A study of the efficacy of organ cultures to examine wood formation in *Pinus radiata* D. Don

Thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

at the

University of Canterbury

by

Tracy L Putoczki

UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

2006

to my mom…

for everything…

Declaration

This thesis contains my original work except where acknowledgement has been provided in the text.

Acknowledgments

This has been quite the journey, and there are a number of people that I must thank for not letting me get lost along the way…

Firstly, to my supervisor Sandra Jackson, a sincere thanks for the many opportunities you have allowed me and for believing in me. Your scribbles were missed in the preparation of this document. Get better soon!

A huge thanks must go to Juliet Gerrard, who was there to adopt me and pick up the pieces when things went dark. You are a wonderful comma fairy. A very sincere thanks must also go to Tony Bacic and his lab group at the University of Melbourne, for taking me in when I needed help, and especially Filomena Pettolino for her time. To the many people that have helped me with bits along the way: Ashley Garrill, Ralf Möller, Mike Jarvis, Philip Harris, Heiner Goldbach, Brownyn Smaill, Juliana Maurer Menestrina, Grant Pearce, Jackie Healy and Brian Butterfield - your time, teachings and/or scribbles have been wonderful. Thanks must also go to Manfred Ingerfeld for always being at the ready when new projects came up, and to Matt Walters for the wonderful posters. Of course, I must also thank WQI Ltd. for my funding, and not letting me starve. To those that have kept me smiling behind the scenes: Linda, Beth, Shayne, Kenny, Sophie, Shiva, the 'tour group', and especially Hema, thank you for not letting me jump out the lab or office window. Your support through everything will always be remembered.

To the friends I have left back in Canada, thank you for always being incredibly and unconditionally supportive of everything that I do. I will visit soon, and there is a Kraft Dinner party to be had!

My love and deepest thanks go to Mike. There is too much to be said. I really couldn't have finished this without you. You are my inspiration.

To my family, your love and support have been the foundation of everything that I have achieved. Thank you for believing in me and the crazy things I do. I will come home… eventually… promise.

Are we there yet?

Table of contents

Abbreviations

%	percent
0BCM	0 μM boron, 0 mM calcium and 0 mM magnesium treatment
α	anomeric configuration
α-GalY	alpha-galactosyl Yariv reagent
ABL	acetyl bromide lignin
AGP	arabinogalactan-protein
Ala	alanine
ANOVA	analysis of variance
Ara	arabinose
Arg	arginine
Asn	asparagine
β	anomeric configuration
β-GlcY	beta-glucosyl Yariv reagent
B	boron
B-RG-II	boron-rhamnogalacturonan-II complex
BSA	bovine serum albumin
°C	degrees Celcius
CAD	cinnamyl alcohol dehydrogenase
cDNA	complementary DNA
cm	centimetre
CML	compound middle lamella
Cys	cysteine
D-	configurational isomer of the sugar
dH_2O	distilled water
ddH_2O	double distilled water

EST	expressed sequence tag
f	furanose form of the monosaccharide
FLA	fascilin-like
FPLC	fast protein liquid chromatography
Fuc	fucose
g	units of gravity
g	gram
Gal	galactose
GA_3	giberellin
GC-MS	gass chromatography-mass spectrometry
Glc	glucose
Glu	glutamate
Gly	glycine
GPI	glycosylphospatidylinositol
ha	hectare
HBCM	1000 μM boron, 100 mM calcium, 100 mM magnesium treatment
HBLC	1000 μM boron, 0 mM calcium treatment
HG	homogalacturonan
His	histidine
HMLC	100 mM magnesium, 0 mM calcium treatment
HRGP	hydroxyproline-rich glycoprotein
Hyp	hydroxyproline
IAA	indolacetic acid
ICP-MS	inductively coupled plasma-mass spectrometry
ICP-OES	inductively coupled plasma-optical emission spectrometry
Ile	isoleucine

kDa	kiloDalton
kg	kilogram
kV	kilovolts
L	litre
L-	conifigurational isomer of the sugar
LBHC	0 μM boron, 100 mM calcium treatment
Leu	leucine
LMHC	0 mM magnesium, 100 mM calcium treatment
Lys	lysine
m/z	mass/charge ratio of the ion fragments
M	moles per litre
Man	mannose
Met	methionine
mg	milligram
mL	millilitre
MWCO	molecular weight cut off
NAA	1-naphthalene acetic acid
Opti	7 μM boron, 5 mM calcium, 5 mM magnesium treatment
OptiLB	0 μM boron, 5 mM calcium, 5 mM magnesium treatment
p	pyranose form of the monosaccharide
P	probability
Phe	phenylalanine
PPT	precipitate
PAGE	polyacrylamide gel electrophoresis
PME	pectin methylesterase
Pro	proline
Py	pyrolysis

RG-I	rhamnogalacturonan-I
RG-II	rhamnogalacturonan-II
Rha	rhamnose
S_1	first secondary wall layer
S_2	second secondary wall layer
S_3	third secondary wall layer
SDS	sodium dodecyl sulfate
Ser	serine
TCA	trichloroacetic acid
TEM	transmission electron microscope
TFA	trifluoroacetic acid
Thr	threonine
Tyr	tyrosine
µg	microgram
µL	microlitre
µm	micron
µM	micromoles per litre
UB	unbound
UV	ultraviolet
v/v	volume:volume ratio
Val	valine
w/v	weight:volume ratio
w/w	weight:weight ratio
Xyl	xylose

Abstract

Pinus radiata D. Don is an economically important plantation species to New Zealand that is susceptible to the wood quality flaw 'intra-ring checking'. Intra-ring checking is a term used to describe radial fractures that can occur in the earlywood portion of a growth ring, altering the appearance and resilience of the wood, thereby decreasing its economic value. This thesis presents a study that was part of a broad, ongoing collaborative investigation directed at understanding wood quality issues, with the long term goal of enhancement of future radiata pine crops. These investigations are funded by the Wood Quality Initiative Ltd., and involve basic science, field trials and engineering studies related to intra-ring checking. Specifically, the present study was designed to establish the effects of the mineral nutrients boron, calcium and magnesium on wood formation, to determine whether they are associated with intra-ring checking. This research was carried out in three stages.

Firstly, the ultra-structural and biochemical properties of wood with intra-ring checking were examined to determine if specific features of the cell wall were associated with the incidence of intra-ring checks. Electron microscopy techniques revealed that the CML/S_1 region of the cell wall often showed a decrease in CML lignin staining and S_1 striations in wood with intra-ring checks. However, Klason and acetyl bromide assays did not show a change in lignin content. In order to understand how changes in the CML/S_1 region of the cell wall may occur, methods were required that would allow for the observation of wood formation in a controlled environment.

In the second stage of this study, an organ culture technique was successfully developed to allow for the growth of radiata pine cambial tissue, sandwiched between phloem and xylem, on a defined nutrient medium. This nutrient medium was manipulated, using ion-binding resins, to control the amount of boron, calcium and magnesium available to the growing tissues, to determine if variations in wood formation could be induced. In the final stage of this research, an extensive comparative examination of different techniques that could be used for the observation and measurement of selected wood properties was undertaken, in order to determine the efficacy of the organ cultures to study wood formation in an altered nutrient environment. Wood properties were examined for various stages of xylogenesis, beginning with cell division and expansion, followed by cell wall deposition, and lastly with the onset of lignification in order to define the success of the culture technique. Electron microscopy investigations suggested that in the presence of very little boron the CML/S_1 wall showed darker striation deposits, while an increase in calcium availability, resulted in a more defined CML/S_1/S_2 wall region compared to the controls. Further examination of the cell walls suggested that pectin esterification and possibly lignification could also be increased by limited boron availability.

However, in many of the observed and measured parameters of wood properties, a great deal of complex 'between-tree' and 'within-culture' variation was observed. The results show that elucidation of the association between nutrient availability and the incidence of intra-ring checking can not be established from this organ culture study.

In a concurrent study, the preliminary investigation of arabinogalactan-proteins (AGPs) in radiata pine was undertaken. Radiata pine AGPs were positioned in the compound middle lamella of xylem cells, suggesting potential roles in cell-cell adhesion or cell-cell signalling. For the first time, radiata pine AGPs were isolated and characterized in terms of their protein and carbohydrate composition, both of which yielded features typical of AGPs in other plant species. Unique to radiata pine AGPs was the presence of a large proportion of 5-linked arabinose. While the precise function(s) of AGPs are unknown, the results obtained in this research have established a basis for further investigation into the potential for their involvement in wood formation.

Overall, new tools have been established to facilitate future research on radiata pine, a commercially important species, and novel results have been obtained concerning the mechanisms of wood formation therein.

Chapter One

Introduction

The subject of this thesis is the coniferous gymnosperm *Pinus radiata* D. Don, an economically important pine species in New Zealand that is prone to the wood quality flaw known as intra-ring checking.

The purpose of the work presented was to establish an organ culture technique for radiata pine and determine its efficacy in the examination of wood formation. In particular, the impact of the mineral nutrients boron, calcium and magnesium on cell-wall formation was investigated by manipulation of the levels of these nutrients provided to the cultures, in order to determine whether these nutrients are associated with intra-ring checking. The validity of the culture technique was assessed using a range of microscopy and biochemical techniques allowing for the study of wood formation *in vitro*. Preliminary work on the isolation and characterization of radiata pine arabinogalactan-proteins, a potential biological target for altering wood quality, is also presented.

Understanding the differentiation of tracheids is vital to improving wood quality, as their ultra-structure and composition determines softwood properties. Therefore, the establishment of an organ culture technique and appropriate methods for assessment of the affects of different variables, such as nutrition, on wood development will lead to a greater understanding of the fundamentals of wood formation in radiata pine.

1.1 Radiata pine

Pinus radiata D. Don (Coniferopsida: Pinaceae), also known as Monterey pine and commonly referred to as radiata pine, is native to the coast of southern California, USA and two small islands off the coast of Mexico, where its natural distribution is limited [1-3]. Radiata pine is, however, grown extensively as an exotic timber species in several southern hemisphere countries, such as New Zealand, Australia, Chile and South Africa [3]. Large plantations can also be found in northern hemisphere

countries including Spain, Portugal, Greece, Turkey, Italy, and Russia [3, 4]. In 1997, radiata pine covered a worldwide area of 3.8 million ha [2, 5-7].

1.1.1 The importance of plantation forests

Despite the development of alternative materials, wood is still a major commercial product and energy source, with a continually growing world wide demand [2, 8, 9]. As natural forest resources are depleted, the wood supplied by plantation forests is becoming an increasingly important substitute.

In 2005, there were just under four billion ha of forest world wide, of which approximately 124 million ha were plantation forests [6, 7]. While plantation forests occupy a small percentage (~4%) of total forest area, in 2000, plantation forests were estimated to supply approximately 35% of global roundwood [6]. In New Zealand, 6.3 million ha (~23%) of the total land area is occupied by natural forests, with only 1.8 million ha (~7%) of the total land area dedicated to plantation forestry [10-12]. However, in 1997, 99% of the industrial roundwood and commercial wood in New Zealand was obtained from plantation forests, demonstrating that wood harvested from plantation forests, if properly managed, has the potential to replace wood harvested from natural forests [6, 10, 13]. Some of the most important species used in plantation forests are fast growing pines, which cover approximately 37 million ha (~20%) of the area occupied by plantation forests world wide [6, 10]. Among these species is radiata pine.

