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Physiological and molecular analysis of the interaction between the conifer pathogen, *Heterobasidion annosum* s.l. and *Phlebiopsis gigantea*

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ACADEMIC DISSERTATION

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Abstract

Prophylactic stump treatment with *Phlebiopsis gigantea* has been used for a long time in the biological control of the conifer pathogen, *Heterobasidion annosum* sensu lato. However, the mechanisms underlying the biocontrol process are poorly understood. A total of 64 isolates of *P. gigantea* from different geographical regions were screened for fungal traits (growth rate, enzyme production, wood decay capabilities and antagonism) important for the biocontrol properties. The genomic sequence and the expression profile of *P. gigantea* hydrophobin encoding genes 1 and 2 in a selected set of isolates of the biocontrol fungus was analysed. Additionally, the distribution and the regulatory patterns of different hydrophobin encoding genes in the ecologically important fungi, *P. gigantea* and *H. irregulare* were investigated alongside the evolutionary forces driving hydrophobin gene evolution.

The results show that different growth media had a huge influence on the outcome of the interaction between *P. gigantea* and *H. annosum* s.s. and the ability to degrade the different structural components of wood could partly explain the higher competitive advantage over *H. annosum* s.s. The antagonistic ability of *P. gigantea* also correlated positively with the transcript levels of hydrophobin 1 and 2 encoding genes (*Pgh1* and *Pgh2*) but there was no relationship between the antagonistic ability and the sequence variants of either *Pgh1* or *Pgh2*. Furthermore, the regulatory patterns of *Pgh1* and *Pgh2* suggest a role in the formation of aerial hyphea during the growth and development of *P. gigantea.*

Hydrophobin encoding genes are redundant in both fungal species studied in the thesis research. However, in *H. annosum* s.s*.,* each gene coding for hydrophobins seemed to be regulated by different environmental factors. Hydrophobin encoding genes were found to have witnessed a considerable expansion in both *P. gigantea* and *H. annosum* s.l. as well as in other basidiomycetes while a massive contraction of the hydrophobin encoding genes has occurred in the ascomycetes. Evidence of positive selection was also observed in *P. gigantea* and *H. annosum* s.l. hydrophobins and the hydrophobins from other fungal species. Finally, to understand the effect of secondary metabolites produced by *P. gigantea* on the gene expression profile of *H. annosum* s.s., transcriptomics analysis was carried out using microarray expression method. Also macroarray analysis was used to compare the transcriptomics profile of different strains of *P. gigantea* (competitively effective wild type, less competitively effective wild type and their progeny) against the commercial isolate, Rotstop F® when cultivated on artificial growth medium. The metabolites from *P. gigantea* effectively repressed some genes involved in diverse metabolic pathways in *H. annosum* s.s. Aditionally, several genes were found to be uniquely expressed in the progeny strain of *P. gigantea,* an indication that breeding could serve as an alternative for improving the *P. gigantea* isolates for a better biocontrol property. Generally, the results reported in this study have highlighted some of the physiological, biochemical and molecular mechanisms governing the biological control of the conifer pathogen, *H. annosum* s.l., by *P. gigantea.*

Contents

List of original publications and submitted manuscripts

This thesis is based on the following publications referred in the text with Roman Numerals I-IV. Unpublished results are also presented.

I Anthony C. Mgbeahuruike, Hui Sun, Petra Fransson, Risto Kasanen, Geoffrey Daniel, Magnus Karlsson, Frederick O. Asiegbu. 2011. Screening of *Phlebiopsis gigantea* isolates for traits associated with biocontrol of the conifer pathogen *Heterobasidion annosum* . Biological Control 57 (2): 118 – 129.

II Anthony C. Mgbeahuruike, Magnus Karlsson, Frederick O. Asiegbu. 2012. Differential expression of two hydrophobin genes (*Pgh1* and *Pgh2*) from the biological control agent *Phlebiopsis gigantea.* Fungal Biology 116 (5): 620–629.

III Anthony C. Mgbeahuruike, Hongxin Chen, Wimal Ubhayasekera, Frederick O. Asiegbu. Gene contraction and expansion in hydrophobin genes of ecologically important fungi *(Phlebiopsis gigantea* and *Heterobasidion annosum* s.l.*)-Submitted*

IV Anthony C. Mgbeahuruike, Hui Sun, Annegret Kohler, Francis Martin, Frederick O. Asiegbu. Expression analysis of the impact of secondary metabolites from biocontrol agent (*Phlebiopsis gigantea*) on the conifer pathogen (*Heterobasidion annosum* s.l.) transcriptome-*Submitted.*

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Author's contribution to the research and publications

I FOA & **ACM** conceived and designed the study**. ACM** conducted all the laboratory work and wrote the manuscript. **ACM** did a major part of the statistical analysis and result interpretation. MK contributed in the statistical analysis. PF contributed in the principal component analysis, DG contributed in the wood microscopy, HS contributed in the field data and the data on spore production, RK helped in editing the manuscript and provided the *P. gigantea* isolates for the study. FOA & MK contributed in drafting the manuscript.

II FOA, MK & **ACM** conceived and designed the study. **ACM** did all the laboratory work, **ACM** carried out all the statistical analysis, interpreted the results and did the writing of the manuscript. FOA & MK contributed in drafting the manuscript

III FOA & **ACM** conceived and designed the study. **ACM** carried out the bioinformatic and statistical analysis, did the microarray work and drafted the manuscript. HC contributed in the microarray work, WU did the work on protein modeling. FOA contributed in drafting the manuscript.

IV FOA & **ACM** conceived and designed the study. **ACM** generated the samples for the microarray studies and carried out the bioinformatic and data analysis; AK and FM contributed in the data analysis, **ACM** also wrote the manuscript. HS generated the macroarray data, FOA contributed in drafting the manuscript.

Abbreviations

ABA	Abscisic acid
BLAST	Basic Local Alignment Search Tool
BioEdit	Biological sequence alignment Editor
cAMP	Cyclic adenosine monophosphate
C3/C4	Cysteine residues 3 and 4
CCD	Charge Coupled Devise
cDNA	Complementary Deoxyribonucleic acid
DC	Digital Camera
dN/dS	Ratio of Non-synonymous to Synonymous nucleotide substitutions
d.p.i	days post inoculation
DNA	Deoxyribonucleic acid
FDR	False Discovery Rate
GH28	Glucoside hydrolase family 28
gL^{-1}	Grammes per Litre
GLM	General Linear Model
GO	Gene Ontology
Hah1	*Heterobasidion annosum* hydrophobin 1
Hah2	*Heterobasidion annosum* hydrophobin 2
IM	Image Manager
ISG	Intersterility group
JGI	Joint Genome Institute
JTT	Jones, Taylor and Thornton model for nucleotide substitutions
LSD	Least Significant Difference
MEA	Malt Extract Agar
MUSCLE	Multiple Accurate and Fast Secquence Comparison
MEGA	Molecular Evolutionary Genetics Analysis
MFS	Major Facilitator Superfamily
mm day^{-1}	Milimeters per day
M/N	Modified Norkrans
MoCo	Molybdenium cofactor
NCBI	National Center for Biotechnology Information
NJ	Neighbour Joining
NM	Norkrans medium
OD	Optical Density
PCA	Principal Component Analysis
PDB	Protein Data Bank
PCR	Polymerase Chain Recation
Pgh1	*Phlebiopsis gigantea* hydrophobin 1
Pgh2	*Phlebiopsis gigantea* hydrophobin 2
PKA	Protein kinase A
PSI-BLAST	Position Specific Iterative Basic Local Alignment Search Tool
qPCR	Quantitative Polymerase Chain Reaction

RT-qPCR	Real Time quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
SAS	Statistical Analysis System
$\%wv^{-1}$	Percentage weight per volume
UV	Ultraviolet light

1 Introduction

1.1 Forest tree and disease management

Forest trees play crucial roles in ecosystem maintenance by forming canopies for the ground vegetation (fauna and flora). Products from forest trees contribute substantially to the revenue generation of most countries in Europe and other parts of the world. In the northern hemisphere and other regions, the annual income generated from conifer trees and the associated industry is over 300 billion US dollars (http://english.forestindustries.fi). In Europe, the annual income from timber alone is estimated at 100 billion US dollars (http://english.forestindustries.fi). Forest trees are increasingly exposed to diverse range of microorganisms; some may be beneficial symbionts (mycorrhiza) or harmful (pathogens). In Europe and most countries in the northern hemisphere, a major disease of high economic importance in most conifer tree plantations is the root and butt rot disease caused by *Heterobasidion annosum* (Fr.) Bref. sensu lato (s.l). *H. annosum* is a necrotrophic white rot fungus that causes extensive decay in most conifer tree species and has been identified as a serious threat to the economic growth of the timber industry in the Northern hemisphere (Woodward et al.,1998; Asiegbu et al., 2005). The socio-economic impact of *H. annosum* in the forest industry across the globe is exceedingly huge. In Europe, losses incurred from the devastating effects of this pathogen are estimated at 800 million euros. In Finland alone, the annual economic loss could be over 50 million euros (Finland's National Forest Programme 2015, 2008). In addition, cost of the preventive chemical or biological control of the disease during cuttings is over 5 million EUR per year (Finnish Statistical Yearbook of Forestry, 2009). Although *H. annosum* exacts a huge negative impact on the profitability of forest and forest products, the fungus on the other hand plays a crucial role in creating forest conditions necessary for nutrient cycling, regeneration and succession (Garbelotto, 2004). Therefore *H. annsoum* has a strong ecological role, directly or indirectly driving natural processes and anthropogenic activities.

Several disease management approaches have been put in place to tackle the menace caused by this pathogen. Control mechanisms such as silvicultural approaches, use of chemical fungicides or natural antagonist have been employed. In silvicultural control of *Heterobasidion species*, several practices such as replacement of susceptible tree species, decreasing the number of thinnings /stand rotation, early harvesting of trees and cutting of trees during the winter period have been adopted. Silvicultural control of *H. parviporum* could be quite effective by growing other tree species in combination with conifer trees (Lygis et al., 2004). However, in areas where stumps and roots are already infected, silvicultural control (replacing tree species, changes in cutting practices etc.) is in practice limited by the lack of resistant tree species which would be suitable for the site fertility and feasible to grow.

Enormous positive results in terms of reducing the severity and dispersal of the disease have been obtained from chemical fungicides such as urea (Oliva et al., 2008) and borates (Pratt, 2000). However, there has been increasing concern on the residual effects of chemical agents such as borates in the environment. There have been reported cases of chemical fungicide effects on non target organisms (Ragsdale & Sisler, 1994). There is also the concern that continuous use of chemical agents in the management of forest tree and agricultural plant diseases could result in resistance build up and possible loss of effectiveness in disease control (Lennox & Spotts, 2003). The use of antagonistic microbes (biocontrol agents) in the control of the conifer tree pathogen has been suggested as a potential replacement for chemical control agents (Elad & Zimand, 1993). Campbell, (1989) suggested that the ecological relationship that exists between an antagonistic microbe and a pathogen could offer opportunities for biological control processes. According to Cook & Baker (1983), biological control involves the use of an organism, the antagonist, against another organism, the pathogen, so as to reduce the amount of inoculums or the disease-producing capacity of the pathogen. Biological control is a naturally occurring process, as could be evidenced in the rhizosphere (region of soil surrounding plant roots) of many plants. This region of plant is enriched with exudates which attract and enhance the colonization of the roots by plant growth promoting rhizobacteria (PGPR) (Whipps, 2001; Wulff et al., 2002a; 2002b). However, human activities involving the introduction of a host-specific biocontrol agent into an environment harbouring a pathogen is common (Roderick & Navajas, 2003).

Biological control processes in both agricultural and forest practices have numerous potentials in disease control as well as in environmental sustainability. Residues from biocontrol agents are readily biodegradable and cases of persistence in the environment have not been reported (Andrew, 1990). Furthermore, biological control interaction encompases the deployment of complex traits of the biocontrol agent and the combined action of such traits reduces the risk of resistance build-up by the target (Burge, 1998). Because of the relatively high public acceptance of biological control, several microbial antagonists have been formulated for the control of plant diseases (**Table 1**). It is generally thought that biocontrol agents have less negative environmental impact when compared to chemical pesticides (Dahlsten & Dreistadt, 1991). However, despite all the numerous advantages inherent in biological control, the disease control process has some limitations. Microbial agents used in most biocontrol processes are vulnerable to changes in the environmental conditions such as temperature or humidity, and the shelf life of the organisms in most cases is very short (Holdenrieder & Greig, 1998). There has been reports of some negative effects in some biocontrol systems where some resident flora and fauna that were not targeted in the biocontrol programme appeard to be adversely affected by the biocontrol agent (Westlund & Nohrsted, 2000; Vasiliauskas et al., 2004). The metabolites from some biological control agents could act as toxins and allergens in the environment thereby constituting a potential risk to human health (Cook & Baker, 1983). Conclusively, biological control programmes if executed properly could lead to sustainability in forest practices by protecting the forest environment from the harmful effects of chemicals as well as reducing losses due to pathogens infestation.

