COMPOSITION OF WOOD PECTINS AND THE ENZYMATIC CONTROL OF PECTIC POLYSACCHARIDES IN MECHANICAL PAPER PULPS

by

Berdine Coetzee

Submitted in partial fulfilment of the requirements for the degree of

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Department of Microbiology and Plant Pathology
Faculty of Natural and Agricultural Science
University of Pretoria
Pretoria
South Africa

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Supervisor: Dr J.F. Wolfaardt

DECLARATION

I, the undersigned, declare that the thesis/dissertation, which I hereby submit for the degree of Doctor of Philosophy at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

.....	.....
Berdine Coetzee	**Date**

Science is not a sacred cow. Science is a horse. Don't worship it. Feed it.

Aubrey Eben

Dedicated to my father

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SUMMARY

COMPOSITION OF WOOD PECTINS AND THE ENZYMATIC CONTROL OF PECTIC POLYSACCHARIDES IN MECHANICAL PAPER PULPS

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Degree: Ph.D.

Pectin is a complex class of polysaccharides that occur in the plant cell-walls. It is concentrated in the middle lamella and cell corners with a gradual decrease from the primary cell-wall to the plasma membrane. The pectic polysaccharides consist of a diversity of monosaccharides that make them heterogeneous in composition, structure and molecular weight. Current knowledge on the structure of these pectic polysaccharides is based on intact pectins extracted from soft plant tissue. Intact pectin can, however, not be extracted from woody material by the same methods. Very little is, therefore, known about the occurrence of the pectic polysaccharides in wood and it was speculated that between 10 to 40 mg/g of the total dry weight of wood consists of pectin.

The occurrence and composition of pectin in wood are of importance in the pulp and paper industry, due to its influence in pulping and papermaking processes. The current work was, therefore, the first attempt to quantify and elucidate the possible

domain structure of pectin from woody tissue. Wood samples were hydrolysed and the pectic monosaccharides (D-galacturonic acid, D-galactose, L-arabinose and L-rhamnose) quantified using high performance liquid chromatography. Through the addition of all pectic monosaccharides, it was determined that eucalyptus wood contained between 15.2 and 25.8 mg/g pectin. It was shown that *Eucalyptus macarthurii* contained significantly more pectin than *E. grandis* and *E. nitens*. The wood tissue type also influenced the occurrence of total pectin and the cambium contained higher concentrations of pectin than sapwood and heartwood.

The molar concentration of the pectic monosaccharides was expressed as a relative amount to the total pectin in wood and it was determined that D-galacturonic acid occurred in the highest concentrations in eucalyptus pectin, followed by D-galactose, L-arabinose and L-rhamnose. Models of the pectic domains were constructed on the basis of these results and homogalacturonan (HG) was the predominant pectic domain, contributing up to seven times more to the total backbone component than rhamnogalacturonan-I (RG-I). The macromolecular composition of the pectin in different eucalyptus species proved to be very diverse. Future studies should include a bigger pool of species to get a more comprehensive understanding of the occurrence and structure of pectin in the *Eucalyptus* genus and also other hardwoods. The tissue type of the wood also had a significant influence on the macromolecular composition of the eucalyptus pectin, confirming that a shift occurred in composition of pectin as the wood tissue aged. Information on the occurrence and composition of pectin in eucalyptus will improve understanding of the influence of these polymers on pulping and papermaking processes and this knowledge can, therefore, be applied to develop biotechnological approaches to improve these processes.

The pectins in wood are released as polygalacturonic acid (PGA) when mechanical pulps are bleached with peroxide under alkaline conditions and contribute to approximately 50% of the anionic substances in the pulp and water. It was demonstrated that pectinase can efficiently depolymerise the PGA into monomeric galacturonic acid and consequently reduce the cationic demand (CD) of mechanical pulp. High-brightness softwood pulp from a chemi-thermo mechanical pulp mill was identified as the pulp source that contributed proportionally the highest amount of CD to the stock going onto a paper machine. A pectinase (Nalco 74303) was applied during a short mill trial to reduce the CD across the storage tower for high-brightness pulp. The enzyme treatment reduced the CD to levels comparable to that achieved with the

usual alum treatment at the mill. The enzyme treatments did not have a negative effect on any of the strength properties of the pulp and future trials should be conducted to determine the impact of the pectinase on the wet-end processes over an extended period.

PREFACE

Woody tissue is generally described to consist of cellulose, hemicelluloses and lignin, without referring to pectin as a significant constituent (Koch, 2006; Sjöström, 1993). The pectic polysaccharides are a highly complex, heterogeneous group of acidic polymers that is a major component of the cell-walls of dicotyledonous plants and gymnosperms (Goycoolea & Cárdenas, 2003; Ridley *et al*., 2001). The highest concentration of pectin occurs in the middle lamellae and cell corners with a gradual decrease from the primary cell-wall to the plasma membrane (Goycoolea & Cárdenas, 2003; Mohnen, 1999), where they play an important role in maintaining intercellular adhesion between plant cells or wood fibres (Jarvis, 1984; Willats *et al*., 2001). These polysaccharides are also present in the junction zone between the secondary walls of fibre cells in wood (Mohnen, 2008), but pectin concentration is greatly reduced or absent in secondary cell-walls (Willats *et al*., 2001).

The fine structure of extractable pectic substances is well known; however, almost no information is available on the occurrence of pectin in wood or the composition of pectins that are associated with lignin, celluloses and hemicelluloses. The difficulties to extract pectin from woody tissue are mainly due to the complexity of their chemical structure, their low concentrations, their insolubility in water and their strong association with the other cell-wall polymers (Anderson, 1936; Rumpunen *et al*., 2002). The association of pectin with other cell-wall polymers includes the covalent linkages to hemicellulose (Cumming *et al*., 2005) and cellulose networks (Oechslin *et al*., 2003) via the reducing termini of their neutral side-chains. The D-galacturonic acids of pectins also form covalent linkages with lignin subunits to form lignin carbohydrate complexes (Cathala *et al*., 2001).

The occurrence of pectin in wood had become important in the pulp and paper industry, due to its influence in pulping (Peng *et al*., 2003) and papermaking processes (Ricard *et al*., 2005a; 2005b; Thornton *et al*., 1996). A major challenge in mechanical pulping was to reduce the energy consumption in the energy intensive refining process and Peng and co-workers (2003) reduced refining energy by implementing a pre-treatment of softwood chips with a pectinase prior to refining. In this process, the

pectinase degraded the pectins in the junction zone between the fibre cells in the wood and resulted in savings in refining energy of up to 7% due to improved fibre separation.

Pectin was also identified as the source of at least 50% of the detrimental anionic substances in the pulp and process water of mills producing bleached chemi-thermo mechanical pulp (BCTMP) (Reid & Ricard, 2000; Thornton, 1994). The pectins are released into the pulp and water as polygalacturonic acid (PGA) A pectinase was effectively applied to depolymerise PGA into monomeric galacturonic acid and consequently reduce the cationic demand (Reid & Ricard, 2000; Thornton, 1994).

At present, eucalyptus wood is the main fibre source for the pulp and paper industry in many parts of the world (Pinto *et al*., 2005; Pisuttipiched, 2004; Quoirin & Quisen, 2006; Ramírez *et al*., 2009; Silvério *et al*., 2007). This increased interest in the *Eucalyptus* genus as a source of fibre is due to their fast growth rates, short rotations and their wood properties in terms of pulping, bleaching behaviour and pulp quality (Gullichsen & Paulapuro, 2000; Sjöström, 1993). It was, therefore, important to develop the necessary analytical tools to determine the occurrence and composition of pectin in eucalyptus, because this information may aid in the understanding of the influence of pectins on pulping and papermaking processes. It may also assist to develop biotechnological approaches to improve these processes.

Pectin is a heterogeneous group of cell-wall polysaccharides with a high degree of structural complexity. The structure of the pectic domains and how the structure and interactions of these domains with each other and other cell-wall polymers relate to cellular functions were described in the literature overview (Chapter 1). This overview also described the biosynthesis and the degradation of the different pectic domains.

It is important to quantify pectin in wood and an analytical procedure to determine the concentrations of the pectic monosaccharides in eucalyptus wood was developed. The pectin content of the samples was calculated through the addition of all pectic monosaccharides (Chapter 2). The following chapters provided information on the monosaccharide (Chapter 3) and macromolecular (Chapter 4) composition of the eucalyptus pectin. Wood from a third species, namely *E. macarthurii* was compared with *E. grandis* and *E. nitens* (Chapter 5) to elucidate the occurrence and structure of pectin in different eucalyptus species.

Information on the occurrence and structure of pectin in wood may aid the development of biotechnological approaches, to improve pulping and papermaking processes. A case study was conducted (Chapter 6) to evaluate alternative treatments to reduce the cationic demand (CD) of the pulp and water at a BCTMP mill that produced pulp from spruce and poplar wood. At the time of the present study, eucalytus wood was not used at any of the project sponsor's mills for production of BCTMP. The case study was followed by a short-mill trial (Chapter 7) where a pectinase was compared with the control treatment with alum to reduce the CD across the storage tower for high-brightness pulp.

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CHAPTER

1

STRUCTURE, BIOSYNTHESIS AND DEGRADATION OF PECTIN: A REVIEW

ABSTRACT

Plant cell-walls are multilayered and are composed of a number of complex polysaccharides and proteins. These layers have distinctly different chemical compositions and structures. The four main components of the dicotyledonous cell-walls of growing cells are cellulose, hemicellulose, the pectic polysaccharides and glycoproteins. The pectic polysaccharides are the most complex class of these cell-wall polysaccharides and are found in the primary cell-walls and the middle lamellae but are greatly reduced or absent in secondary cell-walls. These polysaccharides are a complex group of polymers that all contain D-galacturonic acid and four pectic polysaccharide domains have been identified namely; homogalacturonan, rhamnogalacturonan-I, rhamnogalacturonan-II and xylogalacturonan. These four domains are covalently linked to form the pectic macromolecule. The formation of further covalent and non-covalent cross-links between some of the glycosyl residues in the macromolecule, results in the three-dimensional pectic network. The pectin network is the major constituent of the cell-wall matrix in which the cellulose-hemicellulose network is embedded. The high degree of structural complexity and heterogeneity of the pectic network is established during the biosynthesis of the pectic polysaccharides in the Golgi apparatus and by modification and degradation after their integration into the cell-wall. The complex structure of the pectic polysaccharides, together with the large number of enzymes that are required to synthesise and degrade them reflect their important role in a number of cellular functions that are related to plant growth and development.

INTRODUCTION

Pectin is a collective term to describe all the polysaccharides that contain D-galacturonic acid residues in their backbones (O'Neill *et al*., 1990). This group includes the methyl-esterified pectins, the de-esterified pectic acids and pectates (Goycoolea & Cárdenas, 2003). Pectin has been described in 1825 by the French chemist Henri Braconnot who identified this group of polysaccharides in many plants but mainly focussed his studies on their gelling properties. These polysaccharides were named "pectic acid", which is the direct translation of the Latin "coagulum" (Guillotin, 2005).

Pectins form a highly complex, heterogeneous group of acidic polysaccharides that have been isolated from the cell-walls of many higher plants and were found to be a major component of the cell-walls of dicotyledonous plants and gymnosperms (Goycoolea & Cárdenas, 2003; Ridley *et al*., 2001). These polymers are also abundant in the cell-walls of monocots (O'Neill *et al*., 1990). The pectic polysaccharides are the most abundant class of macromolecules within the primary cell-wall and are also located in the middle lamella, where they function in a number of growth and developmental processes (Mohnen, 1999). The highest concentration of pectin occurs in the middle lamella and cell corners with a gradual decrease from the primary cell-wall to the plasma membrane (Goycoolea & Cárdenas, 2003). Pectin concentration is greatly reduced or absent in the secondary cell-wall, which makes it the only major cell-wall polysaccharide that is restricted to the primary cell-wall (Willats *et al*., 2001b). These polysaccharides are also present in the junction zone between cells with secondary walls, including xylem and the fibre cells in woody tissue (Mohnen, 2008). These polysaccharides are especially abundant in the cell-walls of growing and dividing plant cells and are usually produced during the initial stages of cell growth (Goycoolea & Cárdenas, 2003; Mohnen, 2008).

Pectins greatly influence general properties of the cell-wall and play an important role in plant development, especially in the regulation of cell expansion and adhesion (Jarvis *et al*., 2003; Willats *et al*., 2001a). Many of the functionalities of pectins are related to their molecular mass and distribution, their D-galacturonic acid and neutral monosaccharide content and the distribution of their substituents (Yapo *et al*., 2007). These polysaccharides are of great importance to the food and cosmetic industries because of their natural gelling and stabilising properties (Gnanasambandam & Proctor, 1999; Guillotin, 2005). Pectins are also considered to be important to the

pharmaceutical industry, because of the positive effects on human health (Inngjerdingen *et al*., 2007). These polysaccharides are used in the production of many different speciality products, including edible and degradable films, adhesives, paper substitutes, foams and surface modifiers that are used in medical devices (Mohnen, 2008).

The aim of the present review was focussed on the structure of the different pectic domains and how the structure and interactions of these domains with each other and other cell-wall polymers relate to cellular functions. The biosynthesis and the degradation of the different pectic domains were also discussed.

STRUCTURE OF PECTIN

Pectin is the most complex class of polysaccharides occurring in the plant cell-wall. These acidic polysaccharides consist of a diversity of monosaccharide residues (Figure 1-1) that make them heterogeneous in composition, structure and molecular weight (O'Neill *et al*., 1990). No unambiguous structure of this plant polysaccharide has yet been developed and its complexity is compounded by the variation between different plant species, cell types within the plant tissue and changes associated with cell development (Mohnen, 1999; O'Neill *et al*., 1990). Despite its structural diversity, pectin can be biochemically defined as a group of polysaccharides that are rich in D-galacturonic acid that forms approximately 70% its molecular mass (Mohnen, 2008; O'Neill *et al*., 1990; 2004). These polysaccharides are generally divided into three polysaccharide domains, namely homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II) (Anthon & Barrett, 2008; O'Neill *et al*., 1990; 2004; Yapo *et al*., 2007). The pectic macromolecule may, however, also include a fourth domain, namely xylogalacturonan (XG) (Coenen *et al*., 2007; Schols *et al*., 1995). These four domains are linked to form the pectic network that is distributed throughout the middle lamella and the matrix of the primary cell-wall (Figure 1-1) (Scheller *et al*., 2007; Willats *et al*., 2001a).

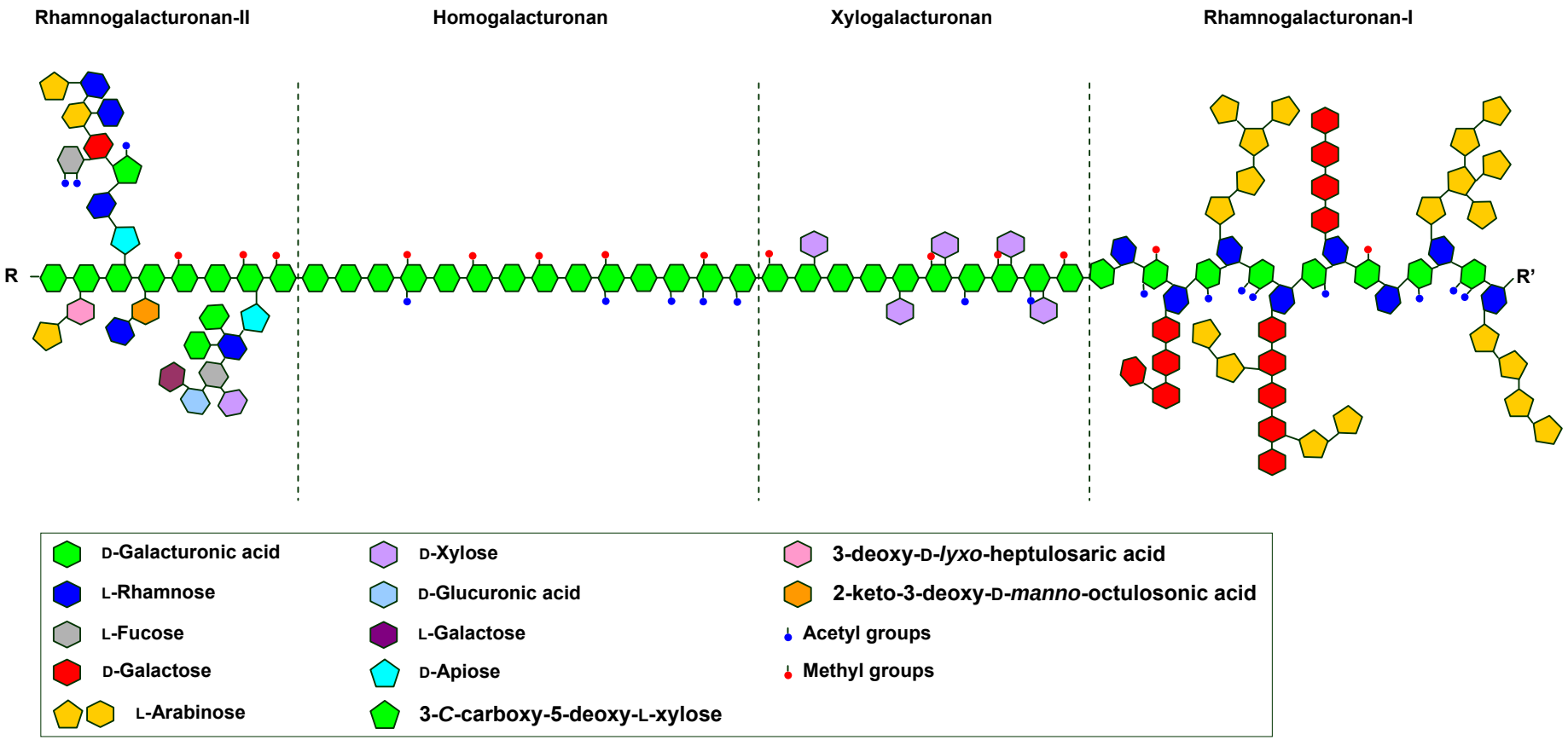


Figure 1-1: Schematic representation of pectin showing the four domains, namely homogalacturonan, xylogalacturonan, rhamnogalacturonan-I and rhamnogalacturonan-II (adapted from Scheller *et al*., 2007).

Structure of homogalacturonan

Approximately 65% of the pectic network consists of HG. Untill recently, this widespread pectic domain was described as a linear homopolymer that consists of 100 to 170 α-(1→4)-linked D-galacturonic acid residues (Thibault *et al*., 1993). Homogalacturonan was also referred to as the smooth region of pectin because of the absence of any saccharide side-chains (O'Neill *et al.*, 1990). However, a recent study that used atomic force microscopy (AFM) confirmed that tomato pectin contains both linear and sparsely branched HG domains (Round *et al*., 2010). It is hypothesised that branching occur at the C-2 and/or C-3 positions of the D-galacturonic acid in the backbone (Ovodova *et al*., 2006). The AFM results also indicated that the HG domains of pectin consist of an average of 320 D-galacturonic acid residues (Round *et al*., 2010).

The D-galacturonic acid residues of this domain can also be substituted with methyl and acetyl groups and the HG domains of different pectins may differ significantly from each other in the type and number of their substituents (Ridley *et al*., 2001). The HG domain, depending on its source can be methyl-esterified to different degrees at the C-6 carboxyl groups (Mort *et al*., 1993; O'Neill *et al.*, 1990) and can also be acetyl esterified at the *O*-2 and/or *O*-3 position of the D-galacturonic acid residues (Figure 1-2) (Ishii, 1997; Perrone *et al*., 2002). It is likely that biosynthetic modifications involving these substituents can change the functional properties of the HG domain (Willats *et al*., 2001a).

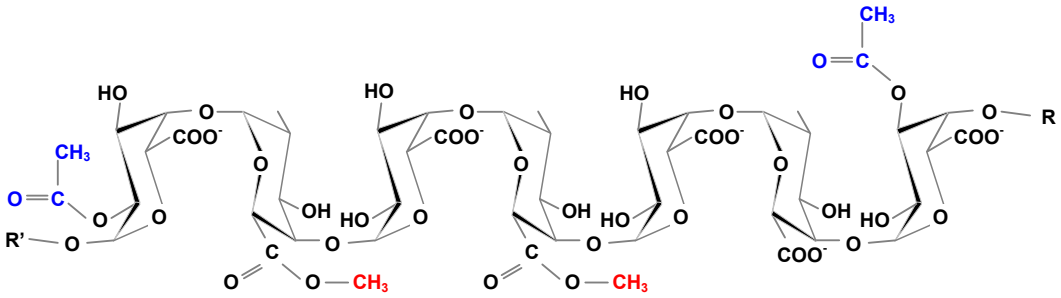


Figure 1-2: Schematic representation of the primary structure of homogalacturonan consisting of α-(1→4)-linked D-galacturonic acid residues. The acetyl esters at the *O*-2 and *O*-3 hydroxyl groups are indicated in blue and methyl esters at the C-6 carboxyl group in red.

Structure of rhamnogalacturonan-I

Rhamnogalacturonan-I refers to the branched, heteropolymeric domains that represent between 20% and 35% of pectin (Mohnen, 2008). The backbones of these domains are formed by up to a 100 units of the repeating disaccharide consisting of D-galacturonic acid and L-rhamnose (Lau *et al*., 1985). The L-rhamnose residues in RG-I backbones function as sites for further glycosylation and between 20% and 80% of these residues are substituted at the C-4 position with neutral chains of arabinan, galactan or arabinogalactan (Figure 1-3) (Mohnen, 1999; O'Neill *et al*., 1990). These side-chains can vary in size from a single glycosyl residue to 50 or more (Albersheim *et al*., 1996). The highly branched nature of RG-I has resulted in it being named as the hairy region of pectin (Schols & Voragen, 1994).

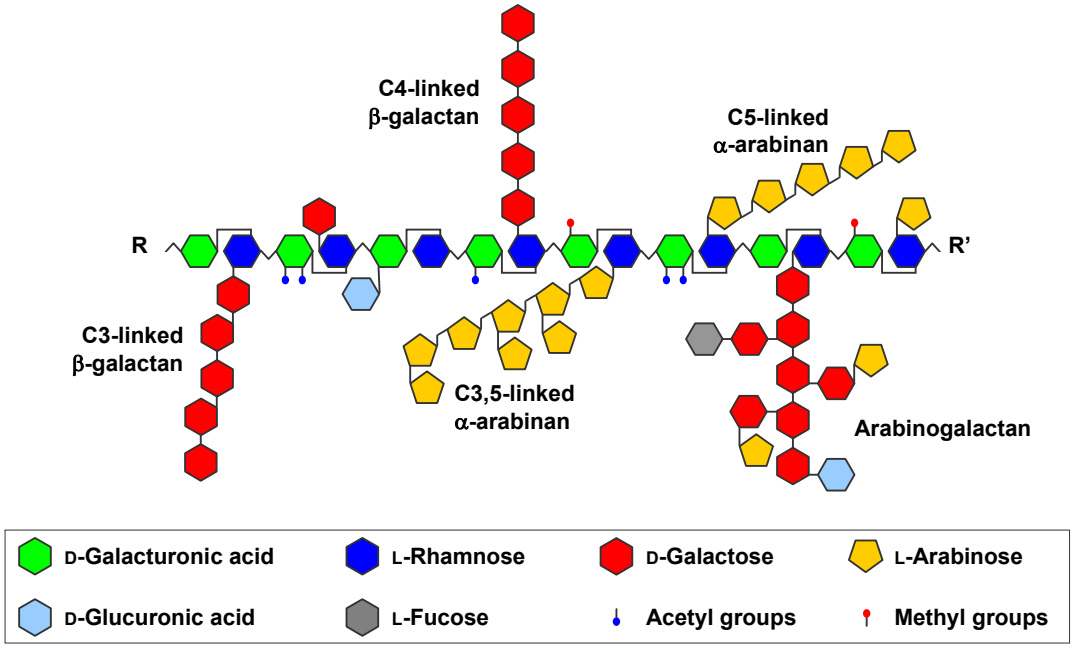


Figure 1-3: Schematic representation of the major structural features of rhamnogalacturonan-I. The backbone is composed of units of the [→4)-α-D-galacturonic acid-(1→2)-α-L-rhamnose-(1→] disaccharide. Branched and linear oligosaccharides composed of L-arabinose and D-galactose residues, are linked to C-4 of some of the L-rhamnose residues.

The arabinan side-chains are generally composed of α-(1→5)-linked L-arabinose residues, which are frequently branched with L-arabinose at the C-3 or C-2 positions, but also α-(1→3)-linked L-arabinose residues (Figure 1-3) (Carpita & Gibeaut, 1993; Oechslin *et al*., 2003). The galactan side-chains are mostly linear and can either be composed of β-(1→4)-linked D-galactose residues or β-(1→3)-linked

D-galactose residues (Carpita & Gibeaut, 1993; Mohnen, 1999), while two types of arabinogalactan side-chains are present in RG-I. The Type-I side-chains consist of linear β-(1→4)-linked D-galactose residues, which are branched with terminal arabinose units at the C-3 position (Carpita & Gibeaut, 1993, Mohnen, 1999; Vincken *et al*., 2003b). Type-II side-chains represents a group of short β-(1→3) and β-(1→6)-D-galactose chains, containing side-chains of α-(1→6)-L-arabinose-[β-(1→6)-D-galactose]$_n$ units where n = 1, 2 or 3. The side-chains of RG-I can also be substituted with D-xylose, D-mannose and D-glucuronic acid in addition to the L-arabinose and D-galactose (Guillon & Thibault, 1989; Renard *et al*., 1991; Vincken *et al*., 2003b). The type and number of monosaccharides, oligosaccharides or branched oligosaccharides that are attached to the backbone of RG-I are highly dependant on the cell type and developmental process (Mohnen, 2008; Ridley *et al*., 2001; Willats *et al*., 2001a).

The D-galacturonic acid residues of the RG-I backbone are usually not substituted with side-chains, but some studies showed that single D-glucuronic acid residues can be linked to the C-3 position (Figure 1-3) (Renard & Jarvis, 1999). The D-galacturonic acid residues of the backbone can also be acetylated on the *O*-2 and/or *O*-3 position (Ishii, 1997) or methyl-esterified on C-6 (Rihouey *et al*., 1995).

Structure of rhamnogalacturonan-II

Rhamnogalacturonan-II has a wide distribution and has a highly conserved structure. Only 1% to 5% of pectin is comprised of RG-II (Ridley *et al*., 2001), but it is one of the most complex pectic domains occurring in the plant cell. This domain is not structurally related to RG-I, but it does contain single L-rhamnose residues in its side-chains. The RG-II domain consists of a backbone of at least eight α-(1→4)-linked D-galacturonic acid residues always carrying four side-chains with conserved structure and length (Matsunaga *et al*., 2004; O'Neill *et al*., 2004; Whitecombe *et al*., 1995). These heteropolymeric side-chains consist of thirteen different glycosyl residues (Figure 1-1), including D-galacturonic acid (D-GalA), D-glucuronic acid (D-GlcA), L-rhamnose (L-Rha), L-fucose (L-Fuc), D-galactose (D-Gal), D-xylose (D-Xyl) and L-arabinose (L-Ara). The L-arabinose that occurs in the side-chains of this domain can either have a furanose (*f*) or puranose (*p*) configuration. Rare sugars such as L-galactose (L-Gal), L-apiose (L-Api), 3-deoxy-D-*lyxo*-heptulosaric acid (Dha), 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo) and 3-*C*-carboxy-5-deoxy-L-xylose (AceA) also

occur in these side-chains (O'Neill *et al*., 1996; Spellman *et al*., 1983; Vidal *et al*., 2000). The RG-II may also contain some methyl-etherified sugars, namely 2-*O*-methyl D-xylose and 2-*O*-methyl L-fucose, which is rarely observed in other cell-wall polysaccharides (Shin *et al*., 1998; Whitecombe *et al*., 1995). These residues are interconnected by more than 22 different types of glycosidic bonds (Figure 1-4) (O'Neill *et al*., 1996; Vidal *et al*., 2000).

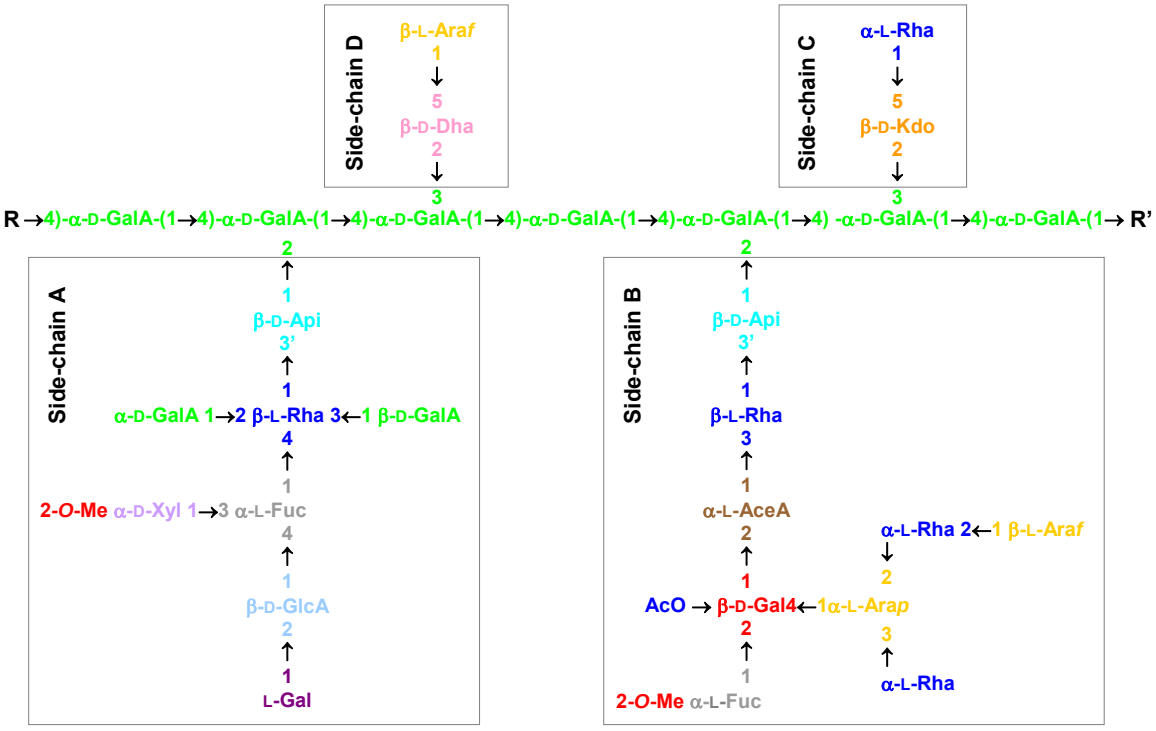


Figure 1-4: Schematic representation of the four side-chains (A-D) on the backbone of rhamnogalacturonan-II (adapted from Matsunaga *et al*., 2004 and O'Neill *et al*., 2004).

Side-chains named A and B are attached to the C-2 position of the D-galacturonic acid residues in the backbone via a β-D-apiose residue (Figure 1-4) (Matsunaga *et al*., 2004; O'Neill *et al*., 2004; Vidal *et al*., 2000). Selective cleavage studies showed that 2-keto-3-deoxy-D-*manno*-octulosonic acid and 3-deoxy-D-*lyxo*-heptulosaric acid-containing disaccharides (Chains C & D) are directly attached to C-3 position of the backbone (Figure 1-4) (Matsunaga *et al*., 2004; Vidal *et al*., 2000). The *O*-acetyl substituents may occur on the 2-*O*-methyl fucosyl and 3-*C*-carboxy-5-deoxy-L-xylose residues that are present in the side-chains of RG-II (Whitecombe *et al*., 1995). The D-galacturonic acid residues in the backbone of this domain can also be methyl-

esterified to different degrees at the C-6 carboxyl groups (Mort *et al*., 1993; O'Neill *et al.*, 1990).

Structure of xylogalacturonan

The XG polysaccharide was originally isolated from the pollen of mountain pine trees (Willats *et al*., 2004). This pectic domain has subsequently been identified from a number of other sources (Coenen *et al*., 2007; Le Goff *et al*., 2001; Schols *et al*., 1995; Yu & Mort, 1996) and can thus, not be ignored as an important pectic domain. The XG domain consists of an α-(1→4)-linked D-galacturonic acid backbone, which is highly substituted with β-D-xylose at the C-3 position (Figure 1-5) (Aspinall & Baillie, 1963). This substitution of the galacturonic acid backbone with D-xylose residues is known to inhibit the formation of pectic gels and also protects the pectic polysaccharide network against the action of *endo*-polygalacturonase (Yu & Mort, 1996). Oligomeric side-chains have been detected in the XG domains of a number of plants and are composed of β-(1→4)-linked D-xylose residues (Oechslin *et al*., 2003), β-(1→4) and β-(1→2)-linked D-xylose (Zandleven *et al*., 2005) and β-(1→2) and β-(1→3)-linked D-xylose (Le Goff *et al*., 2001). The D-galacturonic acid backbone can also be methyl-esterified to different degrees at the C-6 position (Zandleven, 2006).

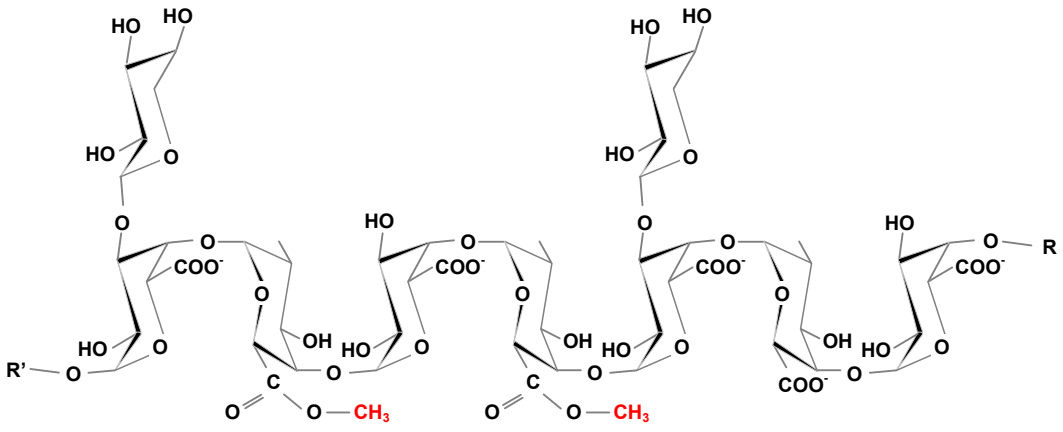


Figure 1-5: Schematic representation of the primary structure of xylogalacturonan consisting of a backbone of α-(1→4)-linked D-galacturonic acid residues substituted at the C-3 position with β-D-xylose. The methyl esters at the C-6 carboxyl groups are indicated in red (adapted from Zandleven *et al*., 2005).

INTERACTIONS AND LINKS OF THE PECTIC NETWORK

The different pectic domains are linked to each other and to other macromolecules through covalent bonds as well as non-covalent bonds. The individual non-covalent bonds are relatively weak, but are numerous enough to ensure strength. These bonds include the Ca^{2+} bridges between the HG domains of different pectic chains (Figure 1-6) and the ionic cross-links between alkaline glycoproteins and acidic pectins. The covalent bonds include the borate di-ester cross-links between pairs of RG-II domains (Figure 1-6) (O'Neill *et al*., 1996), and the linkages between lignin and pectin (Wallace *et al*., 1995). A number of interpolymeric glycosidic bonds can also link individual pectic domains via their reducing termini to one another, hemicelluloses or to protein molecules (Mort, 2002; Thompson & Fry, 2000). Since only one reducing terminus exists per polysaccharide molecule, the polysaccharides can only be linked together as long chains or as tree-like structures (Thompson & Fry, 2000). Separate trees can, therefore, not be cross-linked to each other through glycosidic bonds between their branches (Fry, 2004).

