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Department of Forest Sciences
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Environmental Impact of Using *Phlebiopsis gigantea* in Stump Treatment Against *Heterobasidion annosum sensu lato* and Screening Root Endophytes to Identify Other Novel Control Agents

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public examination in lecture room B6, B-building (Latokartanonkaari 9), on September 4th 2015, at 12 o´clock

Helsinki 2015

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Cover: Interaction between *Heterobasidion parviporum* and root endophytes *in vitro* 14 days post-inoculation (upper pictures). *Heterobasidion parviporum* hyphae after exposed to extracted metabolites of root endophytes (bottom pictures).

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To my kids Inka and Roni

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ABBREVIATIONS

C-endophytes	Clavicipitaceous endophytes
DAMP	Damage associated molecular patterns
DSE	Dark septate endophytes
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
ETI	Effector-triggered immunity
HR	Hypersensitive response
HRMS	High resolution mass spectrometry
IPM	Integrated pest management
ITS	Internal transcribed spacer
LMW	Low-molecular weight
MAMP	Microbe associated molecular pattern
MEA	Malt extract agar
NC-endophytes	Non-clavicipitaceous endophytes
NGS	Next generation sequencing
NMR	Nuclear magnetic resonance
OPLS-DA	Orthogonal partial least squared discrimination analysis
OTU	Operational taxonomic unit
PAC	*Phialocephala fortinii sensu lato*-*Acephala applanata* species complex
PAMP	Pathogen associated molecular pattern
PCA	Principal component analysis
PCD	Programmed cell death
PCR	Polymerase chain reaction
PP-cells	Polyphenolic parenchyma cells
RDA	Redundancy analysis
RFLP	Restriction fragment length polymorphism
UPLC-QTOF/MS	Ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry
16S rRNA	16S ribosomal ribonucleic acid

LIST OF ORIGINAL PUBLICATIONS AND SUBMITTED MANUSCRIPTS

This doctoral thesis is based on the following publications, which are referred to in the text by their Roman numerals. All the articles are reprinted with the kind permission of the publishers.

I. **Terhonen E,** Sun H, Búee M, Paulin L, Kasanen R, Asiegbu FO. (2013). Effects of the use of biocontrol agent (*Phlebiopsis gigantea*) on fungal communities of *Picea abies* stumps. Forest Ecology and Management 310: 428–433.

II. Sun H, **Terhonen E**, Koskinen K, Paulin L, Kasanen R, Asiegbu FO. (2012). The impacts of treatment with biocontrol fungus (*Phlebiopsis gigantea*) on bacterial diversity in Norway spruce stumps. Biological Control 64: 238–246.

III. **Terhonen E**, Keriö S, Sun H, Asiegbu FO. (2014). Endophytic fungi of Norway spruce roots in boreal pristine mire, drained peatland and mineral soil and their inhibitory effect on *Heterobasidion parviporum in vitro*. Fungal Ecology 9: 17–26.

IV. **Terhonen E**, Sipari N, Asiegbu FO. (2015). Inhibition of *Heterobasidion parviporum*, *Phytophthora pini*, *Botrytis cinerea* and *Cryphonectria parasitica* by secreted metabolites by the root endophytes of Norway spruce. (Submitted).

AUTHOR CONTRIBUTION

I. The author collected the field samples and performed the laboratory work together with HS. The author contributed in the experimental design, analysed the data, interpreted the results and wrote the article. FOA formulated the study and contributed in the experimental design and in drafting the article. RK contributed to the experimental design as well as in drafting the article. MB contributed in analysing the data and interpreting the results. LP contributed in the experimental design and the 454- sequencing.

II. The author collected the field samples and performed the laboratory work together with HS. The author contributed to the article draft. FOA formulated the study and contributed in the experimental design and in drafting the article. HS and KK analysed the data and interpreted the results. RK contributed in the experimental design and in drafting of the article. LP contributed to the experimental design and generation of the 454- sequence data.

III. The author formulated the study, collected the field samples with SK and performed the laboratory work together with HS. The author analysed the data, interpreted the results and wrote the article. FOA contributed in the experimental design and drafting of the article. SK selected the sites and contributed in drafting the article.

IV. The author and FOA planned the experiment. The author performed the laboratory work, analysed the data, interpreted the results and wrote the article. FOA contributed in drafting the article. NS performed the UPLC-QTOF/MS analysis, interpreted the results of the chemical analysis and contributed in drafting the article.

ABSTRACT

Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) cover large areas in boreal regions with significant economic importance to Finnish forest industry. Approximately 15% of the spruce trees felled in Finland are rotten and thus commercially less valuable. The majority of this conifer wood decay is due to the root and butt rot pathogen *Heterobasidion annosum sensu lato*. Extensive logging of conifer forests has changed the environment into favouring this pathogen in stands where it originally has been rare. The proportion of diseased forest stands and associated production losses are expected to increase in the foreseeable future due to year-round logging. The disease is currently controlled by the use of chemicals, biocontrol agent and silvicultural measures. The saprotrophic fungus *Phlebiopsis gigantea* has for several years been used as a biocontrol agent against *H. annosum s.l.* in spruce and pine stumps. A major problem is that, although the effectiveness of *P. gigantea* as a biocontrol agent has empirically been shown, the long-term biological effect of this fungus on other decomposing wood microbiota has not been proven. The first objective of this thesis is to evaluate the impact of the only biocontrol agent used against root and butt rot fungus (*H. annosum s.l.*) on other resident microflora of Norway spruce stumps. An additional objective is to screen and identify other potentially novel bioagents that can be deployed for the biocontrol of the conifer pathogen. To find out whether the *P. gigantea* treatment impacts the overall diversity of other non-target stump microbes we used the 454-pyrosequencing approach. Samples were collected from forest sites previously pre-treated with *P. gigantea* either one, six or 13 years ago, DNA was isolated and the PCR products of fungal internal transcribed spacer (ITS) and bacterial 16S of ribosomal DNA, regions were pyrosequenced. Similarly samples were

collected from untreated stumps within the same forest site over the same period of time. The results revealed that initial application of the biocontrol agent influenced the fungal species composition, but the overall fungal diversity was not affected and no statistical differences were observed between treated and non-treated stumps in the mycobiota. The biocontrol treatment significantly decreased the initial bacterial richness in the stumps, but the bacterial community gradually recovered and the negative effect of *P. gigantea* was attenuated.

In parallel to the above studies, I further explored the potential of finding other novel biocontrol agents for use in managing the disease caused by the root rot pathogen *Heterobasidion parviporum*. This necessitated isolation studies of fungal root endophytes from forestry sites such as pristine mires and drained peatlands where the spread of *H. annosum s.l.* species have not been commonly reported. The reasons why *H. annosum s.l.* are not commonly observed in peatland still remains unclear. A possible reason for the suppression of *H. annosum s.l.* in peatlands is the diverse microbial community and their antifungal substances. Draining of pristine mires is likely to change the water balance of the sites, possibly transforming the microbial communities in plant roots, which might facilitate the spreading of the pathogen (*H. annosum s.l.*). Consequently, I sampled non-mycorrhizal *P. abies* roots and isolated endophytes from a pristine mire, a drained peatland and mineral soil and investigated the potential inhibitory effect of a subset on the root rot pathogen *H. parviporum*. A total of 113 isolates of fungal root endophytes were obtained from non-mycorrhizal *P. abies* roots, which were assigned to 15 different operational taxonomic units (OTUs). Most of the isolates consisted of dark septate endophytes (77%); the *Phialocephala fortinii s.l.-Acephala applanata*

species complex was the most dominant group, comprising 52% of all isolates. Nineteen of the isolates (17%) inhibited the growth of the conifer root rot pathogen *in vitro*. From these, two isolates were further used to test the potential inhibitory effects during interaction, *in vitro*, with *H. parviporum* as well as three other phytopathogenic fungi (*Phytophthora pini*, *Botrytis cinerea*, *Cryphonectria parasitica*). Additionally, the metabolites secreted by the selected root endophytes were extracted and the inhibitory effects on these pathogenic fungi were assayed. The root endophytes identified as *Cryptosporiopsis* sp. and *Phialocephala* sp. were able to form inhibition zones in paired cultures with the phytopathogenic fungi. Secreted metabolites from the endophytes also had similar inhibitory effects. The secreted metabolites were further chemically analysed using ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS). The higher numbers of unique metabolites were observed within *Cryptosporiopsis* sp., further suggesting that the stronger inhibitory effect observed could be due to acquisition of a more diverse metabolite pool. Overall, these projects represent an applied and basic scientific investigation with obvious strategic relevance to the forestry and environmental sector not only in Finland but worldwide. The results provide new information to facilitate better management and protection of these forest sites from *H. annosum s.l.* as well as support the continued used of *P. gigantea* for stump pre-treatment in Finnish forests.

1. INTRODUCTION

1.1. Global forestry and tree health

The estimated total forest area is four billion hectares, which covers ca. 30% of the total land area of the world (FRA 2010). Forests currently absorb billions of tonnes of CO_2 (Canadell & Raupach 2008) every year. Forest ecosystems store these large reservoirs of absorbed carbon permanently in their biomass (289 gigatonnes of carbon) (Canadell & Raupach 2008, FRA 2010). These major facts make forests important in climate change protection (Canadell & Raupach 2008). Consequently, forestry is beneficial not only for mitigating the climate change effect, but also as a potential bioenergy source as well as for conserving biological diversity. According to estimates (FRA 2010), the demand for wood and forest products is expected to continue growing in the next decade. Pests and diseases are a major threat to the numerous benefits of forestry outlined above. Changes in climatic conditions are likely to favour certain pathogens in forests (La Porta et al. 2008) and nurseries (Lilja et al. 2010). Global plant trade combined with climate change is introducing new non-indigenous tree pathogens with resulting disease outbreaks into ecosystems (see review by Loo 2009). The need to sustain timber quality gives new challenges in the area of forest biotechnology, particularly in tree health protection.

Forest trees and fungi share overlapping habitats with dynamic balanced relationships. These relationships vary from commensalism to mutualism and fatal pathogenic infections. The balance depends on a diverse scope of factors ranging from host type to ecological and environmental disturbances. This brings us to the disease triangle concept, a conceptual model that examines the

impacts of pathogens, hosts and the environment in the outcome of plant disease (McNew 1960) (Fig. 1). A disease is able to develop when a pathogen meets a susceptible host under favourable environmental conditions (Fig. 1). Over the last century, a number of devastating pathogenic tree infections have been documented, such as chestnut blight (Anagnostakis 1987, Dutech et al. 2012), dutch elm tree disease (Santini & Faccoli 2015), root and butt rot diseases (Asiegbu et al. 2005), sudden oak death (Garbelotto & Hayden 2012), ash dieback (Kowalski 2006) and many other tree diseases that cannot be outlined here. To intervene and manage these pathogenic tree infections, a fundamental understanding of at least one of the factors listed in the triangle (Fig. 1: host, pathogen and/or environment) is required. In the case of the most destructive fungal pathogen in Finland, *Heterobasidion annosum sensu lato*, the extensive logging of conifer forests has changed the environment into favouring this pathogen in stands where it has originally been rare. The economic loss to Finnish forest industry due to *H. annosum s. l.* wood decay is approximately 50 million euros annually (KMO 2015). The spreading of this pathogen is rapid due to new inoculation sources (conifer stumps) provided by the loggings. Infections can be prevented by the application of chemical or biological control agents directly on the stumps after tree felling (Rishbeth 1963, Holdenrieder & Greig 1998, Pratt et al. 1998). A major concern is that we do not know the long-term impact of biocontrol on other stump decomposing microbial species. An understanding of the consequences on wood microbe diversity would be important for knowledge concerning the effect of biocontrol application to the environment. Modern biotechnology offers the opportunity for a deeper understanding of the interspecific interactions between the non-target microbes, the biocontrol agent and the pathogen. This could form the basis for developing environmentally friendly and durable control strategies. In papers **I** and **II**, I

investigated the impact of biocontrol treatment on wood microbes by comparing results from treated and non-treated stumps, over a time period of one, six and 13 years after treatment using the 454 -sequencing approach.