Table 1: Representative examples of some biological control agents (BCAs[a]), the pathogens and their plant hosts and the diseases they cause

Disease /Host	Pathogen	Antagonist or BCAs	Mechanism of action	Reference[b]
Rhizoctonia root rot and bare patch in wheat	*Rhizoctonia* spp.	*Pseudomonas* species	Secretion of antifungal compounds , PGPR	Mavrodi et al., 2012
Pythium root rot in cereals	*Pythium* spp. (oomycetes)	*Pseudomonas* species	Secretion of antifungal compounds, PGPR	Mavrodi et al., 2012
Fusarium head blight disease of maize and barley	*Fusarium* spp.	*Bacillus mojavensis*	Toxic biosurfactant production, reduction of mycotoxins	Bacon et al., 2012
Gray mould disease of strawberry, tomato etc	*Botrytis cinerea*	*Sporidiobolus pararoseus* (yeast)	Competition for limited nutrients and space	Huang et al., 2012
Root necrosis in tomato seedlings and sorghum	*Fusarium oxysporum* and *Phythophthora parasitica* (oomycete)	*Paenibacillus* sp.	Hyperparasitism/predation, disorganization of fungal cell walls through the release of hydrolytic enzymes	Budi et al., 2000
Fusarium head blight of wheat	*Fusarium graminearum*	*Lysobacter enzymogenes*	Lytic enzymes secretion (chitinase and β-1,3-glucanases)	Li et al., 2008
Vascular disease of strawberry and other fruits	*Botrytis cinerea*	*Serratia plumuthica*	Lytic enzymes secretion (chitinase)	Frankowski et al., 2001
Attacks maize	*Fusarium oxysporum*	*Bacillus amyloliquefaciens*	Antibiosis (Bacillomycin, fengycin)	Koumoutsi et al., 2004
White root rot of avocado	*Rosellinia necatrix*	*Pseudomonas pseudoalcaligenes*	Competition for niche	Pliego et al., 2008
Disease of Cucumber	Different pathogens	*Pseudomonas putida*	Competition for iron and siderophore scavenging	Loper and Henkels, 1999
Potato bight	*Phytophthora infestans* (oomycete)	*Pseudomonas putida*	Induces host resistance (Phytohormone-mediated induction)	van Loon, 2007
Damping off of sugar beet	*Pythium* spp.	*Enterobacter cloacae*	Production of waste products like Ammonia	Howell et al., 1988
Crown gall in many plant hosts	*Agrobacterium tumefaciens*	*Agrobacterium radiobacter*	Antibiosis (Agrocin 84)	Vicedo et al., 1993
Root necrosis in tomato	*Fusarium oxysporum*	*Collimonas fungivorans*	Competition for exudates	Kamilova et al., 2007

[a]= Biological control agent [b] = Source of the information

The first suggested use of a natural antagonist against a tree pathogen was reported by John Rishbeth of Great Britain. He observed that *Phlebiopsis gigantea* (Fir.) Jűlich, a saprotrophic white rot fungus sharing the same ecological niche with *H. annosum*, could replace the pathogen on a freshly cut stump (Rishbeth, 1952). He therefore proposed the use of *P. gigantea* as the biocontrol agent against *H. annosum*. This observation made by Rishbeth aroused a new interest in the search for ways of improving the biological control process. Berglund et al. (2006) compared the efficacy of five strains of Swedish isolates of *P. gigantea* with the commercial Finnish Rotstop isolates and *Trichoderma* spp. Sun et al. (2009a) investigated the competitive abilities of several isolates of *P. gigantea* from different geographical sources. The studies reported variations in the efficacy of different *P. gigantea* isolates during competition with *H. annosum*. Although the practical application of *P. gigantea* in the control of *H. annosum* is already technically and feasibly integrated in harvesting operations, the actual mechanisms governing this biological process still remains obscure. Unlike in other biocontrol systems where secretion of growth inhibitory compounds, competition for nutrients, lytic enzyme secretion, induced resistance and mycoparasitism have been reported (Gupta & Utkhede, 1986; Leifert et al., 1995; Monhamed & Caunter, 1995; Walker et al., 1996; Asiegbu et al., 2005; Adomas et al., 2006), the *H. annosum* - *P. gigantea* biocontrol system still presents a huge challenge due to the poor understanding of the mechanisms underlying the biological process. A relatively good effort has been made in the understanding of the biology and ecology of both fungal species but the genetics and the molecular aspects of the interaction still remain poorly understood. Understanding the genetic and molecular factors underlying the interaction between *P. gigantea* and *H. annosum* could help to arm the biocontrol fungus by incorporating improved traits in molecular breeding in order to produce progeny strains with improved biological control abilities.

Recently Adomas et al. (2006) reported the differential expression of two *P. gigantea* hydrophobin encoding genes, *Pgh1* and *Pgh2* at the zone of interaction between *P. gigantea* and *H. parviporum*, and the study concluded that *Pgh1* and not *Pgh2* may be involved in the competitive interaction between the two fungal species. Hydrophobins are cell wall secreted proteins that have been shown to be involved in different stages of fungal developments. Hydrophobins control interfacial forces and this plays a crucial role in the adaptation of fungi to their environment. Hydrophobins have been reported to be involved in the emergence of fungal hyphae from an aqueous medium by lowering the surface tension of water (**Figure 1a-c**; van der Vegt et al., 1996; de Vries et al., 1999; Wösten et al., 1999; Lumsdon et al., 2005; Askolin et al., 2006). Wessels et al. (1991b) suggested that emerging fungal hyphae are coated with hydrophobins as the hyphae grow through the air-water interface into the air. Aerial hyphae and spores of most fungi are lined with hydrophobins which are assembled into a mosaic pattern of rod-like structures of about 10 nm in width, and these rod-like protein structures are called rodlets (Stringer et al. 1991; Bell-Pedersen et al., 1992; Lauter et al., 1992; Wösten et al., 1993). The rodlet coatings are hydrophobic and they protect the fungal aerial structures from wetting. Air channels in fungal aerial structures have been shown to be lined with hydrophobins and these hydrophobic coatings were thought to

protect the air channels from wetting and to ensure free gas exchange (Lugones et al., 1999; van Wetter et al., 2000). .

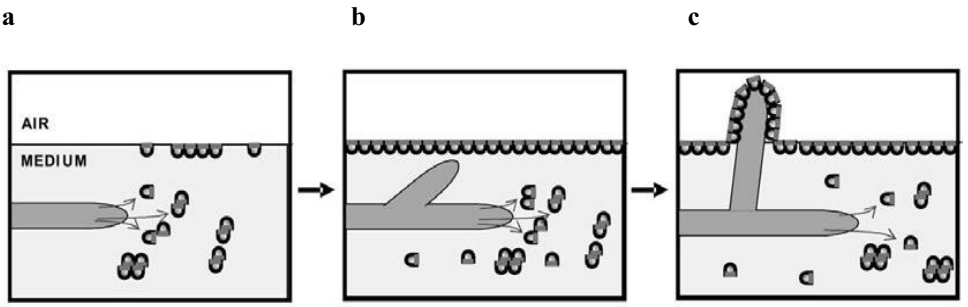

Figure 1: The role of hydrophobins in the emergence of fungal hyphae from an aqueous medium (Wosten et al., 1999). (**a**) During the submerged growth of fungal hyphae in an aqueous medium, hydrophobins are secreted into the medium from the fungal cell wall. (**b**) The hydrophobins adsorb to the air-water interface and lower the surface tension of the water. (**c**) Due to the lowered surface tension, the fungal hyphae penetrate the air-water interface and grow into the air (Wosten et al., 1999).

Hydrophobins have also been implicated in fruit body formation in fungi (Wessels et al., 1991a; Lugones et al., 1996; de Groot et al., 1997). Attachment of fungal hyphae to solid substrates has been shown to be mediated by hydrophobins (Wösten et al., 1994). In addition, hydrophobins play a role in the adsorption of pathogenic fungi to the surfaces of their host organism (St. Leger et al., 1992; Talbot et al., 1993; Talbot et al., 1996; Kazmierczak et al., 2005). Hydrophobins are divided into two classes based on their physical properties and hydropathy patterns (Wessels, 1994). Class I hydrophobins are more stable and form insoluble membranes in water, 2% SDS and most organic solvents. Members of class II hydrophobins form membranes which are easily dissolved in 60% ethanol and 2% SDS (Wessels, 1997). Class I hydrophobins have been reported in ascomycetes and basidiomycetes, whereas class II hydrophobins seem to be found only in ascomycetes (Wösten, 2001).

1.2: *Heterobasidion annosum* s.l. as a conifer tree pathogen

1. 2. 1 Biology

Systematically, *Heterobasidion annosum* (Fr.) Bref. sensu lato (s.l.) is a basidiomycetous fungus classified in the family Bondarzewiecae in the order Russulales, under the class Agaricomycetes in the subphylum Agaricomycotina, phylum Basidiomycota (Matheny et al., 2006; Hibbett et al., 2007). Members of the genus *Heterobasidion* are necrotrophic white rot fungi. They cause root rot and stem decay in conifer tree species mostly in the temperate regions, and the economic losses attributable to the disease are quite huge (Woodward et al., 1998). The pathogen was initially thought to be a monophyletic group with *Heterobasidion annosum* s.l. regarded as single species (Otrosina & Cobb, 1989; Woodward et et al., 1998). However, mating experiments revealed that this fungal group is composed of a species complex known as intersterily groups (ISGs) (Carpretti et al., 1990; Chase & Ullirch, 1998). In Europe, Korhonen (1978) found two ISGs of the pathogen (S and P groups) which have special preferences for spruce (*Picea abies*) and pine (*Pinus sylvestris*), respectively. Another ISG, the F type which mostly attacks fir (*Abies*) species was found in Italy by Capretti et al. (1990). Recently, the European F and S types were placed into separate taxonomic species groups, *Heterobasidion abietinum* and *Heterobasidion parviporum*, respectively (Niemelä & Korhonen, 1998) whereas the P type is known as *Heterobasidion annosum* sensu stricto (s.s.). The P type (*H. annosum* sensu stricto (s.s.) Niemelä and Korhonen) whose major host is pine can also attack other tree species such as- birch (*Betula pendula*), alder (*Alnus incana*) and juniper (*Juniperus communis*). Although the S type *H. parviporum* Niemelä and Korhonen infects *P. abies* as the primary host, it can as well attack Siberian fir (*Abies sibirica*) (Korhonen et al., 1997).

1.2.2 Distribution of *Heterobasidion* species

Heterobasidion annosum (Fr.) Bref. sensu lato (s.l.) is widely distributed in most countries in the Northern Hemisphere. In Europe, the species *H. annosum* (s.s) is found mostly in countries in the Nordic region and Southern Europe; and also in the Altai region of Southern Siberia (Carpretti et al., 1990; LaPorta et al., 1997; Korhonen & Dai, 2004). *H. parviporum* is prevalent in the Southern and central parts of Finland, in the Baltic countries, Poland, Germany, Czech Republic, and in most countries in the Southern Europe. It is also found in the Eastern part of France, the Ural Mountains and in other places where the host tree species (*Picea abies*) predominantly exist. *H. abietinum* mostly occurs in Southern and Central Europe, western Turkey and Russian Caucasus. It is also common in areas where *P. abies* is cultivated in mixed strands with *A. alba* (Korhonen et al., 1998; Dogmus-Lehtijärvi et al., 2006; Sanchez et al., 2007; Zamponi et al., 2007).

The North American *Heterobasidion* P (*H. ireggulare*) and S (*H. occidentale*) types have also been identified (Harrington et al., 1989; Chase & Ullrich, 1990a; Otrosina et al., 1993,

Ottrosina &Garbelotto 2010). Mating experiments have shown that the North American S and P groups have a higher degree of interfertility than the Finnish S and P groups (Chase & Ullrich, 1990a). Furthermore, the North American S group has a broader host range than the P type and may attack *Picea, Abies, Pseudotsuga, Tsuga* and *Sequoiadendron* species whereas the P group is mostly associated with *Pinus* species, although it can also attack other tree species in the genera *Juniperus* and *Colocedrus* in the eastern and western forests of the northern part of America (Garbelotto et al., 1996). Morphological and genetic studies have revealed that the North American *Heterobasidion* S-type (*H. occidentale*) and P-type (*H. irregulare*) are single biological species (Ottrosina & Garbelotto, 2010).

The North American P-type (*H. irregulare*) was suggested to be introduced into Europe through woody materials during the Second World War by the American troops (Gonthier et al., 2004; D'Amico et al., 2007; Gonthier et al., 2007). The population structure of the North American and European *H. annosum* s.l ISGs have been analyzed using isozyme studies (Otrosina et al., 1993). According to the findings, the European S, P, and F type ISGs showed high level of divergence from the North American S and P types of ISGs. Other genetic studies using several markers have been carried out and such studies showed that the American and European types of *H. annosum* s.l are two distinct groups (Harrington et al., 1989; Johannesson & Stenlid, 2003; Asiegbu et al., 2005; Ota et al., 2006; Linzer et al., 2008). The North American S and P ISGs have been shown to have high genetic divergence (Otrosina et al. 1993). Based on laboratory pairings, the interfertility between the two groups (North American S and P-types) was found to be 18 % (Chase & Ullrich 1990a; Harrington et al. 1989). *H. irregulare* is widely distributed in some parts of Canada such as Quebec and Ontario, most parts of the USA, Mexico, Cuba and the Dominican Republic where pine hosts are present (Anonymous, 1980). *H. occidentale* is hardly found in eastern North America, but colonizes and fruits within pine stumps in western North America where it almost resembles *H. irregulare* in gross morphology and habit (Filip & Morrison 1998; Otrosina & Cobb 1989).

The basidiomata of the two North American *Heterobasidion annosum* species (*Heterobasidion irregulare* and *Heterobasidion occidentale*) has been shown to have morphological plasticity and their locations are governed by both ecological circumstances and the inherent host–pathogen influence (Ottrosina & Garbelotto, 2010). This has made morphological distinctions between the two biological species difficult especially when both species are found growing on host trees they are known to specialize on. This situation is common in western North America where basidiomata of both *H. irregulare* and *H. occidentale* are most times found on pine stumps (Filip & Morrison 1998; Harrington et al. 1989; Otrosina et al. 1992, 1993; Garbelotto et al., 1996). However, strong specifity for some conifer tree species has been reported and such host preferences have been suggested to be the driving factor for the genetic differentiation of *H. irregulare* and *H. occidentale* (Filip & Morrison, 1998; Harrington et al., 1989; Otrosina et al., 1992, 1993; Garbelotto et al., 1996). A more consistent morphological feature for distinguishing *Heterobasidion irregulare* and

Heterobasidion occidentale is the pore density (Ottrosina & Garbelotto, 2010). Other features such as pore shape and size appeared to be less dependable, since both structures are affected by the hymenial growth and angle of development (Ottrosina & Garbelotto, 2010). In Japan and China, *H. annosum* s.s and *H. insulare* (Murrill) Ryvarden have been identified (Ito, 1955; Teng, 1964). Reports have shown that the Chinese *H.annosum* species is closely related to the European *H. parviporum (*Dai et al., 2006). Phylogenetic studies have confirmed the close relationship between the Japanese *H. annosum* s.l with the European and Chinese *H. parviporum* populations (Ota et al., 2006).