1.1.2 The importance of radiata pine to New Zealand

Radiata pine was first introduced to New Zealand in the mid-1800s, shortly after which the first stands were planted, and subsequently felled, giving radiata pine a 150 year history of cultivation in New Zealand [2, 11, 14]. As shown in figure 1.1, radiata pine is now the major plantation forest species in New Zealand, covering an area of approximately 1.6 million ha, nearly 90% of New Zealand's plantation forests [10, 15-17]. The remainder of New Zealand's plantation forests are composed of Douglas fir and a variety of other softwoods and hardwoods (figure 1.1).

Radiata pine was chosen as the main plantation species in New Zealand because it grows faster here than in any other country, achieving growth rates as high as 40 m^3/ha/yr that can be maintained over several decades [2]. The maritime, temperate

climate in New Zealand encourages this growth, by allowing radiata pine to grow almost year round (although this is dependent on where in New Zealand it is planted), with a height growth greatest in the spring and least in the winter, meaning that it does not achieve proper dormancy and is vulnerable to frost [2, 3, 18]. As a result of its fast growth, radiata pine can be harvested in a relatively short time (< 30 years), allowing for 20 to 30 year rotations [2].

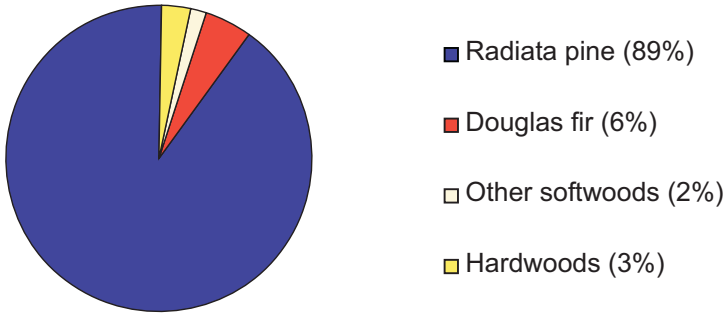


Figure 1.1 Distribution of New Zealand's plantation forest species (Ministry of Agriculture and Fisheries, New Zealand. Statistics as of April 1 2004).

Radiata pine is used to produce a range of products such as timber, furniture and pulp and paper [19]. In 2004, the export of forestry products in New Zealand was worth more than 3.6 billion dollars, making forestry products New Zealand's third largest export [2, 11, 13]. As such, the maintenance of plantation forests that produce quality timber is of great importance to the New Zealand economy.

Since the 1950s, conventional breeding methods have been used to select radiata pine genotypes with features such as superior growth, and form [2, 20, 21]. However, because many of the desirable wood quality traits are only visible at maturity, and radiata pine has a long breeding cycle, traditional breeding programs take a long time and are becoming impractical [22]. The selection of traits by conventional breeding, without understanding the processes and genes involved, has resulted in little progress in further improving wood quality, in part because wood formation is not well understood [9]. In the last decade, genetic and molecular research, coupled to culture techniques, have become popular, as they have the potential to quickly modify cellulose content, lignin content and/or composition, and other important wood cell characteristics, ultimately changing wood quality, and allowing for a better understanding of these processes, in a shorter time [20, 23-25].

1.1.3 Wood quality flaw: intra-ring checking

While radiata pine has many advantages as a plantation species, it is susceptible to the wood quality flaw 'intra-ring checking', a name used to describe radial fractures in the earlywood portion of a growth ring [26]. Wood quality is a subjective term, whose definition can change depending on the context of its use [27]. In this thesis, the term 'wood quality flaw' refers to the attributes that result in a reduction in the value of wood for a given end use [27]. Intra-ring checking results in alterations in the appearance of wood, as well as its strength, when it is present in a severe form, thereby decreasing the overall economic value of the wood [26]. Intra-ring checking results in a loss of profit for both the growers, as a result of the lower prices for check prone logs, and the processors, who must incur the cost of segregating check prone wood prior to processing, or reject the final product after expensive processing is complete, because checked wood is not suitable for high-valued clearwood products [28-30]. The result is a loss of approximately 15 million dollars per year to the New Zealand forest industry [28, 30].

While radiata pine has undergone nearly 60 years of extensive and well funded research, new research efforts have gone into characterizing, predicting and preventing this particular wood quality flaw [2, 26]. The work presented in this thesis is part of a broad, ongoing collaborative investigation directed at understanding wood quality issues such as intra-ring checking. Other studies suggest that intra-ring checking is the result of a combination of environmental variables, genetic composition, and processing procedures [26, 29, 31, 32]. In order to predict and minimize the damage caused by these three factors, it is crucial to understand wood formation. In order to do so, it is critical to examine the molecular composition of wood in order to determine where the predisposition to form intra-ring checks may initiate.

1.2 Overview of wood formation

In conifers, the process of xylogenesis (wood formation) incorporates division of the meristematic vascular cambium and subsequent xylem cell differentiation, and culminates in cell death, marking the formation of a mature tracheid (figure 1.2). The vascular cambium is composed of two types of cells: fusiform initials, which divide to

give the axial secondary vascular tissue (including xylem cells) as well as the axial phloem cells, and ray initials, which divide to give the radially oriented ray cells in both the xylem and phloem [33, 34]. Through periclinal cell division (vertical divisions in the transverse plane) the vascular cambium maintains itself, and produces outer layers of phloem cells and inner radial files of xylem cells. The production of more xylem than phloem accounts for the formation of wood in trees (figure 1.2). These newly formed xylem cells undergo differentiation whereby they expand and elongate [33]. After this process, maturing tracheids produce secondary walls that thicken as cellulose microfibrils are deposited in organized layers and they are impregnated with lignin [35]. These xylem cells ultimately undergo autolysis of the cell contents, which marks their final transition to a mature tracheid [35].

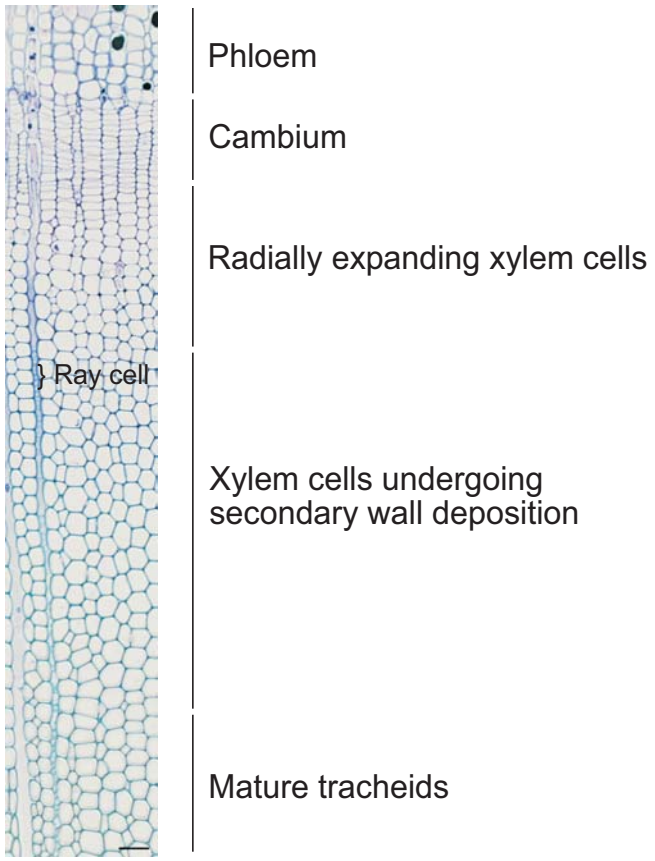


Figure 1.2 The formation of wood in radiata pine. The vascular cambium divides to maintain itself, and to produce outer radial cell files of phloem and inner radial cell files of xylem. Ray cells are also produced. The production of more xylem than phloem accounts for the formation of wood. Transverse section of radiata pine stained with toluidine blue. The image was collected with a Ziess Axioshop camera. Scale bar = 50 µm.

This process occurs every year, increasing the diameter of a tree, and in temperate countries creates the growth rings seen on a transversely cut log. The wood cells created in the beginning of a growing season are referred to as earlywood, characterized by large cells, with thin walls. The wood cells created at the end of a growing season are referred to as latewood, which is composed of smaller cells with thick walls. This thesis focuses on the earlywood portion of radiata pine, where intra-ring checking occurs, and new wood growth, as a result of periclinal division of the vascular cambium during organ culture.

1.3 The structure of the tracheid cell wall

Tracheids, at maturity, are non-living cells that conduct water and provide mechanical strength to the vascular system of a tree [34, 36-38]. Tracheid cell walls are complex: their components maintain the cell's shape, they participate in adhesion, and they can be involved in cell-cell signalling [39]. The components of a tracheid cell wall generally fall into three categories: the substances that create a framework for the tracheid; the substances that comprise a matrix within the framework; and the substances that fill the spaces [40]. Specifically, the framework substances include cellulose; the matrix substances include the pectic polysaccharides; and the main substance that fills the spaces is lignin [40]. In radiata pine, 95% (by volume) of the vascular cells are tracheids, making their cell wall components an obvious target for improving wood quality [35, 41].

1.3.1 The middle lamella

The middle lamella forms the interface between primary walls of neighbouring cells, and is composed primarily of proteins, pectic polysaccharides, and lignin when the tracheid is mature [34, 36, 42, 43]. The middle lamella is thought to adhere the primary cell walls of adjacent cells to each other, thus preventing movement of the xylem radial cell files [36, 44]. Since the middle lamella is difficult to differentiate from the primary cell wall, especially if it contains high lignin levels, the term 'compound middle lamella' (CML) will be used in this thesis in reference to both the primary cell wall and the middle lamella [36].

1.3.2 The primary cell wall

The primary cell wall is the first wall formed in a developing tracheid, and establishes the framework for deposition of the secondary wall [36, 43]. The primary cell wall is composed of cellulose embedded in a matrix, which includes pectic polysaccharides, polysaccharides, protein, and lignin [35, 39, 45]. The elastic properties of the primary cell wall are believed to permit tracheid growth and radial expansion [36, 46].

1.3.2.1 Cellulose

Cellulose is the most abundant carbohydrate in the cell walls of wood, with the secondary cell walls containing the highest concentration [47, 48]. Cellulose is a linear homopolymer of β-(1→4)-D-glucopyranose (Glc*p*) residues (figure 1.3) [39, 49]. The adjacent glucans are oriented parallel to each other creating a cellobiose unit, which is the repeating unit in the chain [34, 43, 47]. These linear glucan chains can aggregate, held together by *inter*-molecular hydrogen bonds, to form long ordered 'crystalline' cellulose microfibrils [39, 43, 47]. The tight *intra*-molecular bonds formed on the same cellulose microfibril chain make them one of the strongest components of the cell wall [39, 47]. Individual glucan chains may be about 2 to 3 μm long and begin at different positions to create the long microfibrils [43]. These cellulose microfibrils can be of any length, and can be found in varying degrees of order depending on where they are observed in the cell wall [39]. In the primary cell wall, microfibrils generally appear dispersed, an arrangement that is likely to allow for the elongation and expansion of the tracheids during development [40, 50]. Cellulose microfibrils can also link, *via* hydrogen bonds, to polysaccarides that are composed of xylans, mannans, galactans, or a combination of the three [47], which, in turn, may be covalently bound to lignin [47].

