DOCTOR OF PHILOSOPHY

Endophytic fungi in Elms:implications for the integrated management of Dutch Elm Disease

Blumenstein, Kathrin

Award date:
2016

Awarding institution:
Bangor University
Swedish University of Agricultural Sciences

Endophytic Fungi in Elms

Implications for the Integrated Management of Dutch Elm Disease

Kathrin Blumenstein
Faculty of Forest Sciences
Department of Southern Swedish Forest Research
Alnarp
&
Bangor University,
School of Environment, Natural Resources
and Geography, UK

Doctoral Thesis
Swedish University of Agricultural Sciences
Alnarp & Bangor University, UK 2015

Acta Universitatis agriculturae Sueciae
2015:106

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)

ISSN 1652-6880
ISBN (print version) 978-91-576-8410-3
ISBN (electronic version) 978-91-576-8411-0

Print: Repro, Alnarp 2015

Endophytic fungi in elms: Implications for the Integrated Management of Dutch Elm Disease

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.

Author's address: Kathrin Blumenstein, SLU, Department of Southern Swedish Forest Research, P.O. Box 49, 230 53 Alnarp, Sweden and Bangor University, School of Environment, Natural Resources and Geography (SENRGY), Deniol Road, Gwynedd, LL57 2UW, UK
E-mail: Kathrin.Blumenstein@slu.se

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

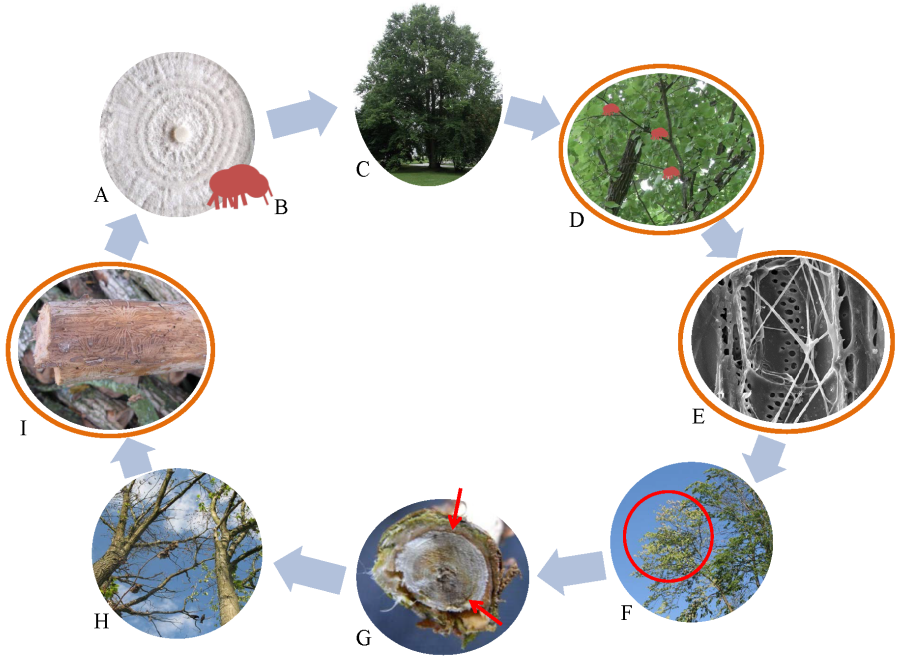


Figure 1. Disease cycle of DED. The pathogen *Ophiostoma novo-ulmi* (A) is transported via bark beetles (B) to healthy elms (C) while the beetle feeds in the tree's twig crotches (D). Spores grow into a mycelium within the xylem vessels (E). Disease symptoms such as wilting in the tree crown (F) or the 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).

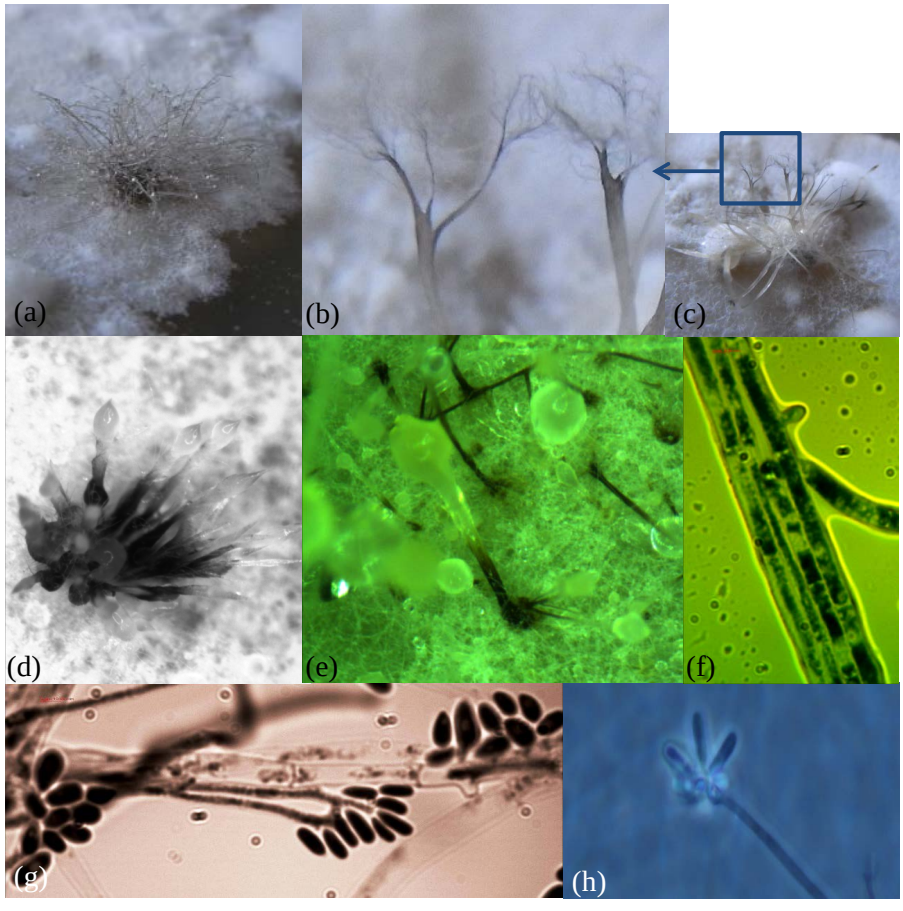


Figure 2. Saprophytic, anamorph (*Graphium*) phase of the pathogen producing synnematal structures (coremia) of~ 2 mm length with conidia-bearing sticky droplets (a-e); (f) coremia composed of fused dark conidiophores and (g, h) conidiophores with conidia (3-5µm length). Photos: Kathrin Blumenstein.

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

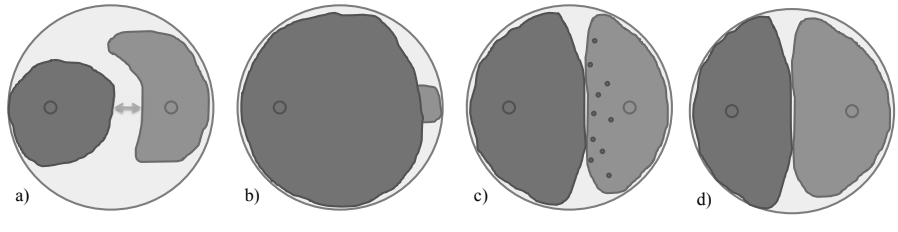


Figure 3. (a) antibiosis (arrow indicates reaction zone); (b) one fungus growth faster than the other; (c) mycoparasitism, (d) neutral / mutually intermingling growth.

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.

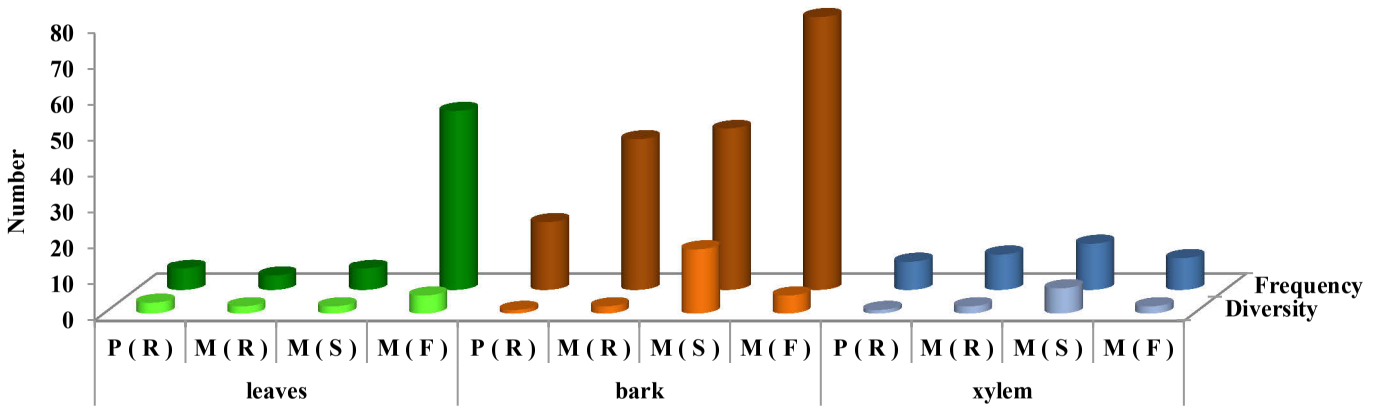


Figure 4. Differences in the frequency (number of isolates) and diversity (number of morphotaxa) among tree organ and groups: P(R) = resistant *U. pumila* clones from the breeding centre, M(R) = resistant *U. minor* clones from the breeding centre, M(S) = susceptible *U. minor* clones from the breeding 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).

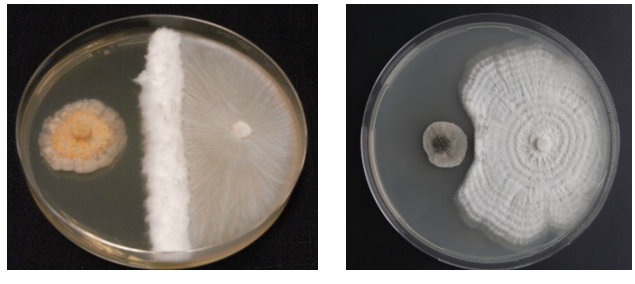


Figure 5. Monographella nivalis (left plate) and *Pyreonachaeta cava* (right plate) growing in dual culture with the Dutch elm disease pathogen, *Ophiostoma novo-ulmi* (white colony on the right side of each plate). A clear reaction zone can be observed between the colonies, indicating an antibiosis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

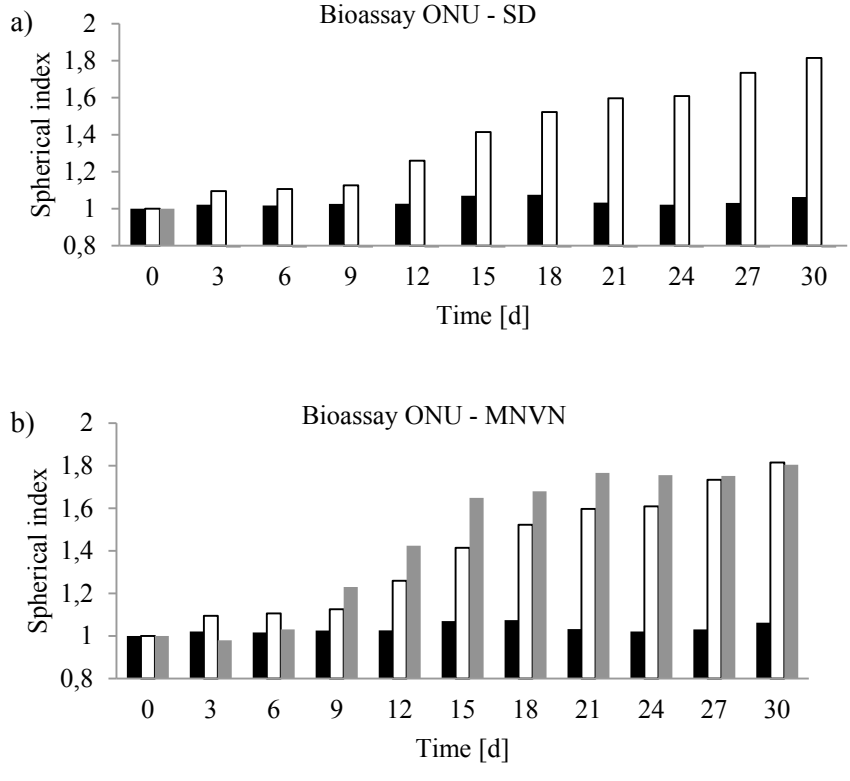


Figure 7. Influence of the presence (dual culture) or absence (single culture) of an opponent fungus on the colony shape of *O. novo-ulmi* (ONU). Dual culture partners were the endophyte (a) S. fimicola (SD) or (b) *M. nivalis* var. *neglecta* (MNVN), respectively. Black bars: ONU in single culture;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

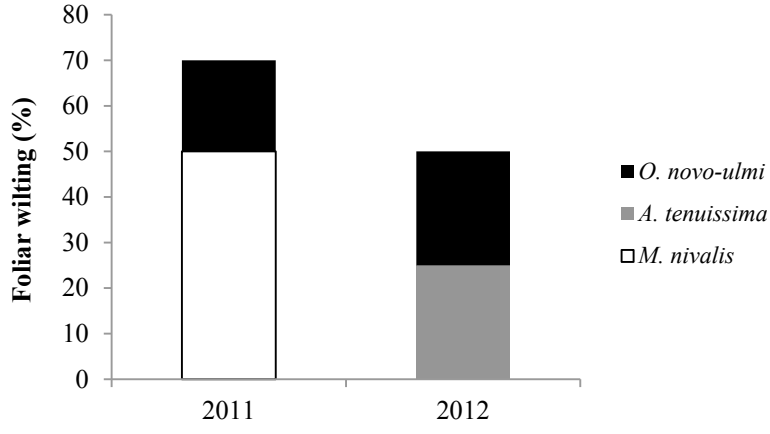


Figure 8. Significant effects on the disease symptoms for the first experiment from 2011 and the second experiment from 2012, each after 120 days. In the first experiment, *O. novo-ulmi* effected ~70% foliar wilting, whereas the treatment with *M. nivalis* prior to infection with the pathogen reduced 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.

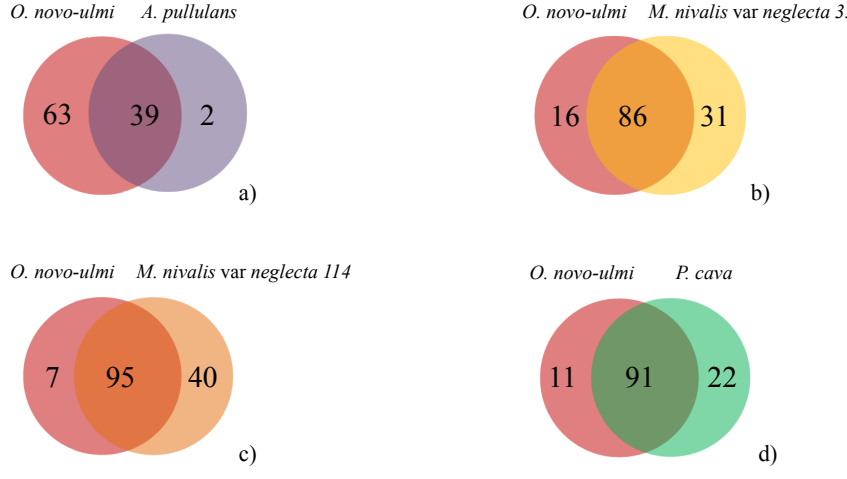


Figure 9. The venn-diagrams show the relation of shared substrates. a) the pathogen and *A. pullulans* shared most of the control fungus' utilized substrates, b) *M. nivalis* var. *neglecta* 33 shared 73.5% of its utilized sources with the pathogen and, c) *M. nivalis* var. *neglecta* 114 shared 70.37%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

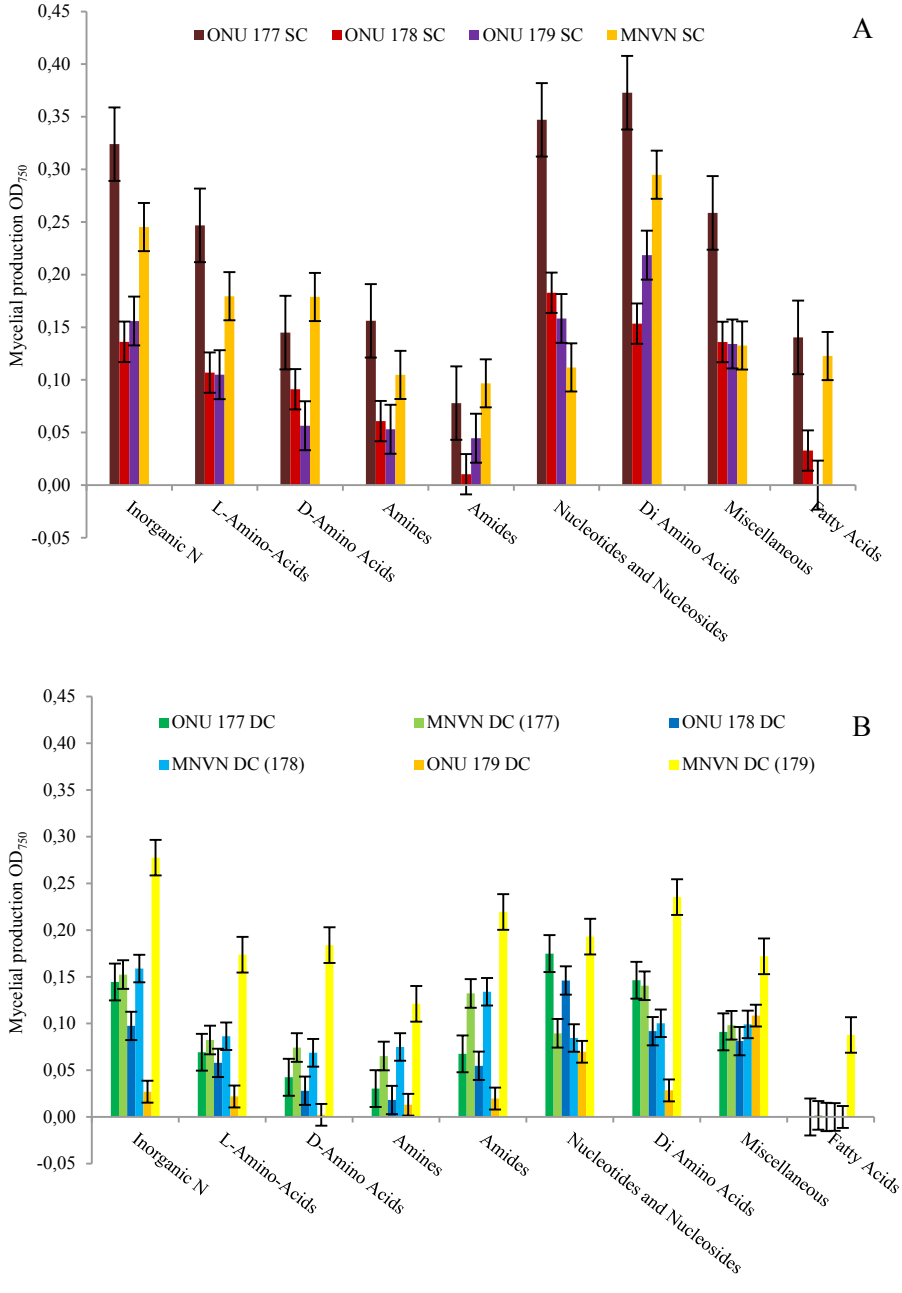


Figure 10. Mycelial production, measured as OD_{750} (120h post inoculation) in Phenotype MicroArrays with nitrogen compounds by three isolates *O. novo-ulmi* (ONU) 177, 178 and 179, and the endophyte *M. nivalis* var. *neglecta* 114, (A) grown in single culture (SC) or (B) dual culture (DC) before inoculations 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.

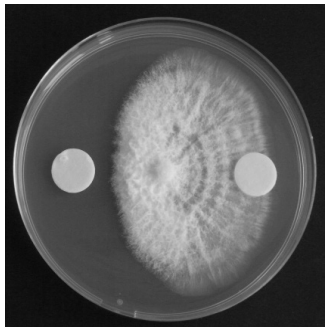


Figure 11. Paper disk tests: In the centre grows *O. novo-ulmi*. The paper disk on the right side is the control (disk soaked with phosphate buffer); the pathogen's growth development is not influenced. The disk on the left side is soaked with the extracted chemicals from *M. nivalis* var. *neglecta* 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).

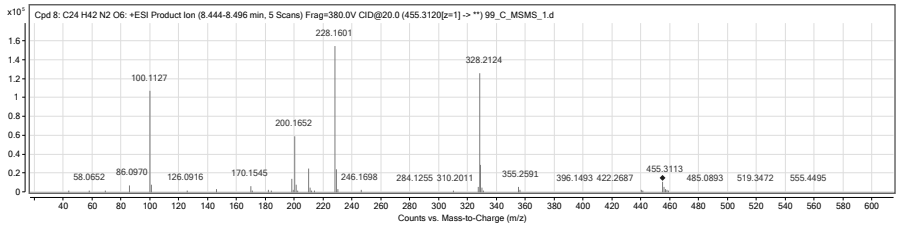


Figure 12. MS/MS spectrum for one of the compounds that correlated with activity. A possible identification suggests $C_{24}H_{42}N_2O_6$, IUPAC name: ethyl 4-[2-[(2-methylpropan-2-yl)oxy]-1-(1,4-oxazepan-4-yl)-2-oxo-1-pyrrolidin-1-ylethoxy]cyclohexane-1-carboxylate.

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

References

Albrectsen, B.R., Björkén, L., Varad, A., Hagner, Å., Wedin, M., Karlsson, J. & Jansson, S. (2010). Endophytic fungi in European aspen (*Populus tremula*) leaves - diversity, detection, and a suggested correlation with herbivory resistance. *Fungal Diversity,* 41(1), pp. 17-28.

Albrectsen, B.R. & Witzell, J. (2012). Disentangling functions of fungal endophytes in forest trees. In: Paz Silva, A. & Sol, M. (eds) *Fungi: Types, environmental impact and role in disease* Nova Science Publishers, pp. 235-246.

Anderson, P.K., Cunningham, A.A., Patel, N.G., Morales, F.J., Epstein, P.R. & Daszak, P. (2004). Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology and Evolution,* 19(10), pp. 535-544.

Aoun, M., Jacobi, V., Boyle, B. & Bernier, L. (2010). Identification and monitoring of *Ulmus americana* transcripts during *in vitro* interactions with the Dutch elm disease pathogen *Ophiostoma novo-ulmi*. *Physiological and Molecular Plant Pathology,* 74(3-4), pp. 254-266.

Arnold, A.E., Maynard, Z., Gilbert, G.S., Coley, P.D. & Kursar, T.A. (2000). Are tropical fungal endophytes hyperdiverse? *Ecology Letters,* 3(4), pp. 267-274.

Arnold, A.E., Maynard, Z. & Gilbert, G.S. (2001). Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. *Mycological Research,* 105(12), pp. 1502-1507.

Arnold, A.E., Mejía, L.C., Kyllo, D., Rojas, E.I., Maynard, Z., Robbins, N. & Herre, E.A. (2003). Fungal endophytes limit pathogen damage in a tropical tree. *PNAS,* 100(26), pp. 15649-15654.

Arnold, A.E. & Lutzoni, F. (2007). Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? *Ecology,* 88(3), pp. 541-549.

Atanasova, L. & Druzhinina, I.S. (2010). Global nutrient profiling by Phenotype MicroArrays: a tool complementing genomic and proteomic studies in conidial fungi. *Journal of Zhejiang University-Science B (Biomedicine & Biotechnology),* 11(3), pp. 151-168.

Bale, J.S., van Lenteren, J.C. & Bigler, F. (2008). Biological control and sustainable food production. *Philosophical Transactions: Biological Sciences,* 363(1492), pp. 761-776.

Bettucci, L. & Alonso, R. (1997). A comparative study of fungal populations in healthy and symptomatic twigs of *Eucalyptus grandis* in Uruguay. *Mycological Research,* 101(9), pp. 1060-1064.

Bills, G.F. & Polishook, J.D. (1992). A new species of *Mycoleptodiscus* from living foliage of *Chamaecyparis thyoides*. *Mycotaxon,* 43, pp. 453-460.

Bleiker, K.P. & Six, D.L. (2009). Effects of water potential and solute on the growth and interactions of two fungal symbionts of the mountain pine beetle. *Mycological Research,* 113, pp. 3-15.

Blodgett, J.T., Herms, D.A. & Bonello, P. (2005). Effects of fertilization on red pine defense chemistry and resistance to *Sphaeropsis sapinea*. *Forest Ecology and Management,* 208(1-3), pp. 373-382.

Blodgett, J.T., Eyles, A. & Bonello, P. (2007). Organ-dependent induction of systemic resistance and systemic susceptibility in *Pinus nigra* inoculated with *Sphaeropsis sapinea* and *Diplodia scrobiculata*. *Tree physiology,* 27(4), pp. 511-517.

Bochner, B.R. (1989). Sleuthing out bacterial identities. *Nature,* 339(6220), pp. 157-158.

Bochner, B.R. (2003). New technologies to assess genotype-phenotype relationships. *Nature Reviews Genetics,* 4(4), pp. 309-314.

Bochner, B.R. (2009). Global phenotypic characterization of bacteria. *FEMS Microbiology Reviews,* 33(1), pp. 191-205.

Borowicz, V.A. (2001). Do arbuscular mycorrhizal fungi alter plant-pathogen relations? *Ecology,* 82(11), pp. 3057-3068.

Boyd, I.L., Freer-Smith, P.H., Gilligan, C.A. & Godfray, H.C.J. (2013). The consequence of tree pests and diseases for ecosystem services. *Science,* 342(6160), pp. 1235773-1 - 1235773-8.

Brasier, C.M. (1983a). A cytoplasmically transmitted disease of *Ceratocystis-ulmi*. *Nature,* 305(5931), pp. 220-223.

Brasier, C.M. (1983b). The future of Dutch elm disease in Europe. In: Burdekin, D.A. (ed.) *Research on Duth Elm Disease in Europe.* Forestry Commission Bulletin 60 (HMSO, London), pp. 96-104.

Brasier, C.M. (1987). Recent genetic changes in the *Ophiostoma ulmi* populations: the threat to the future of the elm. In: Wolfe, M.S. & Caten, C.E. (eds) *Populations of plant pathogens.* Oxford: Blackwell Scientific Publications, pp. 213-226.

Brasier, C.M. (1990). China and the origins of Dutch elm disease - an appraisal. *Plant Pathology,* 39(1), pp. 5-16.

Brasier, C.M. (1991). *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. *Mycopathologia,* 115(3), pp. 151-161.

Brasier, C.M. (2000). Intercontinental spread and continuing evolution of the Dutch elm disease pathogens. In: Dunn, C.P. (ed.) *The elms: Breeding, Conservation and Disease Management.* Boston: Kluwer Academic Publishers, pp. 61-72.

Brasier, C.M. & Kirk, S.A. (2001). Designation of the EAN and NAN races of *Ophiostoma novo-ulmi* as subspecies. *Mycological Research,* 105(5), pp. 547-554.

Brasier, C.M. & Kirk, S.A. (2010). Rapid emergence of hybrids between the two subspecies of *Ophiostoma novo-ulmi* with a high level of pathogenic fitness. *Plant Pathology,* 59(1), pp. 186-199.

Buisman, C.J. (1932). *Ceratostomella ulmi*, de geslachtelijke vorm van *Graphium ulmi* Schwarz. *Tijdschrift over Plantenziekten,* 38(1), pp. 1-8.

Bultman, T.L. & Murphy, J.C. (2000). Do fungal endophytes mediate wound-induced resistance? *Microbial Endophytes*, pp. 421-452.

Caddick, M.X., Peters, D. & Platt, A. (1994). Nitrogen regulation in fungi. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology,* 65(3), pp. 169-177.

Carroll, G. (1988). Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. *Ecology,* 69(1), pp. 2-9.

Clay, K. (2004). Fungi and the food of the gods. *Nature,* 427(6973), pp. 401-402.

Clay, K. & Holah, J. (1999). Fungal endophyte symbiosis and plant diversity in successional fields. *Science,* 285(5434), pp. 1742-1744.

Cardarelli, E., Meriggi, A., Brangi, A. & Vidus-Rosin, A. (2011). Effects of arboriculture stands on European hare *Lepus europaeus* srping habitat use in an agricultural area of northern Italy. *Acta Theriologica*, 56(3), pp. 229-238.

Cogolludo-Agustín, M.A., Agundez, D. & Gil, L. (2000). Identification of native and hybrid elms in Spain using isozyme gene markers. *Heredity,* 85(2), pp. 157-166.

Conn, V.M., Walker, A.R. & Franco, C.M.M. (2008). Endophytic actinobacteria induce defense pathways in *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions,* 21(2), pp. 208-218.

European Commission (2004). Towards a European knowledge-based bioeconomy. RDT info. Office for Official Publications of the European Communities. *ec.europa.eu/research/bioeconomy/e-library/index_en.htm*

Dale, V.H., Joyce, L.A., McNulty, S., Neilson, R.P. & *et al*. (2001). Climate change and forest disturbances. *Bioscience,* 51(9), pp. 723-734.

Das, S. & Deb, B. (2015). DNA barcoding of fungi using ribosomal ITS marker for genetic diversity analysis: a review. *International Journal of Pure & Applied Bioscience,* 3(3), pp. 160-167.

De Lorenzo, G. & Ferrari, S. (2002). Polygalacturonase-inhibiting proteins in defense against phytopathogenic fungi. *Current Opinion in Plant Biology,* 5(4), pp. 295-299.

Deacon, J.W. (1997). *Modern Mycology*. 3rd edition. Blackwell Science, University of Edinburgh.

Díez, J.J. & Gil, L. (1998). Variability in phenylalanine ammonia-lyase activity among cell cultures of elm clones inoculated with *Ophiostoma novo-ulmi* spores. *Plant Pathology,* 47(6), pp. 687-692.

Djonović, S., Vargas, W.A., Kolomiets, M.V., Horndeski, M., Wiest, A. & Kenerley, C.M. (2007). A proteinaceous elicitor Sm1 from the beneficial

fungus *Trichoderma virens* is required for induced systemic resistance in maize. *Plant Physiology,* 145(3), pp. 875-889.

Downing, K.J., Leslie, G. & Thomson, J.A. (2000). Biocontrol of the sugarcane borer *Eldana saccharina* by expression of the *Bacillus thuringiensis cry1Ac7* and *Serratia marcescens chiA* genes in sugarcane-associated bacteria. *Applied and Environmental Microbiology,* 66(7), pp. 2804-2810.

Druzhinina, I.S., Schmoll, M., Seiboth, B. & Kubicek, C.P. (2006). Global carbon utilization profiles of wild-type, mutant, and transformant strains of *Hypocrea jecorina*. *Applied and Environmental Microbiology,* 72(3), pp. 2126-2133.

Duchesne, L.C., Jeng, R.S. & Hubbes, M. (1985). Accumulation of phytoalexins in *Ulmus americana* in response to infection by a nonaggressive and an aggressive strain of *Ophiostoma-ulmi*. *Canadian Journal of Botany,* 63(4), pp. 678-680.

Dvořák, M., Palovčíková, D. & Jankovský, L. (2006). The occurrence of endophytic fungus *Phomopsis oblonga* on elms in the area of southern Bohemia. *Journal of Forest Science,* 52(11), pp. 531-535.

ECPA (2015). Integrated Pest Management (IPM) - Combining biological, chemical & cultural measures for the sustainable management of pests. *http://www.ecpa.eu/dashboard/life-cycle/ipm*

Elgersma, D.M. (1970). Length and diameter of xylem vessels as factors in resistance of elms to *Ceratocystis ulmi*. *Netherlands Journal of Plant Pathology,* 76(3), pp. 179-182.

Endo, A., Nakamura, T. & Shima, J. (2009). Involvement of ergosterol in tolerance to vanillin, a potential inhibitor of bioethanol fermentation, in *Saccharomyces cerevisiae*. *FEMS Microbiology Letters,* 299(1), pp. 95-99.

Eyles, A., Bonello, P., Ganley, R. & Mohammed, C. (2010). Induced resistance to pests and pathogens in trees. *New Phytologist,* 185(4), pp. 893-908.

FAO (2015). AGP- integrated pest management. Pest and pesticide management. *http://www.fao.org/agriculture/crops/thematic-sitemap/theme/pests/ipm/en/*

Flint, M.L. & van den Bosch, R. (1981). *Introduction to Integrated Pest Management*. New York: Plenum Press.

Franken, P. (2012). The plant strengthening root endophyte *Piriformospora indica*: potential application and the biology behind. *Applied Microbiology and Biotechnology,* 96(6), pp. 1455-1464.

Fröhlich, J. & Hyde, K.D. (1999). Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic? *Biodiversity and Conservation,* 8(7), pp. 977-1004.

Fu, L. & Xin, Y. (2000). Elms of China. In: Dunn, C.P. (ed.) *The elms: Breeding, Conservation and Disease Management.* Boston: Kluwer Academic Publishers, pp. 21-44.

Ganley, R.J., Sniezko, R.A. & Newcombe, G. (2008). Endophyte-mediated resistance against white pine blister rust in *Pinus monticola*. *Forest Ecology and Management,* 255(7), pp. 2751-2760.

Garland, J.L. & Mills, A.L. (1991). Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Applied and Environmental Microbiology,* 57(8), pp. 2351-2359.

Gartland, J.S., McHugh, A.T., Brasier, C.M., Irvine, R.J., Fenning, T.M. & Gartland, K.M.A. (2000). Regeneration of phenotypically normal English elm (*Ulmus procera*) plantlets following transformation with an *Agrobacterium tumefaciens* binary vector. *Tree Physiology,* 20(13), pp. 901-907.

Gause, G.F. (1934). *The Struggle for Existence.* Williams & Wilkins, Baltimore.

Gennaro, M., Gonthier, P. & Nicolotti, G. (2003). Fungal endophytic communities in healthy and declining *Quercus robur* L. and *Q. cerris* L. trees in northern Italy. *Journal of Phytopathology,* 151(10), pp. 529-534.

Grassl, J., Westbrook, J.A., Robinson, A., Borén, M., Dunn, M.J. & Clyne, R.K. (2009). Preserving the yeast proteome from sample degradation. *Proteomics,* 9(20), pp. 4616-4626.

Greetham, D. (2014). Phenotype MicroArray technology and its application in industrial biotechnology. *Biotechnology Letters,* 36(6), pp. 1153-1160.

Grizzle, H.W. & Zak, J.C. (2006). A microtiter plate procedure for evaluating fungal functional diversity on nitrogen substrates. *Mycologia,* 98(2), pp. 353-363.

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

Guo, L.D., Huang, G.R., Wang, Y., He, W.H., Zheng, W.H. & Hyde, K.D. (2003). Molecular identification of white morphotype strains of endophytic fungi from *Pinus tabulaeformis*. *Mycological Research,* 107(6), pp. 680-688.

Guries, R.P. & Smalley, E.B. (2000). Once and future elms: classical and molecular approaches to Dutch elm disease resistance. In: Dunn, C.P. (ed.) *The elms: Breeding, Conservation and Disease Management.* Boston: Kluwer Academic Publishers, pp. 231-248.

Haack, S.K., Garchow, H., Klug, M.J. & Forney, L.J. (1995). Analysis of factors affecting the accuracy, reproducibility, and interpretation of microbial community carbon source utilization patterns. *Applied and Environmental Microbiology,* 61(4), pp. 1458-1468.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W.F. & Kloepper, J.W. (1997). Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology,* 43(10), pp. 895-914.

Harfouche, A., Meilan, R. & Altman, A. (2011). Tree genetic engineering and applications to sustainable forestry and biomass production. *Trends in Biotechnology,* 29(1), pp. 9-17.

Harrison, M.J. (2005). Signaling in the arbuscular mycorrhizal symbiosis. *Annual Review of Microbiology,* 59, pp. 19-42.

Haugen, L. (1998). How to identify and manage Dutch elm disease. USDA Forest Service, Northeastern Area State and Private Forestry, St. Paul, Minnesota. *http://www.na.fs.fed.us/spfo/pubs/howtos/ht_ded/ht_ded.htm*

Helander, M.L., Neuvonen, S., Sieber, T. & Petrini, O. (1993). Simulated acid rain affects birch leaf endophyte populations. *Microbial Ecology,* 26(3), pp. 227-234.

Helander, M.L., Sieber, T.N., Petrini, O. & Neuvonen, S. (1994). Endophytic fungi in Scots Pine needles - spatial variation and consequences of simulated acid-rain. *Canadian Journal of Botany,* 72(8), pp. 1108-1113.

Helander, M., Ahlholm, J., Sieber, T.N., Hinneri, S. & Saikkonen, K. (2007). Fragmented environment affects birch leaf endophytes. *New Phytologist,* 175(3), pp. 547-553.

Heybroek, H. M. (1993a). Why bother about the elm? In: Stricklen, M.B. & Sherald, J.L. (eds). *Dutch elm disease research: cellular and molecular approaches.* Springer-Verlag, NY, pp. 1-8.

Heybroek, H.M. (1993b). The Dutch elm breeding program. In: Sticklen, M.B. & Sherald, J.L. (eds) *Dutch elm disease research: cellular and molecular approaches.* Springer-Verlag, NY, pp. 16-25.

Heybroek, H.M., Elgersma, D.M. & Scheffer, R.J. (1982). Dutch elm disease - an ecological accident. *Outlook on Agriculture,* 11(1), pp. 1-9.

Höjer, O. & Hultgren, S. (2004). Åtgärdsprogram för särskilt skyddsvärda träd I kulturlandskapet. *Swedish Environmental Protection Agency Report 5411*, pp. 1-79.

Hollingsworth, P.M., Hollingsworth, M.L. & Coleman, M. (2000). The European elms: molecular markers, population genetics and biosystematics. In: Dunn, C.P. (ed.) *The elms: Breeding, Conservation and Disease Management.* Boston: Kluwer Academic Publishers, pp. 1-20.

Hoog, G.S.d. & Scheffer, R.J. (1984). *Ceratocystis* versus *Ophiostoma*: a reappraisal. *Mycologia*, 76(2), pp. 292-299.

Hubbes, M. (2004). Induced resistance for the control of Dutch elm disease. *Investigación Agraria: Sistemas y Recursos Forestales,* 13(1), pp. 185-196.

Hunt, M.D. & Ryals, J.A. (1996). Systemic acquired resistance signal transduction. *Critical Reviews in Plant Sciences,* 15(5-6), pp. 583-606.

Jumpponen, A. & Trappe, J.M. (1998). Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. *New Phytologist,* 140(2), pp. 295-310.

Jumpponen, A. & Jones, K.L. (2009). Massively parallel 454 sequencing indicates hyperdiverse fungal communities in temperate *Quercus macrocarpa* phyllosphere. *New Phytologist,* 184(2), pp. 438-448.

Knoester, M., Pieterse, C.M.J., Bol, J.F. & Van Loon, L.C. (1999). Systemic resistance in Arabidopsis induced by rhizobacteria requires ethylene-dependent signaling at the site of application. *Molecular Plant-Microbe Interactions,* 12(8), pp. 720-727.

Korhonen, K. (1978). Intersterility groups of *Heterobasidion annosum*. *Communicationes Instituti Forestalis Fenniae,* 94, pp. 1-25.

Lamit, L.J., Lau, M.K., Sthultz, C.M., Wooley, S.C., Whitham, T.G. & Gehring, C.A. (2014). Tree genotype and genetically based growth traits structure twig endophyte communities. *American Journal of Botany,* 101(3), pp. 467-478.