1.2.3 Infection and spread of *H. annosum* species

The infection mode of *H. annosum* s.l. is by aerial basidiospores released on stump surfaces or through wounds on roots or stem. Spread to the adjacent trees is through root contacts and from infected to healthy trees (Redfern & Stenlid, 1998). Basidiospore deposition could occur within hundreds of kilometers to freshly cut stump surfaces (Rishbeth, 1959a; Gonthier et al., 2001). *H. annosum* s.l. individuals can survive for many decades on the stumps and can act as a source of infection for several generations (Piri, 1996; Lygis et al., 2004; Piri & Korhonen, 2007). The asexual conidia of *H. annosum* s.l. can be carried by insect vectors or wind and the spores can travel several meters away from the source (Kadlec et al., 1992). *H. annosum* s.l. has been reported to secrete different extracellular enzymes such as cellulase, manganese peroxidase, laccase, pectinase and proteases during colonization (Asiegbu et al., 1998; Olson et al., 2012). Although the precise roles of these enzymes in pathogenicity are still unknown, the enzymes help to degrade and detoxify the structural and soluble components of wood (Asiegbu et al., 1998). Some metabolites such as fommanoxin, fommanosin, fommanoxin acid, oosponol and oospoglycol have been reported to be produced by *H. annosum* s.l. It is possible that these metabolites contribute to the phytopathogenicity of the fungus (Asiegbu et al., 1998). The recently published data on the genome sequence of *H. irregulare* revealed a trade off between saprotrophy and pathogenesis in *H. irregulare* (Olson et al., 2012). According to the study, *H. irregulare* expresses few genes encoding carbohydrate-active enzymes and transporters during pathogenic growth on living conifer trees than during saprotrophic growth on wood. The study also revealed that *H. irregulare* employs a wide arsenal of metabolic repertoir that are involved in different biological processes such as toxin production, protection against plant defenses, processing of low oxygen pressure and other abiotic stresses during pathogenic colonizatioon of living wood (Olson et al., 2012) .

1.3. The different control methods of *H. annosum* s.l. infection

To effectively control the spread of a pathogen, control measures must be targeted at the phases of the life cycle of the organism starting from the time of spore deposition, adhesion to substrate surface, establishment of infection thread, infection and spread of hyphae (hyphal colonization). As a tree pathogen, *H. annosum* s.l. infection management is aimed mostly at preventing the infection process as curative measures are difficult and currently unavailable. One of the major strategies involves preventing basidiospore deposition, germination and growth. Different methods of the infection control have been adopted and these include silvicultural, chemical and biological control.

1.3.1 Silvicultural control

H. annosum s.l. infection is more common in conifer tree species, while the broad leaf trees are less commonly affected. As a strategy to reduce the incidence of the attack, planting tree species that are less susceptible is encouraged (Delatour et al., 1998; Korhonen et al., 1998b; Lygis et al., 2004a; Lygis et al., 2004b). Some studies have suggested planting tree species that are susceptible to *H. annosum* s.l. infection in combination with the less susceptible species (Piri et al., 1990; Linden & Vollbrecht, 2002). In addition, cutting trees in winter when the spore load and spread is quite minimal has been suggested as a possible way of reducing the infection spread (Möykkynen et al., 2000; Möykkynen & Miina, 2002). Stump removal is another silvicultural method for controlling *H. annosum* s.l. infection. The pathogen can survive for over 60 years in infected stumps and roots; and can act as a source of infection for generations (Piri, 1996). Uprooting the infected stumps alongside the fine roots could likely remove the infection source thereby preventing spread to the next generation (Stenlid, 1987; Laflamme, 2010). Stump removal has been applied in the management of other root rot pathogens, such as *Amillaria* and *Phellinus* and the success rate is quite high (Stuurock, 2000; Greg, 2001; Gibbs et al., 2002). Although this control method has proved to be very effective, 100% efficiency has hardly been achieved due to long survival period of the pathogen (Korhonen et al., 1998b; Stenlid, 1987).

1.3.2 Chemical control

Prophylactic stump treatments with chemicals such as urea and borates have been successfully used in the control of *H. annosum* s.l infection. Impressive results have been achieved with both chemicals and the success rate is over 80% (Thor & Stenlid, 1997). Several studies have tried to understand the mode of action of urea and borates against *H. annosum* s.l. on the stump surface (Lloyd, 1997; Johannsson et al., 2002). According to Lloyd (1997), the primary mode of action of borates is targeted on the metabolism of the basidiomycetes. On the other hand, urea is hydrolysed by urease in the living wood tissues resulting in the formation of ammonia and elevated $_{P}H$ which subsequently inhibits the germination of *H. annosum* spores (Johannsson et al., 2002). Pratt et al. (1998a) suggested that in wetter climates where the

heartwood is targeted in preference to the sapwood, the success rate of urea may be low due to the absence of urease activity in the dead heartwood. Some studies have tried to evaluate the long term (Oliva et al., 2008) and short term effects of urea on the stump (Pratt & Redfern, 2001; Nicolotti & Gonthier, 2005; Thor & Stenlid, 2005). Urea treated stumps were reported to have up to 97.3% protection 15 years after treatment (Oliva et al., 2008). Although a very good success has been recorded in the use of both chemicals, the public concern is increasingly high. The ammonia generated through the hydrolysis of urea could be phytotoxic and can have adverse affects on common ground species such as vascular plants and brophytes (van der Eerden, 1982; Westlund & Nohrsted, 2000). According to reports from other studies, urea has the capacity of changing fungal community structures with the major fungal taxa, zygomycetes and basidiomycetes being the most adversely affected (Vasiliauskas et al., 2004). This change in mycodiversity may affect the ecosystem functioning and it may also affect nutrient cycling by fungi leaving in the forest soil. As a result of these pitfalls, a better and more environmentally friendly way of controlling the conifer pathogen was proposed. Biological control with a natural antagonist was suggested as a replacement for chemical control.

1.3.3 Biological control

Due to the increasing concern on the possible adverse effects of chemical fungicides such as borates on the environment, a biological control approach was proposed for the management of the conifer pathogen, *Heterobasidion annosum* s. l. Several fungal species such as *P. gigantea, Fomitopsis pinicola*, *Bjerkandra adusta*, *Trichoderma spp*. and *Resinus bicolor* have been tested as competitors and antagonist against *H. annosum* s. l. *(*Holdenrieder and Greig, 1998; Holmer & Stenlid, 1996; Nicolotti & Verse, 1996; Nicolotti et al., 1999). However *P. gigantea* was found to be the candidate with a better potential for biocontrol of *H. annosum* s.l. Several commercial preparations of *P. gigantea* are available in the market today.

1.4. *Phlebiopsis gigantea* as a biocontrol agent- Historical perspective

Phlebiopsis gigantea (Fir.) Jűlich, like its pathogenic relative (*H. annosum* s.l.) is a basidiomycetous fungus which belongs to the family phanerochaetecae in the class, agaricomycetes and order, polyporale (Hibett et al., 2007). Phylogenetic studies involving multilocus sequence analysis (18S rRNA and ITS region) showed that *P. gigantea* is more closely related to *Phanerochaete*, *Nematoloma and Hypholoma* species (.Hilděn et al., 2008). *P.gigantea* is a saprotrophic white rot fungus that shows antagonistic behaviour against *H. annosum* s. l. on the stump surface (Rishbeth, 1952). This observation triggered a new interest in the understanding of the interaction between *P. gigantea* and *H. annosum* s. l. In United Kingdom, PG® suspension is produced and marketed by Surrey Forest Research, UK. In

Poland, PG IBL® (Biofood S.C., Walcz, Poland), is available. In Scandinavia, a heterokaryotic strain of *P. gigantea* was isolated by Korhonen in 1991 from spruce wood (*Picea abies*). The oidia of this strain was formulated into a dry powder and was tested on Norway spruce stumps in the Nordic countries where it is currently being produced and marketed commercially as RotstopF® by Kemira AB (Finland). This Rotstop® preparation has been intensively applied to over 62500 hectare stumps in Fennoscandia annually (Thor, 2003; Finnish Statistical Year book of Forestry, 2009) and more than 200, 000 ha in Europe (Thor, 2003). Studies have shown that *P. gigantea* outcompetes *H. annsoum* s.l.on the stump by rapid colonization of the the stump surface (Korhonen et al., 1994; Bailey et al., 2003).

1.4.1 Biology and ecology of *Phlebiopsis gigantea*

*P. gigantea (*syn. *Phanerochaete gigantea, Phlebia gigantea)* like its competitor- *H. annosum* s.l. is a white rot fungus belonging to the phylum basidiomycota. *P. gigantea* is commonly found in dead wood in most of Boreal forests (Käärik & Rennerfelt, 1957; Meredit, 1959). Both *H. annosum* s.l. and *P. gigantea* share the same ecological niche where they compete for nutrients on freshly cut stump surface (Rishbeth, 1963; Korhonen et al., 1994; Bailey et al., 2003). However, while *H. annosum* s.l. is a white rot necrotrophic pathogen that attacks living cells during carbon scavenging, *P. gigantea* is a saprotroph which derives its carbon from already dead cells. *P. gigantea* has not shown any sign of pathogenesis on mature woody trees, however, evidence of colonization of non-suberized spruce seedling roots has been demonstrated, suggesting that *P. gigantea* may act as a potential facultative (although weak) necrotrophic pathogen (Asiegbu et al.,1996). *P. gigantea* is capable of forming structures in spruce wood that are similar to the mycorrhizal mantle on the tree roots (Vasiliauskas et al. , 2007). *P. gigantea* is spread through aerial basidiospores on stump and woody tissues however vegetative spread of the fungus through root contacts has not been reported. Several genetically distinct *P. gigantea* individuals can be found in infected sites several months to one year after infection (Annesi et al., 2005) and this suggest that *P. gigantea* has a very large population size. The fungus can also spread through asexual anthroconidia (oidia). Although the asexual conidia are not capable of travelling distances, however, evidence of the anthroconidia being vectored by bark beetles has been demonstrated in North America (Hunt & Cobb, 1982; Hsiau & Harrington, 2003). Both homokaryotic and heterokaryotic strains of *P. gigantea* have been identified and each form of the fungus has been reported to exist in a multinucleate state (Korhonen & Kauppila, 1988). Different heterokaryotic isolates of *P. gigantea* from diverse geographical regions behave differently under varying treatment conditions (Sun et al., 2009a). Morphological studies have also shown that *P. gigantea* is a single biological species throughout the boreal forest of the Northern Hemisphere and globally (Vaartaja, 1968; Lundquist, 1986; Korhonen et al., 1997; Roy et al., 1997; Hood et al., 2002). Although *P. gigantea* is capable of homokaryotic fruiting *in vitro*, the mating system of the fungus is however heterothallic and bipolar (Korhonen & Kauppila, 1988). Studies by Grillo

et al. (2005) showed no evidence of geographic differentiation between different populations.of *P. gigantea*; the study further reported that the North American and European populations of *P. gigantea* were highly interfertile (Grillo et al., 2005). Mating experiments have shown no evidence of IS groups between the European and American populations of *P. gigantea* (Grillo et al., 2005). *P. gigantea* from different geographical regions have been shown to have very high genetic diversity but low geographic differentiation within continents (Vainio et al., 1998; Vaino & Hantula, 2000).

1.5 Mechanisms of Biocontrol of *H. annosum* s.l. by *P. gigantea*

In agricultural crop plants where the biological control of fungal pathogens have been well documented, different modes of action of the biocontrol agents such as mycoparasitism, antibiosis and competition for nutrients have been identified (Elad, 1996). In mycoparasitism, the biocontrol agent uses cell-wall-degrading enzymes such as chitinases and glucanases to dissolve the hosts'cell walls before penetrating the cell (Elad, 1996). However, competition for nutrients is also an effective mode of action of most biocontrol agents but it is only possible when the pathogen needs exogenous nutrients for the germination and elongation of its germ-tube (Blakeman, 1993). Antibiosis has been documented in the *Botrytis cinerea – Bacillus spp* biocontrol system (Leifert et al., 1995; Seddon & Schmitt, 1999). In addition, synergistic interactions between different forms of antagonism have been reported. In *B. cinerea,* inhibition of conidia germination through antibiosis was reported to be in synergism with the release of cell-wall-degrading enzymes (Lorito et al., 1993a, b). In *Trichoderma* biocontrol system, antibiosis was reported to be coupled with the release of cell wall degrading enzymes (Elad et al., 1983; Baker, 1987). Additionally, other mechanisms such as induced systemic resistance mediated by jasmonic acid and ethylene (Paulitz & Matta, 2000), hypovirulence, mediated through fungal viruses (Taylor et al., 1998) and inhibition of enzymes involved in plant pathogenicity (Kapat et al.,1998) have been reported. Although several mechanisms of actions of biocontrol agents have been documented for other biocontrol systems, not much is known about the *P. gigantea - H. annosum* system. Previous studies have reported competition for nutritional sources (Adomas et al., 2006) as one of the likely mechanisms of biocontrol of *H. annosum* s.l by *P. gigantea.* Ikediugwu (1976) reported structural changes to the hyphae of *H. annosum* s.l. in contact with the hyphae of *P. gigantea.* This phenomenon which is known as hyphal interference has been reported in most interspecific fungal interactions (Ikediugwu &Webster, 1970b; Ikediugwu, 1976). Adomas et al. (2006) also reported the increased expression of genes in *P. gigantea* encoding proteins such as hydrophobins and other genes involved in carbohydrate and protein metabolism during the interaction of *P. gigantea* with *H. parviporium.* The study concluded that the likely mechanism of the *P. gigantea- H. annosum* complex biocontrol system could be competitive exclusion of *H. annosum* s.l. by *P. gigantea* through nutrient acquisition.

