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The effect of lignin content and lignin modification on Norway spruce wood properties and decay resistance

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ABSTRACT

This thesis describes studies with three different Norway spruce cutting clones in three growing environments differing in soil and climatic conditions within the boreal zone. The main aim was to follow variation in the radial growth rate, wood properties and lignin content and to modify wood lignin with a natural monolignol, coniferyl alcohol, by making use of inherent wood peroxidases. In addition, the incorporation of chlorinated anilines into lignin was studied with synthetic model compounds and synthetic lignin preparations to show whether unnatural compounds originating from pesticides could be bound in the lignin polymer.

Annual ring width, latewood proportion and wood properties such as weight density, modulus of rupture (MOR) and modulus of elasticity (MOE) were determined. Fourier transform infrared (FTIR) spectroscopy was applied to the quantitative estimation of lignin in heartwood, sapwood and earlywood samples by using a principal component regression (PCR) technique. Wood blocks were treated with coniferyl alcohol by using a vacuum impregnation method. The effect of impregnation was assessed by FTIR and by a fungal decay test.

Trees from a fertile site showed the highest growth rate and sapwood lignin content and the lowest latewood proportion, weight density and MOR. Trees from a medium fertile site had the lowest growth rate and the highest latewood proportion, weight density, MOE and MOR. The most rapidly growing clone showed the lowest latewood proportion, weight density, MOE and MOR. The slowest growing clone had the lowest sapwood lignin content and the highest latewood proportion, weight density, MOE and MOR. Fairly large variation was found between the individual trees and between the growing seasons but much less variation between the sites and between the clones.

The coniferyl alcohol impregnation increased the content of different lignin-type phenolic compounds in the wood as well as wood decay resistance against a white-rot fungus, *Coriolus versicolor*. Also the incorporation of chlorinated anilines into lignin was shown to be possible and the intermediate quinone methide was seen to be trapped by 3,4-dichloroaniline during the dehydrogenative polymerization of coniferyl alcohol, which resulted in a dimer of coniferyl alcohol with a β-O-4 structure and with dichloroaniline bound by a benzylamine bond.

In conclusion, in this thesis it was shown that the different Norway spruce cutting clones maintained clone-dependent wood properties in the different growing sites although variation between tree individuals was high and climatic factors affected growth. It was also shown that the natural monolignol, coniferyl alcohol, and chlorinated anilines could be incorporated into the lignin polymer *in vivo* and *in vitro*, respectively. The coniferyl alcohol impregnation experiment resulted in the improved resistance of the wood blocks to fungal decay, while chlorinated anilines were covalently bound into the lignin polymer and they could not be released by mild acid hydrolysis.

Keywords: Coniferyl alcohol, FTIR, lignin, growth rate, strength properties, weight density

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LIST OF THE ORIGINAL PUBLICATIONS

This thesis is based on the following publications. In the text they are referred to by their roman numerals (I–V).

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II Raiskila, S., Pulkkinen, M., Laakso, T., Fagerstedt, K., Löija, M., Mahlberg, R., Paajanen, L., Ritschkoff, A.-C. & Saranpää, P. 2007. FTIR spectroscopic prediction of Klason and acid soluble lignin variation in Norway spruce cutting clones. Silva Fennica 41(2): 351–371.

III Raiskila, S., Fagerstedt, K., Laakso, T., Saranpää, P., Löija, M., Paajanen, L., Mahlberg, R. & Ritschkoff, A.-C. 2006. Polymerisation of added coniferyl alcohol by inherent xylem peroxidases and its effect on fungal decay resistance of Norway spruce. Wood Science and Technology 40(8): 697–707.

IV Brunow, G., Raiskila, S. & Sipilä, J. 1998. The incorporation of 3,4-dichloroaniline, a pesticide metabolite, into dehydrogenation polymers of coniferyl alcohol (DHPs). Acta Chemica Scandinavica 52: 1338–1342.

V Brunow, G., Raiskila, S. & Björk, H. 2002. The incorporation of 3,4-dichloroaniline, a pesticide metabolite, into dehydrogenation polymers of coniferyl alcohol (DHPs). Part 2. Identification of a dimeric adduct. Holzforschung 56: 73–75.

The author's contribution in the articles:

I The author participated in planning the research work with the co-authors, interpreted the results of laboratory analyses and wrote the abstract, experimental part, results, discussion and conclusions.

II The author participated in planning the research work with the co-authors, carried out the FTIR analyses for the model estimation and testing and the earlywood lignin content prediction, wrote the experimental part of paper concerning the material, Klason lignin determination, FTIR and statistical analyses and the results and the discussion concerning the lignin content variation.

III The author participated in planning the research work with the co-authors, performed the preliminary impregnation tests, interpreted the results of laboratory analyses, wrote the experimental part of paper concerning the preliminary impregnation tests, peroxidase activity, Klason lignin determination, FTIR transmission and GC-MS analyses, the results and discussion concerning the GC-MS analysis and Klason lignin determination and the conclusions.

IV, V The author participated in planning the research work with the co-authors, carried out the organic syntheses, 1D NMR and HPSEC analyses, interpreted the results of laboratory analyses, wrote the experimental parts of papers and participated in writing the introduction and the results and discussion.

CONTENTS

ABBREVIATIONS

AR	Annual ring
ATR	Attenuated total reflectance
CA, DCA	Chloroaniline, dichloroaniline
CAD	Cinnamyl alcohol dehydrogenase
CEL	Cellulolytic enzyme lignin
$CDCl_3$	Deuteriochloroform
DHP	Dehydrogenation polymer
DIBALH	Diisobutylaluminum hydride
DRIFT	Diffuse reflectance infrared Fourier transform
FTIR	Fourier transform infrared
EW, LW	Earlywood, latewood
GC-MS EI^+	Gas chromatography-mass spectrometry, electron ionization
H_2O_2	Hydrogen peroxide
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
HOHAHA	Homonuclear Hartmann-Hahn
HPSEC	High performance size exclusion chromatography
HRMS EI^+	High resolution mass spectrometry, electron ionization
HRP	Horseradish peroxidase
HSQC-TOCSY	Heteronuclear single quantum coherence-total correlation spectroscopy
HW, SW	Heartwood, sapwood
IR	Infrared
KBr	Potassium bromide
LC-MS $APCI^+$	Liquid chromatography-mass spectrometry, air pressure chemical ionization
LiP	Lignin peroxidase
M_w	Mass average molecular mass
MnP	Manganese peroxidase
MOE, MOR	Modulus of elasticity, modulus of rupture
MS-MS ES^+	Tandem mass spectrometry, electrospray ionization
MWL	Milled wood lignin
NIR	Near infrared
NMR	Nuclear magnetic resonance
PAS	Photoacoustic spectroscopy
PCD	Programmed cell death
PCR, PLS	Principal component regression, partial least squares
RH	Relative humidity
RMSE, RMSPE	Root mean square error, root mean square prediction error
RSCL	Released suspension culture lignin

1. INTRODUCTION

1.1 Structure and chemical composition of Norway spruce stem wood

Norway spruce (*Picea abies* (L.) Karst.) grows mainly in the boreal zone (Sarvas 1964). At the primary stage of stem growth (longitudinal growth) the pith is formed from the apical, primary meristem, and it is mainly composed of parenchyma cells and the cells of primary xylem and phloem. At the secondary stage of growth (stem thickening) the secondary meristem (vascular cambium), xylem and phloem are formed around the pith. The cells of the vascular cambium divide and differentiate into the cells of the xylem inside and the phloem outside (Schweingruber et al. 2006). The secondary xylem consists mainly of tracheids, which have thick lignified cell walls. It also contains a few longitudinal resin ducts and horizontal uniseriate rays and multiseriate rays with resin ducts (Fagerstedt et al. 2004). Water flows from one tracheid to the other through bordered pits from the roots to the crown. The torus in the middle of the pit is not lignified (Schweingruber et al. 2006). The rays consist of parenchyma cells and tracheids. The rays transport water and photosynthesis products between the xylem and phloem and serve as storage tissue. The resin ducts are surrounded by epithelial cells (Fagerstedt et al. 2004). The secondary phloem, which transports the photosynthesis products throughout the tree, is composed of sieve cells, parenchyma cells and sclereids. At the third stage of growth, the epidermis surrounding the stem is replaced by periderm or bark (Schweingruber et al. 2006).

Norway spruce has distinct annual ring boundaries, and the annual rings are divided into wide, light earlywood and narrow, dense latewood zones (Schweingruber et al. 2006). The first annual rings (ca. 10) around the pith are called juvenile wood, and they are formed by the young cambium. The annual rings of juvenile wood are wide and the latewood percentage is low; juvenile wood changes to mature wood over several years (Saranpää 2003). The outer layer of the stem is called sapwood, and heartwood is formed in the inner part of the stem during ageing. In heartwood all the cells are dead but sapwood contains living parenchyma and epithelial cells, which participate actively in cellular metabolism of the tree (Schweingruber et al. 2006). The living parenchyma cells store, among others, triglycerides and starch while the canal resin is formed by the living epithelial cells (Back 2000). In dead heartwood the pits are aspirated, and its content of phenolic compounds and lignin is higher than in sapwood (Magel 2000). Reaction wood (compression wood) is formed on the lower side of branches and horizontally leaning stems. Compression wood has a high lignin content, wood density and compression strength (Timell 1986).