Larran, S., Simón, M.R., Moreno, M.V., Siurana, M.P.S. & Perelló, A. (2016). Endophytes from wheat as biocontrol agents against tan spot disease. *Biological Control,* 92, pp. 17-23.

Lee, H.B. & Magan, N. (1999). Environmental factors and nutritional utilization patterns affect niche overlap indices between *Aspergillus ochraceus* and other spoilage fungi. *Letters in Applied Microbiology,* 28(4), pp. 300-304.

Lee, S., Flores-Encarnacion, M., Contreras-Zentella, M., Garcia-Flores, L., Escamilla, J.E. & Kennedy, C. (2004). Indole-3-acetic acid biosynthesis is deficient in *Gluconacetobacter diazotrophicus* strains with mutations in cytochrome C biogenesis genes. *Journal of Bacteriology,* 186(16), pp. 5384-5391.

Martín, J.A., Solla, A., Coimbra, M.A. & Gil, L. (2008). Metabolic fingerprinting allows discrimination between *Ulmus pumila* and *U. minor*, and between *U. minor* clones of different susceptibility to Dutch elm disease. *Forest Pathology,* 38(4), pp. 244-256.

Martín, J.A., Fuentes-Utrilla, P., Gil, L. & Witzell, J. (2010). Ecological aspects of Dutch elm disease complex in Europe – a review. *Ecological Bulletins* 53:209-224.

Martín, J.A., Witzell, J., Blumenstein, K., Rozpedowska, E., Helander, M., Sieber, T.N. & Gil, L. (2013). Resistance to Dutch elm disease reduces presence of xylem endophytic fungi in elms (*Ulmus* spp.). *PLoS ONE,* 8(2).

Martín, J.A., Macaya-Sanz, D., Witzell, J., Blumenstein, K. & Gil, L. (2015a). 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.

Martín, J.A., Solla, A., Venturas, M., Collada, C., Domínguez, J., Miranda, E., Fuentes, P., Burón, M., Iglesias, S. & Gil, L. (2015b). Seven *Ulmus minor* clones tolerant to *Ophiostoma novo-ulmi* registered as forest reproductive material in Spain. *iForest - Biogeosciences and Forestry,* 8, pp. 172-180.

Mejía, L.C., Rojas, E.I., Maynard, Z., Van Bael, S., Arnold, A.E., Hebbar, P., Samuels, G.J., Robbins, N. & Herre, E.A. (2008). Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. *Biological Control,* 46(1), pp. 4-14.

Melching, J.B. & Sinclair, W.A. (1975). Hydraulic conductivity of stem internodes related to resistance of American elms to *Ceratocystis ulmi*. *Phytopathology,* 65(5), pp. 643-645.

Melin, E. & Nannfeld, J.A. (1934). Researches into the blueing of ground wood pulp. *Svensk Skogsvardsför Tidskrift,* 32, pp. 397-616.

Miller, H.J. & Elgersma, D.M. (1976). Scanning electron microscopical examination of development of aggressive and non-aggressive strains of *Ophiostoma ulmi* in elms. *Acta Botanica Neerlandica,* 25(3), pp. 255-255.

Moreau, C. (1952). Coexistence des formes *Thielaviopsis* et *Graphium* chez une souche de *Ceratocystis major* (van Beyma) nov. comb. Remarques sur les variation des *Ceratocystis*. *Revue mycol, Supplément Colonial,* 17(1), pp. 17-25.

Müller-Schärer, H., Schaffner, U. & Steinger, T. (2004). Evolution in invasive plants: implications for biological control. *Trends in Ecology and Evolution,* 19(8), pp. 417-422.

Neely, D. & Himelick, E.B. (1963). Root graft transmission of Dutch elm disease in municipalities. *Plant Disease Reporter*, 47, pp. 83-85.

Newcombe, G. (2011). Endophytes in forest management: four challenges. In: Pirttilä, A.M. & Frank, A.C. (eds) *Endophytes in forest trees: biology and applications*. Dortrecht: Springer, pp. 251-262.

Nürnberger, T. & Brunner, F. (2002). Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. *Current Opinion in Plant Biology,* 5(4), pp. 318-324.

Orr, D. (2009). Biological control and integrated pest management. In: Peshin, R. & Dhawan, A.K. (eds) *Integrated pest management: innovation-development*. Springer Science and Business Media B.V., pp. 207-239.

Pal, K.K. & McSpadden Gardener, B. (2006). Biological control of plant pathogens. *The plant health instructor*. DOI: 10.1094/PHI-A-2006-1117-02.

Pedrotti, L., Mueller, M.J. & Waller, F. (2013). *Piriformospora indica* root colonization triggers local and systemic root responses and inhibits secondary colonization of distal roots. *Plos One,* 8(7), p. e69352.

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

Petrini, O. & Fisher, P.J. (1986). Fungal endophytes in *Salicornia perennis*. *Transactions of the British Mycological Society,* 87, pp. 647-651.

Petrini, O. & Fisher, P.J. (1990). Occurrence of fungal endophytes in twigs of *Salix fragilis* and *Quercus robur*. *Mycological Research,* 94(8), pp. 1077-1080.

Petrini, O., Sieber, T.N., Toti, L. & Viret, O. (1992). Ecology, metabolite production, and substrate utilization in endophytic fungi. *Natural toxins,* 1(3), pp. 185-196.

Pieterse, C.M., van Wees, S.C., van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J. & van Loon, L.C. (1998). A novel signaling pathway controlling induced systemic resistance in Arabidopsis. *The Plant cell,* 10(9), pp. 1571-80.

Pirttilä, A.M., Joensuu, P., Pospiech, H., Jalonen, J. & Hohtola, A. (2004). Bud endophytes of *Scots pine* produce adenine derivatives and other compounds that affect morphology and mitigate browning of callus cultures. *Physiologia Plantarum,* 121(2), pp. 305-312.

Promputtha, I., Lumyong, S., Dhanasekaran, V., McKenzie, E.H.C., Hyde, K.D. & Jeewon, R. (2007). A phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. *Microbial Ecology,* 53(4), pp. 579-590.

Ragazzi, A., Moricca, S., Capretti, P., Dellavalle, I. & Turco, E. (2003). Differences in composition of endophytic mycobiota in twigs and leaves of healthy and declining *Quercus* species in Italy. *Forest Pathology,* 33(1), pp. 31-38.

Rayner, A.D.M. & Webber, J. (1984). Interspecific mycelial interactions - an overview. In: Jennings, D.H. & Rayner, A.D.M. (eds) *The ecology and physiology of the fungal mycelium*. Cambridge University Press, Cambridge, pp. 383-417.

Redman, R.S., Dunigan, D.D. & Rodriguez, R.J. (2001). Fungal symbiosis from mutualism to parasitism: who controls the outcome, host or invader? *New Phytologist,* 151(3), pp. 705-716.

Rodriguez, R.J. & Redman, R.S. (1997). Fungal life-styles and ecosystem dynamics: Biological aspects of plant pathogens, plant endophytes and *dvances in Botanical Research*, 24, pp. 169-193.

Rodríguez, J., Elissetche, J.P. & Valenzuela, S. (2011). Tree endophytes and wood biodegradation. In: Pirttilä, A.M. & Frank, A.C. (eds) *Endophytes in forest trees: biology and applications*. Dortrecht: Springer, pp. 81-93.

Rosenblueth, M. & Martínez-Romero, E. (2006). Bacterial endophytes and their interactions with hosts. *Molecular Plant-Microbe Interactions,* 19(8), pp. 827-837.

Rotblat, B., Enshell-Seijffers, D., Gershoni, J.M., Schuster, S. & Avni, A. (2002). Identification of an essential component of the elicitation active site of the EIX protein elicitor. *Plant Journal,* 32(6), pp. 1049-1055.

Rudinsky, J.A. (1962). Ecology of Scolytidae. *Annual Review of Entomology,* 7, pp. 327-328.

Ryan, R.P., Germaine, K., Franks, A., Ryan, D.J. & Dowling, D.N. (2008). Bacterial endophytes: recent developments and applications. *FEMS microbiology letters,* 278(1), pp. 1-9.

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

Saikkonen, K., Helander, M., Ranta, H., Neuvonen, S., Virtanen, T., Suomela, J. & Vuorinen, P. (1996). Endophyte-mediated interactions between woody plants and insect herbivores? *Entomologia Experimentalis Et Applicata,* 80(1), pp. 269-271.

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

Saikkonen, K., Ahlholm, J., Helander, M., Poteri, M. & Tuominen, J. (2001). Experimental testing of rust fungus-mediated herbivory resistance in *Betula pendula*. *Forest Pathology*, 31, pp. 321-329.

Santamaría, O., Pajares, J.A. & Diez, J.J. (2004). Physiological and morphological variation of *Gremmeniella abietina* from Spain. *Forest Pathology,* 34(6), pp. 395-405.

Santamaría, O. & Diez, J.J. (2005). Fungi in leaves, twigs and stem bark of *Populus tremula* from northern Spain. *Forest Pathology,* 35(2), pp. 95-104.

Santini, A., Fagnani, A., Ferrini, F., Mittempergher, L., Brunetti, M., Crivellaro, A. & Macchioni, N. (2003). Elm breeding for DED resistance, the Italian clones and their wood properties. In: Gil, L., Solla, A. & Ouellette, G. (eds) *New Approaches to Elm Conservation.* Instituto Nacional de

Investigatión y Tecnologia Agraria y Alimentaria (INIA), Madrid, Spain, pp. 179-184.
Santini, A., Ghelardini, L., De Pace, C., Desprez-Loustau, M.L. & *et al*. (2013). Biogeographical patterns and determinants of invasion by forest pathogens in Europe. *New Phytologist,* 197(1), pp. 238-250.
Santini, A. & Faccoli, M. (2014). Dutch elm disease and elm bark beetles: a century of association. *iForest-Biogeosciences and Forestry,* 8, pp. 126-134.
Scheffer, R.J. (1990). Mechanisms involved in biological control of Dutch elm disease. *Journal of Phytopathology,* 130(4), pp. 265-276.
Scheffer, R.J., Heybroek, H.M. & Elgersma, D.M. (1980). Symptom expression in elms after inoculation with combination of an aggressive and a non-aggressive strain of *Ophiostoma-ulmi*. *Netherlands Journal of Plant Pathology,* 86(6), pp. 315-317.
Scheffer, R.J., Voeten, J.G.W.F. & Guries, R.P. (2008). Biological control of Dutch elm disease. *Plant Disease,* 92(2), pp. 192-200.
Schiebe, C., Hammerbacher, A., Birgersson, G., Witzell, J., Brodelius, P.E., Gershenzon, J., Hansson, B.S., Krokene, P. & Schlyter, F. (2012). Inducibility of chemical defenses in Norway spruce bark is correlated with unsuccessful mass attacks by the spruce bark beetle. *Oecologia,* 170(1), pp. 183-198.
Schulz, B., Boyle, C., Draeger, S., Rommert, A.K. & Krohn, K. (2002). Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycological Research,* 106, pp. 996-1004.
Schulz, B. & Boyle, C. (2006). What are endophytes? In: Schulz, B., Boyle, C. & Sieber, T.N. (eds) *Microbial Root Endophytes*. Heidelberg, Germany: Springer Verlag.
Schwarz, M.B. (1922). Das Zweigsterben der Ulmen, Trauerweiden und Pfirsichbaume: eine vergleichend-pathologische Studie. *Mededelingen Phytopathologische Laboratorium „Willie Commelin Scholten“* , 5, pp. 1-73.
Sevilla, M., Burris, R.H., Gunapala, N. & Kennedy, C. (2001). Comparison of benefit to sugarcane plant growth and $^{15}N_2$ incorporation following inoculation of sterile plants with *Acetobacter diazotrophicus* wild-type and Nif mutant strains. *Molecular Plant-Microbe Interactions,* 14(3), pp. 358-366.
Shoresh, M., Yedidia, I. & Chet, I. (2005). Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by *Trichoderma asperellum* T203. *Phytopathology,* 95(1), pp. 76-84.
Sieber, T.N. (2007). Endophytic fungi in forest trees: are they mutualists? *Fungal Biology Reviews,* 21, pp. 75-89.
Singh, M.P. (2009). Application of Biolog FF MicroPlate for substrate utilization and metabolite profiling of closely related fungi. *Journal of Microbiological Methods,* 77(1), pp. 102-108.
Singh, D. & Smalley, E.B. (1969). Nitrogenous and carbohydrate compounds in the xylem sap of Ulmaceae species varying in resistance to Dutch elm disease. *Canadian Journal of Botany,* 47(2), pp. 335-339.

Smalley, E.B. (1963). Seasonal fluctuations in susceptibility of young elm seedlings to Dutch elm disease. *Phytopathology,* 53(7), pp. 846-853.
Smalley, E.B. & Lester, D.T. (1973). "Sapporo Autumn Gold" elm. *Hortscience,* 8(6), pp. 514-515.
Smalley, E.B., Ehlke, N.S., Clark, E.F. & Mai, S.H. (1982). Cambial reactions in *Ulmus* following inoculation with *Ceratocystis ulmi*. *Phytopathology,* 72(7), pp. 981-981.
Smalley, E.B. & Guries, R.P. (1993). Breeding elms for resistance to Dutch elm disease. *Annual Review of Phytopathology,* 31, pp. 325-352.
Smalley, E.B. & Guries, R.P. (2000). Asian elms: Sources of disease and insect resistance. In: Dunn, C.P. (ed.) *The elms: Breeding, Conservation and Disease Management.* Boston: Kluwer Academic Publishers, pp. 215-230.
Solla, A., Burón, M., Iglesias, S. & Gil, L. (2000). Spanish program for the conservation and breeding of elms against DED. In: Dunn, C.P. (ed.) *The elms: Breeding, Conservation and Disease Management.* Boston: Kluwer Academic Publishers, pp. 295-303.
Solla, A. & Gil, L. (2002). Xylem vessel diameter as a factor in resistance of *Ulmus minor* to *Ophiostoma novo-ulmi*. *Forest Pathology,* 32(2), pp. 123-134.
Solla, A. & Gil, L. (2003). Evaluating *Verticillium dahliae* for biological control of *Ophiostoma novo-ulmi* in *Ulmus minor*. *Plant Pathology,* 52(5), pp. 579-585.
Solla, A., Bohnens, J., Collin, E., Diamandis, S., Franke, A., Gil, L., Buron, M., Santini, A., Mittempergher, L., Pinon, J. & Broeck, A.V. (2005a). Screening European elms for resistance to *Ophiostoma novo-ulmi*. *Forest Science,* 51(2), pp. 134-141.
Solla, A., Martín, J.A., Corral, P. & Gil, L. (2005b). Seasonal changes in wood formation of *Ulmus pumila* and *U. minor* and its relation with Dutch elm disease. *New Phytologist,* 166(3), pp. 1025-1034.
Solla, A., Martín, J.A., Ouellette, G.B. & Gil, L. (2005c). Influence of plant age on symptom development in *Ulmus minor* following inoculation by *Ophiostoma novo-ulmi*. *Plant Disease,* 89(10), pp. 1035-1040.
Solla, A., Dacasa, M.C., Nasmith, C., Hubbes, M. & Gil, L. (2008). Analysis of Spanish populations of *Ophiostoma ulmi* and *O. novo-ulmi* using phenotypic characteristics and RAPD markers. *Plant Pathology,* 57(1), pp. 33-44.
Solla, A., López-Almansa, J., Martín, J. & Gil, L. (2014). Genetic variation and heritability estimates of *Ulmus minor* and *Ulmus pumila* hybrids for budburst, growth and tolerance to *Ophiostoma novo-ulmi*. *iForest - Biogeosciences and Forestry,* 8, pp. 422-430.
Stener, L.-G. (2013) Clonal differences in susceptibility to the dieback of *Fraxinus excelsior* in southern Sweden. *Scandinavian Journal of Forest Research*, 28(3), pp. 205-216.
Stirling, M. & Stirling, G. (1997). Disease management: biological control. In: Brown, J.F. & Ogle, H.J. (eds) *Plant Pathogens and Plant Diseases.* Rockvale Publications, pp. 427-440.

Stone, J.K. (1987). Initiation and development of latent infections by *Rhabdocline parkeri* on Douglas-Fir. *Canadian Journal of Botany,* 65(12), pp. 2614-2621.

Stone, J.K. & Petrini, O. (1997). Endophytes of forest trees: a model for fungus-plant interactions. In: Carroll, G. & Tudzynski, P. (eds) *The Mycota. V. Plant Relationships, Part B*. Berlin: Springer Verlag, pp. 129-140.

Strobel, G. & Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiol and Molecular Biology Reviews,* 67(4), pp. 491-502.

Strobel, G., Daisy, B., Castillo, U. & Harper, J. (2004). Natural products from endophytic microorganisms. *Journal of Natural Products,* 67(2), pp. 257-268.

Sturz, A.V., Christie, B.R., Matheson, B.G. & Nowak, J. (1997). Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. *Biology and Fertility of Soils,* 25(1), pp. 13-19.

Sturz, A.V., Christie, B.R. & Nowak, J. (2000). Bacterial endophytes: potential role in developing sustainable systems of crop production. *Critical Reviews in Plant Sciences,* 19(1), pp. 1-30.

Tan, X.Y., Hurek, T. & Reinhold-Hurek, B. (2003). Effect of N-fertilization, plant genotype and environmental conditions on *nifH* gene pools in roots of rice. *Environmental Microbiology,* 5(10), pp. 1009-1015.

Tanzer, M., Arst, H., Skalchunes, A., Coffin, M., Darveaux, B., Heiniger, R. & Shuster, J. (2003). Global nutritional profiling for mutant and chemical mode-of-action analysis in filamentous fungi. *Functional & Integrative Genomics,* 3(4), pp. 160-170.

Taylor, J.E., Hyde, K.D. & Jones, E.B.G. (1999). Endophytic fungi associated with the temperate palm, *Trachycarpus fortunei*, within and outside its natural geographic range. *New Phytologist,* 142(2), pp. 335-346.

Tiilikkala, K., Fagernas, L. & Tiilikkala, J. (2010). History and use of wood pyrolysis liquids as biocide and plant protection product. *The Open Agriculture Journal,* 4, pp. 111-118.

Tippet, A.W. (2009). Reducing the Incidence of Pressure Ulcers in Nursing Home Residents: A Prospective 6-Year Evaluation. *Ostomy Wound Management,* 55(11), pp. 52-58.

Townsend, A.M. (2000). USDA genetic research on elms. In: Dunn, C.P. (ed.) *The elms: Breeding, Conservation and Disease Management.* Boston: Kluwer Academic Publishers, pp. 271-278.

Turner, J.T., Lampel, J.S., Stearman, R.S., Sundin, G.W., Gunyuzlu, P. & Anderson, J.J. (1991). Stability of the delta-endotoxin gene from *Bacillus thuringiensis* subsp. *kurstaki* in a recombinant strain of *Clavibacter xyli* subsp. *cynodontis*. *Applied and Environmental Microbiology,* 57(12), pp. 3522-3528.

Unterseher, M. (2011). Diversity of fungal endophytes in temperate forest trees. In: Pirttilä, A.M. & Frank, A.C. (eds.) *Endophytes in forest trees: biology and applications*. Dortrecht: Springer, pp. 31-46.

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

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

Unterseher, M., Petzold, A. & Schnittler, M. (2012). Xerotolerant foliar endophytic fungi of *Populus euphratica* from the Tarim River basin, Central China are conspecific to endophytic ITS phylotypes of *Populus tremula* from temperature Europe. *Fungal Diversity*, 54, pp. 133-142.

Vallad, G.E. & Goodman, R.M. (2004). Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop Science,* 44(6), pp. 1920-1934.

Van Loon, L.C. & Van Strien, E.A. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology,* 55(2), pp. 85-97.

Vanneste, J.L., Yu, J. & Beer, S.V. (1992). Role of antibiotic production by *Erwinia herbicola*-eh252 in biological-control of *Erwinia amylovora*. *Journal of Bacteriology,* 174(9), pp. 2785-2796.

Van Wees, S.C.M., Van der Ent, S. & Pieterse, C.M.J. (2008). Plant immune responses triggered by beneficial microbes. *Current Opinion in Plant Biology,* 11(4), pp. 443-448.

Varma, A., Verma, S., Sudha, Sahay, N., Butehorn, B. & Franken, P. (1999). *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. *Applied and Environmental Microbiology,* 65(6), pp. 2741-2744.

Venturas, M., Lopez, R., Martín, J.A., Gasco, A. & Gil, L. (2014). Heritability of *Ulmus minor* resistance to Dutch elm disease and its relationship to vessel size, but not to xylem vulnerability to drought. *Plant Pathology,* 63(3), pp. 500-509.

Verma, S.C., Ladha, J.K. & Tripathi, A.K. (2001). Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. *Journal of Biotechnology,* 91(2-3), pp. 127-141.

Waage, J.K. & Greathead, D.J. (1988). Biological control: challenges and opportunities. *Philosophical Transactions: Biological Sciences,* 318(1189), pp. 111-128.

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Huckelhoven, R., Neumann, C., von Wettstein, D., Franken, P. & Kogel, K.H. (2005). The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proceedings of the National Academy of Sciences of the United States of America,* 102(38), pp. 13386-13391.

Webber, J. (1981). A natural biological control of Dutch elm disease. *Nature,* 292(5822), pp. 449-451.

Webber, J.F. & Brasier, C.M. (1984). The transmission of Dutch elm disease: a study of the processes involved. In: Anderson, J.M., Rayner, A.D.M. &

Walton, D.W.H. (eds) *Invertebrate-microbial interactions*. Cambridge University Press, pp. 271-306.

Weiß, M., Sykorova, Z., Garnica, S., Riess, K., Martos, F., Krause, C., Oberwinkler, F., Bauer, R. & Redecker, D. (2011). *Sebacinales* everywhere: previously overlooked ubiquitous fungal endophytes. *Plos One,* 6(2).

Wicklow, D.T. (1981). Interference competition and the organization of fungal communities. In: Wicklow, D.T. & Carrol, G.C. (eds) *The Fungal Community and its Organization and Role in the Ecosystem*. Marcel Dekker, New York, pp. 351–375.

Wiegrefe, S.J., Sytsma, K.J. & Guries, R.P. (1994). Phylogeny of elms (*Ulmus*, Ulmaceae): molecular evidence for a sectional classification. *Systematic Botany,* 19(4), pp. 590-612.

Wilson, M. & Lindow, S.E. (1994). Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. *Applied and Environmental Microbiology,* 60(12), pp. 4468-4477.

Witzell, J. & Martin, J.A. (2008). Phenolic metabolites in the resistance of northern forest trees to pathogens - past experiences and future prospects. *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere,* 38(11), pp. 2711-2727.

Witzell, J., Martín, J.A. & Blumenstein, K. (2014). Ecological aspects of endophyte-based biocontrol of forest diseases. In: Verma, V.C. & Gange, A.C. (eds) Advances in endophytic research. Berlin: Springer, pp. 321-333.

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

Acknowledgements

My deepest gratitude belongs to my principle supervisor Johanna Witzell. It is because of you, Johanna, that I enjoy research, learned to enjoy challenges and that I found the strength to develop my professional and personal skills as a researcher and woman in the world of natural sciences. Since the beginning of our collaboration you provided me with safety and freedom. You were always there when I needed your advice. Conversations with you gave me new energy and confidentiality during all these years. You showed me that obstacles are manageable when working as a team. I think we had fun during the last almost seven years that we know each other, planning the experiments, discussing our papers or attending seminars and conferences. You always planned to my advantage and with foresight to my future and I am so thankful to you for everything.

I like to thank my co-supervisors for their time and effort they spent on helping me with my thesis, writing articles and for the good personal discussions we had. Benedicte, I never would have come to SLU in Alnarp without you and I am very grateful that you arranged the contacts at that time and even became my co-supervisor on top. Malin, you helped me in the lab in my first years, always providing great ideas and solutions, taking time to explain new methods to me or discussing new ideas. Juan, you and Johanna initiated the DED project and you gave the base material of the project. You are the first author of two of the articles in my thesis and I am very grateful I could include them. Mark, thanks to your flexibility and interest in my project you became my co-supervisor when I needed a new supervisor from Bangor University. Thank you very much for helping me to sort out all the regulations and forms necessary with topics ranging from maternity leave to double degrees.

During my years at the Southern Swedish Forest Research Center I was lucky to meet so many wonderful (PhD-) colleagues and friends. In the order of the first meeting: thank you LiYing, Anna G., Rolf, Eric, Jonas, Lindsey, Lars, Rebecka, Giulia, Naran, Ida, Marta, Renats and Iryna, Anna F., Géraldine, Maria, Guillermo, Inga, Michelle and all other colleagues at the department who contributed to an unforgettable working atmosphere!

Thank you Kent, for being the first person I dared to speak Swedish to. You started at the same time as I did and your positive mentality and kindness contributed to having a good time at the department. Violeta, your helpfulness, patience and flexibility, always kind even when asking things again, meant a lot to me.

Thank you all my dear friends from the FONASO programme and at Bangor University!

I like to thank the financers of this project: the Erasmus Mundus Joint Doctoral Programme FONASO, the Department of Southern Swedish Forest Research (SLU), Formas, Stiftelsen Konsul Faxes Donation and KSLA (Kungl. Skogs- och Lantbruksakademien).

Finally, I like to thank my family: my parents who supported any of my decisions in life and who awakened my love to nature and to Sweden, my brother and his fiancé Elina who share the fascination for natural sciences, and of course my husband Michael who experienced, tolerated and supported me during the joy and stress of becoming a biologist, who followed me to Sweden and who has always been the stable pillar on my side. Little Kristian, thanks to you, life became so much richer, funnier and more complex. You made me keep my balance, laugh so much and show me what really matters in life.

I

OPEN ACCESS Freely available online

PLOS ONE

Resistance to Dutch Elm Disease Reduces Presence of Xylem Endophytic Fungi in Elms (*Ulmus* spp.)

Juan A. Martín[1], Johanna Witzell[2]*, Kathrin Blumenstein[2], Elzbieta Rozpedowska[3], Marjo Helander[4], Thomas N. Sieber[5], Luis Gil[1]

1 Departamento de Silvopascicultura, Escuela Técnica Superior de Ingenieros de Montes, Universidad Politécnica de Madrid, Madrid, Spain, **2** Southern Swedish Forest Research Centre, Faculty of Forest Sciences, Swedish University of Agricultural Sciences, Alnarp, Sweden, **3** Chemical Ecology, Swedish University of Agricultural Sciences, Alnarp, Sweden, **4** Department of Biology, Section of Ecology, University of Turku, Turku, Finland, **5** Institute of Integrative Biology, Eidgenössische Technische Hochschule (ETH) Zurich, Zurich, Switzerland

Abstract

Efforts to introduce pathogen resistance into landscape tree species by breeding may have unintended consequences for fungal diversity. To address this issue, we compared the frequency and diversity of endophytic fungi and defensive phenolic metabolites in elm (*Ulmus* spp.) trees with genotypes known to differ in resistance to Dutch elm disease. Our results indicate that resistant *U. minor* and *U. pumila* genotypes exhibit a lower frequency and diversity of fungal endophytes in the xylem than susceptible *U. minor* genotypes. However, resistant and susceptible genotypes showed a similar frequency and diversity of endophytes in the leaves and bark. The resistant and susceptible genotypes could be discriminated on the basis of the phenolic profile of the xylem, but not on basis of phenolics in the leaves or bark. As the Dutch elm disease pathogen develops within xylem tissues, the defensive chemistry of resistant elm genotypes thus appears to be one of the factors that may limit colonization by both the pathogen and endophytes. We discuss a potential trade-off between the benefits of breeding resistance into tree species, versus concomitant losses of fungal endophytes and the ecosystem services they provide.

Citation: Martín JA, Witzell J, Blumenstein K, Rozpedowska E, Helander M, et al. (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

Editor: Gregory A. Sword, Texas A&M University, United States of America

Received March 26, 2012; **Accepted** January 16, 2013; **Published** February 28, 2013

Funding: This work was supported by the Swedish Research Council FORMAS (www.formas.se), project 2008-1090; the Crafoord Foundation, Sweden (www.crafoord.se), grant 20070906; Stiftelsen Konsul Faxes Donation, Sweden (http://skogstradsforadling.se/stiftelsen-konsul-faxes-donation), projects KF 23 and KF 29; Ministerio de Ciencia e Innovación, Spain, project AGL2009-09289; Ministerio de Economía y Competitividad, Spain (http://www.mineco.gob.es), project CTQ2011-28503-C02-02; the Spanish elm breeding program (Ministerio de Agricultura, Alimentación y Medio Ambiente; Universidad Politécnica de Madrid); and the Joint Doctoral Program "Forest and Nature for Society", FONASO (www.fonaso.eu).The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: johanna.witzell@slu.se

Introduction

Fungal communities play key roles in global carbon sequestration and nutrient mineralization [1] and, for example, the importance of mycorrhizal symbionts for the growth of forest trees has been long established. A less well characterized group of fungal symbionts of forest trees are the endophytic fungi that live at least part of their lives within the aerial tissues of their hosts without causing symptoms [2,3]. Over time, and with conditioning from host-intrinsic and environmental factors, the nature of the tree-endophyte interaction can change and there is a continuum, ranging from neutral association to mutualistic, pathogenic or saprotrophic interactions [4–6]. Given suitable conditions, certain fungi can adopt any one of these life-styles [7], adding a further dimension of functional complexity to this layer of biodiversity inside plants.

Endophytes may provide their host plants with an epigenetic mechanism of adaptation to environmental stress [8,9]. Moreover, some fungal endophytes seem to protect plants against pathogens [10] and herbivores [11,12]. As primary colonizers some endophytes can be actively involved in the degradation of dead tissues [13,14]. Endophytic fungi may thus significantly contribute to the support and regulation of ecosystem services in forests. However, we still lack basic knowledge about regulation and functions of endophytic communities in forest ecosystems. For instance, it is not known whether the resistance status of a tree genotype against aggressive pathogens affects the establishment of endophytic fungi within it. This is a crucial issue for sound evaluation of the goals and approaches applied in forest conservation, restoration and tree breeding because resistance may then have environmental trade-off effects, potentially cascading from individuals to trophic levels and communities. Thus, alterations in endophytic communities in resistant trees could lead to modifications of ecosystem services (e.g. nutrient cycling) (cf. [15]).

In order to explore the possible trade-off between disease resistance and endophyte diversity in forest trees, it is necessary to study the endophytic communities in tree genotypes that express basal resistance or susceptibility to an aggressive pathogen. Elms (*Ulmus* spp.) are forest and amenity trees that are severely affected by the Dutch elm disease (DED) pathogen, *Ophiostoma novo-ulmi* Brasier, and they provide a suitable model system to study the links between pathogen resistance and endophyte colonization in forest trees. *Ulmus minor* Mill., the

main elm species studied in this work, has usually a dominant role in riparian forests of southern Europe. The vascular pathogen *O. novo-ulmi* is introduced into healthy elms by elm bark beetles and moves within xylem tissues, ultimately resulting in the development of a wilt syndrome [16]. DED has killed over 1 billion elm trees in Europe and North America. To assist attempts to conserve elm genetic resources, elm genotypes exhibiting high or low susceptibility to DED have been selected and are maintained as clones [17,18]. This material allows detailed investigations of factors, such as the endophytic flora, that contribute to the phenotypic resistance of elms to DED.

The basal resistance of elms to DED does not follow a major-gene pattern, but is polygenic (quantitative) in nature [19], and the traits behind this type of resistance are still poorly understood. One polygenic trait potentially contributing to plants' resistance to pathogens are phenolic compounds, defensive and signalling metabolites [20,21]. Their involvement in DED-induced responses has been demonstrated [22–24], but the role of constitutively expressed phenolics in the DED-resistance of elms is still unclear, and we do not know if the endophytic communities in elms are affected by them. Furthermore, other polygenic traits may be important for the DED resistance. In comparison to major-gene resistance, polygenic resistance is often considered more durable [25] and thus appears to be an attractive goal for resistance breeding. However, the drawbacks of quantitative resistance include the necessity of vegetative propagation [26], which could lead to a risk of low genetic variability in the propagated population. Moreover, polygenic resistance is also inevitably more non-specific than major-gene resistance [25,27], with potential to affect a broad spectrum of invading genotypes. Thus, it is conceivable that efforts to breed polygenic DED resistance into elms could have unintentional effects on the endophytic communities.

In the presented study, we hypothesized that elms with a high tolerance to DED host a less rich endophytic community than highly susceptible elms, due to their stronger defences, which are conferred by multiple genes. It should be noted that this hypothesis does not exclude the possibility that the phenotypic resistance shown by a given elm tree might be conferred by specific endophytes [28–30], either via direct antagonistic effects on pathogens or indirectly via the induction of specific plant responses, such as the production and release of defensive metabolites. Our study system allowed us to compare the constitutive phenolic profiles of elm genotypes with different degrees of DED resistance, and to evaluate the importance of tissue-specific phenolic status with respect to both pathogens and endophytes. The significance of the results for tree breeding and biodiversity conservation is discussed.

Methods

Ethics Statement

All necessary permits were obtained for the described field studies. One of the study sites, the Rivas-Vaciamadrid site is privately-owned. Oral permission for collection of samples was obtained from the landowner, Ms. Ana María Hernández Ros. For the activities at the Forest Tree Breeding Centre no specific permission was required. The Centre is governmentally-owned and the studies were conducted as part of the regular research activities under supervision of the Head of the Center, Mr. Salustiano Iglesias. The studies did not involve endangered or protected species.

Sites and Plant Material

Elms from two sites in the vicinity of Madrid, Spain were studied (Fig. 1). The first site is located at the Forest Breeding Centre in Puerta de Hierro (40°27′N, 3°46′W), and comprises 205 elm clones randomly planted with a spacing of 4×4 m in a conservation plot (152×36 m) with uniform microclimatic conditions. Each clone was represented by a single 14-year-old ramet. The distance between the selected clones ranged from 16 to 100 m, without any spatial grouping among resistant and susceptible clones. For our study, four *U. minor* and two *U. pumila* clones with low susceptibility to DED were selected (hereafter referred to as *resistant* clones), along with four *U. minor* clones that are highly susceptible to DED (*susceptible* clones; Table 1). The number of clones selected for study was determined by the availability of resistant trees (*U. minor* and *U. pumila*) of the same age and information of their different genetic background [31,32]. The soil has a sandy loam texture and was amended annually with organic matter to enhance moisture retention. The plot was irrigated by sprinklers during spring and summer to avoid water stress.

The second study site is a semi-natural riparian elm stand located in the municipality of Rivas-Vaciamadrid (40°20′N, 3°33′W) consisting of about 270 *U. minor* trees, all of which are between 65 and 75 years old [33]. It is the best-conserved elm stand in Madrid where *U. minor* is the dominant tree species. With a distance of 30 km it is also the closest stand to the Breeding Centre with *U. minor* as the dominant tree species. Most of the trees in the Rivas-Vaciamadrid stand belong to the unique, highly susceptible *U. minor* var. *vulgaris* clone [32]. This taxon presents very low genetic variability, probably because it originates from a single *U. minor* tree, the Atinian elm [32]. Thus, these trees are genetically close to the *U. minor* var. *vulgaris* clone UPM171 at the Breeding Centre. Since 2001, *O. ulmi* and *O. novo-ulmi* have been isolated from several trees of the stand [34], and *Scolytus* bark beetles are abundant in the area [33]. Despite these factors, the spread of DED in the stand is slow, suggesting environmental control of the disease. The Rivas-Vaciamadrid elm stand has historically been used as cattle raising area, where disinfectant products based on phenolic compounds (mainly phenol) were repeatedly applied to the cattle or to the soil to prevent insect bites and hoof infections. The same compounds have been shown to have a strong antifungal activity against *O. novo-ulmi* and induce the accumulation of suberin-like compounds in xylem tissues [35–37]. Seven *U. minor* var. *vulgaris* trees were selected from the stand on the basis of their similar dendrometric features (20.00±3.11 m in height; mean ± SD), good health condition and availability of information about their taxonomy [33].

The four tree groups were coded as follows: **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; and **M (F)**, *U. minor* trees from the Rivas-Vaciamadrid field population.

Sampling of Leaves and Twigs, and Isolation and Characterization of Endophytes

In mid May 2008, one terminal shoot (30 cm long) was cut from the lower half of the crown (at a height of 2 - 3 m) from each of the four cardinal points of the compass (i.e. four shoots from each elm tree). Four leaves were detached from each shoot in order to isolate endophytes (16 leaves per tree). Four 2-year-old twig segments (4 cm in length) were also cut from each shoot (16 twigs per tree) in order to isolate endophytes from bark and xylem tissues. Samples were transported in glass vials to the laboratory.

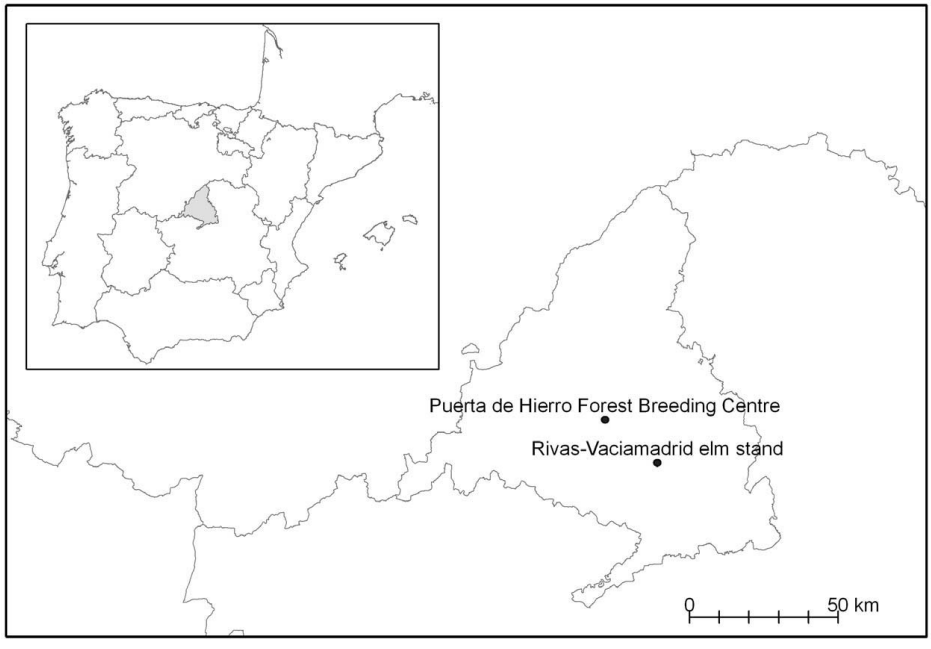


Figure 1. Location of the two study areas in central Spain.
doi:10.1371/journal.pone.0056987.g001

The leaves were surface-disinfected by dipping in 75% ethanol (30 s), 4% Na-hypochlorite (1 min) and 75% ethanol (15 s) [38]. After air drying (4 min), a disc with a diameter of 10-mm was cut aseptically from a randomly selected region of each leaf and placed on 2% (w/v) malt extract agar with no added antibiotics in Petri dishes. Twig segments (8–10 mm in diameter) were surface-disinfected following the same procedure as used for leaves save that they were immersed in the Na-hypochlorite solution for 5 min rather than one. After air drying (8 min), one 4×4×10 mm (thickness, width, length) slice (including bark and xylem tissues) was cut aseptically from each twig segment. The bark (about 2 mm thick) was separated from the xylem, and both tissues were

Table 1. Specifications of the plant material growing at *Puerta de Hierro* Forest Breeding Centre, Madrid, Spain [P (R) = resistant *U. pumila* clones; M (R) = resistant *U. minor* clones; M (S) = susceptible *U. minor* clones].

