

Composition of Lignin in Outer Cell-Wall Layers

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Light microscopy cover illustrations: Top left: Developing spruce xylem in June. Top right: Developing poplar phloem in June. Bottom left: Spruce annual ring. Bottom right: Poplar xylem

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ABSTRACT

The composition of lignin in the outer cell-wall layers of spruce and poplar has been studied and the data obtained have been compared with those of the mature reference wood in which the secondary cell wall predominates. Materials with exclusively or predominantly outer cell-wall layers were examined. Accurate data relating to the lignin monomer composition and the number of β -O-4' bonds were obtained from pure middle lamella/primary cell wall lignin.

Firstly, a 10 000 year old white spruce material, with most of the secondary cell wall missing, was studied. The aged lignin was composed of guaiacyl units only, and was slightly more condensed but otherwise similar to the reference lignin.

Secondly, the developing xylem of a Norway spruce clone was analyzed during a growth season. In spring and early summer, growth is very rapid and the intention was to sample tissues in which the secondary cell-wall layers had not yet lignified, but where the outer layers at least had started to lignify. Microscopy, Klason lignin and carbohydrate analyses showed that the lignin in the developing xylem of samples from mid-June was located exclusively in the middle lamella. The lignin was more condensed, was composed of guaiacyl units only and contained more end-groups than the reference Norway spruce wood.

Thirdly, the cambial tissues of a Balsam poplar clone were surveyed during a growth season. Both the phloem side and the xylem side of the cambial region were examined. The Klason lignin content and carbohydrate monomer distribution showed that in June and August the tissues on the phloem side contained material with mainly middle lamella/primary walls. In June, the xylem side in the cambial region contained mainly middle lamella/primary walls, and in August the secondary cell wall carbohydrates were being deposited. Both tissues contained lignin that was more condensed and had more end-groups than the reference lignin. In mid-June, the developing xylem had a ratio of syringyl to guaiacyl units of 0.6, whereas the ratio for the reference wood was 1.3.

In the final study, lignin from the primary cell walls from a hybrid aspen cell suspension culture was investigated. The lignin contained only guaiacyl units which were more condensed than those observed in the reference poplar wood.

Keywords: Lignin, thioacidolysis, primary wall, middle lamella, *Populus balsamifera*, *Populus tremula* L. x *P. tremuloides* Michx., *Picea abies*, *Picea glauca*,

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Till Kyllikke

List of papers

This thesis is based on the following papers, which in the text are referred to by their roman numerals:

- I. Christiernin, M., N. Shannon, L. Zhang, T. Nilsson, and G. Henriksson, *Comparison between 10 000 year old and contemporary spruce lignin*, Manuscript.
- II. Christiernin, M., *Composition of lignin in developing xylem of spruce*. Manuscript.
- III. Christiernin, M., *Composition of lignin in cambial tissues of poplar*. Submitted.
- IV. Christiernin, M., A.B. Ohlsson, T. Berglund, and G. Henriksson, *Lignin isolated from primary walls of hybrid aspen cell cultures indicates significant differences in lignin structure between primary and secondary cell wall*. *Plant Physiology and Biochemistry*, 2005. **43**(8): p. 777-785.

Related conference proceedings:

- i. Christiernin, M., L. Zhang, T. Nilsson, and G. Henriksson. *Analysis of lignin with secondary cell wall removed*. in *13th ISWFPC (International Symposium on Wood, fibre and Pulping Chemistry)*. 2005. **3**(73-80), Auckland, New Zealand. Reviewed
- ii. Christiernin, M., A.B. Ohlsson, T. Berglund, and G. Henriksson, *Lignin isolated from primary walls of hybrid aspen cell suspension cultures is different from secondary cell wall lignin*. in *13th ISWFPC (International Symposium on Wood, fibre and Pulping Chemistry)*. 2005. **3**(81-86), Auckland, New Zealand. Reviewed

In addition, I have been involved in research projects which go beyond the theme of the thesis. These have resulted in the following publications:

- iii. Christiernin, M., *Biological Role and Technical application of Xyloglucan endotransglycosylase and Xyloglucan*, Licentiate thesis, in *Department of Biotechnology*. 2002, Royal Institute of Technology: Stockholm. p. 53.
- iv. Yan, H., T. Lindström, and M. Christiernin, *Some ways to decrease fibre suspension flocculation and improve sheet formation*. *Nordic Pulp & Paper Research Journal*, 2006. **21**(1): p. 36-43.
- v. Henriksson, G., M. Lawoko, M. Christiernin, and M. Henriksson, *Monocomponent endoglucanases – An excellent tool in wood chemistry and pulp processing*. in *13th ISWFPC (International Symposium on Wood, fibre and Pulping Chemistry)*. 2005. **2**(503-508) Auckland, New Zealand. Reviewed
- vi. Henriksson, G., M. Christiernin, and R. Agnemo, *Monocomponent endoglucanase treatment increases the reactivity of softwood sulphite dissolving pulp*. *Journal of Industrial Microbiology & Biotechnology*, 2005. **32**(5): p. 211-214.
- vii. Christiernin, M. and H. Yan, *Improvement of paper properties by xyloglucan*. in *59th Appita Annual Conference Pre-Symposium; Chemistry and performance of composites and natural plant fibres*. 2005. **A9**: p 41-45, Rotorua, New Zealand. Reviewed
- viii. Christiernin, M., G. Henriksson, M.E. Lindström, H. Brumer, T.T. Teeri, T. Lindström, and J. Laine, *The effects of xyloglucan on the properties of paper made from bleached kraft pulp*. *Nordic Pulp & Paper Research Journal*, 2003. **18**(2): p. 182-187.
- ix. Bourquin, V., N. Nishikubo, H. Abe, H. Brumer, S. Denman, M. Eklund, M. Christiernin, T.T. Teeri, B. Sundberg, and E.J. Mellerowicz, *Xyloglucan endotransglycosylases have a function during the formation of secondary cell walls of vascular tissues*. *Plant Cell*, 2002. **14**(12): p. 3073-3088.
- x. Berglund, P., M. Christiernin, and E. Hedenström, *Enantiorecognition of chiral acids by Candida rugosa lipase: two substrate binding modes evidenced in an organic medium*. *ACS Symposium Series*, 2001. **776** (Applied Biocatalysis in Specialty Chemicals and Pharmaceuticals): p. 263-273.

List of contributions to the papers in this thesis:

Paper I ;

Shannon Notley and Maria Christiernin carried out the AFM imaging, Liming Zhang interpreted the NMR spectra. Thomas Nilsson contributed with the 10 000 year old spruce material, Gunnar Henriksson was supervisor. All other work was done by Maria Christiernin.

Paper II and III

Anni Hagberg embedded and cut all samples for microscopy and carried out light microscopy. All other work was done by Maria Christiernin.

Paper IV;

Anna Ohlsson and Torkel Berglund cultivated the cell suspensions, carried out enzyme activity assays and the phloroglucinol staining of cultures, Gunnar Henriksson was supervisor. All other work was done by Maria Christiernin.

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1 BACKGROUND

Second to cellulose, lignin is the most abundant biopolymer on earth. Its composition affects the properties of wood when it is used as a construction material and the properties of the fiber with respect to, for example, pulping quality and forage digestibility. In order to improve the properties of plants in specific applications it is essential to reach a deeper understanding of both the molecular components and the structure of the plant fibers. In the following paragraphs, the evolution of plants is briefly described. Commercial forestry with tree clones is introduced, and an overview of the cell wall architecture and constituents is given. Biosynthesis of monolignols is presented and the literature regarding lignin analysis from transgenic and natural plants is briefly surveyed. In section four there is a glossary over terms and abbreviations.

1.1 Plant evolution

The first terrestrial plants were bryophytes, of which mosses are the most commonly known. They lack lignin and true vascular tissue with specialized cells that can transport water and nutrients efficiently within the plant. The earliest plants with vascular tissue containing lignin developed 400 million years ago, of which club mosses, ferns and horsetails are surviving plants today, *Figure 1.1.A*. The next step in evolution resulted in plants reproducing by means of seeds, *Figure 1.1.B*. They can be separated into gymnosperms and angiosperms that have a more sophisticated differentiation of their tissues and cells, *Figure 1.1.C*. These plants have flowers and seeds covered with a protecting hull that can survive in some cases for thousands of years, and they have become the dominating class of plants today. Most angiosperms belong to one of two classes; monocotyledons and eudicotyledons. Grass, cereals, bamboo and palms are examples of the monocotyledonous class that contains 65 000 species. The eudicotyledonous class contains approximately 165 000 species, of which 25 000 are hardwood trees. Conifers comprise approximately 500 species (Raven *et al.*, 1999).

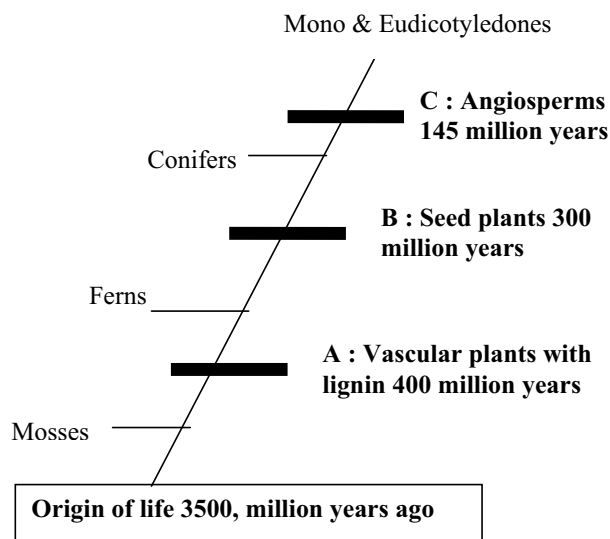


Figure 1.1. Evolution of Plants

1.2 Trees as a commercial crop

In farming, crop selection of improved plants has been practiced for thousands of years, but in the case of trees this development started only in the 1930's with plantations of selected spruce trees with improved quality. The first hybrid aspen *Populus tremula* × *Popula tremuloides* was produced in 1939 in Sweden, the research being carried out to find better

qualities for making matches. The trials ended in the 1960's since it was cheaper to produce the wood outside Sweden. In the 1980's there was an increase in short rotation forestry research due to the 500 000 ha of surplus farming land available when the Swedish government decided to cut back agriculture subsidies (Elfving, 1986). Some 300 hybrid aspen clones were used in trials at the Forestry research Institute of Sweden (Skogforsk) with the aim of producing high quality material for tree plantations. It was successful in terms of finding clones that grew well, 25 m³/ha·year with a rotation period of 20-25 years, and with few trees attacked by pathogenic fungi (Rytter *et al.*, 2002). These figures can be compared to growth rates of 16m³/ha·year from clones obtained in the 1940-1950 period (Johnsson, 1952). In spite of this success, there are still no commercial plantations of hybrid aspen or birch in Sweden, even though pulp and paper industries are now importing birch and poplar for paper pulp production. One reason for this is the resistance towards planting trees on farmland. Another is that in areas with a large elk population it is necessary to fence in the plantations to avoid grazing damage. Research to improve deciduous trees for timber production and to increase biodiversity are projects at Skogforsk that have presently reached field trials of thousands of hectares of poplar, birch, alder, oak, beech, ash, wild cherry, lime, mountain ash and maple. Hardwood forestry on agricultural land or former softwood land may improve the economy for the pulp, paper and timber industry and also increase the recreational value of these areas (Karacic, 1999).

Since the time to harvest ranges from 14 years for poplar to 80 years for spruce in Sweden, it takes a long time before the quality aspects can be truly evaluated. These include high resistance to pathogens, high growth rates as well as a high quality of the wood. In trials with 5000 Norway spruce clone seedlings in mid-Sweden during the 1990's, it was found that the best clones showed a 39% better growth rate than average seed plants (Sonesson and Almqvist, 2002). Poplar and some other trees can be propagated with vegetative techniques such as tree cuttings, rootsucklings and hormone treatments of plant tissue cultures, all giving genetically cloned tree plants. Vegetative propagation is however much more expensive than seed plantations. If one plants poplar trees and finds improved individuals say 10 years later, it is at least possible to use vegetative propagation methods to produce clones at an elevated cost. This is not feasible for spruce, where tree cuttings only sprout roots when they are taken from a very young plant and, to my knowledge, tissue cultures cannot be induced to form spruce seedlings. Spruce flowers naturally at the age of 20 to 30 years in Sweden. After this age, it flowers every 3-4 years provided the weather is warm for some weeks after midsummer during the previous year. Therefore, the recent finding of the *Flowering Locust T (FT)* gene in *Arabidopsis* (Huang *et al.*, 2005) is important, as it affords a new possibility of inducing flowering early for tree breeding.

To speed up the process of spruce breeding and clone production, somatic embryogenesis has been investigated during the last 20 years, a technology which introduces a paradigm shift for the improvement of softwoods. With this method, it may be possible to produce improved clones on a large scale at a cost that approaches that of ordinary spruce seedlings. Somatic embryogenesis involves saving immature seed embryos of chosen trees in liquid nitrogen until they are needed. When plantation trials have been evaluated, each individual cell from the seed embryo can be propagated to a cloned seedling. It is not however easy to ensure that the cells survive to be seedlings, and different clones can require different methods in order to survive (Högberg *et al.*, 1998). Commercial technologies are available for producing "manufactured seeds" from somatic embryogenesis. One option is to have a bio-reactor that delivers somatic embryos into manufactured seed hulls in a fully automated system (Weyerhaeuser, 2003). These "seeds" can germinate in a nursery or directly at the plantation

site. Another possibility is to use a system where naked embryos without hulls are germinated (Sutton, 2002). The somatic embryogenesis technology together with the possibility of inducing early flowering will greatly enhance tree breeding possibilities in the future.

1.3 Plant morphology

Plant growth is initiated in the meristems, which have the capacity to produce new plant cells throughout the life of the plant. When initial cells divide, one of the cells remains meristematic while the other differentiates. The plant tissues are organized into three tissue systems; dermal tissue, vascular tissue and ground (fundamental) tissue. The dermal tissues make up the surface layer of plants, and the vascular tissue transports water and nutrients. In leaves the ground tissue is the mesophyll where photosynthesis takes place, whereas in the stem it is pith and cortex, and in roots cortex only. Ground tissue is composed of parenchyma, collenchyma and sclerenchyma cells. Parenchyma are living cells with different sizes and different wall thickness. Sclerenchyma tissues are dead cells that lack protoplast when they are fully developed. They have thickened lignified secondary cell walls and exist as two types: fibers, which are long and slender, and sclereids, which are shorter and make up seed coats and the shells of nuts.

The vascular cambium is a cylindrical sheet of undifferentiated meristematic cells from which the vascular tissue originates. These cells differentiate into secondary phloem outwardly and secondary xylem inwardly *i.e.* wood. The xylem transports water through the plant and the phloem transports nutrients, generated in the photosynthetic regions of the plant. In gymnosperms, the water-transporting cells of the xylem are known as tracheids. In angiosperms, the cells are more diversified, and the water-conducting cells, vessels, have perforations at the ends that promote water transport. Phloem cells are called sieve cells in gymnosperms and sieve tube elements in angiosperms. *Table 1.3.1* lists the mature differentiated cell types present in vascular tissue.

Table 1.3.1. Mature differentiated cells of the xylem and phloem.

ML=middle lamella P=primary wall SEC=secondary cell wall

Cell types in vascular tissue	Function	Cell-wall layer in mature cell
Xylem		
Tracheids (gymnosperms and angiosperms)	Transportation of water, support, dead at maturity	ML, P, SEC
Vessels (angiosperms)	Transportation of water, dead at maturity	ML, P, SEC
Fibers	Support sometimes storage	ML, P, SEC
Parenchyma	Storage, live at maturity	ML, P
Phloem		
Sieve cells (gymnosperms)	Long distance transportation of nutrients, live at maturity	ML, P
Sieve-tube elements (angiosperms)		ML, P
Sclerenchyma	Support sometimes storage, dead at maturity	
Fibers		ML, P, SEC
Sclerids		ML, P, SEC
Parenchyma	Storage, live at maturity	ML, P

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When the growth season begins in the spring, cell division is very rapid in the cambial zone, and the resulting cells walls are rather thin (early wood). Later in the season, cell division slows down and cell walls become thicker (late wood). This is clearly seen in softwood but is less evident in hardwoods (Brett and Waldron, 1996; Raven *et al.*, 1999).

Microscopy images obtained with different techniques show what the cambium and adjacent xylem and phloem of Norway spruce look like, *Figure 1.3.1 A-E*. Image A shows lignin visualized by immunolocalization with lignin antibodies. The phloem is labeled in certain areas, whereas late-wood xylem cells are outlined. Ray cells and the resin canal are most intensely labeled. Image B is a thin section from June stained with acriflavin. Close to the bottom, the annual ring can be seen and above it the thin-walled early-wood xylem cells the phloem is green in color. Image C is a magnification of A showing the intense lignin labeling of the middle lamella and S3 layer, but the lignin is not visible in the phloem at this magnification. Image D is a fresh thin section from the same area as A stained with acriflavin but imaged with only one laser beam. The phloem is out of focus, since it is softer than the xylem. The variation in color shows that the composition varies in different areas; the thick-walled xylem ray cell with its many pits is clearly shown. In image E an annual ring is shown, where the difference in cell wall thickness between an early-wood fiber (top) and a late-wood fiber is seen.

Figure 1.3.2 A-F shows microscopy images of balsam poplar. The same types of techniques have been used as in *Figure 1.3.1*. Image A (collected in April) and B (collected in October) look similar except for the conducting phloem cells which are clearly revealed only with acriflavin staining (B). Also note the cambium and that a number of new phloem cells have been formed during the growth season (B). The phloem fiber cells with their thick walls are intensely labeled in both images. The many uneven large cells are the vessels, characteristic of hardwoods. At a higher magnification (image C) the immunolabelling shows a less clear image than acriflavin staining (D). In October, cell debris is seen in the phloem and xylem of the vascular cambium (E), and the difference in wall thickness between late-wood and early-wood is evident in the annual growth ring (F).