1.5.1 Physiological and biochemical traits of *P. gigantea* related to its biocontrol activity

On freshly cut stump surface where the pathogen, *H. annosum* s.l. and the biocontrol agent, *P. gigantea* compete for resource capture, two kinds of nutrients which are readily available have been reported to exist; soluble sugars (Asiegbu, 2000) and lignified cellulosic constituents (Cowling & Kirk, 1976). The possession of traits relevant for lignin modification and nutrient metabolism by either the pathogen or the biocontrol fungus could confer a competitive advantage over the other. Studies have shown that *P. gigantea* has the ability of high growth rate as well as rapid colonization of the stump surface (Asiegbu et al., 2005; Adomas et al., 2006; Turby et al., 2008). In addition, *P. gigantea* has been reported to secrete high level of lignin modifying enzymes such as laccases (Asiegbu et al., 2005). Recently, it was demonstrated that different isolates of *P. gigantea* could degrade both spruce and pine wood chips at different rates; implying that the ability to degrade wood could be a crucial factor in the interaction between the pathogen and the biocontrol fungus (**Paper I**; Sun et al 2009a). Sun et al. (2009a); showed that variation in spore production and high growth rate were among the several traits that could increase the competitive ability of *P. gigantea* over *H. annosum* s.l. Most of these studies were either conducted under *in vitro* conditions using artificial media rich in soluble sugars (Asiegbu et al., 2005; Adomas et al. , 2006) or field like conditions (Sun et al., 2009a). Other authors have also studied interactions between organisms using sugar rich media to understand the basis for biological control and the dynamics of fungal competition (Kallio, 1971; Carruthers & Rayner, 1979; Magan & Lacey, 1984a; 1984b). However, there is no known study comparing the competitive abilities, enzyme secretion, growth rate and wood decay capabilities of several isolates of *P. gigantea* from different geographical sources using an artificial media like sawdust that mimics the natural substrate of the fungus. Such screening approach could be helpful in identifying variations in some physiochemical traits relevant for the biocontrol of the conifer pathogen-*Heterobasidion annosum* s.l. among the different *P. gigantea* isolates. It could also offer an opportunity for a rapid *in vitro* screening process for most antagonistic interactions involving wood and the results from such screening could be extrapolated to what happens in the field.

1.5.2 Hydrophobin genes may contribute to the biocontrol activity of *P. gigantea*

Hydrophobins are cell wall secreted proteins with eight conserved cysteine residues, produced by filamentous fungi and dimorphic yeasts (Wösten, 2001; Elliot & Talbot, 2004). Hydrophobins have been reported to be involved in several biological processes in the fungal life cycles; ranging from the formation of aerial structures, initiation of fungal morphogenesis and interaction of fungal hyphae with potential hosts or symbiotic partners (Talbot, 1999; Elliot & Talbot, 2004; Sakamoto et al., 2007). Hydrophobins are also involved in cell wall assembly in most pathogenic interactions where the monomers act as elicitors and toxins (Tucker & Talbot, 2001). Studies by Adomas et al. (2006) revealed the up-regulation of the *P.*

gigantea hydrophobin-1 encoding gene (*Pgh1*) at the zone of interaction between *P. gigantea* and *H. parviporium,* the study suggested that *Pgh1* and not *Pgh2* could possibly be involved in the interaction between the biocontrol fungus and the pathogen. In contrast, other confrontation assays involving *Hypocrea atroviridea* and *Botrytis cinerea*, revealed the down-regulation of three hydrophobin encoding genes (*hfb-1b*, *hfb-2c*, and *hfb-6a)* in the biocontrol agent-*Hypocrea atroviridea* (Mikus et al., 2009). The study concluded that *H. atroviridea* hydrophobins are not involved in the antagonism. However, due to the lack of DNA transformation system for the biocontrol fungus (*P. gigantea*), there is no functional knockout studies presently available to delineat the actual roles of *Pgh1* and *Pgh2* in the biocontrol of the conifer pathogen, *H. annosum* s.l. However gene transcript analysis using isolates of *P. gigantea* with varying antagonistic abilities will help to provide the first initial insight on the regulatory patterns of these two genes under diverse nutrient and experimental conditions. Additionally, investigations involving the analysis of sequences of *Pgh1* and *2* from different isolates of *P. gigantea* could give further insights on the antagonistic roles of both genes in various *P. gigantea* isolates.

1.5. 3 Hydrophobin gene contraction and expansion and fungal ecological strategy

Gene contraction by gene loss and expansion by duplication is an important evolutionary force driving gene family evolution in most organisms. These events have been reported in several gene families including chitinases (Karlsson & Stenlid, 2008) and glucoside hydrolases (GH28 family) (Daniel et al., 2011). In the hydrophobin encoding gene family, there is a huge variation in the number of hydrophobin genes across many fungal taxa, ranging from 1 in *Acremonium alcalophilum* to 40 genes in *Trametes versicolor (***Table 2**). Studies involving nucleotide sequence analysis of hydrophobin encoding genes from *P. gigantea* revealed a high level of sequence divergence in *Pgh1* and *Pgh2* (Adomas et al., 2006). A similar observation was made in the hydrophobin encoding genes (*Hah1* and *Hah2*) from *H. irregulare* the North American P-type (Karlsson et al., 2007). A crucial question therefore arises on whether this rapid differentiation in the hydrophobins of ecologically important fungi could influence their ecological habits. Another question is whether the hydrophobins of these fungi are under any form of selection pressure. Evolution of new genes through duplication events is an important biological process for an organism's complexity, adaptation and diversification to closely related strains and species (Prince & Pickett, 2002; Long et al., 2003). Studies have shown that newly duplicated genes could show functional diversification and different gene expression patterns (Ohno, 1970). Gene duplication creates genetic redundancy and duplicated genes have been reported to be more prone to selection pressure (Prince & Pickett, 2002; Ohno, 1970). Currently, there is little information on the fate of duplicated genes and the forces driving their fixation and divergence (Long et al., 2003). However, recent studies have

shown that duplicated genes with deleterious effects are purged from the genome through purifying selection but genes with enhanced functions are fixed in the population by positive or diversifying selection (Hughes, 2002, Lynch & Conery, 2000). Additionally, it is possible that the differences in sizes between the genomes of some fungal species could depend on the relative rates of gene duplication, gene loss and possible horizontal transfer events occurring in some gene families in the fungi. However, these evolutionary processes and the mechanisms driving them have not been properly investigated in the *P. gigantea* and *H.annosum* s.l. hydrophobin encoding genes.

1.5.4 *Phlebiopsis gigantea* metabolites as a potential mechanism of biocontrol

Most studies on the biological control of the forest pathogen *H. annosum* s.l. have focused on either the biological or the ecological parameters controlling the biocontrol process. Only recently that interest in the molecular aspects of the interaction between *H. annosum* s.l. and *P. gigantea* gained a renewed attention. Data obtained from various gene expression studies during the interaction of *P. gigantea* and *H. parviporium* revealed differential expression of some *P. gigantea* genes involved in different biological processes (Adomas et al., 2006). High levels of wood degrading enzymes were previously reported to be secreted by *P. gigantea* and it was suggested that these enzymes could play a role in the biocontrol activity of the fungus (Asiegbu et al., 2005). Some studies have investigated the role of secreted compounds such as secondary metabolites from the biological control fungus *P. gigantea* on the growth of *H. annosum* s.l. Sun et al. (2011) reported that *P. gigantea* was able to cause induced resistance against *H. annosum* s.s. infection in *Pinus sylvestris*. The study also reported a reduction in lesion length and spread of *H.annosum* s.l. caused by secreted metabolites from the host tree due to prior exposure to *P. gigantea*. However, the role of secondary metabolites produced by biocontrol agents during interactions with pathogens is still poorly understood (Vinale et al., 2008a; b). Ouimet et al. (1996) reported growth inhibition in *Venturia inaequalis* ascomycetes known to be the casual agent for apple scab disease due to the repressing effects of diffusible metabolites from different antagonistic fungi. In plants, the interaction of biological control agents with plant roots has been reported to promote growth and improve the resistance of the plant to diseases (Whipps, 2001). Vinale et al. (2008a; b) reported that this phenomenon could be related to the production of a wide range of compounds such as secondary metabolites which could have a substantial effect on the plant's metabolism. In *Trichoderma spp.* different forms of metabolites have been identified to act as elicitors or plant growth promoters (Harman et al., 2004).

Although so much progress has been made on the understanding of the effects of secondary metabolites produced by biocontrol agents during interaction with pathogens, there is no currently available documented report on the impact of secondary metabolites from *P. gigantea* on the gene expression profile of *H. annosum* s.l. In addition, no study has used a global gene expression approach to understand how secondary metabolites from this

biocontrol fungus could affect the transcript profile of the pathogen. A gene transcript analysis using a genome wide expression approach could help to give a more general overview of the transcriptional changes in the pathogen when treated with secondary metabolites from the biocontrol agent *P. gigantea*.

1.5.5 Breeding as a potential method of improving the genetic diversity of *P. gigantea* for biocontrol application

Evidence of natural breeding of *P. gigantea* has been documented in the wild among the European populations of the fungus (Vainio et al., 1998; Vaino & Hantula, 2000). Studies by (Grillo et al., 2005) showed that the North American and European populations of *P. gigantea* are highly interfertile. Mating experiments have not shown any evidence of ISGs between the European and American populations of *P. gigantea* (Grillo et al., 2005), indicating that isolates of *P. gigantea* from different geographical regions belong to the same biological species. However, due to the need for more effective biocontrol isolates, several screening approaches including laboratory breeding and molecular characterizations have been used to identify isolates of P. gigantea with improved biocontrol properties (Sun et al., 2009b; Hermosa et al., 2000; Rey et al., 2001; Lübeck et al., 2002). For example, in the synergistic interaction of different biocontrol isolates of *Trichoderma* species, isolates with improved biocontrol efficiency have been identified using different molecular techniques (Hermosa et al., 2000). In Finland and other Scandinavian countries, a single isolate of the biocontrol fungus Rotstop® has been routinely used for over ten years for the biocontrol of *H. annosum* s.l. and there is a general notion that constant use of one biocontrol isolate over a long time could result in loss in the effectiveness of the fungus. In addition, recent study by Samils et al. (2008) reported the possibility of resistance build up by *H. annosum* s.l. against *P. gigantea* after a long time application. Furthermore in the UK, two new double-stranded RNA elements (mycoviruses) which were thought to enhance the biocontrol ability of *P. gigantea* isolate TW-2 were identified, but the fungus later lost its efficacy (Kozlakidis et al., 2009). However, mycoviruses are known to have a reducing effect on the virulence of fungal pathogens as well as causing changes in metabolic pathways of some fungi (Pearson et al., 2009). In the filamentous fungus, *Sphaeropsis sapinea* a well known pathogen of pines, totiviruses were reported to co-infect the fungus.

Breeding programs aimed at producing more effective *P. gigantea* isolates for the biocontrol of the conifer pathogen have been reported (Sun et al., 2009b). The progeny strains resulting from such programs were found to be more effective than the wild type in terms of growth rate on wood chips, spore production and competitive ability against *H. annosum s.s.* (Sun et al., 2009b). However, the genetic basis of the improved traits reported in the progeny isolates has not been properly investigated. A transcriptomic approach involving global gene

expression studies can help to unravel the genetic basis for the physiological differences between the wild type and the progeny isolates.

1.6 The importance and application of molecular genetics tools used in this study

Studies on gene expressions have previously relied on Northern blot method which was used to quantify specific mRNA transcripts. However, Northern blot method is time consuming and requires large amounts of mRNA. Other mRNA quantification methods such as semi-quantitative RT-PCR and real-time quantitative RT–PCR (QRT–PCR) were introduced to complement Northern blot analysis. Real-time quantitative RT–PCR is a more user friendly mRNA expression method which uses labeled molecular detection probes. Although the assay is rapid, relatively cheap and requires a minute amount of mRNA, it is more suitable for a single-gene mRNA expression analysis. For the examination of a huge number of different mRNAs in a single assay, micro and macroarray expression analysis are used.

Microarray is a powerful tool used in global gene transcript analysis (Colebatch et al., 2002). Microarray technology is a genome-wide expression method whose basic principle relies on the hybridization of molecular probes complementary to specific mRNA sequences, fixed onto a solid phase, such as glass slide. In microarray preparation, probe synthesis may be directly done on the solid phase by a photolithographic technique (Affymetrix) or in some cases, it can be printed (PCR products or oligonucleotides) onto the solid phase. Microarray can be used on mRNA from any cell or tissue without prior information on the genes that are important in the specific sample. Although previous methods of sample labeling for microarray analysis required large amounts of starting materials (30-100 ng total RNA), new amplification methods and labeling procedures have provided possibilities to perform microarray on minute amounts of RNA (Park et al., 2004). Microarray technology has been used to identify and characterize new genes into different functional categories (Hughes et al., 2000; Giaever et al., 2002). Microarray analysis has also been used to monitor changes in gene expression during fungal interaction with the host environment (Acioli- Santos et al., 2008; Deveau et al. 2008; Jakupovic et al., 2006). Furthermore, changes in gene expression during different stages of fungal development have been studied using microarray analysis (Nowrousian et al., 2005).

Another genome wide expression method that has a useful application in molecular genetics research is macroarray technologies. Macroarray technique is a high throughput method of gene expression studies and it can be used to study hundreds of samples simultaneously in a relatively short time (Lievens & Thomma, 2005; Gilbert et al., 2008). Although both micro-and microarrays could be used to study thousands of genes in one assay, The membrane-based macroarray is cost-effective and uses a more flexible platform when compared to the glass-based, high-density microarray. Macroarray technology is commonly used in most diagnostics to monitor changes in gene expression of targets (Lievens & Thomma, 2005; Gilbert et al., 2008; Zhang et al., 2008; Maoka et al., 2010). In addition,

macroarray results are visualized with an unaided eye, which makes it a more simplistic method when compared to microarrays.

