Endophytic Fungi in Elms

Implications for the Integrated Management of Dutch Elm Disease

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Cover: Endophytic fungi isolated from *Ulmus* spp. and other microorganisms; below: endophyte *Monographella nivalis* var. *neglecta* (left side) inhibiting growth of Dutch elm disease pathogen *Ophiostoma novo-ulmi* (right side).
(Photos: K. Blumenstein)

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Abstract

Integrated pest management calls for new biocontrol solutions in management of forest diseases. Endophytic fungi that are commonly found in tree tissue may have potential in biocontrol. However, the links between endophyte status and disease tolerance are still unclear, and we know little about the mechanisms by which the endophytes can influence tree pathogens.

The first goal of the thesis was to compare the endophyte status in elm (*Ulmus* spp.) trees with low vs. high susceptibility to Dutch elm disease (DED), caused by *Ophiostoma* fungi, and to find correlations between endophytes and the susceptibility pattern of the trees. The second goal was to investigate the potential mechanisms of antagonism by the endophytes towards the pathogen. Thus, endophytes were isolated from leaves, bark and xylem of elms that differed in DED susceptibility. The isolates were screened for their potential to counteract the pathogen in dual cultures. Selected strains were investigated using Phenotype MicroArrays to obtain the substrate utilization profiles that reflect the endophytes' ability to compete with the pathogen for a nutritional niche. To test for a protective effect against the disease, promising isolates were injected into young elms. Preliminary analyses were done to identify the extracellular chemicals that some of the endophytes released into the growth medium.

The results showed that the frequency and diversity of endophytes was higher in xylem of elms with high susceptibility to DED. Some endophytes deadlocked the pathogen with extracellular chemicals *in vitro*, while others had a faster growth rate. Several endophytes were able to utilize substrates more effectively than the pathogen. A preventive treatment with endophytes protected elms against DED, but the effect was unstable across years. Bioactive fungal extracts had a complex chemical profile, and the individual compounds in the extracts remain to be identified. Because endophytes antagonized the pathogen through different mechanisms, I suggest that an endophyte-based biocontrol of DED could be best achieved through a synergistic effect of several endophyte strains.

Keywords: Biocontrol, Phenotype MicroArray, Nutrient utilization, Fungus-fungus interactions, Antagonism, Niche differentiation, *Ophiostoma* spp., *Ulmus* spp.

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Dedication

To my husband Michael who supported me in every possible way and to our son Kristian, my little sunshine.

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Martín, J.A., Witzell, J., Blumenstein, K., Rozpedowska, E., Helander, M., Sieber, T., Gil, L. (2013). Resistance to Dutch elm disease reduces presence of xylem endophytic fungi in elms (*Ulmus* spp.). *PLoS ONE* 8(2), e56987. doi: 10.1371/journal.pone.0056987.

II Martín, J.A., Macaya-Sanz, D., Witzell, J., Blumenstein, K., Gil, L. (2015). Strong *in vitro* antagonism by elm xylem endophytes is not accompanied by temporally stable *in planta* protection against a vascular pathogen under field conditions. *European Journal of Plant Pathology*, 142(1), pp. 185-196. doi: 10.1007/s10658-015-0602-2.

III Blumenstein, K., Macaya-Sanz, D., Martín, J.A., Albrectsen, B.R., Witzell, J. (2015). Phenotype MicroArrays as a complementary tool to next generation sequencing for characterization of tree endophytes. *Frontiers in Microbiology*, 6:1033. doi: 10.3389/fmicb.2015.01033.

IV Blumenstein, K., Albrectsen, B.R., Martín, J.A., Hultberg, M., Sieber, T.N., Helander, M., Witzell, J. (2015). Nutritional niche overlap potentiates the use of endophytes in biocontrol of a tree disease. *BioControl*, 60(5), pp.655–667. doi: 10.1007/s10526-015-9668-1.

V Papers I-IV are reproduced with the permission of the publishers.

The contribution of Kathrin Blumenstein (KB) to the papers included in this thesis was as follows:

I KB was involved in developing the idea and hypothesis with Juan A. Martín and Johanna Witzell. She contributed with 10% to the planning, with 20% to the laboratory work, with 10% to the summary and analysis of results and with 15% to writing the manuscript.

II KB contributed with 15% in developing the idea and hypothesis, 10% in planning of the work, 25% of the laboratory work, 10% of the analysis and summary of the results, and 15% with writing the manuscript.

III The idea and hypothesis were developed by KB to 40% and 65% of the planning of the work. KB performed 60% of the laboratory work. She did 50% of the analysis and summary of the results. As one of the two first authors, KB wrote 45% of the manuscript and contributed to 30% in the correspondence with the journal.

IV KB developed 70% of the idea and hypothesis and 95% of the planning of the work. All laboratory work was completed by KB. 80% of the analysis and summary of results was done by KB and 95% of writing the manuscript. KB was responsible for the entire correspondence with the journal.

Abbreviations

AWCD	Average well colour development
DC	Dual culture
DDT	Dichlorodiphenyltrichloroethane
DED	Dutch elm disease
DNA	Deoxyribonucleic acid
IPM	Integrated Pest Management
ISR	Induced systemic resistance
ITS	Internal transcribed spacer region
LC-MS	Liquid chromatography-mass spectrometry
M(F)	*Ulmus minor*, field population
M(R)	*Ulmus minor*, resistant
M(S)	*Ulmus minor*, susceptible
MEA	Malt extract agar
NGS	Next-generation-sequencing
NOI	Niche overlap index
OD	Optical density
ONU	*Ophiostoma novo-ulmi*
OU	*Ophiostoma ulmi*
PCR	Polymerase chain reaction
PCA	Principal Component Analysis
P(R)	*Ulmus pumila*, resistant
PM	Phenotype MicroArray
rRNA	Ribosomal ribonucleic acid
SAR	Systemic acquired resistance
SC	Single culture

1 Introduction

In the future, a changing climate may increase the frequency and intensity of natural disturbances in forest ecosystems, some of which may involve damage due to pests and pathogens (Dale *et al.*, 2001). In addition, changes in crop management practices such as intensification may create new conditions that promote chronic and epidemic diseases or pests in plant systems (Anderson *et al.*, 2004). The intensive global trade of plants and plant-based materials provides rapid dispersal routes for pathogens (viruses, bacteria, fungi, oomycetes) and insect herbivores (Boyd *et al.*, 2013) to new habitats, creating opportunities for these organisms to establish and thrive in geographic areas that have been unreachable earlier (Santini *et al.*, 2013). Thus, the need for sustainable tree and forest protection solutions is likely to increase in the coming decades, especially as society moves towards a bio-based economy and aims to reduce the use of environmentally hazardous pesticides and fungicides (Tiilikkala *et al.*, 2010).

In forest protection, great expectations have been placed on breeding resistant plant cultivars using traditional selection processes (Martín *et al.*, 2015a) or genetic modifications (Gartland *et al.*, 2000; Harfouche *et al.*, 2011) and on boosting plant resistance through the use of chemical or biological inducers (Blodgett *et al.*, 2005; Eyles *et al.*, 2010; Albrectsen & Witzell, 2012). However, none of these alone may eliminate all plant protection problems that result from dynamic, biological interactions between the plants and their microbial biota. To acknowledge this, the concept of *integrated pest management (IPM)* has been gaining acceptance as a sustainable option for crop protection, and has been embraced by EU (ECPA, 2015). Central to IPM are ecologically-based control strategies, relying on natural mortality factors and broad spectrum of control actions (Flint & van den Bosch, 1981). Thus, the more control method options there are for a certain disease, the better are the chances to control it. In order to ensure a multitude of effective options, new

control methods, based on biological mechanisms, need to be developed continuously. A potential source of new control methods offering crop protection without toxic chemicals is the use of beneficial organisms, such as symbiotic fungi and bacteria that can directly reduce the negative effects of pests or pathogens, e.g., through chemical antagonism or competition (Arnold *et al.*, 2003; Bale *et al.*, 2008; Albrectsen *et al.*, 2010).

The research described in this thesis aims to add to the knowledge-base regarding the use of beneficial organisms as tools in integrated management of the diseases of forest trees. Specifically, the thesis addresses the role of a group of potentially beneficial fungi, *endophytes*, in resistance of elm (*Ulmus* spp.) trees to a vascular disease, Dutch elm disease (DED), caused by pathogenic *Ophiostoma* species. Particular attention is given to the potential mechanisms of antagonism between the putatively beneficial elm endophytes and *Ophiostoma*-pathogens.

1.1 Endophytes

Symbiotic, endophytic bacteria and fungi colonize the internal tissue of their host plant, either intercellularly or intracellularly, without inducing external signs of infection in the host (Carroll, 1988; Clay, 2004; Schulz & Boyle, 2006). Most plant species worldwide are considered to host at least one endophytic organism (Strobel *et al*., 2004; Rosenblueth & Martínez-Romero, 2006).

Endophytic bacteria are found in roots, stems, leaves, seeds, fruits, tubers, ovules, and legume nodules (Hallmann *et al*., 1997; Sturz *et al*., 1997). The population density of endophytes is highly variable, depending mainly on the bacterial species and host genotypes but also in the host developmental stage, inoculum density, and environmental conditions (Tan *et al*., 2003). In most plants, roots have the higher numbers of bacterial endophytes compared with above-ground tissues (Rosenblueth & Martínez-Romero, 2006). Many seeds carry a diversity of endophytic bacteria (Hallmann *et al*., 1997), and plants that propagate vegetatively (such as potatoes or sugarcane) can transmit their endophytes to the next generation. Bacterial endophytes do not inhabit living vegetal cells, but colonize intercellular spaces and xylem vessels (Ryan *et al*., 2008). Endophytic bacteria can establish a mutualistic association with their hosts (Hallmann *et al*., 1997), and increase crop yields, degrade contaminants and produce novel substances or fixed nitrogen (Rosenblueth & Martínez-Romero, 2006). Endophytic bacteria can promote plant growth through nitrogen fixation (e.g., Sevilla *et al*., 2001), production of phytohormones, by enhancing nutrient availability (Sturz *et al*., 2000; Verma *et*

al., 2001; Lee *et al*., 2004; Pirttilä *et al*., 2004) or by biocontrol of phytopathogens in the root zone (through production of antifungal or antibacterial agents, siderophore production, nutrient competition and induction of systematic acquired host resistance or immunity) or in the vascular system (Hallmann *et al*., 1997). Competition experiments with bacterial endophytes have shown that some endophytes are more aggressive colonizers and displace others (Rosenblueth & Martínez-Romero, 2006). It is unknown if bacterial communities inside plants interact, and it has been speculated that beneficial effects are the combined effect of their activities (Rosenblueth & Martínez-Romero, 2006). A future crop protection application may be to use genetically engineered endophytes with biological control potential in agricultural crops. The endophytic bacteria *Herbaspirillum seropedicae* and *Clavibacter xylii* have been genetically modified to produce and excrete the δ-endotoxin of *Bacillus thuringensis* to control insect pests (Turner *et al*., 1991; Downing *et al*., 2000).

Fungi of several clades colonize plant roots, including arbuscular mycorrhizal fungi of the phylum Glomeromycota which are obligate biotrophs (Harrison, 2005). Nevertheless, mycorrhizae are distinguished from endophytic fungi by lacking external hyphae or mantels (Saikkonen *et al*., 1998) and are therefore not further described here.

Roots of terrestrial plants are often associated with nonmycorrhizal, root-endophytic fungi, which have been suggested to impact plant growth and development (bioregulation), plant nutrition (biofertilisation) and plant tolerance and resistance to abiotic and biotic stresses (bioprotection) (Borowicz, 2001; Franken, 2012). Dark septate endophytes are a group of root endophytes (Jumpponen & Trappe, 1998), which contain mycorrhiza-forming and nonmycorrhizal root colonisers and occur worldwide (Weiß *et al*., 2011). One of the best studied members is the species *Piriformospora indica* (Franken, 2012). Its plant growth-promoting effects have been revealed for various hosts, and its application to plant production has been proposed (Varma *et al*., 1999). For instance, barley plants colonised by *P. indica* were more resistant to pathogens and more tolerant to salt stress and showed higher yield (Waller *et al*., 2005). It has been suggested that *P. indica* may protect a wide variety of plants against fungal pathogens: root pathogens might be directly inhibited by antagonistic activities of the endophyte, which is able to produce ROS (reactive oxygen species) and synthesize antioxidants (Waller *et al*., 2005). It has been demonstrated that *P. indica* root colonisation systemically induces resistance, which may provide protection against pathogens in the above-ground plant parts (Waller *et al*., 2005).

In forest trees, asymptomatic infections by fungal endophytes have been found to be ubiquitous in leaves, bark, wood, seeds, and roots (Carroll, 1988; Petrini & Fisher, 1990; Saikkonen, 2007; Sieber, 2007). These infections result from wind- or water dispersed spores, originating from the environment (Arnold *et al*., 2003; Clay, 2004). The environmental transmittance of endophyte infections is referred to as horizontal transmission, as opposite to the vertical transmission through seeds which is commonly observed in grasses (Petrini *et al*., 1992; Clay & Holah, 1999). Taxonomically, the tree endophytes are with few exceptions Ascomycetes, but within the Ascomycetes they are very diverse (Petrini & Fisher, 1986). In general, endophyte communities of forest trees seem to be highly diverse and only a fraction of the endophyte diversity has probably been described so far (Unterseher, 2011).

In the internal tissues of their host trees, endophytic fungi are bound to interact with other microbes, including con-specific, saprophytes as well as pathogens, which all co-habit the same hosts at the same time. Competitive interactions between fungi are likely to be common, and can be either for the primary resource capture (colonization of unoccupied habitat) or the secondary resource capture (colonization of habitat that is already occupied) (Rayner & Webber, 1984). A species can persist on a resource at a stable level, or in competitive exclusion where the winner is the species that can survive on the lowest level of a resource (Bleiker & Six, 2009). These interactions are complex to investigate *in vivo*, because endophytic infections are usually highly localized (Carroll, 1988; Petrini *et al*., 1992; Saikkonen *et al*., 1998) and non-systemic, because they are restricted to disjunctive microthalli which may consist of only a few cells (Stone, 1987). Due to the horizontal spreading, the colonization of perennial tissues is a result of seasonal accumulation of local and independent fungal colonies (Helander *et al*., 1993; Stone & Petrini, 1997). The infection patterns of endophytes in trees are variable in dispersion and density and depend on the availability, viability and infection success of fungal spores, which in turn is influenced by the surrounding vegetation and topography, plant density and architecture, weather conditions and the microclimate within or near the plant (Helander *et al*., 1994; Saikkonen *et al*., 1996).

The interaction between an endophyte and its host plant are described as non-static (Saikkonen *et al*., 1998). Some endophytes have the ability to establish a mutualistic or commensalistic symbiosis with their hosts (Sieber, 2007). After the weakening of the host by any abiotic or biotic stressor, the stage may shift to be parasitic or saprophytic (Promputtha *et al*., 2007; Saikkonen, 2007; Rodríguez *et al*., 2011). Some fungi might be pathogenic on the main host species, but symptomless endophytes on other hosts. This

differential behaviour may result from differences in fungal gene expression in response to the plant, or from the differences in the ability of the plant to respond to the fungus (Sieber, 2007).

The high diversity and omnipresence of the symbiotic endophytes in trees (Arnold *et al*., 2000) has raised questions about the consequences of these infections to trees. Endophytes may affect population dynamics and community structure of plants and their associated species (Saikkonen *et al*., 1998). In the internal tissues of the hosts, the endophytes are bound to be affected by the chemical and physical traits of the host cells. On the other hand, it is considered that the endophytic fungi have a potential to influence the physiology, metabolism, and ecological interactions of trees in various ways (Witzell *et al*., 2014). The symptomless endophytes may provide protection against drought tolerance, or enhance the growth of the host plants (Redman *et al*., 2001). While the evidence is not explicit, the results of several studies indicate that the presence, diversity, or frequency of tree endophytes may be linked to phenotypic patterns of defence or expression of resistance against natural enemies in forest trees (Bettucci & Alonso, 1997; Arnold *et al*., 2003; Gennaro *et al*., 2003; Ragazzi *et al*., 2003; Clay, 2004; Santamaría & Diez, 2005; Ganley *et al*., 2008; Mejía *et al*., 2008; Albrectsen *et al*., 2010). Specifically, endophytes may function as competitors or antagonists to forest tree pathogens (Arnold *et al*., 2003; Mejía *et al*., 2008) that occupy the same tissues. The antagonistic potential of endophytes against pathogenic fungi indicates that endophytes may be used as IPM tools in forest protection (Newcombe, 2011). To put such prospects into practice, however, necessitates thorough understanding of the mechanisms of interaction between endophytes, their hosts and the targeted pathogens.

