A study of the traits associated with the biocontrol activity of *Phlebiopsis gigantea*

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Licentiate Thesis
Swedish University of Agricultural Sciences
Uppsala 2009

Acta Universitatis Agriculturae Sueciae

ISBN 978-91-86197-24

SLU Service/Repro, Uppsala, 2009.

Abstract

Mgbeahuruike A.C. 2009. **A study of the traits associated with the biocontrol activity of *Phlebiopsis gigantea*.**

Licentiate Thesis

Phlebiopsis gigantea has routinely been used for the biocontrol of the conifer pathogen, *Heterobasidion annosum* s.l. but the mechanism for the biocontrol action has not been properly understood. In the present work, 64 isolates of *P. gigantea* were screened for traits important for the biocontrol of *H. annosum*. Growth rate and the interaction patterns of *H. annosum* s.l. and *P. gigantea* were studied in both carbon rich (Hagem) and low carbon media (Norkrans). Laccase assay and wood degradation capabilities were performed on the 64 isolates. Data was analyzed with multiple regression and principal component analysis. Results showed a significant effect of culture composition on the outcome of the interaction, 90 % of the isolates were able to displace *H. annosum* s.s. in sawdust media after 20 days, as compared with only 4% recorded in the glucose rich Hagem media. High growth rate on sawdust, a lignified carbon source, correlated with high growth rate in ferulic acid, a lignin precursor ($P = 0.078$), high growth rate in xylan, a hemicellulose ($P = 0.001$) and percentage weight loss in pine ($P = 0.01$). Interaction in sawdust correlated with high wood degradation capability in pine and spruce with *P*-values ($P = 0.01$, $P = 0.03$) respectively, high growth rate in xylan ($P = 0.01$), laccase production ($P = 0.08$), interaction in Hagem ($P = 0.01$) and mean growth rate at 10 °C ($P = 0.001$). Additionally, the role of hydrophobin in the competitive interaction was further investigated. The genomic sequence of *Phlebiopsis gigantea* hydrophobins 1 and 2 (*Pgh1* and *Pgh2*) from a subset of isolates selected on the basis of geographical origins and antagonistic abilities was investigated. Similarly, the expression of *Pgh1* and *Pgh2* under different substrate conditions was also studied using quantitative PCR. Sequence analysis was performed with Clustal W and inspected with Megalign (DNA Star). Expression data was analyzed using the relative quantification method- $2^{-\Delta\Delta Ct}$ and tested for effects of isolates, genes and culture conditions

using the general linear model (GLM) procedure in SAS. There was a close sequence similarity between hydrophobin genes of isolates having different antagonistic capabilities and from different geographical sources. Higher transcript levels of *Pgh1* and *Pgh2* were recorded in submerged cultures compared with aerial conditions. The effect of substrate on the expression of the two genes (*Pgh1* and *Pgh2*) was statistically significant ($P = 0.0001$). Differences in transcript levels of *Pgh1* and *Pgh2* were also observed among isolates belonging to different antagonistic categories. Overall, the results suggests that the antagonistic and competitive advantage of *P. gigantea,* hinged on the ability of the isolates to degrade the different structural components of wood. A significant correlation was also found between some high antagonistic isolates and the expression of hydrophobin genes (*Pgh1* and *Pgh2).* The significance of these results in the biological control is discussed.

Key words: Biocontrol, Interaction, *Heterobasidion annosum*, *Phlebiopsis gigantea,* hydrophobins, Quantitative PCR, sawdust.

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Dedication

To all who have worked hard for the betterment of humanity.

Contents

List of Manuscripts in the thesis

This thesis is based on these two papers referred to by Roman numerals in the text:

I. Differential screening of *Phlebiopsis gigantea* isolates for traits associated with biocontrol of the conifer pathogen *Heterobasidion annosum* (Submitted)

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

1 Introduction

Control of plant and forest diseases using chemical agents has been successful in reducing disease severity and spread. However, some harmful environmental effects such as effects on non target organisms and persistence of residues in the environment have been reported (Ragsdale & Sisler, 1994). Continuous use of chemical agents in the management of forest and crop diseases could result in disease resistance and loss of effectiveness in disease control (Lennox & Spotts, 2003). Therefore, there is a need for alternative disease control measures that are less prone to resistance build-up and have no adverse effects on ecological biodiversity or the environment. The use of microbial antagonists (biological control agents) in the control of plant and crop diseases has been recommended as an alternative to chemical control methods (Elad & Zamman, 1993). According to Campbell, (1989), the existence of an organism in an ecosystem is partly determined by its ecological relationship with the antagonistic species in that particular habitat. Such antagonist-pathogen relationships could offer opportunities for biological control processes. By definition, biological control involves the use of an organism (antagonist) against another organism (pathogen) so as to reduce the amount of inoculum or the disease-producing capacity of the pathogen (Cook & Baker,1983).Biological control processes in agricultural practice has been a naturally occurring phenomenon evident in the rhizosphere (region of soil surrounding plant roots) of plants. The rhizosphere is rich in exudates released by plant roots and such exudates facilitate the growth and colonization of plant roots by plant growth promoting rhizobacteria (PGPR), which confer protection to plants against pathogenic invaders (Whipps, 2001; Wulff et al., 2002a; 2002b). However, classical biological control which involves the introduction of a host-specific

biocontrol agent into an environment where there is an existing pathogen is common in agricultural practices (Roderick & Navajas, 2003). In forest practice, the first evidence of biological control of forest pathogens was reported in England by John Rishbeth, who showed that *Phlebiopsis gigantea* (Fir.) Jűlich is a strong antagonist of *Heterobasidion annosum* sensu lato (s.l.) (Rishbeth, 1952).

The antagonistic interaction between biological control agents and their pathogenic counterparts involves several potential mechanisms such as secretion of growth inhibitory compounds, competition for nutrients, lytic enzyme secretion, induced resistance and mycoparasitism (Gupta & Utkhede, 1986; Leifert et al., 1995; Monhamed & Caunter, 1995; Walker et al., 1996; Asiegbu et al., 2005; Adomas et al., 2006). In the *H. annosum* - *P. gigantea* biocontrol system, the ability to colonize the stump surface rapidly and high growth rate under different conditions were reported to be partly responsible for the biocontrol ability of *P. gigantea (*Asiegbu et al., 2005; Adomas et al., 2006; Turby et al., 2008). Secretion of wood degrading enzymes such as laccase by the biocontrol fungus has also been reported as a factor in the control of the conifer pathogen (Asiegbu et al., 2005). Furthermore, genes encoding hydrophobins, a surface active protein, and other genes involved in nutrient metabolisms have been reported to be highly expressed at the zone of interaction between *P. gigantea* and *H. annosum* (Adomas et al., 2006). A thorough understanding of the mechanisms of interaction between a biocontrol agent and a pathogen will help to reduce the efficacy variability associated with biocontrol agents.