→4)-β-Glc*p*-(1→4)-β-Glc*p*-(1→4)-β-Glc*p*-(1→4)-β-Glc*p*-(1→

Figure 1.3 The partial structure of a β-(1→4)-D-glucopyranose chain of cellulose.

1.3.2.2 Xyloglucan

Xyloglucan is a branched polymer consisting of a backbone of β-(1→4)-D-glucopyranose residues that has short side chains composed of xylose, galactose and fucose [43, 49, 51-53]. Xyloglucan can form hydrogen bonds with the surface of

cellulose, holding adjacent microfibrils together, resulting in restrictions on cell expansion [39, 52-56]. Xyloglucans have also been found covalently attached to the pectin network in suspension-cultured rose cells, through the arabinan/galactan on rhamnogalacturonan-I [57].

Xyloglucans are the main non-pectic and non-cellulosic polysaccharides in the primary wall of coniferous gymnosperms [51, 58], and have been isolated in, *Cryptomeria japonica* D. Don (Sugi, Japanese cedar) [59, 60], suspension cultures of *Pseudotsuga menziesii* (Douglas fir) [61] and the hypocotyls of radiata pine [62]. They have a similar structure to the most common xyloglucans in the primary walls of dicotyledons [59].

1.3.2.3 Glucomannans and/or galactoglucomannans

Glucomannans and/or galactoglucomannans are composed of a linear backbone of randomly associated β-(1→4)-D-glucopyranose and β-(1→4)-D-mannopyranosyl residues [43, 63, 64]. Glucomannans with galactose residues attached to them are often called galactoglucomannans [43, 63]. Glucomannans and/ or galactoglucomannans are generally present in only small amounts in the primary cell walls, and are the major non-cellulosic polysaccharide in the secondary cell wall of coniferous gymnosperms [34, 48, 62, 65, 66]. They have been found in the primary cell walls of differentiating Japanese cedar [60], *Pinus sylvestris* [66] and isolated hypocotyls and cotyledons in radiata pine [62].

1.3.2.4 Heteroxylans

Heteroxylans have a linear backbone of β-(1→4)-D-xylopyranosyl residues, to which glucuronic acid and arabinose are attached [67]. They are generally a minor component of the primary cell wall of gymnosperms [60].

1.3.2.5 Pectic polysaccharides

The pectic polysaccharides are a heterogeneous group of non-cellulosic polysaccharides that characteristically contain galacturonic acid [43]. There are three major pectic polysaccharides that can be further categorized into two groups: those with a homopolymer backbone of galactopyranosyluronic acid (Gal*p*A) residues, such as homogalacturonan (HG); and rhamnogalacturonan-II (RG-II), and those with a

heteropolymer backbone of galactopyranosyluronic acid residues interspersed with rhamnopyranosyl residues (Rha*p*), such as rhamnogalacturonan-I (RG-I) [39].

Pectins are abundant in the CML, where they are believed to be involved in cell adhesion, are linked to the regulation of cell growth, and are gaining increased recognition for their role in the structure and stability of the cell wall [55, 68-70]. In addition, pectic polysaccharides are considered to form a gel like matrix, in which cellulose and other polysaccharides are embedded, allowing the pectins to act as a hydrophilic filler that prevent the aggregation and collapse of the cellulose network [39, 43, 71-73].

Pectins are a major constituent of the primary cell walls of conifers, and are assumed to be present in the primary cell wall of all land plants [55, 60, 61, 74-78]. Pectic polysaccharides are not present in the secondary cell wall, making them the only major class of polysaccharides restricted to the primary cell walls [55].

Homogalacturonan

Homogalacturonan (HG) has the simplest structure of all of the pectins, consisting of a linear homopolymer of α-(1→4)-D-galactopyranosyluronic acid (figure 1.4) [39]. In dicotyledons, a portion of the carboxyl groups present may be methyl-esterified, and sometimes *O*-acetylated, creating 'blocks' of esterified and un-esterified pectin [44, 55, 79, 80]. These charged blocks can be cross-linked by calcium ions, imparting strength to the primary cell wall [55].

→4)-α-D-Gal*p*-A-(1→4)-α-D-Gal*p*-A-(1→4)-α-D-Gal*p*-A-(1→4)-α-D-Gal*p*-A-(1→

Figure 1.4 The partial structure of homogalacturonan (HG).

HG is present in the primary cell walls of conifers, and has been found in suspension cultured Japanese cedar [60, 75], and Douglas fir [61]. The oligogalacturonides containing terminal 4-linked galactopyranosyluronic acid, isolated from the primary cell walls of differentiating xylem in Japanese cedar, were determined to be structurally similar to the HG in dicotyledons [75].

Rhamnogalacturonan-I

Rhamnogalacturonan-I (RG-I) is the second and most abundant type of pectin, and is composed of a heteropolymer backbone of repeating units of α-(1→4)-D-galactopyranosyluronic acid residues and α-(1→2)-L-rhamnopyranosyl residues (figure 1.5) [39, 43, 44, 55]. The rhamnopyranosyl residues in the backbone are often substituted with long neutral side chains of arabinans, galactans and arabinogalactans, which are believed to be covalently attached to RG-I (figure 1.5) [39, 44, 55]. Like homogalacturonan, the galacturonic acid residues of RG-I may be methyl-esterified, or *O*-acetylated [44, 79-81]. RG-I is also believed to be glycosidically attached to HG [55]. The presence of side chains on RG-I has lead it to be recognized as the 'hairy' portion of pectin, while HG, which does not have side chains, is recognized as the 'smooth region' [55].

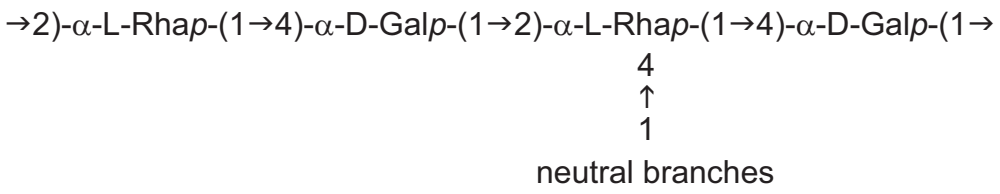


Figure 1.5 The partial structure of rhamnogalacturonan-I (RG-I), showing the neutral pectic polysaccharide branches of the rhamnopyranosyl residues.

RG-I is also present in the primary cell walls of conifers, and has been found in suspension cell cultures of Douglas fir [61] as well as the primary cell wall of differentiating xylem in Japanese cedar [75]. The structure of RG-I from the pectin fraction extracted from the primary cell walls of differentiating xylem in Japanese cedar, using endo-α-1,4-polygalacturonase digestion followed by gel-permeation chromatography, showed that the distribution of the arabinan side chains was different from the dicotyledon plant *Acer pseudoplatanus* L. (sycamore) [82]. Despite this structural detail, RG-I is still considered to be similar to dicotyledons [82].

Rhamnogalacturonan-II

Rhamnogalacturonan-II (RG-II) has the richest diversity of sugar linkages found in the primary cell walls, and is not believed to be present in the middle lamella [43, 55, 80]. RG-II is a polysaccharide composed of a backbone of α-(1→4)-D-

galactopyranosyluronic acid residues, to which four oligosaccarhide side chains are attached at unknown positions (figure 1.6) [80]. RG-II contains monosaccharide units belonging to twelve different families of glycosyl residues, including the rare sugars 3-deoxy-D-manno-2-octulosonic acid (Kdo), 3-deoxy-D-lyxo-heptulosaric (Dha), aceric acid (AceA) and apiose (Api) [43, 70, 72].

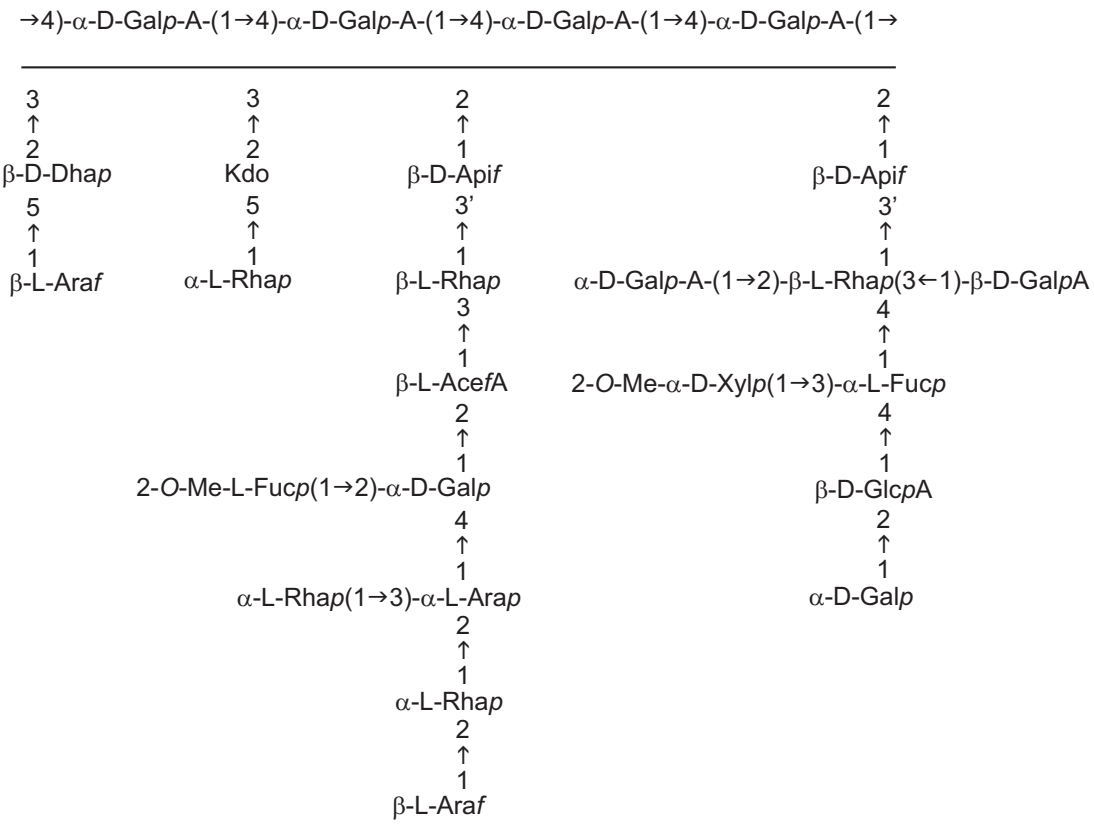


Figure 1.6 The partial structure of rhamnogalacturonan-II (RG-II) and its four oligosaccharide side chains [70].

RG-II was first isolated from the cell wall of suspension cultured sycamore cells, when it was identified as a pectic polysaccharide resistant to endo-polygalacturonase hydrolysis [83]. It has since been found in the primary cell walls of many species, including conifers such as Douglas fir suspension cultures [61, 84, 85] and Japanese cedar, from which is was concluded that this RG-II was structurally similar to dicotyledon RG-II [74].