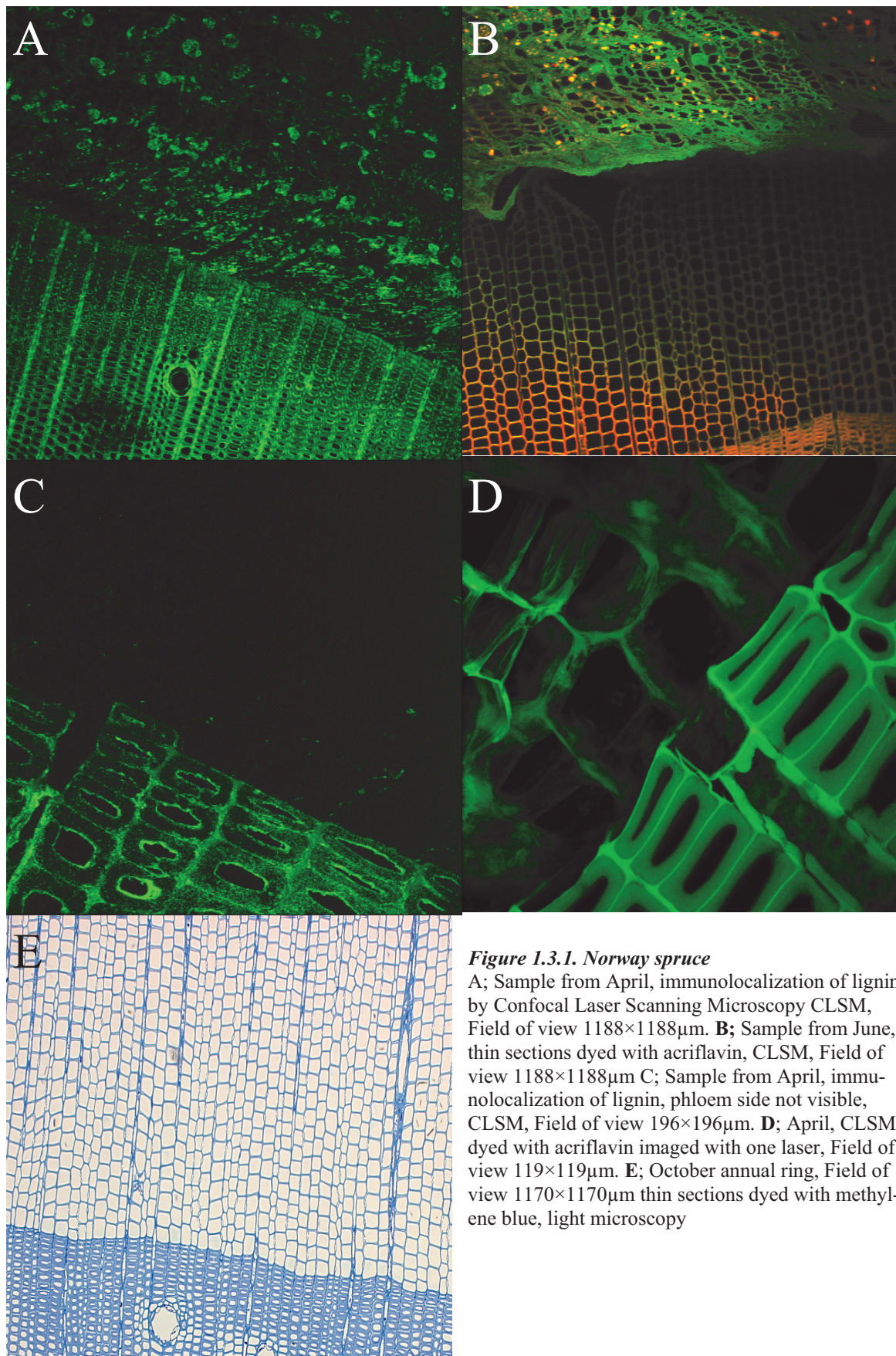


Figure 1.3.1. Norway spruce

A; Sample from April, immunolocalization of lignin by Confocal Laser Scanning Microscopy CLSM, Field of view $1188 \times 1188 \mu\text{m}$. **B**; Sample from June, thin sections dyed with acriflavin, CLSM, Field of view $1188 \times 1188 \mu\text{m}$ **C**; Sample from April, immunolocalization of lignin, phloem side not visible, CLSM, Field of view $196 \times 196 \mu\text{m}$. **D**; April, CLSM dyed with acriflavin imaged with one laser, Field of view $119 \times 119 \mu\text{m}$. **E**; October annual ring, Field of view $1170 \times 1170 \mu\text{m}$ thin sections dyed with methylene blue, light microscopy

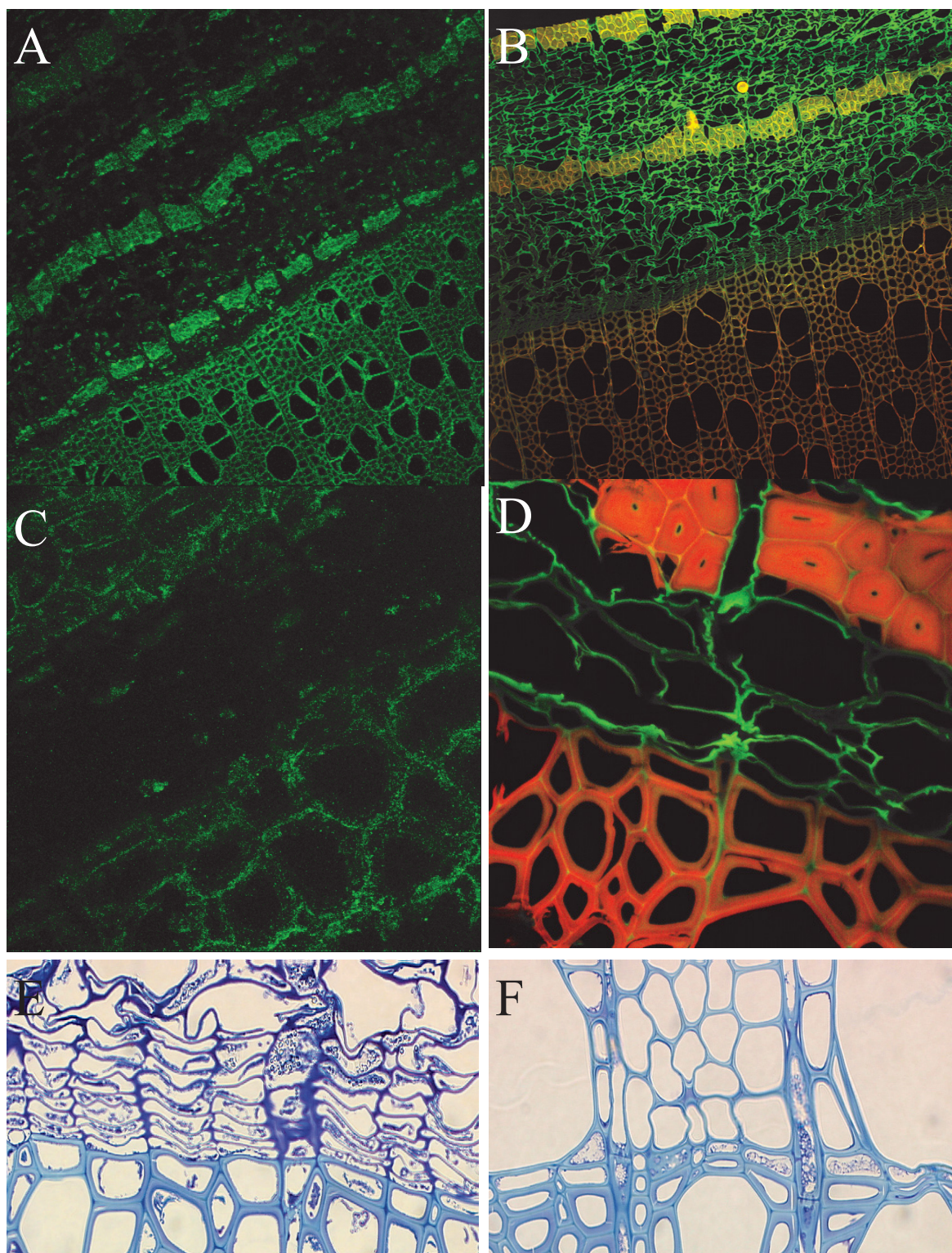


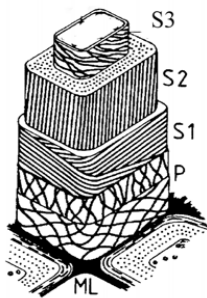
Figure 1.3.2. *Balsam poplar*

A; Sample from April, immunolocalization of lignin, CLSM, Field of view $1188 \times 1188 \mu\text{m}$. **B;** Sample from October, thin sections dyed with acriflavin, CLSM, Field of view $1188 \times 1188 \mu\text{m}$ **C;** Sample from April, immunolocalization of lignin, phloem side visible, CLSM Field of view $119 \times 119 \mu\text{m}$. **D;** April, CLSM dyed with acriflavin Field of view $119 \times 119 \mu\text{m}$. **E;** Sample from October, thin sections dyed with methylene blue, developing xylem and conducting phloem, light microscopy, Field of view $1170 \times 1170 \mu\text{m}$. **F;** October annual ring, Field of view $1170 \times 1170 \mu\text{m}$

1.4 Cell wall architecture

1.4.1 Plant cell wall

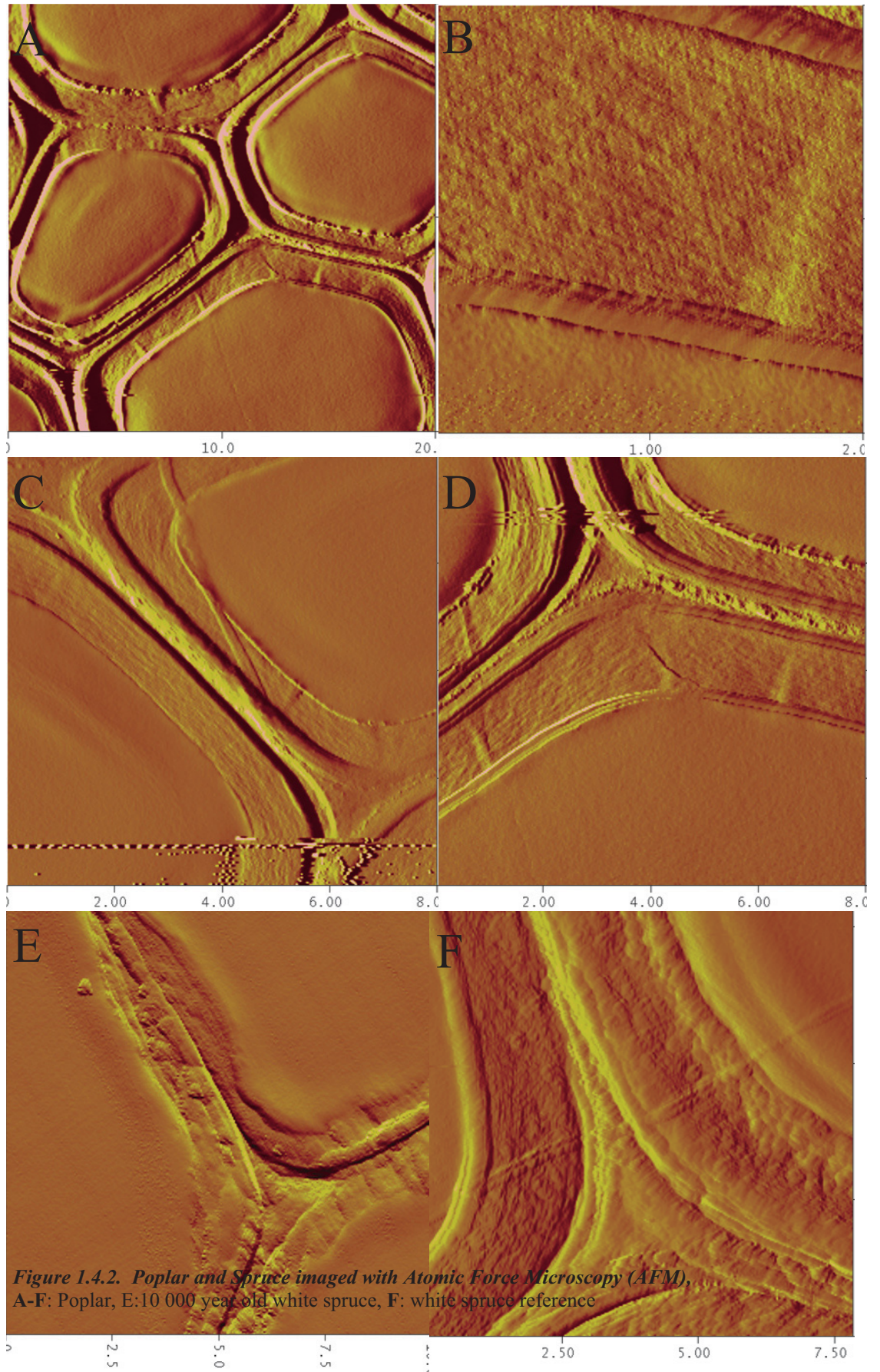
The cell wall gives shape and strength to the cell, but it permits a metabolic turnover of some of its constituents especially during seed germination, fruit ripening, abscission and ageing. The structure of the cell wall efficiently protects the plant from pathogenic attack. Furthermore, the cell deposits new wall material such as lignin and callose in response to tissue damage and environmental stress. The cell wall consists of several main layers: middle lamella, primary wall and secondary wall. The outermost layer is the middle lamella that is formed during cell division. Thereafter the primary wall is laid down, and this continues to be deposited as long as the cell is growing in size. According to the multinet growth hypothesis (Roelofsen and Houwink, 1953), the newly laid down cellulose layer on the inner surface of the cell wall is positioned transversely to the growth axis of the cell. As the cell elongates, the layers become extended in the direction of growth, so that the fibrils closest to the plasma membrane tend to be transversal and the outer layers random or longitudinal with respect to the cell growth direction (Preston, 1982). This is particularly evident in parenchyma cells. Other cell types, for example epidermal cells, exhibit alternating layers in a crossed polylamellate structure (Chafe, 1972). Some cells have only middle lamella and primary wall, but others continue to build up a secondary cell wall when the primary wall has finished expanding. The secondary wall consists of an outer layer (S1), a middle layer, which is the thickest, (S2) and an inner layer (S3), the latter bordering on the plasma membrane as shown



in *Figure 1.4.1*. The outermost layer is laid down first, followed by consecutive layers terminating with the S3 layer. *Figure 1.4.2* shows images of poplar and spruce wood cells obtained by atomic force microscopy (AFM), amplitude images. The lines passing through the cell wall are artifacts introduced during microtoming of the thin sections. Images A-D are poplar, E is a 10 000 year old White spruce that lacks most of the secondary cell wall, and F is a White spruce reference cell corner. Middle lamella/primary wall, S2 and S3 are clearly seen. In the S2 layers, and the cellulose fibrils can be observed. Interestingly, the primary wall and the S3 layer appear to be similar in the AFM images. The scale is marked in μm under each image.

Figure 1.4.1. Schematic picture of the principal layers of the cell wall

Middle lamella (ML), primary wall (P) and secondary wall (S). The secondary wall consists of outer (S1), middle (S2) and inner (S3) layers; the cellulose fibrils have different angles of orientation in the different layers of the cell wall, (adapted from Fengel and Wegener, 1984)



1.5 Cell wall constituents

The layers in the cell wall consist of bundles of cellulose fibrils in a matrix of pectins, hemicelluloses, proteins, lignins and small amounts of other phenolic compounds. The resulting dynamic network is a three-dimensional structure, which varies in composition depending on cell type, species, age and layer in the cell wall (Keegstra *et al.*, 1973; McNeil *et al.*, 1984; Varner and Lin, 1989; Talbott and Ray, 1992; Carpita and Gibeaut, 1993).

1.5.1 Cellulose

The cellulose fibrils consist of bundles of 30–100 unbranched glucopyranose chains connected by β -D-(1 \rightarrow 4) glycosidic bond, with a degree of polymerization of up to at least 15000 (Brett and Waldron, 1986). The fibrils are highly crystalline, but X-ray diffraction patterns and NMR-studies suggest that the surface area is less ordered than the core (Newman, 1998; Wickholm *et al.*, 1998). The fibrils are held together by hydrogen bonds both within the polymer chains and between neighboring chains. When the cell grows, the internal osmotic pressure, turgor, is the driving force. Since the primary cell walls are relatively thin, the tensile force on the cellulose fibrils is several orders of magnitude greater than the cell turgor pressure (Nobel, 1974). It is accepted that the different orientations of the fibrils in separate wall layers, together with the matrix compounds, impart the necessary tensile strength to the cell wall. In addition, the orientation of the fibrils controls the direction of extension of the cell (Carpita and Gibeaut, 1993).

1.5.2 Hemicelluloses

Hemicelluloses are mainly xylans, glucomannans, glucuronomannans, galactomannans, arabinogalactan II and xyloglucan. They are defined as the material extractable from the cell wall by alkali treatment, and their composition varies depending on species, cell type and cell-wall layer. It is thought that most hemicelluloses bind strongly to the cellulose fibrils through hydrogen bonding (Brett and Waldron, 1996).

Xylans have a backbone of xylose residues substituted by 4-O-methylglucuronic acid on some xylose residues and by arabinose on others. Diverse side chains consisting of arabinose and xylose or galactose, xylose and arabinose have been reported. The primary walls of most monocotyledonous plants contain arabinoxylan as the main hemicellulose, glucuronoarabinoxylan is the major hemicellulose in the secondary walls. The principal hemicellulose in the secondary wall of eudicotyledonous plants is glucuronoxylan and they contain very little arabinose.

Glucomannan is the major hemicellulose in the secondary cell wall of conifers. The polymer has a backbone of glucose and mannose residues in a ratio of 1:3. When the main chain is substituted with single residues of galactose, the polysaccharide is called galactoglucomannan.

Xyloglucan is the main hemicellulose in the primary walls of conifers and eudicotyledonous plants, and it is also a storage polysaccharide in certain seed endosperm cell walls. It has a backbone consisting of D-glucose units connected by β (1 \rightarrow 4)-glycosidic bonds, with side groups of xylose attached through α (1 \rightarrow 6)-glycosidic linkages. Certain xylose residues are substituted with galactose or with the disaccharide Fucose α -D-(1 \rightarrow 2)-Galactose- β -D-(1 \rightarrow 2). The xyloglucan polymer is long enough to form tethers between two or more cellulose fibrils (Pauly *et al.*, 1999). In the primary wall, xyloglucan is believed to play an important role during cell wall expansion. Xyloglucan binds specifically to cellulose fibrils by hydrogen

bonds (Valent and Albersheim, 1974), and it is believed that transglycosylating enzymes, XETs, modify xyloglucan by internal cleavage followed by coupling of the new end to another xyloglucan polymer. By cutting certain xyloglucan tethers between cellulose fibrils and inserting new material, it mediates the controlled cell-wall loosening necessary for plant growth and cell wall modification (Smith and Fry, 1991).

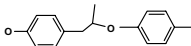
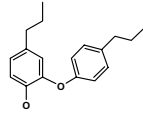
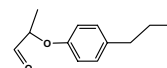
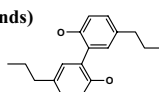
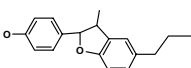
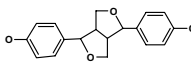
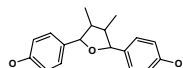
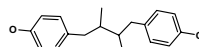
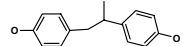
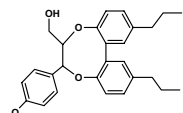
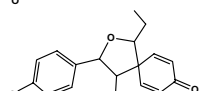
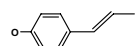
1.5.3 Pectin

Pectins are polysaccharides rich in galacturonic acids, rhamnose, arabinose and galactose. They influence the pH and the ion balance in the wall, and they determine the cell wall porosity which affects the accessibility of the cell for the intrinsic exchange of molecules, enzymes and pathogens. Pectins have been defined as the material extractable from the cell wall by hot water containing Ca^{2+} chelators such as EDTA or hot dilute acids. Integrated mainly in the middle lamella and in the primary cell wall, they form a network predominantly independent of the cellulose-hemicellulose network. Pectins are complex polymers and include homogalacturonan, HGA, which is composed of (1-4) α -D-galacturonic acids. Xylogalacturonans are HGA's substituted with α -D xylose units. Rhamnogalacturonan I is a repeating disaccharide of $\rightarrow 2) \alpha$ -D-Rhamnose-(1 \rightarrow 4) α -D-(1 \rightarrow Galacturonic acids that may be esterified with methanol. This disaccharide is further substituted on some rhamnose residues with arabinans, galactans and arabinogalactans (Brett and Waldron, 1996).

1.5.4 Lignin

Lignin strengthens the cell wall, allows plants to grow tall and also protects them against pathogenic infections. It is a 3-dimensional polymer synthesized by radical coupling of mainly three 4-hydroxyphenylpropanoids; *coumaryl alcohol*, *coniferyl alcohol* and *sinapyl alcohol* (Erdtman, 1933, 1957; Freudenberg and Neish, 1968; Adler, 1977). These monolignols are linked with bonds of either the carbon-carbon (condensed bond) or the ether type (non-condensed), *Table 1.5.4*. The monomer compositions can vary depending on plant species, cell type and even cell-wall layer, resulting in what appears to be a racemic heterogeneously linked random polymer. In softwood, lignin consists mainly of guaiacyl units, i.e., structures derived from coniferyl alcohol, whereas in hardwoods the major unit is syringyl, derived from sinapyl alcohol (Sarkanen and Hergert, 1971).