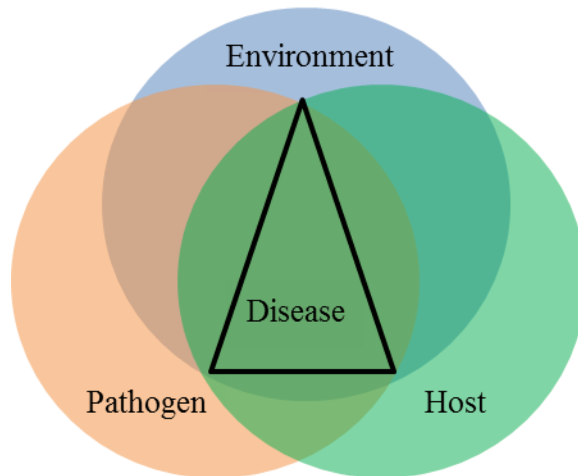

Figure 1. The Disease Triangle (represents the dynamics between the pathogen, susceptible host and favourable environment for a disease to occur).

1.2. Tree-pathogen interactions

Plants, including trees, protect themselves from environmental challenges, predators (insects, nematodes) and pathogens (bacteria, fungi and viruses) with mechanical barriers and both constitutive and induced defences. Constitutive defence is present in the plant whether the predator attacks or not, whereas activation of induced defences is usually preceded by the recognition of an invader (Dixon & Lamb 1990, Hückelhoven 2007). Dead cells only exhibit constitutive defences, whereas living cells have both constitutive and active defences. For trees, an important defensive challenge is to protect the bulk of dead cells from microbial invasion, and to maintain the integrity of the

relatively thin layer of living cells in the cambium and phloem. The plants' innate immunity against pathogens has evolved through the recognition of molecular signatures present in certain classes of pathogens but absent in the host. These signatures are proposed to be called pathogen associated molecular patterns (PAMPs), microbe associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs) (Mackey & McFall 2006, He et al. 2007, Boller & Felix 2009, Jones & Dangl 2006). Recognition results in effector-triggered immunity (ETI) in plants (Göhre & Robatzek 2008), which often culminates in a hypersensitive response (HR) and programmed cell death (PCD) (Stakman 1915, Mur et al. 2007, Robert-Seilaniantz et al. 2011, Dou & Zhou 2012). A disease develops if the plant defence fails, ultimately leading to host death. Conifers consequently rely on biochemical (Asiegbu et al. 1994; 1995; 1998, Kovalchuk et al. 2013) and structural defense (Woodward 1992). The most effective structural defence of conifers is the outer bark that protects the critically important living tissues of the inner bark, i.e., the cambium and phloem (Pearce 1996, Asiegbu et al. 1998, Woodward et al. 2007). Most conifers produce phenols and terpenes that are stored in resin ducts and polyphenolic parenchyma cells (PP -cells) (Franceschi et al. 2005). When the bark is wounded, resin flows out sealing the wound (Phillips & Croteau 1999). In induced defence conifers synthesise a wide range of secondary metabolites including toxic, antimicrobial low-molecular weight (LMW) compounds (phenols, stilbenes, terpenoids and alkaloids) (Woodward 1992, Pearce 1996, Eyles et al. 2010, Zulak & Bohlmann 2010). The pathogen *H. annosum s.l.* invades conifer seedling roots mainly through the cortex into the apical root meristem (Asiegbu et al. 1994, Fossdal et al. 2003). Conifer fine roots have been shown to be rarely infected by *H. annosum s.l.* in nature (Siepmann 1981, Schönhar 1992). However, a recent report has shown that *H. annosum s.l.* can

remain viable for at least seven years in roots of 15 mm in diameter and to vegetatively infect nearby Norway spruce seedlings (Piri & Hamberg 2015). Piri & Hamberg (2015) suggested that root fragment size is not the major factor restricting the infection. Other studies have demonstrated that conifer fine roots are equally susceptible under *in vitro* conditions (Asiegbu et al. 1993; 1994, Adomas et al. 2007) and in non-suberised lateral roots (Heneen et al. 1994). These results suggest that this pathogen can infect roots of all ages (Li and Asiegbu 2004, Asiegbu et al. 2005). Consequently, protecting conifer roots at a very early stage during their development with possible new biocontrol agents against *H. annosum s.l.* and other non-indigenous root pathogens deserves exploring.

There are several ways how fungal endophytes can protect host roots against pathogens: 1) defensive reactions of the host can be triggered by endophytes; 2) endophytes are able to produce antifungal substances or 3) their heavy colonisation of the roots can inhibit the invasion of harmful pathogens (Schulz et al. 1999, Mandyam & Jumpponen 2005). Endophytes have been noted to promote the root growth of conifer seedlings and cuttings (Hietala et al. 1994, Grönberg et al. 2006). If endophytes can promote host root growth and/or protect host roots against invaders, the screening of endophytes for their biocontrol abilities is of biotechnological relevance (see papers **III** and **IV**). The root endophytic community might differ between various forest sites. In that sense, the screenings should concentrate on sites in suppressive soils where the pathogen might be present but the disease is not heavily expressed (see papers **III** and **IV**).

1.3. Species complex *Heterobasidion annosum sensu lato*

The conifer pathogen *H. annosum s. l.* is the main cause of root and butt rot in Norway spruce (*Picea abies* (L.) H. Karst.) and Scots pine (*Pinus sylvestris* L.) (See reviews by Asiegbu et al. 2005, Gonthier & Garbelotto 2013). The *H. annosum* species complex has a wide geographical distribution, Finland presenting the most northern distribution line. This species complex consists of three species found in Europe, *Heterobasidion annosum sensu stricto* (Fr.) Bref., *Heterobasidion parviporum* Niemelä & Korhonen and *Heterobasidion abietinum* Niemelä & Korhonen, and two in North America: *Heterobasidion irregulare* Otrosina & Garbelotto and *Heterobasidion occidentale* Otrosina & Garbelotto (Korhonen 1978, Capretti et al. 1990, Otrosina & Garbelotto 2010). Two species of *Heterobasidion* are known in Finland: *H. annosum s.s.* and *H. parviporum* (Korhonen et al. 1998). All *Heterobasidion* spp. have different but partially overlapping host preferences mainly associated with spruce, fir and pine (Korhonen 1978, Capretti et al. 1990, Garbelotto & Gonthier 2013). The distribution of *H. annosum s.s.* is extended to central Finland (Korhonen et al. 1998). The *H. parviporum* distribution area seems to follow its main host Norway spruce (*P. abies*) to the northern region of Finland (Korhonen et al. 1998, Korhonen & Lipponen 2001). In Finland *H. annosum s.s.* mainly attacks Scots pine, but can also infect Norway spruce and deciduous trees like birch species (Korhonen 1978, Korhonen & Piri 1994).

1.4. Infection cycle of *H. annosum s. l.*

In boreal regions *H. annosum s.l.* infects freshly cut stump surfaces and wounds by airborne basidiospores (Rishbeth 1959a, Isomäki & Kallio 1974, Redfern &

Stenlid 1998). Spore deposition is followed by rapid germination and colonisation of the wood material. The homokaryotic mycelia developing from basidiospores are multinucleate, weakly virulent and unable to produce fruiting bodies. They may live on stumps for many years without causing disease in a living tree (Stenlid & Redfern 1998). However, in a recent paper, Keriö et al. (2015) demonstrated that homokaryons are capable of causing infection in field conditions. In nature, two compatible homokaryons in wood material usually merge to form heterokaryotic mycelium that contains nuclei from both parents (Korhonen & Stenlid 1998). The heterokaryotic mycelium of *Heterobasidion* has the capability of attacking living trees and producing fertile sexual fruiting bodies (Platt et al. 1965, Korhonen & Stenlid 1998, Oliva et al. 2011). Both mycelium types can produce conidiospores (Korhonen & Stenlid 1998). Basidiospores are produced through meiosis and released actively in the air in temperatures above + 5 ˚C (Rishbeth 1959a, Korhonen & Stenlid 1998). Conidiospores are additionally static and need a mechanical force such as wind, rain or animals to be released (Korhonen & Stenlid 1998). Following stump colonisation, the pathogen also spreads from infected to healthy trees by mycelia via root contacts (Rishbeth 1959b, Oliva et al. 2011) (Fig. 2).

Figure 2. A schematic illustration of the infection biology of *H. annosum s. l.* in a natural conifer forest habitat. Spores fall on freshly cut stumps (arrows), germinate, form infective hyphae (red colour) and invade the stumps, spreading to neighbouring healthy trees by root to root contact. (Asiegbu et al. 2005).

1.5. *H. annosum s.l.* occurrence in peatlands

The forestland area in Finland is 26.1 million hectares, with a total mire area of 9 million hectares (Peltola & Ihalainen 2011). Of this total mire area approximately 5.5 million hectares are drained peatlands (Päivänen & Hånell 2012), most of which will be available for forestry in the near future. Most of the spruce-dominated pristine mires in Southern Finland have been drained for forestry (a total of 1.5 million hectares) (Hökkä et al. 2002, Päivänen & Hånell 2012). Approximately 15–20% of spruce trees cut in Southern Finland are rotten and commercially less valuable, largely caused by *H. annosum s.l.* (Mattila & Nuutinen 2007, Peltola & Ihalainen 2011). Observations show that *H. annosum s.l.* do not occur as frequently in peatland forests compared to those on mineral soils (Mattila & Nuutinen 2007). Redfern (1998) concluded that peat soil inhibits the transmission of the disease by root contacts. However, the reasons why *H. annosum s.l.* are not commonly observed in peatland remains unclear. Other explanations besides root contacts may include the different soil types and their chemical properties (pH, nutrients etc.) that could restrict the growth of this pathogen or the biodiversity of endophytic fungi and bacteria associated with conifer roots/soil that could be different in peatlands compared to those in mineral soils. Peatland draining changes the soil water balance accompanied with a change in understorey vegetation (Päivänen & Hånell 2012), possibly transforming the microbial communities that might facilitate the spread of the pathogen. In paper **III**, I investigated the composition of root endophytes in different boreal forest sites including pristine mire, drained peatland and mineral soil, and explored the potential inhibitory effect of a subset of the endophytes on *H. parviporum*.

1.6. Control of *H. annosum s.l.* in Finland

1.6.1. Chemical control

The only chemical used in stump treatment against *H. annosum s.l.* in Finland is urea suspension (Finnish Forest Research Institute 2014). The effectiveness of the chemical treatment is based on the hydrolysis of the urea into ammonia, by bacterial and urease activity, raising the pH (pH=7) of the stump surface to toxic levels for the growth of *H. annosum s.l.* (Johansson et al. 2002). In the absence of hydrolysis, urea acts as a fertiliser and may even enhance the growth of the pathogen in diseased stumps (Pratt & Redfern 2001). The urea treatment of freshly cut stumps of Norway spruce under Scandinavian conditions is a reliable protection method against *H. annosum s.l.* (Brandtberg et al. 1996, Thor & Stenlid 2005, Oliva et al. 2008). Chemical treatment generally gives good results, but may have some collateral effects e.g. the negative shift in the fungal community inhabiting the spruce stumps (Vasiliauskas et al. 2004), or cause damage to ground vegetation, especially bryophytes (Westlund & Nohrstedt 2000). One of the main objectives of agricultural and forestry policies is to strive, if possible, to replace the use of chemical fungicides with biocontrol agents. Biological stump treatment has presently almost entirely replaced urea as a stump protectant in Finland (Korhonen & Lipponen 2001).