However, both micro- and macro-array technologies have some limitations. For microarray, most instruments that are used for the assay can only read a maximum of only two dyes. Such restriction can only allow for comparison between two treatments in a single experiment in which Cy-3 and Cy-5 dyes are used for labeling. Another limitation in the two techniques is the unavailability of arrays for some non model organisms. Most non model organisms are yet to have their genomes fully sequenced and characterized. However, the advent of the genomic era has shifted sequencing efforts from model organisms like yeast to non model organisms and pathogens. The availability of information from such genome sequences will help researchers to make full use of the potentials inherent in genome wide expression technologies for the purpose of gene discovery

1.7 Current Genome information of the fungal species used in this study and other ecologically important fungi

The JGI Fungal Genomics Program has focused on fungal genome sequencing projects as one of its primary objectives. Under this initiative, most fungal genomes have been sequenced and characterized. The first white rot fungi that was sequenced and published under the Whole Genome sequencing project was *Phanerochaete chrysosporium* (Martinez et al., 2004). The sequence data provided more insights into the molecular genetics of lignin degrading white rot fungi. The genome sequence of other basidiomycetes followed suit, for example the crop plant pathogen, *Ustilago maydis* was sequenced and annotated (Kämper et al., 2006). The human pathogen, *Cryptococcus neoformans* (Loftus et al., 2005) and some model fungal species such as *Sacchromyces cerevisea* (Kellis et al., 2003) and *Coprinopsis cinerea* (Stajich et al., 2010) were all sequenced and annotated. Other ecologically important fungal species such as *Lacaria bicolor,* a symbiont has been sequenced and annotated (Martin et al., 2008). Interestingly, the genome of *H. irregulare*, one of the fungal species studied in this work was recently added to the pool of genomic data in JGI (Olson et al., 2012). Futhermore, the genome of *P. gigantea* has been recently sequenced and is currently being annotated (http://genome.jgi.doe.gov/Phlgi1/Phlgi1.home.html). Additionally, over 30 fungal species including ascomycetes and basidiomycetes have been sequenced and assembled (**Table 2-** http://genome.jgi.doe.gov/programs/fungi/index.jsf). Recently, Floudas et al. (2012) reported a massive expansion of the genes encoding lignin-degrading peroxidases in the lineage leading to the ancestor of the Agaricomycetes (white rot fungi) and a contraction of the same gene in the parallel lineages leading to brown rot and mycorrhizal species. This finding suggests that lignin degrading ability in the Agaricomycetes may have been acquired during evolution. From the accumulated genome sequence data, the genome size and the distribution

of hydrophobin encoding genes in ecologically and economically important fungi can be ascertained.

Table 2: Fungal species, ecological strategies, genome sizes and hydrophobin distribution

Fungal taxa	Fungal species	Lifestyle	Ecological strategy[a]	Genome size (MB)	Number of genes[b]	Reference[c]
Basidiomycota	*Auricularia delicata*	Saprotroph/ White rot	NP	74.92	5	http://genome.jgi.doe.gov/Aurde1/Aurde1.home.html
Basidiomycota	*Coprinopsis cinerea*	Saprotroph	NP	37.5	33	40
Basidiomycota	*Pleurotus ostreatus*	Saprotroph/ White rot	NP	35.6	25	http://genome.jgi.doe.gov/PleosPC9_1/PleosPC9_1.home.html
Basidiomycota	*Phlebia brevispora*	Saprotroph/ White rot	NP	49.96	26	http://genome.jgi.doe.gov/Phlbr1/Phlbr1.home.html
Basidiomycota	*Phlebiopsis gigantea*	Saprotroph/ White rot	NP	30.14	18	http://genome.jgi.doe.gov/Phlgi1/Phlgi1.home.html
Basidiomycota	*Agaricus bisporus*	Saprotroph	NP	30.2	22	http://genome.jgi.doe.gov/Agabi_varbisH97_2/Agabi_varbisH97_2.home.html
Basidiomycota	*Laccaria bicolor*	Mycorrhiza/ Symbiont	NP	60.71	13	22
Basidiomycota	*Ceriporiopsis subvermispora*	Saprotroph/ White-rot	NP	39.0	25	http://genome.jgi.doe.gov/Cersu1/Cersu1.home.html
Basidiomycota	*Serpula lacrymans*	Saprotroph/ Brown rot	NP	42.4	17	62
Basidiomycota	*Punctularia strigosozonata*	White rot/saprotroph	NP	34.17	27	http://genome.jgi.doe.gov/Punst1/Punst1.home.html
Basidiomycota	*Dichomitus squalens*	Saprotroph / White rot	NP	42.75	21	http://genome.jgi.doe.gov/Dicsq1/Dicsq1.home.html
Basidiomycota	*Fomitiporia mediterranea*	Pathogen / White rot	PP	63.35	19	http://genome.jgi.doe.gov/Fomme1/Fomme1.home.html
Basidiomycota	*Trametes versicolor*	Saprotroph / White rot	NP	44.79	40	http://genome.jgi.doe.gov/Trave1/Trave1.home.html
Basidiomycota	*Heterobasidion irregulare*	Pathogen / White rot	PP	33.7	13	63

Basidiomycota	*Fomitopsis pinicola*	Saprotroph / Brown rot	PP;NP	46.30	7	http://genome.jgi.doe.gov/Fompi1/Fompi1.home.html
Basidiomycota	*Phanerochaete carnosa*	Saprotroph / White-rot	PP	46.29	15	http://genome.jgi.doe.gov/Phaca1/Phaca1.home.html
Basidiomycota	*Postia placenta*	Saprotroph / Brown rot	NP	33.0	3	64
Basidiomycota	*Ustilago maydis*	Pathogen	PP	20.0	2	65
Basidiomycota	*Stereum hirsutum*	Saprotroph/White rot	PP	46.51	0	http://genome.jgi.doe.gov/Stehi1/Stehi1.home.html
Basidiomycota	*Tremella mesenterica*	Saprotroph / White rot	NP	28.6	0	http://genome.jgi.doe.gov/Treme1/Treme1.info.html
Basidiomycota	*Cryptococcus neoformans*	Pathogen	HP	19.5	0	66
Basidiomycota	*Melampsora larici-populina*	Biotrophic/path-ogenic	PP	101.1	0	http://genome.jgi.doe.gov/Mellp1/Mellp1.home.html
Basidiomycota	*Sporobolomyces roseus*	Saprotroph	NP	21.2	0	http://genome.jgi.doe.gov/Sporo1/Sporo1.info.html
Basidiomycota	*Rhodotorula graminis*	Saprotroph	NP	21.0	0	http://genome.jgi.doe.gov/Rhoba1_1/Rhoba1_1.info.html
Basidiomycota	*Puccinia graminis*	Biotroph	PP	88.84	0	http://genome.jgi.doe.gov/Pucgr1/Pucgr1.home.html
Ascomycota	*Neurospora tetrasperma*	Saprotroph	NP	39.1	2	http://genome.jgi.doe.gov/Neute_matA2/Neute_matA2.info.html
Ascomycota	*Neurospora discreta*	Saprotroph	NP	37.3	2	http://genome.jgi.doe.gov/Neudi1/Neudi1.info.html
Ascomycota	*Neurospora crassa*	Saprotroph	NP	41.04	2	67
Ascomycota	*Acremonium alcalophilum*	Saprotroph	NP	54.42	1	http://genome.jgi.doe.gov/Acral2/Acral2.home.html
Ascomycota	*Saccharomyces cerevisiae*	Saprotroph	NP	12.07	0	68
Ascomycota	*Pichia stipitis*	Saprotroph	NP	15.4	0	http://genome.jgi.doe.gov/Picst3/Picst3.info.html
Ascomycota	*Hansenula polymorpha*	Saprotroph	NP	8.97	0	http://genome.jgi.doe.gov/Hanpo2/Hanpo2.home.html
Ascomycota	*Wickerhamomyces anomalus*	Saprotroph	NP	14.15	0	http://genome.jgi.doe.gov/Wican1/Wican1.info.html
Ascomycota	*Aspergillus nidulans*	Saprotroph	NP	30.48	3	http://genome.jgi.doe.gov/Aspnid1/Aspnid1.home.html
Ascomycota	*Chaetomium globosum*	Saprotroph / pathogen	HP; NP	34.9	0	http://genome.jgi.doe.gov/Chagl_1/Chagl_1.home.html
Ascomycota	*Pichia membranifaciens*	Saprotroph	NP	11.58	0	http://genome.jgi.doe.gov/Picme2/Picme2.home.html
Ascomycota	*Thielavia terrestris*	Saprotroph	NP	36.91	1	http://genome.jgi.doe.gov/Thite2/Thite2.home.html
Zygomycota/ Mycoromycotina	*Mucor circinelloides*	Saprotroph	NP	36.5	0	http://genome.jgi-psf.org/Mucci1/Mucci1.home.html

Ascomycota	*Aspergillus carbonarius*	Saprotroph	NP	36.3	4	http://genome.jgi.doe.gov/Aspca3/Aspca3.home.html
Ascomycota	*Aspergillus niger*	Saprotroph	NP	34.85	4	69
Ascomycota	*Aspergillus aculeatus*	Saprotroph	NP	35.4	3	http://genome.jgi.doe.gov/Aspac1/Aspac1.home.html
Zygomycota / Mucoromycotina	*Phycomyces blakesleeanus*	Saprotroph	NP	55.9	0	http://genome.jgi-psf.org/Phybl1/Phybl1.home.html
Ascomycota	*Magnaporthe grisea*	Hemibiotroph/ phytopathogens	PP	41.70	4	70
Ascomycota	*Trichoderma virens*	mycoparasite	NP	39.0	11	71
Ascomycota	*Trichoderma atroviride*	mycoparasite	NP	36.1	11	71
Ascomycota	*Fusarium oxysporum*	Saprotroph/ pathogen	PP	61.36	3	http://genome.jgi-psf.org/Fusox1/Fusox1.home.html
Ascomycota	*Fusarium graminearum*	Pathogen	PP	36.49	2	http://genome.jgi-psf.org/Fusgr1/Fusgr1.home.html
Ascomycota	*Alternaria brassicicola*	Pathogen/ saprotroph	PP	30.3	2	http://genome.jgi psf.org/Altbr1/Altbr1.info.html
Ascomycota	*Dothistroma septosporum*	Pathogen/ saprotroph	PP	30.21	3	http://genomeportal.jgi-psf.org/Dotse1/Dotse1.home.html
Ascomycota	*Leptosphaeria maculans*	Pathogen/saprotroph	PP	44.89	1	http://genomeportal.jgi-psf.org/Lepmu1/Lepmu1.home.html
Ascomycota	*Batrachochytrium dendrobatidis*	Pathogen	AP	24.3	0	http://genome.jgi-psf.org/Batde5/Batde5.home.html

a= (NP= Non phytopathogen, PP= Phytopathogen, AP= Animal pathogen)

b = No of hydrophobin encoding genes

c= Number of hydrophobins from unpublished references are based on automated results from the sequence assembly

2. Aims of the study

A relatively reasonable success has been recorded in the management of the conifer pathogen, *H. annosum* s.l. using *P. gigantea*. However, poor understanding of the mechanisms of interactions between the pathogen and the biocontrol fungus has hampered current initiatives of producing better isolates of *P. gigantea* with improved biocontrol activities. Another problem created by this poor knowledge of the biocontrol mechanism is the inconsistencies and variabilities inherent in the biocontrol process. A proper understanding of the physiological, biochemical and molecular mechanisms underlying this biological process is important for identification of markers that could be used to improve biocontrol capability of the isolates. This study therefore centered on dissecting the physiological, biochemical and the molecular factors that govern this important biological process.

The main objectives of the study were

1). To screen isolates of *P. gigantea* from different geographic regions for biocontrol-related traits, such as wood decay capabilities, enzyme production, growth rates and antagonistic abilities (**Paper I**).

2). To test if *P. gigantea* hydrophobin encoding genes (*Pgh1* and *Pgh2*) are differentially expressed during antagonism and hyphal growth under different substrate conditions. In addition, correlation between sequence variability of *Pgh1* and *Pgh2* and antagonistic ability was tested (**Paper I**).

3). To test for contraction and expansion in the hydrophobin encoding genes of *P. gigantea* and *H. annosum* s.l (**Paper III**).

4). To investigate the impact of secondary metabolites from *P. gigantea* on the growth and gene expression profile of the conifer pathogen, *H. annosum* s.l. (**Paper IV)**

3. Material and methods

A summary of the materials and methods used in this study are shown in Tables 1 and 2, for more detailed descriptions, please refer to **Papers I-1V.**

Table 3: Materials used in this study

Materials	Papers
Fungal isolates	
P. gigantea isolates	**I, II, III & IV**
H. annosum s. s. isolates (FP5)	**I, II, III & IV**
Media for fungal cultivation	
modified Norkrans media	**I & II**
Hagem agar media	**I & II**
Hagem liquid media	**II & III**
Wood samples	
Pinus sylvestris wood chips	**I**
Picea abies wood chips	**I**
Bark, sapwood and heartwood particles from pine tree	**III**

Table 4: Methods

Methods	Papers
Mycelial growth rate measurements	**I**
Interaction of *P.gigantea* and *H. annosum* s.s	**I & II**
Wood decay and light microscopy	**I**
Enzyme assay	**I**
DNA extraction	**II**
Sequencing	**II**
RNA extraction	**II, II & IV**
cDNA synthesis	**II, III & IV**
Real-time RT-PCR	**II**
Phylogenetic analysis	**II & III**
Protein modeling	**III**
Statistical analysis	**I, II, III & IV**
Microarray and macroarray analysis	**III & IV**

4. Results and Discussion

4.1 Screening of *Phlebiopsis gigantea* isolates for traits associated with biocontrol of the conifer pathogen *Heterobasidion annosum* s.l (Paper I)

4. 1. 2 Fungal isolates, culture conditions and screenings

Sixty four isolates of *P. gigantea* including the two commercial isolates, RotStop® S and RotStop® and wild types collected from different geographical locations, Finland, Sweden, Lithuania and Latvia were kindly provided by Kari Korhonen (Finnish Forest Research Institute, Metla) for this study (see **Paper I**, **Table 3**). The *P. gigantea* isolates were maintained on Hagem agar media (Stenlid, 1985) plates at 20˚C and were screened in different Norkrans media (Norkrans, 1963) at different temperature condition.