Species	Tree group	Code	Origin	Susceptibility to DED (% wilting[a])
U. pumila[b]	P (R)	201	Nanyiang, Henan, China	low (7±14)
		203	Shangqiu, Henan, China	low (24±18)
U. minor	M (R)	UPM007[c]	Alatoz, Albacete, Spain	low (27±10)
		UPM072	Cazorla, Jaén, Spain	low (31±12)
		UPM093	Dehesa de la Villa, Madrid, Spain	low (25±12)
		UPM130	Pedrizas, Málaga, Spain	low (28±11)
	M (S)	UPM045	Ruidera, Ciudad Real, Spain	high (94±13)
		UPM068	Huélago, Granada, Spain	high (90±15)
		UPM158	San Nicolás, Sevilla, Spain	high (80±18)
		UPM171[d]	Puebla de Montalbán, Toledo, Spain	high (91±8)

[a]Values obtained from a previous susceptibility test [84].
[b]Provided by the Institute of Forestry and Nature Research (Wageningen, The Netherlands).
[c]Morphologically appears to be *U. minor* × *U. pumila*.
[d]*U. minor* var. *vulgaris* (= *U. procera*).
doi:10.1371/journal.pone.0056987.t001

placed in separate Petri dishes containing 2% (w/v) malt extract agar with no added antibiotics. The sizes of the leaf, xylem and bark samples were selected so as to ensure that each tissue sample had a similar weight (30–40 mg). The Petri dishes were sealed with Parafilm. The isolation method used resulted in the growth of endophyte colonies which were counted and sub-cultured 2 weeks after incubation at 20°C. The efficacy of the sterilization method was previously tested by direct comparison of the rate and number of fungal colonies that grew from sterilized and unsterilized tissue samples. The results from these tests indicate that in over 90% of the cases, rapidly-growing epiphytic fungi could be removed by the sterilization process and that the recovered isolates thus represent mainly the tissue internal fungal communities.

The endophytes were grouped into morphotaxa on the basis of vegetative features that conservatively reconstruct species boundaries [29,39]. In each tree group [P (R), M (R), M (S) or M (F)], *endophyte frequency* was calculated as the average of the number of endophytes colonies growing in each Petri dish divided by the total number of tissue samples placed in the dish (four samples per dish; i.e. four samples per tissue and cardinal point). *Endophyte diversity* of each 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 [38]. To describe and compare the fungal communities in different sample groups, we used diversity indices [40]. First, to compare the *diversity*, we calculated the Shannon-Weaver index [$H' = -$ sum (P_i ln[P_i]) where P is the proportion of taxon i] and used it to calculate Pielou's index for *evenness* [$J' = H'/H'_{max}$, where $H'_{max} = \log(S)$ and S = number of taxa]. Higher values of H' indicate higher diversity and less competition between the taxa, and higher values of J' indicate low variation in the distribution of taxa across the community. Endophytic communities were also compared among tree tissues, genotypes and sites using the classical Jaccard's *similarity* index, based on binary information (presence/absence), as described by Anderson et al. [41]. This index allows us to quantify the degree of overlap between the taxa in the two communities. The Jaccard's index (J) was calculated as J = A/(A+B+C) where A = number of taxa common to both communities; B = the number of taxa present in community 1 but not 2; C = the number of taxa present in community 2 but not 1. Higher values indicate higher similarity between the two communities.

Identification of Endophytic Fungi

Macro- and microscopic examination of morphological traits was used to tentatively assign isolates to morphotaxa. In addition, the molecular identity of one representative isolate per morphotaxon was determined as described below, for more precise information on the identity of the fungal isolates. The criterion used when selecting isolates was that they had to clearly exhibit the vegetative traits of the morphotaxon they exemplified.

For isolation of DNA, the fungal isolates were incubated on 2% malt extract liquid medium (20 g l^{-1} malt extract) at 25°C for 4–7 days. The hyphal mass was centrifuged down (10060 g, 2 min). After washing with water, 200 µl of the lysis buffer (2% Triton X-100, 1% SDS, 0.1 M NaCl, 0.001 M EDTA, 0.01 M pH 8 Tris buffer), 200 µl of a phenol:chloroform:isoamyl alcohol mixture (25:24:1) and 100 µl of acid-washed glass beads were added to the fungal pellet. The resulting mixture was vortexed for 10 min and 200 µl of pH 8 TE buffer (10 mM pH 7.5–8 Tris, 1 mM pH 8 EDTA) was added. The suspension was centrifuged for 10 min at 10060 g and then 10 µl RNase A (10 mg ml^{-1}) was added to the aqueous phase, which was incubated for 45 min at 37°C. The DNA was precipitated with 1 ml ice cold 96% ethanol and 3 M sodium acetate (1/10 volume). The mixture was centrifuged for 10 min at 10060 g at 4°C. The pellet was washed with ice cold 70% ethanol, air-dried and resuspended in 40 µl TE buffer (pH 8).

The internally transcribed spacer (ITS) region of the rDNA and the small ribosomal subunit (SSU) were amplified using the ITS1/ITS4 and NS5/NS6 primer pairs, respectively [42]. The polymerase chain reaction was run under the following conditions: 94°C, 5 min followed by 30 cycles of 95°C for 30 sec, 50°C for 45 sec and 72°C for 45 sec followed by a final ten minute extension step at 72°C. The PCR products were purified using the GeneJET PCR Purification kit (Fermentas, cat. no K0702) and sequenced using PCR primers by MWG Operon (Ebersberg, Germany). The sequences were identified by comparison with GenBank database using nucleotide megablast search (Table 2, Table S1) [43].

Chemical Analyses of Leaf, Bark, and Xylem Tissues

Additional leaf and twig samples were collected following the same procedure as described for the isolation of endophytes. The bark was separated from xylem using a knife and the samples were allowed to dry in paper bags at room temperature. The samples were then milled into a homogenous powder and 10 mg (leaves and bark) or 300 mg (wood) per tree were extracted with methanol an analysed by HPLC [44]. The peak area data was collected at 320 nm. The quantitative data is expressed as peak area ($AU \times 10^{-5}$) normalized against sample weight per injection. In order to explore the type of phenolic compounds present in the samples, UV-absorbance scanned at 200 to 400 nm was compared to spectral data in an in-house standard compound library. A more comprehensive identification of all compounds was not deemed crucial to fulfil the objectives of this study because we were mainly interested in screening the general patterns among the studied trees, their resistance and endophyte status.

Statistical Analyses

Endophyte frequency and diversity were analyzed using a generalized linear model (GLM) approach to ANOVA with type III sum of squares, considering the effects of the *group* P (R), (M (R), M (S), and M (F), the *tree* nested within the *group*, the *organ* (leaf, bark, and xylem), the *orientation* (North, South, East and West), and the two-fold interactions between organ and orientation. The normality of the data was confirmed using the Shapiro-Wilks statistic [45]. The mean frequency and diversity values were compared by means of multiple range tests using Fisher's least significant difference (LSD) intervals ($\alpha = 0.05$). Linear regression analyses were made between the susceptibility to DED of each elm clone at the Breeding Centre (% leaf wilting) and the frequency and diversity of endophytes in xylem tissues. A non-metric multidimensional scaling (MDS) analysis based on the Jaccard similarity index matrix of any given pair of samples was performed to visualize any grouping in the data set.

To compare morphotaxa richness in tree groups with different sample sizes, and to summarize the completeness of the sampling effort, sample-based rarefaction curves [46] (hereafter referred to as endophyte accumulation curves) of the endophyte morphotaxa (abundance data) were constructed with EstimateS 8.2.0 software using 100 randomizations, sampling without replacement and default settings for upper incidence limit for infrequent species [47].

In order to compare the phenolic profiles of leaf, bark, and xylem samples from each tree group, the results obtained from HPLC analysis were tested using a discriminant function analysis (DFA). The chemical profile of each sample was defined on basis of a characteristic pattern of chromatogram peaks (13 peaks for

Table 2. Identification of representative isolates of the morphotaxa (1–16) on basis of the top three BLAST hits (based on nucleotide megablast of ITS rDNA sequences) with corresponding GenBank taxa identity, characteristic morphological colony traits and literature.

Morphotaxon	Suggested taxon
1	*Pyrenochaeta cava* (syn. *Phoma cava*) [85,86]
2	*Monographella nivalis* (syn. *Fusarium nivale, Gerlachia nivalis, Microdochium nivale*) [75,87]
3	*Aureobasidium pullulans* [78,88]
4	*Alternaria* sp. *(A. tenuissima)*
5	*Cochliobolus cynodontis (*anam. *Bipolaris cynodontis)*
6	*Fusarium* sp.
7	*Alternaria alternata*
8	*Biscogniauxia nummularia (*syn. *Hypoxylon nummularium)*
9	*Xylaria* sp.
10	*Cladosporium cladosporioides*
11	*Phomopsis* sp.
12	*Sordaria fimicola*
13	*Coniochaeta* sp. *(*anam. *Lecythophora)*
14	*Apiospora* sp. *(*anam. *Arthrinium)*
15	*Botryosphaeria sarmentorum*
16	*Leptosphaeria coniothyrium*

doi:10.1371/journal.pone.0056987.t002

leaf and bark samples, and 10 peaks for xylem samples), whose normalized areas were used as input variables with a priori information about sample grouping in the data (tree groups). This information was used to produce measures of within-group variance and between-group variance and then to define optimised discriminant functions (DFs) for distinguishing between profiles originating from different groups of trees. In order to estimate the discriminating power of the DFs, Wilks' Lambda tests were performed. The coefficients by which the original variables (peak retention times) are multiplied to obtain the DFs are called loadings. Since the numerical value of a loading of a given variable on a DF indicates how much the variable has in common with that DF, loadings were used to identify the peaks that were most important in discriminating between samples. The areas of these significant peaks were compared within groups of trees by means of one-way ANOVA. Fisher's least significant difference (LSD) procedure was used to compare averages ($\alpha = 0.05$). All statistical analyses were performed using Statistica 7.0 software package (Tulsa, OK, USA).

Results

Endophyte Frequency and Diversity

The ANOVA of the endophyte *frequency* revealed that the tree group [P (R), M (R), M (S) and M (F)], the tree nested within the group, and the organ all had significant effects on endophyte frequency ($P<0.04$), but the orientation and the organ-orientation interaction did not ($P>0.70$). Considering all tree groups, the endophyte frequency in bark tissues (0.66 ± 0.03; mean $\pm$ SE) was higher ($P<0.001$) than in leaves (0.19 ± 0.04) and xylem tissues (0.10 ± 0.03). The ANOVA of the endophyte *diversity* showed that the tree group, the tree nested within the group, and the organ had significant effects on endophyte diversity ($P<0.01$), but the orientation and the organ-orientation interaction did not ($P>0.77$). Considering all tree groups, the endophyte diversity in bark tissues (0.47 ± 0.03) was higher ($P<0.001$) than in leaves (0.14 ± 0.03) and in xylem tissues (0.07 ± 0.02).

The total number of fungal isolations obtained from the different plant tissues and tree groups is specified in Table 3. A total of 274 isolations were obtained from the 816 plant samples incubated on MEA. The endophytic fungi were classified into 16 different morphotaxa. Six of these were isolated exclusively from bark, three from bark and leaves, and three from bark and xylem; the remaining four morphotaxa were isolated from all tissue types. According to the Shannon-Weaver index (H′, Table 3), the leaf-associated isolates showed a markedly higher diversity and evenness in M (F) trees, as compared to those from the Breeding Centre. For bark tissues, the differences in H′ and J′ values among the tree groups were not as pronounced as they were for leaf or xylem tissues, and the highest diversity and evenness were found for the M (S) group (Table 3). Also for xylem tissues, the H′ and J′ indices suggest highest diversity and evenness, and thus lowest competition, in the M (S) group (Table 3).

The sample-based rarefaction curves constructed for individual tissues showed different patterns: within each tree group, the curves for bark tissue increased at highest rate and reached the highest end points, whereas the curves constructed for xylem and leaf samples increased slower and remained at lower levels throughout the empirical range of samples (Fig. 2). Within this range, the curves constructed for bark tissues approached asymptote in all tree groups, and those for the xylem and leaves clearly reached a plateau in M (F) group. The highest end points of the curves constructed for bark and xylem, as well as for all tissues, were found in M (S) group (Fig. 2). The sample-based rarefaction curves based on non-singletons of all tissues reached an asymptote in all tree groups (Fig. 2). After an initial increment, the number of singletons diminished progressively as the number of twigs processed increased (Fig. 2). The initial level of singletons was lowest in M (F) group, reaching zero when the number of processed twigs was 26.

Table 3. Number of tissue samples (incubated on MEA at 20°C), fungal isolates and morphotaxa obtained, and associated diversity indices: H′ = Shannon-Weaver and J′ = Pielou's evenness index [tree groups: P (R) = resistant *U. pumila* clones from Puerta de Hierro Forest Breeding Centre; M (R) = resistant *U. minor* clones from Puerta de Hierro Forest Breeding Centre; M (S) = susceptible *U. minor* clones from Puerta de Hierro Forest Breeding Centre; and M (F) = *U. minor* trees from Rivas-Vaciamadrid field site].

Organ	Indices	P (R)	M (R)	M (S)	M (F)
Leaf	Number of tissue samples	32	64	64	112
	Number of isolates	6	4	6	50
	Number of morphotaxa	3	2	2	5
	H′	0.56	0.26	0.34	1.44
	J′	0.51	0.37	0.49	0.89
Bark	Number of tissue samples	32	64	64	112
	Number of isolates	19	42	45	76
	Number of morphotaxa	8	10	13	9
	H′	1.65	1.96	2.31	1.8
	J′	0.79	0.85	0.90	0.82
Xylem	Number of tissue samples	32	64	64	112
	Number of isolates	1	2	18	5
	Number of morphotaxa	1	2	7	2
	H′	0.15	0.17	0.94	0.22
	J′	0	0.24	0.48	0.32
All tissues	Number of tissue samples	96	192	192	336
	Number of isolates	26	48	69	131
	Number of morphotaxa	8	11	14	11
	H′	1.81	1.95	2.28	2.18
	J′	0.87	0.81	0.86	0.91

doi:10.1371/journal.pone.0056987.t003

The endophyte frequency and diversity for each group of trees and tree organs were compared on basis of mean values and multiple range test comparisons (Fig. 3). In leaf tissues, the M (F) group showed a higher endophyte frequency than the other groups ($P<0.05$; Fig. 3a), and a higher endophyte diversity than the M (R) and M (S) groups ($P<0.05$; Fig. 3b). In bark tissues, no significant differences in endophyte frequency were observed between the groups ($P>0.12$; Fig. 3c), while M (S) showed higher diversity than the field population ($P<0.05$; Fig. 3d). In xylem tissues, both frequency and diversity values were higher in M (S) than in the rest of tree groups ($P<0.05$; Fig. 3e, f).

The MSD graph obtained from the Jaccard's similarity matrix showed a clear distinction in leaf endophyte community between the M (F) trees and the trees from the Breeding Centre (Fig. 4a). The same analysis applied to the bark endophytes revealed a higher overlap among tree groups than in leaf or xylem tissues (Fig. 4b). However, M (F) samples were grouped in the positive horizontal semi-axis together with a M (S) tree from the Breeding Centre. This M (S) tree is the UPM007 clone (Table 1), belonging to the *U. minor* var. *vulgaris* complex, which also includes the trees studied at the field population. For the xylem-associated endophyte communities (Fig. 4c), a clear distinction was again observed

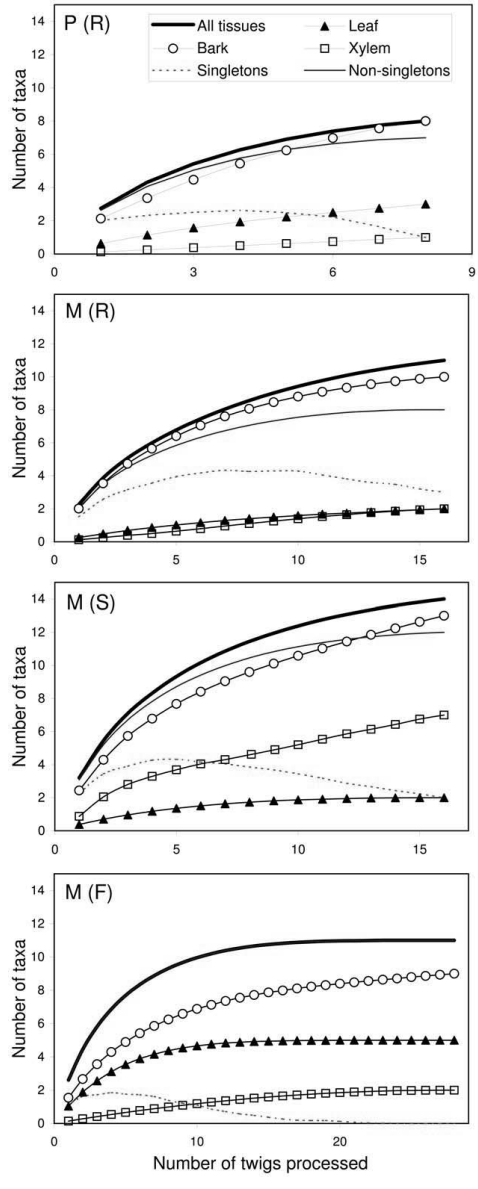


Figure 2. Accumulation curves of elm endophytic fungi. Accumulation curves indicating the number of endophyte morphotaxa isolated per number of twigs processed (four twigs per tree, and four leaf, bark and xylem samples per twig) in each tree group [P (R) = resistant *U. pumila* clones from Puerta de Hierro Forest Breeding Centre; M (R) = resistant *U. minor* clones from Puerta de Hierro Forest Breeding Centre; M (S) = susceptible *U. minor* clones from Puerta de Hierro Forest Breeding Centre; and M (F) = *U. minor* trees from Rivas-Vaciamadrid field site].
doi:10.1371/journal.pone.0056987.g002

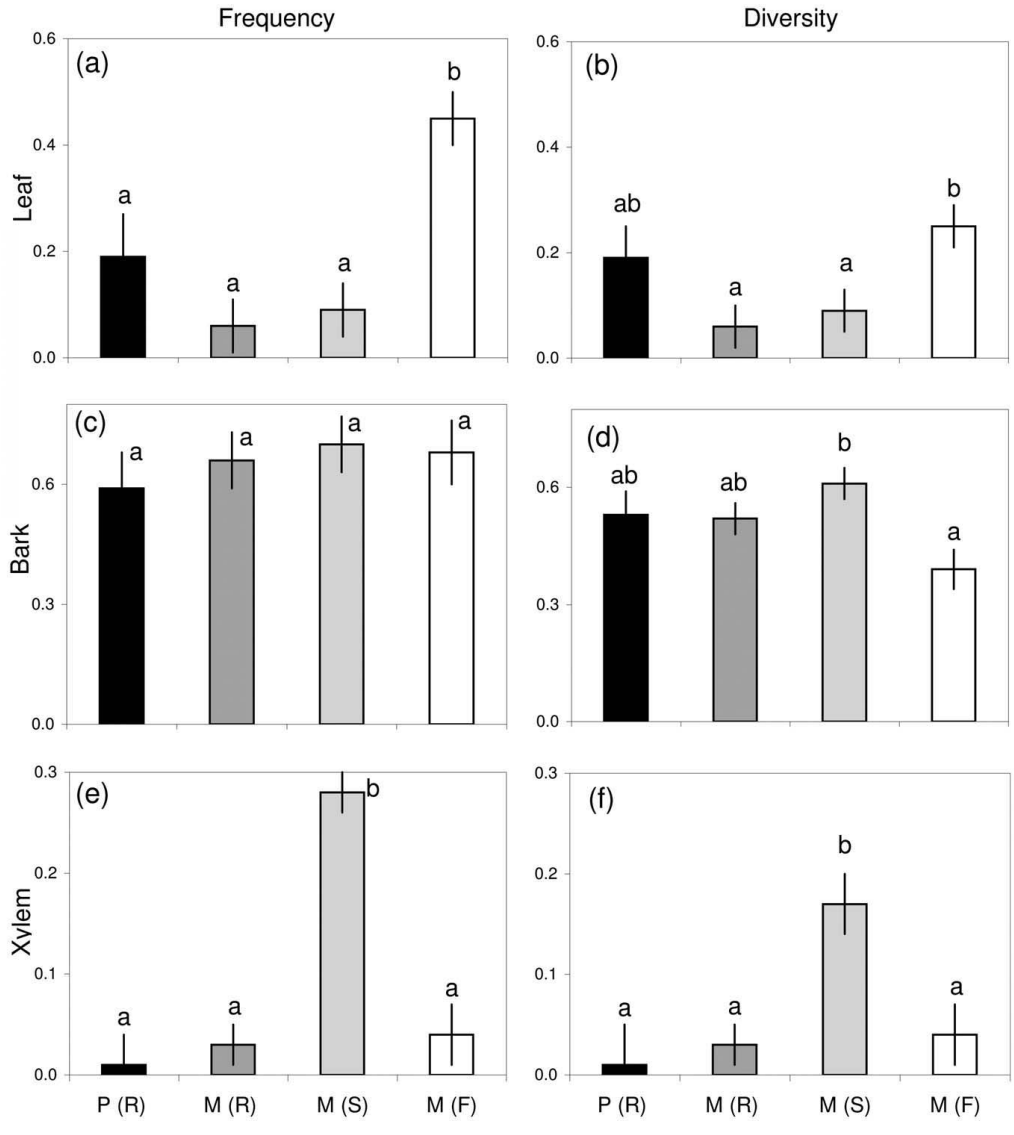


Figure 3. Endophyte frequency and diversity in elms. Mean values of endophyte frequency (a, c, e) and endophyte diversity (b, d, f) of leaf (a, b), bark (c, d), and xylem (e, f) tissues from different groups of elm trees: P (R) = resistant *U. pumila* clones from Puerta de Hierro Forest Breeding Centre; M (R) = resistant *U. minor* clones from Puerta de Hierro Forest Breeding Centre; M (S) = susceptible *U. minor* clones from Puerta de Hierro Forest Breeding Centre; and M (F) = *U. minor* trees from Rivas-Vaciamadrid field site. Different letters indicate differences among groups of trees ($P<0.05$), and bars represent standard errors.
doi:10.1371/journal.pone.0056987.g003

between M (F) and the trees from the Breeding Centre. Furthermore, a clear distinction in endophyte diversity was observed between the M (R) trees on the one hand, and the M (S) and P (R) trees on the other hand (Fig. 4c).

Morphotaxa 3, 4, and 8 were isolated from all tree groups from the Breeding Centre, but not from the field population. Morphotaxon 13 was only isolated from one resistant *U. minor* clone (UPM007) and from one resistant *U. pumila* clone (201). Morphotaxon 7 was exclusive to *U. minor* var. *vulgaris*, since it was

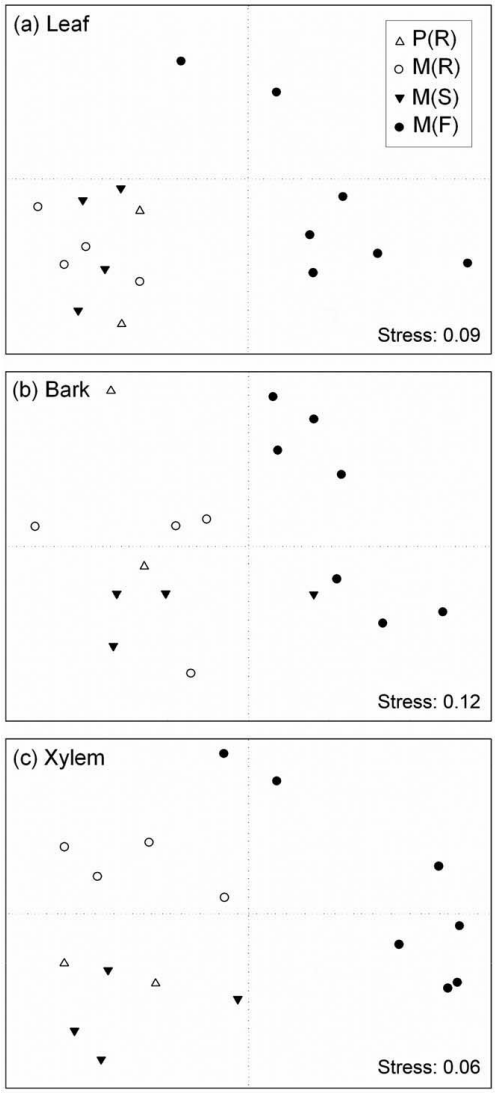


Figure 4. Two-dimensional ordination using non-metric multi-dimensional scaling (MDS) based on Jaccard's similarity measures. Each point represents the fungal endophyte community of an individual tree. Endophytes were isolated from leaf (a), bark (b) or xylem (c) tissues. Groups of elm trees: P (R) = resistant *U. pumila* clones from Puerta de Hierro Forest Breeding Centre; M (R) = resistant *U. minor* clones from Puerta de Hierro Forest Breeding Centre; M (S) = susceptible *U. minor* clones from Puerta de Hierro Forest Breeding Centre; and M (F) = *U. minor* trees from Rivas-Vaciamadrid field site.
doi:10.1371/journal.pone.0056987.g004

only isolated from the UPM171 clone and trees from the field site. Morphotaxon 14 was exclusively isolated from the resistant *U. minor* clone UPM007, while morphotaxa 15 and 16 were only isolated from the susceptible *U. minor* clones UPM045 and UPM068. It is noteworthy that five endophytic morphotaxa (3, 4, 6, 10 and 15) were isolated from the xylem of susceptible *U. minor* clones from the Breeding Centre, but not from the xylem of other tree groups (data not shown). However, four of these morphotaxa (3, 4, 6 and 10) were not restricted to susceptible *U. minor* clones, as they were also isolated from leaf or bark tissues from other tree groups.

The three most common fungal morphotaxa were characterized by *Pyrenochaeta cava* (morphotaxon 1), *Monographella nivalis* (morphotaxon 2), and *Aureobasidium pullulans* (morphotaxon 3) (Table 2, Table S1). Of these, *M. nivalis*, isolated mainly from bark and occasionally from xylem, was predominantly associated with resistant *U. minor* clones and *U. minor* trees from the field population, *P. cava* was primarily associated with the resistant *U. pumila,* and *A. pullulans* was primarily associated with susceptible *U. minor* clones. According to the molecular analysis, the other morphotaxa included species from the genera *Alternaria* (morphotaxa 4 and 7), *Bipolaris* (morphotaxon 5), *Fusarium* (morphotaxon 6), *Biscogniauxia* (morphotaxon 8), *Xylaria* (morphotaxon 9), *Cladosporium* (morphotaxon 10), *Phomopsis* (morphotaxon 11), *Sordaria* (morphotaxon 12), *Coniochaeta* (morphotaxon 13), *Apiospora* (morphotaxon 14), *Botryosphaeria* (morphotaxon 15), and *Leptosphaeria* (morphotaxon 16) (Table 2, Table S1). The Dutch elm disease pathogen was not isolated from any sampled tree in this study.

In xylem tissues, the endophyte frequency and diversity of each genotype at the Breeding Centre were directly related with their mean susceptibility to DED (Fig. 5) ($r = 0.659$, $P = 0.038$; $r = 0.727$, $P = 0.017$, respectively).

Chemical Discrimination of Leaf, Bark, and Xylem Tissues

Quantitative and qualitative differences between the different tissues' phenolic profiles were identified. Several phenolic acids (coumaric acids and chlorogenic acids) were tentatively identified in the leaf samples, along with flavonoids (quercetin and kaempherol derivatives). Bark tissues contained several compounds whose UV-spectra resembled those of catechins and eriodictyol, along with quercetin and kaempherol-type flavonoids, albeit at lower concentrations than were observed in the leaves. The phenolic acid pool in the xylem samples was rich in compounds identified as rosmarinic acid, vanillic acid and chlorogenic acid.

The DFA of the chemical variables (chromatogram peaks) was used to obtain the scatter plot of the scores from the first two DFs (Fig. 6). For the leaf samples (Fig. 6a), DF1 was significant at $P<0.001$, and could be used to distinguish between *U. minor* (positive scores) and *U. pumila* (negative scores) samples (Fig. 6a). DF2 ($P = 0.02$) could be used to distinguish between *U. minor* samples from the Breeding Centre [both M (R) and M (S)] and those from the field site [M (F)].

A similar discrimination pattern was observed with bark tissues (Fig. 6b): DF1 ($P<0.001$) could be used to distinguish between *U. minor* (positive scores) and *U. pumila* (negative scores) samples, while DF2 ($P = 0.01$) separated the *U. minor* samples from the Breeding Centre [both M (R) and M (S)] and those from the field site [M (F)].

For the xylem samples (Fig. 6c), DF1 ($P<0.001$) could be used to distinguish between M (S) and M (F) samples from P (R) and M (R) samples (negative scores), while the discriminating power of DF2 was not statistically significant ($P = 0.15$).

The chromatogram peak at 24.47 min, identified as rosmarinic acid derivative, was one of the most significant peaks in discriminating between xylem samples. The area of this peak

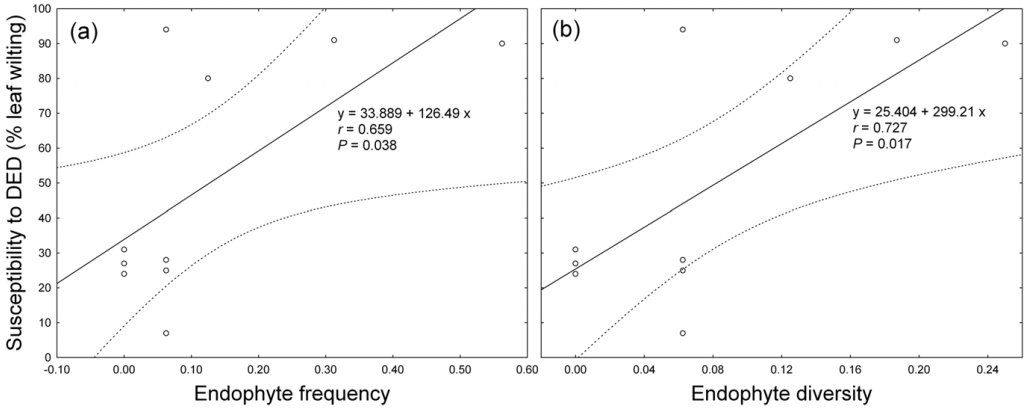


Figure 5. Relation between endophytes and susceptibility to DED in elms. Relations between the mean susceptibility to DED (% leaf wilting) of each elm genotype at the Breeding Centre and its endophyte frequency (a) and diversity (b) in xylem tissues. Solid lines are linear regressions and dotted lines are 95% confidence limits. Wilting values were obtained from a previous susceptibility test [84].
doi:10.1371/journal.pone.0056987.g005

showed higher mean values for genetically susceptible trees [M (S) and M (F)] than for resistant trees [M (R) and P (R)] (Fig. 7).

Discussion

Our study shows that DED-susceptible *U. minor* clones may harbour a greater range and higher densities of endophytic fungi in their xylem tissues than resistant *U. minor* and *U. pumila* clones. Since the DED pathogen develops in xylem [16], the genetic features that increase the constitutive resistance of elms to *O. novo-ulmi* may also negatively affect endophytic fungi in their xylem, leading to a trade-off between fungal biodiversity and DED resistance. The highest end points of the accumulation curves for xylem in M (S) group also support the view that these trees, with high susceptibility to DED, sustain richer endophyte communities in their woody tissues than the more resistant trees. However, the higher fungal diversity and evenness, which indicates an environment with lower level of competition between different fungi, found in the xylem of susceptible trees (see the Shannon's and Pielou's indices in Table 3), should not be generalized to other tissues, as most fungal morphotypes isolated from the xylem of susceptible trees were also isolated from bark or leaf tissues of resistant trees. Furthermore, these results should not be generalized to the entire fungal community of the elm trees, as our study was restricted to endophytic fungi isolated in malt extract agar. This medium permits the growth of most species of fungi once they are obtained in a pure culture. However, the initial growth and isolation of some slow-growing fungi may have been inhibited by the rapidly growing fungi. To achieve the isolation of the total culturable community, isolation conditions should permit equal expression of the entire array of fungal groups present; e.g. by restriction of the rapidly growing fungi by means of destructive chemical and physical procedures to support slow-growing fungi [48]. However, despite the limitations of our isolation protocol, we were able to find differences between susceptibility groups. The endophyte accumulation curves suggest that the sampling effort of 16 processed twigs from four elm trees (64 tissue samples) captured well the majority of the culturable endophytes. However, a more exhaustive sampling of about 20 twigs (i.e. 5 trees in the sampling design) can improve the catchment of the more rare or transient morphotypes. For extensive comparisons of the total fungal communities, pyrosequencing could be applied in future studies [49,50].

Our results emphasize the strong effect of tree genotype on endophyte communities. It is noteworthy that the UPM007 clone from the Breeding Centre appeared in the MDS graph of bark tissues (Fig. 4b) close to the M (F) trees. All these trees belong to the *U. minor* var. *vulgaris* complex and therefore are genetically close to each other. This finding underlines the importance of maintaining the genetic diversity in tree populations. The significance of genetic variation of trees as a factor shaping the fungal assemblages has also been shown in the phyllosphere of European beech (*Fagus sylvatica*) [51]. Moreover, although the benefit of restoring elm stands through resistance breeding is obvious, the putatively high importance of endophytic fungi in forest ecosystems warrants careful consideration of the effect of resistance breeding. Previously, non-targeted effects of improved resistance have been studied mainly in transgenic plants. Newhouse et al. [26] found no negative effects of transformation with a gene encoding a synthetic antimicrobial peptide on mycorrhizal colonization in young elms (*U. americana* L.). Similar results have also been found in some other studies of plant-pathogen systems [52], but others have found that transgenic resistance may be accompanied by unintentional alterations in mutualistic fungal community [53]. Thus, results of increasing resistance transgenically have been mixed in this respect. However, compared to genetic modifications that only involve a limited number of genes, alterations in quantitative resistance traits may potentially cause more profound alterations in endophytic community.

In plant-endophyte interactions, immunity triggered by microbe-associated molecular patterns (MAMPs) does not ward off the interacting endophyte, as it remains hosted by the plant. Endophytes that have evolved closely with their host plants [54,55] might produce MAMPs that activate signalling networks similar to those activated by beneficial microbes [56], resulting in only a mild induction of the plant's immune responses [57]. Systemic resistance induced by these beneficial organisms appears to be predominantly based on priming for enhanced defence, rather than on direct activation of defence [57]. Further studies using

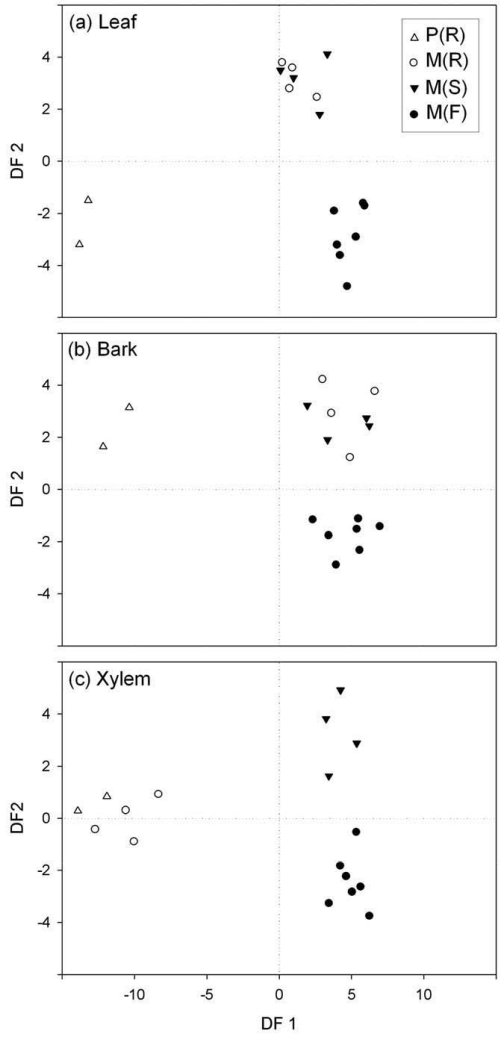


Figure 6. Separation of elm trees on basis of tissue specific phenolic profiles. Discriminant function analysis score scatter plot for the HPLC chromatogram peaks of samples taken from leaf (a), bark (b), and xylem (c) tissues from different groups of trees: P (R) = resistant *U. pumila* clones from Puerta de Hierro Forest Breeding Centre; M (R) = resistant *U. minor* clones from Puerta de Hierro Forest Breeding Centre; M (S) = susceptible *U. minor* clones from Puerta de Hierro Forest Breeding Centre; and M (F) = *U. minor* trees from the Rivas-Vaciamadrid site.
doi:10.1371/journal.pone.0056987.g006

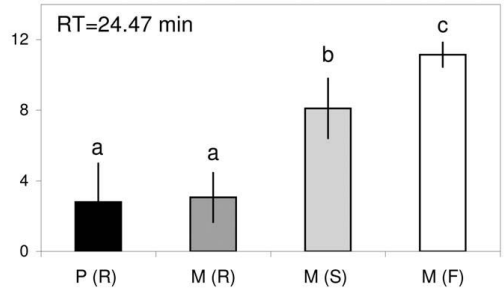


Figure 7. Quantitative patterns of a rosmarinic acid derivative in elms. Mean peak area (AU $\times 10^{-5}$) of one of the HPLC chromatogram peaks (RT = 24.47 min) of xylem samples that was important in discriminating between tree groups: P (R) = resistant *U. pumila* clones from *Puerta de Hierro* Forest Breeding Centre; M (R) = resistant *U. minor* clones from *Puerta de Hierro* Forest Breeding Centre; M (S) = susceptible *U. minor* clones from Puerta de Hierro Forest Breeding Centre; and M (F) = *U. minor* trees from Rivas-Vaciamadrid field site. Different letters indicate differences between groups of trees ($P<0.05$); bars represent standard errors [n = 4 for M(R) and M(S), 2 for P(R) or 7 M(F)].
doi:10.1371/journal.pone.0056987.g007

in vitro model systems [58] are needed to clarify the biochemical interactions between trees and their endophytic fungi.

Anatomical features of the xylem may play a key role in elm resistance to DED [59,60], but the variations in host anatomy alone cannot fully explain the variations in degrees of elm resistance to DED [60,61]. A potentially contributory factor, although often neglected in studies on plant quality, is that endophytes may modify the chemical quality of plants [62]. We found that the phenolic profiles of xylem samples from resistant *U. minor* and *U. pumila* clones grouped together in the DFA, suggesting a link between xylem's chemicals and DED. Further, in xylem tissues, some endophytic morphotaxa were exclusively found in susceptible *U. minor* genotypes, which also had high xylem concentrations of a compound identified as rosmarinic acid. It is possible that certain endophytes stimulate the accumulation of specific compounds in host tissues. For example, rosmarinic acid has been found to be induced by symbiotic mutualistic fungi (arbuscular mycorrhiza) in herbaceous plants [63]. However, other studies have provided evidence for a negative relation between polymeric phenolics (condensed tannins) and fungal endophyte infections in bark [64]. Obviously, the relation between fungal colonizers and phenolic end products can be multifaceted, because the phenolics could both affect, and be affected, by the fungi, and because structurally and functionally different phenolics might have different roles in host-endophyte interactions [21]. Moreover, some endophytes may be latent pathogens [5,28] and be differently affected by the host chemicals at different physiological phases of their life-style continuum. A detailed identification of the compounds involved in the chemical discrimination of resistant and susceptible elm clones is in progress to further explore the relationships between these chemicals, endophytes and resistance in elms. While the host tree's chemical quality may be an important factor affecting the endophytes, it should also be noted that the diversity of endophytes can also be strongly affected by several other factors, such as genotype or geographic differences. In addition to highlighting the potential importance of intrinsic factors in plant-endophyte interactions, our results underline the significance of environmental factors for endophyte diversity in trees. The frequency and diversity of the endophytic fungi (Fig. 3), and the Shannon's and Pielous's indices (Table 3) were rather similar in the xylem of the Rivas-Vaciamadrid elms, which are genetically susceptible but phenotypically resistant to *O. novo-ulmi*, and in the resistant genotypes growing at the Breeding Centre. This could be because the establishment of some xylem

endophytes is hampered at Rivas-Vaciamadrid by the intensive application of phenolic cattle disinfectants that also prevent *O. novo-ulmi* spread there [33,35–37]. However, the trees growing in the field at Rivas-Vaciamadrid had higher leaf endophyte frequencies, diversity and evenness (Fig. 3, Table 3), which may be explained by differences in the availability of fungal inocula. At the Breeding Centre, the soil is periodically ploughed and amended to enhance soil water retention and eliminate competition from herbaceous vegetation. This soil treatment buries plant materials, which probably reduces the availability of fungal inocula. Other environmental factors, such as the higher humidity associated with the riparian habitat of the Rivas Vaciamadrid elm stand may also favour a higher abundance of leaf endophytic fungi [65].

