraxini		9				11	3	6	1		21	1	7	1		22	6	5	6		15	114
Alternaria infectoria						1	2	5	7		10	1	5	5		9	4	18	15		16	98
Alternaria alternata						2	1	5	4		7	1	7	2		5	4	15	13		11	77
Cladosporium spp.								2			3		3	1		3		13	15		9	49
MT0901		2	1			2		3			5		1	2		5		1			8	30
Septoria sp.							1	1	1	1	1			4	1	1	1		8	7	1	28
Xylaria sp.					1			2					4	1	1		1	2	1		3	16
Colletotrichum gloeosporioides								1					1			3		3	4		3	15
Pleospora herbarum								1		1	1	1				2		1	2		4	13
Aureobasidium pullulans				1				2					3						1			7
Lecythophora hoffmannii					1		1	1										1	1	2		7
Phoma sp.									1			1		1		3		1				7
Phomopsis sp.			1										2	1				1	1		1	7
Epicoccum nigrum		1											1							3		5
MT0909							1		1		1		2									5
MT0906								1				1									2	4
MT0907																			2	1		3
Nodulisporium sp.											1									1	1	3
Sordaria fimicola																		1			2	3
Acrodontium sp.													1						1			2
Basidiomycetuous yeasts								1					1									2
MT0903														2								2
MT0908																				2		2
Chaetomium sp.	1																					1
MT0902		1																				1
MT0905																					1	1
MT0910								1														1
MT0912								1														1
MT0913																					1	1
MT0914																				1		1
MT0915								1														1
MT0916													1									1

Table C.3: (continued)

Sampling date	19.05.09		02.06.09	16.06.09			30.06.09	14.07.09				28.07.09	11.08.09				25.08.09	14.09.09				
Trees	**1-5**	**26-30**	**1-5**	**1-5**	**6-10**	**26-30**	**6-10**	**1-5**	**6-10**	**11-15**	**26-30**	**11-15**	**6-10**	**11-15**	**16-20**	**26-30**	**16-20**	**11-15**	**16-20**	**21-25**	**26-30**	**total**
MT0917																					1	1
MT0918			1																			1
MT0919								1														1
MT0920						1																1
MT0921											1											1
MT0922																				1		1
MT0923											1											1
MT0924						1																1
MT0925														1								1
MT0926																1						1
MT0927						1																1
Nigrospora sphaerica																				1		1
Phialophora sp.											1											1
Ramularia endophylla																					1	1
undetermined						1		3			1			1		3		3	3	3	6	24
total		13	3	1	2	20	9	38	15	2	54	6	39	22	2	57	16	65	73	22	86	546

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