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Heterobasidion annosum s.l. and wood degradation of Norway spruce (*Picea abies*): the effects of sectioning, crown type and wood properties

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Academic dissertation

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ABSTRACT

Heterobasidion annosum s.l. is a serious forest pathogen that causes significant economic losses, especially in the Northern Hemisphere. In Finland, the *H. annosum* s.s. and *H. parviporum* within the *Heterobasidion* complex affect the growth and wood properties of Norway spruce (*Picea abies* (L.) Karst.) and its suitability for different industrial purposes. The main aim of this PhD thesis was to study the wood degradation (WD) caused by *H. annosum* s.l. and enzymatic hydrolysis under laboratory conditions in wood specimens representing different genotypes and crown types of Norway spruce, and at positions along the stem. Furthermore, the relationship between WD and different traits such as tree growth, physical properties and wood chemical composition, as well as the production of reducing sugars (RS), were also studied.

The WD differed between normal-crowned Norway spruce clones, but not between normal- and narrow-crowned trees. *H. annosum* s.s. caused a higher WD than *H. parviporum.* The WD was also affected by the position within the wood discs, along the stem and by the age of the sample trees. In older trees, samples taken near the pith were more degraded than those taken near the bark and vice versa. In younger trees, wood density correlated negatively with WD. Positive correlations were also observed between RS and WD, suggesting that genotypes susceptible to WD may represent desired raw material for some other industries in which the hydrolysis of the main wood components is essential (i.e., biorefining). To conclude, this thesis showed that the genotypic variation observed in breeding material may represent an advantage for forest tree breeding to control and avoid *Heterobasidion* attacks, especially when studied along with other important traits and considering silvicultural practices such as the thinning regime and rotation length that may affect WD risk by *H. annosum*.

Keywords: Wood decay, wood sections, wood density, growth traits, reducing sugar yield.

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Joensuu, July 2013

LIST OF ORIGINAL ARTICLES

This doctoral thesis is based on the following four articles, which are referred to in the text by the Roman numerals I-IV. Articles I, II and IV are reproduced with the kind permission of the publishers. Article III is the author's version of a submitted manuscript.

I Puentes Rodriguez Y., Zubizarreta Gerendiain A., Pappinen A., Peltola H. & Pulkkinen P. 2009. Differences in wood decay by *Heterobasidion parviporum* in cloned Norway spruce (*Picea abies*). Canadian Journal of Forest Research 39(1): 26-35.
doi: 10.1139/X08-159

II Puentes Rodríguez Y., Morales L., Willför S., Pulkkinen P., Peltola H. & Pappinen A. 2013. Wood decay caused by *Heterobasidion parviporum* in juvenile wood specimens from normal- and narrow-crowned Norway spruce. Scandinavian Journal of Forest Research 28(4): 331-339.
doi: 10.1080/02827581.2012.746387

III Puentes Rodriguez Y., Ahlholm J., Pulkkinen P., Degefu Y., Jaatinen R. & Pappinen A. Wood decay by *Heterobasidion annosum* s.l. in normal- and narrow-crowned Norway spruce and the effects of crown competition and stilbenes concentration on the decay rate and fungal growth. Manuscript.

IV Puentes Rodriguez Y., Puhakka-Tarvainen H., Pastinen O., Siika-aho M., Alvila L., Turunen O., Morales L. & Pappinen A. 2012. Susceptibility of pretreated wood sections of Norway spruce (Picea *abies*) clones to enzymatic hydrolysis. Canadian Journal of Forest Research 42(1): 47-58.
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Yohama Puentes Rodriguez was primarily responsible for the study design, execution, data analysis and writing of papers I, II and IV. In paper IV, the study design and laboratory work was performed together with Helena Puhakka-Tarvainen MSc. Chemical analyses were carried out by Professor Willför (extractives) for paper II, and Dr. Leila Alvila (lignin), Dr. Matti Siika-aho and Dr. Ossi Pastinen (carbohydrates) for paper IV. The design, execution, data analysis and writing of paper III was performed together with Dr. Jouni Alholm. The other co-authors of the papers contributed mainly by commenting on the manuscripts and by participating in some of the statistical analysis.

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ABBREVIATIONS

13C	Carbon-13
ASE	Accelerated Solvent Extractor
CPMAS	Cross-Polarization Magic Angle
DNS	3,5 dinitrosalicylic acid
GC	Gas Chromatography
H.	Heterobasidion
HPLC	High Throughput Liquid Chromatography
i.d.	Internal diameter
KGy	Kilogray
mbar	Millibar
MS	Mass spectrometry
nm	Nanometer
NMC	Nuclear Magnetic Resonance
p	Probability
RSY	Reducing Sugars Yield
RS	Reducing Sugars
SEM	Scanning Electron Microscopy
s.l.	sensu lato
spp.	species (plural)
SPSS	Statistical Package for Social Sciences
s.s.	sensu stricto
WD	Wood decay or degradation

1. INTRODUCTION

1.1. Wood decay by micro-organisms such as *H. annosum* s.l.

Wood is a valuable renewable raw material with a huge number of uses in construction, furniture industries, fuel for energy, pulp and paper, etc. The main wood components (i.e., cellulose, hemicelluloses and lignin) can be deteriorated through the enzymatic action of specific microorganisms (such as fungi, bacteria, and insects) in a process defined as wood decay. However, decay fungi are regarded as the causes of the greatest wood damages (Highley and Dashek 1998), mainly from the loss or reduction of wood properties (Sandberg 2009). The mechanisms of wood decaying fungi may vary according to the microorganisms and hosts involved. Some fungi are able to degrade only cellulose and hemicelluloses, but cannot depolymerize lignin (brown-rot; Kubicek 2013), or the lignin would appear to be slightly degraded (soft-rot; Schwarze et al. 2000). According to Eastwood et al. (2011), the absence of ligninolysis as a wood decay mechanism in brown-rot fungi has been replaced by an initial attack mechanism involving the internal breakage of cellulose by OH radical action. In contrast, ligninolysis is crucial in the wood decay process for some fungi (white-rot fungi), and the three main components of wood are consequently degraded. Basidiomycetes are the most important group of fungi to cause this type of decay, although some ascomycetes may also be designated as white-rot fungi. This decay is characterized by wood bleaching (Eaton and Hale 1993), and it has a spongy or fibrous appearance with pockets or lines that are separated by apparently firm and strong wood (Tsoumis 1991).

Two types of white-rot decay have been identified, i.e., simultaneous rot and selective delignification, based on the ways in which the main wood components are degraded. Simultaneous rot affects broad-leaved trees but seldom conifers and the decay of the major cell wall components occurs at the same time and at a similar rate (Eaton and Hale 1993). Due to the progressive degradation of the cellulose-rich secondary wall, this rot produces a brittle fracture in the wood (Schwarze et al. 2000). *Fomes fomentarius* (L.) Fr. is one of the fungi that cause this type of decay. On the other hand, selective delignification occurs in both broad-leaved trees and conifers. Lignin and hemicelluloses degradations occur first, and the cellulose is also degraded afterwards. There is a ductile fracture at the initial stages followed by a slight increase in impact bending strength (Schwarze et al. 2000). *Ganoderma* spp. P. Karst. and *Heterobasidion annosum* (Fr.) Bref., are examples of fungi that cause this type of wood decay.