1.2 Overview of biological control systems

The term biological control refers to the use of naturally occurring or introduced microbial antagonists, so called biological control agents, to suppress diseases by reducing the amount or the effect of pathogens, or the use of host specific pathogens to control weed populations (Stirling & Stirling, 1997; Pal & McSpadden Gardener, 2006). Natural products extracted from various sources can also be considered as biocontrol agents. Such products can be mixtures of natural ingredients with specific activities on the host or the target pest or pathogen (Pal & McSpadden Gardener, 2006). Biological control can result from many different types of interactions between organisms, nevertheless, in all cases, pathogens are antagonized by the presence and

activities of other organisms or their extracellular products (Stirling & Stirling, 1997; Pal & McSpadden Gardener, 2006).

Biological control systems are based on the three following antagonism types: competition for nutrients or space, the production of antimicrobial substances, or parasitism (hyperparasitism or mycoparasitism) and predation. Most types of antagonism involve the synergistic action of several mechanisms (Stirling & Stirling, 1997). The degree of success of the different biocontrol agents or control systems is dependent on the type of pathogen that is targeted, but also the mode of dispersion of the agent and its distribution and survival within the host. The production of antimicrobial substances is common for microorganisms as most of them produce secondary metabolites. These compounds are often toxic to other microorganisms (e.g., antibiotics and mycotoxins). They may be volatile or non-volatile (Strobel & Daisy, 2003; Strobel *et al*., 2004). Some of the most studied biocontrol agents are found in the genus *Trichoderma*, and have the ability to penetrate resting structures such as sclerotia or may parasitize growing hyphae by coiling around them. Formulations of some species are commercially available and are used to control fungal pathogens in the soil and on aerial plant surfaces (Stirling & Stirling, 1997).

One of the best known examples of a commercial biological control of a forest pathogen by an antagonistic fungus is the use of *Phlebiopsis gigantea* (Fr.) Jül in control of butt and stem rot disease of conifer trees, caused by the soil-borne pathogens *Heterobasidion parviporum* Niemelä & Korhonen and *H. annosum sensu stricto* (s.s.) (Fr.) Bref. (Korhonen, 1978). To prevent infection the biological agent *P. gigantea* (Fr.) Jül, or urea, are commonly applied to freshly cut stumps. The biocontrol fungus then colonises the whole stump faster than *Heterobasidion*, which is a poor competitor. Another example of biological control of a tree disease, not related to forestry but with importance to the fruit industry, involves antagonism of the fireblight pathogen *Erwinia amylovora* by a closely related bacterium *Erwinia herbicola* (Vanneste *et al*., 1992). The pathogen overwinters in cankers and inoculum is carried to flowers by insects and rain splash. Blossom infection results in reduced yields because fruit-bearing spurs are killed. Application of *E. herbicola* is done as a spray during flowering. Initial results suggest that it may also be possible to utilise bees to disperse the antagonist to blossoms (Stirling & Stirling, 1997).

Most broadly, biological control is the suppression of damaging activities of one organism by one or more other organisms, often referred to as natural enemies. With regards to plant diseases, suppression can be accomplished in many ways. If growers' activities are considered relevant, cultural practices such as the use of rotations and planting of disease resistant

cultivars (whether naturally selected or genetically engineered) would be included in the definition. Because the plant host responds to numerous biological factors, both pathogenic and non-pathogenic, induced host resistance might be considered as a form of biological control (Pal & McSpadden Gardener, 2006).

1.2.1 Specifics on the role endophytes could play in priming host defence responses

Endophytic fungi may activate defensive mechanisms in their hosts (Bultman & Murphy, 2000; Van Wees *et al*., 2008). Two basic forms of induced resistance have been described, systemic acquired resistance (SAR), linked to activation of salicylic acid pathway, and induced systemic resistance (ISR), in which jasmonate and ethylene are active (Pieterse *et al*., 1998; Knoester *et al*., 1999; Vallad & Goodman, 2004). In both, SAR and ISR, a conditioning infection or other environmental stimuli activate signal transduction pathways, resulting in enhanced resistance or tolerance against subsequent attacks by pathogens or pests (Hunt & Ryals, 1996; Vallad & Goodman, 2004). Plants may recognize microbes on the basis of microbial surface-derived compounds, *elicitors*, which induce plant defense responses in both host and non-host plants (Nürnberger & Brunner, 2002). Conn *et al*. (2008) found that endophytic actinobacteria in wheat tissues were capable of suppressing wheat fungal pathogens by activating the hosts' SAR and ISR pathways. A study by Rotblat *et al*. (2002) described the mechanisms by which *Trichoderma* spp. elicit resistance in their host plants by producing cellulose and xylanase. Similarly, the hydrophobin-like elicitor Sm1 of *T. virens* has been found to induce ISR in maize (Djonović *et al*., 2007). Cucumber plants preinoculated with *T. asperellum* T203 developed an ISR that was associated with potentiated gene expression in response to pathogen challenge (Shoresh *et al*., 2005). The apparent potential of endophytes to stimulate their hosts' resistance has increased interest in exploitation of endophytic bacteria and fungi in integrated pest and pathogen management in agricultural and greenhouse systems (Waller *et al*., 2005). Whether such mechanisms could be utilized also in forest protection, remains to be investigated.

1.3 Dutch elm disease

During recent decades, the potential role of endophytes in tree diseases has been studied for example in the tropical tree *Theobroma cacao* (Arnold *et al.* 2003), and in elms (*Ulmus* spp.) (Webber, 1981; Dvorák *et al.*, 2006; Martín *et*

al. 2010; Martín *et al.*, 2013). Elms are a large and important group of forest trees with approximately 45 species divided among six taxonomic sections. They grow mainly in the north temperate regions. Most elm species occur in Asia (25 to 28 species) and North America (9 species) as natural species (Wiegrefe *et al.*, 1994; Fu & Xin, 2000; Hollingsworth *et al.*, 2000). Throughout their current distribution area, elms are severely threatened by DED. This vascular disease has killed billions of adult elms in Europe and North America (Brasier & Kirk, 2010) during two large-scale pandemics.

The first outbreak occurred from around 1910 until 1940 in north-west Europe and from around 1930 in North America (Brasier, 1983b; Brasier, 1987; Brasier, 1991; Brasier, 2000; Brasier & Kirk, 2001; Scheffer *et al.*, 2008). According to Heybroek *et al.* (1982) the first outbreak started in north-western France or Belgium, and spread eastwards to central and southern Europe, and westwards to Britain and North America. It also spread later to southwest and central Asia (Brasier, 1991).

The second, current pandemic started simultaneously in Europe and North America, probably already in the 1940s, even though it was recorded for the first time in the 1970s (Brasier, 1991; Brasier, 2000). As a consequence of the epidemic, the importance of elms as raw material for forestry and as a landscape or urban tree has drastically decreased.

The causal agents of DED are pathogenic fungi in the genus *Ophiostoma* (Ophiostomaceae) (Fig. 1A). These fungi do not disperse vegetatively, using mycelium, but are unit-restricted dispersers: the spores are carried to the healthy trees by vector insects, bark boring beetles (Fig. 1B) of the genus *Scolytus* spp. Geoffroy, or the species *Hylurgopinus rufipes* (Eichhoff) (Rudinsky, 1962; Webber & Brasier, 1984; Santini & Faccoli, 2014). The beetles emerge from the diseased trees and fly to healthy ones in spring and early summer (Fig. 1C). During the maturation feeding, the beetles wound the twig crotches and introduce pathogen spores into the phloem and vascular system of the tree (Fig. 1D). The twig crotches are a potential location where the bark beetles might get in contact with repellent biochemicals that have been released by the trees as an induced response to a targeted infection with biocontrol organisms. The pleomorphic pathogen spreads in the xylem vessels through a yeast-like multiplication phase (Fig. 1E) (Webber & Brasier, 1984), causing vessel cavitation. Endophytes could be used at this stage in the vascular tissues as antagonists to the growing and spreading fungus. Discoloration and wilting of leaves in a few branches in the crown ("flagging") in the early summer is often the first external symptom of DED in elms (Fig. 1F). The wilting spreads throughout the canopy during summer. An internal

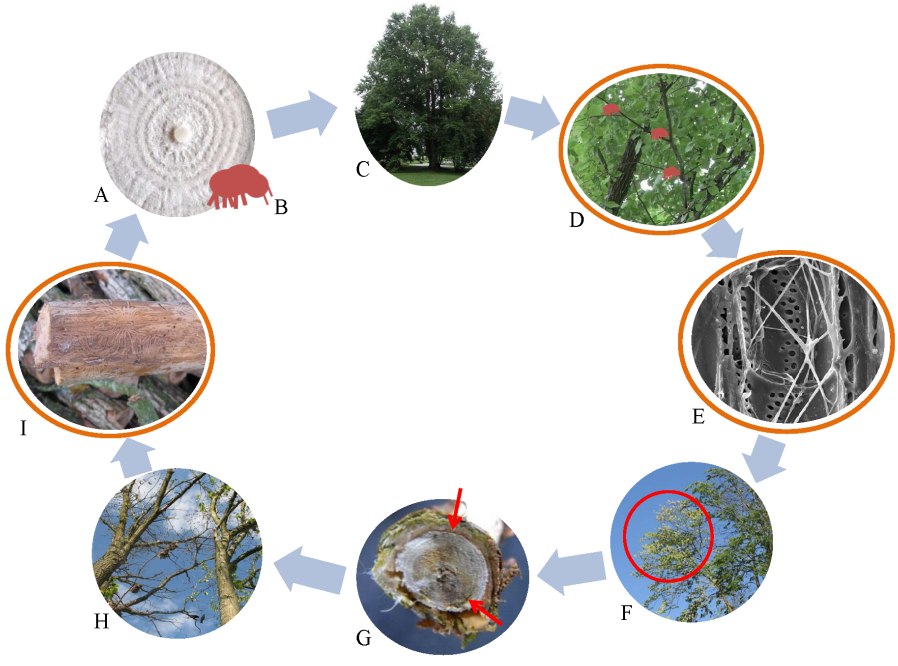

Figure 1. Disease cycle of DED. The pathogen *Ophiostoma novo-ulmi* (A) is transported via bark beetles (B) to healthy elms (C) while the beetle feeds in the tree's twig crotches (D). Spores grow into a mycelium within the xylem vessels (E). Disease symptoms such as wilting in the tree crown (F) or the discoloration of the xylem (G) occurs. The tree dies due to the wilting (H). Bark beetles lay eggs in dead elms and the larvae build breeding galleries (I). The new beetle generation transports fungal spores, present in the breeding galleries to healthy trees for maturation feeding. Photos A, C and G from Kathrin Blumenstein; D, F, H and I from Johanna Witzell and E from Spanish elm breeding programme. The orange frames around picture D, E and I indicate stages in the disease cycle, where endophytes could be used as biocontrol agents.

disease symptom, vascular discoloration, is visible as dark stains in transverse cuts (Fig. 1G). Often, the tree dies within a few weeks or during one season (Fig. 1H) (Scheffer *et al.*, 2008).

The fungus grows saprophytically and produces fruiting bodies in the inner bark and phloem of dying elms. It also grows into maternal galleries (Fig. 1I) where the bark beetle larvae develop (Rudinsky, 1962; Scheffer *et al.*, 2008). The galleries are a place where an interaction based on the potential spatial and temporal co-occurrence of insects, pathogens and bark living endophytes could take place, counteracting the pathogen's growth, the bark beetle larvae or emerging beetles. Female beetles that infest the bark may introduce new pathogen genotypes that can outcross with those that originate from maturation feeding of the original vector beetles in the host tree (Santini

& Faccoli, 2014). Endophytes could also here inhibit the establishment of the growth of new introduced pathogens. When the new beetle generation emerges, they carry fungal conidia and ascospores on their bodies (Fig. 1A+B) and complete the disease cycle by flying to healthy elms for maturation feeding (Webber & Brasier, 1984). In addition, DED also spreads through root contacts (Neely & Himelick, 1963).

Table 1. *Characterization of* O. ulmi *and* O. novo-ulmi *according to Brasier (1991).*

	Ophiostoma ulmi	*Ophiostoma novo-ulmi*	
growth on malt extract agar (MEA) mm day^{-1} at 20℃	(1.5-) 2.0-3.1 (-3.5)	in darkness (2.8-) 3.1-4.8 (-5.7)	
growth optimum	(25-) 27.5-30℃	20-22℃	
maximum	35℃	32-33℃	
pathogenicity on 2 m tall *Ulmus procera*	weak	strong	
defoliation	(2-) 10-35 (-40)% followed by recovery	60-100% with rarely recovery	
habitat	discoloured xylem and the bark of elms (*Ulmus* spp.); particularly in and around breeding galleries of vector beetles	discoloured xylem and the bark of elms (*Ulmus* spp.); particularly in and around breeding galleries of vector scolytid beetles	
subspecies	none	*O. novo-ulmi* subsp. *novo-ulmi*	*O. novo-ulmi* subsp. *americana*
growth rate of subspecies	(3.1-4.4 mm d^{-1})	(3.2-4.8 mm d^{-1}) (Brasier & Kirk, 2001)	

In 1919, the Dutch researcher Marie Beatrice Schwarz isolated and identified the causal agent in its synnematal state (Fig. 2) as *Graphium ulmi* Schwarz (Schwarz, 1922). Somewhat later, Christina Buisman discovered the sexual state of the fungus and changed the name to *Ceratostomella ulmi* (Schwarz) Buisman (Buisman, 1932). Two years later, Elias Melin and Johann A. Nannfeld classified the fungus as *Ophiostoma ulmi* (Buisman) Nannf. (Melin & Nannfeld, 1934) (characteristics in Tab. 1). For a few decades, the fungus was called *Ceratocystis ulmi* in literature, after Claude Moreau had changed the name again (Moreau, 1952), but de Hoog and Scheffer (Hoog & Scheffer, 1984) stated *O. ulmi* to be the correct name for the pathogen. The *Graphium* conidial state was referred to *Pesotum*, while the mycelial conidia have been described to *Sporothrix* (Brasier, 1991 and refs wherein). The aggressive form, responsible for the recent outbreak, was later introduced as a new species, *O.*

novo-ulmi (Brasier, 1991) (characteristics in Tab. 1). *Ophiostoma novo-ulmi* rapidly replaced the almost extinct *O. ulmi* because of their competitive interaction (Brasier, 1983b; Brasier, 1987).

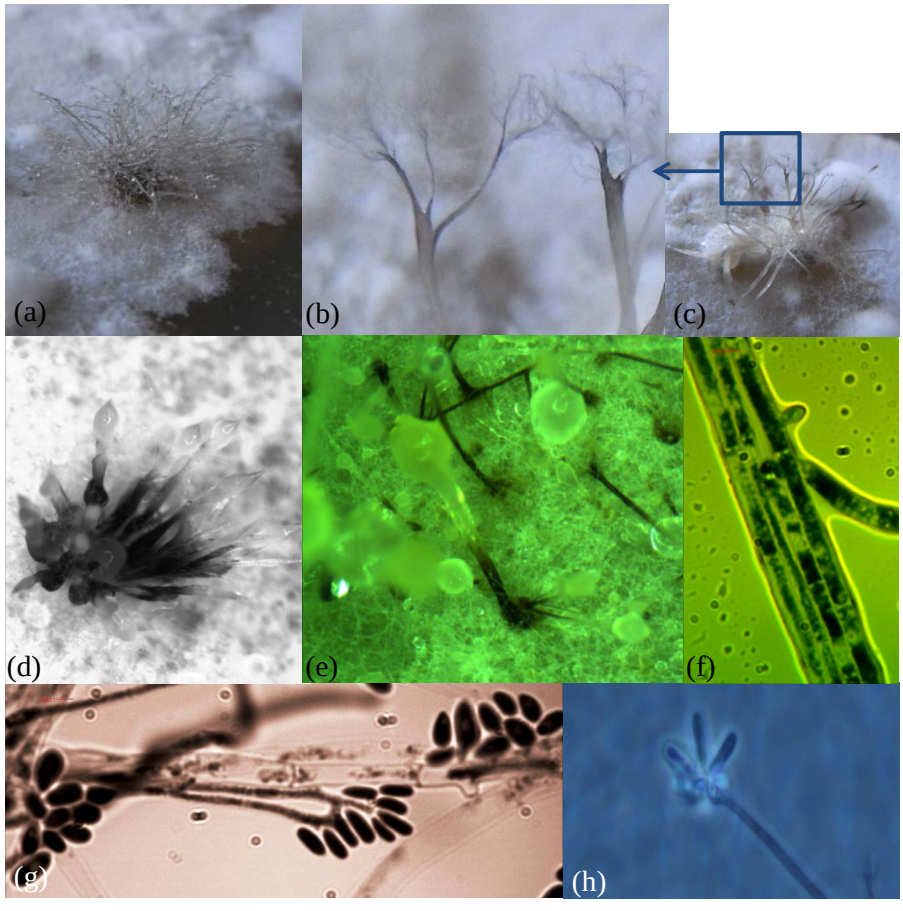

Figure 2. Saprophytic, anamorph (*Graphium*) phase of the pathogen producing synnematal structures (coremia) of~ 2 mm length with conidia-bearing sticky droplets (a-e); (f) coremia composed of fused dark conidiophores and (g, h) conidiophores with conidia (3-5µm length). Photos: Kathrin Blumenstein.