4.1.3 Variations in spore production and growth rates are related to *P. gigantea* biocontrol activity

In **Paper I**, a considerable effect of temperature on the growth rate of the the 64 isolates of *P. gigantea* screened was observed. The highest average growth rate was recorded at 20 °C (**Table 1, Paper I**). Growth rate was also significantly affected by the composition of the substrates. The highest average growth rate was observed in the Norkrans media supplemented with sawdust (NM/sawdust) followed by the glucose rich, complex Hagem agar media (**Table 1, Paper I**). A significantly positive interrelationship was found between growth rate on NM/sawdust and growth rate on NM/xylan (P= 0.0001) but a negative significant correlation was observed between growth rate on NM/sawdust and growth rate on the medium supplemented with the lignin precursor, ferulic acid (P= *0.03*), which may probably indicate that the dynamics of the interaction changes when the different wood components are encountered during *P. gigantea* growth on wood. Conifer wood tissues are usually lignified with very high amounts of phenolic compounds such as ferulic acid or hydroxycinnamic acid that contribute to the bonding between carbohydrates or between hemicelluloses and lignin (Hartley & Ford, 1989). The ability of *P. gigantea* to overcome the toxic effects of the different phenolic compounds in the search for host nutrient is probably the reason for its higher aggressive colonization of the stump. A similar finding was reported by Sun et al. (2009a, b). Interestingly, a comparison of the hyphal growth rate of 10 best performing isolates of *P. gigantea* under *in vitro* condition with the growth rate of the same set of isolates under field condition (Sun et al., 2009a) showed that the growth rate was higher in the artificial substrates for all the tested isolates (**Figure 1, Paper I**).

This could be due to more resource availability and steady *in vitro* incubation conditions compared to the fluctuating nature of wood tissue. Other studies have reported faster growth rates and high spore production as traits relevant for the control efficacy of *P. gigantea* against *H. annosum* s.l. (Korhonen, 2001; Berglund & Rönnberg, 2004; Berglund et al., 2006; Sun et al., 2009a). Holdenreider & Greig (1998), however reported higher growth rate of *H. annosum* on spruce wood when compared with the growth rate of *P. gigantea* on the same substrate. Additionally, a significantly higher spore production was recorded in the homokaryotic isolates of *P. gigantea* screened in this study when compared with the heterokaryon (**Table 2, Paper I**). The biological significance of this high spore production by the homokaryons is however not known as there is no available data on the competitive abilities of the homokaryons. However, homokaryons from the pathogen, *H. annosum* s.l. and the biocontrol fungus, *P.gigantea* have been reported to have a very short life span and could degenerate easily in nature (Garbelotto et al., 1996; Sun et al., 2009b). Some studies have suggested that the ratio of the basidiospores of *Heterobasidion* s.l. to that of *P. gigantea* on the stump surface could influence the outcome of the interaction (Berglund & Rönberg, 2004).

4.1.4 The antagonistic property of *P. gigantea* is related to its oxidoreductive activity and wood degradation

The initial contact of *P. gigantea* and *H. annosum* s.s. on the agar plate was made between 6-10 d.p.i. and this resulted to the formation of a barrage zone (an area formed by the contact made by *P. gigantea* and *H. annosum* s.s.). The size of the barrage zone varied from one time point to the other as the incubation time increased. Among the 64 isolates of *P. gigantea* screened in this study (**Paper I**), high variation in antagonism, laccase like-enzyme activity, wood decay capability and other variables was observed (**Table 3**, **Paper I)**. The outcome of the interaction was highly influenced by the nutrient source; 90 % of the isolates were able to displace *H. annosum* s.s. in NM/sawdust media after 20 days (**Figure 4a, Paper I**), as compared with only 4% observed in the complex Hagem agar (**Figure 4c, Paper I**). This could imply that the antagonistic ability on NM/sawdust (and possibly on the stump surface) may possibly be connected with the ability of *P. gigantea* to degrade the different components of wood. To further support this argument, a positive interdependence between antagonism on Norkrans/sawdust, pine and spruce wood degradation capability and laccase-like oxidation activity on guaiacol as substrate was observed (**Table 4, Paper I**). Laccase is a multicopper oxidase involved in lignin depolymerisation and detoxification of the monomeric phenolic components of wood (Have & Teunissen, 2001; Baldrian, 2006). It has a wide distribution in nature and has been reported in insects, plants, bacteria and fungi (Alexandre & Zhulin, 2000; Mayer & Staples, 2002; Claus, 2004). The variation in the laccase-like oxidation activity observed in the different strains of *P. gigantea* could suggest differences in the ability to degrade wood

during antagonism. Interactions between organisms using artificial media to understand the basis for biological control and the changes during fungal competition have been analysed by different authors (Kallio, 1971; Carruthers & Rayner, 1979; Magan & Lacey, 1984a; 1984b). Woods et al. (2005) reported that competitive interactions between fungi using utilizable sugar substrates could sometimes give results comparable to those obtained under field conditions. The behaviour of the 64 isolates of *P. gigantea* in the artificial sawdust media was most comparable to the observations recorded by Sun et al. (2009a) under natural field conditions for the same isolates of *P. gigantea.* Contrasting results on interactions carried out on artificial media with high sugar concentration and natural woody substrates have also been reported (Nicolotti & Varese, 1996; Highley, 1997).

4.2 Differential expression of two hydrophobin encoding genes (*Pgh1* and *Pgh2*) from the biological control agent *Phlebiopsis gigantea* (Paper II)

4.2.1 *P. gigantea* hydrophobins 1 and 2 may be involved in the formation of hyphal structures during growth and development

Seventeen isolates of *P. gigantea* were randomly selected from the 64 isolates screened in study **I**. The isolates were categorized into strong and weak antagonistic groups (**Table 1, Paper II)** for further studies. The expression patterns of *P. gigantea* hydrophobin encoding genes, *Pgh1* and *Pgh2* were monitored in both the strong and weak antagonistic isolates of the fungus using NM/sawdust, a substrate that mimics the natural environment of the fungus. Both the strong and weak antagonistic isolates showed a high expression of *Pgh1* at the early and late stages of the fungal growth. However, a much higher transcript abundance of the gene was observed in the strong antagonistic group when compared to the weak antagonistic group. The transcript level of *Pgh1* dropped in both the strong and weak antagonistic isolates at the intermediate stage of the fungal growth. A statistically significant difference was observed in the expression of *Pgh1* between the strong and weak antagonistic isolates at 20 d.p.i. (P= 0.01, **Figure 3a, Paper II).** A high induction of *Pgh2* was evident in the strong antagonistic isolates at the early (10 d.p.i) and intermediate stages (15 d.p.i) of the fungal growth, but the transcript level became reduced at the late stage of the growth. However, the induction level was low when compared to *Pgh1* and was statistically significant at days 10 and 15 which corresponds to the early and intermediate stages of growth (P= 0.003, 0.03) respectively (**Figure 3b, Paper II**). The relatively high induction of *Pgh1* in both the strong and weak performing isolates at the early stage (10 d.p.i) of the fungal growth could probably suggest a role of *Pgh1* in the attachment of fungal structures during the early stage of development. At hydrophilic/hydrophobic interfaces; the hydrophobin proteins self-assemble and mediate the interaction between the fungal surfaces and the substrate (Wösten et al., 1993; 1994a; 1994c; 1995). The high transcript level of *Pgh1* observed during the late stage of the growth could

possibly suggest a role in fruit body development. However, the decreased level of transcripts of *Pgh1* recorded at the intermediate stage of the growth could not be explained.

To further understand the effects of substrates on the regulatory patterns of *Pgh1* and *Pgh2,* the transcript levels of the two genes were monitored in a subset of strong and weak performing isolates of *P. gigantea.* Both *Pgh1* and *Pgh2* showed high transcript levels in the submerged conditions except for two isolates (01074 and 04135) that showed higher expression of *Pgh2* in the surface agar culture when compared with the submerged condition (**Table 4, Paper II**). However, this result was in contrast to findings reported by Karlsson et al. (2007). They reported higher transcript levels of *H. irregulare* hydrophobin encoding genes 1 and 2 (*Ha1 and Ha2*) in aerial cultures when compared to the submerged condition. The differences in result might be related to the timing of the sample collection culture which has been shown to have impact on hydrophobin gene expression by other studies (Muñoz et al., 1997). It is also possible that at late time points as in this study, most of the hyphae must have expressed enough hydrophobin to enable it overcome the barrier posed by the air water interphase for formation of emergent aerial hyphae. Differences in gene expression within isolates were further analysed between submerged and surface agar culture condition, *Pgh1* expression was statistically significant for isolate 04135 and *Pgh2* was statistically significant for isolates 01074 (**Table 4, Paper II**). The ability of *P. gigantea* to express high level of hydrophobins on wood could be influenced by the nutrient composition of the stump. Freshly cut stump surface has been shown to be rich in either soluble sugars (Asiegbu, 2000) or lignified cellulosic constituents (Cowling & Kirk 1976).

4.2.2 *P. gigantea* hydrophobin genes (*Pgh1* and *Pgh2*) analysis and the expression during competitive interaction with *H. annosum* s.l.

To further clarify the earlier report that *Pgh1* and *Pgh2* were differentially regulated at the barrage zone formed by *P. gigantea* and *H. annosum* (Adomas et al., 2006), we monitored the expression of both genes in strong and weak antagonistic isolates of the fungus during interaction on NM/sawdust. Specific primers were designed from ESTs of published *Phlebiopsis gigantea* hydrophobin encoding genes 1 and 2. High induction levels of *Pgh1* and *Pgh2* were observed at the early stage of the interaction between *P. gigantea* and *H. annosum* s.s, with higher transcript fold changes of both genes being recorded in the strong performing isolates than in the weak performing isolates (**Table 5, Paper II**). It is possible that the induction of *Pgh1* and *Pgh2* was provoked by the confrontation of *P. gigantea* by *H. annosum* s.s. However, the induction level of both genes reduced as the antagonism progressed, suggesting that the two genes may be more involved during the early stage of the antagonism. Although high fold changes of both genes were observed at the early stage of the interaction (10 d.p.i), higher transcript levels of *Pgh2* may be more critical during the biological interaction (**Table 5, Paper II**).

As indicated in our previous study (**Paper I**), the interaction between *P. gigantea* and *H. annosum* s.s. is characterized with the formation of barrage zone and competitive exclusion of *H. annosum* by *P. gigantea* from the media surface. There has not been any reported evidence of antibiotic production at the zone of interaction between *P. gigantea* and *H. annosum* s.l. In other biocontrol agents, for example, *Trichoderma* species, production of antibiotics and secretion of cell wall degrading enzymes like chitinases have been documented. In *P. gigantea- H. annosum* s.l. system, a number of genes encoding enzymes involved in nutrient acquisition were reported to be up-regulated at the zone of interaction between *P. gigantea* and *H. parviporium (*Adomas et al., 2006*)*. The study concluded that resource control could be the likely mechanism of biocontrol of *H. annosum* s.l. by *P. gigantea.* The evidence of *P. gigantea* hydrophobin encoding genes 1 and 2 transcripts at the barrage zone and the high transcript abundance of both genes at 10 d.p.i further supports their roles in the biocontrol process during the early stages of the interaction.

The analysis of sequences of *Pgh1* and *Pgh2* obtained from the genomic DNA of a randomly selected set of isolates of *P. gigantea* showed that the ORF of both *Pgh1* and *Pgh2* has 108 amino acids with eight cysteine residues arranged in a strictly conserved motif, a feature that is common to all fungal hydrophobins. The size of introns in both genes (*Pgh1* and *Pgh2)* and their positions were quite conserved, which suggests an evidence of duplication event. Phylogenetic analysis (**Figure 2, Paper II**) of the nucleotide sequence of the genes also indicates that this duplication event could be recent propably after a split between *P. gigantea* and *Grifola frondosa.* It is possible that the duplication of these hydrophobin encoding genes is specific for *P. gigantea* and the evolutionary forces driving their selection may be related to the specific ecology of *P. gigantea.* In addition, cysteine spacing and the hydrophobicity patterns of *Pgh1* and *Pgh2* showed that both genes encode proteins belonging to class I hydrophobins (**Figure 2**). The variable sequences of *Pgh1* and *Pgh2* obtained from the different isolates of *P. gigantea* could not show any relationship with their antagonistic properties.