The differences found between the two study sites in terms of their fungal communities can be attributable to environmental factors, but also to differences in tree age or even to tree age × site interactions. It has been shown that plant age can affect the degree of plant colonization by endophytes. For instance, the infection density in leaves of woody plants tends to increase with leaf age [66–68]. In *Populus* × *euramericana*, endophyte richness in leaves and twigs was higher in young stands than in adult stands. Furthermore, the differences in richness between ages depended on the site quality [69]. In our study, it is not possible to ascertain how the tree age affected fungal communities, because all the trees within each location (Breeding Centre or Rivas-Vaciamadrid) were of approximately same age. However, it is possible that the strong "site" effect observed in fungal diversity (Fig. 4) was at least partly due to differences in plant age.

The faster increment of the accumulation curve for bark samples indicates greater richness or evenness of culturable endophyte morphotaxa in bark tissues, as compared to leaves and xylem. In all of the studied trees, also the frequency and diversity of endophytic fungi in the bark tissues was substantially greater than in the leaves, as has previously been reported [70]. This could be expected because bark tissues are colonized on a cumulative basis, with fungi persisting from year to year, whereas leaves are gradually colonized over the course of a single growing season. Conversely, xylem tissues are colonized more selectively [71].

The most abundant morphotypes were *P. cava*, *M. nivalis* and *A. pullulans*. Earlier, *P. cava* has been reported as an endophytic species involved in the aetiology of decline of Mediterranean *Quercus* trees [72,73]. The fungus *M. nivalis* is a snow mold with a temperature minimum of −5°C for growth [74]. This fungus can cause severe damage on cereals and other grasses [75]. Its appearance as an endophyte in elm bark could be explained by the likely commonness of the species in the pasture lands surrounding the studied elms. The fungus *A. pullulans*, on the other hand, was an expected finding because it is a very abundant colonizer of plant surfaces and often isolated as an endophyte in trees [76,77]. This polymorphic, yeast-like fungus is well-adapted to a broad range of habitats and is exploited for its ability to produce the biodegradable extracellular polysaccharide pullulan [78]. Similarly, several of the other tentatively identified genera, e.g. *Alternaria*, *Xylaria*, and *Phomopsis* have been reported as tree endophytes in earlier studies [29,79–81].

The observed spatial variations in the diversity and frequency of fungal endophytes in elms, along with their associations with the elms' chemical and resistance characteristics, emphasise the potential importance of endophytic fungi as dynamic modulators of tree phenotype. Nevertheless, it is difficult to assess whether the endophytic fungi are significant determinants of the phenotypic resistance observed at the Rivas-Vaciamadrid field site. We have recently found that *M. nivalis* (morphotaxon 2), predominantly associated with Rivas-Vaciamadrid trees and resistant *U. minor* clones, releases extracellular metabolites that in vitro inhibit *O. novo-ulmi* (K. Blumenstein et al., unpublished) and reduces the symptoms caused by *O. novo-ulmi* inoculation in elm trees previously challenged with the endophyte (Martín et al., unpublished). The presence of this endophyte could limit the spread of *O. novo-ulmi* in the inner bark of diseased trees, the compartment where the vector insects, elm bark beetles, become contaminated by spores. The potential of a bark endophyte (*Phomopsis oblonga*) to hamper the breeding of elm bark beetles has been previously reported [79,80]. Our results from studies with *M. nivalis* indicate the existence of multiple mechanisms whereby endophytes can influence the DED transmission and the resistance of elms to *O. novo-ulmi* in field conditions.

In conclusion, we found support for our initial hypothesis: the resistant elm genotypes had a more limited endophytic flora in xylem tissues than the susceptible genotypes. However, a significant genotype effect was observed and not all susceptible genotypes showed higher values of endophyte frequency and diversity in xylem tissues than resistant genotypes (Fig. 5). Thus, it would be necessary to characterize the variation of the endophytic community of each genotype in greater detail, using 4–6 tree replicates per genotype. Currently, however, such elm material is not available in an adult stage, and to create it clonal propagation of the existing single genotypes would be necessary. Despite this reservation, our results imply that improving DED resistance in elm trees may have non-targeted effects on fungal biodiversity, and the re-introduction of elms to forest ecosystems with the assistance of breeding for quantitative resistance to DED may involve a trade-off between the goals of ecosystem restoration and fungal biodiversity conservation. As endophyte diversity may contribute to various ecosystem benefits from forests in a similar way than rhizospheric diversity, this issue should be addressed in environmental impact analyses of forest restoration and tree breeding efforts (see also [82,83]). Obviously, the priority of elm breeding is to re-establish elm populations, and the re-introduction of resistant elms to the forests should increase potential habitats for endophytic fungi. Moreover, other plant species in the forest may act as reservoirs of cosmopolitan endophytes that inhabit also the susceptible elms. However, if the susceptible elm genotypes harbour specialist endophytes, a large-scale enrichment of resistant elm genotypes could impede their conservation. If these specialist endophytes are of particular relevance for wood degradation or ecological interactions, the ecosystem processes of the forest might change consequently. Future studies should thus explore further the diversity and ecological functions of the endophyte communities, including the non-culturable species, in elm genotypes differing in their resistance to DED.

Supporting Information

Table S1 The top three BLAST hits (based on nucleotide megablast of ITS rDNA sequences) with corresponding GenBank taxa identity and characteristic morphological colony traits of representative isolates for each morphotaxa (1–16) ("-" = not determined).
(DOCX)

Acknowledgments

We thank the personnel working in Puerta de Hierro Forest Breeding Centre (Madrid) and Dr. Stephen Burleigh (SLU Alnarp) for the technical assistance.

Author Contributions

Conceived and designed the experiments: JAM LG JW. Performed the experiments: JAM JW KB ER MH. Analyzed the data: JAM JW KB ER MH TS. Wrote the paper: JAM JW KB ER MH TS LG.

References

1. McGuire KL, Treseder KK (2010) Microbial communities and their relevance for ecosystem models: Decomposition as a case study. Soil Biol Biochem 42: 529–535.
2. Saikkonen K (2007) Forest structure and fungal endophytes. Fungal Biol Rev 21: 67–74.
3. Sieber TN (2007) Endophytic fungi in forest trees: are they mutualists? Fungal Biol Rev 21: 75–89.
4. Ahlholm J, Helander M, Elamo P, Saloniemi I, Neuvonen S, et al. (2002) Microfungi and invertebrate herbivores on birch trees: fungal mediated plant–herbivore interactions or responses to host quality? Ecol Lett 5: 648–655.
5. Schulz B, Boyle C (2005) The endophytic continuum. Mycol Res 109: 661–686.
6. Álvarez-Loayza P, White JF Jr, Torres MS, Balslev H, Kristiansen T, et al. (2011) Light converts endosymbiotic fungus to pathogen, influencing seedling survival and niche-space filling of a common tropical tree, *Iriartea deltoidea*. PLOS ONE 6(1): e16386. doi:10.1371/journal.pone.0016386.
7. Rodriguez RJ, Redman RS (1997) Fungal life-styles and ecosystem dynamics: Biological aspects of plant pathogens, plant endophytes and saprophytes. Adv Bot Res 24: 169–193.
8. Rodriguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, et al. (2008) Stress tolerance in plants via habitat-adapted symbiosis. ISME J 2: 404–416.
9. Knapp DG, Pintye A, Kovács GM (2012) The dark side is not fastidious – dark septate endophytic fungi of native and invasive plants of semiarid sandy areas. PLOS ONE 7(2): e32570. doi:10.1371/journal.pone.0032570.
10. Mejia LC, Rojas EI, Maynard Z, Van Bael S, Arnold AE, et al. (2008) Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. Biol Contr 46: 4–14.
11. Miller JD, Sumarah MW, Adams GW (2008) Effect of a rugulosin-producing endophyte in *Picea glauca* on *Choristoneura fumiferana*. J Chem Ecol 34: 362–368.
12. Sumarah MW, Adams GW, Berghout J, Slack GJ, Wilson AM, et al. (2008) Spread and persistence of a rugulosin-producing endophyte in *Picea glauca* seedlings. Mycol Res 112: 731–736.
13. Griffith GS, Boddy L (1990) Fungal decomposition of attached angiosperm twigs. 1. Decay community development in ash, beech and oak. New Phytol 116: 407–415.
14. Promputtha I, Lumyong S, Dhanasekaran V, McKenzie EHC, Hyde KD, et al. (2007) A phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. Microb Ecol 53: 579–590.
15. Whitham TG, Bailey JK, Schweitzer JA, Shuster SM, Bangert RK, et al. (2006) A framework for community and ecosystem genetics: from genes to ecosystems. Nature Rev Gen 7: 510–523.
16. Ouellette GB, Rioux D, Simard M, Cherif M (2004) Ultrastructural and cytochemical studies of host and pathogens in some fungal wilt diseases: retro- and introspection towards a better understanding of DED. For Syst 13: 119–145.
17. Collin E, Bilger I, Eriksson G, Turok J (2000) The conservation of elm genetic resources in Europe. In: Dunn CP, editor. The elms: breeding, conservation and disease management. Boston: Kluwer Academic Publishers. 281–293.
18. Mittempergher L, Santini A (2004) The history of elm breeding. For Syst 13: 161–177.
19. Aoun M, Jacobi V, Boyle B, Bernier L (2010) Identification and monitoring of *Ulmus americana* transcripts during in vitro interactions with the Dutch elm disease pathogen *Ophiostoma novo-ulmi*. Physiol Mol Plant Pathol 74: 254–266.
20. Nicholson RL, Hammerschmidt R (1992) Phenolic compounds and their role in disease resistance. Annu Rev Phytopathol 30: 369–389.
21. Witzell J, Martín JA (2008) Phenolic metabolites in the resistance of northern forest trees to pathogens - past experiences and future prospects. Can J For Res 38: 2711–2727.
22. Rioux D, Ouellette GB (1991) Barrier zone formation in host and nonhost trees inoculated with *Ophiostoma ulmi*. I Anatomy and histochemistry. Can J Bot 69: 2055–2073.
23. Aoun M, Rioux D, Simard M, Bernier L (2009) Fungal colonization and host defense reactions in *Ulmus americana* callus cultures inoculated with *Ophiostoma novo-ulmi*. Phytopathology 99: 642–650.
24. Bernier L, Aoun M, Jacobi V, Boyle B (2010) Identification and monitoring of *Ulmus americana* transcripts during in vitro interactions with the Dutch elm disease pathogen *Ophiostoma novo-ulmi*. Physiol Mol Plant Pathol 74: 254–266.
25. Brun H, Chèvre AM, Fitt BD, Powers S, Besnard AL, et al. (2010) Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. New Phytol 185: 285–299.
26. Newhouse AE, Schrodt F, Liang H, Maynard CA, Powell WA (2007) Transgenic American elm shows reduced Dutch elm disease symptoms and normal mycorrhizal colonization. Plant Cell Rep 26: 977–987.
27. Ganley RJ, Sniezko RA, Newcombe G (2008) Endophyte-mediated resistance against white pine blister rust in *Pinus monticola*. For Ecol Manage 255: 2751–2760.
28. Schulz B, Rommert AK, Dammann U, Aust HJ, Strack D (1999) The endophyte-host interaction: a balanced antagonism? Mycol Res 103: 1275–1283.
29. Arnold AE, Mejia LC, Kyllo D, Rojas EI, Maynard Z, et al. (2003) Fungal endophytes limit pathogen damage in a tropical tree. Proc Natl Acad Sci USA 100: 15649–15654.
30. Mucciarelli M, Scannerini S, Bertea C, Maffei M (2003) In vitro and in vivo peppermint (*Mentha piperita*) growth promotion by nonmycorrhizal fungal colonization. New Phytol 158: 579–591.
31. Cogolludo-Agustín MA, Agúndez D, Gil L (2000) Identification of native and hybrid elms in Spain using isozyme gene markers. Heredity 85: 157–166.
32. Gil L, Fuentes-Utrilla P, Soto A, Cervera MT, Collada C (2004) English elm is a 2,000-year-old Roman clone. Nature 431: 1053–1053.
33. Martín JA, Solla A, Buron M, Lopez-Almansa JC, Gil L (2006) Historical, ecological, taxonomic and health characterization of the relict elm stand of Rivas-Vaciamadrid (Madrid). For Syst 15: 208–217.
34. Solla A, Dacasa MC, Nasmith C, Hubbes M, Gil L (2008) Analysis of Spanish populations of *Ophiostoma ulmi* and *O. novo-ulmi* using phenotypic characteristics and RAPD markers. Plant Pathol 57: 33–44.
35. Martín JA, Solla A, Domingues MR, Coimbra MA, Gil L (2008) Exogenous phenol increase resistance of *Ulmus minor* to Dutch elm disease through formation of suberin-like compounds on xylem tissues. Env Exp Bot 64: 97–104.
36. Martín JA, Solla A, Gil L, Garcia-Vallejo MC (2010) Phenological and histochemical changes of *Ulmus minor* due to root absorption of phenol: Implications for resistance to DED. Env Exp Bot 69: 175–182.
37. Martín JA, Solla A, Witzell J, Gil L, Garcia-Vallejo MC (2010) Antifungal effect and reduction of *Ulmus minor* symptoms to *Ophiostoma novo-ulmi* by carvacrol and salicylic acid. Eur J Plant Pathol 127: 21–32.
38. Helander M, Ahlholm J, Sieber TN, Hinneri S, Saikkonen K (2007) Fragmented environment affects birch leaf endophytes. New Phytol 175: 547–553.
39. Arnold AE, Maynard Z, Gilbert GS, Coley PD, Kursar TA (2000) Are tropical fungal endophytes hyperdiverse? Ecol Lett 3: 267–274.
40. Atlas RM, Bartha R (1998) Microbial Ecology: Fundamentals and Applications. Benjamin/Cummings Publishing Co, Menlo Park, CA 593 p.
41. Anderson MJ, Crist TO, Chase JM, Vellend M, Inouye BD, et al. (2011) Navigating the multiple meanings of b diversity: a roadmap for the practicing ecologist. Ecol Lett 14: 19–28.
42. White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TL, editors. PCR protocols: a guide to methods and applications. New York: Academic Press. 315–322.
43. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410.
44. Srivastava V, Schinkel H, Witzell J, Hertzberg M, Torp M, et al. (2007) Downregulation of high-isoelectric-point extracellular superoxide dismutase mediates alterations in the metabolism of reactive oxygen species and developmental disturbances in hybrid aspen. Plant J 49: 135–148.
45. Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). Biometrika 52: 591–611.
46. Gotelli NJ, Colwell RK (2011) Estimating species richness. In: Magurran AE, McGill BJ, editors. Frontiers in measuring biodiversity. Oxford University Press, New York. 39–54
47. Colwell RK (2005) EstimateS: Statistical estimation of species richness and shared species from samples. Version 8.2.0. User's Guide and application published at: EstimateS website. Available: http://purl.oclc.org/estimates. Accessed 2012 Nov 20.
48. Bills GF (1996) Isolation and analysis of endophytic fungal communities from woody plants. In: Redlin SC, Carris LM, editors. Endophytic fungi in grasses and woody plants: systematics, ecology and evolution. St. Paul: APS Press. 31–65.
49. Amend AS, Seifert KA, Bruns TD (2010) Quantifying microbial communities with 454 pyrosequencing: does read abundance count? Mol Ecol 19: 5555–5565.
50. Blaalid R, Carlsen T, Kumar S, Halvorsen R, Ugland KI, et al. (2012) Changes in the root-associated fungal communities along a primary succession gradient analysed by 454 pyrosequencing. Mol Ecol 21: 1897–1908.
51. Cordier T, Robin C, Capdevielle X, Desprez-Loustau M-L, Vacher C (2012) Spatial variability of phyllosphere fungal assemblages: genetic distance predominates over geographic distance in a European beech stand (*Fagus sylvatica*). Fungal Ecol 5: 509–520.
52. De Souza Vieira PD, De Souza Motta CM, Lima D, Torres JB, Quecine MC, et al. (2011) Endophytic fungi associated with transgenic and non-transgenic cotton. Mycology 2: 91–97.
53. Stuart RM, Romão AS, Pizzirani-Kleiner AA, Azevedo JL, Araújo WL (2010) Culturable endophytic filamentous fungi from leaves of transgenic imidazoli-

none-tolerant sugarcane and its non-transgenic isolines. Arch Microbiol 192: 307–313.
54. Clay K (1988) Fungal endophytes of grasses - a defensive mutualism between plants and fungi. Ecology 69: 10–16.
55. Gennaro M, Gonthier P, Nicolotti G (2003) Fungal endophytic communities in healthy and declining *Quercus robur* L. and *Q. cerris* L. trees in northern Italy. J Phytopathol 151: 529–534.
56. Mucciarelli M, Scannerini S, Bertea CM, Maffei M (2002) An ascomycetous endophyte isolated from *Mentha piperita* L.: biological features and molecular studies. Mycologia 94: 28–39.
57. Van Wees SCM, Van der Ent S, Pieterse CMJ (2008) Plant immune response triggered by beneficial microbes. Curr Opin Plant Biol 11: 443–448.
58. Li YC, Tao WT (2009) Interactions of Taxol-producing endophytic fungus with its host (*Taxus* spp.) during Taxol accumulation. Cell Biol Internat 33: 106–112.
59. Sinclair WA, Zahand JP, Melching JB (1975) Anatomical marker for resistance of *Ulmus americana* to *Ceratocystis ulmi*. Phytopathology 65: 349–352.
60. Martín JA, Solla A, Esteban LG, de Palacios P, Gil L (2009) Bordered pit and ray morphology involvement in elm resistance to *Ophiostoma novo-ulmi*. Can J For Res 39: 420–429.
61. Solla A, Martín JA, Corral P, Gil L (2005) Seasonal changes in wood formation of *Ulmus pumila* and *U. minor* and its relation with Dutch elm disease. New Phytol 166: 1025–1034.
62. White JF, Torres MS (2010) Is plant endophyte-mediated defensive mutualism the result of oxidative stress protection? Physiol Plant 138: 440–446.
63. Bais HP, Walker TS, Schweizer HP, Vivanco JM (2002) Root specific elicitation and antimicrobial activity of rosmarinic acid in hairy root cultures of *Ocimum basilicum* L. Plant Physiol Biochem 40: 983–995.
64. Bailey JK, Deckert R, Schweitzer JA, Rehill BJ, Lindroth RL, et al. (2005). Host plant genetics affect hidden ecological players: links among *Populus*, condensed tannins, and fungal endophyte infection. Can J Bot 83: 356–361.
65. Colhoun J (1973) Effects of environmental factors on plant disease. Annu Rev Phytopathol 11: 343–364.
66. Bernstein ME, Carroll GC (1977) Internal fungi in old growth Douglas fir foliage. Can J Bot 55: 644–653.
67. Petrini O, Stone J, Carroll FE (1982) Endophytic fungi in evergreen shrubs in western Oregon – a preliminary study. Can J Bot 60: 789–796.
68. Rodrigues KF (1994) The foliar fungal endophytes of the Amazonian palm *Euterpe oleracea*. Mycologia 86: 376–385.
69. Martín-García J, Espiga E, Pando V, Diez JJ (2011) Factors influencing endophytic communities in poplar plantations. Silva Fenn 45: 169–180.
70. Kowalski T, Kehr RF (1996) Fungal endophytes of living branch bases in several European tree species. In: Redlin SC, Carris LM, editors. Endophytic fungi in grasses and woody plants: systematics, ecology, and evolution. St Paul: American Phytopathological Society. 67–86.
71. Fisher PJ, Petrini O (1990) A comparative study of fungal endophytes in xylem and bark of *Alnus* species in England and Switzerland. Mycol Res 94: 313–319.
72. Ragazzi A, Moricca S, Capretti P, Dellavalle I (1999). Endophytic presence of *Discula quercina* on declining *Quercus cerris*. J Phytopathol 147: 437–440.
73. Linaldeddu BT, Sirca C, Spano D, Franceschini A (2005) Physiological responses of cork oak and holm oak to the infections of pathogens involved in oak decline. J Plant Pathol 87: 296–297.
74. Hoshino T, Xiao N, Tkachenko OB (2009) Cold adaptation in the phytopathogenic fungi causing snow molds. Mycoscience 50: 26–38.
75. Sieber T, Riesen TK, Muller E, Fried PM (1988) Endophytic fungi in four winter wheat cultivars (*Triticum aestivum* L.) differing in resistance against *Stagonospora nodorum* (Berk.) Cast. & Germ. (= *Septoria nodorum* (Berk.) Berk.). J Phytopathol 122: 289–306.
76. Albrectsen BR, Bjorken L, Varad A, Hagner A, Wedin M, et al. (2010). Endophytic fungi in European aspen (*Populus tremula*) leaves - diversity, detection, and a suggested correlation with herbivory resistance. Fungal Div 41: 17–28.
77. Martín-García J, Müller MM, Diez JJ (2012). ITS-based comparison of endophytic mycota in twigs of native *Populus nigra* and cultivated *P.* x *euramericana* (cv. I-214) stands in Northern Spain. Ann For Sci 69: 49–57.
78. Zalar P, Gostincar C, de Hoog GS, Ursic V, Sudhadham M, et al. (2008). Redefinition of *Aureobasidium pullulans* and its varieties. Stud Mycol 61: 21–38.
79. Webber J (1981) A natural biological control of Dutch elm disease. Nature 292: 449–451.
80. Dvorák M, Palovcíková D, Jankovský L (2006) The occurrence of endophytic fungus *Phomopsis oblonga* on elms in the area of southern Bohemia. J For Sci 52: 531–535.
81. Guo LD, Xu L, Zheng WH, Hyde KD (2004). Genetic variation of *Alternaria alternata*, an endophytic fungus isolated from *Pinus tabulaeformis* as determined by random amplified microsatellites (RAMS). Fungal Div 16: 53–65.
82. Newcombe G (2011) Endophytes in forest management: four challenges. In: Pirttilä AM, Frank AC, editors. Endophytes in forest trees: biology and applications. Dortrecht: Springer. 251–262.
83. Smith SA, Tank DC, Boulanger L-A, Bascom-Slack CA, Eisenman K, et al. (2008) Bioactive endophytes warrant intensified exploration and conservation. PLOS ONE 3(8): e3052. doi:10.1371/journal.pone.0003052.
84. Martín JA, Solla A, Coimbra MA, Gil L (2008) Metabolic fingerprinting allows discrimination between *Ulmus pumila* and *U. minor*, and between *U. minor* clones of different susceptibility to Dutch elm disease. For Pathol 38: 244–256.
85. De Gruyter J, Woudenberg JH, Aveskamp MM, Verkley GJ, Groenewald JZ, et al. (2010) Systematic reappraisal of species in *Phoma* section *Paraphoma*, *Pyrenochaeta* and *Pleurophoma*. Mycologia 102: 1066–1081.
86. Boerema GH, Dorenbosch MMJ (1973) The *Phoma* and *Ascochyta* species described by Wollenweber and Hochapfel in their study of fruit rotting. Stud Mycol 3: 1–50
87. Gams W, Müller E (1980) Conidiogenesis of *Fusarium nivale* and *Rhynchosporium oryzae* and its taxonomic implications. Neth J Pl Path 86: 45–53.
88. de Hoog GS (1977) *Rhinocladiella* and allied genera. Stud Mycol 15: 1–140.

II

Eur J Plant Pathol (2015) 142:185–196
DOI 10.1007/s10658-015-0602-2

ORIGINAL RESEARCH

Strong *in vitro* antagonism by elm xylem endophytes is not accompanied by temporally stable *in planta* protection against a vascular pathogen under field conditions

Juan A. Martín · David Macaya-Sanz · Johanna Witzell · Kathrin Blumenstein · Luis Gil

Accepted: 21 January 2015 / Published online: 6 February 2015

Abstract Some endophytic fungi provide their host plants with protection against abiotic and biotic stressors, including pathogens. Endophyte-mediated mechanisms might be behind the environmental resistance shown in the field by some adult *Ulmus minor* trees to the Dutch elm disease (DED) pathogen, *Ophiostoma novo-ulmi*. We isolated and characterized seven endophyte fungi from the xylem of three adult *U. minor* trees that have survived the DED epidemics within areas in Spain ravaged by the disease. The antagonism of the isolated endophytes towards *O. novo-ulmi* was evaluated *in vitro* by means of dual culture assays. Six of the studied endophytes hindered the pathogen growth through antibiosis, competition for the substrate, or a combination of both mechanisms. Four of these endophytes were selected for *in vivo* tests where their protective effect was evaluated in field experiments during three successive years (2011–2013). The conditioning inoculation of two endophytes (*Monographella nivalis* and *Alternaria tennuissima*) reduced DED symptoms in 2011 and 2012, respectively. However, the same isolates did not show any prophylactic effect in 2013, which suggests that the repeatability of the treatments is low. A significant treatment×clone interaction was found, showing that the effectiveness of the treatments depended on the tree clone. The future use of endophytes in biocontrol strategies might be oriented towards taking into consideration the whole fungal microbiome in forest breeding programs rather than the external application of particular endophyte strains.

Keywords Endophytes · Dutch elm disease · *Ophiostoma novo-ulmi* · Tree resistance · *Ulmus*

J. A. Martín (✉) · D. Macaya-Sanz · L. Gil
Escuela Técnica Superior de Ingeniería de Montes, Forestal y del Medio Natural, Universidad Politécnica de Madrid, Ciudad Universitaria s/n. 28040, Madrid, Spain
e-mail: juan.martin.garcia@upm.es

J. Witzell · K. Blumenstein
Faculty of Forest Sciences, Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, Box 49, 230 53 Alnarp, Sweden

J. Witzell
School of Forest Sciences, University of Eastern Finland, P.O. BOX 111, 80101 Joensuu, Finland

Introduction

Several studies have addressed the use of microorganisms for biocontrol of Dutch elm disease (DED), caused by the highly virulent vascular fungus *Ophiostoma novo-ulmi*. For example, it has been shown that the resistance of elms to *O. novo-ulmi* can be enhanced by artificial inoculation of elms with weakly pathogenic *Verticillium* sp. strains (Solla and Gil 2003; Scheffer et al. 2008), or strains of *Ophiostoma ulmi* (Hubbes 2004), a less aggressive DED pathogen. To date, the most successful biocontrol treatment against *O. novo-ulmi* has probably been the *V. albo-atrum* strain WCS850, which has been shown to significantly reduce DED incidence in adult elms when administered as preventive injections (Scheffer et al. 2008). However, as a consequence of its limited spread and survival in the

tree, injections with WCS850 must be annually repeated to be effective and the method thus has limited relevance in large-scale forestry.

Endophytic fungi live inside plant tissues in symbiosis with their hosts without causing disease symptoms (Carroll 1988). The type of symbiosis may vary among endophyte species, but also within species throughout their life cycles. For example, certain endophytes may establish a mutualistic or commensalistic symbiosis with their hosts, but after the weakening of the host by any abiotic or biotic stressor, may shift to a parasitic stage (Saikkonen 2007). Other endophytes, after the death of the plant or any of its organs, change from a dormant state to another of primary colonizers in decomposing tissues (Promputtha et al. 2007; Rodríguez et al. 2011). Some endophytes have been described as competitors or antagonists to forest tree pathogens, and therefore have an interesting potential in biocontrol of tree diseases (e.g., Arnold et al. 2003; Mejía et al. 2008). The development of treatments or disease management practices that are based on utilization of the tree's natural microbiome could be a valuable part of sustainable, integrated pest management strategies. An example of such treatment is the rugulosin-producing endophytes that reduce the incidence of spruce budworm in *Picea glauca* (Sumarah et al. 2008).

During the past and present century, two DED pandemics have nearly eliminated the adult native elms in Europe and North America (Brasier and Kirk 2010). However, a small number of mature elms have survived the pandemics within areas ravaged by the disease. The clonal replicates of most of these elms show severe disease symptoms when they are artificially inoculated with *O. novo-ulmi* in experimental plots, and thus seem to be genetically susceptible to the pathogen. The possibility that these trees are not detected by elm bark beetles, vectors of *O. novo-ulmi*, has been discarded (e.g., Martín et al. 2006). Thus, site-specific environmental factors seem to explain the resistance of the surviving elms. We hypothesize that the horizontally-spreading endophytic fungi might be an environmental factor that contributes to the phenotypic resistance of these elms.

In an earlier paper, we investigated the frequency and diversity of elm endophytes in elm trees showing low or high susceptibility to DED (Martín et al. 2013). The present work aims to gain a deeper insight into the potential role of elm endophytes in host defence against *O. novo-ulmi*. To accomplish this goal, the interactions between an aggressive strain of *O. novo-ulmi* and seven fungi, isolated after surface sterilization from the xylem of three adult elms that have survived the DED epidemics in Spain were evaluated *in vitro*. Then, the ability of a subset of endophytes to enhance tree resistance was assessed *in vivo* during three consecutive years of field experiments.

Materials and methods

Field sampling

Three adult *U. minor* individuals from Spain were sampled. These trees were naturally selected as they survived the DED epidemics, despite the high disease incidence in the area since the 1980s. Concretely, we sampled the ancient tree known as *Somontes* elm, located within a semi-natural forest area close to Madrid city (40°29′N, 3°45′W). The tree is 155 cm in diameter at breast height and 27 m in height, with an estimated age of more than 140 years. A susceptibility *ex situ* test had been performed with 10 clonal replicates of this tree, in order to tests its genetic resistance level to *O. novo-ulmi*, and the replicates were found to be highly susceptible to DED (Spanish elm breeding program, unpublished results). The second studied elm individual is located at the municipality of Rivas-Vaciamadrid (40°20′N, 3°33′ W), in the elm stand known as *Casa Eulogio*, a stand that has survived the last DED epidemic in spite of the fact several trees in the surroundings have been infected and killed by *O. novo-ulmi* (Martín et al. 2006). This tree is 87 cm in diameter at breast height and 19 m in height, with an estimated age of around 80 years. The genetic resistance of this tree to *O. novo-ulmi* was not tested separately, because it belongs to *U. minor* var. *vulgaris* complex which shows low genetic variability and high susceptibility to *O. novo-ulmi* (Gil et al. 2004). The third elm individual was located in Majorca Island, at *Albufera Natural Park* (39°47′N, 3°6′E). The tree is 60 cm in diameter at breast height and 17 m in height, and is symptomless in spite of being surrounded by numerous elms affected by DED. The genetic resistance level of this tree to *O. novo-ulmi* is, however, not established in systematic tests. In 2011, four 3-year-old twigs (around 20–30 cm length and 1–1.5 cm diameter) were taken from each cardinal point and lower half of the crown of each tree (48 twigs in total). Samples were

kept in a refrigerator at 4° C and processed in the laboratory within 72 h after the field collection.

Isolation of endophytic fungi

The isolation process took place in a laminar flow chamber under axenic conditions. Pieces of the original twigs (3 cm length) were surface sterilised by immersing them in 70 % ethanol (30 s), 4 % sodium hypochlorite (5 min) and in 70 % ethanol (15 s) (Helander et al. 2007). Then, they were allowed to dry for 15 min. After removal of the bark, a xylem fragment (0.5×1 cm of surface and 0.5 mm thickness) was carved from each twig with a flame-sterilized scalpel.

To evaluate the potential role of endophytes in environmental resistance, the isolation of fungi was restricted to xylem endophytes, given that *O. novo-ulmi* develops in xylem tissues during its pathogenic phase (Ouellette et al. 2004; Martín et al. 2009), and because the xylem-associated elm endophyte assemblages have shown the strongest linkage to the susceptibility level of host trees (Martín et al. 2013). Xylem fragments were cultured in Petri dishes containing 2% malt extract agar (MEA) without added antibiotics. All plates were sealed with Parafilm and incubated in the dark at 22° C. After 7 and 14 days of incubation, each emerged fungal colony was subcultured into a new dish, for isolation. Once complete isolation of endophyte strains was confirmed, they were stored at 4° C in sterilized distilled water.

Identification of endophytes and in vitro dual culture assays

After isolation of *ca.* 1 μg of total genomic DNA by the method described by Cenis (1992) with small modifications, the ITS region of ribosomal DNA of each isolate was amplified by Polymerase Chain Reaction (PCR) using universal primers ITS1 and ITS4, and following a simplified protocol based on the one described by White et al. (1990). DNA amplification products were Sanger sequenced. To infer strain identity, we used the algorithm 'megablast' (derived from the algorithm 'BLAST'; Altschul et al. 1990) to find the most similar sequences available in the database Genbank (NCBI, MD, USA).

From the isolates growing on Petri dishes and under sterile conditions, fragments of fungal mycelium (4× 4 mm) were taken from the actively growing colony edge and subcultured into new plates containing 2 % MEA. The dual cultures were then established by placing a similar fragment of *O. novo-ulmi* ssp. *novo-ulmi* on the same plate with each endophyte, with a 6 cm distance between the fragments and with six replicate plates per interaction. The *O. novo-ulmi* isolate was ZA-RG, isolated in 2002 from an infected *U. minor* tree in Riego del Camino (Zamora, Spain; 41°05′ N 5°46′ W) (Solla et al. 2008). The plates were incubated in darkness in a growth chamber at 22 °C, and the interactions were evaluated after 48, 96, and 168 h of incubation. Interactions were quantified by measuring the growth of each fungus in three directions: one connecting the centre of the inoculum fraction, and two at +45° and -45° angles to the former. Dual assays were also qualitatively evaluated by assessing the type of interaction: i) antibiosis (growth inhibition, determined by the presence of a reaction zone), ii) substrate competition (higher growth of one fungus relative to the other), and 3) mycoparasitism (direct mycelial parasitism of one fungus over the other) (Mejía et al. 2008).

In vivo experiments

Taking into account the results from the *in vitro* experiment and the availability of suitable plant material, four endophytes were selected for subsequent *in vivo* tests where their potential to enhance plant resistance against DED was evaluated. The plants were at least 4 years-old, the minimum age required to evaluate with scientific rigour their resistance to *O. novo-ulmi* (Solla et al. 2005). Tests were conducted on three plots at Puerta de Hierro Forest Breeding Centre (Madrid, Spain). Experimental details of each test are summarized in Table 1.

In 2011, the effect of two selected endophytic strains, identified as *Monographella nivalis* and *Sordaria* sp., was tested in 6-year-old trees growing in the same plot. The tested trees belonged to two *U. minor* clones (PM-TR2 and AB-AL1) and one *U. minor*×*U. pumila* clone (M-DV5×M-IN6.4), with 30 replicates per clone randomly distributed over the plot. Five treatments were applied: (i) and (ii), trees inoculated with the endophyte *M. nivalis* or *Sordaria* sp., respectively, on April 29 (negative control I and II, respectively); (ii), trees inoculated with *O. novo-ulmi* 15 days later (positive control); (iii) and (iv), trees inoculated with the endophyte *M. nivalis* or *Sordaria* sp., respectively, on April 29 and with *O. novo-ulmi* 15 days later. Each treatment was applied to six trees per genotype and was randomized in the plot.

Table 1 Experimental details of three *in vivo* experiments performed in *Puerta de Hierro* Forest Breeding Centre (Madrid, Spain) to evaluate the protective effects of several pre-treatments with elm endophytes against challenge inoculations with *O. novo-ulmi*

Year	Clones (species)	Age of trees	Total N of trees	N replicates per clone and treatment	Inoculation treatments	
					Pre-treatment[a]	Challenge[b]
2011	PM-TR2 and AB-AL1 (*U. minor*) M-DV5 x M-IN6.4 (*U. minor x U. pumila*)	6	*90*	6	*M. nivalis*	H_2O
				6	*Sordaria sp.*	H_2O
				6	H_2O	*O. novo-ulmi*
				6	*M. nivalis*	*O. novo-ulmi*
				6	*Sordaria sp.*	*O. novo-ulmi*
2012	V-JRI (*U. minor*)	4	*40*	8	*P. crustosum*	H_2O
				8	*A. tenuissima*	H_2O
				8	H_2O	*O. novo-ulmi*
				8	*P. crustosum*	*O. novo-ulmi*
				8	*A. tenuissima*	*O. novo-ulmi*
2013	PM-TR2 and ZA-TR5 (*U. minor)*	5	126	5	*M. nivalis*	H_2O
				5	*A. tenuissima*	H_2O
				5	*M. nivalis+ A. tenuissima*	H_2O
				12	H_2O	*O. novo-ulmi*
				12	*M. nivalis*	*O. novo-ulmi*
				12	*A. tenuissima*	*O. novo-ulmi*
				12	*M. nivalis+ A. tenuissima*	*O. novo-ulmi*

[a] Pre-treatment dates: April 29, 2011; May 10, 2012; April 24, 2013

[b] Challenge dates: 15 days after pre-treatments; Symptom evaluation: 30, 60 and 120 days after challenge

The *M. nivalis* and *Sordaria* sp. inoculum consisted of an aqueous suspension of conidia at 10^6 spores/ml, adjusted using a haemacytometer. The methodology described by Browne and Cooke (2004) was followed to produce *M. nivalis* spores. Sterilized distilled water was poured on the surface of the MEA plates where the *Sordaria* sp. grew. The mycelial surface was rubbed with a cotton swab for 5 min and the water containing spores was then filtered through sterile gauze to remove mycelial fragments. The inoculum of *O. novo-ulmi* (same isolate as in the *in vitro* experiment) consisted of an aqueous suspension of budding cells at 10^6 cells/ml that were obtained following the methodology described by Tchernoff (1965). The endophytes were inoculated in the trunk base (3 cm above the ground level) on the north side, through an incision made with a sharp razor blade to reach the xylem. Then, 0.2 ml of inoculum were poured on the blade surface and rapidly absorbed by the sap flow. The pathogen was inoculated using the same methodology, but on the opposite side and 15 cm above the endophyte inoculation wound. Negative controls received sterilized water instead of *O. novo-ulmi* 15 days after endophyte inoculation, while positive controls received water instead of endophyte inoculum 15 days before pathogen inoculation. The symptom assessment was performed at 30, 60 and 120 days after *O. novo-ulmi* inoculation (hereafter "dai") by recording the percentage of the crown affected by leaf wilting.

In 2012, the effect of two other endophytes, identified as *Penicillium crustosum* and *Alternaria tenuissima,* was tested in 4-year-old trees growing in a second plot of the Breeding Centre. All trees belonged to the *U. minor* clone V-JR1, with 40 replicates. Five treatments were applied: (i) trees inoculated with *Penicillium* on May 10 (negative control I), (ii) trees inoculated with *A. tenuissima* on May 10 (negative control II), (iii) trees inoculated with *O. novo-ulmi* 15 days later (positive control), (iv) trees inoculated with *P. crustosum* on May 10 and with *O. novo-ulmi* 15 days later, and (v) trees inoculated with *A. tenuissima* on May

10 and with *O. novo-ulmi* 15 days later. Treatments were applied randomly in the plot on eight trees per treatment. The inoculum consisted of endophytes in an aqueous suspension of 10^6 conidia/ml obtained with the same methodology as in the case of *Sordaria* sp. Inoculation of the pathogen and endophytes and symptom evaluation were performed following the same methodology described above. Here again, negative and positive controls received sterilized water instead of *O. novo-ulmi* or endophyte inoculum, respectively. The delay in the inoculation date in comparison to the previous year was motivated by a delayed plant phenology, since the window of peak susceptibility to *O. novo-ulmi* largely depends on plant phenology (Ghelardini et al. 2010; Martín et al. 2010a).