1.3.1 Strategies to control DED

Over the decades several measures, including chemical, biological or silvicultural methods, have been used in attempts to control DED. In principle, the protection can be through elimination of vectors in spreading of the disease, or elimination/restriction of the pathogen in trees.

An effective control strategy could be based on reducing the population and feeding by the vector insects. Chemicals like DDT[1] were used during the first epidemic to eliminate bark beetle populations, but DDT was banned for environmental reasons in the 1960s (Scheffer *et al.*, 2008). Pheromone traps are used as monitoring tools for beetle populations in Europe, North America and New Zealand (Scheffer *et al.*, 2008). While the traps can catch millions of beetles, they cannot control the disease.

So far, the most important tool in DED management has been eradication, which aims to destroying the breeding material of the beetles. Dying and dead elms are removed or pruned, and the wood is debarked before the insects emerge in spring and early summer (Scheffer *et al.*, 2008). This measure is difficult to apply in forest and field populations of elm, because of the problem of detecting and removing diseased trees in such complex environments (Scheffer *et al.*, 2008). Eradicative pruning can work effectively, if an infection in the crown is detected at an early stage (less than 5% of affected crown) (Haugen, 1998). In order to locate the fungal infection, the bark of the infected branch needs to be removed to find the discoloration that indicates the fungal infection (Haugen, 1998). In practice, this is not feasible especially when it comes to large trees in forest environments.

The second control strategy is targeting the pathogen, but is also challenging. In the United States, benzimidazoles and sterol biosynthesis inhibitors have been used as fungicide treatment as systemic injections since 1977. Thiabendazole is registered as Arbotect 20-S (Syngenta Crop Protection, Inc., Greensboro, NC) for control of DED as well as sycamore anthracnose. According to Scheffer *et al.* (2008), when properly adjusted and injected in a timely fashion, Arbotect 20-S protects elm trees for up to 3 years after treatment. Because elms often develop root grafts with neighbouring elms (Neely & Himelick, 1963), the use of above ground fungicides does not protect elms when the pathogen infects the tree through the root system (Scheffer *et al.*, 2008). Even though fungicides with systemic actions exist and fungicides that can be applied to protect roots, the application is currently restricted to nurseries and arboriculture stands[2] in Europe. Infections through root grafts are likely to be frequent especially in the monoculture conditions created along boulevards and canals in urban areas. Control treatments for root graft transmission focus on trenching and cutting roots between trees, but such treatments are labour-intensive and expensive. Experiments in the Netherlands with metam-sodium to kill the roots did stop root transmission, but registration

[1] DDT = dichlorodiphenyltrichloroethane

[2] Arboriculture stands are cultivated areas for, e.g., wood production such as groves or short-rotation forestries (Cardarelli *et al*., 2011).

of the product for this purpose was not feasible because of its ecotoxicological effects, especially its potential for groundwater pollution (Scheffer *et al.*, 2008).

Because of the increasing awareness of the harmful side effects of fungicides and pesticides in the environment, there is a growing interest in finding biologically sound and sustainable solutions for control of DED. A lot of efforts are made to breed for increased resistance (see 1.3.2), but also other solutions seem necessary. Biological control (see 1.2), which entails the use of living organisms to regulate pests, is an attractive option and a part of integrated pest management (IPM) strategies (Bale *et al.*, 2008). The concept of biological control was reviewed by Waage and Greathead (1988). By using specialist enemies of the pest to be controlled (Müller-Schärer *et al.*, 2004), the probability of non-targeted effects can be minimized. The main benefits of biocontrol include the safety to people and animals and the possibility to reduce the use of pesticides, such as fungicides in the environment (Pal & McSpadden Gardener, 2006). On the other hand, when directly applying a microbe to the ecosystem, one needs to be aware of the non-targeted effects through the introduction of a new organism to the system. In addition to the use of natural enemies of the target pest or pathogen, protective effect can also be achieved through biological stimulation of tree's own resistance mechanisms (see 1.2.1) (Eyles *et al.*, 2010).

Biological mechanisms have been explored also for the potential in control of DED. For example, it has been shown that the resistance of elms to *O. novo-ulmi* can be enhanced or induced by artificial inoculation with a mixture of *O. ulmi* and *O. novo-ulmi*: The treatment resulted in less symptom development than inoculation with only *O. novo-ulmi* (Scheffer *et al.*, 1980) or only strains of *O. ulmi* (Hubbes, 2004).

Weakly pathogenic *Verticillium* sp. strains have also shown to enhance resistance in elms (Solla & Gil, 2003; Scheffer *et al.*, 2008). *Verticillium* is a vascular wilt pathogen, expected to survive within the tree and to elicit a resistance response. The *V. albo-atrum* isolate WCS850[3] is able to significantly suppress disease development in 'Commelin' elms and susceptible field elms (Scheffer, 1990; Scheffer *et al.*, 2008). Scheffer *et al.*, (2008) found that the fungus could only be re-isolated from injected trees around the site of injection in the trunk. This indicated that the translocation of the isolate was minimal and that direct interaction between the fungus and the pathogen was not likely to occur. Hence, elms respond physiologically to *Verticillium,* which leads to induction of the protection against the DED pathogen.

[3] *Verticillium albo-atrum* isolate WCS850 is nonpathogenic. It does not cause wilt syndromes in elms and even not in *Verticillium* susceptible species like ash (Scheffer *et al.*, 2008).

The strain WCS850 has been developed into a commercial vaccination product called Dutch Trig®. This product is injected to the trunk as a conidial aqueous suspension in late spring. The injection has to take place before infection, because the tree needs time to build a resistance response. The treatment must be repeated annually because of the short-time survival of WCS850 in the elms (Scheffer *et al.*, 2008). The method has been successfully used in preventive treatment of valuable trees in urban areas, but it has only limited relevance in large-scale forestry, because of the laborious application of the repeated injections (Martín *et al.*, 2015a).

In 1983, a virus infecting *O. ulmi* was discovered (Brasier, 1983a). The virus was characterized as a devirulence factor (d-factor) that damages the growth and viability of conidia, resulting in a reduced production of perithecia[4] (Brasier, 1983a). It was then suggested that the virus could be utilized in DED control by releasing elm bark beetles that transport virus-infected spores, compatible with the local pathogen clone as a biocontrol strategy (Brasier, 1983a). Scheffer *et al.* (2008), however, categorized this approach as too radical since pathogen-infested beetles could interfere with sanitation and preventing biological or chemical control programs.

1.3.2 Breeding programs

The degree of DED susceptibility has been found to vary among elm species and genotypes. For example the Siberian elm, *U. pumila,* is rather tolerant to DED whereas *U. minor* is a highly susceptible species (Smalley, 1963; Solla *et al.*, 2005a; Martín *et al.*, 2008). The variation in susceptibility may depend on their anatomy and physiology, such as the proportion of large vessels in the earlywood (Elgersma, 1970; Solla & Gil, 2002), the hydraulic conductivity of twigs (Elgersma, 1970; Melching & Sinclair, 1975), the speed of the browning response of freshly exposed cambium after inoculation (Smalley *et al.*, 1982), the activity of phenylalanine ammonia-lyase (Díez & Gil, 1998) and phytoalexin production (Duchesne *et al.*, 1985). It has been proposed that resistance of *U. pumila* is related to specific anatomical or physiological adaptations to xeric environmental conditions (Brasier, 1990). Thus, earlywood vessels of small diameter, mostly isolated within the xylem, have been related with the greater resistance of *U. pumila* to xylem embolism caused by water-stress or by *O. novo-ulmi*, in comparison to *U. minor* (Solla *et al.*, 2005b). The enhanced levels of starch and cellulose found in *U. pumila* in comparison to *U.*

[4] Fungi belonging to the phylum Ascomycota, such as Ophiostomataceae, produce fruiting bodies called ascocarps after sexual reproduction. They contain millions of asci with ascospores inside. If the shape of the ascocarp is flask-like shaped, it is called perithecium (Encyclopedia Britannica).

minor are consistent with the higher resistance of *U. pumila* to water-stress and to *O. novo-ulmi* (Martín *et al.*, 2008).

The genotypic variation in DED susceptibility raises the hope of success in breeding for DED resistance. Resistance breeding, although slow in its traditional form, is attractive because of its sustainability (Witzell *et al.*, 2014). The first program for elm resistance breeding began in the Netherlands in 1928 (Heybroek, 1993b). Later on, breeding programmes in several European countries and the United States followed. For instance, in the 1930s, considerable efforts were made to identify resistant individual *U. americana* trees (Smalley & Guries, 1993). The second disease pandemic in Europe, however, decimated many surviving native populations and some of the early "resistant" cultivars (e.g., 'Commelin') (Brasier, 2000). Asian elms, including *U. pumila* (Scheffer *et al.*, 2008)*, U. chenmoui*, *U. davidiana* var. *japonica*, *U. wallichiana* (Heybroek, 1993b; Smalley & Guries, 2000), *U. japonica* and *U. parvifolia* (Scheffer *et al.*, 2008), have been the main sources of resistance in the Dutch, American and Italian elm breeding programmes. These species are crossed with native elms, resulting in hybrid clones of varying tolerance levels and genetic backgrounds (Martín *et al.*, 2015b). About 25 elm cultivars with different levels of resistance to DED are already available in Europe and North America offering possibilities for replacement of urban elms which were lost (Scheffer *et al.*, 2008).

More recent elm breeding efforts in Spain and Italy have emphasized the use of the native European species *U. glabra* and *U. minor,* while still relying on the Siberian elm as a source of disease resistance genes (Solla *et al.*, 2000; Santini *et al.*, 2003).

In 1986, a large elm breeding and conservation programme was launched in Spain as an agreement between the Spanish Environmental Administration and the Technical University of Madrid, School of Forestry Engineering (Solla *et al*. 2000). The aim was to conserve remaining genetic resources, to find tolerant native elm genotypes and transmit the variability of tolerant native elms to future generations obtained through breeding; i.e., hybridisation of selected progenitors (Martín *et al.*, 2015b). As part of the programme, susceptibility trials on thousands of elm genotypes were conducted at the clonal bank of the breeding centre, Madrid (Martín *et al.*, 2015b). In the beginning of the programme, *U. pumila* was used as the main source of resistance, giving rise to 10 crossings tolerant to *O. novo-ulmi* (Solla *et al.*, 2000). However, the uncontrolled spread of *U. pumila* in Spain and its extensive hybridisation with the native *U. minor* (Cogolludo-Agustín *et al.*, 2000) led to conservation concerns. Already in the 1990s, native elms, mainly *U. minor*, were included in the *O. novo-ulmi* susceptibility trials. In the

following decade the programme focused mainly on selecting native elms (Martín *et al.*, 2015b).

Susceptibility tests revealed seven *U. minor* clones to be tolerant to *O. novo-ulmi* and these clones are already registered by the Spanish Environmental Administration for the use in forest environments. For the susceptibility tests, local strains of *O. novo-ulmi* were used to evaluate the tolerance level of the clones through artificial inoculations (Solla *et al.*, 2005c). After pathogen inoculation, the seven clones showed leaf wilting values similar to or lower than "Sapporo Autumn Gold"[5]. In all tests, the susceptible control clone UPM089 showed wilting values above 70%, while the most tolerant clone (Dehesa de Amaniel) showed wilting values below 5% (Martín *et al.*, 2015b). Some other clones had high ornamental scores and are therefore promising trees for the use in urban environments and material for tree breeding for ornamental quality (Martín *et al.*, 2015b).

Elm tolerance to *O. novo-ulmi* has been shown to be inheritable (Guries & Smalley, 2000; Townsend, 2000; Venturas *et al.*, 2014; Solla *et al.*, 2014) and polygenic (quantitative) in nature (Aoun *et al.*, 2010). Basically, it can be assumed that the more resistance mechanisms are gathered in the same genotype, the higher are the chances of overcoming an infection. Thus, it would be desirable to perform controlled crossings between genotypes that express different, and preferably complementary, defence mechanisms (Martín *et al.*, 2015b).

[5] "Sapporo Autumn Gold", highly tolerant to *O. novo-ulmi* (Smalley & Lester 1973)

2 Objectives of thesis

During recent decades, growing concerns about the state of the environment have increased acceptance for IPM as a strategy for sustainable crop production and protection (FAO, 2015). In IPM, the goal is to minimize the use of environmentally hazardous chemicals and instead utilize different management practices and natural mechanisms to promote the health of crop plants (ECPA, 2015; European Commission, 2004). Biological control, i.e., utilization of beneficial organisms in control of pests or pathogens, is a valuable tactic in IPM (Orr, 2009), but successful application of biological control necessitates knowledge about the mechanisms of interactions between the biological antagonists and the target pests or pathogens. Fungal endophytes have been identified as a group of potent biocontrol agents against crop diseases, including those of forest trees (Arnold *et al*., 2003; Newcombe, 2011; Albrectsen & Witzell, 2012). However, little is still known about the potential influence of endophytes in disease resistance of large, perennial plants such as forest trees. In particular, the detailed mechanisms through which the endophytes may influence tree pathogens are still poorly investigated.

The goal of this thesis was to improve our understanding of the potential of fungal endophytes as a part of IPM of the vascular tree disease DED, and to provide new insights in the ecological interactions between endophytic and pathogenic fungi that share a habitat (host tree) in time and space. To investigate these aspects, the culturable endophytic mycoflora was studied in a collection of elm trees showing differential susceptibility to DED. The studies were focused on the culturable fraction of endophytes in order to capture strains that could be studied further in the laboratory and eventually be used in biocontrol solutions.

Two overarching research questions were addressed:

1) Are there qualitative or quantitative differences in the cultivable fraction of fungal endophytes between elm trees that differ in their susceptibility to Dutch elm disease? (Paper I).

2) What kind of mechanisms could explain the possible antagonism between elm endophytes and DED pathogens? (Papers II, IV)

In addition, Phenotype MicroArrays (PM) was tested as a promising technique to study the competitive interactions between endophytes and pathogens. (Paper III).

Some preliminary, unpublished results are presented and discussed in the thesis.

3 Material and Methods

The studied endophytic fungi originated from four different locations in Spain, three of them in the vicinity of Madrid and one from Majorca Island. In order to associate endophytes with the resistance patterns of the elms, the endophytes were isolated from elms with different degrees of susceptibility. The isolations were done from leaves, bark and xylem (paper I) in 2008 or xylem (paper II) in 2011. For an overview of the sampling approach and host tree materials, see Table 2; for the fungi and methods used in the thesis, see Table 3.

3.1 Sample collection sites

- The first site is located at the Forest Breeding Centre in Puerta de Hierro (hereafter referred to as breeding centre), close to Madrid, Spain (Martín *et al.*, 2015b). Over 200 clonal trees of *U. minor* and *U. pumila* were planted in 1986 as part of the Spanish elm breeding program, e.g., Solla *et al.* (2000). Leaf, bark and wood samples of 14-year-old trees were collected for endophyte isolations.
- The second site is a semi-natural riparian elm stand in the municipality of Rivas-Vaciamadrid, where most trees belong to the highly susceptible *U. minor* var. *vulgaris* (= *U. procera*) complex. *Ophiostoma novo-ulmi* has been isolated from the stand and *Scolytus* bark beetles are abundant in the area, but the spread of DED is abnormally slow. The trees were selected on the basis of their dendrometric features and good health condition.
- The third site is a forest area close to Madrid. Sampling was done from one, centenary *U. minor* tree, selected on basis of its tolerance to DED in the field, despite its high susceptibility when inoculated in experimental plots.
- The fourth site is located at the Albufera Natural Park, Majorca Island. One *U. minor* individual, symptomless despite the fact that surrounded trees showed symptoms of infection, was selected.

Table 2. *Overview of the plant material used for isolation of endophytes.*

Paper	Tissue	Sites and location	Elm species (no of sampled individuals); symbols used in the study are given in capital letters.		Susceptibility of individuals	Age of elms (yrs.)	Width at breast height (cm) / height (m) of tree	Material (samples)
I	Leaves, bark and xylem	1 breeding centre (40°27'N, 3°46'W)	*U. minor* (4)	M (R)	Low	14	-	One terminal shoot (30 cm length) from lower half of the crown (2-3 m height) from each cardinal point.
			U. pumila (2)					
			U. minor clones (4)	P (R)	Low			Four leaves from each shoot.
				M (S)	High			
		2 Rivas-Vaciamadrid (40°20'N, 3°33'W)	*U. minor* var. *vulgaris* (7)	M (F)	Low	65-75	-	Four 4 cm long twig segments from the 2-year-old twigs of each shoot. →In total 16 twigs and leaves per tree = 272 twigs and leaves.
II	Xylem	3 Semi-natural forest area (40°29'N, 3°45'W)	*U. minor* (1) *"Somontes"*		Low	≥140	155 / 27	Four 3-year-old twigs (20-30 cm length and 1-1.5 cm diameter) Lower half of the crown; from each cardinal point. In total 48 twigs.
		2 Rivas-Vaciamadrid *Casa Eulogio* (40°20'N, 3°33'W)	*U. minor* var. *vulgaris* (1)		Low	80	87 / 19	
		4 Majorca Island, *Albufera Natural Park* (39°47'N, 3°6'E)	*U. minor* (1)		Low	-[1]	60 / 17	

[1] unknown, but estimated in around 40 years

3.2 Isolation of endophytic fungi

The isolation was done in a laminar flow hood, using the surface sterilisation protocol described in Helander *et al.* (2007). Small sections of surface sterilized leaf, bark and xylem tissues were cut, separated and placed on malt extract agar (MEA) in Petri dishes. Over a period of two weeks, emerging endophyte colonies were counted and transferred to fresh medium. The average number of endophyte colonies growing in each Petri dish was divided by the total number of tissue samples placed in the dish to get a measure for endophyte *frequency* per trees in each susceptibility group (paper I, II). The endophyte *diversity* per tree group was estimated as the average of the number of different morphotaxa observed in each Petri dish divided by the number of tissue samples placed in the dish (paper I, II).