Table 1.5.4. Types and percentage of linkages between monolignols present in wood.
The percentage vary depending on analysis method (Henriksson, 2005)

Name	Bonds	Structure*	Frequency per 100 aromatic rings Softwood	Hardwood
Ether bonds				
β -aryl ether	β -O-4'		35 - 50	50 - 60
Diaryl ether	4-O-5'		<4	7
Glyceraldehyde aryl ether			<1	<1
Carbon-carbon bonds (condensed bonds)				
Dihydroxy biphenyl	5-5'		10	5
Phenyl coumarane	β -5'		9- 12	6
Pinoresinol	β - β '		2 - 3	3- 4
Neo-olivil	β - β '		<3	-
Secoisolaricinol	β - β '		<3	-
Diaryl propane 1,3-diol	β -1'		1 - 2	1
Other structures				
Dibenzodioxocin	5-5'-O-4		4 - 5	Trace
Spiro-dienone	β -1' α -O- α '		1 - 3	2 - 3
End group			1 - 6	Trace - 6

* Note that only the "carbon skeleton" in the structures is shown. In the complete structures, hydroxyls, methoxy groups and ether- and carbon-carbon bonds to other monolignols shall be added

1.5.5 Monolignol synthesis

The first step in lignin monomer formation is the synthesis of phenyl alanine and tyrosine in the Shikimic acid pathway. The second step uses several enzymes in the phenyl propanoid pathway to convert these amino acids into their hydroxy cinnamic acid derivatives. In the third step, the monolignols are formed (Grisebach and Hahlbrock, 1974; Higuchi, 1990; Whetten and Sederoff, 1995; Lewis *et al.*, 1999). For a simplified scheme see Figure 1.5.5.

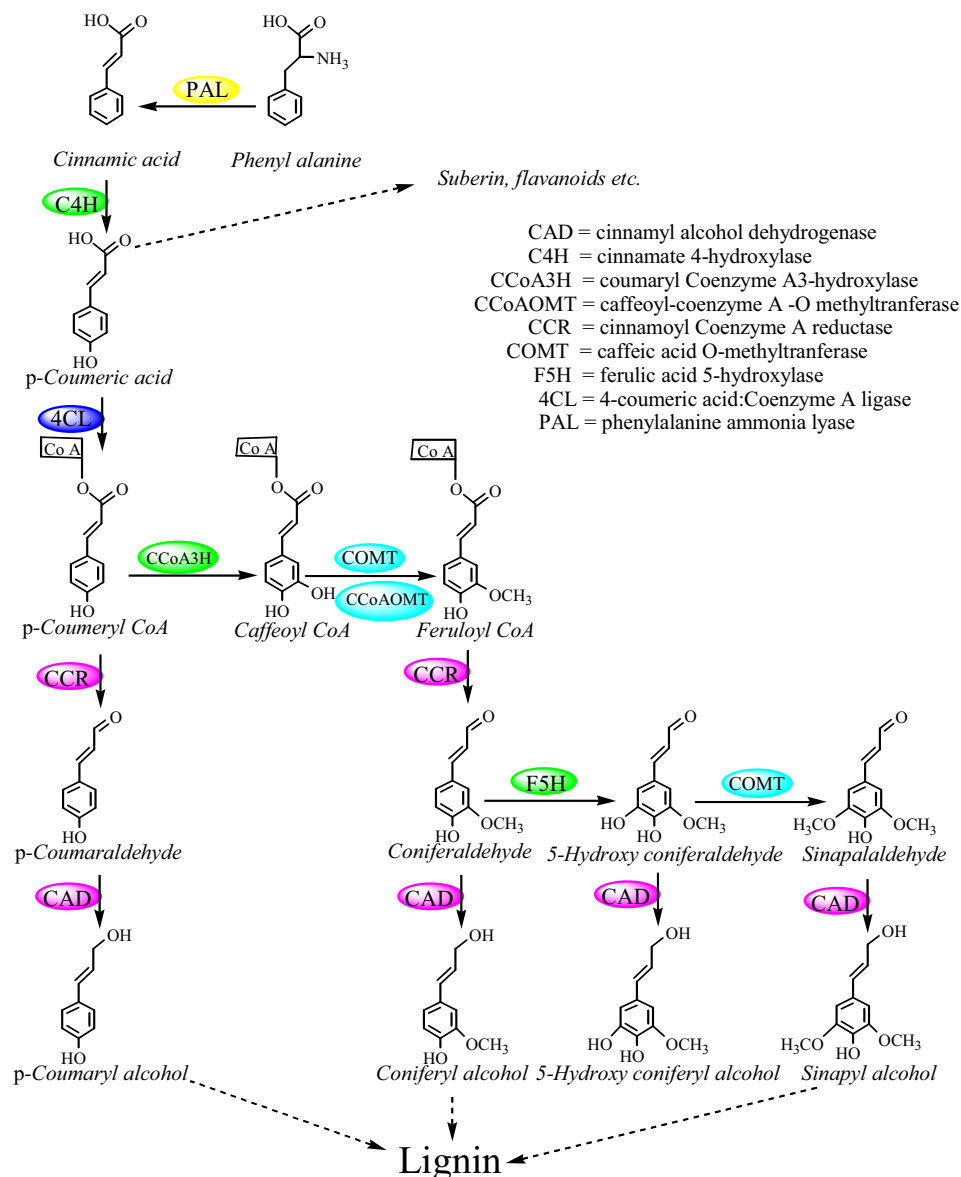


Figure 1.5.5. A simplified scheme of the biosynthesis of monolignols from the amino acid phenyl alanine (Henriksson, 2005)

1.5.6 Plants with genetic modifications in the monolignol biosynthesis pathway

To understand the biochemical processes in the plant, genetic modification is a useful tool (Reiter, 1998). There are many studies in which genes that code for various enzymes in the monolignol biosynthesis pathways has been down- or up-regulated. For example, tobacco plants were down-regulated with respect to CAD and CCR, (see *Figure 1.5.5* for enzyme abbreviations) and this led to a lower lignin content and plants that seemed weaker (Ralph *et al.*, 1998). NMR characterization of the lignin showed that it contained less guaiacyl and syringyl units and higher levels of benzaldehydes, cinnamaldehydes and products from their radical coupling. In another investigation, CCoAOMT and COMT were down-regulated, both separately and together. Inhibition of COMT did not change the phenotype of the plant, but the syringyl units in the lignin were reduced. Repression of CCoAOMT gave both phenotype changes and a reduction in syringyl units. Single mutants changed only the composition of lignin, but double mutants also halved the lignin content of the plant (Pincon *et al.*, 2001). Transgenic alfalfa plants with respect to COMT and caffeoyl CoA 3-O-methyl transferase CCOMT showed that down-regulation of the former led to a lower lignin content, with a small amount of guaiacyl units and no syringyl units in the lignin. In contrast, down-regulation of CCOMT gave a lower lignin content with less guaiacyl units but normal amounts of syringyl units (Guo *et al.*, 2001). Transgenic poplars have been produced by down-regulating COMT, giving trees with lower lignin contents, more condensed bonds and almost no syringyl units in the resulting lignin. Kraft pulp was produced with a higher yield, but it was more difficult to remove the lignin from the transgene pulp than from the reference pulp (Jouanin *et al.*, 2000). Later, transgene mutant trees have been produced that have lignin which is more easily removed (Pilate *et al.*, 2002). More recently, effects of double mutants with respect to COMT and CAD in poplar and *Arabidopsis thaliana* have been assessed (Jouanin *et al.*, 2004). One problem in deriving knowledge concerning the mechanisms behind plant regulation from transgenic plants is the plasticity of plants. When one or several genes are down-regulated, others that were not noticeable before are up-regulated, so that there are no or small visible effects.

1.5.7 Lignification of cell walls

There is some debate as to what controls the lignification of the cell wall. One hypothesis is that lignin polymerization begins on a very limited set of dirigent sites on the cell wall where short primary lignin sequences are polymerized. Each primary sequence acts as a template for further polymerization creating well-defined sets of lignin chains (Guan *et al.*, 1997; Chen and Sarkanen, 2004; Davin and Lewis, 2005). Another hypothesis is that the lignification of plants is controlled by the cell through the synthesis of monolignols, the chemical reactions involved, the radical-generating capacity, and the conditions in the cell wall (Ralph *et al.*, 2004). The radicals can be generated by peroxidases (Harkin and Obst, 1973), activity of which has been found both in cells and in culture media of, for example, Slender goldenbush, (Bredemeijer and Burg, 1986) and Norway spruce (Karkonen *et al.*, 2002). Oxidases such as laccases were early recognised as taking part in lignin polymerization (Freudenberg *et al.*, 1958). Later however, this hypothesis was dismissed when it was shown that laccase could not form synthetic lignin *in vitro* (Nakamura, 1967). Laccase came back on the arena though, when it was found that when excreted from sycamore cell suspension cultures it could polymerize monolignols into dehydrogenative polymers, DHPs, (Sterjiades *et al.*, 1992; Sterjiades *et al.*, 1993). Many investigations have suggested that laccase is involved in lignin polymerization (Dean *et al.*, 1998) but there is still no definitive proof that laccase is active in the polymerization of lignin *in vivo*. Other enzymes that have been implicated in lignification are a laccase-type oxidoreductase in the differentiating xylem of *Populus euramericana*

which was spatially related to lignin deposition and capable of oxidizing coniferyl alcohol (Sterjiades *et al.*, 1996).

Lignification of the cell wall begins when the primary wall has finished expanding. Generally it starts in the cell corner in the middle lamella and proceeds towards the lumen, filling up pores in the already deposited polysaccharide network (Wardrop, 1957; Saka and Thomas, 1982; Donaldson, 1991, 1992). The lignification continues until the protoplast disintegrates and the cell dies. It is generally believed that the structure of lignin varies between the cell-wall layers and different morphological regions of the plant. One explanation for this could be that the carbohydrate matrix in the various cell-wall layers affect lignification in different ways (Donaldson, 1994). Several early studies demonstrated that the middle lamella has a higher concentration of lignin than the secondary cell wall (Crocker, 1921; Ritter, 1925; Bailey, 1936). The middle lamella contains 50-70% lignin whereas the secondary wall has approximately 20%, but since the latter is much thicker, most of the lignin in wood is located in the secondary wall.

Two techniques have been used to investigate lignin composition in different cell-wall layers. One is based on microscopy, either by direct observation of the UV-absorbance, or by techniques involving labeling or staining and analysis with CLSM, SEM, SEM-EDXA and TEM, sometimes in combination with immunolocalization. The other employs fractionation of wood and subsequent chemical analysis. It is difficult, however, to obtain enough material for chemical lignin analysis from pure separate cell-wall layers.

In one study, a sieving technique was used on milled spruce wood to separate the middle lamella from the secondary cell wall, and data were obtained suggesting that middle lamella lignin is rich in *p*-hydroxyphenyl units and has a larger number of condensed bonds than secondary cell wall lignin (Whiting and Goring, 1982). Another investigation of fractionated spruce wood meal also showed that the middle lamella lignin was more condensed, but only trace amounts of *p*-hydroxyphenyl units were found (Westermarck, 1985). In a study of the xylem in pine shoots, radioactively labeled lignin precursors were supplied and the resulting lignin was analyzed (Terashima and Fukushima, 1988). In this investigation highly condensed *p*-hydroxyphenyl lignin and a condensed guaiacyl lignin were observed mainly in the cell corners and in the middle lamella in early cell wall differentiation. Lignin containing syringyl units was deposited during the late cell wall differentiation and primarily in the inner S₂ layer. These results could be representative of native lignin in trees, but they could also be due to lignin structures that appear as a response to the tissue damage caused by wounding the pine shoots and/or as a result of supplied monomers.

Immunolocalization of a condensed lignin substructure, dibenzodioxocin, in the developing xylem of a mature Norway spruce tree showed that the structure was not present before the formation of the S₂ layer. During the secondary cell wall deposition dibenzodioxocin was most abundant in the S₂ layer (Kukkola *et al.*, 2003). In a similar study of mature cells, it was shown that the structure was primarily present in the S₃ layer of Norway spruce and silver birch xylem (Kukkola *et al.*, 2004).

Early investigations of typical hardwoods have shown that vessels contain mostly guaiacyl type lignin whereas fiber-wall lignin is composed mainly of syringyl units (Fergus and Goring, 1970; Terashima *et al.*, 1986). More recently it has been shown by immunolocalization (Grunwald *et al.*, 2002) that condensed lignin units of guaiacyl and mixed guaiacyl/syringyl types were present in cell corners in the developing xylem before S₁

formation in three year old hybrid aspen plants (*Populus tremula* L. \times *P. tremuloides* Michx.). As deposition of carbohydrates progressed, lignification followed, with the S1 layer being lignified while S2 layer carbohydrates were being laid down. β -O-4' lignin units were present in the later stages of lignin development but only in the secondary cell-wall layer. In the same study, UV microscopy of thin sections also demonstrated that lignification was lagging behind the polysaccharide deposition. Approximately 25 cell layers from the cambium deposition of carbohydrates were completed but lignification was still taking place, albeit with a smaller amount of lignin present in the inner cell-wall layer. Rationalizing from absorbance maxima at different wavelengths for different monolignols, it was suggested that the aspen fibers in an early stage of differentiation contained mostly guaiacyl lignin in the middle lamella. During later stages syringyl and p-hydroxyphenyl units increased in the middle lamella, and in the secondary walls mostly syringyl units were detected (Grunwald *et al.*, 2002).

1.6 Chemical lignin analysis in a historical perspective

In 1838, Anselme Payen found that if wood was treated with concentrated nitric acid, part of the material dissolved and this fraction was named lignin. The residue was found to be carbohydrates. The Kraft pulping process was introduced in the 1850's and Peter Klason discovered in 1897 that lignin was associated with coniferyl alcohol. A search for lignin on SciFinder in March 2006 gave 52110 references. The earliest was a complete analysis of animal forage. After a series of extractions with ether, boiling with alcohol, treatment with water, boiling with sulphuric acid and subsequently boiling again with alkali, the *lignic acids* were determined by drying and weighing the soluble material. The residue was treated with chlorine and boiled with alkali and sodium sulfite and the dissolved substance was believed to be a more condensed form of lignin than the *lignic acids* (Browne and Beistle, 1901). Many early publications were related to digestibility of fodder (Browne, 1904; Furstenberg *et al.*, 1907; Konig, 1907), and this matter is still of great interest today.

Many standard methods are available for determining the lignin content in a sample (Dence, 1992a), but information regarding lignin composition in plants is a much more difficult task to tackle. Lignin is an intrinsic part of the plant cell wall and no procedures are yet available for extracting pure lignin in its native form. At present, there are no methods to obtain complete information regarding lignin when it is still encased in an intact plant cell wall either. Therefore, nobody actually knows what the composition or degree of polymerization of lignins are. All available methods are geared to acquiring certain information. Combining such data one tries to paint the whole picture. There are three main approaches:

- Isolation and analysis of polymeric lignin
- Degradation and analysis of soluble products
- Analysis of intact lignocellulosic samples

Milled wood lignin (MWL) is isolated from extracted finely ground wood, by ball milling and subsequent extraction in an organic solvent. Approximately 25% of the total lignin content in wood can be extracted in this way, but it can be questioned how representative it is compared to total cell wall lignin. Chemical changes induced by milling are, for example, a reduction in the degree of polymerization, side-chain oxidations and degradations (Lapierre and Lundquist, 1999; Ikeda *et al.*, 2002). Nuclear Magnetic Resonance (NMR) has been used since the 1960's for lignin analysis (Ludwig, 1971). It is the only method available today providing detailed information regarding linkages and structures in polymeric lignin. Usually, solution state lignin NMR is based on MWL, (Ralph *et al.*, 1999), but complete dissolution of

ball milled whole wood is also possible (Lu and Ralph, 2003). To avoid the isolation procedure, solid state NMR is an option (Gil and Pascoal Neto, 1999). The signal-to-noise ratio can be increased by feeding growing plants with ^{13}C enriched coniferin (Terashima *et al.*, 1997; Terashima *et al.*, 2002). Apart from solid state NMR, the most frequently used methods for analyzing intact wood or plant tissues are various microscopic methods, some of which were discussed in section 1.5.7.

Chemical degradation methods include acidolysis, nitrobenzene oxidation, permanganate oxidation, hydrogenolysis, thioacidolysis (Dence, 1992b), and the DFRC method (Lu and Ralph, 1997a, b). After degradation, the products are analyzed by GC and/or GC-MS or HPLC. The degradation methods give information about the monomer and oligomer composition in the sample. Since the methods selectively cleave certain bonds, information may be obtained concerning specific linkages present in the sample. The most widely used degradation method during the last 15 years has been thioacidolysis (Lapierre *et al.*, 1985). The method is sensitive and reproducible and requires only a small amount of material (5 mg lignocellulosic sample). It selectively degrades the $\beta\text{-O-4'}$ bonds in lignin so that, by quantifying the monomers obtained, one can estimate the frequency of such bonds. The remaining linkages must be of the condensed (carbon-carbon) or 4-O-5 type. Another advantage of the thioacidolysis technique is that since it is so widely used, one can easily compare new results with results reported in the literature.

1.7 Aim of investigations

With more detailed information regarding the lignin composition in the outer cell-wall layers, it might be possible to develop more efficient methods for selective lignin removal during chemical pulping. The composition of the lignin in the outer cell-wall layer of plants is also of general interest. For example, it may be possible to genetically modify annual plants for increased digestibility or improved compatibility when cellulose fibers are to be used in composite materials.

The aim of the work summarized in this thesis has been to elucidate the composition of lignins in the middle lamella and in the primary wall. Since it is difficult to separate the middle lamella/primary wall from the secondary cell wall, materials with exclusively or at least predominantly outer cell-wall layers were examined. Firstly, a 10 000 year old spruce material, with most of the secondary cell wall missing, was studied. Secondly, the developing xylem of a Norway spruce clone was analyzed during a growth season. Thirdly, the cambial tissues of a Balsam poplar clone were surveyed during a growth season. In spring and early summer, growth is very rapid and the intention was to sample tissues where the secondary cell-wall layers had not yet lignified, but where the outer layers at least had started to lignify. Since it was necessary to use whole trees and to compare these during the growth season, genetic variations were avoided by examining identical tree clones. In the final study, the primary cell walls from a hybrid aspen cell suspension culture were investigated.

2 RESULTS AND DISCUSSION

2.1 Lignin composition in Spruce, papers I & II

2.1.1 10 000 year old white spruce material

The aged white spruce sample, *Picea glauca*, was part of a forest that was rapidly buried in an upright position by glacial damming in Michigan, USA, where it had remained under semi-anaerobic conditions until it was found. When the aged wood was examined under a light microscope, it appeared that the secondary cell wall was partly missing, probably having been selectively removed by microorganisms. Chemical analysis showed that the cell wall constituents were rather unchanged except that the carbohydrates and fatty acids had been depleted (Hughes and Merry, 1978; Meyers *et al.*, 1980; Meyers *et al.*, 1995). The sample was chosen because, if the lignin located in the secondary wall had been removed together with the rest of the secondary cell wall constituents, the material might be representative of the middle lamella lignin from spruce. This 10 000 year old white spruce material, was compared to a reference white spruce wood. Figure 1.4.2 E and F show AFM images of the aged and the reference spruce, where the differences in cell wall thickness are clearly visible. The White spruce reference was obtained from a wood collection at TräTek, Stockholm.

2.1.2 Norway Spruce Clone

The particular clone used in this study was propagated from a seed plant that originally grew in Slovakia. This clone was somewhat better than an average of 490 evaluated clones with respect to growth rate, survival, number of cones, resistance to pathogens and frost damage (Sonesson, 1996). The plants were grown in squares with 120 plants, each seedling 2 m apart. Two 17 year-old-trees each were harvested in mid-April, mid-June and mid-August 2004 at Släsby, Uppsala, Sweden. A sample for microscopy was also harvested in mid-October. The diameter 0.6 m above ground varied between 57 and 84 mm. For microscopy, samples were cut approximately 0.6 m above ground and were frozen until the examination. The logs used for chemical analysis (two for each time) were frozen overnight and de-barked by hand. The xylem side of the log was scraped very lightly and the phloem side with moderate pressure, applied with a scalpel. This material was compared to the same clone wood, where care was taken to avoid the outer growth rings and the heartwood. The phloem side of the vascular area was chemically analyzed but no lignin monomers or dimers were detected. Coniferin is known to be present in large amounts in the phloem of spruce, and this shows that the extraction procedure prior to the analysis eliminated coniferin from the sample.

2.1.3 Klason lignin and thioacidolysis degradation products

The protein content of the 10 000 year old spruce was slightly higher than that of the reference wood. In the developing xylem specimens, the protein content was highest in the June sample, 10.6% w/w. Primary walls are generally considered to have 10% cell-wall-bound proteins, and this may therefore be an indication that the material contains mainly primary walls. The same trend was seen in the lignin content of the developing xylem during the growth season indicating that at least the June sample contains lignin that is located exclusively in the middle lamella/primary wall Table 2.1.3. Acid-soluble lignin increased during the growth season, but there may be other substances than lignin present that adsorb at 205 nm and this result may therefore be an over-estimation. The monomer content was much

lower in the developing xylem, indicating that the material contained a larger number of condensed bonds than the reference material. The Klason lignin was 60% in the aged White spruce and 25% in the reference White spruce. It is known that middle lamella contains 50-80% lignin, and the lignin examined may therefore come from this layer. However, it is also known that fossilized wood is depleted of carbohydrates even though all the cell-wall layers seem to be intact when the material is examined with TEM (Ucar *et al.*, 1996; Ucar *et al.*, 2005). It cannot be excluded that some of the lignin located in the S2 layer has remained. Another possibility is that some of the lignin in the S2 layer may have been eliminated due to the degradation of carbohydrates. Under anaerobic conditions, oxidative degradation of lignin cannot take place. Even though the aged spruce had been subjected to a semi-anaerobic environment, NMR analysis showed that there were no oxidized structures such as α -carbonyls present (*Paper I*), which indicates that most of the remaining lignin was in its native form. After thioacidolysis, the lignin monomer content of the aged spruce was 9% lower than that of the reference, presumably due to a larger number of condensed bonds in the aged lignin, *Table 2.1.3*.

Table 2.1.3. Lignin (Klason), acid-soluble lignin and monomer composition of thioacidolysis products quantified by GC-FID from; Ref A=Reference *Picea abies* Clone wood; April, June, August=Developing Xylem of *Picea abies* clone, Ref G= Reference *Picea glauca* wood, Aged G= 10 000 year old *Picea glauca* maximum deviation between samples 7%.

	Ref.A	April	June	Aug	Ref G	Aged G
Protein*	0.4	6.6	10.6	8.6	0.4	1.3
Lignin	25%	20%	5%	10%	25%	60%
Acid sol. lignin	0.3%	1.5%	1.5%	2.5%	0.6%	0.7%
Guaiacyl**	1005	640	525	636	1043	921

*Protein in o.d. extracted material **Yields of major lignin monomer products after thioacidolysis (μmol monomer unit g^{-1} Klason lignin in sample)

The molecular weight distribution of thioacidolysis products obtained from SEC analysis (*Figure 2.1.3*) confirmed the GC-FID analysis, *i.e.* that the 10 000 year old spruce after thioacidolysis contained a somewhat larger amount of condensed bonds resulting in a larger number of lignin oligomers than from the reference white spruce.

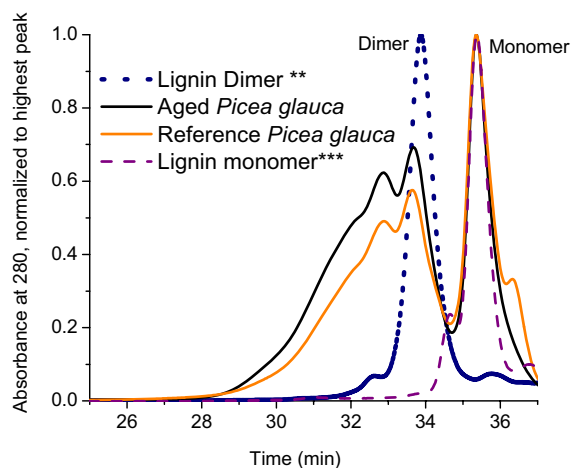


Figure 2.1.3. SEC of acetylated thioacidolysis products from 10 000 year old spruce wood and white spruce reference wood,

** Pinoresinol and ***Guaiacylglycerol beta guaiacyl ether as a lignin monomer and dimer size marker

2.1.4 Mass spectrometry of thioacidolysis degradation products

The mass spectra of the different softwood samples were similar and the peaks have previously been identified for Norway spruce wood by others (Lapierre *et al.*, 1991; Önnérud and Gellerstedt, 2003). The mass fragments are presented in Table 2.1.4.1, and the structures derived from these fragments are shown in Figures 2.1.4.1 and 2.1.4.2. With one exception, the structures that vary between the samples represent rather small peaks in the mass spectra. A total of 18 dimers were identified and 7 of them contained end groups (double bonds between α and β carbon). From the sample of the developing xylem from August, 4 dimers with end groups were identified. This sample also showed structure 2 as the main monomer in the GC spectrum by MS. A possible explanation for these findings is that, at this time of year, lignin polymerization takes place in a bulk fashion. End-wise polymerization means that single monomers are attached one by one onto a growing polymer which would give few end groups in the resulting lignin. Bulk polymerization means that monolignols, dimers and other oligomers are linked together, which would result in more end groups being incorporated into the lignin. This is consistent with an earlier study on the differentiation of xylem in larch (Eom *et al.*, 1988). The main problem with this explanation is that the monomer and dimers were detected after thioacidolysis and Raney Nickel desulphuration. It cannot be excluded that double bonds were introduced during these reactions. Even though the same procedure was used for all the samples, the material showed a variation in composition which could induce different reactions during the work-up. In the 10 000 year old White spruce, only one end group was found, whereas in the White spruce reference three end groups were detected.

Table 2.1.4.1. Selected mass fragments at 70 eV for elucidation of lignin monomers and dimers after thioacidolysis followed by desulphuration and acetylation from Norway spruce and White spruce

A=Reference *Picea abies* Clone wood; B,C,D=Developing Xylem of *Picea abies* clone, R= Reference *Picea glauca*, Q= 10 000 year old *Picea glauca*. A grey background shows that the fragment contains a double bond between the α and β carbons

Tissue/sample	Structure	M ⁺	Fragment					
A;B;C;D; Q;R	1	208(4)	166(44)	137(100)				
B;C;D; Q;R	2	206(10)	164(100)	149(30)				
A;B;C;D; Q;R	3	266(5)	224(58)	164(100)	149(42)	137(80)	133(38)	
A;B;C;D	4	400 miss	358(22)	316(100)	287(24)			
A;B;C;D; Q;R	5	372(8)	330(100)	301(25)	150(90)	122(30)	121(28)	
C;D	6	372(5)	330(30)	315(10)	167(38)	149(100)		
A;B;C;D; R	7	358(0.1)	316(10)	274(18)	137(100)	122(10)		
A;BC;D; Q	8	414(0.1)	372(25)	330(100)	301(38)			
C	9	370(20)	328(70)	137(100)	134(90)	mass from		
A;B;C;D; Q;R	10	400(0.1)	358(25)	316(40)	179(38)	137(100)		
D R	11	368(30)	326(100)	297(25)				
A;B;C;D Q;R	12	472(0.1)	430(10)	370(20)	328(10)	251(20)	191(100)	179(8)
			163(10)	149(20)	137(80)			
Q	13	400(6)	358(24)	344(16)	316(18)	179(100)	151(28)	137(56)
A;B;C;D Q;R	14	428(12)	368(18)	326(100)	311(40)			
A;B;C;D Q	15	458(0.1)	416(10)	374(25)	356(5)	237(10)	177(10)	137(100)
B; D	16	426(15)	324(35)	149(100)				
A;B;C;D; R	17	428(0.1)	386(35)	344(25)	137(100)			
D	18	396(10)	354(20)	312(100)				
Q	19	412(0.1)	370(20)	328(100)	299(24)	267(14)		
Q	20	430(0.1)	388(14)	328(10)	286(16)	209(36)	150(62)	137(100)
R	21	398(10)	382(12)	356(22)	340(34)	177(100)	145(60)	137(44)

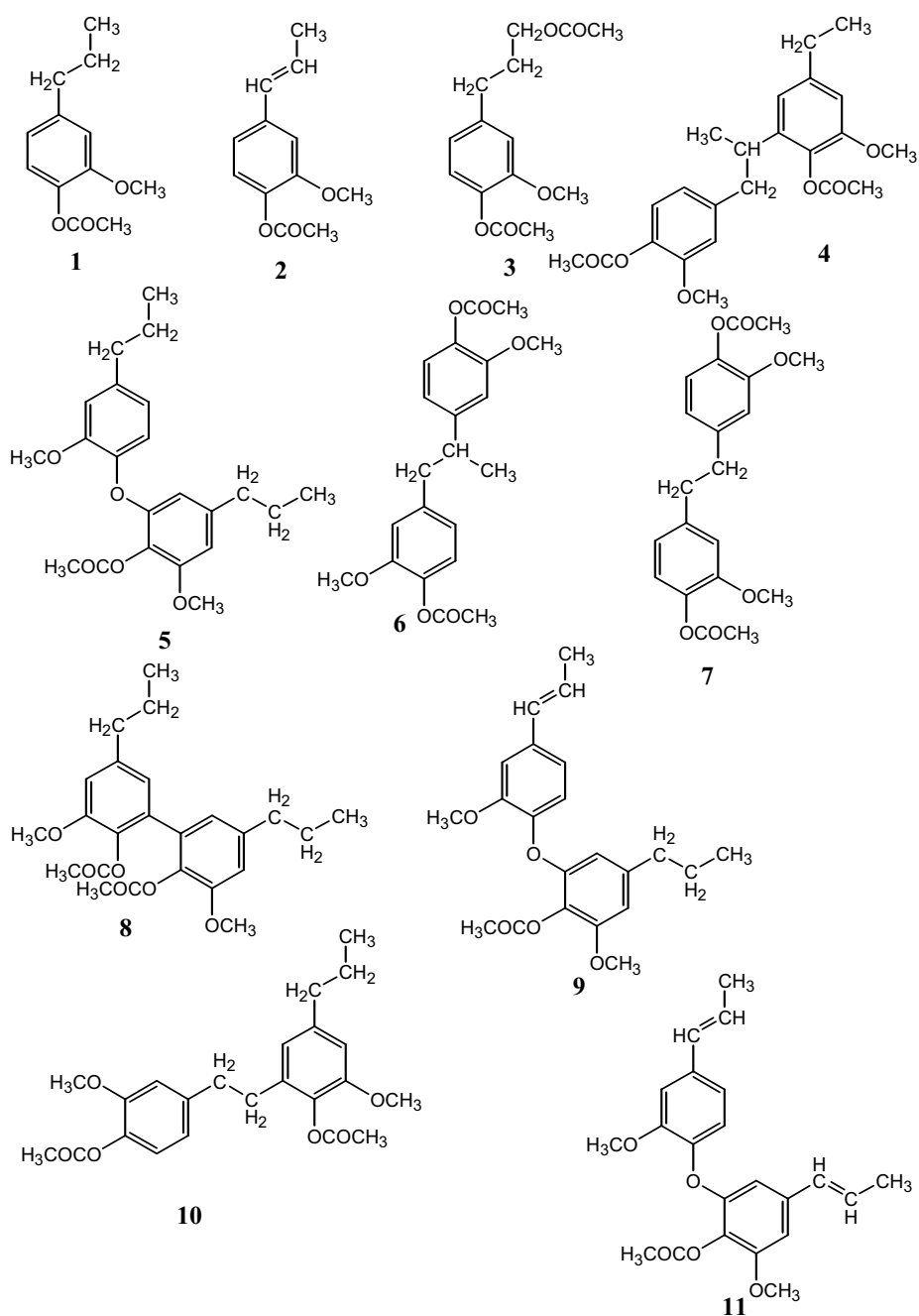


Figure 2.1.4.1. Lignin Structures derived from MS fragments after thioacidolysis followed by Raney-Nickel desulphuration and acetylation

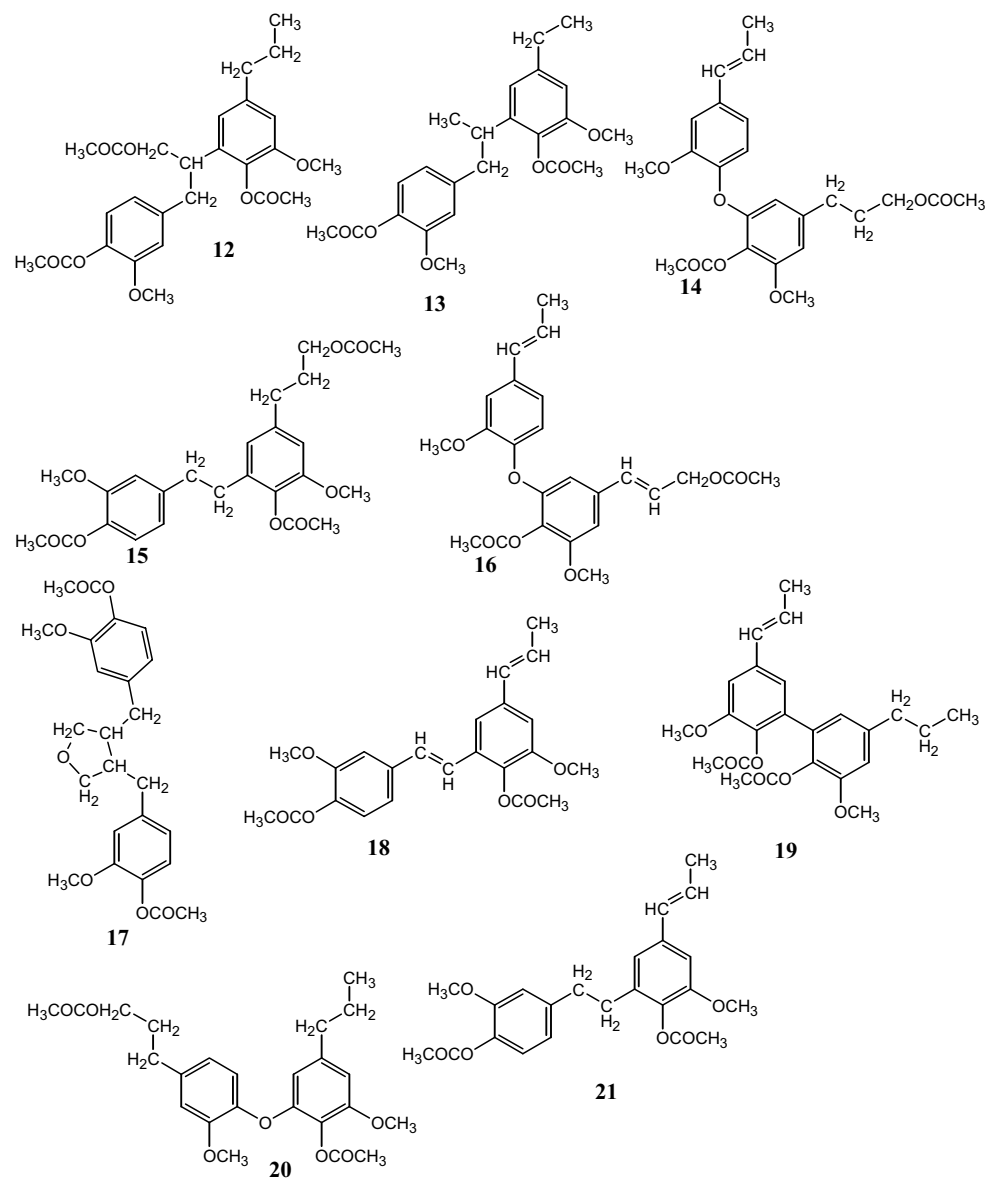


Figure 2.1.4.2. Lignin Structures derived from MS fragments after thioacidolysis followed by Raney-Nickel desulphuration and acetylation

2.1.5 Carbohydrate monomer distribution

Different carbohydrates quantitatively dominate in different cell-wall layers. Therefore, the carbohydrate monomer distribution of samples can be used to distinguish what cell-wall layer is predominating in a sample. The galactose, arabinose and rhamnose contents were higher in the 10 000 year old spruce sample than in the contemporary white spruce reference, *Figure 2.1.5*, which indicates a higher level of pectin, and that this is a major component in the primary cell wall (Thomas *et al.*, 1987; Edashige and Ishii, 1996). This shows that the aged material had a composition of hemicelluloses different from that in the secondary cell wall of the reference, which had glucomannan as the main hemicellulose and glucuronoxylan as the second most abundant hemicellulose (Sjöström, 1981).

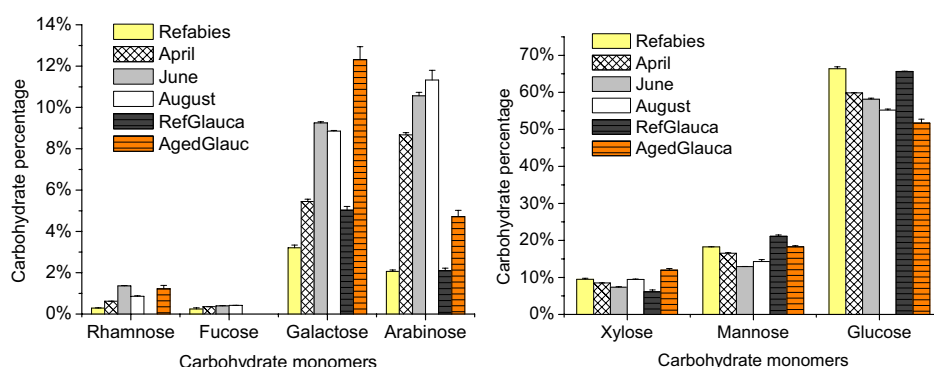


Figure 2.1.5. Carbohydrate monomer distribution in Norway spruce xylem and White spruce
 Refabies=Reference Norway spruce Clone wood; April, June, August=Developing Xylem of Norway spruce clone, RefGlauca= Reference White spruce, Aged Glauca= 10 000 year old White spruce, bars on columns show the maximum deviation between samples

In the xylem developing in spruce during the growth season, the galactose and rhamnose contents increased in April and June but decreased somewhat in August. Fucose, a carbohydrate present in small amounts in xyloglucan, which is the major *hemicellulose* component of softwood and hardwood primary cell walls, increased in the developing xylem but was present only in trace amounts in White spruce. Arabinose increased and glucose decreased in the developing xylem, whereas mannose decreased in April and June but increased in August, *Figure 2.1.5*. Previous authors have shown that the primary cell walls of softwood developing xylem contain 30% pectin (rhamnogalacturonan and homogalacturonan) 10% cell-wall-bound protein, hemicellulose (most abundantly xyloglucan, minor components are xylan and glucomannan) and cellulose, (Edashige *et al.*, 1995). It is therefore reasonable to interpret the carbohydrate monomer distribution during the growth season in developing xylem of Norway spruce as indicating that in June the cell walls contained predominantly primary cell walls whereas in August the secondary cell wall formation had progressed further than in the June specimen.

2.1.6 Microscopy

The specimens used for chemical analysis were also subjected to microscopy to show what the cell walls looked like at the time of harvest. Acriflavin fluorescence was applied to examine tissue samples with CLSM. Using this technique, variations in the lignin-to-carbohydrate ratio is revealed in the images as different color intensities. Methylene blue

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stains all the material in the cell wall, and this was used for light microscopy instead of the Acriflavin staining. *Figure 2.1.6.1* shows the Norway spruce clone in April. Sap was already present in the phloem, which aided de-barking but there were no apparent newly formed xylem cells in the vascular cambium. All the cells on the xylem side seemed to be fully formed and lignified and they looked very similar to those in the mid-October samples (not shown). No cell debris was visible in the xylem in any microscopy image of spruce in mid-April or October. *Figure 2.1.6.1* E and F Norway spruce clone from mid-April shows the annual ring with thin-walled early-wood and thick late-wood cells. Bordered pits with torus are seen, in E, and both half bordered pits and bordered pits in F.

In the mid-June sample, the developing xylem encompasses approximately 25 cell layers from the cambial layer down to the annual ring. Cell debris is seen in all these cells but to a lesser degree in the 4-5 cell layers closest to the annual ring. The thickness of the two neighboring walls and their middle lamella was approximately 1.5 μm when stained with acriflavin (*Figure 2.1.6.2 Norway spruce clone in June F*). With light microscopy and methylene blue staining, the same walls were on the average 3-4 μm thick (*Figure 2.1.6.1 June C*). These results indicate that carbohydrate deposition has begun in the secondary cell wall and that lignification has started only in the middle lamella/primary cell wall.

In *Figure 2.1.6.3 Norway spruce clone in August*, late-wood is forming with secondary cell wall deposition taking place in approximately 20 cell layers adjacent to the cambium. Cell debris can be seen in this area but it seems to be missing in the early-wood region (towards bottom). August C shows the cells closest to the cambium. The cell walls are thin, but they are thickening progressively towards the bottom. In August C, D and E, both staining techniques reveal that the cell walls are roughly 5 μm across two walls, indicating that secondary cell wall lignification has only started. These cells, within 20 cell-wall layers from the cambium, are destined to have dimensions of approximately 18 μm across 2 cell walls (*Figure 2.1.6.1 April C and D*) so that more than 70% of the secondary cell wall carbohydrates in this region had not yet been deposited. The developing xylem in August contained lignin equivalent to 40% of the reference lignin. Even though a large part of this lignin was probably located in the middle lamella, these results indicate that lignification follows the secondary cell wall deposition more closely than most literature data suggest.

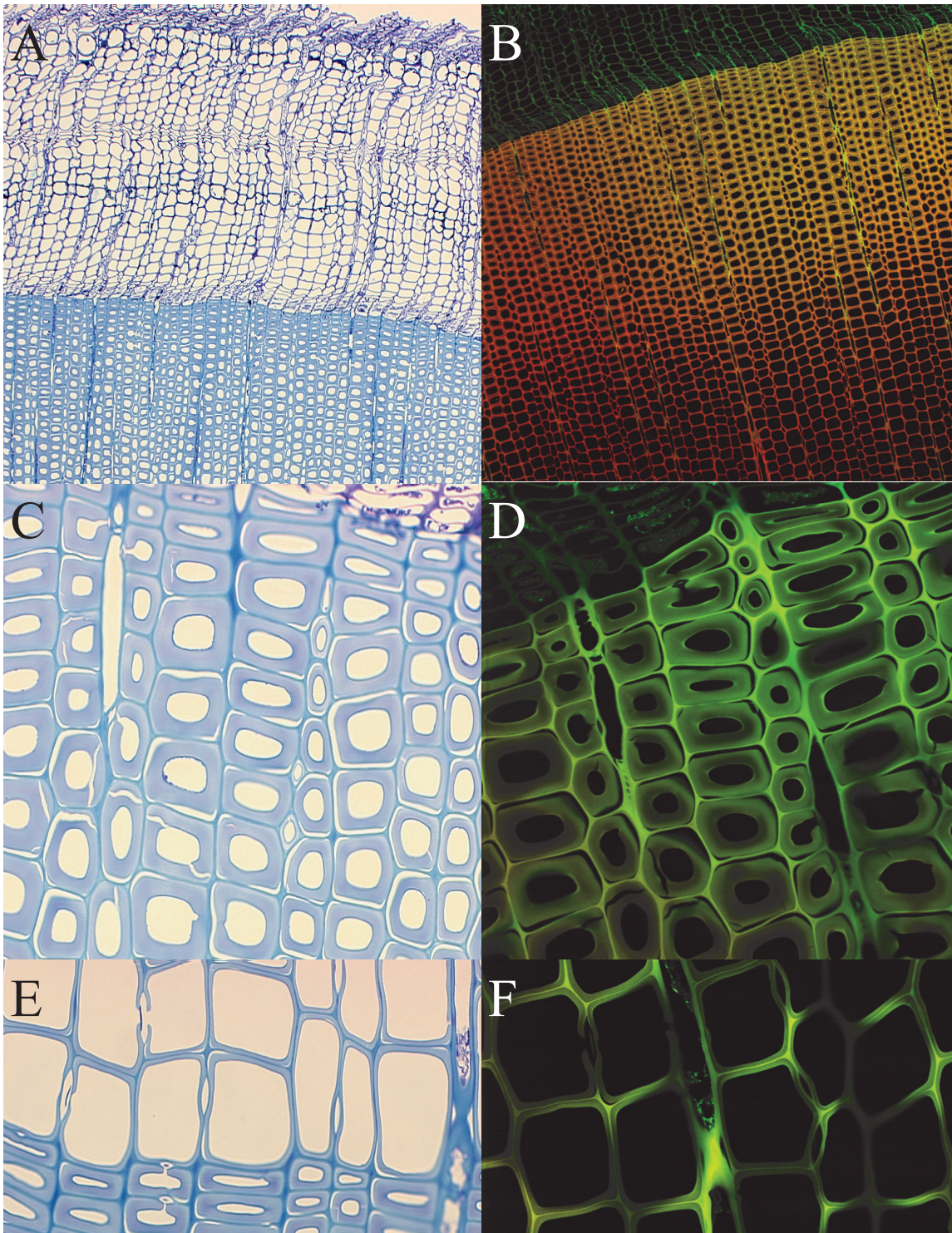


Figure 2.1.6.1 Norway spruce clone in April

Light (left) and CLSM (right) microscopy of samples dyed with methylene blue for light microscopy and with acriflavin for CLSM. April **A**; Cambial layer in the middle phloem and bark on upper side, xylem on the lower side, Field of view $1170 \times 1170 \mu\text{m}$. April **B**; Thick-walled late-wood cells close to cambium thinner early-wood towards the bottom, Field of view $1188 \times 1188 \mu\text{m}$. April **C**; Phloem cells in the right top corner late-wood xylem cells next to cambial layer, Field of view $185 \times 185 \mu\text{m}$ April **D**; Phloem cells in left top corner late-wood xylem cells next to cambial layer, Field of view $196 \times 196 \mu\text{m}$ April **E**; last annual ring early-wood top late-wood bottom, Field of view $185 \times 185 \mu\text{m}$ April **F**; Early-wood, Field of view $196 \times 196 \mu\text{m}$

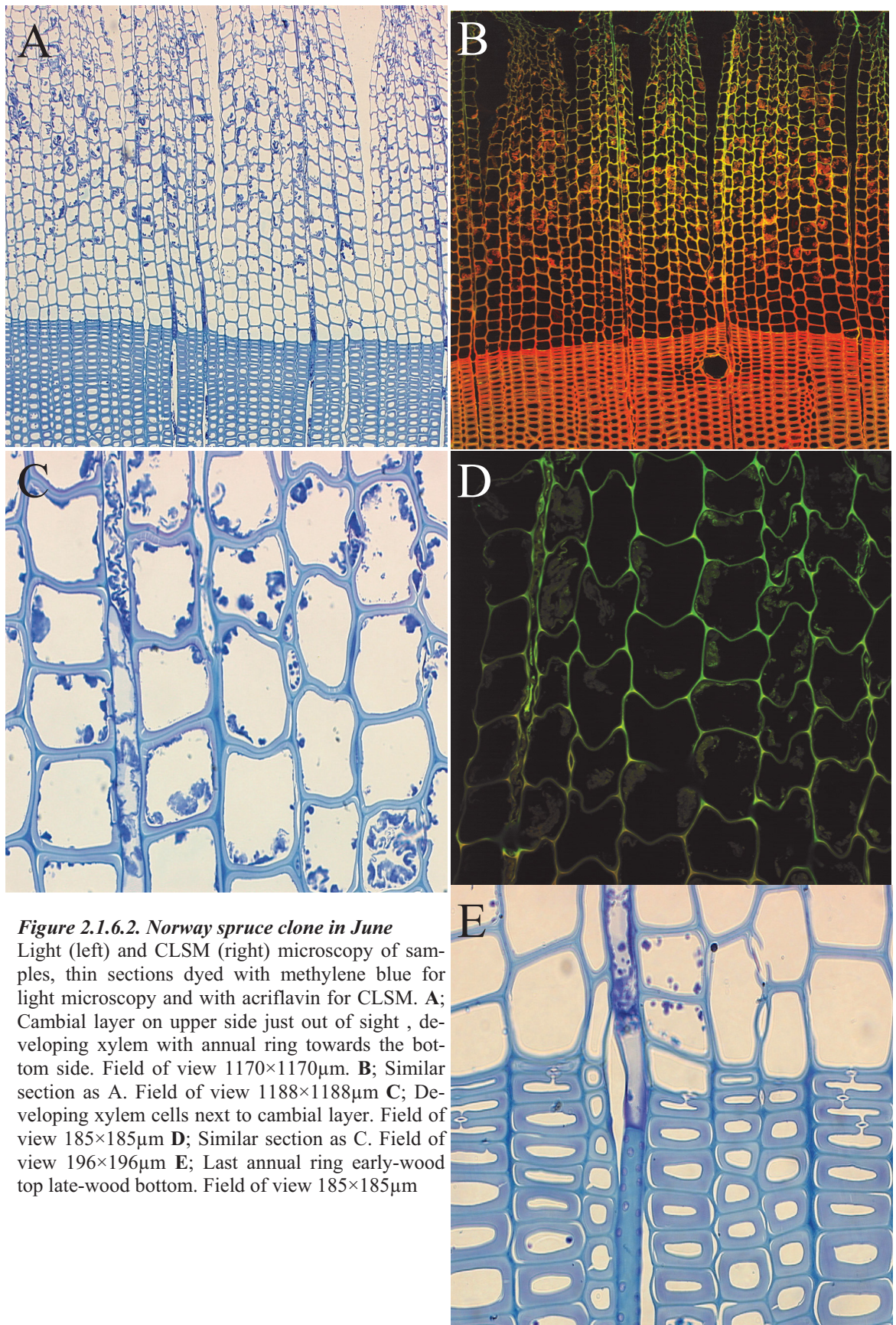


Figure 2.1.6.2. Norway spruce clone in June

Light (left) and CLSM (right) microscopy of samples, thin sections dyed with methylene blue for light microscopy and with acriflavin for CLSM. **A**; Cambial layer on upper side just out of sight, developing xylem with annual ring towards the bottom side. Field of view 1170×1170μm. **B**; Similar section as A. Field of view 1188×1188μm **C**; Developing xylem cells next to cambial layer. Field of view 185×185μm **D**; Similar section as C. Field of view 196×196μm **E**; Last annual ring early-wood top late-wood bottom. Field of view 185×185μm

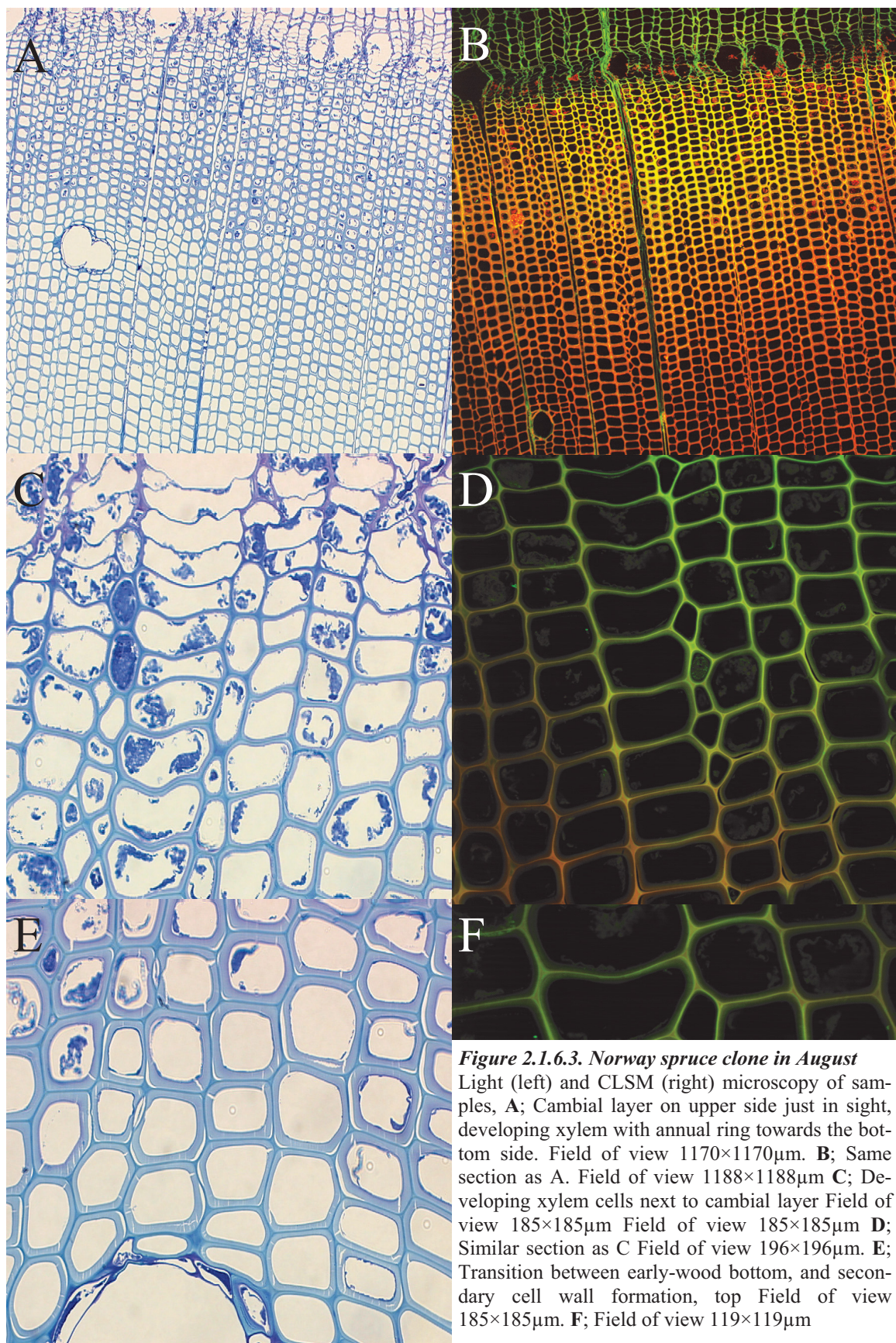


Figure 2.1.6.3. Norway spruce clone in August

Light (left) and CLSM (right) microscopy of samples, **A**; Cambial layer on upper side just in sight, developing xylem with annual ring towards the bottom side. Field of view 1170×1170μm. **B**; Same section as A. Field of view 1188×1188μm **C**; Developing xylem cells next to cambial layer Field of view 185×185μm Field of view 185×185μm **D**; Similar section as C Field of view 196×196μm. **E**; Transition between early-wood bottom, and secondary cell wall formation, top Field of view 185×185μm. **F**; Field of view 119×119μm

2.2 Lignin composition in Poplar, papers III & IV

2.2.1 Hybrid aspen cell cultures

A hybrid aspen (*Populus tremula* L. x *P. tremuloides* Michx.) culture was established and cultured as described by Ohlsson *et al.*, (2006). The culture used in this investigation was grown as a fine suspension in the dark and sub cultivated weekly for maintenance growth. For lignin analysis, cultures were harvested after 7, 14 and 21 days of growth. At day 7, the culture was in its exponential growth phase with active growth. At days 14 and 21, the stationary phase had been reached and parts of the cells were dying or dead, as shown in *Figure 2.2.1.1*. When chemical analysis was carried out, the reference wood for the cell suspension cultures was a fresh aspen tree, *Populus tremula*, from which hand chips were made avoiding heartwood and outer annual rings. Peroxidase and laccase activities were measured in cell extracts and culture media from hybrid aspen cultures, *Figure 2.2.1.1*. Both these enzymes have been involved in the lignification process, and their activities, like the lignin content, increased during growth. Laccase showed the largest increase in activity at day 21, when the lignin content was highest. Most of the activity of this enzyme was found in the culture medium. Peroxidase activity in the culture medium (extracellular) often increases at the same rate as the growth (Melo N.S. *et al.*, 1995). Such an early increase also occurred in the present culture (not shown) and the peroxidase activity was already high at day 7 (*Figure 2.2.1.1*). Many peroxidases are identified and they fulfil various functions in the cell, among them polymerization of lignin. In cell cultures, they are often found both inside the cells and in the culture medium (Bredemeijer and Burg, 1986; Karkonen *et al.*, 2002); this was also found here.

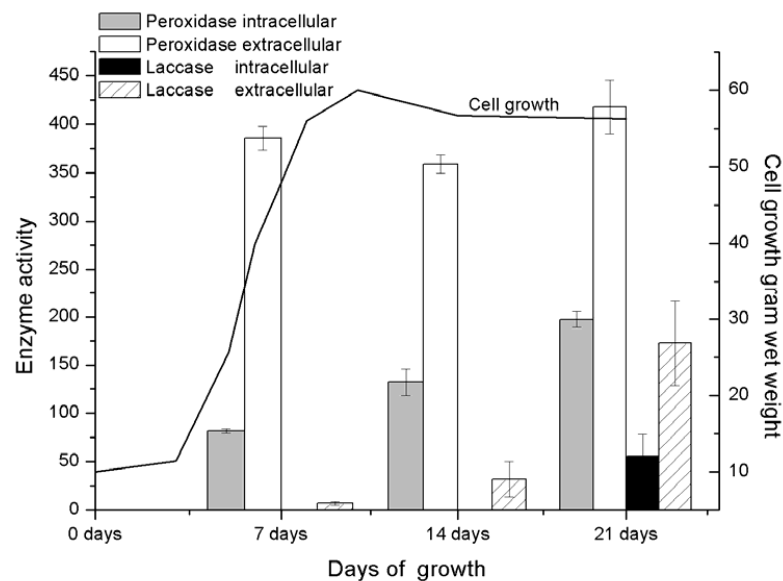


Figure 2.2.1.1. Hybrid aspen cell cultures.

Cell growth and enzyme activity during growth. Peroxidase measured as $\Delta A_{470}/\text{min}$, g wet weight cells (intracellular) and $\Delta A_{470}/\text{min}$, ml medium (extracellular). Laccase measured as $10^3 \Delta A_{420}/\text{min}$, g wet weight cells (intracellular) and $10^3 \Delta A_{420}/\text{min}$, ml medium (extracellular).

2.2.2 Poplar clone

The Balsam poplar clone, *Populus balsamifera* BE 183, was earlier obtained by Swedish University of Agriculture Sciences from Norway where a number of American balsam poplar clones had been investigated (Langhammer, 1974). Originally the tree grew in Alaska. The tree clones for this study were harvested from a trial spot 15 km east of Uppsala, Sweden. Two 17-year old trees each were harvested in mid-June and mid-August 2004. Samples for microscopy were also harvested in mid-April and mid-October. However, at this time of year the trees could not be de-barked by hand. The diameter 1.5 m above ground varied between 137 and 150 mm. For microscopy samples were cut approximately 1.5 m above ground and they were kept frozen until examination. The logs were treated in the same way as described under paragraph 2.1.2 for spruce clones. Since lignin monomers and dimers were found in the phloem, these data are also presented.

2.2.3 Klason lignin and thioacidolysis degradation products

The lignin content in the cell cultures was low, but it increased during cultivation (Table 2.2.1.2) and the lignin in the developing xylem of *P. balsamifera* was also low, 2%, in mid June and 8% in the August sample. The acid-soluble lignin content in both the cell cultures and the developing phloem and xylem of the *P. balsamifera* clone were higher than in the references. The protein content in the balsam poplar material indicates that it contains mostly primary walls/middle lamella, and further evidence of this was obtained by carbohydrate analysis. It has been shown by others using microscopy techniques that lignin with exclusively condensed bonds is deposited in the middle lamella in the early stages of xylem differentiation in poplar wood (Grunwald *et al.*, 2002). The lignin composition showed that guaiacyl or mixed guaiacyl/syringyl (GS) lignin was deposited prior to the S1 formation. Furthermore, non-condensed GS lignin subunits were absent in cell corners and in the middle lamella but abundant in the S2 layer of fiber cells (Grunwald *et al.*, 2002).

In the present study, GC-FID quantification of major lignin degradation products after the thioacidolysis of cell culture samples showed that they contained only guaiacyl monomers, in contrast to poplar wood which has syringyl as the main monomer. The ratio of the lignin monomer to the Klason lignin was lower than that of the poplar wood reference, and the monomer content decreased progressively as cultivation continued which means that the number of condensed bonds increased with cultivation time (Table 2.2.1.2).

It was found that the samples from the Balsam poplar cambial tissues contained approximately 30% fewer lignin monomer units than the reference, indicating a larger number of condensed bonds in the developing zone. The monomer composition was also different from that of the reference clone wood. In developing phloem and xylem from June and developing phloem from August, the synapyl/guaiacyl (S/G) ratio was on average 0.57 whereas it was 1.28 for the clone reference. In the developing xylem of the August sample, the ratio was 0.83, a value somewhat closer to the reference. Since sieve tube elements that have only primary walls when they are mature are a major component of phloem tissue, it is not surprising that this tissue contained predominantly guaiacyl units, Table 2.2.1.1.

Table 2.2.1.1. Lignin (Klason), acid-soluble lignin and monomer composition of thioacidolysis products quantified by GC-FID, Ref= *Populus balsamifera* clone wood, Ph=developing phloem, X=developing xylem, maximum deviation between samples 7%.

	Ref	Ph June	X June	P Aug.	X Aug.
Protein*	0.6	12.9	15.5	11.3	10.6
Acid sol. lignin	3%	6%	7%	7%	5%
Lignin	23%	2%	3%	2%	8%
Guaiacyl**	692	624	542	766	489
Syringyl**	886	344	346	405	406
Total**	1578	960	888	1171	895
S/G	1.28	0.55	0.64	0.53	0.83

* Protein in o.d. extracted material

**Yields of major lignin monomer products after thioacidolysis ($\mu\text{mol monomer unit g}^{-1}$ Klason lignin)

Table 2.2.1.2. Lignin (Klason), acid-soluble lignin and monomer composition of thioacidolysis products quantified by GC-FID, hybrid aspen cell suspension cultures and a poplar wood reference; maximum deviation between samples 7%. n.d.= not detected

	Cells 7 days	Cells 14 days	Cells 21 days	Poplar Wood
Acid sol.lignin	9.8 %	8.6 %	8.4 %	3.7 %
Lignin	0.7 %	2.2 %	3.9 %	18 %
Guaiacyl**	575	337	194	672
Syringyl**	nd	nd	nd	1260
Total**	575	337	194	1932
S/G	n.d	n.d	n.d	1.9

**Yields of major lignin monomer products after thioacidolysis ($\mu\text{mol monomer unit g}^{-1}$ Klason lignin)

In other studies of birch xylem it was found that the lignin was more condensed and contained almost 50% p-hydroxyphenyl units (Eom *et al.*, 1987; Kim *et al.*, 1994). The carbohydrate and lignin contents in these studies however, indicate that they include mature cells with fully formed lignified walls.

2.2.4 Mass spectrometry of thioacidolysis degradation products from poplar

As mentioned previously, only guaiacyl monomer units were present in the cell suspension cultures and the three dimers identified were also of the guaiacyl type. Compounds 32, 1, 2 and 8 (Figure 2.2.1.3) were previously found in lignin released into the media by spruce cell suspension cultures (Brunow *et al.*, 1993). None of the structures identified in the cell cultures contained double bonds. The structures 1, 2, 22 and 23 were found in both the references and the developing phloem and xylem, with the exception of compound 2 that was not found in the reference clone wood. A total of 12 dimers were found in the references and in the differentiating area of poplar wood. In the references, 8 dimers were found, one of which had an end group. In the differentiating zone, 6 dimers were found 3 of which had end groups in them, Table 2.2.1.3, Figure 2.1.4.1, Figure 2.1.4.2 and Figure 2.2.1.3.

Table 2.2.1.3. Selected mass fragments at 70 eV from hybrid aspen cell suspension cultures and Cambial tissues of Balsam poplar.

Lignin degraded by thioacidolysis followed by Raney-Nickel desulphuration and acetylation displayed as relative abundance of base peak. The grey base color shows that the fragment contains a double bond between the α and the β carbons

Specimen*	Struct.	M ⁺	Fragments				
A;X1;P1;X2;P2; C;R	1	208(4)	166(60)	137(100)			
X1;P1;X2;P2; R	2	206(10)	164(100)	149(30)			
A;X1;P1;X2;P2; R	22	238(5)	196(75)	167(100)			
A;X1;P1;X2;P2; R	23	236(10)	194(100)	179(25)			
A	5	372(10)	330(100)	301(40)	150(35)	122(30)	
A;X1; X2;P2; R	7	358(0.1)	316(10)	274(20)	137(100)		
A; X2;P2;C;R	8	414(1)	372(20)	330(100)	301(40)		
A;X1; X2;P2	24	402(2)	360(30)	328(15)	299(5)	180(100)	152(70)
A;	10	400(1)	358(15)	316(30)	179(30)	167(40)	137(100)
X1; X2;P2	11	368(30)	326(100)	297(20)			
P1;	25	442(25)	400(40)	358(100)	343(20)		
A; R	26	418(0.1)	388(5)	376(10)	346(15)	167(100)	
R	27	388(0.1)	358(12)	346(16)	316(18)	304(17)	179(18)
			167(100)	151(4)	137(74)	122(6)	
X1; X2;P2	28	470(1)	428(10)	398(40)	386(15)	356(100)	
A;	29	472(10)	430(80)	388(100)	301(10)	234(80)	
C	30	428(10)	368(14)	326(100)	311(44)	294(20)	265(16)
			221(10)	191(20)	153(10)	137(18)	
C	31	430(0.1)	388(12)	328(35)	267(45)	209(74)	137(100)
C;R	32	194(1)	152(66)	137(4)	122(16)	106(100)	

*Specimen; A=Reference Balsam poplar Clone wood; P1=Phloem June; X1=Developing Xylem June; P2=Phloem August; X2=Developing Xylem August; C=hybrid aspen cell culture, R=reference aspen wood

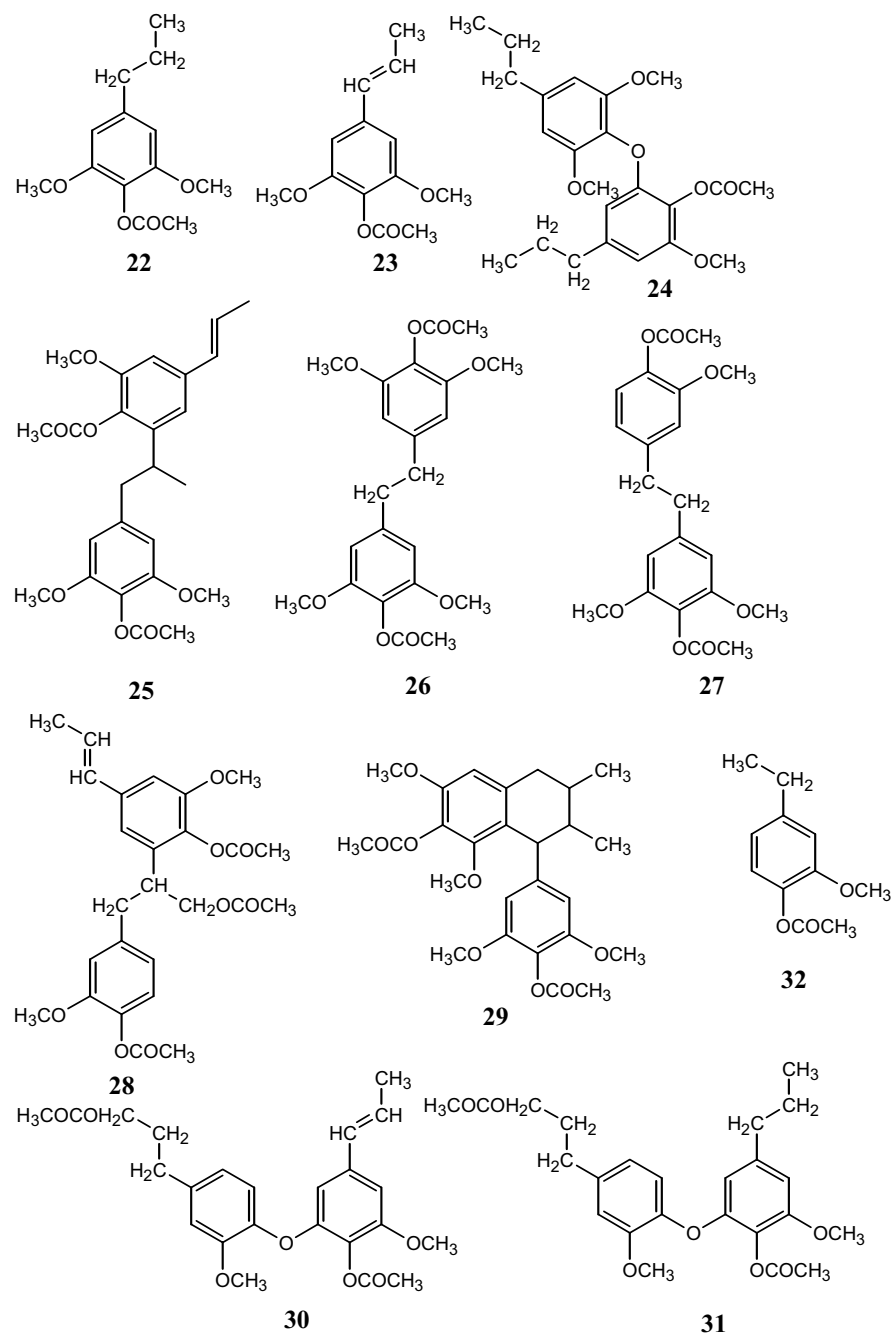


Figure 2.2.1.3. Lignin Structures derived from MS fragments after thioacidolysis followed by Raney-Nickel desulphuration and acetylation

2.2.5 Carbohydrate monomer distribution

In the literature, poplar cambial tissue with only primary walls contains 40% pectin, 12% arabinogalactan, 6% xyloglucan, 10% glucuronoxylan, 1% glucomannan, 21% cellulose and 10-15% protein (Simson and Timell, 1978b). In their study, Simson and Timell assessed the presence of secondary walls by observing the arabinose/xylose ratio and lignin content. Birch wood, in which the S2 layer quantitatively dominates, contains 22% lignin, 2% glucomannan, 28% glucuronoxylan, 41% cellulose, and 7% other components (Sjöström, 1981). Fucose is present in poplar xyloglucan, with L-fucose, D-galactose, D-xylose, and D-glucose in a molar ratio of 4:23:28:45 (Simson and Timell, 1978a). Since fucose is a constituent unique to xyloglucan and this hemicellulose is a major component of the primary cell wall, the detection of fucose is indicative of the extent to which primary walls are dominating in the sample. In the cell cultures (only day 21 is plotted, but all cultures were similar with respect to carbohydrates) fucose was abundant whereas it was missing in the reference aspen wood. In the differentiating zone of the balsam poplar clone, the fucose content reached a peak value in the developing xylem in the June sample. The clone reference also contained some fucose but the developing xylem in the August sample had a value closest to that of the Balsam clone reference. The arabinose/xylose ratios for the samples indicate that cell culture specimens had only primary cell walls. For the Balsam poplar clone tissues, the ratios indicate that, with the exception of the August developing xylem sample, the specimens contained predominantly middle lamella/primary cell walls. The ratio in the August developing xylem sample indicates that secondary cell walls were present. Rhamnose is a constituent of pectin abundant in the middle lamella and primary wall, and this carbohydrate is also indicative of outer cell-wall layers. The contents of rhamnose, mannose, xylose, galactose and glucose point in the same direction, *i.e.* that the secondary cell walls are fully formed only in the references and almost fully formed in the August developing xylem sample *Figure 2.1.5*.

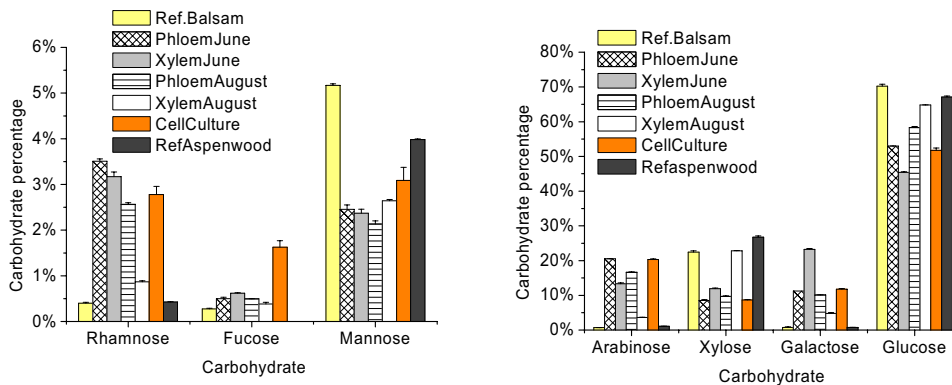


Figure 2.1.5. Carbohydrate monomer distribution in spruce xylem and cell culture

2.2.6 Microscopy

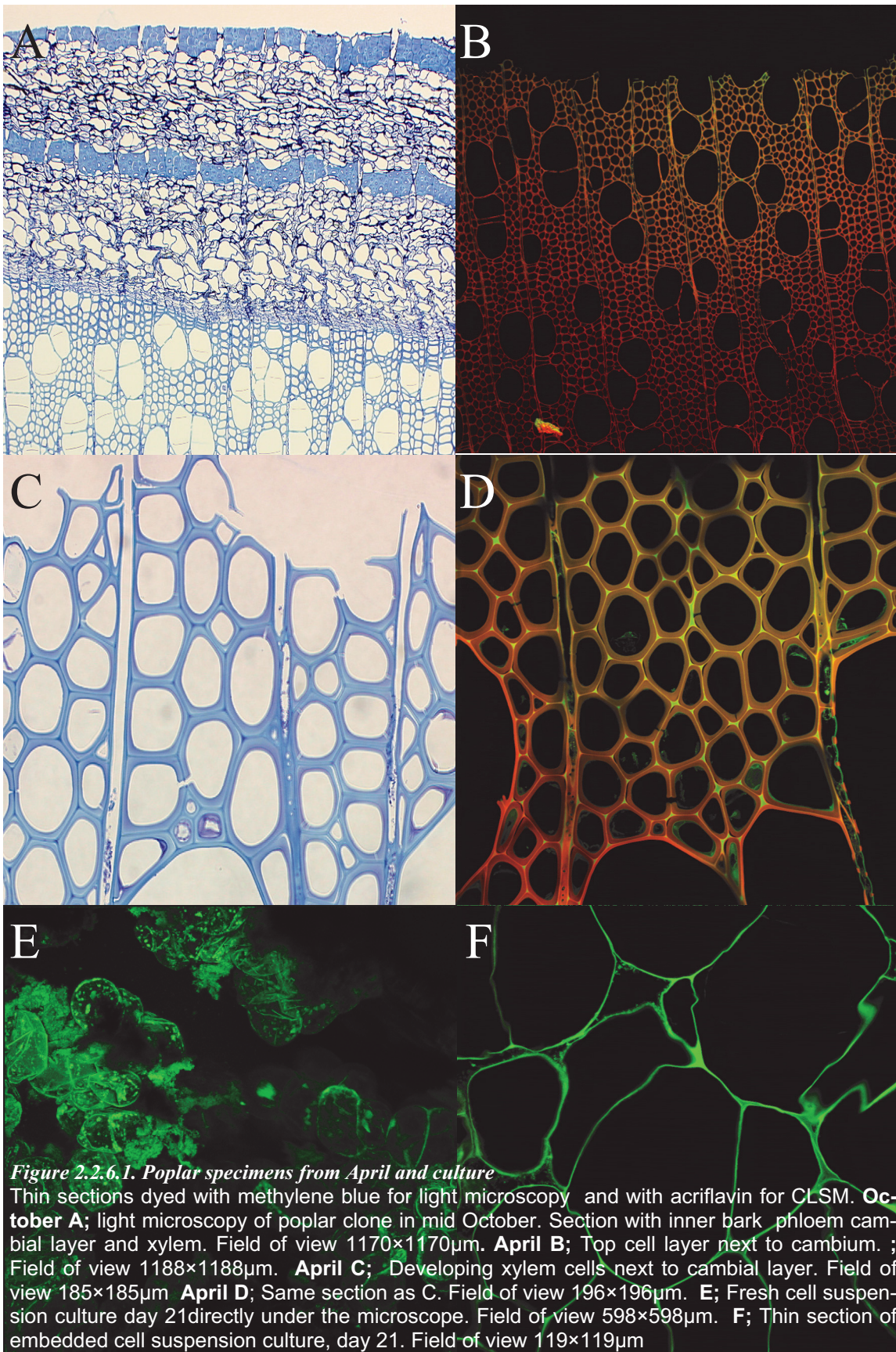
The Balsam poplar clone in April is presented in figure In *Figure 2.2.6.1 B-D*. Image A is from mid-October. Sap was not present in the phloem in amounts that would permit debarking in either April or October samples. In contrast to spruce, where no cell debris could be seen in samples from April or October, both these samples seemed to contain cell debris in the 15 cell layers closest to the cambium. Microscopy of the cell suspension at 21 days showed that the cells were rather uniform and round and that the walls were thin, *Figure*

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2.2.6.1. E, F. No cell differentiation or secondary cell walls were detected and the stained material occurred in a rather irregular way.

In the case of poplar, no apparent difference in cell wall thickness was observed using methylene blue or acriflavin staining of the developing xylem. Four cell-wall layers down from the cambial zone in the June specimen, two neighboring cell walls and their middle lamella spanned a distance of 1,5 μm (*Figure 2.2.6.2. A*), corresponding to the thicknesses found in early-wood mature cells (*Figure 1.3.2 F*). In late-wood cells, this area ranged from 2,5 to 7,3 μm . The developing xylem cells, imaged in *Figure 2.2.6.2 C* and *D*, may have reached the mature thickness of early-wood poplar xylem cells. Nevertheless, the cells look uneven and the abundance of cell debris together with carbohydrate analysis indicate that secondary cell wall carbohydrates were still being deposited. *Figure 2.2.6.2. E* and *F* are imaged close to the last annual ring and here the cells seem to be fully formed and mature.

In the mid-August sample the cambial and the first developing phloem cell layers were very soft and they were therefore somewhat crushed and dislodged from the xylem side during embedding *Figure 2.2.6.3 A, B*. The cells in the developing xylem were clearly more mature, *C* and *D*, than the similar area in June. Light shadows at a higher magnification, (*Figure 2.2.6.3 E*) show that the secondary cell wall was not fully lignified and that cell debris is still present in the xylem cells.



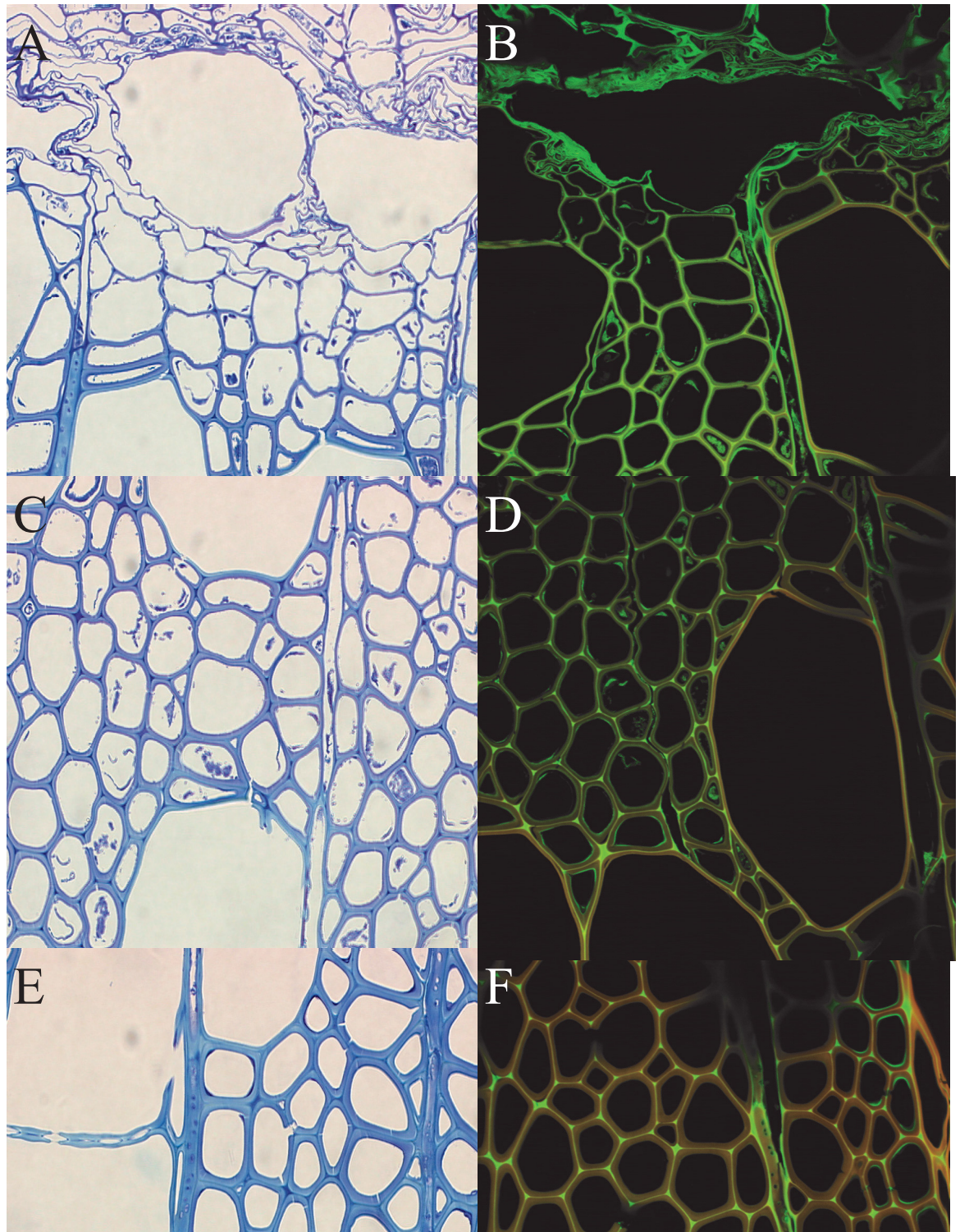


Figure 2.2.6.2. Microscopy of Balsam poplar clone from mid-June

Thin sections dyed with methylene blue for light microscopy and with acriflavin for CLSM. **June A**; Cambial layer, phloem on upper side developing xylem towards bottom. Field of view $185 \times 185 \mu\text{m}$ **June B**; Same section as A. Field of view $185 \times 185 \mu\text{m}$. **June C**; Developing xylem just under image A. Field of view $185 \times 185 \mu\text{m}$ **June D**; Same section as C. Field of view $196 \times 196 \mu\text{m}$ **June E**; Top cell layer approximately 15 cell layers from cambium. Field of view $185 \times 185 \mu\text{m}$ **June F**; similar section as E. Field of view $119 \times 119 \mu\text{m}$

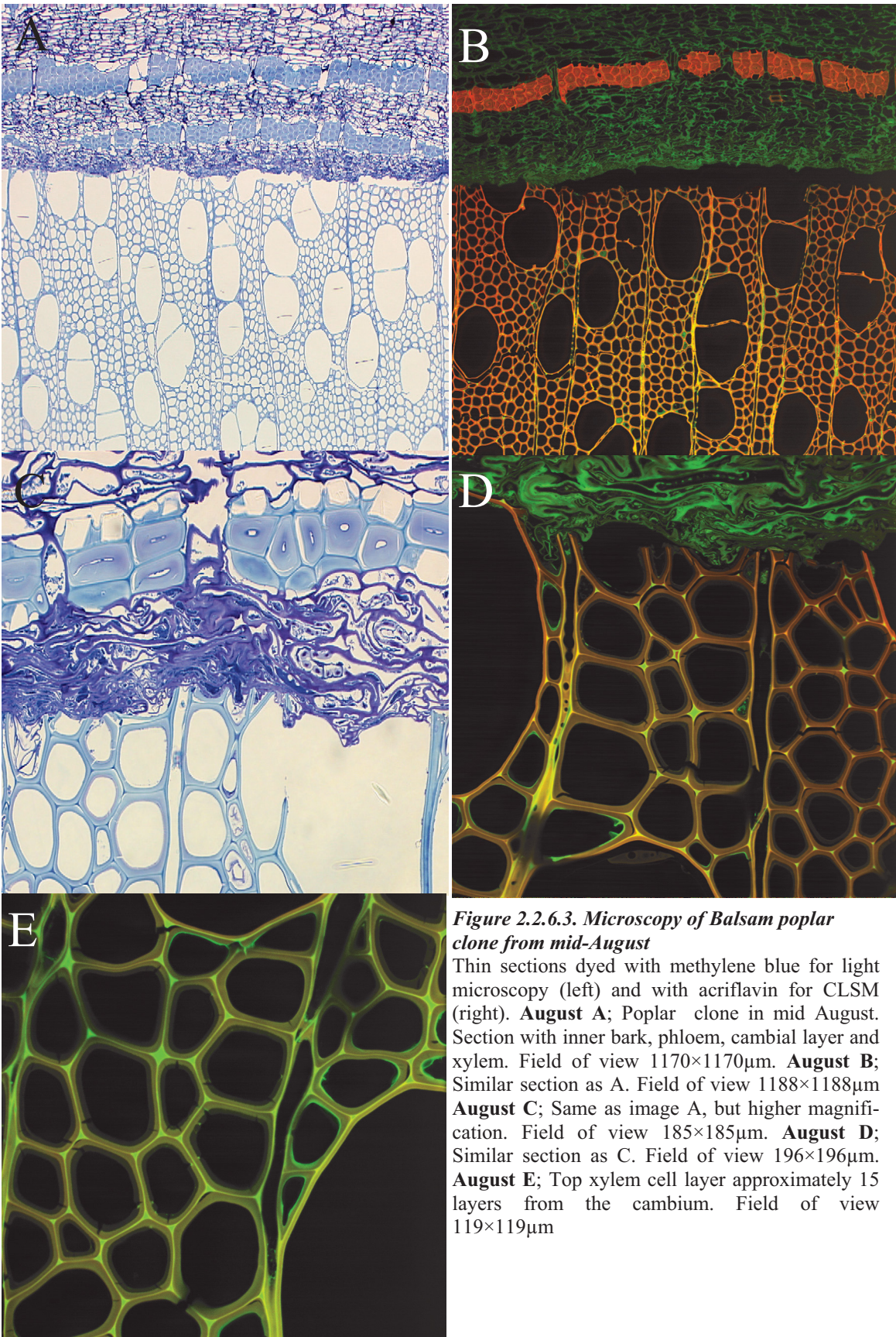


Figure 2.2.6.3. Microscopy of Balsam poplar clone from mid-August

Thin sections dyed with methylene blue for light microscopy (left) and with acriflavin for CLSM (right). **August A**; Poplar clone in mid August. Section with inner bark, phloem, cambial layer and xylem. Field of view $1170 \times 1170 \mu\text{m}$. **August B**; Similar section as A. Field of view $1188 \times 1188 \mu\text{m}$. **August C**; Same as image A, but higher magnification. Field of view $185 \times 185 \mu\text{m}$. **August D**; Similar section as C. Field of view $196 \times 196 \mu\text{m}$. **August E**; Top xylem cell layer approximately 15 layers from the cambium. Field of view $119 \times 119 \mu\text{m}$

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3 CONCLUSIONS

The aim of this work was to elucidate the composition of developing lignin in the middle lamella/ primary cell wall of Norway spruce (*Picea abies*), White spruce (*Picea glauca*), hybrid Aspen (*Populus tremula* L. x *P. tremuloides* Michx.) and Balsam poplar (*Populus balsamifera*) and to compare these data with that of the mature reference wood in which the secondary cell wall predominates.

For the first time, accurate data relating to the lignin monomer composition and the number of β -O-4' bonds were obtained from pure middle lamella/primary wall lignin. The sampling during the growth season gives a snapshot of the lignification at the time of collection. In previous investigations of unmodified middle lamella in wood, various microscopic and spectroscopic methods were employed that are less accurate in terms of quantification than the thioacidolysis method used in this work. Many investigations have been reported where monolignols of various kinds have been added to the developing xylem or cell cultures, but these methods have the drawback that it is impossible to know whether the resulting lignin is within the natural variations in the cell or whether it is a response to what has been added and/or the experimental conditions. For example, the presence of *p*-hydroxyphenyl units in the middle lamella of spruce and poplar reported earlier may have been induced by the experimental conditions, or could be due to contamination. Of course, *p*-hydroxyphenyl units may be present in the middle lamella of stressed trees or of other species than those investigated here.

Poplar

The Klason lignin content in the mid-June sample of the developing xylem of Balsam poplar was so low that it is reasonable to assume that the lignin is confined to the middle lamella/ primary wall region. Analysis of the carbohydrate monomer distribution supported this view, whereas microscopy was inconclusive. Lignin from phloem and developing xylem in both the June and August samples was more condensed and contained more guaiacyl than syringyl units than the mature wood reference. In contrast, no *p*-hydroxyphenyl units were found. It is interesting to note the difference between the Norway spruce phloem and the phloem of Poplar. The Norway spruce phloem seemed to lack lignin in the areas examined, whereas the Poplar phloem contained lignin similar to that found in the developing xylem. A comparison of the lignin degradation products, analyzed by mass spectrometry, from the developing vascular tissue and from the reference wood indicated that there are many more end groups present in the vascular tissue of Balsam poplar than in the Balsam poplar reference wood. This indicates that the lignification at this time of the year takes place in a bulk fashion in the area analyzed. Since it is possible that double bonds are introduced in the degradation procedure, this finding needs to be supported by an analysis of polymeric lignin, for example by NMR of MWL. The hybrid aspen cell suspension cultures definitely had exclusively primary cell walls that are lignified, and this lignin contained only guaiacyl units which are more condensed than those observed in the reference poplar wood. However, no conclusion can be drawn regarding the similarity between this primary cell wall lignin and the lignin present in the primary walls of mature wood cells.

Spruce

Microscopy, Klason lignin and carbohydrate analyses showed that the lignin in the developing xylem of samples from mid-June was located exclusively in the middle lamella. The distribution of the carbohydrates and the lignin monomer composition in the developing

xylem during the growth season were significantly different from those of the reference clone wood material. Moreover, the lignin in the developing xylem was more condensed than the reference lignin. This is surprising, considering the fact that the mid-April xylem adjacent to the cambium appeared to be similar to the mature late-wood cells from the previous fall. The April sample contained 20% lignin compared to 25% in the reference. Thus, a significant portion of the final lignification of these cells may take place during early spring concomitantly with the development of the new early-wood xylem cells. Even if all the lignin bonds remaining to be introduced were β -O-4' bonds, it seemed that the almost mature late-wood cells contained more condensed lignin units than wood in general. In addition, the hemicelluloses differed from the average values for wood. Only guaiacyl units were detected in Norway spruce middle lamella. Comparing the lignin data of developing xylem with the lignin data from the aged wood, it appears that some of the secondary cell wall lignin was still present in the aged material, and the aged spruce material may not therefore be truly representative of middle lamella lignin. It is interesting to note that no degradation or modification of lignin seems to have occurred in the 10 000 year old specimen, even though most of the secondary cell wall was removed.

Most important findings in this thesis

- The pure middle lamella/primary cell wall lignin of Balsam poplar and Norway spruce contained fewer β -O-4' bonds and a larger number of condensed bonds than lignin from wood where secondary cell walls quantitatively dominate
- In mid-June, the developing xylem of Norway spruce contained only guaiacyl units in the middle lamella/primary cell wall
- In mid-June, the developing xylem of Balsam poplar has a ratio between syringyl and guaiacyl units of 0.6 in the middle lamella/primary cell wall but it was 1.3 for the Balsam poplar clone reference wood.
- In mid-June and mid August, lignin polymerization probably takes place in a bulk fashion during early lignification of Balsam poplar and Norway spruce, resulting in the incorporation of a larger number of end groups in the lignin formed.
- Lignin located in latewood Norway spruce tracheids may contain lignin with more condensed bonds than average spruce wood fibers.

4 GLOSSARY

- abscission**, 9; dropping off of plant parts
AFM, 9; atomic force microscopy
anaerobic, 19; without oxygen
angiosperm, 3; having covered seeds (fruit)
callose, 9; complex branched polysaccharide. Common wall constituent in sieve elements, may develop in response to tissue wounding
callus, 31; undifferentiated tissue culture
cambial zone, 5; area of undifferentiated meristematic cells between the secondary phloem and xylem; consists of cambial initials and their recent derivatives
clones, 19; specimens with identical genetic make up
CLSM, 6; confocal laser scanning microscopy
collenchyma cell, 5; elongated cells beneath epidermis (surface layer of plants) with soft non-lignified primary walls, live when mature
Coniferin, 20; coniferyl alcohol with a glucose residue attached, other names; Abietin, Coniferoside; Laricin; 4-hydroxy-3-methoxy-1-(γ -hydroxypropenyl)benzene-4-Dglucoside
Conifer, 3; trees that have cones, also called softwood
deciduous tree, 4 trees that shed leaves at certain times during the year
DFRC, 19; derivatization followed by reductive cleavage. Degradation method for lignin analysis
DHP, 16; dehydrogenative polymers, synthetic lignin produced for scientific research *in vitro*
eudicotyledonous, 3; Germinating with two leaves, one of 2 classes of angiosperms, formerly named dicotyledons,
fiber wall, 17; cell walls of one of the the major cell type of hardwoods; the fiber cell
fibril, 9; bundles of 30-100 glucopyranose chains with a degree of polymerization of approximately 15 000
forage, 3; food for animals especially when taken by browsing or grazing
GC, 19; gas chromatography
GC-FID, 33; gas chromatography flame ionization detector
GC-MS, 19; gas chromatography mass spectrometer
gymnosperm, 3; with naked seeds, as for example in cones
hardwood, 3; common name for a tree from the eudicotyledonous or magnoliid class
HPLC, 19; high pressure liquid chromatography
immunolocalization, 6; the use of antibodies to determine where in a specimen the group recognized by the antibody is situated
Klason lignin, 33; direct gravimetric method for determining lignin content, hydrolysis and solubilization of carbohydrates leaving lignin as a residue
meristem, 5; undifferentiated forever young tissue from which new cells arise
middle lamella, 9; pectin-rich outmost layer of the plant cell wall; formed during cell division, it occupies the space between adjacent cells
monocotyledonous, 3; germinating with one leaf, one of two classes of angiosperms
MWL, 18; milled wood lignin
NMR, 15; nuclear magnetic resonance
parenchyma cell, 5; living cell of various sizes involved in activities that require a living cell; photosynthesis, storage and secretion, may play a role in the transport of water and nutrients within the plant
primary phloem, 5; derived from the procambium, meristematic cells and their recent derivatives

primary xylem, 5; derived from the procambium, meristematic cells and their recent derivatives

protoplast, 5; living substance of an individual cell within the plasma membrane

ray cell, 5; horizontally oriented, mostly parenchyma cells. Vascular rays serve as pathways for transporting water from the xylem to the phloem and nutrients from the phloem to the xylem

sclereid, 5; seed coats and shells of nuts, typically short, thick lignified walls, dead at maturity

sclerenchyma, 5; supporting tissue consisting of sclerids and fibers having thick lignified walls, dead at maturity

secondary phloem, 5; produced outwardly by primary phloem in the vascular cambium

secondary xylem, 5; wood; produced inwardly by primary xylem in the vascular cambium

SEM, 17; scanning electron microscopy

SEM-EDXA, 17 scanning electron microscopy coupled with energy dispersive X-ray analysis

Sieve cell, 5; long-distance transport of nutrients in gymnosperms. Lack sieve plates found in the sieve tube elements of angiosperms

Sieve-tube elements, 5; long distance transport of nutrients in angiosperms. The word sieve refers to the collection of pores through which the protoplast of adjoining cells connect in an end-over-end manner creating continuous tubes within the plant. The walls of sieve elements are generally described as

primary and the cells are living when they are mature. However, when differentiating, they lose their nucleus, ribosomes, golgi complex and cytoskeleton. The rest of the protoplast (plasma membrane, smooth endoplasmatic reticulum, plastids, mitochondria and phloem protein) is distributed along the cell wall and thus creates an efficient transportation pathway within the plant

softwood, 3; common name for conifers, trees with cones

somatic embryogenesis, 5; refers to the initiation of embryos from previously differentiated somatic cells. Unlike cells of other eucaryotes, almost all plant cells have the capacity to become embryogenic under defined conditions. In addition to a model system for understanding embryo development, somatic embryos may be used as a convenient way to propagate large numbers of genetically identical individuals.

TEM, 20; transmission electron microscopy

tether, 12; something (as a rope or chain) by which an animal is fastened so that it can range only within a set radius

tracheids, 5; supportive tissue, transport of water and main celltype in gymnosperms

vascular cambium, 5; cylindrical sheet of meristematic cells, the division of which produces secondary xylem and phloem.

vessel, 5; transports water in the xylem of angiosperms. Tube-like structure of long cells (sieve tube elements) connected by perforations at the end

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6 REFERENCES

- Adler, E.** (1977). Lignin Chemistry - Past, Present and Future. *Wood Science and Technology* **11**, 169-218.
- Bailey, A.J.** (1936). Lignin in Douglas fir-composition of the middle lamella. *Industrial and Engineering Chemistry, Analytical Edition* **8**, 52-55.
- Bredemeijer, G.M.M., and Burg, H.C.J.** (1986). Peroxidase in suspension cultures of *Haplopappus gracilis*. *Acta Botanica Neerlandica* **35**, 153-159.
- Brett, C., and Waldron, K.** (1986). The molecular components of the cell wall. In *Physiology and biochemistry of plant cell wall*, M. Black and B. Charlwood, eds (London: Chapman & Hall), pp. 5-43.
- Brett, C., and Waldron, K.** (1996). *Physiology and biochemistry of plant cell wall*. (London: Chapman & Hall).
- Browne, C.A., Jr.** (1904). The hydrolytic products of sugar-cane fiber. *Journal of the American Chemical Society* **26**, 1221-1235.
- Browne, C.A., Jr., and Beistle, C.P.** (1901). The complete analysis of feeding materials. *Journal of the American Chemical Society* **23**, 229-236.
- Brunow, G., Kilpelainen, I., Lapierre, C., Lundquist, K., Simola, L.K., and Lemmetyinen, J.** (1993). The chemical structure of extracellular lignin released by cultures of *Picea abies*. *Phytochemistry* **32**, 845-850.
- Carpita, N.C., and Gibeaut, D.M.** (1993). Structural Models of Primary-Cell Walls in Flowering Plants - Consistency of Molecular-Structure with the Physical-Properties of the Walls During Growth. *Plant Journal* **3**, 1-30.
- Carpita, N.C., and McCann, M.C.** (2000). The Cell Wall. In *Biochemistry & Molecular Biology of Plants*, pp. 52-110.
- Chafe, S.** (1972). Fine structural observation on epidermis I. The epidermis cell wall. *Planta* **107**, 269-278.
- Chen, Y.-r., and Sarkanen, S.** (2004). Macromolecular lignin replication: A mechanistic working hypothesis. *Phytochemistry Reviews* **2**, 235-255.
- Crocker, E.C.** (1921). An experimental study of the significance of lignin color reactions. *Journal of Industrial and Engineering Chemistry (Washington, D. C.)* **13**, 625-627.
- Davin, L.B., and Lewis, N.G.** (2005). Lignin primary structures and dirigent sites. *Current Opinion in Biotechnology* **16**, 407-415.
- Dean, J.F.D., LaFayette, P.R., Rugh, C., Tristram, A.H., Hoopes, J.T., Eriksson, K.-E.L., and Merkle, S.A.** (1998). Laccases associated with lignifying vascular tissues. *ACS Symposium Series* **697**, 96-108.
- Dence, C.W.** (1992a). The determination of lignin. In *Methods in lignin chemistry*, S.Y. Lin and C.W. Dence, eds (Heidelberg: Springer-Verlag), pp. 33-61.
- Dence, C.W.** (1992b). Characterization in Solution Chemical degradation methods. In *Methods in lignin chemistry*, C.W. Dence and L.S. Lin, eds (Heidelberg: Springer-Verlag), pp. 289--368.
- Donaldson, L.A.** (1991). Seasonal-Changes in Lignin Distribution During Tracheid Development in *Pinus-Radiata* D Don. *Wood Science and Technology* **25**, 15-24.
- Donaldson, L.A.** (1992). Lignin Distribution During Latewood Formation in *Pinus-Radiata* D Don. *Iawa Bulletin* **13**, 381-387.
- Donaldson, L.A.** (1994). Mechanical Constraints on Lignin Deposition During Lignification. *Wood Science and Technology* **28**, 111-118.

- Edashige, Y., and Ishii, T.** (1996). Structures of cell-wall polysaccharides from suspension-cultured cells of *Cryptomeria japonica*. *Mokuzai Gakkaishi* **42**, 895-900.
- Edashige, Y., Ishii, T., Hiroi, T., and Fujii, T.** (1995). Structural-Analysis of Polysaccharides of Primary Walls from Xylem Differentiating Zones of *Cryptomeria-Japonica* D-Don. *Holzforschung* **49**, 197-202.
- Elfving, B.** (1986). odlingsvärdet av björk, asp och al på nedlagd jordbruksmark i Sydsverige. Skogsvårdsförbundets tidskrift, 86.
- Eom, T.J., Meshitsuka, G., Ishizu, A., and Nakano, J.** (1987). Chemical characteristics of lignin in the differentiating xylem of a hardwood. III. *Mokuzai Gakkaishi* **33**, 716-723.
- Eom, T.J., Meshitsuka, G., Ishizu, A., and Nakano, J.** (1988). Chemical Characteristics of Lignin in the Differentiating Xylem of Larch Wood. *Cellulose Chemistry and Technology* **22**, 211-219.
- Erdtman, H.** (1933). Dehydrierungen in der Coniferylreihe. II. Dehydrodi-isieugenol. *Annalen*, 283-294.
- Erdtman, H.** (1957). Outstanding problems in lignin chemistry. *Ind. Eng. Chem.* **49**, 1385-1386.
- Fengel, D., and Wegener, G.** (1984). Ultrastructure of the cell wall. In *Wood Chemistry, Ultrastructure Reactions* (Berlin, New York: Walter de Gruyter), pp. 13-17.
- Fergus, B.J., and Goring, D.A.I.** (1970). Location of guaiacyl and syringyl lignins in birch xylem tissue. *Holzforschung* **24**, 113-117.
- Freudentberg, K., and Neish, A.** (1968). *Constitution and Biosynthesis of Lignin*. (Berlin, Heidelberg, New-York: Springer-Verlag).
- Freudentberg, K., Harkin, J.M., Reichert, M., and Fukuzumi, T.** (1958). Enzymes participating in lignification. Dehydrogenation of sinapin alcohol. *Chemische Berichte* **91**, 581-590.
- Furstenberg, A., Murdfield, R., and König, J.** (1907). The Chemical and Physiological Properties of Cell Walls. *Landw. Vers.-Sta.* **65**, 55-110.
- Gil, A.M., and Pascoal Neto, C.** (1999). Solid-state NMR studies of wood and other lignocellulosic materials. *Annual Reports on NMR Spectroscopy* **37**, 75-117.
- Grisebach, H., and Hahlbrock, K.** (1974). Enzymology and regulation of flavonoid and lignin biosynthesis in plants and plant cell suspension cultures. *Recent Advances in Phytochemistry* **8**, 21-52.
- Grunwald, C., Ruel, K., Kim, Y.S., and Schmitt, U.** (2002). On the cytochemistry of cell wall formation in poplar trees. *Plant Biology* (Stuttgart, Germany) **4**, 13-21.
- Guan, S.-Y., Mlynar, J., and Sarkanen, S.** (1997). Dehydrogenative polymerization of coniferyl alcohol on macromolecular lignin templates. *Phytochemistry* **45**, 911-918.
- Guo, D.J., Chen, F., Inoue, K., Blount, J.W., and Dixon, R.A.** (2001). Downregulation of caffeic acid 3-O-methyltransferase and caffeoyl CoA 3-O-methyltransferase in transgenic alfalfa: Impacts on lignin structure and implications for the biosynthesis of G and S lignin. *Plant Cell* **13**, 73-88.
- Harkin, J.M., and Obst, J.R.** (1973). Lignification in trees. Indication of exclusive peroxidase participation. *Science* (Washington, DC, United States) **180**, 296-298.
- Henriksson, G.** (2005). Chapter 6 Lignin. In *The Ljungberg textbook (Fiber & Polymer technology* Royal Institute of Technology.

- Higuchi, T.** (1990). Lignin Biochemistry - Biosynthesis and Biodegradation. Wood Science and Technology **24**, 23-63.
- Huang, T., Boehlenius, H., Eriksson, S., Parcy, F., and Nilsson, O.** (2005). The mRNA of the *Arabidopsis* Gene FT Moves from Leaf to Shoot Apex and Induces Flowering. Science (Washington, DC, United States) **309**, 1694-1696.
- Hughes, J., and Merry, W.** (1978). Marquette buried forest 9850 years old. In 144th National AAAS meeting.
- Högberg, K.A., Ekberg, I., Norell, L., and von Arnold, S.** (1998). Integration of somatic embryogenesis in a tree breeding programme: a case study with *Picea abies*. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere **28**, 1536-1545.
- Ikeda, T., Holtman, K., Kadla, J.F., Chang, H.M., and Jameel, H.** (2002). Studies on the effect of ball milling on lignin structure using a modified DFRC method. Journal of Agricultural and Food Chemistry **50**, 129-135.
- Johnsson, H.** (1952). Hybridaspens ungdomsutveckling och ett försök till framtidsprognos (Föreningen för växtförädling av skogsträd).
- Jouanin, L., Goujon, T., Sibout, R., Pollet, B., Mila, I., Leple, J.C., Pilate, G., Petit-Conil, M., Ralph, J., and Lapierre, C.** (2004). Comparison of the consequences on lignin content and structure of COMT and CAD downregulation in poplar and *Arabidopsis thaliana*. Plantation Forest Biotechnology for the 21st Century, 219-229.
- Jouanin, L., Goujon, T., de Nadai, V., Martin, M.T., Mila, I., Vallet, C., Pollet, B., Yoshinaga, A., Chabbert, B., Petit-Conil, M., and Lapierre, C.** (2000). Lignification in transgenic poplars with extremely reduced caffeic acid O-methyltransferase activity. Plant Physiol. **123**, 1363-1373.
- Karacic, A.** (1999). Dimensional relations and early production of hybrid aspen and hybrid poplar on former agricultural land (Uppsala: Department of short rotation forestry at SLU).
- Karkonen, A., Koutaniemi, S., Mustonen, M., Syrjanen, K., Brunow, G., Kilpelainen, I., Teeri, T.H., and Simola, L.K.** (2002). Lignification related enzymes in *Picea abies* suspension cultures. Physiologia Plantarum **114**, 343-353.
- Keegstra, K., Talmadge, K., Bauer, W., and Albersheim, P.** (1973). Structure of plant cell walls. III. A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. Plant Physiol. **51**, 188-197.
- Kim, Y.S., Meshitsuka, G., and Ishizu, A.** (1994). Structural heterogeneity of lignin. Contribution of carbon-carbon bonds. Mokuzai Gakkaishi **40**, 407-413.
- Konig, J.** (1907). Determination of Cellulose, Lignin and Cutin in Crude Fibre. Second Contribution. Z. Nahr.-Genussm. **12**, 385.
- Kukkola, E.M., Koutaniemi, S., Gustafsson, M., Karhunen, P., Ruel, K., Lundell, T.K., Saranpaa, P., Brunow, G., Teeri, T.H., and Fagerstedt, K.V.** (2003). Localization of dibenzodioxocin substructures in lignifying Norway spruce xylem by transmission electron microscopy-immunogold labeling. Planta **217**, 229-237.
- Kukkola, E.M., Koutaniemi, S., Poellaenen, E., Gustafsson, M., Karhunen, P., Lundell, T.K., Saranpaa, P., Kilpelainen, I., Teeri, T.H., and Fagerstedt, K.V.** (2004). The dibenzodioxocin lignin substructure is abundant in the inner part of the secondary wall in Norway spruce and silver birch xylem. Planta **218**, 497-500.

- Langhammer, A.** (1974). Ungdomsvekst og utvikling hos kloner av amerikansk balsampoppel under forskjellige klimaforhold i Norge. Meldinger fra Norges landbrukshogskole **53**.
- Lapierre, C., and Lundquist, K.** (1999). Investigations of low molecular weight and high molecular weight lignin fractions. Nordic Pulp & Paper Research Journal **14**, 158-162.
- Lapierre, C., Monties, B., and Rolando, C.** (1985). Thioacidolysis of Lignin - Comparison with Acidolysis. Journal of Wood Chemistry and Technology **5**, 277-292.
- Lapierre, C., Pollet, B., Monties, B., and Rolando, C.** (1991). Thioacidolysis of Spruce Lignin - Gc-MS Analysis of the Main Dimers Recovered after Raney-Nickel Desulfuration. Holzforschung **45**, 61-68.
- Lewis, N.G., Davin, L.B., and Sarkanen, S.** (1999). The nature and function of lignins. Comprehensive Natural Products Chemistry **3**, 617-745.
- Lu, F., and Ralph, J.** (1997a). DFRC Method for Lignin Analysis. 1. New Method for b-Aryl Ether Cleavage: Lignin Model Studies. Journal of Agricultural and Food Chemistry **45**, 4655-4660.
- Lu, F., and Ralph, J.** (1997b). Derivatization Followed by Reductive Cleavage (DFRC Method), a New Method for Lignin Analysis: Protocol for Analysis of DFRC Monomers. Journal of Agricultural and Food Chemistry **45**, 2590-2592.
- Lu, F., and Ralph, J.** (2003). Non-degradative dissolution and acetylation of ball-milled plant cell walls: high-resolution solution-state NMR. Plant Journal **35**, 535-544.
- Ludwig, C.H.** (1971). Magnetic resonance spectra. In Lignins, Occurrence, Formation, Structure and Reactions, L. Sarkanen and C.H. Ludwig, eds (New York: Wiley-interscience), pp. 299-344.
- McNeil, M., Darvill, A.G., Fry, S.C., and Albersheim, P.** (1984). Structure and Function of the Primary-Cell Walls of Plants. Annu. Rev. Biochem. **53**, 625-663.
- Melo N.S., J.M.S., C., and M.P., F.** (1995). Extracellular peroxidases from cell suspension cultures of *Vaccinium myrtillus*. Purification and characterization of two cationic enzymes. Plant Sci. Lett., 177-184.
- Meyers, P.A., Leenheer, M.J., and Bourbonniere, R.A.** (1995). Diagenesis of vascular plant organic matter components during burial in lake sediments. Aquatic Geochemistry **1**, 35-42.
- Meyers, P.A., Leenheer, M.J., Erstfeld, K.M., and Bourbonniere, R.A.** (1980). Changes in Spruce Composition Following Burial in Lake-Sediments for 10,000 Yr. Nature **287**, 534-536.
- Nakamura, W.** (1967). Biosynthesis of lignin. I. Disproof against the catalytic activity of laccase in the oxidation of coniferyl alcohol. Journal of Biochemistry (Tokyo, Japan) **62**, 54-61.
- Newman, R.** (1998). Evidence of assignment of ¹³C NMR signals to cellulose crystallite surfaces in wood, pulp and isolated celluloses. Holzforschung **52**, 157-159.
- Nobel, P.** (1974). Introduction to Biophysical Plant Physiology. (San Francisco: W.H. Freeman).
- Ohlsson, A.B., Djerbi, S., Winzeli, A., Bessueille, L., Ståldal, V., Li, X.G., Blomqvist, K., Bulone, V., Teeri, T.T., and Berglund, T.** (2006). Cell suspension cultures of *Populus tremula x tremuloides* exhibit a high level of

- cellulose synthase gene expression that coincides with increased in vitro cellulose synthase activity. *Protoplasma*.
- Pauly, M., Albersheim, P., Darvill, A., and York, W.S.** (1999). Molecular domains of cellulose/xyloglucan network in the cell wall of higher plants. *The Plant Journal* **20**, 629-639.
- Pilate, G., Guiney, E., Holt, K., Petit-Conil, M., Lapierre, C., Leple, J.C., Pollet, B., Mila, I., Webster, E.A., Marstorp, H.G., Hopkins, D.W., Jouanin, L., Boerjan, W., Schuch, W., Cornu, D., and Halpin, C.** (2002). Field and pulping performances of transgenic trees with altered lignification. *Nature Biotechnology* **20**, 607-612.
- Pincon, G., Maury, S., Hoffmann, L., Geoffroy, P., Lapierre, C., Pollet, B., and Legrand, M.** (2001). Repression of O-methyltransferase genes in transgenic tobacco affects lignin synthesis and plant growth. *Phytochemistry* **57**, 1167-1176.
- Preston, R.** (1982). The case for multinet growth in growing walls of plant cells. *Planta* **155**, 356-363.
- Ralph, J., Hatfield, R.D., Piquemal, J., Yahiaoui, N., Pean, M., Lapierre, C., and Boudet, A.M.** (1998). NMR characterization of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamyl-alcohol dehydrogenase and cinnamoyl-CoA reductase. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 12803-12808.
- Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P.F., Marita, J.M., Hatfield, R.D., Ralph, S.A., Christensen, J.H., and Boerjan, W.** (2004). Lignins: natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. *Phytochemistry Reviews* **3**, 29-60.
- Ralph, J., Marita, J.M., Ralph, S.A., Hatfield, R.D., Lu, F., Ede, R.M., Peng, J., Quideau, S., Helm, R.F., Grabber, J.H., Kim, H., Jimenez-Monteon, G., Zhang, Y., Jung, H.-J.G., Landucci, L.L., MacKay, J.J., Sederoff, R.R., Chapple, C., and Boudet, A.M.** (1999). Solution-state NMR of lignins. *Advances in Lignocellulosics Characterization*, 55-108.
- Raven, P., Ray, E., and Eichhorn, S.** (1999). *Biology of plants*. (New York: W. H. Freeman and company Worth Publishers).
- Reiter, W.D.** (1998). *Arabidopsis thaliana* as a model system to study synthesis, structure, and function of the plant cell wall. *Plant Physiology and Biochemistry* **36**, 167-176.
- Ritter, G.J.** (1925). Distribution of lignin in wood. *Journal of Industrial and Engineering Chemistry (Washington, D. C.)* **17**, 1194-1197.
- Roelofsen, P., and Houwink, A.** (1953). Architecture and growth of the primary plant cell wall in some plant hairs and in the *Phycomyces sporangiophore*. *Acta Bot. Neerl.* **2**, 218-225.
- Rytter, L., Stener, L.-G., and Werner, M.** (2002). Hybridasp-ett lönsamt alternativ som passa i det nya skogsbruket. In Resultat nr 10 (Uppsala: Stiftelsen Skogsbrukets Forsknings institut), pp. 1-4.
- Saka, S., and Thomas, R.J.** (1982). A Study of Lignification in Loblolly-Pine Tracheids by the Sem-Edxa Technique. *Wood Science and Technology* **16**, 167-179.
- Sarkanen, K., and Hergert, H.L.** (1971). Classification and distribution. In *Lignins occurrence, formation and reactions*, K. Sarkanen and C.H. Ludwig, eds (New York: Wiley-Interscience), pp. 43-94.

- Simson, B.W., and Timell, T.E.** (1978a). Polysaccharides in cambial tissues of *Populus tremuloides* and *Tilia americana*. II. Isolation and structure of a xyloglucan. *Cellulose Chemistry and Technology* **12**, 51-62.
- Simson, B.W., and Timell, T.E.** (1978b). Polysaccharides in cambial tissues of *Populus tremuloides* and *Tilia americana*. I. Isolation, fractionation, and chemical composition of the cambial tissues. *Cellulose Chemistry and Technology* **12**, 39-50.
- Sjöström, E.** (1981). Hemicelluloses. In *Wood Chemistry fundamentals and application*, E. Sjöström, ed (San Diego: Academic Press), pp. 63-72, appendix.
- Smith, R.C., and Fry, S.C.** (1991). Endotransglycosylation of xyloglucans in plant cell suspension cultures. *Biochem. J.* **279**, 529-535.
- Sonesson, J.** (1996). Grankloner ur Hilleshögs klonskogspogram. In *Avelsvärden* (Uppsala: Skogforsk).
- Sonesson, J., and Almqvist, C.** (2002). Från klonskogsbruk till bulksticklingar. In *Report*, J. Fryk, ed (Hållsta: Skogforsk).
- Sterjiades, R., Dean, J.F.D., and Eriksson, K.E.L.** (1992). Laccase from Sycamore Maple (*Acer-Pseudoplatanus*) Polymerizes Monolignols. *Plant Physiol.* **99**, 1162-1168.
- Sterjiades, R., Ranocha, P., Boudet, A.M., and Goffner, D.** (1996). Identification of specific laccase isoforms capable of polymerizing monolignols by an "In-gel" procedure. *Analytical Biochemistry* **242**, 158-161.
- Sterjiades, R., Dean, J.F.D., Gamble, G., Himmelsbach, D.S., and Eriksson, K.E.L.** (1993). Extracellular laccases and peroxidases from sycamore maple (*Acer pseudoplatanus*) cell-suspension cultures. Reactions with monolignols and lignin model compounds. *Planta* **190**, 75-87.
- Sutton, B.** (2002). Commercial delivery of genetic improvement to conifer plantations using somatic embryogenesis. *Annals of Forest Science* **59**, 657-661.
- Talbott, L.D., and Ray, P.M.** (1992). Molecular-Size and Separability Features of Pea Cell-Wall Polysaccharides - Implications for Models of Primary Wall Structure. *Plant Physiol.* **98**, 357-368.
- Terashima, N., and Fukushima, K.** (1988). Heterogeneity in Formation of Lignin .11. An Autoradiographic Study of the Heterogeneous Formation and Structure of Pine Lignin. *Wood Science and Technology* **22**, 259-270.
- Terashima, N., Fukushima, K., and Tsuchiya, S.** (1986). Heterogeneity in Formation of Lignin .7. An Autoradiographic Study on the Formation of Guaiacyl and Syringyl Lignin in Poplar. *Journal of Wood Chemistry and Technology* **6**, 495-504.
- Terashima, N., Atalla, R.H., and Vanderhart, D.L.** (1997). Solid state NMR spectroscopy of specifically C-13-enriched lignin in wheat straw from coniferin. *Phytochemistry* **46**, 863-870.
- Terashima, N., Hafren, J., Westermarck, U., and VanderHart, D.L.** (2002). Nondestructive analysis of lignin structure by NMR Spectroscopy of specifically C-13-enriched lignins Part 1. Solid state study of Ginkgo wood. *Holzforschung* **56**, 43-50.
- Thomas, J.R., McNeil, M., Darvill, A.G., and Albersheim, P.** (1987). Structure of plant cell walls. XIX. Isolation and characterization of wall polysaccharides from suspension-cultured Douglas fir cells. *Plant Physiol.* **83**, 659-671.

- Ucar, G., Staccioli, C.G., and Stoll, M.** (1996). Chemical composition and ultrastructure of a fossil wood from the genus of ancestral *Sequoia*. *Holz als Roh- und Werkstoff* **54**, 411-421.
- Ucar, G., Meier, D., Faix, O., and Wegener, G.** (2005). Analytical pyrolysis and FTIR spectroscopy of fossil *Sequoiadendron giganteum* (Lindl.) wood and MWLs isolated hereof. *Holz Als Roh-Und Werkstoff* **63**, 57-63.
- Valent, B.S., and Albersheim, P.** (1974). The structure of plant cell wall. V. On the binding of xyloglucan to cellulose fibers. *Plant Physiol.*, 105-108.
- Wardrop, A.** (1957). The phase of lignification in the differentiation of wood fibers. *Tappi* **40**, 225-243.
- Varner, J.E., and Lin, L.S.** (1989). Plant-Cell Wall Architecture. *Cell* **56**, 231-239.
- Westermarck, U.** (1985). The Occurrence of P-Hydroxyphenylpropane Units in the Middle-Lamella Lignin of Spruce (*Picea-abies*). *Wood Science and Technology* **19**, 223-232.
- Weyerhaeuser, G.** (2003). Biotechnology in forestry: The promise and the economic reality. In *Solutions*, pp. 28-30.
- Whetten, R., and Sederoff, R.** (1995). Lignin Biosynthesis. *Plant Cell* **7**, 1001-1013.
- Whiting, P., and Goring, D.A.I.** (1982). Chemical Characterization of Tissue Fractions from the Middle Lamella and Secondary Wall of Black Spruce Tracheids. *Wood Science and Technology* **16**, 261-267.
- Wickholm, K., Larsson, P., and Iversen, T.** (1998). Assignment of non-crystalline forms in cellulose I by CP/MAS ¹³C NMR spectroscopy. *Carbohydrate Research* **312**, 123-129.
- Önnerud, H., and Gellerstedt, G.** (2003). Inhomogeneities in the chemical structure of spruce lignin. *Holzforschung* **57**, 165-170.