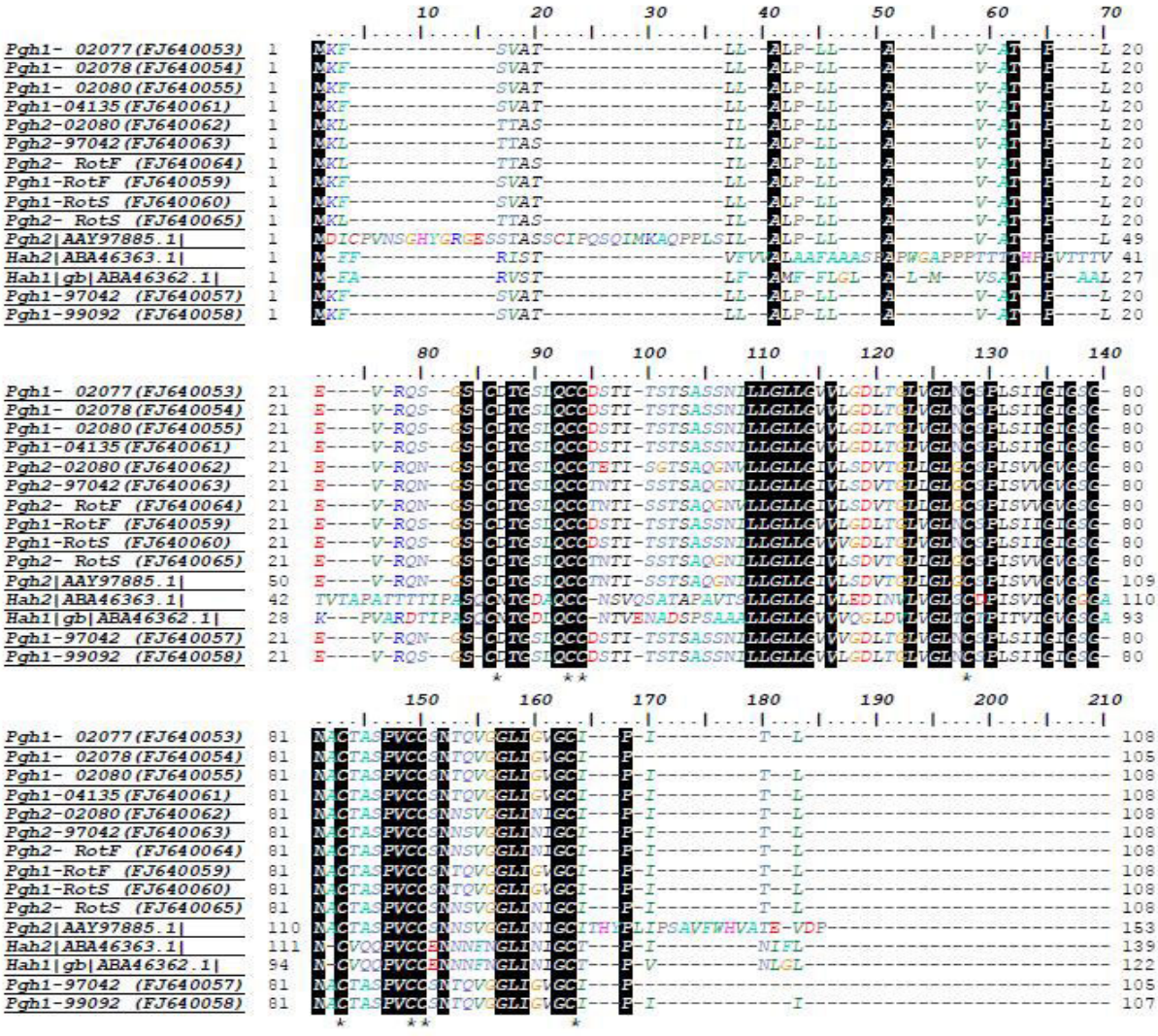

Figure 2: Alignment of protein sequences (aas) of hydrophobins from 10 isolates of *P. gigantea* and two hydrophobins (*Ha1* and *Ha2*) from *H.irregulare* (Karlsson et al., 2007).

Alignment was done with ClawstalW using the Biologigical sequence alignment editor (BioEdit) Win95/98/NT/2K/XP/7. Amino acid residues with 100% conservation are written in white letters but marked in black, conserved cysteine residues are marked with astericks.

4.3 Contraction and expansion in hydrophobin genes of ecologically important fungi *(Phlebiopsis gigantea* and *Heterobasidion annosum* s.l. - Paper III*)*

4.3.1 Phylogeny and classification of hydrophobins from the ecologically important fungi, (*P. gigantea* and *H. annosum* s.l.)

To understand the relationship of hydrophobins from the two ecologically important fungi, *P. gigantea* and *H. annosum* s.l., phylogenetic analysis of the gene was done with hydrophobins from other fungal taxa. The hydrophobins were also placed into different classes based on their cysteine spacing, hydropathy pattern and differences in the number of amino acids between the eight cysteine residues. The classification showed that hydrophobins from *P. gigantea* and *H. irregulare* were members of class I hydrophobins (**Figures 1 & 2, Paper III**). From the alignment, members of class I proteins showed long stretch of amino acids at the C3/C4 region (**Figures 1, Paper III**; Kershaw & Talbot, 1998). Similar finding has been reported for hydrophobins in other fungal species (Linder et al., 2005; Sunde et al., 2008). Additionally, hydrophobins from the two fungi, *P. gigantea* and *H. irregulare* showed higher hydrophobicity stretch when compared to other hydrophobins in class II category (**Figure 2b-d, Paper III**). In *P. gigantea* and *H. irregulare* hydrophobins, the cysteine doublets were followed by stretches of hydrophilic amino acids, however in the class II hydrophobins, the cysteine doublets were followed by hydrophobic amino acids. However hydrophobins from the two fungi (*Thielavia terrestris* and *Ustilago maydis)* which were included in the analysis did not conform with either the class I or class II consensus and we placed hydrophobins from such fungi into a different class (**Figure 1, Table 1, Paper III**). Seidl-Seiboth et al. (2011) observed a similar deviation from the known consensus of class I and class II in *Trichoderma* hydrophobins and they proposed a new classification system that could incorporate new members from different fungal taxa. However, unlike in the present study (**Paper III**), the classification method used in Seidl-Seiboth et al. (2011) included the solubility characteristics of the studied proteins.

Results from the phylogenetic analysis showed that hydrophobins from *P. gigantea* and *H. irregulare* were distantly related despite their close sequence similarities. Phylogenetic grouping further separated hydrophobins from other fungal taxa into class I and class II, with class I hydrophobins mostly found in basidiomycetes while the ascomycetes shared both classes of protein (**Figures 3a & b, Paper III**). Similar conclusion has been made in other studies (Jones et al., 1992; Britt et al., 2010). Separation of fungal hydrophobins into class I and class II using phylogenetic grouping has been reported in other studies (Wosten, 2001; Britt et al., 2010).

4.3.2 Hydrophobin gene distribution in ecologically important fungi may influence their ecological habits

A considerable variation exists in the number of hydrophobin encoding genes across different fungal taxa (**Figure 4a-b, Paper III**). In *Acremonium alcalophilum,* a fungus with a very high affinity for alkaline pH (9.0-9.2), one hydrophobin encoding gene has been reported whereas in *Trametes versicolor,* the Turkey tail fungus, 40 hydrophobin encoding genes have been reported (Grigoriev et al., 2011) http://genome.jgi.doe.gov/programs/fungi/index.jsf. In the members of *Sacchromycetes* species, such as *S. cerevisiae, Pichia stipitis, Hansenula polymorpha* and *Wickerhamomyces anomalus* with yeast-like or monocentric growth pattern (non filamentous growth pattern), no hydrophobin encoding gene exist (**Table 2**). We have also reported in our earlier studies a high level of sequence divergence in hydrophobins (*Pgh1* and *Pgh2)* from different isolates of *P. gigantea* obtained from various geographical regions (**Paper II),** a similar finding was made in the hydrophobin encoding genes from the pathogenic fungus; *Heterobasidion irregulare, Hah1* and *Hah2* (Karlsson et al., 2007). These differences in the number of hydrophobin encoding genes and their sequence variants in both *P. gigantea* and *H. annosum* s.l. raised questions on whether hydrophobin genes are under a dynamic evolutionary process. To understand the evolutionary forces driving hydrophobin gene evolution, different selection tests were conducted and results showed high dN/dS ratio (> 1) in both *P. gigantea* and *H. annosum* s.l. hydrophobin encoding genes, an indication that hydrophobins from these ecologically important fungi may be under positive selection.

However, the possibility that the evolution of this gene may be due to recombination and duplication events was not ruled out. We have earlier demonstrated the existence of duplication events in hydrophobins from both *P. gigantea* (**Paper II**) and *H. irregulare* (Karlsson et al., 2007). Gene duplication is a strong evolutionary force that shapes organism's complexity, adaptation and diversification to closely related strains and species (Prince & Pickett, 2002; Long et al., 2003). Duplication events create genetic redundancy and genes resulting from such process tend to be more prone to selection pressure (Ohno, 1970). Duplication event is a common feature in hydrophobin gene evolution (Kubicek et al., 2008) and in the case of *P. gigantea*, it may be relevant to the specific ecology of the fungus. The evidence of duplication events in *P. gigantea* and *H. annosum* s.l. hydrophobins may suggest that hydrophobins from both fungi may have evolved through the birth and death model (Nei & Rooney, 2005). In this model, newly duplicated gene copies are either retained in the genome if they have vital functions or purged from the genome through purifying selection if they are deleterious to the organism's life. In **Paper III**, we found a very high number of substitutions in the sequences of hydrophobin encoding genes from *P. gigantea* isolates obtained from different geographical regions (**Table 4, Paper III**), we suggested that geography could account for this high number of mutations as the different isolates may have been challenged with different selection pressure due to geographical conditions.

We further analyzed hydrophobins from the two sister lineages of dikarya fungi (ascomycetes and basidiomycetes), we found a considerable expansion of the number of hydrophobin encoding genes among the basidiomycetes (P= 0.002) but contraction of the number of genes, loss of paralogs was more evident in the ascomycota fungi. In contrast, Karlsson & Stenlid (2008) reported a massive expansion in chitinase encoding gene family GH18 among the ascomycetes. The expansion of hydrophobin gene family among the basidiomycetes could suggest that hydrophobins may have been selected for during evolution due to their importance in the fitness of the fungal species in these taxa. It is also possible that the high numbers of this gene may be important for nutrient acquisition, competition, growth, fruit body formation and morphology of the fungi in this group. We therefore investigated the relationship between hydrophobin gene number acquisition and ecological strategy, a statistically significant relationship between the number of hydrophobin encoding genes and ecological strategy was observed (P=0.0001), with more numbers of the gene being present in non-pathogenic fungi (P=0.0001, R^2= 0.971) when compared to the pathogenic ones (P =**0.1; Figure 5b, Paper III**). Although hydrophobins have been reported to play a crucial role in fungal pathogenesis (Felipe et al., 2003; Karlsson et al., 2007; Fedorova et al., 2008; Kubicek et al., 2008), it is possible that more numbers of the gene may be needed during biological processes involving non pathogenic fungi such as during mycorrhiza formation (Martin et al., 2008), symbiosis (Trembley et al., 2002), and interspecific antagonistic fungus-fungus interactions (Adomas et al., 2006; **Paper II**), fruit body formation (Yamada et al. , 2005; Ohm et al., 2010) and emergence of hyphal structures (Mankel et al., 2002; Cho et al., 2007; **Paper II**) .

4.3.3 Hydrophobin protein structural modeling and the regulatory patterns of different *H. annosum* s.l. hydrophobin encoding genes during saprotrophic growth on wood

As a multigene family, large numbers of hydrophobin encoding genes with different regulatory roles exist in several fungal taxa (**Table 2**). In the biocontrol fungus, *P. gigantea*, over 18 different hydrophobin encoding genes are currently undergoing manual curration. In the conifer pathogen, *H. irregulare*, 13 hydrophobin encoding genes have been identified and characterized (Olson et al., 2012). However the regulatory patterns of each gene in either *P. gigantea* or *H. annosum* s.l. during different biological processes is not known. A global gene expression approach involving microarray was used to study the regulatory patterns of the multiple hydrophobin encoding genes in *H. annosum* s.s. during saprotrophic growth on bark, sapwood and heart wood as well as during growth in secondary metabolites produced by *P. gigantea.* Out of the 13 individual hydrophobin gene transcripts identified in *H. annosum* s.s., transcript number 5 showed a high expression during saprotrophic growth on bark, sapwood and heartwood (**Table 5, Paper III**). Transcript numbers 11 and 13 showed up regulation during growth on sapwood and heartwood but were down regulated during saprotrophic growth on bark. However, the transcript abundance of copy number 9 was high in all the tested conditions (saprotrophic growth on bark, sapwood, heart wood and during growth on

secondary metabolite produced by *P. gigantea*). The observed differences in the expression patterns of the individual hydrophobin transcripts during saprotrophic growth suggest that *H. annosum* s.s. employs different hydrophobins during growth on diverse wood components. It was evident to see that the secondary metabolite produced by *P. gigantea* had a repressing effect on the individual transcripts of hydrophobins from *H. annosum* s.s. However, the high abundance of transcript number 9 in the metabolite environment may suggest a role in resistance against *P. gigantea* during antagonistic interaction.

The post-genomic era has provided opportunities for determining several protein structures through the structural genomic initiative. In hydrophobins and other proteins, the hydrophobic and hydrophilic properties of the amino acids in the protein sequences determine the protein structure and fold. The protein 3D homology modeling of hydrophobins from ecologically divergent fungal species revealed that the surface of the proteins are covered with patches of hydrophobic residues (**Figure 3**), which may probably suggest a role in the formation of amphiphilic membranes as reported in other studies ((Hakanpää et al., 2004). Conserved cysteine residues were also prominent and may possibly indicate a preserved structural feature of the hydrophobin proteins. However, the models only covered parts of the relevant hydrophobin proteins. Attempts to construct complete models of the protein were hindered by lack of template structures. Protein modeling could help to determine the functional and biological roles of proteins by comparing the query sequence of the protein with the existing sequences and structural databases (Bairoch, 1999; Attwood et al., 1999; Mewes et al., 1999). Some studies have also tried to ascertain the relationship between protein structure and function using hydropathy indices (Damodharan & Pattabhi, 2004).