3.3 Identification and characterization of endophytes

In order to evaluate the diversity of endophytic fungi present in the elm tissues, all endophytes were grouped into morphological groups (morphotypes or morphotaxa) (paper I, II). Macro- and microscopic examination of the morphological traits was used to group the isolates into morphotaxa (Fröhlich & Hyde, 1999; Taylor *et al.*, 1999; Arnold *et al.*, 2001; Guo *et al.*, 2003). Criteria for the grouping were vegetative features that conventionally constitute species limitations (Guo *et al.*, 1998; Arnold *et al.*, 2000; Arnold *et al.*, 2003). The main features observed were colony surface texture, colours of the colonies and the surrounding media and the growth rates on MEA. In addition, special features such as the formation of fruiting bodies or the accumulation of droplets or coloured spores were recorded and used in the grouping. The molecular identity of one representative isolate per morphotype was determined from the endophyte collection isolated in 2008 (paper I). From the xylem derived endophytes (paper II), every isolate was identified by molecular techniques.

A selection of identified isolates that were statistically related to elms with low susceptibility (paper I) were chosen for further experiments because their potential to contribute to the resistance of their host trees was evaluated to be high. In the further experiments, the mechanisms of chemical antagonism (paper II) and competitive interactions (paper IV) were explored though *in vitro* approaches, and the biocontrol (preventive) potential of endophytes was explored through *in vivo* tests (paper II).

3.3.1 Antagonism assays *in vitro* dual culture and *in vivo* tests

The antagonistic potential of selected endophytes was determined using *in vitro* dual culture tests (paper II, K. Blumenstein, unpublished results). Based on the results of these studies, endophytes were selected for *in vivo* tests where young elms were inoculated with endophytes before they were infected with the DED pathogen (paper II).

Prior to the dual culture tests, all fungi were cultivated on MEA and transferred to fresh plates to guarantee actively growing mycelium and the same age of the colonies in bioassays. Plugs (diam. 5 mm) from one of the endophytes and from the *O. novo-ulmi* ssp. *novo-ulmi* colony were placed with a 6 cm distance on Petri dishes containing MEA. The colonies were allowed to develop in an incubator (22 ℃, darkness) and the interactions were evaluated by measuring the colony growth at three time-points (2, 4, 7 days post inoculation). In paper II, the colony growth was measured in three directions: one direction connecting the centre of the inoculum fraction, and two at +45° and -45° angles to the former, and in an unpublished study from K. Blumenstein the measurements were performed along two axes, i.e., the colony diameter and the perpendicular diameter according to Santamaria *et al.* (2004). A spherical index was applied in order to receive the relative measure of the colony shape:

Diameter b / Diameter a = Spherical-Index.

If the index equals one, the colony has a spherical shape. If the index is greater than one, the colony's growth developed more along the b-axis, away from the opponent. If the index is smaller than one, the fungus grows more along the a-axis, towards the opponent.

Based on the growth responses, the type of interaction between an endophyte and a DED pathogen was assessed according to Mejía *et al.* (2008) into three categories: a) antibiosis (chemical reaction) through growth inhibition; presence of a reaction zone (Fig 3a and Fig 2a in paper II), b) substrate competition by a higher growth of one fungus relative to the other (Fig. 3b and Fig 2b in paper II) and c) mycoparasitism, when mycelium of one

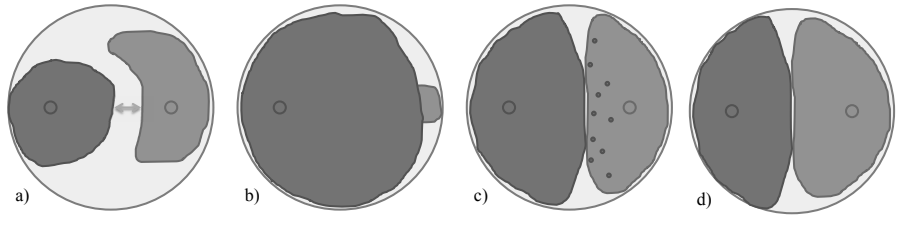

Figure 3. (a) antibiosis (arrow indicates reaction zone); (b) one fungus growth faster than the other; (c) mycoparasitism, (d) neutral / mutually intermingling growth.

fungus growth on the other (Fig. 3c). A fourth category was added indicating that no obvious interaction has happened, called neutral or "mutually intermingling growth" after Larran *et al.* (2016) (Fig 3d).

The results from the *in vitro* experiment allowed the choice of four endophytes to be tested in further antagonism experiments in the elm trees for the *in vivo* tests (paper II), where the aim was to test the potential for enhancing plant resistance against DED by preventive endophyte treatments.

3.3.2 Nutritional profiling with Phenotype MicroArrays

In order to compare the competitive capacity of selected endophytes as compared with the DED pathogens, the ability of the fungi to utilize an array of 285 carbon and nitrogen sources was examined using Biolog Phenotype MicroArray™ technology (paper III and IV). The carbon and nitrogen sources were of particular interest because of their essential importance for heterotrophic fungi (Deacon, 1997). Commercially available (Biolog Inc., Hayward, CA) pre-filled 96-well microtiter plates containing 190 different carbon sources and 95 different nitrogen sources were used in the studies. A detailed list of the compounds in the plates can be found in the Appendix 6.3.

The PM method was optimized and further developed for tree endophyte studies (paper III). In particular, a procedure for preparation of inoculum was optimized by culturing the fungi on a semi-permeable cellophane membrane to facilitate the harvest of fungal material. After homogenizing the fungal mass, it was pressed through cotton wool and an aliquot was taken for the preparation of inoculum. Moreover, an in-house designed PM plate was developed in order to test the sensitivity of endophytes to carbon sources mixed with inhibitory substrates, such as phenolic compounds.

3.3.3 Effect of single vs. dual culture conditions on nutritional phenotypes

Pilot experiments were carried out to test whether the nutrient utilization patterns of a fungal isolate differ depending on whether the fungal cells are collected from a single or dual culture. The hypothesis was that the enzymatic capacity of endophytes or pathogens change in their ability to utilize substrates more effectively after they have been grown in dual culture assays (as described above) as compared to them being cultured in single conditions. Biolog nitrogen PM3B plates were inoculated with the fungi *O. novo-ulmi*, *O. ulmi*, *Monographella nivalis* var. *neglecta, Trichoderma harzianum* and *Aureobasidium pullulans*, three isolates each, after regular single culture

growth (paper III) and after the pathogens had been grown in dual cultures with each of the endophytes (K. Blumenstein, unpublished results).

3.3.4 Preparation of inoculum and inoculation of the microplates

For the carbon-source study (paper IV), mycelium was scraped from the agar and transferred into a tissue grinder to gently fragment the biomass for a homogenous inoculum. An aliquot was transferred into an inoculating fluid provided by Biolog (FF-IF).

For the nitrogen studies, fungi were cultured on a semi-permeable cellophane membrane. The inoculum contained FF-IF, glucose solution, sodium sulphate and potassium phosphate (see Appendix, Table 5). Three replicate plates were prepared per plate type of the carbon plates and two replicates for the nitrogen plates.

3.3.5 Data reading and analysis

The optical density of the wells was measured using a spectrophotometer. Measurements were taken at T=0 and afterwards every 24 h for ten days (paper III, IV). The wavelength chosen for the carbon plates was 590 nm (paper IV) and 750 nm for the nitrogen plates (paper III and unpublished study). Hierarchical clustering was applied in order to find the time-point that best separated the fungal isolates according to their consumption of different substrates and Principal Component Analysis (PCA) was used to show the similarity of the three technical replicates (paper IV). The average well colour development (AWCD), based on the optical density (OD) values, was calculated for each fungal isolate and source across the replicate plates (paper III and IV). The carbon sources tested were divided into 14 substrate groups (Garland & Mills, 1991) and the nitrogen substrates into 9 groups (Grizzle & Zak, 2006) based on their chemical properties. The average substrate utilization was determined for each substrate group (paper III and IV). In study IV, the isolate-specific AWCD value was used to compare the carbon-utilization patterns of the endophytes and the pathogen (Haack *et al.*, 1995).

A niche overlap index (NOI) and an endophyte competitiveness index were calculated to compare the pathogen's carbon-utilization patterns to those of the endophytes (Wilson & Lindow, 1994; Lee & Magan, 1999).

3.3.6 Chemical analyses of extracellular fungal products

A selection of elm endophytes showing strong chemical antagonism against DED pathogens *in vitro* (paper II), were chosen for a more detailed analysis of extracellular products. Colonies from Petri dishes were transferred to ethyl

acetate. After 4 hours of extraction the agar was removed by filtration through paper filters. After this an evaporator was applied to separate the organic solvent from the extracted compound. The dissolved extract was applied to paper disks and arranged in bioassays as in the previous described dual culture tests but with the paper disk replacing the endophyte used. Growth development was measured and reaction of the pathogen evaluated over time.

Based on the results from the bioassays, extracts from three endophyte species, five isolates each, were prepared. Two species, *M. nivalis* var. *neglecta* and *P. cava* and their isolates had shown high degrees of bioactivity (high or moderate) in previous antagonism assays. *Alternaria alternata* was included as a control, based on the observation that it showed no bioactivity in the bioassays to allow a separation of the possible bioactive from the non-bioactive compounds. The extracts were sent to the Swedish Metabolomics Centre for liquid chromatography-mass spectrometry (LC-MS) analysis to screen the extracts for their compounds and to attempt an identification of the single compounds. Data was analysed with the MassHunter™ and Mass Profiler Professional™ software from Agilent.

Table 3. *Overview of fungi and methods used in the thesis.*

Data	Endophytes species/strains	Tested pathogen	Experimental approach	Statistical analyses
Paper I	*Pyrenochaeta cava, Monographella nivalis, Aureobasidium pullulans, Alternaria* sp., *Cochliobolus cynodontis, Fusarium* sp., *A. alternata, Biscogniauxia nummularia, Xylaria* sp., *Cladosporium cladosporioides, Phomopsis* sp., *Sordaria fimicola, Coniochaeta* sp., *Apiospora* sp., *Botryosphaeria sarmentorum, Leptosphaeria coniothyrium*	-	Isolation through surface sterilization and axenic cultures of endophytes. Morphotyping. Molecular identification of specimen (ITS). HPLC analysis of phenolics.	GLM ANOVA, Shapiro-Wilks statistic, multible range tests (Fisher's Least Significant Difference (LSD) intervals, Linear regression Shannon-Index, Pielou's index for evenness, MDS analysis (Jaccard's index) Rarefaction curves DFA

Paper II	Ascomycetes - Dothideomycetes: *A. pullulans, Alternaria tenuissima* and *Neofusciccocum luteum;* Sordariomycetes: *Fusarium sp., M. nivalis and Sordaria* sp.*;* Eurotiomycetes: *Penicillium crustosum,*	*O. novo-ulmi* ssp. *novo-ulmi*	Isolation through surface sterilization and axenic cultures of endophytes. Molecular identification of specimen (ITS). Dual culture bioassays. *In vivo* inoculations.	One-way and repeated measures ANOVAs, Fisher's Least Signifficant Difference (LSD), Shapiro-Wilks test
Paper III	*M. nivalis* var. *neglecta, P. cava, A. pullulans, Trichoderma harzianum,* two Sordariomycetes, *incertae sedis,* eleven Dothideomycetes: four Dothioraceae, three Pleosporaceae, Phaeosphaeriaceae, Lophiostomataceae, Botryosphaeriaceae, Davidiellacea; *Trichoderma* sp.; Basidiomycetes: *Pycnoporus sanguineus, Trametes* sp.	*O. novo-ulmi,* *O. ulmi*	Phenotype MicroArray (nitrogen) Phenotype MicroArray (in house)	ANOVA, Multivariate statistics, Pearson correlation, one-factor ANOVA, Principal Component Analysis (PCA)
Paper IV	*P. cava, M. nivalis* var. *neglecta, A. pullulans*	*O. novo-ulmi* ssp. *americana*	Phenotype MicroArray (carbon)	Hierarchial Cluster Analysis PCA Niche Overlap Index
Unpublished	*P. cava, M. nivalis* var. *neglecta, A. pullulans*, *S. fimicola, T. harzianum*	*Ophiostoma novo-ulmi, O. ulmi*	Dual culture assays. Phenotype MicroArrays (nitrogen) with prior dual cultures. Ethylacetate extraction of extracellular chemicals. Analysis (LC-MS)	-

4 Main results

The described results focus on the main findings from the four publications (I-IV). Unpublished data was added to this chapter.

4.1 Origin and isolation of the endophyte collection

A total of 274 isolates were recovered from 816 plant samples (paper I). The endophyte frequency and diversity were significantly affected by the tree group [P(R), M(R), M(S) and M(F)] and the respective organ (leaf, xylem and bark), whereas the orientation (the four cardinal points of the compass) used for the isolation had no effect. Leaf-associated fungi were more diverse and frequent in M(F) trees than in trees from the breeding centre. Endophyte frequency and diversity in bark tissues was generally higher than in leaves and xylem tissues. No significant difference was found in the bark tissue for the frequency between groups, but M(S) samples had higher endophyte diversity than samples from the field population. The highest diversity was found for the M(S) group in the bark samples. In the xylem samples, different indices suggest the highest diversity and frequency in the M(S) group (Fig. 4).

In the subsequent study (paper II) that aimed to gain a deeper insight into the potential role of elm endophytes in host defence against *O. novo-ulmi*, seven endophytic fungi were isolated from the xylem of the three selected *U. minor* trees.

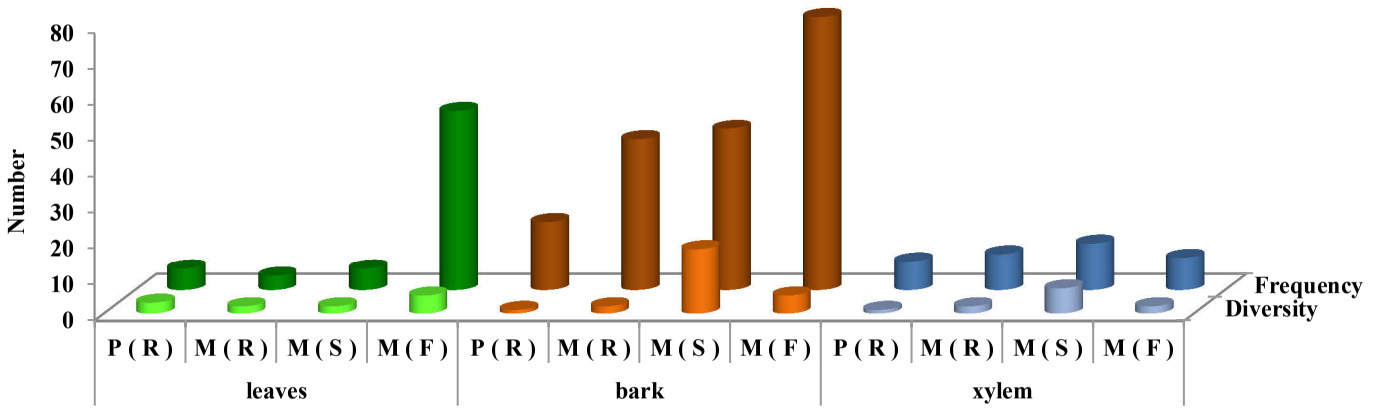

Figure 4. Differences in the frequency (number of isolates) and diversity (number of morphotaxa) among tree organ and groups: P(R) = resistant *U. pumila* clones from the breeding centre, M(R) = resistant *U. minor* clones from the breeding centre, M(S) = susceptible *U. minor* clones from the breeding centre, M(F) = *U. minor* trees from Rivas-Vaciamadrid field site. Data from Martín *et al*., 2013 (paper I, table 3). See also Tab. 2 in Material and Methods.

4.2 Identification and characterization of endophytes

Endophyte isolates were initially grouped into 16 morphotaxa (paper I). Fungi from six groups came solely from bark, three from bark and leaves and three from bark and xylem. Endophytes from four groups originated from all tissue types. The representatives of the three most common fungal morphotaxa were identified based on the internal transcribed spacer region (ITS) sequence as *Pyrenochaeta cava*, *Monographella nivalis* and *Aureobasidium pullulans*. *Monographella nivalis* was isolated from bark and xylem, mainly from resistant *U. minor* clones and trees from the field population. *Pyrenochaeta cava* came mainly from resistant *U. pumila* and *A. pullulans* from susceptible *U. minor* trees.