In 2013, in view of the results of the former experiments, the effect of two previously tested endophytes (*M. nivalis* and *A. tenusissima*) was tested on 5-year-old *U. minor* trees growing in a new plot. Two elm clones (PM-TR2 and ZA-TR5) were present in the plot, with 63 replicates per clone randomly distributed. Seven treatments were randomly applied on the plot: Treatments (i), (ii), and (iii), corresponded to trees inoculated with *M. nivalis*, *A. tenuissima*, and a mixture of them, respectively, on April 24 (negative controls I, II and III, respectively; N=5 per clone); (iv) trees inoculated with *O. novo-ulmi* 15 days later (positive control; N=12 per clone); and (v), (vi), and (vii), trees inoculated with the endophytes *M. nivalis*, *A. tenuissima*, and a mixture of them, respectively, on April 24 and with *O. novo-ulmi* 15 days later (N=12 per clone). The inoculations were performed with the same inoculum concentration and methodology, and using water instead of *O. novo-ulmi* or endophyte inoculum in positive and negative controls, as in the previous years (Table 1).

Statistical analysis

In order to evaluate endophyte-pathogen interactions *in vitro*, the growth of *O. novo-ulmi* in the dual plate assays was analyzed using one-way ANOVA, considering the endophyte isolate as factor. Mean values were compared through Fisher's Least Significant Difference (LSD) test (α=0.05).

Wilting percentages shown by plants in *in vivo* tests were analyzed by repeated measures ANOVAs. In 2011 and 2013 experiments, the treatment, elm clone and time after inoculation were taken as factors, as well as treatment×clone interactions. In 2012, only the treatment and time factors were considered as factors, due to the fact there was a single tree genotype. Since negative controls did not show any disease symptoms during the experiments, they were excluded from the ANOVAs. The wilting values were compared among treatments using Fisher's LSD test (α=0.05). Normality of data was confirmed using the Shapiro-Wilks test. All statistical analyses were performed using STATISTICA program, version 7.0 (StatSoft Inc., OK, USA).

Results and discussion

A total of seven endophytic fungi were isolated from the xylem of the three selected *U. minor* trees. Their taxonomic identification is shown in Table 2. All endophytes were ascomycetes belonging to the classes Dothideomycetes (*Aureobasidium pullulans*, *Alternaria tenuissima* and *Neofusiccocum luteum*), Sordariomycetes (*Fusarium* sp., *Monographella nivalis* and *Sordaria* sp.), and Eurotiomycetes (*Penicillium crustosum*).

Six out of the seven endophytes significantly reduced the growth of the pathogen *in vitro* (P<0.05) (Fig. 1). The mechanism by which this reduction occurred differed among isolates. *M. nivalis* and *P. crustosum* inhibited pathogen growth through antibiosis, whereas *N. luteum* and *Sordaria* sp. did so through competition for the substrate. The endophytes *A. pullulans* and *A. tenuissima* showed combined yet weaker effects of antibiosis and competition for the substrate, while *Fusarium* sp. did not display any antagonistic reaction towards *O. novo-ulmi*. The results of the *in vitro* dual cultures concur with previous studies in which most of the tested fungi showed some degree of antagonism against DED pathogens (e.g., Webber and Hedger 1986; Yang et al. 1993). The endophyte that exerted the strongest antibiotic effect against *O. novo-ulmi* was *M. nivalis*, which induced the formation of a thick reaction barrier in the pathogen (Fig. 2a) in agreement with a previous study which also confirmed that this fungus produces extracellular metabolites that inhibit *O. novo-ulmi* growth (Blumenstein 2010). The highest inhibition of *O. novo-ulmi* growth, caused by *Sordaria* sp. and *N. luteum*, occurred due to the faster endophyte growth in MEA compared to the pathogen (competition for the substrate), thus suffocating the expansion of *O.novo-ulmi* colony (Figs. 1 and 2b).

Taking into account the results from the *in vitro* experiment, four endophytes were selected to test their

Table 2 Identification of endophyte isolates on basis of the top three BLAST hits (based on nucleotide MEGABLAST of ITS rDNA sequences) with corresponding GenBank taxa identity

Accesion number GenBank	Description	Coverage / identity	Tentative identification	Host tree location
JX462673.1	*Aureobasidium pullulans*	100/99	*Aureobasidium pullulans*	Somontes (Madrid)
JX462671.1	*Aureobasidium pullulans*	100/99		
JX290145.1	*Aureobasidium* sp.	100/99		
JX869565.1	*Penicillium crustosum*	100/99	*Penicillium crustosum*	Somontes (Madrid)
HQ262518.1	*Penicillium crustosum*	100/99		
HQ832993.1	*Penicillium* sp.	100/99		
JX860514.1	*Alternaria tenuissima*	99/99	*Alternaria tenuissima*	Somontes (Madrid)
JN624884.1	*Alternaria tenuissima*	99/99		
JX648307.1	*Alternaria* sp.	99/99		
HQ637287.1	*Fusarium* sp.	64/100	*Fusarium* sp.	Rivas-Vaciamadrid
AB470905.1	*Fusarium* sp.	64/100		(Madrid)
EF611091.1	*Fusarium* sp.	64/100		
JQ809674.1	*Fusarium* sp.	98/97	*Monographella nivalis* [a]	Rivas-Vaciamadrid
JQ693397.1	*Fusarium lateritium*	98/97		(Madrid)
EU255803.1	*Gibberella avenacea*	98/97		
JX298886.1	Sordariaceae sp.	100/100	*Sordaria* sp.	Rivas-Vaciamadrid
JQ759666.1	*Sordariomycetes* sp.	100/100		(Madrid)
AM262364.1	*Sordaria* sp.	100/100		
JX073038.1	*Neofusicoccum luteum*	65/98	*Neofusicoccum luteum*	Albufera de Mallorca
HQ529764.1	*Neofusicoccum luteum*	65/98		(Mallorca)
HQ529761.1	*Neofusicoccum luteum*	65/98		

[a] Identification completed by morphological methods (Martín et al. 2013)

potential to inhibit *O. novo-ulmi in vivo*. *M. nivalis* and *P. crustosum* isolates were selected because they showed the strongest antibiotic activity towards *O. novo-ulmi*, *Sordaria* sp. because it strongly inhibited *O. novo-ulmi* through competition for the substrate, and *A. tenuissima* because it inhibited *O. novo-ulmi* by two mechanisms, competition for the substrate and antibiosis.

In the 2011 *in vivo* experiment, the effect of the treatment on wilting values was significant (ANOVA; $P<0.05$; Table 3). The trees pre-treated with the *M. nivalis* isolate and challenged with *O. novo-ulmi*

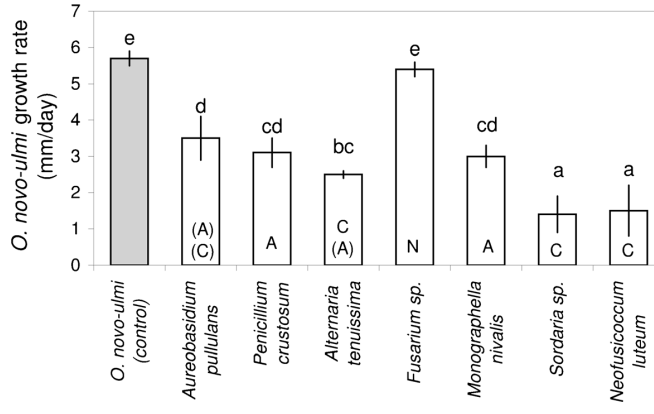


Fig. 1 Average growth of *O. novo-ulmi* in *in vitro* dual cultures with different endophytes. *Lowercase letters* indicate significant differences between treatments ($P<0.05$). *Capital letters* indicate the effect of the endophyte on *O. novo-ulmi* in accordance with the following code: A=antibiosis (formation of a reaction zone without mycelial contact), C=competition for the substrate (higher growth rate), N=neutral effect; *letters in parentheses*=moderate effect; *letters without parentheses*= marked effect. *Vertical bars* show standard errors

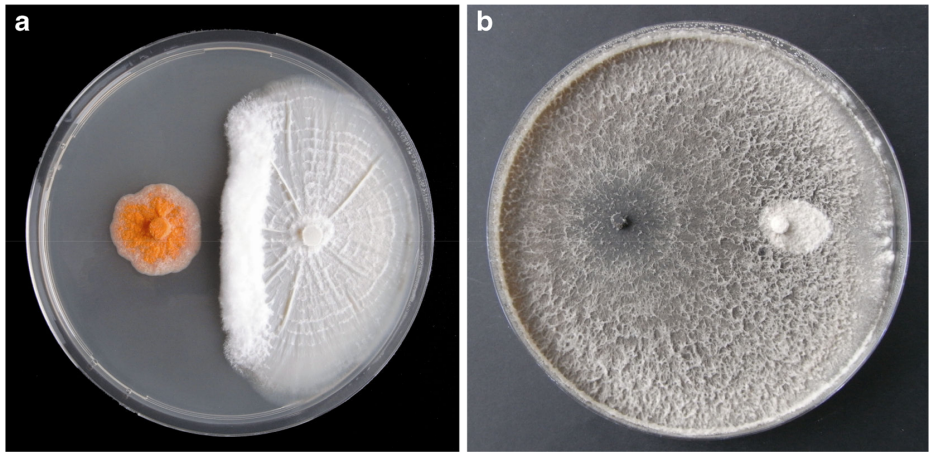


Fig. 2 Dual cultures between *O. novo-ulmi* (on the right side of plates) and two elm endophyes (on the left side of plates): (**a**) *Monographella nivalis*, showing antibiotic activity against *O. novo-ulmi*; (**b**) *Sordaria* sp. inhibiting *O. novo-ulmi* by competition for the substrate. Photos were taken after 14 days of incubation in the dark at 22 °C

showed lower leaf wilting symptoms at 60 and 120 dai, as compared to trees inoculated only with the pathogen, while the trees pre-treated with *Sordaria* sp. showed reduced symptoms only at 120 dai (Fig. 3a). The average reduction of wilting symptoms due to pre-treatment with *M. nivalis* at the end of the growing season was of 23.3% in comparison with the positive control. There was a significant treatment×clone interaction (Table 3), showing that the reduction of symptoms by pre-treatment with *M. nivalis* or *Sordaria* sp. was significant in the PM-TR2 (*U. minor*) and M-DV5×M-IN6.4 (*U. minor*×*U. pumila*) clones, but not in the AB-AL1 clone (*U. minor*) (Fig. 4a). Plants inoculated only with endophytes (negative controls) did not show any external symptoms.

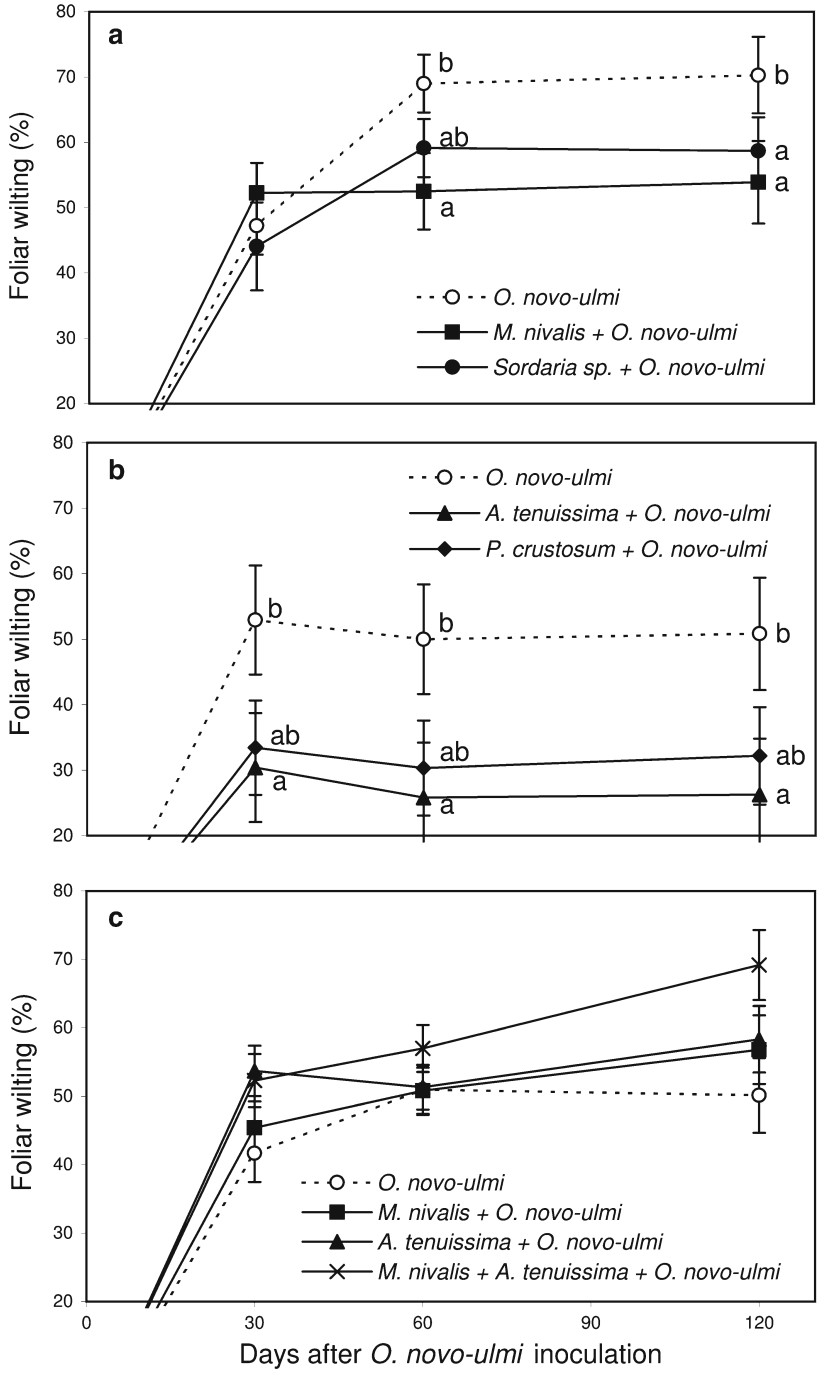
In the 2012 experiment, the effect of the treatment was not significant (ANOVA; P=0.068; Table 3). However, the plants conditioned with the endophyte *A. tenuissima* and afterwards challenged with *O. novo-ulmi* tended to exhibit lower wilting symptoms than plants inoculated only with the pathogen, regardless of the dai (Fig. 3b). The average reduction of wilting symptoms at the end of the growing season was 48.3 %, in comparison with the positive control. The *P. crustosum* isolate did not exert any protective effect against the pathogen. Trees inoculated only with the endophytes (negative controls) showed no sign of leaf wilting or other external symptoms.

To test the repeatability of the experiments performed in 2011 and 2012, in 2013 the effects of *M. nivalis* and *A. tenuissima* isolates were tested again in two additional *U. minor* clones with a higher number of replicates. Furthermore, the protective effect of the combined inoculation of both endophytes into the same tree was evaluated. However, the pre-treatments did not show any prophylactic effect (Figs. 3c, 4b). Again, the trees

Table 3 Results of the repeated measures ANOVA of leaf wilting (repeated variable) shown by the plants 30, 60, and 120 days after *Ophiostoma novo-ulmi* inoculation

Experiment: year (endophytes tested as preventive treatments)	Source of variation			
	Time	Treatment (T)	Tree clone (C)	T×C
2011 (*M. nivalis*, *Sordaria* sp.)	<0.001	0.004	< 0.001	0.002
2012 (*A. tenuissima*, *P. crustosum*)	0.383	0.068	–	–
2013 (*M. nivalis*, *A. tenuissima*)	0.936	0.154	< 0.001	0.757

Different preventive treatments with endophytes were tested each year


a
b
c
Foliar wilting (%)
Days after O. novo-ulmi inoculation
O. novo-ulmi
M. nivalis + O. novo-ulmi
Sordaria sp. + O. novo-ulmi
A. tenuissima + O. novo-ulmi
P. crustosum + O. novo-ulmi
M. nivalis + A. tenuissima + O. novo-ulmi
0
30
60
90
120
20
30
40
50
60
70
80
ab
a

◀ **Fig. 3** Temporal evolution of wilting symptoms displayed by control plants inoculated with *O. novo-ulmi*, and plants conditioned with endophytes and subsequently challenged with *O. novo-ulmi*. (**a**) 2011 experiment in which conditioning treatments with *Monographella nivalis* and *Sordaria* sp. were tested; (**b**) 2012 experiment in which conditioning treatments with *Alternaria tenuissima* and *Penicillium crustosum* were tested; (**c**) 2013 experiment in which conditioning treatments with *M. nivalis*, *A. tenuissima* and a combination of both were tested. *Different letters* indicate significant differences between treatments for the same day (Fisher's LSD test; α=0.05). *Vertical bars* show standard errors

inoculated with the endophytes only showed no external symptoms.

The results show that the endophytes tested *in vivo* provided a variable prophylactic effect against *O. novo-ulmi*. The experiments performed in 2011 and 2012 suggest that the artificial inoculation of *M. nivalis* and *A. tenuissima* isolates, and to a lesser extent *Sordaria* sp., could reduce the elm susceptibility against *O. novo-ulmi*. The experiment in 2013, however, questions the former evidence. The influence of environmental factors and the clone × treatment interaction detected in the 2011 experiment might explain these ambiguous results. The different behaviour of the PM-TR2 clone depending on the inoculation year (2011 or 2013; Fig. 4) suggests that annual variability in environmental conditions may have affected both plant susceptibility to *O. novo-ulmi* and the efficacy of the preventive treatments. The influence of environmental factors, such as climate and soil parameters, on elm susceptibility to DED is well documented (e.g., Kais et al. 1962; Sutherland et al. 1997; Solla and Gil 2002) and often related to seasonal changes in tree phenology (Ghelardini et al. 2009; Martín et al. 2010b). For instance, the rainfall in April 2011 was about 50 % higher in Madrid than normal values for this month, while in April 2013 the rainfall was lower than normal values (AEMET 2015). This may have affected both earlywood formation and tree susceptibility to *O. novo-ulmi* (Solla and Gil 2002). On the other hand, it seemed that the clone AB-AL1 was not sensitive to the preventive treatments. A marked dependence of the effectiveness on the elm clone was also reported in previous experiments that used *Pseudomonas* bacteria as preventive treatments against DED (e.g., Shi and Brasier 1986; Scheffer et al. 1989).

Another important issue, not addressed in the present study, was the spread and survival of the inoculated endophytes within the plant. Contrary to the DED pathogens, which spread systemically throughout the plant, previous studies have reported only local spread and limited survival of inoculated fungal treatments against DED (e.g. Bernier et al. 1996; Scheffer et al. 2008). The monitoring of the inoculated endophytes in plant tissues through classical methods of re-isolation and identification would have some difficulties, as the fungal species inoculated might be present as endophytes in different tree parts before inoculation. The use of molecular fingerprinting techniques, which allow the monitoring of specific fungal strains, appears more adequate for this task. Research is in progress to monitor the survival and spread of *M. nivalis* and *A. tenuissima* within plant tissues, by means of nested PCRs with strain-specific primers designed from the ITS region, and PCR fragment resolution on agarose gel.

O. novo-ulmi spreads through the tree vascular system, causing cavitation of xylem vessels and alterations

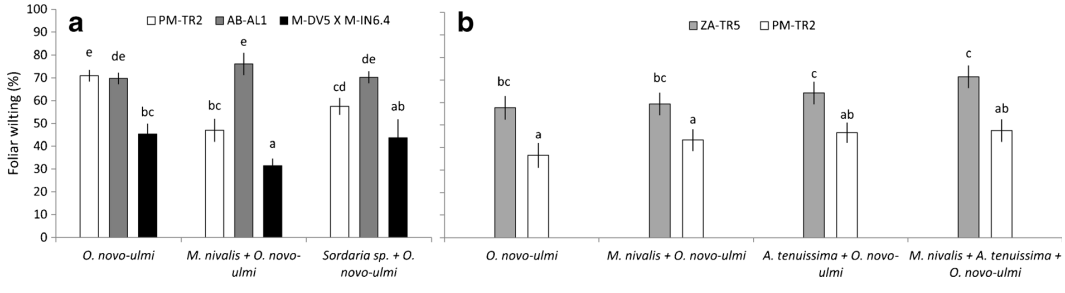


Fig. 4 Average foliar wilting per clone and treatment displayed by plants inoculated with *O. novo-ulmi* (control treatment), and plants conditioned with endophytes and subsequently challenged with *O. novo-ulmi* (conditioning treatments). (**a**) 2011 experiment in which conditioning treatments with *Monographella nivalis* and *Sordaria* sp. were tested in clones PM-TR2, AB-AL1, and M-DV5 × M-IN6.4; (**b**) 2013 experiment in which conditioning treatments with *M. nivalis*, *A. tenuissima* and a combination of both were tested in clones PM-TR2 and ZA-TR5. *Different letters* indicate significant differences between clones (Fisher's LSD test; α=0.05). *Vertical bars* show standard errors

of parenchyma cells (Ouellette et al. 2004; Martín et al. 2009). During its spread inside xylem tissues, the pathogen should suppress or overcome the plant defence responses, such as the release of antifungal metabolites or the presence of lignified or suberized barrier zones that limit pathogen advance (Aoun et al. 2009; Martín et al. 2008a). Also, it should interact with the endophytic microbiome inhabiting xylem tissues. The mechanisms by which the endophytes reduced DED symptoms in 2011 and 2012 are unknown, but at least two hypotheses can be raised. First, the protective effect may have come from the release of antibiotic metabolites by endophytes against *O. novo-ulmi* spread, since both, *M. nivalis* and *A. tenuissima* showed antibiotic activity *in vitro*. Currently, we are working to identify bioactive fractions in *M. nivalis* extract using chemical and transcriptome analysis of the reaction zone. A complementary or alternative possibility is that the massive insertion of endophyte spores within plant tissues triggered induced resistance mechanisms (Eyles et al. 2010), a process already described in preventive inoculations with *O. ulmi* or *Verticillium* sp. strains (Hubbes and Jeng 1981; Scheffer et al. 2008). The fact that one of the endophytes (*P. crustosum*), which showed strong antibiotic activity towards *O. novo-ulmi*, did not reduce the DED symptoms, suggests that other mechanisms besides direct antibiosis were involved.

Although an increasing number of studies have evidenced the possible involvement of the endophyte species composition and abundance in tree resistance against natural enemies (e.g. Arnold et al. 2003; Clay 2004; Mejía et al. 2008; Albrectsen et al. 2010; Martín et al. 2013), the development of practical control strategies based on endophytes would require further knowledge on the functional roles of the endophytic microbiome. For instance, the relative contribution of endophytes and host genotypes to tree phenotypes, including the resistance to pathogens, is still unknown (Newcombe 2011). It would be important to explore the possible role of endophytes in the expression of quantitative resistance in plants. A recent study based on next generation sequencing has revealed that elm clones that were highly resistant to *O. novo-ulmi* have a different endophyte species composition in stem tissues from medium and low resistant clones of the same species (authors, unpublished results). Thus, the future use of endophytes in biocontrol strategies might be oriented towards taking into consideration a larger fraction of, or the whole fungal microbiome in forest breeding programs (e.g., by selecting clones for their ability to host a particular microbiome) rather than the external application of particular endophyte strains.

The variable results obtained in the field experiments impede the acceptance or refusal of the initial hypothesis, concerning the involvement of xylem endophytes in the environmental resistance to *O. novo-ulmi*. Other environmental factors, such as the chemical environment (Martín et al. 2008b, 2010a, b; Vivas et al. 2012) or the action of less aggressive *Ophiostoma* strains inducing tree resistance (Hubbes and Jeng 1981; Jeng et al. 1983) should not be discarded as factors involved in environmental resistance. A deeper study of the fungal microbiome and its spatial distribution over the tree architecture, including seeds, could help to identify candidate mutualistic endophytes in elms.

Acknowledments The authors are very grateful to Jorge Domínguez (Universidad Politécnica de Madrid), and all the personnel working at Puerta de Hierro Forest Breeding Centre for their technical assistance. This work was supported by the research projects CTQ2011-28503-C02-02 and AGL2012-35580 (Ministerio de Economía y Competitividad) and by an agreement established between DGDRyPF (Ministerio de Agricultura, Alimentación y Medio Ambiente) and ETSI de Montes (Universidad Politécnica de Madrid).

References

AEMET (State Meteorology Agency of Spain) (2015). Monthly climate reports. http://www.aemet.es/es/portada. Accessed 7 January 2015.

Albrectsen, B. R., Bjorken, L., Varad, A., Hagner, A., Wedin, M., Karlsson, J., & Jansson, S. (2010). Endophytic fungi in European aspen (*Populus tremula*) leaves – diversity, detection, and a suggested correlation with herbivory resistance. *Fungal Diversity, 41*, 17–28.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*, 403–410.

Aoun, M., Rioux, D., Simard, M., & Bernier, L. (2009). Fungal colonization and host defense reactions in *Ulmus americana* callus cultures inoculated with *Ophiostoma novo-ulmi. Phytopathology, 99*, 642–650.

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

Bernier, L., Yang, D., Ouellette, G. B., & Dessureault, M. (1996). Assessment of *Phaeotheca dimorphospora* for biological control of the Dutch elm disease pathogens, *Ophiostoma ulmi* and *O. novo-ulmi. Plant Pathology, 45*, 609–17.

Blumenstein, K. (2010). Characterization of endophytic fungi in the genus *Ulmus*: putative agents for the biocontrol of Dutch Elm Disease (DED). Diploma thesis, University of Kassel, 114 p.

Brasier, C. M., & Kirk, S. A. (2010). Rapid emergence of hybrids between two subspecies of *Ophiostoma novo-ulmi* with a high level of pathogenic fitness. *Plant Pathology, 59*, 186–199.

Browne, R. A., & Cooke, B. M. (2004). A new method for producing mycelium-free conidial suspensions from cultures of *Microdochium nivale*. *European Journal of Plant Pathology, 110*, 87–90.

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

Cenis, J. L. (1992). Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Research, 20*, 2380–2380.

Clay, K. (2004). Fungi and the food of the gods. *Nature, 427*, 401–402.

Eyles, A., Bonello, P., Ganley, R., & Mohammed, C. (2010). Induced resistance to pests and pathogens in trees. *New Phytologist, 185*, 893–908.

Ghelardini, L., Santini, A., Black-Samuelsson, S., Myking, T., & Falusi, M. (2010). Bud dormancy release in elm (*Ulmus* spp.) clones - a case study of photoperiod and temperature responses. *Tree Physiology, 30*, 264–74.

Gil, L., Fuentes-Utrilla, P., Soto, A., Cervera, M. T., & Collada, C. (2004). English elm is a 2,000-year-old Roman clone. *Nature, 431*, 1053.

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

Hubbes, M. (2004). Induced resistance for the control of Dutch elm disease. *Forest Systems, 13*, 185–196.

Hubbes, M., & Jeng, R. S. (1981). Aggressiveness of *Ceratocystis ulmi* strains and induction of resistance in *Ulmus americana*. *European Journal of Forest Pathology, 11*, 257–64.

Jeng, R. S., Alfarenas, A. C., Hubbes, M., & Dumas, M. (1983). Presence and accumulation of fungitoxic substances against *Ceratocystis ulmi* in *Ulmus americana*: possible relation to induced resistance. *European Journal of Forest Pathology, 13*, 239–44.

Kais, A., Smalley, E., & Riker, A. (1962). Environment and development of Dutch elm disease. *Phytopathology, 52*, 1191–1196.

Martín, J. A., Solla, A., Buron, M., Lopez-Almansa, J. C., & Gil, L. (2006). Historical, ecological, taxonomic and health characterization of the relict elm stand of Rivas-Vaciamadrid (Madrid). *Forest Systems, 15*, 208–217.

Martín, J. A., Solla, A., Coimbra, M. A., & Gil, L. (2008a). Metabolic fingerprinting allows discrimination between *Ulmus pumila* and *U. minor*, and between *U. minor* clones of different susceptibility to Dutch elm disease. *Forest Pathology, 38*, 244–256.

Martín, J. A., Solla, A., Coimbra, M. A., Domingues, M. R., & Gil, L. (2008b). Exogenous phenol increase resistance of *Ulmus minor* to Dutch elm disease through formation of suberin-like compounds on xylem tissues. *Environmental and Experimental Botany, 64*, 97–104.

Martín, J. A., Solla, A., Esteban, L. G., de Palacios, P., & Gil, L. (2009). Bordered pit and ray morphology involvement in elm resistance to Dutch elm disease. *Canadian Journal of Forest Research, 39*, 420–429.

Martín, J. A., Solla, A., Witzell, J., Gil, L., & García-Vallejo, M. C. (2010a). Antifungal effect and reduction of *Ulmus minor* symptoms to *Ophiostoma novo-ulmi* by carvacrol and salicylic acid. *European Journal of Plant Pathology, 127*, 21–32.

Martín, J. A., Solla, A., Gil, L., & García-Vallejo, M. C. (2010b). Phenological and histochemical changes in *Ulmus minor* due to root absorption of phenol: implications for resistance to DED. *Environmental and Experimental Botany, 69*, 175–182.

Martín, J. A., Witzell, J., Blumenstein, K., Rozpedowska, E., Helander, M., Sieber, T. N., & Gil, L. (2013). Resistance to Dutch elm disease reduces presence of xylem endophytic fungi in elms (*Ulmus* spp.). *Plos One, 8*(2), e56987.

Mejía, L. C., Rojas, E. I., Maynard, Z., Van Bael, S., Arnold, A. E., Hebbar, P., Samuels, G. J., Robbins, N., & Herre, E. A. (2008). Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. *Biological Control, 46*, 4–14.

Newcombe, G. (2011). Endophytes in forest management: Four challenges. In A. M. Pirttilä & A. C. Frank (Eds.), *Endophytes of forest trees: Biology and application . Forestry sciences 80* (pp. 251–262). Berlin / Heidelberg / New York: Springer.

Ouellette, G. B., Rioux, D., Simard, M., & Cherif, M. (2004). Ultrastructural and cytochemical studies of host and pathogens in some fungal wilt diseases: retro- and introspection towards a better understanding of DED. *Forest Systems, 13*, 119–145.

Promputtha, I., Lumyong, S., Dhanasekaran, V., Huge, E., McKenzie, C., Hyde, K. D., & Jeewon, R. (2007). A phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. *Microbial Ecology, 53*, 579–590.

Rodríguez, J., Elissetche, J. P., & Valenzuela, S. (2011). Tree endophytes and wood biodegradation. In A. M. Pirttilä & A. C. Frank (Eds.), *Endophytes of forest trees: Biology and applications. Forestry sciences 80* (pp. 81–93). Berlin / Heidelberg / New York: Springer.

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

Scheffer, R. J., Elgersma, D. M., De Weger, L. A., & Strobel, G. A. (1989). Pseudomonas for biological control of Dutch elm disease. I. labelling, detection and identification of Pseudomonas isolates injected into elms: comparison of various methods. *Netherlands Journal of Plant Pathology, 95*, 281–92.

Scheffer, R. J., Voeten, J. G. W. F., & Guries, R. P. (2008). Biological control of Dutch elm disease. *Plant Disease, 92*, 192–200.

Shi, J. L., & Brasier, C. M. (1986). Experiments on the control of Dutch elm disease by injection of *Pseudomonas* species. *European Journal of Forest Pathology, 16*, 280–92.

Solla, A., & Gil, L. (2002). Influence of water stress on Dutch elm disease symptoms in *Ulmus minor*. *Canadian Journal of Botany, 80*, 810–817.

Solla, A., & Gil, L. (2003). Evaluating *Verticillium dahliae* for biological control of *Ophiostoma novo-ulmi* in *Ulmus minor*. *Plant Pathology, 52*, 579–585.

Solla, A., Martín, J. A., Ouellette, G., & Gil, L. (2005). Influence of plant age on symptom development in *Ulmus minor* following inoculation by *Ophiostoma novo-ulmi*. *Plant Disease, 89*, 1035–1040.

Solla, A., Dacasa, M. C., Nasmith, C., Hubbes, M., & Gil, L. (2008). Analysis of Spanish populations of *Ophiostoma ulmi* and *O. novo-ulmi* using phenotypic characteristics and RAPD markers. *Plant Pathology, 57*, 33–44.

Sumarah, M. W., Adams, G. W., Berghout, J., Slack, G. J., Wilson, A. M., & Miller, J. D. (2008). Spread and persistence of a rugulosinproducing endophyte in *Picea glauca* seedlings. *Mycological Research, 112*, 731–736.

Sutherland, M. L., Pearson, S., & Brasier, C. M. (1997). The influence of temperature and light on defoliation levels of elm by Dutch elm disease. *Phytopathology, 87*, 576–581.

Tchernoff, V. (1965). Methods for screening and for the rapid selection of elms for resistance to Dutch elm disease. *Acta Botanica Neerlandica, 14*, 409–452.

Vivas, M., Martín, J. A., Gil, L., & Solla, A. (2012). Evaluating methyl jasmonate for induction of resistance to *Fusarium oxysporum*, *F. circinatum* and *Ophiostoma novo-ulmi*. *Forest Systems, 21*, 289–299.

Webber, J. F., & Hedger, J. N. (1986). Comparison of interactions between *Ceratocystis ulmi* and elm bark saprobes in vitro and in vivo. *Transactions of the British Mycological Society, 86*, 93–101.

White, T. J., Bruns, T., Lee, S., & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. L. White (Eds.), *PCR protocols: A guide to methods and applications* (pp. 315–322). New York: Academic.

Yang, D., Plante, F., Bernier, L., Piché, Y., Dessureault, M., Laflamme, G., & Ouellette, G. B. (1993). Evaluation of a fungal antagonist, *Phaeotheca dimorphospora*, for biological control of tree diseases. *Canadian Journal of Botany, 71*, 426–433.

III

frontiers in Microbiology

METHODS
published: 28 September 2015
doi: 10.3389/fmicb.2015.01033

Phenotype MicroArrays as a complementary tool to next generation sequencing for characterization of tree endophytes

*Kathrin Blumenstein[1†], David Macaya-Sanz[2†], Juan A. Martín[2], Benedicte R. Albrectsen[3,4] and Johanna Witzell[1,5]**

[1] Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, Alnarp, Sweden, [2] Department of Natural Systems and Resources, School of Forest Engineers, Technical University of Madrid, Madrid, Spain, [3] Department of Plant Physiology, Umeå Plant Science Centre, Umeå University, Umeå, Sweden, [4] Department of Plant and Environmental Sciences, University of Copenhagen, Copenhagen, Denmark, [5] School of Forest Sciences, Faculty of Science and Forestry, University of Eastern Finland, Joensuu, Finland

OPEN ACCESS

Edited by:
Mysore V. Tejesvi, University of Oulu, Finland

Reviewed by:
Vijai Kumar Gupta, NUI Galway, Ireland
Liliana Maria Cano, North Carolina State University, USA

***Correspondence:**
Johanna Witzell, Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, Box 49, 23053 Alnarp, Sweden
johanna.witzell@slu.se

†These authors are joint first authors.

Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Microbiology

Received: *17 May 2015*
Accepted: *11 September 2015*
Published: *28 September 2015*

Citation:
Blumenstein K, Macaya-Sanz D, Martín JA, Albrectsen BR and Witzell J (2015) Phenotype MicroArrays as a complementary tool to next generation sequencing for characterization of tree endophytes. Front. Microbiol. 6:1033. doi: 10.3389/fmicb.2015.01033

There is an increasing need to calibrate microbial community profiles obtained through next generation sequencing (NGS) with relevant taxonomic identities of the microbes, and to further associate these identities with phenotypic attributes. Phenotype MicroArray (PM) techniques provide a semi-high throughput assay for characterization and monitoring the microbial cellular phenotypes. Here, we present detailed descriptions of two different PM protocols used in our recent studies on fungal endophytes of forest trees, and highlight the benefits and limitations of this technique. We found that the PM approach enables effective screening of substrate utilization by endophytes. However, the technical limitations are multifaceted and the interpretation of the PM data challenging. For the best result, we recommend that the growth conditions for the fungi are carefully standardized. In addition, rigorous replication and control strategies should be employed whether using pre-configured, commercial microwell-plates or in-house designed PM plates for targeted substrate analyses. With these precautions, the PM technique is a valuable tool to characterize the metabolic capabilities of individual endophyte isolates, or successional endophyte communities identified by NGS, allowing a functional interpretation of the taxonomic data. Thus, PM approaches can provide valuable complementary information for NGS studies of fungal endophytes in forest trees.

Keywords: fungal phenotype, nutrient utilization, fungus–fungus interactions, phenolic compounds, Biolog PM

Introduction

The increasing interest in endophyte communities of plants, including those of forest trees, is fueled by the apparent potential of the endophytes to shape and modulate the stress tolerance in host plants, directly by priming or elevating defence responses in the plant (Rodriguez and Redman, 2008; Eyles et al., 2010; Albrectsen and Witzell, 2012; Witzell et al., 2014) or indirectly through competition for substrates (Blumenstein et al., 2015). Moreover, endophytes are explored as a potent source of new solutions, based on metabolites and enzymes, for industrial, pharmaceutical,

or agricultural purposes (Rodrigues et al., 2000; Schulz et al., 2002; Rančić et al., 2006; Gaur et al., 2010). Investigations of endophytes have traditionally been constrained by the difficulty of deciphering the global endophyte communities that are hidden inside the plants and capturing the target species to cultures for functional studies at organismal level. Recent methodological advances may solve some of these problems. For instance, improvements in standard isolation and culturing processes are feasible (Kaewkla and Franco, 2013), and the accumulating information from next generation sequencing (NGS) studies is likely to support discovery of new endophytic species (Toju et al., 2013), and provide information about their characters (Lim et al., 2010). However, while the genomic NGS analyses now provide powerful tools for global high throughput analyses of endophyte communities (Unterseher et al., 2012), they are still limited by the need of conducting a destructive extraction procedure that creates a snapshot of the point-in-time status in the samples (Greetham, 2014). There is also an increasing need to calibrate the NGS-community profiles with relevant taxonomic identities of the microbes, and to further associate these identities with the corresponding functional traits.

The functional traits of organisms are expressed as phenotypic attributes, jointly defined by the genome and the environment (cf. Houle et al., 2010). In fact, it is the phenotypic characters that make up the desired outcome of any selection process and also the key to understanding the biological complexity (Houle et al., 2010; Cabrera-Bosquet et al., 2012). Adequate and reliable phenotyping is thus a crucial task, if we want to utilize the functional traits of endophytes for practical applications. In general, modern strategies for high throughput phenotyping of organisms (phenomics) include application of computer vision, imaging of cell traits using visible light, NIR and fluorescent imaging technology and reporter gene expression (Houle et al., 2010), but effective application of these methods in studies of the functional traits of endophytes is not uncomplicated. The taxonomic and morphological diversity and the physiological versatility of fungal endophytes further complicate the acquirement of biologically relevant information about their phenotypes. For instance, a major challenge in studies of fungal phenotypes is the indeterminate growth of the fungi, with mycelium that forms colonies and is composed of hyphae as the basic units (Davidson, 1998; Falconer et al., 2005). Moreover, the analysis is complicated by the fact that the fungal hyphae may behave collectively rather than as isolated modules, reacting to the conditions across the whole colony (Falconer et al., 2005). In the case of endophytic fungi, these challenges are magnified further, because the endophytes thrive inside the tissues and are often reluctant to be cultured on artificial media. Consequently, any phenotyping of fungal isolates is usually done using low throughput morphological or physiological measurements that despite their great value are often cumbersome.