Of particular interest are the glycosyl linkages that RG-II forms with borate, which can bind to OH-2 and OH-3 on two of the four rare 4,3-linked apiosyl residues of separate monomeric RG-II (only the apiosyl residue on one side chain is involved)

creating a boron-RG-II complex (section 1.6.1) [72, 80, 83, 86-89]. RG-II is the only known borate binding polysaccharide in the cell wall, and accounts for between 1 and 4% of the primary cell wall of gymnosperms [72, 89-91]. This linkage is thought to play a critical role in cell wall structure and stability [92]. B-RG-II has been isolated in sugar beet pulp [91-93], radish roots [94] sycamore cells, pea stems [72], bamboo [95] and *Pinus densiflora* (Japanesee red pine) [76], suggesting that it is conserved amongst most plant species.

1.3.2.6 Formation of a pectin matrix

It is postulated that the three major pectic polysaccharides, HG, RG-I and RG-II are covalently linked to one another, creating a pectin matrix throughout the CML (figure 1.7) [55, 90].

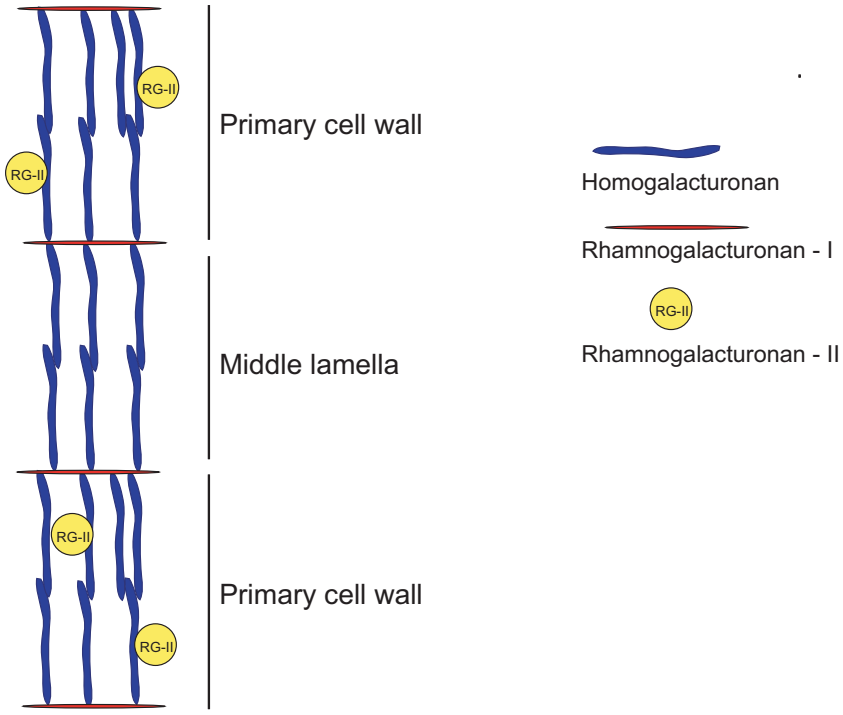


Figure 1.7 Simplified schematic representation of a covalently bound pectin matrix [96].

Cleavage of pectic material to generate RG-I and RG-II when it is digested with endo-polygalacturonase suggests a covalent attachment to HG [55, 92]. In addition, the use of size exclusion columns to characterise solubilised pectin material have found sugars characteristic of HG, RG-I and RG-II [71, 97]. The idea of a coherent pectin matrix that can be modified by cell-wall based enzymes to alter its structure, allowing

space between components, is significant to cell development as it allows, for example, the passage of cell signals through the wall [55]. The presence of a pectic matrix could also alter microfibril deposition, and change the pH and ionic status of the CML [55]. It is not known how HG, RG-I and RG-II are distributed in this network [55]. A detailed review of the structure and function of the pectic polysaccharides and their side chains is given in Perez et al. (2000) [70], Willats et al. (2001) [55] and Ridley et al. (2001) [80].

1.3.2.7 Additional cell wall polysaccharides

Arabinans

Arabinans in both dicotyledons and conifers have a homopolymer backbone of α-(1→5)-L-arabinofuranosyl units [61, 70, 82]. Arabinans occupy approximately 20% of the primary cell wall of radiata pine, where they contain a high proportion of 3-linked, 3,5-linked and 5-linked branches, [98]. RG-I purified from differentiating primary cell walls of Japanese cedar were not found to contain 3,5-linked arabanin residues [82]. However, higher proportions of 5-linked arabinan residues were in found Japanese cedar [82] and Douglas fir [61] compared to the amount found in dicotyledons. This suggests that the branching pattern of the arabinofuranosyl residues may vary between species.

Type-I arabinogalactans

Type-I arabinogalactans are found only associated with pectin [43, 70]; however, they have not been well characterized in conifers. In the primary cell wall of dicotyledons they are comprised of β-(1→4)-D-galactopyranose chains with α-L-arabinofuranosyl units attached to the galactan [43, 70, 73].

Type-II arabinogalactans

Type-II arabinogalactans contain short 1,3- and 1,6-linked β-D-galactopyranose chains [43, 70, 73]. The β-(1→6)-D-galactopyranose chains may have further branches of 1,3- and/or 1,5-linked α-arabinofuranosyl residues [70, 99]. Type-II arabinogalactans also occur in arabinogalactan-proteins [55, 73].

1.3.2.8 Cell wall proteins

Coniferous gymnosperms have primary cell walls that are rich in proteins. Douglas fir for example, has a primary cell wall protein content of between 20 and 32% [61, 77, 100]. Generally, the primary cell wall has more protein than the secondary cell wall [101]. Most of these proteins are glycosylated [102]. Cassab (1998) [103] provides a review of cell wall proteins and their potential functions.

Glycine rich proteins

Glycine rich proteins (GRP), as their name suggests, are predominately composed of glycine, arranged in short amino acid repeats, have smaller amounts of serine, and are only slightly glycosylated [103, 104].

Proline rich proteins

Proline rich proteins (PRP) essentially contain one repeating amino acid motif (Pro-Hyp-Val-Tyr-Lys)$_n$, and are only slightly glycosylated [103]. PRPs are mainly present in the xylem of dicotelydons, suggesting a role in differentiation or lignification [103].

Hydroxyproline rich glycoproteins

Hydroxyproline rich glycoproteins (HRGPs), are a family of proteins which, as their name suggests, have a high hydroxyproline content, and side chains that characteristically contain arabinose and galactose [101, 104, 105]. Included in this family are the extensins and arabinogalactan-proteins. These proteins are distinguished from each other by their amino acid composition, in particular repeating amino acid motifs, and their carbohydrate moieties [103]. For an extensive review of HRGPs refer to Sommer-Knudson et al. (1998) [105].

Extensins are HRGPs abundant in the cell walls of dicotyledons, and are believed to be cross-linked to the cell wall by covalent bonds [73, 103]. Extensins have characteristic Ser-(Hyp)$_4$ repeats, and most of their hydroxyproline residues are glycosylated with short chains of one to four arabinofuranosyl residues [43, 103, 105]. Some serine residues may be glycosylated with single galactopyranosyl residues [103]. The expression of extensins is regulated in dicotyledons, and increases during development, wounding or pathogen invasion [103]. An extensin containing Ser-

$(Hyp)_4$ has been isolated in Douglas fir [106], and an additional extensin rich in proline has also been isolated in loblolly pine [107].

Arabinogalactan-proteins (AGPs) are HRGPs that are mainly *O*-glycosylated and found in most, if not all, plant species [103]. The carbohydrate moiety usually accounts for greater than 90% by weight of the molecule, with the remaining 10% attributed to protein [105]. The carbohydrate component is usually a type-II arabinogalactan (section 1.3.2.7) characterized by an extensively branched β-1,3,-linked galactosyl backbone [105, 108]. While the widespread distribution and structural conservation of AGPs implies that they are important to plant growth and development, their function remains largely unknown [104, 105]. AGPs are not covalently linked to the cell wall, and as such, are not believed to have a structural function [103]. Studies using *Zinnia* mesophyll cultures suggest a role for AGPs in xylogenesis [109]. AGPs have been isolated in loblolly pine xylem [110], suggesting that they may have a role in wood formation.

1.3.2.9 Cell wall enzymes

There are many enzymes active during cell expansion and development [42], that are discussed in reviews by Fry (1986, 1995) [111, 112], Cosgrove (1997) [39], and Walton (1994) [113]. These include enzymes such as pectinase, (which include endo- and exo- polygalacturonases) that are involved in dissolution of the cell wall [113]. A select few enzymes involved in major components of cell wall synthesis and/or modification are described below:

Cellulose synthase

Cellulose synthases are involved in the production of cellulose, and are believed to form a rosette of multiple protein sub-units associated with the cytoplasmic face of the plasma membrane [114]. Full length *CesA* (cellulose synthase genes) have been isolated from the cDNA of developing loblolly pine [115].

Expansin

Expansins are thought to disrupt the non-covalent bonding of matrix polysaccharides to the microfibrils, allowing the wall to expand when turgor pressure increases [39].

An expansin has been cloned in loblolly pine, leading researchers to believe that they are conserved in conifers [116].

Pectin methylesterase

Pectin is in a methylesterified form when it is transported to the cell wall in Golgi vesicles, to prevent premature ionic interactions with its carboxyl groups [117]. Once in the cell wall, the methyl esters are removed by pectin methylesterases [68, 117] allowing for the formation of a covalently linked macromolecular pectin network (section 1.3.2.6) [72, 90]. These methylesterases may themselves be developmentally regulated [55], as esterification has been shown to change during cell wall maturation, with a decrease in esterified pectin being linked to the cessation of growth and the induction of wall stiffening, as a result of the formation of calcium cross links [92, 96, 118].

1.3.2.10 Structural models of primary cell wall organization

The primary cell wall of coniferous gymnosperms is likely to be organized in a similar manner to dicotyledons, as their components are similar (figure 1.8).

Cellulose-xyloglucan model

The cellulose-xyloglucan model of the primary cell wall was proposed by Keegstra et al. (1973) [119] based on their work on suspension-cultured sycamore cells. They believed that the cellulose microfibrils were coated with xyloglucan chains attached by hyrdrogen bonding [119]. They also believed that the xyloglucan chains were attached to arabinogalactan side chains on RG-I, which were covalently linked to structural proteins [119]. Basically, in this model, non-cellulosic polysaccharides and the protein components were considered as a gigantic macromolecule holding the cellulose microfibrils in place.

Cellulose-xyloglucan model independent of the pectin network

The cellulose-xyloglucan model independent of the pectin network is the most commonly referenced wall model and is extensively reviewed by Cosgrove (2001) [120]. This model was based on the work by McCann et al. (1990) [56] with *Allium cepa* L. (onion) cell walls, which have a similar structure to those of dicotyledons [56]. This model suggests that the primary cell wall of dicotyledons has three

entwined, but independent, structural features. The first of these is the cellulose-xyloglucan network, where the xyloglucan chains bind microfibrils via hydrogen bonds, creating aggregated microfibrils [73, 120]. Studies on *Pinus pinaster* have demonstrated this tight binding between xyloglucan and microfibrils [58]. This network is then believed to be embedded in a pectin matrix, and, finally, the structural proteins prevent movement of the primary cell wall components when expansion has ceased [73, 120]. This model suggests that cellulose-xyloglucan association occurs; however, unlike the cellulose-xyloglucan model, there is no covalent attachment of xyloglucan to pectin [73].