4.2.1 Antagonism assays *in vitro* dual culture and *in vivo* tests

Dual culture tests demonstrated that six out of seven endophytes reduced the growth of the pathogen *in vitro* (paper II). The visual evaluation of the interactions between endophytes and pathogen suggested that endophytes could antagonize the pathogen through several mechanisms (see below).

Antibiosis (chemical reaction)

In cases where a reaction barrier between endophyte and pathogen could be observed already before they physically met, the mode of interaction was classified as antibiosis (Fig. 5). In repeated tests, *M. nivalis* inhibited the growth of the pathogen through antibiosis (paper II, K. Blumenstein, unpublished results). This type of interaction was characterized by the formation of a thick reaction barrier in the pathogen colony facing the endophyte (Fig. 5, left plate). A similar response was induced in the pathogen by *Penicillium crustosum* (paper II), and also by *Pyrenochaeta cava,* although in the latter case the response was less pronounced (Fig. 5, right plate).

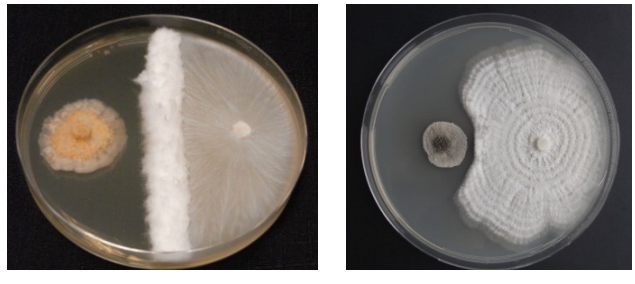

Figure 5. Monographella nivalis (left plate) and *Pyreonachaeta cava* (right plate) growing in dual culture with the Dutch elm disease pathogen, *Ophiostoma novo-ulmi* (white colony on the right side of each plate). A clear reaction zone can be observed between the colonies, indicating an antibiosis effect of the endophyte on the pathogen. Photos: Kathrin Blumenstein.

Competition for substrate

Neofusiccocum luteum (paper II) and *Sordaria* sp. (Fig. 6a) (paper II, K. Blumenstein, unpublished results) suffocated the expansion of the pathogen's colony through a faster growth by competition for the substrate. *Aureobasidium pullulans* and *A. tenuissima* showed combined but weaker effects of antibiosis and competition for the substrate (paper II).

Mycoparasitism

In addition to antibiosis, *M. nivalis* var. *neglecta* also demonstrated mycoparasitic behaviour: it was growing on the pathogen's colony at a late state of the dual culture assays (Fig. 6b) (K. Blumenstein, unpublished results).

Neutral reaction / mutually intermingling growth

Some of the tested fungi, such as *Fusarium* sp., did not show any reaction towards *O. novo-ulmi* (paper II). In some cases, the character of the interaction seemed to change with time. For instance, at an earlier stage of the experiment, *A. pullulans* had no effect on the pathogen and was therefore classified at neutral (Fig. 6c), but later on, signs of antibiosis could be observed (K. Blumenstein, unpublished results).

Figure 6. (a) The endophyte *Sordaria fimicola* (grey mycelium) has almost overgrown the pathogen *Ophiostoma novo-ulmi* (white colony on the right), hindering its further growth on the plate. (b) The orange spots of the endophyte *Monographella nivalis* were found on the colony of the pathogenic *Ophiostoma novo-ulmi* in later stages of dual culture tests, indicating mycoparasitism by the endophyte. (c) Neutral interaction between *A. pullulans* (left side) and *O. novo-ulmi* (right side). Photos: Kathrin Blumenstein.

Development of an index for the colony form responses in dual cultures

The visual evaluation of the growth of the fungi in single and dual cultures demanded a mathematical value for more accurate comparisons. For this purpose an index was developed that allowed the calculation of a spherical index per setting (K. Blumenstein, unpublished results). *Ophiostoma novo-ulmi*

growing in single culture had a spherical index of 1, indicating that the colony grew regularly to all directions, resulting in a round (spherical) colony (Fig. 7a and b, black bars). When two *O. novo-ulmi* colonies were placed opposite to each other on a same Petri dish, they grew slightly to the sides, resulting in an index value that gradually differed from 1 over time, reaching an index of 1.8 after 30 days (Fig. 7a and b, white bars). In dual culture with *S. fimicola* the colony of the pathogen was surrounded by the endophyte already after 3 days and could therefore not further expand (Fig. 7a; compare with Fig. 6a). The influence of the endophyte *M. nivalis* var. *neglecta,* on the pathogen's colony shape was not measurable during the first 6 days. Afterwards the shape became more non-spherical because the formation of the reaction zone and a decreased growth facing towards the endophyte (Fig. 7b; compare with Fig. 5 left side).

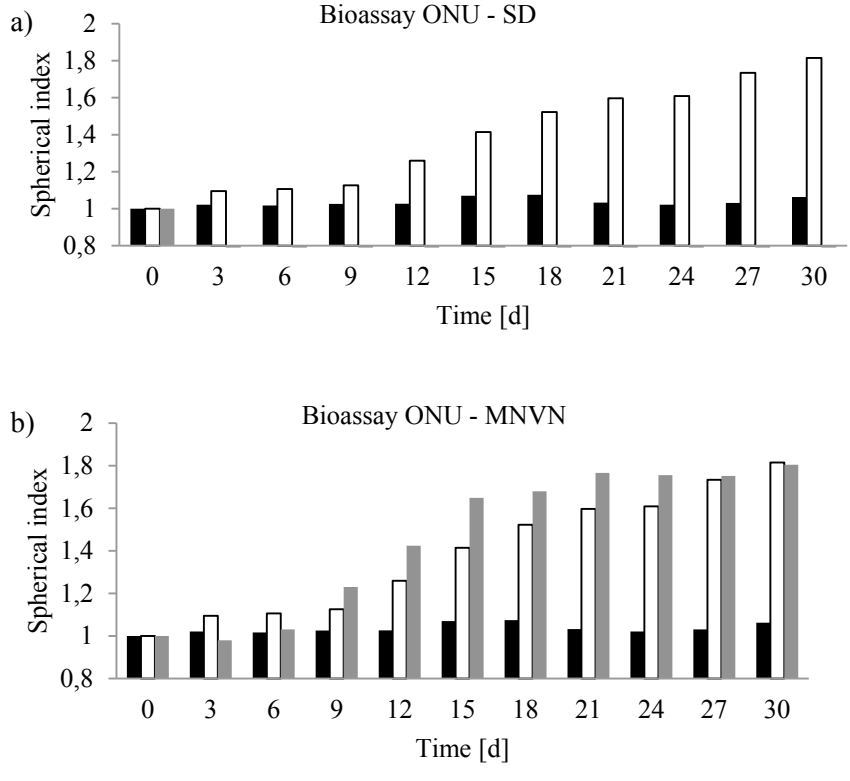

Figure 7. Influence of the presence (dual culture) or absence (single culture) of an opponent fungus on the colony shape of *O. novo-ulmi* (ONU). Dual culture partners were the endophyte (a) S. fimicola (SD) or (b) *M. nivalis* var. *neglecta* (MNVN), respectively. Black bars: ONU in single culture; white bars: ONU in dual culture with another ONU; grey bars: ONU in dual culture with an endophyte.

In vivo *antagonism tests*

Four endophytes were selected to be tested for their potential protective effect against DED as preventive inoculations in an *in vivo* study (paper II). The fungi were selected because they had shown the strongest antibiotic activity or because they inhibited the pathogen through competition for substrate or by a combination of the two mechanisms. In 2011 and 2012, the trees pre-treated with *M. nivalis* or *A. tenuissima* injections and then challenged with ONU showed lower leaf wilting symptoms at the end of the season as compared to the control treatments (Fig. 8). However, in 2013 the measured decreased wilting symptoms were not significantly different compared to the control treatment.

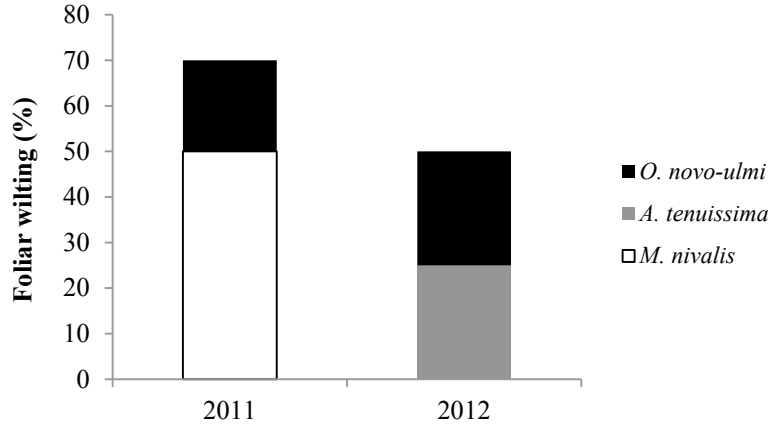

Figure 8. Significant effects on the disease symptoms for the first experiment from 2011 and the second experiment from 2012, each after 120 days. In the first experiment, *O. novo-ulmi* effected ~70% foliar wilting, whereas the treatment with *M. nivalis* prior to infection with the pathogen reduced the symptoms by ~20%. In the second experiment from 2012, *O. novo-ulmi* caused ~50% foliar wilting. The pre-treatment with the endophyte *A. tenuissima* reduced the wilting symptoms by ~25%. Data from paper II.

4.2.2 Nutritional profiling of endophytes with Phenotype MicroArrays – carbon and nitrogen substrates

When analysing the results from the nutritional profiling, differences could be observed in the quality (= the spectrum of utilized substrates) and the quantity (= the utilization effectiveness as shown by the value of AWCD) for the different fungi (papers III and IV).

The experiments described in paper III focused on the evaluation of the suitability of the PM method for the study of tree endophytes, the

improvement of selected steps in the preparation of the plates, and suggesting solutions for difficulties when working with endophytic fungi in PM technology. Part I in that paper was done with pre-configured Biolog plates filled with nitrogen sources. Experiences from paper IV, which was performed earlier in time, were applied to design and optimize this study.

In paper IV, the utilization of carbon sources was tested. The pathogen *O. novo-ulmi* utilized 54% of the available carbon sources (Fig. 9). The generalist *A. pullulans* used 22% (Fig. 9a). A total of 29 of these substrates overlapped with the ones utilized by the pathogen (Fig. 9a). The two endophytes *M. nivalis* var. *neglecta* 33 and 114 used 62% and 71% of the available substrates, respectively, (Fig. 9b and c). Only 16 and 7 sources (for isolates 33 and 114, respectively) were specific for the pathogen compared to the two endophytes. *Pyrenochaeta cava* used 59% of the tested carbon sources and also overlapped to a high extend with the pathogen (Fig. 9d): a total of 91 substrates were used by both the pathogen and the endophyte.

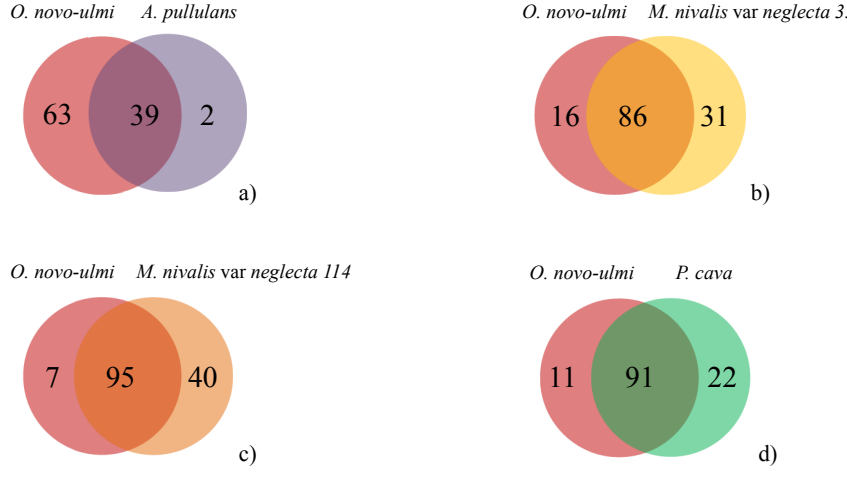

Figure 9. The venn-diagrams show the relation of shared substrates. a) the pathogen and *A. pullulans* shared most of the control fungus' utilized substrates, b) *M. nivalis* var. *neglecta* 33 shared 73.5% of its utilized sources with the pathogen and, c) *M. nivalis* var. *neglecta* 114 shared 70.37% of the 135 utilized in total and d) *P. cava* and the pathogen shared 80.53% of the 113 the endophyte uses in total.

The niche overlap index (paper IV) revealed which of the tested endophytes had a disadvantage compared to the pathogen in utilizing the compound group of interest: If the index was higher than 0.9 the pathogen had a superior capacity to utilize this group. The generalist *A. pullulans* had a comparatively high index for most compound groups, whereas the other endophytes had not

(Tab. 4). In the same line, the generalist fungus *A. pullulans* did not show specifically high values of the competitiveness index, but the three other endophytes did for the most substrate groups.

Table 4. *Niche overlap and endophyte competitiveness of the four tested endophytes [*M. nivalis *var.* neglecta *(Mn) isolate 33 and 114,* P. cava *(Pc) and* A. pullulans *(Ap)] in relation to the pathogen. A NOI value of 0.9 or higher (in bold) indicates a high degree of niche overlap and a competitive disadvantage for the endophyte. The competitiveness index of ≥1 (bold fields) indicates that the endophyte is more effective at utilizing the compound group. Table modified from paper IV.*

Compound group	Niche Overlap Index (NOI)[2]				Endophyte competitiveness index[3]			
	Mn33	Mn114	Pc	Ap	Mn33	Mn114	Pc	Ap
Sugar alcohols	**1.00**	**1.00**	**1.00**	**1.00**	0.86	0.86	1.00	0.71
Sacc. Phosphates		n.a.[4]				n.a.		
Tri- and tetra sacc.	**1.00**	**1.00**	**1.00**	**1.00**	1.00	1.00	1.00	1.00
Polysaccharides	**1.00**	0.89	0.88	**1.00**	1.00	**1.13**	1.00	0.63
Monosaccharides	0.86	**1.00**	**0.93**	**1.00**	1.00	0.86	1.00	0.57
Methyl-saccharides	0.25	0.33	0.25	0,50	**4.00**	**3.00**	**4.00**	**2.00**
Disaccharides	0.89	0.80	0.80	**1.00**	**1.13**	**1.25**	**1.25**	0.75
Phenolics	0.75	0.60	0.60	0.75	**1.33**	**1.67**	**1.67**	**1.33**
Amino acids	0.64	0.63	0.75	**1.00**	**1.47**	**1.60**	**1.33**	0.07
Alcohols	0.67	0.50	**1.00**	**1.00**	**1.50**	**2.00**	1.00	1.00
Acids	0.62	0.60	0.80	**1.00**	**1.26**	**1.52**	0.87	0.04
Nucleosides		n.a.				n.a.		
Miscellaneous	0.89	**0.92**	0.86	**1.00**	0.64	0.93	1.00	0.21
Surfactants	**1.00**	**1.00**	**1.00**	n.a.	0.67	1.00	1.00	n.a.

Preliminary tests were conducted to observe whether the ability of endophytes to utilize nitrogen compounds would be affected by the presence of an opponent fungus growing in the vicinity (K. Blumenstein, unpublished results). As an example, *O. novo-ulmi* (ONU) isolates 177, 178 and 179 and *M. nivalis* var. *neglecta* (MNVN), cultured in single cultures (SC) (Fig. 10, A) were compared with the cultures of the same strains that, prior to their inoculation in the PM plates, had been grown in dual cultures (DC) (Fig. 10, B). When the pathogen (ONU) had been grown in SC, the isolate 177 was clearly the strongest utilizer compared to the other isolates. Second most utilizer in SC was MNVN (Fig. 10, A). In the DC, MNVN was the most successful utilizer for all nitrogen compounds (Fig. 10, B). It was the only fungus that utilized fatty acids in the DC. Except for the miscellaneous group, ONU isolate 179 was the weakest utilizer.