Biological control processes in both agricultural and forest practices are associated with numerous advantages. The residues from biological control agents tend to be readily biodegradable and there has not been any reported case of persistence in the environment (Andrew, 1990). In addition, the biocontrol interaction ability is a complex trait, which reduces the risk of resistance build-up by the target organism (Burge, 1998). Biological control attracts a wide public acceptance because it is viewed to be less harmful to the environment than chemical pesticides (Dahlsten & Dreistadt, 1991). However, notwithstanding these numerous advantages associated with biological control, some problems and possible adverse effects still exist. Biological control agents appear to be very sensitive to environmental changes such as temperature and humidity, and the shelf life tends to be

short (Holdenrieder & Greig, 1998). Some biological control agents have been reported to cause unwanted shift in ecological biodiversity by adversely affecting the non target microorganisms including the resident population of the antagonist (Westlund & Nohrsted, 2000; Vasiliauskas et al., 2004). The metabolites of some biological control agents could as well constitute toxins and allergens to humans thereby posing a potential health hazard during inoculum production and application (Cook & Baker, 1983). A thorough understanding of the mechanisms of interaction between biocontrol agents and their pathogenic counterparts can aid in improving the efficacy and consistency of biological control. A properly executed biological control program can increase the ecological and economic sustainability in forestry by reducing the losses due to pathogens as well as protecting the environment from the harmful effects of chemicals.

2 The conifer tree pathogen- *Heterobasidion annosum*

2.1 Biology

Heterobasidion annosum (Fr.) Bref. (s.l.), is a necrotrophic white rot fungus which constitutes a major threat to the forest industry in the Northern hemisphere (Woodward, 1998; Asiegbu et al., 2005a). The fungus causes root rot and stem decay in most coniferous species in the temperate region and the annual economic losses attributable to the disease in Europe is over 790 million Euros (Woodward et al., 1998). Mating experiments have revealed that *H. annosum* s.l. consists of a species complex of intersterility groups (IS) (Carpretti et al., 1990; Chase & Ullirch, 1998). In Europe, three IS groups (species) have been identified with differences in host preference, these include the P-type (*H. annosum* sensu stricto (s.s.) Niemelä and Korhonen) which has a broad host range including Scots pine (*Pinus sylvestris*), birch (*Betula pendula*), alder (*Alnus incana*) and juniper (*Juniperus communis*). The S-type, *H. parviporum* Niemelä and Korhonen, infects *P. abies* as the primary host but can also attack Siberian fir (*Abies sibirica*) (Korhonen et al., 1997). The F-type, *H. abietinum* Niemelä and Korhonen mainly infects fir (*Abies alba*) (Niemelä & Korhonen, 1998).

2.2 Distribution

In Europe, *H. annosum* s.s has a wide geographical distribution among the countries in the Nordic region, spanning through Southern Europe down to the Altai region of Southern Siberia (Carpretti et al., 1990; LaPorta et al., 1997; Korhonen & Dai, 2004). *H. parviporum* is commonly found in

areas where Norway spruce, the major host, predominantly exists. Its prevalence has been reported from Northern parts of Finland, down to Greece in Southern Europe, from Eastern part of France to the Ural Mountains (Korhonen, 1998). *H. abietinum* is restricted to Central and Southern Europe, western Turkey and Russian Caucasus, and in areas where *P. abies* is grown in mixed strands with *A. alba* (Korhonen et al., 1998; Dogmus-Lehtijärvi et al., 2006; Sanchez et al., 2007; Zamponi et al., 2007).

In North America, two species known as the American P and S types have been identified (Harrington et al., 1989; Chase & Ullrich, 1990a; Otrosina et al., 1993). The North American P-type attacks mostly *Pinus* species, *Juniperus* and *Colocedrus* in the eastern and western forest, though it is less common in the central part of North America (Garbelotto et al., 1996). The S-type shows a broader host range, infecting several conifer species such as *Picea, Abies, Pseudotsuga, Tsuga* and *Sequoiadendron* (Garbelotto et al., 1996). The North American P-type has been reported to be introduced to 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).

2.3 Infection and Spread

H. annosum s.l. infection is by aerial basidiospores which are deposited on freshly cut stump surfaces or wounds on roots or stem. The fungus is known to spread from the infected stumps to adjacent tree stands through root to root contacts as well as from the infected to healthy trees (Redfern & Stenlid, 1998). The basidiospores could travel few meters to hundreds of kilometers to infect freshly cut stump surfaces (Rishbeth, 1959a; Gothier et al., 2001). Individual *H. annosum* s.l. genets can survive for several decades in conifer stumps thereby constituting a source of infection for several generations (Piri, 1996; Lygis et al., 2004; Piri & Korhonen, 2007). Asexual conidiospores of *H. annosum* could be carried by wind under high humidity and mist and such spores have been reported to be carried by insect vectors as well (Kadlec et al., 1992). During stump colonization, *H. annosum* s.l. has been reported to secrete numerous extracellular enzymes (cellulase, manganese peroxidase, laccase, pectinase and proteases) which degrade and detoxify the structural and soluble components of wood, although the precise role of the enzymes in pathogenicity is unknown (Asiegbu et al.,

1998). In addition, *H. annosum* s.l. has been reported to secrete low molecular weight compounds such as fommanoxin, fommanosin, fommanoxin acid, oosponol and oospoglycol which may contribute to the pathogenicity of the fungus (Asiegbu et al., 1998).

3 The different control methods of *H. annosum* infection

3.1 Silvicultural control

Conifer tree species are the most susceptible to *H. annosum* s.l. infection and the frequency of attack on broad leave trees is lower. Planting trees species with low susceptibility can help to reduce the spread of infection (Korhonen et al., 1998b; Lygis et al., 2004a; Lygis et al., 2004b). A mixed stand approach which exploits the advantage of low attack on the deciduous species could be a possible way of minimizing the spread of infection (Piri et al., 1990; Linden & Vollbrecht, 2002). Another silvicultural approach for the control of *H. annosum* s.l. involves stump removal, although 100% efficiency is hardly achieved because the fungus can survive and carry over infection to another stand (Korhonen et al., 1998b; Stenlid, 1987).