Revised cellulose-xyloglucan model

Recently, evidence has been presented that suggests a covalent linkage does occur between the pectic polysaccharides and xyloglucan in the primary cell wall [121-123]. Moreover, only a small percentage of xyloglucan is now thought to be associated with cellulose [122]. As a result of these findings, a new cell wall model has been proposed, that incorporates both of the discussed cell wall models with less xyloglucan and cellulose interactions [122].

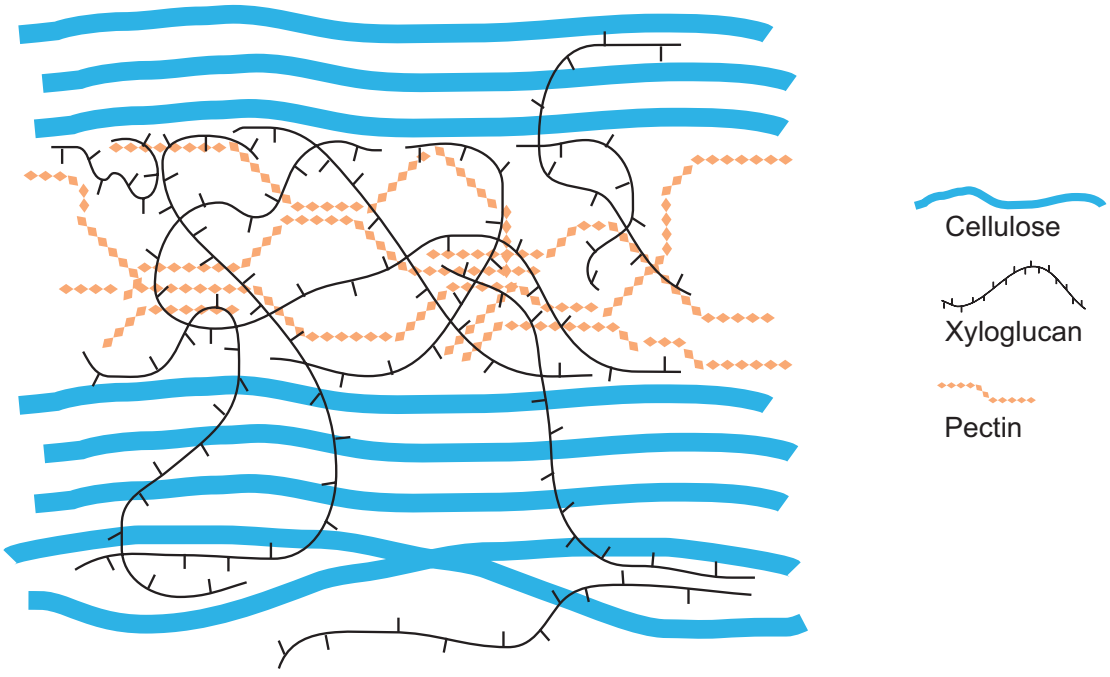


Figure 1.8 Schematic illustration of the primary cell wall. Pectin forms a space-filled hydrophillic gel between microfibrils. Xyloglucan binds to cellulose, cross-linking adjacent microfibrils [39].

1.3.3 The secondary cell walls

Once cell growth is complete, the secondary cell wall, composed of three layers denoted S_1, S_2 and S_3, is deposited [35, 46]. The secondary cell wall contains more cellulose and lower concentrations of lignin, but higher total amounts, than the primary cell wall, providing it with inelastic properties that fix the shape of the cell [33, 44].

1.3.3.1 S_1, S_2 and S_3 secondary cell wall layers

The first of the secondary wall layers, S_1, forms adjacent to the primary cell wall and is recognized by the nearly helical orientation of its microfibrils (figure 1.9) [33]. In Norway spruce tracheids, S_1 microfibrils are oriented nearly perpendicular to the cell axis [124]. The S_2 layer is the thickest of the secondary wall layers, it is deposited after the S_1 layer and its microfibrils are oriented nearly parallel to the cell axis (figure 1.9) [33, 40]. This wall layer contains the majority of the cellulose, other polysaccharides and lignin [47, 125]. The microfibrils of the final layer to be deposited, S_3, which is closest to the cell lumen, form nearly helical patterns (figure 1.9) [33].

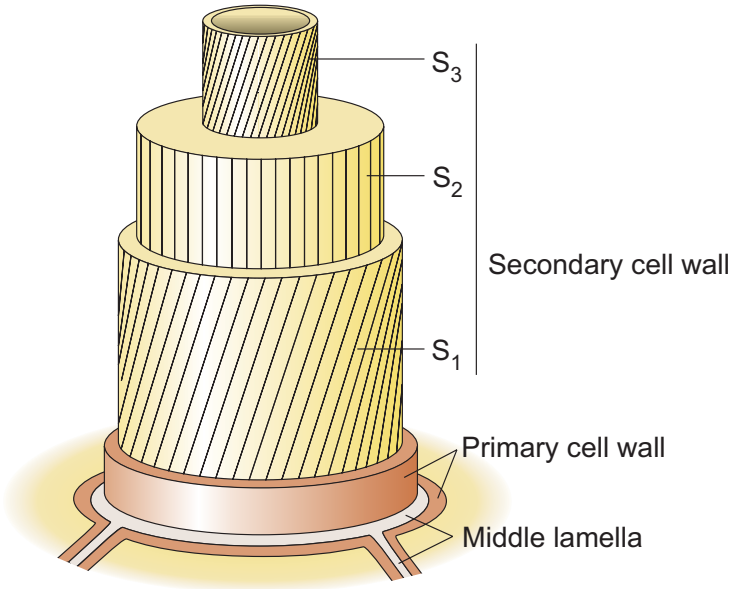


Figure 1.9 Schematic diagram of the organization of the secondary cell wall layers illustrating the change in orientation of the cellulose microfibrils [44, 125].

1.3.3.2 Cellulose

The molecular composition of cellulose in the secondary wall is identical to that in the primary wall (section 1.3.2.1); however, the secondary wall contains more cellulose than the primary cell wall [45, 48].

1.3.3.3 Glucomannans and/or galactoglucomannans

Glucomannans and/or galactoglucomannans have the same structure in the secondary cell wall as they do in the primary cell wall (section 1.3.2.3)[66], and are present in the lignified secondary wall of conifers [64]. Radiata pine contains 15% glucomannan (by weight) [65].

1.3.3.4 Heteroxylan

The main heteroxylan in conifers is a 4-*O*-methylglucuronoxylan, which has a linear backbone of β-(1→4)-D-xylopyranosyl residues, on which single α-4-*O*-methylglucopyranosyluronic acid residues are linked [48, 126]. α-L-Arabinofuranosyl residues can also be linked to the xylopyranosyl residues. [48]. In maize, covalent linkages have been found between heteroxylan chains and lignin [127].

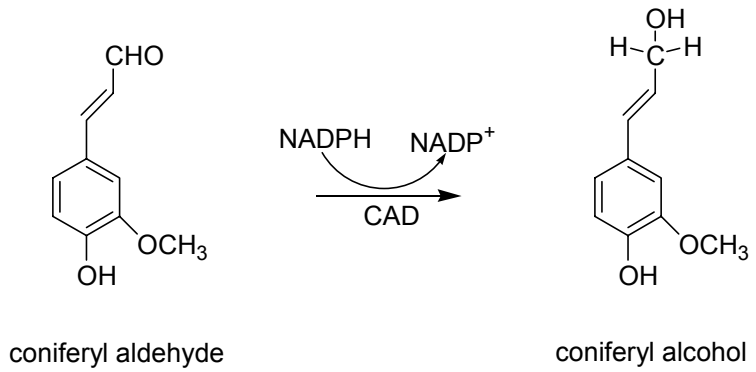
1.3.3.5 Cell wall proteins

Proteins are considered to aid in maintaining the structure of the cell walls, as was proposed in studies on the earlywood of loblolly pine, where an extensin-like protein was isolated from the secondary walls [107].

Studies on suspension cultured *Zinnia* mesophyll cultures localized an AGP to the secondary cell wall of tracheids [128]. This suggests that AGPs may also be present in the secondary walls of coniferous gymnosperms. AGPs have been purified from differentiating xylem of loblolly pine [110]; however, it is not known how much of the extracted material contained secondary walls.

1.3.3.6 Cell wall enzymes

There are many enzymes involved in cell wall formation and modification (section 1.3.2.9).



coniferyl aldehyde coniferyl alcohol

Figure 1.10 The conversion of coniferyl aldehyde to coniferyl alcohol by CAD is the final step in the monolignol specific pathway.

Enzymes involved in the polymerization of monolignols have been isolated in the developing walls of angiosperms [129]. For example, cinnamyl alchohol dehydrogenase (CAD) is a key enzyme in lignification, as it catalyzes the final step in monolignol synthesis, the conversion of cinnamaldehydes to their corresponding alcohol (figure 1.12) [130].

1.3.3.7 Lignin

Lignin is a complex hydrophobic aromatic polymer derived from the enzyme mediated coupling of oxygenated cinnamyl alcohols, or monolignols, to a growing polymer chain [129, 131]. The proportion of monolignols [coniferyl (3-methoxy-4-hydroxy-cinnamyl), sinnapyl (3,5-dimethoxy-4-hydroxy-cinnamyl) and *p*-coumaryl (4-hydroxy-cinnamyl) alcohol] polymerised varies in different plant species, resulting in a polymer that is heterogeneous in both structure and composition (figure 1.11) [129, 132]. The final composition of lignin is determined by the availability of the building units required for polymerization [133]. It is also believed that other phenolics can be incorporated into lignin [131]. In gymnosperms such as radiata pine, coniferyl alcohol units are the predominating monolignol and are termed ‘guaiacyl lignins’ [129, 130, 132].

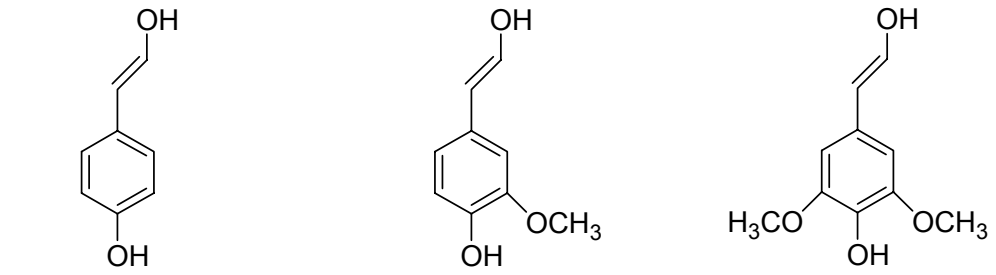


Figure 1.11 The structure of the three monolignols: p-*coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol.*

Lignin is present across the cell wall layers, including the primary wall and middle lamella [125]. Lignin acts as a cement to brace adjacent cells, preventing their movement relative to each other, provides additional strength to the cell wall preventing collapse of cellulose microfibrils under axial load or when exposed to water stress, allows the transport of water and prevents the degradation of polysaccharides [125, 131, 134, 135]. This makes lignin not only a filler substance in the wall, but also a mechanism of defence against insects, pathogens and herbivores [125, 131, 134, 135].

