

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

STRUCTURAL STUDIES ON CELL WALLS
OF PINUS RADIATA WITH PARTICULAR
REFERENCE TO CALLUS CULTURED CELLS

A THESIS PRESENTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

AT

MASSEY UNIVERSITY

NEW ZEALAND

DALLAS R. FENEMOR

1982

ABSTRACT

A study of the composition of the primary cell wall of Pinus radiata has been undertaken.

1. Preliminary work with hypocotyl tissue showed that hemicelluloses of hypocotyl consisted of a xylan (probably (4-O-methylglucurono)xylan) and a xyloglucan. Acidic sugars examined, showed that galacturonic acid was the main component, and that 4-O-MeGlcA was present.
2. Cell walls were prepared from callus tissue either by wet sieving in 80% ethanol (Batch 1) or by disruption in a French Pressure cell and washing with aqueous potassium phosphate buffer; (Batch 2).

Each batch was submitted to a series of extractions with different reagents in order to investigate the mode of bonding of polymers within the walls. The polysaccharide and protein components of each fraction were studied by monosaccharide and amino acid analysis.

Fractions of Batch 1 were assayed for lignin and selected fractions from both batches were studied by methylation analysis.

3. The results of investigations led to the following major conclusions. The non-cellulosic components recognised in the wall preparations were: -
 - a) A(1→3)-linked galactan and an arabino-3,6-galactan which were largely extractable from the cell walls by hot water and may be only loosely bound in the cell wall.
 - b) The pectic components consisting of;
 - i) pectin, a galacturonate polymer containing a linear (1→4)-galacturonan back bone interspersed with branched rhamnose residues,
 - ii) branched (1→5)-arabinan and
 - iii) linear (1→4)-galactan,

which occurred together in cell wall fractions and were not all extracted by classical extractants (such as hot aqueous EDTA), some being tightly bound in the cellulosic residue after alkali extraction.

- c) A fucogalactoxyloglucan some of which was extracted by water or EDTA, but the majority was extracted by subsequent treatment with either alkali or in part by a strong chaotropic reagent (6M GTC). Thus the fucogalactoxyloglucan was probably bound in the cell wall by strong hydrogen bonding. Some other bonding may be involved in the GTC-resistant fraction,
- d) A branched xylan which was removed by GTC and alkali, the larger level being removed by GTC.
- e) A galactoglucomannan, identified only by 4-linked mannose residues in hot water extracts and strong alkali fractions.
- f) Hydroxyproline-containing protein which was extracted from the cell wall by a variety of reagents but hydroxyproline-rich protein remains tightly bound after alkali extraction.
- g) Lignin which was tentatively identified in the cell wall. It appeared likely that cross-linking with lignin would be responsible for the non-extractability of some polysaccharides and protein from the walls. A mild acid chlorite treatment followed by alkali extraction removed most of the residual pectic components, xylan and protein from the walls.

A basis has been laid for the further investigation of the wall structure and isolation of polysaccharides.

ACKNOWLEDGEMENTS

I wish to express my appreciation to my supervisors, Dr I.G. Andrew for his interest, guidance and appraisal of this work, and Dr G.G. Pritchard for his interest and advice.

My thanks are also due to Bruce Christie and Hugh Neilson for their advice and encouragement in the growth of the callus cultures at Massey.

Thanks are also due to the staff of the electron microscopy unit, D.S.I.R., Palmerston North.

Helpful suggestions have also been received from the Forestry Research Institute, Rotorua, N.Z. from whom I gratefully acknowledge the receipt of a research grant to assist in the completion of this work.

Lastly, to my family for support and interest, and to Mrs V. Fieldsend for typing this thesis.