The tracheid cell wall is divided into a thin primary cell wall and a thick secondary cell wall, which consists of three layers (S1, S2, S3), the S2 layer being the thickest (Boerjan et al. 2003, Fagerstedt et al. 2004). The cell wall is mainly composed of cellulose, hemicelluloses and lignin (Fagerstedt et al. 2004). The cellulose microfibrils are irregularly oriented in the primary wall and regularly oriented in the secondary wall. Lignin is situated in inter-fibrillar cavities of cellulose (Schweingruber et al. 2006). The thin middle lamella between the cells consists of pectin and lignin (Fagerstedt et al. 2004). Mature wood tracheids are larger and have thicker walls and smaller cellulose microfibril angle than juvenile wood tracheids (Saranpää 2003). Latewood tracheids are longer and have thicker walls than earlywood tracheids (Timell 1986). If compression wood is present, its tracheids are short, circular in cross-section and very thick-

walled lacking the S3 layer, and inter-cellular cavities exist (Timell 1986, Schweingruber et al. 2006). The microfibril angle is larger than that in normal wood (Gindl 2002). The lignified cell walls are thought to give mechanical support to the tissues (Fagerstedt et al. 2004) and the phenolic compounds are known to prevent fungal growth (Schweingruber et al. 2006).

1.1.1 Synthesis and reactivity of lignin

Lignin is a phenolic polymer, which is formed through dehydrogenative polymerisation of *p*-hydroxycinnamyl alcohols (*p*-coumaryl, coniferyl and sinapyl alcohols) (monolignols). The synthesis is thought to initiate by the enzymatic dehydrogenation of monolignol followed by coupling of a resulting resonance-stabilized monolignol radical (Fig. 1) mostly at its most reactive β-position with a phenoxy radical at the growing polymer. The dehydrogenation is a one-electron transfer reaction catalysed by peroxidases or laccases in the presence of hydrogen peroxide (H_2O_2) or oxygen (O_2), respectively (Freudenberg 1968, Adler 1977, Baucher et al. 1998, Brunow et al. 1998a, Boerjan et al. 2003, Ralph et al. 2004). Intermediate quinone methides then react further with water, phenolic groups, polysaccharides (Freudenberg 1968) and possibly also with 'xenobiotics', such as pesticide residues, during the biosynthesis in nature (Trenck et al. 1981, Still et al. 1981, Sandermann et al. 1983).

Lignification takes place during the secondary wall formation at the final stage of xylem cell differentiation (Boerjan et al. 2003). The polymer formation starts at the cell corners and in the middle lamella and proceeds through the primary and secondary walls (Boerjan et al. 2003) partially at the same time as polysaccharides are deposited (Ruel et al. 2002). The orientation of the polymer is affected by the cellulose microfibrils. Spherical structures are formed in the middle lamella and primary wall and lamellas in the secondary wall (Boerjan et al. 2003). Mainly benzyl ether and ester bonds but also glycoside and acetal bonds (Watanabe 2003) may be formed between lignin and hemicelluloses (Boerjan et al. 2003).

The differentiation of xylem cells includes the cell division, cell enlargement, cell wall thickening and lignification followed by the cell death (Timell 1986). The ageing and death of cells are genetically determined processes called programmed cell death (PCD). "The earlywood tracheids survive for only a few days, whereas the parenchyma cells are able to survive for decades" (Schweingruber et al. 2006). The parenchyma cells can remain alive for a long time and die only during the heartwood formation, and at that point phenolic compounds are accumulated. An enzymatically initiated copolymerisation between the phenolic compounds and cell wall lignin may be involved in the heartwood formation (Magel 2000).

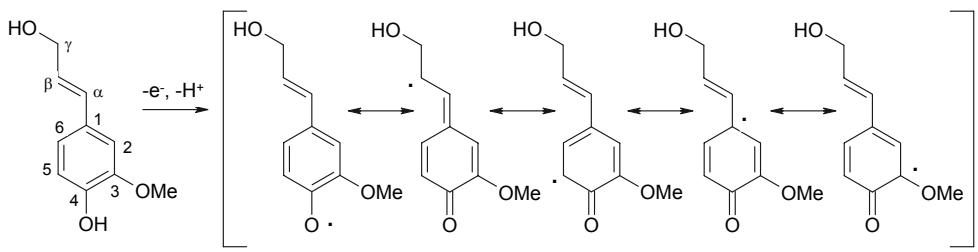

Figure 1. Dehydrogenation of coniferyl alcohol, modified from Adler (1977).

1.1.2 Chemical composition and content of lignin

Softwood (gymnosperm) lignin (Fig. 2) is composed mainly of guaiacyl units, hardwood (angiosperm) lignin of guaiacyl and syringyl units and grass lignin of *p*-hydroxyphenyl, guaiacyl and syringyl units. Hence lignin is called *p*-hydroxyphenyl, guaiacyl and syringyl lignin consisting of *p*-coumaryl, coniferyl and sinapyl alcohol, respectively. The monomers

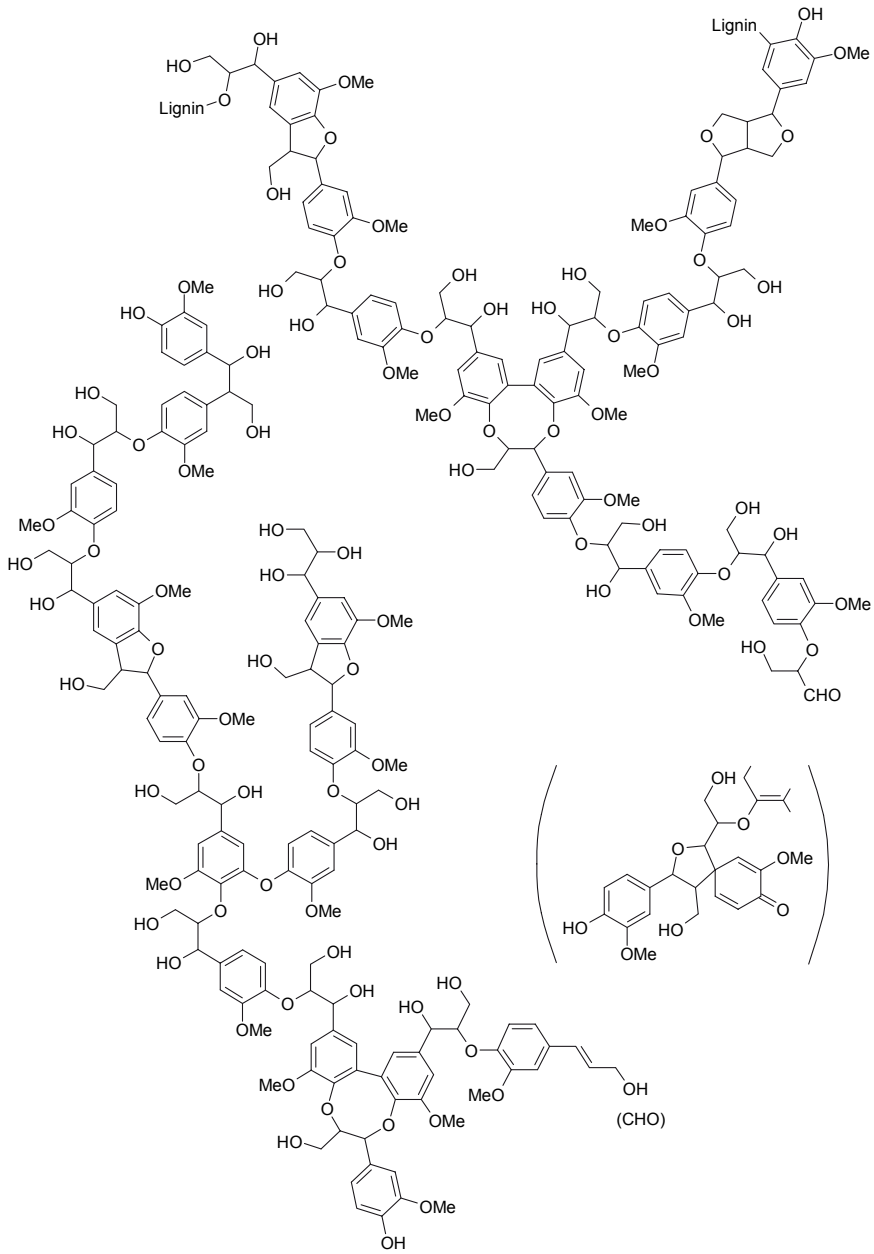

Figure 2. Structural scheme for softwood lignin, redrawn from Brunow et al. (1998a).

differ in the extent of methoxyl groups (Baucher et al. 1998, Boerjan et al. 2003). In Norway spruce, the proportion of the guaiacyl units is 98% and *p*-hydroxyphenyl units only 2%. The monomeric units of the polymer are linked by different carbon-oxygen and carbon-carbon bonds (Baucher et al. 1998). A β-O-4 bond is the most abundant structure in softwood lignin, and it is responsible for ca. 48% of the monomeric units in spruce. The other bond types found in spruce are β-5 (9–12%), 5-5 (9.5–11%), 5-O-4 (3.5–4%) and β-β (2%) (Adler 1977). A cyclic dibenzodioxocin structure has been observed in pine milled wood lignin (MWL) (Karhunen et al. 1995), and it is found in released suspension culture lignin (RSCL) from spruce and as a dehydrogenation polymer of coniferyl alcohol (DHP) (Brunow et al. 1998b). The dibenzodioxocin structure has been found to localise mainly in the S3 layer of the tracheid secondary wall in spruce (Kukkola et al. 2004). A β-1 bond has been observed in MWL but not in RSCL (Ede and Brunow 1992) and in DHP with nuclear magnetic resonance (NMR) spectroscopy (Brunow et al. 1998b). Non-cyclic α-O-4 structures may exist in softwood lignin, though no clear evidence has been found in MWL (Kilpeläinen et al. 1994), RSCL (Ede and Brunow 1992) or in DHP (Brunow et al. 1998b). The number of coniferyl alcohol and coniferaldehyde (3–4 per 100 monomeric units) end groups is rather limited in spruce lignin, while the number of free phenolic hydroxyl groups is < 20 per 100 monomeric units (Adler 1977). Compression wood lignin contains more *p*-hydroxyphenyl units and carbon-carbon bonds and fewer ether bonds than normal wood lignin (Timell 1986). Hardwood lignin contains more β-O-4 structures than softwood lignin (Adler 1977). Softwood lignin contains about equal amounts of *erythro* and *threo* isomers of the β-O-4 structures, whereas in hardwood lignin the *erythro* form predominates (Akiyama et al. 2005).

Softwood contains typically 42 ± 2% cellulose, 27 ± 2% hemicellulose, 28 ± 3% lignin and 3 ± 2% extractives of the wood dry weight. About three quarters of total lignin is found in the secondary wall of tracheids and a fourth in the middle lamellae and cell corners (Walker 1993). The lignin content of the S2 layer has been found to be 22–24% in spruce normal wood and 35% in compression wood (Gindl 2002). The lignin content of the middle lamellae is about 70–75% (Walker 1993). The lignin content of knots (33%) and branches (32%) is higher than that of stem wood, which is due to the variable amounts of lignin rich compression wood. Earlywood has higher lignin content than latewood, because the earlywood cells have thinner walls (Timell 1986). The lignin content in xylem decreases from the pith to the bark (Panshin and de Zeeuw 1980). The Klason lignin content of heartwood is higher than that of sapwood, since heartwood lignin may be contaminated with polyphenolics (Timell 1986). The dead parenchyma cells have higher lignin content (43%) than the tracheids (26%) in spruce (Back 2000). The lignin content of a wood sample depends on the lignin content of the individual cell wall layers and on the ratio of the highly lignified middle lamellae to the less lignified S2 layers of the secondary wall (Gindl 2002). The lignin content may be affected also by the relative proportions of earlywood and latewood (Anttonen et al. 2002).

The chemical composition and content of lignin vary between different plant species, plant individuals, plant parts, tissue types, cell types and cell wall layers (Baucher et al. 1998). Lignin is mainly deposited in the tissues giving mechanical support (compression wood) and conducting water (xylem) (Baucher et al. 1998) but also in the dead parenchyma (Back 2000), in the sclerenchyma and in the periderm (Baucher et al. 1998). In addition, defence mechanisms (e.g. responses to wounding and pathogen infection) (Baucher et al. 1998) are based on extreme cell wall growth and lignification (Schweingruber et al. 2006).

1.1.3 Incorporation of chlorinated anilines into lignin

Chlorinated anilines are released into the environment in the biotic and abiotic degradation of acylanilide, phenylurea and carbamate pesticides (Winkler and Sandermann 1989, Lao et al. 2003, Giacomazzi and Cochet 2004). In the higher plants chlorinated anilines are metabolised to polar compounds and accumulate in vacuoles (Brazier-Hicks and Edwards 2005) or are incorporated into the cell wall macromolecules (Sandermann 2004). 3,4-Dichloroaniline (DCA) has been found to react predominantly with glucose and malonic acid by forming N-malonyl-DCA in soybean (Winkler and Sandermann 1989, Gareis et al. 1992, Bockers et al. 1994, Lao et al. 2003), N-glucosyl-DCA in wheat (Winkler and Sandermann 1989, Bockers et al. 1994) and in *Arabidopsis thaliana* (Lao et al. 2003, Loutre et al. 2003). In *Arabidopsis* root cultures and soybean plants DCA is rapidly taken up and metabolised to N-glucosyl-DCA and N-malonyl-DCA, respectively, and the metabolites are then partly exported from the roots (Lao et al. 2003). The reactions with glucose and malonic acid are catalyzed with glucosyltransferases (Loutre et al. 2003, Sandermann 2004, Brazier-Hicks and Edwards 2005) and malonyltransferases (Winkler and Sandermann 1989, Sandermann et al. 1991, Lao et al. 2003).

3,4-Dichloroaniline, 3-chloroaniline and 4-chloroaniline are incorporated predominantly into the cell wall lignin in rice plants (Still et al. 1981, Sandermann et al. 1983), in wheat plants (Arjmand and Sandermann 1986) and in the cell suspension cultures of wheat (Winkler and Sandermann 1989). Evidence for the formation of covalent bonds has been found in several studies both *in vivo* and *in vitro* (Trenck et al. 1981, Still et al. 1981, Sandermann et al. 1983, Hoque 1995, Brazier-Hicks and Edwards 2005). Incorporation has been suggested involving the nucleophilic addition of aromatic amine to quinone methide formed as the intermediate during the lignin biosynthesis by forming a benzylamine bond (Trenck et al. 1981). Bonding to the aromatic ring or merging into the molecular network of lignin without chemical bonding are other types of incorporation that have been proposed (Still et al. 1981). The possibility of purifying soils by the covalent incorporation of xenobiotics (e.g. pesticide residues) into the lignin of growing plants has been studied earlier; lignin has been thought to be indigestible in animals and thus the bound pesticide residues generally have a very low bioavailability for animals (Sandermann et al. 1983, 1990, 1992, Sandermann 1994, 2004, Lange et al 1998).

1.2 Wood properties, decay resistance and modification

The cell walls provide shape and strength to the living plants but little is known about a relationship between the structure and strength properties. The thin primary walls are flexible, elastic, capable of growth and adapted to withstand tension created by turgor pressure inside the cell and cell separation forces at the cell corners and tensile stress falling on the stem e.g. in the wind. The thick lignified secondary walls are rigid and adapted to withstand low pressure within the xylem created by transpiration and compressive stress falling on the stem on the gravity to avoid collapsing. The rigidity probably arises both from the crystallinity of cellulose in the microfibrils and from the cross-linking of lignin in the matrix (Jarvis and McCann 2000). The compressive strength is provided by lignin (Walker 1993), and it has been hypothesized (Abreu et al. 1999) that the β-O-4 structures of lignin could play a role in wood flexibility. The tensile strength is contributed mainly by the glycoside structures of cellulose (Walker 1993, Jarvis and McCann 2000). The cells of the grass (e.g. tobacco) xylem have only one distinguishable layer in the secondary wall while the tracheids of the

tree xylem have three distinct secondary wall layers (S1, S2, S3) with different cellulose microfibril angles with the smallest angle in the S2 layer which comprises ca. 85% of the cell wall thickness (Hepworth and Vincent 1998). The microfibrils form bundles and lignin is distributed unhomogeneously in the S2 layer of spruce tracheids (Singh and Daniel 2001). The monomeric units of lignin have been found to align largely along the cell longitudinal axis in spruce pulp fibres (Åkerholm and Salmén 2003) and the aromatic rings of the monomeric units in the plane of the cell wall in black spruce (Atalla and Agarwal 1985, Agarwal and Atalla 1986). Lignin shows more viscoelastic properties than polysaccharides in pulp fibres (Åkerholm and Salmén 2003). The high compression strength of compression wood is probably due to the high lignin content and large microfibril angle (Timell 1986). The effect of lignin content on the mechanical strength is, however, difficult to observe over the impact of the microfibril angle (Gindl 2002). The condensed nature of lignin (more carbon-carbon bonds and fewer ether bonds than in normal lignin) probably follows as an adaptation to the heavy compression conditions, and this rigidity is also contributed by a lack of ester bonds (Sarkanen et al. 1967, Timel 1986). Pectin of the middle lamellae forms a gel-like liquid interpenetrating the microfibril layers of the primary walls in hydrated plant tissues (Jarvis and McCann 2000) and a solid pectin matrix in waterless tissues (Ha et al. 1997). In wood the middle lamellae are highly lignified (70–100%) (Timell 1986). The different structural units of lignin have different physical properties, and they differ in their chemical reactivity under different conditions among other things in pulping and in biodegradation (Brunow et al. 1998a, Ralph et al. 2004).

The decay resistance of wood is provided by the lignified cell walls (Zabel and Morrell 1992), and it is affected by the growth rate and site (Fagerstedt et al 2004). ”White-rot fungi are the only known organisms that are capable of completely degrading lignin to carbon dioxide and water” has been stated by Highley and Dashek (1998). Lignin is usually utilized at faster relative rate than cellulose and hemicelluloses (Highley and Dashek 1998). The degradation of crystalline cellulose is carried out by a multicomponent enzyme complex by the interaction of individual components (endoglucanases, cellobiohydrolases and β-glucosidases) to degrade cellulose to glucose (Eriksson and Pettersson 1975). The lignin degradation is carried out by a variety of oxidative enzymes; the white-rot fungi produce two extracellular heme-containing peroxidases, manganese peroxidase (MnP) and lignin peroxidase (LiP) and laccases, and a H_2O_2-generating system is needed for the degrading process (Périé and Gold 1991). The properties and catalytic mechanisms of lignin-degrading enzymes have been studied by using simple model compounds but the question how they attack native lignin still remains (Highley and Dashek 1998).

Generally, the purpose of wood chemical modification is to improve wood material properties by providing protection against decay, water, ultraviolet (UV) and thermal degradation by interfering with the chemistry of degradation (hydrolysis, oxidation, reduction, dehydration and free radical reactions) (Rowell 2006). The chemical modification involves a chemical reaction between a reagent and the cell wall polymers (lignin, hemicelluloses, cellulose) resulting in covalent bond formation which prevents leaching of the reagent. E.g. acetylation of hydroxyl groups with acetic anhydride on the wood surface or within the cell wall has been extensively studied and it is now in a process of commercialization. Impregnation involves a treatment of the wood cell wall e.g. with a monomer solution that diffuses into the cell wall followed by polymerisation. Wood enzymatic modification can be achieved by activation of lignin. Phenol oxidases, peroxidases and laccases are able to catalyse one-electron oxidations of phenolic OH groups, while they reduce H_2O_2 or O_2 to produce phenoxy radicals and water (Hill 2006).

The wood density and mechanical strength are the most important properties which determine a value of wood raw material in the wood industry, building industry and energy production. The wood density and strength are affected by the growth rate, age and genetic factors (Saranpää et al. 2002), and the radial growth and latewood density are affected by the climatic conditions (Schweingruber et al. 2006). Lignin must be removed from wood with harmful chemicals in the pulp and paper making, and this process consumes a large amount of energy, while durable timber is needed in the building industry (Walker 1993). In the living plants the cell walls are capable of growth and adapted to resist the internal and external mechanical stress. The primary walls are needed to be strong in tension and the secondary walls rigid in compression. These functional characteristics of the walls arise from the details of the polysaccharide and lignin structures, conformations and ordering (Jarvis and McCann 2000). The rigidity, strength (Jarvis and McCann 2000) and resistance of the plant structures are largely determined by lignin (Zabel and Morrell 1992). Modification of the lignin chemical composition or content is mechanistically possible e.g. by the incorporation of different monomers into lignin *via* nucleophilic addition to intermediate quinone methides formed during the lignification or *via* copolymerisation with the normal monolignols by free-radical coupling (Ralph et al. 1999b, 2004). Lignin biosynthesis modification by genetic transformation, which has been done especially in poplar and radiata pine, is not dealt with here as it goes beyond the scope of this thesis.

1.3 Generally used methods for lignin research

The lignin content of wood is usually determined by wet chemical methods e.g. with an acetyl bromide method (spectrophotometric method) suitable for a small amount of sample (Iiyama and Wallis 1988, Hatfield et al. 1999, Fukushima and Hatfield 2001, Hatfield and Fukushima 2005) and with a Klason lignin (gravimetric method) and acid soluble lignin (spectrophotometric method) measurement suitable for a large amount of sample (Dence 1992, Hatfield and Fukushima 2005). In the first method, the ground extracted wood is dissolved in acetyl bromide in acetic acid containing perchloric acid and lignin is determined by measuring absorbance at 280 nm (Iiyama and Wallis 1988). In the latter methods, the ground extracted wood is treated with 72% sulphuric acid followed by heating with dilute acid. Klason lignin is determined gravimetrically and acid soluble lignin is determined by measuring absorbance at 205 nm (Dence 1992).

The chemical structure of lignin can be studied e.g. with acidolysis, thioacidolysis, ozonolysis, permanganate, nitrobenzene and cupric oxide oxidation (degradative methods) and with NMR, UV, infrared (IR) (Dence 1992) and Raman spectroscopy (nondegradative methods) (Barsberg et al. 2006). The structural characterisation is often performed in synthetic lignin such as DHP, RSCL (Brunow et al. 1998b), differently isolated lignin samples such as MWL and cellulolytic enzyme lignin (CEL) by comparing the data of model compounds with the data of natural lignin (Dence 1992).

1.3.1 FTIR spectroscopy in the quantitative evaluation of lignin

In Fourier transform infrared (FTIR) spectroscopy a molecule absorbs photon energy at an infrared region of the electromagnetic spectrum. The infrared radiation covers wavelength (λ) ranges about 780–2 500 nm (near infrared), 2 500–50 000 nm (mid infrared) and 50 000–1 000 000 nm (far infrared). A unit called wavenumber, which is reciprocal of radiation

wavelength ($1/\lambda$) expressed in cm^{-1}, is commonly used (Colthup et al. 1975). The most useful ranges are the mid infrared region between 4 000–500 cm^{-1} and the near infrared region between 10 000–4 000 cm^{-1} (Faix 1992). The molecule absorbs radiation energy at frequencies which match the natural vibration frequencies of the molecule by increasing its own vibration energy (Colthup et al. 1975). "In order to absorb infrared radiation, the molecular vibration must cause a change in a dipole moment of a molecule" has been stated by Colthup et al. (1975). The symmetric vibrations about a centre of symmetry are infrared inactive (Williams and Fleming 1989). In infrared spectroscopy these different molecular vibrations (bond stretching and bending), in which bond lengths and bond angles between atoms continuously change, are measured (Colthup et al. 1975). Absorption bands in a spectrum represent the vibration frequencies which are characteristic of the covalent bonds or functional groups (e.g. O–H, N–H, C–H, C=O, C–O) and the whole molecule (Williams and Fleming 1989). In the spectra of macromolecules, such as lignin, the bands are broad and overlapping. Band assignments to a spectrum of spruce MWL are presented e.g. in Faix (1991, 1992).

A potassium bromide (KBr) transmission technique is the most common tool for the quantitative evaluation of lignin and suitable for routine work. A diffuse reflectance infrared Fourier transform (DRIFT) method is suitable for wood surface investigation and lignin evaluation in wood though its reproducibility is considered poor. Attenuated total reflectance (ATR) and photoacoustic spectroscopy (PAS) are less used in lignin research. Among different multivariate techniques principal component regression (PCR) and partial least squares (PLS) methods are the most important for a calibration of FTIR data in practice. In the calibration, the FTIR data set is related to a measurement data set obtained with the wet chemical methods (Faix 1992). An introduction to two-dimensional (2D) infrared spectroscopy, a new technique based on time-resolved infrared spectroscopy, has been presented by Noda (1989).

Mid infrared spectroscopy by using the KBr transmission technique has been applied for studying esters of aromatic acids (e.g. vanillic, *p*-hydroxybenzoic, ferulic acids) and acetyl groups on the lignin monomeric units in softwood MWL from normal and compression wood (Sarkanen et al. 1967, Timell 1986), for estimating a percentage of β-O-4 structural units in MWL from tropical woods (Abreu et al. 1999), for estimating a lignin content in Sitka spruce (Costa e Silva et al. 1999) and for analysing structural changes in MWL from recent and fossil wood (*Sequoiadendron giganteum*) (Uçar et al. 2005) and in chemical components of Scots pine and beech wood exposed to brown-rot and white-rot fungi (Pandey and Pitman 2003). Static and dynamic measurement techniques have been used to determine an orientation of lignin and its mechanical interaction with other cell wall components in spruce pulp fibres (Åkerholm and Salmén 2003). Wood density and chemical composition of Pinus radiata (Meder et al. 1999), Sitka spruce, Scots pine and tropical hardwoods have been studied with DRIFT technique (Nuopponen et al. 2006), and variation of lignin content between pulp fibres was studied with ATR technique by Jääskeläinen et al. (2003). Near infrared (NIR) spectroscopy has been used recently to predict a chemical composition of wood in loblolly pine (Jones et al. 2006) and in transgenic aspen (Yamada et al. 2006) and to analyse a chemical composition of lignin (H/G ratio) in maritime pine (Alves et al. 2006) and to study within tree variation in a lignin content, extractives and a microfibril angle in longleaf pine (Via et al. 2007).

1.3.2 NMR spectroscopy in the structural studies of lignin

In nuclear magnetic resonance (NMR) spectroscopy, an atomic nucleus absorbs energy at a radio frequency region of the electromagnetic spectrum in a strong external magnetic field. Some atomic nuclei behave as bar magnets because of their nuclear spin. The charged

and spinning nucleus generates a small magnetic field. The nuclei of proton ^{1}H and carbon isotope ^{13}C (nuclear spin quantum number I=½) can be observed with NMR spectroscopy while the abundant isotope ^{12}C (I=0) is not suitable for NMR measurement. The nucleus with the spin I can occupy 2I+1 energy levels in the applied external magnetic field (Friebolin 1998, Robert 1992). The nuclear spins orient themselves either parallel (low energy state) or anti-parallel (high energy state) to the field. When energy of radio waves is absorbed at the right frequency the parallel nuclear spins align themselves against the magnetic field and the nuclei are promoted to a higher energy state. A position at which the atom absorbs in a spectrum depends on an electronic environment of atom within the molecule and is presented as a chemical shift, δ (ppm). Each atom resonates at the specific frequency (Friebolin 1998, Ämmälahti 1999).

^{1}H NMR spectroscopy is an important tool in the structural studies of lignin because of high natural abundance (99.98%) and detection sensitivity of the nucleus. A proton spectrum is quantitative but signals are, however, broad and overlapping due to the stereochemical complexity of structures hindering the use of coupling information that makes the interpretation of the chemical shifts difficult. An analysis of acetylated lignin in deuteriochloroform ($CDCl_3$) is a general method (Lundquist 1992, Ralph et al. 1999b, Ämmälahti 1999). ^{13}C NMR spectroscopy is a powerful tool in the lignin study though the natural abundance of carbon isotope ^{13}C (1.11%) is low. The resolution of carbon spectrum is better than that of the proton spectrum but the carbon nucleus is less sensitive (difficult to observe) and data accumulation times are long (Robert 1992, Ralph et al. 1999b, Ämmälahti 1999).

Two-dimensional (2D) NMR techniques, proton-carbon and proton-proton correlation spectroscopy, e.g. heteronuclear multiple quantum coherence (HMQC) and homonuclear Hartmann-Hahn (HOHAHA also called TOCSY) spectroscopy have been applied to an analysis of some minor structural units in RSCL (Ede and Brunow 1992) and pine MWL (Kilpeläinen et al. 1994) and also dibenzodioxocin structures in pine MWL (Karhunen et al. 1995). Heteronuclear multiple bond correlation (HMBC) spectroscopy has been applied to study esters such as *p*-coumarates on the lignin monomeric units and ferulates cross-linking lignin and polysaccharides in grasses (Ralph et al. 1999b, 2004). Two-dimensional (2D) HMQC-TOCSY has been used for identifying aldehydes, dihydroconiferyl alcohol units (Ralph et al. 1997) and guaiacylpropane-1,3-diol units (Ralph et al. 1999a) in lignin of a CAD-deficient loblolly pine mutant and acetylated monomeric units in kenaf lignin (Ralph et al. 1999b, 2004). Three-dimensional (3D) HMQC-HOHAHA is well suited for studying the side chain structures of lignin because of better resolution of the overlapping chemical shifts and sensitivity of the method but ^{13}C-labelling is often needed to shorten a measuring time (Ämmälahti et al. 1999, Brunow et al. 1998b). Three-dimensional (3D) heteronuclear single quantum coherence-total correlation spectroscopy (HSQC-TOCSY) has been used in an analysis of non-labelled technical lignin samples by Liitiä et al. (2003).

2. AIMS OF THE STUDY

The general aim of this work was to follow variation in the radial growth rate, wood properties and lignin content among the three Norway spruce (*Picea abies* (L.) Karst.) cutting clones growing in the different environments. The Norway spruce clones used in this study were planted in the 1970's with the aim of following the growth of the same clones in different growing sites. They are now giving an excellent opportunity to assess the effect of the environment on the composition of wood. In addition to the comparisons of chemical and mechanical wood properties, the resistance to fungal decomposition was determined. As it was predicted that higher lignin content may hinder rot caused by fungi, wood samples were modified with the natural monolignol, coniferyl alcohol, and its effects on wood decay resistance were studied. Another aim was to investigate chemical grounds for the incorporation of chlorinated anilines, which are formed from pesticides, into lignin. Evidence for the formation of covalent bonds has been found in several studies with NMR and mass spectrometry both *in vivo* and *in vitro* (Trenck et al. 1981, Still et al. 1981, Sandermann et al. 1983).

The objectives of sub-studies were:

- to study variation in the growth rate and wood properties of the three different Norway spruce cutting clones growing in three different soil and climatic conditions. (I)

- to study variation in the amount of lignin of the Norway spruce cutting clones growing in the different sites by using the FTIR spectroscopic method. (II)

- to use Norway spruce wood peroxidases to polymerise added coniferyl alcohol and to see whether the coniferyl alcohol impregnation affects on the wood decay by the white-rot fungus. The hypothesis was that internal wood peroxidases would oxidise the added monolignol and bind it stably into the cell wall, which could hinder fungal decay. (III)

- to study the incorporation of pesticide metabolites 3,4-dicloroaniline (DCA) and 4-chloroaniline (CA) into lignin with the lignin model compounds and synthetic lignin preparations. A hypothesis was that chlorinated anilines could be trapped by the intermediate quinone methides during the lignin biosynthesis in nature. (IV, V)

3. MATERIAL AND METHODS

3.1 Norway spruce cutting clones

3.1.1 Growth rate and wood properties

The growth rate and wood properties were studied in the three different Norway spruce cutting clones (A, B, C) growing on three different sites in Loppi, Imatra and Kangasniemi within the boreal zone. The cuttings had been planted on the three sites differing in the soil and climatic conditions in the 1970's. The growth sites in Loppi and Imatra were fertile old agricultural lands and the site farther north in Kangasniemi a medium fertile *Myrtillus*-type

forest according to Cajander (1926). The cuttings originated from three seedlings, which were the progeny of three mother trees, which had been chosen for rejuvenation on the basis of good growth, good stem form and vigour from natural forests in Finland. Sample trees (5 trees/clone/site) were 26 (Loppi), 28 (Imatra) and 24 (Kangasniemi) years old at the time of felling in 2001–2003. Altogether 44 stems were sampled at breast height (about 1.3 m).

The annual ring width, weight density and mechanical strength were studied in samples of 44 stems representing three cutting clones from three sites. Before measurements, the moisture content of the samples was stabilised at 20 °C at relative humidity (RH) of 65%. The annual ring width and weight density measurements were performed on strips of wood (5 mm thick) from pith to bark by using an X-ray densitometric method (Saikku 1975) according to Jaakkola et al. (2005). The modulus of elasticity (MOE) and modulus of rupture (MOR) measurements were performed on mature wood samples (20 mm × 20 mm × 341 mm) with a standard bending test (Kučera 1992) according to Saranpää and Repola (2001). Also the weight density of the test samples (ρ_{12}) was determined by measuring dimensions and weights of the samples stabilized at 65% RH. The measurement results for the annual ring width, latewood proportion, annual ring density and latewood density were analysed statistically with an analysis of covariance using a mixed model in a program SPSS for windows. The MOE, MOR and weight density (ρ_{12}) measurement results were analysed with a one-way analysis of variance (ANOVA) followed by a Tukey HSD test and also tested with a Student's t-test. (I)

3.1.2 FTIR spectroscopic prediction of Klason lignin and acid soluble lignin

The amount of lignin was studied in heartwood and sapwood samples of 44 stems (88 samples) representing three cutting clones from three sites (Loppi, Imatra, Kangasniemi). FTIR spectra were recorded on potassium bromide (KBr) pellets of unextracted sieved wood powder (3 mg) by using the transmission technique in the mid infrared region (4 000–500 cm^{-1}) according to Jaakkola et al. (2007). The first the principal component regression (PCR) model was built on total lignin contents (Klason lignin + acid soluble lignin) and the normalised spectra (1 850–500 cm^{-1}) of 18 wood samples of 9 stems for a calibration of FTIR data as published by Jaakkola et al. (2007). The acid insoluble lignin content was determined with the Klason method (Browning 1967, Dence 1992) and the acid soluble lignin content with a spectrophotometric method (Maekawa et al. 1989, Dence 1992, Hatfield and Fukushima 2005) from extracted samples and then calculated for unextracted wood: Klason lignin $\%=p \times (100-e) / m$ and acid soluble lignin $\%=A \times (100-e) / K \times m \times D-B$, where p=dry weight of precipitate, e=extractive content, m=calculated dry weight of extracted sample, A=absorbance, K=lignin absorptivity, D=dilution ratio and B=correction for carbohydrates. The second, the PCR model was tested with 6 samples of 3 stems (Jaakkola et al. 2007) and then used to predict the total lignin content in the rest 64 samples of 32 stems. Altogether 272 candidate models were built with all-subset regressions from the principal components estimated from differently treated transmission spectra of the samples; the model with the best combination of root mean square error (RMSE) 0.61% and root mean square prediction error (RMSPE) 0.69% in the estimation data and RMSPE 0.53% in the test data was selected as the final model; it showed an adequate fit in the estimation data (R^2=0.74) and a good prediction performance in the test data (R^2_P=0.90) (Jaakkola et al. 2007). In addition, the model was used to predict the total lignin content in earlywood samples of 45 individual annual rings of 9 stems (5 rings per stem). The annual rings with a very high and low weight density (years 1999, 1995, 1994, 1992, 1987) (rings 1–5) were chosen in trees with a high, average and low growth rate from

each of the three clones (3 trees per clone) from one site (Loppi) in order to maximise density variation. Loppi was selected by reason of the highest growth rate and high variation between trees. Finally, the measured total lignin (24 samples of 12 stems) and predicted total lignin (64 samples of 32 stems) contents were combined and analysed statistically with a univariate ANOVA using a mixed model followed by the Tukey HSD test. The predicted total lignin contents of earlywood (45 samples of 9 stems) were analysed with the one-way ANOVA followed by the Tukey HSD test. (II)

3.1.3 Coniferyl alcohol impregnation and fungal decay test

Green sapwood blocks (15 mm × 15 mm × 5 mm) were impregnated with an acetone-water solution of coniferyl alcohol (Fluka) containing hydrogen peroxide (H_2O_2) by using a standard vacuum impregnation method followed by a 20-hour incubation time at room temperature to allow for any reaction catalysed by inherent wood peroxidases. After impregnation, penetration of coniferyl alcohol and its reaction products in the sample blocks were studied with FTIR photoacoustic spectroscopy (PAS). A ratio of lignin and cellulose was determined from a ratio of intensities of two absorption bands (1267/1160 cm^{-1}, guaiacyl unit/cellulose) measured from the spectra of inner and outer parts of the blocks. Dimers of coniferyl alcohol (Fig. 3, compounds **1–3**) were identified in acetone extracts of powdered wood with a gas chromatography-mass spectrometric (GC-MS EI^+) method (Tiimonen et al. 2005), and the Klason lignin (Browning 1967, Dence 1992) and acid soluble lignin (Maekawa et al. 1989, Dence 1992) contents were determined. The coniferyl alcohol treated and non-treated sample blocks were exposed to the white-rot fungus (*Coriolus versicolor*) in a standardised fungal decay test. Weight loss of the samples was determined after the test. (III)

3.2 Lignin model compounds and synthetic lignin preparations

The model compounds 3-(3,4-dichloroanilino)-3-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy) propanol (Fig. 4, compound **6**) and 3-(4-chloroanilino)-3-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy) propanol (Fig. 4, compound **7**) were prepared by a reaction of quinone methide with 3,4-dicloroaniline (DCA) and 4-chloroaniline (CA) in dichloromethane under nitrogen at room temperature by using a method of Ralph and Young (1983). Quinone methide (Fig. 4, compound **5**) was prepared from 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol, guaiacylglycerol-β-guaiacyl ether (Fig. 4, compound **4**), by treatment first with bromotrimethylsilane in dichloromethane under nitrogen at room temperature and then with saturated sodium bicarbonate (Ralph and Young 1983, Brunow et al. 1989). The guaiacylglycerol-β-guaiacyl ether was synthesized from 4'-hydroxy-3'-methoxyacetophenone *via* a synthesis route comprising six steps (Adler and Eriksoo 1955, Landucci et al. 1981, Toikka and Brunow 1999). Then the methylated model compounds 3-(3,4-dichloroanilino)-3-(3,4-dimethoxyphenoxy)-2-(2-methoxyphenoxy) propanol (Fig. 4, compound **8**) and 3-(4-chloroanilino)-3-(3,4-dimethoxyphenoxy)-2-(2-methoxyphenoxy) propanol (Fig. 4, compound **9**) were prepared by treatment of the phenolic model compounds with dimethyl sulfate in the presence of sodium hydroxide in dioxane-water at 64–66 °C (Larsson and Miksche 1969). After that the model compounds were characterized with ^1H and ^{13}C NMR spectroscopy and with high resolution mass spectrometry (HRMS EI^+). $^1H/^{13}C$ NMR ($CDCl_3$): (**4**, acetylated) 6.1/74 (α), 4.6/80 (β), 4.1/4.4/63 (γ). (**6**, acetylated) 4.6/59 (α), 4.4/82 (β), 4.1/4.4/62 (γ).

The dehydrogenation polymers of coniferyl alcohol (DHPs) were prepared by slowly adding with peristaltic pumps a dioxane-buffer solution of coniferyl alcohol and a buffer solution of H_2O_2 to a reaction mixture containing a horseradish peroxidase (HRP) catalyst in disodium hydrogen phosphate buffer (pH 6.5) under argon at room temperature within 20 hours (Kirk and Brunow 1988). Coniferyl alcohol was polymerised together with and without DCA and CA. Coniferyl alcohol was prepared by the treatment of methyl ferulate with DIBALH (diisobutylaluminum hydride) in cooled toluene under argon (Quideau and Ralph 1992). Methyl ferulate was prepared by a reaction of ferulic acid with methanol in the presence of an acid catalyst at room temperature. Ferulic acid was synthesized from vanillin and malonic acid in pyridine-piperidine at room temperature (Pearl and Beyer 1951). The relative molecular mass distribution profiles of the acetylated DHPs with and without DCA were determined with high performance size exclusion chromatography (HPSEC). After that a DHP-DCA adduct (Fig. 5, compound **10**) was identified in the dehydrogenation mixture by comparing it with the model compounds with NMR spectroscopy (^{1}H, ^{13}C and HSQC-TOCSY) and with mass spectrometric methods (LC-MS APCI$^+$, MS-MS ES$^+$ and HRMS EI$^+$). HSQC-TOCSY ($CDCl_3$): (**10**, acetylated) 4.6/59 (α), 4.4/82 (β), 4.1/4.4/62 (γ). Finally, resistance to the mild acid hydrolysis was tested. (IV, V)

4. RESULTS AND DISCUSSION

4.1 Norway spruce cutting clones

4.1.1 Variation in the growth rate and wood properties

We studied variation in the growth rate, weight density and mechanical strength properties (MOE, MOR) of the Norway spruce cutting clones (A, B, C) growing in different soil and climatic conditions in Loppi, Imatra and Kangasniemi. In the whole material studied the average annual ring width was 3.15 mm, latewood proportion 14.12%, annual ring density 0.461 g cm^{-3}, latewood density 0.750 g cm^{-3} and earlywood density 0.414 g cm^{-3}. The mean modulus of elasticity (MOE) was 9.88 GPa, modulus of rupture (MOR) 67.51 MPa and weight density of the test samples (ρ_{12}) 414 kg m^{-3} in mature wood (Table 1a). Minima, maxima and standard deviations (SD) between the individual trees are presented in Table 1a. Minima, maxima and SDs between the individual annual rings are presented in Table 1 in the Publication I. A comparison of the standard deviations in Table 1c showed higher variation between the individual annual rings or trees than between the clones or sites. An average annual ring width of mature Norway spruce wood has been found to be 2.3 ± 0.8 mm (variation between 1.1–5.5 mm), latewood proportion 15.2 ± 6.7%, weight density (ρ_{12}) 462 ± 46 kg m^{-3}, MOE 12.6 ± 2.0 GPa and MOR 82.3 ± 12.5 MPa in trees growing in monoculture or mixed with birch in Finland, Sweden and Norway (Saranpää and Repola 2001). The growth rate of this clone material was higher than the reported average growth rate of Norway spruce. A mean MOR of *Picea abies* grown in northern Norway has been found to be 67.7 MPa, MOE 9.7 GPa and weight density (ρ_{12}) 410 kg m^{-3} (Okstad and Kårstad 1985), which was well in accordance with our results. Increased growth rate caused by thinning leads to only slight reduction of mean annual ring density but large variation has been found between annual rings and between trees in experiments with Norway spruce stands growing in southeastern Finland (Jaakkola et al. 2005). Increased growth rate caused by fertilisation

Table 1. Norway spruce cutting clones: a) Variation in the growth rate, wood properties, sapwood (SW) and heartwood (HW) lignin contents between the clones (A, B, C), sites (Loppi, Imatra, Kangasniemi) or individual trees (minimum, maximum, standard deviation in parenthesis). Variation in the earlywood (EW) lignin content between the individual annual rings with the very high and low weight density chosen in the three clones in Loppi. Significant (p≤0.05) differences (Tukey test) between the clones, sites or selected individual annual rings were marked with different letters (a, b, c). [i)]n=14, [j)]n=13, [k)]n=43. AR=annual ring, LW=latewood, TS=test sample.

	n	Stem Ø at 1.3 m mm	Stem Ø at 6.0 m mm	Stem height m	First living branch whorl m	MOE GPa	MOR MPa	HW lignin %		SW lignin %
Clone A	15	137.4 (25.3)	98.2 (22.6)	12.54 (1.21)	2.87 (1.81)	9.89 (1.24) ab[i)]	68.22 (11.39) ab[j)]	26.17 (0.53) a		25.84 (0.68) b
Clone B	15	123.3 (26.5)	78.5 (28.8)	11.29 (2.24)	2.57 (1.73)	10.60 (1.61) b	73.96 (10.45) b	26.08 (0.98) a		24.86 (1.37) a
Clone C	14	130.6 (21.4)	85.2 (19.3)	11.31 (1.44)	2.43 (1.25)	9.11 (1.01) a	59.89 (8.29) a	26.45 (0.82) a		25.56 (0.97) b
Loppi	15	140.3 (25.4)	101.7 (20.9)	12.80 (0.98)	3.24 (0.93)	9.63 (1.47) a	63.20 (9.01) b	26.19 (0.89) a		26.31 (0.62) a
Imatra	15	143.9 (12.0)	98.1 (12.0)	12.55 (0.78)	3.73 (1.25)	9.50 (0.94) a	64.52 (7.12) b	26.27 (0.82) a		24.91 (1.31) b
Kangasn.	14	105.4 (13.1)	60.5 (16.6)	9.67 (1.37)	0.79 (0.53)	10.62 (1.64) a[j)]	75.94 (14.05) a[j)]	26.21 (0.73) a		25.01 (0.60) b
Mean	44	130.4 (24.7)	87.4 (24.9)	11.72 (1.76)	2.63 (1.59)	9.88 (1.43) [k)]	67.51 (11.50)[k)]	26.23 (0.80)		25.42 (1.10)
Max.	–	186.0	147.0	14.20	5.60	12.47	97.65	28.48		27.79
Min.	–	85.0	36.5	7.80	0.20	6.96	40.42	24.80		23.17
Median	–	131.3	91.5	12.25	2.75	9.99	67.05	26.01		25.53
	n	AR width mm	LW proportion %	AR density g cm^{-3}	LW density g cm^{-3}	EW density g cm^{-3}	TS density, ρ_{12} kg m^{-3}		n	EW lignin %
Clone A	15	3.24 (0.57) ab	14.93 (1.71) b	0.465 (0.045) b	0.758 (0.061) b	0.417 (0.042)	417 (38) b	Ring 1	9	26.06 (0.49) b
Clone B	15	2.92 (0.44) a	15.34 (2.29) b	0.486 (0.038) b	0.776 (0.030) b	0.434 (0.040)	441 (43) b	Ring 2	9	26.47 (0.76) ab
Clone C	14	3.30 (0.62) b	11.80 (2.02) a	0.428 (0.030) a	0.711 (0.044) a	0.389 (0.025)	380 (30) a	Ring 3	9	25.95 (0.98) b
Loppi	15	3.70 (0.57) a	13.29 (2.83) a	0.426 (0.032) c	0.708 (0.048) b	0.377 (0.022)	403 (29) b	Ring 4	9	25.67 (0.78) b
Imatra	15	3.00 (0.21) b	14.33 (2.06) a	0.462 (0.024) b	0.750 (0.025) b	0.420 (0.022)	386 (22) b	Ring 5	9	27.14 (0.93) a
Kangasn.	14	2.76 (0.35) b	14.71 (2.68) a	0.497 (0.046) a	0.795 (0.047) a	0.447 (0.039)	456 (49) a			
Mean	44	3.15 (0.56)	14.12 (2.56)	0.461 (0.045)	0.750 (0.054)	0.414 (0.040)	414 (44)	Mean	45	26.26 (0.93)
Max.	–	4.86	19.42	0.562	0.845	0.504	533	Max.	–	28.77
Min.	–	2.15	7.02	0.361	0.630	0.399	340	Min.	–	24.64
Median	–	3.05	14.31	0.453	0.751	0.407	408	Median	–	26.20

b) Results of the covariance and variance analyses. p≤0.05 *, p≤0.01 **, p≤0.001 ***.

	F values									
	AR width	LW proportion	AR density	LW density	MOE	MOR	TS density, ρ_{12}	HW lignin	SW lignin	EW lignin
Site	14.278 ***	1.326	20.202 ***	14.401 ***	2.724	6.339 **	15.347 ***	0.031	17.845 ***	–
Clone	3.531 *	13.040 ***	20.314 ***	20.568 ***	4.635 *	7.023 **	10.157 ***	0.928	7.517 **	–
Site-clone	0.158	2.466	3.072 *	4.122 **	–	–	–	1.115	4.578 **	–
Year	20.928 ***	6.393 ***	16.983 ***	66.872 ***	–	–	–	–	–	4.536 **
Site-year	18.406 ***	6.645 ***	15.670 ***	22.721 ***	–	–	–	–	–	–
Clone-year	3.933 ***	1.575 **	4.271 ***	3.932 ***	–	–	–	–	–	–
Site-clone-year	2.411 ***	1.320	1.085	1.794 ***	–	–	–	–	–	–

c) Comparison of the standard deviations (SD).

	n	SD										
		AR width mm	LW proportion %	AR density g cm^{-3}	LW density g cm^{-3}	EW density g cm^{-3}	TS density kg m^{-3}	MOE GPa	MOR MPa	HW lignin %	SW lignin %	EW lignin %
Individual rings	45	–	–	–	–	–	–	–	–	–	–	0.93
Individual rings	843	1.32	7.34	0.077	0.125	0.070	–	–	–	–	–	–
Individual stems	44	0.56	2.56	0.045	0.054	0.040	44	1.43 [k)]	11.50 [k)]	0.80	1.10	–
Sites	3	0.49	0.74	0.036	0.044	0.035	36	0.61	7.00	0.04	0.78	–
Clones	3	0.20	1.94	0.029	0.033	0.023	31	0.74	7.07	0.19	0.51	–

leads to reduction of annual ring density and especially latewood density, and large variation has been found between individual trees in a nutrient optimisation experiment with a Norway spruce stand in northern Sweden (Mäkinen et al. 2002a). Faster growth rate increases both the latewood proportion and especially the relative proportion of earlywood (Mäkinen et al. 2002a). The increased growth rate may lead also to changes in tracheid dimensions such as

to an increase of lumen diameter and to a decrease of cell wall thickness and tracheid length (Mäkinen et al. 2002b) even though only small changes have been found in fertilisation and thinning experiments with Norway spruce stands in central and eastern Finland (Jaakkola et al. 2007). No clear correlation was found either between the annual ring width and annual ring density or between the latewood proportion and latewood density which agrees with reported studies (Saranpää 2003, Jaakkola et al. 2005). The MOR had a stronger linear correlation (R^2=0.76, n=43) with the weight density (ρ_{12}) than the MOE (R^2=0.47, n=43) which is also in accordance with earlier results (R^2_{MOR}=0.79, R^2_{MOE}=0.48) (Saranpää and Repola 2001).

The site was found to have a statistically significant effect on the annual ring width, weight density and MOR but not on the latewood proportion of annual rings and the MOE (Table 1b). Trees from the fertile site in Loppi showed significantly higher growth rate (ring width 3.70 mm) than trees from Imatra (3.00 mm) or Kangasniemi (2.76 mm). Loppi produced wood, which had lower latewood proportion, annual ring density, latewood density, earlywood density and MOR than that of wood grown in the two other sites. The medium fertile site farther north in Kangasniemi produced wood, which had higher latewood proportion, annual ring density, latewood density, earlywood density, MOR and MOE than that of wood grown in the two other sites. The latewood density and MOR varied significantly between Kangasniemi and the two other sites. The annual ring density varied significantly between all the sites (Table 1a).

The clone was found to affect significantly the growth rate, weight density and mechanical strength properties (MOE, MOR) (Table 1b). Clone B showed significantly lower growth rate (2.92 mm) than the clone C (3.30 mm). Clone B produced wood, which had higher latewood proportion, annual ring density, latewood density, earlywood density and mechanical strength properties than wood of the two other clones. The differences in the mechanical strength properties between the clone B and clone C were significant. Clone C showed significantly higher growth rate than the clone B, and produced wood which had lower latewood proportion, annual ring density, latewood density, earlywood density and mechanical strength properties than wood of the two other clones. The differences in the latewood proportion, annual ring density and latewood density between the clone C and two other clones were significant (Table 1a).

The site-clone interaction had a significant effect on the weight density but not on the growth rate. However, a pairwise comparison was omitted in the limited material of only five trees per clone per site. The site-clone-year interaction had a significant effect on the annual ring width and latewood density but not on the latewood proportion and annual ring density. The site-year and clone-year interactions had a significant influence on the growth rate and weight density. The annual variation in the growth rate and weight density was significant (Table 1b). (I)

4.1.2 Variation in the lignin content

Variation in the amount of lignin in Norway spruce has been studied recently among fertilised and non-fertilised trees (Anttonen et al. 2002), in a young wood among full-sib families (Wadenbäck et al. 2004) and within annual rings along a cross section of stem (Bertaud and Holmbom 2004) by using traditional wet chemical methods. We studied variation in the total lignin content among the three cutting clones and three different growing environments with calibrated mid infrared spectroscopy.

In the whole material the sapwood lignin content varied from 23.17% to 27.79% and heartwood lignin content from 24.80% to 28.48% between the individual trees. The lignin

content varied between 24.64–28.77% in the earlywood proportions of individual annual rings with the very high and low weight density in Loppi. The average lignin content of heartwood (26.23%) was slightly higher than that of sapwood (25.42%). The mean earlywood lignin content was 26.26% in the selected annual rings (Table 1a). The standard deviations between the individual trees are presented in Table 1a and compared in Table 1c. In heartwood (28.3 ± 0.3%) and earlywood (30.6 ± 1.7%) the lignin content is generally higher than in sapwood (27.7 ± 0.1%) and latewood (27.5 ± 1.0%), respectively, but no variation has been observed between annual rings (Bertaud and Holmbom 2004). The lignin content has been found not to vary among families of the same age but to increase with age (Wadenbäck et al. 2004).

The site, clone and site-clone interaction were found to have a significant effect on the sapwood lignin content (Table 1b). Trees from the fertile site in Loppi showed significantly higher sapwood lignin content (26.31%) than trees from Imatra (24.91%) or Kangasniemi (25.01%). The slowest growing clone B showed significantly lower sapwood lignin content (24.86%) than clone A (25.84%) and clone C (25.56%). No significant differences were found in the heartwood lignin content between the clones and between the sites (Table 1a). The lignin content of Norway spruce wood (27.6%) grown on a forest land has been found to be slightly lower than that of wood (28.9%) grown on an agricultural land in central Sweden (Brolin et al. 1995), which was in accordance with our results. Increased growth rate caused by fertilisation may lead to increased lignin content (by 7%) as has been found in a nutrient optimisation experiment with a Norway spruce stand in northern Sweden (Anttonen et al. 2002) even though only a slight increase (1–2%) has been found in fertilisation and thinning experiments with Norway spruce stands in central and eastern Finland (Jaakkola et al. 2007). The annual variation in the earlywood lignin content was significant (Table 1b). No correlation was found either between the lignin content and weight density of earlywood or between the sapwood lignin content and the mechanical strength properties (MOE, MOR) of mature wood. The lignin content in softwood, however, tends to increase with the decreasing density (Gindl 2002). (II)

The observations show that the growth rate, wood properties and lignin content vary more between the growing seasons and between the individual trees than between the clones and between the growing environments in this material. The fertile site in Loppi, however, showed higher growth rate and sapwood lignin content and produced wood with lower weight density and MOR compared with the two other sites. The medium fertile site in Kangasniemi showed lower growth rate and produced wood with higher weight density and strength properties compared with the two other sites. Clone C showed higher growth rate and lower weight density and strength properties whereas clone B showed lower growth rate and sapwood lignin content and higher weight density and strength properties than the two other clones. The environment had a large effect while the three clones differed from each other similarly in the different sites, e.g. the fastest growing clone was the fastest on all sites. Climatic factors affected growth of all the clones on all the three sites similarly. (I) (II)

4.1.3 Modification of lignin: Coniferyl alcohol impregnation

We impregnated the Norway spruce wood blocks with the natural monolignol, coniferyl alcohol, in the presence of H_2O_2 in order to utilise inherent wood peroxidase activity in the coniferyl alcohol polymerisation. This was based on the fact that xylem peroxidases remain active in the xylem cell walls for decades (Fagerstedt et al. 1998). A comparison of total ion chromatogram (TIC) signals from the acetone extracts of untreated wood and the dehydrogenation polymers of coniferyl alcohol (DHPs) with the TIC from the acetone extracts of impregnated wood

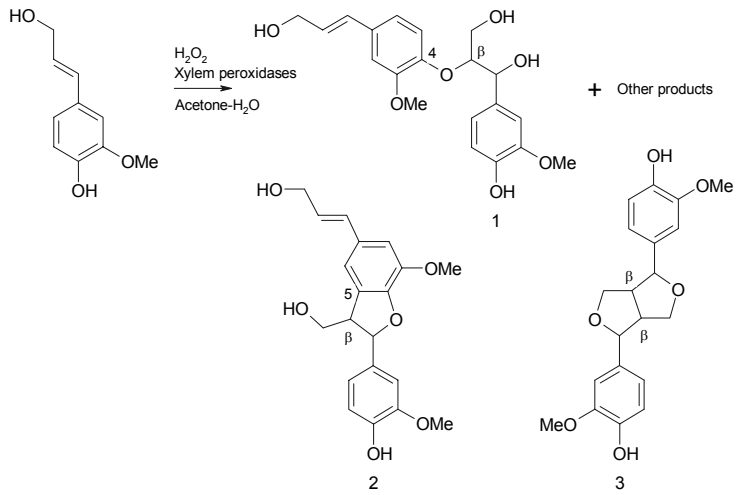

Figure 3. Preparation of guaiacylglycerol-β-coniferyl ether (1), phenylcoumaran (2) and pinoresinol (3).

suggested that the dimerisation or polymerisation products of coniferyl alcohol were formed during the impregnation. We identified the dimers guaiacylglycerol-β-coniferyl ether (β-O-4 structure, Fig. 3, compound **1**), phenylcoumaran (β-5, Fig. 3, compound **2**) and pinoresinol (β-β, Fig. 3, compound **3**) in the acetone extracts of impregnated wood with GC-MS EI^+. The β-O-4, β-5 and β-β structures are important structural units also in the lignin polymer. The Klason lignin and acid soluble lignin determinations showed that the lignin content was increased slightly during the coniferyl alcohol treatment. Coniferyl alcohol or its reaction products were found at or near the surface of the wood blocks with FTIR PAS and coniferyl alcohol was well penetrated into the wood. This treatment hindered fungal decay significantly in the standardised decay test with the white-rot fungus, *Coriolus versicolor*, and led to the dry weight loss of 8.8% in comparison with 19.9% in the control.

These observations show that there are possibilities to impregnate wood with natural phenolic compounds in order to increase its decay resistance. Peroxidases persist in an active form in Norway spruce wood throughout the year (Marjamaa et al. 2003) and for decades even in heartwood (Fagerstedt et al. 1998). It has been found previously that isolated differentiating xylem can be used to polymerise coniferin by enzymes naturally present in a spruce soft xylem (Hafrén et al. 2002). An impregnation of pine sapwood with vanillin and tannin has been found to lead to decreased weight losses caused by fungi but a leaching of reagents is a problem (Rättö et al. 2004). (III)

4.2 Incorporation of 3,4-dichloroaniline and 4-chloroaniline into lignin: Studies with the lignin model compounds and synthetic lignin preparations

The possibility of purifying soils by the covalent incorporation of xenobiotics, such as pesticide residues, into the lignin of growing plants has been studied earlier both *in vivo* and *in vitro* (Sandermann et al. 1983, 1990, 1992, Lange et al. 1998). We studied the incorporation of pesticide metabolites 3,4-dicloroaniline (DCA) and 4-chloroaniline (CA) into lignin with the lignin model compounds and synthetic lignin preparations. We found that DCA and CA

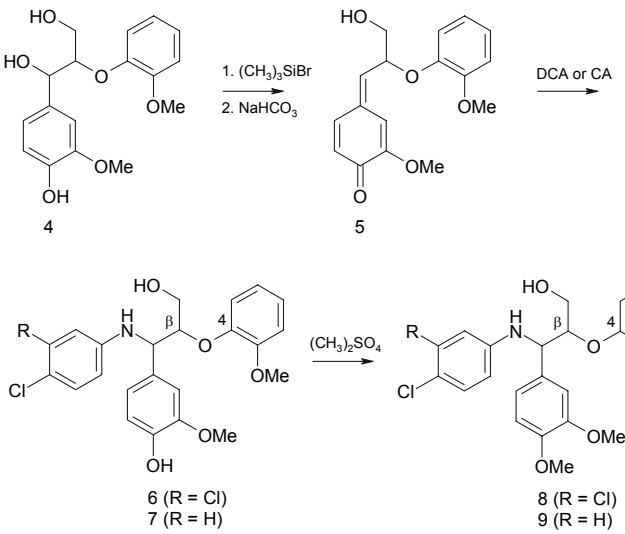

Figure 4. Preparation of the model compounds (6, 7) and methylated model compounds (8, 9).

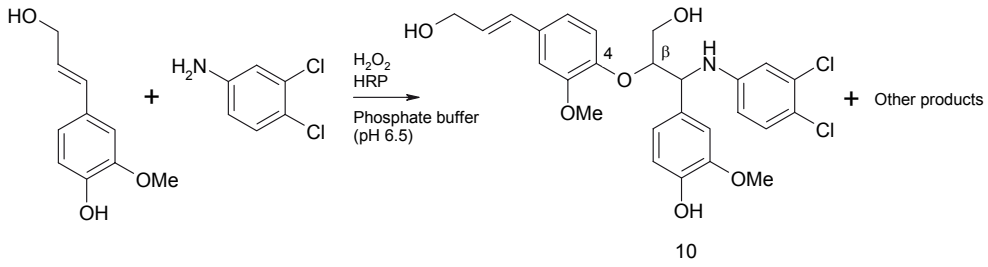

Figure 5. Preparation of the DHP-DCA adduct (10).

became bound by the benzylamine bond to the arylglycerol-β-guaiacyl ether structures in the polymer during the dehydrogenative polymerization of coniferyl alcohol. A comparison of the NMR spectra of the phenolic model compounds (Fig. 4, compounds **6, 7**) and etherified model compounds (Fig. 4, compounds **8, 9**) with the spectra of DHP-DCA and DHP-CA adducts proposed that most of the units in DHPs bound to chlorinated anilines were phenolic. The DHP with covalently bonded DCA had lower mass average molecular mass (M_w) than the pure DHP. The DHP-DCA adduct (Fig. 5, compound **10**) was identified in the dehydrogenation mixture with the mass spectrometric methods (LC-MS APCI$^+$ and MS-MS ES$^+$). The structure of this adduct was found to be the dimer of coniferyl alcohol with the β-O-4 structure and DCA bound by the benzylamine bond. The adduct seems not to react further with coniferyl alcohol during the dehydrogenation, thus the adduct formation causes the termination of the polymer growth.

These observations, the lower molecular mass values and the phenolic nature of the DCA and CA adducts, can be explained by the intermediate quinone methide being trapped by

3,4-dichloroaniline or 4-chloroaniline during the DHP preparation, which resulted in the formation of the dimer of coniferyl alcohol with the β-O-4 structure and chlorinated anilines bound by the benzylamine bond. The benzylamine bond was found to be resistant to mild acid hydrolysis under simulated stomach conditions, which indicates that residues bound in this way are not readily bioavailable in animals. (IV, V)

5. CONCLUSIONS

We studied the three different Norway spruce cutting clones in order to follow variation of the growth rate, wood properties and lignin content in three different growing environments, and in order to make use of wood peroxidases in the lignin modification with the natural monolignol, coniferyl alcohol. In addition, the chemical grounds for the incorporation of chlorinated anilines into lignin were studied with the lignin model compounds and synthetic lignin preparations. The known techniques, FTIR spectroscopy and PCR, were applied to the estimation of total lignin content in the heartwood, sapwood and earlywood samples. The FTIR-PCR based method developed and applied during this thesis work was shown to be a quick, reproducible and reliable tool in the estimation of lignin amount in softwoods.

Trees from the fertile site in Loppi showed the highest growth rate and sapwood lignin content and the lowest latewood proportion, weight density and strength (MOR). Trees from the medium fertile site in Kangasniemi had the lowest growth rate and the highest latewood proportion, weight density and mechanical strength properties (MOE, MOR). Clone C showed the highest growth rate and the lowest latewood proportion, weight density and mechanical strength properties. Clone B had the lowest growth rate and sapwood lignin content and the highest latewood proportion, weight density and mechanical strength properties. The weight density and lignin content are affected by the growth rate of trees, even though no correlation was found between them in the material studied here. However, the weight density and MOR correlated linearly. Due to the large variation in the lignin contents between the individual trees and between the growing seasons no clear distinctions, although some statistically significant differences, were found between the clones and between the growing environments.

The coniferyl alcohol impregnation lead to the increased content of different lignin-type phenolic compounds in the wood and improved the wood decay resistance against the white-rot fungus. Also chlorinated anilines were shown to incorporate into lignin *in vitro*; the intermediate quinone methide was seen to be trapped by 3,4-dichloroaniline or 4-chloroaniline during the DHP preparation, which resulted mainly in the dimer of coniferyl alcohol with the β-O-4 structure and with chlorinated anilines bound by the benzylamine bond.

Future investigations could comprise an application of FTIR spectroscopy for a large-scale screening of variation in the lignin content and chemical composition in Norway spruce and other tree species to find individuals, clones or mutants with an altered lignin content and/or quality, which can be used in a tree breeding and for various industrial purposes.

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