1.3.4 The mature tracheid

Prior to the formation of the secondary wall, the tracheids contain protoplasts that are alive and contain nuclei and other cytoplasmic structures [33, 45, 136]. The final stage of tracheid differentiation is programmed cell death (PCD), whereby the vacuole collapses [137]. At this point in development, a tracheid is considered to be mature. For a detailed review of the biochemical components of a tracheid refer to Harris (2005) [138].

1.4 Model systems for xylem development

As introduced in section 1.1.2, the progress towards understanding the molecular, biochemical and genetic processes regulating xylem formation and subsequent development in trees has been slow. Recent advances in forest biotechnology include large scale EST-sequencing [139, 140], microarray analysis [141], the successful creation of transgenic trees [130, 142] and proteomic analysis [143-145]. One of the major obstacles for the advancement of forest biotechnology is the trees themselves.

Their slow growth makes the production of mutants laborious and impractical. As such, many researchers are looking for model systems to examine the molecular, biochemical and genetic control of xylem formation so that they can examine these processes more efficiently. This section presents a selection of some of the model systems employed to date.

1.4.1 Arabidopsis mutants

With the appropriate growth conditions, Arabidopsis is capable of secondary growth in the hypocotyl, including the development of a vascular cambium [146]. In the early secondary xylem developmental phase, only vessel elements are formed, while the later developmental state forms vessel elements and fibres [146]. Having a completely sequenced genome [147], and numerous Arabidopsis mutants available for manipulation of desirable wood quality traits, such as lignin and cellulose content, Arabidopsis is one of the xylem development model systems of choice. A select few Arabidopsis mutants are described below:

1.4.1.1 Irregular xylem mutants

The *irx* mutants (irregular xylem), which have recessive mutations at three loci [148], grow and expand normally; however, at later stages in development, the xylem elements collapse as a result of the negative pressure generated in the cell [146, 148]. This collapse late in development is consistent with the defect being related to secondary wall formation, which normally maintains the shape of the cell [148, 149].

1.4.1.2 Cellulose deficient mutants

The *rsw1* mutant (radial swelling), which is defective in cellulose synthesis, fails to properly develop its primary cell wall [150]. Three of the *irx* mutant lines are defective in secondary wall cellulose synthesis; however, they grow normally, suggesting that the primary wall in these plants in unaffected [148, 149].

1.4.1.3 Lignin deficient mutants

In an additional *irx* mutant, there is no apparent difference in cellulose deposition, but lignin deposition is altered [149]. In these mutants, only 50% of the lignin content compared to the wild type is obtained [149]. Lignin is present in mutants examined

early in secondary wall formation, but not later in development, suggesting that the defect occurs later in differentiation [149].

The presence of vessel elements and fibres in Arabidopsis resembles the anatomy of an angiosperm tree, and, as such, it is not the best model for gymnosperms such as radiata pine.

1.4.2 Transgenic tobacco plants *(Nicotiana tabacum)*

Tobacco plants were initially considered to be an ideal model of xylem formation as they produce xylem cells in their stems, have short generation times, and their DNA has been extensively mapped [151].

1.4.2.1 CAD supression

CAD was one of the first enzymes to be purified, cloned and manipulated in tobacco plants [129, 152]. These plants were genetically modified using Agrobacterium-mediated transformation to express CAD cDNA inserted in the antisense orientation between the promoter and terminal sequence of the nopaline synthase gene [129, 151, 152]. Despite the reduction in CAD activity, the plants grew normally [129, 152]. The only apparent difference was the colour of the lignin in the xylem, which appeared red/brown compared to the controls [129, 152]. It was determined by pyrolysis-mass spectrometry that when CAD is inhibited, aldehyde groups are incorporated into lignin in the place of the alcohols [129, 152]. These studies did not succeed in decreasing the amount of lignin, but rather modified its structure [152].

Despite the successful creation of transgenic tobacco plants, it has been determined that tobacco plants are not a good model for wood formation, as their cells are different to those found in trees, in that they only have two cell wall layers, primary and secondary [151].

1.4.3 Transgenic poplar (*Populus*)

Poplar is an angiosperm that has emerged as the main model system for trees, often modified to study xylogenic processes because of its small genome size, the success of genetic transformation and its fast growth [145, 153, 154].

1.4.3.1 CAD supression

Both antisense and sense genes have been used to suppress CAD in poplar [130, 142]. The antisense and sense transgenic poplars both had approximately 70% reduced CAD activity, that was associated with a red colouration of the xylem [130]. As in the CAD suppressed tobacco plants, there was no change in the amount or monomeric composition (syringly/guaiacyl) of lignin [130]. Furthermore, these studies did not succeed in decreasing the amount of lignin, but rather modified its structure.

The main lignins in angiosperms include guaiacyl and syringyl units, unlike gymnosperms, which contain mainly guaiacyl lignins. While transgenic angiosperms provide us with insight into many aspects of wood formation, their composition differs from gymnosperms and, as such, they are not ideal models gymnosperm xylem development.

1.4.4 Culture systems

Numerous culture systems have been employed in cell development studies. Three culture systems are described below:

1.4.4.1 *Zinnia* cultures (*Zinnia elegans L.*)

Zinnia cultures were established from individual mesophyll cells isolated from young leaves and maintained in liquid media [109, 155-157]. Manipulation of the culture media can induce trans-differentiation of the cells to 'tracheary elements', cells that are composed of xylem vessels characterized by the formation of secondary cell wall thickenings [109, 157-159]. This system is popular because it is possible to visually follow the process of differentiation through limited cell-cell interaction in a single cell system, and isolation of signal molecules is possible [109, 128, 136, 155]. *Zinnia* cultures have been used to study the ultra-structural changes associated with cell wall thickening [160], and the enzymes involved in cell wall formation [161]. However, its relevance to true xylogenesis in an intact tree is questionable [162].

1.4.4.2 Callus cultures

Recently, a radiata pine callus culture system has been established whereby induction of differentiated tracheids can occur [163]. These cultures can successfully be established from hypocotyl segments, or xylem strips, and can be used to observe

direct responses to alterations in media components representing environmental changes. The undifferentiated xylem derived calli in these cultures can also be stably transformed, allowing for the selection of transgenic lines that can be used to study the effects of introduced genes, such as CAD, on cell wall biosynthesis [163].

1.4.4.3 Organ cultures

These explants are termed 'organ' culture because of the presence of more than one tissue [164]. Organ cultures involve explanting cambium, sandwiched between phloem and xylem, from a standing tree. With the exception of the establishment of *Larix Laricina* (larch) [165, 166] and *Eucalyptus globules* Labill organ cultures [167], there has been very little work with culturing cambium that is greater than a few years in age [165, 167]. Frequently, non-tree species are used to establish cultures that produce results that aren't necessarily applicable to gymnosperms. Moreover, culture systems that are easy to manipulate are often used in favour of natural systems utilizing the portion of the tree of interest [162]. If material is used that originates from a tree, the explants used to establish cultures often originate from petioles, leaves, callus, seedlings or young stem cuttings collected from older trees [165].

1.5 Advantage of using an organ culture technique to study wood formation

The economic and ecological importance of trees has placed an impetus on the development of model systems to study wood formation [154]. In the past, culture systems were overlooked in forestry because a stronger emphasis was placed on selection techniques for macro-feature improvement of trees [168]. *In vitro* culture systems can grow for a few weeks to a few months, making processes involved in wood formation easier to study, when compared to the management of a whole tree [164]. An organ culture system provides a means of studying wood formation in a controlled environment, where interfering factors from the whole tree are removed, and changes to a defined media can result in a controlled response [164]. Organ culture also provides an opportunity to examine the wood qualities that are deemed important in plantation trees, in a short time. In addition, organ cultures afford the advantage of being able to trace the origin of a cell back to its starting point, as radial

cell files can be observed, meaning that every stage of differentiation can be examined simultaneously in the tree species of interest.

1.6 Can wood formation be altered by changing nutrient availability?

One of the goals of this research was to determine if boron, calcium or magnesium could be involved in intra-ring checking.

1.6.1 Why study boron nutrition?

Many researchers suggest that boron nutrition plays a critical role in the regulation of plant development. In particular, boron may be involved in maintaining the strength and integrity of the cell wall matrix, and/or the production and deposition of cell wall material [169]. Boron may, therefore, be involved in intra-ring checking through alterations in the cell wall.

Boron is an essential micronutrient required throughout the plant life cycle, as it is not generally retranslocated to new plant growth once it is bound [170, 171]. Boron mobility within the phloem is negligible, the majority of transport is thought to be facilitated through the xylem [169, 172]. Some researchers suggest the possibility of boron entering the xylem *via* a boron transporter [173, 174]. Other studies have demonstrated a high permeability of cell membranes to boron in the form of undissociated boric acid at the pH present in soil [175, 176]. Boric acid is highly soluble in water, likely to be soluble in lipid membranes, and may be passively absorbed into the cell wall, where it can complex with polysaccharides such as pectin [86, 170, 176, 177]. A high exogenous supply of boron may result in an influx of boric acid into the cell wall [178]. The higher internal pH in the cell, compared to the extracellular matrix, is thought to convert boric acid to borate [174, 179], which has the potential to form monoesters and diesters with compounds containing *cis*-hydroxyl groups, including RG-II (section 1.3.2.5 and figure 1.12) [172, 178, 180].

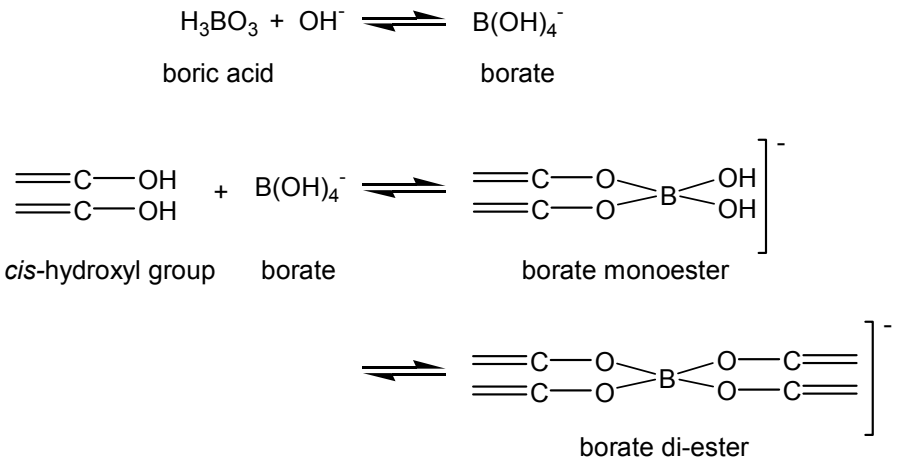


Figure 1.12 Conversion of boric acid to borate and subsequent monoester and di-ester formation [72, 132, 172, 180, 181].

While the need for adequate boron nutrition is agreed in the scientific community, the role of boron within the plant cell is not. In studies on sunflower, Brown and Hu (1994) [169] found that boron may possess a structural role stemming from the formation of "non-exchangeable boron complexes" in the cytoplasm and the cell wall [170]. In tobacco cells, more than 90% of boron was found to occur in the cell walls, nearly 80% of which was bound to RG-II [182]. It is believed that the swelling of the cell wall characteristic of boron deficiency was due to a lack of RG-II cross-links with borate, rather than an increase in density of the cell wall [183]. The general consensus has been that this boron-RG-II complex is responsible for incurring strength and rigidity to the cell (figure 1.13), which in the context of this study, may alter wood quality.