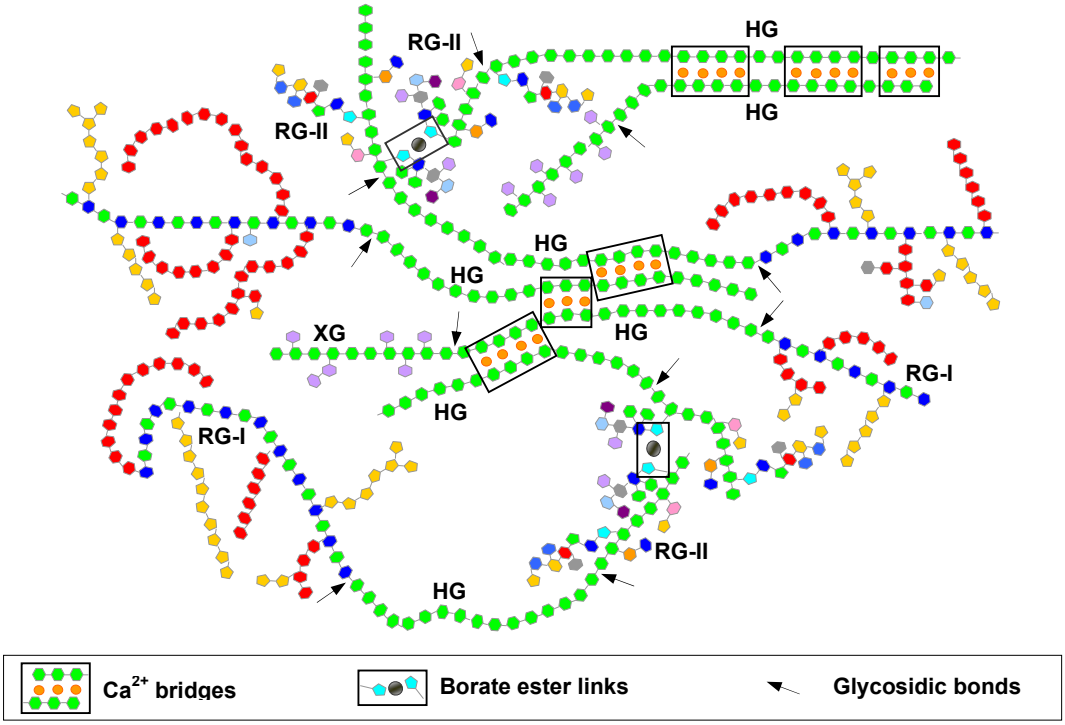
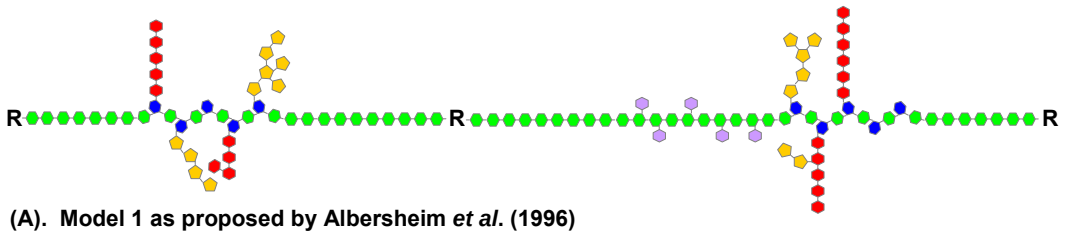


Figure 1-6: Schematic representation of the macromolecular structure of pectin. Homogalacturonan (HG), rhamnogalacturonan-II (RG-II), xylogalacturonan (XG) and rhamnogalacturonan-I (RG-I) can be bound via glycosidic bonds (indicated by arrows) Different polymeric chains can also be linked via Ca^{2+} bridges between different HG domains or borate-ester links between different RG-II domains (indicated by boxes).

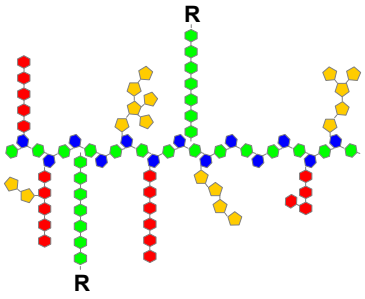
Macromolecular structure

Links between pectic domains: The interactions of the four pectic domains within the pectic network are highly complex and their absolute distribution is still unknown. It is generally assumed that HG, XG and RG-II are covalently attached to one another, since they all have backbones that consist of α-(1→4)-linked D-galacturonic acid residues (Figure 1-6) (O'Neill *et al*., 1990; Whitecombe *et al*., 1995). Rhamnogalacturonan-I has also been isolated from HG-rich cell-wall material, indicating that this domain was glycosidically linked to the HG domain (Coenen *et al*., 2007; Ishii & Matsunaga, 2001). It was also recently demonstrated that XG and RG-I were covalently linked when an oligomer consisting of these two domains were identified (Coenen *et al.*, 2007). It is not known whether RG-I and RG-II can be directly bound through glycosidic bonds or whether they are only attached to the HG domain.

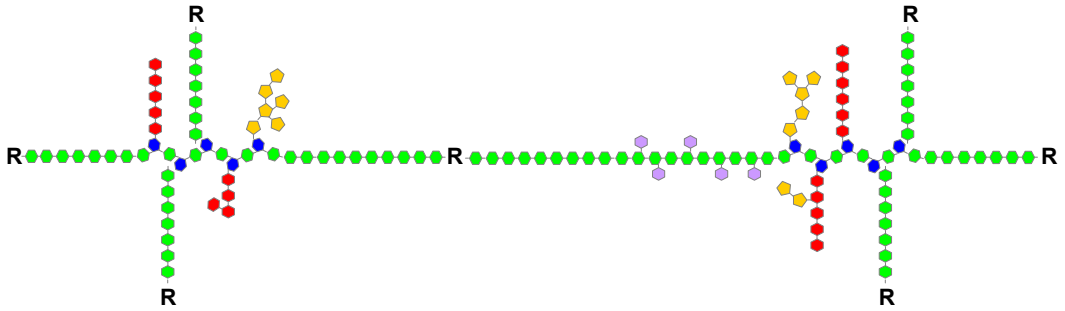
Four possible models for pectin structure have been proposed, namely the linear model (Albersheim *et al*., 1996), the branched-RG-I model (Vincken *et al*., 2003a; 2003b), the combination model (Schols *et al*., 2009) and the branched-HG model (Round *et al*., 2010). The linear model suggested that the pectin molecule consisted of contiguous sections of HG (smooth regions) and XG interspersed by RG-I (hairy regions) that were connected via a D-galacturonic acid-(1→2)-α-L-rhamnose linkage (Albersheim *et al*., 1996; Coenen *et al*., 2007) (Figure 1-7 A). In the second model it was suggested that the RG-I domains acted as a scaffold to which single HG chains were attached, in addition to the D-galactose and L-arabinose containing chains. It was, however, unclear if the HG side-chains were attached to the L-rhamnose or D-galacturonic acid residues in the RG-I backbone (Figure 1-7 B) (Vincken *et al*., 2003a). The third model proposed that the HG domains occurred both in-line and as side-chains of the RG-I domains (Figure 1-7 C) (Schols *et al*., 2009). The fourth model was consistent with the linear model of pectin, but suggested that the HG domains were branched with HG-chains at the C2 or C3 position of the backbone residues (Figure 1-7 D) (Round *et al*., 2010). This model also differed from the other three models in suggesting that the HG polymers consisted of approximately 320 α-(1→4)-linked D-galacturonic acid residues (Round *et al*., 2010) compared to the 100 to 170 residues reported by other authors (Coenen *et al*., 2007; Thibault *et al*., 1993; Yapo *et al*., 2007).



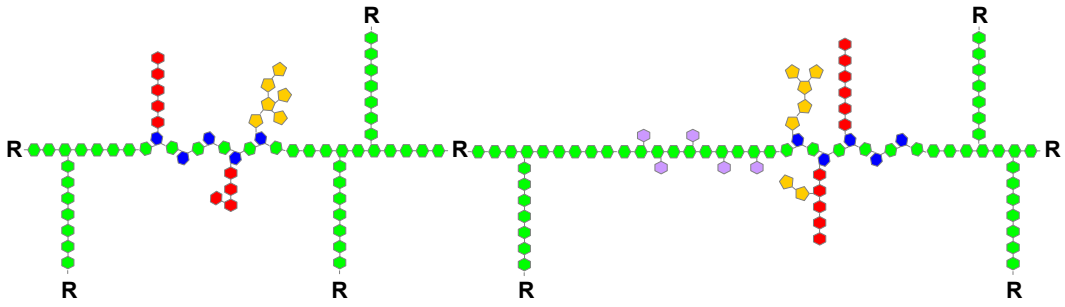
(A). Model 1 as proposed by Albersheim *et al*. (1996)



(B). Model 2 as proposed by Vincken *et al*. (2003a; 2003b)



(C). Model 3 as proposed by Schols *et al*. (2009)



(D). Model 4 as proposed by Round *et al*. (2010)

Figure 1-7: Schematic representation of four hypothetical models for the structure of a pectin molecule.

***Anionic cross-links*:** From a polymeric perspective, pectin is considered to be a complex, branched polyelectrolyte with a predominantly anionic nature. Many of the material properties of these anionic polysaccharides, especially HG, are determined by interactions with inorganic cations (Ca^{2+}) that are available from the apoplast of the cell (Jarvis, 1984; Morris *et al*., 1982). The ionic interactions between HG and Ca^{2+} lead to chain association and the formation of three-dimensional pectic-gel aggregates (Jarvis, 1984). A block sequence of at least ten de-methylated D-galacturonic acid residues is, however, required to build stable cross-links between HG chains (Figure 1-6 and Figure 1-8) (Vincken *et al*., 2003b). These pectic gels provide a crucial structural component of the cell-wall that contributes to the mechanical strength of the cell-wall matrix (Carpita & Gibeaut, 1993; Willats *et al*., 2001b).

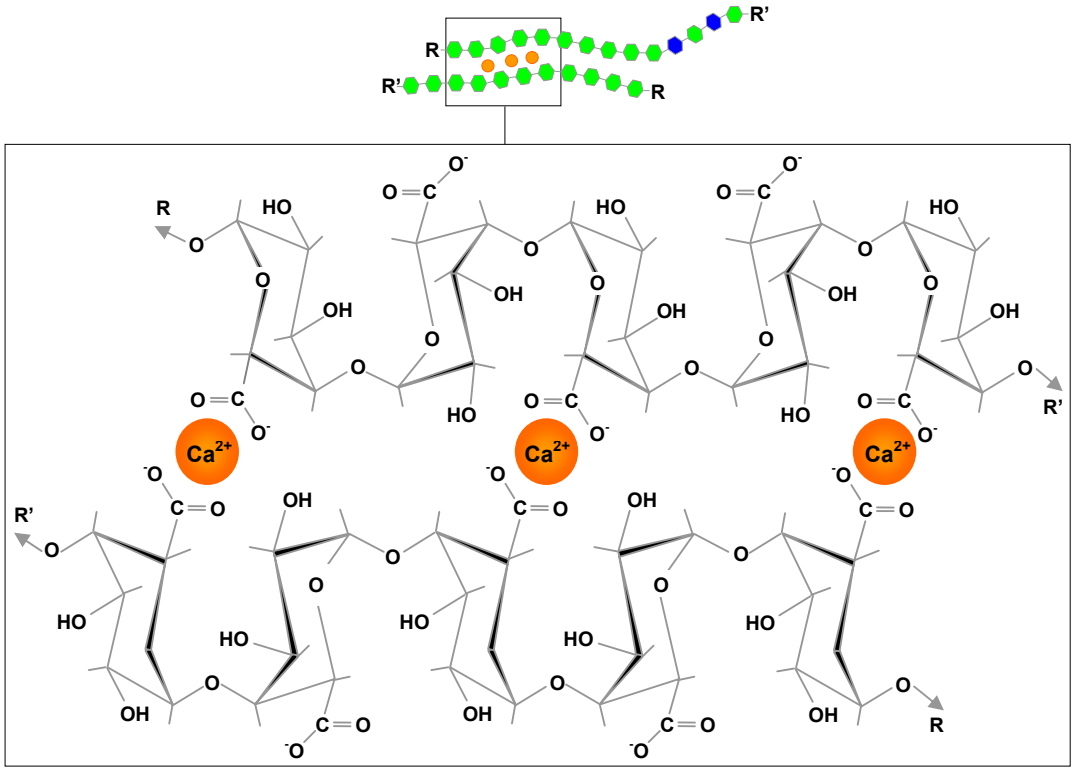


Figure 1-8: Linking of the de-esterified carboxyl groups of the galacturonosyl residues of two homogalacturonan chains through insertion of Ca^{2+} ions (adapted from Vincken *et al*., 2003b).

The "egg-box" model has become the established model to describe the interaction between Ca^{2+} ions and the de-esterified portions of HG chains (Morris *et al*., 1982). According to this model, the inter-junction segments are single chains while the junction zones are pairs of HG chains (Jarvis & Apperley, 1995; Morris *et al*., 1982). The carboxyl groups of two D-galacturonic acid residues in these junction zones, form

negatively charged pockets that can accommodate Ca^{2+} cations (Figure 1-8) (Willats *et al*., 2001a). These pairs of linked chains will aggregate into larger sheets, retaining the egg-box conformation when Ca^{2+} is available in excess (Morris *et al*., 1982).

***Borate-ester links*:** Rhamnogalacturonan-II predominantly exists as dimers, where two domains are covalently linked by means of borate-diol esters (Figure 1-6) (Ishii *et al*., 1999; Kobayashi *et al*., 1996). This domain is the only biological polysaccharide that contains boron (Fleischer *et al*., 1998; Ishii & Matsunaga, 1996; Kobayashi *et al.*,1996). Borate esters are formed between subunits with a β-D-erythro-furanose configuration such as the D-apiose occurring in RG-II (O'Neill *et al*. 2004; Pellerin *et al*., 1996). The cross-links are thus, formed between hydroxyl groups at C-2 and C-3 of the D-apiosyl residues in different of RG-II domains (Figure 1-9) (Ishii *et al*., 1999; Pellerin *et al*., 1996). A single borate cross-links the four hydroxyls of the D-apiosyl residues that are present in the dimer. The borate-diol links between different pectin chains thus, contributes to the formation of macromolecular pectic networks within the plant cell-walls (Ishii *et al*., 1999; O'Neill *et al*., 1996). It is not clear if the cross-linking occurs during synthesis of RG-II in the Golgi or if it occurs when RG-II is incorporated into the primary cell-wall (O'Neill *et al*., 2004). The widespread occurrence of these borate esters in cross-linked networks and its limited association with D-apiose suggest that boron is essential for the development, regulation and the mechanical and biological properties of a normal cell-wall (O'Neill *et al*., 1996).

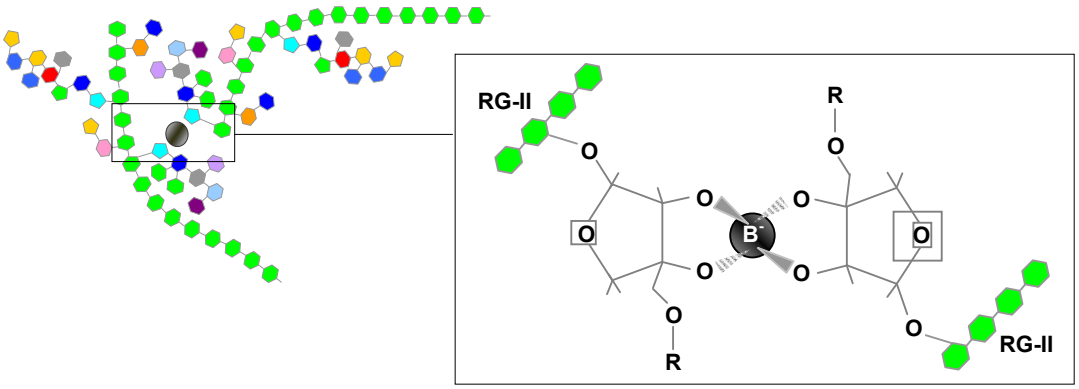


Figure 1-9: Schematic representation of the borate 1:2 diol ester that cross-links two D-apiose units of rhamnogalacturonan-II at the C-2 and C-3 position. "R" represents the rest of side-chain A that is linked to the D-apiose residue (adapted from Ridley *et al*., 2001).

Other cross-links

***Cellulose-hemicellulose network*:** Xyloglucan is the most abundant hemicellulose in the primary cell-walls of dicotyledons and is strongly linked to cellulose via hydrogen bonds to form cellulose-hemicellulose networks (Lima *et al*., 2004). These networks are embedded in a matrix of pectic polysaccharides, other hemicelluloses, lignin and proteins (Carpita & Gibeaut, 1993).

Recent studies proved that pectin is covalently linked to xyloglucan in the cell-walls of plants (Femenia *et al*., 1999; Popper & Fry, 2005; Thompson & Fry, 2000). It was suggested that these two polysaccharides are covalently linked via the arabinan side-chains of the RG-I domain (Figure 1-10) (Cumming *et al*., 2005; Thompson & Fry 2000). Both pectin and xyloglucan are synthesised in the Golgi apparatus and they have been regarded to be structurally independent polymers (Baydoun *et al*., 2001; Keegstra *et al*., 1973). During *in vitro* studies it was, however, demonstrated that nascent pectin present in the Golgi membranes was already linked to xyloglucan (Abdel-Massih *et al*., 2003). Further studies provided evidence that the pectin-xyloglucan complexes were a general feature of the cell-wall and that the nascent pectin-xyloglucan complexes remain linked during the further assembly of the cell-wall (Cumming *et al*., 2005).

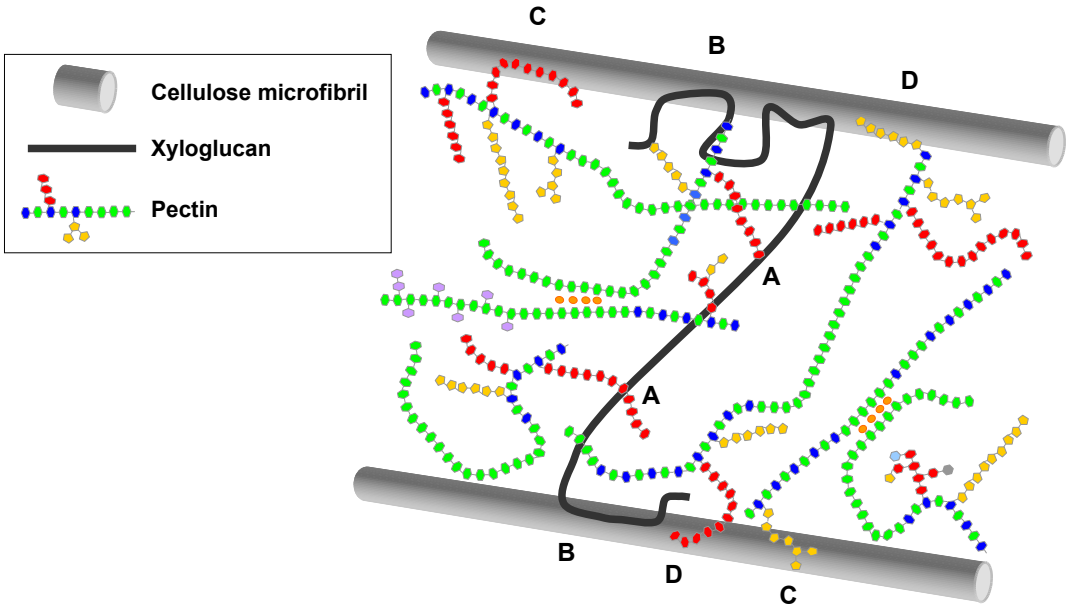


Figure 1-10: Schematic representation of the cellulose-hemicellulose networks that occur in primary cell-walls. The hydrogen links between the xyloglucan and pectin (A), cellulose microfibrils and xyloglucan (B), hypothetical connections between the cellulose microfibrils and the pectic polysaccharides (C) and side-chains of pectic molecules cross-linking two cellulose microfibrils (D) are illustrated (adapted from Zykwinska *et al*., 2005).

The neutral side-chains of pectin can be bound to cellulose (Figure 1-10) and the different pectic polymers that are part of this association have been identified through the treatment of cell-wall fragments with different enzymes (Oechslin *et al*., 2003; Vignon *et al*., 2004; Zykwinska *et al*., 2005). Both RG-I and XG were present in the cellulose fraction but the XG domain was only linked to the RG-I domain and not involved in the actual linkage to cellulose. It was subsequently proposed that the association between RG-I and cellulose is due to interactions of the galactan side-chains (Oechslin *et al*., 2003). Strong interactions also exist between the arabinan side-chains of RG-I and the crystalline domains of cellulose (Vignon *et al*., 2004). The extent of the binding of pectin to cellulose, therefore, varies with respect to the nature and the structure of the neutral side-chains of RG-I. The adsorption of linear arabinan side-chains was, for example, higher than that of galactan and branched arabinan side-chains (Vignon *et al*., 2004; Zykwinska *et al*., 2005). The bonding strength of the linear arabinan side-chains of RG-I and cellulose was also comparable to the strength of bonds between xyloglucan and cellulose (Zykwinska *et al*., 2005).

***Lignin*:** Lignin is a heterogeneous aromatic polymer that is embedded in the cellulosic network of the plant cell-wall. The incorporation of lignin into the cell-walls provides strength and stability to the conductive tissue as well as the entire plant body (Boerjan *et al*., 2003). The lignification of the plant cells starts at the end of cell growth in the cell corners and the middle lamella, which are rich in pectin, especially HG (Cathala *et al*., 2005; Donaldson, 2001; Wi *et al*., 2005). The D-galacturonic acids of this pectic domain can form covalent linkages with lignin subunits to form lignin carbohydrate complexes (Cathala *et al*., 2001). These lignin subunits are formed during the lignin polymerisation process when different mesomeric radicals polymerise with each other to form 8-*O*-4 quinone methide. This electrophilic compound reacts with the free carboxylic groups of the D-galacturonic acid residues, to form an ester linkage on the *O*-7 position of the lignin monomer resulting in the lignin-pectin complex (Figure 1-11) (Cathala & Monties, 2001). Formation of these linkages increases the solubility of the oligomer to promote further polymerisation reactions (Terashima *et al*., 1996).

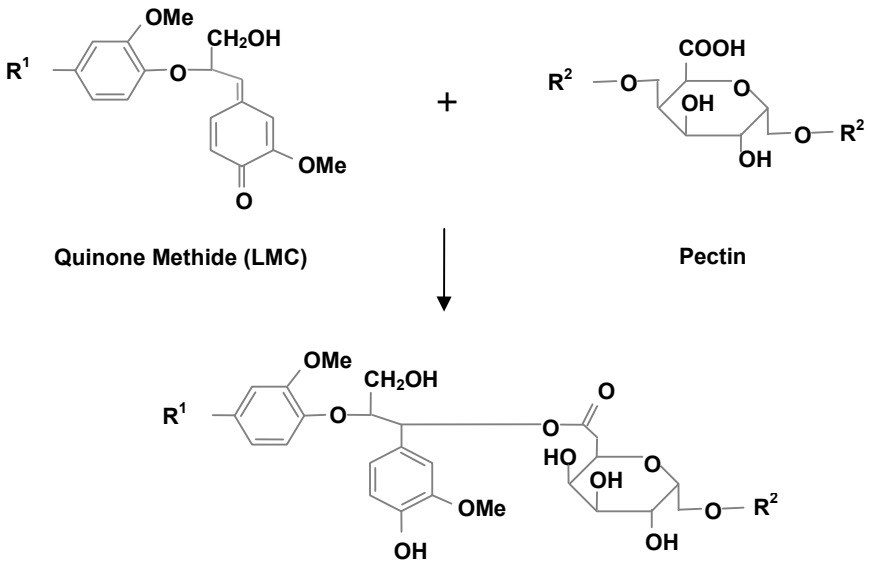


Figure 1-11: The formation of benzyl-ester linkages between a lignin model compound (LMC) and pectin via nucleophilic addition. R^1 represents the lignin polymer and R^2 the pectic polysaccharide (adapted from Cathala & Monties, 2001).

During experiments in which the first steps of lignification were reconstructed artificially in bacterial cells, it was found that the formation of cellulose-lignin composites was profoundly different in cells containing pectin (Cathala *et al*., 2005). The lignin nodules of the composites that contained pectin displayed a more dispersed distribution, which proved that pectin plays a major role in lignification (Cathala *et al*., 2005; Hafrén *et al*., 2000). It was also established that the pectin architecture in the middle lamella influenced the distribution of lignin in the cell-walls and that a relationship existed between the distribution of pectin and lignin in corresponding regions of the middle lamellae (Wi *et al*., 2005).

Proteins: A wide range of proteins and glycoproteins that are bound to the structural polysaccharides are present in the cell-wall (Cassab, 1998). The capacity of these proteins to bind to the pectic polysaccharides and influence its properties and functions has not been studied extensively, but proteins may play an important role in the functionality of pectin (Mollet *et al*., 2000; Willats *et al*., 2001a). It is, for example, known that the interaction between the RG-II domains of pectin and hydroxyproline-rich protein contributes to wall strength and integrity (Bonilla *et al*., 1997). Other proteins that are associated with pectins include extensins (Qi *et al*., 1995), cationic

isoperoxidases (Penel & Greppin, 1996) and a number of wall-associated kinases (Kohorn, 2000).

FUNCTION AND DISTRIBUTION OF THE PECTIC DOMAINS

The complex nature and the ubiquitous occurrence of pectin in the cell-walls, as well as the large number of pectin-modifying enzymes produced by plants and their pathogens reflects the importance of pectin in plant physiology (Willats *et al*., 1999b; Williams & Benen, 2002). These polysaccharides are multifunctional, but it is difficult to relate specific structural domains within the pectic network to a wider biological function in the growth and development of plant cells. The difficulty arises from fact that the properties of the different domains, the position and linkage between domains (Bonnin *et al*., 2002a; Yapo *et al*., 2007) and the interactions with other macromolecules and smaller entities contribute to matrix properties (Willats *et al*., 2001a). Two other important issues concerning the function pectic polysaccharide domains include their distribution within tissues and their location in the cell-wall (Willats *et al*., 2001b).

Function and distribution of homogalacturonan

The HG domain is implicated in influencing a number of cell-wall properties that have an impact upon cell expansion, cell development, intercellular adhesion and separation, as well as defence mechanisms (Jarvis, 1984; Willats *et al*., 2001b). The ability of this domain to form pectic gels also plays an important role in maintaining the structural and physiological integrity of the cell-wall and the middle lamella. An example of structural importance of pectic gels is their role in resistance to turgor pressure (Jarvis, 1984). One of the physiological functions of pectic gels is its contribution to the porosity of the cell-wall (Rondeau-Mouro *et al*., 2008). Pectic gels also assist to maintain intercellular adhesion between plant cells at the middle lamella (Jarvis, 1984; Willats *et al*., 2001b).

The capacity of HG to participate in the formation of gels is regulated by pectin methylesterase. The de-esterification of this domain is a complex and highly regulated process that occurs in distinct regions of the cell-walls or tissues, resulting in differences in methyl-esterification between regions (Femenia *et al*., 1998). De-esterified HG is generally localised on the inner surface of the primary cell-walls adjacent to the plasma membrane, in the middle lamella, the cell corners and around intercellular spaces. In contrast, methyl-esterified HG is distributed throughout the

cell-wall (Bush & McCann, 1999; Knox *et al*., 1990; Orfila & Knox, 2000). Changes in methyl-esterification of certain areas of this domain are also correlated with specific developmental stages of the plants for example during fruit ripening (Willats *et al*., 1999a; 1999b).

The function of HG can also be influenced through other types of biochemical modification and the location of these modifications in the cell-wall determines the function of this domain. The acetylation and methyl-esterification of HG is, for example, known to limit the enzymatic breakdown of pectin (Renard & Jarvis, 1999). High levels of substitution are also associated with reduced gelling and a consequent decrease in intercellular adhesion (Sobry *et al*., 2005). The HG also plays an important role in intramolecular signalling, because this domain is the source of biologically active oligogalacturonides, which function in plant morphogenesis and as elicitors in the host-pathogen interactions (Darvill *et al*., 1992; Weber *et al*., 1996).

Function and distribution of rhamnogalacturonan-I

The RG-I domain is highly variable in its fine structure and its distribution within the cell-walls. The most significant feature of this domain is that there is no conserved RG-I structure in cell-walls (Bush & McCann, 1999; Willats *et al*., 1999b) and forms of this domain that differ in the ratio of galactan and arabinan occur (Ermel *et al*., 2000; McCartney *et al*., 2000; Willats *et al*., 1999b).

The second significant feature of RG-I is that galactan-rich and arabinan-rich forms have restricted locations within the cell-wall (Willats *et al*., 2001a). A consistent feature of galactan-rich RG-I is that it is absent from cell-walls in the regions of pit fields containing plasmodesmata. Rhamnogalacturonan-I that is rich in arabinan is, however, abundant close to pit fields (Bush & McCann, 1999; Orfila & Knox, 2000) reflecting the specific mechanical properties around the plasmodesmata (Willats *et al*., 2001a). Rhamnogalacturonan-I that is rich in galactan is also most abundant in meristematic cells, while a form that is rich in arabinan only appears during cell differentiation (Vicré *et al*., 1998; Willats *et al*., 1999b). The development of side-chains in this domain is, therefore, related to the regulation of mechanical properties of the cell-wall.

The arabinan and galactan side-chains of RG-I are also involved in intercellular adhesion (together with de-methylated HG). Electron microscopy showed that the concentration of pectin in weakly-attached mutant callus tissue was very low compared

to the normal callus tissues of *Nicotiana plumbaginifolia* (Iwai *et al*., 2001). The ratio of L-arabinose to D-galactose was also low in pectins isolated from the cell-walls in the mutant callus (Iwai *et al*., 2001; Popper & Fry, 2005; Thompson & Fry, 2000). This L-arabinose-rich RG-I was also strongly associated with cellulose-xyloglucan complexes (Popper & Fry, 2005; Thompson & Fry, 2000).

Function and distribution of rhamnogalacturonan-II

The RG-II domain occurs widely in all primary cell-walls, but is absent from the middle lamellae (Williams *et al*., 1996). The widespread occurrence of RG-II in cell-walls and its conserved structure indicates that this domain is essential in the development of normal cell-walls and in the regulation of the mechanical and biological properties of the cell-wall (O'Neill *et al*., 1996; 2001). Rhamnogalacturonan-I is also the only pectic domain that may play a role in the binding of hydroxyproline-rich proteins to the cell-wall (Bonilla *et al*., 1997) and this domain, therefore, functions to maintain normal cell size and growth (Showalter, 1993).

The inclusion of boron in the ester cross-links between RG-II fully accounts for the boron requirement for normal plant growth and development (Fleischer *et al*., 1998; 1999). These cross-links play an important role in the regulation and maintenance of cell-wall properties such as pore structure generation of size-exclusion limits (Fleischer *et al*., 1999). Cells that lack this ability are unable to control absorption and undergo excessive cell enlargement that may lead to cell-wall rupture (Fleischer *et al*., 1998). The presence of the cross-linked complexes in the pectic network is also important to ensure that the cell-wall matrix is packed in a thin wall (Ishii *et al*., 2001; Vidal *et al*., 1999; Willats *et al*., 2001a). Studies with biosynthetic mutants proved that the tensile properties of the cell-wall depend on the cross-linking of RG-II (Ryden *et al*., 2003).

Function and distribution of xylogalacturonan

Xylogalacturonan occurs in various types of plant tissue, but until recently, this domain was only reported from storage and reproductive tissue of plants (Albersheim *et al*., 1996; Le Goff *et al*., 2001; Nakamura *et al*., 2002; Schols *et al*., 1995; Vincken *et al*., 2003b; Zandleven *et al*., 2006). This domain is associated with the loosely attached inner parenchyma of these tissues, indicating that XG is specifically associated with separation of cells that occurs in abscission zones (Willats *et al*., 2004). This domain also occurs in the cell-walls of other than storage or reproductive tissue. Xylogalacturonan was recently isolated from the stems, roots, seeds and

leaves of *Arabidopsis thaliana* (Zandleven *et al*., 2007). Further investigation into the presence of XG in other plant varieties is, however, necessary to elucidate the relationship between the structure and function of this domain.

BIOSYNTHESIS OF PECTIN

The structure of pectin in the cell-wall is the result of two processes, namely biosynthesis within the intracellular membrane system and its modification in the cell-wall after synthesis (Doong *et al*., 1995). The specific enzyme activities in membrane fractions and the use of antibodies against pectin epitopes established that pectin biosynthesis occurs in the Golgi vesicles (Moore *et al*., 1991). Pectin synthesis occurs in an assembly line across the Golgi stacks in a process mediated by enzymes localised in specific areas of these stacks (Mohnen, 2008).

Polymerisation starts in the *cis*-Golgi stack and continues into the *medial*-Golgi (Moore *et al*., 1991), while esterification occurs in the *medial*- and *trans*-Golgi. The more extensive branching of pectin also occurs in the *trans*-Golgi (Zhang & Staehelin, 1992). The newly synthesised polysaccharides are then transported to the plasma membrane in Golgi-derived vesicles and are inserted into the wall, often as highly methyl-esterified polymers (Carpita & Gibeaut, 1993; Dolan *et al*., 1997). Due to the complexity of the pectic polysaccharides, a large number of biosynthetic enzymes are required for its assembly, including at least 67 glycosyltransferases and a number of methyltransferases and acetyltransferases (Mohnen, 2008). In addition to these biosynthetic enzymes, other auxiliary enzymes are involved in the stepwise synthesis of pectin (Figure 1-12).

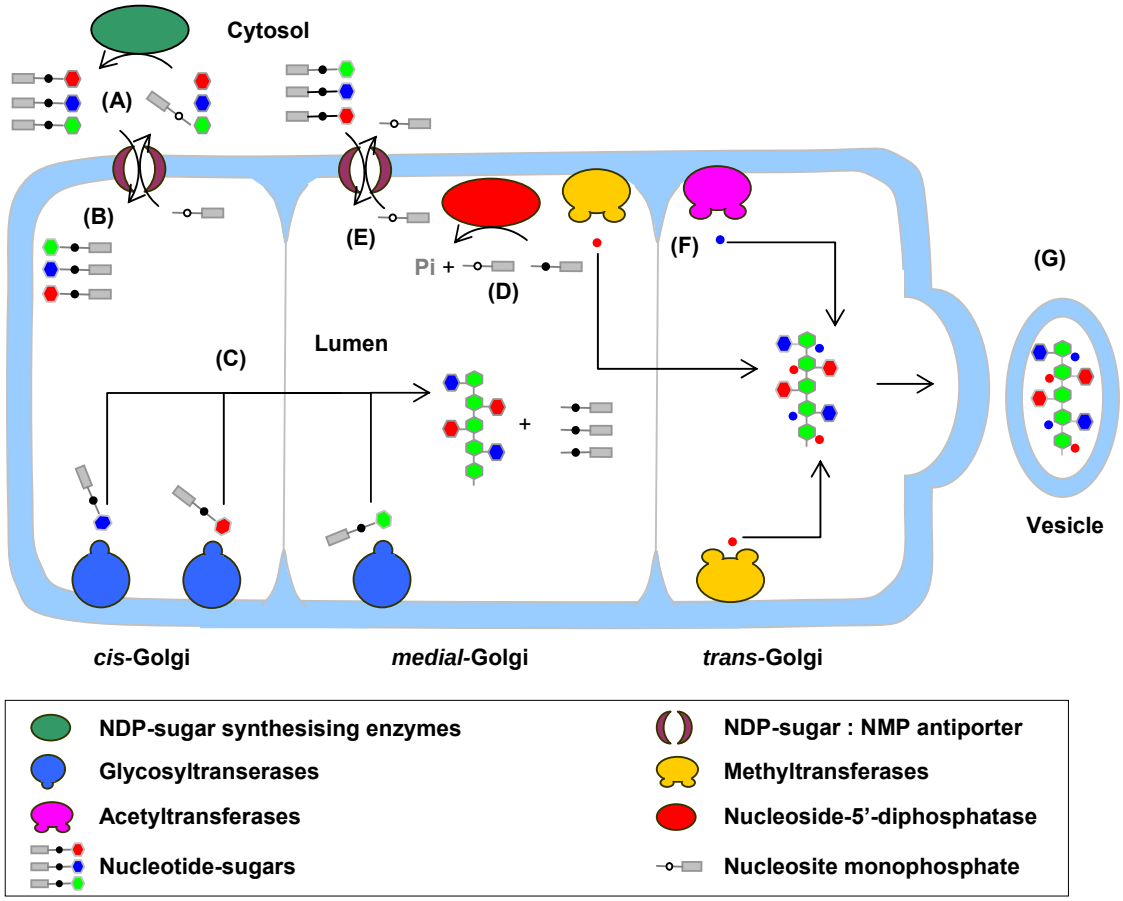


Figure 1-12: Schematic representation of pectin biosynthesis that occurs in the Golgi apparatus.

The nucleotide-sugars that act as substrates for the glycosyltransferases are produced on the cytosolic side of the Golgi through the action of nucleotide-sugar synthesising enzymes (Figure 1-12 A). The nucleotide-sugars are transported across the Golgi membrane into the lumen through specific nucleotide-sugar : nucleosite monophosphate antiporters (Figure 1-12 B) (Muňoz *et al*., 1996; Orellana *et al*., 1997).