Heterobasidion annosum sensu lato (s.l.) is regarded as one of the most serious and important forest pathogen that causes butt and root rot, especially in boreal forests (Hietala et al. 2009). This basidiomycete (Russulales, Bondarzewiaceae) represents the first "sequenced plant pathogenic homosidiomycete" bearing a complete genome sequence (Olson et al. 2012; Garbelotto and Gonthier 2013). Five intersterility groups have been

identified within the *H. annosum* s.l. complex; two occur in North America, i.e., *H. irregulare* Otrosina & Garbelotto and *H. occidentale* Otrosina & Garbelotto (Dalman et al. 2010), and three in Europe (Niemelä and Korhonen 1998). The European species are *H. annosum* (Fr.) Bref. sensu stricto (s.s., P type), *H. parviporum* Niemelä & Korhonen (S type) and *H. abietinum* Niemelä & Korhonen (F type). These intersterility groups have been named after their main host preferences. Thus, the P type comes from pine, S type from spruce and F type from fir (Niemelä and Korhonen 1998; Olson et al. 2005). Although *H. annosum* s.s. has been mainly associated with pines, it can also attack some other tree species (i.e., Norway spruce (*Picea abies* (L.) Karst.). In Finnish forests, both *H. annosum* s.s. and *H. parviporum* are able to colonize Norway spruce, but *H. annosum* occurs less frequently in relation to *H. parviporum.* In fact, *H. parviporum* is found approximately 10 times more often than *H. annosum* s.s. in the Norway spruce forests in southern and western Finland (Korhonen and Piri 1994; Korhonen et al. 1998).

H. annosum s.l. can colonize both dead (saprotrophic) and living trees (pathogen) (Olson et al. 2012). This fungus can not only use a range of carbon sources, such as starch, cellulose and pectin (Asiegbu et al. 1998; Olson et al. 2005), but also lignin via enzymatic mechanisms. Specific enzymes (i.e. cellulases, xylanases, and peroxidases) are required to degrade the main components of wood. In this sense, the recently reported fungal genome sequence of *H. annosum* s.l. showed a wide range of encoded enzymes crucial for cellulose, hemicelluloses and pectin digestion (Olson et al. 2012).

Heterobasidion infection can occur both by geminating spores or by mycelium already present in the roots (Schmidt 2006). The fungus spreads primarily via spores through fresh wounds on stems or newly cut stumps and roots to form a mycelium. Then, it colonizes the stump and spreads through the root system to uninfected surrounding trees (Swedjemark et al. 1997; Swedjemark and Stenlid 1997). At the Norway spruce early decay stage, only the compound middle lamella of tracheids, and middle lamella, primary and secondary wall of xylem rays, are broken down, resulting in the separation of tracheids from their matrix (Schwarze et al. 2000).

The inner bark of the tree must be colonized by the fungus that is spreading to a living root system and subsequently invades the sapwood (Karlsson and Swedjemark 2006). *H. annosum* s.l. has the ability to produce certain metabolites (i.e., phytotoxic dihydrobenzofuran aldehyde fomannoxin) to inhibit protein biosynthesis in the protoplasm of *Picea abies* (Karlovsky 1999). However, trees are also able to defend themselves, and the fungus should overcome that defense. Plants generally defend themselves through a combination of two mechanisms, namely by physical barriers in the form of structural characteristics and biochemical reactions, which produce toxins or phytoalexins. This combination varies according to "different host-pathogen systems" (Agrios 2005). These mechanisms can be constitutive or induced (Keeling and Bohlmann 2006). According to Kwon et al. (2001), the xylem parenchyma is also involved in the synthesis and secretion of extractives (i.e., lignans) into the heartwood of conifers (Franceschi et al. 2005), thereby providing defense against wood-decaying fungus.

Activating the living parenchyma cells in response to fungal attack or injury may result in the modification of wood and the formation of the so-called reaction zone (RZ) (Oliva et al. 2012). In *Picea abies*, this zone inhibits *H. annosum* growth within the tree in response to the high pH (i.e., ~8) and the presence of phenolic compounds (Oliva et al. 2010 and references therein; Nagy et al. 2012). However, tree radial growth may be compromised by defense resource allocations (Oliva et al. 2012). The RZ is an important defense mechanism against *Heterobasidion* in living trees, whereas the mechanisms involved in the defense of dead wood are basically constitutive.

In addition, wood anatomy (i.e., structure) is very important when considering wood decay (Schwarze et al. 2000) because it can be a limiting factor for microbial colonization and also influence the active defense (Pearce 1996). Wood density may affect the wood decay rate in response to the compact structure that prevents fungal growth into the wood. However, contrasting results including different tree species (i.e., *Larix siberica* Ledeb., *Pinus taeda* L.) have revealed no clear wood density effect on wood decay resistance (Garren 1939; Venäläinen et al. 2001; Yu et al. 2003). This relationship is most likely linked to the chemical composition of the wood and, more specifically, to chemical extractives acting as fungistatic or fungitoxic compounds. Xylem elements (i.e. fibers or vessels) may also affect wood decay. For instance, according to Eckstein et al. (1979), large vessels could contribute to easier pathogen dissemination within the tree (Pearce 1996). However, not much information is available in the literature regarding this issue. In addition to wood structure and chemical compounds, tree growth rate is another factor affecting the decay resistance of living trees (Yu et al. 2003). Thus, tree height and diameter may most likely correlate with decay resistance as well. For example, Piri and Korhonen (2001) have suggested a strong correlation between the proportion of advance-growth Norway spruce infected by *Heterobasidion* with the mean height and diameter of trees.

1.2. Wood degradation with fungal enzymes for industrial purposes

In general, wood degradation by fungal enzymes should not be viewed only as a negative process because it can also have a positive impact on nutrient cycling processes in natural ecosystems (Watkinson et al. 2006) and can be used in lignocellulose-based biotechnologies (Malherbe and Cloete 2002). In fact, enzymatic processes similar to those occurring in nature (i.e., ligninolysis) have been reproduced in recent decades under laboratory conditions and even at an industrial scale to produce, for instance, bioethanol (Kamei et al. 2012), or in pulping processes (Malherbe and Cloete 2002). The utilization of lignocellulosic materials and fungal enzymes has also increased over the years, mainly in response to global environmental concerns (Dashtban et al. 2009), and new technologies and products have been developed.

For instance, the separation of wood into fibers (i.e., pulp) in the pulp and paper industry has been carried out mainly by chemical pulping with sulfite and kraft or by sulfate processing with sodium hydroxide and sodium sulfide (Shmulsky and Jones 2011). However, environmental concerns have led to new alternatives such as biopulping using white-rot fungi (i.e., *Ceriporiopsis subvermispora)* to improve delignification and to therefore reduce both energy costs and environmental impacts (Malherbe and Cloete 2002). Bio-bleaching represents another process in which either hemicellulolytic enzymes (i.e., xylanases) or lignin-degrading fungi and their enzymes (Bajpai 2012) are used to bleach the pulp color to a whiter tone.