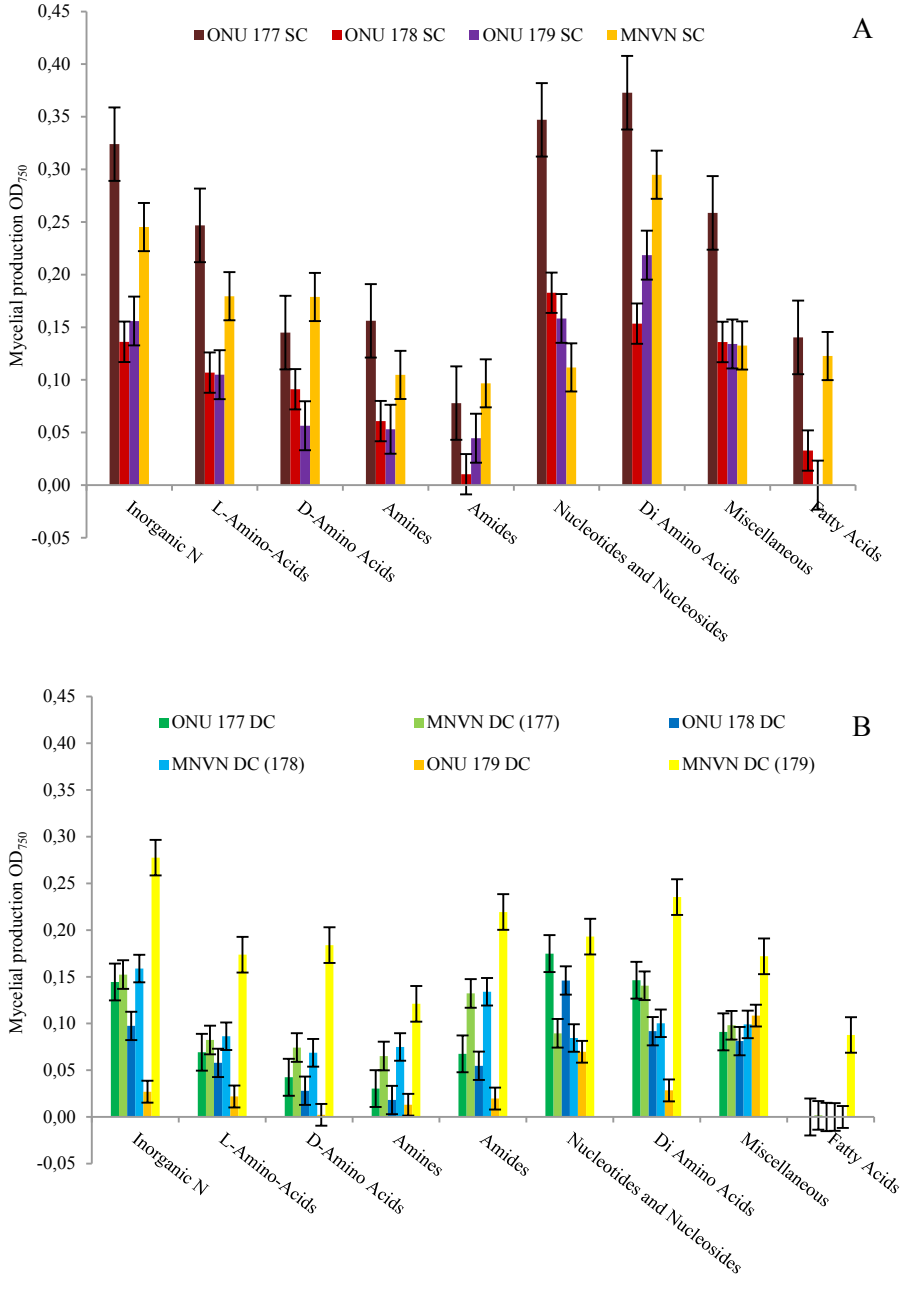

Figure 10. Mycelial production, measured as OD_{750} (120h post inoculation) in Phenotype MicroArrays with nitrogen compounds by three isolates *O. novo-ulmi* (ONU) 177, 178 and 179, and the endophyte *M. nivalis* var. *neglecta* 114, (A) grown in single culture (SC) or (B) dual culture (DC) before inoculations of the plates. The vertical bars show the average OD_{750} values for the nitrogen sources ordered in chemical groups. Values of each individual well (= source) were subtracted by control value. Negative values were set to 0; error bars show standard error.

4.2.3 Chemical analyses of extracellular fungal products

The extracts from the culture medium of *M. nivalis* var. *neglecta* and *P. cava* inhibited the growth of the pathogen as can be seen in Figure 11. Because no living fungus was needed to prevent the pathogen's growth, the inhibition through antibiosis was confirmed.

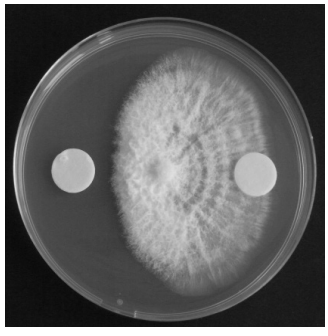

Figure 11. Paper disk tests: In the centre grows *O. novo-ulmi*. The paper disk on the right side is the control (disk soaked with phosphate buffer); the pathogen's growth development is not influenced. The disk on the left side is soaked with the extracted chemicals from *M. nivalis* var. *neglecta* dissolved in phosphate butter. The pathogen is inhibited to grow further towards the compounds on the disk. Photo: Kathrin Blumenstein.

Analysis with LC-MS detected 114 components in the extracts of *M. nivalis* var. *neglecta* and *P. cava*. Many correlated with activity. So far, none of the individual compounds in the extract has been identified. A few compounds gave tentative results such as $C_{24}H_{42}N_2O_6$ for the peaks in Fig. 12. Further work is needed in this area (K. Blumenstein, unpublished results).

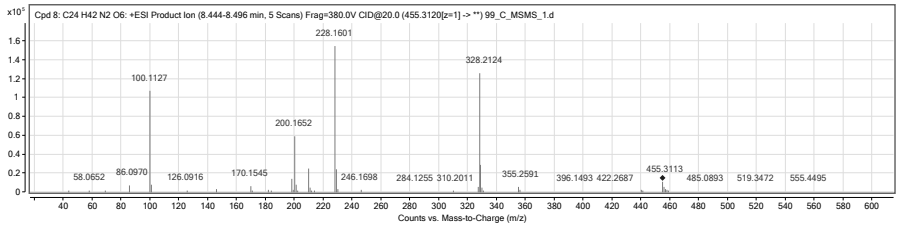

Figure 12. MS/MS spectrum for one of the compounds that correlated with activity. A possible identification suggests $C_{24}H_{42}N_2O_6$, IUPAC name: ethyl 4-[2-[(2-methylpropan-2-yl)oxy]-1-(1,4-oxazepan-4-yl)-2-oxo-1-pyrrolidin-1-ylethoxy]cyclohexane-1-carboxylate.

5 Discussion

5.1 General discussion

5.1.1 DED complex as a system for endophyte studies

The overall goal of my thesis was to contribute to the knowledge about the mechanisms of interactions between selected endophytic fungi and the DED pathogen *O. novo-ulmi*. The DED complex was used as a model system for endophyte-pathogen interactions because of its well-known disease cycle and the access to the Spanish collection of elm trees with documented susceptibility level to the disease.

Because of the extensiveness of DED, the elms are often considered as a "lost case" in forestry, and thus the need of further research on this study system can be questioned. Yet, there are many reasons for continued research on elms. Throughout the temperate deciduous forests, elms are an integral part of natural ecosystems, with high arboricultural and amenity importance. Elms harbour a rich biodiversity (fungi, insects, lichens) that can be endangered along with their hosts (Heybroek, 1993a; Höjer & Hultgren 2004). My studies on elm endophytes are thus highly timely, considering that we might lose some of elm-associated endophyte diversity in coming decades. Moreover, the damages that DED causes destroy valuable landscapes, e.g., the riparian forests that protect water quality and provide other ecosystem services and income opportunities for landowners (Martín *et al*., 2010). In southern Sweden, for instance, forestry is missing elms as an alternative in forest regeneration, in particular after another, more recent tree disease, the ash decline (Stener, 2013) has further reduced the regeneration alternatives for the more moist sites. While the future of elms in forestry seems dark, it is motivated to attempt to conserve the existing genetic resources and associated biodiversity. In long-term, increased knowledge about the fungal components in the DED-system may also help to prevent other tree-disease epidemics.

5.1.2 Biocontrol as an option in DED control

The findings of my studies indicate that certain endophytic fungi have a high capacity to inhibit or suppress the growth of the DED pathogens through different mechanisms, confirming the promise these fungi have for the biological control of DED. Yet, there are only few successful examples of biological control of tree diseases, and several practical problems can be foreseen in biocontrol of DED using endophytes. In practice, inclusion of these fungi into IPM against DED would necessitate that the trees to be protected should be treated with the fungi (viable spores or mycelium) or their extracellular metabolites, and preferably the endophyte infections should be established in elms for a more permanent protective effect. This could be feasible in nurseries, but the nursery treatment would have to be effective a long time after planting to be of practical importance. Currently, however, very little is known about the spatial scale (within the trees) or temporal longevity of the endophyte infections (Newcombe, 2011; Witzell *et al*., 2014).

Although the practical solutions for use of endophytes in DED control are still far away, more emphasis on the attempts to use a combination of several control measures seem warranted when fighting tree diseases such as DED. Employment of multiple methods seems reasonable also because even though biological control provides many benefits, there are also drawbacks that need to be mentioned. One aspect is the slowness of biological control, although it is not likely to be as disturbing in the long-lived trees as it can be for example in greenhouse environments (Bale *et al*., 2008). The costs of the production and the application of biocontrol agents are much higher as compared to fast and reliable / proven chemical treatments (Bale *et al*., 2008). Moreover, the potential non-targeted effects, and the difficulty in predicting them, are concerns in the use of any biocontrol (Witzell *et al*., 2014), and could be especially pronounced if cocktails of multiple species or strains would be applied. Clay (2004) suggests using endophytes in biocontrol by spraying trees with fungal spores or by growing inoculated trees alongside other trees that could serve as sources of fungal inoculum. The host specificity of endophytes or, rather, the lack of it would make uncontrolled host shifts by nonspecialized endophytes possible (Witzell *et al*., 2014). The interdependency between tree genotype and endophyte is also mentioned by Newcombe (2011), who points out that host shifting or invasiveness can occur if endophytes are introduced into new areas. Endophytes of one tree species can be pathogens for others, but common tree pathogens with high virulence do not occur as endophytes in other plant species (Sieber, 2007). A further threat to forest health is the dispersal of alien pathogens to forests, which increases the probability of hybridizations between alien and native species, possibly creating progenies

that are more aggressive than the parents of forest pathogens (Witzell *et al*., 2014).

Because of its power and robustness, tree breeding is seen as a more reliable control measure as compared to biocontrol, especially with the current possibilities to enhance tree resistance using gene technologies (Harfouche *et al*., 2011). As an alternative to biocontrol methods, chemically or biologically induced plant responses that increase the resistance can be considered, because of their environmental friendliness and high potential to suppress pests and pathogens (Solla & Gil, 2003; Hubbes, 2004; Blodgett *et al*., 2007; Schiebe *et al*., 2012). However, in many cases the regulation and reliability of induction can be problematic.

5.2 Methodological considerations

5.2.1 Culturable vs. "total" communities

My study focused on the work with culturable fractions of endophytic fungi isolated from bark, leaves and xylem tissues. Endophytic fungi with significant antagonistic potential *in vitro* and *in vivo* were found (paper I) and were further investigated for their modes of interaction (papers II and III). Working with endophytes that are easily culturable on artificial media allowed us to maintain such isolates conveniently. Similar procedure has been used in many studies with success (e.g., Arnold *et al*., 2000; Helander *et al*., 2007; Albrectsen *et al*., 2010). However, the isolation of endophytes was not comprehensive, and alternative isolation procedures, such as the dilution-to-extinction technique (Unterseher & Schnittler, 2009) would most likely have given different results. In particular, the diversity and frequency of fungi inhabiting the elm tissues, including the non-culturable fungi, are likely to be considerably higher than what could be detected using the described isolation technique.

Varying the incubation temperature may influence the isolated diversity of fungi. Generally, the preferred temperature-ranges between the isolated fungi can be assumed to be comparatively narrow, since the endophytes originated from the same habitat and were therefore likely to be to a certain temperature range. The length between the sub-cultivating steps is a crucial decision, because slow growing fungi can be overgrown by fast growing fungi, creating a bias. The peptone rich and slightly acidic MEA is commonly used for the isolation and cultivation of endophytic fungi. Fungi basically need a sugar source as the minimum growth requirement and can synthesize all cellular compounds from that (Deacon, 1997). The use of water agar favours the growth of slow growing fungi if sub-cultivation is undertaken

in regular distances. Nutrients will exhaust after a while since the piece of plant material is the only source. Bills and Polishook (1992) have demonstrated that the use of different media may yield distinct species richness's or greater or smaller numbers of isolates. They suggest that selective media may help to increase diversity of endophytes recovered from leaves or twigs. Comparative experiments performed with other tree species in Europe have demonstrated that incubation of the plant material under different drying regimes before the isolation of the endophytes takes place may yield distinct endophyte assemblages and can thus be an effective method to detect endophyte diversity in a given host tissue (Petrini *et al*., 1992).

The endophytes of trees are transmitted horizontally (from the environment) and infest the tree's tissues locally in most cases. They may spread in the xylem and phloem tissues, but it can be assumed that the speed and success of spreading varies. A full overview about the inhabiting endophytic fungi across the whole tree is therefore difficult to gain with any currently available technique. A concern raised by Arnold and Lutzoni (2007) is that common endophytes such as *Phomopsis*, *Xylaria*, *Colletotrichum*, *Fusarium*, and *Botryosphaeria* often tend to be generalists in terms of broad host ranges, and these endophytes dominate culturable fractions, whereas more specialized species may be found in the slow growing or unculturable fractions. Despite the limitations of the isolation technique, quite a big collection of endophytes was managed to be isolated including the slow growing species *Monographella nivalis* and *Pyrenochaeta cava* that antagonized the pathogen (paper I, II). Thus, the isolation technique proved adequate for the purpose of finding endophytes with antagonistic characteristics, and it enabled to obtain viable isolates that could be investigated for their interactions with the pathogen. If, however, the goal is to provide a comprehensive list of the entire mycobiota in the elm trees, molecular techniques such as pyrosequencing should be used.

5.2.2 Dual cultures

In studies of fungus-fungus interactions, a practical and cheap method are antagonism assays, also called dual culture tests. Under controlled conditions, such as temperature, light and media concentrations, the fungal interactions can easily be studied over time. The dual culture experiments served as the base experiment of my studies, allowing a first phenotypic classification of the isolated endophytes. In accordance to the observed and measured results, the further potential of the endophytes was evaluated. This method proved its effectivity, because it allowed a clear distinction between the different groups of interactions, while being rather easy to accomplish. If there was no visible

interaction ("neutral" interaction), the endophyte was not investigated further and thus could be excluded at an early stage of the investigations. If the endophyte showed superiority over the pathogen by a faster growth, resulting in suppression of the pathogen's growth, the next consequence for further investigations was to gain more information about the fungal nutritional preferences. The reason was that the potential of this endophyte as a biocontrol organism was classified as high due to its direct antagonism, and therefore those endophytes were included in the *in vivo* study (paper II) or the Phenotype MicroArrays (papers III and IV). If chemical antagonism was observed, the endophytes' potential as a possible biocontrol organism was also classified as high since this group of interaction was also a direct interaction. Those endophytes were included in the *in vivo* study (paper II) and some were further investigated for their nutrient utilization preferences in paper III and IV. Though, the dual cultures are an artificial *in vitro* study and force the opponent fungi to interact with each other. *In planta*, however, it is not known how big the actual size of the fungal thallus is that might interact or compete with the surrounding organisms. In addition, the growth medium used to accomplish the dual culture assays is malt extract agar, a common fungal growth medium. In the trees, fungi have to cope with facing a heterogeneous chemical environment, with different types of substrates in varying concentrations. The ability of fungi to interact with their surroundings is very likely dependent on their ability to utilize these substrates, resulting in different growths speeds, among other factors. Even though the *in vitro* results do not necessarily translate directly to what occurs *in planta*, *in vitro* studies and their results are particularly useful for identifying likely candidates for biocontrol and for making educated guesses concerning the mechanisms by which they reduce pathogen damage (Mejía *et al*., 2008). Nevertheless, my studies using Phenotype MicroArrays to give a proxy for the *in planta* situation served as the first step to mimic the natural situation. The preliminary experiments, where the endophytic fungi were exposed to co-cultures with the pathogen represent development of the technique towards increased correspondence with the *in planta* conditions.

5.2.3 Possibilities in the application of genomics for the research of endophyte communities

While the focus of my work was on the cultured fraction of endophytic communities, more detailed information about the diversity of endophyte communities could be gained through genomic approaches. The opportunities and the range of available molecular methodologies that can be implied in studies with endophytic fungi are diverse. Arnold *et al*. (2007) raised three

main points where genomics are mainly applied for in this research field: (1) identification purposes, especially of sterile endophytes that cannot be identified with macroscopic techniques, (2) restricting functional taxonomic units and (3) to avoid biases executed by culturing techniques.

Molecular sequence data from the multi-copy nuclear ribosomal internal transcribed spacer region (ITS) can be used to identify fungal isolates and to analyse species richness (Arnold *et al*., 2007; Unterseher & Schnittler, 2010). Because of the fast rate of evolution in the spacer regions, ITS data is useful in these purposes. It can be recovered rather easily and is highly present in GenBank (Arnold *et al*., 2007). A "biodiversity fingerprint" of a sampling site can be created (Promputtha *et al*., 2007; Jumpponen & Jones, 2009). The 16S rRNA (ribosomal ribonucleic acid) is the genomic region to be compared for the study of prokaryotic endophytes, microbial communities inhabiting stems, roots and tubers of plants (Ryan *et al*., 2008). Furthermore, DNA (deoxyribonucelid acid) barcoding has become a standardized tool for the assessment of global biodiversity patterns and it can allow diagnosis of known species as well as unknown species to non-taxonomists. It is a fast, accurate, and standardized method for species level identification, by using short DNA sequences. The method is not yet fully established for fungi (Das & Deb, 2015).