1.6.2. Biological control

The saprotrophic fungus *Phlebiopsis gigantea* (Fr.) Jülich is currently used for the biocontrol of the root rot pathogen with very good success (Holdenrieder & Greig 1998, Tubby et al. 2008) as it has been shown to reduce *H. annosum s.l.*

stump infections by 50–100%, compared to untreated stumps (Korhonen et al. 1994, Berglund & Rönnberg 2004, Berglund et al. 2005, Nicolotti & Gonthier 2005, Thor & Stenlid 2005, Rönnberg et al. 2006). The fungus (*P. gigantea*) is a common saprotrophic wood decay basidiomycete, and its biocontrol ability is due to rapid colonisation of the stumps outcompeting the pathogen in wood infected by both fungi (Korhonen et al. 1994, Holdenrieder & Greig 1998, Bailey et al. 2003).

Rishbeth (1952) in Great Britain was the first to discover that *P. gigantea* was able to replace *H. annosum s.s.* on pine stumps and proposed the use of the fungus for biological control (Rishbeth 1952; 1963). In Finland a heterokaryotic strain of *P. gigantea* was isolated from Norway spruce (*P. abies*) stump (Korhonen et al. 1994), and after formulation into a dry powder the oidia of this isolate were tested on Norway spruce stumps in the Nordic countries with great success (Korhonen et al. 1994, Rönnberg et al. 2006). This oidia preparation is now commercially produced and marketed in Finland as a pesticide (by Verdera AB as Rotstop®) for the control of *H. annosum s.l.* in both spruce and pine stumps. Presently, stumps in 25% (or 117 000 ha) of final felling forest areas in Finland are treated with the "Rotstop" isolate (Finnish Forest Research Institute 2014).

1.6.3. Impact of *P. gigantea* stump treatment on other microbiota

Compared to chemical treatments, biological control is considered more environmentally friendly. However, a major concern in the continued use of a single strain of *P. gigantea* in biocontrol is the potential effect on fungal species richness and biodiversity of colonised stumps (Vasiliauskas et al. 2004; 2005).

Furthermore, although the success of *P. gigantea* as a stump protectant is well documented, knowledge about its long-term effect on other wood mycota or bacteria is still insufficient. Bacteria are initial colonisers of wood material, having an important role in the decay processes (see review by Clausen 1996). Vasiliauskas et al. (2004) reported that urea and biological control (Rotstop) treatments decreased species richness in *P. abies* stumps soon after application. In Finland, the effects of *P. gigantea* treatment on fungal communities of conifer stumps have also been studied one and six years after treatment with some evidence that the use of the biocontrol has an effect on the species composition of other fungi (Vainio et al. 2005). *P. gigantea* (Rotstop) was shown to dominate the fungal community of *P. abies* stumps (Vasiliauskas et al. 2005) in Sweden four years after application. However, no study has examined the influence of using *P. gigantea* on the bacterial community in conifer stumps or the influence to microbes during a period longer than six years. Additionally not much is known about how persistent these observed shifts on species diversity are.

1.6.3.1. Microbiota identification

The methods used in previous studies on fungi (Vainio et al. 2005, Vasiliauskas et al. 2005) and bacteria (Kowalchuk et al. 1997, Yrjälä et al. 2010), have used culture-based and/or molecular methods (deoxyribonucleic acid (DNA) extraction, polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), sequencing) for species identification. The problem with the direct culture method is that many fast-growing microbes will be easily isolated, masking slow-growing microbes (Hyde & Soytong 2007, 2008). Un-culturable microbes also inevitably escape detection (Guo et al. 2001, Duong et

al. 2006, Hyde & Soytong 2007). Several authors suggest that next generation sequencing (NGS) could be used to overcome such limitations (Duong et al. 2006, Nilsson et al. 2009). Short pyrosequencing reads have proven to be a highly useful tool for microbial community analysis (Liu et al. 2007) and have been applied to study fungal and bacterial diversity in soils (Buée et al. 2009, Jones et al. 2009). Applying the high-throughput sequencing method could help reveal a higher diversity of stump microbes when compared to culture-based methods. In papers **I** and **II**, I investigated the impact of *P. gigantea* treatment on treated and non-treated stump microbes at different time points (one, six and 13 years after biocontrol treatment) using the 454 -sequencing approach.

1.6.4. Regeneration of diseased forest sites with silvicultural methods

The silvicultural control of *H. annosum s.l.* is difficult because it spreads through root contacts to neighbouring trees (Rishbeth 1959b, Oliva et al. 2011). *H. annosum s.l.* can remain viable and infective in stumps for decades (Piri 1996, Stenlid & Redfern 1998, Piri & Korhonen 2007), resulting in an inoculum source for new tree generations (Piri 2003). Preventing germination on freshly cut stumps without stump treatment could be achieved with winter cuttings instead of summer cuttings (Möykkynen & Miina 2002), but the constant need for timber is relevant all year round. It is recommended that the first thinning of forest sites should at least be performed during the winter time, decreasing damages to tree roots due to heavy machinery. If thinnings are reduced to one winter cutting and the rotation length is shortened, it is possible to prevent heavy disease in the next tree generation even in already diseased sites (Piri 2003). Removing stumps and larger roots decreases the *H. annosum s.l.* infections by 20–72% (Cleary et al. 2013). Although removing stumps is an

effective control method, the use of the biocontrol agent *P. gigantea*, is considered more cost-effective in reducing the frequency of *H. annosum s.l.* (Cleary et al. 2013). Disturbance to the environment due to stump removal (Laurén et al. 2008) should also be assessed and evaluated.

A mixed forest of conifer stands could serve as a preventive measure against this disease (Piri et el. 1990, Lindén & Vollbrecht 2002, Möykkynen & Pukkala 2010), as the use of more resistant or non-preferred host trees would slow down the infection of the pathogen through root contacts. Changing tree species is the best method to control decay losses in the subsequent tree generation in heavily diseased spruce sites (Lindén & Vollbrecht 2002, Piri 2003). Scots pine (*P. sylvestris*) and silver birch (*Betula pendula* Roth) are fairly resistant to *H. parviporum* and are recommended for the regeneration of infested sites (Korhonen 1978, Piri 1996; 2003, Möykkynen & Pukkala 2010). The problems with replacing a tree species with others are that spruce sites are too fertile for pine and equally, pine and birch plantations are often impractical because of the high risk of browsing damage by moose (*Alces alces* L.) (Heikkilä & Raulo 1987, Heikkilä & Härkönen 2007, Wam & Hofstad 2007, van Beest et al. 2010, Edenius et al. 2011). Deciduous trees have been reported to be less susceptible to *H. annosum s.l.* infection compared to conifers (Korhonen 1978, Piri 1996, Korhonen & Stenlid 1998). Aspen (*Populus tremula* L.) is considered highly resistant (Swedjemark & Stenlid 1995) and favouring these trees when planting next to infected spruce stumps could provide some protection to Norway spruce rotation (Piri 2003). However, mixed forests with deciduous trees are not always a guarantee for the total prevention of *H. annosum s.l.* as this fungus has been reported to be transferable from conifer stumps to healthy silver birch trees (Piri 1996). Preventing the infection caused by *H. annosum s.l.* is the most

important control method against this pathogen. This is easier to achieve in forestry if the planted trees are perfectly adapted to the site and thinnings are performed during the wintertime (avoiding injuries to standing trees).

1.7. Fungal endophytes

The term “endophyte” is used to describe microbes that live asymptotically inside the plant tissues for the entire or at least a significant part of their life cycle without causing any clear negative harm to the host (Petrini 1991, Saikkonen et al. 1998). Fungal endophytes may turn parasitic (Saikkonen et al. 1998, Sieber 2007, Rodriguez & Redman 2008) during host senescence or saprotrophic (Korkama-Rajala et al. 2008, Sun et al. 2011) in dead host tissues. It is an acknowledged fact that fungal endophytes can be found virtually on every terresrtial plant (Arnold & Lutzoni 2007, Rodriguez & Redman 2008, see Rodriguez et al. 2009 and Sieber & Grünig 2013). Endophytes have received notable attention due to their great potential as a major source of biologically active compounds with unique chemical structures (Tan & Zou 2001, Schulz et al. 2002, Strobel 2003, Yu et al. 2010, Mousa & Raizada 2013). Thus, less investigated endophytic microorganisms from diverse and unique ecosystems still harbour enormous amounts of novel natural products (Gunatilaka 2006). The considerable diversity of endophytes within individual hosts has increased the studies examining the ecological roles of fungal endophytes. Presently, the researches on endophytes have focused mostly on the better understanding of the interactions between endophytes and their host as well as their unique natural products. Future studies on detailed documentations of individual species and their infection cycles are needed to understand the functions of these ubiquitous symbionts.

1.7.1. Conifer tree endophytes

A majority of the biodiversity studies on fungal endophytes in the needles of *Pinus* and *Picea* are concentrated in boreal and temperate regions where coniferous forests cover large areas and are of significant economic importance. These fungi have been studied since the 1970's (Carroll et al. 1977, Carroll & Carroll 1978). The endophyte communities in the same plant family are dominated by closely related species while endophyte species diversity among conifer trees is high (Sieber 2007). Most of the dominant endophytic fungal species found in the aerial tissues of conifer trees (Pinaceae) belong to the class Leotiomycetes and the order Helotiales (Sieber 2007 and references within). The needles of *Pinus* species have been extensively studied (Carroll et al. 1977, Carrol & Carrol 1978, Kowalski 1982, Legault et al. 1989, Kowalski 1993, Helander et al. 1994, Helander 1995, Hata & Futai 1995; 1996, Jurc et al. 1996, Hata et al. 1998, Sieber et al. 1999, Deckert & Peterson 2000, Deckert et al. 2002, Ganley et al. 2004, Martín et al. 2004, Ganley & Newcombe 2006, Guo et al. 2008, Zamora et al. 2008, Botella et al. 2010, Botella & Diez 2011, Terhonen et al. 2011, Larkin et al. 2012) with less attention given to the roots (Wang & Wilcox 1985, Wilcox & Wang 1987b, O'Dell et al. 1993, Ahlich & Sieber 1996, Hoff et al. 2004, Menkis et al. 2006, Reay et al. 2010, Stenström et al. 2014). A similar pattern can be observed for *Picea* trees where the species composition and diversity of needle endophytes have received considerable attention (Carroll et al. 1977, Carrol & Carrol 1978, Sieber 1988, Johnson & Whitney 1992, Magan & Smith 1996, Müller & Hallaksela 1998, Stefani & Bérubé 2006a;b, Müller & Hallaksela 2000, Müller et al. 2001, Lorenzi et al. 2004, Müller et al. 2007, Sokolski et al. 2007, Korkama-Rajala et al. 2008, Koukol et al. 2012, Rajala et al. 2013; 2014) again with less attention given to

the roots (Wilcox & Wang 1987b, Holdenrieder & Sieber 1992, Ahlich & Sieber, 1996, Kernaghan et al. 2003, Grünig et al. 2006, Menkis et al. 2006, Kernaghan & Patriquin 2011, Stenström et al. 2014).

1.7.1.1. Norway spruce endophytes

Norway spruce (*P. abies*) is one of the dominant tree species in temperate and boreal regions of Europe, occupying 24% of forested areas in Finland alone (Peltola 2008). Norway spruce together with Scots pine (*P. sylvestris*) forms the basis for one of the largest industries in Finland contributing yearly net income of several billion EUR (Ministry of Agriculture and Forestry 2015). In Finland 159 million forest tree seedlings are produced in nurseries annually; Norway spruce being the most important species with 105 million seedlings (Finnish Food Safety Authority 2014). Interest is thus high in the biology and ecology of fungal endophytes in forest ecosystems with special emphasis on sustainable management strategies for forestry.

