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Tracheid cell-wall compositions and properties in compression woods of young trees of *Pinus radiata* D. Don

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Abstract

Compression wood (CW) forms on the underside of tilted stems of coniferous gymnosperms and opposite wood (OW) on the upperside. More is known about the most severe form of CW, severe CW (SCW), but mild CWs (MCWs) also occur widely. In this thesis, two grades of MCWs, MCW1 and MCW2, as well as SCW and OW were identified in the stems of slightly tilted radiata pine (*Pinus radiata*) by the distribution of lignin in their tracheid walls. The tracheid walls of the four wood types differed structurally and chemically. The presence of an S3 wall layer was a marker for very MCW (MCW1) and the presence of helical cavities in the S2 wall layer for SCW. Using immunomicroscopy, (1→4)-β-D-galactans and (1→3)-β-D-glucans were detected in the tracheid walls of all CWs, but in only trace amounts in OW. The areas and intensities of labelling increased with CW severity. The four wood types were identified using the immunolabelling method on whole stem sections with the antibody for (1→4)-β-D-galactans. Microscopically well-characterized discs (0.5 mm diameter) of the different wood types were used to determine the neutral monosaccharide compositions of the non-cellulosic polysaccharides, lignin concentrations and lignin monomer compositions. Lignin concentration and the proportion of p-hydroxyphenyl units (H-units) relative to guaiacyl (G-units) increased with CW severity. Raman and FTIR micro-spectroscopies coupled with principal component analysis (PCA) were used to differentiate the four different wood types. The longitudinal, tangential and radial shrinkage of tracheids in the four wood types was investigated using variable-pressure scanning electron microscopy. The longitudinal shrinkage of SCW tracheids was ~300% greater than that of OW tracheids, with the MCW tracheids being intermediate. A statistical association was found between longitudinal shrinkage and the content of lignin and galactosyl residues in the cell-wall matrix.
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Abbreviations used

Abbreviations used were those of the “International System of Units” (SI). Standard notations for chemical elements and formulae were used.

- **ABSL**: acetyl bromide soluble lignin
- **ANOVA**: analysis of variance
- **AO**: acridine orange
- **Araf**: arabinofuranosyl
- **Arap**: arabinopyranosyl
- **BCIP/NBT**: bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium
- **BSA**: bovine serum albumin
- **CCD**: charge-coupled device
- **CDA**: canonical discriminant analysis
- **CLSM**: confocal laser scanning microscopy
- **CML**: compound middle lamella
- **CV**: canonical variate
- **CW**: compression wood
- **ESEM**: environmental scanning electron microscope
- **FEG**: field emission gun
- **FTIR**: Fourier transform infrared
- **GaLA**: galacturonic acid
- **GALp**: galactopyranosyl
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>HG</td>
<td>homogalacturonan</td>
</tr>
<tr>
<td>Glcp</td>
<td>glucopyranosyl</td>
</tr>
<tr>
<td>GlcpA</td>
<td>glucopyranosyluronic acid</td>
</tr>
<tr>
<td>GSED</td>
<td>gaseous secondary electron detector</td>
</tr>
<tr>
<td>G-unit</td>
<td>guaiacyl-derived unit of lignin</td>
</tr>
<tr>
<td>HPAEC-PAD</td>
<td>high-performance anion-exchange chromatography with pulsed amperometric detection</td>
</tr>
<tr>
<td>H-unit</td>
<td>$p$-hydroxyphenyl-derived unit of lignin</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
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<td>MANOVA</td>
<td>multivariate analysis of variance</td>
</tr>
<tr>
<td>Manp</td>
<td>mannopyranosyl</td>
</tr>
<tr>
<td>MCW</td>
<td>mild compression wood</td>
</tr>
<tr>
<td>MCW1</td>
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</tr>
<tr>
<td>MCW2</td>
<td>mild compression wood 2</td>
</tr>
<tr>
<td>MFA</td>
<td>microfibril angle</td>
</tr>
<tr>
<td>ML</td>
<td>middle lamella</td>
</tr>
<tr>
<td>MLCC</td>
<td>middle lamella at cell corners</td>
</tr>
<tr>
<td>MP-PBS</td>
<td>phosphate buffered saline containing milk powder</td>
</tr>
<tr>
<td>NW</td>
<td>normal wood</td>
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MeGlc pA  4-O-methyl-α-D-glucuronic acid
OW    opposite wood
PBS   phosphate buffered saline
PC    principal component
PCA   principal component analysis
PIPES 1,4-piperazinediethanesulfonic acid
PLS   partial least square
pH    potential of hydrogen
PM    pit membrane
Py-GC/MS pyrolysis gas chromatography and mass spectrometry
RG I  rhamnogalacturonan I
RG II rhamnogalacturonan II
RH    relative humidity
Rhap  rhamnopyranosyl
S2i   inner region of S2 layer
S2L   outer region of S2 layer
SCW   severe compression wood
S-unit syringyl-derived unit of lignin
TEC   thermoelectrically cooled
TEM   transmission electron microscopy
TFA   trifluoroacetic acid
<table>
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<th>Definition</th>
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<tbody>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>VP-SEM</td>
<td>variable-pressure scanning electron microscopy</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
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<tr>
<td>Xylp</td>
<td>xylopyranosyl</td>
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Chapter 1 – General introduction

1.1 Types of woods

Wood (secondary xylem) is one of the world’s most abundant resources. It is used both structurally as a timber and for pulping and paper production (Plomion et al., 2001). Wood develops from the vascular cambium of trees during secondary growth (Butterfield and Meylan, 1980; Timell, 1986). The physical and chemical properties of wood are determined by the structure and composition of the secondary cell walls (Novaes et al., 2010).

Woods are commonly classified into two groups: softwoods from gymnosperms and hardwoods from angiosperms. The terms softwood and hardwood come from the traditional timber trade, as softwoods are generally easier to work than hardwoods (Butterfield and Meylan, 1980). These two wood types are quite different in their anatomical structures and cell-wall compositions, with hardwoods being more complex and variable than softwoods. Softwoods have a relatively simple structure, which is formed mostly of axial tracheids and some rays (Figure 1-1) (Butterfield and Meylan, 1980). I will focus on softwoods in this thesis. The gymnosperms are often divided into four groups, Cycadidae (the cycads), Ginkgoidae (Ginkgo biloba), Gnetidae (the gnetophytes) and Pinidae (the conifers) (Christenhusz et al., 2011). The subclass Pinidae, comprises the conifers, which include most of the commercially important gymnosperms. The conifers number some 629 species (Christenhusz and Byng, 2016). As well as the wood that is normally formed, which I shall refer to as normal wood, both gymnosperms and angiosperms produce reaction woods.
Figure 1-1. Scanning electron micrograph of the softwood *Pinus nigra* Schneid. (Pinaceae). The wood is formed mainly of axial tracheids (T), and radial rays (R) including ray parenchyma and horizontal ray tracheids. Figure shows both axial and radial resin canals (RC). Figure modified from Butterfield and Meylan (1980).

1.1.1 Reaction woods

Reaction woods form in tilted stems or branches of both softwoods and hardwoods as the response to mechanical stress to restore vertical growth. In softwoods, they are called compression woods (CWs), and form on the lower side of the leaning stems or branches exerting a force to push the stems or branches back to the upright direction. In hardwoods, they are called tension woods, and form on the upper side of the leaning stems or branches pulling the stems or branches to the upright direction. In both softwoods and hardwoods, the woods formed on the opposite sides of the stems or branches to the reaction woods are called opposite woods (OWs), and are generally chemically and anatomically similar to normal woods (NWs) (Scurfield, 1973; Timell, 1986).

1.1.1.1 Compression woods

CWs have been noticed for many centuries and found to form in the conifers (subclass Pinidae) and *Ginkgo biloba* (subclass Ginkgoidae) associated with geotropic and other orientation movements in stems and branches (Timell, 1986). The formation of CW is more common in young than old trees and may be caused by high growth rates (Wardrop and Dadswell, 1950; Wadenbäck et al., 2004). The mechanism of CW formation is not clear, but
plant hormones, including auxin and ethylene, are believed to be involved in regulating CW formation (Du and Yamamoto, 2007).

CW is vital to the tree as a tree that lacked the ability to form CW could probably not survive very long (Westing, 1965). However, CW is considered a serious defect for the timber industry due to its unusual properties. The hardness and brittleness of CW make it more difficult to work with tools (Butterfield, 2006). On drying, the longitudinal shrinkage of CW is greater than that of NW. The presence in timber of CW adjoining OW or NW can lead to twisting, warping and sometimes even splitting resulting from differential shrinkage of the wood types (Westing, 1965; Timell, 1986; Chauhan et al., 2006). The high lignin content of CWs reduces their desirability for the paper and pulping industry (Timell, 1986).

1.2 Anatomy of softwood OWs and CWs

1.2.1 Axial cell system of NW and CW

The axial cell system of both softwood NWs and CWs consists mostly of axial tracheids (more than 90% of the total wood volume), and sometimes of axial parenchyma cells and axial resin canals (Butterfield and Meylan, 1980; Bamber and Burley, 1983). Axial tracheids are thick-walled elongated cells with pointed ends with two functions: to transport water and to provide mechanical strength (Butterfield and Meylan, 1980; Evert, 2006). Axial tracheids produced by the vascular cambium early in the growing season form the earlywood, which accounts for 40-80% of the width of a growth ring. Axial tracheids formed towards the end of the growing season form the latewood (Butterfield and Meylan, 1980; Domec and Gartner, 2002). The distinction between earlywood and latewood is obvious (Figure 1-2). The earlywood tracheids have larger lumens and thinner walls than latwood tracheids which have smaller lumens and thicker walls. The two types of tracheids are separated by the growth ring boundary (Butterfield and Meylan, 1980). Earlywood transports up to 90% of the total water flow and is less vulnerable to embolism than latwood (Domec and Gartner, 2002). Sudden changes in water availability and other environmental factors may lead to the production of an additional false ring, and this is more likely to happen when the trees are young (Wimmer et al., 2000; Copenheaver et al., 2006).
Figure 1-2. Scanning electron micrograph of a growth ring boundary in *Chamaecyparis lawsoniana* Parl. (Cupressaceae). The latewood (LW) tracheids have smaller lumens and thicker walls. The earlywood (EW) tracheids have larger lumens and thinner walls. The two wood types are divided by the growth ring boundary (GRB). The pith (P) is on the left hand side direction and the cambium (C) is at the right hand side direction of the image. Figure modified from Butterfield and Meylan (1980).

Normal wood (NW) or OW tracheids are usually 2-5 mm long and 0.01-0.08 mm in the radial direction depending on the species and on their positions in the stem. They have both a primary and a thick secondary cell wall. Between individual tracheids, a highly lignified thin layer called the middle lamella (ML) tightly glues the cells together. The primary cell wall is generally very thin and it can often not be distinguished from the ML; the term compound middle lamella (CML) is often used to describe the combined ML and primary wall (Butterfield and Meylan, 1980). The ML first develops as the cell plate onto which is deposited the primary wall (Vincken et al., 2003). After cell enlargement is complete, the secondary wall is deposited on the inside of the primary wall (Timell, 1967; Harris, 2006). The secondary cell walls of NW and OW tracheids typically have three distinct layers, the S1, S2 and S3 layers, with the S2 layer being the thickest (Figure 1-3) (Butterfield and Meylan, 1980; Harris, 2006). The cellulose microfibrils in the secondary walls are more densely packed and highly ordered than those in the primary walls, and are aligned at different angles in each layer. They usually have a uniform and steep helical organization in the thick S2 layer (Donaldson, 2008) (Figure 1-3).
Figure 1-3. Cell wall layers of a typical NW tracheid containing cellulose microfibrils arranged at different angles. Figure from Donaldson (2008).

CW tracheids are shorter, rounder and thicker walled than NW tracheids. They also have truncated or bent tips, and have intercellular spaces present at the corners of adjoining cells (Timell, 1986; Groover, 2016). These tracheids have a highly lignified, outer S2 layer (S2L) that is continuous around the perimeter of the cell with helical grooves in the inner S2 layer, and they lack the innermost S3 layer (Yumoto and Ishida, 1982; Yumoto et al., 1983; Timell, 1986). CW tracheid walls have a greater microfibril angle (often > 45° from the long axis of the cell) than mature NW or OW tracheid walls, which may result from a shift in microtubule orientation during the CW formation (Barnett and Bonham, 2004; Fagerstedt et al., 2014). However, corewood or juvenile OW also has a large MFA, which is not significantly different from that in the CW (Burdon et al., 2004; Donaldson 2008; Brennan et al., 2012).

Adjacent tracheids are connected by circular bordered pits that are initiated before the secondary wall begins to form (Figure 1-4a) (Evert, 2006; Choat et al., 2008). The secondary wall surrounding the pit forms a pit chamber, and the primary wall becomes the pit membrane, with a centrally thickened torus, suspended by a margo of cellulose microfibrils (Figure 1-4b) (Bamber and Burley, 1983). The torus and margo allow water to pass between two functional tracheids while preventing air flow out from an embolized tracheid (Bauch et al., 1972; Butterfield and Meylan, 1980; Hacke et al., 2004). CW tracheids have fewer and
smaller bordered pits with narrower pit apertures in their radial walls than those in OW tracheids (Westing, 1968; Tarmian et al., 2011).

Figure 1-4. (a) Scanning electron micrograph of bordered pits in the radial walls of earlywood tracheids in *Pinus radiata* D. Don (Pinaceae). Water passes from one tracheid to the next through the pit aperture (PA) of one cell, into the pit chamber (PC), through the pit membrane and into the pit chamber of the second tracheid and finally out into the second tracheid. The electron micrograph is modified from Butterfield and Meylan (1980). (b) Scanning electron micrograph of a bordered pit membrane of a conifer, *Calocedrus decurrens*, with a central thickened torus (T) and a very porous outer margo (M). Figure modified from Choat and Pittermann (2009).

Axial parenchyma cells have thinner walls than axial tracheids, and have protoplasts that often live for many years. There are two types of axial parenchyma cells: true axial parenchyma cells, which occur in single strands and axial parenchyma cells, which are associated with resin canals. The true axial parenchyma cells are not common in softwoods, and are absent from the genera *Taxus, Pinus, Picea* and most *Daacrydium sp.* (Butterfield and Meylan, 1980; Evert, 2006).

Axial resin canals are commonly present in many genera of conifers, including *Pinus, Picea, Cathaya, Larix* and *Pseudotsuga*, but they are never been found in the woods of *Juniperus* and *Cupressus* (Evert, 2006). These canals are composed of epithelial resin-secreting cells surrounding the canal lumen, parenchyma support cells and strand tracheids (Cown et al., 2011). The sizes and the structures of resin canals vary among the softwood species. *Pinus* has the largest resin canals (30-300 µm in diameter), but *Larix, Picea* and *Pseudotsuga* have
much smaller resin canals (less than 80 µm in diameter). The epithelial cells remain thin-walled in mature wood of *Pinus*, but secondary walls occur in the epithelial cells in mature wood of *Larix, Picea* and *Pseudotsuga* (Larson, 1994; Evert, 2006). The structure of axial resin canals in OW and CW are the same. However, there are fewer axial resin canals in CW than in OW (Westing, 1968; Lee and Eom, 1988; Donaldson et al., 2015).

### 1.2.2 The ray system of NW and CW

Softwood rays are usually one cell wide (uniseriate) or sometimes two cells wide (biseriate), and up to 20 cells long. They contain either only ray parenchyma cells, or parenchyma cells and horizontal ray tracheids, depending on the genus (Evert, 2006). Ray parenchyma cells usually have only thin primary walls, and no bordered pits. However, ray parenchyma cells with thick secondary walls have been reported to rarely occur in *P. radiata* (Donaldson and Knox, 2012). Ray tracheids are commonly found in *Pinus, Picea, Larix, Pseudotsuga* and *Tsuga* (Butterfield and Meylan, 1980). They are located at the top and bottom margins of the rays in one or two rows, and are different from ray parenchyma cells by having secondary walls with bordered pits. However, their bordered pits are smaller than those of the axial tracheids and lack margos (Bamber and Burley, 1983). CWs contain more and larger rays than OW (Timell, 1972; Lee and Eom, 1988). However, the structures of the ray parenchyma cells and ray tracheids are similar in NW (or OW) and CW (Westing, 1965; Timell, 1972; Donaldson et al., 2015).

Radial resin canals are also found in *Larix, Picea, Pinus* and *Pseudotsuga*, and always occur in the centre of the rays, which are called fusiform rays (Figure 1-1). They are usually continued from one growth ring to another and are always associated with several or many axial resin canals (Larson, 1994). In NW, there are usually more radial resin canals than axial resin canals (Larson, 1994; Ilvessolo-Pfäffli, 1995), but they are three or four times smaller in diameter (Cown et al., 2011). In *Pinus koraiensis*, the CW had twice as many radial resin canals as OW (Lee and Eom, 1988).
1.3 Chemical compositions of normal softwoods and CW

The cell walls of normal softwoods mainly consist of cellulose, non-cellulosic polysaccharides and lignin. CWs are different from those of NW or OW, not only in the proportions of these major components, but also in their distributions in the tracheid walls (Timell 1986; Altaner et al., 2010; Donaldson and Knox, 2012). The tracheid walls of CW contain more lignin, but less cellulose, heteromannans and heteroxylans than those of NW (Westermark, 1985; Timell, 1986; Brennan et al., 2012; 2013). Two other characteristic polysaccharides of tracheid walls of CW are (1→4)-β-D-galactans (Altaner et al., 2007a; Altaner et al., 2010; Donaldson and Knox, 2012; Brennan et al., 2012), and the (1→3)-β-D-glucans (Altaner et al., 2007b; 2010).

1.3.1 Cellulose [(1→4)-β-D-glucan]

Cellulose is one of the major components of wood cell walls (~40-45% of softwoods) (Timell, 1967). It is an unbranched, linear polysaccharide consisting of β-glucopyranosyl residues (Glcₚ) linked (1→4) (Figure 1-5) (Harris, 2006; Harris and Stone, 2008). Cellulose molecules interact laterally by hydrogen bonds and Van der Waals forces to form cellulose microfibrils. Cellulose is produced by a six-lobed rosette of particles referred to as the cellulose-synthesizing complex within the plasma membrane. Each lobe is now considered to contain three cellulose synthases (CESAs) (Figure 1-6) (Jarvis, 2013; Newman et al., 2013; Nixon et al., 2016; Vandavasi et al., 2016). The movement of the rosettes in the plane of the plasma membrane is guided by cortical microtubules, and thus the orientation of cellulose microfibrils is the same as these microtubules (Paredez et al., 2006). Within secondary lignified walls, such as those of softwood tracheids, there is evidence for aggregation of microfibrils to form macrofibrils (Donaldson, 2007; Xu et al., 2007).
Figure 1-6. Diagram illustrating the synthesis of a cellulose microfibril. A six-lobed cellulose-synthesizing complex with eighteen cellulose molecules emerging from the eighteen cellulose synthases. Figure from Jarvis (2013).

1.3.2 Non-cellulosic polysaccharides

Tracheid walls of softwoods also contain polysaccharides other than cellulose. In tracheid cells walls, the cellulose microfibrils are embedded in a matrix of these non-cellulosic polysaccharides (Timell, 1967; Harris and Stone, 2008). In contrast to cellulose, the non-cellulosic polysaccharides, with the exception of callose, are synthesized in the Golgi apparatus before being moved in secretory vesicles to the cell wall (Doblin et al., 2010). In most softwood species, the predominant non-cellulosic polysaccharides are heteromannans, followed by heteroxylans (Harris, 2006).

1.3.2.1 Heteromannans (O-acetyl-galactoglucomannans)

Heteromannans (O-acetyl-galactoglucomannans) are the most abundant non-cellulosic polysaccharides in the tracheid walls of normal softwoods, and can make up ~12-18% of the wood (Timell, 1967; Harwood, 1973; Harris and Stone, 2008). They have a linear chain of randomly arranged (1→4)-linked β-D-mannopyranosyl (Manp) and β-D-glucopyranosyl (Glcp) residues, with α-D-galactopyranosyl residues attached by (1→6)-linkages to some of both the Manp and Glcp residues and acetyl groups irregularly attached at the C(O)2 or C(O)3 positions of some of the Manp residues (Figure 1-7) (Sjöström and Westermark, 1999; Harris and Stone, 2008). The galactose to glucose to mannose ratio varies among species with *P. radiata* NW galactoglucomannans containing galactose, glucose and mannose in the ratio 0.1:1.0:3.7 (Harwood, 1973). Most of the galactoglucomannans in the tracheid walls of
normal softwood are associated with cellulose, and only small proportions are associated with lignin (Åkerholm and Salmén, 2001; Fahlén and Salmén, 2005).

![Figure 1-7. Structure of galactoglucomannan.](image1)

### 1.3.2.2 Heteroxylans (4-O-methylglucuronoarabinoxylans)

In tracheid cell walls of normal softwoods, heteroxylans (4-O-methylglucuronoarabinoxylans) are usually the second most abundant non-cellulosic polysaccharides (7-15% of the wood) (Timell, 1967; Harris and Stone, 2008). They have a linear backbone of (1→4)-linked β-D-xylopyranosyl residues, with 4-O-methyl-α-D-glucuronic acid (MeGlcP A) residues attached to every sixth xylosyl residue, which is two xylosyl residues away from the α-L-arabinofuranose substitutions (Figure 1-8) (Busse-Wicher et al., 2016). These polysaccharides in conifers lack acetylation (Busse-Wicher et al., 2016). P. radiata NW heteroxylans contain MeGlcP A, xylose and arabinose in the ratio 1.0:5.8:1.1 (Harwood, 1972).

![Figure 1-8. Structure of 4-O-methylglucuronoarabinoxylans.](image2)

### 1.3.2.3 (1→3)-β-D-Glucans (callose or laricinan)

These are linear polysaccharides of (1→3)-linked-β-D-glucopyranose residues and are some of the most dynamic polysaccharides that are widely distributed in nature (Figure 1-9) (Parre and Geitmann, 2005; Harris, 2006). These polysaccharides are present in only minor amounts in the walls of normal softwood tracheids, but up to ~3% is present in CW of Larix laricina, which is sometimes referred to as laricinan (Hoffmann and Timell, 1970). Using the monoclonal antibody BS-400-2, which is specific for (1→3)-β-glucans, the polysaccharides
were located in the helical cavities in the inner S2 region of CW tracheids towards the cell lumen (Altaner et al., 2007; 2010). (1→3)-β-Glucans swell when they absorb water, and have been regarded as a cell-wall sealant (Boyd, 1978; Wloch and Hejnowicz, 1983). They provide mechanical resistance to compression stresses and lowering the amount of (1→3)-β-glucans in cell walls reduced cellular stiffness and increased viscoelasticity (Parre and Geitman, 2005).

(1→3)-β-Glucan (callose or laricinan)

\[-\beta-D-Glcp(1→3)-\beta-D-Glcp(1→3)-\beta-D-Glcp(1→3)-\beta-D-Glcp(1→-)]

Figure 1-9. Structure of (1→3)-β-glucan.

1.3.2.4 Type II Arabinogalactans

Arabinogalactans (Type II) are highly branched, water-soluble polysaccharides present in large proportions in larches (Larix spp.) OW and CW (Côté et al., 1966). However, these polysaccharides occur only in small amounts (less than 1-2%) in the OWs and CWs of other conifer genera (Timell, 1967; 1986). Larch arabinogalactans contain a backbone of (1→3)-linked β-D-Galp residues with many of the residues carrying a side chain linked (1→6)-. Some of these side chains are shown in Figure 1-10 (Harris, 2005).
Figure 1-10. Structure of Type II arabinogalactans found in *Larix* spp.. $R = \beta$-D-Galp-(1→ or $\alpha$-L-Araf-(1→ or $\beta$-L-Arap-L-Araf-(1→ (Ponder and Richards, 1997).

1.3.2.5 Pectic polysaccharides

Pectic polysaccharides are present in only small proportions in softwoods, where they occur in the primary walls and ML of tracheid walls including the membranes of bordered pits (Harris, 2005; Rowell et al., 2005; Choat et al., 2008).

They are complex polysaccharides containing a high proportion of galacturonic acid (GalA). They consist of three major domains: homogalacturonan (HG), rhamnogalacturonan I (RG I), and rhamnogalacturonan II (RG II) (Harris and Stone, 2008). Among these, the HG and RG I domains are the most abundant. The HG domain is composed of linear chains of $\alpha$-D-GalpA acids linked (1→4)- (Figure 1-11). The RG I domain has a backbone of alternating $\alpha$-D-GalpA and $\alpha$-L-Rhap residues. Between 20 and 80% of the $\alpha$-L-Rhap residues are substituted with oligosaccharides or polysaccharides containing linear and branched Araf and Galp residues, including (1→5)- $\alpha$-arabinans, (1→4)-$\beta$-galactans, Type I arabinogalactans and small proportions of arabinono-3,6-galactans (Figure 1-11) (Harris, 2005). RG-II domains have a very complex structure, which have the same backbone structure as HG, but are substituted with four different side chains containing at least 12 different glycosyl residues linked together by more than 20 different glycosidic linkages (O’Neill et al., 2004; Harris and Stone, 2008).
1.3.2.5.1 (1→4)-β-D-Galactans

(1→4)-β-Galactans consist of a linear, unbranched chain of (1→4)-linked β-D-galactopyranosyl residues (Figure 1-12) (Timell, 1986; Jones et al., 1997) and are generally found as one of the side chains of pectin RG I (see Section 1.3.2.5) (Thomas et al., 1987; Edashige and Ishii, 1997). (1→4)-β-Galactans are present in small amounts in OW, where they are sparsely distributed in the primary walls of the tracheids. However, these polysaccharides constitute ~10% (w/w) of CW (Brennan et al., 2012), and are found in the secondary walls of the tracheids (Altaner et al., 2010; Donaldson and Knox, 2012). Their amounts in CW have been correlated with the high longitudinal shrinkage of this wood type (Floyd, 2005; Brennan et al., 2012). Their high water-binding capacities may be relevant to their shrinkage (Brennan et al., 2012). The physiological and mechanical roles of (1→4)-β-galactans in CW remain unclear, but they have been considered to participate in the strengthening of the secondary walls (Fagerstedt et al., 2014).

1.3.3 Lignins in normal softwoods and CWs

Lignin is another major component of wood, accounting for ~27-33% of normal softwoods, but can account for up to ~40% of CW (Timell, 1967; Fagerstedt et al., 2014). In addition to
providing mechanical strength, it provides hydrophobic surfaces for water conduction in the tracheids, and acts as a physico-chemical barrier against insects and pathogens (Wagner et al., 2012). In softwoods, lignin is a complex aromatic polymer synthesized mainly from the oxidative coupling of two \( p \)-hydroxycinnamyl alcohol monomers (monolignols) and involves the actions of peroxidases and/or laccases to form free radicals, which then polymerize non-enzymatically. The monolignols are \( p \)-coumaryl and coniferyl alcohols, and these give rise to \( p \)-hydroxyphenyl (H) and guaiacyl (G) units of the lignin polymers, respectively (Figure 1-13) (Boerjan et al., 2003; Ralph et al., 2008; Vanholme et al., 2010). In NW and OW, the lignin usually consists mostly of G-units with only small proportions of H-units (Vanholme et al., 2010). However, in CW, the lignin contains significantly higher proportions of H-units (Brennan et al., 2013).

![Figure 1-13. The main building blocks of softwood lignins: \( p \)-hydroxyphenyl (H lignin) and guaiacyl (G lignin) units produced from \( p \)-coumaryl alcohol and coniferyl alcohol, respectively (Vanholme et al., 2010).](image)

Lignin deposition mainly takes place during secondary cell wall formation, which is the last developmental phase of wood cell formation (Boerjan et al., 2003). In NW, lignification is first initiated at the cell corner regions of the ML and the primary wall, and then further proceeds through the secondary wall when the formation of the polysaccharide matrix of the S2 layer is completed (Timell, 1967; Kutscha and Schwarzmann, 1975; Boerjan et al., 2003). Lignification in the walls of CW tracheids occurs in a similar order to that in NW tracheids. However, lignification of the S2L region takes place after the completion of secondary wall formation, and lignification of the S1 layer of the secondary wall of CW tracheids is considerably reduced compared to those of NWs (Donaldson, 2001). The lignification process is controlled on an individual cell basis (Wagner et al., 2012) and it may start in the cells adjacent to rays before those more distant from the rays (Donaldson, 2001).
1.3.4 Extractives and inorganic components

There are minor amounts of extraneous materials present in softwoods, mostly in the form of organic extractives and inorganic components. Extractives, such as terpenes, resin acids, starch, fats, waxes, tannins, and a large number of polyphenols, such as the lignans, and trofolones, are present in the resin canals, ML, and only occasionally occur within the cell wall. They contribute to wood properties including colour, odour, and decay resistance (Pettersen, 1984; Timell, 1986). CWs generally contain greater amounts of extractives than OW (Timell, 1986), which are probably correlated to their darker red colour. The inorganic components in softwoods are normally low (~1%) and are determined as ash (Pettersen, 1984; Sjöström and Westermark, 1999). CW contains similar or slightly higher amounts of ash than NW (Timell, 1986).

1.3.5 Proteins

Wood cell walls contain small proportions of proteins, mostly in the form of glycoproteins. They are classified into four groups based on the abundance of certain amino acids or glycan motifs in their sequences, including glycine-rich proteins (GRPs), proline-rich proteins (PRPs), arabinogalactan-rich proteins (AGPs) and hydroxyproline-rich glycoproteins (HRGPs or extensins) (Cassab, 1998; Kumar et al., 2016). The majority of the cell wall proteins are considered to be cross-linked in the wall and probably have structural functions, as well as participate in morphogenesis (Cassab, 1998). However, the exact functions of most of the cell-wall proteins and their interactions with other cell-wall components still remain unclear (Kumar et al., 2016).

1.4 Mild compression woods (MCWs)

CW as described in Sections 1.1 to 1.3 above is more accurately described as severe compression wood (SCW) (Donaldson and Singh, 2013). CWs with severities intermediate between SCW and OW have also been described and are often referred to as mild compression wood (MCW). MCWs occur widely and are usually found on the lower sides of slightly tilted stems or in the transition zones between OW and CW (Yumoto et al., 1983).
However, the term MCW was first used with a different meaning: a wood sample containing only a small proportion of SCW (Pillow, 1941; Donaldson and Singh, 2013). Much less is known about MCWs than SCW.

Researchers have attempted to classify CWs into different grades. For example, CWs of *P. radiata* have been classified into 5 grades based on their opacity and reddish colour (Burdon, 1975). The CWs of *Picea glauca* have been classified into six grades based on anatomical features (Yumoto et al., 1983). Different grades of MCWs of *P. radiata* have been recognised based on anatomical features and on the distribution of lignin in the tracheid cell walls (Donaldson et al., 1999). However, a particular MCW grade may not extend over a large area of wood and so studies of MCWs that have involved large wood samples may include a mixture of different grades recognised by anatomical features and lignin distribution in tracheid walls.

Lignin distribution in the tracheid walls is considered to be the most reliable feature to classify CWs and can be determined using fluorescence microscopy based on the autofluorescence of lignin (Donaldson and Singh, 2013). The first feature to occur in the mildest MCWs is an increase in lignification of the outer S2 (S2L) wall layer at the cell corners with less lignification of the ML. In more severe grades of MCWs, the highly lignified S2L layer extends around the whole tracheid and the lignification of the ML is further reduced, with intercellular spaces occasionally appearing at the cell corners (Donaldson et al., 1999; Donaldson and Singh, 2013).

### 1.4.1 Detection of CWs

SCW can sometimes be visually detected in fresh cut stems based on the eccentric shape of the stem and its darker reddish colour compared to that of NW or OW (Timell, 1986). However, the chemical basis for the different colour of SCW is unknown, and the coloration of SCW and OW varies among species. For example, there are some species such as *Juniperus* spp. and *Pseudotsuga menziesii* that have highly coloured OW as well as SCW (Timell, 1986).

SCWs have often been detected by examining moistened whole transverse stem sections using transmitted light; the SCW appears opaque (darker) compared to the rest. At least part
of the reason for this is that the inner S2 region of the SCW tracheids contain helical cavities that absorb more light and scatter less light than the equivalent region in OW tracheids walls (Pillow, 1941). This method has been used to estimate the areas of SCWs, as well as MCWs, in stem discs of Norway spruce (*Picea abies*) using computer imaging software based on the colour of each wood type in the image. The MCWs were considered to have intermediate colour between the SCW and the OW (Andersson and Walter, 1995). However, large errors in estimating the amount of CWs occurred and the method often confuses CW, especially MCWs with latewood (Donaldson and Singh, 2013). The transmitted light method also failed to detect CW in Sitka spruce (*Picea sitchensis*) and Scots pine (*Pinus sylvestris*) due to the dark coloration of the heartwood (Gardiner and Macdonald, 2005).

Other methods have also been used to detect CWs. For example, SCWs and MCWs have been detected in Norway spruce (*P. abies*) by hyperspectral image analysis using reflected light in the wavelength range 400-1000 nm, based on reference spectra obtained from SCW and MCW samples with severity grades defined by tracheid anatomy (Duncker and Spiecker, 2005). Areas of stem sections of saplings of radiata pine (*P. radiata*) occupied by SCW were assessed by scanning wet sections and measuring the darker coloured regions using Photoshop software (Chauhan et al., 2013).

1.5 Detection and quantification of wood cell-wall components

1.5.1 Immunolabelling of polysaccharides

The presence of specific polysaccharides in individual plant cell walls is often determined using immunomicroscopy with monoclonal antibodies that recognize polysaccharide-specific epitopes (Hervé et al., 2011). Indirect immunomicroscopy is commonly used. This involves using a primary monoclonal antibody followed by a secondary antibody that is labelled with a fluorochrome or colloidal gold particles, and then detecting these using a fluorescence microscope or transmission electron microscope, respectively (Harris, 2006). Specific cell-wall polysaccharides in much larger sections can be detected by labelling in a similar way, but using a secondary antibody conjugated to an enzyme, such as alkaline phosphatase. After labelling, the position of the enzyme is determined by treating the section with a soluble, coloured substrate of this enzyme that is converted to an insoluble, coloured product (Bratthauer, 2009).
There have been a number of monoclonal antibodies raised and used to detect specific polysaccharides in tracheid cell walls. For example, the monoclonal antibody LM 5 specifically recognises \((1\rightarrow 4)\)-\(\beta\)-galactans, and was raised using a neoglycoprotein (galactotetraose-bovine serum albumin (BSA)). The epitope of LM5 is more than three contiguous \((1\rightarrow 4)\)-linked \(\beta\)-galactosyl residues (Jones et al., 1997). The monoclonal antibody BS 400-2 specifically recognizes \((1\rightarrow 3)\)-\(\beta\)-glucans, and was raised against a laminarin-haemocyanin conjugate. It binds optimally to five to six \((1\rightarrow 3)\)-linked \(\beta\)-glucosyl residues (Meikle et al., 1991). The monoclonal antibodies LM 10 and LM 11 specifically detect heteroxylans with low and high degrees of substitution, respectively (McCartney et al., 2005). The monoclonal antibodies LM 21 and LM 22 have been used specifically to detect heteromannans in the tracheid walls of \textit{P. radiata} OW and SCW (Donaldson and Knox, 2012).

1.5.2 Chemical analysis of cell-wall polysaccharides

Cell-wall polysaccharides of wood are often investigated by determining their monosaccharide compositions. This involves treating the wood samples with acid solutions to hydrolyse the component polysaccharides to monosaccharides, which are then identified and quantified chromatographically. The yield of monosaccharides can be influenced by the choice of acids and the reaction time (Fengel and Wegener, 1979; Pettersen, 1991; Vuorinen and Alén, 1999). Sulfuric acid has most commonly been used to hydrolyse wood samples in a two-stage process. The primary hydrolysis uses a 72\% w/w sulfuric acid solution, followed by dilution with water and a secondary, high temperature (100-125 °C) hydrolysis (Saeman et al., 1963; Vuorinen and Alén, 1999). Trifluoroacetic acid (TFA) has been used to hydrolyse the non-cellulose polysaccharides of wood by using a 2 M solution at 121 °C for 1 h (Albersheim et al., 1967; Mankarios et al., 1979). TFA is a highly volatile organic acid which can be completely removed by evaporation (Albersheim et al., 1967).

The monosaccharides in cell-wall hydrolysates can be separated, identified and quantified using gas chromatography (GC) (Harris et al., 1988) or high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Brennan et al., 2012). GC requires derivatization steps to make the monosaccharides volatile before chromatography (Blakeney et al., 1983). HPAEC-PAD has the advantage that it can be used
without derivatization. It separates anionic analytes which are either anions in their common form or analytes that can be ionized at high pH values using sodium hydroxide eluent (Currie and Perry, 2006).

1.5.3 Histochemical detection of lignin

Various histochemical methods have been used to detect and determine the distribution of lignin in tracheid cell walls. Lignin is autofluorescent in ultraviolet (UV) and blue radiation and its distribution in tracheid walls can be determined by fluorescence microscopy and by confocal laser scanning microscopy (CLSM) (Donaldson et al., 1999; 2004). CLSM has the advantage of removing light coming from out-of-focus planes (Hepler and Gunning, 1998; Muller, 2006).

Various bright-field dyes have been used to stain lignified walls. For example, safranine has often been used to stain lignified walls red. This staining is often followed by a counter stain, such as fast green to stain the non-lignified cell walls green (Johansen, 1940). More recently, it has been recognised that may such bright-field stain, including safranine and acridine orange, fluorescence in UV or blue radiation and can be used as fluorochromes (Kutscha and McOrmond, 1972; Haseloff, 2003; Bond et al., 2008).

Different colour reagents have also been used to detect lignin. The Wiesner reagent (Wiesner, 1878; Pomar et al., 2002), which involves treatment of sections with a solution of phloroglucinol and concentrated hydrochloric acid, gives a red coloration with lignified cell walls in angiosperms and coniferous gymnosperms. The Mäule reagent, which involves treatment with an aqueous solution of potassium permanganate, hydrochloric acid, and ammonia, gives a red colouration with lignified cell walls in angiosperms, but a dark brown colouration with lignified cell walls in coniferous gymnosperms (Iiyama and Pant, 1988). The Cross and Bevan reagent, which involves treatment with chlorine water and aqueous sodium sulfite, produces a similar colouration to the Mäule reagent (Lewis and Yamamoto, 1990; Nakano and Meshitsuka, 1992). Both the Mäule and the Cross and Bevan reagent produce the red colouration by reaction with syringyl groups in angiosperm lignin.
In addition, lignin can be detected and its distribution determined using transmission electron microscopy (TEM) after staining the sections with potassium permanganate (Bland et al., 1971; Singh et al., 2003).

### 1.5.4 Chemical quantification and characterization of lignin

Lignin has often been quantified as Klason lignin, which is the insoluble residue left after cell walls, including wood cell walls, have been hydrolysed by the two-stage sulfuric acid method (Saeman et al., 1963; Vuorinen and Alén, 1999). Another method of lignin quantification is the acetyl bromide assay (Hatfield et al., 1999; Hatfield and Fukushima, 2005). This is a convenient method of quantifying lignin in small amounts of wood, with sample sizes of 1-2 mg. The wood is solubilized in acetyl bromide in acetic acid and the absorbance of the solution determined at 280 nm by spectrophotometry. The amount of lignin is calculated from the extinction coefficient, which varies among species (Johnson et al., 1961; Iiyama and Wallis, 1988).

The main monomeric units in lignin are often determined by chemical degradation methods (Dean, 1997; Whetten et al., 1998; Vanholme et al., 2010). One of the most common methods of determining the proportions of different monomers is thioacidolysis (Lapierre and Rolando, 1988; Rolando et al., 1992; Lapierre 2010). The reaction is done in a mixture of dioxane and ethanethiol with boron trifluoride diethyl etherate as the acid catalyst (100 °C, 4 h). Cleavage occurs at β-O-4 linkages, and after trimethylsilylation, the monomers are separated, identified and quantified by gas chromatography-mass spectrometry. Using this method, very small samples, e.g., ~0.1 mg (Nakashima et al., 2008) can be analysed.

In addition to chemical degradation, pyrolysis (500 °C in the absence of air) in combination with gas chromatography and mass spectrometry (Py-GC/MS) has been used to analyse lignin structures in *P. radiata* OWs and CWs (Brennan et al., 2013). The Py-GC/MS method involves little sample preparation, including drying and milling, and small samples (μg range) can to be analysed (Meier and Faix, 1992; Brennan et al., 2013).
1.5.5 Use of FTIR and Raman spectroscopy in analysing cell-wall polysaccharides and lignin

Spectroscopic techniques have been widely used to characterise wood cell walls. These techniques include Fourier transform infrared (FTIR) spectroscopy (Pandey, 1999; Silva et al., 1999; Colom et al., 2003) and Raman spectroscopy (Agarwal and Atalla, 1993; Agarwal and Ralph, 1997; Agarwal et al., 2011). Both FTIR and Raman spectroscopies depend on sampling the same molecular vibrational states. However, the two techniques have different mechanisms and complement each other (Séné et al., 1994; Matthäus et al., 2008). FTIR spectroscopy is based on the vibration of the atoms in a molecule after absorption of IR radiation. The functional groups that have large permanent dipole moments, such as carbonyl and hydroxyl groups, give strong IR absorption, whereas nonpolar bonds absorb IR radiation only weakly (Stuart, 2004). Raman spectroscopy probes vibrational transitions indirectly by light scattering, and the vibrational modes that are weak in IR are generally strong in Raman (Séné et al., 1994; McCreery, 2000; Agarwal et al., 2011). Raman spectroscopy is more sensitive to extractives, lignin and carbon-hydrogen bonds of polysaccharides than FTIR spectroscopy (Agarwal and Atalla, 1993).

Both FTIR and Raman spectroscopies coupled with light microscopes can be used to collect spectra from specific regions of wood samples (Hori and Sugiyama, 2003; Nuopponen et al., 2003; Agarwal, 2014). FTIR micro-spectroscopy can be used to collect spectra from area as small as ~50 μm × 50 μm in sections (Gorzsás et al., 2011), but Raman micro-spectroscopy is able to analyse much smaller areas (~1 μm × 1 μm) (Wiley and Atalla, 1987). Sample preparation for FTIR micro-spectroscopy, which is used in the transmission mode is critical. These sections have to be thin enough to avoid band saturation (no more than ~30 μm) (Naumann et al., 2005). The absorbed water in the samples absorbs IR radiation strongly (McCreery, 2000). However, Raman spectroscopy is insensitive to water and so wet wood samples can be analysed. It works in the diffuse reflectance mode therefore sample thickness is not critical (Stenius and Vuorinen, 1999). Near infrared excitation is often used in Raman spectroscopy of wood to avoid sample fluorescence when using excitation of visible light (Agarwal et al., 2011).

FTIR and Raman spectroscopies have been widely used to quantify the chemical compositions, including the lignin and polysaccharide components of wood based on calibration and prediction rather than on direct measurement (Boeriu et al., 2004; Zhou et al.,...
Linear regression methods have been used to predict lignin contents. Calibration equations are obtained from the intensities of the bands characteristic of lignin (1510 cm\(^{-1}\) in FTIR spectra, 1600 cm\(^{-1}\) in Raman spectra), and the lignin content can be obtained from reference samples (Rodrigues et al., 1998; Agarwal, 2011). In addition, statistical multivariate methods, e.g., partial least squares (PLS) model, have been used to calibrate the spectra against wet chemical compositional data, and to predict the chemical composition of unknown samples. This method can be both fast and reliable (Zhou et al., 2011; McLean et al., 2014).

Principal component analysis (PCA) is another statistical multivariate method often used for spectral analysis. It has been used to identify groupings within sample populations, with the principal component loadings showing the variabilities between the sub-groups (Chen et al., 1998). For example, FTIR in combination with PCA has been used to identify different classes of Arabidopsis mutants and to characterise the altered cell-wall components compared with the wild type (Chen et al., 1998; Mouille et al., 2003). It has also been used to investigate chemical variation between earlywood tracheids, latewood tracheids, and earlywood ray parenchyma cells in 15 softwood species (Hori and Sugiyama, 2003).

### 1.6 Radiata pine in plantations

Radiata pine (*Pinus radiata* D. Don) (Figure 1-14), which is native to California, USA, is an important plantation species of coniferous gymnosperm. It is now distributed mostly in the Southern Hemisphere, including New Zealand, Chile, Australia, South Africa and Argentina, but also in parts of the Northern Hemisphere, including Spain, small areas of Turkey, Ireland and Great Britain (Rogers, 2002). Radiata pine wood is extremely versatile and suitable for both interior and exterior uses, but the quality of these woods is mediocre and matches only the lowest EU grade for structural timber (Walker, 2013). Breeders have attempted to improve the wood quality through selective breeding to produce timber with sufficient stiffness and low level of distortion (Moore, 2012).
1.7 Aims

SCW has been well studied, but less is known about MCWs. This thesis aims to identify and characterise the compositions and properties of three different grades of CWs and OW in slightly tilted radiata pine sapling stems:

1. In chapter 2, I aim to identify three different grades of CWs based on the distribution of lignin in their tracheid walls by its autofluorescence. I aim to determine the distributions of the polysaccharides (1→4)-β-galactan and (1→3)-β-glucan in tracheid walls in all three CWs and OW using indirect immunofluorescence microscopy and immunogold microscopy in conjunction with the monoclonal antibodies LM5 [for (1→4)-β-galactans] and BS 400-2 [for (1→3)-β-glucans]. I aim to locate the (1→4)-β-galactans in whole stem transverse sections by indirect immunofluorescence labelling with the secondary antibody labelled with an enzyme. I also aim to determine the neutral monosaccharide compositions of the non-cellulosic cell-wall polysaccharides in the four wood types using microscopically characterised small (0.5 mm diameter) discs.

2. In chapter 3, I propose to determine the distribution of lignin in the tracheid walls of the different wood types using the fluorescent dye acridine orange. I aim to quantify the lignin contents of the walls of the different wood types using the acetyl bromide
method with small, well characterised discs as described above. These discs will also be used to determine the lignin monomer compositions by thioacidolysis. Raman and FTIR micro-spectroscopy, in combination with PCA, will be used to differentiate the four wood types also using similar discs.

3. In chapter 4, I propose to determine the shrinkage of tracheids of three grades of CWs and OW in the longitudinal, radial and tangential directions using variable pressure scanning electron microscopy. In addition, the dimensional changes of the transverse (tangential and radial) wall thicknesses and the structural changes of other wood wall features will be examined. I also aim to statistically correlate the shrinkage of tracheids of the four wood types with their cell-wall compositions.
Chapter 2 – Tracheid cell-wall structures and locations of (1→4)-β-D-galactans and (1→3)-β-D-glucans in compression woods of radiata pine (*Pinus radiata* D. Don)

2.1 Abstract

Compression wood (CW) forms on the underside of tilted stems of coniferous gymnosperms and opposite wood (OW) on the upperside. The tracheid walls of these wood types differ structurally and chemically. Although much is known about the most severe form of CW, severe CW (SCW), mild CWs (MCWs), also occur, but less is known about them. In this study, tracheid wall structures and compositions of two grades of MCWs (1 and 2) and SCW were investigated and compared with OW in slightly tilted radiata pine (*Pinus radiata*) stems. The four wood types were identified by the distribution of lignin in their tracheid walls. Only the tracheid walls of OW and MCW1 had a S3 layer and this was thin in MCW1. The tracheid walls of only SCW had a S2 layer with helical cavities in the inner region (S2i). Using immunomicroscopy, (1→4)-β-D-galactans and (1→3)-β-D-glucans were detected in the tracheid walls of all CWs, but in only trace amounts in OW. The (1→4)-β-D-galactans were located in the outer region of the S2 layer, whereas the (1→3)-β-D-glucans were in the inner S2i region. The areas and intensities of labelling increased with CW severity. The antibody for (1→4)-β-D-galactans was also used to identify the locations and relative amounts of these galactans in whole stem cross sections based on the formation of an insoluble dye. Areas containing the four wood types were clearly differentiated depending on colour intensity. The neutral monosaccharide compositions of the non-cellulosic polysaccharides of these wood types were determined on small, well defined discs, and showed the proportion of galactose was higher for CWs and increased with severity. The presence of an S3 wall layer is a marker for very MCW and the presence of helical cavities in the S2 wall layer for SCW. The occurrence and proportions of (1→4)-β-D-galactans and (1→3)-β-D-glucans can be used as markers for CW and its severity. The proportions of galactose were consistent with the labelling results for (1→4)-β-D-galactans.
2.2 Introduction

Cell walls of the secondary xylem of woody plants are of considerable commercial importance. In addition to being the major component of solid wood, they are used to produce pulp for making paper and second generation liquid biofuels. When the growth of stems of such woody plants is displaced from the vertical, for example by wind or snow, a special type of secondary xylem is formed known as reaction wood, which restores normal, vertical growth (Scurfield, 1973; Timell, 1986). In coniferous gymnosperms (softwoods), this reaction wood is formed on the underside of tilted stems and is known as compression wood (CW) (Timell, 1986). The cell walls in this wood contain more lignin and less cellulose than normal wood (NW) (Timell, 1982; Brennan et al., 2012; Fagerstedt et al., 2014). The wood formed geometrically opposite to CW, is known as opposite wood (OW) and the cell walls of this wood type are structurally and chemically similar to those of NW (Brennan et al., 2012). Lignin hinders the production of chemical pulps and biofuels, and consequently the presence of CW reduces their yields (Vanholme et al., 2012). Additionally, the presence of CW affects the quality of solid wood. On drying, CW shrinks longitudinally more than NW, and when CW occurs with NW, the differential shrinkage causes warping and other distortions (Langrish and Walker, 2006).

The wood of coniferous gymnosperms consists mostly of tracheids that have a dual role: they conduct water up the stem and they provide mechanical support. Tracheid cell walls are composed of a thin primary wall layer and a thick secondary wall. The individual tracheids adhere to one another by a thin middle lamella (ML), and this together with the two adjacent primary walls are often referred to as the compound middle lamella (CML) (Butterfield, 2006; Evert, 2006). Tracheid walls of CW differ both structurally and chemically from NWs and OWs (Timell, 1986; Altaner et al., 2010; Brennan et al., 2012; Donaldson and Singh, 2013). In tracheids of NW and OW, the secondary walls are composed of three layers, S1, S2 and S3, with the S2 layer the thickest and the S3 layer adjacent to the cell lumen (Butterfield, 2006; Evert, 2006). In CW, the tracheid walls are thicker, but lack the S3 layer. The S2 layer often has helical cavities on the inner side nearest the cell lumen. CW also differs from NWs and OWs in the presence of intercellular spaces at the corners of adjacent tracheids.

Chemically, the tracheid walls of NWs and OWs are composed of cellulose, lignin and the non-cellulosic polysaccharides heteromannans (O-acetyl-galactoglucomannans) and smaller proportions of heteroxylans [arabino(4-O-methylglucurono)xylans] (Harris and Stone, 2008;
In addition to containing less cellulose and more lignin, the tracheid walls of CW contain less heteromannans, and heteroxylans. These walls also contain significant proportions of \((1\rightarrow4)-\beta\)-d-galactans (up to 10%) and small proportions (\(~3\%) of \((1\rightarrow3)-\beta\)-d-glucans (callose or laricinan) (Timell, 1986; Hoffmann and Timell, 1970; Brennan et al., 2012). The greater longitudinal shrinkage of CW has been correlated with these \((1\rightarrow4)-\beta\)-galactans (Floyd, 2005; Brennan et al., 2012).

In addition, the distribution of lignin in tracheid walls of CW differs from that in NW and OW. Lignin is autofluorescent in ultraviolet and blue radiation and can be localized using fluorescence microscopy. Using this technique it has been found that lignin occurs at high concentrations in the ML and primary walls of tracheids in OW and NW. However, in CW, the lignin concentrations in the ML and primary walls are lower, but are higher in the outer region of the S2 layer (S2L) (Donaldson et al., 1999). CW is also often darker in colour than NWs and OWs.

CW, as described above, is more accurately described as severe CW (SCW), but a continuum of wood types (grades) occur between this and NW or OW, with the intermediate types being referred to as mild CWs (MCWs) (Donaldson and Singh, 2013). As many as five major grades of CWs (one severe and four mild) have been described in white spruce (\(Picea glauca\)) (Yumoto et al., 1983). Different CW grades can most reliably be recognized based on the distribution of lignin in tracheid walls as determined by its autofluorescence using fluorescence microscopy (Donaldson and Singh, 2013). However, there is currently no other good method available to accurately detect and classify CW on a larger scale, i.e. with the naked eye or low-power (sterio) microscope. Compared with SCW, there have been only a few studies of mild CWs and relatively little is known about the relationship between structural and chemical features in the walls of different grades of CW. However, one study correlated structural features with chemical analyses in radiata pine (\(Pinus radiata\)), but quite large samples of wood (2-3 g) were analysed, and within this, the structural features were so highly variable that only one MCW grade was identified (Nanayakkara et al., 2009).

Here, I describe a study in which saplings of radiata pine were grown tilted from the vertical to induce the formation of CW. A small tilt angle (~8-20º) to the vertical was used to try to maximize the formation of MCW rather than SCW. Four wood types, OW, SCW and two MCWs, were identified based on the distribution of lignin in their tracheid walls using fluorescence microscopy. Light and electron microscopy were used to compare the structures
of the tracheid walls of the four wood types and immunomicroscopy with monoclonal antibodies was used to specifically locate \(1\rightarrow4\)-β-galactans and \(1\rightarrow3\)-β-glucans in relation to structural features in the walls. Immunolabelling with an enzyme labelled secondary antibody was used to examine the distribution of \(1\rightarrow4\)-β-galactans in whole cross sections of stems. Pure, synthetic aniline blue fluorochrome that binds specifically to \(1\rightarrow3\)-β-glucans (Stone et al., 1984) was also used with fluorescence microscopy to locate this polysaccharide in the tracheid walls. Additionally, the neutral monosaccharide compositions of the non-cellulosic cell-wall polysaccharides of the four wood types were determined using small discs (0.5 mm diameter) each containing only a single wood type as determined by fluorescence microscopy.

2.3 Materials and methods

2.3.1 Wood samples

Saplings of *Pinus radiata* (D. Don) (radiata pine) (Forest Genetics Ltd, Rotorua, New Zealand) were grown outside at Harewood, Christchurch, New Zealand. Saplings were planted, in September 2011, in 100 litre bags of potting mix containing slow-release fertiliser and were irrigated regularly. Three saplings were used: clone 30 ramets 1 and 2, and clone 17, which are referred to as Trees 1, 2, and 3, respectively. They were grown upright for 6 months and then, in February 2012, tilted by staking at ~8-20° from the vertical to produce CW and OW; they were harvested in June 2013. The exact angle of tilting, measured at harvest, was for Tree 1 ~20°, Tree 2 ~13° and Tree 3 ~8°. A segment (~10 cm long) was sawn from each stem ~20 cm above the potting mix and used for all the experiments. Lengths (1 cm) were cut transversally from the segment using a band saw, and the surface smoothed using a sliding microtome (Model HN 40, Jung, Heidelberg, Germany), moistened with water and photographed in reflected light using a digital camera (Canon model EOS 40D) with a macro lens (EF 100 mm 1:28 usm ultrasonic motor) (Canon Corp., Tokyo, Japan). Transverse sections (1 mm thick) were also cut from the segment with a band saw, moistened with water, illuminated by transmitted light and photographed (as above).
2.3.2 Identifying and obtaining samples of the four wood types

Lengths (1 cm) were cut from the stem segments with a band saw, softened by soaking in water at 4°C for three days, and transverse sections (60 µm thick) cut using the sliding microtome. These sections were cut into two equal halves with one half being a darker colour (CW) than the other. Each half was then divided again giving four quadrants, each of which was then scanned by fluorescence microscopy (see below) to identify the locations of the four wood types based on the lignin distribution in the tracheid walls. Then discs were cut using a Harris Uni-Core™ micro-punch sampler (diameter 0.5 mm) (ProSciTech, Thuringowa Central, QLD 4817, Australia) from areas containing only one wood type and were again checked by fluorescence microscopy, with discs containing more than one wood type being rejected. These discs were used to determine the neutral monosaccharide compositions of the cell-wall polysaccharides (see below).

2.3.3 Fixation, embedding and sectioning

Samples (~1 mm tangential width × ~1 mm radial width × ~10 mm long) were cut from the 1 cm stem lengths at locations where each wood type had been identified in the 60 µm thick sections (see above). These samples were fixed using 2% (w/v) paraformaldehyde and 0.1% (w/v) glutaraldehyde in 100 mM sodium 1,4-piperazinediethanesulfonic acid (PIPES) buffer (pH 7.2) for 2 h at room temperature under vacuum, and dehydrated in an aqueous ethanol series (30, 50, 70, 90, 95, and 100% (twice)) for 15 min in each concentration. All samples were then infiltrated with a 1:2 (v/v) mixture of medium grade LR White resin (London Resin Co. Ltd, Basingstoke, UK) and ethanol at room temperature on a rotator for 1 h, then with a 2:1 (v/v) mixture of resin and ethanol for 1 h, and finally in pure resin for 18 h. The resin was polymerized for 24 h at 60°C. Sections (100 and 500 nm thick) were cut with a diamond knife using an ultramicrotome (Model EM UC6: Leica, Vienna, Austria). Sections (500 nm thick) from each block were checked for wood type using fluorescence microscopy (see below). Any block containing more than one wood type was discarded. In addition samples containing the two false growth rings, and normal and traumatic resin canals were fixed and embedded in the same way.
2.3.4 Indirect immunofluorescence microscopy

Indirect immunofluorescence microscopy was done with resin sections (500 nm thick) collected on poly-L-lysine coated slides (Biolab Scientific, Auckland) and dried at 55°C for 30 min. The sections were incubated in phosphate buffered saline (PBS) (0.01 M sodium phosphate buffer, pH 7.4; 0.14 M NaCl) containing 5% (w/v) milk powder (0.1% fat; Alpine, Dairyworks Ltd, Christchurch, New Zealand) (MP-PBS) for 1 h to block nonspecific binding sites. After washing in PBS (5×), the sections were incubated with the monoclonal antibody LM5 (0.2 mL; 1:10 dilution) (PlantProbes, Leeds, UK) or the monoclonal antibody BS 400-2 that specifically recognizes (1→3)-β-glucans (Meikle et al. 1991) (Biosupplies, Parkville, VIC, Australia) (0.2 mL; 1:10 dilution of the solution obtained by reconstituting the freeze-dried antibody according to the manufacturer’s instructions) in MP-PBS, and then with the secondary antibodies goat anti-rat IgG (H+L) conjugated to Alexa Fluor® 546 (Molecular Probes Inc, Eugene Or, USA) (used with LM5) and goat anti-mouse IgG (H+L) conjugated to Alexa Fluor® 546 (Molecular Probes Inc) (used with BS 400-2) (0.2 mL; 1:200 dilution). Both primary and secondary antibodies were incubated for 2 h, and after each incubation the sections were washed with PBS (5×). Sections were then washed with water, mounted in AF1 antifadent (Citifluor Ltd, London, UK) and examined by confocal laser scanning microscopy. Control experiments were done with the primary antibody omitted and where the BS 400-2 was pre-incubated with laminarin (100 µg/ml), a (1→3)-β-glucan, (Sigma-Aldrich, St. Louis, MO, USA) for 30 min (Meikle et al., 1991).

2.3.5 Indirect immunolabelling of (1→4)-β-galactans in whole-stem sections

Transverse sections (60 µm thick) of whole, debarked stems were treated with LM5 as described above, except incubations with MP-PBS and the antibodies were done at 30°C and not at room temperature. Also the secondary antibody was goat anti-rat IgG (H+L chains) secondary antibody conjugated with alkaline phosphatase (0.2 mL; 1:100 dilution) (Invitrogen, New Zealand) in MP-PBS, and after the last PBS wash, the sections were treated for 15 min with substrate solution containing bromo-4-chloro-3-indolyl phosphate (BCIP), nitro-blue tetrazolium (NBT) and water (1:1:8 v/v) (BCIP/NBT substrate kit, Invitrogen). Alkaline phosphatase reacts with the substrate to produce an insoluble blue product. The sections were then washed in water (5×) before mounting in Aqua-Mount medium (Thermo...
Control experiments were also done with the primary antibody omitted and the images recorded using a digital camera (see above).

2.3.6 Light microscopy

Tracheid wall features were examined in transverse sections (60 µm thick) mounted in 75% aqueous glycerol by differential interference optics using a Leica microscope (model DMR; Leica, Wetzlar, Germany). Similar sections mounted in the same way were used to examine lignin distribution in tracheid walls by its autofluorescence using this microscope fitted with an I3 filter block (excitation filter BP450-490 nm, chromatic beam splitter 510 nm, and emission filter LP515 nm) and using a confocal laser scanning microscope (model TCS SP2; Leica) with the 488 nm line of an Ar/Ar-Kr laser for excitation and emission >530 nm. The DMR microscope was also used to check the distribution of lignin autofluorescence in resin sections (500 nm thick).

Sections for immunofluorescence microscopy were examined using a confocal laser scanning microscope with the 561 nm line of a diode-pumped solid state laser used for excitation and emission > 590 nm. A Leica microscope (model TCS SP2) was used for the microscopy of all the tracheid walls and a Zeiss (Oberkochen, Germany) microscope (model LSM 710) was used for the microscopy of the other cell types.

Resin sections (500 nm thick) were also stained with an aqueous solution of the pure, synthetic aniline blue fluorochrome (0.033 mg/ml) (Biosupplies Australia Pty Ltd, Victoria, Australia) for 1 h at room temperature. After washing in water, the sections were examined using the Leica confocal laser scanning microscope with the 458 nm line of the Ar/Ar-Kr laser for excitation and emission > 493 nm. Unstained, control sections were also examined. The 458 line, rather than the 488 line, was used to reduce lignin autofluorescence.

2.3.7 Indirect immunogold microscopy and transmission electron microscopy

Ultrathin sections (100 nm thick) of samples of resin-embedded wood (see above) were cut using an ultramicrotome and collected on 200 mesh square nickel grids (ProSciTech). Sections were incubated in 5% MP-PBS for 1 h at room temperature to block nonspecific
binding sites. After washing in PBS (2×), the sections were incubated with the monoclonal antibody LM5 or BS 400-2 (both at 1:10 dilution in 5% MP-PBS) at 4°C overnight. After washing in PBS (5×), the sections were incubated with the following secondary antibodies: goat anti-rat IgG (H+L) (with LM5) or goat anti-mouse IgG (H+L) (with BS 400-2) (both at 1:10 dilution in 5% MP-PBS), both conjugated to 15 nm diameter colloidal gold particles (Electron Microscopy Science, Hatfield, PA, USA) at room temperature for 2 h. Then all the sections were washed in PBS (5×) followed by water (2×). Control experiments were done in which the primary antibody was omitted and in which BS400-2 was pre-incubated with laminarin (100 µg/ml) for 30 min (Meikle et al., 1991).

For studies not involving immunolabelling, freshly cut sections were treated with 2% aqueous uranyl acetate for 20 min, washed with water (5×), stained with a solution of lead citrate for 2 min, washed with water (5×), and dried.

All sections were examined with a transmission electron microscope (Model CM12, Philips, Eindhoven, The Netherlands) operated at 80 kv.

2.3.8 Determining the neutral monosaccharide compositions of the cell-wall polysaccharides

Neutral monosaccharide compositions of the non-cellulosic polysaccharides in the walls of the four wood types were determined using hydrolysates of small discs (0.5 mm diameter each containing ~610 tracheids) that had been checked by fluorescence microscopy to ensure that all tracheids walls had the appropriate lignin distributions (Figure 2-1). A total of three discs for each wood type were cut from each section. Each disc was transferred to a separate glass vial, dried over silica gel for 16 h, and hydrolysed with 2 M trifluoroacetic acid (TFA) (0.1 mL, 121°C, 1 h) in a sealed tube under argon (Albersheim et al., 1967; Smith and Harris, 1995; Chavan et al., 2015). After cooling, the 2 M TFA was evaporated in a stream of air, the residues dissolved in water (0.2 mL) and filtered using a PTFE filter (pore size 0.2 µm; WhatmanTM, Maidstone, Kent, UK). The neutral monosaccharides were separated and quantified using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex BioLC system (Dionex, Sunnyvale, CA, USA) fitted with an ED 50 electrochemical detector and a GP 50 gradient pump. A CarboPac PA 20 guard column (3 × 30 mm) and a CarboPac PA20 analytical column (3 × 150 mm)
were used. Column temperature was kept at 25°C by a TCC-100 thermostatted column compartment. Neutral monosaccharides were separated using isocratic elution (1 mM NaOH for 30 min). The column was washed for 5 min with 200 mM NaOH before equilibration for 10 min with 1 mM NaOH. The injection volume was 20 µL, and the flow rate was 0.4 mL min⁻¹. The order of elution of monosaccharides was confirmed by running solutions of individual reference monosaccharides. Before the hydrolysate runs, a water blank was run following by a reference solution containing 0.01 mg mL⁻¹ of each of L-arabinose, D-galactose, D-glucose, D-xylose and D-mannose, which was used to determine the relative responses of equal weighs of each monosaccharide. Mean neutral monosaccharide compositions were calculated from the compositions of the three discs of each wood type.

Figure 2-1. Fluorescence micrograph of a 0.5 mm diameter disc cut from MCW1 using a micro-punch sampler. The micrograph shows the disc containing ~610 tracheids, with autofluorescent walls. The disc was from Tree 1 and the micrographs obtained using a DMR microscope. Scale bar = 100 µm.

The neutral monosaccharide compositions were examined statistically. Since the data are multivariate (five monosaccharides), a 2-way factorial multivariate analysis of variance (MANOVA) was first done to search for differences between the wood types and individual trees. The canonical discriminant analysis was used to display the differences between the centroids by plotting the centroids in two dimensions with a minimum loss of information (Manly 2004). All statistical analysis was performed in R (version 3.0.1) (R Core Team, 2013).
2.4 Results

2.4.1 Three grades of CW were identified by lignin distribution in tracheid walls

Three grades of CW were identified in transverse sections of stems of tilted saplings of radiata pines based on the distribution of lignin autofluorescence in their tracheid walls: MCW 1 and 2, and SCW (Table 2-1, Figure 2-2). In OW tracheids, lignin autofluorescence was most evident in the CML and in the ML at cell corners (MLCC), and in the S3 layer of the secondary walls (Figure 2-2a). In MCW1 tracheids, some autofluorescence was present in the S2L layer at the cell corners (Figure 2-2b). However, in the MCW2 tracheids, this autofluorescence of the S2L layer at the cell corners was more evident and this layer was also present around the cells (Figure 2-2c). In SCW tracheids, the intensity of the S2L layer fluorescence was greater and there was no fluorescence of the CML, either at the cell corners (MLCC) or around the cells (CML) (Figure 2-2d). In addition, intercellular spaces were identified only between MCW2 and SCW tracheids, and the tracheids became increasingly circular in transverse section on going from OW to SCW.
Table 2-1. Characteristics of the three types of compression woods (CW) and opposite wood (OW).

<table>
<thead>
<tr>
<th>Wood types</th>
<th>Lignification of compound middle lamella at cell corners</th>
<th>Lignification of S2L at cell corners</th>
<th>Lignification of S2L around cells</th>
<th>Intercellular spaces</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opposite wood (OW)</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mild compression wood 1 (MCW1)</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mild compression wood 2 (MCW2)</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Severe compression wood (SCW)</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

-, no lignin autofluorescence / intercellular spaces absent; +, weak lignin autofluorescence; ++, moderate lignin autofluorescence / moderately frequent intercellular spaces; ++++, strong lignin autofluorescence / numerous intercellular spaces.
Figure 2-2. Fluorescence micrographs of transverse sections of OW and three CWs showing lignin autofluorescence. In OW tracheid walls (a) lignin autofluorescence is strongest in the compound middle lamella (CML), the middle lamella at the cell corners (MLCC), and in the S3 layer of the secondary walls. In MCW1 (b), there is moderate autofluorescence in the middle lamella at the cell corners, and some autofluorescence in the S2L region (S2L) at the cell corners. In MCW2 (c), autofluorescence of the S2L layer at the cell corners is more evident and this layer is also fluorescent around the cell. In SCW (d) there is a highly fluorescent S2L layer all around the tracheids and there is no fluorescence of the CML. Helical cavities (HC) are present on the inner region of the S2 layer (S2i). Intercellular spaces (IS) are present in MCW 2 and SCW. Sections were from Tree 1 and the micrographs obtained using a Leica confocal microscope. Scale bar = 10 μm.

When transverse sections of whole sections of the tilted saplings were examined in reflected light, areas of the sections appeared darker coloured than the rest (Figure 2-3a, b). When sections of these darker coloured areas were examined by fluorescence microscopy, the lignin
distribution in the tracheid walls indicated they were SCW. These darker coloured areas also appeared darker when the sections were examined in transmitted light (Figure 2-3c, d). However, the boundary of the SCW could not be accurately determined by colour. Furthermore, MCWs could not be reliably distinguished from SCW or OW by colour.

Figure 2-3. Photographs of whole-stem transverse sections of tilted radiata pine saplings. Photographed in reflected light Tree 3 (a) and Tree 1 (b) and in transmitted light Tree 3 (c) and Tree 1 (d). Tree 1 was tilted at ~20° to the vertical and Tree 3 at ~8°. The darker coloured areas (arrows) contained SCW, determined by the distribution of lignin in the tracheid walls using fluorescence microscopy. Scale bars: 5 mm.
2.4.2 The three grades of CW have different tracheid wall structures

Examination of transverse sections of tracheid walls of all four wood types using transmission electron microscopy showed differences in wall structures (Figure 2-4). Total tracheid wall thickness increased progressively in the order OW, MCW1, MCW2 and SCW. In all the wood types, the ML and primary wall could be differentiated and were densely stained. S1 and S2 secondary wall layers were also evident in all wood types. A well-defined S3 layer was present in OW (Figure 2-4a) and a very thin S3 layer could just be discerned in MCW1 (Figure 2-4b), but no S3 layers were found in MCW2 or SCW (Figure 2-4c, d). Helical cavities were present in the inner region of the S2 layer (S2i) only in SCW (Figure 2-4d). Warts were observed on the tracheid wall surface adjacent to the cell lumen only in OW (Figure 2-4a).

Differential interference micrographs (insets in Figure 2-4) also showed the different layers in the tracheid secondary tracheid walls of the different wood types. These micrographs particularly showed the S1 layer in the tracheid walls in all the wood types, the S3 layer in OW and even in MCW 1, as well as the helical cavities in the S2i region of SCW.
Figure 2-4. Micrographs of transverse sections of OW and three CWs showing tracheid wall structures. The main panels show transmission electron micrographs of OW (a), MCM1 (b), MCW2 (c) and SCW (d). In all wood types, the middle lamella is clearly differentiated from the primary wall (P) around the cells and at the cell corners (MLCC). All tracheid walls have a S1 and S2 layer, but an S3 layer is present in only OW (a) and MCW1 (b). Warts (W) are observed on the tracheid wall surface adjacent to the cell lumen in only OW (a). Helical cavities (HC) are present in the inner region of the S2 layer (S2i) in only SCW. Intercellular spaces (IS) are present between tracheids in only MCW 2 (c) and SCW (d). Micrographs obtained using a differential interference microscopy. Scale bar = 1 µm. The insets show differential interference contrast micrographs. These particularly show the S1 and S3 layers in OW (a) and MCW1 (b) and helical cavities in SCW (d). All sections were from Tree 1. Scale bar = 5 µm.
2.4.3 (1→4)-β-Galactans occur as a band in the S2L region of tracheid walls in all three grades of CW, with the band becoming wider with increasing severity

Immunofluorescence microscopy with the monoclonal antibody LM5, which specifically recognizes (1→4)-β-galactans, showed extremely weak labelling of the tracheid walls in OW (Figure 2-5a). Computer enhanced brightening of the selected region of the image showed that the CML, probably the primary wall, was the structure that was labelled (see inset in Figure 2-5a). In the MCW1 tracheid walls, there was a thin band of labelling corresponding to the outer region of the S2 layer, with the brightest labelling at the cell corners (Figure 2-5b). In the MCW2 tracheid walls, the band was wider and brighter than in MCW1, with the brightest part again at the cell corners (Figure 2-5c). The position of the band corresponds to the S2L layer. In the SCW tracheid walls, the band was even wider and brighter than in the MCW2 tracheid walls. Furthermore, in the SCW tracheid walls, the band was of similar brightness all around the cell (Figure 2-5d). Thus, although only small amounts of (1→4)-β-galactan labelling were found in the tracheid walls in OW, in the CWs, more intense and greater areas of labelling were found in the outer layer of the S2 with increasing CW severity. No labelling was detected in micrographs from control experiments in which the primary antibody (LM5) was omitted.
Figure 2-5. Immunofluorescence micrographs of transverse sections of tracheids of OW and three CWs labelled with LM5. OW (a) MCW1 (b), MCW2 (c) and SCW (d). There is extremely weak fluorescence of the tracheid walls in the tracheid walls of OW (a). Computer enhanced brightening of the selected region shows the CML, probably the primary walls, is the structure labelled (inset in (a) at the same scale). In MCW1, there is a thin band of fluorescence in the outer region of the S2 layer (S2L) which is brighter at the cell corners (b). In MCW2, this fluorescent band is wider and brighter than in MCW1, with the brightest part again at the cell corners (c). In SCW, this fluorescent band is even wider and brighter, but is of similar brightness all around the cell (d). All sections were from Tree 1. Scale bar = 10 µm.

Immunogold microscopy with LM5 showed a similar pattern of labelling (Figure 2-6). The OW tracheid walls showed occasional particles over only the CML region, probably over the primary wall (Figure 2-6a circled), but all CW tracheids showed labelling as a band in the
outer region of the S2 layer (S2L), with a smaller amount of labelling in the S1 layer (Figure 2-6b-d). The band of labelling progressively increased in width from MCW1, MCW2 to SCW, with the labelling density increasing particularly from MCW1 (Figure 2-6b) to MCW2 (Figure 2-6c). The labelling in the SCW walls extended into the region with helical cavities (Figure 2-6d). In MCW1 and MCW2 there was more labelling at the cell corners than around the cells (Figure 2-6b, c). No labelling was found in micrographs from control experiments in which the primary antibody was omitted.
Figure 2-6. Immunogold micrographs of transverse sections of tracheids of OW and three CWs labelled with LM5. OW (a) MCW1 (b), MCW2 (c) and SCW (d). In the OW, only occasional particles (circled) are present over the CML, probably the primary wall of OW (a). In MCW1, particles are present as a band in the outer S2 layer (S2L) particularly at the cell corners, with smaller amounts in the S1 layer (b). The labelling density of the band increases with CW severity, particularly from MCW 1 to MCW 2 (c) in the S2L. The band of labelling in the SCW wall extends into the regions with helical cavities (HC) (d). All sections were from Tree 1. Scale bar = 1 µm.

2.4.4 (1→3)-β-Glucans occur as a band in the S2i region of tracheid walls in all three grades of CW, with the band becoming wider with increasing severity

Immunofluorescence microscopy with the monoclonal antibody BS 400-2, which specifically recognizes (1→3)-β-glucans, showed very weak labelling of the tracheid walls in the OW (Figure 2-7a). Computer enhanced brightening of the selected region of the image showed
that the S2 tracheid wall layer was the structure labelled (see inset in Figure 2-7a). The S2i region was weakly labelled in the MCW1 tracheid walls (Figure 2-7b). This region was labelled brighter in the MCW2 tracheid walls (Figure 2-7c), but the brightest labelling was in the same region of the SCW tracheid walls (Figure 2-7d). In these SCW walls, the labelling was banded, corresponding to the helical cavities in this wall region. No labelling was found in micrographs from control experiments in which the primary antibody was omitted, or from control experiments using BS 400-2 that had been pre-incubated with laminarin.
Figure 2-7. Immunofluorescence micrographs of transverse sections of OW and three CWs labelled with BS 400-2. OW (a) MCW1 (b), MCW2 (c) and SCW (d). In OW, there is very weak labelling of the tracheid walls (a). Computer enhanced brightening of the selected region (inset in (b) at the same scale) shows the S2 layer is the structure labelled. In MCW1, there is weak labelling of the inner region of the S2 layer (S2i) (b). This region is labelled brighter in MCW 2 (c). The brightest labelling is found in the same region in the SCW tracheid walls and is in the helical cavities. Sections were from Tree 1 and the micrographs obtained using a Leica confocal microscope. Scale bar = 10 µm.

Immunogold microscopy with BS 400-2 showed a similar labelling pattern (Figure 2-8). In the OW, occasional particles were found in the S2 tracheid wall layer. These were located in the mid region of this layer (inset Figure 2-8a) rather than in the inner (S2i) or outer region (Figure 2-8a). No label was present in the S1 or the S3 layers. Much more labelling was
found in the MCW1 and this was present as a band in only the S2i region (Figure 2-8b). Greater labelling density was found in the same wall region in the MCW2 tracheid walls (Figure 2-8c). In the SCW, the labelling was again confined to the S2i region, and was mainly located in the helical cavities (Figure 2-8d). No particles were found in micrographs from control experiments in which the primary antibody was omitted, or from control experiments using BS 400-2 that had been pre-incubated with laminarin.

Figure 2-8. Immunogold micrographs of transverse sections of OW and three CWs labelled with BS 400-2. OW (a) MCW1 (b), MCW2 (c) and SCW (d). In OW tracheid walls, there are sporadic particles in the mid region of the S2 wall layer (see inset). In MCW1, there is denser labelling in the inner region of the S2 layer (S2i). Even greater density of labelling is found in the same wall region in MCW2. In SCW, there is abundant labelling of the S2i layer, which is mostly confined to within the helical cavities. Sections were from Tree 1. Scale bar = 1 µm (inset scale bar = 0.5 µm).
The presence of (1→3)-β-glucans in the S2i region of the tracheid walls of all three grades of CW was also shown by staining sections with pure, synthetic aniline blue fluorochrome, which specifically binds to (1→3)-β-glucans. Because lignin autofluoresces at the wavelengths used for this fluorochrome, fluorescence images were compared from sections of each wood type stained with the fluorochrome with unstained control sections. Lignin autofluorescence was also reduced by using the 458 nm laser line for excitation rather than the 488 nm line. For OW, there were no obvious differences between the micrographs of stained and unstained sections (Figure 2-9a, b). However for the CWs, there was staining by the fluorochrome of the S2i region of the tracheid walls, with the staining intensity greatest for the SCW (Figure 2-9g, h) and least for the MCW1 (Figure 2-9c, d).
Figure 2-9. Fluorescence micrographs of transverse sections of OW and three CWs stained with aniline blue fluorochrome. OW unstained control (a) and stained (b); MCW1 unstained control (c) and stained (d); MCW2 unstained control (e) and stained (f); SCW unstained control (g) and stained (h). For the OW, there is no obvious difference between micrographs of the unstained, control section (a) and the stained section (b). For MCW1, MCW2 and SCW, the stained sections (d, f, h) show more fluorescence than the controls (c, e, g), with the most fluorescence being in the S2i region. Fluorescence intensity of this region was least in MCW1 (d) and most in SCW (h). Intercellular spaces (IS) are present only in MCW2 and SCW. Sections were from Tree 1 and the micrographs obtained using a Leica confocal microscope. Scale bar = 5 µm.
2.4.5 (1→4)-β-Galactans labelling in whole-stem sections of tilted stems co-locates with CW

LM5 was also used in conjunction with a secondary antibody conjugated with an enzyme (alkaline phosphatase) to examine the distribution of (1→4)-β-galactans in whole sections of the tilted stems. The formation of an insoluble blue dye marked the locations of the (1→4)-β-galactans, which could be observed with the naked eye or low-power (stereo) microscope. On adjacent sections to ones used for immunolabelling, the locations of the four different wood types were determined based on lignin distributions in tracheid walls using fluorescence microscopy. Comparison of the distribution of blue coloration and its intensity among the wood types showed that OW gave no blue colour, MCW1 labelled light blue (outlined in green), MCW2 labelled mid blue (outlined in red) and SCW labelled dark blue (outlined in yellow) (Figure 2-10). Labelled sections from Tree 3 (Figure 2-10a), which was tilted at ~8° from the vertical, contained only small areas of SCW, but large areas of MCW1 and MCW2, whereas sections from Tree 1 (Figure 2-10b), tilted at ~20°, contained large areas of SCW, but only small areas of MCW1 and MCW2.

Figure 2-10. Immunolabelling of whole-stem transverse sections of tilted radiata pine saplings using LM5. Tree 3 (a) and Tree 1 (b). Blue coloration indicates the presence of (1→4)-β-galactans in cell walls. SCW labels dark blue (outlined in yellow), MCW 2 labelled mid blue (outlined in red) and MCW 1 labelled light blue (outlined in green). The grades of CWs were determined by the distribution of lignin in the tracheid walls using fluorescence microscopy. The cambium and adjacent differentiating tracheids (DT), normal resin canals (RC), traumatic resin canals (TRC), and two false growth rings (FR) were also labelled. Rays were also labelled and can just be seen as fine, light-blue, radial lines, particularly in (b). Scale bar = 5 mm.
In addition to CW, some other tissues were labelled. These included the cambium and adjacent differentiating tracheids before the formation of the secondary walls or the deposition of lignin; this occurred adjacent to both OW and CW. They also included resin canals, where the walls of the parenchyma cells surrounding the canals were labelled blue. Two types of resin canals were recognized in both the OW and CW: one type occurred singly and scattered, which was considered to be normal resin canals and the other, occurring in pairs in poorly defined rings, which was considered to be traumatic resin canals (Figure 2-11). To further investigate the occurrence of (1→4)-β-galactans in the walls of the parenchyma cells of resin canals, immunofluorescence microscopy (using a confocal laser scanning microscope) was carried out with LM5 on the OW side of the sections where the canals are present. This confirmed that (1→4)-β-galactans occurred sparsely in the thin walls of the epithelial cells surrounding the canals, but abundantly in the surrounding parenchyma cells of both normal and traumatic resin canals (Figure 2-11b, d). However, labelling was not present in the walls of adjacent tracheids. Autofluorescence micrographs of the same areas obtained using the 488 nm laser line for excitation showed the distribution of lignin and other fluorescent materials; it showed the cell walls of the tracheids, the resin canal epithelial and parenchyma cells (Figure 2-11a, c).
Figure 2-11. Fluorescence and immunofluorescence micrographs of transverse resin sections of resin canals in OW labelled with LM5. Fluorescence micrographs of a normal resin canal (a) and a pair of traumatic resin canals (c) and immunofluorescence micrographs the same normal resin canal (b) and pair of traumatic resin canals (d). The fluorescence micrographs shows the distribution of lignin and other autofluorescent materials. The epithelial and parenchyma walls of both normal and traumatic resin canals and the tracheid walls show autofluorescence. The thin-walled epithelial cells (E) in both normal and traumatic resin canals are sparsely labelled with LM5. The walls of the parenchyma cells (P) in both types of canals are brightly labelled. The ray (R) cell walls are also brightly labelled. Sections were from Tree 1 and the micrographs obtained using a Zeiss confocal microscope. Scale bar = 20 µm.

Ray cell walls were also labelled with LM5. In the immunolabelling of the whole-stem sections, these showed as light-blue radial lines throughout the sections, even in the dark blue
regions. That these lines were due to the labelling of ray cell walls was confirmed using immunofluorescence microscopy (Figure 2-11b, d, Figure 2-12b). Labelling of the ray cell walls was much brighter than the walls of adjacent OW tracheids. However, the ray cell walls in SCW, labelled less brightly than the S2L layer of the walls of neighbouring tracheids (Figure 2-12d). Autofluorescence micrographs of the same areas obtained using the 488 nm laser line, as indicated above, showed the walls of the tracheids adjacent to the rays (Figure 2-12a, c).

Figure 2-12. Fluorescence and immunofluorescence micrographs of transverse resin sections of rays in OW and SCW labelled with the monoclonal antibody LM5. Fluorescence micrographs of ray cells and adjacent tracheids of OW (a) and SCW (c), showing the distribution of lignin and other autofluorescent materials. The walls of the tracheids (T) are clearly seen and there is autofluorescent material in some of the ray cells (R). Immunofluorescence micrograph of OW (b) shows labelling of the ray cell walls, and of SCW (d) shows strong labelling of the S2L layer of the tracheid walls and weaker labelling of the ray cell walls. Sections were from Tree 1 and the micrographs obtained using a Zeiss confocal microscope. Scale bar = 50 µm.

Two concentric rings also labelled blue in the whole-stem sections (Figure 2-10), both of which were considered to be false growth rings. The inner ring (labelled dark blue) was in the latewood zone of first year growth and the outer ring (labelled light blue) was in the earlywood zone at the beginning of the second year growth. Both rings apparently contained traumatic tissue, including incompletely developed tracheids with thin walls and incomplete lignification and showed evidence of collapse (Figure 2-13a inner ring, c outer ring). In
addition, the ray cells were expanded within these rings. Immunofluorescence microscopy with LM5 of both rings showed labelling of the walls of these abnormal tracheids and ray cells, which is consistent with the blue labelling of the rings in the whole-stem sections (Figure 2-13b inner ring, d outer ring).

Figure 2-13. Fluorescence and immunofluorescence micrographs of transverse resin sections of two false growth rings labelled with the monoclonal antibody LM5. Fluorescence micrographs of the inner false growth ring (a) and the outer false growth ring (c), showing the distribution of lignin and other autofluorescent materials. Both false growth rings contain abnormal tissues, including thin-walled incompletely lignified tracheids (ILT), collapsed tracheids (CT), and expanded ray (ER) cells. Immunofluorescence micrographs of the inner false growth ring (b) and the outer false growth ring (d) show strong labelling of these abnormal tissues. Sections were from Tree 1 and the micrographs obtained using a Zeiss confocal microscope. Scale bar = 50 µm.
2.4.6 The percentage of galactose in acid hydrolysates is higher in CWs and indicates CW severity

There were significant differences among the neutral-monosaccharide compositions of the non-cellulosic polysaccharides of the four wood types (Table 2-2). In particular, the percentages of galactose was lowest in the OWs hydrolysates (8.4-8.8%) and highest in those of SCW (49.7-50.5%), with intermediate percentages in MCW1 and MCW2, showing that the percentages of galactose indicate CW severity. Even in the milder of the two MCWs, MCW1, the percentages of galactose in the hydrolysates (29.4-31.6%) were much higher than in those of the OW. The percentages of mannose, xylose and arabinose all decreased with wood type in the order OW, MCW1, MCW2, and SCW. Mannose had the highest percentage of all neutral monosaccharides in the OW (36.5-40.4%), but the lowest percentage of the SCW (16.1-18.9%). The percentage of glucose remained approximately similar among the wood types. There were some relatively small differences in the neutral monosaccharide percentages among the three different trees (P = 2.49×10^-6). This was largely due to the percentage of glucose (Table 2-2). However, the situation was complicated by clear evidence that the differences between individual trees depends on the wood type (interaction P = 1.31×10^-5). To display these differences, the plot of canonical variates 1 and 2 is shown in Figure 2-14. This shows clearly the trend among the wood types (CV 1) and the differences between the individual trees (CV 2). The circles around each centroid are approximate confidence ellipses (95%). The interaction effect that the differences between trees depend on the wood type is especially visible in MCW1, where the differences between the trees virtually vanish. Interestingly, in the other three wood types (OW, MCW2 and SCW) the two ramets from the same clone (Trees 1 and 2) are consistently different on CV2. This CV is largely associated with differences in the percentage of glucose. Table 2-3 shows the correlations between the neutral monosaccharide percentages and the canonical variate.
Table 2-2. Neutral monosaccharide composition (% w/w of all neutral monosaccharides) of the non-cellulosic polysaccharides in the four wood types from three trees.

<table>
<thead>
<tr>
<th>Tree</th>
<th>Wood type</th>
<th>Arabinose</th>
<th>Xylose</th>
<th>Galactose</th>
<th>Mannose</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tree 1</td>
<td>OW</td>
<td>9.9±0.6</td>
<td>30.3±1.18</td>
<td>8.4±1.4</td>
<td>36.5±0.9</td>
<td>14.9±1.0</td>
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<tr>
<td></td>
<td>(Tilted at MCW1)</td>
<td>6.8±0.5</td>
<td>20.9±0.34</td>
<td>31.6±0.7</td>
<td>27.7±0.2</td>
<td>13.0±1.3</td>
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<tr>
<td></td>
<td>~20°</td>
<td>5.7±0.6</td>
<td>18.0±0.86</td>
<td>40.8±0.6</td>
<td>20.8±1.0</td>
<td>14.8±1.5</td>
</tr>
<tr>
<td></td>
<td>SCW</td>
<td>4.9±0.6</td>
<td>15.3±0.90</td>
<td>49.7±0.5</td>
<td>16.1±1.4</td>
<td>14.0±0.3</td>
</tr>
<tr>
<td>Tree 2</td>
<td>OW</td>
<td>11.0±1.0</td>
<td>31.0±1.1</td>
<td>8.8±0.3</td>
<td>38.2±0.6</td>
<td>11.0±0.5</td>
</tr>
<tr>
<td></td>
<td>(Tilted at MCW1)</td>
<td>6.3±0.3</td>
<td>23.6±0.2</td>
<td>30.4±0.9</td>
<td>28.6±0.7</td>
<td>11.1±0.3</td>
</tr>
<tr>
<td></td>
<td>~13°</td>
<td>5.7±0.5</td>
<td>17.8±0.5</td>
<td>41.0±0.9</td>
<td>20.7±0.6</td>
<td>14.8±1.4</td>
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<tr>
<td></td>
<td>SCW</td>
<td>4.1±0.2</td>
<td>14.2±0.7</td>
<td>50.5±1.1</td>
<td>18.9±0.4</td>
<td>12.3±0.8</td>
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<tr>
<td>Tree 3</td>
<td>OW</td>
<td>8.8±0.4</td>
<td>30.1±0.2</td>
<td>8.7±0.7</td>
<td>40.4±1.1</td>
<td>12.1±0.1</td>
</tr>
<tr>
<td></td>
<td>(Tilted at MCW1)</td>
<td>6.2±0.8</td>
<td>23.4±1.1</td>
<td>29.4±0.2</td>
<td>30.3±0.3</td>
<td>10.7±0.3</td>
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<tr>
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<td>~8°</td>
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<td>16.7±0.9</td>
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<td>15.9±0.5</td>
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<tr>
<td></td>
<td>SCW</td>
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<td>49.9±1.1</td>
<td>18.4±0.2</td>
<td>11.6±0.9</td>
</tr>
</tbody>
</table>

Means and standard errors of determination on three hydrolysates.
Figure 2-14. Canonical discriminant analysis plot. The four wood types from three trees defined by the first two canonical variates (CV 1 and CV2) obtained from canonical discriminant analysis conducted on all neutral monosaccharide variables combined. The centroids for each wood type of each tree with approximate 95% confidence regions are separated based on differences in the monosaccharide compositions between wood types, and not trees. The percentage in the axis labels refers to the proportion of the total between-centroid variance summarised by that canonical variate.

Table 2-3. Structural coefficients (correlations) for the first two canonical variates (CV 1 and CV 2) of the differences between the neutral monosaccharide compositions of the non-cellulosic polysaccharides of the four wood types.

<table>
<thead>
<tr>
<th>Monosaccharides</th>
<th>CV1</th>
<th>CV2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>-0.962</td>
<td>0.201</td>
</tr>
<tr>
<td>Xylose</td>
<td>-0.992</td>
<td>-0.06</td>
</tr>
<tr>
<td>Mannose</td>
<td>-0.984</td>
<td>-0.17</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.999</td>
<td>-0.011</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.243</td>
<td>0.844</td>
</tr>
</tbody>
</table>
2.5 Discussion

The present study showed that the two grades of MCWs which were identified based on the distribution of lignin in the tracheid walls, had tracheid wall structures and polysaccharide compositions intermediate between those in OW and SCW. A thin S3 wall layer similar to that found in the mildest grade, MCW1, has been reported in some samples of MCWs of the same species (Singh and Donaldson, 1999; Singh et al., 2003). Such a layer was also reported in the tracheid walls of very MCW in white spruce (Picea glauca). This finding came from a study, using ultraviolet-microscopy, of the transition between NW and SCW (Yumoto et al., 1983). No S3 layer was found later in CW development and there have been no reports of it in the tracheid walls of SCW. However, another structural feature of tracheid walls, helical cavities in the S2i region, appears to be confined to SCW. The tracheid walls of neither of the two MCWs, MCW1 and MCW2, showed evidence of such cavities, and, as far as I am aware, there are no literature reports of these in the walls of MCW tracheids. Thus, in terms of tracheid wall structures in CWs, a S3 layer occurs in only the very mildest MCW, and helical cavities occur in the S2i region of only SCW.

In the present study, immunofluorescence and immunogold microscopy using the monoclonal antibody LM5, which is specific for (1→4)-β-galactans, showed these polysaccharides were located mostly as a band in the S2L layer of the tracheid walls of all three CW severities, although the band width increased with severity. Similar results have previously been reported for SCW in P. radiata using immunofluorescence microscopy (Schmitt et al., 2006; Mast et al., 2009; Donaldson and Knox, 2012) and immunogold microscopy (Mast et al., 2009; Altaner et al., 2010), in Sitka spruce (Picea sitchensis) also using both types of microscopy (Altaner et al., 2007a, 2010) and in Norway spruce (Picea abies) using immunogold microscopy (Altaner et al., 2010). However, in none of these studies was MCW examined that had been defined using the distribution of lignin in the tracheid walls. Nevertheless, in the immunofluorescence microscopy study of Altaner et al. (2007a), some labelling was reported in the tracheid walls of what was described as “moderate CW” defined using only tracheid morphology. Interestingly, it was found that at least some (1→4)-β-galactans were present in the tracheid wall S2L layer in even the mildest grade, MCW1, in which lignin was detected in the outer region of the S2L layer only at the cell corners. The presence of this polysaccharide is thus a characteristic feature of the S2L layer of tracheid walls in all grades of CW and not just SCW.
In contrast to the CWs, OW showed only weak labelling of the tracheid wall and this was at a different location, the compound middle lamella. This may represent labelling of (1→4)-β-galactan side chains of the pectic polysaccharide rhamnogalacturonan I (RG I), which is known to occur in the primary cell walls of coniferous gymnosperms (Harris, 2005). RG I has been chemically characterized from the primary walls of cell suspension cultures of Douglas fir (*Pseudotsuga menziesii*) (Thomas et al., 1987) and from cell walls in the differentiating xylem zone of Japanese cedar (*Cryptomeria japonica*) (Edashige and Ishii, 1997). Both studies indicated that the RG I side chains contained much smaller proportions of (1→4)-β-galactans than of (1→5)-α-arabinans. Similar weak labelling of the primary wall with LM5 has been reported in tracheid walls of OW of radiata pine using immunofluorescence microscopy (Donaldson and Knox, 2012) and of radiata pine, Sitka spruce and Norway spruce using immunogold microscopy (Altaner et al., 2010). Consistent with this, only small amounts of (1→4)-β-galactans have been found in 6 M sodium hydroxide extracts of radiata pine OW compared with SCW (Chavan et al., 2015). These polysaccharides were also found in smaller amounts in OW than SCW in the same species using 2D NMR spectroscopy of cell-wall gels of finely milled wood (Brennan et al., 2012). The tracheid walls of OW therefore differed from those of CWs in only weakly labelling with LM5 and at a different location, the primary cell walls, where the labelling was probably due to (1→4)-β-galactans side chains on RG I.

Immunolabelling with LM5 of whole stem sections using an enzyme labelled secondary antibody showed the locations of CW tracheids and the different grades of CW present could be differentiated from one another. The labelled SCW also corresponded to the dark areas in sections viewed by reflected or transmitted light. The method would be more reliable than reflected or transmitted light methods which rely on SCW often having a darker colour than NW or OW (Duncker and Spiecker, 2005; Gardiner and Macdonald, 2005; Chauhan et al., 2013). The chemical basis of this coloration is unknown, and the colour intensity of SCW varies with species (Timell, 1986). Transmitted light methods, in particular, have confused CWs with latewood (Donaldson and Singh, 2013) and with heartwood (Gardiner and Macdonald, 2005). Although immunolabelling of whole stem sections is labour intensive, it is a promising method for the reliable detection and location of different grades of CWs in coniferous gymnosperms and could potentially be partially automated and used commercially. It could be particularly useful in detecting MCW, large areas of which were formed in the tree tilted at only ~8°. The angle of tilt is likely to be the major factor in
determining the area of MCW formed relative to SCW. The sections from the tree tilted at ~20° contained very much less MCW, but this tree was from a different clone and so it is formally possible that clonal difference may, at least partly, affect the proportions of MCW.

Immunolabelling with LM5 of whole stem sections also showed labelling of other features, including cambial cells and adjacent differentiating cells, producing both OW and CW, as well as rays, and resin ducts. However, this labelling could easily be distinguished from labelling of CW. Cambial and differentiating tracheids have primary walls, which would be expected to contain RG I with some (1→4)-β-galactans side chains. However, the labelling was brighter than would be expected when compared with the immunofluorescence and immunogold labelling of the primary walls of mature tracheids. It is possible that the high lignin concentrations in the primary walls of mature tracheids may partially mask the epitope resulting in weaker labelling. Further examination of the ray cells using immunofluorescence microscopy with LM5 showed labelling of their walls, which are non-lignified primary walls, again indicating the likely presence in these walls of (1→4)-β-galactan side chains on RG I.

Similar immunofluorescence labelling with LM5 of ray cell walls has previously been reported in OW and SCW in Sitka spruce (Altaner et al., 2007a) and radiata pine (Donaldson and Knox, 2012). Normal and traumatic resin canals were also labelled in whole stem sections. Both types of canals when examined by immunofluorescence microscopy with LM5, showed that the walls of the epithelial cells lining the canals labelled weakly, whereas the walls of the surrounding parenchyma cells labelled brightly. Similar labelling with LM5 of normal resin canals in radiata pine has previously been reported by Donaldson and Knox (2012). Traumatic resin canals occurring in pairs in poorly defined rings have been reported in many species of the family Pinaceae, including radiata pine, in response to a variety of stresses, including wounding and drought stress (Fahn et al., 1979; Cown et al., 2011). The occurrence of these traumatic resin canals in the present study is likely to be in response to the tilting of the trees (Lepage and Begin, 1996). In the present study, the walls of the parenchyma and epithelial cells of these traumatic resin canals labelled in a similar way to those of normal resin canals. Thus, immunolabelling of whole stem sections with LM5 labelled cambial and ray cells, as well as resin ducts, with the labelling probably being due to (1→4)-β-galactans side chains of RG I in non-lignified primary walls.

Two false growth rings were also labelled by LM5 in the whole stem sections. Such rings have been described previously as occurring after a variety of stresses, including water stress
Given the sapling trees in the present study were well watered, drought is not a likely cause of the false growth ring. However, frosts are known to occur in the area where the trees were grown, and so it is possible they are frost rings. LM5 labelling of such rings has not previously been reported, but abnormal tracheid development resulting in collapsed cells, with poorly developed lignification, has been reported in these rings, together with the occurrence in them of expanded rays due to the proliferation of parenchyma cells in the false growth rings. The collapsed tracheids lacked fully developed secondary walls suggesting that differentiation of the tracheid stopped prematurely (Barnett, 1976; Donaldson, 2002) and the irregular lignification may relate to a failure in the mechanism of lignification (Lee et al., 2007). The RG I in the primary walls of the abnormal cells may have higher proportions of \((1\rightarrow4)\)-\(\beta\)-galactan side chains than in the equivalent walls of normal cells, resulting in greater LM 5 labelling. Interestingly, cold stress has been reported to cause an increase in the proportion of \((1\rightarrow4)\)-\(\beta\)-galactan side chains in RG I in pea (\textit{Pisum sativum}), with these side chains possibly acting as a cold protectant (Baldwin et al., 2014; Le Gall et al., 2015). The LM5 labelling of the two false growth rings thus resulted from the labelling of primary walls in abnormal cells, possibly containing higher proportions of \((1\rightarrow4)\)-\(\beta\)-galactan side chains on RG I.

Immunofluorescence and immunogold microscopy with the monoclonal antibody BS 400-2 specifically detected \((1\rightarrow3)\)-\(\beta\)-glucans (callose or laricinan) in the S2i region of tracheid walls in all the CWs, with most labelling being detected in the SCW and the least in MCW1. The presence and location of \((1\rightarrow3)\)-\(\beta\)-glucans in the walls of SCWs tracheids has previously been reported using the same antibody (Altaner et al. 2007b, 2010). Using immunofluorescence microscopy \((1\rightarrow3)\)-\(\beta\)-glucans were shown to be located in or between helical cavities in the inner region of the S2 layer of the of the trach eid walls in SCW of Sitka spruce (\textit{Picea sitchensis}) (Altaner et al., 2007b). A later study using immunogold microscopy with the three species radiata pine, Sitka spruce, and Norway spruce (\textit{Picea abies}) showed the \((1\rightarrow3)\)-\(\beta\)-glucans were located within these helical cavities in the S2 wall layer of tracheids in SCW (Altaner et al., 2010). The present immunogold microscopy studies also showed that in the SCW, the \((1\rightarrow3)\)-\(\beta\)-glucans were within the helical cavities. Interestingly in the two MCWs, \((1\rightarrow3)\)-\(\beta\)-glucans were also present in the S2i region despite there being no cavities. This has implications for the possible functions of \((1\rightarrow3)\)-\(\beta\)-glucans in these walls. They have been suggested as acting as permeability barriers and leak sealants (Currier, 1957;
Waterkeyn, 1981) and more recently as functioning to resist compressive stresses (Parre and Geitmann, 2005). The finding of (1→3)-β-glucans within the cavities led to the suggestion that they may act as sealants for these cavities (Altaner et al., 2010), but because in the MCWs the (1→3)-β-glucans occur in the absence of cavities, this suggests this is not their only role. Another likely role of (1→3)-β-glucans in CW tracheid walls is their ability to resist gravitropic stresses generated on tilting stems. The presence of (1→3)-β-glucans is thus a characteristic feature of the inner S2i layer of tracheid walls in all grades of CWs and not just SCW.

Before the availability of monoclonal antibodies to (1→3)-β-glucans, fluorescence microscopy after staining with decolorized aniline blue had been used to detect and locate these polysaccharides in the tracheid walls of SCW. This method was used to locate the polysaccharide in the S2i region of tracheid walls in Pinus strobus (Wloch and Hejnowicz, 1983) and in 19 other species of gymnosperms (Brodzki, 1972). The method relies on the presence in aniline blue preparations of a fluorochrome that is produced during the manufacture of the dye. The fluorochrome binds specifically to (1→3)-β-glucan (Evans and Hoyne, 1982; Evans et al., 1984). However, the decolorized aniline blue is used at a high pH which increases the intensity of lignin autofluorescence, making it difficult to locate (1→3)-β-glucans in lignified walls (Smith and McCully, 1978). To reduce this problem, a pure, synthetic preparation of the fluorochrome in water (Stone et al., 1984) was used, and, as with immunofluorescence microscopy with BS 400-2, it was found that (1→3)-β-glucans occur in the S2i region of tracheid walls in all CW grades. Thus, staining with the pure, synthetic fluorochrome in water is a fast, convenient method of detecting (1→3)-β-glucans in the tracheid walls of all CW severities.

The neutral monosaccharide compositions of the four wood types examined in the present study showed increasing proportions of galactose from OW, through MCW1 and MCW2 to the SCW. To determine these compositions, trifluoroacetic acid (TFA) was used under conditions that are known not to hydrolyse crystalline cellulose (Mankarios et al., 1979) and is well suited to study non-cellulosic polysaccharides. As TFA can easily be removed, it allowed very small samples to be used which were examined before hydrolysis by fluorescence microscopy to determine the lignin distribution in the tracheid walls, and hence the wood category could be confirmed. By using relative percentages of neutral monosaccharides in the hydrolysate rather than absolute yield of each monosaccharide on a
dry weight basis, accurate weighing of samples was unnecessary. Even in hydrolysates of MCW1, where lignification of the tracheid S2L layer was apparent only at the cell corners, the percentage galactose was over three times greater than in OW hydrolysates. Intermediate amounts of galactose (on a g/100 g oven dry wood basis) were also reported in hydrolysates of MCW of radiata pine using the traditional two-stage sulphuric acid method, which also hydrolyses crystalline cellulose (Nanayakkara et al., 2009). Although determining monosaccharide compositions cannot of course differentiate between different galactose-containing polysaccharides, labelling of the tracheid walls of MCW1 with LM5 is consistent with the majority of the galactose coming from (1→4)-β-galactans. Another potential source of galactose in the form of arabino-3,6-galactans have been shown not to be present in similar wood samples (Brennan et al., 2012). Lower proportions of mannose in hydrolysates of the radiata pine CWs in the present study are consistent with decreases in the proportions of the most abundant non-cellulosic polysaccharides in OW, O-acetyl-galactoglucomannans, which contain galactose, glucose and mannose in the ratio 0.1:1.0:3.7 (Harwood, 1973). Lower proportions of arabinose and xylose in the same CW hydrolysates are consistent with decreases in the proportions of the second most abundant non-cellulosic polysaccharides in OW, arabino(4-O-methylglucurono)xylans, which contain 4-O-methylglucuronic acid, xylose and arabinose in the ratio of 1.0:5.8:1.1 (Harwood, 1972). However, given the glucose content of the O-acetyl-galactoglucomannans, it is interesting that the proportions of glucose remained similar in the hydrolysates of all four wood types. Higher proportions of (1→3)-β-glucans in the CWs may at least partly offset the decrease in glucose from the heteromannans. The neutral monosaccharide compositions of the four wood types showed higher proportions of galactose in the CW polysaccharides and are consistent with the greater labelling with LM5 of the CW tracheid walls than those of OW, and with the extent of labelling being related to CW severity.

The (1→4)-β-galactans in CW tracheid walls may, as suggested above for (1→3)-β-glucans, act to resist the gravitropic stresses generated on tilting stems. The (1→4)-β-galactans are co-located with lignin, and both may act together. Both (1→4)-β-galactans and (1→3)-β-glucans swell markedly on hydration and this may play a role in resisting these stresses in the tree and also result in the greater longitudinal shrinkage on drying of SCW than NW or OW (Brennan et al. 2012; Fagerstedt et al., 2014), which is of considerable commercial importance.
2.6 Conclusions

Two grades of MCW, MCW1 and MCW2, as well as SCW and OW were identified in radiata pine and characterized. An S3 tracheid wall layer was present in only OW and the mildest MCW, MCW1, and helical cavities were present only in the inner region of the S2 tracheid wall layer in SCW. (1→4)-β-Galactans were a characteristic feature of the outer S2L tracheid wall layer of all grades of CW and not just SCW, with the proportions increasing with CW severity. However, the tracheid walls of OW contained only small proportions of these galactans, which were located in the primary walls. The neutral monosaccharide compositions of the non-cellulosic polysaccharides of the four wood types were consistent with these different proportions of (1→4)-β-galactans. Antibody labelling for (1→4)-β-galactans can also be used with whole stem sections to identify regions occupied by the four different wood types with the naked eye or low-power microscope. (1→3)-β-Glucans were a characteristic feature of the S2i region of tracheid walls in all grades of CWs and not just SCW, with the proportions increasing with CW severity. Thus, (1→3)-β-glucans and (1→4)-β-galactans were present in the tracheid walls of even the mildest grade of CW (MCW1). If these polysaccharides play a causal role in SCW shrinking longitudinally more than NW or OW, then it is likely that this will also be true for MCW, and be of considerable commercial importance.
Chapter 3 - Location and characterization of lignin in tracheid cell walls of radiata pine (*Pinus radiata* D. Don) compression woods

3.1 Abstract

Tilted stems of softwoods form compression wood (CW) and opposite wood (OW) on their lower and upper sides, respectively. The structures and chemical compositions of the tracheid walls of these two wood types differ. More is known about the most severe form of CW, severe CW (SCW), but mild CWs (MCWs) also occur widely. In a previous study, two grades of MCWs, MCW1 and MCW2, as well as SCW and OW were identified in the stems of radiata pine (*Pinus radiata*) that had been slightly tilted. Tracheid wall structures and cell-wall polysaccharide compositions in the four wood types were compared. The present study is an extension of this investigation and focused particularly on lignin. The four wood types were identified by the distribution of lignin in the tracheid walls determined by fluorescence microscopy. In addition to using lignin autofluorescence, a solution of the fluorescent dye acridine orange (AO) (0.02% at pH 6 or 7) was shown to metachromatically stain the tracheid walls. The lignified walls fluoresced orange to yellow depending on the lignin concentration. Microscopically well-characterized discs (0.5 mm diameter) of the wood types were used to determine lignin concentrations and lignin monomer compositions using the acetyl bromide method and thioacidolysis, respectively. Lignin concentration and the proportion of *p*-hydroxyphenyl units (H-units) relative to guaiacyl (G-units) increased with CW severity, with <1% H-units in OW and up to 14% in SCW. Similar discs were also examined by Raman and FTIR micro-spectroscopies coupled with principal component analysis (PCA) to determine if these techniques can be used to differentiate the four different wood types. Both techniques were able to do this, particularly Raman micro-spectroscopy. AO staining can be used to determine lignin distribution in tracheid walls and hence different severities of CW. Lignin H-units can be used as a marker for CW and CW severity. Raman and FTIR micro-spectroscopies in combination with PCA, can be used to differentiate the four wood types, with Raman micro-spectroscopy being the preferred technique.
3.2 Introduction

Cell walls of wood (secondary xylem) make a major contribution to plant biomass and are of considerable economic importance. They are extensively utilized in the construction industry as solid timber, in the manufacture of pulp and paper, and increasingly in the production of second generation biofuels such as ethanol. When trees are tilted from the vertical, for example by wind, weight of snow or because of the slope of the land, a special type of wood is formed, known as reaction wood, that functions to correct this tilt (Scurfield, 1973; Groover, 2016). The reaction wood in hardwood (angiosperm) trees is known as tension wood (Felten and Sundberg, 2013), whereas that in softwood (coniferous gymnosperm) trees as compression wood (CW) (Timell, 1986).

Here, the focus was on CW. This type of wood is formed on the under sides of stems and is accompanied by opposite wood (OW), which is formed on the opposite (upper) sides of stems and is similar to normal wood (NW). Most research has been carried out on a type of CW that is more accurately described as severe compression wood (SCW) (Donaldson and Singh, 2013). This differs from NW and OW both in its cell-wall structures and chemical composition. The predominant cell type in softwoods is the tracheid, which conducts water and provides mechanical support. Tracheids are elongated cells with thick secondary walls that overlie thin primary walls, and the individual tracheids adhere to one another by a thin middle lamella (ML). This, together with the two adjacent primary walls, is often referred to as the compound middle lamella (CML). The secondary walls of NW and OW tracheids consist of three distinct layers known as S1, S2 and S3, with the S2 layer being the thickest. In contrast, SCW tracheids have secondary walls that are thicker, lack the S3 layer, and have helical cavities in the inner region of the S2 layer (the S2i region). SCW tracheids are also shorter, more rounded in transverse section and have intercellular spaces between them (Timell, 1986).

Chemically, the tracheid walls of NW and OW are composed mostly of the polysaccharides cellulose and heteromannans (O-acetyl-galactoglucomannans), with smaller proportions of heteroxylans (arabino(4-O-methylglucuronoxylans), together with the aromatic polymer lignin (Harris and Stone, 2008). Softwood lignin is formed by the oxidative coupling of the two p-hydroxycinnamyl alcohol monomers (monolignols) coniferyl and p-coumaryl alcohols, which give rise to the guaiacyl (G) and p-hydroxyphenyl (H) units of lignin, respectively. Most softwood lignins, unlike hardwood lignins, contain no syringyl (S) units formed from
sinapyl alcohol (Ralph et al., 2004; 2007). In contrast to the tracheid walls of NW and OW, those of SCW contain higher proportions of lignin, but lower proportions of cellulose, heteromannans and heteroxylans (Timell, 1986; Brennan et al., 2012). Additionally, the walls contain (1→4)-β-D-galactans (up to 10%) and small proportions (3%) of (1→3)-β-D-glucans (callose or laricinan) (Timell, 1986; Brennan et al., 2012; Hoffmann and Timell, 1970). SCW lignin differs in structure from that in NW and OW. It contains significant proportions of H units, whereas OW and NW lignin contains only trace amounts of H units (Bland, 1958; Westermark, 1985; Nanayakkara et al., 2009; Brennan et al., 2013). The distribution of lignin in SCW tracheid walls also differs from that in NW and OW tracheid walls. This has been determined using fluorescence microscopy based on the autofluorescence of lignin in ultraviolet and blue radiation (Donaldson and Singh, 2013). In OW and NW tracheid walls, lignin occurs in high concentrations in the ML and primary walls. However, in SCW tracheid walls, the lignin concentration is highest in the outer region of the S2 layer (S2L region), lower in the inner region of the S2 layer (S2i region), and lowest in the S1 layer, and CML (Zhang et al., 2016).

SCW is of considerable commercial importance for a number of reasons. First, the presence of SCW in solid timber lowers its quality and is regarded as a defect. On drying, SCW exhibits much greater longitudinal shrinkage than does NW or OW. When SCW and NW or OW occur adjacent to one another, this results in the timber warping and distorting in shape (Langrish and Walker, 2006; Xu et al., 2009). Second, the higher lignin content of SCW has a negative impact on the efficient production of chemical pulps and second generation biofuels such as bioethanol (Vanholme et al., 2012). Third, in contrast to these negative aspects of SCW, the heating value on combustion of SCW is higher than that of OW or NW. This is because the carbon content of lignin (~64%), present in higher proportions in CW, is higher than that of cellulose (~44%) (White, 1987; Lewandowski et al., 2003; Ishida et al., 2004).

In addition to SCW, a continuum of wood types (grades) occur between SCW and OW or NW and are referred to as mild CWs (MCWs) (Donaldson and Singh, 2013). There have been fewer studies on MCWs and less is known about them, but they are also likely to be of considerable commercial importance. In a recent study (Zhang et al., 2016), slightly tilted (~8-20° to the vertical) saplings of radiata pine (Pinus radiata) were used to maximize the formation of MCW rather than SCW. In this study, four wood types, OW, SCW and two grades of MCW, MCW1 and MCW2, were identified based on the distribution of lignin in
their tracheid walls. The study showed that only the tracheid walls of OW and MCW1 had a S3 layer, and this was thin in MCW1, and only the SCW had a S2 layer with helical cavities in the S2i region. By using immunomicroscopy with monoclonal antibodies, (1→4)-β-galactans and (1→3)-β-glucans were detected in the tracheid walls of all the CWs, although in different locations. The (1→4)-β-galactans were found in the S2L region, and the (1→3)-β-glucans in the S2i region. The areas and intensities of labelling increased with CW severity. Furthermore, immunolabelling was used on whole stem cross sections to determine the locations and relative amounts of (1→4)-β-galactans, which allowed areas containing the four wood types to be clearly differentiated. In addition, the neutral monosaccharide compositions of the non-cellulosic polysaccharides were determined of the four wood types on small (0.5 mm diameter), microscopically well-defined discs. This showed that the proportion of galactose was higher for CWs and increased with severity, which was consistent with the immunomicroscopy results.

In addition to immunological and wet chemical techniques, Fourier transform infrared (FTIR) and Raman micro-spectroscopies carried out on similar discs to those described above, in combination with principal component analysis (PCA), could potentially be used to identify CW and determine its severity. These are rapid, non-destructive techniques that probe molecular vibrations by different means, with FTIR spectroscopy based on an absorption process and Raman spectroscopy on a scattering effect (Séné et al., 1994; Gierlinger and Schwanninger, 2007). They complement one another, with Raman spectroscopy being more sensitive to the symmetric ring stretch vibrations of lignin, and FTIR spectroscopy showing greater intensity for the carbon-hydrogen stretching modes of polysaccharides (Agarwal and Atalla, 1993; Stenius and Vuorinen, 1999). FTIR micro-spectroscopy has been widely used to examine primary cell walls, and with PCA to identify cell-wall mutants (Séné et al., 1994; Chen et al., 1998; McCann et al., 2007). More recently, it has been used to examine wood cell walls (Naumann et al., 2007). For example, in combination with PCA, it has been used to differentiate between latewood and earlywood tracheids of a range of softwoods based on the compositions of their walls (Hori and Sugiyama, 2003). In contrast, Raman micro-spectroscopy has only recently been used to examine plant cell walls, including those in compression and opposite woods (Agarwal, 2014). It has also been used in combination with PCA to differentiate wood cell wall layers of black poplar (Populus nigra) (Zhang et al., 2015).
Here, an extension of my previous study (Zhang et al., 2016) is reported in which the tracheid walls of the four wood types were further characterized, particularly in relation to lignin. First, the use of the fluorescent dye acridine orange (AO) (Horobin, 2002) was investigated, to give bright, fluorescence staining of lignin and provide a simple, yet effective, alternative method to autofluorescence to determine lignin distribution in tracheid walls. Second, the lignin content of small (0.5 mm diameter), microscopically well-defined discs of the four wood types was determined and the relative proportions of the monomeric units in the lignin was determined using the thioacidolysis method. Third, FTIR and Raman microspectroscopies in combination with PCA were used to determine whether they can differentiate the four wood types from one another.

3.3 Materials and methods

3.3.1 Wood samples

Saplings of *Pinus radiata* (D. Don) (radiata pine) (Forest Genetics Ltd, Rotorua, New Zealand) were used to obtain the four wood types and were grown outside at Harewood, Christchurch, New Zealand, in 100 litre bags containing potting mix, as described in detail by Zhang et al. (2016). Briefly, saplings were grown upright for six months, and then tilted by staking at ~8-20° from the vertical to induce the formation of CW and OW, and harvested 16 months later. Three saplings were used: clone 30, ramets 1 and 2, and clone 17, which are referred to as Trees 1, 2, and 3, respectively. The exact angle of tilting, as measured at harvest, was ~20° for Tree 1, ~13° for Tree 2, and ~8° for Tree 3. At harvest, a segment (~10 cm long) was sawn from each stem, ~20 cm above the potting mix, and used for the experiments involving the four wood types.

Another sapling (clone 15, referred to as Tree 4) was used for staining experiments using acridine orange at different pHs. This was planted as a 12-month-old sapling in potting mix and grown in an unheated glasshouse (temperature range 13-27°C) at the School of Biological Sciences, University of Auckland. After 16 days, the sapling was tilted by staking at ~45° to induce the formation of CW and OW, and harvested after five months. A segment was obtained from the stem by cutting at the first node above the soil and 2 cm below this. Midway along this segment samples (~1 mm tangential width × ~1 mm radial width × 10 cm
long) were cut from the lower part of the tilted region of the stem. The wood in this region had a reddish hue and subsequent microscopy showed it was SCW.

### 3.3.2 Identifying and obtaining samples of the four wood types

This was done as described in detail by Zhang et al. (2016). Briefly, transverse sections (60 µm thick) were cut with a sliding microtome (Model HN 40, Jung, Heidelberg, Germany) and the locations of the four wood types identified based on the lignin distributions in the tracheid walls. Lignin was detected by its autofluorescence using a Leica microscope (model DMR; Leica Microsystems, Wetzlar, Germany) fitted with an I3 filter block (excitation filter BP450-490 nm, chromatic beam splitter 510 nm, and emission filter LP515). Discs were cut from areas containing only one wood type using a Harris Uni-Core™ micro-punch sampler (diameter 0.5 mm) (ProSciTech). Each disc was again checked by fluorescence microscopy and any disc containing more than one wood type was rejected. The discs were used to determine lignin content, to examine lignin monomer composition by thioacidolysis, and for Raman and FTIR micro-spectroscopy. The discs were all cut from the earlywood of year two.

### 3.3.3 Fixation, embedding and sectioning for microscopy

Samples from the four wood types, identified as described above, and from the SCW (from Tree 4) used for staining experiments using AO at different pH values, were fixed in 2% (w/v) paraformaldehyde and 0.1% (w/v) glutaraldehyde in 100 mM sodium 1,4-piperazinediethanesulfonic acid (PIPES) buffer (pH 7.2) for 2 h (or 3 h for the sample for staining experiments using acridine orange at different pH values), dehydrated in an aqueous ethanol series, and embedded in LR White resin (London Resin Co. Ltd, Basingstoke, UK) as described in detail in Zhang et al. (2016). Sections (500 nm thick) were cut with a diamond knife using an ultramicrotome (Model EM UC6: Leica Microsystems, Wetzlar, Germany).

### 3.3.4 Acridine orange (AO) staining and microscopy

The effect of pH on AO staining was examined by staining sections (500 nm thick) of SCW from Tree 4 with 0.02% (w/v) (660 µM) AO (as the hydrochloride hydrate) (C.I. 46005;
Raymond A Lamb, Wembley, Middlesex, London, UK) in a buffer solution at pHs from 2-8 for 10 min, washed in the relevant buffer for 30 s, and mounted in the same buffer. Control sections were prepared by mounting unstained sections in the relevant buffer. The buffer solutions were as follows: pH 2, 0.1 M glycine buffer; pH 3, 0.1 M citric acid-sodium citrate buffer; pH 4, 5 and 6 0.1 M sodium acetate-acetic acid buffer; pH 7 and 8, 0.1 M sodium phosphate buffer. The sections were examined with a Leica microscope (Model DMR; Leica, Wetzlar, Germany) fitted for epifluorescence with an I3 filter block (excitation filter BP450-490 nm, chromatic beam splitter 510 nm, and emission filter LP515 nm), photographed with a Leica camera (Model DC500). AO is an animal mutagen and appropriate safety measures need to be used in handling this compound (Horobin, 2002).

Sections (500 nm and 60 µm thick) of the four wood types (from Tree 1) were stained with AO as described above using the pH 6 and 7 buffers, but were examined with an inverted confocal laser scanning microscope (Model LSM 710; Zeiss, Oberkochen, Germany) with the 488 nm line from an argon-ion gas laser for excitation and imaged using two adjustable bandwidth detection channels: 515-560 nm was set for channel 1, and wavelengths >560 nm set for channel 2. Channel 1 was assigned to green and channel 2 assigned to red, and combined to produce coloured images that simulated those obtained when the same sample was examined with the Leica fluorescence microscope fitted with the I3 filter block (see above).

3.3.5 Quantification of lignin using the acetyl bromide assay

Discs (~2 mg) (obtained as described above) of each of the four wood types of all three trees were dissolved in 25% v/v acetyl bromide in 17.4 M acetic acid (0.2 mL) and heated at 50°C for 2 h with occasionally mixing (Hatfield et al., 1999; Fukushima and Hatfield, 2001). After cooling, 2 M NaOH (1 mL) and 0.5 M hydroxylamine hydrochloride (0.175 mL) were added and made up to 10 mL with 17.4 M acetic acid and the absorbance at 280 nm measured using UV/VIS/NIR spectrophotometer (Shimadzu UV-3600 plus, Shimadzu Corp., Kyoto, Japan). The content of acetyl bromide soluble lignin was calculated using the extinction coefficient of 20.0 liter·g⁻¹·cm⁻¹, which has previously been used for *P. radiata* (Iiyama and Wallis, 1988; Wagner et al., 2007). The significance of the effects of the different wood types and trees on differences in lignin content were investigated by the two-way analysis of variance (ANOVA) using R software (version 3.0.1) (R Core Team, 2013).
3.3.6 Thioacidolysis followed by gas chromatography-mass spectrometry (GC-MS) of the derivatized lignin monomers

Triplicate samples were used of each of the four wood types, each consisting of 10 discs. This was done essentially as described by Méchin et al. (2014). Each sample was placed into a 30 mL glass tube fitted with a Teflon-lined screwcap, together with freshly prepared thioacidolysis reagent (1 mL), and of heneicosane (C21) (20.72 µg) and nonadecane (C19) (18 µg) internal standards in dichloromethane (40 µL). The thioacidolysis reagent was prepared by adding ethanethiol (10 mL) and boron trifluoride diethyl etherate (2.5 mL) to a 100 mL volumetric flask containing dioxane (20 mL) and then adjusting the final volume to 100 mL with dioxane. The closed tubes were then heated in an oil bath at 100°C for 4 h, with occasional gentle shaking. After cooling in ice water, 0.2 M sodium bicarbonate (1 mL) was added to each tube to quench the excess boron trifluoride diethyl etherate. Then, HCl (6 M) (0.1 mL) was added to ensure that the pH of the reaction mixture was < 3, before addition of dichloromethane (2 mL) extraction solvent and mixing the tube. Most of the lower organic phase was removed with a Pasteur pipette and dried over anhydrous sodium sulfate. The sample was finally redissolved in dichloromethane (0.2 mL) and an aliquot (20 µL) of this was trimethylsilylated (TMS) with BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide/pyridine mixture (10/1, v/v) (40 µL) for a few minutes at room temperature. The TMS sample was injected (1 µL) onto a gas chromatograph (Varian 4000, Varian, Les Ulis, France) fitted with an autosampler, a splitless injector (280°C), and an ion trap mass spectrometer operating in the electron impact mode with the ion source at 220°C, the interface at 280°C, and a 50-650 m/z scanning range. The column was a VF-1 ms 15 m × 0.25 mm i.d. dimethylpolysiloxane capillary column (0.25 µm film thickness) (Agilent Technologies, Les Ulis, France) operated in the temperature program mode (from 45 to 180°C at +30°C/min, then 180 to 260°C at +3°C/min), with helium carrier gas at a flow rate of 1.5 mL/min. The derivatized monomers were quantified using reconstructed ion chromatograms at m/z 239 and 269 for the H- and G-units, respectively, with the base peak of their mass spectra corresponding to the benzyl ion. These chromatograms were compared with the internal standard hydrocarbons using a reconstructed ion chromatogram at m/z (57+71+85) (Méchin et al., 2014). The significance of the effects of the different wood types and trees on differences in the percentage of lignin H-units were investigated by the two-way analysis of variance (ANOVA) using R software (version 3.0.1) (R Core Team, 2013).
3.3.7 Raman micro-spectroscopy

Raman spectra were recorded using a Renishaw System1000 Raman microprobe (Renishaw plc, Wotton-under-Edge, Glos, UK) with a Leica microscope (Wetzlar, Germany) attachment, a thermoelectrically cooled (TEC) charge-coupled device (CCD) detector and a 1200 g/mm grating. Excitation radiation was provided by a Renishaw solid-state diode laser emitting a line in the near infrared region at 785 nm at 25 mW, with the integration time 60 s and the slit width 50 µm. A holographic notch filter was used to filter out the Rayleigh scattered light. The instrument was controlled by the WiRE (Windows-based Raman Environment) software and GRAM S32 (PANalytical, Boulder, CO, USA) data analysis software. Samples were analysed as discs (0.5 mm diameter, 60 µm thick, in triplicate) cut from the four wood types for Trees, 1, 2 and 3. Each disc was placed on a glass microscope slide covered with aluminium foil to avoid interference bands from the glass. A ×50 objective lens (numerical aperture. 0.85) was used which focused the laser to a line width of 10 x 2 µm. Spectra were collected between 3500-250 cm⁻¹ as shown in Figure 3-1a from transverse sections of double walls of adjacent tracheids in eight randomly selected regions in each of the discs (i.e. a total of 24 spectra for each wood type). Spectra were baseline-corrected and smoothed using the OMNIC spectroscopic software (version 7.3, Thermo Fisher Scientific). The spectra were then area-normalized, and the eight spectra obtained from each disc were averaged and used for PCA analysis with the Unscrambler® X software (version 10.3; CAMO Software AS, Oslo, Norway).
3.3.8 FTIR micro-spectroscopy

The IR spectra were recorded in transmission mode using a Thermo Electron Nicolet™ 8700 FTIR spectrometer (Thermo Fisher Scientific, Waltham, MA., USA) equipped with a Continuum™ FTIR microscope. Discs (0.5 mm diameter, 60 µm thick, in triplicate) of the different wood types were analysed. A diamond micro-compression cell (Thermo Fisher Scientific) was used to compress and flatten the discs to uniform thickness. FTIR spectra were collected from areas of 100 x 100 µm, each containing cross sections of ~10 tracheids (Figure 3-1b). Three spectra were collected from each disc at different locations. Each
spectrum was an average of 128 scans over the range 4000-649 cm\(^{-1}\) at a spectral resolution of 4 cm\(^{-1}\), using the OMNIC software (as above). The spectra were truncated to the finger print region (1850-690 cm\(^{-1}\)), and baseline corrected using the OMNIC software. They were then normalized to the same height for the CH\(_3\) deformation band at 1374 cm\(^{-1}\). The triplicate-averaged spectra from each disc were then subjected to PCA analysis using the Unscrambler® X software (as above) excluding the saturated region between 1189 and 941 cm\(^{-1}\) and the region of absorbed water between 1681 and 1529 cm\(^{-1}\) (Marchessault, 1962).

### 3.4 Results

#### 3.4.1 Fluorescence colour of cell-wall staining with acridine orange (AO) depends on pH and lignin concentration

The fluorescence colour of SCW tracheid walls after staining with AO varied depending on the pH of the dye solution. Moreover, at some pH values, different regions of these walls gave different fluorescence colours, i.e. the staining was metachromatic (Figure 3-2). The fluorescence staining colour of a wall region can be related to its lignin concentration as previously investigated with similar walls using lignin autofluorescence (Zhang et al., 2016). Autofluorescence indicated that in SCW tracheids, the outer region of the S2 wall layer (S2L) was highly lignified, whereas the inner S2 region was less lignified, with the S1 layer and CML having low lignification. AO staining of the highly lignified outer S2L region was most clearly differentiated from the inner S2 region and the S1 layer at pHs 6 and 7. At these pHs, the S2L region was stained orange, the less lignified inner S2 region, orange/yellow, and the even less lignified S1 layer, yellow. At pH 8, the colours were deeper, but the differentiation between the regions and layers was less clear. At pHs 4 and 5, the fluorescence colour was yellow/green, with even less differentiation. At pHs 2 and 3, the fluorescence colour was green with no differentiation. The non-lignified walls of the cambium and early tracheid derivatives showed a green fluorescence at all pHs. Control experiments in which similar unstained sections were examined and photographed, at each pH value, using the same exposure times to the stained sections, showed no or only very weak (pH 4 and 5) autofluorescence (Figure 3-2). Thus, staining with AO at pHs 6 or 7 is metachromatic, and can be used to differentiate wall regions of different lignin concentrations, as well as well as non-lignified walls.
Figure 3-2. Fluorescence micrographs of resin sections (500 nm thick) of SCW tracheids of Tree 4 stained with AO at different pHs. As the pH increased from 2 to 8, the SCW tracheid walls stained differently. At pHs 2 and 3, the fluorescence colour is green with no differentiation between wall regions. At pH 4 and 5, the fluorescence colour is yellow/green, with little differentiation between regions. At pHs 6 and 7, there is clear differentiation between the highly lignified, orange S2L region, the less lignified, orange/yellow, inner S2 region, and the even less lignified, yellow S1 layer. At pH 8, the colours are deeper, but the differentiation between the regions and layers is less clear. Control experiments in which similar unstained sections were examined and photographed, at each pH value, show no or only very weak autofluorescence (pHs 4 and 5) at the same exposure times to the stained sections. Scale bar = 20 µm.

3.4.2 Acridine orange (AO) staining can be used to determine wood types

Fluorescence staining of lignified walls with AO can be used as an alternative to using lignin autofluorescence to determine lignin distribution in tracheid walls and hence wood types, as shown in Table 3-4. Using AO has the advantage of giving a brighter fluorescence image and colour differences can be used to distinguish regions of different lignin concentrations. Fluorescence micrographs of resin sections of the three grades of CWs (SCW, MCW2 and MCW1) and OW stained with AO are shown in Figure 3-3 and Figure 3-4. Staining colour differentiation of regions within tracheid walls was most clearly shown at pH 7 (Figure 3-3), and slightly less clearly at pH 6 (Figure 3-4). In the walls of the OW tracheids (Figure 3-3a), the CML, including the CML at the cell corners (MLCC), was stained orange, indicating that it was highly lignified. The S1 and S3 layers were stained orange/yellow, indicating they were less lignified than the CML, and the S2 layer was stained yellow, indicating an even lower lignin concentration. In the walls of the MCW1 tracheids, the orange fluorescence was present in the S2L region at the cell corners (Figure 3-3b). However, in the walls of the MCW2 tracheids, this orange fluorescence of the S2L layer at the cell corners was more evident and this layer was also present around the cells (Figure 3-3c). In the walls of the SCW tracheids, the S2L region stained orange (as described above) (Figure 3-3d). The pit membranes (PM) in the bordered pits in all of the wood types fluoresced green, which indicated they were non-lignified.
Table 3-1. Lignin distribution in the walls of the tracheids of the three grades of compression woods (CW) and opposite wood (OW) determined by lignin autofluorescence and fluorescence colour after staining with acridine orange (AO) at pH 7, and the presence or absence of intercellular spaces.

<table>
<thead>
<tr>
<th>Characters</th>
<th>Opposite Wood (OW)</th>
<th>Mild Compression Wood 1 (MCW1)</th>
<th>Mild Compression Wood 2 (MCW2)</th>
<th>Severe Compression Wood (SCW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lignification of compound middle lamella at cell corners</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>NA(^1)</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>O/Y</td>
<td>Y</td>
<td>NA(^1)</td>
</tr>
<tr>
<td>Lignification of S2L at cell corners</td>
<td>NA(^2)</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>NA(^2)</td>
<td>Y</td>
<td>O/Y</td>
<td>O</td>
</tr>
<tr>
<td>Lignification of S2L around cells</td>
<td>NA(^2)</td>
<td>NA(^2)</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>NA(^2)</td>
<td>NA(^2)</td>
<td>O/Y</td>
<td>O</td>
</tr>
<tr>
<td>Intercellular spaces</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

-\(^{\text{-}}\), intercellular spaces absent; \(+\), weak lignin autofluorescence; \(\text{+}+\), moderate lignin autofluorescence / moderately frequent intercellular spaces; \(\text{+++}\), strong lignin autofluorescence / numerous intercellular spaces.

Y, yellow; O/Y, orange/yellow; O, orange.

NA\(^1\), not applicable as no CML at cell corner; there is an intercellular space.

NA\(^2\), not applicable as no S2L region in these walls.
Figure 3-3. Confocal laser scanning micrographs of resin sections (500 nm thick) of the four wood types of Tree 1 after staining with AO at pH 7. (a) In the walls of the OW tracheids, the highly lignified CMLs, including the CMLs at the cell corners (MLCCs), are stained orange, the less lignified S1 and S3 layers are stained orange/yellow and the even less lignified S2 layers are stained yellow. (b) In the walls of the MCW1 tracheids, the orange fluorescence is present in the S2L region at the cell corners. (c) In the tracheid walls of MCW2, orange fluorescence of the S2L region at the cell corners is more evident and this region is also present around the cells. (d) In the tracheid walls of SCW, the highly lignified S2L layers around the whole tracheid are stained orange. The less lignified S2i regions are stained orange/yellow and the even less lignified S1 layers are stained yellow. The helical cavities (HC) are present only in SCW tracheid walls. Intercellular spaces (IS) occur only in MCW2 and SCW. Pit membranes (PM) are stained green indicating they are non-lignified. IS, intercellular space. MLCC, middle lamella at the cell corner. Scale bar = 20 μm.
Figure 3-4. Confocal laser scanning micrographs of resin sections (500 nm thick) of the tracheid walls of the four wood types (Tree 1) after staining with AO at pH 6. Fluorescence staining is similar to that at pH 7 (Figure 3-3), but with less colour differentiation between the wall regions. The most lignified regions are stained orange, and the least lignified regions yellow. CML, compound middle lamella; MLCC, middle lamella at the cell corner. Scale bar = 20 µm.

Additionally, AO staining (at pHs 7 and 6) of unembedded sections (60 µm) of the four wood types showed similar results, but with less resolution as would be expected for thicker sections (Figure 3-5 and Figure 3-6).
Figure 3-5. Confocal laser scanning micrographs of sections (60 µm thick) of the tracheid walls of the four wood types of Tree 1 after staining with AO at pH 7. Lignified wall regions are stained orange to yellow as Figure 3-3. The most lignified regions are stained orange, and the least lignified regions yellow. MLCC, middle lamella at the cell corner. Scale bar = 20 µm.
Figure 3-6. Confocal laser scanning microscopy micrographs of sections (60 µm thick) of the tracheid walls of the four wood types of Tree 1 after staining with AO at pH 6. Fluorescence staining is similar to that at pH 7 (Figure 3-5). The highly lignified regions are stained orange, and the least lignified regions yellow. MLCC, middle lamella at the cell corner. Scale bar = 20 µm.

3.4.3 Lignin content of the tracheid walls increases with CW severity

The content of acetyl bromide soluble lignin (ABSL) of the four wood types was lowest for OW (~26%) and highest for SCW (~35%), with the two MCW grades intermediate (MCW1 ~28% and MCW2 ~32%) (Table 3-2). A two-way analysis of variance (ANOVA) was carried out on the lignin content of the four wood types and in the three trees. Significant differences were found in the lignin contents between the four wood types (P = 2 × 10^{-16}), but there were
no detectable differences in the lignin content between the different trees (P = 0.65). An interaction plot (Figure 3-7) shows the relationship between the lignin contents of the four wood types in the three trees. Greater variation in lignin content was related to CW severity and the differences in lignin content that followed CW severity were similar in the three trees.

Table 3-2. Acetyl bromide soluble lignin (ABSL) content of the four wood types from three *P. radiata* trees.

<table>
<thead>
<tr>
<th>Tree</th>
<th>Opposite Wood (OW)</th>
<th>Mild Compression Wood 1 (MCW1)</th>
<th>Mild Compression Wood 2 (MCW2)</th>
<th>Severe Compression Wood (SCW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tree 1</td>
<td>25.9 ± 0.3</td>
<td>28.8 ± 0.03</td>
<td>31.7 ± 0.3</td>
<td>35.1 ± 0.6</td>
</tr>
<tr>
<td>Tree 2</td>
<td>26.1 ± 0.3</td>
<td>28.3 ± 0.07</td>
<td>31.3 ± 0.5</td>
<td>35.0 ± 0.2</td>
</tr>
<tr>
<td>Tree 3</td>
<td>26.4 ± 0.08</td>
<td>28.1 ± 0.5</td>
<td>32.0 ± 0.4</td>
<td>34.5 ± 0.05</td>
</tr>
</tbody>
</table>

| Mean   | 26.1 ± 0.1          | 28.4 ± 0.2                     | 31.7 ± 0.2                     | 34.9 ± 0.2                    |

Expressed as mean % oven-dry weight of wood ± standard error.
3.4.4 Thioacidolysis shows the proportion of H-units in lignin increases with CW severity

During thioacidolysis of lignin, the β-O-4 bonds are cleaved and thioethylated monomers released as a ~50/50 erythro/threo mixture of diastereoisomers (Rolando et al., 1992; Lapierre, 2010). The partial reconstructed GC-MS ion chromatograms at m/z (239+269) (Figure 3-8) show the separation and relative proportions of the thioethylated monomers derived from the two lignin monomers (H- and G-units) in the four wood types. The relative molar percentages of these lignin H- and G-units are shown in Table 3-3. OW lignin contained almost all G-units, with only extremely small proportions of H-units (< 1%).
Interestingly, the milder of the two MCWs, MCW1, had lignin with the proportion of H-units ~12 times higher than in OW lignin, although the lignin content was only ~2.3% higher than in OW (Table 3-2). The proportion of H-units was even higher in MCW2 lignin and highest in SCW lignin, ~23 times that of OW lignin. A two-way analysis of variance (ANOVA) was done on the percentages of lignin H-units between the four wood types and the two trees (Trees 1 and 2). There were significant differences in the proportions of lignin H-units in the four wood types ($P = 1.10 \times 10^{-12}$), but there was no detectable difference in the proportions between the trees ($P = 0.403$).

![Diagram showing partial GC-MS reconstructed ion chromatograms at m/z (239+269) to show the separation of the thioethylated monomers derived from lignin H- and G-units and their relative proportions in the four wood types (Tree 1). Red trace: SCW; brown trace: MCW2; orange trace: MCW1; green trace: OW. Part of the OW chromatogram is enlarged (see insert) to show more clearly the very small peak given by the thioethylated monomer derived from the lignin H-units.](image)

Figure 3-8. Partial GC-MS reconstructed ion chromatograms at m/z (239+269) to show the separation of the thioethylated monomers derived from lignin H- and G-units and their relative proportions in the four wood types (Tree 1). Red trace: SCW; brown trace: MCW2; orange trace: MCW1; green trace: OW. Part of the OW chromatogram is enlarged (see insert) to show more clearly the very small peak given by the thioethylated monomer derived from the lignin H-units.
Table 3-3. The relative molar percentages (± standard error) of the thioethylated monomers derived from lignin H- and G-units, and the H/G molar ratio (± standard error) of Trees 1 and 2.

<table>
<thead>
<tr>
<th>Wood type</th>
<th>Tree</th>
<th>% H-units</th>
<th>% G-units</th>
<th>H/G ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe Compression</td>
<td>1</td>
<td>13.3 ± 0.77</td>
<td>86.7 ± 0.77</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>Wood (SCW)</td>
<td>2</td>
<td>14.2 ± 0.83</td>
<td>85.8 ± 0.83</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>13.8 ± 0.65</td>
<td>86.2 ± 0.65</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>Mild Compression</td>
<td>1</td>
<td>11.3 ± 0.38</td>
<td>88.7 ± 0.38</td>
<td>0.13 ± 0.006</td>
</tr>
<tr>
<td>Wood 2 (MCW2)</td>
<td>2</td>
<td>10.7 ± 0.34</td>
<td>89.3 ± 0.34</td>
<td>0.12 ± 0.005</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>11.0 ± 0.31</td>
<td>89.0 ± 0.31</td>
<td>0.12 ± 0.006</td>
</tr>
<tr>
<td>Mild Compression</td>
<td>1</td>
<td>8.2 ± 0.25</td>
<td>91.8 ± 0.25</td>
<td>0.089 ± 0.004</td>
</tr>
<tr>
<td>Wood 1 (MCW1)</td>
<td>2</td>
<td>6.3 ± 0.48</td>
<td>93.9 ± 0.048</td>
<td>0.067 ± 0.007</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>7.2 ± 0.51</td>
<td>92.8 ± 0.51</td>
<td>0.078 ± 0.008</td>
</tr>
<tr>
<td>Opposite Wood</td>
<td>1</td>
<td>0.6 ± 0.03</td>
<td>99.4 ± 0.03</td>
<td>0.0057 ± 0.00033</td>
</tr>
<tr>
<td>(OW)</td>
<td>2</td>
<td>0.7 ± 0.01</td>
<td>99.3 ± 0.01</td>
<td>0.0067 ± 0.00033</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>0.6 ± 0.026</td>
<td>99.4 ± 0.026</td>
<td>0.0062 ± 0.00031</td>
</tr>
</tbody>
</table>

When the ratio of H- and G-units was plotted against the averaged lignin contents of all wood types of both Trees 1 and 2, it showed a strong linear correlation ($R^2 = 0.95$) (Figure 3-9a).
3.4.5 Raman micro-spectroscopy differentiates the four wood types

Raman spectra of the four wood types are shown in Figure 3-10a. The intensities of the band at 1600 cm\(^{-1}\), attributed to lignin (Table 3-4) increased in the order OW (the lowest), MCW 1, MCW 2 and SCW (the highest). This is consistent with an increase in lignin content of the tracheid cell walls with the increasing CW severity. Smaller differences occurred in other bands between the four wood types, particularly in the region below 1400 cm\(^{-1}\).

To obtain more information about compositional differences between the four wood types, digital subtraction spectra were obtained by subtracting the spectrum of OW from the spectrum of each of the CW types. The resulting subtraction spectra in Figure 3-11 (a-c)
reflected the compositional differences between each of the CW severities and the OW. The most obvious difference was the lignin band at 1600 cm$^{-1}$, with the highest intensity in the SCW-OW spectrum, and the lowest in the MCW 1-OW spectrum, indicating that the lignin content in the CW increased with CW severity. There were also two smaller positive bands, 1256 and 825 cm$^{-1}$ that were most pronounced in the SCW-OW spectrum. These were assigned to H-units of lignin (Table 3-4), indicating that all three CW severities contained higher proportions of H-units in their lignin compared with OW. The decrease in intensities of these two bands in Figure 3-11b and 3-11c indicated that the proportions of H-units in the CW lignin increased with CW severity. In addition, there were three negative bands, 1377, 1096 and 380 cm$^{-1}$, the first two were assigned to cellulose, heteromannans or heteroxylans, and the last to cellulose (Table 3-4).
Figure 3-10. (a) Baseline-corrected and area-normalized Raman spectra of the four wood types of Tree 1 (light blue, SCW; green, MCW 2; red, MCW 1; dark blue, OW). The spectrum of each wood type was obtained by averaging the 24 spectra obtained from triplicate wood discs of Tree 1 (8 spectra from each disc). The intensities of the 1600 cm⁻¹ lignin band are in the order OW (lowest) to SCW (highest) (see insert). (b) The score plots of PC1 and PC2. (c) PC 2 and PC 3 from PCA of triplicate-averaged spectra of three discs from each of the four wood types of Trees 1, 2, and 3. The four wood types cluster into four
separate groups along PC2. (d) The PC2 loadings with wave numbers of bands that characterize the chemical differences between the four wood types.

Table 3-4. Assignment of the main bands in PC2 loadings of PCA analysis of Raman spectra.

<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>Band Origin</th>
<th>Possible P. radiata wood components</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2887</td>
<td>CH and CH(_2) stretching</td>
<td>Cellulose, heteromannans, heteroxylans, lignin</td>
<td>Blackwell, 1977; Agarwal and Ralph, 1997; Edwards et al., 1997; Kačuráková et al., 1999</td>
</tr>
<tr>
<td>1600</td>
<td>Aromatic ring stretch, symmetric</td>
<td>Lignin</td>
<td>Agarwal and Ralph, 1997; Edwards et al., 1997; Agarwal et al., 2011</td>
</tr>
<tr>
<td>1479</td>
<td>CH(_2) scissors</td>
<td>Cellulose, heteromannans, heteroxylans</td>
<td>Blackwell, 1977; Agarwal and Ralph, 1997; Edwards et al., 1997</td>
</tr>
<tr>
<td>1413</td>
<td>CH(_2) bending</td>
<td>Cellulose, heteromannans, heteroxylans</td>
<td>Blackwell, 1977; Agarwal and Ralph, 1997; Edwards et al., 1997</td>
</tr>
<tr>
<td>1377</td>
<td>CH(_2) bending</td>
<td>Cellulose, heteromannans, heteroxylans</td>
<td>Blackwell, 1977; Agarwal and Ralph, 1997; Edwards et al., 1997</td>
</tr>
<tr>
<td>1256</td>
<td>Car–O stretching</td>
<td>Lignin H-units</td>
<td>Larsen and Barsberg, 2010</td>
</tr>
<tr>
<td>1241 (weak shoulder)</td>
<td>Car–O stretching and ring deformations</td>
<td>Lignin G-units</td>
<td>Larsen and Barsberg, 2010</td>
</tr>
<tr>
<td>1214</td>
<td>ring deformation, Car–C</td>
<td>Lignin H-units</td>
<td>Larsen and Barsberg, 2010</td>
</tr>
<tr>
<td>1173</td>
<td>Car–OH in-plane binding</td>
<td>Lignin H-units</td>
<td>Saariaho et al., 2003; 2005; Larsen and Barsberg, 2010</td>
</tr>
<tr>
<td>Line</td>
<td>Description</td>
<td>Compound</td>
<td>Reference(s)</td>
</tr>
<tr>
<td>------</td>
<td>--------------------------------------------------</td>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>1155</td>
<td>C–C ring breathing, asymmetric</td>
<td>Cellulose</td>
<td>Blackwell, 1977; Agarwal and Ralph, 1997; Gierlinger et al., 2008</td>
</tr>
<tr>
<td>1136</td>
<td>CH bending</td>
<td>Lignin</td>
<td>Agarwal et al., 2011</td>
</tr>
<tr>
<td>1122</td>
<td>COC glycosidic, symmetric</td>
<td>Cellulose/heteromannans/heteroxylans</td>
<td>Blackwell, 1977; Agarwal and Ralph, 1997; Gierlinger et al., 2008</td>
</tr>
<tr>
<td>1096</td>
<td>COC glycosidic, asymmetric</td>
<td>Cellulose/heteromannans/heteroxylans</td>
<td>Blackwell, 1977; Agarwal and Ralph, 1997; Gierlinger et al., 2008</td>
</tr>
<tr>
<td>936</td>
<td>CCH wagging</td>
<td>Lignin G-units</td>
<td>Saariaho et al., 2003; Agarwal, 1999</td>
</tr>
<tr>
<td>859</td>
<td>Aromatic breathing mode</td>
<td>Lignin H-units</td>
<td>Saariaho et al., 2003; Larsen and Barsberg, 2010</td>
</tr>
<tr>
<td>825</td>
<td>C_{ar–H2}, C_{ar–H3} out-of-plane bending</td>
<td>Lignin H-units</td>
<td>Saariaho et al., 2003; Larsen and Barsberg, 2010</td>
</tr>
<tr>
<td>639</td>
<td>Ring deformation</td>
<td>Lignin H-units</td>
<td>Saariaho et al., 2003; 2005; Larsen and Barsberg, 2010</td>
</tr>
<tr>
<td>434</td>
<td>CCO ring</td>
<td>Cellulose</td>
<td>Agarwal and Ralph, 1997; Edwards et al., 1997</td>
</tr>
<tr>
<td>380</td>
<td>CCC ring</td>
<td>Cellulose</td>
<td>Edwards et al., 1997; Gierlinger et al., 2008</td>
</tr>
<tr>
<td>330</td>
<td>CCC ring, twisting</td>
<td>Cellulose</td>
<td>Agarwal and Ralph, 1997; Edwards et al., 1997</td>
</tr>
</tbody>
</table>
Figure 3-11. Digital subtraction spectra obtained by subtracting the averaged Raman spectra of each of the four wood types in Trees 1, 2 and 3 (72 spectra were averaged for each wood type). (a) SCW minus OW, (b) MCW 2 minus OW, and (c) MCW 1 minus OW. The positive bands (greater in CWs) indicate the higher proportions of lignin in CWs. The bands
at 1256 and 825 cm$^{-1}$ were assigned to lignin H-units, indicating higher proportion of lignin H-units in the CWs; these two bands are obvious in the subtracted spectra, MCW 2 minus OW (b) and SCW minus OW (c).

PCA was performed to extract additional information from the complex original spectra about compositional differences between the four wood types. The PCA score plots of principal components (PC) 1 and 2 (Figure 3-10b), and PC 2 and PC 3 (Figure 3-10c) showed the four wood types clustered into four distinct groups. Although PC1 accounted for 66% of the variance, it appeared to reflect only the variability within each wood type, even though there were no detectable differences between the trees. The OW and three grades of CWs were well differentiated by PC 2 (accounting for 20% of the variance) and the wood types were separated according to CV severity along this PC, with SCW (light blue) at the high positive end of PC2 and OW (dark blue) at the high negative end of PC2 (Figure 3-10b). The separation along PC2 is better shown in the PC3 vs PC2 score plot (Figure 3-10c). The PC2 loadings (Figure 3-10d, Table 3-4) showed the sources of the variance between the four wood types. The positive loadings correspond to the bands for lignin (Table 3-4). The 1600 cm$^{-1}$ lignin band had the highest loading on PC2 and was the basis for differentiating between the four wood types. This reinforced the observation in the subtracted spectra that the SCW contained the highest content of lignin and that the lignin content decreased with decreasing CW severity. The high negative loadings on PC2 corresponded with bands for cellulose, including those at 1155, 434, 380 and 330 cm$^{-1}$ (Table 3-4). The bands at 1479, 1413, 1377, 1122 and 1096 cm$^{-1}$ were assigned to either cellulose, heteromannans or heteroxylans (Table 3-4). These observations are consistent with OW containing the highest proportions of cellulose, heteromannans and heteroxylans, and indicated that the content of these polysaccharides decreased with increasing CW severity. Another major difference evident between the four wood types related to lignin structure. Several positive bands in the PC2 loadings were assigned to H-units of lignin: 1256, 1214, 1173, 859, 825 and 639 cm$^{-1}$ (Table 3-4). This indicated that the lignin of CWs was enriched in H-units, and the proportion of these was highest in SCW. Two other positive bands in the PC2 loadings were assigned to G-units of lignin: the weak shoulder at 1241 and 936 cm$^{-1}$ (Table 3-4).
3.4.6 FTIR micro-spectroscopy differentiates the four wood types

FTIR micro-spectroscopy was used to complement those vibrational modes of CW and OW that appear weakly in the Raman spectra. The finger print region (1850-690 cm\(^{-1}\)) of the spectra of the four wood types were similar and are shown in Figure 3-12a, after baseline correction and the intensities normalized to the 1374 cm\(^{-1}\) methyl deformation band. Assignment of the bands are given in Table 3-5. Bands in the 1189-941 cm\(^{-1}\) region were not able to be resolved as they were saturated, despite the use of a micro-compression cell in an attempt to reduce the thickness of the sample. The intensities of the band at 1513 cm\(^{-1}\), assigned to lignin (Table 3-5), varied with the order OW (the lowest), MCW 1, MCW 2 and SCW (the highest), reflecting the corresponding increase in lignin contents of these wood types.
Figure 3-12. (a) FTIR spectra of the four wood types of Tree 1 after baseline correction and normalization to the CH$_3$ deformation band at 1374 cm$^{-1}$, and averaging (9 spectra averaged for each wood type). The spectra of the four wood types appear similar, except the band at 1513 cm$^{-1}$. The intensity of this band increases from OW to SCW. (b) The score plots of PC1 and PC2, and (c) PC 2 and PC 3 obtained by PCA of triplicate-averaged spectra from three
discs of each of the four wood types of Trees 1 to 3. The four wood types are separated into four clusters on PC2. (d) The PC2 loadings showing the chemical differences between the four wood types.

Table 3-5. Assignment of the main bands in PC2 loadings of PCA analysis of FTIR spectra.

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Band Origin</th>
<th>Possible P. radiata wood components</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1734</td>
<td>C = O stretching of acetyl groups and carboxylic acids</td>
<td>Acetyl groups on heteromannans; glucuronic acid of heteroxylans</td>
<td>Marchessault, 1962; Harrington et al., 1964; Simonović et al., 2011</td>
</tr>
<tr>
<td>1513</td>
<td>Benzene ring stretching vibration in lignin</td>
<td>Lignin</td>
<td>Marchessault, 1962; Harrington et al., 1964; Faix 1991</td>
</tr>
<tr>
<td>1455</td>
<td>CH₂ deformation vibration and benzene vibration</td>
<td>Lignin, heteroxylans</td>
<td>Marchessault, 1962; Harrington et al., 1964</td>
</tr>
<tr>
<td>1421</td>
<td>CH₃ bending vibration in lignin, CH₂ scissoring vibration in cellulose</td>
<td>Lignin, cellulose</td>
<td>Harrington et al., 1964; Blackwell, 1977</td>
</tr>
<tr>
<td>1384</td>
<td>CH and OH bending vibration</td>
<td>Cellulose</td>
<td>Marchessault, 1962; Harrington et al., 1964; Åkerholm and Salmén, 2001</td>
</tr>
<tr>
<td>1353</td>
<td>CCH deformation</td>
<td>Cellulose</td>
<td>Blackwell, 1977; Åkerholm and Salmén, 2001</td>
</tr>
<tr>
<td>1328</td>
<td>OH in-plane bending</td>
<td>Cellulose</td>
<td>Marchessault, 1962; Åkerholm and Salmén, 2001</td>
</tr>
<tr>
<td>1311</td>
<td>CH₂ wagging</td>
<td>Cellulose</td>
<td>Marchessault, 1962; Harrington et al., 1964; Blackwell, 1977; Åkerholm and Salmén, 2001</td>
</tr>
<tr>
<td>1268</td>
<td>C = O stretching vibration in lignin</td>
<td>Lignin</td>
<td>Marchessault, 1962; Harrington et al., 1964</td>
</tr>
</tbody>
</table>
For PCA of the spectra, the saturated region at 1189-941 cm\(^{-1}\) and the absorbed water region at 1681-1529 cm\(^{-1}\) were excluded. The PCA score plots of PC1 and PC2 (Figure 3-12b), and PC2 and PC3 (Figure 3-12c) showed that the four wood types from the three trees were clustered into four separate groups. PC 1 accounted for 45% of the variance, which reflected only the variability within each wood type, as also found in the PCA of the Raman spectra. The four wood types were differentiated in the expected order by PC 2 (SCW, MCW2, MCW1 and OW), which explained 16% of the variance in the data. The high PC 2 loadings (Figure 3-12d) represented the bands of the major components in the spectra that were the dominant factors determining the compositional differences between the four wood types. The high positive loadings at 1513, 1268 and 1224 cm\(^{-1}\) were for bands assigned to lignin (Table 3-5). This was consistent with SCW having the highest lignin content, which decreased in the order SCW, MCW 2, MCW 1 and OW. The positive loading at 1455 cm\(^{-1}\) was assigned to both lignin and heteroxylans, and the positive band at 1421 cm\(^{-1}\) was
assigned to both lignin and cellulose (Table 3-5). The positive loading at 838 cm\(^{-1}\) was assigned to H-units of lignin (Table 3-5), and was consistent with the higher proportions of H-units in the lignins of CWs, with SCW containing the highest proportion. The highest negative loading at 1734 cm\(^{-1}\) was assigned to the acetyl groups on heteromannans and the glucuronic acid residues of heteroxylans (Table 3-5). The strong negative loading at 904 cm\(^{-1}\) was assigned to the vibration of the \(\beta\)-glycosidic linkages between the monosaccharide residues of \(\beta\)-linked polysaccharides (Table 3-5); however, this band has been reported to be very weak for cellulose (Blackwell, 1977). The two negative loadings at 869 and 804 cm\(^{-1}\) were assigned to the mannosyl residues in the heteromannans (Table 3-5). These negative loadings were consistent with heteromannans and heteroxylans being present in the highest proportions in OW and in decreasing proportions with increasing CW severity. High negative loadings were also associated with cellulose bands, including 1384, 1353, 1328, 1311 and 717 cm\(^{-1}\), which indicated that cellulose was present in the highest proportion in OW and decreased with increasing CW severity. The loading at 717 cm\(^{-1}\) may correspond to the cellulose I\(\beta\) allomorph (Table 3-5) (Sugiyama et al., 1991; Kataoka and Kondo, 1999), suggesting that OW contained a high proportion of cellulose I\(\beta\) that decreased with increasing CW severity.

3.5 Discussion

The four wood types OW, MCW1, MCW2 and SCW used in the present study were initially identified based on the distribution of lignin in the tracheid walls determined with fluorescence microscopy using lignin autofluorescence (Zhang et al., 2016). In the present study, it has been shown that the distribution of lignin in the tracheid cell walls of these wood types can also be quickly and easily shown by staining with a solution of AO buffered at pH 6 or 7. Compared with lignin autofluorescence, AO stained sections gave a much brighter fluorescence. In addition, at the AO concentration used (0.02%) (660 µM) and at these pHs, there was metachromatic staining, with the fluorescence colours differentiating regions of varying lignin concentrations (orange, orange/yellow, yellow) in the tracheid walls, as well as walls with no or low concentration of lignin (green). Similar fluorescence staining of tracheid walls to that found in the present study have been reported for sections of white spruce (\textit{Picea glauca}) stained with AO at pH 7 at a concentration of 50 µM (Houtman et al., 2016). As well as autofluorescence, other techniques that do not rely on staining have previously been used
to determine the distribution of lignin in tracheids of normal and SCWs of conifers and the results are all consistent with the present results. These techniques include UV microscopy (Wardrop and Davies, 1964; Wood and Goring, 1971) and interference microscopy (Donaldson, 1985; Donaldson et al., 1999). Nevertheless, details of the binding of AO to cell-wall components and the causes of the metachromatic staining are complex and not fully resolved. At low concentrations (1 µM or less) AO occurs only as the monomer, which fluoresces green (Li and Reeve, 2004), but at higher concentrations (5 µM and greater) forms dimers as well as oligomers, which fluoresce red (Liu et al., 1999; Houtman et al., 2016). Only one colour (orthochromatic) (green) staining occurs at the low AO concentrations, where the AO exists only as the monomer, whereas metachromatic staining occurs at the higher AO concentrations. In addition to staining lignified walls AO, at a concentration of 0.02% and unbuffered, has been used to stain the non-lignified, pectin-rich walls of apple (Malus domestica), kiwifruit (Actinidia deliciosa) and tomato (Lycopersicon esculentum), giving an intense green fluorescence (Redgwell et al., 2008); the AO was thought to bind to the pectin in the cell walls.

The lignin concentrations of the OW and SCW discs determined in the present study using the acetyl bromide assay are similar to the concentrations of these wood types determined by the Klason method in an earlier study using radiata pine saplings of approximately the same age (Brennan et al., 2012). As in the present study, this wood was corewood, often referred to as juvenile wood, which is formed in the first ten growth rings from the pith (Burdon, 2004). However, the acetyl bromide assay has the advantage that it was done on samples weighing only 2 mg, consisting of small discs (0.5 mm diameter and 60 µm thick) that were each quickly examined by fluorescence microscopy using lignin autofluorescence to ensure the lignin distribution in all the tracheids was consistent with the appropriate wood type. This would have been impossible with the weights of sample needed to determine the content of Klason lignin (100 mg or more). The lignin concentrations are also similar to those determined in a study of radiata pine wood, which included OW, SCW and one grade of MCW, of various ages and from branches as well as stems (Nanayakkara et al., 2009). As in the present study, they found the lignin content increased with CW severity, but the lignin content at which MCW first became evident using fluorescence microscopy was 31%, whereas the milder grade of MCW (MCW1) had a lignin content of ~28%.
In addition to an increase in lignin content with CW severity, an increase in the proportion of H-units in the lignin was found using thioacidolysis, with the H/G ratio showing a linear relationship with lignin concentration. A similar linear relationship has previously been reported (Nanayakkara et al., 2009). As OW contains only very small proportions of H-units (<1% of total H- and G-units), the presence of H-units can be used as a marker for CW, as well as its severity. The increase in lignin concentration and proportion of lignin H-units corresponds to a change in lignin distribution in tracheid cell walls, with increasing amounts of lignin being located in the S2L region. This suggests that the lignin in this layer is enriched in H-units, and evidence supporting this has been obtained by examining sections of CW from Norway spruce (*Picea abies*) using time-of-flight secondary ion mass spectrometry (Tokareva et al., 2007). The lignin composition in this layer is likely determined by a relatively greater flow through the pathway leading to *p*-coumaryl alcohol than coniferyl alcohol and the pattern of lignin deposition in the walls being determined by spatially localized peroxidases and/or laccases (Vanholme et al., 2010; Schuetz et al., 2014).

It has also been shown using immunomicroscopy that (1→4)-β-galactans occur as a band in the S2L region of CW with the band becoming wider with increasing CW severity (Zhang et al., 2016). Consistent with this, neutral monosaccharide compositions of the non-cellulosic polysaccharides of the tracheid walls of these different wood types showed increasing proportions of galactose as a percentage of the total neutral monosaccharides from OW, through MCW1 and MCW2 to SCW (Zhang et al., 2016). There is a straight line relationship between galactose and lignin percentages (Figure 3-9b). Lignin and (1→4)-β-galactans are thus co-located in the tracheid walls of CWs. Partial delignification of radiata pine SCW resulted in increased amounts of (1→4)-β-galactans that can extracted from the walls with 6 M sodium hydroxide, suggesting they are covalently linked to lignin (Chavan et al., 2015). Consistent with this is the report of a complex containing (1→4)-β-galactans and lignin being isolated from SCW of Japanese red pine (*Pinus densiflora*) (Watanabe et al., 1989). The cross links between lignin and (1→4)-β-galactans are probably formed during lignin synthesis by reactions involving quinone methide intermediates (Ralph et al., 2007).

In the present study, it has been shown that FTIR micro-spectroscopy in combination with PCA can be used to differentiate the four different wood types. However, transmission FTIR micro-spectroscopy is very sensitive to sample thickness, and even though a diamond micro-compression cell was used throughout this study to compress and flatten the 60 µm discs, this
was not able to prevent saturation of the strong absorbance between 1189 and 941 cm\(^{-1}\), so that this region was excluded from PCA. A study using FTIR micro-spectroscopy on beech (\textit{Fagus sylvatica}) wood found that a section thickness of 30 \(\mu\text{m}\) or less was required to avoid saturation (Naumann et al., 2005). In the present study, it was found that sections of this thickness were too fragile to handle. Another disadvantage of FTIR micro-spectroscopy was its sensitivity to water and the resulting need to exclude the absorbed water region (~1681 to 1529 cm\(^{-1}\)) from PCA. Nevertheless, FTIR micro-spectroscopy has been used in the reflective mode to scan increment cores of two species of spruce, Sitka (\textit{Picea sitchensis}) and Norway (\textit{P. abies}) (Altaner et al., 2009). Only poor quality spectra were obtained from the wood surfaces and to obtain better quality spectra sawdust was produced in a process that maintained spatial orientation. A CW severity indicator, which required spectra to be analysed individually, was calculated from the ratio of peaks in two spectral areas representing aromatic and carboxyl groups.

Clearer differentiation of the four wood types was achieved in the present study by using Raman micro-spectroscopy in combination with PCA and, with separation being based on lignin signals, is the preferred technique. In contrast to FTIR micro-spectroscopy, it is insensitive to the presence of water and to sample thickness. Further research using this technique is needed on other conifer species, on outerwood as well as corewood, and on trees grown in a range of locations. Using defined samples of OW and SCW, this technique has the potential to be used commercially to quickly identify and determine the severity of CWs from the PCA models. The recent advances that have been made in laser and spectrometer optics of Raman systems further make Raman micro-spectroscopy an attractive potential technique for the wood industry.

### 3.6 Conclusions

A solution of AO buffered at pH 6 or 7 can be used to stain sections of radiata pine wood to determine the distribution of lignin in the tracheid walls, and hence the severity of CW. The fluorescence is brighter than lignin autofluorescence and is metachromatic, with high concentrations of lignin stained orange, low concentrations stained yellow and walls with no lignin or only trace amount stained green. This method can be used more easily than lignin autofluorescence. However, it cannot of course be used if the discs are subsequently used in
analyses. Discs of the four wood types, OW, MCW1, MCW2 and SCW, characterized by autofluorescence, were analysed for lignin content and found have lignin contents that increased with CW severity. Analysis by thioacidolysis showed the lignin structure also altered with CW severity with OW containing G-units with only a trace of H-units to SCW which contained ~14% H-units. Interrogation of these discs using FTIR and Raman micro-spectroscopy in combination with PCA, showed that both techniques can be used to successfully differentiate the four wood types. Raman micro-spectroscopy afforded clearer differentiation and so was the preferred technique and shows promise to be used commercially to identify and determine the severity of CWs.
Chapter 4. Dimensional changes of tracheids during drying of radiata pine (*Pinus radiata* D. Don) compression woods: a study using variable-pressure scanning electron microscopy (VP-SEM)

4.1 Abstract

Variable-pressure scanning electron microscopy was used to investigate, on wetting and drying, the dimensional changes in longitudinal, tangential and radial directions, of tracheids of opposite wood (OW) and three grades of compression woods (CWs), including severe CW and two grades of mild compression wood (MCW) (MCW1 and MCW2) of radiata pine (*Pinus radiata*). The CW was formed on the underside and OW on the upperside of slightly tilted stems. In the longitudinal direction, the shrinkage of SCW tracheids was ~300% greater than that of OW tracheids, with the shrinkage of the MCW tracheids being intermediate. Longitudinal swelling was also investigated and hysteresis was demonstrated for the tracheids of all wood types, with the extent of hysteresis increasing with CW severity. A statistical association was found between longitudinal shrinkage and the content of lignin and galactosyl residues in the cell-wall matrix. The galactosyl residues are present mostly as (1→4)-β-D-galactans, which are known to have a high capacity for binding water and swell on hydration. The small proportions of (1→3)-β-D-glucans in the CWs have similar properties. These polysaccharides may play a functional role in the longitudinal shrinking and swelling of CW tracheids. Tangential shrinkage of tracheids was greater than radial shrinkage, but both were greatest for OW and least for SCW, with the MCWs being intermediate. Shrinkage of the radial and tangential walls appears to determine the shrinkage in the tangential and radial directions, respectively.

4.2 Introduction

In coniferous gymnosperms (softwoods), a type of wood known as compression wood (CW) is formed when stems are tilted from the vertical during growth as the result of various factors such as the weight of snow, prevailing winds and the slope of the land. CW forms on the lower side of the tilted stem to correct the growth to the vertical (Timell, 1986; Chauhan et al., 2006; Brennan et al., 2012). The wood type formed on the opposite side of the stem is known as opposite wood (OW), which is very similar in structure and composition to normal
wood (NW). Although the formation of CW is beneficial to the tree, CW is regarded as a defect in the timber industry. When wood is dried and rewetted, it shrinks and swells and this dimensional instability is a major obstacle in the use of wood, particularly for structural purposes (Spear and Walker, 2006). CW shows particularly high longitudinal shrinkage and swelling (Timell, 1986). Because of the differences in longitudinal shrinkage and swelling between CW and OW or NW, where these wood types occur adjacent to one another in timber, severe warping can occur with a loss of economic value (Spear and Walker, 2006). CW also shows differences from OW or NW in tangential and radial shrinkage, but the differences are much less pronounced than for longitudinal shrinkage (Timell, 1986).

The water in wood occurs in two locations: adsorbed within the cell walls, where it is known as bound water; and in the lumens of the component cells or in intercellular spaces, where it is known as free (or absorbed) water (Walker, 2006; Glass and Zelinka, 2010). During drying, wood shrinkage occurs only when all the free water has been removed, a point known as the fibre saturation point (Walker, 2006). Wood shrinkage and swelling depend on only the amount of bound water, and thus cell walls play a pivotal role in these processes. In softwoods, these cell walls are mostly of tracheids because softwoods are composed predominantly of this one cell type (Evert, 2006).

Tracheids are thick-walled, elongated cells. In CW, they are rounder, shorter and have thicker walls than in OW and NW. In contrast to OW and NW tracheids, which have three distinct secondary wall layers (S1, S2 and S3), CW tracheids have only two wall layers; the third (inner) layer (S3) is missing (Butterfield, 2006; Evert, 2006). As in all plant cell walls, these wall layers consist of a fibrillar phase in the form of cellulose microfibrils set in a matrix phase. The angle the cellulose microfibrils make to the vertical axis of the tracheid varies in each secondary wall layer. The angle of the cellulose microfibrils in the thickest of these layers, the S2 layer, is known as the cellulose microfibril angle (MFA). In the stems of Pinus radiata (radiata pine), two regions of wood are often recognized: corewood, which is formed in the first ten growth rings from the pith; and outerwood, which is formed further out (Burdon et al., 2004). The MFA of CW formed in the outerwood region, is higher than in NW or OW formed in the same region. This high MFA is thought to be related to the high longitudinal shrinkage of CW (Meylan, 1968). The MFA of CW formed in the corewood of radiata pine is also high (> ~35° from the long axis of the cell), but has been found to be either the same as or similar to that of NW and OW in this region (Donaldson et al., 2004;
Brennan et al. 2012). Thus, even though a high MFA may be necessary for high longitudinal shrinkage, it is not sufficient, and polymers in the cell-wall matrix are likely to play an important role (Yamamoto et al., 2001). Tracheid cell walls of CW have been shown to contain lower proportions of cellulose than the tracheid cell walls of NW and OW (Timell, 1986; Brennan et al. 2012).

The cell-wall matrix of NW and OW tracheids are composed of the non-cellulosic polysaccharides heteromannans (O-acetyl-galactoglucomannans) and smaller proportions of heteroxylans [arabino(4-O-methylglucurono)xylans], in addition to lignin (Harris and Stone, 2008; Brennan et al., 2012). In comparison, the cell-wall matrix of CW tracheids contains lower proportions of heteromannans and heteroxylans, but higher proportions of lignin containing high proportions of p-hydroxyphenyl (H-units) that are present in only trace amounts in NW and OW lignin. Additionally, the cell-wall matrix of CW tracheids contains (1→4)-β-galactans and small proportions of (1→3)-β-glucans (Brennan et al., 2012; Zhang et al., 2016; 2017). Positive statistical associations have been found between longitudinal shrinkage and the content of (1→4)-β-galactans in CW in the corewood of radiata pine (Brennan et al., 2012). A similar association has also found between longitudinal shrinkage and lignin content. Thus, positive associations have been found between longitudinal shrinkage of CW and two cell-wall matrix components.

As described above, CW is more accurately referred to as severe CW (SCW). However, a continuum of wood types or grades occur between SCW and NW or OW, and the intermediate types are referred to as mild CWs (MCWs) (Donaldson and Singh, 2013; 2016). MCWs have been much less studied than SCW. However, I have recently reported two studies (Zhang et al. 2016; 2017) using radiata pine corewood of similar age to that used by Brennan et al. (2012) in which I identified two grades of MCW, MCW1 and MCW2, as well SCW and OW, based on the distribution of lignin in the tracheid cell walls. Because of the likely involvement of the tracheid cell-wall matrix polymers in the longitudinal shrinkage of CW, I focussed on these. Immunomicroscopy was used to determine the distributions of (1→4)-β-galactans and (1→3)-β-glucans in the tracheid cell walls. In addition, the lignin contents and lignin monomeric compositions and neutral monosaccharide compositions of the matrix polysaccharides were determined on small (0.5 mm diameter), microscopically well-defined discs cut from transverse wood sections. These studies showed that the two MCWs had distributions of (1→4)-β-galactans and (1→3)-β-glucans intermediate between those of
OW and SCW. Intermediate results for the MCWs were also found for the lignin contents and monomeric compositions and for the neutral monosaccharide compositions of the matrix polysaccharides. From this, because the polymer compositions of the tracheid cell-wall matrix of the MCWs are intermediate between OW and SCW, we hypothesize that the longitudinal shrinkage of the two grades of MCWs will also be intermediate.

In this study, the similar, small discs cut from the same four wood types were used and the longitudinal shrinkage and swelling of the tracheids, as well as their radial and tangential shrinkage were determined, by variable-pressure scanning electron microscopy (VP-SEM), sometimes referred to as environmental scanning electron microscopy (ESEM). VP-SEM is an excellent technique for this purpose because the samples can be imaged without pre-treatment. The samples can be hydrated (adsorption) and dehydrated (desorption) by altering the water vapour pressure and hence the relative humidity in the chamber (Donald, 2003). The technique has been used to study tracheid shrinkage in earlywood and latetwood of Norway spruce (Picea abies), but CW has not been examined (Almeida et al., 2014).

4.3 Material and methods

4.3.1 Tree growth and wood samples

In spring, seedlings of radiata pine (Pinus radiata D. Don) (Forest Genetics Ltd, Rotorua, New Zealand) were planted and grown outside in bags with regular irrigation at Harewood, Christchurch, New Zealand, as described by Zhang et al. (2016; 2017). Three saplings were used: clone 30 ramets 1 and 2, and clone 17, which are referred to as Trees 1, 2, and 3, respectively. After growing upright for six months, they were tilted by staking at ~8-20° from the vertical to produce CW and OW and harvested after another 16 months. The exact angle of tilting, measured at harvest, was for Tree 1 ~20°, Tree 2 ~13°, and Tree 3 ~8°. A segment (~10 cm long) was sawn from each stem ~20 cm above the surface of the soil. The pieces were dried at 35°C to constant weight, and stored at ambient temperature. Segments (1 cm thick) were cut from each dried piece using a band saw and immersed in water at 4°C to soften. All the stems contained one annual growth ring boundary and all the tracheids examined were of earlywood from the second year.
Transverse sections (~200 µm thick) were cut from the softened segments using a sliding microtome (Model HN 40, Jung, Heidelberg, Germany). Regions within these sections containing SCW, MCW1, MCW2 and OW were identified based on the distribution of lignin in the tracheid walls as determined by its autofluorescence using fluorescence microscopy. This was done with a fluorescence microscope (model DMR; Leica, Wetzlar, Germany) equipped with an I3 filter block (excitation filter BP450-490, chromatic beam splitter 510, and emission filter LP515) (Zhang et al., 2016).

Discs, each containing only one of the four wood types, were excised from the 200-µm thick transverse sections of each tree, using a 0.5 mm Harris Uni-core™ micro-puncher (ProSciTech) and the purity of each wood type was again checked by fluorescence microscopy. Triplicate discs of each wood type from each tree were used “as cut” or were trimmed under a stereo microscope with a double sided razor blade as shown in Figure 4-1, so that they could be more easily placed on the Peltier stage to observe the tracheid radial longitudinal walls because only these walls have bordered pits (Figure 4-2). All the discs were kept fully saturated by immersing them in water before viewing.

Figure 4-1. A diagram showing the preparation of a trimmed disc (100 µm tangential × 200 µm longitudinal × 500 µm radial) from an untrimmed (“as cut”) disc (0.5 mm diameter).
4.3.2 Variable-pressure scanning electron microscopy (VP-SEM)

Discs of the four wood types were examined using a FEI Quanta 200 FEG ESEM (FEI Company, Eindhoven, The Netherlands) used in the ESEM imaging mode with a gaseous secondary electron detector (GSED). Imaging parameters were kept constant: 10.0 KV, 4.0 spot size, 400 x magnification and the working distance was the same in all experiments. Moisture sorption experiments were done in the microscope chamber, maintained at 2°C, with the percentage relative humidity (RH) in the chamber being controlled by altering the water vapour pressure (Table 4-1) (Messier and Vitale, 1993).
Table 4-1. VP-SEM water vapour pressures used to obtain specific % relative humidities at 2 °C (Messier and Vitale, 1993).

<table>
<thead>
<tr>
<th>Steps</th>
<th>Water vapour pressure (Torr)</th>
<th>% Relative humidity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 19</td>
<td>5.3</td>
<td>100</td>
</tr>
<tr>
<td>2 and 18</td>
<td>4.7</td>
<td>90</td>
</tr>
<tr>
<td>3 and 17</td>
<td>4.2</td>
<td>80</td>
</tr>
<tr>
<td>4 and 16</td>
<td>3.7</td>
<td>70</td>
</tr>
<tr>
<td>5 and 15</td>
<td>3.2</td>
<td>60</td>
</tr>
<tr>
<td>6 and 14</td>
<td>2.6</td>
<td>50</td>
</tr>
<tr>
<td>7 and 13</td>
<td>2.1</td>
<td>40</td>
</tr>
<tr>
<td>8 and 12</td>
<td>1.6</td>
<td>30</td>
</tr>
<tr>
<td>9 and 11</td>
<td>1.1</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
<td>10</td>
</tr>
</tbody>
</table>

N.B. Steps 1-10 are desorption steps. Steps 11-19 are adsorption steps.

To measure the longitudinal shrinkage and swelling of tracheids, fully water saturated, triplicate, trimmed discs of each wood type (from Tree 1) were placed on their longitudinal surfaces on the cooled Peltier stage. The RH was adjusted from fully saturated (100% RH) to 10% RH in 10 desorption steps, and then back to 100% RH in 9 adsorption steps (Table 4-1). The 10% RH corresponds to a ~2.6% moisture content for the wood (Hailwood and Horrobin, 1946). At each step, images were taken every minute of the longitudinal surface of each of the three discs, until the distance d (~150 µm) between two references points (edges of bordered pit apertures on the same radial longitudinal wall of a tracheid) (Figure 4-3) on one of the discs, measured using the microscope software, remained constant (~4-5 mins). Images of all three discs were then captured and the distances between similar reference points measured using eighteen tracheids (six tracheids in each of the triplicate discs) with ImageJ 1.47v software (National Institutes of Health, USA) and the percentage dimensional changes calculated. Images from this experiment were also used to determine the shrinkage of bordered pits and their apertures by measuring the changes in the diameters of the bordered pits (bdp, Figure 4-4), and of the pit apertures (pad, Figure 4-4) from 100% RH to 10% RH. The diameters of thirty bordered pits and pit apertures were measured for each wood type.
Figure 4-3. Diagram showing the measurement of the longitudinal shrinkage of a tracheid. The distance (d) between the two referencing points (edges of bordered pit apertures) was measured. B, Pit border; BP, bordered pit; PA, pit aperture; T, tracheid.
Longitudinal shrinkage, but not swelling, was measured using trimmed discs of all wood types from Trees 2 and 3 by changing the RH from 100% to 10% in only one step. As described above, images were taken every minute of the longitudinal walls of the tracheids until the distances between the two reference points were constant, when images for analysis were taken and analysed. Radial and tangential shrinkage, but not swelling, was also measured in a similar way except that “as cut” discs were used. The discs were placed with one of their transverse surfaces on the cooled Peltier stage. Radial and tangential shrinkage was determined by measuring the dimensional changes between two reference points spanning three or four tracheids, with the reference points being the CML (Figure 4-5). The transverse surfaces of the discs were also used to measure changes on going from RH 100% to 10% in the lumen diameters of tracheids in the radial and tangential directions. These surfaces were also used to measure transverse wall shrinkage of the radial and tangential tracheid walls, by measuring the reduction in thickness (widths) of these walls (measured as double walls of adjacent tracheids). Transverse wall shrinkage of the radial and tangential walls represents shrinkage in the tangential and radial directions, respectively (Figure 4-5). All measurements were done on six tracheids, chosen at random, for each of the three discs. Imaging and measurements were done as described above.
Figure 4-5. VP-SEM micrographs of cut transverse surfaces of discs showing the tracheids from the four wood types in Tree 1. (a) OW at 100% RH, (b) OW at 10% RH, (c) MCW1 at 100% RH, (d) MCW1 at 10% RH, (e) MCW2 at 100% RH, (f) MCW2 at 10% RH, (g) SCW at 100% RH, (h) SCW at 10% RH. At 100% RH, free water fills some of the tracheid lumens (arrow heads). OW tracheids have the thinnest walls, but the largest lumen diameters. The wall thickness increases and the lumen diameter decreases with increasing CW severity. The tracheids in all four wood types are better aligned radially than tangentially. Shrinkage in the radial direction (red line in a) and in the tangential direction (green line in a) was investigated by measuring the dimensional changes between two reference points spanning three or four tracheids. Transverse wall shrinkage was also measured of tangential walls (light blue line) and of radial walls (yellow line) was also measured; the thickness of the double wall of two adjacent tracheids was measured. Scale bar = 50 µm.
4.3.3 Statistical analysis

Statistical analyses were done in R (version 3.0.1) (R Core Team, 2013). Shrinkage of the tracheids in the longitudinal, tangential and radial directions of the four wood types was examined statistically. Since the data are multivariate (three directions), a two-way factorial multivariate analysis of variance (MANOVA) was performed to look for differences between wood types and trees. To display the differences between the centroids, canonical discriminant analysis (CDA) was used to plot the centroids in two dimensions with a minimum loss of information (Manly 2004). A similar analysis was also done to examine the shrinkage of the tracheid walls: longitudinal wall shrinkage, which is exactly the same measurement as the longitudinal shrinkage of the tracheids as a whole; transverse wall shrinkage of the radial walls, which is wall shrinkage in the tangential direction (Figure 4-5); and transverse wall shrinkage of the tangential walls, which is wall shrinkage in the radial direction (Figure 4-5). The significance of differences between wood types in the diameters of tracheid bordered pits and their apertures, and the changes in these caused by going from RH 100% to 10% were investigated using one-way analysis of variance (ANOVA). The post hoc Duncan’s multiple range test (Sokal and Rohlf, 2012) was then used to determine the significance of differences between pairs of wood types. The significance of difference between wood types and between trees in the thicknesses of the radial and tangential walls, and in the lumen diameters in the radial and tangential directions were investigated using three-way analysis of variance (ANOVA).

The multivariate approach of canonical correlation analysis (Manly 2004) was performed to investigate possible relationships between shrinkage of the tracheids and the tracheid cell-wall matrix compositions of the four wood types. Two sets of random variables were involved, set 1 represented the shrinkage variables (longitudinal, tangential and radial) and set 2 represented the chemical composition variables (lignin and the non-cellulosic neutral monosaccharides arabinose, xylose, galactose, and mannose, obtained by acid hydrolysis) (Zhang et al. 2016; 2017) (Table 4-2). Three pairs of canonical variables were defined. The overall canonical correlation analysis provided an overview of the relationship between two groups of variables and the follow-up regression analysis tested the relative importance of variables in each set. A similar correlation analysis was also performed to investigate possible relationships between tracheid wall shrinkage (longitudinal wall shrinkage, and transverse...
wall shrinkage of the radial and tangential walls). The longitudinal wall shrinkage is, of course, the same as the longitudinal tracheid shrinkage.

Table 4-2. The chemical data for the four wood types of three trees used for the multivariate approach of canonical correlation analysis.

<table>
<thead>
<tr>
<th>Tree</th>
<th>Wood types</th>
<th>Lignin&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Arabinose&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Galactose&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Xylose&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mannose&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tree 1</td>
<td>OW</td>
<td>25.86</td>
<td>9.89</td>
<td>8.4</td>
<td>30.34</td>
<td>36.47</td>
</tr>
<tr>
<td></td>
<td>MCW1</td>
<td>28.74</td>
<td>6.79</td>
<td>31.55</td>
<td>20.94</td>
<td>27.69</td>
</tr>
<tr>
<td></td>
<td>MCW2</td>
<td>31.74</td>
<td>5.71</td>
<td>40.75</td>
<td>17.95</td>
<td>20.79</td>
</tr>
<tr>
<td></td>
<td>SCW</td>
<td>35.14</td>
<td>4.89</td>
<td>49.72</td>
<td>15.31</td>
<td>16.11</td>
</tr>
<tr>
<td>Tree 2</td>
<td>OW</td>
<td>26.11</td>
<td>11.01</td>
<td>8.81</td>
<td>31.04</td>
<td>38.17</td>
</tr>
<tr>
<td></td>
<td>MCW1</td>
<td>28.26</td>
<td>6.3</td>
<td>30.39</td>
<td>23.59</td>
<td>28.57</td>
</tr>
<tr>
<td></td>
<td>MCW2</td>
<td>31.25</td>
<td>5.71</td>
<td>40.99</td>
<td>17.81</td>
<td>20.71</td>
</tr>
<tr>
<td></td>
<td>SCW</td>
<td>35.03</td>
<td>4.09</td>
<td>50.53</td>
<td>14.16</td>
<td>18.89</td>
</tr>
<tr>
<td>Tree 3</td>
<td>OW</td>
<td>26.43</td>
<td>8.76</td>
<td>8.65</td>
<td>30.05</td>
<td>40.44</td>
</tr>
<tr>
<td></td>
<td>MCW1</td>
<td>28.14</td>
<td>6.21</td>
<td>29.42</td>
<td>23.43</td>
<td>30.29</td>
</tr>
<tr>
<td></td>
<td>MCW2</td>
<td>31.99</td>
<td>5.25</td>
<td>41.07</td>
<td>16.74</td>
<td>21.07</td>
</tr>
<tr>
<td></td>
<td>SCW</td>
<td>34.46</td>
<td>3.47</td>
<td>49.93</td>
<td>16.64</td>
<td>18.36</td>
</tr>
</tbody>
</table>

<sup>a</sup> Lignin content of the four wood types were determined by the acetyl bromide assay (Zhang et al., 2017).

<sup>b</sup> The neutral monosaccharide compositions of the four wood types were determined by 2 M trifluoroacetic acid (TFA) hydrolysis as a percentage of the total neutral monosaccharides (Zhang et al., 2016). The percentage of glucose is not given as it was not used in the statistical analysis.

4.4 Results

4.4.1 Longitudinal shrinkage and swelling of the tracheids increases with CW severity and shows hysteresis

Longitudinal dimensional changes at the 10 desorption and 9 absorption steps of the tracheids (measured as dimensional changes in the longitudinal walls) of the four wood types in Tree 1 are shown in Figure 4-6. Non-linear relationships were observed between percentage dimensional change and relative humidity for all the wood types, with SCW showing the greatest change, OW the least, and MCW1 and MCW2 intermediate. There was considerable
hysteresis observed between swelling and shrinkage for all the wood types and the closing of all the hysteresis loops indicated that shrinkage and swelling were reversible. For any given RH, the dimensional changes were always greater during desorption than adsorption, and SCW and MCW 2 showed more pronounced hysteresis than MCW 1 and OW.

Figure 4-6. Plot showing the percentage longitudinal dimensional changes at the 10 desorption (from 100% to 10% RH) and 9 absorption steps (from 10% to 100% RH) in 10% steps of tracheids of the four wood types (SCW, MCW2, MCW1 and OW) in Tree 1. The SCW showed the greatest change, OW the least, and MCW1 and MCW2 were intermediate. The shrinkage and swelling of all the wood types was reversible. Considerable hysteresis was shown between swelling and shrinkage for all the wood types, and the dimensional changes were always greater during desorption than absorption for any given RH.

Longitudinal shrinkage, but not swelling, of tracheids of the four wood types in Trees 2 and 3 was also determined, and the percentages were similar to those obtained in Tree 1 (Table 4-3). For each of the trees, the percentage shrinkage increased from OW, which showed the least percentage shrinkage (~0.95%), to SCW, which showed the most (~3.8%), with the increase following the CW severity (Table 4-3). This represented an increase of ~300% increase in shrinkage from OW to SCW. Statistical analysis of these results and those of tangential and radial shrinkage of the tracheids is shown below.
Table 4-3. Average\textsuperscript{a} shrinkage of tracheids in three directions for all four wood types of three trees.

<table>
<thead>
<tr>
<th>Tree</th>
<th>Wood types</th>
<th>Longitudinal Shrinkage%</th>
<th>Tangential Shrinkage%</th>
<th>Radial Shrinkage%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tree 1\textsuperscript{b}</td>
<td>OW</td>
<td>0.99 ± 0.03</td>
<td>6.48 ± 0.08</td>
<td>3.21 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>MCW1</td>
<td>1.85 ± 0.07</td>
<td>5.64 ± 0.05</td>
<td>2.94 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>MCW2</td>
<td>2.71 ± 0.02</td>
<td>5.22 ± 0.03</td>
<td>2.23 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>SCW</td>
<td>3.82 ± 0.16</td>
<td>4.09 ± 0.04</td>
<td>2.08 ± 0.03</td>
</tr>
<tr>
<td>Tree 2</td>
<td>OW</td>
<td>0.89 ± 0.06</td>
<td>6.66 ± 0.07</td>
<td>3.09 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>MCW1</td>
<td>2.12 ± 0.05</td>
<td>5.81 ± 0.07</td>
<td>2.62 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>MCW2</td>
<td>2.97 ± 0.07</td>
<td>5.18 ± 0.06</td>
<td>2.30 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>SCW</td>
<td>3.89 ± 0.08</td>
<td>4.24 ± 0.04</td>
<td>2.10 ± 0.03</td>
</tr>
<tr>
<td>Tree 3</td>
<td>OW</td>
<td>0.92 ± 0.07</td>
<td>6.76 ± 0.06</td>
<td>3.22 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>MCW1</td>
<td>1.93 ± 0.05</td>
<td>5.89 ± 0.05</td>
<td>2.89 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>MCW2</td>
<td>2.78 ± 0.06</td>
<td>5.21 ± 0.04</td>
<td>2.51 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>SCW</td>
<td>3.72 ± 0.07</td>
<td>4.15 ± 0.04</td>
<td>2.16 ± 0.03</td>
</tr>
</tbody>
</table>

\textsuperscript{a}18 tracheids were measured and averages of tracheids ± standard errors.
\textsuperscript{b}Both longitudinal shrinkage and swelling were determined for tracheids of Tree 1.

4.4.2 Tangential and radial shrinkage of the tracheids decreases with CW severity, with tangential shrinkage greater than radial shrinkage

The tangential and radial shrinkage of tracheids in the four wood types of Trees 1, 2 and 3 is also shown in Table 4-3. Unlike the longitudinal shrinkage, both the tangential and radial shrinkage of the tracheids in each wood type decreased with increasing CW severity. The OW tracheids had the highest percentage shrinkage in both the tangential and radial directions, and the SCW tracheids had the least. The percentage tangential and radial shrinkage of MCW was intermediate between that of SCW and OW, with MCW1 showing more shrinkage than MCW2. Tangential shrinkage of the tracheids was approximately twice that of the radial shrinkage in all the wood type. The tangential and radial shrinkage of OW was ~6.6\% and ~3.1\%, and of SCW was ~4.2\% and ~2.1\%, representing decreases in shrinkage of ~37\% and 32\%, respectively. The shrinkage of the tracheids forming the four different wood types was thus anisotropic.
4.4.3 Statistical analysis of tracheid shrinkage by two-way factorial MANOVA confirmed the differences between wood types

The effects of wood type and tree number on the tracheid longitudinal, tangential and radial shrinkage was examined by a two-way factorial MANOVA. This showed a highly significant effect of wood type ($P = 6.89 \times 10^{-11}$) and a less significant effect of tree number ($P = 2.77 \times 10^{-10}$). However, the situation was complicated by clear evidence that the differences between the trees depends on the wood type (interaction $P = 1.04 \times 10^{-4}$). The canonical discriminant analysis (CDA) plot (Figure 4-7a) shows that the four wood types line up on a single axis (canonical variate 1, CV1), with the SCW at the positive end being associated with high values of longitudinal and low values of tangential and radial shrinkage, and with the OW at the negative end being associated with low values of longitudinal and high values of tangential and radial shrinkage. The confidence ellipses on each point emphasises how separate they are. The correlation coefficients of all the variables for CV1 and CV2 are listed in Table 4-4.

Table 4-4. Canonical discriminant analysis (CDA) structure coefficients for the first two canonical variates of the correlations between the shrinkage of tracheids and between the shrinkage of the tracheid walls in three directions, in the four wood types of the three trees.

<table>
<thead>
<tr>
<th></th>
<th>CV1</th>
<th>CV2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tracheid shrinkage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Longitudinal tracheid shrinkage (%)</td>
<td>0.99</td>
<td>0.016</td>
</tr>
<tr>
<td>Tangential tracheid shrinkage (%)</td>
<td>-0.99</td>
<td>-0.148</td>
</tr>
<tr>
<td>Radial tracheid shrinkage (%)</td>
<td>-0.97</td>
<td>0.213</td>
</tr>
<tr>
<td><strong>Tracheid wall shrinkage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Longitudinal shrinkage (%) of tracheid walls</td>
<td>1.00</td>
<td>0.028</td>
</tr>
<tr>
<td>Transverse shrinkage (%) of tangential tracheid walls</td>
<td>-0.88</td>
<td>0.333</td>
</tr>
<tr>
<td>Transverse shrinkage (%) of radial tracheid walls</td>
<td>-0.913</td>
<td>-0.143</td>
</tr>
</tbody>
</table>

*aThe longitudinal tracheid shrinkage (%) is the same as the longitudinal wall shrinkage (%).
4.4.4 Micrographs of transverse surfaces show that tracheids become less well ordered, more rounded, and their walls thicker with CW severity

As shown in the micrographs of the transverse surfaces of discs of the four wood types (Figure 4-5), the tracheids are all well-ordered in the radial direction, but are very much less ordered in the tangential direction. As the CW severity increases, the shapes of the tracheids in transverse view gradually changes from the rectangular or polygonal shape of the OW tracheids (Figure 4-5a, b) to the more rounded or oval shape of the SCW tracheids.
(Figure 4-5g, h). The thicknesses of the tracheid walls and the lumen diameters also change with severity of the CW. These were quantified on micrographs and the thicknesses of the radial and tangential tracheid walls in a particular wood type were not significantly different (P>0.05), but both increased with CW severity (Table 4-5). Lumen diameters were significantly greater in the radial direction than the tangential (P<0.01), but both diameters decreased with CW severity. The OW had the thinnest walls and largest lumens, whereas the SCW had the thickest walls and the smallest lumens.
Table 4-5. Dimensional changes\textsuperscript{a} on drying of tracheid lumen diameter and wall thickness\textsuperscript{b} of the four wood types.

<table>
<thead>
<tr>
<th>Tree</th>
<th>Wood types</th>
<th>Lumen diameter (μm)</th>
<th>Wall thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Radial</td>
<td>Tangential</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100% RH Shrinkage %</td>
<td>10% RH Shrinkage %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27.81 ± 0.45</td>
<td>27.38 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>Tree1 OW</td>
<td>1.55 ± 0.07</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.24 ± 0.57</td>
<td>23.78 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>MCW1</td>
<td>1.02 ± 0.11</td>
<td>1.02 ± 0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.99 ± 0.59</td>
<td>23.39 ± 0.59</td>
</tr>
<tr>
<td></td>
<td>MCW2</td>
<td>1.02 ± 0.11</td>
<td>1.02 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>SCW</td>
<td>0.71 ± 0.04</td>
<td>0.71 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20.60 ± 0.73</td>
<td>20.30 ± 0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.49 ± 0.49</td>
<td>0.49 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>Tree2 OW</td>
<td>1.43 ± 0.08</td>
<td>1.43 ± 0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.61 ± 0.68</td>
<td>22.31 ± 0.68</td>
</tr>
<tr>
<td></td>
<td>MCW1</td>
<td>0.98 ± 0.03</td>
<td>0.98 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.23 ± 0.79</td>
<td>23.03 ± 0.79</td>
</tr>
<tr>
<td></td>
<td>MCW2</td>
<td>0.94 ± 0.06</td>
<td>0.94 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>SCW</td>
<td>0.90 ± 0.05</td>
<td>0.90 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20.50 ± 0.72</td>
<td>20.30 ± 0.72</td>
</tr>
<tr>
<td></td>
<td>Tree3 OW</td>
<td>1.51 ± 0.08</td>
<td>1.51 ± 0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.88 ± 0.62</td>
<td>23.68 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>MCW1</td>
<td>1.03 ± 0.04</td>
<td>1.03 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26.50 ± 0.56</td>
<td>26.30 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>MCW2</td>
<td>0.90 ± 0.05</td>
<td>0.90 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>SCW</td>
<td>0.67 ± 0.03</td>
<td>0.67 ± 0.03</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Obtained from the transverse surface.

\textsuperscript{b} Thickness of double wall of two adjacent tracheids.
4.4.5 Transverse wall shrinkage decreased with CW severity and was greater for radial walls than tangential tracheid walls

Comparison of micrographs of exactly the same transverse surface areas at 100% and 10% RHs show differences, mostly associated with the presence of free water in the 100% RH micrographs. For example some of the tracheids contained free water (see arrows in Figure 4-5). However, at 10% RH, all the free water and most of the bound water was removed. Changes as a result of drying were quantified on micrographs (Table 4-5). The lumen diameters of the tracheids showed the greatest shrinkage in OW and least in SCW, with shrinkage of the tangential diameters being greater for a given wood type than of the radial diameters. Transverse tracheid wall shrinkage, determined from changes in wall thickness (width), was greatest in the OW and least in the SCW, with shrinkage of the radial walls being greater for a given wood type than the tangential walls. Shrinkage of the radial and tangential walls of OW was ~4.7% and ~2.14%, and of SCW ~2.0% and ~1.1%, representing differences between the two wood types of ~58 and 48%, respectively. For all the wood types, tangential shrinkage was about twice that of radial.

The effects of wood type and tree number on longitudinal and transverse tracheid wall shrinkage was also examined statistically by two-way factorial MANOVA. The results were similar to the MANOVA shown above for overall tracheid shrinkage, with the wood type showing a highly significant effect (P = 9.61 X 10^{-7}), but the between tree variation is much less (P = 0.0067). The CDA plot is shown in Figure 4-7b and the correlation coefficients of all the variables for CV1 and CV2 are listed in Table 4-4.

4.4.6 Canonical correlation analysis showed that longitudinal tracheid shrinkage is positively correlated with lignin content and galactose percentage

To investigate the relationship between tracheid shrinkage (longitudinal, tangential and radial) of the four wood types and their cell-wall compositions, a canonical correlation analysis was conducted. In the overall canonical correlation analysis, the association between the shrinkage variables set and the chemical variables set was assessed by establishing the canonical correlation coefficient for each pair of linear composites derived from the data. The analysis yielded three functions with canonical correlations of 0.992, 0.468 and 0.164, with
only the first two functions being considered noteworthy in the context of this study (99.2% and 46.8% of the shared variance, respectively). This suggested a strong linear relationship between the shrinkage variables set and the chemical variables set. To determine the most important variables in a given pair of canonical variates, the canonical variate scores were analysed (Table 4-6). The structure coefficients (Manley 2004) of the first variate (CV1) indicated that the longitudinal shrinkage is positively associated with lignin content and percentage galactose (in an acid hydrolysate), but negatively associated with arabinose, xylose and mannose percentages. Tangential and radial shrinkage were both negatively associated with the lignin contents and galactose percentages, but positively associated with arabinose, xylose and mannose percentages. Neither the CV 2 nor the CV 3 correlations are particularly large for both sets of variables, and so these canonical variables yielded little information about the data, and will not be considered further. To see the relationship, a canonical variate plot was generated (1, OW; 2, MCW1; 3, MCW2; 4, SCW) (Figure 4-8a) based on an analysis of the combined variables in the two sets of data. This plot indicated considerable separation among the four wood types along the first canonical variate.
Figure 4-8. The canonical variate plot (a) Plot showing the separation of the four wood types (1, OW; 2, MCW1; 3, MCW2; 4, SCW) from three trees defined by the first two canonical variates (CV1 and CW2) obtained from canonical correlation analysis conducted on a combination of tracheid shrinkage in the three directions (longitudinal, tangential and radial) and the chemical data (lignin content; percentage arabinose, galactose, xylose and mannose). (b) Plot showing the separation of the four wood types from three trees defined by the first two canonical variates (CV1 and CW2) obtained from canonical correlation analysis conducted on a combination of tracheid wall shrinkage (longitudinal shrinkage and transverse shrinkage of the radial and of the tangential walls) and the chemical data (lignin content; percentage arabinose, galactose, xylose and mannose).
Table 4-6. Correlations between the tracheid shrinkage and chemical variables, and between the tracheid wall shrinkage and chemistry variables, and their canonical variables.

<table>
<thead>
<tr>
<th></th>
<th>CV1</th>
<th>CV2</th>
<th>CV3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tracheid shrinkage variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Longitudinal tracheid shrinkage%</td>
<td>-0.994</td>
<td>-0.091</td>
<td>0.068</td>
</tr>
<tr>
<td>Tangential tracheid shrinkage%</td>
<td>0.992</td>
<td>0.045</td>
<td>0.119</td>
</tr>
<tr>
<td>Radial tracheid shrinkage%</td>
<td>0.969</td>
<td>-0.189</td>
<td>-0.159</td>
</tr>
<tr>
<td><strong>Chemical variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AcBr-soluble lignin\textsuperscript{a}</td>
<td>-0.985</td>
<td>-0.043</td>
<td>-0.134</td>
</tr>
<tr>
<td>Arabinose\textsuperscript{b}</td>
<td>0.902</td>
<td>0.229</td>
<td>-0.306</td>
</tr>
<tr>
<td>Galactose\textsuperscript{b}</td>
<td>-0.981</td>
<td>-0.029</td>
<td>0.181</td>
</tr>
<tr>
<td>Xylose\textsuperscript{b}</td>
<td>0.953</td>
<td>0.014</td>
<td>-0.191</td>
</tr>
<tr>
<td>Mannose\textsuperscript{b}</td>
<td>0.974</td>
<td>-0.156</td>
<td>-0.148</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>CV1</th>
<th>CV2</th>
<th>CV3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tracheid wall shrinkage variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Longitudinal shrinkage (%) of tracheid walls</td>
<td>-0.992</td>
<td>0.046</td>
<td>-0.121</td>
</tr>
<tr>
<td>Transverse shrinkage (%) of tangential tracheid walls</td>
<td>0.952</td>
<td>-0.286</td>
<td>-0.105</td>
</tr>
<tr>
<td>Transverse shrinkage (%) of radial tracheid walls</td>
<td>0.997</td>
<td>0.059</td>
<td>-0.044</td>
</tr>
<tr>
<td><strong>Chemical variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AcBr-soluble lignin\textsuperscript{a}</td>
<td>-0.981</td>
<td>0.172</td>
<td>-0.077</td>
</tr>
<tr>
<td>Arabinose\textsuperscript{b}</td>
<td>0.955</td>
<td>0.226</td>
<td>-0.133</td>
</tr>
<tr>
<td>Galactose\textsuperscript{b}</td>
<td>-0.990</td>
<td>-0.067</td>
<td>0.074</td>
</tr>
<tr>
<td>Xylose\textsuperscript{b}</td>
<td>0.974</td>
<td>0.021</td>
<td>-0.190</td>
</tr>
<tr>
<td>Mannose\textsuperscript{b}</td>
<td>0.975</td>
<td>-0.049</td>
<td>-0.103</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Lignin content of the four wood types have been determined by the acetyl bromide assay (Zhang et al., 2017).

\textsuperscript{b} The neutral monosaccharide compositions of the four wood types have been determined by 2 M trifluoroacetic acid (TFA) hydrolysis (Zhang et al., 2016).

A second, related canonical correlation analysis was conducted to specifically investigate the relationship between tracheid cell-wall shrinkage (longitudinal, and transverse of the radial and of tangential walls) and chemical composition. The longitudinal tracheid wall shrinkage was, of course, the same as the longitudinal tracheid shrinkage, which has already been...
considered. As in the first analysis, this analysis yielded three functions with canonical correlations of 0.998, 0.787 and 0.522, which suggested a strong linear relationships between the two sets of variables. The canonical variate scores were further analysed (Table 4-6), with similar results being obtained to those in the first analysis, with longitudinal tracheid wall shrinkage, unsurprisingly, being positively associated with the lignin contents and the galactose percentages, but negatively associated with arabinose, xylose and mannose percentages. The transverse wall shrinkage of the radial and tangential tracheid walls were both negatively associated with lignin contents and galactose percentages, but positively associated with arabinose, xylose and mannose percentages. As above, a canonical variate plot was generated to show the considerable separation among the four wood types along the first canonical variate (Figure 4-8b).

4.4.7 Diameters of tracheid bordered pits did not change significantly on drying, but the diameters of the pit apertures decreased with increasing CW severity

The diameters of the bordered pits and their apertures in the tracheids of the different wood types of Tree 1 are shown in Table 4-7, which also shows their percentage changes on drying. One-way ANOVA and the post-hoc Duncan’s test were used to statistically compare the diameters of the bordered pits and their apertures in the different wood types and before and after drying (i.e. on going from 100% RH to 10% RH) (Table 4-8). Before drying, the bordered pits of OW and MCW1 had similar diameters (P > 0.1), which were larger than those of the bordered pits of MCW2 (P < 0.05), and SCW had the smallest diameter bordered pits (P < 0.001) (Table 4-7). The apertures of the bordered pits of SCW were also the smallest of the different wood types (P < 0.001). On drying, the diameters of the bordered pits of the different wood types showed little change. However, the diameters of the pit apertures decreased with increasing CW severity. The percentage decrease was greatest in the SCW (4.69%) and least in the OW (1.61%), with MCW1 and MCW2 intermediate. There were significant differences in the changes of the pit aperture diameters on drying between the four wood types (P < 0.001), in which the differences between the MCW2 and MCW1 were less detectable (P < 0.05) (Table 4-8). Comparison of micrographs of transverse surfaces of MCW1 at 100% and 10% RH showed the chambers of the bordered pits collapsed during drying (Figure 4-9). This collapse probably accounts, at least in part, for the decrease in aperture diameter after drying.
Table 4-7. Diameters of the tracheid bordered pits and their apertures in the four wood types of Tree 1 before and after drying (100% and 10% RH).

<table>
<thead>
<tr>
<th>Wood types</th>
<th>Diameters of bordered pits (μm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Width of single border&lt;sup&gt;b&lt;/sup&gt; (μm)</th>
<th>Diameters of apertures (μm)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100% RH</td>
<td>10% RH</td>
<td>Dimensional changes%</td>
</tr>
<tr>
<td>OW</td>
<td>10.87 ± 0.22</td>
<td>10.86 ± 0.22</td>
<td>-0.10 ± 0.11</td>
</tr>
<tr>
<td>MCW 1</td>
<td>10.89 ± 0.12</td>
<td>10.87 ± 0.12</td>
<td>-0.16 ± 0.14</td>
</tr>
<tr>
<td>MCW 2</td>
<td>10.17 ± 0.27</td>
<td>10.17 ± 0.27</td>
<td>0.01 ± 0.09</td>
</tr>
<tr>
<td>SCW</td>
<td>8.72 ± 0.20</td>
<td>8.71 ± 0.21</td>
<td>-0.10 ± 0.26</td>
</tr>
</tbody>
</table>

<sup>a</sup>Diameters of 30 bordered pits.
<sup>b</sup>By subtracting aperture diameter from pit diameter and dividing by two.

Table 4-8. One-way ANOVA followed by the post-hoc Duncan multiple range test set at 95% significance level of the diameters of tracheid bordered pits and their apertures in the four wood types of Tree 1 before and after drying (100% and 10% RH).

<table>
<thead>
<tr>
<th>P value of one-way ANOVA</th>
<th>Post hoc contrasts by Duncan</th>
</tr>
</thead>
<tbody>
<tr>
<td>OW-MCW1</td>
<td>OW-MCW2</td>
</tr>
<tr>
<td>OW</td>
<td>MCW2 - MCW1</td>
</tr>
<tr>
<td>SCW</td>
<td>SCW - OW</td>
</tr>
<tr>
<td>SCW-MCW1</td>
<td>SCW-MCW2</td>
</tr>
</tbody>
</table>

| Significant levels: ***0.001 **0.01 *0.05. |
Figure 4-9. VP-SEM micrographs showing the cut transverse surface of a disc of MCW1 from Tree 1 (a) at 100% RH and (b) at 10% RH. The chamber (arrow) of the tracheid (T) bordered pits (BP) has collapsed on drying. Scale bar = 20 µm.

Comparison of micrographs of transverse surfaces of SCW at 100% and 10% RH also showed major changes of the resin canals and associated cell types on drying (Figure 4-10). The thin-walled epithelial and parenchyma cells collapsed. In addition, the thin-walled, radial ray tracheids shrunk tangentially after drying and the distance between the two tangential walls of the ray tracheids (arrows) became much smaller (Figure 4-11).
Figure 4-10. VP-SEM micrographs of the cut transverse surface of a disc of SCW from Tree 1 (a) at 100% RH and (b) at 10% RH. The structure of the resin canal (RC) and associated cell types change on drying. The thin-walled epithelial cells (E) around the resin canal and parenchyma cells (P) are collapsed after drying to 10% RH. R, ray tracheid. T, tracheid. Scale bar = 50 µm.

Figure 4-11. VP-SEM micrographs showing the cut transverse surface of a disc of MCW1 from Tree 1 (a) at 100% RH and (b) at 10% RH. The thin-walled, radial ray tracheid (R) shrank tangentially after drying to 10% RH. The distance between the two longitudinal walls of the ray tracheid (arrows) became much smaller after drying. T, tracheid. Scale bar = 50 µm.
4.5 Discussion

As we hypothesized, the present study showed that the longitudinal shrinkage of the tracheids of the two grades of MCW, MCW1 and MCW2, was intermediate between that of OW and SCW, with the shrinkage of MCW1 being less than MCW2. The percentage longitudinal shrinkage of OW and SCW tracheids was similar to that reported by others for radiata pine in which the shrinkage of small strips (e.g. 3 mm radial x 10 mm tangential x 100 mm longitudinal) of wood was measured using micrometer calipers (Harris, 1977; Xu et al., 2009; Chauhan et al., 2013). The shrinkage was also similar to the longitudinal swelling of OW, NW and SCW reported by Brennan et al. (2012) also using similar strips of radiata pine corewood. Interestingly, one of these studies (Xu et al., 2009) included wood strips that contained MCW, although the size of the strips would preclude examining all the tracheids to check if they were indeed MCW. Nevertheless, the MCW strips also showed longitudinal shrinkage intermediate between SCW and NW.

Our longitudinal shrinkage results are consistent with the tracheid walls of the MCWs MCW1 and MCW2 containing intermediate proportions of (1→4)-β-galactans determined both by immunomicroscopy, which showed they were present in the S2L region of the tracheid walls, and by determining the neutral monosaccharide compositions of the cell-wall matrix polysaccharides (Zhang et al., 2016). Statistically, there was a positive association between longitudinal shrinkage and the proportions of galactosyl residues as a percentage of the total neutral monosaccharides released by acid hydrolysis conditions that did not hydrolyse cellulose. Similar associations have previously been reported between longitudinal swelling and the proportions of galactose in acid hydrolysates of OW, NW and SCW of corewood radiata pine (Brennan et al., 2012) and between longitudinal shrinkage and the proportions of galactose in acid hydrolysates of logs of 24-year-old, plantation-grown loblolly pine (Pinus taeda) (Floyd, 2005). (1→4)-β-Galactans are known to have a high capacity for binding water and swell on adsorbing water (hydration) (Ryden et al., 2000; Turnbull et al., 2005; Harris and Smith, 2006). These polysaccharides may thus play a functional role in the longitudinal shrinkage and swelling of CWs.

A statistically positive association was also found in the present study between longitudinal shrinkage and lignin contents. A similar association has also been reported between lignin content and longitudinal swelling of SCW (Brennan et al., 2012). However, lignin is a hydrophobic molecule and does not have a high capacity for binding water (Ralph et al.,
Lignin therefore probably does not play a functional role in the longitudinal shrinkage and swelling of CWs. Nevertheless, the formation and location of lignin and (1→4)-β-galactans are closely related and it is possible that this close relationship results in the statistical association. Earlier studies showed that with increasing CW severity, the band of (1→4)-β-galactans in the S2L region of the tracheid walls increased in width and, in the same region, lignin staining by the fluorescent stain acridine orange also increased (Zhang et al., 2016; 2017). The (1→4)-β-galactans are therefore co-located with lignin and together increase in concentration with CW severity. The two components are probably covalently linked as partial delignification of radiata pine SCW resulted in increased amounts of (1→4)-β-galactans that could be extracted using 6 M sodium hydroxide (Chavan et al., 2015). This finding is consistent with the isolation of a complex containing (1→4)-β-galactans and lignin from Japanese red pine (*Pinus densiflora*) (Watanabe et al., 1989). The synthesis of the (1→4)-β-galactans probably slightly precedes lignification in the S2L region, with the covalent cross links between the two components probably being formed by reactions involving quinone methide intermediates during lignin synthesis (Ralph et al., 2007).

In addition to (1→4)-β-galactans, small proportions of (1→3)-β-glucans were found in all grades of CW and increase with increasing CW severity (Zhang et al., 2016). They were detected by immunomicroscopy and by staining with pure, synthetic aniline blue fluorochrome, which specifically binds to (1→3)-β-glucans, and were found to be located in the inner region of the S2 layer (S2i region). Like (1→4)-β-galactans, these polysaccharides have a high capacity for binding water and are known to swell on adsorbing water (hydration) (Vithanage et al., 1980; Piršelová and Matušíková, 2013; Stone, 2005). Consequently, they may also contribute to the longitudinal shrinkage and swelling of CW. My previous immunogold microscopy study (Zhang et al., 2016) showed that, in SCW, these polysaccharides were located in the helical cavities in the inner region of the S2 layer. A previous immunogold microscopy study (Altaner et al., 2010) also reported the same location of (1→3)-β-glucans in the tracheid walls in SCW of radiata pine, and of Sitka spruce (*Picea sitchensis*) and Norway spruce (*Picea abies*). Interestingly, in the two MCWs of radiata pine (Zhang et al., 2016), the (1→3)-β-glucans were also located in the S2i region of the tracheid walls, despite there being no cavities (Zhang et al., 2016). The presence of the glucans in the helical cavities of the SCW walls led to the suggestion they may act as sealants for these cavities (Altaner et al., 2010), but the absence of cavities in the MCW walls suggests this is not their only function.
The hysteresis found in the present study for the sorption curves for the four different wood types has previously been reported for softwoods (Walker, 2006). The water adsorbed within the tracheid walls (also referred to as bound water), and which is responsible for the dimensional changes, is thought to be mostly hydrogen bonded to the hydroxyl groups of the non-cellulosic polysaccharides. Hysteresis being explained by it being more difficult for water to penetrate tracheid walls, with low water contents, and hydrogen bond to hydroxyl groups of the non-cellulosic polysaccharides (adsorption) than for these hydrogen bonds to be broken and the water leave the walls (desorption). The degree of hysteresis was greater for CW and increased with CW severity. This may be related to the differences in the cell-wall matrix compositions of the different wood types. Alternatively, it may be related to the differences in the tracheid wall thickness, which also increased with increasing CW severity. The degree of hysteresis in transverse shrinkage (tangential and radial) has previously been related to tracheid wall thickness in Norway spruce (Picea abies) (Derome et al., 2012).

As all the tracheids in softwoods are joined through their MLs, it is not surprising that if there is shrinkage in the longitudinal direction, there will also be dimensional changes in the tangential and radial directions. Indeed, in all the wood types, the tracheids also showed tangential and radial shrinkage, with the tangential shrinkage being about twice that of the radial shrinkage. Similar higher values for tangential than for radial shrinkage have been reported for NW of softwood species, including radiata pine (Spear and Walker, 2006). Interestingly, when the transverse surfaces of the discs were examined, the shrinkage in thickness of the radial walls, which is shrinkage in the tangential direction, was about twice that of the shrinkage in thickness of the tangential walls, which is shrinkage in the radial direction. Thus, the relative shrinkage of the tangential and radial walls appears to play a major part in determining the anisotropy of transverse shrinkage. What actually determines these shrinkage differences between the radial and tangential walls is less clear. One factor may be the presence of bordered pits in the radial but not in the tangential walls. The cellulose microfibrils in the radial walls deviate around the pits, probably affecting the wall shrinkage (Barber and Meylan, 1964). This is consistent with the finding in the present study that the diameters of the bordered pits remain almost unchanged on drying in all the wood types. A factor that may also cause less radial than tangential shrinkage of tracheids is the arrangement of the tracheids. As was also shown in the present study, they are regularly ordered in the radial direction, but less so in the tangential direction. In hardwoods, the rays have also been found to restrain shrinkage in the radial direction (Skaar, 1988; Spear and
Walker, 2006). However, in the softwood *Pinus sylvestris*, cross sections of wood with and without rays showed no difference in shrinkage, suggesting they are not a limiting factor in softwood shrinkage (Boutelje, 1962).

In the present study, it was found that in addition to the tracheids of SCW having a much higher (~300% higher) longitudinal shrinkage than the tracheids in OW, the tangential and radial shrinkage of the tracheids was less by ~37% and ~32%, respectively. Similar, lower tangential and radial shrinkage values for SCW than NW (or OW) have been reported for a number of softwood species (Timell, 1986). The results in the present study extend this information by showing that the tangential and radial shrinkage of the tracheids of the two mild CWs was intermediate between that of OW and SCW. In a similar way to the tangential and radial shrinkage of OW, the relative shrinkage of the tangential and radial walls appears to play a major part in determining the anisotropy of transverse shrinkage of the CWs. The lower shrinkage of the tangential and radial walls of the CWs, relative to that of OW, may result from the much greater longitudinal shrinkage driven by the presence of the (1→4)-β-galactans and possibly (1→3)-β-glucans.

### 4.6 Conclusions

Our VP-SEM study showed that the tracheids in discs of radiata pine MCW exhibited longitudinal shrinkage and swelling intermediate between that found in SCW and OW, with the dimensional changes increasing with CW severity. A statistical positive association was found between the galactosyl content of the wall-matrix and longitudinal shrinkage of CWs. Where MCWs occurs adjacent to OW or NW, the differences in longitudinal shrinkage and swelling between the two wood types may thus result in warping similar to that reported when SCW and OW or NW are adjacent. Tangential and radial shrinkage of the tracheids also occurred on drying, with the tangential shrinkage being greater than radial shrinkage for all wood types. However, unlike longitudinal shrinkage, tangential and radial shrinkage decreased with CW severity.
Chapter 5. General conclusion

CW forms on the lower side of the stems of leaning coniferous gymnosperms and helps the trees resist the gravitropic forces of the lean while new growth is being restored in a more vertical orientation. *Pinus radiata* is widely grown for lumber, however the presence of CW in the sawn timber is regarded as a defect, as it tends to warp and distort (Timell, 1986) on drying, resulting in a loss of commercial value. CW forms in different degrees of severity. Most is known about the severe form with much less being known about milder forms which also exist. In this thesis, 21-month-old radiata pine stems were tilted slightly from the vertical during their growth to induce the formation of milder forms of CW as well as severe. Subsequently, three grades of CW (SCW, MCW1 and MCW2) together with OW were classified based on the distribution of lignin in their tracheid cell walls. In collecting material for analysis, special attention was paid to ensuring each grade of CW or OW was exclusive in its cellular composition. This was achieved by carefully dissecting out 0.5 mm discs from the different woods and examining them microscopically. This gave unambiguous sample identity and confidence of sample uniformity in the subsequent microanalyses.

The three grades of CWs were different from each other anatomically, in their tracheid wall structures, wall thickness and cell shapes. The other cell types, including rays and resin canals, were anatomically very similar among the different wood types. As the CW severity increased, the tracheid walls became thicker and rounder in cross section. The middle lamella became less lignified and the highly lignified S2L layer began to form. In MCW1 tracheids, the S2L layers were only formed at the cell corners regions. The width of this layer increased in tracheids of MCW2, becoming thickest in SCW where these layers surrounded the whole tracheid. S3 layers were present in tracheid walls of only OW and MCW1, and were much thinner in MCW1. Other features, such as helical cavities, which were present in the inner S2 regions of only SCW tracheids, and intercellular spaces, which appeared between tracheids in both MCW2 and SCW.

As the anatomy of the CWs changed with increasing CW severity, so did their chemical compositions. The lignin, (1→4)-β-D-galactan and (1→3)-β-D-glucan contents increased with increasing CW severity, but those of cellulose and the non-cellulosic polysaccharides O-acetyl-galactoglucomannans and arabino(4-O-methylglucurono)xylans decreased. The two
mild grades of CWs had chemical compositions intermediate between those of SCW and OW.

Since the monosaccharide compositions indicated that the galactose content of the cell wall polysaccharides increased with increasing CW severity, it was of interest for me to find out where in the tracheid walls these were located. The precise location of (1→4)-β-galactans in the tracheid walls was determined by immunolabelling using the monoclonal antibody LM5, which indicated these polysaccharides were located in the outer region (S2L) of the S2 layer of the tracheid walls of all the grades of CWs, not just SCW, and were co-located with lignin. The majority of these galactose residues were considered to originate from (1→4)-β-galactans, and only a small proportion from O-acetyl-galactoglucomannans.

The (1→4)-β-galactans in SCWs have a higher degree of polymerisation than those in OW (Brennan et al., 2012). Nevertheless in the four wood types, these (1→4)-β-galactans are probably sidechains on the pectic polysaccharide rhamnogalacturonan I (RG-I). Further immunolabelling experiments could be conducted using the monoclonal antibody INRA-RU2, which is specific for the RG-I backbone (Ralet et al., 2010), to determine if the (1→4)-β-galactans co-locate with the RG-I backbone in CWs. Such a co-localization of the RG-I backbone and (1→4)-β-galactans has been demonstrated in the cell walls of hybrid aspen (Populus tremula × Populus tremuloides) tension wood fibres (Gorshkova et al., 2015).

(1→3)-β-Glucans have previously been found in or between helical cavities in the inner region (S2i) of the S2 layer of the SCW tracheids (Altaner et al., 2007b; 2010). In the present study I have shown that (1→3)-β-glucans also occur in the inner region of the S2 layer of tracheids of OWs and MCWs, where the helical cavities were absent. This was also achieved using an immunolabelling method but with the monoclonal antibody BS 400-2 and as well as by staining with the pure, synthetic fluorochrome from aniline blue. As was found for (1→4)-β-galactans, the concentrations of (1→3)-β-glucans increased with increasing CW severity. Possibly both these polysaccharides, which are both known to swell on hydration, assist the tree in resisting the gravitropic stress generated on tilting stems.

The distribution of lignin in tracheid walls can be characterized using a solution of the fluorescent dye acridine orange at pH 6 or 7. This is better than simply observing lignin autofluorescence because it provides a brighter fluorescence and is metachromatic, allowing a greater understanding of the concentration and location of lignin in the walls. The
microscopic findings of increased lignin distribution in the tracheid walls of CWs were consistent with the lignin concentrations quantified using the acetyl bromide method. The mildest CW, MCW1, with a greater lignin distribution in the S2L layer only at the cell corner regions contained much more lignin than OW. Not only did the lignin contents increase with increasing CW severity, but the composition of the lignin among the woods changed. OW contained only very small proportions of H-units, but the proportions of these increased dramatically with CW severity, with even the mildest form, MCW 1, having 12 times the H-units as those in OW. There was a strong correlation found between H/G ratio and the lignin content ($R^2 = 0.95$), which suggested that the lignins formed in the CWs under stress have a specific structure. Therefore, the synthesis of H-unit lignin may be one of the early physiological responses to gravitropic stress, besides the synthesis of \((1 \rightarrow 4)\)-β-galactan and \((1 \rightarrow 3)\)-β-glucan. The exact location of the H-units in tracheid walls of mild CWs is unknown. However, this could be investigated using confocal Raman microscopy for chemical imaging. The differences in cell wall compositions between and within the cell wall layers can be imaged with high spatial resolution (Gierlinger and Schwanninger, 2006).

In the present study, I sought to examine the walls using techniques which might be useful commercially. The first of these was using an immunolabelling method with LM 5 and an enzyme-labelled secondary antibody on whole stem sections. This allowed the different grades of CWs, especially mild CWs, to be successfully identified and distinguished from one another based on the resultant colour produced by the enzyme reacting with a colourless substrate to produce an insoluble colour product. The second, was to use the spectroscopic techniques FTIR and Raman micro-spectroscopies, in combination with PCA, which was able to detect the chemical differences between the wood types, and the results were consistent with the wet chemistry findings. Both methods have overcome problems associated with visual detection methods for CWs which were based on the different coloration of CWs under transmitted or reflected light, and have failed to detect MCWs, especially the mildest, MCW1, which has been shown to have a similar colour to OW.

In accordance with other sections of this thesis in which very small samples were used, the shrinkage of the tracheids was also investigated on very small samples using VP-SEM. OW shrank most in the tangential direction, which was almost twice that in the radial direction, and was the most stable in the longitudinal direction. Conversely, SCWs shrank extensively in the longitudinal direction (~300% more than the OW), but shrank less in both the
tangential and radial directions. The two mild grades of CWs had shrinkage characteristics intermediate between the OW and SCW in all three directions. These findings are important for understating the properties and dimensional stability of timber containing any grades of CWs. The excessive longitudinal shrinkage is of greatest concern for timber to be used for structural purposes. But even the mildest CW, MCW1, had longitudinal shrinkage about twice that of OW. Therefore, it appears that *Pinus radiata* timber containing any grade of CW has the potential to distort. The different shrinkage behaviours of the tracheids in the four wood types were statistically correlated with their wall matrix compositions.

The excessive longitudinal shrinkage of CWs probably related to the increased amounts in their tracheid walls of (1→4)-β-galactans, which are known to have a high water binding capacity. The gene and protein involved in the biosynthesis of (1→4)-β-galactans has been identified in *Arabidopsis thalinan* and the protein is referred to as (1→4)-β-galactan synthase (GALS1) (Liwanag et al., 2012). Co-overexpression of this gene and a gene encoding a UDP-glucose 4-epimerase (UGE) in *A. thaliana* resulted in a large increase in the amount of (1→4)-β-galactans in the secondary walls of sclerenchyma fibres (Gondolf et al., 2014). It would be interesting to examine the shrinkage and swelling of these cell walls using VP-SEM and compare these with wildtype cell walls.

Overall this study has increased the understanding of the properties of CWs, particularly of MCWs, in their anatomy, chemical compositions, and shrinkage behaviors at the tracheid level. In particular, the results in this thesis have highlighted that the high concentrations of the (1→4)-β-galactans, (1→3)-β-glucans, and H-unit lignins are the characteristic features for all grades of CWs and can be used as markers for the detection of CWs. In addition, using the immunolabelling method on whole stem sections with the monoclonal antibody LM5 and using Raman and FTIR spectroscopic methods in combination with PCA are alternatives for accurately identifying the CWs and their severities.
References