ITS data can also be used to evaluate morphotaxon boundaries. The morphotype concept is a rapid and reliable approach to assess species richness of cultivable foliar endophytic fungi, but it might not provide taxonomic information of the isolated community (Arnold *et al*., 2007). Morphotaxon boundaries are estimated on the basis of ITS BLAST matches to different taxa, comparisons of ITS sequence divergence, or phylogenetic relationships of endophytes and closely related species (for which ITS data can be aligned) (Unterseher & Schnittler, 2010). However most fungi are not represented in GenBank and some GenBank records are misidentified (Arnold *et al*., 2007). For testing variations in fungal endophyte communities, such as the diversity within individual trees, within sites, between adjacent sites that differ in nutrient availability, the culture-independent, high-throughput barcoded amplicon pyrosequencing can be used to quantify patterns of variation. This method is successful in detecting very high levels of diversity and can help to answer questions such as how the variation in endophyte diversity among sites reflects environmental characteristics (Zimmerman & Vitousek, 2012). In a study by Lamit *et al*. (2014), the fungal communities associated with *Populus angustifolia* James (Salicaceae; narrowleaf cottonwood) twigs were examined to understand how endophytes respond to genotypic differences in their host.

For estimating endophyte diversity and species composition, environmental PCR of plant material can be implied (Arnold *et al*., 2007). Compared to the culture based-method, the advantage of this method is the higher yield of genotypes (Sieber, 2007), to discover endophytes with obligate host associations, species that grow slowly or that do not grow on standard media and species that lose in competitive interactions during the culturing process. Nevertheless, it has also been reported that some commonly isolated fungi were never found by this method (Arnold *et al*., 2007).

As demonstrated above, many useful methodologies exist to study endophyte communities, to identify endophytes and to overcome biased conclusions of comprehension due to chosen culture techniques. Genomic tools can thus provide completely new insights into structures of endophyte communities. So far, however, even highly advanced genomic tools are still limited by the point-in-time nature of the analysis, and complementary methods are needed to decipher the functional aspects of individual endophytes, or endophyte communities, in trees.

5.2.4 Phenotype MicroArrays – possibilities and limitations

Modern pest and disease management is founded on a broad scientific base that is rapidly developing, advanced in particular by modern molecular technologies (Boyd *et al*., 2013). The analyses of genomes (genomics), proteins (proteomics) and metabolomes (metabolomics) have become the standard in investigations on the responses of cells to certain environmental conditions (Greetham, 2014). In general, genomics, proteomics and metabolomics analyses represent snapshots of the cellular physiology at the point of material collection (Endo *et al*., 2009; Grassl *et al*., 2009). However, for understanding the cellular responses in a given environment, the physiological state of a cell is additional useful information. This information can be gained by phenotyping.

The term ‘phenotype’ includes any cell property, including ‘molecular phenotypes’ such as the mRNA level of a single gene, whereas growth phenotypes define if and how fast a microorganism will grow (Bochner, 2009). Phenotype is the manifested attribute of an organism, the joint product of its genes and their environment during ontogeny (Atanasova & Druzhinina, 2010). Phenotype MicroArrays (PM) (phenomics) were originally designed for bacteria; the indicator system to measure the quantitative and qualitative utilization profiles for selected nutrients or chemicals was developed for these organisms (Bochner, 1989). Pre-filled PM plates are available for the analysis of cellular pathways of 200 different assays of carbon-source metabolisms, 400 assays of nitrogen metabolism, 100 assays of phosphorous and sulphur

metabolism, 100 assays of biosynthetic pathways, 100 assays of ion effects and osmolality, 100 assays of pH effects and pH control with deaminases and decarboxylases, and 1000 assays of chemical sensitivity. In the chemical sensitivity assays, there are 240 diverse chemicals, each at four concentrations (Bochner, 2009) demonstrating the diverse set of available options to choose from. In my work I chose the available plates for the analysis of catabolic pathways for carbon-sources and one plate of the nitrogen sources. Macronutrients such as carbon and nitrogen sources are of essential importance for fungi, being the major determinant of the fungal phenotype (Atanasova & Druzhinina, 2010). These macronutrients are found in elm tissues where most nitrogen in the xylem sap is in the form of amino acids, amides, amines and ammonia, nitrate is usually absent and sucrose, glucose and fructose are the most abundant sugars (Singh & Smalley, 1969). The concentrations of the nutrients vary in the sap dependent on the time of the year, the available soil nutrient and the susceptibility status of the elm phenotype (Singh & Smalley, 1969). It is, however, important to keep in mind that PMs do not accurately mimic the nutritional niche *in planta*. Nevertheless, the PM arrays provide a proxy for the enzymatic capacity of the investigated fungal strains *in vitro,* and by allowing us to bring all the tested fungi to a similar environment, it permits the comparison of their phenotypic reactions at a higher throughput than other currently available methods.

Clearly, fungi are capable of metabolizing a wide variety of nutrients (Caddick *et al.*, 1994; Tanzer *et al.*, 2003). Without time and financial restriction, testing the other available nitrogen plates and the phosphorous- and sulphur-plates would have been of interest as well. The three plate types chosen for my studies provided distinct utilization patterns for all tested fungi what has been the objective of such tests.

The evaluation of substrate utilisation using PM technology has been used to optimise growth media for all types of microorganisms as for instance filamentous fungi (Singh, 2009). PMs are attracting increasing attention due to their versatile applications. The investigation of the metabolic profile of cells through PM is not a new method though. According to Greetham (2014), the Dutch microbiologist Dooren de Long was the first to describe the identification of a microbe based on its carbon source utilisation in the 1920s. A further advantage of PM technology is that the target cells can be exposed to different conditions, which enables dynamic studies, such as investigation of proteins whose genes coding for pathways of secondary metabolites are often only turned on under a specific set of conditions for many microorganisms (Bochner, 2009; Greetham, 2014). Functional characteristics of cells can be used to complement mechanistic, biochemical and molecular, studies

(Bochner, 2009). Yet, the PM analysis also has limitations and cannot discover all cellular phenotypes. For instance, many microbial cells have phenotypes that involve intracellular structures that PM technology cannot measure; besides, the effects of certain genes might be cryptic or have a function under highly specific conditions (Bochner, 2003).

Intriguingly, while the PM technology has been used in a range of studies with filamentous fungi (see above), there is no standard for the preparation of inoculum. Despite the protocol available from Biolog, diverse modifications have been published and research groups seem to modify the procedure. According to Tanzer *et al*. (2003) the utilization of high-throughput microtiter plate growth methods has not been readily adopted for filamentous fungi because non-uniform growth typically leads to highly variable OD measurements. Thus, one of the objectives in my work was to refine an inoculation method for endophyte isolates, to gain reproducible optical density measurements for the growth of endophyte cells in microtiter wells.

My experiments showed that when preparing the fungal cultures, all fungi should be carefully adapted to the same cultural conditions before being transferred to the microplates. A temperature of 25 ℃ meets the average of the preferred fungi included in the studies. This temperature is recommended in the manufacturer's protocol. In an optimal case, each fungal culture, and later each microplate inoculated with the cells of that fungus, should be incubated at that particular fungus' optimal temperature for growth. However, this is hardly feasible in standard laboratory conditions. Besides, in nature, fungi do not meet their optimal favoured climate conditions either. In regard to fungi growing in elms, the active, pathogenic phase of the pathogen happens from spring until summer, usually meeting a temperature of 20-25 ℃ in most European countries. Therefore, in terms of external temperatures, the experimental conditions can be considered as rather close to reality.

In my study, the optical density for inoculum preparation was determined according to the manufacturer's instructions: adjusting the density of the IF-FF fluid to the standard of 62% turbidity in a turbidimeter provided by Biolog worked well in my study and led to reproducible results.

There are many alternatives to analyse and display the Biolog PM data. A practical approach to visualize the results is via so-called "heat maps" where the intensity of a colour indicates the degree of utilization at a certain measurement time, or the mycelial growth as OD against time or against the tested compounds. Alternatively a heat map can be combined with a hierarchical clustering of the compounds (Tanzer *et al*., 2003; Druzhinina *et al*., 2006; Atanasova & Druzhinina, 2010).

In study IV, I present the data as a mean value between selected time-points in the growth development of the fungi. This is in accordance to Atanasova and Druzhinina (2010) who argue that contrary to endpoint assays absorbance data need to be collected over the incubation period to generate complete growth curves for the nutrients. This is necessary because, for example, different carbon sources result in different growth kinetics, and assessing growth only at a single time point would eventually be indicative of the early growth phase in one case and the phase of already terminated growth in another. Nevertheless, I suggest that a single point data can be also meaningful if it represents the exponential growth phase, rather than the endpoint for the studied fungi (see the results section, Fig. 10).

5.3 Qualitative and quantitative differences in fungal endophytes between elm trees that differ in their susceptibility to Dutch elm disease

One of the questions that was raised in my thesis work was whether the culturable fractions of endophyte communities would reflect the susceptibility patterns of the host elms. Assuming a defensive function to endophytes, one could expect that the most susceptible trees would harbour fewer endophytes. Intriguingly, however, the results presented in paper I suggest that the less susceptible genotypes showed a lower frequency and diversity of fungal endophytes in the xylem tissues. This finding was especially interesting, taking into consideration that the DED pathogen develops within the xylem tissue. A plausible explanation could be that the defensive mechanisms of the resistant genotypes limit the colonization of all kinds of fungi in xylem, including both the pathogen and the endophytes. This assumption is indirectly supported by the fact that based on the profile of phenolic compounds, plant metabolites that have been associated with defensive and stress responses in trees (Witzell & Martín, 2008), the least susceptible genotypes were indeed grouped together and separated from the most susceptible genotypes. If negative correlation exists between the disease tolerance of the trees and their quality for xylem mycoflora, an enrichment of "resistant elms" in a landscape could have negative effects on the fungal biodiversity in it, especially if the xylem-bound endophytes were rare species.

It is intriguing that those endophytes that showed high antagonisms against the pathogen (paper II and IV) by chemical and nutritional superiority were originally isolated from the xylem tissues of elms with low susceptibility (paper I), and at that *M. nivalis* was most frequent in these trees. This could reflect a long evolution of elms and their endophytes. The communities in host

species of the same plant family tend to be dominated by closely related endophyte species, and it can be assumed that dominant endophytes have co-evolved with their hosts for more than 300 Ma. (Sieber, 2007).
Taken together, the findings in paper I suggest that the relation of endophytes to the host resistance is multifaceted. In addition, indirect support was found for the assumption that certain endophytes add to the trees' tolerance. The data presented in this thesis support my suggestion that the solution for successful tree disease control should consist of a combination of several measurements as this seems to be the natural case for the tolerant trees.

5.4 Mechanisms of antagonism between elm endophytes and DED pathogens

Earlier research on endophytes' defensive role in woody plants has been strongly focused on endophytes that are able to inhibit the growth of herbivores through mycotoxins and enzymes (e.g., Carroll, 1988; Petrini *et al*., 1992; Saikkonen *et al*., 2001; Albrectsen *et al*., 2010). My work, however, demonstrates that the endophytes, as a group, may counteract pathogenic fungi through multifaceted mechanisms. Chemical antibiosis and the endophytes' ability to compete successfully with the pathogen for resources, observed in my studies (paper II and paper IV) may add an extra layer to the phenotypic resistance of host trees. The existence of multifaceted and specialized mechanisms to compete against co-existing pathogens seems logical, taking into account that woody plant endophytes are closely related to pathogenic fungi, and may have evolved from them via an extension of latency periods and a reduction of virulence (Petrini *et al*., 1992). This view is supported by the fact that the endophytes are able to infect their hosts, but remain in a quiescent state inside the plant. The initial steps of host infection are, however, the same as those for pathogens (recognition, germination and penetration) (Sieber, 2007). Those fungi that manage to establish a symbiosis overcome preformed and induced plant defence mechanisms (Sieber, 2007). The inducible defences such as programmed cell death, papillae formation, phytoalexins, pathogenesis related proteins (Van Loon & Van Strien, 1999), e.g., peroxidases, chitinases, RNases, proteases and protease inhibitors (e.g., polygalacturonase inhibitor proteins) (De Lorenzo & Ferrari, 2002), might not be properly activated. Further, most natural populations may be mosaics of unique endophyte-host plant genotypic combinations that are adapted to the local biotic and abiotic environment (Saikkonen *et al*., 1998). Once living in their host plants' tissues, fungi are limited by their hosts' resources on which they depend.

5.4.1 Extracellular chemicals produced by endophytes

Fungi offer an enormous potential for new pharmaceutical and agrochemical industry products (Schulz *et al*., 2002). The search for bioactive fungal secondary metabolites was also of interest in my study for the endophytes that showed chemical antagonism towards the pathogen, such as *M. nivalis* var. *neglecta* and *P. cava*. Paper disk tests proved that the ethyl acetate extracts from these fungi had a strong antagonistic effect on the pathogen (K. Blumenstein, unpublished results). In the subsequent chemical analysis, attempts were made to elucidate the composition of the extract. The preliminary results show that a huge number of bioactive compounds, majority of which were unknown ones, could be detected in extracts. Their identification requires, however, more investigations than what was feasible within this thesis project.

The extracts with antifungal effect on DED pathogen could provide material for a bio-based, DED-control product. On average, however, 10 000 natural products need to be screened in order to receive one commercial product, and a chemical product would need to pass a rigorous testing for an environmental consequence analysis. Such a development process would take approximately 12 years (Schulz *et al*., 2002). Thus, more resources, time and the application of further techniques would bring clearer results and might enable a discrimination between bioactive and non-active compounds. When the active compounds are identified, further studies on their necessary concentrations and rate of degradation are needed. Further, it would be interesting to test if the bioactivity of the compounds is only effective in the mixture as the fungus produces it, or if only single compounds show the effect.

5.4.2 Competition between fungi – nutritional niches

I found evidence that endophytes may be effective utilizers of many organic compound groups, and that some endophytes in fact could utilize a broader range and higher amounts or organic compounds that the pathogen (paper IV). This finding leads to two hypotheses: first, endophytes might have a potential to utilize a considerable part of plant resources, and second, the great success of the pathogen might not be explained by it possessing a superior battery of degrading enzymes and thus winning over the mutualistic fungal flora in trees. Several aspects of the competitive interactions between fungi that in time and space share the elm tissues thus remain to be addressed in future studies.

The fact that more than one endophyte species can be isolated from the same tissue (Petrini, 1986) indicates that the plant tissues host complex fungal communities. Rodriguez & Redman (1997) distinguish between four classes of endophytic fungi as defined by their behaviour in plant tissues: (1)

fungi that actively grow through host tissues, resulting in extensive colonization; (2) fungi that actively grow through host tissues but only result in limited colonization; (3) fungi that are inhibited from colonization by plant defence responses or metabolic inhibitors, and remain metabolically quiescent until the host becomes senescent; and (4) fungi that are inhibited as described in (3) but that are metabolically active. Despite their status, most endophytic fungi are likely to compete with one another and with other groups of fungi (e.g., pathogens, saprophytes) at some stage in their life when sharing the same habitat, such as leaves, bark or xylem tissues in trees, in time and place.

According to Wicklow (1981), competition occurs when one species negatively affects another by consuming a common limited resource (exploitation) or controlling access to a limited resource (interference). Competition may result in species persisting on a resource at some equilibrium level, or it may lead to competitive exclusion where the winner is the species that can survive on the lowest level of a resource (e.g., Gause, 1934). The overall competitive ability of a species is probably attributable to a combination of factors including growth rates, metabolite production, niche overlap and interactions with environmental conditions (Lee & Megan, 1999). Lee & Megan (1999) suggest that environmental factors might exert selective pressures which influence community structure and the dominance of individual species. Competition between fungi has been categorized as either primary resource capture (colonization of unoccupied habitat) or secondary resource capture (colonization of habitat that is already occupied) (Rayner & Webber, 1984). Primary resource capture occurs at the beginning of the *in vitro* dual culture assays, when the fungal plugs are transferred to new petri dishes. The same situation might be found in young trees or saplings when the mycobiota is not fully established in a young plant or in new leaves when they are occupied by horizontally spreading endophytes in the beginning of the growth season. In the perennial parts of trees, such as bark and wood, where the endophyte infections are likely to accumulate, fungi might compete mainly in the secondary resource capture mode. It might be that the expression of competitive mechanisms occurs at higher rate or diversity in fungi that principally compete in this mode: while the colonization of unoccupied habitats probably demands fast and effective enzymatic capacity that enables the fungus to win over the plant defences, a secondary competition situation might demand more, and more specific, mechanisms that allow the fungus to combat also an array of other fungal occupants. To test this hypothesis with elm endophytes, more dual (and multi-) culture tests, as well as PM-analyses, studies should be conducted with several fungal species and strains, the tissue-specificity of which is known.