Enzymatic processes have also been implemented for biomass conversion (i.e., second-generation transport fuels). This process involves a pre-treatment step, the hydrolysis of hemicelluloses and cellulose to monomer sugars, fermentation and finally the product separation and purification (Dashtban et al. 2009). However, there are many limiting factors in the efficient hydrolysis of cellulose, such as cellulose crystallinity, degree of polymerization, lignin content and distribution, and surface area of cellulose accessible to enzymes (Chandra et al. 2009). The pore structure and substrate surface area are among the physical barriers that negatively affect cellulose hydrolysis (Jeoh et al. 2007). Several studies have been carried out to improve the conditions for a more cost-effective biomass conversion (Malherbe and Cloete 2002, Lange 2007, Kumar et al. 2009). However, there is still a great deal to be performed and learned in this research field.

The degradation of the primary wood components for biomass conversion mainly requires the action of different enzymes; for instance, the cooperation of three classes of cellulolytic enzymes (endo β-1,4-glucanases, cellobiohydrolases and β-glucosidases) is crucial for cellulose hydrolysis (Viikari et al. 2007). In the case of hemicelluloses, the actions of endo-type enzymes, side-group cleaving enzymes and exo-type enzymes are required (Maijala 2000). For instance, the hemicellulose xylan is hydrolyzed by endo-1,4-β-xylanase and xylan 1,4-β-xylosidase; whereas mannan is broken down by mannan endo-1,4-β-mannosidase and β-mannosidase (Schmidt 2006). A variety of oxidative enzymes are involved in lignin degradation, such as the oxidoreductases lignin peroxidase and manganese peroxidase (Schmidt 2006).

1.3. Wood decay by *H. annosum* s.l. in *Picea abies*

Norway spruce is the second most common tree species after *Pinus sylvestris* in Finnish forests (FAO 2010) and it is the most important commercial tree species for the pulp and paper industry in Scandinavia. However, Norway spruce wood decay by *H. annosum* s.l causes significant economic losses to European forestry. For instance, wood decay caused by *H. parviporum* decreases the quality of Norway spruce as pulp wood and saw logs. It also subsequently affects wood processing by decreasing the pulping mass quality (e.g.,

fiber properties and pulp strength) and pulp yield. The use of decayed wood also requires more effective alkali for kraft pulping (Korhonen and Stenlid 1998).

In this sense, a better understanding of Norway spruce resistance mechanisms against wood decay caused by microorganisms such as *H. annosum* s.l. may also offer means for engaging in practical forestry and forest tree breeding. It would, for example, be important to know whether often studied growth and wood property traits (e.g., stem volume, wood density and fiber properties) correlate with susceptibility to decay.

Norway spruce field trials established in southern Finland during 1970-90s have been used to study the differences in growth and yield traits, wood density traits and fiber properties between and within normal- and narrow-crowned Norway spruce (see Zubizarreta Gerendiain et al. 2007, 2008a, b, 2009). The narrow-crowned type of Norway spruce (*Picea abies* f. pendula) is in fact a rare mutant discovered in the 1950s in southern Finland. It was expected to have a large potential as raw pulp and paper industry material because it can be grown with narrow spacing, and it provides a large quantity of uniform wood per unit area even without thinning and a short rotation (Pulkkinen 1991). If higher resistance against *H. annosum* s.l. decay than in normal-crowned tress is also seen in this mutant, its utilization in other industries (i.e., biorefining) may also be possible. However, wood decay by *H. annosum* s.l. should be analyzed with both crown types along with different factors involved in the decay process (e.g., wood density, fiber properties, etc.) before drawing any conclusion.

1.4. Aims of the study

The main aim of this PhD thesis was to study the wood degradation caused by *H. annosum* s.l. and enzymatic hydrolysis under laboratory conditions in wood specimens representing different genotypes and crown types and positions along the stem in Norway spruce. Furthermore, the relationships between wood degradation and different traits such as tree growth traits (i.e., height, diameter, and stem volume), wood physical properties (i.e., wood density, fiber properties), wood chemical composition, and the production of reducing sugars (RS) were also studied.

The following hypotheses were established within this PhD thesis:

1. There are differences in the wood decay caused by *H. annosum* s.l. in different Norway spruce crown types and genotypes, on average, and between sections of wood discs taken at the same height (Papers I and II), or at various heights along the tree stem (Paper III).
2. The growth/yield traits (Papers I, II and III), physical traits (Papers I and II: wood density, Paper I: fiber properties) and chemical traits (Paper II: wood extractives) as well as crown competition index (Paper III) correlate with the wood decay rate.
3. The CCI (crown competition index) affects *H. annosum* s.l. wood decay.

4. There are wood decay differences between *H. annosum* s.l. intersterility groups (Paper III) and between strains within the same intersterility group (Paper I).
5. Wood susceptibility to enzymatic hydrolysis is affected by the wood section and particle size (Paper IV)
6. The reducing sugar yield correlates with the wood decay caused by *H. parviporum* (Paper IV).

2. MATERIALS AND METHODS

2.1. Experimental setup and study materials

The wood samples used in this study were harvested from three different Norway spruce field trials. The first trial (Table 1, Set I) consisted of 119 clones replicated in 20 blocks (4 repetition trees per block) on agricultural soil with site fertility conditions typical for Norway spruce. This trial was established in 1974 in Imatra, in south-eastern Finland (61°08'N, 28°48'E, 60 m above sea level) with a plantation spacing of 2.5 m x 2 m. In 2004, nine to ten sample trees from twenty clones of southern and central Finnish origins were randomly selected and harvested. In Paper I, wood disc samples taken at 1-1.3 m height from the stem base were used for detailed analyses of wood decay, wood density and fiber properties. Based on the susceptibility of different sample trees to *H. parviporum* wood decay, ten sample trees were used for further analyses to address their susceptibility to enzymatic hydrolysis after acid pre-treatment based on wood discs taken at 0.5 m heights from the stem base (Table 1, Set I, Paper IV).

The second study material (Set II) was harvested from a trial established in Karkkila, southern Finland (60°32'N, 24°12'E, 75 m above sea level). The trial was located on a medium to fertile forest soil (consisting of *Myrtillus* (MT) and *Oxalis-Myrtillus* (OMT) type), with typical fertility conditions for Norway spruce at 2 m x 1.5 m spacing. In this trial, 98 genotypes were replicated in 8 blocks (12 ramets per clone per block); 90% of the trees were narrow-crowned and 10% were normal-crowned trees. The normal-crowned genotypes originated from seeds collected from commercial forest stands located in southern Finland (60°40'N-63°22'N). The narrow ones represented controlled crosses between 1) narrow-crowned spruces from a Mäntsälä stand (60°40'N, 25°15'E) and 2) between the narrow-crowned spruces from the Mäntsälä stand and normal-crowned spruces from southern to central Finland (60°45'N-64°58'N). In Paper II, the data analyses were based on wood discs taken at 1-1.3 m high from the stem base from four to six sample trees from each of three normal- and nine narrow-crowned genotypes. The sample trees were randomly selected and harvested in 2007.