3.2 Chemical control

Stump treatment using chemicals such as urea and borate have been used in the control of *H. annosum* s.l. Both chemicals have been reported to inhibit the germination and growth of *H. annosum* s.l. spores with protection efficacy ranging between 80%-100% (Thor & Stenlid, 1997). The effectiveness of urea in the control of *H. annosum* s.l. on the stump surface has been evaluated in other studies (Pratt & Redfern, 2001; Nicolotti & Gonthier, 2005; Thor & Stenlid, 2005). Although such studies aimed at evaluating the short term effect of urea on the stump, Oliva et al. (2008) have investigated the long term effect of urea on stump surface.

According to the report, stump protection in urea treated plot after 15 years was 97.3% compared to untreated plot where 33.3% rotting of living trees was observed. The efficacy of urea in the control of *H. annosum* s.l. has been attributed to the elevated pH > 7 caused by the hydrolysis of urea to ammonia (Johansson et al., 2002). Although both chemicals have been shown to be highly efficacious in the control of *H. annosum* s.l., there has been environmental concerns on the use of such chemicals. Ammonia from urea is phytotoxic and adversely affects common ground species like brophytes and vascular plants (van der Eerden, 1982; Westlund & Nohrsted, 2000). In addition, urea has been reported to have more adverse effects than *P. gigantea* on fungal community structure with the zygomycetes and basidiomycetes being the most affected groups (Vasiliauskas et al., 2004). The effect of urea on mycodiversity may affect the stability of the forest ecosystem by interfering with nutrient recycling and other ecological roles played by fungi. Hence, a more environmentally friendly approach is needed for the treatment and control of the spread of *H. annosum* s.l. Biological control with *P. gigantea* has therefore been suggested as an option that will protect the environment and at the same time reduces the spread of the disease.

3.3 Biological control with *P. gigantea*

P. gigantea (Fr.) Jűlich is a saprotrophic wood decay fungus, found as a primary colonizer of stumps, fallen trunks and remnants of dead conifers in both temperate and boreal forests (Holdenrieder & Greg, 1998; Rönnberg et al., 2006b). It is a basidiomycete causing extensive decay in infected wood. Although not pathogenic to living tree tissues, reports have shown that non-suberized spruce seedling roots can be colonized by high oidiospore inocula of *P. gigantea* under *in vitro* condition. This suggests that it could act as a facultative but weak necrotrophic pathogen (Asiegbu et al., 1996). Evidence of formation of structures resembling mychorrizal mantles has been reported in spruce seedlings inoculated with *P. gigantea* (Vasiliauskas et al., 2007). *P. gigantea* is a primary competitor of *H. annosum* s.l on the stump surface and has been widely used as a biocontrol agent against the pathogen (Rishbeth, 1952). Several commercial preparations of *P. gigantea* are available in the market. The fungal preparations are commercially marketed as Rotstop® in Scandinavia, PG suspension® in the UK and PG IBL® in Poland.

The *P. gigantea* preparations are efficient in protecting the stump surface against *H. annosum* s.l. infection (Thor & Stenlid, 2005). Colonization of the

stump by *H. annosum* s.l. was drastically reduced by 89-99% in the stumps treated with the biocontrol fungus, compared to untreated stumps. However, different strains of the biocontrol fungus showed variable efficacy against *H. annosum* s.l. spore infections under different environmental conditions (Rönnberg et al., 2006). Stump infection by *P. gigantea* is through aerial spores which are capable of traveling long distances to facilitate stump colonization (Rishbeth, 1950b). However, unlike its competitor, *H. annosum* s.l., *P. gigantea* has not been reported to show any evidence of vegetative spread through root contacts.

P. gigantea has a large population size due to its highly efficient spore dispersal capability. Studies based on morphological observations and molecular techniques such as random amplified microsatellite (RAMS) fingerprinting have shown that *P. gigantea* is a single species across Europe (Korhonen et al., 1997; Vaino et al., 1998). Mating experiments have not been able to show any evidence of IS groups between the European and American populations of *P. gigantea* (Grillo et al., 2005). Studies on *P. gigantea* from different geographical regions have shown that the fungus has a very high genetic diversity but low geographic differentiation within continents (Vainio et al., 1998; Vaino & Hantula, 2000). Furthermore, the effect of Rotstop® treatment on the resident population of *P. gigantea* has been investigated (Oliva et al., 2008; Vaino et al., 2001). According to both studies, Rotstop® treatment had a significant effect on the genetic diversity of the resident *P. gigantea* population only in the surrounding areas where the biocontrol agent was applied.

However, the exact mechanism by which *P. gigantea* is able to control *H. annosum* s.l. is still not known. Although several biological control mechanisms such as secretion of growth inhibitory compounds, lytic enzyme secretion, induced resistance and mycoparasitism have been reported in other biocontrol systems (Gupta & Utkhede, 1986; Leifert et al., 1995; Mohammed & Caunter, 1995; Walker et al., 1996), none of these mechanisms has been reported in the *P. gigantae* - *H. annosum* system. Earlier studies have reported competition for resource (Adomas et al., 2006) as the likely mechanism of interaction between *P. gigantea* and *H. annosum*. Furthermore, *P. gigantea* hyphae has been reported to cause structural changes to the hyphae of *H. annosum* when they come in contact with each other, resulting in cytoplasmic vacuolation, granulation and loss of opacity (Ikediegwu, 1976). This phenomenon known as hyphal interference has been shown to be common in most interspecific fungal interactions.

4 Aims of the study

The overall aim of this study was to identify and study traits in *P. gigantea* that are important for biological control of *H. annosum* s.l. More specifically the objectives were:

I. To test for correlations between antagonistic ability of different strains of *P. gigantea* with wood degradation capability, growth rate and enzyme production.

II. To test if *P. gigantea* hydrophobin genes (*Pgh1* and *Pgh2*) are differentially expressed during antagonism on wood and under different nutrient sources. In addition, correlation between sequence variability of *Pgh1* and *Pgh2* and antagonistic ability was tested.

5 Materials and Methods

This thesis comprised two papers, **Paper I** and **Paper II**, the materials and methods for each of the papers is fully described in the respective papers. However, this section gives a brief description of the different methods employed in the current work.

5.1 Fungal isolates, culture conditions and screenings- Paper I

Sixty four isolates of *P. gigantea* including 2 Rotstop® isolates (RotStop® S and RotStop® F) collected from different geographical locations, Finland, Sweden, Lithuania and Latvia (Tabe 1, **Paper I**) were maintained on Hagem (Stenlid, 1985) media at 20°C.