The Golgi lumen is the site where the transferase reactions and the modifications of the polysaccharide backbones and side-chains occur. The glycosyltransferases act on specific nucleotide-sugars and transfer single glycosyl residues from the substrate to the non-reducing end of the growing polysaccharide chain (Figure 1-12 C) (Mohnen, 1999). The released nucleoside diphosphate is hydrolysed by a nucleoside-5'-diphosphatase into nucleoside monophosphate and inorganic phosphate (Figure 1-12 D), which is transported out of the Golgi into the cytosol via the nucleotide-sugar : nucleosite monophosphate antiporter (Figure 1-12 E)

(Orellana *et al*., 1997). Some of the glycosyl residues of polymerised product are modified with methyl or acetyl-substituents through the action of either methyltransferases or acetyltransferases (Figure 1-12 F) before it is released in Golgi-derived vesicles (Figure 1-12 G) to the cell-wall (Ishikawa *et al*., 2000; Pauly & Scheller, 2000).

The process described above is a general overview of pectin biosynthesis and apply to all four pectic domains. The specific transferases, their receptors and their substrates involved in the biosynthesis of the specific domains will be described in the following sections.

Biosynthesis of homogalacturonan

At least three enzymes, namely a homogalacturonan-galacturonosyltransferase, a homogalacturonan-methyltransferase and a homogalacturonan-acetyltransferase are involved in the synthesis of HG (Bourlard *et al*., 1997b; Ohashi *et al*., 2007; Pauly & Scheller, 2000; Sterling *et al*., 2006; Villemez *et al*., 1965). The substrates and acceptors for these enzymes are summarised in Appendix 1-A. The homogalacturonan-galacturonosyltransferase is a membrane bound enzyme located in the Golgi lumen (Goubet & Mohnen, 1999a; Sterling *et al*., 2006) and it requires oligomers with more than nine galacturonides to act as exogenous acceptors (Doong & Mohnen, 1998; Scheller *et al*., 1999).

The HG is deposited in the cell-wall in a form where 70 to 80% of the D-galacturonic acid residues are methyl-esterified on the C-6 carboxyl groups (Mohnen, 1999; O'Neill *et al*., 1990). This methyl-esterification functions to prevent premature cross-linking with Ca^{2+} during the secretion of the HG into the cell-wall (Kim & Carpita, 1992). The amount and the pattern of methyl-esterification play an important role in the growth and development of the cell-wall. The degree of methyl-esterification of this domain is controlled by a homogalacturonan-methyltransferase in the Golgi (Vannier *et al*., 1992). This enzyme is bound to the membrane on the luminal side of the Golgi (Goubet *et al*., 1998) and a number of different homogalacturonan-methyltransferases are required for the synthesis of HG, but their substrate specificities have not been determined yet (Goubet & Mohnen, 1999b; Sterling *et al*., 2006). At least one homogalacturonan-methyltransferase shows a preference for partially esterified HG in stead of polygalacturonic acid (Ishikawa *et al*., 2000). It is thus, possible that different

isoforms may exist that either initiates the methyl-esterification or enhances the further methyl-esterification of HG.

Coordination of homogalacturonan-methyltransferase and homogalacturonan-galacturonosyltransferase is an important aspect of the synthesis and methyl-esterification of HG. The activity of the homogalacturonan-methyltransferase is stimulated by the addition of 5'-diphosphate-D-galacturonic acid, indicating that the monomeric components must be present or the actual synthesis of HG must be in process to induce endogenous methyl-esterification (Goubet *et al*., 1998). The synergetic action of these two enzymes, (either sequential or as a complex), is supported by the fact that both enzymes are situated in the lumen of the Golgi (Goubet & Mohnen, 1999b; Sterling *et al*., 2006).

It is still unclear whether homogalacturonan-acetyltransferase is responsible for the acetylation of the D-galacturonic acid residues at the *O*-2 and/or *O*-3 position of HG. Alternatively, acetylation may be due to acetyltransferases that acts on one RG-I or RG-II that are covalently linked to the HG (Pauly & Scheller, 2000; Ridley *et al*., 2001).

Biosynthesis of xylogalacturonan

The XG is a portion of HG that is highly substituted on C-3 and C-4 with D-xylose (Mohnen, 2008) and it is, therefore, speculated that the backbone of this domain is synthesised by the same galacturonosyltransferase that is responsible for HG synthesis (Doong *et al*., 1995; Doong & Mohnen, 1998, Mohnen *et al*., 2008). The backbone is then substituted with D-xylose by different xylogalacturonan-xylosyltransferases (Jensen *et al*., 2008; Scheller *et al*., 2007). The specific enzyme activities that are involved in the biosynthesis of XG and their substrates and acceptors are summarised in Appendix 1-B.

Biosynthesis of rhamnogalacturonan-I

The biosynthesis of RG-I is not as well understood as that of HG, because of the complexity of the backbone and the highly branched nature of its side-chains. Antibodies that were directed against RG-I epitopes indicated that synthesis of this domain is initialised in the *cis*-Golgi and continues into the *medial*-Golgi (Zhang & Staehelin, 1992). At least two types of glycosyltransferases, namely galactosyltransferases (Geshi *et al*., 2000) and arabinosyltransferases (Skjøt *et al*.,

2002) are involved in the biosynthesis of this domain (Appendix 1-C). A methyltransferase (Bourlard *et al*., 1997a) as well as an *O*-acetyltransferase (Pauly & Scheller, 2000) may also be involved in the substitution of some of the backbone-residues of RG-I (Appendix 1-C).

The backbone of RG-I is the only plant polysaccharide that is composed of repeating disaccharide units (Lau *et al*., 1985). No evidence exists for this backbone of repeating D-galacturonic acid and L-rhamnose units to be synthesised by glycosyltransferases and the mechanism for synthesis is, therefore, still unknown. The side-chains of RG-I predominantly consist of linear and branched α-(1→5)-L-arabinose and β-(1→4)-D-galactose residues (Albersheim *et al*., 1996; Mohnen, 1999) and different isoforms of galactosyltransferase and arabinosyltransferase are required for the initiation as well as elongation of these side-chains (Geshi *et al*., 2000; Pauly & Scheller, 2000). The galactosyltransferases are associated with the membranes of a number of plants (Geshi *et al*., 2000; Goubet & Morvan, 1994; Ishii *et al*., 2004). A rhamnogalacturonan-I arabinosyltransferase has also been isolated and characterised recently (Harholt *et al*., 2006).

An enzyme with possible rhamnogalacturonan-I methyltransferase activity has been identified, but it is not clear if the methyl-esterification occurred on the backbone of RG-I or HG (Bourlard *et al*., 1997a; Ridley *et al*., 2001). It is thus, possible that the activity may result from the methyl-esterification of HG tails that are covalently linked to RG-I. The D-galacturonic acid residues in the RG-I backbone is highly acetylated at the *O*-2 and *O*-3 positions (Komalavilas & Mort, 1989; Schols & Voragen, 1994) and the reaction is catalysed by a rhamnogalacturonan-I acetyltransferase (Pauly & Scheller, 2000). All the enzyme activities that are involved in the biosynthesis of RG-I are summarised in Appendix 1-C.

Biosynthesis of rhamnogalacturonan-II

The biosynthesis of RG-II also requires the action of the three groups of transferasese. The first group consists of at least 22 different glycosyltransferases, which accounts for almost half of the number of glycosyltransferases that are required for pectin synthesis (Ridley *et al*., 2001; O'Neill *et al*., 2004). The second and third groups include a number of methyltransferases (Bourlard *et al*., 1997a) and *O*-acetyltransferases (Pauly & Scheller, 2000) that are involved in the substitution of the backbone and some of the side-chain residues of RG-II. The different forms of

these enzymes and their substrates and acceptors are described in Appendix 1-D. The backbone of RG-II is possibly synthesised by the same galacturonosyltransferase that is responsible for synthesis of HG (Ridley *et al*., 2001; Mohnen *et al*., 2008). The four side-chains that are attached to this backbone are also synthesised by glycosyltransferases that transfer the monosaccharide constituents of the side-chains from the activated precursors onto the non-reducing end of acceptors (O'Neill *et al*.,2004). Only three (Scheller *et al*., 2007) of these glycosyltransferases, namely a glucuronsosyltransferase (Iwai *et al*., 2002), and two xylosyltransferases (Egelund *et al*., 2006) have been isolated and identified.

The side-chains of RG-II consist of thirteen different monosaccharides, as described above. Ten of these monosaccharide constituents are transferred from nucleotide-sugar precursors (Reiter & Vanzin, 2001; Tanner, 2001) and the remaining three are transferred from other activated molecules. The first of these three monosaccharides is 3-*C*-carboxy-5-deoxy-L-xylose and its activated form has not yet been identified. This residue is structurally related to 5-deoxy-3-*C*-formyl-L-*lyxo*-furanose and it is possible that the active precursors of these molecules are synthesised in similar ways (Yamese *et al*., 2000). The activated form of the second of these monosaccharides, namely of 2-keto-3-deoxy-D-*manno*-octulosonic acid is synthesised from phosphoenol pyruvate and D-arabinose-5-phosphate. The third monosaccharide (3-deoxy-D-*lyxo*-heptulosaric acid) is structurally related to 2-keto-3-deoxy-D-*manno*-octulosonic acid and these two monosaccharides may, thus, have similar biosynthetic origins (O'Neill *et al*., 2004).

The methyltransferase and *O*-acetyltransferase responsible for the substitution of HG are possibly also involved in the methyl-esterification and acetylation of the backbone of RG-II (Ridley *et al*., 2001). The side-chains of RG-II are also substituted by different methyltransferase and *O*-acetyltransferase isoforms. Single monosaccharides in the side-chains are methyl-esterified by pectin methyltransferases that use the L-fucose and D-xylose residues in the side-chains as acceptors (Bourlard *et al*., 1997a). The activity of two *O*-acetyltransferases that substitute the 2-*O*-methyl fucosyl and 3-*C*-carboxy-5-deoxy-L-xylose residues in the side-chains of RG-II have also been confirmed (Whitecombe *et al*., 1995).

A number of questions concerning the primary structure of RG-II and its distribution in the pectic polymer need to be addressed to get a completed

understanding of the biosynthesis of this domain. It is also not clear whether the RG-II molecule is synthesised by the sequential transfer of monosaccharides onto HG backbone or whether the oligosaccharide side-chains are assembled and then transferred to the backbone (O'Neill *et al*., 2004).

ENZYMATIC DEGRADATION OF PECTIN

The adhesion between and separation of neighbouring cells are fundamental characteristics of all multicellular organisms and are central aspects to plant morphogenesis. Pectic networks are, therefore, important, since they are actively involved in cell adhesion. Pectins are specifically localised in the primary cell-walls, cell corners and middle lamellae to be effective in this role (Knox, 1992; Lord & Mollet, 2002). Plant cells undergo dramatic changes in shape and developmentally-regulated cell separation when these networks are disassembled. The pectic network is thus, a target for specific modifications during developmental processes (Jarvis *et al*., 2003; Ridley *et al*., 2001; Roberts *et al*., 2000). The pectin in the cell-walls is also a major target for degradation during the pathogenic attack by bacteria and fungi and pectinases are in many cases the factors that determine the virulence of pathogens (Rogers *et al*., 2000; Willats *et al*., 2001a).

Due to its complex structure, the modification and disassembly of the pectin polymer requires the combined action of numerous pectinolytic enzymes. These enzymes can be divided into the esterases and the depolymerases (Hoondal *et al*., 2002; Kashyap *et al*., 2001). Pectin esterases hydrolyse methyl and acetyl groups from pectic domains (Oosterveld *et al*., 2000; Willats *et al*., 1999a; 2001b), while the depolymerases hydrolyse the glycosidic bonds between saccharide moieties. The depolymerases include the lyases and the hydrolases (Hoondal *et al*., 2002; Kashyap *et al*., 2001). The lyases cleave the glycosidic bonds in polysaccharide chains by means of a β-elimination mechanism, which results in the formation of a double bond between the C-4 and C-5 at the non-reducing end of the polymer (Kashyap *et al*., 2001; Solbak *et al*., 2005; Soriano *et al*., 2006). The hydrolases degrade the pectic backbone and side-chains by the hydrolytic cleavage of the glycosidic bonds (Markovič & Janeček, 2001).

Degradation of homogalacturonan

The pectinolytic enzymes that are responsible for the degradation of HG are summarised in Table 1-1 and a schematic representation of their points of attack is presented in Figure 1-13. Under the esterases, pectin methylesterase hydrolyses the methyl-ester groups attached to the C-6 of the D-galacturonic acid residues of HG to release methanol (Willats *et al*., 1999a; 2001b). This enzyme does not have the ability to solubilise the cell-walls by itself, but reduces the degree of methyl esterification, thus regulating the cleavage of this domain by other pectinases (Steele *et al*., 1997; Willats *et al*., 2001b). De-esterification also facilitates the formation of pectic gels through calcium bridges between stretches of de-esterified HG (Morris *et al*., 1982). Pectin acetylesterase specifically cleaves acetyl-ester groups from the *O*-2 and/or *O*-3 of the D-galacturonic acid residues of HG (Oosterveld *et al*., 2000; Vercauteren *et al*., 2002). The action of these enzymes diminishes the hydrophobic nature of pectin to increase its solubility in water. The removal of the acetyl groups from the linear polymer also makes the polysaccharide more accessible to the action of other pectinolytic enzymes (Vercauteren *et al*., 2002).

Table 1-1: Classification of different enzymes involved in the modification and degradation of homogalacturonan.

		Pectinolytic enzymes	**References**
Esterases		Pectin methylesterase (EC 3.1.1.11)	Willats *et al*., 1999a; 2001b
		Pectin acetylesterase (EC 3.1.1.6)	Oosterveld *et al*., 2000; Vercauteren *et al*., 2002
Depolymerases	**Lyases**	Pectate lyases	
		Endo-pectate lyase (EC 4.2.2.2) *Exo*-pectate lyase (EC 4.2.2.9)	Soriano *et al*., 2006 Payasi *et al*., 2006 Soriano *et al*., 2006 Payasi *et al*., 2006
		Pectin lyase (EC 4.2.2.10)	Sakiyama *et al*., 2001
	Hydrolases	Polygalacturonases	
		Endo-polygalacturonase (EC 3.2.2.15) *Exo*-polygalacturonase (EC 3.2.1.67)	Bonnin *et al*., 2002b Hadfield & Bennett, 1998 Markovič & Janeček, 2001 Kluskens *et al*., 2005

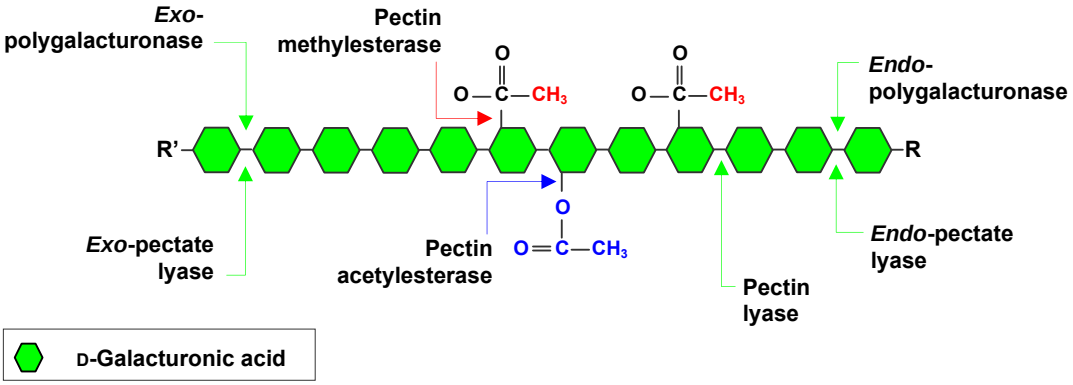


Figure 1-13: Schematic representation of homogalacturonan indicating the point of attack for different pectinases involved in its degradation.

Endo-pectate lyase and *exo*-pectate lyase are specific to HG, while pectin lyase apparently also acts on other pectic domains (Niture *et al*., 2006; Payasi *et al*., 2006; Sakiyama *et al*., 2001). Pectin lyase is the only pectinolytic enzyme that cleaves the α-(1→4)-linkages of highly esterified polygalacturonic acid (Figure 1-13) without being preceded by the action of other enzymes such as pectin methylesterase and pectin acetylesterase (Sakiyama *et al*., 2001). The two pectate lyases, however, only catalyse the cleavage of α-(1→4)-linkages of a demethylated backbone (Figure 1-13) (Payasi *et al*., 2006). Both pectin lyase and pectate lyases produce α-Δ-(4,5)-unsaturated oligogalacturonates (Solbak *et al*., 2005) and are even able to depolymerise pectic gels despite the presence of calcium bridges (Marín-Rodríguez *et al*., 2002).

Polygalacturonases are the most abundant enzymes that degrade HG and have both *exo* and *endo* activities. These enzymes catalyse the hydrolytic cleavage of the glycosyl bonds of α-(1→4)-D-polygalacturonan (Hadfield & Bennett, 1998). *Exo*-polygalacturonase cleaves the HG from the non-reducing end of the molecule (Figure 1-13) to release monogalacturonic acid units (Kluskens *et al*., 2005; Markovič & Janeček, 2001). *Endo*-polygalacturonase attacks HG randomly at the (1→4)-linkages between the adjacent α-D-galacturonic acid residues (Figure 1-13) to produces D-galacturonic acid oligosaccharides of different sizes (Bonnin *et al*., 2002b; Hadfield & Bennett, 1998). The activity of *endo*-polygalacturonase decreases with an increase in the degree of methyl-esterification of the HG domain and requires a sequence of non-esterified D-galacturonic acid residues to attack (Daas *et al*., 2000). The combined action of *endo*-polygalacturonase and pectin methylesterase is thus, necessary for the extensive degradation of HG (Bonnin *et al*., 2002b).

Degradation of rhamnogalacturonan-I

The pectinolytic enzymes responsible for the degradation of RG-I are summarised in Table 1-2 and a schematic representation of their points of attack is presented in Figure 1-14. A number of esterases are likely to be involved in the degradation of RG-I, but only rhamnogalacturonan acetylesterase has been characterised. Rhamnogalacturonan acetylesterase cleaves acetyl groups from the *O*-2 and/or *O*-3 of D-galacturonic acid residues on the RG-I backbone (Mølgaard *et al*., 2000; Oosterveld *et al*., 2000) (Figure 1-14 A). The action of this enzyme reduces the hydrophobic nature of the pectin backbone to increase its solubility in water and makes RG-I more accessible to the action of other pectinolytic enzymes (Vercauteren *et al*., 2002).

Table 1-2: Classification of different enzymes involved in the modification and degradation of rhamnogalacturonan-I.

		Pectinolytic enzymes	**References**
Esterases		Rhamnogalacturonan acetyl esterase (EC 3.1.1.-)	Mølgaard *et al*., 2000; Oosterveld *et al*., 2000
Depolymerases	**Lyases**	*Endo*-rhamnogalacturonan lyase (EC 4.2.2.-)	McDonough *et al*., 2004; Pagès *et al*., 2003
	Hydrolases	*Endo*-rhamnogalacturonan hydrolase (EC 3.2.1.-)	McDonough *et al*., 2004; Pagès *et al*., 2003
		Exo-rhamnogalacturonan rhamnohydrolase (EC 3.2.1.40)	Mutter *et al*., 1994; 1998a
		Exo-rhamnogalacturonan galacturonohydrolase (EC 3.2.1.-)	Mutter *et al*., 1994; 1998a
		Arabinase	Lazan *et al*., 2004
		Endo-α-L-arabinase (EC 3.2.1.99)	Beldman *et al*., 1997; Ralet *et al*., 2005
		Exo-arabinase (EC 3.2.1.-)	Beldman *et al*., 1997; Ralet *et al*., 2005
		α-L-arabinofuranosidases (EC3.2.1.55)	Saha, 2000
		Galactanase	Ralet *et al*., 2005
		Endo-(1,4)-β-D-galactanase (EC 3.2.1.89)	Saha, 2000
		Endo-(1,6)-β-D-galactanase (EC 3.2.1.164)	Ralet *et al*., 2005
		Exo-(1,3)-β-D-galactanase (EC 3.2.1.145)	Ralet *et al*., 2005
		Exo-(1,4)-β-D-galactanase (EC 3.2.1.-)	Ralet *et al*., 2005
		β-D-galactosidase (EC 3.2.1.23)	Lazan *et al*., 2004

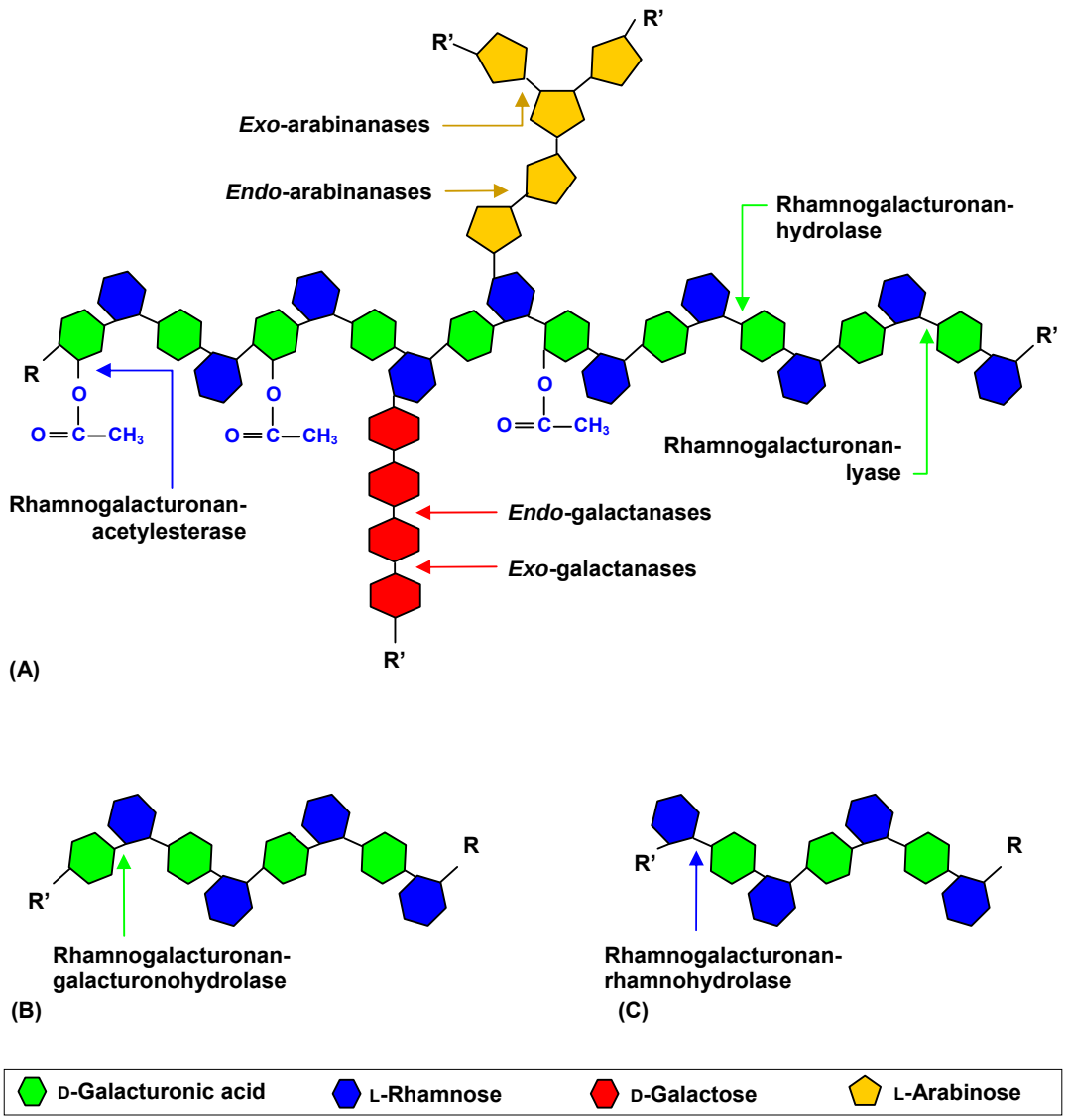


Figure 1-14: Schematic representation of rhamnogalacturonan-I indicating the point of attack for different pectinases involved in its degradation.

The depolymerases attack glycosidic bonds both in the backbone and in the different side-chains of RG-I. The depolymerases that act on the backbone are divided into the rhamnogalacturonan lyases and the rhamnogalacturonan hydrolases (Mutter *et al*., 1998c). Rhamnogalacturonan lyases specifically recognise the α-(1→4) glycosidic bonds between L-rhamnose and D-galacturonic acids in the backbone (Figure 1-14 A) (Mutter *et al*., 1996). The action of this enzyme results in the formation of α-Δ-(4,5)-unsaturated RG-I oligosaccharides that terminates at the non-reducing end with a hex-4-enopyranosyluronic acid residue (Mutter *et al*., 1996; 1998b).

The three hydrolases that cleave the backbone of RG-I include rhamnogalacturonan hydrolase, rhamnogalacturonan rhamnohydrolase as well as rhamnogalacturonan galacturonohydrolase (Ralet *et al*., 2005). Rhamnogalacturonan hydrolase cleaves α-(1→2)-glycosidic bonds between L-rhamnose and D-galacturonic acids in an *endo* fashion (Figure 1-14 A) (McDonough *et al*., 2004). The other two hydrolases are *exo*-enzymes and remove single monosaccharide residues from the non-reducing ends of RG-I. The rhamnogalacturonan rhamnohydrolase releases L-rhamnose residues (Mutter *et al*., 1994) and rhamnogalacturonan galacturonohydrolase release D-galacturonic acid residues (Mutter *et al*., 1998a) from the backbone (Figure 1-14 B and Figure 1-14 C) of this domain.

The side-chains of RG-I are degraded by two groups of hydrolases that specifically act on the different side-chains in this domain. The first group acts on the arabinan-containing side-chains and include the arbinanases and arabinofuranosidases (Beldman *et al*. 1997). *Endo*-α-L-arabinanase randomly act on the α-(1→5)-linkages in the arabinan side-chains (Figure 1-14 A) (Beldman *et al*., 1997; Ralet *et al*., 2005). The oligosaccharides that is released by this enzyme are further hydrolysed by α-L-arabinofuranosidases that act on the α-(1→5) and α-(1→3)-linkages (Saha, 2000). *Exo*-arabinanases release monomeric L-arabinose from the non-reducing end of both α-(1→5) and α-(1→3)-linked arabinan (Figure 1-14 A) (Beldman *et al*., 1997; Ralet *et al*., 2005).

The second group of hydrolases acts on the galactan-containing side-chains of RG-I and include three groups of enzymes, namely *endo* and *exo*-galactanases and a galactosidase. The *endo*-galactanases include a β-D-galactanase that randomly hydrolyse (1→4)-linkages, as well as a galactanase that might hydrolyse the (1→6)-linkages. The resulting galactan and arabinogalactan oligosaccharides are further hydrolysed by *exo*-galactanases and a galactosidase. The *exo*-galactananses are responsible for the hydrolysis of the (1→3) and (1→4)-linkages from the non-reducing end of the galactan chains or released oligosaccharides (Ralet *et al*., 2005) (Figure 1-14 A), while the β-D-galactosidase acts from the reducing ends to release D-galactose residues (Lazan *et al*., 2004).

Degradation of rhamnogalacturonan-II

Commercial pectinases were used to isolate RG-II from different sources and since the structure was similar for all products, this domain was described as an enzyme resistant fraction (Doco *et al*., 1997; Vidal *et al*., 1999). This resistance against enzymes may reflect the prevalence of the unusual linkages and sugar residues in the molecule, combined with a steric hindrance caused by a high level of ramification. However, the abundance and ubiquity of RG-II indicates that corresponding hydrolytic enzymes should be present in natural ecosystems. A strain of *Penicillium daleae* has, for example, been found with the ability to degrade monomeric RG-II (Vidal *et al*., 1999). This organism is thus a potential source of unidentified pectinases with the ability to degrade RG-II.

Degradation of xylogalacturonan

Little is known about hydrolases that are involved in the degradation of XG, but an *exo*-polygalacturonase that removes disaccharides consisting of D-xylose and D-galacturonic acid from XG has been described (Beldman *et al*., 1996). A hydrolase that specifically acts on XG by cleaving the α-(1→4)-D-galacturonic acid linkages in an *endo*-fashion has also been identified (Van der Vlugt-Bergmans *et al*., 2000) and this enzyme has a preference to cause cleaving between two xylosylated D-galacturonic acid units (Zandleven *et al*., 2007). It was further found that this hydrolase also displayed *exo*-activity, because its action produced disaccharides that consisted of D-xylose and D-galacturonic acids. This enzyme acts from the non-reducing end of XG (Figure 1-15) (Zandleven *et al*., 2005). It is also possible that specific hydrolases may attack the glycosidic bonds between the backbone and the side-chains of XG to make the backbone accessible for further degradation by the depolymerases that usually act on the HG domain.

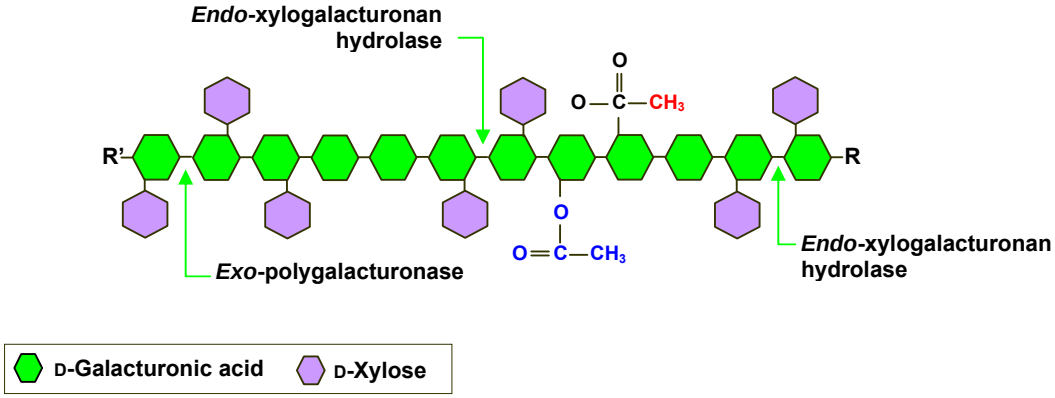


Figure 1-15: Schematic representation of xylogalacturonan indicating the points of attack for different pectinases involved in its degradation.

CONCLUSIONS

Plant cells are enclosed by a primary cell-wall that is composed of cellulose microfibrils that is interwoven and cross-linked with hemicelluloses. This cellulose-hemicellulose network is embedded in a hydrated matrix of polysaccharides and proteins (Carpita & Gibeaut, 1993). The most abundant polysaccharide in this matrix is pectin and it is also found in abundance in the middle lamella between the primary cell-walls of adjacent cells (Willats *et al*., 2001a).

These pectins are a group of acidic heteropolysaccharides that consist of HG, RG-I, RG-II and XG. Experimental evidence suggests that these domains are covalently linked to each other to form pectic chains (O'Neill *et al*., 1990; Whitecombe *et al*., 1995), but the exact molecular arrangement of the pectic molecules is still unclear (Vincken *et al*., 2003b). Different pectic chains can also be linked via covalent bonds between the RG-II domains and ionic interactions between HG domains, which results in the formation of an extensive pectic network (Kobayashi *et al*., 1996; O'Neill *et al*., 1996; Ridley *et al*., 2001). The pectic domains can also form links with the hemicellulose-cellulose complex, lignin and proteins (Ridley *et al*., 2001).

The pectic polysaccharides are involved in a number of cell-wall functions that are related to physiology, growth, development and defence mechanisms. The extensive structural and conformational variety and dynamic nature of the different domains provide the cell-wall matrix with a variety of mechanical properties, ionic and hydration conditions, signalling ability, potential for molecular interactions and capacity to be degraded by pectinases. Pectin should not be considered as a single form that is

structurally integrated throughout the plant but rather as populations of pectic domains with particular functions in specific plant tissues and occurring at specific locations in the cell-walls (Willats *et al*., 2001a).

Both pectin biosynthesis and pectin degradation are regulated in a temporally, spatially and developmentally specific manner. Due to the complex structure of pectin the action of numerous different enzymes are required for its biosynthesis (Mohnen, 1999), but many of these enzymes and their substrates are still unknown (Ridley *et al*., 2001). The modification and disassembly of the pectin polymer also requires the combined action of different pectinolytic enzymes (Kluskens *et al*., 2005; Payasi *et al*., 2006;).

The biotechnological potential of pectinolytic enzymes as biological catalysts has received a great deal of attention in a variety of industrial processes (Hoondal *et al*., 2002; Kashyap *et al*., 2001). This thesis intends to explore the potential of these enzymes and to encourage new pectinase-based technology in the pulp and paper industry.

APPENDICES

Appendix 1-A: List of transferase activities that are required for the biosynthesis of homogalacturonan.

	Type of transferase	Substrate	Acceptor	Ref.
Backbone synthesis	***Glycosylation***			
	α-1,4-D-galactyronosyl-transferase (I)*	UDP-D-GalA	D-GalAα1,4-D-GalA	1
	α-1,4-D-galactyronosyl-transferase (II)		D-GalAα1,2-L-Rha	2
Substitution	***Methyl-esterification***			
	D-galacturonic acid-6-*O*-metyltransferase (I)*	*S*-adenosyl L-methionine	D-GalAα1,4-D-GalA$_{(n)}$	3; 4
	Acetylation			
	D-galacturonic acid-2-O/3-*O*-acetyltransferase (I)*	Acetyl-coenzyme A	D-GalAα1,4-D-GalA$_{(n)}$	5; 6

* Transferases have been enzymatically confirmed

1. O'Neill *et al*.,1990
2. Ridley *et al*., 2001
3. Goubet *et al*., 1998
4. Vannier *et al*., 1992
5. Ishii, 1997
6. Pauly & Scheller, 2000

Appendix 1-B: List of transferase activities that are required for the biosynthesis of xylogalacturonan.

	Type of transferase	Substrate	Acceptor	Ref.
Backbone synthesis	***Glycosylation***			
	α-1,4-D-galactyronosyl-transferase (I)*	UDP-D-GalA	D-GalAα1,4-D-GalA	1
Side-chain synthesis	***Glycosylsylation***			
	β-1,3-D-xylosyltransferase (II)*	UDP-D-Xyl	D-GalAα1,4-D-GalA	2
	β-1,4-D-xylosyltransferase (III)		D-Xylβ1,3-D-GalA	3
	β-1,4-D-xylosyltransferase (IV)		D-Xylβ1,4-D-Xyl	4
	β-1,2-D-xylosyltransferase (V)		D-Xylβ1,3-D-GalA	5
	β-1,2-D-xylosyltransferase (VI)		D-Xylβ1,4-D-Xyl	
	β-1,2-D-xylosyltransferase (VII)		D-Xylβ1,2-D-Xyl	
	β-1,3-D-xylosyltransferase (VIII)		D-Xylβ1,4-D-Xyl	6
	β-1,3-D-xylosyltransferase (IX)		D-Xylβ1,2-D-Xyl	
	β-1,3-D-xylosyltransferase (X)		D-Xylβ1,3-D-Xyl	
Substitution	***Methyl-esterification***			
	D-galacturonic acid-6-*O*-metyltransferase (I)*	*S*-adenosyl L-methionine	D-GalAα1,4-D-GalA$_{(n)}$	7-8
	Acetylation			
	D-galacturonic acid-2-O/3-*O*-acetyltransferase (I)*	Acetyl-coenzyme A	D-GalAα1,4-D-GalA$_{(n)}$	9-10

* Transferases have been enzymatically confirmed

1. O'Neill *et al*., 1990
2. Jensen *et al*., 2008
3. Mohnen, 2008
4. Oechslin *et al*., 2003
5. Zandleven *et al*., 2005
6. Le Goff *et al*., 2001
7. Goubet *et al*., 1998
8. Vannier *et al*., 1992
9. Ishii, 1997
10. Pauly & Scheller, 2000

Appendix 1-C: List of transferase activities that are required for the biosynthesis of rhamnogalacturonan-I.

	Type of transferase	Substrate	Acceptor	Ref.
Backbone synthesis	***Glycosylation***			
	α-1,2-D-galactyronosyl-transferase (I)	UDP-D-GalA	L-Rhaα1,4-D-GalA	1-2
	α-1,4-D-galactyronosyl-transferase (II)		D-GalAα1,2-L-Rha	3-4
	α-1,4-L-rhamnosyl-transferase (I)	UDP-L-Rha	D-GalAα1,2-L-Rha	1-2
	α-1,4-L-rhamnosyl-transferase (II)		D-GalAα1,4-D-GalA	
Side-chain synthesis	***Glycosylation***			
	β-1,4-D-galactosyl-transferase (I)*	UDP-D-Gal	L-Rhaα1,4-D-GalA	1-2
	β-1,4-D-galactosyl-transferase (II)*		D-Galβ1,4-L-Rha	
	β-1,4-D-galactosyl-transferase (III)*		D-Galβ1,4-D-Gal	
	β-1,6-D-galactosyl-transferase (IV)		D-Galβ1,4-D-Gal	2, 5
	β-1,3-D-galactosyl-transferase (V)*		D-Galβ1,3-D-Gal	
	β-1,6-D-galactosyl-transferase (VI)*		D-Galβ1,6-D-Gal	
	β-1,6-D-galactosyl-transferase (VI)*		D-Galβ1,6-D-Galβ1,3-D-Gal	
	α-1,3-L-arabinosyl*f*-transferase (I)	UDP-L-Ara*f*	D-Galβ1,4-L-Rha	5
	α-1,2-L-arabinosyl*f*-transferase (II)		L-Ara*f*α1,3-D-Gal	
	α-1,5-L-arabinosyl*f*-transferase (III)*		L-Ara*f*α1,2-L-Ara	
	L-arabinosyl-transferase (IV)		L-Rhaα1,4-D-GalA	5
	α-1,5-L-arabinosyl*f*-transferase (V)*		L-Ara*f*α1,5-L-Ara*f*	
	α-1,2-L-arabinosyl*f*-transferase (VI)		L-Araα1,5-L-Ara*f*	
	α-1,3-L-arabinosyl*f*-transferase (VII)*		L-Ara*f*α1,5-L-Ara*f*	
	α-1,3-L-arabinosyl*f*-transferase (VIII)*		L-Ara*f*α1,3-L-Ara*f*	
	α-1,3-L-arabinosyl*f*-transferase (IX)*		D-Galβ1,4-D-Gal	2
	α-1,5-L-arabinosyl*f*-transferase (X)		L-Ara*f*α1,3-D-Gal	5
	α-1,6-L-arabinosyl*f*-transferase (XI)		D-Galβ1,6-D-Gal	
	α-1,6-L-arabinosyl*f*-transferase (XII)		D-Galβ1,6-D-Gal	
	α-1,2-L-fucosyl-transferase (I)	UDP-D-Fuc	D-Galβ1,4-D-Gal	6-7
	β-1,6-D-glucoronosyl-transferase (I)	UDP-D-GlcA	D-Gal…	
	β-1,4-D-glucoronosyl-transferase (I)		D-Gal…	
Substitution	***Methyl-esterification***			
	D-galacturonic acid-6-*O* metyltransferase (II)	*S*-adenosyl L-methionine	D-GalAα1,2-L-Rhaα1,4$_{(n)}$	8
	D-glucuronic acid-4-*O*-metyltransferase (I)*		D-GlcAβ1,6-D-Gal	9
	Acetylation			
	D-galacturonic acid-3-*O*/2-O-acetyltransferase (II)*	Acetyl-coenzyme A	D-GalAα1,2-L-Rhaα1,4$_{(n)}$	10

* Transferases have been enzymatically confirmed

1. Lau *et al*., 1985
2. O'Neill *et al.*, 1990
3. Mohnen, 2008
4. Ridley *et al.*, 2001
5. Carpita & Gibeaut, 1993
6. Renard *et al.*, 1991
7. Vincken *et al.*, 2003b
8. Goubet *et al*., 1998
9. Renard & Jarvis, 1999
10. Ishii, 1997

Appendix 1-D: List of transferase activities that are required for the biosynthesis of rhamnogalacturonan-II.

	Type of transferase[a]	Substrate	Acceptor[b]	Ref.
Backbone synthesis	***Glycosylation***			
	α-D-1,4-galactyronosyl-transferase (I)*	UDP-D-GalA	D-GalAα1,4-D-GalA	1
Side-chain synthesis	***Glycosylation***			
	α-1,2-D-galactyronosyl-transferase (II)	UDP-D-GalA	L-Rhaβ1,3'-DApi*f*	2-3
	α-1,3'-D-galactyronosyl-transferase (III)		L-Rhaβ1,3'-D-Api*f*	
	β-1,3-L-rhamnosyl-transferase (I)	UDP-L-Rha	D-Api*f*β1,2-D-GalA	
	α-1,5-L-rhamnosyl-transferase (II)		D-Kdo2,3-D-GalA	
	α-1,2-L-rhamnosyl-transferase (III)		L-Ara*p*α1,4-D-Gal	
	β-1,3-L-rhamnosyl-transferase (IV)		L-Ara*p*α1,4-D-Gal	
	α-1,2-L-galactosyl-transferase (I)	GDP-L-Gal	D-GlcAβ1,2-L-Fuc	
	β-1,2-D-galactosyl-transferase (II)	UDP-D-Gal	L-Ace*f*Aα1,3-L-Rha	3-4
	β-1,5-L-arabinosyl*f*-transferase (I)	UDP-L-Ara*f*	D-Kdo2,3-D-GalA	2-3
	α-1,4-L-arabinosyl-transferase (II)	UDP-L-Ara*p*	D-Galβ1,2-L-AceA*f*	
	β-1,2-L-arabinosyl*f*-transferase (III)	UDP-L-Ara*f*	L-Rhaα1,2-L-Ara	
	α-1,4-L-fucosyl-transferase (I)	GDP-L-Fuc	L-Rhaβ1,3'-D-Api*f*	2-4
	α-1,2-L-fucosyl-transferase (II)		D-Galβ1,2-L-Ace*f*A	
	β-1,2-D-apiosyl*f*-transferase (I)*	UDP-D-Api*f*	D-GalAα1,4-D-GalA	2-3
	α-1,3-D-xylosyl-transferase (I)*	UDP-D-Xyl	L-Fucα1,4-L-Rha	5
	β-1,4-D-glucoronosyl-transferase (III)*	UDP-D-GlcA	L-Fucα1,4-L-Rha	6
	2,3-D-Kdo-transferase (I)	-	D-GalAα1,4-D-GalA	3
	2,3-D-Dha-transferase (I)	-	D-GalAα1,4-D-GalA	
	α-1,3-D-AceA-transferase (I)	-	L-Rhaβ1,3'-D-Api*f*	3-4
Substitution	***Methyl-esterification***			
	D-xylose-2-*O*-metyltransferase (I)*	*S*-adenosyl L-methionine	D-Xylα1,3-L-Fuc	2
	D- fucose-2-*O*-metyltransferase (I)*		L-Fucα1,2-D-Gal	3
	D-galacturonic acid-6-*O*-metyltransferase (I)*		D-GalAα1,4-D-GalA$_{(n)}$	7-8
	Acetylation			
	L-fucose-acetylesterase (I)*	Acetyl-coenzyme A	L-Fucα1,2-D-Gal	7-10
	L-AceA-3-*O*-acetylesterase (I)*		L-Ace*f*Aα1,3-L-Rha	
	D-galacturonic acid-2-O/3-*O*-acetyltransferase (I)*		D-GalAα1,4-D-GalA$_{(n)}$	

* Transferases have been enzymatically confirmed

1. O'Neill *et al*., 1990
2. Carpita & Gibeaut, 1993
3. O'Neill *et al*., 2004
4. Vidal *et al*., 2000
5. Egelund *et al*., 2006
6. Iwai *et al*., 2002
7. Goubet *et al*., 1998
8. Vannier *et al*., 1992
9. Ishii, 1997
10. Pauly & Scheller, 2000

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CHAPTER

2

PECTIC MONOSACCHARIDES AND TOTAL PECTIN CONTENT OF EUCALYPTUS WOOD

ABSTRACT

Very little is known about the occurrence of the pectic polysaccharides in wood and it is speculated that between 10 and 40 mg/g of the total dry weight of wood consists of pectin. However, no literature that describes the accurate quantification of pectins in wood is currently available. This work aimed to provide data on the pectic monosaccharides and pectin content of wood, specifically two species of eucalyptus. The influence of four factors (tree species, yield potential of the site, tree age class and wood tissue type) on the total pectin content of the eucalyptus wood was determined. The wood samples were hydrolysed with H_2SO_4, using the Saeman procedure and the hydrolysates were analysed with two high performance liquid chromatography protocols to quantify the neutral and acidic monosaccharides, respectively. The D-galacturonic acid was found to be the predominant pectic monosaccharide followed by D-galactose, L-arabinose and L-rhamnose. Through the addition of all pectic monosaccharides, it was determined that eucalyptus wood contained between 15.2 and 25.8 mg/g pectin. Wood tissue type was the factor that had the biggest influence on the total pectin content of the samples. The cambium displayed the highest concentration of pectin, because active growth mostly occurs in this tissue. These results on the occurrence of pectic polysaccharides in wood can contribute to the understanding of wood biochemistry and can be useful in the biofuels and paper-making industries.

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By Coetzee, B., Schols, H.A. & Wolfaardt, F.

INTRODUCTION

Very little is known about the occurrence of the pectic polysaccharides in wood, and woody tissue is generally described to consist of cellulose, hemicelluloses and lignin, without referring to pectin as a significant constituent (Koch, 2006; Sjöström, 1993). It is speculated that between 10 and 40 mg/g of the total dry weight of wood consists of pectin (BeMiller, 2001; Green *et al*., 1996; Peng *et al*., 2003). To our knowledge, no recent work is available on the isolation of wood pectins and older literature (Anderson, 1936; Anderson *et al*., 1937; Larson, 1967) does not fully quantify pectins in wood.

The quantification of pectin is important, since pectic polysaccharides are structural components of all primary cell-walls and intercellular regions of plants (Goycoolea & Cárdenas, 2003). The difficulties to extract and quantify these polysaccharides are due to the complexity of their chemical structure and their close association with other cell-wall polymers (Rumpunen *et al*., 2002). Previously, detection and determination of pectins were based on the occurrence and chemistry of D-galacturonic acid, which is the primary monomer of pectin (Gnanasambandam & Proctor, 1999; Sundberg *et al*., 1996). This monosaccharide can be quantified with different techniques including titration and spectrophotometry (Ahmed & Labavitch, 1977; Blumenkrantz & Asboe-Hansen, 1973) or high performance liquid chromatography (HPLC) (Rumpunen *et al*., 2002; Sundberg *et al*., 1996). However, the actual correspondence between the D-galacturonic acid content and the total content of extractable pectins can be variable, as it is influenced by differences between species, genotypes and tissue (Massiot & Renard, 1997; Thomas *et al*., 2000). The only available method to analyse and quantify complex polysaccharides in wood, including the pectic polysaccharides, is to determine their constituent monomers. These are obtained through total acid hydrolysis (Garna *et al*., 2006) or acid methanolysis (Sundberg *et al*., 1996) of the sample, followed by quantification with HPLC or gas chromatography.

Protocols making use of mild conditions such as acid methanolysis resulted in incomplete cleavage of the glycosidic and glucuronosyl linkages, especially from a robust substrate such as wood (Bertaud *et al*., 2002; Biermann, 1988; Garna *et al*., 2006; Sundberg *et al*., 1996). This incomplete hydrolysis may lead to an overestimation of the individual sugars, because oligomers that contain more than the monosaccharide of interest are included in the quantification. Total hydrolysis of the

sample is, however, achieved when using a strong acid at a high temperature as outlined in the Saeman procedure (Adams, 1965; Biermann, 1988). The Saeman hydrolysis of pectin-containing material, however, combines the release of the D-galacturonic acid and its further degradation into lactones (Blake & Richards, 1968; Garna *et al*., 2006; Sundberg *et al*., 1996; Uçar & Balaban, 2003). Standards should, therefore, be subjected to the same hydrolytic conditions to compensate for possible degradation of the released monosaccharides. The total acid hydrolysis of wood, thus, results in more accurate quantification of the monosaccharides.

Many studies investigated the total monosaccharide composition of hardwood species after hydrolysis (Bertaud *et al*., 2002; Brito *et al*., 2008; Kaar *et al*., 1991; Koch, 2006; Sundberg *et al*., 1996; Uçar & Balaban, 2003; Whiting & Goring, 1983) and cellulose and hemicellulose were considered to be the only source of these monosaccharides (Koch, 2006; Tunc & van Heiningen, 2008). The fact that a considerable portion of these monosaccharides may originate from the pectic polysaccharides was ignored. D-Glucose is the most abundant sugar released during hydrolysis and originates mainly from cellulose (Brown, 1999; Koch, 2006). The hemicellulose fractions of hardwoods consist of 4-*O*-methylglucuronoxylan and glucomannans (Grand-Reid, 1997; Rowell *et al*., 2005). It can thus, be assumed that these hemicelluloses are mainly composed of D-glucuroncic acid, D-xylose and D-mannose and that the D-galacturonic acid, L-rhamnose, D-galactose and L-arabinose originates from polysaccharides other than the cellulose and hemicelluloses.

The occurrence of the pectic polysaccharides in wood has not been studied to a significant extent. This study, therefore, focused on the quantification of pectin in hardwoods, specifically *Eucalyptus grandis* and *E. nitens*. An analytical procedure is described to quantify the pectic monosaccharides in wood, including the uronic acids and neutral monosaccharides. The concentrations of the pectic monosaccharides were used to calculate the pectin content of the eucalyptus samples.

MATERIALS AND METHODS

Sampling and preparation of wood samples

Wood cores were sampled at breast height from two eucalyptus species in commercial plantations during the winter of 2006. The wood samples consisted of 36 wood cores from *E. grandis* and *E. nitens* from three age classes (six, ten and fourteen

years) grown on sites that were considered to be high and low in terms of potential wood yield (Appendix 2-A). Cores were collected from three randomly selected trees at each site and each tree represented a replication for the experiment. Sections representing three tissue types, namely cambial tissue, sapwood and heartwood were cut from each core. These samples were finely ground into powder, using coarse sandpaper (no. 60), without heating the core in the process. The wood powder was screened and the fraction that passed through the 635 µm screen but was retained on the 423 µm screen was used for analysis.

Hydrolysis and sample preparation

The wood samples (10 to 20 mg) were weighed into 10-ml screw-cap tubes and hydrolysed with H_2SO_4, using the two-step Saeman procedure (Adams, 1965; Biermann, 1988). Pre-hydrolyses was done with 450 µl H_2SO_4 (72%). After incubation at 30°C for 60 min, 1.00 ml of the internal standard L-fucose (16.2 µg/ml, Sigma-Aldrich) and 6.65 ml HPLC-grade water were added to each tube to reach a final concentration of 4% H_2SO_4. During the second step, the samples were hydrolysed at 100°C for 120 min before cooling to room temperature. Standard solutions containing D-galactose, L-rhamnose, L-arabinose (Sigma-Aldrich) and D-galacturonic acid (Fluka) at four concentrations (0.75, 1.25, 2.25 and 3.25 µg/ml) were subjected to both hydrolysis steps. The standards were exposed to identical hydrolysis conditions to compensate for possible degradation of the monosaccharides released from the wood. The hydrolysates of the samples and the standards were filtered with 0.45 µm polytetrafluoroethylene syringe filters. Undiluted samples of the hydrolysates were used to analyse for D-galacturonic acid and the standards. The hydrolysates were diluted four times with HPLC-grade water to quantify L-rhamnose, D-galactose and L-arabinose.

Analytical method

The monosaccharide composition of the hydrolysates was determined by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD). The separation of the D-galacturonic acid and the neutral monosaccharides was done on a Dionex Ultimate 3000 system with a CarboPAc® PA1 column (250 x 4 mm) in combination with a CarboPAc® PA1 guard column (50 x 4 mm) (Dionex Corporation, Sunnyvale, California). Samples were kept at 5°C until injection (20 µl) and analysis were carried out at 30°C at a flow rate of 1 ml/min. Each sample

was analysed by two separate runs. During the first run, the neutral monosaccharides were eluted with 10 m*M* NaOH for 30 min. The column was washed with 170 m*M* sodium acetate in 80 m*M* NaOH for 5 min followed by equilibration with 10 m*M* NaOH for 30 min after every third injection. The column was then conditioned and equilibrated with 170 m*M* sodium acetate in 80 m*M* NaOH for 60 min prior to the second run during which the uronic acids were analysed. The D-galacturonic acid was eluted with 170 m*M* sodium acetate in 80 m*M* NaOH for 15 min. The pulsed-amperometric detector (PAD) (*esa* Coulochem III; 5040 analytical cell) settings were E_1 = 200 mV, E_2 = -1000 mV, E_3 = 600 mV and E_4 = -100 mV for 500, 10, 10 and 40 ms, respectively at a sensitivity of 100 nC. The delay time was set at 360 ms at a guard cell potential of 0.0 mV. The detector output was digitised by a UCI-50 Universal Chromatography interface (Dionex Corporation, Sunnyvale, California) at a sampling rate of one point per second and analysed using Chromeleon® version 6.80 software (Dionex Corporation, Sunnyvale, California). Neutral monosaccharides and the D-galacturonic acid were quantified by calculating the response factors of the peak areas using L-fucose as an internal standard and D-galacturonic acid as external standard, respectively.

Experimental design and statistical analysis

A completely randomised experiment was designed with four factors (tree species, yield potential of the site, tree age class and wood tissue types) replicated three times. The data were tested in a 2 × 2 × 3 × 3 factorial experiment to investigate the influence of the different factors and interactions between the factors on the total pectin content of the eucalyptus samples. The data were subjected to general linear modelling (GLM) procedures of the SAS/STAT® 9.1 statistical package (SAS Institute Inc., USA). A LSMEAN with standard error and p-values to compare the different LSMEANS was calculated for each factor level combination and means were compared with Scheffé's test.

RESULTS AND DISCUSSION

Separation of monosaccharides

Monosaccharides including D-glucose, D-mannose, D-xylose, D-galactose, L-rhamnose and L-arabinose as well D-galacturonic acid was released during hydrolysis. The D-glucose was considered to originate from the cellulose fraction of the

wood (Sundberg *et al*., 1996), while the D-mannose and D-xylose mainly originated from the hemicellulose (Grand-Reid, 1997; Rowell *et al*., 2005). The pectic polysaccharides were, therefore, considered to be composed of the remaining monosaccharides, namely D-galacturonic acid, D-galactose, L-rhamnose and L-arabinose. These pectic monosaccharides were accurately quantified in two runs (Figure 2-1 and Figure 2-2) by using HPAEC-PAD. Complete separation of the neutral monosaccharides was achieved in the first run (Figure 2-1), except in the case of D-xylose and D-mannose (Appendix 2-B and Appendix 2-C). Separation of these two sugars was not required since D-xylose represents less than 1% of the total pectin (Zandleven *et al*., 2007; Zsivanovits *et al*., 2004) and D-mannose is not a pectic monosaccharide. In the second run with 170 m*M* sodium acetate in 80 m*M* NaOH, complete separation of the uronic acids was achieved (Appendix 2-B). However, D-glucuronic acid was not present at detectable levels in any of the samples (Figure 2-2). The contribution of each monosaccharide to the sample was expressed as the mass of the monosaccharide relative to the mass of wood (mg/g) and the amount of pectin was calculated through addition of all the pectic monosaccharides.

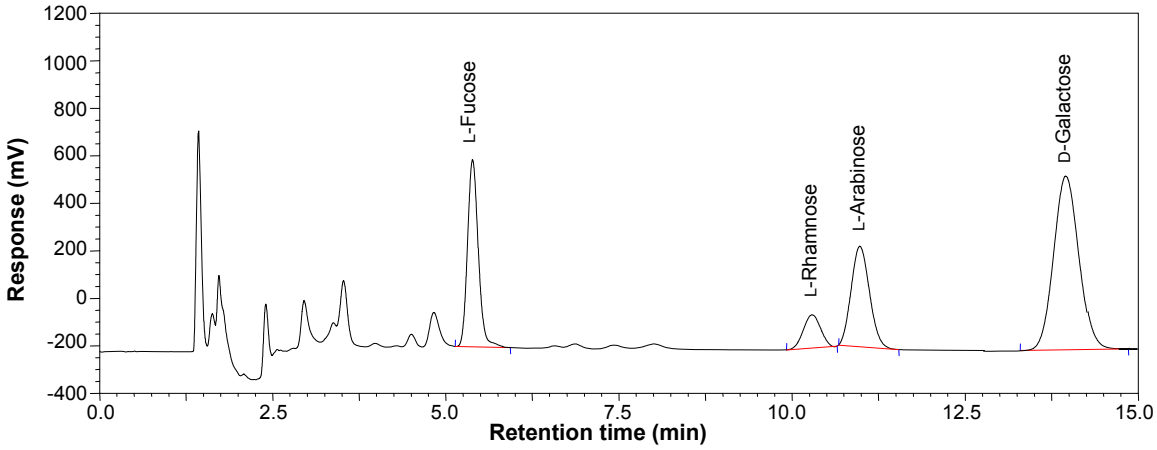


Figure 2-1: HPAEC-PAD chromatogram of the hydrolysate of a wood sample that was diluted (1 : 3) indicating L-fucose (internal standard), L-rhamnose, L-arabinose and D-galactose, eluted with 10 m*M* NaOH.

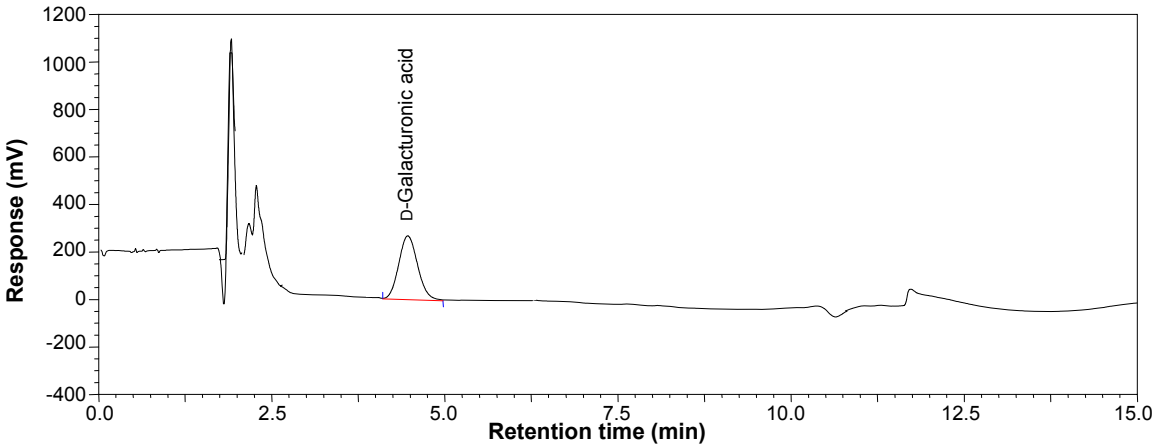


Figure 2-2: HPAEC-PAD chromatogram of the hydrolysate of a wood sample indicating D-galacturonic acid when eluted with 170 m*M* sodium acetate in 80 m*M* NaOH.

Occurrence of pectic monosaccharides in wood

The D-galacturonic acid occurred in higher concentrations than the other pectic monosaccharides at an average of 14.4 mg/g wood (Appendix 2-D). This value was calculated across all four factors (tree species, yield potential of the site, tree age class and wood tissue type) and had a standard deviation (S.D.) of 4.1. Notably higher concentrations (36 to 50 mg/g) of D-galacturonic acid were reported to occur in *Betula* spp. (Koch, 2006; Sundberg *et al*., 1996). The birch wood was, however, subjected to acid methanolysis, probably resulting in an overestimation of the D-galacturonic acid content. In other studies, the total uronic acid content of wood was used to indicate the concentration of D-galacturonic acid at values from 36 to 60 mg/g (Garrote *et al*., 2007; Koch, 2006). This method, however, did not account for the significant amount of D-glucuronic acid originating from hemicellulose (Grand-Reid, 1997; Rowell *et al*., 2005).

The tested eucalyptus samples contained an average of 3.87 mg/g (S.D. = 1.90) D-galactose and 1.64 mg/g (S.D. = 0.600) L-arabinose (Appendix 2-E and Appendix 2-F). These values were again notably less than the 7.0 to 10.1 mg/g D-galactose and 4.0 to 10.0 mg/g L-arabinose reported in literature for other hardwoods (Koch, 2006; Sundberg *et al*., 1996). These reported values may again be an overestimation due to incomplete methanolytic hydrolysis and not necessarily as a result of eucalyptus containing less of these pectic monosaccharides than other hardwoods.

The L-rhamnose fractions of the wood mainly originate from the backbone of the rhamnogalacturonan-I (RG-I) domains (Lau *et al*., 1985), but small quantities of

L-rhamnose are also present in the side-chains of rhamnogalacturonan-II (RG-II) (O'Neill *et al*., 1996; Spellman *et al*., 1983; Vidal *et al*., 2000). The L-rhamnose was the pectic monosaccharide that occurred in the lowest concentrations in the eucalyptus wood and the samples contained an average of 0.797 mg/g (S.D. = 0.215) (Appendix 2-G). This amount was again, notably lower than the values of 3.9 to 6.0 mg/g reported to occur in *Betula* spp. (Koch, 2006; Sundberg *et al*., 1996) and in other hardwoods (Koch, 2006).

Total pectin content

The pectin content of different *E. grandis* and *E. nitens* wood samples was calculated by adding the total amount of pectic monosaccharides (D-galacturonic acid, L-rhamnose, L-arabinose and D-galactose). The total pectin in the wood varied between 15.2 to 25.8 mg/g of dry wood (Table 2-1) and these values compared well with the estimates of 10 to 40 mg/g reported (BeMiller, 2001; Green *et al*., 1996).

Table 2-1: Pectin content (mg/g) measured in different wood samples from two eucalyptus species. Each value represents the mean of three replications (three trees). Data were summarised from Appendix 2-H.

		Eucalyptus grandis		*Eucalyptus nitens*	
Age	**Wood tissue type**	**High-yield site**	**Low-yield site**	**High-yield site**	**Low-yield site**
6 yrs	**Cambial tissue**	20.5	25.1	21.8	24.4
	Sapwood	17.7	22.8	21.6	19.3
	Heartwood	17.2	21.1	21.8	23.0
10 yrs	**Cambial tissue**	20.1	24.8	24.6	19.9
	Sapwood	18.9	18.0	18.1	16.6
	Heartwood	22.8	19.6	25.8	17.3
14 yrs	**Cambial tissue**	24.6	23.3	22.4	19.2
	Sapwood	22.3	20.4	17.0	18.3
	Heartwood	19.8	15.2	17.0	21.4

The *E. grandis* samples with the lowest and the highest concentrations of pectin (15.2 and 25.1 mg/g, respectively), were collected from a site with a low yield potential (Table 2-1). However, the *E. nitens* samples with the lowest and highest concentration of pectin of 17.0 and 25.8 mg/g, respectively, were obtained from the high-yield site. These values did not differ significantly ($p \leq 0.05$) between species, but the wood tissue type had a significant influence. The cambial tissue contained significantly more pectin (22.6 mg/g) than the sapwood (19.2 mg/g) or heartwood (20.4 mg/g), which did not differ. These results, thus, confirm that pectin is closely associated with tissue where active growth occurs (Pallardy & Kozlowski, 1997; Verma, 2001).

The interaction between potential yield of the site and tree age was a significant influence in terms of the pectin concentration, and on low-yield sites, six-year-old trees contained more pectin than older trees (Figure 2-3). The higher pectin content in the younger trees may reflect more actively growing tissue (Mohnen, 1999). A difference between trees of different ages was however, not seen at the high-yield sites and may be as a result of more favourable environmental conditions that allow sustained growth of trees at all ages.

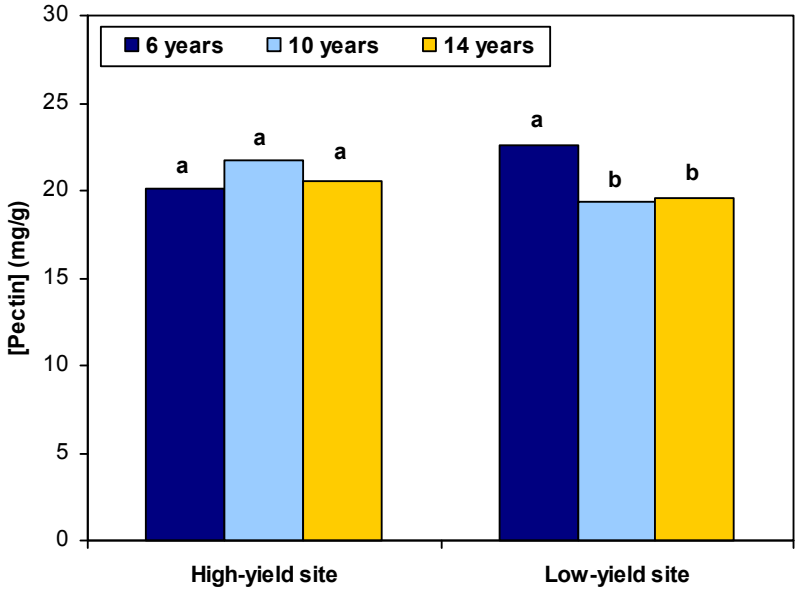


Figure 2-3: Influence of the yield potential of the site and tree age class on the pectin content of eucalyptus trees (bars for each site with the same letters do not differ significantly, Scheffé's test, $p \leq 0.05$)

CONCLUSIONS

The Saeman procedure (Adams, 1965; Biermann, 1988) resulted in complete hydrolysis of the wood polysaccharides and was preferred to protocols where the incomplete cleavage of the glycosidic and glucuronosyl linkages resulted in an overestimation of monosaccharide quantities (Sundberg *et al*., 1996). The hydrolysis of eucalyptus wood resulted in the release of all pectic monosaccharides, as well as monosaccharides originating from cellulose and hemicellulose. Complete separation of the neutral monosaccharides and uronic acids in the hydrolysates was subsequently achieved with two HPLC protocols.

The D-galacturonic acid occurred in the highest concentration of the pectic monosaccharides, reflecting the fact that it is the sole constituent homogalacturonan

(HG) and also 50% of the RG-I backbone. The remainder of the RG-I backbones consisted of L-rhamnose. The tested samples also contained small quantities of D-galactose and L-arabinose, originating from the side-chains of RG-I.

The pectin content of the eucalyptus wood ranged from 15.2 to 25.8 mg/g. Quantification of pectin in wood through determination of its constituent monomers proved to be suitable for hardwoods. However, this technique cannot be applied for softwoods, since their hemicelluloses consist of galactoglucomannan and arabino-4-*O*-methylglucuronxylan that may contribute to the D-galactose and L-arabinose in the hydrolysates (Grand-Reid, 1997; Koch, 2006; Rowell *et al*., 2005).

Wood tissue type proved to have a major influence on the total pectin content of the samples. The cambial tissue contained the highest concentrations of pectin, because active growth mainly occurs here (Pallardy & Kozlowski, 1997), thus, reflecting the importance of pectin in developmental processes (Darvill *et al*., 1992; Jarvis 1984; Weber *et al*., 1996; Willats *et al*., 2001). Species did not have a significant influence on the pectin content of samples and *E. grandis* and *E. nitens* did not differ in their total pectin content. However, the pectin content of more eucalyptus species need be determine before any general conclusions about the relative amounts of pectin across species can be made.

The age class of the trees and the yield potential of the sites did not influence the pectin content directly, but these factors were involved in a number of significant interactions. The interaction between the age class of the trees and the other factors, for example, indicated a possible decrease in the pectin content with an increase in tree age, supporting the theory that actively-growing wood contains more pectin (Pallardy & Kozlowski, 1997; Verma, 2001).

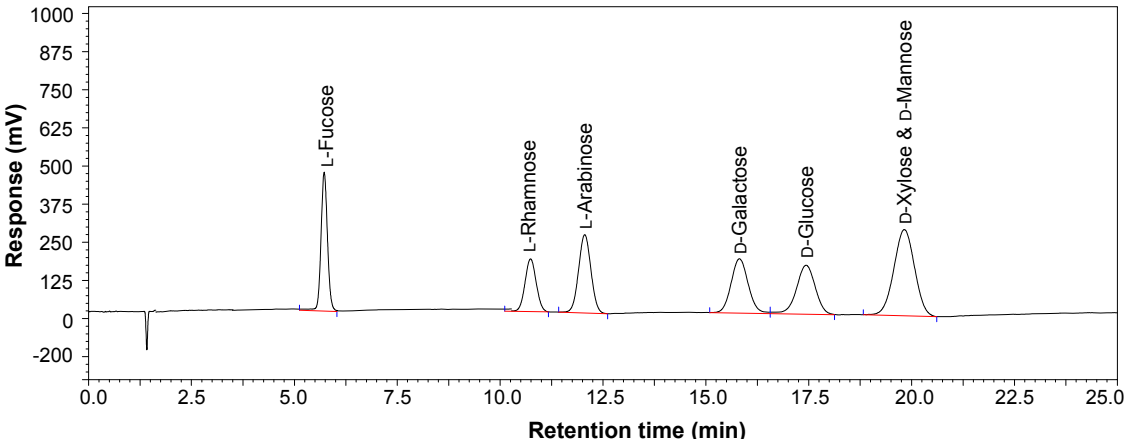
Further work should include reconstruction of the pectic domains based on the molar mass of the monosaccharides contributing to pectin. A better comprehension of the different factors that influence the composition and the structure of the pectin in wood can contribute to the understanding of wood biochemistry. Such information can be especially useful for biomass conversion and fibre utilisation in the biofules and paper-making industries, respectively.

APPENDICES

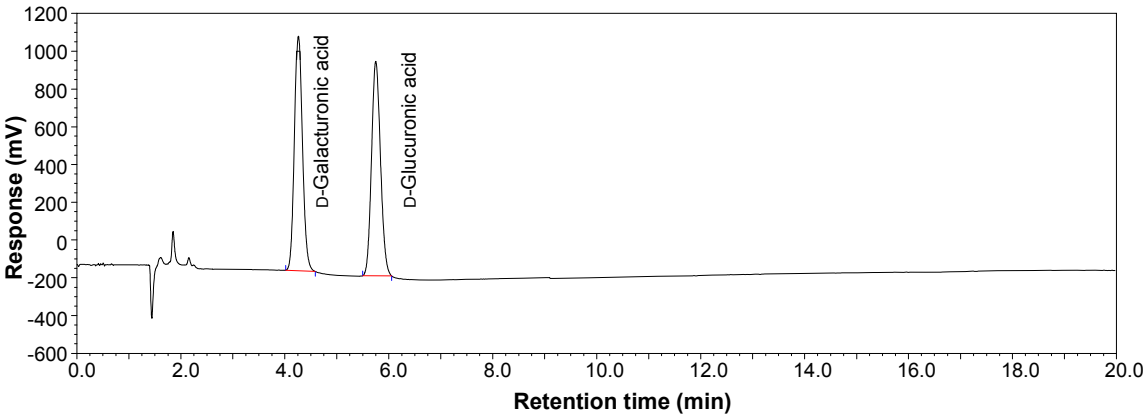
Appendix 2-A: Site and length of wood cores sampled for analyses. Replicates (three trees) from *Eucalyptus grandis* and *E. nitens* were sampled from three age classes (six, ten and fourteen years) of trees that were grown on sites that were considered to be high and low in terms of potential wood yield.

Species	Replicates (tree)	Plantation	Yield potential of the site	Tree Age (years)	Core length (cm)
Eucalyptus grandis	**1**	Riverdale	High	6	22.5
	2				22.5
	3				23.0
	1	Windy Hill	Low	6	17.0
	2				14.0
	3				17.0
	1	Shafton	High	10	19.0
	2				20.5
	3				21.0
	1	Shafton	Low	10	22.5
	2				22.0
	3				22.5
	1	Riverdale	High	14	20.0
	2				18.0
	3				16.5
	1	Riverdale	Low	14	16.0
	2				20.5
	3				21.5
Eucalyptus nitens	**1**	Underberg	High	6	15.0
	2				14.5
	3				17.0
	1	Lothair	Low	6	12.5
	2				13.0
	3				10.5
	1	Epsom	High	10	19.5
	2				20.5
	3				14.5
	1	Hogdsons	Low	10	20.5
	2				21.0
	3				14.5
	1	Lothair	High	14	14.5
	2				17.5
	3				15.5
	1	Hogdsons	Low	14	21.0
	2				18.5
	3				19.0

Appendix 2-B: HPAEC-PAD chromatograms of standard solutions containing (A.) L-fucose, L-rhamnose, L-arabinose, D-galactose, D-glucose and the combined peak of D-xylose and D-mannose (2 µg/ml each) when eluted with 10 m*M* NaOH and (B.) D-galacturonic acid and D-glucuronic acid (5 µg/ml each) when eluted with 170 m*M* sodium acetate in 80 m*M* NaOH.

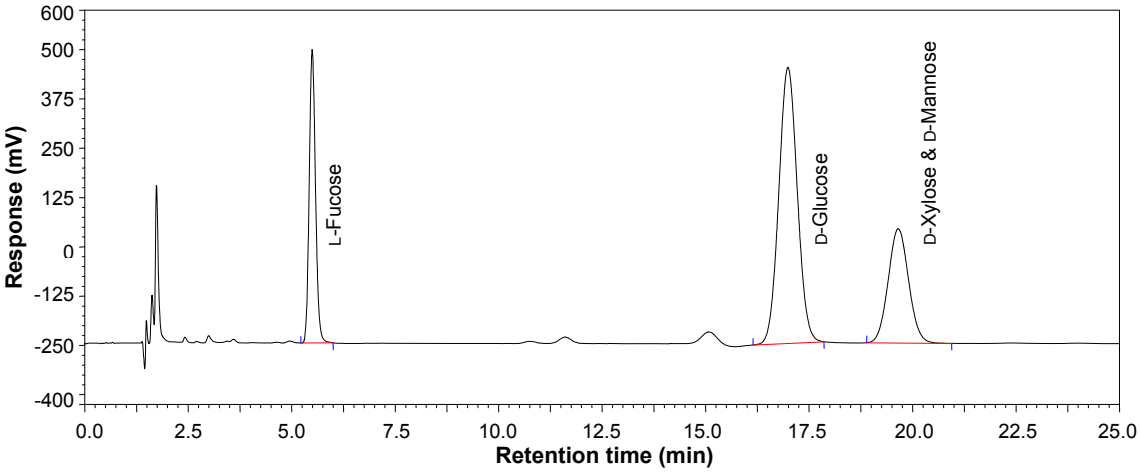


(A.)



(B.)

Appendix 2-C: HPAEC-PAD chromatogram of the hydrolysate of a wood sample that was diluted 1 : 59, showing elution of L-fucose (internal standard), D-glucose and the combined peak of D-xylose and D-mannose, with 10 m*M* NaOH.



Appendix 2-D: The D-galacturonic acid concentration (mg/g) measured in each of the 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
Eucalyptus grandis	6 years	Cambial tissue	19.5	12.7	15.4	9.13	21.6	25.7
		Sapwood	8.28	15.4	13.9	18.0	11.4	21.7
		Heartwood	14.2	11.1	11.6	10.0	19.5	14.6
	10 years	Cambial tissue	17.0	16.2	3.2	15.5	17.6	19.1
		Sapwood	12.7	10.3	16.3	10.8	19.1	11.0
		Heartwood	18.2	19.2	15.5	13.0	16.2	18.2
	14 years	Cambial tissue	14.1	19.3	15.1	13.7	21.6	13.0
		Sapwood	18.4	12.4	15.5	14.6	13.4	13.3
		Heartwood	12.3	8.20	12.7	10.5	10.0	9.62
Eucalyptus nitens	6 years	Cambial tissue	19.5	14.5	17.2	14.8	15.8	21.9
		Sapwood	15.3	18.1	10.7	8.96	16.7	12.9
		Heartwood	13.0	19.2	14.0	9.09	11.8	26.9
	10 years	Cambial tissue	16.2	23.4	15.7	11.7	13.3	9.70
		Sapwood	7.98	11.8	16.3	12.9	12.6	10.5
		Heartwood	15.0	15.6	21.4	12.9	10.8	8.3
	14 years	Cambial tissue	19.8	17.3	11.3	7.58	13.4	15.1
		Sapwood	13.2	10.6	10.8	13.4	9.66	17.0
		Heartwood	12.2	13.2	10.1	18.2	15.3	12.0

Appendix 2-E: The D-galactose concentration (mg/g) measured in each of the 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
Eucalyptus grandis	6 years	Cambial tissue	2.58	2.79	3.71	5.19	3.19	3.01
		Sapwood	2.39	3.05	4.29	3.09	3.82	3.58
		Heartwood	2.40	3.21	3.17	3.40	4.79	4.04
	10 years	Cambial tissue	5.17	4.97	2.90	8.02	5.86	3.53
		Sapwood	2.36	4.34	3.47	4.13	3.07	1.78
		Heartwood	3.80	3.23	2.01	2.31	2.14	2.49
	14 years	Cambial tissue	7.92	5.76	4.44	5.05	2.71	7.02
		Sapwood	3.38	6.81	2.74	2.55	4.85	6.92
		Heartwood	7.43	13.9	4.76	2.55	3.79	2.48
Eucalyptus nitens	6 years	Cambial tissue	2.09	2.38	3.78	5.06	3.94	4.27
		Sapwood	3.18	6.73	1.98	3.89	2.65	3.38
		Heartwood	3.36	3.43	2.78	3.52	2.76	3.68
	10 years	Cambial tissue	3.68	4.41	2.87	8.69	3.50	4.25
		Sapwood	2.89	3.57	2.39	1.56	2.67	2.83
		Heartwood	4.21	4.53	6.35	3.04	3.40	2.84
	14 years	Cambial tissue	2.23	3.96	3.76	7.91	3.55	3.26
		Sapwood	2.79	2.47	3.19	2.42	3.06	2.71
		Heartwood	1.88	3.81	1.91	2.21	3.65	3.97

Appendix 2-F: The L-arabinose concentration (mg/g) measured in each of the 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
Eucalyptus grandis	6 years	Cambial tissue	0.880	0.926	1.15	1.67	1.15	2.31
		Sapwood	1.06	1.09	0.983	1.61	1.39	1.56
		Heartwood	1.18	1.11	1.26	1.45	1.28	1.56
	10 years	Cambial tissue	1.38	1.07	1.00	0.920	1.22	0.857
		Sapwood	0.902	1.18	3.06	0.655	0.952	0.799
		Heartwood	1.43	1.16	0.974	0.751	0.770	1.00
	14 years	Cambial tissue	1.96	1.88	1.29	1.24	1.80	1.22
		Sapwood	1.90	3.06	0.864	0.947	1.57	1.06
		Heartwood	1.61	2.31	1.16	1.21	1.78	1.08
Eucalyptus nitens	6 years	Cambial tissue	1.06	1.34	1.40	1.81	1.65	1.74
		Sapwood	1.96	1.71	1.81	2.21	1.99	2.38
		Heartwood	2.17	2.15	2.32	2.79	2.07	2.95
	10 years	Cambial tissue	1.84	1.78	1.85	2.07	1.88	2.11
		Sapwood	2.11	2.31	2.25	1.12	1.82	2.00
		Heartwood	2.24	2.31	3.08	2.72	2.63	2.33
	14 years	Cambial tissue	2.30	2.00	1.95	2.54	2.07	1.64
		Sapwood	1.94	1.96	2.03	1.45	1.55	1.42
		Heartwood	2.06	2.69	0.852	1.66	2.20	1.71

Appendix 2-G: The L-rhamnose concentration (mg/g) measured in each of the 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
Eucalyptus grandis	6 years	Cambial tissue	0.511	0.592	0.655	0.787	0.674	0.968
		Sapwood	0.843	0.820	1.05	0.466	0.813	0.849
		Heartwood	0.740	0.836	0.818	0.923	0.956	0.710
	10 years	Cambial tissue	0.609	0.324	0.512	0.505	0.717	0.579
		Sapwood	0.322	0.611	1.07	0.480	0.536	0.589
		Heartwood	0.859	1.09	0.914	0.395	0.706	0.760
	14 years	Cambial tissue	0.745	0.774	0.533	0.717	1.20	0.605
		Sapwood	0.619	0.975	0.405	0.541	0.914	0.586
		Heartwood	0.858	0.904	0.957	0.746	1.08	0.737
Eucalyptus nitens	6 years	Cambial tissue	0.637	0.867	0.729	0.826	0.604	0.748
		Sapwood	1.04	1.11	1.04	0.872	0.906	0.994
		Heartwood	0.856	0.978	1.04	0.967	1.09	1.22
	10 years	Cambial tissue	0.814	0.595	0.777	0.879	0.758	0.797
		Sapwood	0.829	0.962	1.01	0.495	0.625	0.628
		Heartwood	0.914	0.949	0.837	1.07	0.949	0.940
	14 years	Cambial tissue	1.00	0.752	0.762	1.70	0.749	0.543
		Sapwood	0.759	0.751	0.604	0.722	0.695	0.680
		Heartwood	0.850	0.801	0.678	1.02	1.26	0.892

Appendix 2-H: The total pectin concentration (mg/g) measured in each of the 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
Eucalyptus grandis	6 years	Cambial tissue	23.5	17.0	21.0	16.8	26.6	32.0
		Sapwood	12.6	20.4	20.2	23.2	17.5	27.7
		Heartwood	18.6	16.3	16.8	15.7	26.6	20.9
	10 years	Cambial tissue	30.2	22.6	17.6	24.9	25.4	24.0
		Sapwood	16.3	16.5	24.0	16.1	23.6	14.1
		Heartwood	24.3	24.7	19.4	16.5	19.8	22.5
	14 years	Cambial tissue	24.7	27.8	21.4	20.7	27.3	21.8
		Sapwood	24.3	23.3	19.5	18.7	20.7	21.8
		Heartwood	22.2	25.3	19.6	15.1	16.7	13.9
Eucalyptus nitens	6 years	Cambial tissue	23.3	19.1	23.1	22.5	22.0	28.6
		Sapwood	21.4	27.6	15.6	15.9	22.2	19.6
		Heartwood	19.4	25.8	20.2	16.4	17.8	34.8
	10 years	Cambial tissue	22.5	30.1	21.2	23.3	19.4	16.9
		Sapwood	13.8	18.6	21.9	16.0	17.7	16.0
		Heartwood	22.3	23.4	31.7	19.7	17.7	14.4
	14 years	Cambial tissue	25.3	24.0	17.8	17.2	19.8	20.6
		Sapwood	18.6	15.8	16.6	18.0	15.0	21.9
		Heartwood	17.0	20.5	13.6	23.1	22.4	18.5

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CHAPTER

3

MONOSACCHARIDE COMPOSITION OF PECTIN FROM EUCALYPTUS WOOD

ABSTRACT

It was determined that pectin comprises between 15.2 and 25.8 mg/g of the total dry weight of eucalyptus wood. Very little information is, however, available on the composition of these pectins, mainly due to the difficulty to isolate the polysaccharides intact from the woody tissue. While the previous chapter focussed on the quantification of pectin in eucalyptus wood, the present work concentrated on the characterisation of these pectins and being the first of its kind, provides information on the composition of pectin from woody substrates. The molar concentration of each of the pectic monosaccharides was expressed relative to the total pectin in the different eucalyptus samples. The D-galacturonic acid occurred in the highest concentrations in the eucalyptus pectin followed by D-galactose, L-arabinose and L-rhamnose. In addition to wood tissue type and tree species, the yield potential of the sites and tree age also had significant influences on the composition of the pectin. *Eucalyptus grandis* contained significantly more D-galacturonic acid and D-galactose than *E. nitens*, while the latter contained significantly more L-rhamnose and L-arabinose. The differences in the D-galacturonic acid and L-rhamnose content may have indicated differences in the relative amounts of rhamnogalacturonan-I (RG-I) and homogalacturonan, while the differences in the D-galactose and L-arabinose concentrations indicated variations in the composition of the RG-I side-chains.

INTRODUCTION

Pectins are a heterogeneous group of acidic polysaccharides that have been isolated from the cell-walls of many higher plants (Goycoolea & Cárdenas, 2003; O'Neill *et al*., 1990; Ridley *et al*., 2001). The highest concentration of pectin occurs in the middle lamella and cell corners with a gradual decrease from the primary cell-wall to the plasma membrane (Goycoolea & Cárdenas, 2003). These polysaccharides are also present in the junction zones between cells with secondary walls, including the fibre cells in woody tissue (Mohnen, 2008). The pectic polysaccharides are the most complex class of polysaccharides occurring in the cell-wall and consist of a diversity of monosaccharides that make them heterogeneous in composition, structure and molecular weight (O'Neill *et al*., 1990). Pectins are relatively easy to extract from most sources to get a better understanding of their composition and structure. It is, however, in some cases difficult to accurately characterise these polysaccharides, due to incomplete extraction from more complex substrates.

Pectins have been extracted with chemicals and enzymes (Gnanasambandam & Proctor, 1999; Marcon *et al*., 2005; Rascón-Chu *et al*., 2009; Renard *et al*., 1991; Saulnier & Thibault, 1987). During chemical extraction, the different fractions of pectins are sequentially extracted from the sources based on their solubility in water or buffers, chelating agents, diluted acids or diluted alkali (Gnanasambandam & Proctor, 1999). The two approaches of enzymatic extraction include the treatment with pectinolytic enzymes (Saulnier & Thibault, 1987) that results in the degradation of the pectic backbone or with non-pectinolytic enzymes that yields pectin in their native state (Oechslin *et al*., 2003). The fine structure of extractable pectic substances is well known; however, almost no information is available on the composition of pectins that are associated with lignin, celluloses and hemicelluloses.

During earlier studies (Anderson, 1936; Anderson *et al*., 1937) attempts were made to characterise pectin from wood, but no recent studies focussed on the extraction of pectin from lingo-cellulosic substrates such as wood. The incomplete isolation of pectin from the woody tissue was mainly due to their low concentrations, their insolubility in water and their strong association with the other cell-wall polymers (Anderson, 1936). It was, however, found that the highest concentration of extractable pectin originated from the cambium, with very low concentrations from the sapwood and heartwood (Anderson *et al*., 1937). Very little is, therefore, still known on the composition and structure of the pectic polysaccharides in wood. Previous work

(Chapter 2) described the quantification of pectin in the eucalyptus wood based on the addition of the pectic monosaccharides. The aim of the present study was to determine the influence of different factors on the composition of the pectin in eucalyptus wood.

MATERIALS AND METHODS

Monosaccharide composition of pectin

The monosaccharide composition of the pectins was determined by calculating the molar weight of its constituent monosaccharides from the individual monosaccharide concentrations (Chapter 2) and expressing it as a percentage of the total pectin.

Experimental design and statistical analysis

The influence of different factors on the monosaccharide composition of eucalyptus pectin was investigated in a completely randomised experiment with four factors (tree species, yield potential of the site, tree age class and wood tissue type). The data were tested in a $2 \times 2 \times 3 \times 3$ factorial experiment and subjected to analysis of covariance (ANCOVA) using the correlation analysis (CORR) and general linear modelling (GLM) procedures (SAS Institute Inc., USA). A LSMEAN with standard error and p-values to compare the different LSMEANS was calculated for each factor level combination and means compared with Scheffé's test.

RESULTS AND DISCUSSION

D-Galacturonic acid

The D-galacturonic acid was identified as the most abundant monosaccharide in the pectin of the tested samples, and it contributed to an average of 63.8% of the pectin (Appendix 3-A). This value was calculated across all four factors (tree species, yield potential of the site, tree age class and wood tissue type) and had a standard deviation (S.D.) of 9.5. These results were comparable to the 80% in pectin from various fruits and vegetables as reviewed by Ridley and co-workers (2001).

The D-galacturonic acid content of the pectin of eucalyptus samples was significantly influenced by the interaction between species and the yield potential of the sites. The pectin from *Eucalyptus grandis*, for example, contained significantly more

D-galacturonic acid than *E. nitens* when the samples from the low-yield sites were compared (Figure 3-1). No significant differences were, however, found between *E. grandis* and *E. nitens* at the high-yield sites. This difference may reflect the relatively fast growth rate of *E. grandis*, compared to *E. nitens*, at sites where growth conditions are not optimal.

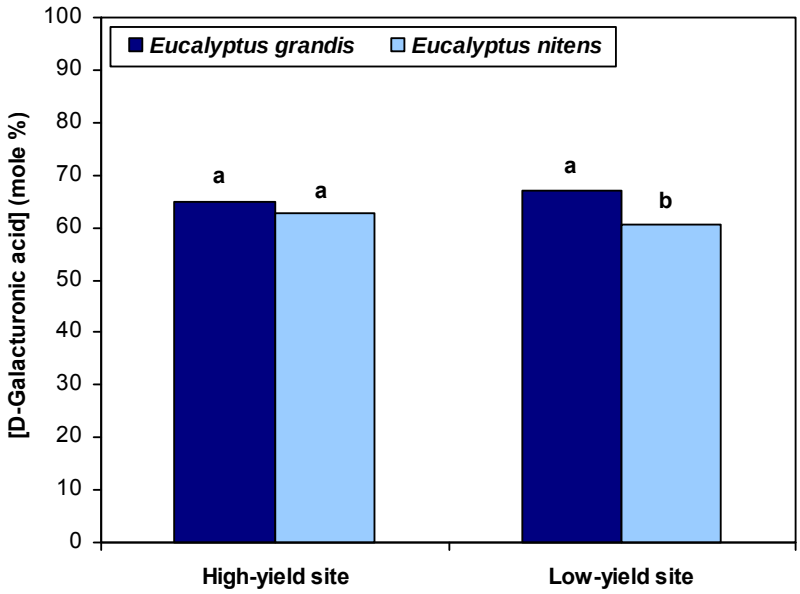

Figure 3-1: Influence of the yield potential of the site and tree species on the D-galacturonic acid content (mole %) of pectin from eucalyptus trees (bars for each site with the same letters do not differ significantly, Scheffé's test, $p \leq 0.05$).

Tree age also had a significant ($p \leq 0.05$) influence on the D-galacturonic acid content of the pectin and in general, the D-galacturonic acid content decreased with an increase in the tree age. The pectin in the six-year-old trees contained significantly more D-galacturonic acid (65.9%) compared to the fourteen-year-old trees (61.5%), but neither of these age classes differed from the ten-year-old trees, with 63.8% D-galacturonic acid. This higher D-galacturonic acid content of younger trees supports the hypothesis that D-galacturonic acid is associated with plants where active growth occurs (Jarvis, 1984; Willats *et al*., 2001). The tissue type did not have a significant influence on the D-galacturonic acid concentration of the pectin, possibly reflecting bad separation of the cambial tissue and the sapwood during sample preparation.

L-Rhamnose

The L-rhamnose was the pectic monosaccharide that occurred in the lowest concentrations in eucalyptus pectin. The pectin contained an average of 4.8%

L-rhamnose (S.D. = 1.5) (Appendix 3-B), while Zsivanovits *et al*. (2004) reported an average of 6.0% occurring in pectin. The pectin from *E. nitens* generally contained significantly more ($p \leq 0.05$) L-rhamnose than *E. grandis* (5.2 versus 4.4%). The L-rhamnose content of the pectin also significantly increased from the cambial tissue (4.2%) to the sapwood (4.9%) and heartwood (5.5%). The change in distribution of L-rhamnose may reflect shifts in the composition of different pectic domains occurring and probably relates to the difference in functions of pectic domains during the different developmental stages of cell-walls (Ridley *et al*., 2001). Neither yield potential nor tree age had a significant influence on the L-rhamnose content of pectins.

L-Arabinose

The pectin from eucalyptus wood contained an average of 10.9% (S.D. = 3.9) L-arabinose (Appendix 3-C). These values were notably lower than the reported values of 29 to 41% occurring in apple fruit (Zsivanovits *et al*., 2004). This difference probably reflects a variation in the composition of monosaccharides occurring in the side-chains of rhamnogalacturonan-I (RG-I).

The L-arabinose content of the pectin from *E. nitens* was significantly ($p \leq 0.05$) higher than that of *E. grandis* (8.6 and 13.1%, respectively). The yield potential of the sites also influenced the L-arabinose concentration and was involved in interactions with both tree age class and wood tissue type. On high-yield sites, the L-arabinose content of the pectin increased with age, however, no differences were observed on the low-yield sites (Table 3-1). Pectin in the cambial tissue contained less L-arabinose than the inactive tissue at high-yield sites, but at the low-yield sites the L-arabinose content significantly increased from the cambial tissue to the heartwood (Figure 3-2).

Table 3-1: Influence of tree age class and yield potential of the site on the L-arabinose content (mole %) of pectin of eucalyptus trees.

Tree age class	High-yield site	Low-yield site
6 years	9.6 b	11.5 a
10 years	11.2 ab	10.5 a
14 years	11.9 a	9.7 a

a,b Mean values in the same columns followed by the same letters do not differ significantly ($p \leq 0.05$), Scheffé's test.

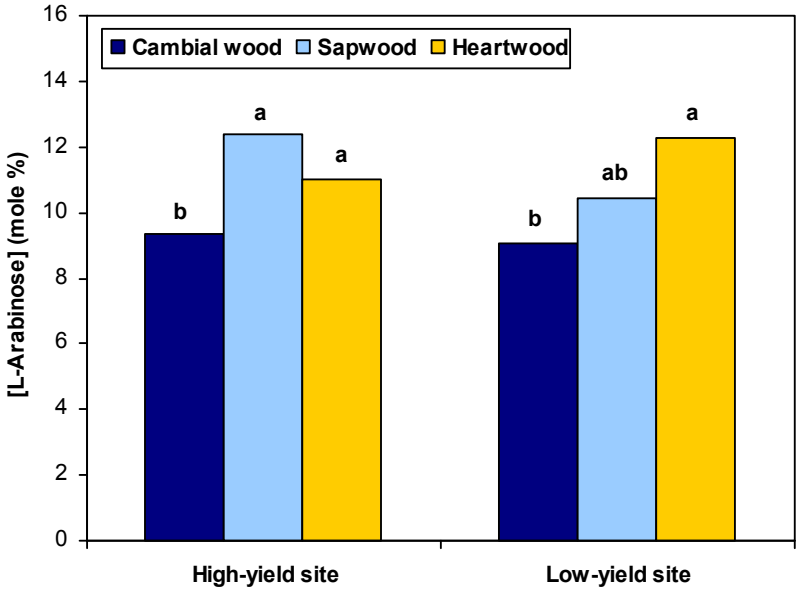

Figure 3-2: Influence of the yield potential of the site and wood tissue type on the L-arabinose content (mole %) of pectin from of eucalyptus trees (bars for each site with the same letters do not differ significantly, Scheffé's test, $p \leq 0.05$).

The interaction between wood tissue type and species also had a significant influence on the L-arabinose content of the pectin. No differences were seen when the different wood tissues of *E. grandis* were compared (Table 3-2). However, in *E. nitens* the pectin in the cambial tissue contained significantly less L-arabinose than the pectin from the sapwood and the heartwood.

Table 3-2: Influence of tree species and wood tissue type on the L-arabinose content (mole %) of pectin of eucalyptus trees.

Wood tissue type	*Eucalyptus grandis*	*Eucalyptus nitens*
Cambial wood	8.1 a	10.3 b
Sapwood	9.0 a	13.8 a
Heartwood	8.8 a	14.6 a

a,b Mean values in the same column followed by the same letters did not differ significantly ($p \leq 0.05$), Scheffé's test.

D-Galactose

The D-galactose contributed an average of 20.7% (S.D. = 8.2) to the pectin of the eucalyptus samples (Appendix 3-D). The D-galactose content of apple pectin was reported as ranging between 49 and 62% (Zsivanovits *et al*., 2004), notably higher than the results presented here. This difference may also reflect variations in the composition of the side-chains of the RG-I as described above.

The pectin of the *E. grandis* samples from the high-yield sites contained significantly more D-galactose than the pectin from *E. nitens*, but the D-galactose content of pectin from these two species did not differ at the low-yield sites (Figure 3-3). In general, an increase in D-galactose concentrations of the pectin was seen from the younger to the older trees. The pectin of the six-year-old samples contained significantly ($p \leq 0.05$) less D-galactose (18.5%) than the pectin from ten-year-old and fourteen-year old trees with 20.7 and 22.7%, respectively. When the different wood tissue types were compared, the cambial pectin (22.9%) contained significantly higher D-galactose concentrations than the pectin from the sapwood or the heartwood (19.3 and 19.8%, respectively).

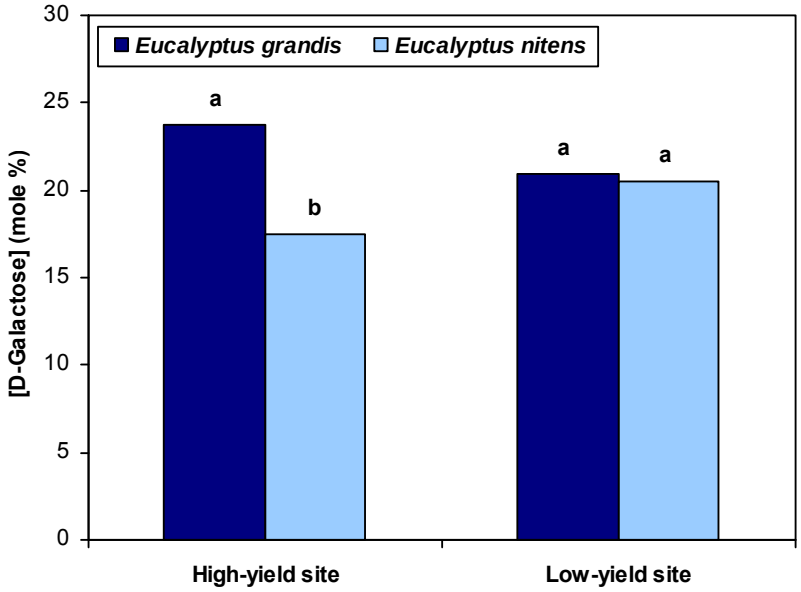

Figure 3-3: Influence of the yield potential of the site and tree species on the D-galactose content (mole %) of pectin from of eucalyptus trees (bars for each site with the same letters do not differ significantly, Scheffé's test, $p \leq 0.05$).

CONCLUSIONS

The occurrence of the pectic monosaccharides in eucalyptus wood was determined in the previous chapter, to calculate the total pectin content of the wood samples (Chapter 2). During the present study, the amounts of pectic monosaccharides measured in these eucalyptus samples were converted into molar concentrations and expressed as percentages (mole %) of the total pectin content of these samples. The results showed that the pectin mainly consisted of D-galacturonic acid and smaller quantities of D-galactose, L-arabinose and L-rhamnose. Wood tissue

type and tree species were identified as the factors that had the biggest influence on the pectin composition of the wood. However, the yield potential of the sites and the tree age class also had a significant impact on the composition of eucalyptus pectin in addition to these two factors.

The tissue type of the wood had a major influence on the distribution of all the monosaccharides that were associated with the RG-I domain. The increase in the concentration of L-rhamnose from the cambial tissue to the heartwood reflected the change from the cambial wood to the sapwood and heartwood of the trees. The change in distribution of L-rhamnose may reflect a shift in the differentiation of pectin from young, actively growing tissue to more mature tissue. The assumption that D-galacturonic acid occurs in higher concentrations in the actively growing tissue (Jarvis, 1984; Willats *et al*., 2001) was, however, not supported by the results of the present study, possibly due to difficulty to separate the cambial tissue and the sapwood during sample preparation. However, the pectin from the younger trees (six-year-old trees) contained significantly more D-galacturonic acid than that of the older trees, which supports the theory that D-galacturonic acid occurred in higher concentrations in young tissue (Pallardy & Kozlowski, 1997; Verma, 2001). The distribution of L-arabinose and D-galactose in the pectin was also significantly influenced by the wood tissue type, tree age class or by the interaction of these two factors. The influence of the different factors on the concentration of the L-arabinose and D-galactose in the pectin was probably due to the difference in functions of pectic domains during the different developmental stages of plants and their cell-walls (Ridley *et al*., 2001).

Species also had a big influence on the composition of the pectin when the monosaccharides that occur in the backbones of the different domains were compared. The present study showed that *E. grandis* contained higher concentrations of D-galacturonic acid in the pectic backbone structures than *E. nitens*. The opposite was true when L-rhamnose was quantified, possibly indicating that these two species differ in the ratio of the RG-I backbone to the other pectic domains. Differences in the composition of the pectic side-chains between *E. grandis* and *E. nitens* are reflected in the differences in L-arabinose and D-galactose content. The macromolecular organisation of the different domains of the wood pectin should, therefore, be investigated.

APPENDICES

Appendix 3-A: The D-galacturonic acid concentration (mole %) measured in pectin of 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
Eucalyptus grandis	6 years	Cambial tissue	79.5	69.9	68.9	48.6	77.3	75.8
		Sapwood	60.1	71.0	63.6	73.1	59.9	73.9
		Heartwood	72.0	62.9	63.4	57.5	68.8	64.6
	10 years	Cambial tissue	51.2	67.1	36.4	57.2	64.4	75.4
		Sapwood	73.7	57.3	62.1	62.4	76.8	73.0
		Heartwood	70.1	73.3	75.5	74.9	77.9	77.0
	14 years	Cambial tissue	51.4	64.5	65.6	60.9	74.4	54.2
		Sapwood	70.7	47.2	75.4	74.0	59.1	55.6
		Heartwood	49.8	27.9	59.5	64.6	54.1	63.6
Eucalyptus nitens	6 years	Cambial tissue	80.0	71.0	69.6	60.4	66.7	71.7
		Sapwood	65.6	60.1	62.8	49.9	69.8	59.4
		Heartwood	61.1	69.4	63.6	48.9	60.5	72.4
	10 years	Cambial tissue	66.5	73.0	68.8	44.5	62.7	51.4
		Sapwood	51.2	57.0	68.6	75.6	65.5	59.7
		Heartwood	61.3	61.0	61.9	58.9	54.2	50.9
	14 years	Cambial tissue	73.0	66.7	57.6	39.1	61.9	68.4
		Sapwood	64.8	61.0	58.9	69.2	58.6	73.3
		Heartwood	65.7	58.2	69.7	74.1	62.5	58.8

Appendix 3-B: The L-rhamnose concentration (mole %) measured in pectin of 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
Eucalyptus grandis	6 years	Cambial tissue	2.74	4.31	3.85	5.51	3.17	3.76
		Sapwood	8.05	4.97	6.36	2.49	5.61	3.80
		Heartwood	4.92	6.23	5.90	7.01	4.44	4.13
	10 years	Cambial tissue	2.41	1.77	7.67	2.45	3.45	3.01
		Sapwood	2.45	4.46	5.37	3.64	2.84	5.15
		Heartwood	4.35	5.51	5.88	3.00	4.47	4.23
	14 years	Cambial tissue	3.58	3.40	3.05	4.19	5.43	3.32
		Sapwood	3.14	4.89	2.60	3.60	5.31	3.23
		Heartwood	4.58	4.06	5.91	6.01	7.66	6.42
Eucalyptus nitens	6 years	Cambial tissue	3.44	5.59	3.88	4.42	3.35	3.23
		Sapwood	5.87	4.88	8.01	6.40	4.99	6.05
		Heartwood	5.30	4.64	6.21	6.85	7.36	4.32
	10 years	Cambial tissue	4.40	2.45	4.47	4.41	4.72	5.56
		Sapwood	7.01	6.14	5.58	3.84	4.27	4.69
		Heartwood	4.92	4.87	3.19	6.43	6.29	7.60
	14 years	Cambial tissue	4.88	3.82	5.11	11.6	4.56	3.23
		Sapwood	4.92	5.69	4.33	4.92	5.55	3.85
		Heartwood	6.05	4.65	6.14	5.43	6.76	5.76

Appendix 3-C: The L-arabinose concentration (mole %) measured in pectin of 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
Eucalyptus grandis	6 years	Cambial tissue	5.16	7.36	7.39	12.8	5.91	9.81
		Sapwood	11.1	7.19	6.48	9.41	10.5	7.66
		Heartwood	8.59	9.05	9.91	12.0	6.50	9.89
	10 years	Cambial tissue	5.99	6.38	16.3	4.89	6.42	4.87
		Sapwood	7.51	9.39	16.8	5.43	5.52	7.64
		Heartwood	7.93	6.36	6.85	6.22	5.33	6.11
	14 years	Cambial tissue	10.3	9.05	8.11	7.96	8.95	7.36
		Sapwood	10.6	16.8	6.06	6.89	10.0	6.40
		Heartwood	9.40	11.3	7.80	10.6	13.8	10.3
Eucalyptus nitens	6 years	Cambial tissue	6.26	9.42	8.16	10.6	10.0	8.23
		Sapwood	12.1	8.19	15.2	17.7	12.0	15.8
		Heartwood	14.7	11.1	15.1	21.6	15.2	11.4
	10 years	Cambial tissue	10.9	8.00	11.6	11.4	12.8	16.1
		Sapwood	19.5	16.1	13.7	9.51	13.6	16.4
		Heartwood	13.2	12.9	12.8	17.9	19.0	20.6
	14 years	Cambial tissue	12.2	11.1	14.3	0.26	13.8	10.7
		Sapwood	13.8	16.2	15.9	10.8	13.6	8.81
		Heartwood	16.0	17.0	8.44	9.71	12.9	12.1

Appendix 3-D: The D-galactose concentration (mole %) measured in pectin of 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
Eucalyptus grandis	6 years	Cambial tissue	12.6	18.5	19.9	33.1	13.7	10.6
		Sapwood	20.8	16.9	23.6	15.0	24.0	14.6
		Heartwood	14.5	21.8	20.8	23.5	20.3	21.4
	10 years	Cambial tissue	40.3	24.7	39.6	35.5	25.7	16.7
		Sapwood	16.4	28.8	15.8	28.5	14.8	14.2
		Heartwood	17.6	14.8	11.8	15.9	12.3	12.6
	14 years	Cambial tissue	34.7	23.0	23.2	26.9	11.2	35.1
		Sapwood	15.6	31.1	16.0	15.5	25.6	34.8
		Heartwood	36.2	56.7	26.8	18.7	24.5	19.7
Eucalyptus nitens	6 years	Cambial tissue	10.3	14.0	18.4	24.7	19.9	16.8
		Sapwood	16.4	26.8	13.9	26.0	13.3	18.7
		Heartwood	18.9	14.9	15.1	22.7	16.9	11.9
	10 years	Cambial tissue	18.1	16.5	15.1	39.7	19.8	27.0
		Sapwood	22.3	20.7	12.1	11.0	16.6	19.3
		Heartwood	20.6	21.2	22.1	16.7	20.5	20.9
	14 years	Cambial tissue	9.88	18.4	23.0	49.0	19.7	17.7
		Sapwood	16.5	17.1	20.9	15.0	22.3	14.0
		Heartwood	12.2	20.1	15.7	10.8	17.8	23.4

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CHAPTER

4

MACROMOLECULAR COMPOSITION OF PECTIN FROM EUCALYPTUS WOOD

ABSTRACT

Current knowledge on the structure of pectic polysaccharides is based on intact pectins extracted from soft plant tissue. Intact pectin can, however, not be extracted from woody material by the same methods. The monosaccharide composition of eucalyptus samples was previously determined and the pectin content calculated. The present study focussed on the reconstruction of the pectic domains to get insight into the macromolecular composition of pectin from *Eucalyptus grandis* and *E. nitens*. The proportional molecular contribution of the different monosaccharides to the pectin and the pectic domains was used to reconstruct and quantify the homogalacturonan (HG) and rhamnogalacturonan-I (RG-I) domains of the pectins. The contribution of each of the domains to the backbone of the pectin was also determined. The present study showed that HG was the predominant pectic domain in both species and contributed up to seven times more to the total backbone component than the RG-I domain. None of the eucalyptus samples differed in their HG or total RG-I content, but the composition of the RG-I domains differed significantly between *E. grandis* and *E. nitens*. The tissue type of the wood also had a significant influence on the macromolecular composition of the eucalyptus pectin, confirming that a shift occurred in composition of pectin between different wood tissues. This work was the first attempt to elucidate the possible domain structure of wood pectin.

INTRODUCTION

The pectic polysaccharides form a class of structurally complex polysaccharides that occur in the primary cell-walls and middle lamellae of all plants. Pectin is divided into four distinctly different structural domains, namely homogalacturonan (HG), rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II (RG-II) and xylogalacturonan (XG) (Anthon & Barrett, 2008; O'Neill *et al*., 1990; 2004; Schols *et al*., 1995; Yapo *et al*., 2007). The interactions of the four pectic domains within the pectic network are highly complex and it is generally assumed that HG, XG and RG-II are covalently interlinked, since they all have backbones that consist of α-(1→4)-linked D-galacturonic acid residues (O'Neill *et al*., 1990; Whitecombe *et al*., 1995). Rhamnogalacturonan-I can be covalently bound to HG (Ishii & Matsunaga, 2001) and XG (Coenen *et al.*, 2007), but it is still unknown whether RG-I is directly bound to RG-II.

The most abundant pectic domain is HG and approximately 65% of pectin is made up by this homopolymer, consisting of α-(1→4)-linked D-galacturonic acid residues (O'Neill *et al.*, 1990; Thibault *et al*., 1993). Recent work indicated that pectin from tomato and sugar beet may contain HG domains that are branched with long chains of D-galacturonic acid (Kirby *et al*., 2008; Round *et al.,* 2010). Two other pectic domains that have backbones consisting of α-(1→4)-linked D-galacturonic acid residues, namely RG-II and XG, occur in much lower concentrations in the pectic network. These two domains cumulatively contribute to less than 10% of the total concentration of pectin (O'Neill *et al*., 2004; Zandleven *et al*., 2007). The fourth domain, namely RG-I, is an extensively branched heteropolymer and up to 35% of pectin consists of this domain (Mohnen, 2008). The backbone of this domain consists of a number of repeating disaccharides of D-galacturonic acid and L-rhamnose (Lau *et al*., 1985). The L-rhamnose residues in the backbones are substituted with neutral chains of arabinan, galactan or arabinogalactan (Mohnen, 1999; O'Neill *et al*., 1990). Rhamnogalacturonan-I is highly variable in its fine structure, and forms of this domain occur that differ in the ratio of galactan and arabinan in their side-chains (Ermel *et al*., 2000; McCartney *et al*., 2000; Willats *et al*., 1999). The second significant feature of RG-I is that galactan-rich and arabinan-rich forms have restricted locations within the cell-wall (Willats *et al*., 2001a). The type, number and nature of the side-chains are, therefore, highly dependant of the cell type and developmental process (Mohnen, 2008; Ridley *et al*., 2001; Willats *et al*., 2001a).

The pectic polysaccharides are multifunctional and have an impact upon cell expansion, cell development, intercellular adhesion and separation, as well as defence mechanisms (Jarvis, 1984; Willats *et al*., 2001b). These polysaccharides are also involved in cell differentiation and the regulation of mechanical properties of the cell wall (Willats *et al*., 1999). The macromolecular composition and structure of the pectic domains, the amount and distribution of their substituents and their molar mass influences many of these functionalities (Axelos & Thibault, 1991). The length of the different domains and the proportion of HG, RG-I and RG-II, may also influence the properties of the pectin (Bonnin *et al*., 2002). The determination of the macromolecular characteristics of the pectic domains can, thus, provide useful information on the properties of pectin from a specific source, as well as specific developmental processes.

The structure of the pectic domains from a number of soft-tissue sources have been studied comprehensively (Lau *et al*., 1985; Oechslin *et al*., 2003; Rascón-Chu *et al*., 2009; Ridley *et al*., 2001; Yapo *et al*., 2007), but pectin from wood tissue has not been extracted or characterised. The current work, therefore, attempted to reconstruct hypothetical pectic polymers based on the amounts of monosaccharide residues released during the total hydrolysis of the wood.

MATERIALS AND METHODS

Macromolecular composition of pectin

The pectic domains (HG and RG-I) of the eucalyptus samples were reconstructed from the monosaccharide data obtained previously (Chapter 2). The constituent monosaccharides of pectin differ substantially in their molecular weight. Rhamnose for example, has a molecular weight of only 164.14 g/mol compared to galacturonic acid with a molecular weight of 216.12 g/mol. Due to difference in molecular weights, the relative masses of the monosaccharides do not give a true illustration of the macromolecular composition of pectin. The contribution of the different fractions to each pectic component was, therefore, calculated as follows:

$$C = (Mc / Mt) \times 100 \qquad \text{(Equation 1)}$$

where C is the amount of a specific component of pectin (%), Mc is the number of molecules in the sample contributing to the component and Mt is the total number of molecules in pectin or the domain of the sample (Table 4-1).

Table 4-1: Terms used in the calculation of the relative contribution of a component to pectin or other smaller components.

Pectic component (C)*	**Molecules contributing to component (Mc)***	**Total molecules (Mt)***
Rhamnogalacturonan-I domain	Rha + GalA (= Rha) + Ara + Gal	GalA + Rha + Ara + Gal
Homogalacturonan domain	Total GalA - RG-I GalA	GalA + Rha + Ara + Gal
Total backbone	GalA + Rha	GalA + Rha + Ara + Gal
Homogalacturonan backbone fraction	Total GalA - RG-I GalA	GalA + Rha
Rhamnogalacturonan-I backbone fraction	Rha + GalA (= Rha)	GalA + Rha
Backbone fraction of rhamnogalacturonan-I	Rha + GalA (= Rha)	Rha + GalA (= Rha) + Ara + Gal
Side-chain fraction of rhamnogalacturonan-I	Ara + Gal	Rha + GalA (= Rha) + Ara + Gal
Galactan fraction of rhamnogalacturonan-I side-chains	Gal	Ara + Gal
Arabinan fraction of rhamnogalacturonan-I side-chains	Ara	Ara + Gal

* Terms substituted in Equation 1

The size of each component was determined by calculating the number of constituent residues from the individual monosaccharide concentrations (Chapter 2). Round *et al*. (2010) proposed that a HG polymer had an average degree of polymerisation (dp) of 320 residues. This dp was used as the basis for developing hypotheitcal pectin polymers. In the hypothetical polymers the dp of each component was expressed proportionally to the HG domain of *E. grandis*, that was assigned a dp of 320 and represented by an estimated number of residues (Re).

The side-chains of RG-I occur as chains of arabinan (frequently branched) (Carpita & Gibeaut, 1993; Oechslin *et al*., 2003), galactan (mostly linear) (Carpita & Gibeaut, 1993; Mohnen, 1999) or arabinogalactan of various lengths attached to the L-rhamnose residues in the backbone (Mohnen, 1999; O'Neill *et al*., 1990). Arabinogalactan consists of chains of D-galactose with one (in Type I arabinogalactan) to three L-arabinose residues (in Type II arabinogalactan) per chain (Carpita & Gibeaut, 1993, Mohnen, 1999; Vincken *et al*., 2003). The small amount of L-arabinose in arabinogalactan was, for the purposes of this study, assigned to the arabinan side-chains and the D-galactose to galactan side-chains.

The RG-II domain was not quantified during this study due to the very low concentrations of the side-chain monosaccharides, while the XG domain was excluded due to the difficulty in distinguishing between D-xylose monomers from XG and from hemicelluloses. The small amount of D-galacturonic acid that possibly originated from these two domains was considered to be part of HG.

Experimental design and statistical analysis

The distribution and the macromolecular composition of the HG and RG-I domains in eucalyptus wood was investigated in a completely randomised experiment with four factors (tree species, yield potential of the site, tree age class and wood tissue type), replicated three times. The data were subjected to analysis of covariance (ANCOVA) using the correlation analysis (CORR) and general linear modelling (GLM) procedures (SAS Institute Inc., USA) and means compared with Scheffé's test.

RESULTS AND DISCUSSION

Molecular contribution of different domains to pectin

Homogalacturonan contributed to an average of 58.9% of pectin residues in all the samples (Appendix 4-A), which is slightly lower than the values reported in apple, citrus and beet of approximately 65% (Thibault *et al*., 1993). The RG-I domain made up the remainder of the pectic residues and values of up to 76.1% measured in the present study (Appendix 4-B) were notably higher than reported values of 20% to 35% for soft plant tissues (Mohnen, 2008). This difference may reflect variation in the amount of side-chains between plant species. None of the tested factors (tree species, yield potential of the site, tree age class or wood tissue type) had a significant influence ($p \leq 0.05$) on the distribution of the pectic molecules between HG and RG-I and the ratio of these two domains were, therefore, relatively constant (Figure 4-1 and Figure 4-2).

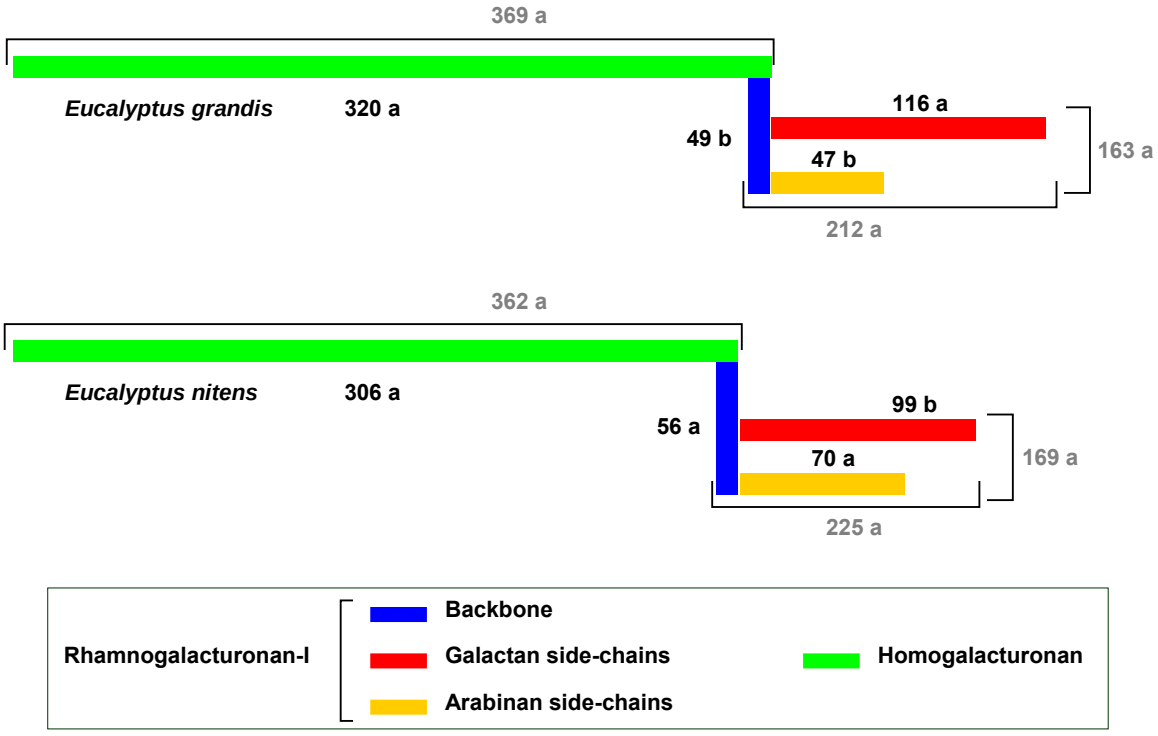

Figure 4-1: Schematic representation of the pectin composition of *Eucalyptus grandis* and *E. nitens* wood. Length of rods represents the average contribution of each component to a hypothetical pectic polymer. Length of rods is expressed as an estimated number of monosaccharide residues (Re). Rods for each component with the same letters did not differ significantly (Scheffé's test, $p \leq 0.05$).

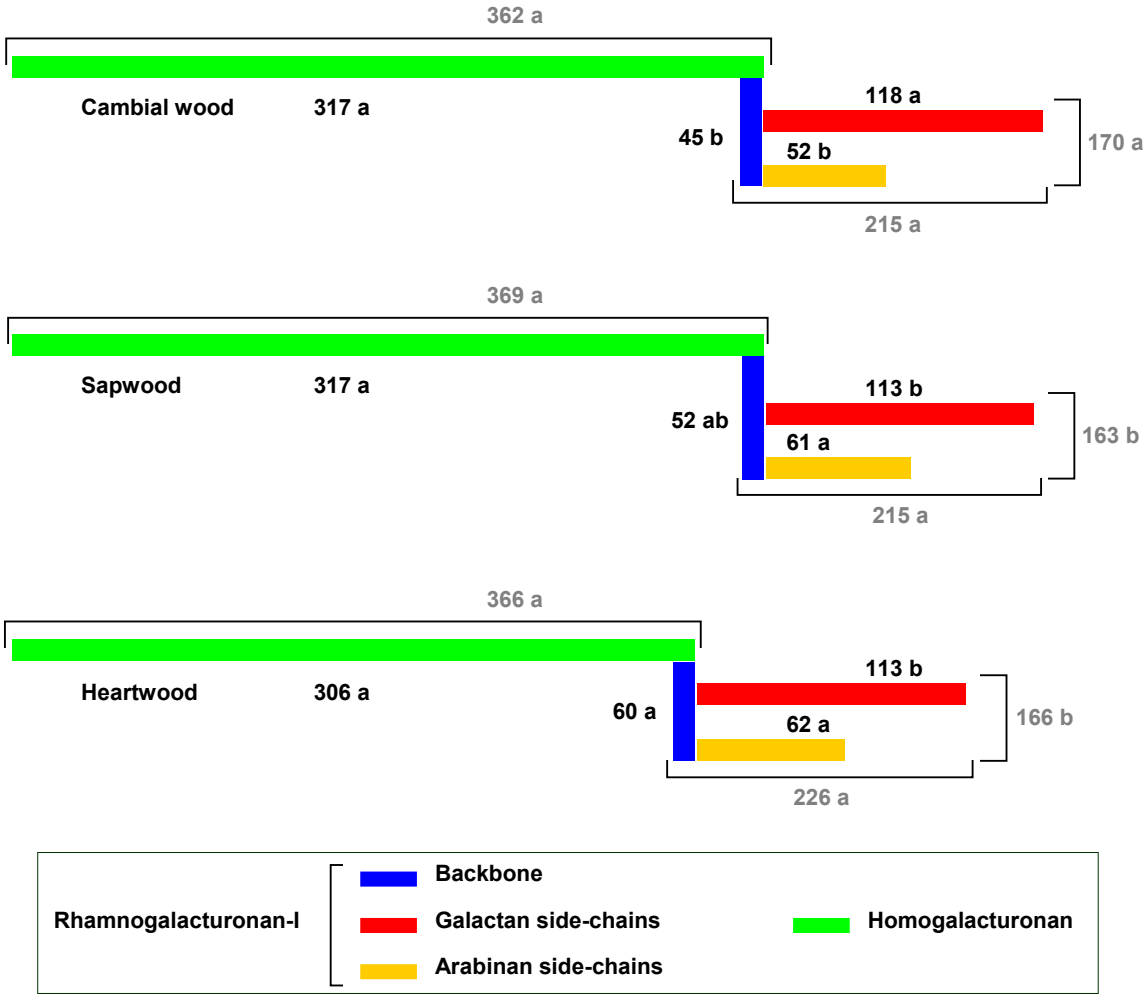

Figure 4-2: Schematic representation of the pectin composition in different wood tissue types. Length of rods represents the average contribution of each component to a hypothetical pectic polymer. Length of rods is expressed as an estimated number of monosaccharide residues (Re). Rods for each component with the same letters did not differ significantly (Scheffé's test, $p \leq 0.05$).

Pectic backbone

The backbone fraction (from both HG and RG-I) contributed to an average of 68.8% (Appendix 4-C) of eucalyptus pectin and none of the tested factors influenced the backbone content of pectin. Although the contribution of HG domain to the backbone was constant, the size of the RG-I backbone differed significantly ($p \leq 0.05$) between the species and the different types of wood tissue (Figure 4-1 and Figure 4-2). The variation resulted in differences in the ratio of the backbone components (HG and RG-I).

Macromolecular composition of rhamnogalacturonan-I

On average, the backbone molecules contributed only 23.9% to the RG-I domain, while the balance of 76.1% consisted of the side-chain molecules (Appendix 4-D and Appendix 4-E). The yield potential of the plantations was the only factor that did not have a significant influence on the ratio of the backbone to side-chain fractions of the RG-I. The RG-I from *E. nitens* (56 Re) contained significantly more backbone molecules and than *E. grandis* (49 Re), but a similar number of side-chain molecules (169 and 163 Re, respectively) (Figure 4-1). A comparison of the tissue types of the wood showed a significant increase in the backbone (and a decrease in side-chains) occurring from the cambial tissue (45 Re) to the sapwood (52 Re) and heartwood (60 Re) (Figure 4-2). This increase in the backbone fraction in older tissue (heartwood) was also observed in fourteen-year-old trees that contained significantly more backbone (60 Re) that the six-year-old and ten-year-old trees (52 and 55 Re, respectively).

The present study indicated that galactan contributed to an average of 64.9% of the side-chains and the remainder consisted of arabinan (Appendix 4-F and Appendix 4-G). The *E. grandis* samples contained significantly more galactan side-chains than *E. nitens* (116 versus 99 Re), while the latter contained significantly more arabinan (70 versus 47 Re) (Figure 4-1). The tissue type of the wood also had a significant influence on the occurrence of arabinan and galactan in the side-chains. The arabinan side-chains increased from the cambial tissue (52 Re) to the sapwood and heartwood (61 Re and 62 Re, respectively) (Figure 4-2). This increase in arabinan from the outside to the inside of the tree was mirrored by a decrease in the amount of galactan side-chains (Figure 4-2). These findings were consistent with literature stating that galactan-rich RG-I is associated with meristematic tissue, while arabinan-rich RG-I is associated older tissue (Vicré *et al*., 1998; Willats *et al*., 1999). Similar changes in the composition of RG-I side-chains has been described for *Populus tremula* (Ermel *et al*., 2000), peas (McCartney *et al*., 2000) and carrot (Willats *et al*., 1999).

CONCLUSIONS

The present study was the first attempt (to my knowledge) to determine the molecular composition of pectin and its domains in wood and it required an alternative isolation procedure. The monosaccharide data obtained previously (Chapter 2) were used to construct hypothetical HG and RG-I domains. These hypothetical domains

illustrated the relative composition of pectic polymers and did not represent the exact structure, seeing that it was impossible to make predictions on the distribution of different RG-I domains in the pectic polymer or the length and composition of the RG-I side-chains. The RG-II and XG domains were not characterised. This limitation was not expected to have a notable influence on the results, because these two domains are considered to to account for less than 10% of the total concentration of pectin (Fleischer *et al*., 1999; Ishii & Matsunaga 2001; Nakamura *et al*., 2002; O'Neill *et al*., 2004; Zandleven *et al*., 2007).

Homogalacturonan consisting only of D-galacturonic acid was the predominant pectic domain in eucalyptus wood. The observed amounts of approximately 60% D-galacturonic acid were consistent with the observations of Ridley *et al*. (2001) and Thibault *et al*. (1993) who considered this monosaccharide to be the predominant pectic component. However, the RG-I content of the eucalyptus wood was notably higher than values reported from soft tissue (Mohnen, 2008). The highly variable nature (length and composition) of the side-chains of this domain may have caused differences in reported amounts (Ermel *et al*., 2000; Mohnen, 2008; Willats *et al*., 1999). The HG and RG-I content in eucalyptus appeared to be conserved, seeing that none of the tested factors influenced the ratio of pectic domains.

The hypothetical polymers illustrated the occurrence of similar amounts of HG and RG-I in pectin of *E. grandis* and *E. nitens*. The composition of the RG-I domains of these to species, however, differed notably. The RG-I from *E. grandis* consisted of shorter backbones with a higher concentration of side-chains, while *E. nitens* had longer backbones and a lower concentration of side-chains. The two species also differed in the ratio of galactan and arabinan in their side-chains.

Different types of wood tissue contained similar amounts of HG and RG-I in their pectin. However, the ratio of the backbone of HG and RG-I changed from the outside to the inside of the trees, due to an increased RG-I backbone. This increased RG-I backbone fraction was also observed in the older trees (fourteen-year-old) trees. The composition of the RG-I domains of the three tissues differed notably, as illustrated by the differences in their backbone and the side-chain content and in the composition of their side-chains.

The findings of this study were consistent with literature that described pectin from different sources to be highly variable in the fine structure and distribution within

the cell-walls, especially that of the RG-I domains (Bush & McCann, 1999; Vicré *et al*., 1998; Willats *et al*., 1999). Future studies to investigate the occurrence and composition of pectin in eucalyptus wood should include more species. This information may aid in the understanding of the influence of pectins on pulping and papermaking processes (Peng *et al.*, 2003; Ricard *et al*., 2005; Thornton *et al*., 1993). It may also assist to develop biotechnological approaches to improve these processes.

APPENDICES

Appendix 4-A: Homogalacturonan fraction (%) of the pectin from 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	**Wood tissue type**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
E. grandis	**6 years**	**Cambial tissue**	76.7	65.6	65.1	43.1	74.1	72.0
		Sapwood	52.0	66.0	57.2	70.6	54.3	70.1
		Heartwood	67.0	56.7	57.5	50.4	64.4	60.4
	10 years	**Cambial tissue**	48.8	65.4	28.7	54.7	61.0	72.4
		Sapwood	71.2	52.8	56.7	58.8	74.0	67.9
		Heartwood	65.8	67.8	69.6	71.9	73.4	72.8
	14 years	**Cambial tissue**	47.9	61.1	62.6	56.7	69.0	50.9
		Sapwood	67.6	42.4	72.8	70.4	53.8	52.4
		Heartwood	45.3	23.9	53.6	58.6	46.4	57.2
E. nitens	**6 years**	**Cambial tissue**	76.6	65.4	65.7	55.9	63.3	68.5
		Sapwood	59.7	55.2	54.8	43.5	64.8	53.4
		Heartwood	55.8	64.7	57.4	42.0	53.2	68.0
	10 years	**Cambial tissue**	62.1	70.6	64.4	40.1	58.0	45.8
		Sapwood	44.2	50.8	63.1	71.8	61.2	55.0
		Heartwood	56.3	56.1	58.7	52.5	47.9	43.3
	14 years	**Cambial tissue**	68.2	62.9	52.5	27.5	57.4	65.2
		Sapwood	59.9	55.3	54.5	64.3	53.1	69.5
		Heartwood	59.7	53.5	63.6	68.6	55.7	53.0

Appendix 4-B: Rhamnogalacturonan-I fraction (%) of the pectin from 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
E. grandis	6 years	Cambial tissue	23.3	34.4	34.9	56.9	25.9	28.0
		Sapwood	48.0	34.0	42.8	29.4	45.7	29.9
		Heartwood	33.0	43.3	42.5	49.6	35.6	39.6
	10 years	Cambial tissue	51.2	34.6	71.3	45.3	39.0	27.6
		Sapwood	28.8	47.2	43.3	41.2	26.0	32.1
		Heartwood	34.2	32.2	30.4	28.1	26.6	27.2
	14 years	Cambial tissue	52.1	38.9	37.4	43.3	31.0	49.1
		Sapwood	32.4	57.6	27.2	29.6	46.2	47.6
		Heartwood	54.7	76.1	46.4	41.4	53.6	42.8
E. nitens	6 years	Cambial tissue	23.4	34.6	34.3	44.1	36.7	31.5
		Sapwood	40.3	44.8	45.2	56.5	35.2	46.6
		Heartwood	44.2	35.3	42.6	58.0	46.8	32.0
	10 years	Cambial tissue	37.9	29.4	35.6	59.9	42.0	54.2
		Sapwood	55.8	49.2	36.9	28.2	38.8	45.0
		Heartwood	43.7	43.9	41.3	47.5	52.1	56.7
	14 years	Cambial tissue	31.8	37.1	47.5	72.5	42.6	34.8
		Sapwood	40.1	44.7	45.5	35.7	46.9	30.5
		Heartwood	40.3	46.5	36.4	31.4	44.3	47.0

Appendix 4-C: Contribution of the backbone fraction (%) to the pectin of 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
E. grandis	6 years	Cambial tissue	82.2	74.2	72.8	54.1	80.4	79.6
		Sapwood	68.1	75.9	69.9	75.6	65.5	77.7
		Heartwood	76.9	69.1	69.3	64.5	73.3	68.7
	10 years	Cambial tissue	53.7	68.9	44.1	59.6	67.9	78.4
		Sapwood	76.1	61.8	67.4	66.1	79.6	78.2
		Heartwood	74.5	78.8	81.4	77.9	82.3	81.3
	14 years	Cambial tissue	55.0	67.9	68.7	65.1	79.9	57.5
		Sapwood	73.8	52.1	78.0	77.6	64.4	58.8
		Heartwood	54.4	32.0	65.4	70.6	61.7	70.0
E. nitens	6 years	Cambial tissue	83.5	76.6	73.5	64.8	70.0	75.0
		Sapwood	71.5	65.0	70.9	56.3	74.8	65.5
		Heartwood	66.4	74.0	69.8	55.7	67.9	76.7
	10 years	Cambial tissue	70.9	75.5	73.3	48.9	67.4	56.9
		Sapwood	58.3	63.1	74.2	79.5	69.7	64.4
		Heartwood	66.2	65.8	65.1	65.4	60.4	58.5
	14 years	Cambial tissue	77.9	70.5	62.8	50.7	66.5	71.6
		Sapwood	69.8	66.7	63.2	74.1	64.2	77.2
		Heartwood	71.8	62.8	75.8	79.5	69.2	64.5

Appendix 4-D: Contribution of the backbone fraction (%) to the rhamnogalacturonan-I domain of the pectin from 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
E. grandis	6 years	Cambial tissue	23.6	25.0	22.0	19.4	24.5	26.9
		Sapwood	33.5	29.2	29.7	16.9	24.5	25.5
		Heartwood	29.9	28.7	27.8	28.3	24.9	20.9
	10 years	Cambial tissue	9.4	10.2	21.5	10.8	17.7	21.8
		Sapwood	17.0	18.9	24.8	17.7	21.8	32.1
		Heartwood	25.5	34.2	38.7	21.3	33.6	31.1
	14 years	Cambial tissue	13.7	17.5	16.3	19.4	35.0	13.5
		Sapwood	19.3	17.0	19.1	24.4	23.0	13.6
		Heartwood	16.7	10.7	25.5	29.1	28.6	30.0
E. nitens	6 years	Cambial tissue	29.4	32.3	22.6	20.1	18.3	20.5
		Sapwood	29.2	21.8	35.5	22.6	28.3	25.9
		Heartwood	24.0	26.3	29.1	23.6	31.4	27.1
	10 years	Cambial tissue	23.3	16.6	25.1	14.7	22.5	20.5
		Sapwood	25.1	25.0	30.2	27.2	22.0	20.8
		Heartwood	22.6	22.2	15.4	27.1	24.1	26.8
	14 years	Cambial tissue	30.6	20.6	21.5	32.0	21.4	18.6
		Sapwood	24.6	25.5	19.1	27.6	23.7	25.2
		Heartwood	30.0	20.0	33.7	34.7	30.5	24.5

Appendix 4-E: Contribution of the side-chain fraction to the rhamnogalacturonan-I domain of the pectin from 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
E. grandis	6 years	Cambial tissue	76.4	75.0	78.0	80.6	75.5	73.1
		Sapwood	66.5	70.8	70.3	83.1	75.5	74.5
		Heartwood	70.1	71.3	72.2	71.7	75.1	79.1
	10 years	Cambial tissue	90.6	89.8	78.5	89.2	82.3	78.2
		Sapwood	83.0	81.1	75.2	82.3	78.2	67.9
		Heartwood	74.5	65.8	61.3	78.7	66.4	68.9
	14 years	Cambial tissue	86.3	82.5	83.7	80.6	65.0	86.5
		Sapwood	80.7	83.0	80.9	75.6	77.0	86.4
		Heartwood	83.3	89.3	74.5	70.9	71.4	70.0
E. nitens	6 years	Cambial tissue	70.6	67.7	77.4	79.9	81.7	79.5
		Sapwood	70.8	78.2	64.5	77.4	71.7	74.1
		Heartwood	76.0	73.7	70.9	76.4	68.6	72.9
	10 years	Cambial tissue	76.7	83.4	74.9	85.3	77.5	79.5
		Sapwood	74.9	75.0	69.8	72.8	78.0	79.2
		Heartwood	77.4	77.8	84.6	72.9	75.9	73.2
	14 years	Cambial tissue	69.4	79.4	78.5	68.0	78.6	81.4
		Sapwood	75.4	74.5	80.9	72.4	76.3	74.8
		Heartwood	70.0	80.0	66.3	65.3	69.5	75.5

Appendix 4-F: Contribution of arabinan to the side-chains of the rhamnogalacturonan-I domain of the pectin from 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
E. grandis	6 years	Cambial tissue	29.0	28.5	27.1	27.8	30.2	48.0
		Sapwood	34.7	29.9	21.6	38.5	30.5	34.4
		Heartwood	37.2	29.3	32.3	33.8	24.3	31.6
	10 years	Cambial tissue	12.9	20.5	29.2	12.1	20.0	22.5
		Sapwood	31.4	24.6	51.4	16.0	27.1	35.0
		Heartwood	31.1	30.0	36.7	28.1	30.2	32.6
	14 years	Cambial tissue	22.9	28.2	25.9	22.8	44.4	17.3
		Sapwood	40.4	35.0	27.5	30.8	28.0	15.5
		Heartwood	20.6	16.7	22.6	36.2	36.0	34.4
E. nitens	6 years	Cambial tissue	37.8	40.2	30.8	30.0	33.5	32.9
		Sapwood	42.5	23.4	52.3	40.5	47.4	45.8
		Heartwood	43.7	42.9	50.1	48.7	47.3	49.0
	10 years	Cambial tissue	37.6	32.6	43.5	22.3	39.2	37.3
		Sapwood	46.7	43.8	53.1	46.3	45.0	45.9
		Heartwood	39.0	37.9	36.8	51.7	48.1	49.7
	14 years	Cambial tissue	55.2	37.7	38.4	0.5	41.1	37.7
		Sapwood	45.5	48.7	43.3	41.8	37.9	38.6
		Heartwood	56.8	45.9	34.9	47.4	42.0	34.1

Appendix 4-G: Contribution of galactan to the side-chain of the rhamnogalacturonan-I domain of the pectin from 108 samples representing tree species, yield potential of the sites, tree age classes and wood tissue types.

			High-yield site			Low-yield site		
	Tree age	Wood tissue type	R1	R2	R3	R1	R2	R3
E. grandis	6 years	Cambial tissue	71.0	71.5	72.9	72.2	69.8	52.0
		Sapwood	65.3	70.1	78.5	61.5	69.5	65.6
		Heartwood	62.8	70.7	67.8	66.2	75.7	68.4
	10 years	Cambial tissue	87.1	79.5	70.8	87.9	80.0	77.5
		Sapwood	68.6	75.4	48.6	84.0	72.9	65.0
		Heartwood	68.9	70.0	63.3	71.9	69.8	67.4
	14 years	Cambial tissue	77.1	71.8	74.1	77.2	55.6	82.7
		Sapwood	59.7	65.0	72.5	69.2	72.0	84.5
		Heartwood	79.4	83.3	77.4	63.8	64.0	65.6
E. nitens	6 years	Cambial tissue	62.2	59.8	69.2	70.0	66.5	67.1
		Sapwood	57.5	76.6	47.7	59.5	52.6	54.2
		Heartwood	56.4	57.1	50.0	51.3	52.7	51.0
	10 years	Cambial tissue	62.4	67.4	56.5	77.7	60.8	62.7
		Sapwood	53.3	56.2	46.9	53.7	55.0	54.1
		Heartwood	61.0	62.1	63.2	48.3	51.9	50.3
	14 years	Cambial tissue	44.8	62.3	61.7	99.5	58.9	62.3
		Sapwood	54.5	51.3	56.7	58.2	62.1	61.4
		Heartwood	43.2	54.2	65.1	52.6	58.0	65.9

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CHAPTER

5

COMPARISON OF THE CONTENT AND COMPOSITION OF PECTIN FROM *EUCALYPTUS* SPP.

ABSTRACT

Eucalyptus grandis and *E. nitens* were compared in previous chapters as part of a study to determine the effect of different factors (tree species, yield potential of the site, tree age class and wood tissue types) on the occurrence and composition of the pectin in eucalyptus wood. A third species, namely *E. macarthurii* was included in the current work and compared with *E. grandis* and *E. nitens* to investigate the occurrence and structure of pectin in different eucalyptus species. During this study, only samples that were collected from sites that were considered to be high in terms of potential wood yield were analysed. Two of the factors namely wood tissue type and tree age class did not cause differentiation between the pectins. The focus of the present study was, therefore, differences in pectin between *E. grandis*, *E. nitens* and *E. macarthurii*. The *E. macarthurii* wood contained significantly more pectin than *E. grandis* and *E. nitens*, while the latter two species, contained more homogalacturonan (HG), as well as rhamnogalacturonan-I (RG-I) backbone residues. Monomers from the RG-I domain of *E. macarthurii* were significantly more than that of *E. grandis* and *E. nitens*. The distribution and composition of the RG-I domain of the three eucalyptus species differed substantially, supporting previous findings that this domain to be highly variable in its fine structure. The content and macromolecular composition of the pectin in different eucalyptus species proved to be very diverse. Future studies should include a bigger pool of species to get a more comprehensive understanding of the occurrence and structure of pectin in the *Eucalyptus* genus and also other hardwoods.

INTRODUCTION

Different types of wood are highly variable in composition and structure and this diversity is pronounced in hardwoods, because the number of species is much higher than in softwoods (Pinto *et al*., 2005; Sjöström, 1993). At present, hardwood species are the most important source of wood for the pulp and paper industry and eucalyptus species represent the main fibre sources in various parts of the world (Pinto *et al*., 2005; Pisuttipiched, 2004; Quoirin & Quisen, 2006; Ramírez *et al*., 2009; Silvério *et al*., 2007). The increasing interest in wood from the *Eucalyptus* genus is due to their fast growth rates, short rotations and their wood properties in terms of pulping, bleaching behaviour and pulp quality (Gullichsen & Paulapuro, 2000; Sjöström, 1993).

It was recently determined that eucalyptus wood contained between 15.2 and 25.8 mg/g pectin (Chapter 2). The highest concentration of these pectins usually occurs in the middle lamella, the cell corners and the primary cell-walls (Goycoolea & Cárdenas, 2003), where they play an important role in maintaining intercellular adhesion between cells (Jarvis, 1984; Willats *et al*., 2001). These polysaccharides are also present in the junction zone between fibre cells in woody tissue (Mohnen, 2008). The occurrence of wood pectin is important in the pulp and paper industry, because it influences the refining energy used during mechanical pulping (Peng *et al*., 2003). Pectin is also the source of polygalacturonic acid that is a substantial part of the anionic trash released during alkaline-peroxide bleaching (Ricard *et al*., 2005a; 2005b; Thornton *et al*., 1993).

Studies that focussed on the biochemistry of different eucalyptus species mainly concentrated on the occurrence and composition of lipophilic extractives (Silvério *et al*., 2007). Other studies touched on the occurrence of lignin and hemicellulose (Garrote *et al*., 2007; Ona *et al*., 1998). No information is, however, available on how species of eucalyptus differ in the occurrence and structure of their pectins. In this chapter, the pectin content and composition of *E. macarthurii* were compared with that of *E. grandis* and *E. nitens*.

MATERIALS AND METHODS

Sampling and preparation of wood samples

Wood cores were sampled at breast height from three eucalyptus species in commercial plantations during the winter of 2006. The wood samples consisted of 27

wood cores from *E. grandis*, *E. nitens* and *E. macarthurii* from three age classes (six, ten and fourteen years) grown on sites that were considered to be high in terms of potential wood yield. Cores were collected from three randomly selected trees and each tree represented a replication for the experiment. Each of the wood cores was cut into sections representing three tissue types, namely cambial tissue, sapwood and heartwood. These samples were finely ground, using coarse sandpaper (no. 60), without heating the core in the process. The wood powder was screened and the portion that passed through the 635 µm screen but was retained on the 423 µm screen was used for analysis.

Analysis and determination of the content and composition of pectin

Wood samples were hydrolysed and the constituent pectic monosaccharides quantified by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) as described previously (Chapter 2). The pectin content of the eucalyptus samples was calculated by adding the total amount of pectic monosaccharides (D-galacturonic acid, L-rhamnose, D-galactose, L-arabinose). The monosaccharide composition of the pectins was then determined from the molar contribution of each monosaccharides and expressing it as a percentage of the total pectin (Chapter 3). Each of the pectic components was constructed and the contribution of the different fractions to these pectic components calculated as described previously (Chapter 4). Hypothetical models of the pectin polymers were constructed by assuming a degree of polymerisation (dp) of 320 residues for the HG domains of tomato fruit (Round *et al*., 2010). In these hypothetical models, the dp of each component was proportional to the largest HG domain (that of *E. nitens*) and expressed as an estimated number of residues (Re).

Experimental design and statistical analysis

A completely randomised experiment with three factors (tree species, tree age class and wood tissue types) was designed and replicated three times. The data were tested in a 3 × 3 × 3 factorial experiment to investigate the influence of the different factors on the content and composition of the eucalyptus pectin. The data were subjected to general linear modelling (GLM) procedures of the SAS/STAT® 9.1 statistical package (SAS Institute Inc., USA). A LSMEAN with standard error and p-values to compare the different LSMEANS was calculated for each factor level combination and means compared with Scheffé's test.

RESULTS AND DISCUSSION

Influence of wood tissue type and tree age class

Inclusion of the data from *E. macarthurii* in the comparison of species confirmed that the tested factors had the same relative influence on the content and composition of the pectin in all three species. Wood from the cambial tissue was significantly different compared to the older tissues in the content and composition of its pectin, while the pectins from the sapwood and heartwood were similar in composition (Table 5-1). Wood tissue type mainly caused differences in the concentrations of the monosaccharides that originated from the RG-I domain, as well as the composition of this domain. The only change between the current results and previous findings was that the D-galactose content of cambium and the older tissues did not differ significantly (Table 5-1).

Table 5-1: Influence of wood tissue type on the pectin content and composition of eucalyptus wood. Data were summarised from Appendix 5-A to Appendix 5-L.

Concentration of component	Cambium	Sapwood	Heartwood
Total pectin (mg/g wood)	24.0 a	20.4 b	21.0 b
Pectic monosaccharides (mole % of pectin)			
D-Galacturonic acid	63.7 a	61.7 a	59.4 a
L-Rhamnose	3.57 b	4.78 a	5.00 a
L-Arabinose	9.27 b	12.1 a	12.3 a
D-Galactose*	23.5 a	21.4 a	23.3 a
Pectic domains and backbone fractions (% of pectin)			
Rhamnogalacturonan-I domain	39.9 a	43.1 a	45.6 a
Homogalacturonan domain	60.1 a	56.9 a	54.4 a
Total backbone	67.3 a	66.5 a	64.4 a
Rhamnogalacturonan-I backbone fraction	7.14 b	9.57 a	9.99 a
Rhamnogalacturonan-I fractions (% of rhamnogalacturonan-I)			
Backbone fraction of rhamnogalacturonan-I	18.9 b	22.2 a	23.2 a
Side-chain fraction of rhamnogalacturonan-I	81.1 a	77.8 b	76.8 b
Rhamnogalacturonan-I side-chain fractions (% of side-chains)			
Galactan fraction of rhamnogalacturonan-I side-chains	69.7 a	63.7 b	64.1 b
Arabinan fraction of rhamnogalacturonan-I side-chains	30.3 b	36.3 a	35.9 a

a,b Mean values in the same row followed by the same letters did not differ significantly ($p \leq 0.05$), Scheffe's test. Values were averaged over tree species and tree age class.
* Changed from previous findings.

The age class of the trees had a significant influence on the occurrence of the monosaccharides and the composition of the RG-I domain in all three species (Table 5-2). The largest influence was seen in the concentrations of the monosaccharides that originated from the side-chains of RG-I domain, as well as the

distribution of this domain between the backbone and the side-chain fractions. The only difference here was that the fourteen-year-old wood contained significantly more backbone residues than the younger trees (Table 5-2).

Table 5-2: Influence of tree age class on the pectin content and composition of eucalyptus wood. Data were summarised from Appendix 5-A to Appendix 5-L.

Concentration of component	6 years	10 years	14 years
Total pectin (mg/g wood)	21.2 a	22.5 a	21.7 a
Pectic monosaccharides (mole % of pectin)			
D-Galacturonic acid	65.0 a	61.0 a	58.8 b
L-Rhamnose	4.82 a	4.37 a	4.16 a
L-Arabinose	10.0 b	11.5 ab	12.1 a
D-Galactose	20.2 b	22.5 ab	25.5 a
Pectic domains and backbone fractions (% of pectin)			
Rhamnogalacturonan-I domain	39.8 a	43.4 a	45.4 a
Homogalacturonan domain	60.2 a	56.6 a	54.7 a
Total backbone*	63.0 b	65.3 b	69.8 a
Rhamnogalacturonan-I backbone fraction	9.64 a	8.74 a	8.31 a
Rhamnogalacturonan-I fractions (% of rhamnogalacturonan-I)			
Backbone fraction of rhamnogalacturonan-I	19.2 b	20.5 b	24.6 a
Side-chain fraction of rhamnogalacturonan-I	80.8 a	79.5 a	75.4 b
Rhamnogalacturonan-I side-chain fractions (% of side-chains)			
Galactan fraction of rhamnogalacturonan-I side-chains	66.0 a	64.7 a	66.8 a
Arabinan fraction of rhamnogalacturonan-I side-chains	34.0 a	35.3 a	33.2 a

a,b Mean values in the same row followed by the same letters did not differ significantly ($p \leq 0.05$), Scheffe's test. Values were averaged over tree species and wood tissue type.

* Changed from previous findings.

Influence of tree species

The total pectin content of *E. macarthurii* (23.6 mg/g) was significantly higher than that of *E. grandis* and *E. nitens* (20.7 and 23.6 mg/g, respectively). The D-galacturonic acid was the monosaccharide that occurred in the highest concentrations in pectin from all three species, followed by lower concentrations of D-galactose, L-arabinose and L-rhamnose. The pectin from *E. nitens* contained significantly more D-galacturonic acid than that of *E. macarthurii*, while *E. grandis* did not differ from either of the other two species (Table 5-3). Significant differences also existed in the concentrations of L-rhamnose, L-arabinose and D-galactose from the RG-I domain (Table 5-3). Both *E. grandis* and *E. nitens* contained significantly ($p \leq 0.05$) more L-rhamnose than *E. macarthurii* (Table 5-3). The *E. macarthurii* pectin contained L-arabinose concentrations comparable to that of *E. nitens* as well as D-galactose concentrations similar to that of *E. grandis* (Table 5-3), which indicated

distinct differences in the composition of the RG-I domains of the three eucalyptus species.

Table 5-3: Concentrations (mole %) of monosaccharides from pectin of three eucalyptus species. Data were summarised from Appendix 5-B to Appendix 5-E.

Component	*E. grandis*	*E. nitens*	*E. macarthurii*
D-Galacturonic acid	62.6 ab	64.8 a	57.4 b
L-Rhamnose	4.53 a	5.05 a	3.76 b
L-Arabinose	9.01 b	12.8 a	11.8 a
D-Galactose	23.8 ab	17.5 b	27.0 a

a,b Mean values in the same row followed by the same letters did not differ significantly ($p \leq 0.05$), Scheffe's test.

The pectin from all three species mainly consisted of HG and a smaller portion of RG-I. *Eucalyptus macarthurii* contained significantly more monomers in its RG-I domain than *E. grandis* and *E. nitens* (248, 225 and 216 Re, respectively) (Figure 5-1). However, the HG domains from the latter two species, contained more residues (311 and 320 Re, respectively) than that of *E. macarthurii* (288 Re) (Figure 5-1). Between 60 and 70% of the total residues in the pectin of the three species originated from the backbone fraction. The pectin from *E. grandis* and *E. nitens* contained more backbone residues than *E. macarthurii* (Figure 5-1). This reduction in the contribution of the RG-I backbone resulted in a significant difference between *E. macarthurii* and the other two species, in the ratio of HG : RG-I (Figure 5-1).

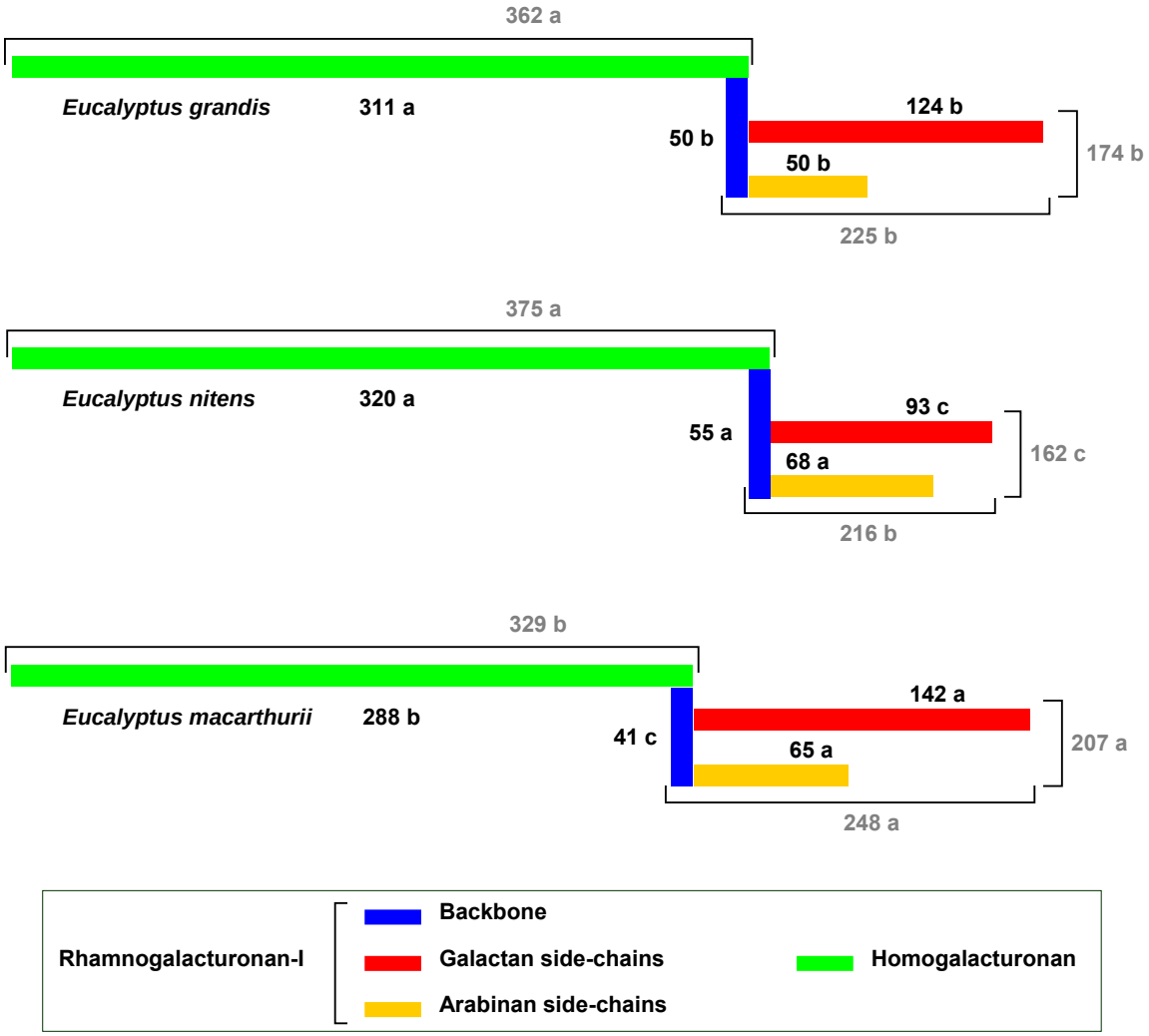

Figure 5-1: Schematic representation of the pectin composition of *Eucalyptus grandis*, *E. nitens* and *E. macarthurii* wood. Length of rods represents the average contribution of each component to a hypothetical pectic polymer. Length of the rods is expressed as an estimated number of monosaccharide residues (Re). Rods for each component with the same letter did not differ significantly (Scheffé's test, $p \leq 0.05$).

The three eucalyptus species differed significantly from each other in the composition of their RG-I domains. The side-chain molecules of *E. grandis* contributed 174 Re to the RG-I domain, while the balance of only 50 Re consisted of backbone molecules (ratio of 3.6 : 1) (Figure 5-1). *Eucalyptus grandis* had a similar ratio of RG-I side-chain molecules to backbone molecules compared to *E. nitens* (3.0 : 1) and both these species differed significantly from *E. macarthurii* (5.2 : 1). Although the ratios of the RG-I components were constant between *E. grandis* and *E. nitens*, the size of the RG-I backbone of all threes species differed significantly (Figure 5-1). *Eucalyptus*

nitens (55 Re) contained more backbone molecules and also less side-chain molecules than *E. grandis* (50 Re) and *E. macarthurii* (41 Re) (Figure 5-1).

Eucalyptus grandis and *E. macarthurii* were similar in the ratio of galactan to arabinan in the RG-I side-chains (2.6 : 1 and 2.3 : 1, respectively), while *E. nitens* differed from the other two species with a ratio of 1.4 : 1 (Figure 5-1). These three species, however, differed significantly in the amount of monomers that occurred in the fractions of their side-chains. *Eucalyptus macarthurii* contained the highest number of galactan monomers (142 Re), followed by *E. grandis* (124 Re) and *E. nitens* (93 Re), while pectin from *E. nitens* and *E. macarthurii* contained significantly more arabinan monomers (68 and 65 Re, respectively) than *E. grandis* (50 Re) (Figure 5-1).

CONCLUSIONS

Very little information is available on the occurrence of the pectic polysaccharides in wood (Koch, 2006; Sjöström, 1993) and an isolation procedure was developed to quantify pectin in hardwood and characterise its pectic domains. During the present study, the pectins from three species, namely *E. grandis*, *E. nitens* and *E. macarthurii* were compared by using these methods. The tissue type of the wood and the age class of the trees had significant influence on the content and composition of the eucalyptus pectin, similar to previous results (Chapter 2, Chapter 3 and Chapter 4). Tree species also had a significant influence on the content and composition of the pectin of the wood samples and the pectin content of *E. macarthurii* was higher than that of *E. grandis* and *E. nitens*. The monosaccharide and macromolecular composition of the pectin of all three species also differed greatly.

Homogalacturonan was the most abundant pectic domain in all three species and the observed amounts were consistent with previous findings (O'Neill *et al.*, 1990; Thibault *et al*., 1993). The HG domain of *E. macarthurii* was shorter than that of *E. grandis* and *E. nitens*, while the latter two species contained less RG-I residues. The total backbone and RG-I backbone of *E. macarthurii* were, however, on average shorter than that of *E. grandis* and *E. nitens*. The RG-I domains of the three species were highly variable in composition and the most significant difference was that forms that differ in the ratio of galactan and arabinan in their side-chains occurred, which was consistent with literature (Ermel *et al*., 2000; McCartney *et al*., 2000; Willats *et al*., 1999).

The RG-I domain of pectin from *E. nitens* consisted of a long backbone with short side-chains, while a pectin polymer from *E. grandis* contained RG-I with medium length backbones and more side-chain residues than *E. nitens*. *Eucalyptus macarthurii* contained RG-I domains that had the shortest backbones and a side-chain fraction with the most residues. The side-chains of *E. macarthurii* were distributed between galactan and arabinan fractions in a ratio comparable to that of *E. grandis*, while *E. nitens* contained notably more galactan than arabinan.

The content and composition of the pectic domains in different *Eucalyptus* species were highly variable, thus supporting findings that described pectin from different sources to be distinctly different in fine structure (Bush & McCann, 1999; Vicré *et al*., 1998; Willats *et al*., 1999). Future studies should include a larger pool hardwood species to get a more comprehensive understanding of the occurrence and structure of pectin in the *Eucalyptus* genus, as well as in other genera and families.

APPENDICES

Appendix 5-A: The total pectin concentration (mg/g) measured in each of the 81 samples that is representative of tree specie, yield potential of the site, tree age class and wood tissue type.

		E. grandis			***E. nitens***			***E. macarthurii***		
Tree age	**Wood tissue type**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
6 yrs	**Cambium**	23.5	17.0	21.0	23.3	19.1	23.1	24.0	27.1	22.5
	Sapwood	12.6	20.4	20.2	21.4	27.6	15.6	28.8	13.7	22.8
	Heartwood	18.6	16.3	16.8	19.4	25.8	20.2	24.0	21.5	26.2
10 yrs	**Cambium**	30.2	22.6	7.6	22.5	30.1	21.2	38.1	32.2	21.3
	Sapwood	16.3	16.5	24.0	13.8	18.6	21.9	27.6	27.5	14.8
	Heartwood	24.3	24.7	19.4	22.3	23.4	31.7	23.2	22.6	8.7
14 yrs	**Cambium**	24.7	27.8	21.4	25.3	24.0	17.8	23.4	33.8	24.4
	Sapwood	24.3	23.3	19.5	18.6	15.8	16.6	21.7	20.6	25.6
	Heartwood	22.2	25.3	19.6	17.0	20.5	13.6	17.4	27.0	15.9

Appendix 5-B: The D-galacturonic acid concentration (mole %) measured in pectin of 81 samples that is representative of tree specie, yield potential of the site, tree age class and wood tissue type.

		E. grandis			*E. nitens*			*E. macarthurii*		
Tree age	**Wood tissue type**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
6 yrs	**Cambium**	79.5	69.9	68.9	80.0	71.0	69.6	67.6	47.2	63.2
	Sapwood	60.1	71.0	63.6	65.6	60.1	62.8	48.4	54.5	63.2
	Heartwood	72.0	62.9	63.4	61.1	69.4	63.6	68.5	72.5	56.0
10 yrs	**Cambium**	51.2	67.1	36.4	66.5	73.0	68.8	77.7	32.3	76.2
	Sapwood	73.7	57.3	62.1	51.2	57.0	68.6	59.9	61.2	63.0
	Heartwood	70.1	73.3	75.5	61.3	61.0	61.9	59.2	60.1	20.3
14 yrs	**Cambium**	51.4	64.5	65.6	73.0	66.7	57.6	69.1	66.5	38.6
	Sapwood	70.7	47.2	75.4	64.8	61.0	58.9	61.5	60.3	63.0
	Heartwood	49.8	27.9	59.5	65.7	58.2	69.7	60.5	31.7	48.6

Appendix 5-C: The L-rhamnose concentration (mole %) measured in pectin of 81 samples that is representative of tree specie, yield potential of the site, tree age class and wood tissue type.

		E. grandis			*E. nitens*			*E. macarthurii*		
Tree age	**Wood tissue type**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
6 yrs	**Cambium**	2.74	4.31	3.85	3.44	5.59	3.88	2.90	2.98	3.30
	Sapwood	8.05	4.97	6.36	5.87	4.88	8.01	3.87	6.06	4.49
	Heartwood	4.92	6.23	5.90	5.30	4.64	6.21	3.57	3.78	4.00
10 yrs	**Cambium**	2.41	1.77	7.67	4.40	2.45	4.47	2.14	2.91	2.05
	Sapwood	2.45	4.46	5.37	7.01	6.14	5.58	3.48	2.94	3.58
	Heartwood	4.35	5.51	5.88	4.92	4.87	3.19	4.91	3.76	9.31
14 yrs	**Cambium**	3.58	3.40	3.05	4.88	3.82	5.11	2.45	3.25	3.55
	Sapwood	3.14	4.89	2.60	4.92	5.69	4.33	3.17	3.00	3.86
	Heartwood	4.58	4.06	5.91	6.05	4.65	6.14	3.23	2.86	6.15

Appendix 5-D: The L-arabinose concentration (mole %) measured in pectin of 81 samples that is representative of tree specie, yield potential of the site, tree age class and wood tissue type.

		E. grandis			*E. nitens*			*E. macarthurii*		
Tree age	**Wood tissue type**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
6 yrs	**Cambium**	5.16	7.36	7.39	6.26	9.42	8.16	9.78	10.9	9.96
	Sapwood	11.1	7.19	6.48	12.1	8.19	15.2	9.56	16.2	10.3
	Heartwood	8.59	9.05	9.91	14.7	11.1	15.1	10.8	10.4	9.72
10 yrs	**Cambium**	5.99	6.38	16.3	10.9	8.00	11.6	6.38	10.3	6.45
	Sapwood	7.51	9.39	16.75	19.5	16.1	13.7	14.3	10.7	12.0
	Heartwood	7.93	6.36	6.85	13.2	12.9	12.8	16.2	13.9	34.7
14 yrs	**Cambium**	10.3	9.05	8.11	12.2	11.1	14.3	8.80	8.85	10.6
	Sapwood	10.6	16.8	6.06	13.8	16.2	15.9	10.8	9.14	10.2
	Heartwood	9.40	11.3	7.80	16.0	17.0	8.44	13.1	8.07	16.6

Appendix 5-E: The D-galactose concentration (mole %) measured in pectin of 81 samples that is representative of tree specie, yield potential of the site, tree age class and wood tissue type.

		E. grandis			*E. nitens*			*E. macarthurii*		
Tree age	**Wood tissue type**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
6 yrs	**Cambium**	12.6	18.5	19.9	10.3	14.0	18.4	19.8	39.0	23.5
	Sapwood	20.8	16.9	23.6	16.4	26.8	13.9	38.2	23.3	22.1
	Heartwood	14.5	21.8	20.8	18.9	14.9	15.1	17.2	13.3	30.2
10 yrs	**Cambium**	40.3	24.7	39.6	18.1	16.5	15.1	13.8	54.4	15.3
	Sapwood	16.4	28.8	15.8	22.3	20.7	12.1	22.3	25.1	21.5
	Heartwood	17.6	14.8	11.8	20.6	21.2	22.1	19.6	22.3	35.8
14 yrs	**Cambium**	34.7	23.0	23.2	9.9	18.4	23.0	19.7	21.4	47.2
	Sapwood	15.6	31.1	16.0	16.5	17.1	20.9	24.5	27.5	23.0
	Heartwood	36.2	56.7	26.8	12.2	20.1	15.7	23.1	57.4	28.7

Appendix 5-F: Homogalacturonan fraction of the pectin (%) from 81 samples that is representative of tree specie, yield potential of the site, tree age class and wood tissue type.

		E. grandis			*E. nitens*			*E. macarthurii*		
Tree age	**Wood tissue type**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
6 yrs	**Cambium**	76.7	65.6	65.1	76.6	65.4	65.7	64.7	44.2	59.9
	Sapwood	52.0	66.0	57.2	59.7	55.2	54.8	44.5	48.4	58.7
	Heartwood	67.0	56.7	57.5	55.8	64.7	57.4	64.9	68.7	52.0
10 yrs	**Cambium**	48.8	65.4	28.7	62.1	70.6	64.4	75.6	29.4	74.2
	Sapwood	71.2	52.8	56.7	44.2	50.8	63.1	56.4	58.3	59.4
	Heartwood	65.8	67.8	69.6	56.3	56.1	58.7	54.3	56.3	10.9
14 yrs	**Cambium**	47.9	61.1	62.6	68.2	62.9	52.5	66.6	63.2	35.1
	Sapwood	67.6	42.4	72.8	59.9	55.3	54.5	58.3	57.3	59.1
	Heartwood	45.3	23.9	53.6	59.7	53.5	63.6	57.3	28.9	42.4

Appendix 5-G: Rhamnogalacturonan-I fraction (%) of the pectin from 81 samples that is representative of tree specie, yield potential of the site, tree age class and wood tissue type.

		E. grandis			*E. nitens*			*E. macarthurii*		
Tree age	**Wood tissue type**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
6 yrs	**Cambium**	23.3	34.4	34.9	23.4	34.6	34.3	35.3	55.8	40.1
	Sapwood	48.0	34.0	42.8	40.3	44.8	45.2	55.5	51.6	41.3
	Heartwood	33.0	43.3	42.5	44.2	35.3	42.6	35.1	31.3	48.0
10 yrs	**Cambium**	51.2	34.6	71.3	37.9	29.4	35.6	24.4	70.6	25.8
	Sapwood	28.8	47.2	43.3	55.8	49.2	36.9	43.6	41.7	40.6
	Heartwood	34.2	32.2	30.4	43.7	43.9	41.3	45.7	43.7	89.1
14 yrs	**Cambium**	52.1	38.9	37.4	31.8	37.1	47.5	33.4	36.8	64.9
	Sapwood	32.4	57.6	27.2	40.1	44.7	45.5	41.7	42.7	40.9
	Heartwood	54.7	76.1	46.4	40.3	46.5	36.4	42.7	71.1	57.6

Appendix 5-H: Contribution of the backbone fraction (%) to the pectin of 81 samples that is each representative of tree specie, yield potential of the site, tree age class and wood tissue type.

		E. grandis			*E. nitens*			*E. macarthurii*		
Tree age	**Wood tissue type**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
6 yrs	**Cambium**	82.2	74.2	72.8	83.5	76.6	73.5	70.5	50.2	66.5
	Sapwood	68.1	75.9	69.9	71.5	65.0	70.9	52.3	60.5	67.6
	Heartwood	76.9	69.1	69.3	66.4	74.0	69.8	72.0	76.3	60.0
10 yrs	**Cambium**	53.7	68.9	44.1	70.9	75.5	73.3	79.9	35.3	78.3
	Sapwood	76.1	61.8	67.4	58.3	63.1	74.2	63.4	64.2	66.6
	Heartwood	74.5	78.8	81.4	66.2	65.8	65.1	64.2	63.8	29.6
14 yrs	**Cambium**	55.0	67.9	68.7	77.9	70.5	62.8	71.5	69.7	42.2
	Sapwood	73.8	52.1	78.0	69.8	66.7	63.2	64.7	63.3	66.9
	Heartwood	54.4	32.0	65.4	71.8	62.8	75.8	63.7	34.6	54.7

Appendix 5-I: Contribution of the backbone fraction (%) to the rhamnogalacturonan-I domain of the pectin from 81 samples that is representative of tree specie, yield potential of the site, tree age class and wood tissue type.

		E. grandis			*E. nitens*			*E. macarthurii*		
Tree age	**Wood tissue type**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
6 yrs	**Cambium**	23.6	25.0	22.0	29.4	32.3	22.6	16.4	10.7	16.5
	Sapwood	33.5	29.2	29.7	29.2	21.8	35.5	14.0	23.5	21.7
	Heartwood	29.9	28.7	27.8	24.0	26.3	29.1	20.3	24.2	16.7
10 yrs	**Cambium**	9.4	10.2	21.5	23.3	16.6	25.1	17.5	8.2	15.8
	Sapwood	17.0	18.9	24.8	25.1	25.0	30.2	16.0	14.1	17.6
	Heartwood	25.5	34.2	38.7	22.6	22.2	15.4	21.5	17.2	20.9
14 yrs	**Cambium**	13.7	17.5	16.3	30.6	20.6	21.5	14.7	17.7	10.9
	Sapwood	19.3	17.0	19.1	24.6	25.5	19.1	15.2	14.1	18.9
	Heartwood	16.7	10.7	25.5	30.0	20.0	33.7	15.1	8.0	21.4

Appendix 5-J: Contribution of the side-chain fraction to the rhamnogalacturonan-I domain of the pectin from 108 samples that is representative of tree specie, yield potential of the site, tree age class and wood tissue type.

		E. grandis			*E. nitens*			*E. macarthurii*		
Tree age	**Wood tissue type**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
6 yrs	**Cambium**	76.4	75.0	78.0	70.6	67.7	77.4	83.6	89.3	83.5
	Sapwood	66.5	70.8	70.3	70.8	78.2	64.5	86.0	76.5	78.3
	Heartwood	70.1	71.3	72.2	76.0	73.7	70.9	79.7	75.8	83.3
10 yrs	**Cambium**	90.6	89.8	78.5	76.7	83.4	74.9	82.5	91.8	84.2
	Sapwood	83.0	81.1	75.2	74.9	75.0	69.8	84.0	85.9	82.4
	Heartwood	74.5	65.8	61.3	77.4	77.8	84.6	78.5	82.8	79.1
14 yrs	**Cambium**	86.3	82.5	83.7	69.4	79.4	78.5	85.3	82.3	89.1
	Sapwood	80.7	83.0	80.9	75.4	74.5	80.9	84.8	85.9	81.1
	Heartwood	83.3	89.3	74.5	70.0	80.0	66.3	84.9	92.0	78.6

Appendix 5-K: Contribution of arabinan to the side-chains of the rhamnogalacturonan-I domain of the pectin from 81 samples that is representative of tree specie, yield potential of the site, tree age class and wood tissue type.

		E. grandis			*E. nitens*			*E. macarthurii*		
Tree age	**Wood tissue type**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
6 yrs	**Cambium**	29.0	28.5	27.1	37.8	40.2	30.8	33.1	21.8	29.8
	Sapwood	34.7	29.9	21.6	42.5	23.4	52.3	20.0	41.1	31.7
	Heartwood	37.2	29.3	32.3	43.7	42.9	50.1	38.5	43.8	24.3
10 yrs	**Cambium**	12.9	20.5	29.2	37.6	32.6	43.5	31.7	16.0	29.6
	Sapwood	31.4	24.6	51.4	46.7	43.8	53.1	39.1	29.9	35.8
	Heartwood	31.1	30.0	36.7	39.0	37.9	36.8	45.3	38.3	49.2
14 yrs	**Cambium**	22.9	28.2	25.9	55.2	37.7	38.4	30.9	29.2	18.4
	Sapwood	40.4	35.0	27.5	45.5	48.7	43.3	30.6	24.9	30.7
	Heartwood	20.6	16.7	22.6	56.8	45.9	34.9	36.2	12.3	36.6

Appendix 5-L: Contribution of galactan to the side-chain of the rhamnogalacturonan-I domain of the pectin from 81 samples that is representative of tree specie, yield potential of the site, tree age class and wood tissue type.

		E. grandis			*E. nitens*			*E. macarthurii*		
Tree age	**Wood tissue type**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
6 yrs	**Cambium**	71.0	71.5	72.9	62.2	59.8	69.2	66.9	78.2	70.2
	Sapwood	65.3	70.1	78.4	57.5	76.6	47.7	80.0	58.9	68.3
	Heartwood	62.8	70.7	67.7	56.3	57.1	49.9	61.5	56.2	75.7
10 yrs	**Cambium**	87.1	79.5	70.8	62.4	67.4	56.5	68.3	84.0	70.4
	Sapwood	68.6	75.4	48.6	53.3	56.2	46.9	60.9	70.1	64.2
	Heartwood	68.9	70.0	63.3	61.0	62.1	63.2	54.7	61.7	50.8
14 yrs	**Cambium**	77.1	71.8	74.1	44.8	62.3	61.6	69.1	70.8	81.6
	Sapwood	59.6	65.0	72.5	54.5	51.3	56.7	69.4	75.1	69.3
	Heartwood	79.4	83.3	77.4	43.2	54.1	65.1	63.8	87.7	63.4

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CHAPTER

6

ENZYMATIC REDUCTION OF POLYGALACTURONIC ACID IN MECHANICAL PULP

ABSTRACT

It was previously determined that hardwood tissue contained between 1.5 and 2.6% pectin. These pectins are released as polygalacturonic acid (PGA) when mechanical pulps are bleached with peroxide under alkaline conditions and contribute to approximately 50% of the anionic substances in the pulp and water. Due to their anionic nature, the PGA bind to the cationic pulping additives and increase the consumption of cationic drainage and retention aids applied to the pulp before the paper machine. Pectinase can be used to degrade these PGAs to smaller oligomers and monomers to reduce the complexation of interfering anionic substances with cationic drainage aids. In the present study, pectinase was successfully applied to reduce the cationic demand (CD) of bleached chemi-thermo mechanical pulp (BCTMP). The enzyme reduced the CD of white water and different types of BCTMP by 50 to 65% when it was applied at an efficient dosage of 4.0 kU/ton. Treatment of pulp with enzyme after adjusting the pH with H_2SO_4 was found to be just as effective as the alum treatment of high-brightness softwood BCTMP. It was determined that enzyme application will be more cost efficient for treatment of mechanical pulp than alum.

INTRODUCTION

It was recently determined that between 1.5 and 2.6% of the dry weight of eucalyptus consisted of pectin (Chapter 2). The pectin occurs in the middle lamella, the cell corners, the primary cell-walls and the junction zone between fibre cells in woody tissue (Goycoolea & Cárdenas, 2003; Mohnen, 2008). Pectins are very susceptible to chemical degradation under alkaline conditions. The methoxy groups and side-chains that are carried on the D-galacturonic acid moieties are saponified and the polygalacturonic acid (PGA) backbone that originated from homogalacturonan (HG), xylogalacturonan (XG) and rhamnogalacturonan-II (RG-II) is split by β-elimination (Renard & Thibault, 1996). Pectins are thus, released as anionic PGA during the peroxide bleaching of mechanical pulps and can bind to cationic retention aids to form polyelectrolyte complexes. The negative charges of PGA originate from the ionisation of the carboxylic acid groups on the galacturonic acid units (Ricard *et al*., 2005a; Thornton, 1994; Thornton *et al*., 1993).

Papermaking is fundamentally a continuous filtration process in which a diluted suspension of fibres, fines and inorganic filler particles are formed into a sheet. Filter fabric with holes that are large enough to allow passage of filler particles and fines are used to assure rapid water drainage. Retention aids are added to the pulp to keep fines and filler particles in the paper sheet and to improve the drainage of water. Cationic polymers with various structures are commonly used as retention aids (Reid & Ricard, 2000). The positively-charged retention aids form bridges between the negatively-charged surfaces of the fines and filler particles and the fibres. However, anionic trash, which is a collective term to describe a wide range of colloidal and dissolved anionic substances present in the pulp and water system bind to the cationic chemicals. The binding of these interfering substances result in an increased demand for cationic additives also referred to as cationic demand (CD) (Linhart *et al*., 1987; Webb, 1997), which is expressed in units of charge concentration (eq/l) (Thornton *et al*. 1993). The amount of anionic trash that is released during bleaching can either be reduced with a step where the bleached pulp is thickened and some of the filtrate is purge to the sewer system or with the addition of cationic chemicals in the papermaking stock to neutralise and agglomerate detrimental substances (Ricard *et al*., 2005a; 2005b; Thornton *et al*., 1993).

Polygalacturonic acids accounts for approximately 50% of the CD that is associated with peroxide-bleached mechanical pulps (Reid & Ricard, 2000; Thornton,

1994). The CD of PGA depends on the degree of polymerisation, because monomers, dimers and trimers of D-galacturonic acid do not cause any measurable CD. Longer PGA chains, however, displayed a high CD (Thornton, 1994). The amount of anionic trash that is released during peroxide-bleaching can, therefore, also be reduced using a third approach, namely to apply pectinases for degradation of PGA (Reid & Ricard, 2000; Ricard *et al*., 2005a; 2005b; Thornton *et al*., 1996). These enzymes are effective to depolymerise PGA into monomeric galacturonic acid and consequently lower the CD of the filtrates from peroxide bleached thermo mechanical pulp (Reid & Ricard, 2000; Thornton, 1994).

Pectinases are a family of enzymes that catalyse the degradation of the pectic polymer (Alkorta *et al*., 1998). Different types of pectinases have different preferences for methylated and de-methylated forms of pectin and may cleave either at the end (*exo*-) or in the interior (*endo*-) of the pectic chain. The enzyme activity of most interest for pulp treatment is *endo*-polygalacturonase (EC 3.2.1.15), because the pectins dissolved by alkaline treatment of mechanical pulps are already de-methylated and *endo*-cleavage are more efficient at lowering the degree of polymerisation than with an *exo*-cleavage (Reid & Ricard, 2000; Renard & Thibault, 1996).

The present chapter describe a case study to evaluate the use of pectinase for reduction of the CD at a mechanical pulp mill as described in the patent by Thornton *et al*. (1996). This technology was successfully applied during various mill trials and the treatment with pectinases decreased the CD and resulted in significant savings in wet-end chemicals and increased strength properties (Ricard *et al*., 2005b).

MATERIALS AND METHODS

Pulp mill

A mill producing chemi-thermo mechanical pulp, bleached with a two stage alkaline peroxide process (BCTMP) was the subject of the present case-study. The mill produced pulp from spruce (*Picea abies*) and poplar (*Populus* spp.) wood. The pulp from different wood species were produced during different campaigns and stored in separate towers, before blending. The mixed-stock generally consisted of 30% hardwood pulp and 70% softwood (either high-brightness or low-brightness) pulp. A single-stage bleaching process was used to produce low-brightness softwood BCTMP, while the two-stage bleaching process produced high-brightness softwood BCTMP.

After bleaching, the pulp (30% consistency) was treated with 10 kg/ton alum [$Al_3(SO_4)_3$] mixed in with white water for dilution to reach a consistency of 12% before a storage tower. The alum was applied to adjust the pH to 5.5 and act as anionic scavenger to reduce CD. A second treatment of 3 kg/ton alum was dosed with the white water after the storage tower, diluting the pulp to 5% consistency in the mixing chest. The second alum treatment was applied to reduce the CD.

Determination of pectinase activity

The polygalacturonase activity of the enzyme formulation was determined using polygalacturonic acid (Merck) as substrate. The concentration of galacturonic acid released by the pectinase was determined according to the Somogyi-Neslon method to quantify reducing sugars (Somogyi, 1952). This method is based on the principle that Cu^{2+} ions are oxidised to Cu^{+} by the reducing-end groups of carbohydrates. The formed Cu^{+} reduces the arsenomolybdate reagent to molybdate blue, which can be measured at 580 nm. A drawback of this method is, however, that the method not only measures the reducing ends of the galacturonic acid monosaccharides, but also that of short oligosaccharides that were released. Galacturonic acid standards (300 µl) at four concentrations (6.0, 10.0, 15.0 and 20.0 µmol/ml), enzyme blanks and reagent blanks were included in each assay batch. The absorbance values of the samples were expressed as the difference in absorbance between the samples and the reagent blank. The galacturonic acid concentrations of the samples were read from the standard curve to calculate the polygalacturonase activity. Enzyme activity was expressed in international Units (U) where one unit of activity was defined as the amount of enzyme transforming 1.0 µmol of substrate in one minute.

Analytical methods

The BCTMP samples were vacuum-filtered through Whatman filter paper (Grade 2.0) immediately after sampling and the filtrates passed through a 1.2 µm Millipore type RA filter prior to analysis. The CD of the samples was determined with a Particle Charge Detector PCD-02 (Mütek, Germany) by titrating 10 ml of sample to isoelectrical point with 1.0×10^{-4} N polydiallyldimethyl-ammonium chloride (Reid & Ricard, 2000). The galacturonic acid concentrations of the samples were determined according to the Somogyi-Nelson method as described previously.

Enzymatic treatment of pulp

Pulp (BCTMP) that was already treated with 13 kg/ton alum was sampled from the mixing chest. Enzyme treatments and subsequent analysis were carried out at pH 5.5, without adjusting the pH of the sample. The pectinase (Nalco 74363) was diluted and applied to reach final dosages of 0.004, 0.04, 0.4, 4.0, 40, 80, 200 and 400 kU/ton pulp and control samples without enzyme were also included. Treatments were done for 30 min at 40°C where after the CD and galacturonic acid concentrations were measured to determine the enzyme efficiency.

Evaluation of enzyme efficiency

The efficiency of the pectinase was tested on three pulp streams and the white water. White water and pulp were sampled from the storage towers for hardwood, high-brightness softwood and low-brightness softwood. All of these pulps were treated with alum (10 kg/ton of pulp) to control the pH and the CD prior to sampling. Enzyme treatments and subsequent analysis were carried out at pH 5.0 to pH 5.3 without adjusting the pH. The enzyme was diluted and applied to reach final concentrations of 0.04, 4.0, 20, 40, 80 and 200 kU/ton pulp and control samples without enzyme were also included. The samples were treated for 30 min at 40°C and the CD and galacturonic acid concentrations determined.

Efficiency of enzyme compared to alum

Currently, the mill applies alum after the alkaline peroxide bleaching to reduce the pH and to control the CD. The following treatments were compared in this experiment:

1. Treatment with alum to reduce the pH and the CD.
2. Treatment with alum to reduce the pH, followed by pectinase to reduce the CD.
3. Treatment with H_2SO_4 to reduce the pH, followed by pectinase to reduce the CD.

Bleached softwood pulp was collected from the screw-press after the second bleaching stage, before any alum was added to the pulp. The pulp was made up to 5% consistency with the tap water and incubated for 120 min at 40°C to release the PGA from the fibre surface into the water. The pH of the pulp was adjusted to pH 5.2 with measured amounts of either H_2SO_4 (1.0 N) or alum (2%) and each set of samples was then subjected to treatment with three different dosages of pectinase and a control without enzyme. Diluted enzyme preparations were added to the pulp to reach a 5-%

consistency and final enzyme concentrations of 4.0, 20, and 40 kU/ton pulp. These treatments were done for 60 min at 40°C and the samples were mixed regularly.

Experimental design and statistical analysis

A completely randomised design with three replications was used in all experiments. The data were subjected to one-way analysis of variance and the means of the different treatments were tested for significant differences with Tukey's test at a 95% confidence level.

RESULTS AND DISCUSSION

Activity of pectinase

The polygalacturonase activity of the enzyme formulation (Nalco 74303) was determined to be 4000 U/ml. This value was used to calculate appropriate enzyme dosages (kU/ton) to be applied in further experiments, since the product sheet (Anonymous) did not contain any information on enzyme activity. The only measurable side-activity in the enzyme formulation was that of a pectin metylesterase that displayed an activity of 500 U/ml. The enzyme, however, did not have an influence on any of the treatments.

Enzymatic treatment of pulp

Cationic demand of the pulp filtrate collected from the mixing chest, was 117.4 µeq/l (Figure 6-1). The CD of the filtrate after enzyme treatment was dependent on the enzyme concentration and decreased significantly as the enzyme dosage was increased to 40 kU/ton of pulp (Figure 6-1). The reduction in CD was due to the degradation of PGA into its monomers and mirrored the release of galacturonic acid, especially at the higher enzyme dosages (Figure 6-1). The largest reduction was achieved with the 40 kU/ton dosage. It was thus, possible to reduce the CD by approximately 42% with a relatively low enzyme dosage. Although, the dosage recommended by the enzyme supplier was 1.6 kU/ton pulp (Anonymous), the 4.0 kU/ton treatment did not reduce the CD to the same extent as the 40 kU/ton treatment.

Enzyme concentrations of 200 and 400 kU/ton resulted in an increase in CD (Figure 6-1). This increase in CD at the higher enzyme dosages may have beeb due to the higher endo-cleavage of the polygalacturonic acid into more reactive galacturonic

acid oligomers rather than less reactive galacturonic acid monosaccharides. These galacturonic acid oligmomers are also anionic in nature, which may result in an increase in CD

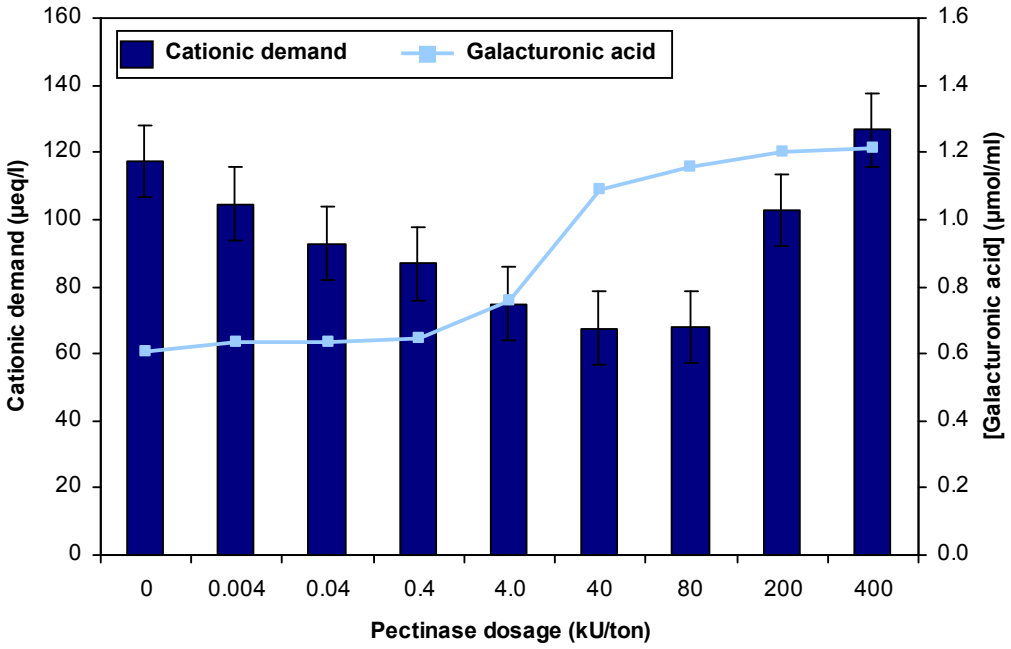

Figure 6-1: Influence of enzyme dosage on cationic demand and galacturonic acid concentration of the filtrate of BCTMP from the mixing chest (Error bars = Q value, $p \leq 0.05$).

Evaluation of enzyme efficiency

The CD in the filtrate from the hardwood pulp (343 µeq/l) was significantly ($p \leq 0.05$) higher than the other pulps and white water. Hardwoods generally contain more uronic acid than softwoods (Koch, 2006) and these uronic acids mainly originate from the PGA-containing backbones of pectin. The higher PGA content was thus, reflected in the higher CD of the hardwood pulp. The CD of the high-brightness softwood (176 µeq/l) was significantly more than that of the low-brightness softwood (100 µeq/l), while the lowest CD was measured in the white water (79 µeq/l).

All the enzymatic treatments resulted in an increase in the galacturonic acid concentration (Figure 6-2) and the reduction in CD mirrored the degradation of PGA. The final concentrations of galacturonic acid released by the enzyme were similar for the different pulps and white water, even if the filtrate from the hardwood pulp contained much higher concentrations of PGA. This may be due to inhibition of the enzyme by a high substrate concentration. It is, however, also possible that the enzyme treatment of the hardwood filtrate resulted in the formation of more reactive

galacturonic acid oligomers. The CD of all the samples after treatment depended on the enzyme dosage and initially decreased with an increase in enzyme dosage (Figure 6-2). Higher enzyme dosages (from 80 kU/ton in the filtrates and 40kU/ton in the white water), however, resulted in the increase in CD, possibly due to an increase in the rate of release of more reactive galacturonic acid oligomers.

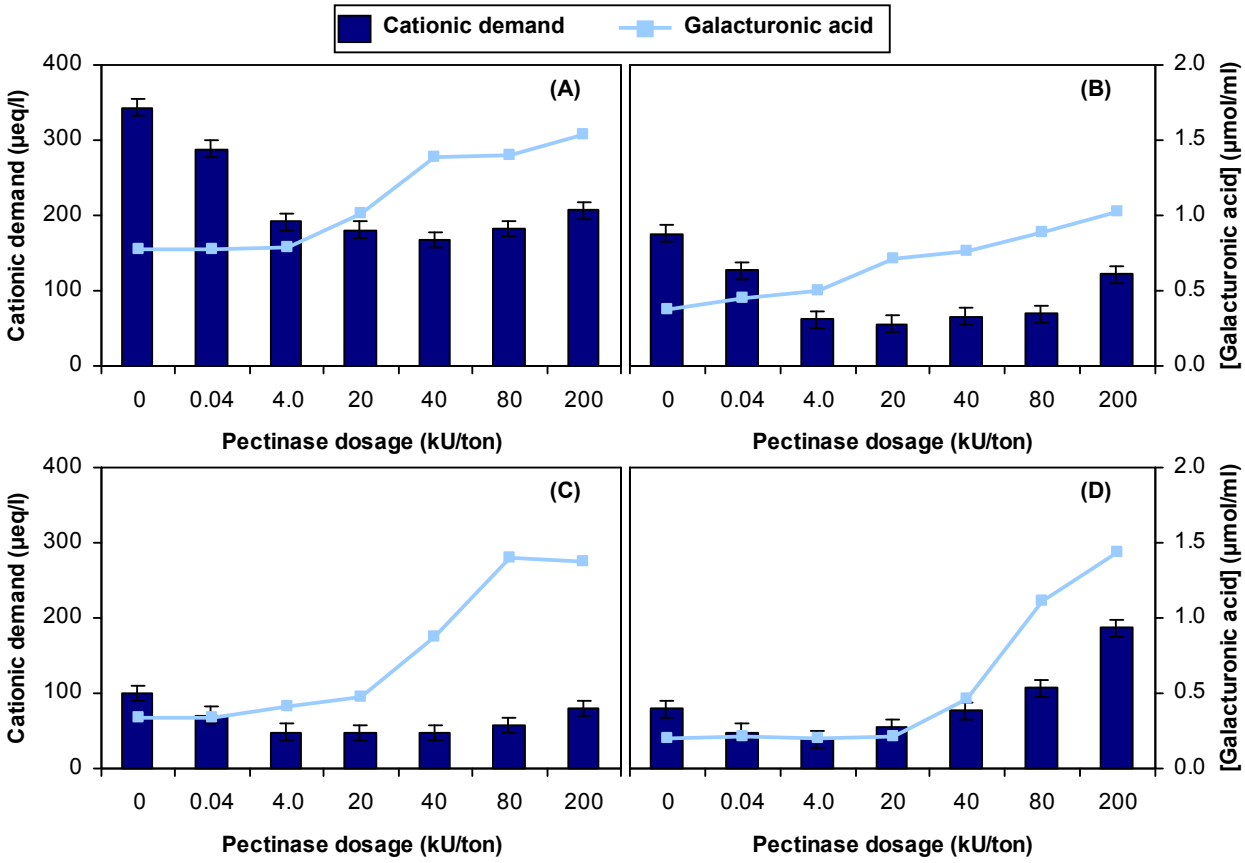

Figure 6-2: Influence of enzyme dosage on cationic demand and galacturonic acid concentration of hardwood BCTMP (A), high-brightness softwood BCTMP (B), low-brightness softwood BCTMP (C) and white water (D) (Error bars = Q value, $p \leq 0.05$).

The CD of the hardwood pulp rapidly decreased with an increase in pectinase dosage (Figure 6-2 A). A dosage of 40 kU/ton was the most effective and resulted in a reduction of 51.3%. At a dosage of 20 kU/ton, the enzyme resulted in reductions of 68.1% and 52.7% in the high-brightness softwood and the low-brightness softwood, respectively (Figure 6-2 B and Figure 6-2 C). When the white water was treated with pectinase, the maximum decrease in CD (50.1%) was seen at a dosage of 4.0 kU/ton enzyme (Figure 6-2 D).

The proportional contributions of the white water, hardwood and softwood and to the final CD of the high-brightness blend at 12% consistency were calculated to be 36, 29 and 35% and, respectively. The contribution of each component to the low-brightness blend was 42, 34 and 23%, respectively. The most effective dosages for hardwood (40 kU/ton), high-brightness softwood (20 kU/ton) and low-brightness softwood (20 kU/ton), however, resulted in reductions of only 9.7, 30.8 and 13.5 meq/kU of enzyme (Table 6-1). However, reductions of 83.4, 146.8, and 66.2 meq/kU could be achieved at a dosage of 4.0 kU/ton of pulp for the respective pulps (Table 6-1). The enzyme at a dosage of 4.0 kU/ton was significantly more efficient in high-brightness softwood to control CD than in the other pulps and it could, therefore, be more cost effective to treat high-brightness softwood pulp.

Table 6-1: Sources of cationic demand (CD) and the calculated efficiency to treat CD in filtrates at different dosages.

			White water	Hardwood	High-bright softwood	Low-bright softwood
CD contribution (meq/ton)			921.7	754.6	903.5	513.3
Contribution high-bright blend (%)			35.7	29.3	35.0	0.0
Contribution low-bright blend (%)			42.1	34.5	0.0	23.4
Dosage (kU/ton)	**4**	Reduction (%)	50.1	44.2	65.0	51.6
		Efficiency (meq/kU)	115.2	83.4	146.8	66.2
	20	Reduction (%)	31.9	47.4	68.1	52.7
		Efficiency (meq/kU)	14.7	17.9	30.8	13.5
	40	Reduction (%)	2.8	51.3	62.5	52.7
		Efficiency (meq/kU)	0.6	9.7	14.1	9.7

Efficiency of enzyme compared to alum

The previous experiments were conducted on the filtrates from pulp treated with 13 kg/t and 10 kg/t alum, respectively. In the current experiment, high-brightness softwood pulp was collected prior to the addition of any alum and the CD of the filtrate from this sample was 617.5 µeq/l (Figure 6-2). This value was approximately four times higher than the values reported for the same pulp after treatment with alum (10 kg/ton) (Figure 6-2 B). When the pH was adjusted to pH 5.2 with 10 kg/ton alum, the CD of the pulp was reduced to 172 µeq/l (Figure 6-3). Further treatment with enzyme reduced the CD to approximately 70 µeq/l, but no significant difference was seen between the different enzyme treatments (Figure 6-3).

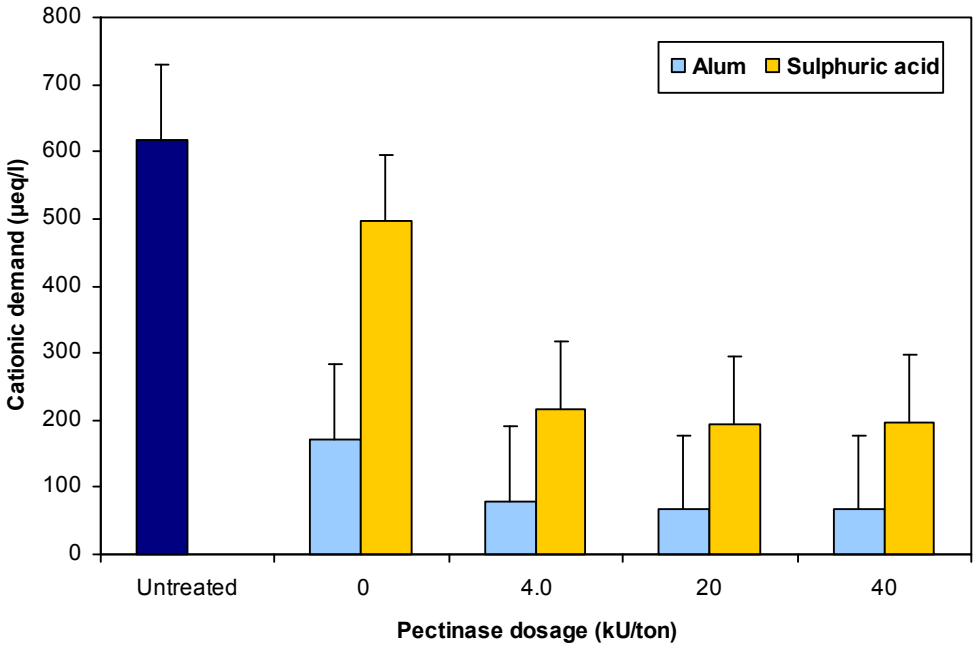

Figure 6-3: Influence of enzyme treatment on the cationic demand of the high brightness BCTMP of which the pH was adjusted with alum and H_2SO_4 (Error bars = Q value, $p \leq 0.05$).

The addition of H_2SO_4 (1.0 N) caused a smaller, but significant reduction of approximately 20% in the CD when it was compared to the alum treatment (Figure 6-3). However, a significant decrease in the CD was seen when the H_2SO_4 pre-treated samples were further treated with enzyme. There was again, no significant difference between the different enzyme dosages (Figure 6-3). Treatment with enzyme decreased the CD to approximately 200 µeq/l. This data support previous findings that an enzyme dosage of 4.0 kU/ton was the most efficient dosage to treat high-brightness softwood pulp (Table 6-2).

CONCLUSIONS

A mechanical pulp mill was the subject of a case study to evaluate the application of pectinase (Nalco 74303) to reduce the CD as described previously (Thornton *et al*., 1996). Three aspects were investigated. During the first experiment, the enzyme resulted in a reduction of 42% in CD of alum treated BCTMP filtrate from the mixed stock due to the degradation of the PGA. This reduction was comparable to the 49% that was achieved in a mill trial where softwood BCTMP was treated with a pectinase (Ricard *et al*., 2005a).

It was determined in the second experiment that the CD of the hardwood pulp was significantly higher than that of the softwood pulps and the white-water, possibly

due to a higher PGA content of hardwoods (Koch, 2006). Enzymatic treatment of the pulp filtrates resulted in reductions in the CD of between 44 and 68%. The level of these reductions reflected an underestimation of the reported levels of 50% PGA in the total detrimental anionic substances (Reid & Ricard, 2000; Thornton, 1994). The dosage of 40 kU/ton was identified as the most efficient treatment and it was determined that it was more efficient to treat the high-brightness softwood pulp than the other pulps or white water.

During the third experiment H_2SO_4 reduced the pH of the pulp to levels similar to that after it has been treated with the alum. When the enzyme treatment was used in combination with the alum, the CD was reduced more than with alum on its own. The enzyme treatment in combination with H_2SO_4 pre-treatment was just as effective as alum treatment to reduce the pH and CD of high-brightness softwood pulp. A techno-economical evaluation should, however, be done to determine and compare the cost efficiency of the three treatments. The present study showed that the pectinase has potential to reduce PGA in the specific mill environment. The effect of the pectinase should, therefore, be evaluated during a mill trial to test its efficiency on a large scale application and to record its impact on the paper properties.

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CHAPTER

7

A SHORT MILL TRIAL TO REDUCE CATIONIC DEMAND

ABSTRACT

The alkaline peroxide-bleaching of mechanical pulps results in the conversion of pectin into anionic polygalacturonic acid (PGA) that binds to cationic chemicals. Previous work demonstrated that pectinase can efficiently depolymerise the PGA into monomeric galacturonic acid and consequently lower the cationic demand (CD) of mechanical pulp. High-brightness softwood pulp was identified as the pulp source that contributed proportionally the highest amount of CD to the stock going onto the paper machine. During the present trial, a pectinase was applied to reduce the CD across the storage tower for high-brightness pulp. The enzyme treatment reduced the CD to levels comparable to that achieved with 13 kg/ton alum. An increase in the galacturonic acid content by an average of 31% indicated that this reduction in CD was due to the enzymatic degradation of PGA. The enzyme treatments did not have a negative effect on any of the strength properties of the pulp. The impact of the pectinase on the wet-end processes is still unclear and the impact of the treatment on zeta potential and turbidity should be closely monitored in future trials.

INTRODUCTION

Pectins are released as anionic polygalacturonic acid (PGA) from peroxide bleached mechanical pulp (BCTMP) and can bind to cationic retention aids to form polyelectrolyte complexes (Ricard *et al*., 2005a; Thornton, 1994; Thornton *et al*., 1993). It has been demonstrated in the previous chapter that pectinase can efficiently depolymerise the dissolved PGA into monomeric galacturonic acid and consequently reduce the cationic demand (CD) of alkaline peroxide-bleached mechanical pulp. It was also determined that the high-brightness softwood pulp contributed the highest proportion of CD to the furnish. When the enzyme treatment was used in combination with the alum treatment that is currently used at the mill, the CD was reduced more than with alum on its own.

The use of pectinase to degrade PGA and, therefore, reduce CD was successfully applied during various mill trials and the enzyme treatment did not only decrease the CD, but it also resulted in significant savings in wet-end chemicals and increased strength properties (Reid & Ricard, 2000; Ricard *et al*., 2005a; 2005b; Thornton *et al*., 1996). The aim of the current trial was to compare the effectivity of the pectinase with alum treatment to reduce the CD of the high-brightness softwood BCTMP and to evaluate the effect of the enzyme on the pulp properties.

MATERIALS AND METHODS

Pectinase was applied to BCTMP in a short mill trial to reduce CD across the storage tower. During normal production of high-brightness softwood BCTMP, pulp was treated with 10 kg/ton of alum to reduce the pH to approximately pH 6.2 and stored in a tower at 12% consistency (control treatment) (Figure 7-1). White water and a further 3 kg/ton of alum were added at the bottom of the tower before the pulp at 5% consistency was pumped into a mixing chest that supplied pulp to a paper machine. During the trial, a further 30 ton of pulp (12% consistency) was treated with 10 kg/ton of alum as well as pectinase (Nalco 74303) and the flow redirected to a second storage tower, where the batch of enzyme-treated pulp was incubated for a minimum of 60 min (Figure 7-1). The pectinase was applied at a rate of approximately 8.0 kU/ton of pulp and was added to the white water for better mixing before application to the pulp in a screw-conveyor. No additional alum was added to the enzyme treated pulp at the bottom of the tower (Figure 7-1). The ability of the enzyme to control CD to the same level as obtained with the additional 3 kg/ton alum applied to the control treatment

could, therefore, be compared. When incubation of the treated pulp was completed, it was mixed with untreated pulp at a ratio of 1 : 9 to reduce the risk of system upsets during paper making.

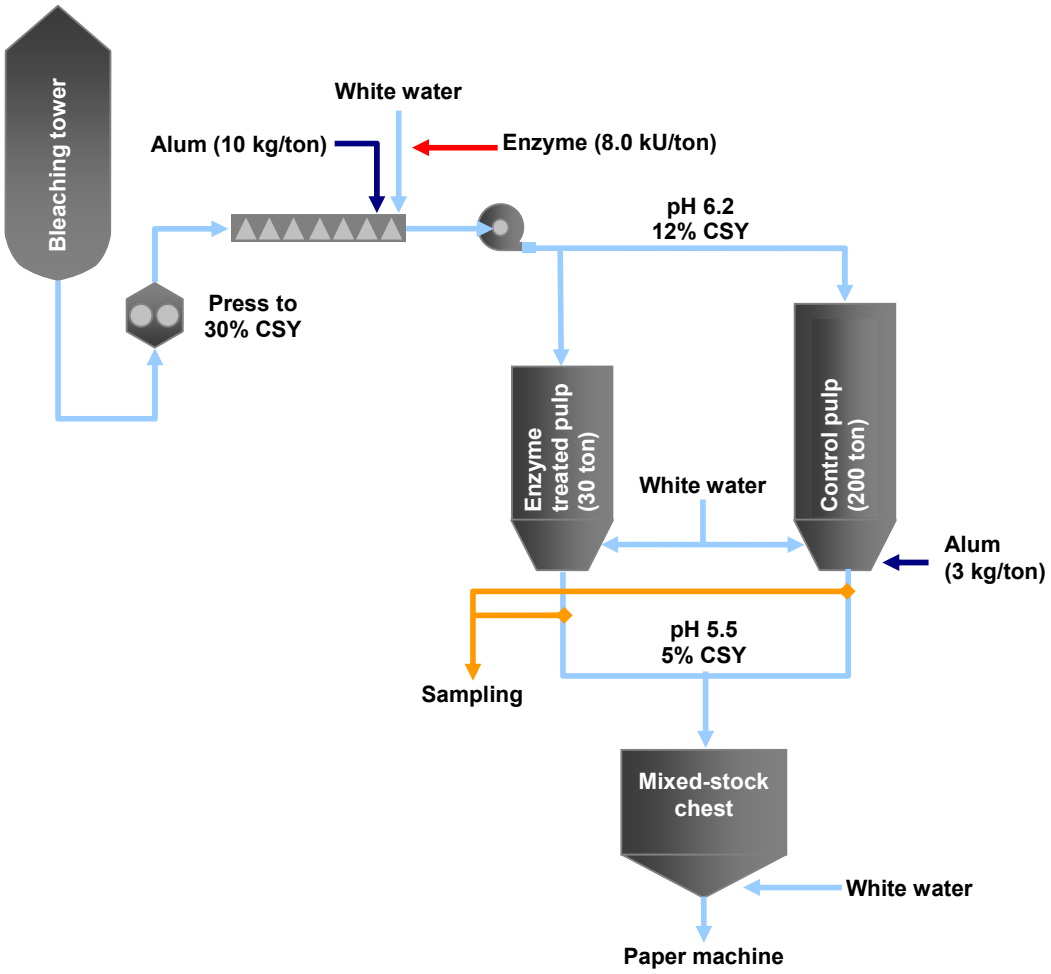

Figure 7-1: Flow of white water and high-brightness BCTMP at different consistencies (CSY), to the paper machine. Arrows indicate the application point for the enzyme (red), sampling points (yellow) and alum treatments (dark blue).

Pulp samples were collected over two days from the outlets of both storage towers (Figure 7-1). Collection of the samples from the control and treated pulps started 3 and 4 h after enzyme treatment began, respectively. The treated pulp was sufficient for a 22 h trial period, but samples were only collected during the day shifts, resulting in seven control samples and six enzyme-treated samples over a period of two days.

Drainage time and zeta potential of the pulp were determined before the samples were filtered. The filtrates were tested for CD, turbidity and chemical oxygen demand (COD). The remainder was boiled to denature the enzyme in order to determine the galacturonic acid content of the filtrates by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) as described previously (Chapter 2). One pulp sample per day was collected from each treatment and used to make 55 g/m^2 handsheets that were tested for physical properties.

RESULTS AND DISCUSSION

The CD of the high-brightness softwood BCTMP before treatment was 445 µeq/l. The alum treatment of 13 kg/ton reduced the CD by 54.1%, while the enzyme treatment reduced the CD by 53.0% (Table 7-1). These results are similar to previous findings where the enzyme was just as effective as the control treatment to reduce the CD (Chapter 6). It was also noted that the extended incubation of the treated pulp overnight did not influence the efficiency of the enzyme and the average CD remained stable at 209 µeq/l, while extended incubation of the control resulted in an increase of 42.9 µeq/l (Table 7-1).

Table 7-1: Wet-end parameters of the filtrate from the control and enzyme treated samples. Each value represents the mean or standard deviation (S.D.) of seven control samples or six enzyme-treated samples.

		Control		Enzyme treated	
		Mean	**S.D.**	**Mean**	**S.D.**
Cationic demand (µeq/l)	Day 1	185.8	14.5	209.7	6.4
	Day 2	228.7	12.1	209.0	23.3
	Average	204.1	26.1	209.3	15.3
pH		5.3	0.1	5.5	0.1
Zeta potential (mV)		-9.9	1.9	-12.4	1.3
Chemical oxygen demand (mg/l)		1202	72	1296*	89*
Transmission (%)		96.6	0.7	87.0	3.1

* One outlier discarded

Addition of more alum was expected to decrease pH, but only a small decrease was observed between the pH values of the control and the treated samples (Table 7-1). At present it is not clear what the impact of such a change might be on the wet-end processes. The enzyme treatment also resulted in a more negative zeta potential, possibly because less alum bound to the fibres. Although this reduction cannot be explained, it could potentially benefit binding of wet-end chemicals.

Degradation of PGA did not influence COD (Table 7-1), possibly since the polymer as well as its monomeric products consume the same approximate amounts of oxygen during degradation. Differences in turbidity were noted between the two treatments and it possibly reflected the ability of alum to cause flocculation and settling of colloidal material. The impact of the treatment on turbidity, therefore, has to be monitored downstream in future trials.

Galacturonic acid is the degradation product of the PGA, which consumes alum and cationic drainage aids. The concentration of galacturonic acid in filtrates from pulp treated with only 10 kg of alum was 0.694 µmol/ml (Figure 7-2). Addition of a further 3 kg of alum to this pulp did not cause a notable change in the concentration of this monosaccharide. However, the enzyme treatment increased the galacturonic acid content by an average of 31% over two days, clearly indicating that the pectinase was effective in the degradation of PGA. These results account for the ability of the enzyme to reduce CD at a lower alum dosage.

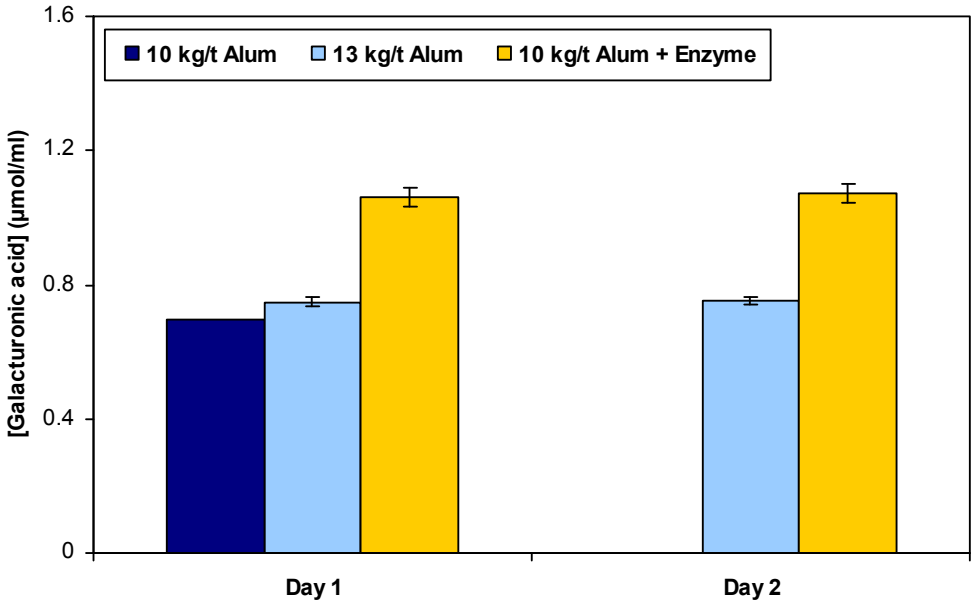

Figure 7-2: Concentration of galacturonic acid in filtrates from alum and enzyme treated pulps collected on two consecutive days during the trial. Error bars reflect the standard deviation for each treatment. The value for the treatment with 10 kg/ton alum is based on a single observation.

With only two pulp samples subjected to physical tests, it was also very difficult to determine the significance of the differences caused by the different treatments (Table 7-2). The most notable changes caused by the enzyme were the slight

increases in breaking length and tear strength. However, none of these changes can be considered as significant.

Table 7-2: Handsheet properties of the control and enzyme-treated pulps collected on two consecutive days during the trial.

	Control		Enzyme treated	
	Day 1	**Day 2**	**Day 1**	**Day 2**
Drainage (CSF) (ml)	112.0	115.0	82.0	95.0
Breaking length (km)	3.7	3.8	4.2	3.9
Breaking strength (kN/m)	2.9	3.0	3.3	3.0
Stretch (%)	1.6	1.7	1.8	1.7
Tear strength (km)	391.0	404.0	417.0	411.0
Residual strength (km)	2.3	2.2	2.4	2.3
Scattering coefficient (%)	53.4	54.2	53.2	54.0
Opacity (%)	90.1	89.9	89.5	89.5
Brightness (%)	73.0	74.0	73.6	73.6

CONCLUSIONS

The pectinase (Nalco 74303) was as effective as the control treatment with the full alum dosage to reduce the CD of the BCTMP. The critical success parameter for the trial was, therefore, met. The enzyme treatments did not have a negative effect on any of the strength properties, but the statistical significance of the slower drainage should be determined in future trials. A proposal should be drafted to conduct a longer trail for enzymatic reduction of CD at the mill. Such a trial should include more frequent sampling and allow the evaluation of the application on the paper machine performance as well as product and effluent quality.

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CHAPTER

8

GENERAL DISCUSSION AND CONCLUSIONS

In a review of literature, an overview of the structure and function of the pectic polysaccharides made it apparent that very little information is available on the occurrence and structure of pectin in woody tissue. The investigations into the occurrence and structure of the pectic polysaccharides mainly focused on pectins from soft tissue sources (fruit and vegetables). During the present work, an analytical method was, however, successfully developed to quantify pectin in eucalyptus wood. The monosaccharide and macromolecular composition of these pectins was also determined, allowing the construction of hypothetical models of eucalyptus pectin. This work was thus, the first attempt at elucidating the possible domain composition of wood pectin. Three species, namely *Eucalyptus grandis*, *E. nitens* and *E. macarthurii* were investigated and the influence of four factors (tree species, yield potential of the site, tree age class and wood tissue type) on the pectin content and composition was determined.

The pectin content (15.2 to 25.6 mg/g) of the eucalyptus samples were calculated through the addition of the concentrations of the pectic monosaccharides (D-galacturonic acid, L-rhamnose, D-galactose and L-arabinose). The concentrations of these monosaccharides were determined with two high performance liquid chromatography protocols after the complete hydrolysis of the wood samples. During this study, it was found that some hydrolysis protocols that made use of mild conditions such as acid methanolysis resulted in incomplete cleavage of the glycosidic and glucuronosyl linkages in wood (Bertaud *et al*., 2002; Biermann, 1988; Garna *et al*., 2006; Sundberg *et al*., 1996). Total hydrolysis of the samples was, however, achieved with the Saeman protocol that applied a strong acid at a high temperature (Adams, 1965; Biermann, 1988). The quantification of pectin in wood through determination of its constituent monomers proved to be suitable for hardwoods but cannot be applied for softwoods, since the hemicelluloses of softwoods are notably different and may

contribute to the D-galactose and L-arabinose in the hydrolysates (Grand-Reid, 1997; Koch, 2006; Rowell *et al*., 2005).

When the monosaccharide composition of the eucalyptus pectins were determined and expressed as percentages of the total pectin (mole %), it was found that eucalyptus pectin mainly consisted of D-galacturonic acid and smaller quantities of D-galactose, L-arabinose and L-rhamnose. Construction of the hypothetical polymer models based on these results was the first illustration of the possible macromolecular composition of eucalyptus pectin. These models were, however, not representative of the exact structure of pectic polymers, because it was impossible to make predictions on the distribution of different RG-I domains in the pectic polymer or the length and composition of the RG-I side-chains. The RG-II and XG domains were also not characterised due to analytical limitations. The constraints to quantify and characterise these domains were not expected to have an influence on the results, seeing that these domains account for less than 10% of the total pectin (Fleischer *et al*., 1999; Ishii & Matsunaga 2001; Nakamura *et al*., 2002; O'Neill *et al*., 2004; Zandleven *et al*., 2007).

The pectin contents of the three eucalyptus species were different and *E. macarthurii*, for example, contained significantly more pectin than *E. grandis* and *E. nitens*. The polymer models illustrated that HG was the most abundant pectic domain and the amounts measured were similar to reported values (O'Neill *et al*., 1990; Thibault *et al*., 1993). The RG-I domains of the three species were also highly variable in composition and the most significant difference was that forms with different ratios of galactan and arabinan in their side-chains occurred, as described in literature (Ermel *et al*., 2000; McCartney *et al*., 2000; Willats *et al*., 1999). Future studies should, however, include a larger pool of hardwood species for a more comprehensive understanding of the occurrence and composition of pectin in the *Eucalyptus* genus, as well as in other genera and families.

Wood tissue type was the only factor, other than tree species, that had a major influence on the content and composition of eucalyptus pectin. The biochemistry of the pectin changed greatly from the younger tissue (cambium) to the older tissues (sapwood and heartwood). Many of the differences between younger and older tissue types were also seen between younger and older trees. The cambium contained the highest concentrations of pectin, because active growth mainly occurs in this tissue (Pallardy & Kozlowski, 1997). The pectin models illustrated that the RG-I domains of

the three tissue types were also significantly different, especially in the composition of their side-chains. Findings of the current study were consistent with the literature that states that galactan-rich RG-I is associated with meristematic tissue, while arabinan-rich RG-I is associated older tissue (Vicré *et al*., 1998; Willats *et al*., 1999).

The current work, therefore, provided information on the biochemistry of wood pectin that improved the understanding of the influence of pectins on pulping and papermaking processes and to assist in the development of biotechnological approaches to improve these processes (Peng *et al.*, 2003; Ricard *et al*., 2005a; 2005b). A mechanical pulp mill was the subject of a case study to evaluate the application of pectinase to degrade polygalacturonic acid (PGA) released from pectins in the wood. In some cases the enzymatic treatment of the pulp filtrates resulted in reductions in the cationic demand (CD) by as much as 68%, indicating that the reported levels of 50% PGA in the total detrimental anionic substances (Reid & Ricard, 2000; Thornton, 1994) might have been underestimated. A number of alternative treatments that were just as effective to reduce the CD in the pulp streams and water systems as the control treatment with alum were identified. It was, however, suggested that a techno-economical evaluation should be done to compare the cost efficiency of these treatments. A short trial at the mill showed that the pectinase was just as effective as the full alum dosage to reduce the CD of bleached chemi-thermal mechanical pulp. Future trials should, however, be longer with more frequent sampling to allow the evaluation of the enzyme application on the paper machine performance as well as product and effluent quality.

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