An emerging method that seems to have a realistic potential to provide at high throughput information about the phenotypes of microbial isolates is the Phenotype MicroArray (PM) technique. This technique relies on microtiter-plate-based substrate utilization assays (Bochner and Savageau, 1977; Bochner, 1989; Bochner et al., 2001). PM technique provides (semi-)high throughput assays for characterization and monitoring the cellular phenotypes of pure cultures or communities in an environmental sample (Borglin et al., 2012). The cellular responses, i.e., respiration or growth, can be monitored over a period of time, which makes it possible to capture some of the metabolic dynamics of the target cells (Bochner et al., 2001). The method thus allows construction of specific metabolic fingerprints that can be used for identification of microbes with desired traits, e.g., for industrial applications (Greetham, 2014). So far, however, the method has mainly been used in studies of bacteria. For example, Dong et al. (2010) applied PM for identification of bacterial strains with capacity for converting a novel precursor into an anti-cholesterol drug. Recently, however, the method has gained popularity also in studies of fungi, including endophytes (Atanasova and Druzhinina, 2010; Blumenstein et al., 2015). For example, PM technology has been used to optimize growth media for production of secondary metabolites by filamentous fungi (Singh, 2009).

The aim of this method paper is to illustrate how PM methods can be applied in studies on the ecology and utilization potential of forest tree endophytes, and validate the performance and reliability of these methods. In particular, we report experiences from two procedures, one where we used pre-configured, commercially available PM arrays to evaluate nutritional niches of endophytes and pathogens sharing the same host plant (Procedure I; part of the results modified from Blumenstein et al., 2015), and another where we used an in-house configured PM array to test the sensitivity of endophytes to a set of carbon sources and inhibitory substrates (phenolic compounds), which were interesting for our research questions but not available among the preconfigured, commercially available arrays (Procedure II). Finally, we discuss the benefits and limitations of the PM approach in studies on the ecological role of tree endophytes and their utilization in practical applications.

Materials and Methods

Procedure I: Utilization of Pre-configured Biolog PM Plates for Comparison of Nutritional Niches of Endophytes and Pathogens

Selection of the Fungi for the Studies

We employed PM technique in studies where the aim was to experimentally explore the competitive interactions between pathogens and endophytes that co-exist in time and space in trees. For this purpose, the carbon and nitrogen substrate utilization patterns of two pathogens (causal agents of Dutch elm disease, *Ophiostoma novo-ulmi* and *O. ulmi*) and four endophyte species (see below) were studied. Three isolates per each of the *Ophiostoma*-species were purchased from CBS-KNAW Fungal Biodiversity Center, Netherlands, or originated from the mycology library of Spanish elm breeding program (Solla et al., 2008). Three of the studied endophytic fungi were isolated earlier from elm trees (*Ulmus* sp.) (Martín et al., 2013). Two of them, *Monographella nivalis* var. *neglecta* (three

isolates) and *Pyrenochaeta cava* (two isolates) have earlier shown chemical antagonism against the pathogenic *O. novo-ulmi*. They both inhabit elm bark and xylem where interspecific competition for the niche might occur (Martín et al., 2015). The third fungus, *Aureobasidium pullulans* (three isolates), was included in the tests as an example of a ubiquitous, "generalist" fungus, with potentially broad nutritional niche. In nitrogen utilization tests, also a common biocontrol fungus, *Trichoderma harzianum* (MB#340299, purchased from CBS - KNAW Fungal Biodiversity Center, Netherlands) was included for comparison. All fungi were cultivated on malt extract agar (MEA) and 26°C following the recommended protocol (Biolog Inc.). Occasional light exposure was not excluded during the experiment period.

Preparation of Inoculum

For preparation of homogenous inoculum, we developed the following protocol that is carried out under sterile conditions. In order to obtain pure fungal mass and to avoid any contamination of agar in the inoculation fluid (IF), fungi were cultivated on semi-permeable cellophane membrane on MEA. After incubation at 26°C (10–15 days depending on species' individual growth rate), the fungal mass was lifted from the cellophane membrane with a cotton swab. Material from 2 to 5 agar plates per isolate was found to contain sufficient fungal material for the tests. The material was transferred into 2 mL Eppendorf vials and manually homogenized with a pestle together with 500 μL of Biolog FF-IF. When a thick suspension was obtained, it was poured over cotton wool on a metal sieve placed over a beaker. By adding 1–3 mL FF-IF, the material was flushed through the cotton. The longer fungal hyphae and bigger cell aggregates were collected into the cotton wool, and a dense, homogenous solution containing fungal spores and small aggregates of mycelial cells was collected underneath the sieve. The viability of the cells in the suspension immediately after the collection procedure was tested by spreading 200 μL aliquots of the cell stock suspension on MEA plates. The viability of the cells in the suspension immediately after the cell collection procedure was tested by spreading 200 μL aliquots of the suspension on MEA plates. After 3–4 days incubation, outgrowing mycelium was visually checked for development and purity.

Using a turbidimeter, the optical density of the inoculum was adjusted to 62% by adding small amounts from the cell suspension. Depending on the species, 400 μL^{-1} mL of cell stock suspension per 17 mL FF-IF was used. Then, following the protocol from the manufacturer, solutions of glucose, sodium sulfate, and potassium phosphate were added. The final inoculum was transferred into a sterile reservoir for multichannel pipettes, and 100 μL of suspension was pipetted into each PM array well. The suspension was added to plates on the same day than it was prepared.

In order to count the colony forming units (CFU) of the inoculum, an aliquot of 100 μL was pipetted into Petri dishes containing MEA, gently tilting the dish with sterile glass beads to evenly distribute the fluid on the agar surface. After 3–4 days incubation, the CFU was determined.

Pre-configured Biolog Phenotype MicroArrays

The commercially available, pre-configured Biolog Phenotype MicroArrays (Biolog Inc., Harvard, CA, USA) are composed of microtiter plates with one negative control well and 95 wells pre-filled with a nutrient source (e.g., C,N,P,S, amino acids) or substrates leading to inhibitory conditions (pH, NaCl, antibiotics) in a dried state. The substrate rehydrates after the target cell suspension, mixed with an IF at a standardized cell density, is inserted in each well. The IF provided by Biolog contains nutrients or chemicals (e.g., C, N, P, S, K, Na, Mg, Ca, Fe, amino acids, purines, pyrimidines, and vitamins) at sufficient levels to maintain cell viability. Through this combination (a nutrient source or an inhibitory compound and IF), unique culture conditions are created for the inoculated cells (Bochner, 2009).

The phenotypic response, i.e., how the cells respond to the conditions, is monitored by the change of color or turbidity in each well. The IF contains a tetrazolium salt, which is reduced by the action of dehydrogenases and reductases of the prokaryote and yeast cells, yielding a purple formazan dye. This color reaction is irreversible, and thus the more intensive the stronger the organism is able to catabolize the provided substrate in the well. In other words, a color reaction indicates that the inoculated cells are actively metabolizing a substrate in the well, while the lack of color change implies that the cells are not able to utilize the substrate. The rate and extent of color formation in each well can be monitored at 490 nm and recorded by the OMNILOG instrument (Bochner, 2003), a specialized instrumentation provided by Biolog. Kinetic response curves can be generated for each well and used for cellular phenotype comparisons. Alternatively, color change can be recorded spectrophotometrically (Atanasova and Druzhinina, 2010), or by visual observations (Bochner et al., 2001). While the color reaction is most convenient for bacteria, the growth response of filamentous fungi can be recorded as change in the optical density at 750 nm (OD750) (Tanzer et al., 2003; Druzhinina et al., 2006; Seidl et al., 2006). Measurements of growth can also be conducted at 590 nm (Blumenstein et al., 2015), which yield results that are comparable to 750 nm.

In our Procedure I, we examined the nutritional niche of endophytes and pathogens with the Biolog plates: PM1 and 2A, that represent 190 carbon sources (95 on each plate) (Blumenstein et al., 2015) and PM3B with 95 nitrogen sources. Three (carbon-source studies, Blumenstein et al., 2015) or two (nitrogen-source studies) replicate plates were used.

Measurements and Data-Analysis

Data for fungal activity was obtained through measurements of OD at 590 nm (PM1 and PM 2A, Blumenstein et al., 2015) or 750 nm (PM3B) using a spectrophotometer with microplate format compatibility (SPECTROstar Nano BMG Labtech) every 24 h for 10 days. The first reading (T = 0) was done at approximately 30 min after the plates were inoculated with the fungal IF. In the PM3A (nitrogen test), the content of six randomly chosen wells of all 30 plates was placed on MEA plates after 360 h when the final reading was done in order to control possible contaminations and vitality of cells. The purity of the

developing mycelium was observed during the seven following days.

Differences in OD for specific substrates were tested using standard ANOVA analyses. Global differences in substrate use were compared by implementing multivariate statistics or by calculation of a niche overlap index (NOI) which compares the number of substrates used and the intensity by which they are used between two strains of fungi (Blumenstein et al., 2015). The competitiveness of the focal fungus against another fungus, or the effectiveness of a potential biocide chemical, may thus be evaluated (Blumenstein et al., 2015).

Examples of the Application Potential of Pre-configured PMs: Nutritional Niche Studies with Elm (Ulmus sp.) Endophytes and Pathogens

Pre-configured PM plates were used to examine whether the carbon-substrate utilization profiles of elm endophytes differ from those of the Dutch elm disease pathogen (Blumenstein et al., 2015). The basic hypothesis to be tested was that endophytes with good potential as biocontrol agents should be able to effectively compete with the pathogen for carbon, but that a successful pathogen might also be superior competitor for nutrients against endophytes. Here, we discuss part of the results from the earlier study by Blumenstein et al. (2015). In ongoing studies, we are applying preconfigures PMs to study the same aspects in competition for nitrogen substances and present here some of the findings from these studies.

Procedure II: In-house Configured PM Array to Test the Sensitivity of Endophytes to a Set of Inhibitory Substrates

Experimental Aim and Selection of the Fungi for the Studies

In order to explore the role of fungal endophytes in the early stages of wood degradation, we employed a combination of NGS and PM approaches. Endophytic fungi were isolated from the wood (including phloem and xylem, but excluding external bark) of *Eucalyptus globulus* and *E. camadulensis* twigs, 1–2 cm in diameter, collected in 2012 and 2013 from different provinces across Spain (five sites in five provinces, three trees per site). The collection was done in spring and the twigs were transported to the laboratory and stored at 4°C. On the same day or the day after, the twigs were surface sterilized with subsequent immersions (30 s in 70% ethanol, 5 min in 4% bleach and 15 s in 70% ethanol), followed by 15 min drying at room temperature. Then, the twigs were peeled with a sterilized scalpel to remove the external bark and 1–2 mm thick slices were excised and placed on 90 mm Petri dishes (four explants per dish). We used five different culture media: MEA, potato dextrose agar, yeast extract agar, rose Bengal chloramphenicol agar, and eucalypt sapwood agar (10% w/v eucalypt sapwood, and 3% w/v agar). During the following two weeks, emerging colonies were transferred into new MEA dishes for preparation of inoculum (see below). By DNA sequencing of the ITS region and searching for matches in the GenBank database (NCBI, Bethesda MD, USA) through BLAST algorithm (Martín et al., 2015) we identified the most probable family of each strain. Fifteen eucalypt endophyte isolates were used for test this procedure, belonging fourteen to the phylum Ascomycota and the remaining one to the phylum Basidiomycota. The ascomycetes were two sordariomycetes (orders Hypocreales and Microascales), one incertae sedis and the rest dothideomycetes. These last belonged to the families Dothioraceae (four strains), Pleosporaceae (three strains), Phaeosphaeriaceae (order Pleosporales), Lophiostomataceae, Botryosphaeriaceae, and Davidiellacea. Additionally, we included in our study *Trichoderma* sp., *Pycnoporus sanguineus* and *Trametes* sp. isolates, commonly used as model fungi, from our mycology library. The species *P. sanguineus* and *Trametes* sp. are basidiomycetes of the order Polyporales.

Preparation of Inoculum

The selected isolates were cultured in Petri dishes on an autoclaved cellophane sheet over MEA medium (darkness, 25°C). After a week, the fungal biomass was harvested by rubbing with a sterilized scalpel and transferring the fungal tissue into a sterile centrifuge tube (15 mL) with a known volume of sterile distilled water. Centrifuge tubes were weighed before and after introduction of the tissue to calculate the weight of the added biomass. Then, the content of the centrifuge tube was homogenized using a sterilized tissue grinder, first with a large clearance pestle and then with a small clearance one (∼20 strokes with each). The homogenate was inserted back into the centrifuge tube and stored at 4°C until use. The concentration of fungal tissue in the suspension was calculated and the suspension was diluted to 1 g/L before pipetting into the PM plates.

In-house Configured PMs

Optical 96-well, round-bottom, sterile polystyrene plates (Deltalab, Barcelona, Spain) were used in the modified PM tests. Each well was first filled with 60 μL of liquid basal culture media (35 μL for inhibition tests; see below), composed by autoclaved Murashige and Skoog (MS) salts (1x; ref. n. 0926230; MP Biochemicals; Santa Ana, CA, USA), Biolog Redox Dye E (2x; ref. n. 74225; Biolog Inc., Hayward, CA, USA), and filtered 1-metoxy-5-methylphenazine methosulfate (1.5 mg/l; ref n. A3799; Applichem, Darmstadt, Germany). We prepared the plates by pouring into each well 50 μL of MS salt solution (2x; i.e., two times as concentrated as the standard recipe; 25 μL of MS salt solution (4x) and glucose 1 M of C atoms for inhibition tests; see below), 10 μL of dye mix, which contained Biolog Redox Dye E (20x; provided by the manufacturer at 100x; this reagent's final concentration was 2x) and of 1-metoxy-5-methylphenazine methosulfate (15 mg/L). MS salts are normally used to plant tissue *in vitro* culture, thus we expected they would also be appropriated for endophyte fungi. Biolog Redox Dye E is recommended by the manufacturer for assays with fungi. It changes its color from transparent to violet when reduced, in a similar way than the classical tetrazolium dye. The mediator 1-methoxy-5-methylphenazine methosulfate enhances the change of color.

After adding these components, each well was supplemented with selected substances (see the details below). Combinations of two groups of substances were tested: carbon sources and inhibitors (phenolic compounds). For testing the effect of seven

different carbon sources (cellobiose, galactose, glucose, sucrose, xylose, pectin, and starch) on the growth of the fungi, we supplemented the media with 20 μL of carbon source solution, to reach a final concentration 0.25 M of C atoms in each well. For testing the possible inhibitory effect on the fungi by 10 phenolic compounds that have been associated to tree resistance as metabolites or external treatments (Witzell and Martín, 2008; Martín et al., 2010): chlorogenic, tannic, and gallic acids; the simple phenolics o-cresol, carvacrol, thymol, and phenyl alcohol, and the flavonoids catechins, myricetin, and quercetin). We supplemented the media with 50 μL of inhibitor solutions (2x). Water-insoluble compounds could dissolved in 10% ethanol (v/v; for stock solution: 2x), 25% methanol (v/v; for the stock solution: 5x), or an alkaline solution (0.01 M NaOH for the stock solution: 5x). To neutralize the alkalinity of the latter media, 10 μL of 0.02 M HCl was added into the wells before inoculation. To test how these solvents affect fungal metabolism, solutions of glucose and sucrose with all these three solvents were prepared to control their possible effect (see Effect of additive solvents).

After addition of the test substances, water was added to fill the volume in each cell to 90 μL. Finally, from a suspension of 1 g/L of homogenized fungal biomass (see above), 10 μL was added into each well, making up a final concentration of 0.1 mg of fungal biomass per mL.

The thermotolerant solutions (carbon sources, tannic, salicylic, and gallic acids) were sterilized by autoclaving and the thermolabile or volatile substances and the substances dissolved in alcoholic solution (chlorogenic acid, flavonoids, and simple phenols) were filtered through disposable, sterile cellulose acetate syringe filters of 0.2 μm pore size. Water was always deionized and autoclaved prior to use. All the operations were done under axenic conditions in a laminar flow chamber.

The PM plates (a total of 30) were composed following four general principles. First, each combination of carbon source or secondary metabolite with a fungal strain was replicated in three separate wells. Second, with few justified exceptions (see Unintentional Chemical Interactions), all wells of a single plate had the same concentration of inhibitory substances, ethanol, or methanol, whenever present. Third, all the treatments included one negative control with the relevant conditions, but fungal inoculum substituted by water, and another one containing inoculum, but the carbon source/secondary metabolite substituted by water. The first was used to calculate the net absorbance (see below), while the second was used as a reference to compare between different endophytic strains. Fourth, all plates were cultivated in the dark at 25°C.

Measurements and Data-analysis

The following aspects that have relevance for the applicability of PMs in our studies on tree endophytes were evaluated from the in-house configurated PMs.

Stability/repeatability

To evaluate the stability and repeatability of the designed configuration, we repeated assays with carbon sources (cellobiose, galactose, glucose, sucrose, xylose, pectin, and starch) with a six months interval, using the freshly prepared inocula of five eucalypt endophyte strains and a model fungus (*Trichoderma sp.*).

Unintentional chemical interactions on PM plates – volatility and unexpected color changes in the medium

Our preliminary tests indicated that certain volatile metabolites might affect cells in neighboring wells in a plate where no such substance had been added. This unwanted effect was evaluated in a plate as described above, where the first three columns were supplemented with 1 g/L (final concentration) of the simple phenol *o*-cresol, while the rest of columns were not. All wells possessed glucose 0.25 M of carbon atoms. In each row one different fungal strain was inoculated, except the last one that was a negative control.

Preliminary tests also indicated that in some inhibitory compound tests the culture media unexpectedly changed color to orange (note that dye should change to violet) when in contact with certain inhibitory substances and certain strains. To explore this phenomenon, we performed a test to infer if this change of color could be because the strains used certain phenolic chemicals as carbon sources. We tested thirteen endophyte strains (selected from the Spanish tree endophyte collection) and the two Polyporales model fungi in media with chlorogenic acid, gallic acid, and tannic acid (1 g/L final concentration, solved in water), salicylic acid (0.02 g/L, in water) and catechins (1 g/L final concentration, solved in ethanol) with glucose. Absorbance was later measured at $\lambda = 405$ nm and $\lambda = 630$ nm.

Effect of additive solvents

Because some of the phenolic compounds had to be dissolved in solvents (see above), we wanted to test if these affected fungal activity. Thus, we tested the same set of strains as in the preceding assay, in four solvents (ethanol, methanol, NaOH+HCl as described above, and water) with added sugar in the form of either glucose or sucrose (0.25 M of carbon atoms). To the basal culture media (MS salts+dye mix) we supplemented with glucose and 5% ethanol or water or with sucrose (0.25 M of carbon atoms) and methanol 5%, NaOH+HCl 0.002 M (i.e., saline solution) or water. Growth in the media with ethanol, methanol, and saline solution were compared to the growth in water.

Data analysis

We defined and calculated the following parameters from the absorbance reads: (i) gross absorbance (A_λ): the mean of the three technical replicates (same conditions in three different wells) for each substance tested, measured at a given λ; (ii) net absorbance ($A'_\lambda = A_\lambda - A_{\lambda\,\mathrm{neg}}$): the difference between the gross absorbance and its negative control (water instead of fungal inoculum, $A_{\lambda\,\mathrm{neg}}$); (iii) cumulated growth ($A^{\mathrm{d}}{}_\lambda = A'_\lambda{}^{\mathrm{d}} - A'_\lambda{}^{0}$): net absorbance at day d ($A'_\lambda{}^{\mathrm{d}}$) minus net absorbance at day 0 ($A'_\lambda{}^{0}$); and (iv) relative growth (ρ_λ): the ratio between the cumulated growth in two different substances ($A^{\mathrm{d}}{}_{\lambda\ \mathrm{subst1}} : A^{\mathrm{d}}{}_{\lambda\ \mathrm{subst2}}$), usually one of them taken as reference (glucose or sucrose). Comparisons between different strains were done in terms of their relative growth, and therefore the negative control was not considered in comparisons. Standard analyses of Pearson correlation, one-factor ANOVA and Principal Component

Analysis were carried out in STATISTICA V8.0 (StatSoft Inc., Tulsa, OK, USA).

Examples of the Application Potential of In-house Configured PMs: Chemical Sensitivity of Eucalypt (*Eucalyptus* sp.) Endophytes

Phenolic compounds have been identified as potential plant internal defenses and as external inducers of plant defenses (Witzell and Martín, 2008; Martín et al., 2010). However, little is still known about the possible responses of endophytes to these chemicals. Thus, the in-house configuration of PMs with phenolic compounds was designed to evaluate the role of these compounds for individual endophyte species, with the underlying hypothesis the compounds would show inhibitory effects on fungi, but that the effect would show strain- and compound-specificity. Specifically, we performed an inhibition test of four phenolic compounds (phenol, *o*-cresol, thymol, and carvacrol) and two flavonoids (quercetin and myricetin) on the same 15 strains tested above plus a negative control without inoculum. Final concentrations were 0.1 g/L for the phenols and 0.01 g/L for the flavonoids. Flavonoids and carvacrol were dissolved in ethanol, whereas the other phenolics were dissolved in water. We incorporated the results on this assay to the ones on the test we did to research on undesired changes of color (inhibitory substances: chlorogenic, salicylic, gallic and tannic acids, and catechins). We only took into account measures at $\lambda = 630$ nm to minimize interferences of undesired color changes.

Optical densities were measured in a microplate absorbance reader ELx808 (BIOTEK, Winooski, VT, USA). We measured at $\lambda = 405$, 490, and 630 nm, and at 0, 1, 2, 3, 4, 5, 7, 9, and 11 days after inoculation (dai). Wavelengths were selected in order to detect if the absorbance shifts were due to an increase in the turbidity, a change of color due to Biolog Redox Dye, or a change of color by other causes. Single measurement was considered sufficient, because variation between repeated, consecutive measurements were found to be negligible in preliminary tests (Macaya-Sanz, personal observation) Absorbance measurements were stored using the software KCjunior provided by the plate reader's manufacturer. The plates were also photodocumented at 0, 5, and 11 dai.

The absorbance values at all the wavelengths, but especially at shorter ones, were due to increase of turbidity of the medium and the cumulative quantity of redox reactions (reflected in the change of color of the Redox Dye), i.e., two interrelated processes, and were thus considered a proxy of the catabolic activity and the vegetative growth of the fungi.

Results

Standardization of Inoculum (Procedure I and II)

Adequate quality inoculum for PM tests was achieved from the studied endophytes through both procedures. In Procedure I, the inoculum concentration was determined by transmittance, whereas in Procedure II, the inoculum was standardized by biomass. Standard culture conditions were used for studied fungi in both Procedures (I and II), resulting in adequate amount of viable fungal biomass.

In Procedure II studies, we found that fungal inocula lost vitality after a month storage at 4°C, showing clearly reduced growth rate (Macaya-Sanz, personal observation). All the inocula were, however, alive after the storage period.

In Procedure I, the test for the CFU in the inoculum gave varying results for the different species. For instance, CFU for *A. pullulans* was about 400 CFU per 100 μL (**Figure 1A**), whereas for *O. ulmi* the number of growing colonies was too dense to be counted (**Figure 1B**). Bacterial or fungal contaminations were not detected among the growth recovered from the randomly chosen wells.

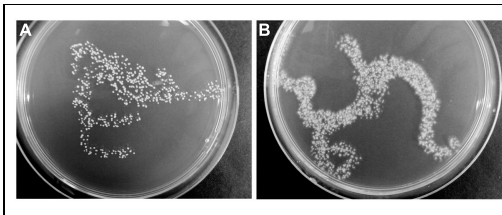


FIGURE 1 | Examples of fungal inoculum (100 μL, i.e., the volume injected into one well) applied to an agar plate for testing the development of CFU. *Aureobasidium pullulans* **(A)** and *Ophiostoma ulmi* **(B)**.

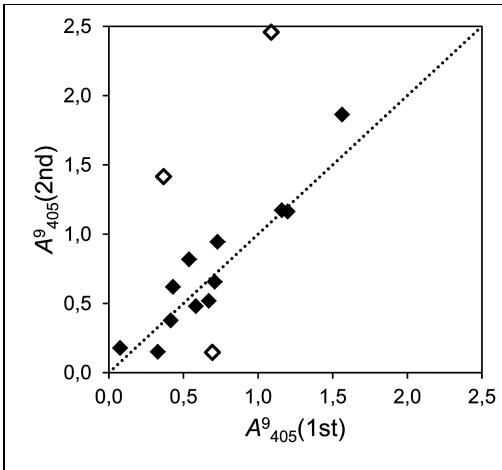


FIGURE 2 | Correlation plot of two independent assays of cumulative growth with glucose as the only carbon source (time point 9 days after inoculation (dai), $\lambda = 405$ nm) with 15 fungal strains, of which 13 were isolated as endophytes ($r^2 = 0.485$; $P = 0.004$). The dotted line represents the bisector of slope $b = 1$. Dots close to this line produced even results in both assays (filled dots). Empty dots indicate deviating strains.

Technical Challenges with In-house Configurated PMs (Procedure II)

The repeated assays showed that the precision of the in-house configurated PMs was moderate. In the experiment which tested 15 strains in glucose (**Figure 2**), the correlation was moderate ($r^2 = 0.485$) but significant ($P = 0.004$), and the slope of the regression line was close to the unity ($b = 1.17$). Nevertheless, ignoring the results of three strongly deviating strains (marked with blank dots in **Figure 2**), the correlation grew to $r^2 = 0.889$ and the regression slope shifted slightly toward one ($b = 1.10$).

The repeated assays where six strains were tested on seven carbohydrates, the precision (measured as correlation of cumulated growth) was extremely high ($r^2 > 0.9$) in some of them, whereas almost negligible in others. Intriguingly, carbon sources where the standard deviation of the absorbance was low (i.e., the different response to the carbon source of the tested strains), displayed a reduced correlation. The carbon sources with high correlation also presented high standard deviation and values of linear regression slope close to the unity (**Table 1**). However, a couple of carbon sources did not follow this pattern (especially, xylose).

Our tests with *o*-cresol indicate that there is a risk that the volatile compounds cause unintentional effects in the neighboring wells: we found that the fungal growth was severely reduced in the adjacent wells and visibly limited in the next columns (**Figure 3**).

Our tests confirmed that unexpected color change (to orange) occurred only in certain combinations of strain and inhibitory substances. The combination of certain strains with the four tested secondary metabolites (salicylic acid, tannic acid, chlorogenic acid, gallic acid, and catechins) resulted in change of color to yellow–orange in last three of them. Occasional change of color was also found in tannic acid assays. This change of color was measurable as a shift in the ratio between absorbance at wavelength $\lambda = 405$ nm and at $\lambda = 630$ nm. In the cases were a change of color occurred, the absorbance at $\lambda = 405$ nm increased abnormally, and the ratio $\lambda = 405$ to $\lambda = 630$ was not conserved (**Figure 4**). Such color change did not occur when other strains were combined with these metabolites or when the strains were growing without these substrates.

Tests with different solvents showed that some of them have a strong effect on the activity of the strains. Alkaline solution, which was neutralized with an acid, did not affect the activity of the strains (**Figure 5A**), while 5% methanol in water (v/v) induced a general decrease in the growth of all the endophytic strains (**Figure 5B**). Ethanol (5% in water, v/v) had an inconsistent effect on strains, decreasing the growth in some of them, but promoting it in others (**Figure 5C**).

TABLE 1 | Correlations between the growth of six fungal strains on seven different carbon sources at 9 days after inoculation (dai; $\lambda = 405$ nm) in two independent assays with identical conditions.

Carbon source	$A^9_{405}(1)$	$A^9_{405}(2)$	σ	r^2	P	b
Cellobiose	1,258	0,830	0.433	0.179	0.403	0.5555
Galactose	0,747	0,849	0.165	0.002	0.940	–0.0361
Glucose	0,916	0,859	0.275	0.039	0.708	0.1372
Sucrose	0,646	0,658	0.381	0.986	0.0001	1.1845
Xylose	1,073	0,723	0.385	0.009	0.857	0.0681
Pectin	0,219	0,474	0.359	0.910	0.012	1.0413
Starch	0,860	0,341	0.441	0.977	0.001	0.6013

The second and third columns display the mean of the cumulated growth of the six strains for the first and second assays, respectively. The fourth column shows the standard deviation (σ) of the pooled measurements from both assays. The fifth and sixth columns display the determination coefficient between two assays (r^2) and its level of significance (P). The last column presents the slope of the regression line (b). Note that carbon sources with lower standard deviation have low correlations between assays. However, not all the carbon sources with higher standard deviation present high correlations.

Application of PM to Forest Tree Endophyte Studies

Procedure I – Comparison of Nutrient Utilization Patterns

With the goal of studying the potential of endophytes in biocontrol, we used PM data to compare the nutritional preferences of a pathogen and endophytes that co-colonize the same host (**Figure 6**, data modified from Blumenstein et al., 2015). The comparison showed that all tested fungi were able to use all of the four tri-and tetrasaccharides tested (**Figure 6**). On the other hand, the endophyte *M. nivalis* was able to use a broader array of available amino acids (96%) and other acids (69%) as compared to the pathogen (56 and 49%, correspondingly) and *A. pullulans* had generally low preference for acids, utilizing 15% of the available amino acids and less than 1% of the other acids (**Figure 6**). Moreover, the endophytes were able to use all available phenolics (5 out of 5) while the pathogen could only use 60% of them (3 out of 5).

Phenotype MicroArray technique also allowed us to observe the effect of substrates on the morphology of the tested fungi. In particular, nitrogen sources seemed to induce varying morphological responses. For instance, only little fungal mass was produced when *T. harzianum* grew on cytidine or cytosine (**Figure 7**). Tyramine and formamide triggered production of in green fungal mass, whereas acetamide resulted in yellow, and adenosine yellow–green, fungal mass. Guanine induced formation of dense, dark green fungal mass in *T. harzianum*.

Procedure II – Targeted Test of Chemical Sensitivity

The measurements of the inhibitory effects of eleven substances on fifteen fungal strains (13 of them endophytes) were analyzed by means of Principal component analysis. The two main principal components collated the fungi following its phylogenetic relations (**Figure 8**).

Discussion

Benefits of PM Approach in Endophyte Studies

Our studies demonstrate that the PM approach is a useful tool to investigate the cellular phenotypes of forest tree endophytes at semi-high throughput rate and in a standardized manner, and to functionally interpret the taxonomic data generated by NGS. For instance, in a recent study exploring the role of endophytes in the

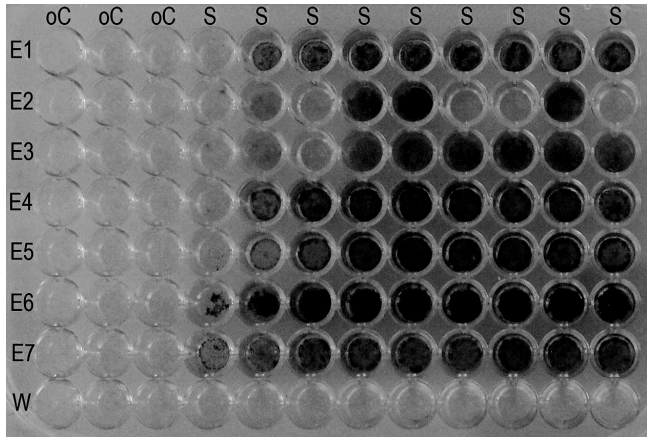


FIGURE 3 | Effect of volatile inhibitory substances on adjacent cells. All the wells of the plate were supplemented with the standard basal medium. In each row, cell suspension of one endophyte strain was added, excluding the last row one where water was added. In the first three columns, the phenolic compound *o*-cresol was incorporated to a final concentration of 1 g/L. Note that the fungi in the fourth and fifth columns were partially inhibited in their growth. E = endophyte; W = water; S = standard basal medium; oC = standard basal medium supplemented with *o*-cresol.

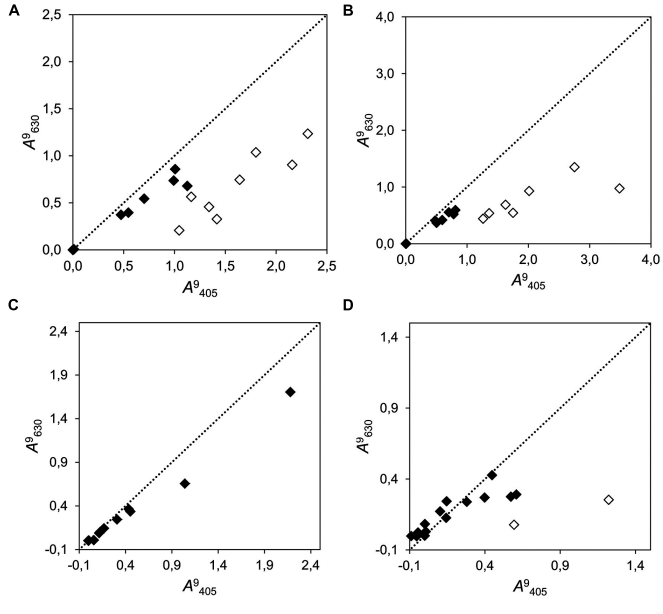


FIGURE 4 | Correlation plots of the cumulative growth (measured at λ = 405 nm vs. λ = 630 nm at time point 9 dai) of 15 fungi in media supplemented with four different phenolic compounds. (A) Chlorogenic acid ($r^2 = 0.683$; $b = 0.430$); **(B)** Gallic acid ($r^2 = 0.788$; $b = 0.321$); **(C)** Salicylic acid ($r^2 = 0.992$; $b = 0.460$); and **(D)** (+)-catechin ($r^2 = 0.407$; $b = 0.240$). The dotted line represents the bisector of slope $b = 1$. Dots close to this line produced even results when measured with both wavelengths (filled dots). Empty dots indicate strains in which an unexpected change of color to yellow–orange was visually evident. Note that it is not expected that points aggregate to the bisector, because the measurements at different wavelengths need not to be alike, regardless of the undesired color change.

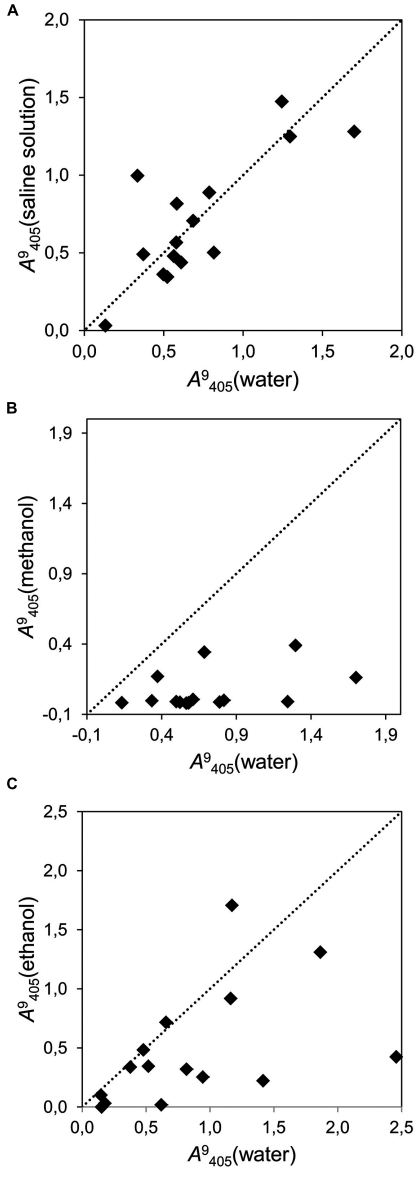


FIGURE 5 | Correlation plot reflecting the effects of different solvents on the cumulative fungal growth (time point 9 dai, λ = 405 nm) of 15 fungal strains. Each dot represents one strain. The dotted line represents the bisector of slope $b = 1$. Placement of dots under this line indicates inhibition by the tested solvent. **(A)** Growth in a saline solvent (NaOH+HCl) versus water with sucrose as carbon source ($r^2 = 0.634$); **(B)** Growth in methanol versus water with sucrose as carbon source ($r^2 = 0.178$); **(C)** Growth in ethanol or water with glucose as carbon source ($r^2 = 0.230$).

Dutch elm disease complex, we were able to identify nutritional niche overlap as a potential mechanism of interaction between the pathogen and potential antagonist endophytes (Blumenstein et al., 2015). This information will assist selection of candidate endophytes for biocontrol purposes. Moreover, we successfully applied PM to characterize the degradation capabilities of the main fungal taxa operating during the first stages of eucalypt wood decay (Macaya-Sanz et al., 2014). In that work, a succession of the most abundant endophyte fungi present during the first 70 days of wood degradation was monitored through pyrosequencing fungal ITS1 region. The resulting operational taxonomic units (OTUs) frequencies varied in time, and certain endophyte OTUs orders were abundant at the beginning of the degradation. Furthermore, the PM analysis showed that these orders are able to effectively degrade lignin-like substances, while other OTUs prevailed at the end and were favored by presence of lignin degradation by-products. This information may be valuable for wood processing industries, but it can also add to the current scientific discourse about the role of microbes as regulators of carbon balance in forest ecosystems (Hiscox et al., 2015) and support the decision making regarding conservation of biodiversity in our forests. Thus, PM techniques are useful tools for both basic and applied research, and can be successfully applied in highly different research fields, such as plant protection, wood material research, and conservation ecology.

Based on our experiences, we conclude that the general benefits of PM approach include its great versatility that allows various research questions to be addressed in a same experiment (e.g., testing of competitive relationships between fungal strains along with gaining information about their sensitivity to individual chemical compounds), testing of a broad array of different compounds and concentrations, and a higher throughput of samples, as compared with earlier methods that have been used in studies of fungal phenotypes (Yourman et al., 2001; Atallah et al., 2011). A great advantage was also that the phenotypic responses are recorded quantitatively and stored electronically (Bochner, 2003; Bochner et al., 2010). If the technical challenges and limitations are properly acknowledged (see below), PM approach opens new experimental possibilities for tree-endophyte research.