In **Paper I,** the 64 isolates of *P. gigantea* were screened for differences in growth rate at different temperature and substrate conditions using both complex, carbon-rich Hagem media and low carbon containing Norkrans medum (Norkrans, 1963). The isolates were also screened for laccase enzyme production using guaiacol. Wood degradation capability of each isolate was tested by inoculating pine and spruce wood blocks with agar plugs of each isolate and incubation was done for 4 months. Microscopic analysis of the degraded wood was performed using a Leitz Wetzlar sliding microtome (Type 1300) and stained with either 1% wv^{-1} lactophenol blue (longitudinal sections) or 1% wv^{-1} safranin (transverse sections). The antagonistic ability of *P. gigantea* was tested by pairing each isolate of *P. gigantea* with *H. annosum* s.s. (strain FP5) on Hagem or Norkrans media supplemented with either sawdust, cellulose or xylan (Fig.1, **Paper I**). Data

obtained from the screening was analyzed with both principal component analysis (McCune & Mefford, 1999) and multiple regression analysis where correlations between different variables were tested.

5.2 Gene expression and sequencing-Paper II

Based on the initial screenings from **Paper I**, isolates representing high, medium and low antagonistic ability as well as different geographical origins, were selected for a detailed study on two different *P.gigantea* hydrophobin genes (*Pgh1* and *Pgh2*). *Pgh1*, but not *Pgh2*, was previously reported by Adomas et al. (2006) to be up-regulated at the zone of interaction between *P. gigantea* and *H. parviporum*. Specific primers were designed and real time quantitative RT-PCR was used to monitor the expression of *Pgh1* and *Pgh2* under different nutrient and interaction conditions. The sequences of *Pgh1* and *Pgh2* in several isolates representing the two categories (antagonism and geography) were analyzed to determine if sequence variation could account for the differences in the antagonistic abilities of these isolates. We also tried to investigate if sequence variation in these two genes (*Pgh1* and *Pgh2*) could be related to geographical origin. Quantitative PCR data was analyzed with the relative gene expression method; $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001), after normalization with the endogenous control, glyceraldehydes-3-phosphate dehydrogenase. Statistical test was performed with ANOVA using the general linear model (GLM) procedure in SAS for Windows (version 9.1). Pair-wise comparisons between means for different effects were performed using least square means with 5 % comparison wise error rate. In addition, Mann-Whitney U-test (Statistica 7.1, StatSoft, OK) was used for comparing the gene expression between aerial and submerged hyphae for individual isolates for one gene at a time. Sequences were manually trimmed and aligned with Clustal W (Thompson et al., 1994) and inspected using MegAlign (DNASTAR, WI).

6 Results and Discussion

Several factors have been suggested to be important for efficient control of *H. annosum* s.l. by *P. gigantea*. Such factors include rapid colonisation of the stump surface (Boddy, 2000; Turby et al., 2008), resource capture (Asiegbu et al., 2005; Adomas et al., 2006) and hyphal interference (Ikediegwu et al., 1976). In the current work we screened 64 different *P. gigantea* isolates for variation in growth rate and their ability to displace *H. annosum* s.s. on different substrates, thereby addressing the questions on rapid colonization or resource capture as two possible biocontrol mechanisms of *P. gigantea*. The variation in growth rate and displacement ability was then correlated with other factors such as substrate preference, wood degradation and enzyme production, in order to identify the traits that are important for high biocontrol ability of *P. gigantea*.

The *P. gigantea* isolates screened in this study showed a wide variation in antagonism, growth rate, laccase enzyme production and wood decay capability. Nutrient source had a substantial effect on the outcome of the interaction; 90 % of the isolates were able to displace *H. annosum* s.s. in sawdust media after 20 days, as compared with only 4% recorded in the nutrient rich Hagem media. One interpretation of this is that antagonistic ability on Norkrans/sawdust (and possibly on the stump surface) is connected with the ability of *P. gigantea* to degrade the different wood components. This argument was further supported by the positive correlation between antagonism on Norkrans/sawdust, pine and spruce wood degradation capability and laccase enzyme production (Table 4, **Paper I**). Laccase is a multicopper enzyme reported to be involved in lignin depolymerisation and detoxification of the chemical

and structural components of wood (Have & Teunissen, 2001; Baldrian, 2006).

The light microscopic examination of the degraded wood showed a typical white rot decay pattern in both Scots pine and Norway spruce, however, there was a shift from simultaneous decay in pine to preferential decay in spruce (Fig.4, **Paper I**). Such simultaneous white rot decay has been reported in other wood rotting fungi including *H. annosum* s.s. (Daniel et al., 1998; Daniel, 2003).

Different nutrient sources also had a substantial effect on growth rate of all the isolates, with Norkrans/sawdust being the media with the highest average growth rate. Previous studies have reported that the efficacy of *P. gigantea* to control *H. annosum* s.l. on wood could be connected with traits such as high spore production and high growth rate ability (Korhonen, 2001; Berglund & Rönnberg, 2004; Berglund et al., 2005; Sun et al., 2009), indicating that rapid colonization of the stump surface is an important mechanism for the biocontrol of *H. annosum* s.l. by *P. gigantea*. Mean growth rate on Norkrans/sawdust was correlated with mean growth rate on Norkrans/xylan and Norkrans/ferulic acid, but not with growth rate on Hagem or Norkrans/Cellulose (Table 5). Again, this illustrates that high wood degrading ability in general, and lignin degradation ability of *P. gigantea* in particular, could be an important factor for high growth rate and rapid coverage of the stump surface. In addition, it also shows that the traits that determine high growth rate in easily utilizable carbon media may be different from those in highly lignified woody tissues. A comparison of the 20 isolates with highest growth rate *in vitro* with the 20 isolates with highest growth rate on spruce wood under field conditions (Sun et al., 2009) showed that Norkrans/sawdust media had the best agreement with results from field conditions, and may be used for *in vitro* screening of isolates for high growth rate.

One candidate gene that may be involved in the antagonistic ability of *P. gigantea* was identified by Adomas et al. (2006) as encoding a hydrophobin (*Pgh1*). That study showed that *Pgh1* and another hydrophobin (*Pgh2*) were highly expressed in *P. gigantea* during several different developmental conditions using carbon rich complex Hagem media. However, the two genes were differentially regulated on carbon-rich Hagem media during interactions with *H. parviporum*; *Pgh1* was up-regulated while *Pgh2* was down-regulated (Adomas et al., 2006). In the current work, we tested if the expression patterns of *Pgh1* and *Pgh2* could be repeated when using a

Norkrans/wood media which has more resemblance with the stump surface where the biocontrol interaction takes place. We also studied the expression of *Pgh1* and *Pgh2* under different nutrient media and growth conditions. Although high transcript levels of both hydrophobin genes were detected during growth on nutrient-rich Hagem-media, no expression was detected during growth on pine wood either in single or dual cultures indicating that nutrient source had a significant influence on the expression of the two genes.

On carbon-rich media, transcript levels of *Pgh1* was higher in hyphae submerged in liquid Hagem-media as compared with transcript levels in aerial hyphae on solid Hagem media in four isolates (RotStopF®, 04135, 04118 and 01074) classified as moderate to high antagonistic isolates. In the only isolate considered to be low performing on Norkrans/sawdust (isolate 02077), the expression of *Pgh1* was higher in aerial hyphae than during submerged growth (**Paper II**, Table 3). These differences in transcript levels of *Pgh1* and *Pgh2* caused by substrate was statistically significant (P = 0.0001 and P = 0.0029) for *Pgh1* and *Pgh2* respectively. Comparison of the different isolates revealed a general pattern, with the isolate with low antagonistic ability (02077) having significantly (P = 0.0001) lower expression of both genes when compared to the other four isolates. There were differences in transcript levels of both genes within isolates in submerged and aerial hyphae. For *Pgh1*, this difference was statistically significant for isolates Rotstop F®, 04118 and 02077 (P = 0.02) and 04135 (P = 0.01) respectively but not for isolate 01074. However in *Pgh2*, such difference was only statistically significant in one isolate, 04135 (P = 0.01). The relevance of these differences is not known. One possible function of these hydrophobins may be related to hyphal emergence, which is a common function of hydrophobins (Wessel, 1997; Wösten et al., 1999; Wösten, 2001).

The translated sequence of both *Pgh1* and *Pgh2* consists of 108 amino acids with eight cysteine residues found in a strictly conserved motif, a feature that is characteristic to all hydrophobins. The position and size of introns in *Pgh1* and *Pgh2* are conserved, which suggests that the two genes are the result from a duplication event. The phylogenetic analysis (Fig.1, **Paper II**) of these genes also suggests that this duplication event is recent, at least after the split between *P. gigantea* and *Grifola frondosa*. Gene duplication is a prominent feature in hydrophobin evolution (Kubicek et al., 2008) and play a role in adaptations to several environmental stresses with relevance to the biological control situation such as carbon starvation (Dunham et al.,

2002) and microbial interactions (Ochman & Moran, 2001; Karlsson & Stenlid, 2008).

7 Conclusions

The results from this work show that the ability of *P. gigantea* to antagonize *H. annosum* s.l. on *in vitro* sawdust media (and possibly on the stump surface) may be connected to high growth rate, laccase enzyme production and wood degradation capability. From the results, it could be deduced that the ability to degrade wood components in general and lignin in particular, is an important trait for high biocontrol ability of *P. gigantea*. The result also suggests that the *in vitro* sawdust media may function as a rapid alternative for screening for high performing *P. gigantea* isolates.

The expression of the two hydrophobin genes, *Pgh1* and *Pgh2*, was highly affected by both substrate and culture condition. The failure to detect expression of either *Pgh1* or *Pgh2* in the sawdust media suggest that other nutrients like nitrogen and sugar could be necessary for the induction of these genes on the stump surface. In submerged culture condition, increased levels of expression of the genes were recorded when compared with a solid media equivalent, suggesting a possible involvement of *Pgh1* and *Pgh2* in aerial hyphae emergence. The results show no correlation between antagonistic abilities of different isolates of *P. gigantea* and sequence variation of the two hydrophobin genes.

8 Prospects for the future

Future studies will aim at testing the results from the *in vitro* studies in natural or field conditions and isolates with proven traits will be used in breeding purposes so as to produce *P. gigantea* progenies with better biocontrol abilities. In addition, it will also be interesting to induce the expression of *Pgh1* and *Pgh2 in vitro* by altering the levels of nitrogen and sugar on the sawdust media since the media used in this study had a limited supply of these two nutrients. Furthermore, the specific roles of *Pgh1* and *Pgh2* in the biology of *P. gigantea* may be studied through new and more specific functional approaches like genetic transformation studies.

9 References

Adomas, A., Eklund, M., Johansson, M., & Asiegbu, F. O. 2006. Identification and analysis of differentially expressed cDNAs during nonself-competitive interaction between *Phlebiopsis gigantea* and *Heterobasidion parviporum*. *FEMS Microbiology Ecology* 57, 26-39.

Andrews, J. M. 1990. Biological control in the phyllosphere: Realistic goal or false hope? *Canadian Journal of Plant Pathology* 12, 300-307.

Asiegbu, F. O., Daniel, G., Johansson, M. 1996. Cellular interaction between the saprotroph *Phlebiopsis gigantea* and non-suberized roots of *Picea abies*. *Mycological Research* 100, 409-417.

Asiegbu, F. O., Johansson, M. & Woodward, S. 1998. Biochemistry of the host-parasite interaction. In: *Heterobasidion annosum,* Biology, Ecology, Impact and Control, (Woodward, S., Stenlid, J., Karjalainen, R. & Hüttermann, A. eds.), pp.167-193. CAB International.

Asiegbu, F. O., Adomas, A. & Stenlid, J. 2005. Conifer root and butt rot caused by *Heterobasidion annosum* (Fr.) Bref. s.l. *Molecular Plant Pathol*ogy 6, 395-409.

Baldrian, P. 2006. Fungal laccases-occurrence and properties. *FEMS Microbiology Review* 30, 215-242.

Berglund, M. & Rönnberg, J. 2004. Effectiveness of treatment of Norway spruce stumps with *Phlebiopsis gigantea* at different rates of coverage for the control of *Heterobasidio*n. *Forest Pathology* 34, 233-243.

Berglund, M., Rönnberg, J., Holmer, L. & Stenlid, J. 2005. Comparison of five strains of *Phlebiopsis gigantea* and two *Trichoderma* formulations for treatment against natural *Heterobasidion* spore infections on Norway spruce stumps. *Scandinavian Journal of Forest Research* 20, 12-17.

Burge, M. N. 1988. Fungi in Biological Control System. *Manchester University Press*, Manchester, 269. pp.

Boddy, L. 2000. Interspecific combative interaction between wood-decaying basidiomycetes. *FEMS Microbiology Ecology* 31, 185-195.

Campbell, R. 1989. Biological control of microbial plant pathogens. *Cambridge University Press,* Cambridge, 218. pp.

Capretti, P., Korhonen, K., Mugni, L. & Romgnoli, C.1990. An intersterility group of *Heterobasidion annosum* specialised to *Abies alba*. *European Journal of Forest Patho*logy 20, 231-240.
Chase, T. E. & Ullrich, R. C. 1990a. Genetic basis of biological species in *Heterobasidion annosum*: Mendelian determinants. *Mycologia* 82, 62-72.
Cook, R. J. & Baker, K. E. 1983. The nature and practice of biological control of plant pathogens. *American Phytopathological Society* XVI, APS Press, St Paul, Minnesota, 539.pp.
Dahlstren, D. L. & Dreistadst, S. H. 1991. Forest pest management sociopolitics. *Forest Ecology and Management* 39, 289-297.
D' Amico, L., Motta, E., Annesi, T., Scire, M. Luchi, N., Hantula, J., Korhonen, K., Carpretti, P. 2007. The North American P group of *Heterobasidion annosum* s.l. is widely distributed in *Pinus pinea* forest of the western coast of central Italy. *Forest Pathology* 37, 303-320.
Daniel, G., Asiegbu, F. O. & Johannson, M. 1998. The saprotrophic and wood degrading abilities of *Heterobasidion annosum* intersterility P and S groups *Mycological Research* 102, 991-997.
Daniel, G. 2003. Microview of wood under degradation by bacteria and fungi. In: *Wood Deterioration and Preservation*: Advances in our changing world. (Goodell, B. Nicholas, D. D. & Schultz, T. P. eds). pp. 34-72. ACS Symposium Series 845, Washington, DC.
Do mus-Lehtijärvi, H. T., Lehtijärvi, A., Korhonen, K. 2006. *Heterobasidion annosum* on *Abies species* in western Turkey. *Forest Pathology* 36, 280-286.
Dunham, M. J., Badrane, H., Ferea, T., Adams, J., Brown, P. O., Rosenzweig, F. & Botstein, D. 2002. Characteristic genome rearrangements in experimental evolution of *Saccharomyces cerevisiae*.*Proceedings of the National Academy of Science,* USA 99, 16144-16149.
Elad, Y. & Zimand, G. 1993. Use of *Trichoderma harzianum* combination and alternation with fungicides to control cucumber grey mould (*Botrytis cinerea)* under commercial greenhouse conditions. *Plant Pathology* 42, 324-332.
Eerden, L. J. M. van der 1982. Toxicity of ammonia to plants. *Agricultural Environment* 7, 223-235.
Garbelotto, M., Ratcliff, A., Bruns, T. D., Cobb, F. W. & Otrosina, W. J. 1996. Use of taxon specific competitive priming PCR to study host specificity, hybridization, intersterility and intergroup gene flow in intersterility groups of *Heterobasidion annosum*. *Phytopathology* 86, 543-551.
Garcia, R., Caltagirone, L. E. & Gutierrez, A. P.1998. Comments on a redefinition of biological control. Roundtable. *BioScience* 38, 692-694.
Gonthier, P., Garbelotto, M. M. & Nicolotti, G. 2001. Seasonal patterns of spore deposition of *Heterobasidion* species in four forest of the western Alps. *Phytopathology* 95, 759-767.

Gonthier, P. Nicolotti, G., Linzer, R., Guglielmo, F. & Garbelotto. M. 2007. Invasion of European pine stands by North American forest pathogen and its hybridization with a native infertile taxon. *Molecular Ecology* 16, 1389-1400.
Gonthier, P., Warner, R., Nicolotti, G. Mazzaglia, A. & Garbelotto, M. 2004. Pathogen introduction as a collateral effect of military activity. *Mycological Research* 108, 468-470.
Grillo, R., Korhonen, K., Hantula, J. & Hietala, A. 2000. Genetic evidence for somatic haploidization in developing fruit bodies of *Armillaria tabescens*. *Fungal Genetics and Biology* 30, 135-145.
Gupta, V. K. & Utkhede, R. S. 1986. Factors affecting the production of antifungal compounds by *Enterobacter aerogenes* and *Bacillus subtilis*, antagonists of *Phytophthora cactorum*. *Journal of Phytopathology* 117, 9-16.
Harrington, T. C., Worrall, J. J. & Rizzo, D. M. 1989. Compatibility among host specialized isolates of *Heterobasidion annosum* from western North America. *Phytopathology* 79, 290-296
Holdenrieder, O. & Greig, B. J. W. 1998. Biological methods of control: In *Heterobasidion annosum,* Biology, Ecology, Impact and Control, (Woodward, S., Stenlid, J., Karjalainen, R. & Hüttermann, A. eds.), pp, 235-258. CAB International.
Have, R. T. & Teunissen, P. J. M. 2001. Oxidative mechanisms involved in lignin degradation by white rot fungi. *Chemical Review* 101, 3397-3413.
Johansson, S. M., Pratt, J. E. & Asiegbu, F. O. 2002. Treatment of Norway spruce and Scots pine stumps with urea against root and butt rot fungus *Heterobasidion annosum*- Possible modes of action. *Forest Ecology and Mangement* 157, 87-100.
Kadlec, Z., Starý, P. & Zumr, V. 1992. Field evidence for large pine weevil, *Hylobius abietis* as a vector of *Heterobasidion annosum*. *European Journal of Forest Pathology* 22, 316-318.
Karlsson, M. & Stenlid, J. 2008. Comparative evolutionary histories of the fungal chitinase gene family reveal non-random size expansions and contractions due to adaptive natural selection. *Evolutionary Bioinformatics* 4, 47-60.
Karlsson, M., Stenlid, J. & Olson, Å. 2007. Two hydrophobin genes from the conifer pathogen *Heterobasidion annosum* are expressed in aerial hyphae. *Mycologia* 99, 227-231.
Korhonen, K. , Kannelsuo, S., Vainio, E. & Hantula. J. 1997. Population structure of *Phlebiopsis gigantea* in Europe. In: Root and Butt Rots of Forest Trees (Ninth Int. Conf. on Root and Butt Rots, Carcans-Maubuisson, Sept. 1-7, 1997). Ed. by Delatour, C., Guillauman, J.J., Lung Escarmant, B., Marcais, B. France: INRA Editions (France), Les Colloques No. 89, Abstr. No. 15, p. 439.

Korhonen, K., Delatour, C., Greg, B. J. W. & Schönhar, S. 1998b. Silvicultural control. In *Heterobasidion annosum*, Biology, Ecology, Impact and Control (Woodward, S., Stenlid, J., Karjalainen, R. & Hüterman, A. eds), pp. 43-71. London: CAB International.

Korhonen, K. 2001. Stimulated stump treatment experiment for monitoring the efficacy of *Phlebiopsis gigantea* against *H. annosum* infection. In: *Root and Butt rots of Forest Trees* (Proc.10th Int.Conf.on Root and Butt Rots. Quebec City, 16-22 Sept. 2001). (Laflamme G, Bèrube J. A. & Bussières G. eds), pp.126-210. Sainte-Foy, Quebec, Canada: Laurentian Forestry Center, Information Report LAU-X-126.

Korhonen, K. & Dai, Y. C. 2004. Genetically identified taxa of *Heterobasidion* and their distribution in Eurasia. In: Manka, M. & Larkomy, P. (eds). Root and Butt Rots of Forest Trees. Proceedings of the 11th International Conference on Root and Butt Rots, Poznan and Bialowieza, Poland, August 16-22, 2004. IUFRO & The August Cieszkowski Agricultural University. Poznan. p. 57-63.

Kubicek, C. P., Baker, S., Gamauf. C., Kenerley, C. M. & Druzhinina, I. S. 2008. Purifying selection and birth-and-death evolution in the class II hydrophobin gene families of the ascomycete *Trichoderma/Hypocrea*. *BMC Evolution Biology* 8, 4.

Ikediugwu, F. E. O. 1976. The interface in hyphal interference by *Peniophora gigantea* against *Heterobasidion annosum*. *Transactions of British Mycological Society* 66, 291-296.

La Porta, N., Carpretti, P., Kammiovirta, K., Korhonen, K. & Karjalainen, R. 1997. The relatedness of Italian F intersterility group of *Heterobasidion annosum* with the S group, as revealed by RAPD assay. *Mycological Research* 101, 1065-1072.

Leifert, C., Chidberee, S. L. H., Hampson, S., Workman, S., Sigee, D., Epton, H. A. S. & Harbour, A. 1995. Antibiotic production and biocontrol activity by *Bacillus subtillis* CL27 and *Bacillus pumilus* CL45. *Journal of Applied Bacteriology* 78, 97-108.

Linden, M. & Vollbrecht, G. 2002. Sensitivity of *Picea abies* to butt rot in pure stands and in mixed stands with *Pinus sylvestris* in Southern Sweden. *Silva Fennica* 36, 767-778.

Livak, K. J. Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-Ct} method. *Methods* 25, 402-408.

Lennox, C. L. & Spotts, R. A. 2003. Sensitivity of populations of *Botrytis cinerea* from pear-related sources to benzimidazole and dicarboximide fungicides. *Plant Disease* 87, 645–649.

Lockwood, J. L. 1992. Exploitation competition. In: The Fungal Community: Its Organization and Role in the Ecosystem (Carrol, G. C. & D.T., eds), pp. 265-274. Marcel Dekker, New York.

Lygis, V., Vasiliuaskas, R. & Stenlid, J. 2004a. Planting *Betula pundula* on pine sites infected by *Heterobasidion aanosum*: disease transfer, silvicultural evaluation and community of wood inhabiting fungi. *Canadian Journal of Forest Research* 34, 120-130.

Lygis, V., Vasiliuaskas, R. Stenlid, J. & Vasiliuaskas, A. 2004b. Silvicultural and pathological evaluation of Scots pine afforestations mixed with deciduous trees to reduce the infection by *Heterobasidion annosum*. *Forest Ecology and Management* 201, 275-285

Maijala, P., Harrington, T. C. & Raudaskoski, M. 2003. A peroxidase gene family and gene trees in *Heterobasidion* and related genera. *Mycologia* 95, 209-221.

McCune, B. & Mefford, M. J. 1999. PC-ORD: multivariate analysis of ecological data. Version 4. User's guide. MjM Software Design, Gleneden Beach, Oregon. pp. 237.

Mohammed, S. & Caunter, I. G. 1995. Isolation and characterization of *Pseudomonas fluorescens* strain suppressive to *Bipolaris maydis*. *Journal of Phytopathology* (Berlin) 143, 111-114.

Niemelä, T. & Korhenen, K. 1998. Taxonomy of the genus *Heterobasidion annosum*. In: Woodward, S. Stenlid, J. Karjalainen, R. & Hüttermann, A. eds. *Heterobasidion annosum:* Biology, Ecology, Impact and Control. CAB International, Wallingford, UK. p. 27-33.

Nicolotti, G. & Gunthier, P. 2005. Stump treatment against *Heterobasidion* with *Phlebiopsis gigantea* and some chemicals in *Picea abies* stands in the Western Alps. *Forest Pathology* 35, 365-374.

Norkrans, B. 1963. Influence of some cultural conditions on fungal cellulose production. *Physiologia Plantarum* 16, 11-19

Ochman, H. & Moran, N. A . 2001. Genes lost and genes found: evolution of bacterial pathogenesis and symbiosis. *Science* 292, 1096-1098.

Oliva, J., Samils. N., Johansson, U. Bendz-Hellgren, M. & Stenlid, J. 2008. Urea treatment reduced *Heterobasidion annosum* s.l. root rot in *Picea abies* after 15 years. *Forest Ecology and Management* 255, 2876-2882.

Ottrosina, W. J., Chase, T. E., Cobb, F. W. & Korhonen, K. 1993. Population structure of *Heterobasidion annosum* from North America and Europe. *Canadian Journal of Botany* 71, 1064-1071.

Piri, T., Korhonen, K. & Sairanen, A. 1990. Occurrence of *Heterobasidion annosum* in pure and mixed stands in Southern Finland. *Scandinavian Journal of Forest Research* 5, 113-125.

Piri, T. 1996. The spreading of the S type of *Heterobasidion annosum* from Norway spruce stumps to the subsequent tree stand. *European Journal of Forest Pathology* 37, 1-8.

Piri T. & Korhonen, K. 2007. Spatial distribution and persistence of *Heterobasidion parviporium* genets on Norway spruce sites. *Forest Pathology* 37, 1-8.

Pratt, J. E. & Redfern, D. B. 2001. Infection of *Sitka spruce* stumps by spores of *Heterobasidion annosum:* control by means of urea. *Forestry* 74, 73-78.

Ragsdale, N. N. & Sisler, H. D. 1994. Social and political implications of managing plant diseases with decreased availability of fungicides in the United States, *Annual Review of Phytopathology* 32, 545–557.

Redfern, D. B. & Stenlid, J. 1998. Spore dispersal and infection. In: *Heterobasidion annosum,* Biology, Ecology, Impact and Control, (Woodward, S. Stenlid, J. Karjalainen, R. & Hüttermann, A. eds.). CAB International, Wallingford, UK. p. 105-124.

Rishbeth, J. 1952. Control of *Formes annosus*. *Forestry* 25, 41-50.

Rishbeth, J. 1959a. Dispersal of *Formes annosus* (Fr.) and *Peniophora gigantea* (Fr.). *Transactions of British Mycological Society* 42, 243-260.

Roderick, G. K. & Navajas, M. 2003. Genes in New Environments: Genetics and Evolution in Biological Control. *Nature Reviews*, 889.

Roush, R. T. & Hopper, K. R. 1995. Use of single – family lines to preserve genetic –variation in laboratory colonies. *Annals Entomology Society of America* 38, 713-717.

Rönnberg, J., Sidorov, E. & Petrylaite, E. 2006. Efficacy of different concentrations of Rostop® F and Rostop® S and imperfect coverage of Rotstop® S against *Heterobasidion* s.l. spore infections on Norway spruce stumps. *Forest Pathology* 36, 422-433.

Stenlid, J. 1985. Population structure of *Heterobasidion annosum* as determined by somatic incompatibility, sexual incompatibility and isozyme patterns. *Canadian Journal of Botany* 63, 2268-2273.

Stenlid, J. 1987. Controlling and predicting the spread of *Heterobasidion annosum* from infected stumps and trees of *Picea abies*. *Scandinavian Journal of Forest Research* 2, 187-198.

Sánchez, M. E., Luchi, N., Jimènez, J. J., de Vita, P., Sánchez, J. E., Tapero, A. & Carpretti, P. 2007. An isolated population of *Heterobasidion abietinum* on *Abies pinsapo* in Spain. *Forest Pathology* 37, 348-356.

Sun, H. , Korhonen, K. , Hantula. J. & Kasanen. R. 2009. Variation in properties of *Phlebiopsis gigantea* related to biocontrol against infection by *Heterobasidion spp*. in Norway spruce stumps. *Forest Pathology* 39, 133-144.

Thompson, J. D. Higgins, D. G. & Gibson, T. J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673-4680.

Thor, M. & Stenlid, J. 1997. *Heterobasidion annosum* infection of *Picea abies* following manual or mechanized stump treatment: In Thor, M. (ed.).Stump Treatment against *Heterobasidion annosum*-Techniques and Biological Effect in Practical Forestry. PhLic. Thesis, 18 pp. Department

of Forest Mycology and Pathology, Swed. Univ. of Agri. Sciences, Uppsala. ISBN 91-576-5372-0.
Thor, M. & Stenlid, J. 2005. *Heterobasidion annosum* infection of *Picea abies* following manual or mechanized stump treatment. *Scandinavian Journal of Forest Research* 20, 154-164.
Turby, K. V., Scott, D. & Webber, J. F. 2008. Relationship between stump treatment coverage using the biological control product PG® suspension and the control of *Heterobasidion annosum* on Corsian pine, *Pinus nigra* spp. laricio. *Forest Pathology* 38, 37-46.
Vainio, E., Korhonen, K. & Hantula, J. 1998. Genetic variation in *Phlebiopsis gigantea* as detected with random amplified microsatellite (RAMS) makers. *Mycological Research* 102, 187-192.
Vainio, E., Hantula, J. 2000. Genetic differentiation between European and North American populations of *P. gigantea*. *Mycologia* 92, 436-446.
Vainio, E., Lipponen, K. & Hantula, J. 2001. Persistence of a biocontrol strain of *Phlebiopsis gigantea* in conifer stumps and its effects on within-species genetic diversity. *Forest Pathology* 32, 285-295.
Vasiliuaskas, R., Lygis, V., Thor, M. & Stenlid, J. 2004. Impact of biological (Rotstop®) and chemical (urea) treatments on fungal community structure in freshly cut *Picea abies* stumps. *Biological control* 31, 405-413.
Vasiliuaskas, R., Menkis, A., Finlay, R. D., Stenlid, J. 2007. Wood decay fungi in fine living roots of conifer seedlings. *New Phytologist* 174, 441-446.
Walker, R., Emsile, K. A. & Allan, E. J. 1996. Bioassay methods for detection of antifungal activity by *Pseudomonas antimicrobica* against the grey mould pathogen *Botrytis cinerea*. *Journal of Applied Bacteriology* 81, 531-537.
Westlund, A. & Nohrstedt, H. Ö. 2000. Effects of stump treatment substances for root-rot control on ground vegetation and soil properties in *Picea abies* forest in Sweden. *Scandinavian Journal of Forest Research* 15, 550-560.
Wessels, J. G. H. 1997. Hydrophobins: proteins that change the nature of fungal surface. *Advances in Microbial Physiology* 38, 1-45
Whipps, J. 2001. Microbial interaction and growth in the rhizosphere. *Journal of Experimental Botany* 52, 487-511.
Woodward S., Stenlid, J., Karjalainen, R. & Huttermann, A. 1998. *Heterobasidion annosum*: Biology Impact and Control. Wallingford, UK and New York, USA, CAB International, pp 589.
Wösten, H. A. B., van Wetter M. A., Lugones, L. G. , van der Mei, H. C., Busscher, H. J. & Wessels, J. G. H. 1999. How a fungus escapes the water to grow into air. *Current Biology* 9, 85-88.
Wösten, H. A. B. 2001. Hydrophobins: multipurpose proteins. *Annual Review of Microbiology* 55, 625-646

Wulff, E. J. Mugni, C. M., Mortensen, C. N., Kweswani, C. L. & Hocknhull, J. 2002a. Biological control of black rot (*Xanthomonas campestris pv. Campestris*) of Brassicas with an antagonistic strain of *Bacillus subtilis* in Zimbabwe. *European Journal of Plant Pathology* 108, 317-325.

Wulff, E. J., Mugni, C. M., Mansfeld-Geiese, K., Fels, J., Lubeck, M. & Hockenhull, J. 2002b. Biochemical and molecular characterization of *Bacillus amyloliquefaciens, B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris* pv. Campestris. *Plant Pathology 51,* 574-584.

Zamponi, L., Paffetti, D., Tehli, S., Larkomy, P., Carprtti, P. 2007. Genetic variation in *Heterobasidion abietinum* populations detected with the M13 minisatellite maker. *Forest Pathology* 37, 433-436.

10 Acknowledgements

I am thankful to all those who have contributed in one way or the other to the success of this work. I give a special thanks to Fred Asiegbu my main supervisor who introduced me to this new world of research and has supported me this far. I sincerely thank Magnus Karlsson, the assistant supervisor, for his scientific expertise and for tirelessly guiding me throughout the period of this work. I am indebted to every member of the department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, for the friendly and conducive atmosphere created for me in the course of this work. I give a special thanks to the head of department, Prof. Roger Finlay and the department's secretary, Karin Bäckstrom for providing the necessary administrative support for the completion of this work. To those who had the time to read the thesis and the manuscripts, especially Malin Efstrand and Sadhna Alström, I say thank you for all your comments. To Ulf Olsson and Petra Fransson, I say thanks for your statistical help. Finally, I will like to thank all my friends, especially Philip Obasi, Uche Obute and Dr. Emeka Asiegbu for all their encouragements. To my family members, I love you all and I am happy that you were all there for me in prayers all through the period of this work. To God almighty, I give the glory.