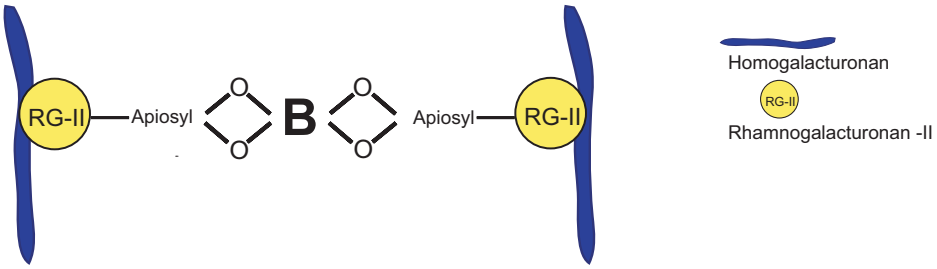


Figure 1.13 A simplified illustration of the 'boron-RG-II dimer'. Boron can bind to OH-2 and OH-3 on two of the rare 3-4 linked apiosyl residues of separate monomeric RG-II, creating a dimer complex.

1.6.2 Why study calcium nutrition?

Calcium is not thought to be re-translocated within a plant once it is bound [175]. As a result, plants require a continuous supply of calcium to maintain normal growth and development [184]. When taken up by a plant, calcium is phloem immobile, resulting in slow accumulation in tissues and transport between cells [175, 185]. As such, the availability of calcium has the potential to alter the development of xylem in radiata pine.

In plants, calcium is localized to the exterior portion of the cell wall, and middle lamella, with additional cytoplasmic concentrations resulting from sequestered salts and precipitates [185]. The localization of calcium to these regions places it in close proximity to unesterified pectins, with which it can form cross-links involving the carboxyl groups on uronic acid residues (section 1.3.2.5, figure 1.14) [55, 86, 111]. Moreover, calcium has been isolated with boron-RG-II dimers, where it has been found to impart stability to the complex and increase its formation *in vitro* [80, 87, 94, 186, 187]. When calcium was removed from the complex, cleavage of dimeric RG-II into monomeric RG-II was observed, suggesting that calcium is a critical component [187]. However, no dimeric RG-II boron complexes were formed when monomeric RG-II was treated with calcium, in the absence of additional boron, demonstrating that calcium alone does not initiate formation of this complex [72].

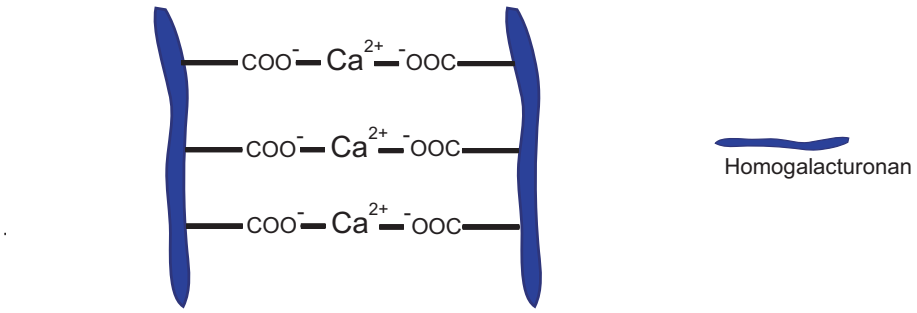


Figure 1.14 Simplified schematic diagram of the interaction of calcium with uronic acid residues on adjacent HG molecules.

1.6.3 Why study magnesium nutrition?

Magnesium is highly mobile in the phloem, although it may be transported in a complex form [184]. Once in the tissue, magnesium can be re-translocated after it is bound and its concentration may vary [175, 185]. When O'Neil et al. (1996) [72]

isolated a boron-RG-II complex in sycamore cells and pea stems, they found calcium and magnesium to be present, suggesting that magnesium may also be involved in the formation of this complex. Other studies suggest that magnesium competitively displaces calcium from the complex (figure 1.15) [188]. In addition, the amount of boron required for maximal growth rates in pine cultures varied with the concentration of calcium and magnesium in the media [188]. Taken together, these studies suggest that a complex inter-relationship between boron, calcium and magnesium may exist.

Very few studies have been completed examining the complex interaction of these nutrients in plants. While there is strong evidence supporting the interactions of these nutrients with RG-II *in vitro,* there is little evidence explaining how these interactions result in the changes researchers have observed in the cell wall [72, 189].

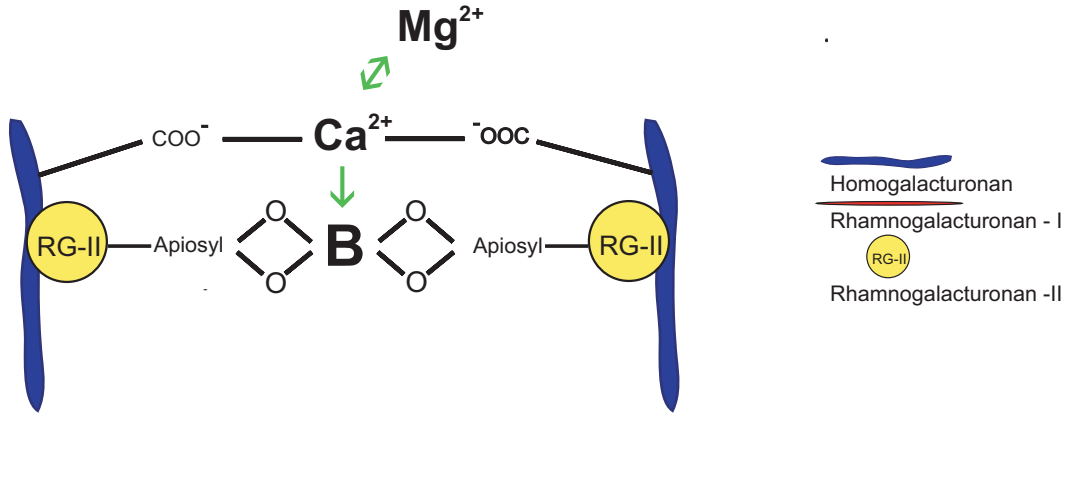


Figure 1.15 Relationship between boron, calcium and magnesium. Calcium can cross-link adjacent HG residues through interactions with carboxyl groups on the uronic acids. Calcium has also been found to stabilize the boron-RG-II complex, while magnesium may competitively displace calcium.

1.7 Can wood formation be altered by protein signals?

A preliminary investigation of arabinogalactan-proteins and their role in wood formation was also undertaken in this thesis.

1.7.1 Why study arabinogalactan-proteins?

Studies utilizing *Zinnia* mesophyll cultures have found an arabinogalactan-protein(s), which they referred to as 'xylogen', to be present in the cell wall of tracheary elements, with an apparent function in differentiation [109, 157, 158]. These AGP(s) accumulate when dedifferentiated cells restrict their competence and become precursors of tracheary elements [109]. The addition of purified AGP(s) to *Zinnia* cell cultures increased the frequency of differentiation, whereas deglycosylation of the AGP(s) did not [109]. In addition, overexpression of homologous genes from Arabidopsis under the control of the cauliflower mosaic virus 35S promoter resulted in the accumulation of these 'xylogen' AGP(s) in the cultures, which also corresponded to increased xylogen activity [109]. Moreover, transfer-DNA (t-DNA) insertion mutants of the homologous Arabidopsis genes *in planta* were used to define the role of these AGP(s) [109]. While none of these mutants showed obvious defects in morphology [109], double knockout mutants did have obvious morphological defects [109]. As a result of this research, it was determined that AGP(s) was not an essential component needed to ensure vascular differentiation, as deficiency did not result in a loss of vascular tissue, but rather AGP(s) may coordinate the events involved to ensure integrity of the system [109].

AGPs have been linked to several regulatory roles such as plant defence and growth [190], cell expansion [191], somatic embryogenesis [192], cell differentiation [193] and cell death [194]. In loblolly pine, AGPs have been localised to differentiating xylem, suggesting an important role in wood formation [110].

1.8 Aims of this research

The overall aim of this research has been to understand the impact of the mineral nutrients boron, calcium and magnesium on cell-wall formation in radiata pine, and whether they are associated with intra-ring checking. The specific aims were as follows:

To establish an organ culture technique of radiata pine to examine wood formation *in vitro* and to apply techniques and media to modify nutrient availability.

To examine different methods that could be utilized to observe and quantify selected wood properties. This included cell measurement using an image analysis program, microscopy techniques to observe cell wall alterations, and biochemical analysis of carbohydrate and lignin content of the cells, to assess the role of boron, calcium and magnesium in wood formation.

To examine the location of arabinogalatan proteins and radiata pine and develop a protocol to isolate and then characterise these proteins.

1.9 Format of this thesis

Chapter two introduces the wood quality flaw, intra-ring checking, and presents the characterization of the ultra-structural and biochemical features of oven dried radiata pine disks showing intra-ring checking. **Chapter three** describes the development of the methods used to establish and manipulate radiata pine organ cultures to study the influence of boron, calcium and magnesium on tracheid development. **Chapter four** describes the viability and growth characteristics of each of the nutrient-treated organ cultures and validation of the methods used. **Chapter five** and **chapter six** examine changes in the structural organization of the cell wall in response to variation in nutrient availability. Specifically, chapter five examines alterations in pectic polysaccharide distribution, while chapter six examines changes in lignification. **Chapter seven** presents the preliminary characterization of isolated radiata pine AGPs and the manipulation of organ cultures to understand their function. **Chapter eight** details an overall summary of the methods developed, their efficacy to study wood formation, and future applications of the work presented in this thesis. **Chapter nine** outlines the experimental methods used in all of the above mentioned chapters.

1.10 References

1. Millar, C.I. 1999. *Evolution and biogeography of Pinus radiata, with a proposed revision of its quarternary history.* New Zealand Journal of Forestry Science, **29**: 335-65.

2. Sutton, W.R.J. 1999. *The need for planted forests and the example of radiata pine.* New Forests, **17**: 95-109.

3. Scott, C.W. 1960. *Pinus radiata*. Rome: Food and Agriculture Organization of the United Nations.

4. Turner, J., Lambert, M.J. 1986. *Nutrition and nutritional relationships of Pinus radiata.* Annual Review of Ecology and Systematics, **17**: 325-350.

5. Johnston, I.G., Ades, P.K., Eldridge, K.G. 1997. *Growth of natural Californian provenances of Pinus radiata in New South Wales, Australia.* New Zealand Journal of Forestry Science, **27**: 23-38.

6. 2001. *Global forest resources assessment 2000 - Main report.* Food and Agriculture Organization of the United Nations: Rome.

7. 2005. *Global forest resources assessment: 15 key findings*. Food and Agriculture Organization of the United Nations: Rome.

8. Whiteman, A. 1999. *Forest product market developments: the outlook for forest product markets to 2010 and the implications for improving management of the global forest estate*. Food and Agriculture Organization of the United Nations, Forestry Policy and Planning Division: Rome.

9. Fenning, T. and Gershenzon, J. 2002. *Where will the wood come from? Plantation forests and the role of biotechnology.* Trends in Biotechnology, **20**: 291-296.