Table 1. Description of Norway spruce study materials used in this research.

Sets of Norway spruce	Genotypes/ trees	Crown type	Age of the trees (years)	No. of trees	Papers
I	C364	Normal	30	10 & 5	I & II
I	C465	Normal	30	10	I
I	C43	Normal	30	9 & 5	I & II
I	C48	Normal	30	9 & 3	I
I	C332	Normal	30	10 & 5	I & II
I	C334	Normal	30	9	I
I	C331	Normal	30	10	I
I	C338	Normal	30	10 & 5	I & II
I	C337	Normal	30	10	I
I	C328	Normal	30	10	I
I	C47	Normal	30	10	I
I	C304	Normal	30	10 & 5	I & II
I	C314	Normal	30	9	I
I	C308	Normal	30	10 & 5	I & II
I	C3152	Normal	30	10	I
I	C3151	Normal	30	10 & 5	I & II
I	C374	Normal	30	10 & 5	I & II
I	C430	Normal	30	10 & 5	I
I	C375	Normal	30	10	I
I	C3155	Normal	30	10	I
II	1A	Normal	19	4	III
II	2A	Normal	19	4	III
II	3A	Normal	19	5	III
II	4B24	Narrow	19	5	III
II	4B29	Narrow	19	6	III
II	5B43	Narrow	19	4	III
II	5B46	Narrow	19	4	III
II	6B70	Narrow	19	4	III
II	6B71	Narrow	19	4	III
II	6B72	Narrow	19	4	III
II	6B75	Narrow	19	4	III
II	6B76	Narrow	19	4	III
III	14 trees	Normal	30	15	IV
III	13 trees	Narrow	30	14	IV

The third study material (Set III) represents material harvested in 2000 from a field trial established on agricultural soil in Kytäjä (60°36'N, 24°37'E, 100 m above sea level), southern Finland in 1972, at a spacing of 2.2 m x 2.2 m. In Paper III, the analysis material

represents 14 normal- and 13 narrow-crowned Norway spruce trees, and discs taken from the butt of the stem and at 1-1.3 m height from the stem base. The crown competition index (CCI) was determined on the basis of crown measurements. Tree heights and diameters at 1.3 and 6 m were available for this work from all the sample material (Papers I-IV).

2.2. Wood decay test

Wood samples were sectioned (1 cm x 1 cm x 0.5 cm) along the wood discs from the pith to the bark (Paper I). However, only the inner (close to the pith) and outer sections (close to the bark) were considered in data analyses for Papers II and IV. These wood samples were subsequently oven-dried for 3 days at 50°C and weighed to obtain the initial weight. Afterwards, they were sterilized by Gammacel^{60} co-radiation (32.8-34.1 kGy). Malt extract agar, which consisted of 22 mL of 2% malt extract and 2.5% agar, was used as fungal culture media. Then, each Petri dish with medium was inoculated with a small piece of a fungal strain. Different *H. parviporum* fungal strains were used for the inoculations. More specifically, strains 5 and 7 were used in Paper I, strain 98 037-7 in Paper II and strain 96020 in Paper III. In addition, one strain (97074) of *H. annosum* s.s. was also used for comparison in Paper III. Fungal strains were provided by the Finnish Forest Research Institute, Finland.

After three (Paper III) and six (Papers I and II) months of incubations under controlled conditions (temperature of 20-23°C and relative humidity of 75-80%), the fungus was brushed off from the wood specimens and the samples were oven-dried. The final oven-dried sample weights were used to calculate the weight loss (WL%) from wood decay caused by the fungus. This was performed by using the formula:

$$WL\% = \frac{W_o - W_f}{W_o} \cdot 100 \quad (1)$$

where W_o is the initial weight before inoculation and W_f is the final wood sample weight after inoculation and incubation.

2.3. Wood density and fiber property measurements

Wood density was measured (Paper I and II) from small rectangular wood specimens (5 mm x 5 mm), representing radial segments from pith to bark. These specimens were cut from the same sample discs used for the wood decay tests. The X-ray measurements were carried out for wood specimens with a stabilized moisture content of 12%, using a direct scanning ITRAX X-ray microdensitometer (Cox Analytical Systems, Göteborg, Sweden). This was performed using a standard X-ray intensity (30 kV, 35 mA), exposure time of 20

ms, and geometric resolution of 40 measurements per millimeter (0.025 mm x 1 mm) (see Bergsten et al. 2001, Peltola et al. 2007, for more details). The resulting X-ray radiographic images were analyzed with Density software (Bergsten et al. 2001) to provide density profiles for each sample from pith to bark.

The fiber lengths and widths were measured (Paper I) using the L&W Fiber tester (AB Lorentzen & Wettre, Kista, Sweden) based on an image analysis. For this purpose, we used samples taken close to the other samples used for wood decay and density analyses, and they were matchstick-sized (for 2 annual ring pairs) and macerated in a boiling 1:1 (v/v) mixture of acetic acid and hydrogen peroxide. In this equipment, the highly diluted suspension flows between two glass plates that are close to one another, and limits the movement of the fibers in one direction but allows them to move in the other two directions. The resulting two-dimensional images permit the separate measurements of fiber lengths and widths.

2.4. Measurement of chemical compounds in wood

Inner and outer wood sections were also used for the appropriate analysis of extractives (Paper II). After being sectioned, the wood was prepared according to Willför et al. (2003). Thus, wood sections were splintered, freeze-dried and ground in an ultracentrifugal mill (RETSCH ZM 100) and passed through a 10-mesh screen. A second freeze-drying cycle was subsequently carried out to ensure the almost complete removal of volatile compounds. The sequential extraction of freeze-dried wood samples was carried out according to Willför et al. (2003, 2005) in an ASE apparatus (Accelerated Solvent Extractor, Dionex Corp.). Lipophilic extractives were extracted first with hexane (at a solvent temperature of 90°C, pressure 13.8 MPa, 2 x 5 min static cycles). Then, an acetone:water (95:5 v/v) mixture (solvent temperature 100°C, pressure 13.8 MPa, 2 x 5 min static cycles) was used to extract the hydrophilic extractives.

After the evaporation of the extract solutions and the silylation of the extractives, further analyses were carried out on a 25 x 0.20 mm i.d. column (HP-1, 0.11 µm film thickness). Steryl esters and triglycerides were analyzed on a short column according to Örså and Holmbom (1994). Heneicosanic acid, betulinol, cholesteryl heptadecanoate and 1, 3-dipalmitoyl-2-oleyl-glycerol were used as internal standards. The identification of individual components was completed by gas chromatography (GC)-mass spectrometry (MS) analysis with an HP-6890/5973 GC-MSD instrument and a similar GC column as mentioned above.