TABLE OF CONTENTS CONTINUED

Page

2.2.4	Determination of Hydroxyproline	41
2.2.5	Determination of Lignin	42
2.2.6	Determination of Starch	42
2.3	Carbohydrate Composition Analysis	46
2.3.1	Neutral Monosaccharide Analysis	46
2.3.1.1	Total Hydrolysis	46
2.3.1.2	Reduction to Alditol Acetates	47
2.3.1.3	Acetylation	48
2.3.1.4	Gas Chromatography of Alditol Acetates	48
2.3.2	Total Monosaccharide Analysis	52
2.3.2.1	Hydrolysis Procedure	53
2.3.2.2	Enzyme Preparation	54
2.3.2.3	Synthesis of Allonic Acid	55
2.3.3	Determination of Extent of Hydrolysis	56
2.3.3.1	Degradation and Depolymerisation Factors	56
2.3.3.2	Assay of Non Reducing Sugar	60
2.3.4	Identification and Isolation of Uronic Acids and Oligosaccharides	61
2.3.4.1	Recovery of Acidic Sugars by Ion Exchange Chromatography	62
2.3.4.2	Paper Chromatography	62
2.3.4.3	The Problem of Lactonisation	64
2.4	Methylation Analysis of Polysaccharide Fractions	64
2.4.1	Theory of Methylation	65
2.4.2	Preparation of Dimethyl Sulphinyl Anion	65
2.4.3	Methylation Procedure	66
2.4.3.1	Methylation by the Standard Hakomori Procedure	66
2.4.3.2	Multiple Methylation Technique	67
2.4.4	Methylation of Uronic Acid-Containing Polysaccharide	68
2.4.5	Hydrolysis and Derivatisation of Methylated Products	71
2.4.6	Gas-Liquid Chromatography of Partially Methylated Alditol Acetates	72

TABLE OF CONTENTS CONTINUED	Page
2.4.7 Quantitation of Results and Mass Spectrometry	73
2.4.7.1 Peak Area Quantitation with Flame Ionisation Detection	73
2.4.7.2 Quantitation by Mass-Spectrometry	73
2.4.7.3 Expression of Data	74
2.4.8 Trial of the Methylation Method	75
2.5 Microscopic Examination of Tissue and Walls	79
2.5.1 Transmission Electron Microscopy	79
2.5.2 Scanning Electron Microscopy	80
2.5.3 Light Microscopy	80
 CHAPTER 3: GROWTH AND PREPARATION OF SAMPLE MATERIAL	 81
3.1 Growth of Hypocotyls	81
3.2 Origin of Callus Cultures	82
3.2.1 Derivation of Callus from Hypocotyl	82
3.3 Maintenance of Callus Cultures	82
3.4 Attempted Growth of Suspension Cultures	84
3.5 Callus Cultures for Cell Wall Preparation	84
3.5.1 Examination of Callus Cultures	84
3.5.2 Electron Microscopic Examination	85
3.6 Purification of Callus Walls	88
3.6.1 Preparation of Wall by Wet Sieving	88
3.6.2 Preparation of Wall by Disruption and Buffer Washing	90
3.6.3 Starch Assays	95
3.6.4 Tannin, Lignin and Phenolic Acids	96
3.7 Examination of Wall Preparations	96
3.7.1 Light Microscopy	96
3.7.2 Electron Microscopy	100
3.8 Synopsis	100

TABLE OF CONTENTS CONTINUED	Page
CHAPTER 4: HYPOCOTYL STUDIES	103
4.1 Introduction	103
4.2 Study of Acidic Components in Hypocotyl Cell Walls	103
4.2.1 Preparation of Hypocotyl Fraction	103
4.2.2 Acid Extraction of Sugars	105
4.2.3 Monosaccharide Compositional Analysis by Gas Chromatography	106
4.2.3.1 Separation and Derivatisation of Neutral and Acidic Fractions for Gas-Chromatography	106
4.2.3.2 Results from Gas-Chromatography Monosaccharide Composition.	106
4.2.4 Extent of Hydrolysis with TFA-Enzyme	109
4.2.5 Paper Chromatography	109
4.2.5.1 Separation of Neutral and Acidic Fractions for Paper Chromatography	109
4.2.5.2 Chromatographic Conditions	110
4.2.5.3 Results	110
4.3 Structure of Hemicellulosic Xylan, by Methylation Analysis	116
4.4 Conclusions	116
 CHAPTER 5: ANALYSIS OF CALLUS CELL WALLS: MAIN COMPONENTS	 117
5.1 Introduction	117
5.2 Extraction Sequences	124
5.2.1 Preliminary Fractionations	124
5.2.2 Fractionation of Batch 1 Walls	126
5.2.2.1 Removal of Water Soluble Polysaccharides	126
5.2.2.2 Removal of Pectic Polysaccharides	126
5.2.2.3 Removal of Hydrogen-bonded Polysaccharides	127
5.2.2.4 Removal of Hemicellulosic Polysaccharides	127
5.2.3 Fractionation of Batch 2 Walls	129
5.2.3.1 Removal of Water Soluble Polysaccharides	129