10. Brown, C. 2000. *The global outlook for future wood supply from forest plantations. Report.*, in *Global forest products outlook study working paper series*. Food and Agriculture Organization of the United Nations, Forestry Policy and Planning Division: Rome.

11. Rhodes, D., Novis, J. 2002. *The impact of incentives on the development of plantation forest resources in New Zealand*, in *Ministry of Agriculture and Fisheries Information Paper No. 45*.

12. Waggner, T. 2001. *Role of plantations as substitutes for natural forests in wood supply-lessons learned from the asian-pacific region*, in *Forest Plantations Thematic Papers*, Mead, D., Editor. Food and Agriculture Organization of the United Nations,: Rome.

13. *Current facts and trends*, in *Forestry Insights*. 2005: New Zealand.

14. 2004. *Radiata Pine*. Fletcher Challenge Forests: Rotorua.

15. 2002. *A national exotic forest description. Report 18.* Ministry of Agriculture and Forestry, New Zealand.

16. Withers, T.M. 2001. *Lymantria monacha (nun moth) and L. dispar (gypsy moth) survival and development on improved Pinus radiata.* New Zealand Journal of Forestry Science, **31**: 66-77.

17. 2004. *A national exotic forest description as at 1 April 2004. Report 21*. Ministry of Agriculture and Fisheries, New Zealand.

18. Harris, M.J. 1991. *Formation of wood and bark*, in *Properties and Uses of New Zealand Radiata Pine*, Kininmonth, J.A., Whitehouse, L.J., Ed. New Zealand Ministry of Forestry, Forest Research Institute: Rotorua.

19. Jayawickrama, K.J.S., Shelbourne, C.J.A., Carson, M.J. 1997. *New Zealand's long internode breed of Pinus radiata.* New Zealand Journal of Forestry Science, **27**: 126-141.

20. Carson, M. 1987. *Improving log and wood quality: the role of the radiata pine improvement programme.* New Zealand Journal of Forestry: 26-30.

21. Shelbourne, C.J.A., Carson, M.J., Wilcox, M.D. 1989. *New techniques in the genetic improvement of radiata pine.* Commonwealth Forestry Review, **68**: 3-7.

22. Dean, J. and Merkle, S. 2000. *Forest tree biotechnology.* Current Opinion in Biotechnology, **11**: 298-302.

23. van Raemdonck, D., Jaziri, M., Boerjan, W., Baucher, M. 2001. *Advances in the improvement of forest trees through biotechnology.* Belgium Journal of Botany, **134**: 64-78.

24. Dean, J., et al. 1997. *Forest tree biotechnology.* Advances in Biochemical Engineering and Biotechnology, **57**: 1-44.

25. Walter, C., Carson, S.D., Menzies, M.I., Richardson, T., Carson, M. 1998. *Review: application of biotechnology to forestry-molecular biology of conifers.* World Journal of Microbiology & Biotechnology, **14**: 321-330.

26. Cown, D., McConchie, D., and McConchie, M. 2003. *Intra-ring internal checking in radiata pine (P. radiata D. Don) in New Zealand*, in *Confidential Report*, APP 1. Wood Quality Initiative Ltd. p. 1-21.

27. Jozsa, L.A., Middleton, G.R. 1994. *A discussion of wood quality attributes and their practical implications*, SP-34. Forintek Canada Corporation: Vancouver. p. 1-42.

28. Gabbitas, B., Saunders, J., and Jonassen, A. 2004. *Fine element modelling of intra-ring checking in radiata pine*, in *Confidential Report*, Young, G., Editor. Wood Quality Initiative Ltd. p. 1-4.

29. Kumar, S., Jefferson, P., Carson, M. 2004. *Variation of intra-ring checking within-and-among sites*, in *Confidential report*, APP 17. Young, G., Editor. Wood Quality Initiative Ltd. p. 1-21.

30. Xue, J., Olykan, S., Beets, P., McConchie, M., Graham, D., Leckie, A., Kimberley, M., Pearce, S. 2004. *Effect of boron, genotype and site on tree growth, intra-ring checking and other wood properties of radiata pine*, in *Confidential Report*, APP 38. Young, G., Editor. Wood Quality Initiative Ltd. p. 1-19.

31. Beets, P., et al. 2003. *Family and clonal variation in intra-ring checking and resin characteristics and relationship with foliar nutrients*, in *Confidential Report*, APP 9. Young, G., Editor. Wood Quality Initiative Ltd. p. 1-20.

32. Cote, W. and Hanna, R. 1983. *Ultrastructural characteristics of wood fracture surfaces.* Wood and Fiber Science, **15**: 135-163.

33. Barnett, J. 1981. *Secondary xylem development*, in *Xylem Cell Development*, Barnett, J., Ed. Castle House Publications Ltd.: Kent. 47-95.

34. Panshin, A.J., de Zeeuw, C. 1980. *Textbook of Wood Technology*. Fourth Edition ed. Sydney: McGraw-Hill Inc.

35. Higuchi, T. 1997. *Biochemisty and Molecular Biology of Wood*. Berlin. 13-42, 263-90.

36. Esau, K. 1953. *Plant Anatomy*. New York: John Wiley and Sons.

37. Bamber, R. 1983. *The wood anatomy of eucalypts and papermaking.* Appita, **38**: 210-216.

38. Torrey, J.G., Fosket, D.E., Hepler, P.K. 1971. *Xylem formation: a paradigm of cytodifferentiation in higher plants.* American Scientist, **59**: 338-352.

39. Cosgrove, D. 1997. *Assembly and enlargement of the primary cell wall in plants.* Annual Review of Cell and Developmental Biology, **13**: 171-201.

40. Wardrop, A.B. 1964. *The structure and formation of the cell wall in xylem*, in *The Formation of Wood in Forest Trees*, Zimmerman, M.H., Ed. Academic Press Ltd.: New York, London. 87-133.

41. Harris, M.J. 1991. *Structure of wood and bark*, in *Properties and Uses of New Zealand Radiata Pine*, Kininmonth, J.A., Whitehouse, L.J., Ed. New Zealand Ministry of Forestry, Forest Research Institute: Rotorua.

42. Brett, C. and KW, W. 1996. *Physiology and Biochemistry of Plant Cell Walls*. London: Chapman and Hall.

43. Carpita, N. and McCann, M. 2000. *The cell wall*, in *Biochemistry and Molecular Biology of Plants*, Buchanan, B., Gruissem, W., and Jones, R., Eds. The American Society of Plant Physiologists: Rockville. 52-157.

44. Taiz, L., Zeiger, E. 2002. *Cell walls: structure, biogenesis, and expansion*, in *Plant Physiology*. Sinauer Associates Inc.: Massachusetts.

45. Samuels, A.L., Rensing, K.H., Douglas, C.J. 2002. *Cellular machinery of wood production: differentiation of xylem in Pinus contorta var. latifolia.* Planta, **216**: 72-82.

46. Dell, B. and Huang, L. 1997. *Physiological response of plants to low boron.* Plant and Soil, **193**: 103-120.

47. Forss, K. and Fremer, K. 2003. *Anabolic formation of wood components*, in *The nature and reaction of lignin-a new paradigm*. Oy Nord Print Ab: Helsinki, Finland.

48. Fengel, D. and Wegener, G. 1983. *Distribution of the components within the wood cell wall*, in *Wood: Chemistry, Ultrastructure, Reactions*, Fengel, D. and Wegener, G., Eds. Walter de Gruyter & Co.: Berlin. 227-238.

49. McNeil, M., Darvill, A.G., Fry, C. 1984. *Structure and function of the primary cell walls of plants.* Annual Review of Biochemistry, **53**: 625-663.

50. Abe, H., et al. 1995. *Changes in the arrangement of microtubules and microfibrils in differentiating conifer tracheids during the expansion of cells.* Annals of Botany, **75**: 305-310.

51. Acebes, J., Moral, R., and Zarra, I. 1993. *Purification and structure of xyloglucan in pine hypocotyls.* Phytochemistry, **33**: 1343-1345.

52. Fry, S. 1989. *The structure and function of xyloglucan.* Journal of Experimental Botany, **40**: 1-11.

53. Hayashi, T. 1989. *Xyloglucans in the primary cell wall.* Annual Review of Plant Physiology, **40**: 139-168.

54. Fry, S., et al. 1993. *Oligosaccharides as signals and substrates in the plant cell wall.* Plant Physiology, **103**: 1-5.

55. Willats, W.G.T., McCartney, L., Mackie, W., Knox, P. 2001. *Pectin: cell biology and prospects for functional analysis.* Plant Molecular Biology, **47**: 9-27.

56. McCann, M.C., Wells, B., Roberts, K. 1990. *Direct visualization of cross-links in the primary plant cell wall.* Journal of Cell Science, **96**: 323-334.

57. Thompson, J.E., Fry, S.C. 2000. *Evidence for covalent linkage between xyloglucan and acidic pectins in suspension-cultured rose cells.* Planta, **211**: 275-286.

58. Acebes, J., et al. 1993. *Pine xyloglucan. Occurance, localization and interaction with cellulose.* Physiologia Plantarum, **89**: 417-422.

59. Kakegawa, K., Edashige, Y., Ishii, T. 2002. *Xyloglucan from xylem-differentiating zones of Cyrptomeria japonica.* Phytochemistry, **47**: 767-771.

60. Edashige, Y., et al. 1995. *Structural analysis of polysaccharides of primary walls from xylem differentiating zones of Cryptomeria Japonica D. Don.* Holzforschung, **49**: 197-202.

61. Thomas, J.R., McNeil, M., Darvill, A.G., Albersheim, P. 1987. *Structure of plant cell walls. XIX. Isolation and characterization of wall polysaccharides from suspension cultured Douglas fir cells.* Plant Physiology, **83**: 659-671.

62. Andrew, I. and Little, J. 1997. *A xyloglucan in etiolated seedings of Pinus radiata.* Phytochemistry, **46**: 203-207.

63. Maeda, Y., Awano, T., Takbe, K., Fujita, M. 2000. *Immunolocalization of glucomannans in the cell wall of differentiating tracheids in Chamaecyparis obtusa.* Protoplasma, **231**: 148-156.

64. Baba, K., et al. 1994. *Localization of hemicelluloses in the cell walls of some woody plants using immuno-gold electron microscopy.* Holzforschung, **48**: 297-300.

65. Uprichard, J.M. 1991. *Chemistry of wood and bark*, in *Properties and Uses of New Zealand Radiata Pine*, Kininmonth, J.A., Whitehouse, L.J., Ed. New Zealand Ministry of Forestry, Forest Research Institute: Rotorua. 2-45.

66. Thornber, J.P., Northcote, D.H. 1962. *Changes in the chemical composition of a cambial cell during its differentiation into xylem and phloem in trees. III. Xylan, glucomannan and alpha-cellulose fractions.* Biochemistry Journal, **82**: 340-346.

67. Evtuguin, D., et al. 2003. *Characterization of an acetylated heteroxylan from Eucalyptus globulus Labill.* Carbohydrate Research, **338**: 597-604.

68. Willats, W.G.T., Orfila, C., Limberg, G., Buchholt, H.C., Van Alebeek, G.W.M., Voragen, G.J., Marcus, S.E., Christensen, T.M.I.E., