Monitoring the Control Methods of *Heterobasidion annosum s.l.* Root Rot

Nicklas Samils
Faculty of Natural Resources and Agricultural Sciences
Department of Forest Mycology and Pathology
Uppsala

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Abstract
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This thesis summarises the author's work on the effectiveness of two control methods against the necrotrophic fungal pathogen *Heterobasidion annosum s.l.* (Fr.) Bref. The studied control methods were stump treatments through the application of a fungal biocontrol agent (*Phlebiopsis gigantea*) and application of urea solution. An additional component to this thesis was to develop a useful *Agrobacterium tumefaciens*-mediated transformation system for the *H. annosum s.l.* The potential risk for a build-up in the capacity of *H. annosum s.l.* to resist overgrowth by *P. gigantea* was investigated by utilising a *P. gigantea* strain used in the commercial preparation Rotstop and different strains of *H. annosum s.l.* The impact on intraspecific genetic diversity from the *P. gigantea* used in Rotstop four years after stump treatment was estimated using both microsatellite markers and AP-PCR. The long-term effect of urea treatment during first rotation thinning of *Picea abies* stands was evaluated 15 years post-treatment. An *A. tumefaciens*-mediated transformation system was developed using two different transformation plasmids and a North American *H. annosum* strain.

The results revealed differences between *H. annosum s.l.* strains in their ability to resist overgrowth by *P. gigantea*. This trait was found to be heritable which suggests that *H. annosum s.l.* has the capacity to develop more tolerant strains against the biocontrol agent if the conditions are met. Also, one QTL effect associated with this trait was identified. Genetic diversity was lower within Rotstop treated plots and the resident population was more genetically similar to the applied *P. gigantea* strain than other populations sampled in Sweden. This effect on genetic structure was not detected 100-200 m from the treated plot which suggests that stump treatments with *P. gigantea* only has a local effect on genetic diversity. Urea treated plots exhibited less rot than untreated plots and the urea treatments also significantly reduced the occurrence of windthrown trees. This shows that urea treatment during thinning operations is an efficient method to reduce root rot in *P. abies* stands. A total of 18 *H. annosum s.l.* transformants were obtained from the *A. tumefaciens*-mediated transformation system. The transformants expressed GFP throughout the mycelia and had a normal growth rate. This system will be very useful for genomic work and interaction studies.

Keywords: Heterobasidion annosum, Phlebiopsis gigantea, Rotstop, biological control, biocontrol, urea, heritability, GFP, *Agrobacterium tumefaciens*

Author's address: Nicklas Samils, Department of Forest Mycology and Pathology, SLU, Box 7026, 750 07 Uppsala, Sweden.
E-mail: Nicklas.Samils@mykopat.slu.se

Dedication

To everyone.

Jag vill inte tröska råg och jag vill inte repa lin, ty den hand som stråken skälver i skall hållas vek och fin. Ni får inte ge mig bannor eller kalla mig för lat, fast jag stundom hellre hungrar än jag spelar för mat.

Dan Andersson

Contents

List of Publications

This thesis is based on the following papers, referred to by Roman numerals:

I. Samils, N., Olson, Å. & Stenlid, J. 2008. The capacity in *Heterobasidion annosum s.l.* to resist overgrowth by the biocontrol agent *Phlebiopsis gigantea* is a heritable trait. *Biological Control* 45, 419-426.

II. Samils, N., Vasaitis, R. & Stenlid J. Impact of the biological control agent *Phlebiopsis gigantea* on genetic structure of resident populations in the Baltic Sea region. *Manuscript*.

III. 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

IV. Samils, N., Elfstrand, M., Lindner-Czederpiltz, D., L., Fahleson, J., Olson, Å., Dixelius, C. & Stenlid, J. 2006. Development of a rapid and simple *Agrobacterium tumefaciens*-mediated transformation system for the fungal pathogen *Heterobasidion annosum*. *FEMS Microbiology Letters* 255, 82-88.

Abbreviations

ASI	Allele sharing index. An index of genetic similarity to a reference genotype. In this thesis ASI is based on microsatellite loci.
BSI	Band sharing index. Same concept as ASI. In this paper, the BSI is based on the PCR products scored on agarose gel after arbitrary primed-PCR.
cM	A unit of genetic distance, used to describe the separation of genes on a chromosome. A distance of one cM equals one recombination in 100 meioses.
GFP	Green Fluorescent Protein. The gene originates from the jellyfish *Aequorea victoria*, and is commonly used in molecular biology when tagging organisms through transformation.
IS-group	Intersterility group. The compatibility of different groups is controlled by intersterility genes. However, in *Heterobasidion sp.* the different groups are not completely intersterile.
QTL	Quantitative trait loci. Regions in the DNA that are linked to given phenotypic traits.

1 Introduction

Sweden is a country with a total area of 41.3 million hectares, and about 23 million hectares are forested. The estimated amount of timber in these forests is 3.2 billion m^3, out of which 41% is Norway spruce (*Picea abies*), 39% Scots pine (*Pinus sylvestris*), 12% Silver birch (*Betula pendula*), 5% other deciduous trees, and the remaining 3% are standing dead trees (Loman, 2007). Due to this abundance of trees, forestry has for hundreds of years been a major resource in Swedish economy. But it was mainly during the 1850's that Swedish forestry had its great expansion. Early sawmills used hydropower, and were therefore restricted to upstream rivers. This geographical constriction made it difficult to transport the sawn products to the harbours, located at the coast of the Baltic Sea. When the use of steam power was adopted it made it possible to locate the sawmills at the coast, and the following boom in forest industry led to a five fold increase in export of wood products 1850-1872. The pulp industry also experienced its first financial upswing during the later part of the 1800's and is still today an important forest industry (Hult *et al.* 1989). The total output of the Swedish forest industry in year 2004 was estimated to 19.3 billion € (Loman, 2007). Losses in the forest industry due to pathogens in the managed forests are of course inevitable. At present, the greatest economical loss in Swedish forests is caused by the necrotrophic fungus *Heterobasidion annosum sensu lato*. Annual losses in Sweden due to this pathogen, were in 1998 estimated to 54 million € and that figure excludes the costs for windthrown trees, silvicultural, chemical and biological treatments (Bendz-Hellgren *et al.* 1998). Control methods aimed to reduce the *Heterobasidion annosum s.l.* infections are an important factor in modern forestry, at present, two control methods presented in this thesis are available for this purpose.

1.1 *Heterobasidion annosum s.l.* -the pathogen

Heterobasidion annosum (Fr.) Bref *sensu lato* (*s.l.*) (Fig. 1) is a species complex of necrotrophic wood decaying basidiomycetes, which in Sweden consists of two different intersterile taxa, referred to as the intersterility groups (IS-groups) P and S. The P-group is represented by the species *H. annosum sensu stricto (s.s.)* that mainly attacks Scots pine (*P. sylvestris*), but has also been reported to attack other tree species like juniper (*Juniperus communis*), birch (*Betula pendula*), alder (*Alnus incana*) and rowan (*Sorbus aucuparia*) (Korhonen & Piri, 1994). The *H. annosum s.s.* has a wide distribution in Europe, ranging from Portugal to Ukraine and from Greece up to central Sweden and Finland (Korhonen *et al.* 1998). Even though its main host, the Scots pine, is naturally distributed in northern Sweden, *H. annosum s.s.* has not been reported from areas further north than central Sweden. The S-group is represented by *H. parviporum* Nimelä & Korhonen, which mainly attacks Norway spruce (*P. abies*), but can also infect *P. sylvestris* and Siberian fir (*Abies sibirica*) (Stenlid & Swedjemark 1988; Korhonen *et al.* 1997). *H. parviporum* mainly follows the distribution of Norway spruce (*P. abies*) and has been reported from the northern parts of Finland to Greece in southern Europe, and from the eastern parts of France to the Ural Mountains (Korhonen *et al.* 1998).

In Europe there is also a third intersterility (IS) group of *H. annosum* which, to this date, has not been reported in Sweden; *H. abietinum* Nimelä & Korhonen. *H. abietinum* attacks and infects fir trees (*Abies* spp.) that are non-resident tree species in Sweden. *Abies* species are abundant in Central and Southern Europe and therefore the distribution of *H. abietinum* has been restricted to these areas. However, if *P. abies* is grown in mixed stands with *Abies alba*, it can be attacked by *H. abietinum* (Korhonen *et al.* 1998). On the North American continent there are at least two known intersterility groups named S and P like their European counterparts, though they belong to their own separate IS-groups. The North American S-group infects host species within *Abies, Picea, Tsuga, Pseudotsuga*, and *Sequoiadendron* and the North American P-group infects *Pinus, Juniperus* and *Calocedrus* (Garbelotto *et al*. 1996).

H. annosum s.l. forms perennial sporocarps on both living and dead trees and these are usually situated close to the ground level on logs, stumps and roots (Greig 1998; Redfern & Stenlid 1998). From the basidiocarps, the fungus produces and spreads the sexual basidiospores during the warm periods of the year. During this time biological or chemical control methods are important to consider when performing logging or thinning operations.

Fig. 1. Heterobasidion annosum s.l. sporocarps on a *Picea abies* stump.

1.2 Infection and interspecific interactions

In managed forest stands, freshly cut stumps are, among many other fungal species, often colonised by airborne *H. annosum s.l.* basidiospores. The

stump surfaces of *P. abies* and *P. sylvestris* are susceptible to infection during approximately the first 3-4 weeks after cutting (Rishbeth 1951; Schönhar 1979). According to Rishbeth (1951) this relatively short period of time to colonise the wood could be due to competition from other competing wood degrading fungi. After successful germination, *H. annosum s.l.* is able to spread to neighbouring trees *via* connecting root systems (Rishbeth 1951; Redfern & Stenlid, 1998). The initial stage of colonisation is therefore a crucial stage of the pathogen life cycle where a control method can be applied to minimise the damages to the managed forest stand. In order to prevent the colonisation of the stump surface, the manager of the stand can choose between two main treatments during thinning and logging operations; the application of a biocontrol agent (*Phlebiopsis gigantea* (Fr.) Jülich) or a chemical compound (urea). Like many organisms, the conifer host trees have defence mechanisms that may slow down or stop invading pathogens. The conifer host defences are mainly constituted of resin production, the production of phenolic compounds, the suberization of tissues, and enzyme production (Asiegbu *et al.* 1998). Although *H. annosum s.l.* is recognised as a very severe pathogen on coniferous trees, the molecular basis of the interactions with its hosts is still relatively poorly understood. However, recent studies have described some proteins and genes involved in the defence mechanisms against *H. annosum s.l.* (Nahalkova *et al.* 2001; Li & Asiegbu 2004; Asiegbu *et al.* 2005). Also, more knowledge about gene function and expression in *H. annosum s.l.* during various biological processes has started to accumulate (Karlsson *et al*. 2003; Abu *et al*. 2004; Iakovlev *et al*. 2004). The actual interactions between *H. annosum s.l.* and the biocontrol agent *P. gigantea* have so far been sparsely investigated and are restricted to microscopy studies and interactions on nutrient agar (Ikediugwu *et al.* 1970; Ikediugwu, 1976). A recent study, however, was conducted on the gene expression of *P. gigantea* during interactions with *H. parviporum*. The study revealed up-regulation of several genes essential for nutrient acquisition suggesting that *P. gigantea* has a competitive advantage on the freshly cut stump surfaces (Adomas *et al.* 2006).

Gene disruption experiments and interaction studies are therefore of great importance for determining the biological role of the encoded proteins with unknown functions and the interactions between the pathogenic fungus and the applied biocontrol agent. Analysing gene activation patterns under different conditions is an additional way to elucidate the role and importance of a gene and its corresponding product. In this context, fusing

the promoter of a gene of interest to a marker gene, such as the gene encoding Green Fluorescent Protein (GFP), is an efficient way to study gene regulation in basidiomycetes (Ma *et al.* 2001). Development of a successful gene delivery system for *H. annosum s.l.* would have a great benefit by enabling gene knock-out studies and make analysis of promoters of genes with unknown functions possible. Furthermore, GFP-tagging of *H. annosum s.l.* strains would make it possible to visualize the tagged individual during microscopic hyphal-hyphal interactions. The most important gain from an optimised transformation system, however, is for future studies when the total *H. annosum s.l.* genome is sequenced (JGI, 2007).

2 Control methods

2.1 *Phlebiopsis gigantea* -the biocontrol agent

The application of biocontrol agents in the service of mankind to prevent pests is an older practice than the application of chemical pesticides. An early example of biocontrol was in Ukraine in 1879 where a entomopathogen (*Metarhizium anisopliae*) was applied to control the wheat cockchafer (*Anisoplia austriaca*) (Bidochka 2001).

Phlebiopsis gigantea (Fr.) Jülich is a common wood degrading basidiomycete fungus found within partially the same ecological niches as the *H. annosum s.l.* (i.e. sharing space on fresh woody tissues) in temperate and boreal forests around the world (Holdenrieder & Greig 1998). *P. gigantea* is considered a primary coloniser of fresh wood where it causes white rot and is recognised by its resupinate and relatively large sporocarps that are coloured white-grey to brownish-yellow (Fig. 2). The first experiments using *P. gigantea* as a biocontrol agent were initiated by John Rishbeth (Rishbeth 1950; Rishbeth 1951) during the middle of the last century. Other fungal species have been tested in experiments for the same purpose (Holdenrieder & Greig 1998; Berglund *et al.* 2005), but *P. gigantea* is still the only available fungus on the market used as a biocontrol agent against *H. annosum s.l.* infections of stumps.

Thor and Stenlid (2005) showed that the treatment reduced *H. annosum s.l.* colonisation on stump surfaces by 89-99% compared to untreated stumps. However, the efficacy of different *P. gigantea* strains against *H. annosum s.l.* spore infections of stumps varies with local environmental conditions (especially humidity) and fluctuating spore dispersal of *H. annosum s.l.*

(Rönnberg *et al*. 2006). There are also reports that there is variation between different strains of *P. gigantea* in the ability to prevent *H. annosum s.l.* infections of stumps (Berglund *et al*. 2005). *P. gigantea* has furthermore, in a microcosm study, been reported to have the ability to colonise fine roots of *P. abies* seedlings and thereby resembling the characteristics of a mycorrhizal fungus. It is able to form a mantle and penetrate intercellularly between the epidermal root cells without causing any visible negative symptoms to the seedling (Vasiliauskas *et al*. 2007). However, in another study, *P. gigantea* was described as a weak necrotrophic pathogen when tested in laboratory experiments on non-suberized roots of *P. abies* seedling roots (Asiegbu *et al.* 1996).

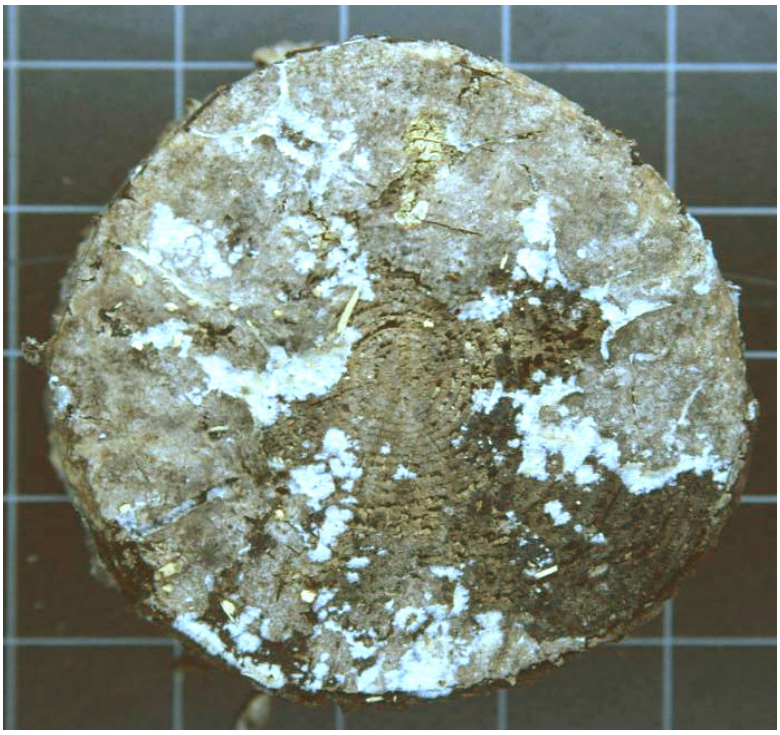

Fig. 2. Phlebiopsis gigantea sporocarps on a *Picea abies* stump. Photo kindly provided by Jan Stenlid.

During the processes of evolution, changes in inherited traits will occur from generation to generation. If a pathogen is treated with a control agent, be it a chemical or biological, the risk that the pathogen will develop resistance or tolerance needs to be evaluated properly. This is crucial in order to prevent the pathogen from developing into strains that are more tolerant to the control method and thereby creating future complications in the field of use. Resistance to chemical fungicides is a common phenomenon among different fungal pathogens (Leroux & Gredt 1997; Gisi *et al*. 2002; Hufbauer & Roderick 2005). But so far, resistance to biological control methods is not known to be as pronounced as the resistance to chemical compounds (Holt & Hochberg 1997). The underlying genetics responsible for the development of resistance to chemical agents are likely to be relatively simple, where a single mutation in a gene or in a cluster of genes, with major effects, may rapidly increase in frequency in the target species (Groeters & Tabashnik 2000). In comparison to the chemical control methods, biological control agents may act on several levels and therefore involve complex genetics (Hufbauer & Roderick 2005). Another important factor in the development of resistance or tolerance in a pathogen population is the population size – the larger the number of individuals in the pathogen population, the higher the probability of a more diverse genetic variation within the target species carrying beneficial genes against the applied control agent.

The Rotstop® formula, formulated in 1991 and applied in Swedish forest management since the early 1990's, was first based on one strain of *P. gigantea* which originally was isolated in southern Finland (Korhonen *et al*. 1994). However, in the summer of 2004, a new strain isolated in Sweden and named Rotstop S was made available on the Swedish market (Berglund *et al*. 2005; Rönnberg *et al*. 2006). But until then, only the Finnish *P. gigantea* strain had been applied at the stump treatments (Korhonen *et al.* 1994). In Sweden about 35000 ha are treated annually with the Rotstop formula (Thor 2003). It is currently known that the *P. gigantea* strain that is applied during the stump treatments later forms sporocarps that disperse viable spores that are able to mate with local individuals (Vasiliauskas *et al*. 2005). This raises the question of whether the genetic diversity will decrease among the resident populations of *P. gigantea* in the areas where the Rotstop treatment has been applied. If there is a loss of genetic diversity, how far from the treated sites can a possible "Rotstop-effect" be traced?

2.2 Urea treatment –the chemical treatment

When urea is applied to a fresh stump surface, the compound is hydrolysed to ammonia which raises the pH in the adjacent wood tissues. Urea itself as a compound is not toxic to *H. annosum s.l.*, but the raise in pH >7 prevents both the growth of *H. annosum s.l.* and the germination of basidiospores on wood (Johansson *et al*. 2002). However, the authors argue that the buffering effect in the bark of the stumps might lead to a harbouring effect, sheltering the *H. annosum s.l.* in these refuges and that the *H. annosum s.l.*, from this point might be able to invade the stump *via* aerial hyphae. Urea treatments have in earlier studies been shown to affect the composition of fungal species in treated stumps where they cause more adverse effects than *P. gigantea*-treated stumps. Especially zygomycetes and basidiomycetes were the groups of fungi most affected by the treatment, where the latter group was found to be almost completely absent in the treated stumps (Vasiliauskas *et al*. 2004). Not only fungi are affected by urea treatments of stump surfaces, another study has shown that bryophytes and vascular plants located close to the treated stumps are adversely affected (Westlund & Nohrstedt 2000).

Earlier work on the effect of urea treatments to prevent *H. annosum s.l.* infections on stumps have mainly focused on a relative short-term effects by scoring the presence of *H. annosum s.l.* on treated and non-treated stumps (Pratt & Redfern, 2001; Nicolotti & Gonthier, 2005; Thor & Stenlid, 2005). The normal rotation time for spruce in Swedish forestry is 65-110 years (depending on the site), therefore evaluation of the long-term effects of stump treatments are required. This decision support is particularly valuable for thinning and logging operations during the warm seasons of the year when the production and release of *H. annosum s.l.* basidiospores is as most active (Kallio 1970; Brandtberg *et al*. 1996). When trees are exposed to root rotting fungi, the tree growth is usually reduced as a response to the infection (Bendz-Hellgren & Stenlid 1995; Mallett & Volney 1999). Furthermore, due to the consequence of the physical degradation of the wood, the rot might also lead to windthrown trees during intensive wind episodes.

3 Objectives

• Investigate if *H. annosum s.l.* has the capacity to become more tolerant against the biocontrol agent *P. gigantea* during a competitive situation, and to what degree this trait is present in the *H. annosum s.l.* (**Paper I**)

• Estimate the spread of genetic material from the *P. gigantea* strain used in the commercial biocontrol agent Rotstop and to investigate the intraspecific genetic variation in the geographical region surrounding the Baltic Sea. (**Paper II**)

• Evaluate the long-term effect of urea treatments in thinned *P. abies* stands, aiming at both rot-frequencies and the frequencies of windthrown trees. (**Paper III**)

• Create an *Agrobacterium tumefaciens*-mediated transformation system for *H. annosum s.l.* in order to develop a tool for future genomic work and to produce GFP-tagged *H. annosum s.l.* strains for microscopy studies. (**Paper IV**)

4 Material and Methods

4.1 Fungal strains

In **paper I** the experimental set of strains used consisted of 91, previously described (Lind *et al.* 2005) homokaryotic *H. annosum s.l.* progeny strains. These strains were derived from a compatible mating between two North American homokaryotic S- and P-types, thus creating a heterokaryotic hybrid strain (AO8), which also was included in the experiment. As wild type material, 23 strains of *Heterobasidion parviporum* isolated at different geographical locations in Fennoscandia (10 homokaryons and 13 heterokaryons) were also used. The *P. gigantea* strain, isolated in Finland and used in the product registered as Rotstop (Korhonen *et al.* 1994) was used as the antagonistic strain in this study.

The *P. gigantea* strains in **paper II** are shown in table 1 and figure 3. In all populations, except for Lunsen and Råberg, pure cultures of *P. gigantea* heterokaryons were isolated from decayed wood of stumps or logs as previously described (Vasiliauskas *et al.* 2004, 2005, 2005b). Briefly, the stumps or logs (in most cases bearing *P. gigantea* sporocarps) were sampled by cutting a 5-10 cm thick wood disc. Cut discs were individually placed into plastic bags and brought to the laboratory, where they were stored at +5 $^{\circ}$C for three to four weeks. In the laboratory, pieces of wood (about 5×5×10 mm in size) were taken from the surface of the discs showing mycelial growth, the surface was sterilised by a flame and placed in Petri dishes containing agar medium. Mycelia that grew out were individually subcultured. Each strain was collected from discrete spatially separated resource unit (stump or log) thus representing different fungal individuals (genets).

In the populations from Lunsen and Råberg, spore prints of *P. gigantea* were collected from 10 and 11 sporocarps respectively, each also originating from different stumps. The procedures of spore collection and isolation of single spore (haploid) strains were largely similar to those used previously (Johannesson *et al.* 2001; Franzén *et al.* 2007). Briefly, basidiospores were collected on a piece of paper pinned onto a surface of a sporocarp, either directly in the field or in the laboratory. The spores were washed from the paper with sterilised water, collected with a pipette and spread onto agar medium in 9 cm Petri dishes. The dishes were examined daily with a microscope, and germinating basidiospores were individually transferred to new agar dishes, where single-basidiospore mycelial cultures were grown out. In total, from 21 sporocarps 126 single spore strains (6 strains per sporocarp) were obtained.

In all populations, except for Palanga, Druskininkai and North America, the strains were collected from *Picea abies* wood. In the populations from Palanga and Druskininkai, they originated from *Pinus sylvestris* and in the North American group of strains they originated from various conifers.

The *P. gigantea* strain used in Rotstop (Korhonen *et al.* 1994) was used as a reference strain when estimating genetic similarity through ASI and BSI.

In **paper III**, *P. abies* stumps in selected stands were, prior to the study, artificially inoculated with *H. annosum s.l.* conidia. Out of the used strains, 11 were *H. parviporum* and 9 were *H. annosum s.s.*

In **paper IV** the North American homokaryotic *Heterobasidion annosum* strain TC 32-1 (intersterility group P) (Chase *et al*. 1985) was used as the recipient fungal strain in the *Agrobacterium*-transformation system.

Table 1.

Population ID	No strains	Origin		Habitat*	Host**
		Location	Year		
A	49	Rämsön	2000	Cut wood placed 1 m from Rotstop treated stumps, four years post treatment	*P. a.*
B	24	Rämsön	2000	Cut wood placed 1 m from untreated stumps. 100-200 m away from population A	*P. a.*
C	9	Rämsön	1996	Stumps colonised by resident	*P. a.*

				population. Sampled in same plot as population B, but before the Rotstop treatment of population A. Situated 400 km from the Rotstop origin area	
D	12	Helsinki	2004	Stumps in the region where Rotstop origins from	*P. a.* & *P.s.*
E	12	Hyytiälä	2004	Stumps situated 100-200 km from the region where Rotstop origins from. Rotstop application in neighbouring stands during 10 years prior to collection	*P.s.*
F	10	Gåsholmen	1998	Cut wood in neighbouring areas to which Rotstop has been introduced 2 years previously. Situated 400 km from the Rotstop origin area	*P. a.*
G	10	Lunsen	1994	Stumps colonised by the resident population prior to introducing Rotstop treatment, situated 400 km from the Rotstop origin area	*P. a.*
H	11	Råberg	1994	Stumps colonised by the resident population prior to introducing Rotstop treatment, situated 400 km from the Rotstop origin area	*P. a.*
I	15	Palanga	2002	Stumps colonised by the resident population in area where Rotstop has never been applied, situated 500 km from the Rotstop origin area	*P.s.*
J	15	Druskininkai	2002	Stumps colonised by the resident population in area where Rotstop has never been applied, situated 700 km from the Rotstop origin area	*P.s.*
K	13	North America	1949-1999	Representatives of different North American populations of *P. gigantea*, genetically separated from European populations of the fungus (Vainio & Hantula 2000)	Various

* Habitat characteristic in relation to treatment with Rotstop *Phlebiopsis gigantea* biocontrol agent

** Host material: *Picea abies, Pinus sylvestris*, and various conifers

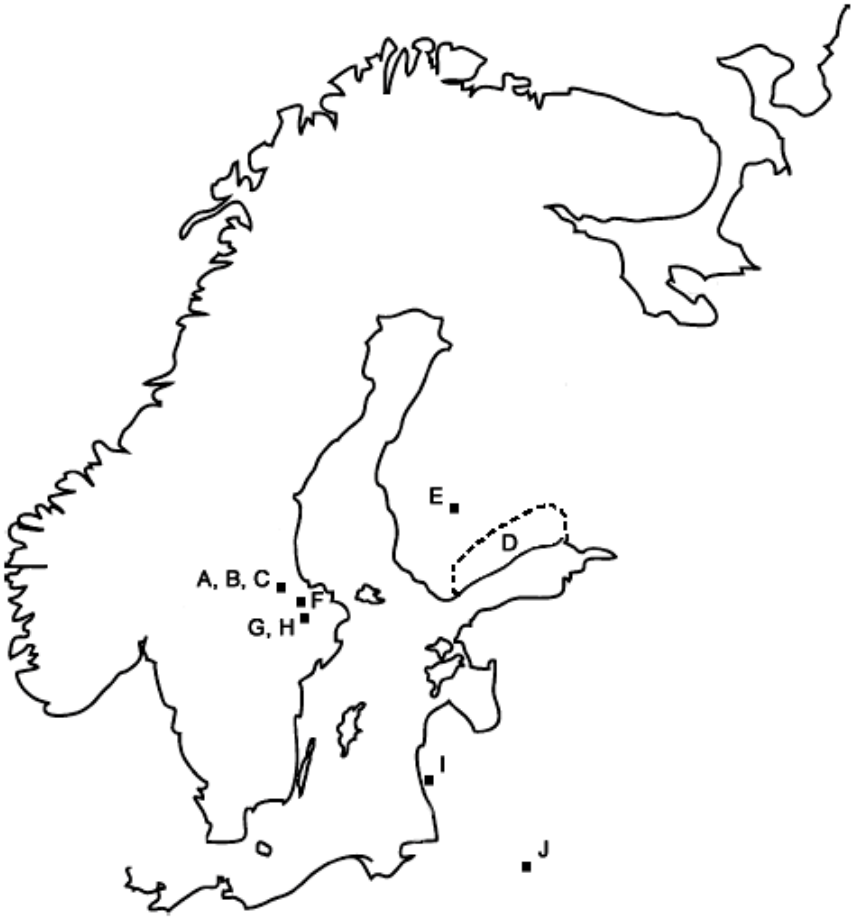

Fig. 3. Map of Northern Europe showing the origin of the sampled *P. gigantea*. The letters denote the identity of sampled populations as in table 1.

4.2 Capacity in *Heterobasidion annosum s.l.* to resist overgrowth by the biocontrol agent *Phlebiopsis gigantea* (Paper I)

The described fungal cultures were cultivated on Hagem medium (Stenlid 1985) for two weeks. Autoclaved wood blocks from *P. abies* were divided in two classes; large and small (5x10x50 mm and 5x10x10 mm, respectively). The large wood blocks were placed on the mycelial mat of the different *H. annosum s.l.* strains and the small wood blocks were placed on the *P. gigantea* strain from Rotstop F until they were fully colonised by the fungal strains. The colonised wood blocks were subsequently transferred to a Petri dish containing water agar and aligned as a bridge over a centralised gap in the water agar in order to force the fungi to grow and interact on the aligned wood blocks. Five replicates were made for the hybrid *H. annosum s.l.* progenies and ten for the natural *H. parviporum* strains. Three days post alignment of the wood blocks, the first scoring of the *P. gigantea* mycelial progress was made, and then further scorings were made every other day during a total of ten days. The scorings were performed by using a stereomicroscope and based on the morphological characters of the two species. Data from the *P. gigantea* overgrowth was grouped and ranked for the homokaryotic progenies and their dikaryotic parental strain. The collected data was treated statistically with one-way ANOVA in order to determine the significant differences in *P. gigantea* overgrowth (mm/day) between and within the groups of the tested *H. annosum s.l.* strains. The heritability traits were estimated as described in Falconer (1989): Genotypic $(\hat{\sigma}^2_{\mathrm{G}})$, environmental $(\hat{\sigma}^2_{\mathrm{E}})$ and phenotypic $(\hat{\sigma}^2_{\mathrm{P}})$ variance components were estimated using a likelihood approach as $\hat{\sigma}^2_{\mathrm{G}} = \hat{\sigma}^2_{\mathrm{g}}$, $\hat{\sigma}^2_{\mathrm{E}} = \hat{\sigma}^2_{\mathrm{e}}$ and $\hat{\sigma}^2_{\mathrm{P}} = \hat{\sigma}^2_{\mathrm{G}} + \hat{\sigma}^2_{\mathrm{E}}$ where $\hat{\sigma}^2_{\mathrm{g}}$ and $\hat{\sigma}^2_{\mathrm{e}}$ are the estimated clonal and residual variances, respectively. The estimates of individual observation broad-sense heritability $(\hat{H}^2)$ were obtained by $\hat{H}^2 = \hat{\sigma}^2_{\mathrm{G}} / \hat{\sigma}^2_{\mathrm{P}}$. In the quantitative trait loci (QTL) analysis, a previously produced genetic linkage map was used. This map was constructed from the progeny of the AO 8 hybrid *H. annosum s.l.* strain and based on 358 AFLP markers, organized in 19 larger and 20 smaller linkage groups was used (Lind *et al.* 2005).

QTL for resistance estimates were based on the *P. gigantea* overgrowth (mm growth/day) of the *H. annosum s.l.* hybrids between day 3 and 10 during the interactions. The analysis was performed by using interval mapping (Lander & Botstein, 1988). For every 5 cM of the *H. annosum s.l.* genetic linkage map associations between AFLP markers and the

resistance, significant at the 1% level, were identified as calculated by the permutation test from the MapQTL 4.0 software (van Ooijen *et al.* 2002).

4.3 Impact of the biological control agent *Phlebiopsis gigantea* on genetic structure of resident populations (Paper II)

All the *P. gigantea* strains were cultivated in Petri dishes containing liquid Hagem medium (Stenlid 1985) and the mycelia were harvested after 10 days for DNA-extraction. The DNA was extracted as in Gardes & Bruns (1993), but omitting the β-mercaptoethanol step. Fluorescent labelled microsatellite primers were designed as described in **Paper II** and only markers that showed polymorphism in eight selected *P. gigantea* strains originating from the Baltic Sea region were used for the study. The primers that amplified non-variable markers were discarded.

The analyses of the fragments amplified by the fluorescent labelled primers were performed on a CEQ 8000 Genetic Analysis System (Beckman Coulter).

The obtained data was grouped according to their origin and both the expected (H_E) and observed heterozygosity (H_O) were calculated manually and used to estimate the F_{ST}-indices of each allele through the *P. gigantea* populations. Allele-sharing indices (ASI) were also calculated manually for every strain in order to estimate every populations genetic similarity to the *P. gigantea* strain used in Rotstop by using the formula: $2S_{ab}/(S_a+S_b)$, where S_a and S_b are the total number of bands in individual a and b respectively. S_{ab} is the number of bands that are shared between the two strains (Lynch 1990).

Also, arbitrary primed PCR (AP-PCR) was performed on all strains by using the core sequence of M13 minisatellite DNA (5'-GAGGGTGGCGGTTCT-3') as a primer (Vasiliauskas & Stenlid 1998). Presence and absence of 17 bands were scored on photographic prints of the gels and the results were used to estimate the band sharing index (BSI) by $2S_{ab} / (S_a+S_b)$ as described above.

4.4 Long-term effect of urea treatment in *Picea abies* stands (Paper III)

This experiment was conducted in two first-rotation *P. abies* stands located on former agricultural lands in southern Sweden (Bertilstorp and Christinehof). Both stands were thinned in 1992 at an age of 22 years. Immediately after the thinning operation, two replicates of each treatment were performed within each stand as follows: a) treatment with a 35% aqueous solution of urea, b) artificial infection with *H. annosum s.l.* conidiophores (using 11 *H. parviporum* and 9 *H. annosum s.s.* strains), c) treatment with urea on half of the stumps, and artificial infection with the *H. annosum s.l.* strains on the other half of the stumps, d) no treatment at all, used as a control that was subjected to natural infection from resident spores. The treatments are referred to as; urea, 100% conidia, 50% conidia, and control, respectively. The plots had been subjected to two major storms, one in year 2005 and the other in the beginning of 2007. Therefore a thinning operation had been performed in 2005, but no stump treatments were performed during this thinning. In the spring of year 2007, wood cores were extracted with a Pressler borer at stump height (20 cm above ground) from all living trees in the plots, recently dead or uprooted trees were also sampled. All wood cores that were suspected of being rotted were kept separate in sterile bottles for the following fungal isolation. Furthermore, all stumps created in the thinning operation performed in year 2005 were inspected for rot symptoms and *H. annosum s.l.* sporocarps. The subsequent isolation of *H. annosum s.l.* was made by picking conidiophores from the mycelia on the stored wood cores and transferring them to Hagem agar (Stenlid 1985). Subsequent identification, to determine whether the isolated fungus was *H. annosum s.l.* or not, was performed using a stereomicroscope and based on the morphological characters of the fungus. The diameter of the trees was measured at breast height (1.30 m) both during the treatments in 1992, and during the core extractions in 2007. Presence-absence variables of rot at tree level were considered as percent variables at plot level. Mean comparison of percent variables between treatments were performed by logistic regression using the GENMOD procedure of SAS/STAT version 8.02 software (Schabenberger & Pierce, 2002). The treatment effect on the diameter was tested by means of the MIXED procedure. The differences in diameter in year 1992, the diameter in year 2007 and the growth (percent referred to the diameter of 2007) were tested between rotted and healthy trees. In these analyses, the treatment and the interaction between the presence of rot and the treatment were included. All trees in the same plot may not behave independently, so the plot was

included as a random factor in order to perform more reliable tests on the fixed factors (Schabenberger & Pierce, 2002).

4.5 Development of an *Agrobacterium tumefaciens*-mediated transformation system for *Heterobasidion annosum s.l.* (Paper IV)

In order to create transformed *H. annosum s.l.* strains, two different transformation plasmids were used in the experiments. The first transformation plasmid, named pCD61 (Fig. 4), was based on the plasmid pAN7-1 (Punt *et al*. 1987), and it contained the promoter of *Aspergillus nidulans GDP* gene (*Pgpd*A) and the coding sequence of a *GFP* gene (*GFP*) under control of a sunflower ubiquitin promoter (*PUbi*), terminated with a nos3' sequence (Nehlin *et al*. 2000). The second transformation plasmid, pJF4 (Fig. 4), was based on the pGPDGFP plasmid (Sexton & Howlett 2001), which was digested and resulted in a fragment carrying the coding sequence of the *EGFP* (*eGFP*) gene under control of the *gpdA* promoter (*PgpdA*). Both transformation plasmids also carried a hygromycin-resistance gene (*hph*) which originated from *Escherichia coli*. This selective marker is necessary in order to select for transformed strains in the sub-culturing where untransformed individuals will be selected against. Both pCD61 and pJF4 were subsequently transferred to the *Agrobacterium tumefaciens* strain C58C1Rif (Deblaere *et al*. 1985).

Prior to the experiment, the lethal hygromycin concentration in Hagem medium was determined to 45 µg/ml for the untransformed *H. annosum s.l.* strain TC 32-1 (Chase *et al*. 1985). Conidia from TC 32-1 were used as the recipient of the transformation plasmids in the experiment. The co-cultivation during the transformation was performed as described in **Paper IV** at 20ºC. Since the growth of *A. tumefaciens* was reduced in low pH; the pH of the co-cultivation medium was stabilized and maintained at 5.6, using 2-(N-morpholino)ethanesulfonic acid (MES) as a buffering agent. Maintaining a stable pH during the co-cultivation phase enabled the *A. tumefaciens* to infect the germinating *H. annosum s.l.* conidia. The concentration of hygromycin in Hagem medium (Stenlid 1985) was set to 50 µg/ml for the selection process and the following sub-culturing. Only strains that grew with a growth rate comparable to the untransformed TC 32-1 and that showed an intense and well-spread GFP-expression

throughout the mycelia were chosen for further studies. The GFP-expression was monitored by exposing the transformants to fluorescent UV-light during microscopy using UV-filter blocks. PCR was used as described in **Paper IV** to amplify the *GFP*, *eGFP*, and *hph* genes in order to verify that the *H. annosum s.l.* transformants harboured the transformation plasmids.

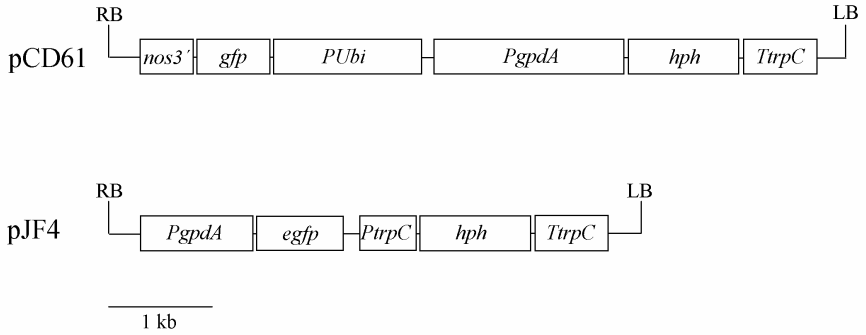

Fig. 4. Schematic drawings of the transformation plasmids used when transforming *H. annosum s.l.* Plasmid pCD61 was created by moving the gene cassettes *PgpdA-hph-TtrpC* and *PUbi-GFP-nos3´* from pAN7-1 (Punt *et al*. 1987) and pLUBIGFP13 (Nehlin *et al*. 2000) respectively, to the binary vector pPZP102 (Hajdukiewicz *et al*. 1994). Plasmid pJF4 was constructed by moving the two gene cassettes *PtrpC-hph-TtrpC* and *PgpdA-eGFP* from the pGPDGFP plasmid (Sexton and Howlett 2001) to the binary vector pPZP102. RB and LB denotes left and right borders of the transformation plasmids in the pPZP102 plasmid. Bar represents 1 kb.

5 Results and discussion

5.1 Capacity in *Heterobasidion annosum s.l.* to resist overgrowth by the biocontrol agent *Phlebiopsis gigantea* (Paper I)

The rate of mycelial overgrowth by the *P. gigantea* strain in Rotstop over *H. annosum s.l.* mycelia was used as a parameter to estimate tolerance in both the *H. annosum s.l.* hybrids and wild-type *H. parviporum*. This method simulates the action of mycelial replacement during interactions on fresh, uncolonised wood substrate. Similar studies have previously been used as a parameter for competition by both Rayner and Boddy (1988), and Holmer *et al.* (1997). It is based on the hypothesis that the competition for substrates among wood degrading fungi can be divided into two different stages: 1) the primary stage is when a fungus is exposed to an uncolonised source of nutrients (*i.e*. fresh stump surfaces, tree wounds, etc.). During this early stage of colonisation, the most important strategies are to: i) capture the uncolonised surface as quickly as possible at the lowest energy cost possible before other competitive strains arrive. ii) When other competitors are present, the need to defend the colonised area against other competing fungi is crucial, and this is where more stress-tolerant strains are likely to prevail. (Rayner & Boddy 1988). 2) In the secondary stage, an already established mycelium is replaced by a new fungal coloniser (Rayner & Boddy, 1988).

The results show that there is a natural variation among *H. parviporum* strains in their trait to tolerate overgrowth by *P. gigantea* and that the average difference between homokaryons and heterokaryons is negligible

(the *P. gigantea* overgrowth was 3.2 and 3.3 mm/day, respectively). However, there were statistically significant differences between both homokaryotic and heterokaryotic *H. parviporum* strains which ranged from 2.1-5.0 mm/day. This result shows differences among the wild-type *H. parviporum* strains in their ability to tolerate *P. gigantea* overgrowth. Among the *H. annosum s.l.* progeny strains, the ability to tolerate the overgrowth ranged between 0.9-8.6 mm/day (Fig. 5). Their average ability to resist the overgrowth was estimated to an average *P. gigantea* growth rate of 3.6 mm/day, which also was equal to the performance of the heterokaryotic parental strain AO8. The results also suggest that there is no measurable difference between homokaryons and heterokaryons in the ability to resist *P. gigantea* overgrowth. The similarity between homo- and heterokaryons in their ability to resist *P. gigantea* was also verified in an earlier study (Korhonen 2001).

The broad sense heritability trait in *H. annosum s.l.* to resist *P. gigantea* overgrowth was estimated to 0.34 in the *H. annosum s.l.* progenies. This shows that *H. annosum s.l.* has the ability to develop more tolerant strains against *P. gigantea* overgrowth if selection for these traits would take place. Comparing what can be achieved under controlled conditions in a laboratory, and what takes place in the natural environment are two separate scenarios. The examined trait in this study is one among many traits that are involved in fungal-fungal interactions and there are many more traits that play an important role during fungal interactions. Small individual mycelia of *H. annosum s.l.* have a low survival rate in the field and might not remain long enough to breed more resistant strains within the *P. gigantea* treated sites since they are likely to be replaced by other competing fungi (Morrison & Redfern 1994). Also, with a rotation period of 65-110 years including up to two thinning operations during that period, the selection pressure is considered as low. However, this study shows that *H. annosum s.l.* has the capacity to develop strains that are more tolerant to the mycelial overgrowth by the species used as a biocontrol agent, if the conditions that favour this selection are met.

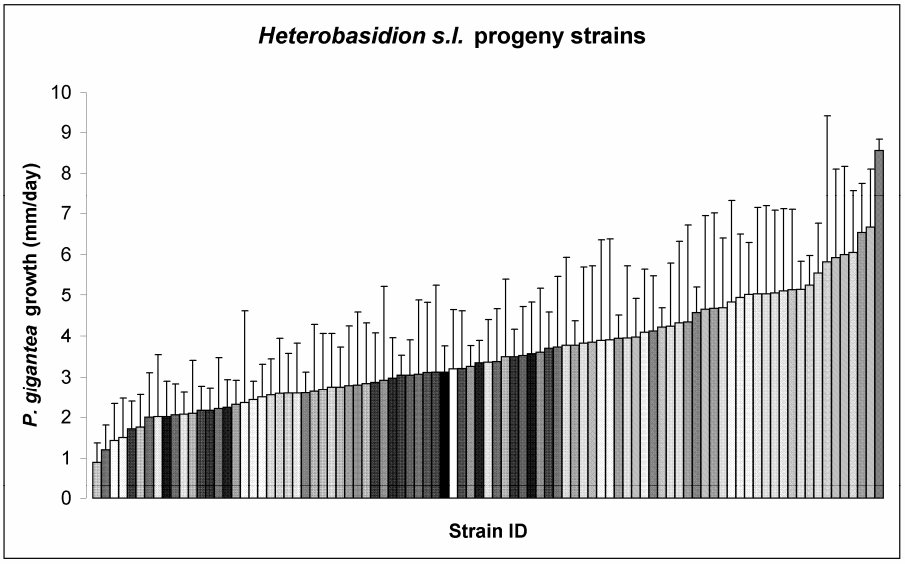

Fig. 5. Growth rate of the *P. gigantea* strain used in Rotstop over 91 *H. annosum s.l.* hybrid progenies. Error bars shows the standard deviation. The average growth rate of the *P. gigantea* strain was 3.6 mm/day.

Interval mapping was performed in order to identify genomic regions that are responsible for controlling the investigated trait. This interval mapping of the segregation data in *H. annosum s.l.* for capacity to resist overgrowth by *P. gigantea* identified a QTL effect in one small linkage group consisting of three AFLP markers. The *H. annosum s.l.* hybrid strains used in this study were earlier used by Lind *et al.* (2005) to create a genetic linkage map. However, additional QTLs that are involved in the examined trait might exist. Furthermore, if other phenotypic traits than the ability to resist mycelial overgrowth by *P. gigantea* had been studied, other QTLs in the *H. annosum s.l.* that are important in the interactions between these species might have been found. It is interesting to note that the ability to resist overgrowth by *P. gigantea* did not correlate to other traits like virulence, somatic incompatibility and intersterility that have previously been mapped in this population (Lind *et al.* 2007; Lind *et al.* 2007b).

5.2 Impact of the biological control agent *Phlebiopsis gigantea* on genetic structure of resident populations (Paper II)

From a total of 22 microsatellite loci developed from the *P. gigantea* strain in Rotstop (Korhonen *et al.* 1994), only five showed polymorphism among the tested strains isolated from the Baltic Sea region. Two of these loci were not present in all examined strains, and where therefore excluded from the H_O, H_E and F_{ST} calculations. However, these two loci were present in the *P. gigantea* strain used in Rotstop; therefore they were used when calculating the allele sharing index (ASI). Two of the five markers (Cov2 and S18) were developed by using North American strains of *P. gigantea* (Ai-zhong *et al.* unpublished).

From the total number of analysed strains, three strains from the Rotstop treated plot S9-Rämsön in Sweden, and one strain from the Helsinki area in south Finland were identical in all five microsatellite loci to the *P. gigantea* strain used in Rotstop. One strain from the Rotstop treated plot in Rämsön was identical in all 17 loci to the *P. gigantea* strain used in Rotstop when examined with the AP-PCR. However, none of the strains were identical to the *P. gigantea* strain in Rotstop when considering the total number of loci amplified with both the microsatellite primers and the AP-PCR.

The allele sharing indices (ASI) and the band sharing indices (BSI) are values on population similarity to the *P. gigantea* strain used in Rotstop. ASI is based on the data collected from the five microsatellite markers and BSI is based on the AP-PCR (Fig. 6). The results in ASI show that the population sampled from the Rotstop treated site in Rämsön is the population genetically most similar to the *P. gigantea* strain in Rotstop F (ASI = 0.69). The population from the Rotstop treated plot is also more similar to the Finnish population from Hyytiälä and the group of strains from the Helsinki area (ASI = 0.53 and 0.40 respectively), than the other Swedish populations. However, the strains from the two populations that were sampled 100-200 m from the Rotstop treated plot in S9-Rämsön, in 1996 and 2000, show little genetic similarity to the *P. gigantea* strain used in Rotstop (ASI = 0.29 and 0.26 respectively). However, they show high similarity to each other. These results show that the effect on intraspecific genetic diversity, when mass-introducing one strain of *P. gigantea*, remains within the treated plots for at least four years, but does not spread to adjacent areas. Also, the treated plots will contain a population with a high genetic similarity to populations from the area where the biocontrol agent strain was isolated. The results in BSI (Fig. 6) differ slightly from the data

derived from the microsatellite markers since the population sampled in Råberg scored a higher genetic similarity to the *P. gigantea* strain in Rotstop (BSI = 0.86) than the population from the Rotstop treated plot in Rämsön (BSI = 0.84). The Finnish population from Hyytiälä scored the highest BSI (BSI = 0.87). The strains from the Helsinki area scored relatively high (BSI = 0.84). The North American strains show least overall genetic similarity to the *P. gigantea* strain used in Rotstop (ASI = 0.21 and BSI = 0.65). Taken together, the results show that the population most similar to the *P. gigantea* strain in Rotstop is the population sampled in Rämsön. This plot was stump treated with Rotstop during a first rotation thinning four years before the population was sampled.

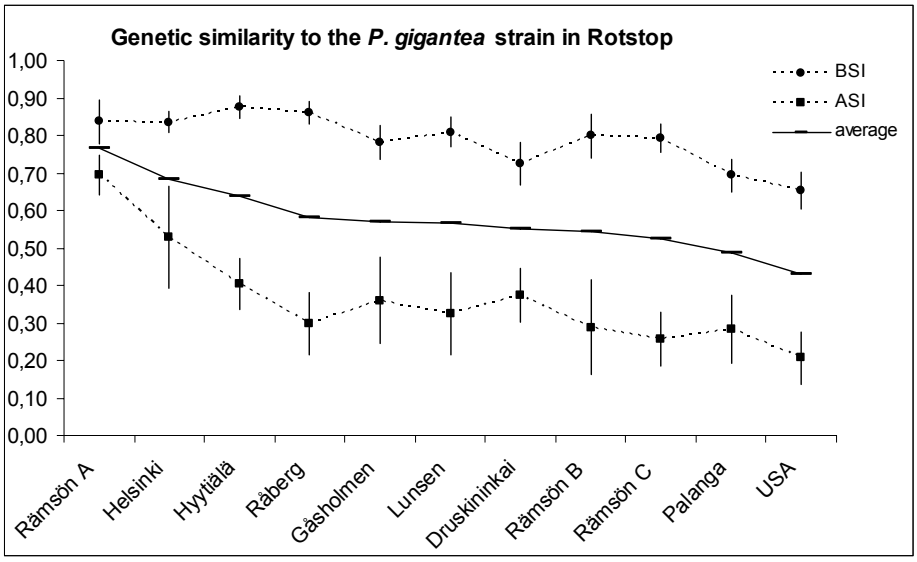

Fig. 6. Genetic similarity of resident populations to the *Phlebiopsis gigantea* strain used in the preparation Rotstop. BSI indicates band sharing index, based on 17 loci from arbitrary primed PCR. ASI indicates allele sharing index, based on five polymorphic microsatellite loci. Bars show confidence intervals at 95%. The populations are described in table 1.

The results indicate that the vast majority of *P. gigantea* basidiospores lands in the vicinity of the sporocarp, which has been previously reported for other wood-decay basidiomycetes (Stenlid 1994; Edman *et al.* 2004). It is likely that the shift in population structure was caused by a massive impact of basidiospores since a perfect match with the Rotstop strain among the individual strains was not detected. This latter result is in contrast to what was previously reported by Vainio *et al.* (2001) who found

exact matching in genotypes and interpreted that as spread by the asexual oidiospores or vectored by insects. However, the power of this analysis is higher since a set of five microsatellite markers were included, and two of these were multiallelic (Pg7 and S18).

The F_{ST} values for each population are presented in table 2. The population from the Rotstop treated plot in Rämsön (A) showed the highest genetic differentiation, with F_{ST} values ranging 0,093-0,194. The Swedish populations showed a moderately low to moderately high genetic differentiation among each other. The two populations that were collected in Lithuania had overall higher F_{ST} values than the Swedish populations, suggesting that they are more differentiated from the Swedish populations. The strains from the Helsinki area do not constitute a defined population since the geographical origin of each strain is too scattered when compared to the geographically restricted populations. The North American group of strains is also shown as a comparison since it does not constitute a defined population. The North American strains also had loci that were not present in the other populations in this study. This indicates that *P. gigantea* populations in North America and Europe are considerably differentiated, as reported by Vainio and Hantula (2000).

Table 2. F_{ST} values for all the population pairs of *Phlebiopsis gigantea* based on three microsatellite loci. The group of strains isolated in North America (K) is shown as a comparison and is not included in the average F_{ST} calculations.

ID	A	B	C	D	E	F	G	H	I	J	K
†	*0,153*	*0,066*	*0,070*	*0,191*	*0,067*	*0,066*	*0,103*	*0,071*	*0,086*	*0,133*	*0,209*
A		0,159	0,164	0,148	0,142	0,093	0,194	0,158	0,182	0,139	0,209
B			0,014	0,185	0,018	0,025	0,041	0,017	0,027	0,110	0,190
C				0,200	0,012	0,034	0,040	0,015	0,023	0,129	0,208
D					0,218	0,165	0,291	0,208	0,266	0,034	0,191
E						0,025	0,022	0,011	0,009	0,145	0,230
F							0,053	0,045	0,051	0,108	0,184
G								0,048	0,016	0,219	0,309
H									0,015	0,126	0,204
I										0,187	0,272
J											0,097

† Average F_{ST} values, based on the microsatellite loci.

The mean observed (H_O) and expected (H_E) heterozygosity, based on the three microsatellite markers Pg 7, Cov2 and S18 are shown together with p-values in table 3. Overall, the H_E and H_O differed significantly according to the Chi^2 test in the population sampled at the Rotstop treated plot in Rämsön and the North American group of strains. The Rotstop treatment resulted in a lower heterozygosity than expected although the other two populations in the close vicinity both were in Hardy-Weinberg equilibrium.

Table 3. The observed (H_O) and expected (H_E) heterozygosities in the different populations and groups of *P. gigantea*. The values are based on three microsatellite loci. P-values are based on Chi^2-tests.

	Pg7			**Cov2**			**S18**		
ID	H_E	H_O	P-value	H_E	H_O	P-value	H_E	H_O	P-value
Rämsön A	0,414	0,224	0,007	0,480	0,327	0,032	0,507	0,277	0,002
Rämsön B	0,722	0,625	0,289	0,435	0,042	0,000	0,776	0,792	0,850
Rämsön C	0,698	0,444	0,097	0,459	0,556	0,562	0,765	0,556	0,138
Helsinki	0,700	0,667	0,801	0,000	0,000	-	0,818	0,833	0,892
Hyytiälä	0,665	0,818	0,282	0,375	0,091	0,052	0,828	0,455	0,001
Gåsholmen	0,643	0,400	0,109	0,490	0,400	0,570	0,765	0,889	0,375
Lunsen	0,612	0,400	0,168	0,278	0,200	0,583	0,805	0,300	0,000
Råberg	0,747	0,636	0,399	0,444	0,364	0,590	0,720	0,364	0,008
Palanga	0,717	0,867	0,198	0,332	0,267	0,589	0,809	0,643	0,121
Druskininkai	0,737	0,867	0,255	0,231	0,000	0,034	0,733	0,867	0,105
USA	0,729	0,231	0,000	0,298	0,077	0,082	0,852	0,333	0,000

5.3 Long-term effect of urea treatment in *Picea abies* stands (Paper III)

In the plots where the stumps had been treated with 100% conidia, 50% conidia and left untreated as controls; 17.4%, 7.1% and 12.9% of the trees were found dead, respectively. By contrast, in the plots where urea treatment had been carried out during the thinning operations, only 3.3% of the trees were found dead. When the frequency of rotted trees in the plots was compared, the 100% conidia plots and the 50% conidia plots were subjected to the highest incidences of rot (60.6% and 41.1% respectively). The untreated control plots had a rot frequency of 33.3%. Again, the urea treated plots were less infected and had only 2.7% of its trees damaged by rot (Fig. 7).

In total, *H. annosum s.l.* was identified in 24.1% of the living trees with rot symptoms and in 31.2% of the dead trees with rot symptoms. *H. annosum*

s.l. was also identified on 16.7% on the stumps that had been created during the thinning operation in year 2005. But no *H. annosum s.l.* was found in the urea treated plots (Fig. 7), neither in extracted wood cores, nor on trees or stumps. One possible reason for not identifying *H. annosum s.l.* in a higher number of the total sampled trees that were rotted could be the method of extracting wood cores from 20 cm above ground and not extracting an additional core further up the tree trunk.

During the storms in 2005 and 2007, 6.6% and 3.0%, respectively, of the trees in all plots were windthrown. Trees showing rot symptoms were more likely to be windthrown than trees that showed no rot symptoms. The median probability of rotted trees to be windthrown was 12.8%, in comparison to trees with no symptoms of rot where the risk of being windthrown was estimated to 3.5%. However, the geographical locality of the plots was of importance regarding the windthrowing incidences, in Bertilstorp fewer trees had been uprooted due to the storms than in Christinehof. The effect of urea treatments on wind damages was apparent; not a single tree was windthrown in the urea treated plots. On the other hand, the number of windthrown trees in the untreated control plots was 8.7% and in the plots that had been treated with 100% *H. annosum s.l.* conidia 11%.

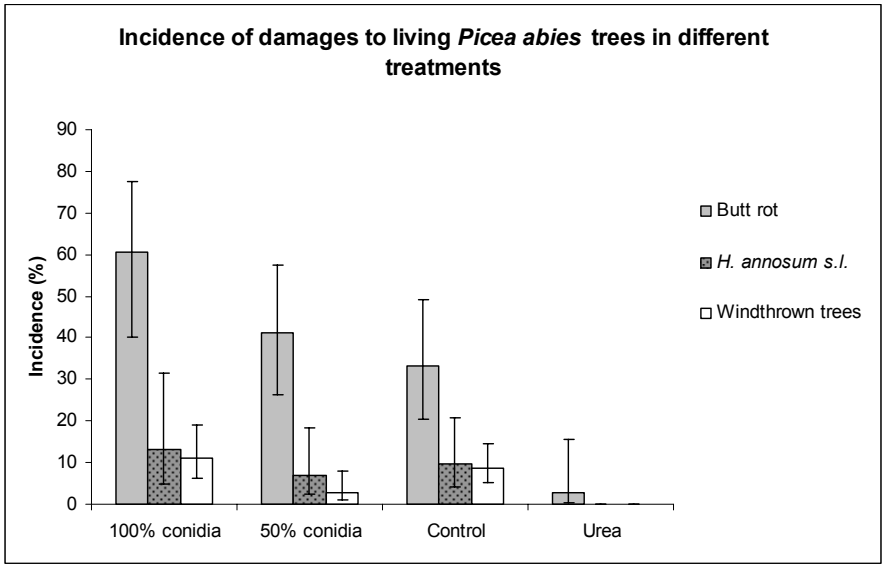

Fig. 7. The incidences of butt rot, *Heterobasidion annosum s.l.* and windthrown trees in living *Picea abies* trees as a result of four different stump treatments during a first rotation thinning 15 years before sampling. Bars denote confidence intervals at 95%.

No differences were found in radial growth when comparing infected and healthy trees. The trees with rot symptoms in year 2007 had a larger diameter than the trees without rot symptoms. The same relationship, in the same plots, between rot and diameter was measured 15 years earlier in 1992.

Studies by Johansson and Brandtberg (1994) and Brandtberg *et al.* (1996) showed an 85% reduction of *H. annosum s.l.* 3-24 months after stump treatment with urea. The infection frequency in the same study was, in the untreated plots, 34% which correlates well with the infection frequency in this study. In another study where 100% of the control stumps were naturally infected, the urea treated stumps were infected at a level of 38% (Nicolotti & Gonthier 2005). Thor and Stenlid (2005) showed that manual and mechanical application of the urea had an effect on the infection rates. In the plots where manual application of urea was performed, 3% of the stumps were infected with *H. annosum s.l.* and in the mechanized treatment 19% of the stumps were infected, but the controls on the other hand had an infection rate of 90%. Obviously, the risk of *H. annosum s.l.* infections varies depending on geographical location, season and climate. But nonetheless, urea treatments substantially reduce the risks of introducing *H. annosum s.l.* infections through fresh stump surfaces of *P. abies*, and the results in paper III show that urea treatments during thinning operations significantly reduce damages caused by root-rotting fungi in *P. abies* stands over time.

5.4 Development of an *Agrobacterium tumefaciens*-mediated transformation system for *Heterobasidion annosum s.l.* (Paper IV)

About 200 *H. annosum s.l.* cultures grew on hygromycin containing Hagem medium after the co-cultivation. After the subsequent transfer to hygromycin containing medium in Petri dishes, 120 of these cultures continued growing on the medium. Seventy-two of the strains originated from co-cultivation with *A. tumefaciens* harbouring the pCD61 plasmid and 48 strains harbouring the pJF4 plasmid. After the UV-light microscopy

screening, 34 strains were selected based on their GFP-activity that was well expressed throughout the mycelia. Eighteen of these 34 strains were selected for further analysis based on growth rate (Fig. 8). Seven strains originated from transformation with pJF4, while eleven originated from pCD61. Of these, eleven strains were analysed for GFP fluorescence after 18 months. Co-cultivation with *H. annosum s.l.* and the untransformed *A. tumefaciens* never resulted in any hygromycin-resistant *H. annosum s.l.* strains.

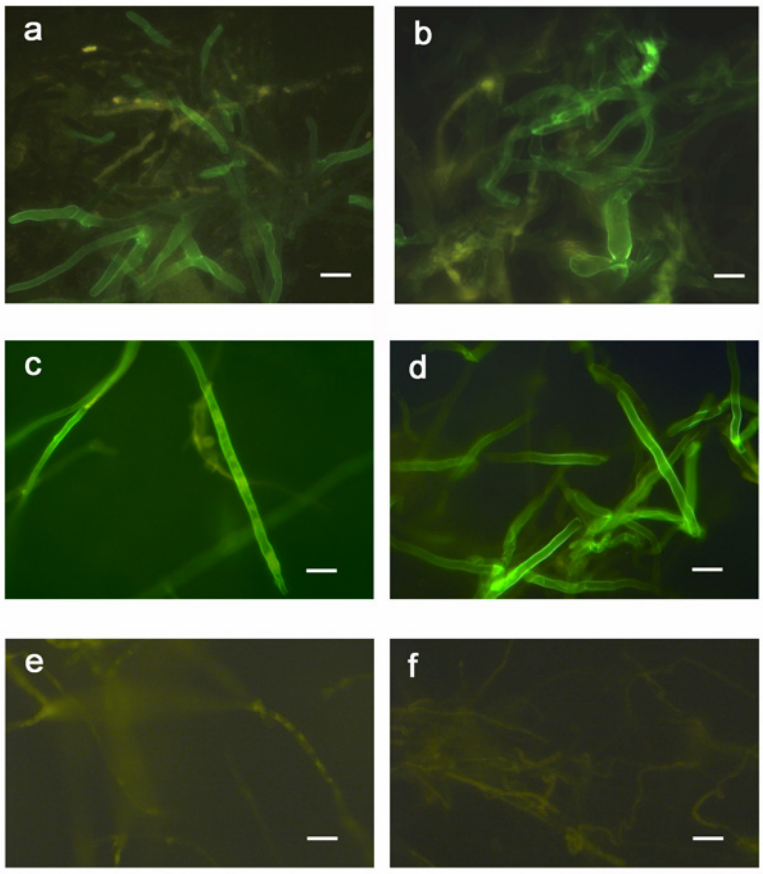

Fig. 8. Photomicrographs of GFP fluorescence in *H. annosum s.l.* transformed with pJF4 (a and c) and pCD61 (b and d) at 3 months post transformation (a-b) and at 18 months post transformation (c-d). Photos e-f displays untransformed *H. annosum s.l.* TC 32-1 incubated on Hagem medium containing 50µg/ml hygromycin B (e) and grown on standard Hagem medium (f). Scale bar represents 20 µm

This is the first successful transformation of a basidiomycete fungus where GFP is expressed in the recipient fungal strain without inserting an intron into the transformation plasmid (Lugones *et al.* 1999; Ma *et al.* 2001; Burns *et al.* 2005). It was discovered that the dominant factor in order to achieve a successful transformation of *H. annosum s.l.* was that the pH needed to be maintained at 5.6. The growth of *A. tumefaciens* was reduced at low pH, as has been reported previously by Hammerschlag *et al.* (1997) and consequently, no *H. annosum s.l.* transformants could be obtained when co-cultivation was setup in a medium with a lower pH. Another possibility to the reduction in growth of *A. tumefaciens* is that *H. annosum s.l.* produces secondary metabolites that are toxic to bacteria. Two known secondary metabolites, fommanoxin and fommanosin, produced in dual cultures, have shown toxicity to bacteria (Sonnenbichler *et al.* 1989). It is vital to keep the transformed strains on a selective medium so that transformed nuclei are favoured before untransformed ones. This is especially important if the experiment is designed to produce transformed *H. annosum s.l.* strains with a high ratio of transformed nuclei to untransformed nuclei.

6 Conclusions

The broad sense heritable trait in *H. annosum s.l.* to tolerate overgrowth by *P. gigantea* in **paper I** was obtained under conditions which are not entirely representative of the natural habitat in the field where several organisms and individuals of the same species are interacting in a fluctuating environment. But the results show that the *H. annosum s.l.* has the capacity to develop tolerance in the examined trait if the conditions are met, although this is unlikely to occur in managed sites where *P. gigantea* is used as a biocontrol agent.

The massive introduction of single *P. gigantea* strains during stump treatments does not seem to affect the intraspecific genetic diversity on a wide spatial scale. However, there is an effect on the genetic diversity within the treated sites that remains at least four years post treatment. The study in **paper II** also shows that it is possible to track a biocontrol agent in the field several years post-release. Regarding the use of *P. gigantea* in forestry, the strains should be changed periodically in order to decrease a possible loss in genetic diversity caused by the use of one strain during a long time. Also, since transatlantic *P. gigantea* strains are considerably differentiated, strains should not be traded for biocontrol purposes in order to avoid the introduction of unfamiliar genotypes.

Urea treatment of stumps during thinning operations is a reliable method to protect *P. abies* stands against root rotting fungi as shown in **paper III**. As an effect from the protection against root rot by urea treatments, the trees within a stand also become more resistant against strong winds and the number of windthrown trees is reduced. At present, a forest manager can choose between urea or *P. gigantea* as stump treatment methods during

logging and thinning operations. Urea has shown some adverse effects on the resident plant and fungal communities in close proximity of the treated stumps. *P. gigantea*, on the other hand, has a milder effect on the resident plant and fungal communities, but reduces the local intraspecific genetic variation. If *P. gigantea* treatment of stumps is as effective in long-term prevention of root rot as urea, than the method of choice will be at the user's discretion.

Paper IV is the first reported successful transformation of a basidiomycete fungus using a non-selective marker (GFP). With an efficient and inexpensive transformation system, future work on the *H. annosum s.l.* genome will be much facilitated. Enabling gene knock-outs and studies of promoters with unknown functions will open up for new knowledge on the fungus. Also, interaction studies between *H. annosum s.l.* and other fungi will be given new possibilities. With a GFP-tagged *H. annosum s.l.* strain, the process of distinguishing between individuals under the microscope by their hyphal features will be easier than when working with untransformed hyphae.

7 Future prospects

As *H. annosum s.l.* has the capacity to become more tolerant to *P. gigantea* it would be interesting to test the tolerance development through real-time trials where conidial isolations can be made from several generations of a *H. annosum s.l.* strain. Each of these generations should be forced to interact with *P. gigantea* in a controlled environment. This design would add more to the knowledge of tolerance build-up and how the *H. annosum s.l.* evolves as a response to interactions with other fungi. Future research should also focus more on the spread of genetic material from the *P. gigantea* in a spatiotemporal scale. One way of studying this could be through sampling periodically at known distances from the treatment in order to clarify how long the "Rotstop effect" is present in the treated sites, and how far the clones from the treatment are spread in the environment. Since the knowledge of the long-term efficacy of stumps treatments with *P. gigantea* against *H. annosum s.l.* infections is limited, there is a need to examine whether this biological control agent has the same long-lasting effect on the treated stands as the chemical agent, urea. Also, studies on the hyphal-hyphal interactions between the two species should be encouraged in order to reveal more about the actual interactions between fungi on a hyphal level. This work would be facilitated by performing microscopy on GFP-tagged strains of *H. annosum s.l.* and, for instance, tagging the corresponding *P. gigantea* strain with a red fluorescent protein. This setup would substantially facilitate the procedure of tracking the hyphae of the two strains during microscopy. Furthermore, with this transformation system, future work on the sequenced *H. annosum s.l.* genome will also be facilitated when undertaking studies regarding promoters with unknown functions. Another future study that could be performed using the transformation system is to knock out selected genes in the *H. annosum s.l.*

and study the changes in interactions with *P. gigantea* or even host trees. However, even though the promoters of the transformation plasmids used in paper IV still functioned in the recipient organism, they were derived from as distant organisms as sunflower and an ascomycete. To improve the transformation system and the following performance of the transformants, future research should aim on using a *H. annosum s.l.* promoter, or at least a promoter from another basidiomycete.

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