The most common needle endophytes of *P. abies* are *Lophodermium piceae* (Fuckel) Höhn. (Sieber 1988, Müller & Hallaksela 1998; 2000, Korkama-Rajala et al. 2008, Rajala et al. 2013), and *Tiarasporella parca* (Berk. & Broome) H. S. Whitney, J. Reid & Piroz (Sieber 1988, Müller & Hallaksela 1998). *Lirula macrospora* (R. Hartig) Darker, the cause of Lirula needle blight, may occasionally be observed as an endophyte (Müller & Hallaksela 1998, Rajala et al. 2014). *Cadophora luteo-olivacea* (J.F.H. Beyma) T.C. Harr. & McNew, *Chalara longipes* (Preuss) Cooke (Rajala et al. 2014) and *Phoma herbarum* (Cooke) Saccardo (Rajala et al. 2013) are the most common needle endophytes from *P. abies* clonal cuttings. *Phacidiopycnis* spp., *Cistella acuum* (Alb. &

Schwein.) Svrcek and *C. longipes* have been documented in wind-fallen mature Norway spruce trees (Koukos et al. 2012). Differences in species composition are most likely due to the different host sources (wind-felled trees, clonal cuttings, seedlings, mature trees) of Norway spruce and from different forest ecosystems that have affected the species distribution and frequency of endophytes.

Dark septate endophytes (DSEs) are the most frequently isolated endophytic fungi from the roots of *P. abies* (Ahlich & Sieber 1996, Grünig et al. 2002, Queloz et al. 2005). DSE hyphae are both septate and melanised and can form specialised structures in the host roots, referred to as microsclerotia (Jumpponen & Trappe 1998a, Mandyam & Jumpponen 2005). The dominant DSE group in conifer (i.e. *P. abies*) roots is formed by members of the *Phialocephala fortinii s.l.-Acephala applanata* species complex (PAC) (Ahlich & Sieber 1996, Grünig et al. 2002; 2004, Queloz et al. 2005). *Phialocephala fortinii* sensu stricto C.J.K Wang & H.E Wilcox seems to not have any host preference (Ahlich & Sieber 1996, Grünig et al. 2006; 2008a, Tejesvi et al. 2010; 2013). *Acephala applanata* Grünig & T.N. Sieber on the other hand is more associated with *P. abies* (Grünig & Sieber 2005, Grünig et al. 2006; 2008a). Members of this species complex are cryptic as they cannot be differentiated based on morphology. Rather, PAC members have been identified to the species level based on multilocus molecular markers such as single-copy restriction fragment length polymorphism (RFLP), microsatellites, sequence loci, or a combination of them (Grünig et al. 2008b, Queloz et al. 2008; 2010). PAC is composed of identified species including *Phialocephala turicensis* Grünig & T.N. Sieber, *Phialocephala letzii* Grünig & T.N. Sieber, *Phialocephala europaea* Grünig & T.N. Sieber *Phialocephala helvetica* Grünig

& T.N. Sieber, *Phialocephala uotilensis* Grünig & T.N. Sieber, *Phialocephala subalpina* Grünig & T.N. Sieber, *P. fortinii* and closely related *A. applanata* (Grünig & Sieber 2005, Grünig et al. 2008). The species complex has also been reported to consist of 21 reproductively isolated lineages (Queloz et al. 2011) including the eight previously described species (Grünig et al. 2008, Queloz et al. 2011).

The endophytes of *P. abies* have been studied in Finland as colonisers of symptomless needles (Müller & Hallaksela 1998; 2000, Müller et al. 2001; 2007) and as primary decomposers of forest litter (Müller et al. 2001, Korkama-Rajala et al. 2008), but to my knowledge, the endophytic composition of *P. abies* roots have not been previously studied in Finland. In paper **III**, I investigated the composition of fungal root endophytes from non-mycorrhizal roots of Norway spruce from three different boreal forest sites including pristine mire, drained peatland and mineral soil.

1.7.2. Functional roles of fungal endophytes

Two major groups of endophytic fungi have been recognised as the clavicipitaceous endophytes (C-endophytes) and the non-clavicipitaceous endophytes (NC-endophytes) (see review by Rodriguez et al. 2009). C-endophytes infect some grasses and their transmission to a new host is primarily vertical, with fungi passing on from plants to offsprings via seed infections (Saikkonen et al. 2002). These C-endophytes have been reported to increase plant biomass and drought tolerance, produce chemicals toxic to animals and decrease herbivory (Clay 1988; 1991, Patterson et al. 1991, Riedell et al. 1991, Saikkonen et al. 2010). However, these benefits are not automatically

mutualistic as they appear to depend on host species, host genotype and environmental conditions (Saikkonen et al. 1999, Faeth & Sullivan 2003, Faeth et al. 2006, Saikkonen et al. 2010). NC-endophytes mainly infect the host plants horizontally (Rodriguez et al. 2009), but some are known to be vertically transmitted (Sieber et al. 1988). An important role of aerial NC-endophytes in the ecosystem may be the switch to saprotrophic habits through the degradation of dead or dying host plants (Oses et al. 2008, Sun et al. 2011) and endophytic fungi have been reported as having a significant role in the process of needle decomposition in boreal forests (Korkama-Rajala et al. 2008). Root endophytes are a strictly horizontally transmitted group representing the assemblage of primarily ascomycetous fungi with poorly defined ecological roles.

The meta-analyses of root-inhabiting DSE fungi have shown that while host growth responses to colonisation by DSE fungi were variable, they tended to be negative (Mayerhofer et al. 2013) or positive (Newsham 2011). Tree endophytes have been shown to play a role in the resistance of their hosts to pathogen damage (Herre et al. 2007, Mejía et al. 2008, Sumarah et al. 2008, Tellenbach & Sieber 2012), reflecting the production of secondary metabolites (Schulz et al. 2002, Sumarah et al. 2009; 2010; 2011, Tellenbach et al. 2013). In many of these cases, endophytes have been implicated in protecting the host plant against herbivory (Miller 2002, Sumarah et al. 2005; 2008; 2009) or phytopathogens (Tellenbach & Sieber 2012). However, it is sometimes difficult to link *in vitro* pathogen inhibition to disease resistance expressed in the field. These endophytic fungi might benefit the host plants or suppress competitor growth in part via the production of bioactive metabolites (Schulz et al. 1999; 2002, Strobel 2003, Mandyam & Jumpponen 2005, Mousa & Raizada 2013). This could partly be an explanation for the existence of these ubiquitous fungi

in their host plants. The ecological roles and functions of these ubiquitous endophytes, especially their general effects on the colonised hosts are difficult to define, and despite their apparent great abundance, have not been fully resolved (Sieber & Grünig 2013).

The host-endophyte relationship in the plant roots is thought to differ from mycorrhizal symbioses as the cellular interface where specialised structures (e.g. arbuscules) occur is lacking (Brundrett 2006). Additionally, there are no significant benefits for both partners (Brundrett 2006). Several hypotheses have tried to explain the observed positive responses of the host to root endophyte colonisation. The two most prominent explanations include the modulation of plant growth via nutrient mineralisation (as in mycorrhizae) (Jumpponen 2001, Mandyam & Jumpponen 2005, Newsham 2011, Reininger & Sieber 2013). The other is the production of plant growth-promoting phytohormones (Schulz et al. 1998; 2002, Schulz & Boyle 2005). Mycorrhizal fungi have many significant functions in ecosystems (Smith & Read 2008) but the root-associated fungal endophytes have received very little attention (Rodriguez et al. 2009). Mycorrhizal fungi in the boreal forest have been intensively studied (Högberg & Högberg 2002, Högberg et al. 2003; 2007; 2008, Nilsson et al. 2005, Yarwood et al. 2009). Helotiales (Yarwood et al. 2009) and DSE (Jones et al. 2012) are commonly observed in these studies. There is a gap in our knowledge on the research of fungal endophytes. It has been estimated that root colonisation by endophytic DSE fungi are possibly as abundant as mycorrhizaes (Mandyam & Jumpponen 2008, Dolinar & Gaberscik 2010, Uma et al. 2010, Zhang et al. 2010), if not more abundant (Mandyam & Jumpponen 2008, Sieber & Grünig 2013).

1.7.3. Biocontrol using root endophytes

Endophyte interactions in conifer trees can range from antagonistic to mutualistic (Jumpponen & Trappe 1998b, Jumpponen et al. 1998, Mandyam & Jumpponen 2005, Sumarah et al. 2009, Tellenbach et al. 2011, Reininger et al. 2012). Despite the demonstrated diversity of endophytic fungi in conifer trees (see reviews by Saikkonen 2007 and Sieber 2007) much is still not known of the functions of endophyte-conifer tree interactions. Some tree fungal endophytes have been noted to suppress the growth of phytopathogenic microbes, and their potential ability as biocontrol agents has been acknowledged (Miller et al. 2002, Arnold et al. 2003, Ganley et al. 2008, Hanada et al. 2010, Miles et al. 2012). Beneficial (Jumpponen et al. 1998), neutral (Wilcox & Wang 1987b, Jumpponen & Trappe 1998b) and sometimes even pathogenic outcomes (Wilcox & Wang 1987a, Tellenbach et al. 2011, Reininger et al. 2012) have been reported in conifer tree roots inoculated with DSE fungi. These results highlight the unknown ecological function of root endophytes in conifer hosts. Tellenbach & Sieber (2012) showed that some strains of *P. subalpina* reduced mortality and disease severity caused by the pathogens *Phytophthora plurivora* T. Jung & T.I. Burgess and *Elongisporangium undulatum* (H.E. Petersen) Uzuhasi, Tojo & Kakish in *P. abies* roots. Tellenbach et al. (2013) also isolated metabolites produced by a PAC member (*P. europaea*) and found two antifungal metabolites, sclerin and sclerotinin A, against the pathogenic oomycetes pathogen. Tellenbach et al. (2013) concluded that these two antifungal metabolites are either individually or synergistically responsible for the growth inhibition of the oomycetes pathogen under *in vitro* experiments. Endophytic fungi might benefit the host plants or suppress the growth of a competitor via the production of secondary

metabolites. Mandyam & Jumpponen (2005) suggested three mechanisms through which DSE may inhibit pathogens; 1) mycorrhizal fungi and rhizosphere-inhabiting pathogens may compete for the plant photosynthates or for colonization sites; 2) compounds inhibitory to pathogens may be produced or 3) DSE colonisation may have prophylactic value by inducing plant defense responses to subsequent pathogen infection. According to previous studies (Miller et al. 2002, Sumarah et al. 2010; 2011, Tellenbach et al. 2013) it can be hypothesised that endophytes can produce antifungal substances in addition to host metabolites (Schulz et al. 1999). A meta-analysis of DSEs on plant performance has revealed positive effects on total, shoot and root biomass, and on shoot nitrogen (N) and phosphorus (P) contents (Newsham 2011 and references within). DSEs have also been noted to suppress pathogens (Tellenbach et al. 2013), which increase their role as possible protectors against root pathogens. Screening root endophytes for biocontrol capabilities is, therefore, of biotechnological relevance. It is obvious that these endophytes and their metabolites possess new possibilities to be utilised in forestry and agriculture against plant pathogens. Especially their potential to control possible new invasive alien pathogenic species makes them a high priority. The screening of local strains or isolates is a primary priority when developing a possible biocontrol. To utilise these root endophytes as biocontrols, the mechanisms behind the possible inhibition of the root pathogen should be determined. We evaluated the potential inhibitory effect of a subset of the isolated root endophytes from different forest sites (pristine mire, drained peatland, mineral soil) on the pathogen *H. parviporum*, the main cause of root rot in Norway spruce in Finland (Paper **III**). This was followed by studies on the inhibitory effects of secreted metabolites from the root endophytes on the phytopathogenic fungi to understand the mechanism and ecological

consequences of the existence of these ubiquitous endophytes in various host roots (Paper **IV**).

1.7.4. Root endophytes in boreal peatlands

Compared to the endophytes of healthy roots, mycorrhizal associations and saprotrophic microorganisms have been more extensively studied in peatlands (Thormann et al. 1999) together with activities of other microbes involved in litter decomposition (Thormann 2006, Myers et al. 2012). Dark septate hyphae have commonly been observed from the roots of different plants in boreal peatland (Thormann et al. 1999). In Sweden, the composition of microfungi in a mire ecosystem (Nilsson et al. 1992) and root endophytes of co-existing ericaceous plant species in a subarctic mire community (Kjøller et al. 2010) have also been studied. Kjøller et al. (2010) observed that the roots of ericaceous plants were dominated by potential ericoid mycorrhizal fungi, but some DSEs such as *P. fortinii* were equally observed. Artz et al. (2007) found that the fungal community of peat changes during vegetational succession and varies significantly in different successional stages when cutover peatlands are regenerated. The compositions of fungal endophytes in the roots of conifers have so far not been studied in different boreal peatlands in Finland. We particularly do not know how these communities respond to disturbances such as the draining of pristine mires. Part of the additional objective for my thesis was to study (Paper **III**) the root endophytes of *P. abies* in different habitats (pristine mire, drained peatland, mineral soil) to investigate whether the draining of peatland, followed by vegetation succession, had an effect on species composition and frequency.

2. AIMS OF THE STUDY

H. annosum s.l. is the main cause of wood rot in Norway spruce (*P. abies*) in Finland, decreasing the commercial value of these trees. The intensive use of a biocontrol, *P. gigantea*, might disturb the microbial community in wood stumps. Understanding the consequences on other wood microbes of the long-term application of *P. gigantea* to living wood tissues would be important for gaining knowledge of the biocontrol effect on the environment. In addition to the above-mentioned stump treatment studies, I explored the potential of finding other novel biocontrol agents to be utilized in managing the disease caused by the root rot pathogen *H. parviporum*. This necessitated exploratory isolation studies of fungal root endohytes from forestry sites such as pristine mires and drained peatlands where the spread of *H. annosum s.l.* has not commonly been reported. Protecting young seedlings using endophytic fungi during the early stages of establishment under field conditions was a primary consideration. I concentrated on *P. abies* fine root endophytes known to be susceptible to infection *in vitro* (Asiegbu et al. 1993; 1994). I consequently sampled a large number of non-mycorrhizal *P. abies* roots and isolated endophytes from a pristine mire, a drained peatland and mineral soil, and investigated the potential inhibitory effect of a subset on the root rot pathogen *H. parviporum* and other phytpathogenic fungi.

The specific aims of this thesis were the followings:

1) To study whether the use of *P. gigantea* as a biocontrol has a negative effect on the fungal and/or bacterial community of wood-inhabiting microbes over

a time period of one, six and 13 years post-treatment using the 454 -sequencing approach (**I**, **II**).

2) To study the composition of fungal root endophytes of *P*. *abies* and explore their potential inhibitory effect against the root and butt rot pathogen *H*. *parviporum* (**III**, **IV**).

3. HYPOTHESIS

Our first hypothesis is that the long-term treatment of conifer stumps with *P. gigantea* has a negative effect on the diversity of the non-target microbial community of conifer stumps (**I**, **II**). Secondly, we also hypothesise that fungal root endophytes from forest sites (peatland), not commonly inhabited by *H. annosum s. l.*, possess an inhibitory effect on the growth and survival of the pathogen *H. parviporum* (**III**, **IV**).

4. MATERIALS AND METHODS

4.1. Study sites and sampling

Wood core samples from Norway spruce stumps in forest sites previously pre-treated with *P. gigantea* either one, six or 13 years previously were collected in May 2010 (**I**, **II**). Similarly, samples from untreated stumps within the same forest site over the same time period were also collected. The one-year-old treated stumps were located in Liesjärvi (southwestern Finland; 60° N, 24° E). The site was a mature thinned Norway spruce stand (age 60 years). These stumps were treated with biocontrol *P. gigantea* (Rotstop) by a single-grip harvester during cutting in May 2009. The harvester sprayed untreated stumps with water. The six-year-old stumps were on a clear-cut stand of Norway spruce located in Karjalohja (southwestern Finland; 60° N, 23° E). A diagonal stand had been subject to stump treatment by a single-grip harvester during final felling in early June 2004. The upper and lower sides of the clear-cut had been left as untreated control areas. The 13-year-old treated stumps were located in Taipalsaari (southeastern Finland; 62° N, 28° E). Norway spruce trees (ca. 50 years old) were felled in August 1997 and the stumps were treated with suspension of biocontrol (Rotstop) immediately and untreated stumps were left as a control. Samples from each site were collected from three treated and three untreated stumps (20–50 m apart from each other), respectively. Five wood cores (1x1x2 cm depth) from each stump surface (one core sample from the center [heartwood] of the stump and four randomly from the sapwood) were sampled and pooled in a sterile falcon tube and stored at -20 ºC (**I**, **II**).

In paper **III**, the roots of *P. abies* were collected from sites located in Lakkasuo (61°47'N, 24°18'E, ca. 150 m a.s.l.), which is a boreal raised bog complex. The sites were a maximum two kilometres apart. The sites had mature (>100-year-old) and naturally regenerated Norway spruce (*P. abies*) -dominated stands. The site represented mineral soil, pristine mire or drained peatland with comparable fertility (rich). Site 1 was a mineral soil stand (*Myrtillus* type) with *Vaccinium myrtillus* L. in the field layer and feather mosses in the undergrowth. Site 2 was minerotrophic pristine mire (*V. myrtillus* spruce swamp) with *V. myrtillus* and other indicator species in the field layer (*Trientalis europea* L., *Linnea borealis* L.) and *Sphagnum* species and feather mosses in the bottom layer. Site 3 was a drained peatland originating from a minerotrophic pristine spruce-dominated mire (*V. myrtillus* type 1), where the ground vegetation consists mainly of mire vegetation. The area was drained in 1966 and the ditches were cleared in 1988. From each site three different spruce trees were chosen. From each sampled tree, three separate major roots were grubbed up until the fine roots were discovered and collected. The samples were collected in September 2010, they were stored at +4°C and processed within 48 hours after collection (**III**).

4.2. Effects of using a biocontrol agent on microbial communities

The wood core samples were homogenised in liquid N_2 with mill grinding and genomic DNA was extracted. Amplicon libraries were performed for the fungal internal transcribed spacer (ITS) region by PCR using primer ITS4B together with different tagged ITS1 (xxx-ITS1 designed for each stump separately) (Gardes & Bruns 1993) and PCR amplification of the bacterial 16S region using primers 27F (xxx-27F designed for each stump separately) and 519R (Lane 1991). The amplicons were sequenced at the Institute of Biotechnology

(Helsinki University, Finland) using the 454 GS-FLX Titanium protocol (454 Life Sciences/Roche Diagnostics, CT, USA). Raw pyrosequencing reads of bacteria and fungi were quality trimmed using Mothur software (Schloss et al. 2009). The bacterial sequences were clustered into operational taxonomic units (OTUs) defined by a 3% distance level. Species richness and evenness was estimated using the non-parametric diversity index (Shannon). To correct for differences in survey effort between samples, the number of sequences from the smallest size among all samples (4386 sequences per sample) or among all treatments (17 224 sequences per treatment) was randomly selected and used for normalisation on calculating species richness and diversity. Redundancy analysis (RDA) was used for calculating the dendrogram describing the similarity between the community structures based on relative bacterial abundance. Multiple sample analysis of molecular variance (AMOVA) in Mothur was used to test the significant difference in genetic diversity within the populations between treatments. The Mann-Whitney analysis was used to test the significance level at 5% in relative bacterial abundance between treatments (**I**, **II**).

The ITS1 sequences were clustered at 97% similarity with the most abundant sequence types serving as cluster seeds. The most frequent sequence type in each cluster was used for the manual BLAST (Altschul et al. 1997) searches against GenBank / NCBI (Sayers et al. 2010) to provide taxonomic identification. All OTUs with fewer than five reads were excluded from further analysis (Unterseher et al. 2011). Diversity indices (Shannon-Wiener) were used to measure the general species diversity (species richness and evenness) of every individual stump at all sites. The similarity indices (Sørensen) and dissimilarity Bray-Curtis index were used to compare OTU composition

between the control and treated stumps at every time point. The Sørensen index was calculated as QS= 2C/(A+B), where A and B are the number of OTUs in treated or control stumps respectively, and C is the number of OTUs shared by treated and control stumps (Sørensen 1948). Bray-Curtis dissimilarity was calculated as $BC_{ij}= 1- 2C_{ij}/(S_i+S_j)$ (Bray & Curtis 1957), where C_{ij} is the sum of the lesser values for shared OTU in both stumps; S_i and S_j are the total observed value of shared OTU in the stumps. To decrease the high abundance effect caused by a few species on the Bray-Curtis index, we repeated the calculations with logarithms (log10) of the data. Principal Component Analysis (PCA) was used to visualise the treatment impact on fungal communities at the different study sites (**I**). Mann-Whitney logarithm analysis of OTU abundance (data transformed to log10) was used to test the significance level at 5% between the treatment and control stumps.

4.3. Endophytic fungi of Norway spruce roots

The fine roots, where no visible mycorrhizae were detected, were rinsed under tap water. Following surface sterilisation, a total of 72 root pieces were placed on Petri plates containing modified Hagem media as well as 36 root pieces on 2% malt extract agar (MEA) plates (see details in paper **III**). The plates were incubated at room temperature in the absence of light for 4-weeks due to the slow growth of the endophytes. The plates were sub-cultured until a total of 113 pure cultures were obtained (~1.05 endophytes/root). These fungal isolates were examined with bright-field microscopy and a stereomicroscope. Based on similar morphology some of the pure cultures originating from the same Petri plate were grouped together. From these morphologically similar groups, 84 representative isolates were selected for molecular identification. DNA

extraction, followed by PCR with primer pair ITS1 and ITS4 (Gardes & Bruns 1993), was used to obtain the entire ITS regions 1 and 2 of ribosomal DNA. The cleaned sequences of ITS regions 1 and 2 were identified using BLAST (Altschul et al. 1997) searches against GenBank/NCBI (Sayers et al. 2010). The sequences with ≥ 98% similarity and ≥ 97% query coverage were set to constrict the OTUs (Arnold & Lutzoni 2007). The novel sequences obtained in this study and the best matches from GenBank were aligned and a phylogenetic tree was generated using the Neighbour-Joining (NJ) analysis with 1000 bootstrap replicates. Diversity indices and evenness were used to compare fungal frequencies for every individual sampled tree. Diversity between sites was estimated using the similarity and species richness indices. Diversity indices characterising fungal communities in the individual sampled trees were compared using one-way ANOVA. Differences in total fungal average frequencies between the three sampled sites were tested with the Kruskall-Wallis test in SPSS 19 (Chicago, IL, USA) (**III**).

I used the cultural method to isolate the endophytes of *P. abies* roots. The species accumulation curve (**III**; **Fig. 2**) showed that endophytic species accumulated more slowly in the mineral soil and pristine mire compared with isolates from drained peatland sites. Observed species richness did not fall within 95% confidence intervals for estimated species richness Chao 1 values (**III**; **Table 2**), which indicates that fungal assemblage was under sampled.

4.4. Antagonism between the endophytes and phytopathogenic fungi

The dual culture method was used to test the inhibitory effect of the isolated endophytes on *H. parviporum*. An endophyte was considered to possess

"inhibitory capability" if *H. parviporum* was not able to overgrow the hyphae of the endophytes or the growth of the pathogen ceased (**III**). Based on the initial screening described above, two isolates of root endophyte strains 513 and 222 were selected. These endophytes were identified based on ITS regions 1 and 2 as *Cryptosporiopsis* sp. (strain 513) and *Phialocephala* sp. (222) (**IV**). The dual culture method was also applied for the other phytopapathogens (*Phytopthora pini* L.H. Leonian, *Botrytis cinerea* Pers. and *Cryphonectria parasitica* (Murrill) Barr)) (**IV**).

The endophytes were inoculated aseptically into 100 ml 2% malt extract and shaken at 50 rpm for 3 months at 21 ˚C. Liquid cultures of each fungal isolate were harvested with Miracloth to remove the fungal hyphae. The filtrate (20 ml) was extracted with 2x equal volumes of ethyl acetate. The extract was weighed and re-suspended to a concentration of 5 mg/ml. Ethyl acetate with 1 mg of extracts were placed on round filter paper discs (ø 6 mm) and as a control, pure ethyl acetate (200µl) was used on the same plate. Ethyl acetate was allowed to evaporate before placing filter papers on the plate. An agar piece (ø 5 mm) with a sample of the pathogen growing on it was placed at the center of the 2% malt agar plate. The filter papers with and without extracted metabolites were placed two centimetres from the pathogen. The growth towards the filter paper discs was measured until the hyphae of the pathogen had reached the control filter paper disc. Pathogen growth under each condition was statistically compared with the Paired T-test (IBM SPSS Statistic version 21). To confirm the persistence of the inhibition effect due to the metabolites the experiment was repeated by placing the filter papers with and without extracted metabolites at a distance of four centimetres from pathogens *H. parviporum* and *Ph. pini* and hyphae growth was followed for 14 days (**IV**).

4. 5. Characterisation of the secreted metabolites

A non-inhibitory endophyte strain 5992 (identified as *Phialocephala* sp. based on ITS 1 and 2) was used as a control to characterise the secondary metabolites. The two inhibitory endophytes (strains 513 and 222) as well as the non-inhibitory endophyte (strain 5992) and *H. parviporum* were inoculated separately in 50 ml of 2% malt extract. They were shaken at 50 rpm for 3 months at 21 °C. The filtrate was extracted with 2x equal volumes of ethyl acetate and then dried. The extracts were re-suspended in acetonitrile and dried under nitrogen. The extracts were filtered and re-dissolved in acetonitrile and screened by UPLC-QTOF/MS using electrospray ionisation in both positive and negative ion mode. Sclerin was used as a reference standard to test whether it was present in any of the samples (Tellenbach et al. 2013) (**IV**).

The UPLC-QTOF/MS data of the metabolites was analysed using two pattern recognition methods, PCA and OPLS-DA to distinguish differences between groups, identify possible outliers and identify metabolites responsible for differences between control non-inhibitory endophyte versus inhibitory endophyte 513 or 222, and pathogen vs. inhibitory endophyte 513 or 222. The UPLC-QTOF/MS analysis showed that results from the positive and negative ion mode did not differ significantly, therefore only results from the positive ion data were analysed. Each mass-retention time combination from the UPLC-QTOF/MS data corresponded to one entry of a metabolite in a score plot. Such data consists of fragments and adducts of the original metabolite (**IV**). This was taken into account and checked when the most significantly differentiated metabolites were chosen (**IV**; **Table 1**, **2**). Based on the statistical differences, ten metabolites were chosen from a sigma plot and manually checked to find

the metabolites only present in the inhibitory *Cryptosporiopsis* sp. (endophyte 513) or *Phialocephala* sp. (endophyte 222). Their tentative molecular formula was elucidated primarily by the analyses of HRMS data. All chemometrics analysis (PCA, OPLS-DA) were performed with MarkerLynx XS V4.1 software (Waters Inc.) (**IV**) (Table 1). The materials, methods and phytopathogenic fungal strains used in this thesis are summarised in Tables 1 and 2. Detailed descriptions can be found in publications **I**–**IV**.

Table 1. Materials and methods used in this study

Materials and methods	Publications
Surface sterilisation	III
Cultivation of endophytic fungi	III, IV
DNA isolation	I, II, III, IV
PCR amplification of the internal transcribed spacer (ITS) region of ribosomal DNA	III, IV
Bar-coded PCR amplification of the ITS region of ribosomal DNA	I
Bar-coded PCR amplification of 16S rDNA region	II
454 -pyrosequencing	I*, II*
Sequence analysis and bioinformatics	I, II*, III, IV
Principal Component Analysis (PCA)	I, IV*
Analysis of variance (ANOVA)	I, III
Kruskal-Wallis test	III
The rarefaction analysis	I, II*, III
Diversity indices (Shannon-Wiener and Simpson's Reciprocal Index 1/D)	I, III
Similarity indices: Sørensen and Bray-Curtis	I, III
Redundancy analysis (RDA)	II*
Screening of inhibitory root endophytes	III
Inhibitory assays against Ascomycete and Oomycete plant pathogens	IV
Extraction of secreted metabolites from growth broth	IV
Metabolite inhibition assays	IV
Microscopy (bright-field microscopy and stereomicroscope)	III, IV
UPLC-QTOF/MS	IV*
Chemometrics analysis (OPLS-DA)	IV*

*Publications with asterisk indicate the methods were conducted by the co-authors.

Table 2. Phytopathogenic fungi used in this study

Fungi	Strain	Publications
Heterobasidion parviporum	Isolate 03014, heterokaryotic	III, IV
Phytophtora pini	Isolate Ph443	IV
Botrytis cinerea	Isolate B05.10	IV
Cryphonectria parasitica	Isolate C2658/LE1093	IV

5. RESULTS AND DISCUSSION

5.1. The impact to wood microbial communities after biocontrol agent application

454 -pyrosequencing of 18 stump samples (90 wood cores) generated a total of 53 117 fungal and 154 453 bacterial raw sequences. After data cleaning, we had 26 127 fungal sequences representing 49% of the original sequences and 123 562 bacterial sequences representing 80% of the original sequences. The biocontrol fungus *P. gigantea* represented 0.43% of all fungal sequences and was only found on stumps one year after post-treatment (**I; Table 2**). The root pathogen *H. annosum s.l.* was not observed in this study.

5.1.1. Impact on wood microbes one year after application

One year post-treatment, a total of 8379 (treated = 4060, control = 4319) fungal sequence reads were observed from six different stumps generating 124 different OTUs: 106 and 119 OTUs from treated and control stumps, respectively (**I**, **Table 1**). Substantially higher number of sequences were observed for bacterial sequences (treated = 17801, control = 17224), generating 3925 OTUs: 2285 and 2692 OTUs from treated and control stumps, respectively (**II**, **Table 1**). Based on the PCA analysis of the fungal OTU composition, a specific aggregation pattern between control and treated stumps could be observed one year post-treatment indicating that *P. gigantea* impacted fungal community structure (**I; Fig. 2**), even the difference between control and treated stumps was not found to be significantly different (p= 0.47). For the bacterial community, the biocontrol application significantly decreased (p=

0.004) the number of individual OTUs in treated stumps compared to control stumps within the first year. Similarity indices (Sørensen) showed high similarity between the control and treated stumps (**I**; **Table 3**) and further confirmed that the main fungal OTUs observed were equally present in the stumps despite the treatment. The Bray-Curtis index (BC, **I**; **Table 3**) was found to be intermediate. This index was recalculated with logarithms of sequence data observed in each OTU (number of sequences in each OTU) (BC= 0.18), indicating that treated and control stumps share similar OTU rate. Vainio et al. (2005) could not find statistical differences between the fungal OTU profiles of the three treated and three untreated Norway spruce stumps one year after "Rotstop" application. Vasiliauskas et al. (2004) discovered that 7 weeks after "Rotstop" application the fungal species richness decreased by 15%. Nevertheless, Vasiliauskas et al. (2004) observed that the Sørensen similarity indices showed treated stumps to be mainly colonised by the same fungi occurring naturally in untreated stumps (Vasiliauskas et al. 2004). The present study is in line with results observed in previous studies (Vasiliauskas et al. 2004, Vainio et al. 2015), indicating that the application of *P. gigantea* on stump surfaces impacts fungal community structure, but this does not appear to be significantly decreased (Vainio et al. 2005, **I**) as the major fungal OTUs are present in the same magnitude (**I**). "Rotstop" application had a clear negative impact on the bacterial community after one year. Obviously the initial application of *P. gigantea* to fresh stumps has an effect on bacterial flora in wood material. Saprotrophic *P. gigantea* (especially the genotype of "Rotstop") acts as a strong competitor, and based on our results this fungus disturbed bacterial OTU composition during the primary decomposition succession of the stumps in our study site in southwestern Finland (Liesjärvi) (**II**).

5.1.2. Impact on wood microbes six years after application

We observed 8388 (4273 = treated, 4115 = control) fungal sequences from six different stumps six years after the stump treatment. Altogether, we documented 153 different OTUs: 134 from control stumps and 119 from treated stumps (**I**, **Table 1**). Substantially higher number of sequences were observed for bacterial sequences (treated = 22342, control = 20620) generating 4628 OTUs: 3467 and 2298 OTUs from treated and control stumps, respectively (**II**, **Table 2**). Vainio et al. (2001) observed that six years after treatment with the "Rotstop" strain of *P. gigantea*, it was still present in *P. abies* stumps. In this study *P. gigantea* ("Rotstop") was not observed six years after stump application. However, a taxon with only 84% sequence similarity to *Phlebiopsis gigantea* was first considered a species of *Phlebiopsis* in paper **I** (Terhonen et al. 2013). Further reblasting and analysis has revealed that *Phlebiopsis* is not a plausible genus name for this isolate (**I**). Vasiliauskas et al. (2005) also observed that the "Rotstop" strain of *P. gigantea* was less frequently isolated from six-years-old Norway spruce stumps compared to four-year-old stumps. A similar decline in the isolation rate for *P. gigantea* could be observed in control stumps that were subjected to natural infections by wild strains of *P. gigantea* (Vasiliauskas et al. 2005). Our results further confirmed previous reports on the natural behaviour of *P. gigantea* that have shown a decrease in older stumps (Hintikka 1993, Vainio et al. 2001, Vasiliauskas et al. 2002, Vainio et al. 2005, Vasiliauskas et al. 2005).

PCA and RDA showed more intermingling between the control and treated samples in fungal and bacterial OTU composition in this study, and no statistical differences were observed (**I**; **Fig. 2**, **II**; **Fig. S3**). Vainio et al. (2015)

observed that the “Rotstop” treatment appeared to mainly influence species composition in the six-year-old Norway spruce stumps, but did not reduce their overall fungal diversity. Vasiliauskas et al. (2005) found that “Rotstop” application led to a significant decrease in overall fungal community structure in Norway spruce stumps six years after treatment. Despite the decrease in species diversity, the majority of species that colonised stumps following “Rotstop” treatment were found to also occur naturally in untreated controls (Vasiliauskas et al. 2005). In the previous study the quantitative Sørensen similarity index showed that species, common in both treated and control stumps occurred at approximately similar rates (Vasiliauskas et al. 2005). The Sørensen similarity index in this study also indicates that the major fungal OTUs were present six years after in the treated and control stumps (**I**; **Table 3**). The Bray-Curtis index (BC, **I**; **Table 3**) was found to be intermediate. This index was recalculated with logarithms of sequence data observed in each OTU (BC= 0.20), indicating that treated and control stumps share similar OTU rates. The observed frequency (number of sequences) and diversity (number of OTUs) of bacterial communities were higher in treated stumps compared to control stumps in this study (**II**; **Table 1**). Based on our results we can conclude that six years after “Rotstop” treatment, the presence of *P. gigantea* has decreased and that major fungal and bacterial OTU composition and frequency (number of sequences) are similar in treated and non-treated stumps.

5.1.3. Impact on wood microbes 13 years after application

Thirteen years post-treatment, we observed 9630 (4338 = treated, 5292 = control) fungal sequences from six different stumps. In total, we recorded 161 different fungal OTUs: 139 from the control stumps and 131 from the treated

stumps (**I**; **Table 1**). Substantially higher number of sequences were observed for bacterial sequences (treated = 23397, control = 22178), generating 6893 OTUs: 3997 and 4431 OTU from treated and control stumps, respectively (**II**; **Table 1**). The bacterial frequency (number of observed sequences) was higher at the treated compared to the control stumps (**II**; **Table 1**). An RDA of bacterial OTUs showed some aggregation between control and treated stumps 13 years post-treatment and no statistical differences were observed (**II**; **Fig**. **S3**). PCA results on fungal species composition similarly showed some aggregation between control and treated stumps 13 years post-treatment and no statistical differences were observed (**I**; **Fig**. **2**). The diversity index (Shannon-Wiener) indicates that species richness in the fungal community reached the same level between the control and treated stumps (**I**; **Table 1**). The Sørensen index was high (0.8), illustrating that the major OTUs were shared between the control and treated stumps (**I**; **Table 1**). The Bray-Curtis index (**I**; **Table 3**) was found to be intermediate for fungal OTUs. This index was recalculated with logarithms of data sequences observed in each OTU (BC= 0.19), indicating that treated and control stumps share similar OTU rates. Based on our results we can conclude that after a long time period the persistence of *P. gigantea* is zero and bacterial and fungal communities have reached the same level in frequency (number of sequencies), species level (number of OTUs) as well as sharing the major OTUs in treated and control stumps (**I**, **II**).

Menkis et al. (2012) concluded that the biocontrol agent *P. gigantea* has little or no impact on the belowground occurrence and persistence of this species in forest ecosystems and consequently has no significant impact on soil fungi. Together with previous findings (Menkis et al. 2012), our results gathered 13 years after "Rotstop" application on the stumps supports the continued use of *P.*

gigantea for stump pre-treatment in Finnish forests against *H. annosum s. l.* when the risk of infection is high.

It was obvious that the microbial community was disturbed at the initial stage of decay (one year) after "Rotstop" application. As the stump-age effect cannot be separated from the site effect we cannot properly say that the negative effects observed one year after would be attenuated over time in the same site (**I**, **II**). This experiment should be repeated at the same site (Liesjärvi) at different time periods to further prove that the "Rotstop" strain of *P. gigantea* is not persistent over the long-term and the negative effect especially towards bacterial flora will be attenuated over time.

5.2. Composition of *P. abies* fungal root endophytes at different boreal forest sites

The diversity and frequency of culturable endophytes of healthy non-mycorrhizal Norway spruce (*P. abies*) roots in different boreal forest sites was investigated. The species diversity of endophytic fungi was low; only 15 different OTUs could be detected and the frequency of singletons was high (20% off all OTUs). A similar pattern was observed by Stenström et al. (2014), as the fungi isolated either once or twice from *P. abies* and *P. sylvestris* roots consisted of ~ 36% of all isolates. Among the mycota, the class Leotiomycetes and the order Helotiales were found to be most dominant (**III**; **Table 1**). DSEs were the most abundant isolates (77 %), consisting mostly of the PAC species complex (52% of all isolates). Other authors have similarly reported that the isolation rate of DSEs and PAC constituted the major isolates (Holdenrieder & Sieber 1992, Ahlich & Sieber 1996, Grünig et al. 2002, Stenström et al. 2014).

In our study, *A. applanata* (27%) and *P. fortinii* (25%) were the most abundant species. *A. applanata* is associated more with *P. abies* (Grünig & Sieber 2005, Grünig et al. 2006; 2008a), but *P. fortinii s.l.* seems not to have a host preference (Ahlich & Sieber 1996, Grünig et al. 2006; 2008a; 2011, Tejesvi et al. 2010; 2013).

Endophyte species composition in different forests stands can be distinct and the same fungal species may occupy different micro-habitats or hosts during the lifecycle of the fungus (Saikkonen et al. 2004a;b, Osono 2006, Saikkonen 2007). For example Helander et al. (2006) observed that the abundance and species composition of endophytic fungi in the leaves of silver birch (*B. pendula*) significantly differed between seedling stands, managed mature forests and natural old forests. Grünig et al. (2006) observed that the number and species composition of the PAC species complex clearly differed between managed and undisturbed forests. Queloz et al. (2011) on the other hand found no evidence of a cryptic biogeographic structure in PAC species comprising more than 5000 isolates of 21 PAC species sampled from across the Northern Hemisphere. In this study drained peatland had the highest number of different OTUs and isolates (**III**; **Table 1**). All the diversity indices (**III**; **Table 3**) and species accumulation curves (**III**; **Fig. 2**) indicated higher diversity in drained peatland compared to mineral soil and pristine mire, although this difference was not significant. Based on the Bray-Curtis value, mineral soil and pristine mire endophyte communities are composed of similar OTU rates while drained peatland differed equally from the other two study sites (**III**; **Table 3**). Artz et al. (2007) showed that when vegetation in different peatlands varied significantly, the composition of fungi also varied. Bougoure et al. (2007) observed a similar trend when the fungi community associated with *Calluna*

vulgaris (L.) Hull root hairs varied along a vegetation gradient: samples were collected from the forest, open heathland and a transition zone between the two. Our study sites were comparable in terms of fertility (rich *Vaccinium* sites) (Päivänen & Hånell 2012). Soil material composition is nevertheless considerably different on these sites as the mineral soil consists of podsol (humus layer, eluviated soil, subsoil and parent material) and pristine mire and drained peatland consist mostly of organic material that is highly decomposed and dark peat. Drainage changes peatland vegetation towards that of mineral soils as the *Sphagnum* species are replaced with feather mosses with time (Päivänen & Hånell 2012). Based on our results the endophytic communities in Norway spruce roots were not statistically different in pristine mire, mineral soil and drained peatland at the fertility level of a rich forest type (**III**). OTUs *A. applanata* and *P. fortinii s.l.* were found from all sites (**III**; **Table 1**). Besides the PAC species complex, only *Phialocephala sphaeroides* B.J. Wilson (16 isolates) and *Meliniomyces variabilis* Hambleton & Sigler (9 isolates) were isolated from all sites (**III**; **Table 1**). It was clear that the same major fungal species could be found from all sites. Although no statistical difference between the study sites was observed, disturbance of the habitat (drainage) leads to plant succession and/or to the alteration of the host plant's reactions, which in turn may affect the occurrence frequency and species communities of endophytes.

5.3. Endophytes screened for antagonism against *H. parviporum*

A total of 19 isolates (17% of all isolates) from four OTUs showed an inhibitory effect against *H. parviporum in vitro* (**III**; **Table 4**). The isolates were considered inhibitory when *H. parviporum* was unable to overgrow the endophyte and its growth had ceased. Most of these "inhibitory" isolates (58%)

were obtained from drained peatland (**III**; **Table 4**), and 21% from both the pristine mire and the mineral soil (**III**; **Table 4**). Most likely the inhibition observed in paper **III** is due to secondary metabolites secreted by these endophytes. Of the endophytes isolated from drained peatland, 25% are expressed as inhibitory against *H. parviporum.* 10% of endophytes from the pristine mire and 13% of isolated endophytes from the mineral soil were considered inhibitory. We can conclude that fungal root endophytes from forest sites (drained peatland) not commonly inhabited by *H. annosum s. l.* possess a strong inhibitory effect on the growth of pathogen *H. parviporum*. It is difficult to draw conclusions for the reasons behind this strong inhibition of endophytes from drained peatland. Disturbance of the habitat (drainage) may lead to alterations of the host plant's reactions, which in turn could affect the endophytes. Removing the inoculum source of *H. annosum s. l.* entirely from diseased forest sites seems impossible as this pathogen can remain viable for at least seven years in 15-mm diameter roots and vegetatively infect nearby seedlings (Piri & Hamberg 2015). This highlights the importance of having new biocontrol agents against *H. annosum s.l.* that could be utilised to protect seedling roots especially at heavily diseased sites. Based on our results some root endophytes can inhibit the root rot pathogen *H. parviporum in vitro*. The next step is to test whether strongly DSE-colonised host plants can escape infection by root rot pathogens *in vitro*. This will be followed by a pilot field trial to evaluate the persistence and performance under natural conditions.

The two inhibitory strains, 222 isolated from the roots of Norway spruce from a minerotrophic pristine mire (*Vaccinium myrtillus* spruce swamp) and 513 isolated from roots from a minerotrophic drained peatland (*Vaccinium myrtillus* type 1), and the non-inhibitory strain 5992 also isolated from drained peatland,

were used for further assays (**IV**). Based on the sequence analyses of ITS 1 and 2, the two inhibitory endophytes grouped with *Cryptosporiopsis ericae* Sigler (strain 513) and *P*. *sphareoides* (strain 222) and the non-inhibitory strain 5992 within the PAC species complex (**III**; **Fig. 1**, **IV**; **Fig. 2**). *C*. *ericae* was originally found from plant roots belonging to Ericaceae (Sigler et al. 2005), but in the boreal region it has been isolated from *Abies balsamea* (L.) P. Mill (Kernaghan & Patriquin 2011) and *Populus tremuloides* Michx. (Wang et al. 2007) roots. Wilson et al. (2004) described *P*. *sphareoides* for the first time, as originating from the roots of diverse hosts (*Betula papyrifera* Marsh., *Rubus idaeus* L., *Smilacina trifolia* L. Sloboda). They obtained *P*. *sphaeroides* only from plants in the highly acidic (pH= 3.9), *Sphagnum*-dominated wetland habitat and not from the same plant species in the less acidic (pH= 6.5), aspen-dominated upland site (Wilson et al. 2004). We observed *P*. *sphaeroides* evenly at each site (**III**; **Table 1**). The mineral soil or peat at our study sites is highly acidic (the pH values of that study area vary from 3.8 to 4.5). *P*. *sphareoides* has been previously isolated from the roots of *Deschampsia flexuosa* (L.) Trin and *Trientalis europaea* L. from mixed forests (acid soil, pH= 4.4) (Tejesvi et al. 2013) and from trees *B*. *papyrifera*, *A*. *balsamea* and *Picea glauca* (Moench) Voss in mature boreal sites (Kernaghan & Patriquin 2011). This endophyte does not appear to have a host preference and seems to prefer acidic habitats. Hereafter the endophytes chosen for further inhibitory assays are referred to as *Phialocephala* sp. 222, *Cryptosporiopsis* sp. 513 or *Phialocephala* sp. 5992.

5.4. Antagonism between the chosen endophytes and pathogens in paired cultures

The pathogens (*H. parviporum*, *P. pini*, *B. cinerea* or *C. parasitica*) and endophytes (*Cryptosporiopsis* sp. 513 or *Phialocephala* sp. 222) were paired in a Petri plate at a distance of 60 mm from each other and incubated at 21 ˚C (**IV**; **Fig. 1**; **3**). Pathogen growth was significantly reduced by the presence of the endophytes (**IV**, **Fig. 3**; **4A**). *Cryptosporiopsis* sp. 513 and *Phialocephala* sp. 222 evidently inhibited and even stopped the growth of pathogenic fungi (**IV**, **Fig. 3**; **4A**). The use of fungal endophytes as biocontrols in conifer trees has been demonstrated on *P. glauca* seedlings following inoculation with the rugulosin producing fungal endophyte, as this fungus was able to reduce the growth and development of the spruce bud worm (*Choristoneura fumiferana* (Clemens)) (Miller et al. 2002). Fungal needle endophytes of *Pinus strobus* L. have been noted to be antifungal against *Saccharomycetes cerevisae* Meyen ex E.C. Hansen (Sumarah et al. 2011) and the potential of these pine endophytes to provide protection to *P. strobus* trees against *Cronartium ribicola* J.C. Fisch in eastern North America is under evaluation (Sumarah et al. 2011). Tellenbach & Sieber (2012) showed that some *P. subalpina* isolates effectively reduced mortality and disease intensity caused by the two pathogenic oomycetes in conifer tree (*P. abies*) roots. Fungal endophytes could play a vital role on host fitness by protecting the tree host against pathogens and pests (Miller 2002, Arnold et al. 2003, Ganley et al. 2008, Li et al. 2012). Results from our inhibitory study have definitely increased the interest towards these root endophytes, their metabolites and the new possibilities for utilising these endophytes as biocontrol agents in forestry (especially in nurseries) against plant pathogens.