Technical Challenges with PM Method

One of the fundamental challenges when working with fungi in the PM procedure is to prepare a representative, homogenous and viable inoculum. Part of this challenge is because the external growth conditions can strongly modulate the quality and quantity of inoculum. Fungi are known to show great phenotypic plasticity in their responses to their immediate growth environment (see, e.g., Rohlfs, 2015, and references therein). In accordance with this expectation, also our results from the nitrogen tests (Procedure I, **Figure 7**) witness how strongly the substrate can affect fungal morphology, which in turn is a product of the fungal metabolism. Moreover, we found a marked loss of vitality during long term storage, possibly because the month of storage promoted the strains to enter latent stages, from which the fungi could not completely recover on the MS medium. Thus, the conditions

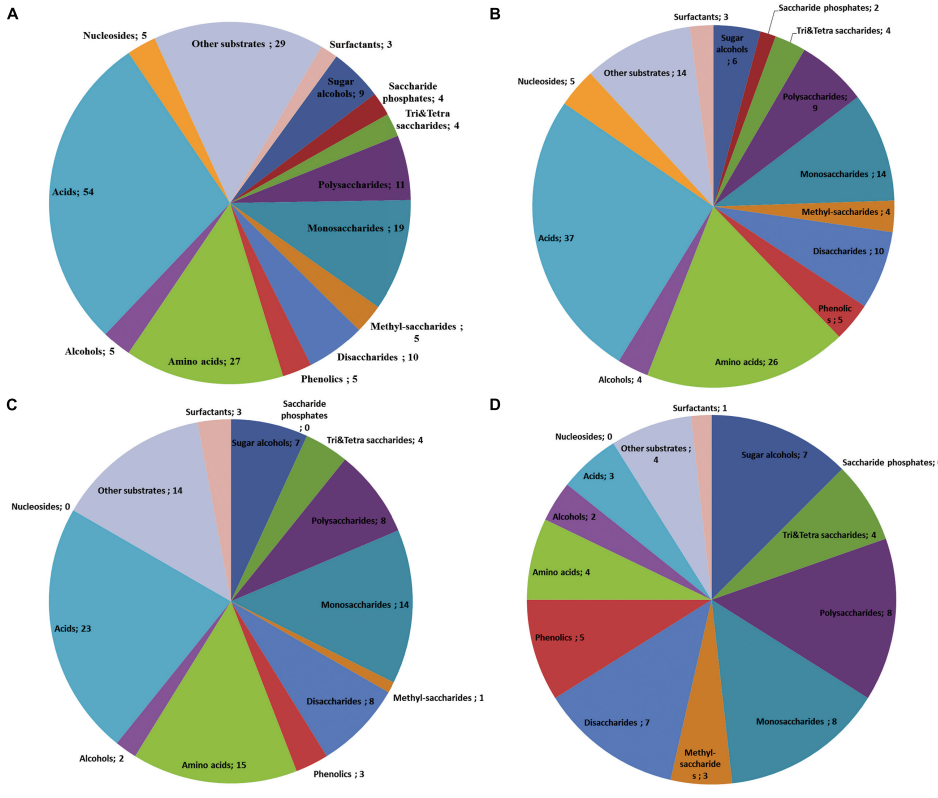


FIGURE 6 | Venn-diagrams show the utilization patterns of carbon sources (divided in chemical groups) of three fungi as indicated by the color development data on BIOLOG plates Phenotype MicroArray (PM) 1 and 2A. All available substrates divided in chemical groups (number of individual compounds after the semicolon; **A**). Substrate utilization pattern by the endophyte *Monographella nivalis* var *neglecta* – a strong *in vitro* competitor of the pathogen, occupying the same niches in the tree and utilizing carbon sources more efficient than the pathogen **(B)**, the pathogen causing Dutch elm disease, *O. novo-ulmi* **(C)** and *Aureobasidium pullulans* **(D)**, a ubiquitous fungus with no detected competitive relation to the pathogen *in vitro* (Data modified from Blumenstein et al., 2015).

before preparation of inoculum may influence the responses of fungal cells on PM plates through metabolic carry-over effects. As a first step when using PM approach, whether it is in-house or pre-configured PM plates, we thus recommend careful standardization and documentation of the pre-inoculum growth conditions for the fungi, to ensure the repeatability of analyses.

Standardization of growth conditions may, however, also add bias to the analysis, given the strain-specific preferences for optimal growth. Indeed, in both procedures (I and II), we found evidence for strain-specificity in fungal responses. For instance, the above mentioned conditioning effects of pre-inoculum preparation growth environment could be highly genotype-specific because the nutritional niches of the strains differ (Blumenstein et al., 2015). Therefore, the possible carry-over effect of culturing conditions may be a factor that needs to be taken into consideration in particular when comparing interspecific differences. In our studies, we pre-cultured all fungi on MEA and at 26°C even though their individual growth preferences may differ. Ideally, the species-specific nutrient utilization patterns detected through PM analysis should be validated using inoculum collected from colonies of the same isolates that have been cultured under a well-defined set of pre-conditions that cover the realistic regimes in physicochemical environment (light, temperature, pH, etc.). Standardization of the pre-inoculum preparation conditions could also be done on strain-specific basis, ensuring the strain-specific maximum growth rate. An interesting possibility with PM could also be to study the possible carryover effects with the goal of gaining a better understanding of how we could better gear the fungal phenotypes for different industrial or pharmaceutical purposes.

Intriguingly, we also observed (Procedure II) how complex strain-specific interactions with the chemical environment were

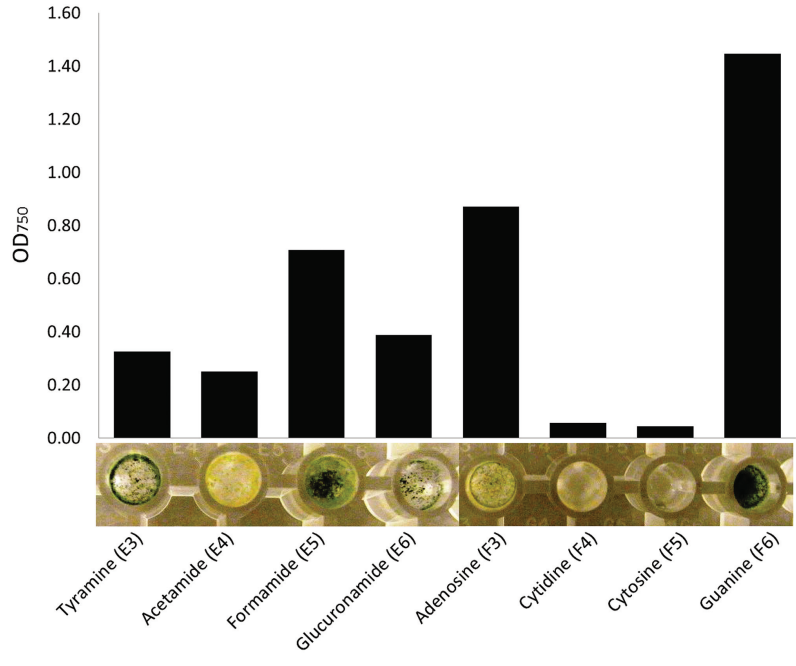


FIGURE 7 | An assortment of nitrogen sources leading to clearly visible differences in optical density (750 nm) and morphology of the fungal mass of *Trichoderma harzianum*. Measurement at 144 h after inoculation to the plates (bars represent the mean of readings from two plates).

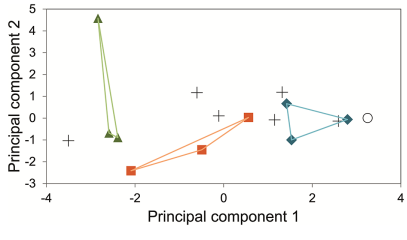


FIGURE 8 | Principal Component analysis of the cumulative growth (time point 9 dai, λ = 630 nm) of 15 fungal strains in an array of in-house PM supplemented with 11 inhibitory substances and glucose. Green triangles indicate three strains of the family Dothioraceae. Orange squares represent members of the order Pleosporales (families Pleosporaceae and Phaeosphaeriaceae). Blue diamonds for the basidiomycetes assayed. Crosses represent a variety of ascomycetous endophytic fungi (Davidiellaceae, Lophiostomataceae, Botryosphaeriaceae, two sordariomycetes, and one *incertae sedis*). Empty circle represents the negative control without inoculum. Principal component 1 explains 42.76% and principal component 2 does 21.50%.

expressed as unexpected color changes in the wells (**Figure 4**). The orange color is characteristic for oxidation of phenols (Holderbaum et al., 2010) and could thus be indication of fungal, extracellular phenolase activity. Another possibility is that the chemical environment induced the fungi to produce colored substances (Velmurugan et al., 2010). Thus, in order to avoid confounding effects of such within-well processes, it is necessary to carefully plan the positive and negative controls in each in-house array. Moreover, new research is needed to better understand the metabolic processes in fungal cells as they grow in the wells.

The second essential step in use of PM approach is to establish an inoculum preparation routine that ensures good viability and accurate and repeatable quantification of inoculum. Our method for preparing a homogenous inoculum (Procedure I) resulted in dense emergence of hyphae on the Petri dishes within 2–4 days, indicating that the process did not negatively affect the viability of the cells. According to the manufacturer's protocol, the density of the IF should be set to 62% transmittance, conveniently measured from inoculum fluid tubes with the original Biolog turbidimeter. Bochner (2009) states that technically one cell per well would be adequate, but recommends 100 cells per 100 μL for the inoculum. For bacterial cells, a concentration of approximately 10^6 cells/mL is a common standard (e.g., Bourne et al., 2012). For fungi, Atanasova and Druzhinina (2010) recommend an adjusted cell density ranging from 1.25×10^5 to 5×10^5 spores/mL, depending on the tested fungus to guarantee repeatable OD measurements.

Insam et al. (1996), however, suggest that instead of total counts, viable counts for microorganisms should be used, or the biomass should be standardized. In our Procedure II, we used biomass standardization. This method is practical in particular for those endophytes that do not readily sporulate in cultures. However, the proportion of metabolically non-active biomass (e.g., non-active hyphal segments) should ideally be controlled as a part of the protocol, by microscopic examination or by determining the CFU of the inoculum. For adequate repeatability of the results, efforts should be made to guarantee that the inoculum prepared from different isolates of a same species contain a comparable CFU/mL and that each well contains at least 100 cells per well (Bochner, 2009).

The third crucial step in PM analyses, in particular when working without the OMNILOG instrumentation and software, is to decide the time points of interest for data collection. There is a temporal dynamic in the substrate use by the cells, which is a fundamental to the evaluation of cell phenotypes. The reaction in the wells is often characterized by a lag-period that can last up to 2–4 days. After that the reaction develops and finally tends to saturate. In the case of the fungi that we have studied the saturation often started after 96 h from the start of the inoculation period. Hierarchical cluster analysis proved to be a useful tool for determination of the appropriate time for downstream analyses of the substrate use by the fungi (Blumenstein et al., 2015). In cases where such clear cluster separation of the replicate measurements does not exist, the readings that represent the highest degree of strain separation could be chosen for further analyses. In our Procedure I studies, we found that the readings for the studied fungi (a pathogen and three fungal endophytes) were most reliable between 168 and 240 h after incubation. We also found that viable cells could be recovered from the wells even after 360 h on the plates. An intriguing option to further utilize PM technique would be to extract the fungal biomass in the wells in the end of an incubation period and study, e.g., using chromatography and molecular approaches, how growth on a single substrate might affect the capacity of fungal cells to accumulate specific chemicals, or express certain genes.

For the in-house configured PMs, we also identify a fourth crucial step: Our Procedure II studies demonstrated that the repeatability and reliability of the in-house arrays can only be ensured through a careful design that acknowledges the specific characters of the studied chemicals. For instance, the volatility of a compound dispensed in a certain well could affect fungal growth in surrounding wells (e.g., observed in adjacent wells to inhibitory, volatile *o*-cresol; **Figure 3**). Thus, when analyzing the chemical sensitivity of volatile substances, a preferable approach seems to be dispensing the same concentration of the volatile substance in question in all the wells of the microplate in order to avoid unintentional cross-well effects.

Challenges in Interpretation of the Biolog Data

The PM technique is a powerful tool to estimate the relative speed of substrate use for particular fungi. However, the interpretation of the between-species difference in the speed of substrate use is not straightforward. A change is absorbance values can be interpreted as a proxy of metabolic activity, but it is risky to propose narrower views. The change of color could be produced by a shift in the tetrazolium dye due to respiration, by an increase of turbidity through fungal body proliferation or even by change of the medium color after production of metabolites by the fungi. Such mélange of processes makes it challenging to contrast different organisms in a fine scale. The inconsistency of results in the repeated assays where six strains were tested on seven carbohydrates (**Table 1**) may reflect such blended processes that interplayed with the inherent variations in the enzymatic activities of the fungi (e.g., van den Brink and de Vries, 2011). Thus, until refinement, the procedure is best suited for studies on general metabolic trends.

Despite the uncertainty, the proxy of metabolic activity can be valuable additional information, e.g., in studies addressing the potential endophyte-based applications (see Benefits of PM Approach in Endophyte Studies). In our recent study (Protocol I; Blumenstein et al., 2015), we used pre-configured PMs to deepen our understanding of the mechanisms of antagonism by potential biocontrol endophytes, identified on basis of field correlations and laboratory tests. In this case, the PM data allowed us to nuance the emerging picture of the potential of endophytes in biocontrol: we could conclude that the antagonistic effect of an endophyte against a pathogen may be due to several, simultaneous or parallel mechanisms (chemical antagonism and competition for nutrients). PM analysis also provided information about the possible ecological strategies of the fungi. In particular, we found that the ubiquitous *A. pullulans* is a slow substrate user with a relatively high tolerance to potentially antifungal compounds such as phenolics (**Figure 6**), whereas the biologically more specialized species *M. nivalis* var. *neglecta* and *P. cava*, were capable of faster catabolism of certain substrates, e.g., monosaccharides, polysaccharides and acids, but were less tolerant to phenolics as compared to *A. pullulans*. These results indicate that while *M. nivalis* var. *neglecta* and *P. cava* may be superior competitors in sugar- and acid-rich environments, *A. pullulans* may have an ecological strategy that permits it to remain active even on nutritionally more reluctant substrates that might cause other fungi to starve or become intoxicated. Given that *M. nivalis* var. *neglecta* and *P. cava* have been identified as potent inhibitors of a vascular pathogen of the host species, elms (Blumenstein et al., 2015), this information might help to set chemical quality targets for the elm trees that are most receptive for biocontrol through these endophytes. Similarly, the protocol II study showed that the phylogeny could be considered as a proxy of the fungal response to plant secondary metabolite. This kind of results, in combination with NGS analyses, can provide novel information about the mechanisms behind the structure and functions of endophyte communities in trees.

The obviously artificial growth environment in PM plate wells, and the distant resemblance of preconfigured plates with the substrate availability under natural conditions, may obviously obscure the interpretations. We only see the response to one isolated substrate in each well on a PM plate. In nature, however, fungal cells inside the plant tissues will exist in a chemical environment that is likely to constantly and gradually change.

This dynamicity will be caused both by the metabolism of the plant and by the endophytic inhabitants, and the composition of the substrate, as well as the fungal community, will continuously be altered. Similar successions have been studied for macro-organisms including fungi and insects in decaying leaf litter (Hättenschwiler et al., 2005) or the succession of saprotrophic organisms in dead woods of forests (Bader et al., 1995, Similä et al., 2003). As one of the functions of endophytic fungi is likely that of a decomposer (Schulz and Boyle, 2005), the conversion from one substrate to another by the help of a fungi will likely determine the succession of endophyte function in a plant both during its life as well as during its after life.

In natural conditions, variations in species-specific infection mode and presence of specific endophytes are also determining the use of substrates by a fungus, which, in turn, will affect the succession of endophytes (Heilmann-Clausen and Boddy, 2005). Thus, the order in which endophytic fungi enter the host plant may also determine the activity and importance of subsequent endophytes in that plant. It further suggests that there is plasticity in how the fungi may make use of the plant, in the sense that some individual species may play different roles depending on when they enter the scene. A certain fungal endophyte may therefore be plastic in the ecological role they play, which may further complicate the interpretations of the biological significance of the phenotypic responses detected in PM plates.

Conclusion

The technical challenges of the PM method are multifaceted and the interpretation of PM data is not straightforward. Thus, ideally, extensive validation through carefully standardized pre-conditions for the fungal growth and careful replication and control well strategies are needed for successful PM analyses, whether the studies use preconfigured or in-house designed PM plates. However, it is evident that the PM technique may significantly help to bridge the genotype–phenotype gaps for the culturable fraction of endophytic fungi. Despite the above-mentioned challenges, PM analyses can provide unique knowledge about functional properties of individual strains and species, and thereby contribute to the knowledge pool that is needed for a more comprehensive understanding of the associations between the NGS-profiles and functional fungal biodiversity.

Acknowledgments

This work was supported by the research project CTQ2011-28503-C02-02 (Ministerio de Economía y Competitividad, Spain) to DM-S and JM, Erasmus Mundus Joint Doctoral Programme FONASO fellowship to KB, and VINNMER Marie Curie International Qualification grant (2011-01350) to JW.

References

Albrectsen, B. R., and Witzell, J. (2012). "Disentangling functions of fungal endophytes in forest trees," in *Fungi: Types, Environmental Impact and Role in Disease*, eds A. Paz Silva and M. Sol (Huntington, NY: Nova Science Publishers), 235–246.

Atallah, Z. K., Maruthachalam, K., Vallad, G. E., Davis, R. M., Klosterman, S. J., and Subbarao, K. V. (2011). Analysis of *Verticillium dahliae* suggests a lack of correlation between genotypic diversity and virulence phenotypes. *Plant Dis.* 95, 1224–1232. doi: 10.1094/PDIS-02-11-0110

Atanasova, L., and Druzhinina, I. S. (2010). Global nutrient profiling by Phenotype MicroArrays: a tool complementing genomic and proteomic studies in conidial fungi. *J. Zhejiang Univ. Sci. B* 11, 151–168. doi: 10.1631/jzus.B1000007

Bader, P., Jansson, S., and Jonsson, B. G. (1995). Wood-inhabiting fungi and substratum decline in selectively logged boreal spruce forests. *Biol. Conserv.* 72, 355–362. doi: 10.1016/0006-3207(94)00029-P

Blumenstein, K., Albrectsen, B., Martin, J., Hultberg, M., Sieber, T., Helander, M., et al. (2015). Nutritional niche overlap potentiates the use of endophytes in biocontrol of a tree disease. *BioControl* 60, 655–667. doi: 10.1007/s10526-015-9668-1

Bochner, B. R. (1989). Sleuthing out bacterial identities. *Nature* 339, 157–158. doi: 10.1038/339157a0

Bochner, B. R. (2003). New technologies to assess genotype-phenotype relationships. *Nat. Rev. Genet.* 4, 309–314. doi: 10.1038/nrg1046

Bochner, B. R. (2009). Global phenotypic characterization of bacteria. *FEMS Microbiol. Rev.* 33, 191–205. doi: 10.1111/j.1574-6976.2008.00149.x

Bochner, B. R., Gadzinski, P., and Panomitros, E. (2001). Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. *Genome Res.* 11, 1246–1255. doi: 10.1101/gr.186501

Bochner, B., Gomez, V., Ziman, M., Yang, S., and Brown, S. D. (2010). Phenotype MicroArray profiling of *Zymomonas mobilis* ZM4. *Appl. Biochem. Biotechnol.* 161, 116–123. doi: 10.1007/s12010-009-8842-2

Bochner, B. R., and Savageau, M. A. (1977). Generalized indicator plate for genetic. Metabolic, and taxonomic studies with microorganisms. *Appl. Environ. Microbiol.* 33, 434–444.

Borglin, S., Joyner, D., Deangelis, K. M., and Et, A. (2012). Application of phenotypic microarrays to environmental microbiology. *Curr. Opin. Biotechnol.* 23, 41–48. doi: 10.1016/j.copbio.2011.12.006

Bourne, C. R., Wakeham, N., Bunce, R. A., Berlin, K. D., and Barrow, W. W. (2012). Classifying compound mechanism of action for linking whole cell phenotypes to molecular targets. *J. Mol. Recognit.* 25, 216–223. doi: 10.1002/jmr.2174

Cabrera-Bosquet, L., Crossa, J., von Zitzewitz, J., Serret, M. D., and Luis Araus, J. (2012). High-throughput phenotyping and genomic Selection: the frontiers of crop breeding converge. *J. Integr. Plant Biol.* 54, 312–320. doi: 10.1111/j.1744-7909.2012.01116.x

Davidson, F. A. (1998). Modelling the qualitative response of fungal mycelia to heterogeneous environments. *J. Theor. Biol.* 195, 281–292. doi: 10.1006/jtbi.1998.0739

Dong, H. P., Liu, Z. Q., Zheng, Y. G., and Shen, Y. C. (2010). Novel biosynthesis of (R)-ethyl-3-hydroxyglutarate with (R)-enantioselective hydrolysis of racemic ethyl 4-cyano-3-hydroxybutyate by *Rhodococcus erythropolis*. *Appl. Microbiol. Biotechnol.* 87, 1335–1345. doi: 10.1007/s00253-010-2584-5

Druzhinina, I. S., Schmoll, M., Seiboth, B., and Kubicek, C. P. (2006). Global carbon utilization profiles of wild-type, mutant, and transformant strains of *Hypocrea jecorina*. *Appl. Environ. Microbiol.* 72, 2126–2133. doi: 10.1128/AEM.72.3.2126-2133.2006

Eyles, A., Bonello, P., Ganley, R., and Mohammed, C. (2010). Induced resistance to pests and pathogens in trees. *New Phytol.* 185, 893–908. doi: 10.1111/j.1469-8137.2009.03127.x

Falconer, R. E., Bown, J. L., White, N. A., and Crawford, J. W. (2005). Biomass recycling and the origin of phenotype in fungal mycelia. *Proc. Biol. Sci.* 272, 1727–1734. doi: 10.1098/rspb.2005.3150

Gaur, R., Singh, R., Gupta, M., and Gaur, M. K. (2010). *Aureobasidium pullulans*, an economically important polymorphic yeast with special reference to pullulan. *Afr. J. Biotechnol.* 9, 7989–7997.

Greetham, D. (2014). Phenotype microarray technology and its application in industrial biotechnology. *Biotechn. Lett.* 36, 1153–1160. doi: 10.1007/s10529-014-1481-x

Hättenschwiler, S., Tiunov, A. V., and Scheu, S. (2005). Biodiversity and litter decomposition interrestrial ecosystems. *Annu. Rev. Ecol. Evol. Syst.* 36, 191–218. doi: 10.1146/annurev.ecolsys.36.112904.151932

Heilmann-Clausen, J., and Boddy, L. (2005). Inhibition and stimulation effects in communities of wood decay fungi: exudates from colonized wood influence growth by other species. *Microbial. Ecol.* 49, 399–406. doi: 10.1007/s00248-004-0240-2

Hiscox, J., Savoury, M., Vaughan, I. P., Müller, C. T., and Boddy, L. (2015). Antagonistic fungal interactions influence carbon dioxide evolution from decomposing wood. *Fungal Ecol.* 14, 24–32. doi: 10.1016/j.funeco.2014.11.001

Holderbaum, D. F., Kon, T., Kudo, T., and Guerra, M. P. (2010). Enzymatic browning, polyphenol oxidase activity, and polyphenols in four apple cultivars: dynamics during fruit development. *HortScience* 45, 1150–1154.

Houle, D., Govindaraju, D. R., and Omholt, S. (2010). Phenomics - the next challenge. *Nat. Rev. Genet.* 11, 855–866. doi: 10.1038/nrg2897

Insam, H., Amor, K., Renner, M., and Crepaz, C. (1996). Changes in functional abilities of the microbial community during composting of manure. *Microbial. Ecol.* 31, 77–87. doi: 10.1007/BF00175077

Kaewkla, O., and Franco, C. M. M. (2013). Rational approaches to improving the isolation of endophytic Actinobacteria from Australian native trees. *Microbial. Ecol.* 65, 384–393. doi: 10.1007/s00248-012-0113-z

Lim, Y., Kim, B., Kim, C., Jung, H., Kim, B.-S., Lee, J.-H., et al. (2010). Assessment of soil fungal communities using pyrosequencing. *J. Microbiol.* 48, 284–289. doi: 10.1007/s12275-010-9369-5

Macaya-Sanz, D., del-Barrio-Duque, A., and Martín, J. A. (2014). Fungal endophyte dynamics and functions in the early steps of degradation of eucalypt twigs. *Presentation at XVI International Congress on Molecular Plant-Microbe Interactions. 6-10 July*, Rhodes. 501.

Martín, J. A., Macaya-Sanz, D., Witzell, J., Blumenstein, K., and 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. *Eur. J. Plant Pathol.* 142, 185–196. doi: 10.1007/s10658-015-0602-2

Martín, J. A., Solla, A., Witzell, J., Gil, L., and Concepcion Garcia-Vallejo, M. (2010). Antifungal effect and induction of elm resistance towards Dutch elm disease fungi by salicylic acid and carvacrol. *Eur. J. Phytopathol.* 127, 21–32.

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

Rančić, A., Soković, M., Karioti, A., Vukojević, J., and Skaltsa, H. (2006). Isolation and structural elucidation of two secondary metabolites from the filamentous fungus *Penicillium ochrochloron* with antimicrobial activity. *Environ. Toxicol. Pharmacol.* 22, 80–84. doi: 10.1016/j.etap.2005.12.003

Rodrigues, K. F., Hesse, M., and Werner, C. (2000). Antimicrobial activities of secondary metabolites produced by endophytic fungi from *Spondias mombin*. *J. Basic Microbiol.* 40, 261–267. doi: 10.1002/1521-4028(200008)40:4<261::AID-JOBM261>3.3.CO;2-4

Rodriguez, R., and Redman, R. (2008). More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. *J. Exp. Bot.* 59, 1109–1114. doi: 10.1093/jxb/erm342

Rohlfs, M. (2015). Fungal secondary metabolite dynamics in fungus–grazer interactions: novel insights and unanswered questions. *Front. Microbiol.* 5:788. doi: 10.3389/fmicb.2014.00788

Schulz, B., and Boyle, C. (2005). The endophytic continuum. *Mycol. Res.* 109, 661–686. doi: 10.1017/S095375620500273X

Schulz, B., Boyle, C., Draeger, S., Rommert, A. K., and Krohn, K. (2002). Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycol. Res.* 106, 996–1004. doi: 10.1017/S0953756202006342

Seidl, V., Druzhinina, I. S., and Kubicek, C. P. (2006). A screening system for carbon sources enhancing β-N-acetylglucosaminidase formation in *Hypocrea atroviridis* (*Trichoderma atroviride*). *Microbiology* 152, 2003–2012. doi: 10.1099/mic.0.28897-0

Similä, M., Kouki, J., and Martikainen, P. (2003). Saproxylic beetles in managed and seminatural Scots pine forests: quality of dead wood matters. *For. Ecol. Manage.* 174, 365–381. doi: 10.1016/S0378-1127(02)00061-0

Singh, M. P. (2009). Application of Biolog FF MicroPlate for substrate utilization and metabolite profiling of closely related fungi. *J. Microbiol. Methods* 77, 102–108. doi: 10.1016/j.mimet.2009.01.014

Solla, A., Dacasa, M. C., Nasmith, C., Hubbes, M., and Gil, L. (2008). Analysis of Spanish populations of *Ophiostoma ulmi* and *O. novo-ulmi* using phenotypic characteristics and RAPD markers. *Plant Pathol.* 57, 33–44. doi: 10.1111/j.1365-3059.2007.01692.x

Tanzer, M. M., Arst, H. N., Skalchunes, A. R., Coffin, M., Darveaux, B. A., Heiniger, R. W., et al. (2003). Global nutritional profiling for mutant and chemical mode-of-action analysis in filamentous fungi. *Funct. Integr. Gen.* 3, 160–170. doi: 10.1007/s10142-003-0089-3

Toju, H., Yamamoto, S., Sato, H., Tanabe, A. S., Gilbert, G. S., and Kadowaki, K. (2013). Community composition of root-associated fungi in a Quercus-dominated temperate forest: "codominance" of mycorrhizal and root-endophytic fungi. *Ecol. Evol.* 3, 1281–1293. doi: 10.1002/ece3.546

Unterseher, M., Petzold, A., and Schnittler, M. (2012). Xerotolerant foliar endophytic fungi of *Populus euphratica* from the Tarim River basin. Central China are conspecific to endophytic ITS phylotypes of *Populus tremula* from temperate Europe. *Fungal Divers.* 54, 133–142.

van den Brink, J., and de Vries, R. P. (2011). Fungal enzyme sets for plant polysaccharide degradation. *Appl. Microbiol. Biotechnol.* 91, 1477–1492. doi: 10.1007/s00253-011-3473-2

Velmurugan, P., Lee, Y. H., Venil, C. K., Lakshmanaperumalsamy, P., Chae, J.-C., and Oh, B.-T. (2010). Effect of light on growth, intracellular and extracellular pigment production by five pigment-producing filamentous fungi in synthetic medium. *J. Biosci. Bioengin.* 109, 346–350. doi: 10.1016/j.jbiosc.2009.10.003

Witzell, J., and Martín, J. A. (2008). Phenolic metabolites in the resistance of northern forest trees to pathogens – past experiences and future prospects. *Can. J. For. Res.* 38, 2711–2727. doi: 10.1139/X08-112

Witzell, J., Martín, J. A., and Blumenstein, K. (2014). "Ecological aspects of endophyte-based biocontrol of forest diseases," in *Advances in Endophytic Research*, eds V. C. Verma and A. C. Gange (Berlin: Springer), 321–333.

Yourman, L. F., Jeffers, S. N., and Dean, R. A. (2001). Phenotype instability in *Botrytis cinerea* in the absence of benzimidazole and dicarboximide fungicides. *Phytopathology* 91, 307–315. doi: 10.1094/Phyto.2001.91.3.307

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

IV

BioControl (2015) 60:655–667
DOI 10.1007/s10526-015-9668-1

Nutritional niche overlap potentiates the use of endophytes in biocontrol of a tree disease

Kathrin Blumenstein · Benedicte R. Albrectsen · Juan A. Martín · Malin Hultberg · Thomas N. Sieber · Marjo Helander · Johanna Witzell

Received: 22 August 2014 / Accepted: 27 March 2015 / Published online: 4 April 2015

Abstract Asymptomatic endophytic fungi are often regarded as potent biocontrol agents in plants, but the competitive interactions between endophytes and other microbes within the same host plant are poorly understood. We tested a hypothesis that as compared to asymptomatic endophytes, an aggressive pathogen inhabiting the same host is able to utilize carbon substrates more efficiently. Using phenotype microarray, we determined the carbon utilization profiles of the highly virulent Dutch elm disease (DED) pathogen *Ophiostoma novo-ulmi*, and four asymptomatic elm (*Ulmus* spp.) endophyte isolates that were selected based on their differential association to the DED-susceptibility pattern of the host elms. The competitive interactions between isolates were evaluated using a niche overlap index. In contrast to our hypothesis, the studied endophytes exhibited extensive niche overlap with the pathogen, suggesting that some endophyte strains might protect elms against DED-pathogen through competition for substrates and provide new tools for biocontrol of DED.

Keywords Carbon utilization profile · Endophytic fungi · Dutch elm disease · Biocontrol · Niche differentiation hypothesis · Niche tradeoff

Handling Editor: Choong-Min Ryu.

K. Blumenstein (✉) · J. Witzell
Faculty of Forest Sciences, Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, P.O. Box 49, 230 53 Alnarp, Sweden
e-mail: Kathrin.Blumenstein@slu.se

J. Witzell
e-mail: Johanna.Witzell@slu.se

B. R. Albrectsen
Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, 90187 Umeå, Sweden
e-mail: benedicte.albrectsen@umu.se

B. R. Albrectsen
Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldensvej 40, 1871 Fredriksberg C, Denmark

J. A. Martín
Department of Natural Systems and Resources, Technical University of Madrid (UPM), Ciudad Universitaria s/n, 28040 Madrid, Spain
e-mail: juan.martin.garcia@upm.es

M. Hultberg
Department of Biosystems and Technology, Faculty of Landscape Architecture, Horticulture and Crop Production Science, Swedish University of Agricultural Sciences, 230 53 Alnarp, Sweden
e-mail: Malin.Hultberg@slu.se

T. N. Sieber
Institute of Integrative Biology, ETH Zurich, 8092 Zurich, Switzerland
e-mail: thomas.sieber@env.ethz.ch

Introduction

Plants' internal spaces are ubiquitously colonized by microbes, some of which are asymptomatic endophytic fungi. Several studies have shown that some endophytes can increase their hosts' resistance to pathogens (Martín et al. 2009; Scheffer et al. 2008; Tellenbach and Sieber 2012), and that the nature and extent of endophyte infections may be inversely correlated with damage caused by herbivores (Ahlholm et al. 2002; Albrectsen et al. 2010; Saikkonen et al. 2001). It has been suggested that endophytes can shape their hosts' resistance to pathogen attacks in various ways, including by stimulating defensive metabolism (Hamilton et al. 2012; White and Torres 2010), competing for resources, and antagonistically by synthesizing secondary metabolites that are toxic to the pathogen (Arnold 2007; Rodriguez and Redman 2008). Endophytes could therefore be valuable tools for the biocontrol of herbivorous insects and pathogens in plants such as forest trees (Albrectsen and Witzell 2012; Newcombe 2011; Witzell et al. 2013). However, before evaluating or exploring the scope for such applications, we need to better understand how endophytes interact with other consumers that co-inhabit the host plants.

One way of studying endophyte interactions with other organisms *in planta* is to investigate niche partitioning. Endophytic fungi share their host plants with an array of pathogenic fungi that also inhabit the plant's internal tissues (Mejía et al. 2008; Saikkonen et al. 1998), and interactions between these fungi are therefore likely to occur on multiple temporal and spatial scales. The principle of competitive exclusion states that two species cannot occupy the same ecological niche: only species with sufficiently differentiated niches can coexist in a given ecological community (Chase and Leibold 2003; Mikkelson 2005; Tilman 2004). Niche partitioning is regarded as a fundamental factor that dictates the assembly, dynamics, and structures of plant endophyte communities (Ernst et al. 2011). The niche partitioning hypothesis explains cohabitation patterns using trade-off based mechanisms of coexistence, and also suggests that antagonistic interactions are molded by species-specific needs for abiotic and biotic factors. This idea is consistent with the endophyte continuum hypothesis (Saikkonen et al. 1998, 2004). An alternative explanation is the neutral hypothesis (Hubbell 2001), which states that niche differences cannot explain why certain competitors can coexist whereas others cannot. Instead, it is contended that diversity is due to speciation, dispersal, and "random ecological drift" in population sizes (Mikkelson 2005; Tilman 2004).

A recent study (Ernst et al. 2011) investigated the coexistence of two related endophyte species in the common reed (*Phragmites australis* (Cav.) Trin. ex Steudel) in light of these two hypotheses. It was concluded that niche partitioning was consistent with the observed differential colonization of the common reed by two endophytes, *Microdochium bolleyi* and *M. phragmitis*, and that the neutral model was unable to explain the assembly of the mycoflora in this plant. However, we still know little about the dynamics of the fungal assemblages in large and long-lived trees. Importantly, the long lifetimes of trees and the accumulation and growth of horizontal infections in their woody parts over time might enhance the importance of the processes on which the viability of neutral hypothesis is based.

In the work reported herein, we investigated the niche partitioning of endophytic and pathogenic fungi in elm trees (*Ulmus* sp.) by studying the fungal utilization of different carbon substrates. The global elm population has been severely affected by a vascular disease, Dutch elm disease (DED), which is one of the most devastating tree diseases described so far (Martín et al. 2010; Santini and Faccoli 2015; Scheffer et al. 2008). The pathogenic fungus *Ophiostoma novo-ulmi* Brasier is responsible for the ongoing outbreak of this disease, whereas an earlier DED outbreak in the 1920s was caused by the less aggressive species *O. ulmi* (Buisman) Nannfeldt (Brasier 1991). The complex cycle of DED comprises spreading of the pathogen into healthy trees with vector insects, maturing beetles of the genus *Scolytus* spp. Geoffroy or *Hylurgopinus rufipes* (Eichhoff) (Rudinsky 1962;

M. Helander
Department of Biology, Section of Ecology, University of Turku, 20014 Turku, Finland
e-mail: helander@utu.fi

M. Helander
Plant Production Research, MTT Agrifood Research Finland, 31600 Jokioinen, Finland

J. Witzell
Faculty of Science and Forestry, School of Forest Sciences, University of Eastern Finland, P.O. Box 111, 80101 Joensuu, Finland

Santini and Faccoli 2015; Webber and Brasier 1984) that feed in the tree crown and introduce pathogen spores into the phloem and vascular system of the tree through the wounds in the twig crotches. The pleomorphic pathogen spreads in the xylem vessels through a yeast-like multiplication phase of the pathogen (Webber and Brasier 1984) causing vessel cavitation and the appearance of wilting symptoms. The fungus grows saprophytically and produces fruiting bodies in the inner bark and phloem of dying elms into maternal galleries where the bark beetle larvae develop (Rudinsky 1962; Scheffer et al. 2008). 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 and Faccoli 2015). When the new beetle generation emerges, they carry fungal conidia and ascospores on their bodies and complete the disease cycle when flying to healthy elms for maturation feeding (Webber and Brasier 1984). In addition, DED also spreads through root contacts (Neely and Himelick 1963).

The effective spreading of DED makes it challenging to control. In addition to eradication and chemical treatments (Martín et al. 2010; Scheffer et al. 2008), fungal communities found within elms have been explored as potential sources of resistance inducers or biocontrol agents against DED (Dvorák et al. 2006; Martín et al. 2010; Webber 1981). Hubbes and Jeng (1981) showed in their study that inoculating DED sensitive elms with low concentrations of *O. ulmi* spores induces resistance against a later *O. novo-ulmi* infection. A study by Bernier et al. (1996) demonstrated that inoculating DED sensitive elms with the endophyte *Phaeotheca dimorphospora* protected them against subsequent infections by the less aggressive *O. ulmi,* but not by *O. novo-ulmi*. Recent studies have shown that certain endophytic fungi found in elms are associated with a low susceptibility to DED (Martín et al. 2013). However, little is known about the mechanisms of protection that fungal endophytes provide against DED.

We hypothesized that fungal endophytes with potential applications as biocontrol agents against DED would out-compete *O. novo-ulmi* within its nutritional niche. To test this hypothesis, we studied the carbon (C) substrate utilization profiles of an aggressive *O. novo-ulmi* isolate and a selection of endophytic isolates from elm trees. Specifically, the carbon utilization patterns of three endophyte isolates representing two species that have been strongly linked to low DED-susceptibility in their host trees were compared to that of the pathogen. For comparative purposes, the study also included an additional isolate from a collection of elm endophytes (Martín et al. 2013), representing a ubiquitous, leaf-associated endophyte species with no obvious connection to the resistance phenotype of the host trees. The carbon utilization patterns of the isolates were studied using the phenotype microarray (PM) technique, which enables the metabolic profiling of cells. The resulting data were used to compute a niche overlap index (NOI) (Lee and Magan 1999). Specifically, we tested the following hypotheses: (1) the C-substrate niche of an aggressively pathogenic strain will be larger than those of typical endophytes, and (2) endophytes that have previously been linked to low phenotypic susceptibility to DED (Martín et al. 2013) will compete effectively with the pathogen for substrates, i.e. they will exhibit niche overlap with the pathogen. The obtained results were used to evaluate the antagonistic potential of endophytes against the DED pathogen. In addition, the applicability of the trade-off based niche partitioning hypothesis and the neutral hypothesis as models for the cohabitation patterns of endophytes and pathogens in trees is discussed briefly.

Materials and methods

Fungal strains

In 2008, around 220 fungal endophytes were isolated from the leaves, bark and xylem of elm trees with different degrees of susceptibility to DED. The initial isolations were conducted at the Forest Breeding Centre in Puerta de Hierro (Madrid, Spain, 40°27′N, 3°46′W) from healthy *U. minor* trees that differed in their susceptibility to DED, and from healthy *U. pumila* trees that are tolerant to DED (Martín et al. 2013). Additionally, branches were collected from *U. minor* trees in Rivas-Vaciamadrid elm stand (Madrid, 40°20′N, 3°33′W). The progress of DED has been slow in this semi-natural habitat despite the apparent availability of DED inocula and insect vectors in the area (Martín et al. 2008).

For sampling and endophyte isolation, leaves were detached from the collected elm shoots. The leaves'

surfaces were disinfected by immersing them in ethanol (75 %) for 30 s, followed by aqueous sodium hypochlorite (4 %) for one minute and then ethanol (75 %) again for 15 s. Finally, the leaves were air-dried under a laminar flow for four minutes, and a disc with a diameter of 10 mm was cut aseptically from a randomly selected region of each leaf. The discs were placed on 2 % (w/v) malt extract agar (MEA) in Petri dishes without added antibiotics. Two-year-old twig segments (4 cm in length and 8–10 mm in diameter) were surface disinfected in the same way as the leaves save that they were immersed in the sodium hypochlorite solution for 5 min rather than 1 min. After air drying (8 min), the twigs were cut into 4-mm-thick and 10-mm-long sections (measured in axial direction) and the bark was separated from the xylem. The separated tissue samples were then placed in Petri dishes containing 2 % (w/v) MEA without added antibiotics as was done for the leaf samples. The dishes were then sealed with Parafilm and incubated at 25 °C. After two weeks, hyphae had developed around most segments. Outgrowing hyphae were sub-cultured on new MEA-dishes. Once in pure culture, the isolates were maintained on MEA.