The carbohydrates were measured (Paper IV) using an analytical total hydrolysis to monosaccharides with sulfuric acid, followed by sugar analysis with high throughput liquid chromatography (HPLC) according to Várnai et al. (2010). An Agilent HP-5 column or a Dionex ICS-3000 gradient HPLC system (Dionex ICS-3000, Sunnyvale, CA) was used with a CarboPac PA-1 column and 1 ml/min eluent flow and a 30°C column temperature.

The lignin content was analyzed and quantified (Paper IV) by nuclear magnetic resonance (NMR) using wood particle size of 20 µm. A Bruker AMX 400 high-resolution NMR instrument operating at a carbon frequency of 110.6 MHz and a temperature of 295.16 K was used to record the cross-polarization magic angle spinning (CPMAS) ^{13}C NMR spectra. The spectrometer was equipped with a multinuclear magic angle spinning probehead. The contact time used was 2 ms (Leary and Newman 1992, Newman et al. 2005), the delay time was 2.5 s (Larsson et al. 1999, Hult et al. 2000), and the number of transients was 30 000. Glycine was used as the external standard for calibrating the chemical shift scale. Signal areas were calculated by deconvolution using both Lorentzian and Gaussian line shapes.

The *in vitro* sensitivity test was carried out with four stilbenes (piceatannol, isorhapontigenin, astringin and isorhapontin) extracted from Norway spruce bark at the Department of Chemistry, University of Jyväskyla (Finland). To test the sensitivity of *H. annosum* s.s. to stilbenes at different concentrations (0.10, 0.20, 0.50, 1.00, 2.00 and 4.00 mg/ml), 100 µl of DCR media with *H. annosum* s.s. (DCR-macro, DCR-micro, MS-micro, inositol, Fe-EDTA, $NiCl_2$, DCR-vit, maltose, casein hydrolysate, glutamine), 50 µl of stilbene solution and 50 µl of conidial suspension were placed into the wells of ELISA (enzyme-linked immunosorbent assay) plates and then mixed. For the control samples, only DCR media and conidial suspension were used. The conidial suspension was checked by using a Burker haemocytometer (depth 0.1 mm) and the quantity of conidia (per ml) was calculated as the average number of conidia (x 10^5) in 10 squares (dimension of each square = 1/25 mm^2). The average was 17.8 x 10^5 conidia/ml. The hyphal growth was verified using a scale from 0 (no growth at all) to 3 (abundant growth).

2.5. Acid pre-treatment and enzymatic hydrolysis

Concentrated acid hydrolysis with sulfuric acid (26% w/w) was applied as the pre-treatment for enzymatic hydrolysis (Paper IV). This was accomplished by following a modified US patent (Saito et al. 2008). Pre-treated samples were subsequently mixed with a cellulase solution of *Trichoderma viride* Pers. (100 U/mL; BDH 390744E, EC 3.2.1.4) (VWR International Ltd.) at a dose of 2000 U/g dry sample in 0.05 mol/L sodium citrate buffer (pH 5). The incubation time was 1 hour at 40°C in a water bath. The resulting supernatants were used to measure susceptibility to enzymatic hydrolysis by DNS reagent (based on 3,5-dinitrosalicylic acid) and were expressed as the reducing sugars yield (RSY) as a percentage of dry matter.

2.6. Scanning electron microscopy (SEM) observations

SEM observations were carried out for wood samples incubated with *H. parviporum* over 3 and 6 months and control samples (Paper II) and in enzymatically hydrolyzed samples with and without pre-treatment and without any treatment (control samples) (Paper IV). Before imaging, the samples were oven-dried for one day (103°C) and subsequently coated with electrically conductive copper. The coating was performed with putter equipment (Emitech K575X) at a pressure of approximately $7x10e^{-2}$mbar. Observations were made under a field emission ccanning electron microscope (LEO 1550 Gemini), with a resolution of approximately 10 nm and an "in lens detector" sensor.

2.7. Statistical data analysis

Statistical analyses were performed with the SPSS statistical program package (SPSS for Windows). Analyses of variance (one- and two-way) were used to find if statistically significant differences ($p < 0.05$) exist in the wood decay caused by *H. annosum* s.l. in different Norway spruce genotypes on average, and between the wood sections taken at the same disc height (Papers I and II) or at various heights along the tree stem (Paper III). The measured growth/yield traits (Papers I, II and III), physical traits (Papers I and II: wood density, Paper I: fiber properties) and chemical traits (Paper II: wood extractives) were also examined to see if they would correlate with the wood decay rate (Pearson's correlation, $p < 0.05$). Regression analyses were also used to study the relationship between wood density and wood decay (Paper II) and the effect of CCI (crown competition index) on wood decay (Paper III). In Papers I and III, differences in wood decay between fungal strains from the same intersterility group and between *H. annosum* s.l. intersterility groups were also analyzed. Furthermore, the susceptibility of wood to enzymatic hydrolysis in relation to by wood section and particle size and the reducing sugar yield correlation with the wood decay caused by *H. parviporum* were also addressed (Paper IV).

3. RESULTS

3.1. Effects of genotype and crown type on wood decay rate by *H. annosum* s.l. in Norway spruce (Papers I, II and III)

The wood decay rate (WD) caused by *H. parviporum* was significantly affected by genotype ($p < 0.05$) in 30-year-old normal-crowned Norway spruce clones in the Imatra trial (Dataset I), and this was the case regardless of the fungal strain (Paper I). Furthermore, clones representing the highest and lowest WD by strain 7 of *H. parviporum* (WD 22% for

C364 and 11% for C48, average over all clones 17.8%) had south-eastern Finnish origins. However, the three other most decayed clones (C430, C374 and C3151) originated from Central Finland. This result was similar to that of strain 5. However, the origin of the mother trees did not affect ($p > 0.05$) the wood decay caused by *H. parviporum* (Paper I).

In comparison, significant differences were not observed in the wood decay rate according to the genotypes or between crown types in 19-years old normal- and narrow-crowned Norway spruce trees at Karkkila trial (Dataset II; Paper II). The wood decay rate was 2.7% on average for normal-crowned trees and 3% for narrow-crowned ones. Similarly, the wood decay rate of *H. parviporum* did not differ in normal- and narrow-crowned trees harvested from the Kytäjä trial, with averages of 6.7% and 7.3%, respectively (Dataset III). Overall, the wood decay rate appeared to be higher on average in the older trees harvested from the Imatra trial, followed by trees harvested in Kytäjä and Karkkila trials, respectively. The trees in the Karkkila trial were also the youngest ones, and had the lowest average wood decay rate, as well (Fig. 1).

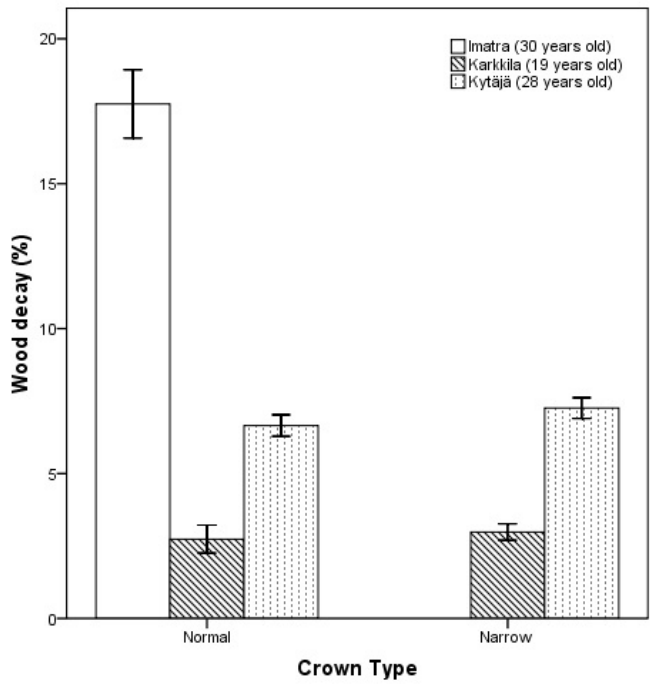

Figure 1. Averages *H. parviporum* wood decay rates in normal- and narrow-crowned Norway spruce trees harvested from Imatra (30 years old), Karkkila (19 years old) and Kytäjä (28 years old) trials at 1-1.3 m height from the stem base. Error bars represent the standard error.

3.2. Sampling position effects along the stem on wood decay rate by *H. annosum* s.l. (Papers I, II and III)

The overall *H. parviporum* wood decay rate differed depending on the sampling positions along the stem, i.e., from pith to bark (at 1.3 m height) and from the stem base towards the tree top. In normal-crowned Norway spruce clones grown for the Imatra trial (Dataset I), the wood decay rate was higher on average in samples taken close to the pith (for an average of 26.1%) than near the bark (with an average of 9.4%). This result was the opposite of the averages observed for wood density and fiber properties (i.e. length and width). In contrast to the findings from the Imatra trial, a higher average wood decay rate was observed in the outer sections (on average 4.9%) than in the inner ones (on average 1%) in normal- and narrow-crowned trees grown for the Karkkila trial. In the outer sections, both wood density (opposite to Dataset I) and lignan contents were also lower, and the free glucose content was higher. When considering the sampling height, the wood decay rate by *H. annosum* s.s. was slightly higher on average in wood specimens taken near the stem base (on average 16%) than in the middle part of tree (on average 6.9%) in narrow- and normal-crowned trees harvested from the Kytäjä trial (Dataset III, Paper III). However, this difference was not statistically significant ($p > 0.05$).

3.3. Inter- and intraspecific variation in *H. annosum* s.l. wood decay and the effects of phenols on fungal growth inhibition (Papers I and III)

The two strains of *H. parviporum* degraded ($p < 0.05$) the wood samples harvested from the Imatra trial in different manners. Strain 7 caused a higher average wood decay rate (16.9%) than strain 5 (1.7%) (Paper I). In addition, *H. annosum* s.s. appeared to degrade the wood more aggressively (on average 14.9%) than *H. parviporum* (on average 7.2%) (Paper III). The fungal growth inhibition was regarded as dose responsive for certain stilbenes (isorhapontigenin and piceatannol) as shown by the *in vitro* sensitivity test. The higher the concentration of the stilbenes were, the lower the fungal growth, and vice versa. Astringin and isohapontin also inhibited the fungal growth, but in a less perceptible way.

3.4. Wood decay by *H. annosum* s.l. and its relationship with other traits (Papers I, II and III)

In normal-crowned trees harvested from the Imatra trial (Dataset I), the wood decay rate did not correlate significantly ($p > 0.05$) with the wood density, fiber properties and growth/yield traits (Paper I, Table 2).

Table 2. Summary of differences observed in the wood decay rate (WD) by *H. annosum* s.l. for sample trees representing different genotypes and/or crown types and positions along the stem, and correlations observed between the wood decay rate and other traits.

Field trial/ Variables	**Dataset I (Imatra)**	**Dataset II (Karkkila)**	**Dataset III (Kytäjä)**
Effects on WD:			
Genotypes	YES	NO	-
Crown types	-	NO	NO
WD higher:			
Near pith	YES	NO	-
Near bark	NO	YES	-
At stem base	-	-	YES
Differences:			
between/within *H. annosum* s.l.	YES	-	YES
WD correlates with:			
Wood density	NO	YES (-)	
Fiber properties	NO	-	-
Lignans	-	NO	-
Stem diameter	NO	NO	NO

Note: WD= wood decay; YES= observed; NO= not observed; - = does not apply; (-) significant negative correlation ($p < 0.05$).

On the other hand, normal- and narrow-crowned trees harvested from the Karkkila trial (Dataset II) had a negative correlation ($p < 0.05$) between the wood decay rate and wood density (Table 2) and glucose content (the latter one was only found in narrow-crowned trees). The wood decay rate by *H. annosum* s.l. could not be explained by the crown competition index in the normal- and narrow-crowned trees harvested from the Kytäjä trial (Dataset III). The average wood decay rate increased in general along with the age of the sample trees ($p < 0.05$), when considering all the datasets (I-III) together (Fig. 1).

3.5. Sectioning and particle size effects on the sugar yields in wood samples from normal-crowned clones following enzymatic hydrolysis (Paper IV)

Sugar yields after enzymatic hydrolysis were generally similar within different pre-treated wood samples for the normal-crowned Norway spruce clones of the Imatra trial (Dataset I, Paper IV). However, wood sectioning affected both the enzymatic action of *H. parviporum* and that of commercial enzymes after acid pre-treatment. Wood sections taken from the inner wood discs parts were more resistant to enzymatic hydrolysis than those taken from the outer sections and the production of reducing sugars was subsequently lower in wood

sections representing the inner part of the wood discs. In addition, the particle size also affected RSY production. Fine particle sizes resulted in a higher yield than coarse particle size. The RSY also negatively correlated with wood density and positively with wood decay rate by *H. parviporum*.

4. DISCUSSION AND CONCLUSIONS

4.1. Effects of the genotype and crown type on *H. annosum* s.l. wood decay

This work addressed differences in *H. annosum* s.l. wood decay in different Norway spruce genotypes and crown types. Sample trees were harvested from three different field trials established during separate time spans in southern Finland. The average wood decay rate of *H. parviporum* was affected by the genotype of normal-crowned Norway spruce trees from the trial located at Imatra (Paper I). The genetic component in Norway spruce susceptibility to *Heterobasidion* (Arnerup et al. 2010) could at least partly explain the differences observed in *H. parviporum* wood decay between the Imatra trial clones. In this study, the least decayed clones originated from the southern part of Finland and the most decayed came mainly from central Finland (Paper I), but the origin of the mother trees did not affect the wood decay caused by this pathogen. This finding was in line with previous studies on lesion length and fungal growth in living Norway spruce genotypes (Treschow 1958, Swedjemark et al. 1997). In contrast, narrow- and normal-crowned Norway spruces harvested from the Karkkila (Dataset II) and Kytäjä trials (Dataset III) did not show significant differences between the clones and trees, in terms of *H. annosum* s.l. wood decay.

Earlier studies in living trees did not show large differences in wood decay between genotypes (Treschow 1958 as cited by von Weissenberg 1975). Karlsson and Swedjemark (2006) did not find significant differences in visible *Heterobasidion* spp. infection for different clones in 15-year-old sample trees from a Norway spruce field trial. In contrast, clonal differences have been found for the lesion length (Swedjemark et al. 1997, 2007), fungal growth in sapwood (Swedejemark and Stenlid 1997, Swedjemark et al. 1997, 2007) and visible infection (Karlsson and Swedjemark 2006). Previous studies (Swedjemark and Stenlid 1997, Swedjemark et al. 1999) have also suggested that approximately 35% of the variation in fungal sapwood colonization in living Norway spruce trees is related to genotypic variation (Arnerup et al. 2010). If so, this factor should be considered by forest tree breeding programs to improve the resistance of Norway spruce against *Heterobasidion.*

Most previous studies dealing with the susceptibility of Norway spruce trees to *Heterobasidion* spp. have been performed in living trees. Although it seems like an oversimplification to compare studies in living and dead wood because the main resistance

mechanisms taking place are different (i.e., induced and constitutive), good correlations between resistance studies under field and laboratory conditions presented by Scheffer and Duncan (1947) as cited by Scheffer and Cowling (1966) suggest that such comparisons are possible.

4.2. Sampling position effects along the stem on *H. annosum* s.l. wood decay

Wood decay by *H. annosum* s.l. caused different patterns of degradation based on the sampling position. In general, inner wood sections (near the pith) appeared to degrade more relative to outer ones (near the bark) in 30-year-old normal-crowned Norway spruce clones harvested from the Imatra trial (Paper I). Thus, it appeared that near the pith (juvenile wood) the wood was less resistant to the wood decay than near the bark (mature wood/sapwood). This difference could not be explained by the presence of heartwood near the pith by visual wood inspection (Pertti Pulkkinen, Finnish Forest Research Institute, pers. comm. in 2013), because of the relative young age of the sample trees at the Imatra and Kytäjä trials, but especially at the Karkkila trial.

The heartwood of living Norway spruce trees is usually the target for *Heterobasidion* attack in wetter climates (Pratt et al. 1998; Asiegbu et al. 2005). This finding may be related to the fact that heartwood is composed of dead cells and their active response to infection is lacking (Pearce 1996). The lower moisture content of heartwood relative to sapwood and the formation of a reaction zone (RZ) may also explain the differences between heartwood and sapwood in living Norway spruce trees (Schwarze et al. 2000). These traits may also result in low O_2 availability in sapwood and thereby restrict the fungal colonization in sapwood (Pearce 1996). In addition, the RZ that formed between colonized heartwood and sapwood (Nagy et al. 2012) acts as a barrier against pathogen development mainly because of the high concentration of extractives (i.e., minerals and lignans) and the high pH (Hietala et al. 2009).

In comparison, the dead wood acts as a passive substrate for fungal infection (Pearce 1996), as was the case for the wood samples used in this work and defense mechanisms are mainly determined by constitutive defence (i.e., lignans) and anatomical structure (i.e., wood density) of wood. In fact, the WD was higher on average and the wood density was lower near the pith in sample trees harvested from the Imatra trial (older trees), in contrast to samples trees harvested, for example, from the youngest Karkkila trial (Paper II), where both the inner and outer wood sections were still in the juvenile phase for the 19-years-old normal- and narrow-crowned trees. However, the wood density did not correlate with wood decay in the Imatra trial. In Norway spruce, the wood density first decreases in the first annual rings near the pith and starts to increase thereafter. In previous studies on wood decay, there has not being any clear pattern connecting wood decay to wood density (Venäläinen et al. 2001; Yu et al. 2003). In this work, wood decay and lignans content did not correlate (Set II, Paper II), but the amount of these phenol extractives was lower in

sections with higher wood decay (outer) and vice versa. In general, lignan content is higher in *Picea abies* heartwood than in sapwood (Pierce 1996) and therefore, its fungicidal properties may possibly be connected to differences between wood sections. In principle, the heartwood/sapwood ratio depends mainly on the age of the tree (Nawrot et al. 2008), and could explain the WD of older living trees. The older the tree, the higher proportion of heartwood and consequently the concentration of certain compounds affecting the decay resistance may also be higher than in sapwood. In Norway spruce, heartwood development typically starts in trees at 25-40 years old (Hietala et al. 2009); however, heartwood was not detected in the older samples used in this study (Imatra trial).

In general, the colonization of living trees by *H. annosum* s.l. may also vary with the age of the tree. For instance, young Norway spruce (< 20- years-old) have a generally higher resistance against *Heterobasidion* attacks than older trees (Korhonen and Stenlid 1998; Piri and Korhonen 2001; Johansson et al. 2004) and heartwood colonization have been reported when tree is approximately 25-years-old (Korhonen and Stenlid 1998). Von Weissenberg et al. (1975) have also suggested that young cuttings would react in a different way to pathogen invasion than older trees. On the other hand even young Norway spruce (< 10-year-old, without heartwood development) can be colonized if established in infested areas (Piri 2003).

The wood decay rate by *H. annosum* s.s. appeared to also be slightly higher on average in wood specimens taken near the stem base than in the middle part of stem (but $p > 0.05$) in narrow- and normal-crowned trees harvested from the Kytäjä trial (Paper III). Hietala et al. (2009) also found higher weight loss at the base of the decay column in mature Norway spruce trees naturally infected by *Heterobasidion.* The differences observed in the wood decay rate along the stem may also be related to differences in morphological and chemical properties of the wood from the stem base to the middle part of the tree**.** In fact, the wood density in Norway spruce varies along the stem, first decreasing for some distance from the butt and then increasing again towards the top (Repola 2006). Wood density has already been used as an indicator of longitudinal variation in Norway spruce stems (Kliger et al. 1995). Based on previous studies, the effect of wood density on wood decay resistance is not yet totally understood as shown in this work.

4.3. Inter- and intraspecific variations in *H. annosum* s.l. wood decay and the effects of phenolic compounds on fungal growth inhibition

Wood degradation caused by *H. annosum* s.l. differed not only between but also within intersterility groups. In this study, *H. annosum* s.s. more aggressively degraded the wood samples than *H. parviporum*; which is in line with previous studies from nature and infection experiments (Olson et al. 2005 and references therein). In addition, Daniel et al. (1998) also found that *H. annosum* s.s could initiate decay earlier and possessed a higher wood-decaying capacity than *H. parviporum*. The differences in the enzymatic activity

between these organisms are also already known. For instance, *H. annosum* s.s. has higher pectic enzyme activity than *H. parviporum*, despite its slower growth rate in living sapwood (Mitchelson and Korhonen 1998). Swedjemark and Stenlid (1995) also found differences between intersterility groups in living trees in terms of fungal growth and extension. *H. annosum* s.s. produced 30% higher growth and 22% higher fungal extension than *H. parviporum* strains.

Interspecific variation was also found between *H. parviporum* isolates in terms of wood decay. One of the strains used in this study (strain 7; Paper I) degraded the wood samples almost 10 times more than the other (strain 5). Differences between *Heterobasidion* isolates have also been found in terms of pathogenicity (virulence) but were measured in living trees as lesion length and fungal growth (Swedjemark and Stenlid 1997). Nevertheless, previous studies have also shown low virulence variation among isolates originating from the same intersterility group (Stenlid and Swedjemark 1988).

4.4 Wood property and growth traits effects on *H. annosum* s.l. wood decay

The influence of different traits, such as wood properties (i.e., wood density, fiber length and width, and chemical composition), tree growth traits (tree height and diameter) and crown competition on the *H. annosum* s.l wood decay rate was also evaluated in this work. As a result, it was found that most of the traits did not generally have a significant influence on the wood decay rate. Wood decay and wood density did not correlate significantly in normal-crowned trees harvested from the Imatra trial, but a negative correlation ($p < 0.05$) was found in normal- and narrow-crowned young trees harvested from the Karkkila trial. Different patterns have also been found for the relationship between wood density and wood decay rate in previous studies (Garren 1939, Venäläinen et al. 2001). Therefore, the wood density may be considered as a secondary trait relative to the chemical composition in terms of *H. annosum* s.l resistance as also stated by Tsoumis (1991).

In fact, phenolic compounds have been previously associated with Norway spruce wood decay resistance to *H. annosum* (Deflorio et al. 2011). Danielsson et al. (2011) recently found that higher concentrations of certain compounds such as piceasides and an unknown phenol glucoside were present in more resistant Norway spruce clones than in less resistant ones. Additionally, previous studies have also shown that the natural resistance of different heartwoods (e.g., western red cedar (*Thuja plicata* Donn ex D.Don) and Scots pine (*Pinus sylvestris* L.) is a result of their chemical composition (DeBell et al. 1999, Harju et al. 2001). However, heartwood does not possess active defense mechanisms against microbes, and lignin provides high resistance (Hietala et al. 2009), and only specialized microorganisms are able to degrade this wood component (e.g., white-rot fungi).

However, the lignans in this study did not have any significant effect on wood decay for the samples tested, although the lignans content was higher in wood samples with lower

wood decay and vice versa. Stilbenes were also analyzed in terms of growth inhibitors. Isorhapontigenin and piceatannol extracted from Norway spruce bark showed a clear dose-response against fungal growth. Astringin and isorhapontin also inhibited the fungal growth but the inhabitation was not directly proportional to the concentration. In previous studies initial concentrations of astringin also negatively correlated with the depth of hyphal penetration into Norway spruce bark (Lindberg et al. 1992; Danielsson et al. 2011).

The tree growth traits and crown competition evaluated in this study did not significantly influence the wood decay rate in any of the study material. Nevertheless, we found some clones from the Imatra trial (C308 and C43), that had higher average stem volumes (Zubizarreta Gerendiain et al. 2007, 2008) and were ranked among the least *H. parviporum* decayed clones. Some clones also had a higher average stem volume and dry stem mass, higher wood density and longer fibers, as associated with a lower wood decay rate.

In terms of practical forestry and considering the tree growth traits and competition between trees, the risk of wood decay by *H. annosum* s.l. could be reduced by avoiding root contact between infected and uninfected trees and also by preventing crown competition. This is true because competition may stress plants and have repercussions for their susceptibility/resistance and reduce infection tolerance (Gilbert 2002).

4.5. Reducing sugar yield (RSY) and its relationship to wood decay

In this study, the enzymatic hydrolysis rate of wood Norway spruce sections (Data Set I) pre-treated by concentrated acid hydrolysis showed that outer sections produced higher RSYs than inner ones, in contrast to the wood decay results from the same data set (but fewer sample trees). This RSY pattern was most likely related to the higher glucan and lower lignin contents found in the outer sections relative to the inner ones. High lignin contents in wood have been regarded as one negative factor influencing the hydrolysis of cellulose and further conversion to biofuels. This limitation is caused by the protective barrier created by lignin in lignocelluloses to prevent cell destruction by microorganisms (i.e., fungi and bacteria) (Kumar et al. 2009), which then confers recalcitrance to the woody material. Despite these differences in the wood sections, wood decay and RSY appeared to be positively correlated in this study, which means that the greater the wood decay is, the greater the production of RSY, and vice versa. Hong-bo et al. (2008) also suggested that the enzymatic hydrolysis rate should be "inversely correlated to the resistance of wood cellulose to degradation". Based on these findings susceptible genotypes could provide a good raw material for industries where the high production of reducing sugars is desired (i.e., biorefining).

4.6. Conclusions

The main aim of this work was to find if there are differences in wood decay rates as caused by *H. annosum* s.l. and how the wood degradation is caused by enzymatic hydrolysis in different clones, crown types and positions along the stem, as well as the effects of different factors on fungal development and wood degradation under laboratory conditions. Several previous studies have also focused on wood degradation, but mainly in normal-crowned living Norway spruce trees under natural conditions. In this work, *in vitro* studies (with dead wood specimens) were carried out using both normal- and narrow-crowned harvested trees.

The *H. annosum* s.l. wood decay rate appeared to be different between intersterility groups but also within groups. In this work and also in previous studies carried out in field conditions, *H. annosum* s.s. was more aggressive in decaying wood than *H. parviporum*. Wood samples were also shown to degrade differently depending on the sample positions along the stem and the age of sample trees. Wood samples taken near the pith were more degraded than those near the bark of older trees and vice versa. Among the wood properties tested, the wood density was negatively correlated with wood decay in younger trees, but not in older ones. Based on these results, the wood density or tree growth traits could not be considered as primary characteristics in relation to *H. annosum* s.l. resistance.

The results also showed that wood decay rates did not differ in general between crown types. In this sense, the narrow-crowned trees may offer potential for practical forestry because they can be grown in very narrow spacing, and they still produce a high biomass yield per unit area, even without thinning and with a short rotation (Pulkkinen 1991). One may expect that short rotation lengths and the avoidance or decreased thinnings would be preferred, especially in areas where the risk by *H. annosum* s.l. is high to avoid economic losses caused by this pathogen. However, further research is required to verify this assumption.

In addition, the fact that wood decay was positively correlated with the production of RSY may be valuable for forest tree breeding. This is because genotypes susceptible to wood decay may still represent useful raw material for some other industries (e.i., biorefining). Until now, there have been many studies on the utilization of lignocellulosic biomass for biorefinery purposes, but the utilization of softwoods has been quite limited because they are very recalcitrant and, therefore, very difficult to hydrolyze. However, further research is still needed, including for field- and *in vitro* experiments, to find out the most suitable genotypes for various industries for connecting desired raw material properties (i.e., volume and high hydrolysis yield) to resistance against fungal decay.

Finally, the genotypic variation found between the breeding materials may be considered advantageous for forest tree breeding to control and avoid *Heterobasidion* attacks. This variation, along with other important tree traits (e.g., growth, wood density, extractives, etc.) in consideration with some silvicultural practices (e.g., thinning regime and rotation length) may provide important information as to how to improve forestry

practices and to use narrow-crowned Norway spruce as a future regeneration raw material and reduce the current negative effects of *Heterobasidion* in Norway spruce forests.

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