5.5. Effect of crude extracts from the fungal endophytes on pathogen growth

We were able to show that the inhibition observed in this study originates from the metabolites secreted in the liquid cultures (**IV**; **Fig. 4 B-C**; **6**), further highlighting the strong prospects of using these endophytes as biocontrol agents. Previous studies have shown that the metabolites extracted from various fungal endophytes have expressed antifungal activity against human and plant pathogens *in vitro* (Strobel et al. 1999, Tellenbach et al. 2013). Our results showed that some fungal endophytes isolated from *P. abies* roots secrete antifungal substances. Metabolites secreted by *Cryptosporiopsis* sp. 513 induced apical swelling in the hyphae tips and along the mycelia of *B. cinerea* hyphae (**IV; Fig. 7**). The *C. parasitica* hyphae in the vicinity of metabolites from *Cryptosporiopsis* sp. 513 were observed to grow abnormally. *H. parviporum* hyphae in contact with the metabolite showed abnormal growth due to more branching and were thicker compared to the control hyphae (**IV**; **Fig. 7**). No morphological change was observed in the case of *Ph. pini* hyphal growth. Some statistically significant differences were detected in the metabolites from *Phialocephala* sp. 222 (**IV; Fig. 4C**, **7**). Morphological changes such as swollen, thick hyphae were noted in the case of *H. parviporum* (**IV; Fig. 7**) and *C. parasitica*. Branching of *B. cinerea* hyphae growing towards metabolites extracted from *Phialocephala* sp. 222 could be observed (**IV**; **Fig. 7**). Fungiside treatments against grey mold (*B. cinerea*) are sometimes needed in nurseries (Lilja et al. 2010). In this study some of the observed metabolites could be derived to antifungal chemicals that could possibly be utilised against *B. cinerea* and other pathogenic fungi in nurseries. However,

the use of any endophyte-derived fungicidal will involve the same environmental concerns as other chemicals (Witzell et al. 2014).

5.6. Chemical analysis of the metabolites

The two inhibitory endophytes (*Cryptosporiopsis* sp. 513 and *Phialocephala* sp. 222), the non-inhibitory control endophyte (*Phialocephala* sp. 5992) and *H. parviporum* shared 432 metabolites and associated fragments and adducts (hereafter mentioned only as metabolites), which is close to half of the individually observed metabolites in each fungus (**IV**; **Fig. 8B**). A total of 214 unique metabolites were detected from *Phialocephala* sp. 222 and 342 unique metabolites from *Cryptosporiopsis* sp. 513. The observed amounts of these metabolites in the crude extracts were found to be minimal in addition to difficulties in isolating them. Fungal endophytes have been noted to secrete various groups of metabolites: amides, amines, peptides, flavonoids, steroids, phenylpropanoids, lignans and terpenoids (Schulz et al. 1999, Tan & Zou 2001, Schulz et al. 2002, Strobel 2003, Yu et al. 2010, Mousa & Raizada 2013). Based on molecular weight and putative chemical formulae it is impossible to assign these metabolites to a specific group. More supporting information from nuclear magnetic resonance (NMR) spectroscopic assays of pure isolated metabolites are needed. *Cryptosporiopsis* sp. 513 possessed the highest number of unique metabolites. This is probably the reason for its stronger inhibitory effect against the pathogens (**IV**; **Fig. 4A-C**). Sumarah et al. (2011) extracted seven major metabolites produced by the foliar fungal endophytes of *P. strobus*, which resulted in the discovery of three antifungal compounds against both the rust *Microbotryum violaceum* (Pers.) G. Deml & Oberw. and *S. cerevisae*. Sumarah et al. (2010) similarly extracted nine major metabolites from the foliar

endophytes of *Picea rubens* Sarg. three of which showed toxicity to *S. cerevisae*. Tellenbach et al. (2013) isolated four major compounds from the root endophyte *P. europaea*. Sclerin and sclerotinin A significantly reduced the growth of *Phytophthora citricola s. lato*. Tellenbach et al. (2013) concluded that the two metabolites are either individually or synergistically responsible for the growth inhibition of the oomycetes pathogen under *in vitro* experiments. Sclerin was detected only in the metabolite profiles of *Phialocephala* sp. 222. It is most likely that the inhibition zones observed in my study are also due to the synergistic effect of unique metabolites (**IV**; **Table 1**, **2**, **Fig**. **8B**)

According to elemental composition analysis of exact mass fragmentation of the mother ion, the loss of water i.e. hydroxyl groups found in the structure as well as known isotope patterns, the structures of metabolites with chlorine isolated from *Phialocephala* sp. 222 could be similar to dichlorodiaportin (Larsen & Breinholt 1999) or cryptosporiopsin (Strunz et al. 1969) that was earlier found to be a novel chlorine-containing antifungal agent. Our results revealed that the extracted metabolites had a profound effect on pathogen growth such as malformation, swelling, coiling and general retardation in hyphal tip extension. The observed metabolites from *Cryptosporiopsis* sp. 513 can be excluded from the alkaloids group because of the absence of nitrogen. Other reports have described a lipopeptide (cryptocandin) isolated from *Cryptosporiopsis* cf. *quercina*, an endophyte of *Tripterigium wiflordii* Hook.f., with demonstrated antifungal activity against some important human pathogenic fungi including *Candida albicans* (C.P.Robin) Berkhout and *Trichophyton* sp. (Strobel et al. 1999). This lipopeptide was also active against a number of plant-pathogenic fungi including *Sclerotinia sclerotiorum* (Lib.) de Bary and *B. cinerea* (Strobel et al. 1999). Similarly echinocandin, a peptide,

isolated from a broth culture of endophytic *Cryptosporiopsis* sp., from *P. sylvestris*, expresses antimicrobial activity against certain yeasts (Noble et al. 1991). Li et al. (2000) were also able to isolate the peptide cryptocin from *Cryptosporiopsis* cf. *quercina* with an inhibitory effect against plant pathogenic fungi. The metabolites cryptocandin, echinocandin or cryptocin described by previous authors are peptides indicating that they are chemically different from the metabolites extracted in the present study. The metabolites observed in this study could belong to sequiterpenes, maleic anhydride moieties or polyketides as described in Sumarah et al. (2010). These results suggest that endophytic *Cryptosporiopsis* sp. appear to be producers of a wide range of secondary metabolites with fungicidal activity as their teleomorph genus *Pezicula* has also been demonstrated to secret secondary metabolites with antibacterial and fungicidal activity (Noble et al. 1991, Schulz et al. 1995).

5.7. Revealing true microbial diversity

The rarefaction curves of fungi and bacteria in papers **I** and **II** did not converge at an asymptote (**I**, **Fig.1**; **II**, **Fig. 1**) indicating that increasing the sampling effort would have revealed more species. Unterseher et al. (2011) highlighted that NGS provides large amounts of data, but undersampling remains a problem in biodiversity research. The sequencing strategy can be modeled in terms of the number of sequenced samples and the per-sample sequencing depth (see Fumagalli 2013). This would decrease the variation within sampling groups and is highly recommended for further studies carried out with NGS. In this study (**I**, **II**), new sequencing methods revealed a dramatic increase of OTUs compared to previous studies and methods, for example six years post-treatment we observed 153 fungal OTUs in the control stumps versus 43 OTUs

through the direct isolation method (Vasiliauskas et al. 2005) and 38–48 OTUs through DGGE, depending on the primer pairs used (Vainio et al. 2005). This new method obviously gives more informative results from the wood fungal community as already reported by Ovaskainen et al. (2010) and Rajala et al. (2012). Nevertheless, we could not identify most of the fungi to the species level. The large proportion of unclassified genus-level sequences (39–60%) suggests that a large diversity of bacteria in the stumps also remains unknown. Similarly, Rajala et al. (2010) could not identify many of the wood-decaying fungi determined by DGGE and sequencing because their DNA sequences did not match any of the identified fungal species deposited in the public gene bank database. Ovaskainen et al. (2010) compared the 454 -sequence data with a reference library containing well-annotated sequences of 1145 species of wood-decaying and mycorrhizal fungi and were unable to identify more than half of the fungi inhabiting dead wood. Ottosson et al. (2015) were able to identify one third of the OTUs obtained from spruce logs by 454- sequencing either to the fungal species or the genus level, using three different databases: UNITE (Abarenkov et al. 2010), SAF (spruce-associated fungi) (Ovaskainen et al. 2010) and BLAST search against GenBank (NCBI) (Altschul et al. 1997). Ottosson et al. (2015) could assign ecological roles to more than half of the amplified DNA sequences. The authors concluded that in addition to wood-decay fungi the fungal communities in decaying wood largely comprise fungal species with a range of other ecological roles. These results show that most of the microbes in dead wood and their mode of action still remain unknown. Hibbet & Taylor (2013) also raise the question of errors and incomplete taxonomic sampling in sequence databases; if an environmental sequence has no match in GenBank, it could still represent a described but un-sequenced species. To overcome these limitations Ovaskainen et al. (2010) suggested

improving the reliability of identifications with sequence information from other regions of the genome and extending the coverage of the reference database (Ryberg et al. 2009).

6. SUMMARY AND CONCLUSIONS

In this thesis, I studied the impact of *P. gigantea* treatment on stump microbes using the 454-pyrosequencing approach. I further explored the potential of finding other novel biocontrol agents against the root rot pathogen (*H. annosum s.l.*) with isolation studies of fungal root endophytes from forestry sites such as pristine mires and drained peatlands where *H. annosum s.l.* spread has not been commonly reported. Additionally, the metabolites secreted by the root endophytes and pathogen *H. parviporum* were extracted and the inhibitory effects of the endophytes metabolites on phytopathogenic fungi were assayed.

Our results 13 years after "Rotstop" application to the stumps supports the continued use of *P. gigantea* for stump pre-treatment in Finnish forests against *H. annosum s. l.* (**I**, **II**). Compared to other control methods (chemical, stump removal, no control), the use of the "Rotstop" strain in the clear-cutting areas to decrease the occurrence of *H. annosum s.l.* is recommended when loggings are performed during summertime to reduce the basidiospore dispersal of this pathogen. Biocontrol application seems to currently have the smallest negative impact on the environment. However, comprehensive studies regarding longer-term monitoring from the same site are still needed to prove that the adverse initial impacts of *P. gigantea* are attenuated over time.

The findings in paper **III** revealed no significant difference between the fungal isolate frequencies or diversity at the various site habitats. However, these results were based only on cultivated endophytes, which may have led to an undersampling of the fungal community and a combination of various methods (direct sequencing of DNA and cultural methods) should be applied in future

research. Diversity studies of root endophytes in different environments provide additional knowledge about their community and host interactions, but we still know little about their precise functional role in the roots of their hosts. Overall, this study provides an initial insight into the major fungal root endophytes of pristine mires, mineral soil and drained peatlands (**III**).

In paper **IV**, a subset of the endophytes with potential inhibitory effects on the growth of *H. parviporum* and other phytopathogenic fungi were identified. The secreted metabolites from these endophytes were also found to possess inhibitory properties. The best way to achieve the set objectives of integrated pest management (IPM) policy would be the exploration of biotechnology application of these beneficial endophytes as inoculants to facilitate plant protection.

7. FUTURE PERSPECTIVES

The new possibilities to protect the valuable seedling roots from root rot pathogens are vital in the foreseeable future in forestry. The biocontrol capability of inhibitory root endophytes documented in this study could serve as an alternative method to restrict and manage the disease caused by the root rot pathogen *H. annosum s. lato*. Root endophytes might serve as the first root protectors of young seedlings after planting, especially on most contaminated clear-cut sites. This may potentially enhance seedling survival during the most critical years against root rot pathogens. Further studies on this are on-going.

Molecular biology is becoming an increasingly important tool in forestry for tackling forest ecology and pathology problems. These new methods and accumulated information provides a better understanding of complex interactions in forest ecology and provide possibilities to go deeper into molecular forest pathology applications in practical forestry. Presently, we still do not fully understand the environmental consequences in the application of a single strain of biocontrol agent *P. gigantea* to freshly cut stump surfaces. The biological and antagonistic activity against non-target microbes in principle implies a potential environmental risk. A deeper understanding on the mechanism of action of *P. gigantea* would be of importance for the further improvement and management of the biocontrol effect. To accomplish this, long-term follow up trials and basic research are still needed. This would form the basis for the development of environmentally friendly and sustainable management strategies for forestry.

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