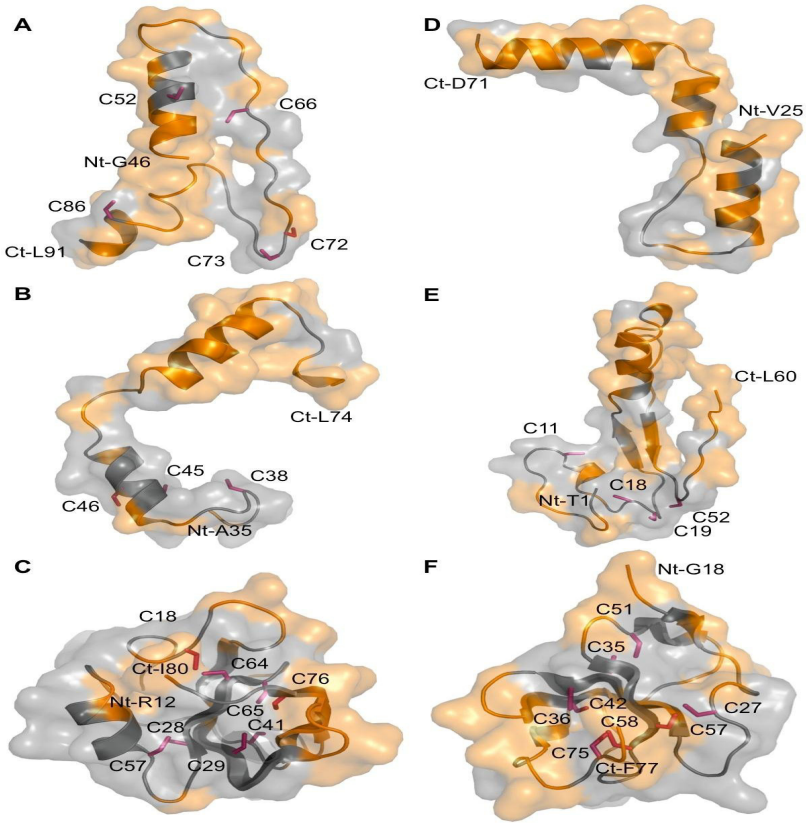

Figure 3: Homology modeling of hydrophobins. Surface and ribbon cartoons of the parts of the hydrophobins from (**A**) *Phlebiopsis gigantea* (**B**) *Heterobasidion annosum* (**C**) *Verticillium dahlia* (**D**) *Ustilago maydis* (**E**) *Laccaria bicolor* (**F**) *Thielavia terrestris*. The hydrophobic areas and conserved cysteine residues are shown in orange and hotpink respectively. N- and C-termini (Nt- and Ct-) are shown with the respective amino acid recidues

4. 4 Expression analysis of the impact of secondary metabolites from the biocontrol agent (*Phlebiopsis gigantea*) on the conifer pathogen (*Heterobasidion annosum s.s.*) (Paper IV)

4.4.1 Metabolites from *P. gigantea* are capable of inducing transcriptional changes in *H. annosum* during *in vitro* growth

In **Paper IV,** microarray technology was used to study the transcriptional responses of *H. annosum* s.s.during growth on metabolites produced by the commercial isolate of the biocontrol fungus (Rotstop®). Oligonucleotide arrays from *H. irregulare* genome sequence (Olson et al., 2012) were probed with labelled cDNAs from *H. annosum* s.s. cultivated in secreted metabolites produced by *P.gigantea*. About 3779 genes were found to be differentially expressed in *H. annosum* s.s. cultivated in extracellular metabolites from *P. gigantea.* Out of the 3779 genes, 2880 genes were differentially up-regulated whereas 899 of the transcripts showed differential down-regulation. Based on GO term analysis, 57.3% of the up-regulated genes were either predicted/hypothetical proteins whereas 35.5% were genes whose functions were unknown. The down-regulated genes followed a similar trend. Genes encoding either hypothetical or predicted proteins accounted for 67.4% of the entire gene transcripts in this category whereas 22.4 % of the genes encoded proteins whose functions are not known. A reasonable proportion (5.4%) of the genes involved in metabolism were down-regulated in *H. annosum* s.s. This finding supported our earlier hypothesis on nutrient acquisition as one of the primary mechanisms of the biocontrol action of *P. gigantea.* It is possible that the metabolites from *P. gigantea* could have a repressing effect on the genes involved in different metabolic processes in *H. annosum* s.l.. Some genes encoding proteins such as, cellobiose dehydrogenase, endo-1.4-B-xylanase A, and the gene encoding FAD binding domain containing protein which are involved in important metabolic processes were significantly down regulated in this study (**Table I, Paper IV**).

However, genes encoding proteins involved in metabolism (2.7%), showed high fold changes. For example; genes encoding beta-fructofuranosidase (FFase) and carboxylesterases had relatively high fold changes (**Supl. Table 1, Paper IV**). Beta-fructofuranosidases are involved in transfructosylation as well as in the hydrolysis of sucrose to glucose and fructose (Antosova & Polakovic, 2001; Nguyen et al., 2005). Carboxylesterases are important for xenobiotics hydrolysis (Krell et al., 1984). However, cytochrome P450, another gene encoding proteins involved in xenobiotics hydrolysis was found in a very low amount. Molybdenum cofactor sulfurase, a gene reported to be involved in stress tolerance in plants (Xiong et al., 2001; Min Huang et al., 2009), showed high fold change. Molybdenum cofactor sulfurase plays a key role in abscisic acid (ABA) biosynthesis. Abscisic acid (ABA)-mediated signaling pathways regulate different abiotic stresses in plants (Narusaka et al., 2003; Zhu et al., 2005). The high transcript fold change of this gene could possibly suggest a role in stress tolerance. An important gene encoding d-4, 5 unsaturated-

glucuronyl hydrolase-like proteins which has been shown to be involved in hydrolysis of glycosidic bonds (Hashimoto et al., 1999), was found to show a relatively high fold change. Also, pyruvate dehydrogenase which converts pyruvate to acetyl-Co A, a key step in citric acid cycle (Jordan et al., 2004) was expressed at high level.

Genes in other functional categories were also found to be either down regulated or upregulated (**Figures 1a &b, Paper IV**). For example, major facilitator superfamily (MFS) polyamine transporters showed a very high fold change (2573-fold change). Polyamines are important molecules needed in some major physiological processes, such as response to various stresses, embryogenesis and cell division (Bagni, 1989; Flores, 1991). The relatively high level of the gene transcript in the array could suggest a role in cell regeneration or remodeling. Ankyrin repeat proteins and adelylate cyclase which are major players in signal transduction were also found in relatively high folds (93.18, 12.20-fold change), respectively. Ankyrin repeat proteins are ubiquitous proteins which play important roles in protein-protein interactions (Andrade et al., 2001; Forrer et al., 2003). Adenylate cyclase is an important gene encoding proteins involved in the regulation of cellular cAMP levels. cAMP activates several important downstream signaling processes in the cell such as phosphorylation of protein kinase A (PKA) and these coordinated activities lead to the phosphorylation and regulation of key enzyme substrates involved in intermediary metabolism (Daniel et al., 1998). However, low transcript fold changes of *H. irregulare* hydrophobins 1 and 2 encoding genes *(Ha1* and *Ha2)* were observed in the array, implying that *Ha1* and *Ha2* expression could possibly be repressed by the metabolites from *P. gigantea.* In contrast, the *P. gigantea Pgh1* and *Pgh2* expression was induced by contact in paired cultures of *P. gigantea* and *H. annosum* s.s. (**Paper II**; Adomas et al., 2006). The up-regulation of some key genes involved in metabolism in this study may suggest some mechanisms of defence by the pathogen (*H. annosum* s.s) against the effect of metabolite from the biocontrol agent. Finally, the paucity of genome sequence information on many fungal species may have accounted for the huge number of uncharacterized or genes with unknown functions found in this study (**Paper IV**).

4.4.2 Macro-array differential screening reveals some unique genes that may account for the phenotypic variation in the progeny isolates of *P. gigantea*

Breeding and genetic engineering have been employed as new methods of improving the biocontrol properties of some biocontrol agents (Lübeck et al., 2002; Sun et al., 2009b). By crossing 10 homokaryotic isolates of *P. gigantea*, Sun et al. (2009b) identified 172 progeny isolates by their ability to fruit within 2-3 months. The identified progeny isolates were reported to show different phenotypic variations when compared with the wild type strains (Sun et al., 2009b). In our study (**Paper IV**), we used macroarray screening to study the genetic basis of the variations observed in the progeny isolates of *P. gigantea* when compared with three wild type isolates. The macroarray results revealed 58 genes which were differentially expressed with reference to the *P. gigantea* Rotstop ® the commercial isolate of

the biocontrol fungus (**Sup Table 3, Paper IV).** The effective progeny strain showed about 10 unique genes that were up-regulated in the array when compared to the other forms of *P. gigantea*, an indication that the progeny strains have acquired some new traits through crossing of homokaryotic wild types. Sun et al (2009b) reported improved growth rate, higher spore production ability and better competitive ability of the progeny isolates when compared with the wild type strains. In addition, some overlaps were observed in the gene expression profile of the isolates (effective wild type, less effective wild type and the progeny strain), suggesting that although the three groups of isolates may have some phenotypic differences, their genetic variation may be low. However most of the differentially expressed genes could not be identified due to the absence of information on the identity of such genes and as a result, the functional roles of such genes could not be ascertained. Breeding programs aimed at improving traits has a long history in agricultural crop plants but little or no applications have been recorded in biological control. Studies on improvement of biocontrol properties of *P. gigantea* have relied on isolate selections based on growth rate, spore production and better competitive abilities (**Paper I**, Sun et al., 2009a), a new approach involving breeding could offer an alternative to screening method. The identification of genes with unique expression in the progeny isolates has given more insights on the basis for phenotypic differences observed in the progeny isolates and the wild type of *P. gigantea.*

5 Conclusions

On a general note, the work reported in this study has provided a sound basis for future studies aimed at a better understanding of the physiological, biochemical, molecular and genetic factors underlying the interaction between *P. gigantea* and *H. annosum* s.l.

In **Paper I**, our results demonstrated wide phenotypic variations in different isolates of *P. gigantea* in relation to growth rate, antagonistic behaviour, wood degrading capability and enzyme production. The results also highlighted some insights on the importance of choosing a proper cultivation media (modified Norkrans sawdust media in this case) and optimal temperature for *in vitro* screening programs of *P. gigantea* isolates. From the results we could also deduce that the ability to degrade wood is an important trait for high biocontrol ability of *P. gigantea*.

In **Paper II**, we showed that the proteins encoded by *P. gigantea* genes (*Pgh1* and *Pgh2*) are new members of class I hydrophobins. Further analysis of these genes in strong and weak isolates of the biocontrol fungus, *P. gigantea*, showed different regulatory patterns during growth and antagonism on wood. While *Pgh1* was expressed at a very high level during *P. gigantea* growth on pine wood (sawdust), both genes (*Pgh1* and *Pgh2*) showed high transcript abundance during the early stages of antagonism on wood. However, from the result, it appeared that higher transcript levels of *Pgh2* may be needed during competition of *P. gigantea* and *H. annosum* s.s. on wood. The expression of the two hydrophobin genes, *Pgh1* and *Pgh2*, was highly affected by the cultivation medium. Although we could not observe any relationship between antagonistic ability and sequence variants of *Pgh1 and Pgh2,* the antagonistic ability of the *P. gigantea* isolates appeared to correlate positively with the transcript levels of *Pgh1* and *Pgh2*. Based on our findings, it could be suggested that the regulatory patterns of both *Pgh1* and *Pgh2* may be related to their roles in antagonism on wood.

In **Paper III,** a survey of hydrophobin distribution and evolution in *P. gigantea* and *H. annosum* as well as in other ecologically important fungi was made. We demonstrated that

high number of hydrophobin genes could play a significant role in the ecological strategy of some fungal species. Our result also showed that a considerable expansion of the number of hydrophobin genes has been witnessed in both *P. gigantea* and *H. annosum* s.l. as well as in other basidiomycetes while the ascomycetes have suffered a loss in the number of hydrophobin encoding genes. However, the expression of the hydrophobin genes from *H. annosum* s.s.was repressed by metabolites produced by the biocontrol fungus, *P. gigantea*.

In the last article, **Paper IV**, it was evident to see that the secreted extracellular metabolites produced by *P. gigantea* can effectively inhibit some metabolic pathways in the pathogen. This was evident in the repression of a good number of genes involved in nutrient metabolism in *H. annosum* s.s. The ability of the extracellular metabolites produced by *P. gigantea* to down-regulate most of the genes involved in metabolism in *H. annosum* s.s. supports our earlier hypothesis of nutrient acquisition as part of the mechanism for the biocontrol action of *P. gigantea*. From this study we could also conclude that the progeny isolates of *P. giagntea* could offer a better alternative for enhancing the genetic diversity of *P. gigantea* for improved biocontrol activity.

6. Future Perspectives

Our future studies will most likely focus on incorporating some of the newly identified biological traits into molecular breeding so as to produce *P. gigantea* isolates with better and improved biocontrol abilities**.** This study has provided an initial insight into the regulatory patterns of *P. gigantea* hydrophobins 1 and 2 under diverse conditions. However, a more definitive study is needed to confirm the actual involvement of these genes in the biological control process; this necessitates the use of functional knockout analysis. However, since no DNA transformation system is presently available for this fungal species, our future aim will be to develop a suitable transformation system that will be used to carry out this confirmatory study.

It will also be interesting to dissect the entire metabolome of *P. gigantea* using some of the state of the art tools in analytical chemistr*y*. In addition*,* the factors controlling the different metabolic pathways need to be properly studied. Furthermore, it will be of immense importance to understand the actual metabolic pathways in the pathogen that are targeted by the metabolites from the biocontrol fungus, a thorough understanding of the pathway will help in the biocontrol process by targeting the active enzymes involved in the biosynthetic pathways in the pathogen.

The global gene expression methods employed in this study have provided an opportunity for investigation into the transcriptomics profile of *P. gigantea* and *H. annosum*

s.l. The approach will facilitate a better understanding of the genetic basis of breeding and to identify the candidate genes that are responsible for the biocontrol efficacy of the progeny isolates of *P. gigantea*. It has also enabled us to understand the effect of metabolites on the gene expression profile of *H. annosum* s.l. However, the lack of information on a huge number of genes identified in this study opens a new frontier for research. The availability of genome sequences of several fungal species including *P. gi*gantea and *H. irregulare* has provided more opportunities for proper annotation and identification of the genes found in this work.

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