TABLE OF CONTENTS CONTINUED	Page
5.2.3.2 Removal of Hot-Water Soluble and Pectic Polysaccharides	129
5.2.3.3 Extraction of Hydrogen-bonded Polymers	129
5.2.3.4 Cleavage of Ester Links in the Wall	131
5.2.3.5 Extraction of Residual Hemicelluloses	131
5.2.3.6 Chlorite Extraction	132
5.2.3.7 Post Chlorite Alkali Extraction	133
5.2.3.8 The Final Residue	133
5.3 Analytical Data	133
5.3.1 Results to Preliminary Wall Fractionations	134
5.3.2 An Overview of Major Components	135
5.3.3 Sugar Analyses. Batch 1 and 2 Walls: Results and Discussion	140
5.3.4 Amino Acid Analyses. Batch 1 and 2 Walls: Results and Discussion	154
5.3.5 Lignin Analyses, to Batch 1 Walls: Results and Discussion	168
5.4 Discussion	170
5.4.1 Comparison Between Batch 1 and 2 Walls	170
5.4.2 Comparison Between Different Extraction Procedures	172
 CHAPTER 6: METHYLATION OF SELECTED FRACTIONS OF CALLUS BATCH 1 and 2 WALLS	 177
6.1 Introduction	177
6.2 Methylation Results for Batch 1 Walls	179
6.3 Methylation Results for Batch 2 Walls	184
6.3.1 Methylation of the 100°C Water Fraction	185
6.3.2 Methylation of 10% KOH and GTC Fractions	190
6.4 Discussion	196
 CHAPTER 7: PURIFICATION OF SOME PECTIC POLYSACCHARIDES BY CHROMATOGRAPHY ON DEAE-CELLULOSE	 204
7.1 Introduction	204
7.2 DEAE-Cellulose Chromatography of 100°C Water Fraction of Batch 2 Walls, Run 1	204
7.3 Methylation of Neutral Galactan	208

TABLE OF CONTENTS CONTINUED		Page
7.4	DEAE-Cellulose Chromatography of 100°C Water Fraction of Batch 2 Walls, Run 2 (large scale)	210
7.5	Analysis of Fractions from DEAE- Cellulose, Run 2	212
7.6	Methylation of "Arabinan"-rich Fraction	215
7.7	Discussion	217
CHAPTER 8: CONCLUSION		219
CHAPTER 9: APPENDICES		228
	Appendix 1: Identification of Running Parameters for Partially Methylated Alditol Acetates and Methods of Quantitation	228
	Part 1 - Procedure (Including Parameter Diagrams)	228
	Part 2 - Gas Chromatograms of some Programmed Runs	237
	Part 3 - Quantitation of Per-Methylated Alditol Acetates, by Gas Chrom- atography - Mass Spectrometry	242
	Part 4 - Quantitation of the Proportion of Dideuterated Derivates arising from Uronic Acids	245
	Appendix 2: Mass Spectral Fragmentation Patterns arising from Partially Methyl- ated Alditol Acetates.	247
	Part 1 - Origin of Fragments	247
	Part 2 - Fragmentations to Primary Fragments	249
	Part 3 - Detailed Mass Spectra	253
REFERENCES		259

LIST OF TABLES

Table	Page
2.1 Relative Retention Times of Alditol Acetates on Stationary Phases, in Gas Chromatography	50
2.2 Peak Height Responses for Sugars on SP2340.	51
2.3 Degradation of Standard Sugars by 0.5M HNO ₃ /0.5% Urea	57
2.4 Factors Involved in Quantitative Recovery of Monosaccharides by Gas Chromatography	60
2.5 R _{xylose} values for sugars in Solvent B	64
2.6 Methylation Data for Standard Polysaccharides	76-78
3.1 Linsmaier and Skoog Growth Medium	83
4.1 Mole % of Sugars in Extracted Fractions of Hypocotyl EDTA Residue	107
4.2 Summative Levels of Reducing and Non-Reducing Sugar Residues in the TFA-Enzyme Hydrolysate	109
4.3 R _{xylose} Values of Mono and Oligosaccharides in the TFA-Enzyme Hydrolysate	111
4.4 Composition of Hypocotyl Xylan	115
5.1 Sugars Recovered as Percentage of Cell Wall Weight to Preliminary Fractionation 1	134
5.2 Sugars Recovered as Percentage of Cell Wall Weight to Preliminary Fractionation 2	135

LIST OF TABLES CONTINUED		Page
5.3	Gross Analysis of Cell Wall Fractions of Batch 1 Walls	136
5.4	Gross Analysis of Cell Wall Fractions of Batch 2 Walls	138
5.5	Sugar Residues in Each Fraction of Green Callus, Batch 1 Walls	141
5.6	Sugar Residues in Each Fraction of Green Callus, Batch 2 Walls	142
5.7	Individual Sugar Residues in Each Fraction of Green Callus Batch 1 Walls μ moles/g 70 ^o C Water Residue	143
5.8	Individual Sugar Residues in Each Fraction of Green Callus Batch 2 Walls μ moles/g 70 ^o C Water Residue	144
5.9	Polysaccharide Sugar Residues; Protein and Hydroxyproline, Batch 2 Walls, % of Totals Accounted for in 70 ^o C Water Residue	152
5.10	Polysaccharide Sugar Residues; Protein and Hydroxyproline, Batch 2 Walls, % of Totals Accounted for in 70 ^o C Water Residue	153
5.11 -	Individual Amino Acid Residues, Mole %	155-158
5.14	Composition and μ moles/g 70 ^o C Water Residue, Batch 1 and 2 Walls	
5.15	Amino Acid Mole % Composition for Analogous Fractions of <u>Pinus</u> , <u>Phaseolus</u> , and Lupin	164
5.16	Mole % Composition of Amino Acids in Arabino-galactan Protein Isolated from Pinus taeda Callus	166

LIST OF TABLES CONTINUED		Page
5.17	Lignin Levels in Batch 1 Walls	169
6.1	Methylated Sugar Residues for Fractions of Batch 1 Walls	183
6.2	Methylated Sugar Residues for the 100°C Water Fraction of Batch 2 Walls	187
6.3	Methylated Sugar Residues for 6M GTC, 10% KOH fractions of Batch 2 Walls	191
6.4	Derivatives Associated with the Xyloglucan	193
6.5	Quantitation of Methylated Derivatives for Polysaccharides of 10% KOH Fraction, Batch 2 Walls	194
6.6	Derivatives as Approximate Mole % Total for Xyloglucan and Xylan	195
6.7	Approximate Levels of Polysaccharides in Batch 1 Walls	200
6.8	Approximate Levels of Polysaccharides in Batch 2 Walls	201
7.1	Sugar Percentage Composition of Fractions to DEAE Run 1	207
7.2	Methylation Data for DEAE Run 1, Fraction 1	209
7.3	Sugar Percentage Composition of Some Fractions of DEAE, Run 2	214
7.4	Amino Acid Composition of Fractions from DEAE Run 2.	214
7.5	Methylation Data of DEAE, Run 2, Fraction 38.	216

LIST OF FIGURES

Figure	Page
1.1 Basic Structure of a Galactoglucomannan from Gymnosperms	7
1.2 Basic Structure of a Representative Arabino-(4-O-methylglucurono)xylan	7
1.3 General Structure of a Fucogalactoxyloglucan	10
1.4 Basic Structure of a Rhamnogalacturonan	14
1.5 Variations of Rhamnogalacturonan Structure	14
1.6 Possible Structure of Cell Wall Glycoprotein Segments	18
1.7 Possible Structural Organisation of Primary Cell Wall	24
2.1 Phenol-Sulphuric Colorimetric Standard Curves for Total Sugar	37
2.2 Meta-hydroxydiphenyl Colorimetric Standard Curves for Uronic Acids	39
2.3 Standard Curve for Spectrophotometric Assay of Hydroxyproline.	39
2.4 Spectral Curves for Lignin	43
2.5 Standard Curves for Spectrophotometric Assay of <u>Pinus radiata</u> Starch by I ₂ /KI	45
2.6 Standard Curves for Spectrophotometric Assay of <u>Pinus radiata</u> Starch by the Amyloglucosidase method.	45

LIST OF FIGURES	Page
2.7 Sugar Recovered with Time for Hydrolysis in HNO ₃ /urea	59
2.8 The β -elimination reaction	70
2.9 The Methylation-reduction Reaction	70
3.1 Transmission Electron Photomicrograph of a Section through Green Callus Cells	86
3.2 Transmission Electron Micrograph of Section through Green Callus Cells, enlargement of Figure 3.1	86
3.3 Cross Section of the Primary Wall, showing Cellulose Microfibrils	87
3.4 Rod-like Material from around Callus bases	87
3.5 Preparation of Batch 2 Walls	91
3.6 Solubilisation and Hydrolysis of <u>Pinus</u> <u>radiata</u> Starch.	93
3.7 Wet Sieved Walls, Batch 1, Fluorescence Microscopy	97
3.8 Light Photomicrograph of Walls Prepared in Phosphate Buffer with PEG treatment	97
3.9 Light Photomicrograph of Batch 1 walls, Stained with congo red	99
3.10 Light Photomicrograph of Batch 2 Walls (without PEG) stained with congo red	99

LIST OF FIGURES	Page
3.11 Light Photomicrograph of Batch 2 Walls, (without PEG) viewed with Nomarski Differential Interference	99
3.12 Scanning Electron Micrograph of Batch 1 Walls	101
3.13 Scanning Electron Micrograph of Batch 1 Walls with a trace of cytoplasmic debris	101
3.14 Scanning Electron Micrograph of Batch 1 Walls showing a bordered pit region from a tracheid	101
4.1 Hypocotyl Extraction Scheme	104
4.2 - Chromatographic Identification of Acidic Sugars	112
4.3 in Hypocotyl TFA-enzyme Hydrolysates	113
5.1 Major Fractionation Scheme for Cell Walls of Batch 1	128
5.2 Fractionation Scheme for Batch 2 Walls	130
5.3 Flow Diagram for Preparation of Fractions containing Protein from <u>Pinus</u> , <u>Phaseolus</u> and Lupin	162
5.4 Protein Composition with Extractant for <u>Pinus</u> , <u>Phaseolus</u> and Lupin	163
7.1 Fractionation of Soluble Polysaccharides of Wall Pectin, by DEAE-cellulose chromatography Run 1	205
7.2 Fractionation of Soluble Polysaccharides of Wall Pectin by DEAE-cellulose chromatography Run 2	211

LIST OF FIGURES	Page
7.3 Fraction PF.2 (from DEAE-run 2) Rechromatographed on DEAE-cellulose	213
APPENDICES	
9.1 Running Parameters for Methylated Alditol Acetates on OV225 Steel Column, Column <u>5</u>	230
9.2 Running Parameters for Methylated Alditol Acetates on OV225 Glass Column, Column <u>2</u>	231
9.3 Running Parameters for Methylated Alditol Acetates on SP2340 Glass Column, Column <u>1</u> and <u>3</u>	232
9.4 Programmed Chromatogram (Column <u>2</u>) of Derivatives from the Fully Methylated 10% KOH Fraction of Batch 2 Walls	238
9.5 Programmed Chromatogram (Column <u>2</u>) of Derivatives from the Fully Methylated EDTA Fraction, Path A, Batch 1 Walls	239
9.6 Effect of Glass Columns and Complete Acetylation on Gas Chromatography Traces	240
9.7 Fragmentations of 2,3,6-Me ₃ -galactitol-tri-acetate	245

ABBREVIATIONS

COMMON ABBREVIATIONS USED:

EDTA	Ethylene diaminetetra-acetic acid
TFA	Trifluoroacetic acid
TFA-enz	Trifluoroacetic acid - <u>Sclerotium rolfsii</u> enzyme treatment
Dimsyl	Dimethyl sulphanyl anion ($\text{CH}_3\text{-S-CH}_2^-$)
PEG	Polyethylene glycol
Amino Acids	Standard abbreviations are used
g.c.	Gas chromatography
g.c.-m.s. (G.C.-M.S.)	Gas chromatography-mass spectrometry
f.i.d./F.I.D.	Flame ionisation detection
m/e	Mass/charge ratio of ion fragments in mass spectrometry
mass spec.	Mass spectrometry
R_{xyl} , R_{xylose}	Coefficient of diffusion relative to xylose in paper chromatography
Inos	Inositol
2dglc - 2 deoxyglucose	2-Deoxy-D-glucose
Monocots	Monocotyledons
Dicots	Dicotyledons
Polysacch	Polysaccharide (only seldom used)
F.R.I.	Forest Research Institute (New Zealand)

SUGARS IN PLANT CELL WALLS:

Rha, L-Rha, L-Rha p	L-rhamnose,	L-rhamnopyranose
Fuc, L-Fuc, L-Fuc p	L-fucose,	L-fucopyranose
Ara, L-Ara, L-Ara f	L-arabinose,	L-arabinofuranose
Xyl, D-Xyl, D-Xyl p	D-xylose,	D-xylopyranose
Man, D-Man, D-Man p	D-mannose,	D-mannopyranose
Gal, D-Gal, D-Gal p	D-galactose,	D-galactopyranose
Glc, D-Glc, D-Glc p	D-glucose,	D-glucopyranose

OLIGOSACCHARIDES

GalA, D-GalA D-GalA p	D-galacturonic acid D-galactopyranosyl uronic acid
GlcA, D-GlcA D-GlcA p	D-glucuronic acid D-glucopyranosyl uronic acid
4-O-MeGlcA	4-O-methylglucuronic acid
4-O-MeGlcA-Xyl 4-O-MeGlcA-(1→2)-Xyl	4-O-methylglucuronosyl-(1→2)-xylose
GalA-GalA GalA-(1→4)-GalA	Galacturonosyl-(1→4)-galacturonic acid
GlcA-GalA GlcA-(1→4)-Gal	Glucuronosyl-(1→4)-galactose
GalA-Rha GalA-(1→2)-Rha	Galacturonosyl-(1→2)-rhamnose
GlcA-Xyl GlcA-(1→4)-Xyl	Glucuronosyl-(1→4)-xylose
MeGlcA-Xyl-Xyl	Methylglucuronosyl-(1→2)-xylose-(1→2)-xylose

POLYSACCHARIDES:

Named in text according to backbone structure. The general descriptions of polysaccharides are given in Chapter 1.

e.g.

β -(1→4)-mannan: mannose (1→4)-linked in β configuration

Arabino-3,6-galactan: polymer of (1→3) and (1→6)-linked β -D-galactopyranose units, to which are attached L-arabinofuranose units.

Arabino-(4-O-methylglucurono)xylan:

(1→4)-linked xylose backbone carrying arabinose, and 4-O-MeGlcA substituents.

METHYLATION DATA:

a) Sugar residues, with position of methyl groups,

e.g. 2,3,6-Tri-O-methyl-D-glucose

= 2,3,6-Me₃-glucose, or 2,3,6-Me₃Glc

- b) Sugar residues indicating linkage positions,
e.g. 4,6-linked glucose, are sometimes abbreviated
to, e.g. 4,6-Glc.