5.5 Future research

The eradication of DED as a goal seems unrealistic when considering the low success of eradication efforts in history and the many possible pathways for the distribution of the pathogen. It is rather desirable to manage the disease to an acceptable level (Scheffer *et al*., 2008) and for that goal biological control is one option. The comprehensive investigation of all endophytes inhabiting the resistant elm genotypes would be the initial step. Next-generation-sequencing (NGS) could be used to screen the trees' mycobiota for all species and sub-species. Further studies on the chemical fractions of the endophytes also seem warranted. Assuming all components of the extracellular compounds of an antagonistic endophyte were identified, a chemical product could be designed and applied to infected elms where it might function as a novel, specific fungicide. However, it can be further criticized that the use of any endophyte-derived chemicals will bring about the same environmental concerns as any other chemicals (Witzell *et al*., 2014). Therefore, the components of a biocontrol product would need to be classified as harmless to the environment: each single compound and as a compound mixture. *In vivo* tests would be necessary to gain knowledge about the required concentrations of such a product and its stability.

Using endophytes in practical forest protection creates challenges. In nature it is likely that endophyte communities rather than just a single endophyte, may contribute to resistant phenotypes. The dynamics of microbial communities over time and space adds to the challenge. If IPM measures to control tree diseases included endophytes, the question remains on how the endophyte community could be engineered in forests. Micropropagated elms, based on the most resistant phenotypes, and inoculated with selected endophytes in nurseries, could be the initial step in such a trial. By this, the tree would be equipped with a strengthening endophyte flora, helping it to defend itself against invading pathogens and at the same time constantly stimulating its immune system, completing the tree's defence strategies. It would be necessary to take into account that different climate conditions might favour the establishment of differing microbiota and also the phenotypic trees' characters might differ in regard to drought or frost tolerance or the susceptibility to bark beetles (Martín *et al*., 2015b).

At the current stage, breeding programmes are the most reliable option for recovery of native elm populations (Martín *et al*., 2015b) and therefore breeding for trees with higher tolerance is the most applied approach to deal with DED. In order to promote endophytology (Unterseher, 2012) it is important, as stated by Newcombe (2011) and Witzell *et al*. (2014), to guarantee the information transfer from research communities to end users and

other relevant professional groups, e.g., arborists, landscape engineers, and nature conservationists. In the long run, as we learn more about endophytes as a functional layer of biodiversity in trees, endophytology might become a natural part of the forest protection agenda, and individual endophytes or their combinations might be developed into potential tools in tree and forest protection and management.

5.6 Concluding remarks

Working with endophytic fungi has fascinated me more and more over the years and the responses I have observed in our tests have strengthened my conviction about the realistic potential of these fungi as biocontrol organisms. The ability of endophytes to change their morphology in response to external conditions is intriguing. As a research object, culturable endophytes have many advantages, since they are relatively easy to maintain on artificial growth media and they grow fast enough to give results in an experiment within a few days. The application range for endophytes is diverse and well explored through scientific investigation. With my work, I aimed to contribute to the characterization of these organisms and their role in trees. The different interaction mechanisms that I discovered show the endophytes' multiple "talents" in engineering their immediate surroundings, while at the same time living in mutualism with their host plants. The underlying hypothesis in many endophyte studies is that while the endophytes gain shelter and nutrients from their hosts, they may, at the same time, provide the hosts with ecological advantages, such as defence against invading pathogens. If we could better comprehend the spatial, temporal and mechanistic complexity of the interactions between the endophytic and pathogenic fungi, we would have stronger possibilities to use endophytes in IPM. The findings of my work demonstrate that certain endophytes have the ability to antagonize the DED pathogen through several mechanisms, such as the proposed occupation of the pathogen's nutritional niche in the host plant or repellence through the production of extracellular chemicals. It seems possible that several endophytes may express their antagonistic mechanisms at the same time and in a same space, and thus they might synergistically influence the pathogen's growth *in planta*. The primary question remaining unanswered is whether, and to what degree, tolerant tree phenotypes may gain their tolerance through an advantageous, endophytic microbiota.

6 Appendix

6.1 Recipes

6.1.1 Fungal culture media

Malt extract agar

20 g malt extract
18 g agar
1000 ml deionized water

PM inoculation media

PM 1 and PM2A
0.05 ml of cell suspension to 23.95 ml inoculating fluid

PM3B

0.125 ml cell suspension to 59.875 ml inoculating fluid

Table 5. *Recipe for PM inoculating fluids from stock solutions.*

PM Stock solution	PM1 and 2A	PM 3B
FF-IF	20.00	50.00
D-glucose 3200 mM	-	1.875
PM additive: potassium phosphate monobasic anhydrous (pH 6.0) 60 mM and sodium sulphate 24 mM	-	5.00
Cells	0.05	0.125
Sterile water	3.95	3.00
total	24	60.00

6.2 List of fungal isolates and their origins

Table 6.

Species name	Isolate no.	Origin	Experiment
M. nivalis var. *neglecta*	33	Xylem, *U. minor*, Rivas-Vaciamadrid	PM, *In vitro* dual cultures, Extractions
M. nivalis var. *neglecta*	114	Bark, *U. minor*, Rivas-Vaciamadrid	PM, *In vitro* dual cultures, Extractions
M. nivalis var. *neglecta*	99	Xylem, *U. minor*, breeding centre	Extractions
M. nivalis var. *neglecta*	JQ809674.1	Rivas-Vaciamadrid	*In vitro* and *in vivo* dual cultures
P. cava	120	Xylem, *U. minor*, low susceptibility	Dual cultures, Extractions
T. harzianum	a	CBS- KNAW Fungal Biodiversity Centre, NL	PM
T. harzianum	b		PM
T. harzianum	c		PM
A. pullulans	27	Leaf, *U. minor*, Rivas-Vaciamadrid	PM, *In vitro* dual cultures
A. pullulans	70	Leaf, *U. minor*, breeding centre	PM, *In vitro* dual cultures
A. pullulans	JX462673.1	Somontes (Madrid)	*In vitro* dual culture
P. crustosum	JX869565.1	Somontes (Madrid)	*In vitro* and *in vivo* dual culture
A. tenuissima	JX860514.1	Somontes (Madrid)	*In vitro* and *in vivo* dual culture
Sordaria sp.	JX298886.1	Rivas-Vaciamadrid	*In vitro* and *in vivo* dual culture
Fusarium sp.	HQ637287.1	Rivas-Vaciamadrid	*In vitro* dual culture
N. luteum	JX073038.1	Albufera de Mallorca	*In vitro* dual culture
O. novo-ulmi ssp. *americana*	177	2002, infected *U. minor* tree in San Sebastián de Gormaz (Soria, Spain, 41°34'N 3° 12'W) (Solla *et al.*, 2008)	PM, *In vitro* dual cultures
O. novo-ulmi ssp. *americana*	178		PM, *In vitro* dual cultures
O. novo-ulmi ssp. *americana*	179		PM, *In vitro* dual cultures
O. novo-ulmi ssp. *novo-ulmi*	ZA-RG	infected *U. minor* tree, Riego del Camino (Zamora, Spain; 41°05' N 5°46' W) (Solla *et al.*, 2008)	*In vitro* and *in vivo* dual culture
O. ulmi	a	CBS- KNAW Fungal Biodiversity Centre, NL	PM
O. ulmi	b		PM
O. ulmi	c	CBS Netherlands (Solla *et al.*, 2008)	PM

6.3 Phenotype MicroArray Plates used in this project

6.3.1 PM 1 MicroPlate™ Carbon sources

A1 Negative Control	**A2** L-Arabinose	**A3** N-Acetyl-D-Glucosamine	**A4** D-Saccharic Acid	**A5** Succinic Acid	**A6** D-Galactose	**A7** L-Aspartic Acid	**A8** L-Proline	**A9** D-Alanine	**A10** D-Trehalose	**A11** D-Mannose	**A12** Dulcitol
B1 D-Serine	**B2** D-Sorbitol	**B3** Glycerol	**B4** L-Fucose	**B5** D-Glucuronic Acid	**B6** D-Gluconic Acid	**B7** D,L-α-Glycerol-Phosphate	**B8** D-Xylose	**B9** L-Lactic Acid	**B10** Formic Acid	**B11** D-Mannitol	**B12** L-Glutamic Acid
C1 D-Glucose-6-Phosphate	**C2** D-Galactonic Acid-γ-Lactone	**C3** D,L-Malic Acid	**C4** D-Ribose	**C5** Tween 20	**C6** L-Rhamnose	**C7** D-Fructose	**C8** Acetic Acid	**C9** α-D-Glucose	**C10** Maltose	**C11** D-Melibiose	**C12** Thymidine
D1 L-Asparagine	**D2** D-Aspartic Acid	**D3** D-Glucosaminic Acid	**D4** 1,2-Propanediol	**D5** Tween 40	**D6** α-Keto-Glutaric Acid	**D7** α-Keto-Butyric Acid	**D8** α-Methyl-D-Galactoside	**D9** α-D-Lactose	**D10** Lactulose	**D11** Sucrose	**D12** Uridine
E1 L-Glutamine	**E2** M-Tartaric Acid	**E3** D-Glucose-1-Phosphate	**E4** D-Fructose-6-Phosphate	**E5** Tween 80	**E6** α-Hydroxy Glutaric Acid-γ-Lactone	**E7** α-Hydroxy Butyric Acid	**E8** β-Methyl-D-Glucoside	**E9** Adonitol	**E10** Maltotriose	**E11** 2-Deoxy Adenosine	**E12** Adenosine
F1 Glycyl-L-Aspartic Acid	**F2** Citric Acid	**F3** M-Inositol	**F4** D-Threonine	**F5** Fumaric Acid	**F6** Bromo Succinic Acid	**F7** Propionic Acid	**F8** Mucic Acid	**F9** Glycolic Acid	**F10** Glyoxylic Acid	**F11** D-Cellobiose	**F12** Inosine
G1 Glycyl-L-Glutamic Acid	**G2** Tricarballylic Acid	**G3** L-Serine	**G4** L-Threonine	**G5** L-Alanine	**G6** L-Alanyl-Glycine	**G7** Acetoacetic Acid	**G8** N-Acetyl-β-D-Mannos-amine	**G9** Mono Methyl Succinate	**G10** Methyl Pyruvate	**G11** D-Malic Acid	**G12** L-Malic Acid
H1 Glycyl-L-Proline	**H2** p-Hydroxy Phenyl Acetic Acid	**H3** m-Hydroxy Phenyl Acetic Acid	**H4** Tyramine	**H5** D-Psicose	**H6** L-Lyxose	**H7** Glucuron-amide	**H8** Pyruvic Acid	**H9** L-Galactonic Acid-γ-Lactone	**H10** D-Galacturonic Acid	**H11** Phenylethyl-amine	**H12** 2-Amino-ethanol

6.3.2 PM 2A MicroPlate™ Carbon sources

A1 Negative Control	A2 Chondroitin Sulfate C	A3 α-Cyclodextrin	A4 β-Cyclodextrin	A5 γ-Cyclodextrin	A6 Dextrin	A7 Gelatin	A8 Glycogen	A9 Inulin	A10 Laminarin	A11 Mannan	A12 Pectin
B1 N-Acetyl-D-Galactos-amine	B2 N-Acetyl-Neuraminic Acid	B3 β-D-Allose	B4 Amygdalin	B5 D-Arabinose	B6 D-Arabitol	B7 L-Arabitol	B8 Arbutin	B9 2-Deoxy-D-Ribose	B10 I-Erythritol	B11 D-Fucose	B12 3-0-β-D-Galacto-pyranosyl-D-Arabinose
C1 Gentiobiose	C2 L-Glucose	C3 Lactitol	C4 D-Melezitose	C5 Maltitol	C6 α-Methyl-D-Glucoside	C7 β -Methyl-D-Galactoside	C8 3-Methyl Glucose	C9 β-Methyl-D-Glucuronic Acid	C10 α-Methyl-D-Mannoside	C11 β-Methyl-D-Xyloside	C12 Palatinose
D1 D-Raffinose	D2 Salicin	D3 Sedoheptu-losan	D4 L-Sorbose	D5 Stachyose	D6 D-Tagatose	D7 Turanose	D8 Xylitol	D9 N-Acetyl-D-Glucosamini-tol	D10 γ-Amino Butyric Acid	D11 δ-Amino Valeric Acid	D12 Butyric Acid
E1 Capric Acid	E2 Caproic Acid	E3 Citraconic Acid	E4 Citramalic Acid	E5 D-Glucosamine	E6 2-Hydroxy Benzoic Acid	E7 4-Hydroxy Benzoic Acid	E8 β-Hydroxy Butyric Acid	E9 γ-Hydroxy Butyric Acid	E10 α-Keto Valeric Acid	E11 Itaconic Acid	E12 5-Keto-D-Gluconic Acid
F1 D-Lactic Acid Methyl Ester	F2 Malonic Acid	F3 Melibionic Acid	F4 Oxalic Acid	F5 Oxalomalic Acid	F6 Quinic Acid	F7 D-Ribono-1,4-Lactone	F8 Sebacic Acid	F9 Sorbic Acid	F10 Succinamic Acid	F11 D-Tartaric Acid	F12 L-Tartaric Acid
G1 Acetamide	G2 L-Alaninamide	G3 N-Acetyl-L-Glutamic Acid	G4 L-Arginine	G5 Glycine	G6 L-Histidine	G7 L-Homoserine	G8 Hydroxy-L-Proline	G9 L-Isoleucine	G10 L-Leucine	G11 L-Lysine	G12 L-Methionine
H1 L-Ornithine	H2 L-Phenyl-alanine	H3 L-Pyroglutamic Acid	H4 L-Valine	H5 D,L-Carnitine	H6 Sec-Butylamine	H7 D,L-Octopamine	H8 Putrescine	H9 Dihydroxy Acetone	H10 2,3-Butanediol	H11 2,3-Butanone	H12 3-Hydroxy 2-Butanone

6.3.3 PM 3B MicroPlate™ Nitrogen sources

A1 Negative Control	**A2** Ammonia	**A3** Nitrite	**A4** Nitrate	**A5** Urea	**A6** Biuret	**A7** L-Alanine	**A8** L-Arginine	**A9** L-Asparagine	**A10** L-Aspartic Acid	**A11** L-Cysteine	**A12** L-Glutamic Acid
B1 L-Glutamine	**B2** Glycine	**B3** L-Histidine	**B4** L-Isoleucine	**B5** L-Leucine	**B6** L-Lysine	**B7** L-Methionine	**B8** L-Phenyl-alanine	**B9** L-Proline	**B10** L-Serine	**B11** L-Threonine	**B12** L-Tryptophan
C1 L-Tyrosine	**C2** L-Valine	**C3** D-Alanine	**C4** D-Asparagine	**C5** D-Aspartic Acid	**C6** D-Glutamic Acid	**C7** D-Lysine	**C8** D-Serine	**C9** D-Valine	**C10** L-Citrulline	**C11** L-Homoserine	**C12** L-Ornithine
D1 N-Acetyl-D,L-Glutamic Acid	**D2** N-Phthaloyl-L-Glutamic Acid	**D3** L-Pyroglu-tamic Acid	**D4** Hydroxylamine	**D5** Methylamine	**D6** N-Amylamine	**D7** N-Butylamine	**D8** Ethylamine	**D9** Ethanolamine	**D10** Ethylene-diamine	**D11** Putrescine	**D12** Agmatine
E1 Histamine	**E2** β-Phenyl-ethylamine	**E3** Tyramine	**E4** Acetamide	**E5** Formamide	**E6** Glucuronamide	**E7** D,L-Lactamide	**E8** D-Glucosamine	**E9** D-Galactos-amine	**E10** D-Mannos-amine	**E11** N-Acetyl-D-Glucos-amine	**E12** N-Acetyl-D-Galactos-amine
F1 N-Acetyl-D-Mannos-amine	**F2** Adenine	**F3** Adenosine	**F4** Cytidine	**F5** Cytosine	**F6** Guanine	**F7** Guanosine	**F8** Thymine	**F9** Thymidine	**F10** Uracil	**F11** Uridine	**F12** Inosine
G1 Xanthine	**G2** Xanthosine	**G3** Uric Acid	**G4** Alloxan	**G5** Allantoin	**G6** Parabanic Acid	**G7** D,L-α-Amino-N-Butyric Acid	**G8** γ-Amino-N-Butyric Acid	**G9** ε-Amino-N-Caproic Acid	**G10** D,L-α-Amino-Caprylic Acid	**G11** δ-Amino-N-Valeric Acid	**G12** α-Amino-N-Valeric Acid
H1 Ala-Asp	**H2** Ala-Gln	**H3** Ala-Glu	**H4** Ala-Gly	**H5** Ala-His	**H6** Ala-Leu	**H7** Ala-Thr	**H8** Gly-Asn	**H9** Gly-Gln	**H10** Gly-Glu	**H11** Gly-Met	**H12** Met-Ala

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