One hundred and forty of the initial isolates are currently being maintained in a stock collection. The isolates were initially classified into 16 morphological groups (e.g., Arnold et al. 2000) based on macromorphological traits such as colony surface texture, the colors of the colonies and the surrounding media, and their growth rates on MEA. Special characters such as fruiting body formation and the accumulation of droplets or colored spores were also recorded. Representative isolates of the morphological groups have been sequenced, and phylogenetic analyses of the internal transcribed spacer (ITS) regions of their ribosomal genes have been conducted (Martín et al. 2013).

This work focused on endophytic isolates representing two species that are frequently found in elms with low DED susceptibility (Martín et al. 2013): *Pyrenochaeta cava* (Schulzer) Gruyter, Aveskamp & Verkley [MB#514652] (growth rate 1.5 mm day^{-1}), which was isolated from xylem samples, and *Monographella nivalis* var. *neglecta* (Krampe) Gerlach [MB#113869], which was represented by two isolates, one originating from xylem (isolate 33; growth rate 1.2 mm day^{-1}) and the other from bark (isolate 114; growth rate 1 mm day^{-1}), i.e. the tissues occupied by the DED pathogen. *M. nivalis* var. *neglecta* is the teleomorph of *Microdochium majus* (Wollenw.) Glynn & S. G. Edwards which is the current name of *Fusarium nivale* var. *majus* Wollenw. (Glynn et al. 2005). This fungus is mainly known as a snow mold and endophyte of grasses and cereals (Dahl 1934; Sieber et al. 1988) but was recently reported to cause symptomless endophytic infections in elms (Martín et al. 2013). According to our hypothesis, these isolates should represent endophyte species with the potential to antagonize the DED pathogen *in planta*, and we anticipated that they would exhibit extensive niche overlap with the pathogen. To validate the proposed relationship between the C-utilization patterns of these endophytes and their effects on the host tree's DED susceptibility, we also studied a third endophyte isolate as a negative control. A single *Aureobasidium pullulans* (de Bary) G. Arnaud [MB#101 771] (growth rate 3.2 mm day^{-1}) isolate originating from a leaf sample was used for this purpose. This species' presence has been shown to have no relation to the host elm's susceptibility to DED (Martín et al. 2013). This species is a ubiquitous endophyte and epiphyte (Albrectsen et al. 2010; Zalar et al. 2008). We therefore expected it to be flexible in its C-utilization preferences and anticipated that it would probably deviate from the pathogen's C-utilization profile. As a representative pathogenic fungus, we selected the aggressive *O. novo-ulmi* subsp. *americana* Brasier & S. A. Kirk isolate SO-SE (Solla et al. 2008). This strain was isolated in 2002 from a DED-infected *U. minor* tree in San Sebastián de Gormaz (Soria, Spain, 41°34′N 3°12′W), and has an in vitro growth rate of 5 mm day^{-1} at 20 °C in MEA.

Phenotype microarrays

We used phenotype microarrays (PMs; Biolog Phenotype MicroArrays^{TM}) to assess the competitive capacity of the selected fungi and determine their carbon (C) utilization profiles. Carbon substrates were considered as particularly interesting in this context because of the essential importance of carbon substrates for heterotrophic fungi.

Phenotype microarray experiments were performed using PM1 and PM2A Biolog MicroArray^{TM} plates in order to cover a broad spectrum of carbon substrates.

Every microarray plate has 96 micro-wells, 95 of which are filled with a pre-determined set of different C-sources, the remaining micro-well contains no substrate and serves as a control. Both the PM1 and PM2A plates have wells containing a wide range of C sources including mono-, di-, tri-, tetra-, and methyl saccharides, saccharide phosphates, phenolic compounds, amino acids, and carboxylic acids. In addition, PM1 plates have wells containing alcohols, nucleosides, and surfactants, while PM2A plates have polysaccharide-containing wells. Each well on a plate also contains a redox dye (tetrazolium violet) as an indicator. If the substrate in a particular well is metabolized by the fungus, the tetrazolium violet is reduced to formazan, resulting in a change in that well's absorbance. The colour change in each well of the microtiter plates is recorded by a reader at user-specified time-intervals (Garland and Mills 1991).

Prior to inoculation on the plates, the fungi were cultivated on MEA for at least one week at 25 °C in the dark in order to obtain adequate amount of fungal material from the mycelial growth phase. The superficial layer of the mycelial mat was scraped with a sterile cotton swab to transfer fungal biomass from the colonies (a mixture of spores and mycelial cells) into sterile glass tubes containing 12 ml of an inoculating fluid provided by Biolog. A tissue grinder (Kontes® Duall® 21, Kimble Chase) was used to gently disrupt the fungal biomass to make the inoculum more homogenous. The transmittance of each inoculum was adjusted to 62 % by adding more fungal biomass or inoculation fluid as required. For each isolate, an aliquot of 0.05 ml of the final inoculum was mixed with 23.95 ml of inoculating fluid in a sterile plastic vial, and 100 µl of the resulting mixture was pipetted into the wells. Three replicate plates were prepared for each isolate. The inoculated plates were kept at 20 °C for 30 min to enable gelatinization, after which they were sealed and incubated at 25 °C in darkness between readings. Substrate absorbance at 590 nm was recorded using a microreader (Dogoscan, Asys, Linz, Austria; software: Digiwin). Readings were performed every 24 h for ten days. Absorbances were calibrated relative to the empty well on each plate and the calibrated absorbances (Ac; range −0.345–2.019) were used in all subsequent analyses. In cases where a negative absorbance was recorded, a value of zero was assumed for analytical purposes (Garland and Mills 1991).

Data analysis

Time point selection

Hierarchical clustering (Ward) was used to identify the time period that best separated the fungal isolates according to their consumption of different substrates. All statistical analyses were performed using JMP Version 7.0.

There was no or poor separation of the isolates during the first four time points (0–72 h) (Fig. 1a), but a higher level of clustering by species was observed subsequently. The greatest separation of the different species based on their C-utilization patterns occurred at 168, 192 and 216 h (Fig. 1b). We therefore focused on data collected between the 168 and 240 h time-points in our analyses (192–240 h for PM 1 plates and 168–240 h for PM 2A plates).

Determination of isolate-specific C-use profiles

Principal component analysis (PCA) revealed that the technical replicates for individual isolates clustered strongly with one-another with respect to the average well color development (AWCD), which represents the isolate's ability to utilize specific carbon sources (data not shown). Therefore, average A_C values ($\bar{A}_C$) were calculated for each fungal isolate and C-source across the three replicate plates. $\bar{A}_C$-values above 0.1 were considered to indicate utilization of the corresponding carbon source. The tested substrates were divided into 14 substrate groups (Table 1) based on their chemical properties (Garland and Mills 1991). The average substrate utilization was then determined for each substrate group across all wells using the following equation: $AWCD_{\text{chemical group}} = \Sigma\bar{A}_C/N$ (number of substrates in a specific group). The isolate-specific $AWCD_{\text{chemical group}}$ value was then used to compare the C-utilization patterns of the endophytes and the pathogen as described previously (Haack et al. 1995).

Competition for resources among fungal isolates

To compare the pathogen's C-utilization patterns to those of the endophytes, a NOI was calculated according to Eq. 1 (Lee and Magan 1999; Wilson and Lindow 1994), in which the endophyte of interest is referred to as the target fungus:

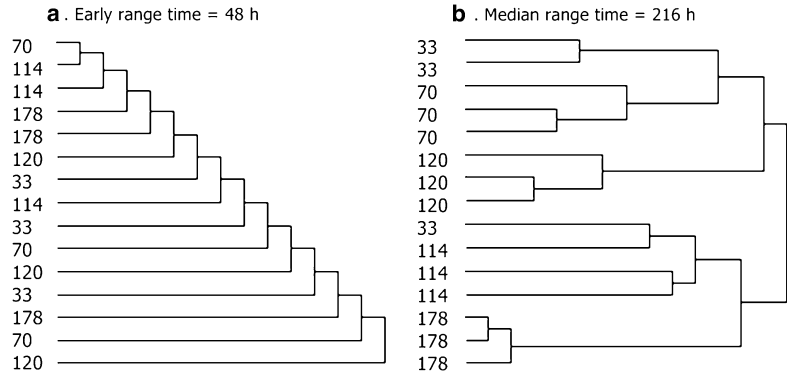


Fig. 1 Representative dendrograms generated via hierarchical clustering analyses of the responses during the early and intermediate stages of the utilization measurements. The highest degree of consistency between replicate analyses of individual fungal isolates was observed at intermediate time points (shown in subfigure **b**, "Median range"). Numbers at the *left side* stand for the studied isolates [*Aureobasidium pullulans* (70), *Monographella nivalis* var. *neglecta* (33 and 114), *Pyrenochaeta cava* (120), and *Ophiostoma novo-ulmi* (178)] and for each of the three replicates

$$\mathrm{NOI} = \frac{\text{number of C - sources shared by both pathogen and endophyte}}{\text{total no C - sources utilized by target fungus}} \quad (1)$$

A NOI value of 0.9 or above indicates a high degree of niche overlap and a competitive disadvantage for the target fungus (Lee and Magan 1999). We also developed a function to quantify each endophyte's ability to compete with the pathogen (Eq. 2). If the value of this function is greater than 1.0, the endophyte exhibits competitive superiority relative to the pathogen.

$$\text{Endophyte competitiveness} = \frac{\mathrm{NOI}_{\text{pathogen}}}{\mathrm{NOI}_{\text{endophyte}}} \quad (2)$$

Results

Isolate-specific niche sizes

The niche size, i.e. the total number of C-sources utilized by the fungus of interest, varied among the endophytic isolates. The endophytic isolate *M. nivalis* var. *neglecta* 114 had the largest niche and used 71 % of the 190 tested substrates. Its conspecific isolate *M. nivalis* var. *neglecta* 33 utilized 62 %, while *P. cava* utilized 60 % of the 190 tested substrates. The pathogenic *O. novo-ulmi* isolate utilized 54 % of the tested 190 substrates, and the endophytic *A. pullulans* had the smallest niche size, utilizing only 22 % of the tested substrates (Table 1).

Isolate-specific selectivity in C-substrate utilization (preferred and non-preferred substrates)

All of the tested fungi had a high capacity for the utilization of tri- and tetrasaccharides (Table 1). The pathogen *O. novo-ulmi* and endophytes *M. nivalis* var. *neglecta* 114 and *P. cava* also utilized all of the tested surfactants. The pathogen and all of the endophytes other than *A. pullulans* were able to utilize a high proportion (over 80 %) of the individual disaccharide compounds. All four of the endophyte isolates were able to utilize 80–100 % of the phenolic compounds, but the pathogen was only able to metabolize 60 % of these substances.

The potentially antagonistic endophyte isolates were also highly capable utilizers of C-substrates in several other compound groups. This was particularly true for *M. nivalis* var. *neglecta* 114, which was able to

Table 1 Utilization of and competition for different carbon sources by the pathogenic fungus *Ophiostoma novo-ulmi* (On) and the endophytic fungi *Monographella nivalis* var. *neglecta* (Mn 33 and 114), *Pyrenochaeta cava* (Pc), and *Aureobasidium pullulans* (Ap)

Compound group	Total (nr)[a]	Substrates utilized (% from total nr)					Niche overlap index (NOI)[b]				Endophyte competitiveness index[c]			
		On	Mn33	Mn114	Pc	Ap	Mn33	Mn114	Pc	Ap	Mn33	Mn114	Pc	Ap
Sugar alcohols	9	78	67	67	78	56	**1.00**	**1.00**	**1.00**	**1.00**	0.86	0.86	1.00	0.71
Sacc. phosphates	4	0	0	50	0	0	n.a.				n.a.			
Tri-and tetra sacc.	4	100	100	100	100	100	**1.00**	**1.00**	**1.00**	**1.00**	1.00	1.00	1.00	1.00
Polysaccharides	11	73	73	82	73	45	**1.00**	0.89	0.88	**1.00**	1.00	**1.13**	1.00	0.63
Monosaccharides	19	74	74	63	74	42	0.86	**1.00**	**0.93**	**1.00**	1.00	0.86	1.00	0.57
Methyl-saccharides	5	20	80	60	80	40	0.25	0.33	0.25	0.50	**4.00**	**3.00**	**4.00**	**2.00**
Disaccharides	10	80	90	100	100	60	0.89	0.80	0.80	**1.00**	**1.13**	**1.25**	**1.25**	0.75
Phenolics	5	60	80	100	100	80	0.75	0.60	0.60	0.75	**1.33**	**1.67**	**1.67**	**1.33**
Amino acids	27	56	81	89	74	4	0.64	0.63	0.75	**1.00**	**1.47**	**1.60**	**1.33**	0.07
Alcohols	5	40	60	80	40	40	0.67	0.50	**1.00**	**1.00**	**1.50**	**2.00**	1.00	1.00
Acids	54	43	54	65	37	2	0.62	0.60	0.80	**1.00**	**1.26**	**1.52**	0.87	0.04
Nucleosides	5	0	60	100	40	0	n.a.				n.a.			
Miscellaneous	29	48	31	45	48	10	0.89	**0.92**	0.86	**1.00**	0.64	0.93	1.00	0.21
Surfactants	3	100	67	100	100	0	**1.00**	**1.00**	**1.00**	n.a.	0.67	1.00	1.00	n.a.
Σ (nr) or Average (%)	190	**54**	**62**	**71**	**59**	**22**								

n.a. not available

[a] Number of substrates on the Biolog PM1 and PM2 plates utilized at an AWCD rate of ≥0.1. Substrates were classified into different chemical groups as described by Garland and Mills (1991) and Lee and Magan (1999)

[b] The niche overlap index (NOI; Lee and Magan 1999) compares the number of substrates utilized by both the pathogen and the endophyte to the total number of substrates utilized by the endophyte. A value of 0.9 or higher (in bold) indicates a high degree of niche overlap and a competitive disadvantage for the endophyte in question (see Eq. 1 in the Materials and Methods section)

[c] Endophyte competitiveness indicates the relative rate of substrate use by the pathogen compared to that of a given endophyte. A value of ≥1 (bold letters) indicates that the endophyte is more effective at utilizing the compound type in question (see Eq. 2 in the Materials and Methods section)

utilize more than 80 % of the individual compounds in five additional groups: polysaccharides, amino acids, alcohols, nucleosides and surfactants (Table 1). The two *M. nivalis* var. *neglecta* isolates and *P. cava* utilized over 80 and 74 %, respectively, of the tested amino acids, while *M. nivalis* var. *neglecta* 33 and *P. cava* utilized over 80 % of the methyl-saccharides.

Some of the compound groups were clearly less preferred by the tested isolates. For instance, only *M. nivalis* var. *neglecta* 114 utilized saccharide phosphates (Table 1). The endophyte *A. pullulans* was the most selective, utilizing at most 10 % of the amino acids, acids, and miscellaneous compounds, and none of the saccharide phosphates, nucleosides, or surfactants (Table 1). The potentially antagonistic endophyte isolates *M. nivalis* var. *neglecta* 114 and 33 and *P. cava* utilized most of the substrate groups effectively, including more than 30 % of the substrate groups for which the extent of their competition with the pathogen could be determined (Table 1).

Isolate-specific rates of C-utilization

Compared to the endophytes, the pathogen, *O. novo-ulmi,* utilized most of the tested C-substrates at an intermediate level, with AWCD values ranging from 0.01 to 0.44 (Fig. 2). The two *M. nivalis* var. *neglecta* isolates (especially no. 114) and *P. cava* utilized C sources at a higher intensity than the pathogen and *A. pullulans,* which had AWCD values ranging from

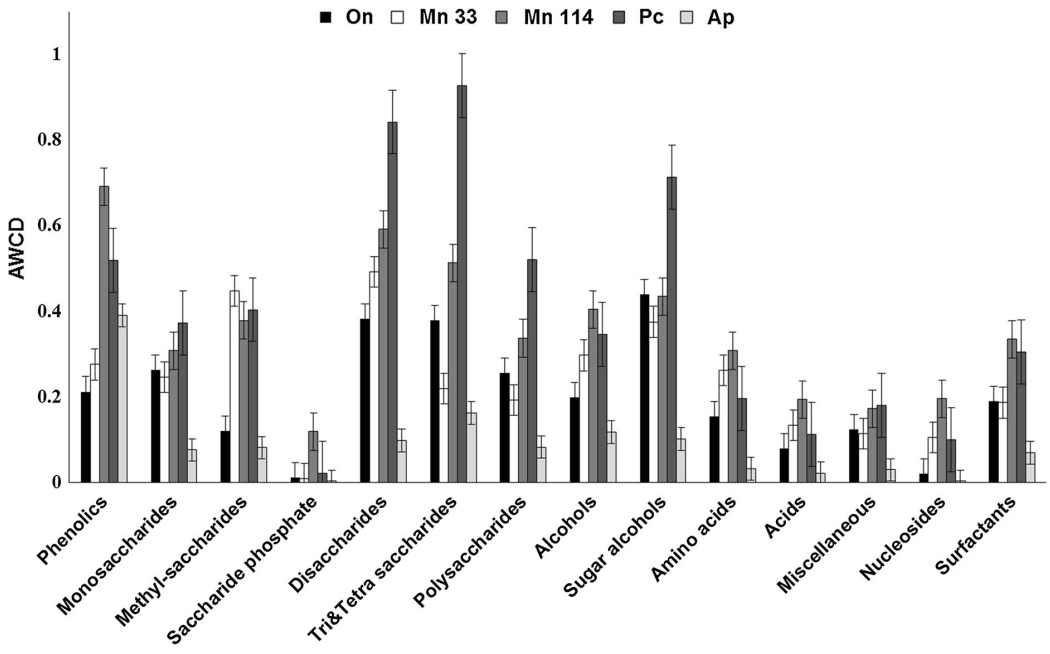


Fig. 2 Utilization of 14 carbon-substrate groups by four species of microfungi found in elm trees (*Ulmus* spp.): the pathogenic *Ophiostoma novo-ulmi* (On) and the endophytic fungi *Monographella nivalis* var. *neglecta* (Mn 33 and 114), *Pyrenochaeta cava* (Pc), and *Aureobasidium pullulans* (Ap). The figure shows the average well color development values (AWCD) for each isolate, as determined using Biolog Phenotype MicroArrays™ (PM 1 and PM 2A plates, n = 3). The *error bars* indicate SE

0.003 to 0.39. The isolate *A. pullulans* utilized most substrates at a low intensity with the exception of phenolics, for which it had an AWCD value of 0.39 (Fig. 2).

Competition for C-substrates between endophytes and the pathogen

There was extensive overlap between the four endophyte isolates and the pathogen with respect to the compound groups they utilized, particularly among the sugar alcohols, monosaccharides and surfactants (Table 1). Two substrate groups were not considered when evaluating the potential competition between the pathogen and the endophytes: the saccharide phosphates, which were generally not utilized, and the nucleosides, which were only utilized by the potential antagonists *M. nivalis* var. *neglecta* and *P. cava*. *A. pullulans* did not utilize surfactants at all.

The pathogen was competitive with or as proficient as the endophytes with respect to the utilization of sugar alcohols, tri- and tetra-saccharides, monosaccharides, surfactants and miscellaneous compounds as demonstrated by the high NOI values and low endophyte competitiveness values (Table 1). All of the endophytes, including the unlikely antagonist *A. pullulans*, were highly competitive with the pathogen for methyl-saccharides and phenolics, as indicated by the low NOI values and high competitiveness values for these substrate groups (Table 1). In addition, the pathogen was found to have a high degree of niche overlap and to be at a competitive disadvantage relative to the two *M. nivalis* var. *neglecta* isolates and *P. cava* with respect to several other compound groups (Table 1). The isolate *M. nivalis* var. *neglecta* 114 was the strongest competitor against the pathogen: in addition to methyl-saccharides and phenolics, this isolate effectively competed with *O. novo-ulmi* for

polysaccharides, disaccharides, amino acids, alcohols and acids (Table 1). The isolate *M. nivalis* var. *neglecta* 33 was competitive for four additional groups of compounds (disaccharides, amino acids, alcohols and acids), while *P. cava* outcompeted the pathogen for two additional groups—disaccharides and amino acids (Table 1).

Discussion

In contrast to our initial hypothesis, the DED pathogen, represented by *O. novo-ulmi* strain 178, was not the most effective utilizer of carbon substrates, either in terms of the range of substrates it utilized or the rate at which it utilized them. Some previous studies of *Ophiostoma*-species (Brasier 1991) and other necrotrophic pathogens have indicated the existence of positive correlations between growth rates and pathogenicity or virulence (Brasier and Webber 1987; Meyer et al. 2010; Pagán et al. 2007; Solheim and Krokene 1998), and between virulence and the production of extracellular enzymes (Annis and Goodwin 1997; Kaur and Padmaja 2009; Tonukari 2003). For example, *O. ulmi* has slower mycelium growth than the more virulent *O. novo-ulmi* at 20 °C (Brasier 1991). We therefore expected the fast-growing, highly virulent pathogen to use a broader array of substrates or to catabolize them at higher rates compared to the less virulent endophytic fungi. Our results show that the slow growing endophytes *P. cava* and *M. nivalis* var. *neglecta* exhibited the most aggressive substrate utilization patterns, and that the generalist fungus *A. pullulans* had the lowest intensity of substrate use and the narrowest niche. This suggests that the relationships between virulence, growth rate and substrate utilization efficiency among microfungi are highly complex. Similar conclusions were drawn from a previous study (Klepzig 1998), in which it was demonstrated that slower growing fungi were more efficient at defending their colonized habitats from invading pathogens. Further information about the relation between growth rate, virulence and exoenzyme production could also be gained through phenotype microarray comparisons of the slow growing, less virulent *O. ulmi* and the highly virulent *O. novo-ulmi* isolates (Brasier 1991).

Our results support our second hypothesis: the endophytes *M. nivalis* var. *neglecta* and *P. cava*, which were both identified as potential DED biocontrol agents in a previous study (Martín et al. 2013), had substrate utilization patterns that overlapped extensively with that of the pathogen. This was particularly true for some substrates that are central to carbon metabolism, such as sugar alcohols, tri- and tetrasaccharides, and monosaccharides, and for fatty acids (surfactants). While the *P. cava* isolate had the highest utilization rates for sugars (monosaccharides, disaccharides, tri- and tetra-saccharides, polysaccharides and sugar alcohols), the *M. nivalis* var. *neglecta* 114 isolate had the broadest niche in terms of C-substrate utilization. The ability of this endophyte to catabolize most of the tested carbon sources suggests that it has a complex arsenal of catabolic enzymes, which may compensate for its rather slow growth. Interestingly, it was recently found that this endophyte also secretes bioactive chemicals into its growth medium and that these chemicals halt the growth of *O. novo-ulmi* colonies in dual cultures (K. Blumenstein et al. unpublished data). These results suggest that endophytes may use multiple tools to compete with other fungi, and that the different components of their arsenals may be deployed sequentially or simultaneously. Fungi that can antagonize pathogen growth in multiple ways can be expected to have considerable potential as biocontrol agents since their antagonism should be both stable and highly effective.

The finding that the generalist fungus *A. pullulans* had a low intensity of substrate use and a narrow spectrum of acceptable substrates was unexpected. As a pleiomorphic fungus, *A. pullulans* (like *O. novo-ulmi)* is known to switch between yeast and mycelial forms in response to its environmental conditions. Because of the industrial interest in the polysaccharide pullulan that *A. pullulans* produces, it is a well-studied fungus (Slepecky and Starmer 2009). Strains of *A. pullulans* produce a variety of enzymes, including amylase, xylanase, and 13-glucosidase (Gaur et al. 2010) and given that this species is omnipresent in environmental samples (Gaur et al. 2010; Slepecky and Starmer 2009; Zalar et al. 2008), we expected it to utilize a broad range of different carbon sources at a high rate. Instead, the generally narrow substrate range and low substrate utilization rate of *A. pullulans* in our tests suggest that this species is adapted to a small number of nutritional sources and that its establishment and survival in diverse environments is instead due to a highly efficient metabolic system. Preliminary

results (data not shown) indicate that at least one other *A. pullulans* isolate has a similar C-utilization pattern, so the narrow substrate range and low intensity C-utilization observed in this work may be characteristic of the species as a whole rather than an isolate-specific phenomenon. However, we cannot exclude the possibility that some other compound or compound class that was not considered in this work might be utilized more extensively by this fungus. In addition to C utilization, it would also be interesting to compare the nitrogen (N)-utilization patterns of endophytes and pathogens, in particular as it has been suggested that the N content differs between resistant and susceptible elms (Singh and Smalley 1969) and because it is known that different N sources influence the pleomorphism in DED fungi (Kulkarni and Nickerson 1981).

We found that all four endophyte isolates utilized phenolic substrates more effectively than the pathogen, suggesting that the efficient metabolization of these compounds may be important for the life strategies of endophytes in general. This result is consistent with the suggested role of phenolic compounds as defensive metabolites that are synthesized to counteract pathogen infections (Witzell and Martin 2008). Notably, such responses have been proposed to occur in elms (Martín et al. 2013). However, the phenolics were the only compound class for which there was a readily apparent pathogen- or endophyte-specific C substrate utilization pattern. Instead, the C substrate utilization profiles seemed to be highly specific for each fungal isolate.

The two conspecific *M. nivalis* var. *neglecta* isolates differed somewhat in their C-utilization patterns, indicating that there is appreciable functional variation within the species and emphasizing the importance of including several isolates of the same species in phenotype microarray screens when seeking species-level information. The intraspecific variation of morphological and physiological traits is well documented also for *O. novo-ulmi* (Brasier 1991) and should thus be accounted for in future microarray-based studies. Only four endophyte isolates were considered in this work, all of which were selected based on their potential effects on the host tree's DED-susceptibility (Martín et al. 2013). While this small number of isolates was sufficient to test our hypothesis, it is clear that a larger number of pathogen and endophyte species and isolates will have to be tested in order to draw general conclusions about the characteristic C-utilization profiles for different fungal life strategies and to more accurately quantify the extent of within-species variation. A deeper understanding of within- and between-species variation in substrate utilization patterns among endophytes could be particularly important for biodiversity conservation efforts. In particular, if there is substantial intraspecific variation in endophytes' functional traits, and if these traits contribute significantly to the ability of a given species to perform ecosystem services (e.g. the degradation of wood), then it may be desirable to take measures in order to maintain endophyte diversity as discussed elsewhere (Brasier 1991; Martín et al. 2013).

The niche concept posits that niches are defined by the requirements and impacts of the species that are present, which in turn determine whether a given set of species can coexist in a given ecological community (Chase and Leibold 2003). Our current understanding of the requirements and effects of most endophytic fungi in trees, including the species studied in this work, is either rudimentary or completely lacking. This presents significant difficulties when analyzing competitive interactions within the *in planta* mycobiome. However, we know that carbon assimilation is essential for all fungi, and the quality and quantity of carbon sources available within the host are widely acknowledged to influence the composition of the fungal communities in plants (Ernst et al. 2011 and refs. within). Moreover, although an array of C substrates on a microtiter plate cannot accurately mimic the *in planta* chemical environment inside the host trees, it can be used to assess the exoenzymatic capacity of the tested fungi and was therefore chosen to be efficient in testing our hypothesis. The observed overlap in the C-utilization profiles of the different fungal isolates examined in this work suggests that at least some of the *M. nivalis* var. *neglecta* isolates and *P. cava* resemble the pathogen *O. novo-ulmi* in terms of their carbon use profiles and are therefore likely to compete with it for C substrates *in planta*. This is important because C is an essential resource for fungi, hence the presence of these endophytes in the pathogen's habitats (i.e. the bark and xylem of elms) may hinder its growth. These habitats are colonized horizontally by endophytes that originate from the tree's external environment, and the invasions accumulate over the tree's long life span (Helander et al.

1994). Established endophytic infections in the bark could be in good position to compete with an additional infestation of the pathogen by conidia that, according to Santini and Faccoli (2015), can be transmitted to the bark by mature beetles.

Stochastic events can therefore be expected to play a major role in the formation of fungal communities inside trees, in keeping with the neutral hypothesis (Hubbell 2001). However, our results provide evidence for the existence of physiological mechanisms that potentiate niche differentiation among members of the tree-associated mycobiome. Future studies on the cohabitant patterns of tree endophytes and pathogens might therefore benefit from the application of the stochastic niche hypothesis (Tilman 2004), which modifies the competitive trade-off hypothesis by including stochastic processes such as those proposed by the neutral theory. Future analyses of cohabitant patterns would also benefit from systematic studies on the spatial and temporal distributions of endophyte communities in trees.

Acknowledgments This work was supported by the Swedish Research Council FORMAS (project 2008-1090); Stiftelsen Konsul Faxes Donation, Sweden (projects KF 23 and KF 29); Ministerio de Ciencia e Innovación, Spain, project AGL2009-09289; Ministerio de Economía y Competitividad, Spain (project CTQ2011-28503-C02-02); the Spanish elm breeding program (Ministerio de Agricultura, Alimentación y Medio Ambiente; Universidad Politécnica de Madrid); and the Joint Doctoral Program "Forest and Nature for Society", FONASO. The English language was edited by Sees editing Ltd, North Somerset, UK. The work was carried out as a part of research aiming to elucidate the role of endophytes in Dutch elm disease (DED) complex, initiated by Johanna Witzell and Juan Martín in 2008. An important goal of the research is to identify endophytes with biocontrol potential against DED.

References

Ahlholm J, Helander M, Elamo P, Saloniemi I, Neuvonen S, Hanhimäki S, Saikkonen K (2002) Micro-fungi and invertebrate herbivores on birch trees: fungal mediated plant-herbivore interactions or responses to host quality? Ecol Lett 5:648–655

Albrectsen BR, Björkén L, Varad A, Hagner Å, Wedin M, Karlsson J, Jansson S (2010) Endophytic fungi in European aspen (*Populus tremula*) leaves—diversity, detection, and a suggested correlation with herbivory resistance. Fungal Divers 41:17–28

Albrectsen BR, Albrectsen BR, Witzell J, Witzell J (2012) Disentangling functions of fungal endophytes in forest trees. In: Paz Silva A, Sol M (eds) Fungi: Types, environmental impact and role in disease. Nova Science Publishers, New York, pp 235–246

Annis SL, Goodwin PH (1997) Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. Eur J Plant Pathol 103:1–14

Arnold AE (2007) Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biol Rev 21:51–66

Arnold AE, Maynard Z, Gilbert GS, Coley PD, Kursar TA (2000) Are tropical fungal endophytes hyperdiverse? Ecol Lett 3:267–274

Bernier L, Yang D, Ouellette GB, Dessureault M (1996) Assessment of *Phaeotheca dimorphospora* for biological control of the Dutch elm disease pathogens, *Ophiostoma ulmi* and *O. novo-ulmi*. Plant Pathol 45:609–617

Brasier CM (1991) *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. Mycopathologia 115:151–161

Brasier CM, Webber JF (1987) Positive correlations between in vitro growth rate and pathogenesis in *Ophiostoma ulmi*. Plant Pathol 36:462–466

Chase JM, Leibold MA (2003) Ecological niches: linking classical and contemporary approaches. University of Chicago Press, Chicago

Dahl AS (1934) Snowmold of turf grasses as caused by *Fusarium nivale*. Phytopathology 24(3):197–214

Dvořák M, Palovčíková D, Jankovský L (2006) The occurrence of endophytic fungus *Phomopsis oblonga* on elms in the area of southern Bohemia. J For Sci 52:531–535

Ernst M, Neubert K, Mendgen KW, Wirsel SGR (2011) Niche differentiation of two sympatric species of *Microdochium* colonizing the roots of common reed. BMC Microbiol 11:242–254

Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Appl Environ Microb 57:2351–2359

Gaur R, Singh R, Gupta M, Gaur MK (2010) *Aureobasidium pullulans*, an economically important polymorphic yeast with special reference to pullulan. Afr J Biotechnol 9:7989–7997

Glynn NC, Hare MC, Parry DW, Edwards SG (2005) Phylogenetic analysis of EF-1 alpha gene sequences from isolates of *Microdochium nivale* leads to elevation of varieties *majus* and *nivale* to species status. Mycol Res 109:872–880

Haack SK, Garchow H, Klug MJ, Forney LJ (1995) Analysis of factors affecting the accuracy, reproducibility, and interpretation of microbial community carbon source utilization patterns. Appl Environ Microb 61:1458–1468

Hamilton CE, Gundel PE, Helander M, Saikkonen K (2012) Endophytic mediation of reactive oxygen species and antioxidant activity in plants: a review. Fungal Divers 54:1–10

Helander ML, Sieber T, Petrini O, Neuvonen S (1994) Ecology of pine needle endophytes: spatial variation and consequences of acid irrigation. Can J Bot 72:1108–1113

Hubbell SP (2001) Monographs in population biology, the unified neutral theory of biodiversity and biogeography. Princeton University Press, Princeton

Hubbes M, Jeng RS (1981) Aggressiveness of *Ceratocystis ulmi* strains and induction of resistance in *Ulmus americana*. Eur J For Path 11:257–264

Kaur G, Padmaja V (2009) Relationships among activities of extracellular enzyme production and virulence against *Helicoverpa armigera* in *Beauveria bassiana*. J Basic Microb 49:264–274

Klepzig KD (1998) Competition between a biological control fungus, *Ophiostoma piliferum*, and symbionts of the southern pine beetle. Mycologia 90:69–75

Kulkarni RK, Nickerson KW (1981) Nutritional control of dimorphism in *Ceratocystis ulmi*. Exp Mycol 5:148–154

Lee HB, Magan N (1999) Environmental factors and nutritional utilization patterns affect niche overlap indices between *Aspergillus ochraceus* and other spoilage fungi. Lett Appl Microbiol 28:300–304

Martín JA, Solla A, Domingues MR, Coimbra MA, Gil L (2008) Exogenous phenol increase resistance of *Ulmus minor* to Dutch elm disease through formation of suberin-like compounds on xylem tissues. Environ Exp Bot 64:97–104

Martín JA, Solla A, Esteban LG, de Palacios P, Gil L (2009) Bordered pit and ray morphology involvement in elm resistance to *Ophiostoma novo-ulmi*. Can J For Res 39:420–429

Martín JA, Fuentes-Utrilla P, Gil L, Witzell J (2010) Ecological factors behind the Dutch elm disease complex in Europe—a review. Ecol Bull 53:209–224

Martín JA, Witzell J, Blumenstein K, Rozpedowska E, Helander M, Sieber TN, Gil L (2013) Resistance to Dutch elm disease reduces xylem endophytic fungi presence in elms (*Ulmus* spp.). PLoS ONE 8:e56987

Mejía LC, Rojas EI, Maynard Z, van Bael S, Arnold AE, Hebbar P, Samuels GJ, Robbins N, Herre EA (2008) Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. Biol Control 46:4–14

Meyer SE, Stewart TE, Clement S (2010) The quick and the deadly: growth vs virulence in a seed bank pathogen. New Phytol 187:209–216

Mikkelson GM (2005) Niche-based vs. neutral models of ecological communities. Biol Phil 20:557–566

Neely D, Himelick EB (1963) Root graft transmission of Dutch elm disease in municipalities. Plant Dis Rep 47:83–85

Newcombe G (2011) Endophytes in forest management: four challenges. In: Pirttilä AM, Frank AC (eds) Endophytes in forest trees: biology and applications. Springer, Dordrecht, pp 251–262

Pagán I, Alonso-Blanco C, García-Arenal F (2007) The relationship of within-host multiplication and virulence in a plant-virus system. PLoS ONE 2:e786

Rodriguez R, Redman R (2008) More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. J Exp Bot 59:1109–1114

Rudinsky JA (1962) Ecology of Scolytidae. Annu Rev of Entomol 7:327–348

Saikkonen K, Faeth SH, Helander M, Sullivan TJ (1998) Fungal endophytes: a continuum of interactions with host plants. Annu Rev Ecol Syst 29:319–343

Saikkonen K, Ahlholm J, Helander M, Poteri M, Tuominen J (2001) Experimental testing of rust fungus-mediated herbivory resistance in *Betula pendula*. Forest Pathol 31:321–329

Saikkonen K, Wäli P, Helander M, Faeth SH (2004) Evolution of endophyte-plant symbioses. Trends Plant Sci 9:275–280

Santini A, Faccoli M (2015) Dutch elm disease and elm bark beetles: a century of association. iForest 8:126–134

Scheffer RJ, Voeten JGWF, Guries RP (2008) Biological control of Dutch elm disease. Plant Dis 92:192–200

Sieber T, Riesen TK, Müller E, Fried PM (1988) Endophytic fungi in four winter wheat cultivars (*Triticum aestivum* L.) differing in resistance against *Stagonospora nodorum* (Berk.) Cast. & Germ. = *Septoria nodorum* (Berk.) Berk. J Phytopath 122:289–306

Singh D, Smalley EB (1969) Nitrogenous and carbohydrate compounds in the xylem sap of *Ulmaceae* species varying in resistance to Dutch elm disease. Can J Bot 47:335–339

Slepecky RA, Starmer WT (2009) Phenotypic plasticity in fungi: a review with observations on *Aureobasidium pullulans*. Mycologia 101:823–832

Solheim H, Krokene P (1998) Growth and virulence of mountain pine beetle associated blue-stain fungi, *Ophiostoma clavigerum* and *Ophiostoma montium*. Can J Bot 76:561–566

Solla A, Dacasa MC, Nasmith C, Hubbes M, Gil L (2008) Analysis of Spanish populations of *Ophiostoma ulmi* and *O. novo-ulmi* using phenotypic characteristics and RAPD markers. Plant Pathol 57:33–44

Tellenbach C, Sieber TN (2012) Do colonization by dark septate endophytes and elevated temperature affect pathogenicity of oomycetes? FEMS Microbiol Ecol 82:157–168

Tilman D (2004) Niche tradeoffs, neutrality, and community structure: a stochastic theory of resource competition, invasion, and community assembly. P Natl Acad Sci USA 101:10854–10861

Tonukari NJ (2003) Enzymes and fungal virulence. J Appl Sci Environ Manag 7:5–8

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

Webber JF, Brasier CM (1984) The transmission of Dutch elm disease: A study of the process involved. In: Anderson JM, Rayner ADM, Walton D (eds) Invertebrate-microbial interactions. Cambridge University Press, Cambridge, pp 271–306

White JF, Torres MS (2010) Is plant endophyte-mediated defensive mutualism the result of oxidative stress protection? Physiol Plant 138:440–446

Wilson M, Lindow SE (1994) Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. Appl Environ Microb 60:4468–4477

Witzell J, Martin JA (2008) Phenolic metabolites in the resistance of northern forest trees to pathogens—past experiences and future prospects. Can J For Res 38:2711–2727

Witzell J, Martín JA, Blumenstein K (2013) Ecological aspects of endophyte-based biocontrol of forest diseases. In: Verma VC, Gange AC (eds) Advances in endophytic research. Springer-Verlag, Heidelberg, pp 321–333

Zalar P, Gostinčar C, de Hoog GS, Uršič V, Sudhadham M, Gunde-Cimerman N (2008) Redefinition of *Aureobasidium pullulans* and its varieties. Stud Mycol 61:21–38

Kathrin Blumenstein is a Ph. D. student focusing on interactions between elm endophytes and *Ophiostoma*-pathogens.

Benedicte R. Albrectsen is an ecologist with special interest in plant resistance to biotic stress.

Juan A. Martín is a forest pathologist with specific expertise in DED and resistance mechanisms in elms.

Malin Hultberg is an expert in microbes in plant production systems and biocontrol.

Thomas N. Sieber is lecturer in forest pathology and a specialist in endophytic fungi of forest trees.

Marjo Helander has specialist expertise in plant-endophyte interactions and their environmental regulation.

Johanna Witzell is a specialist in tree resistance mechanisms and endophytes of forest trees.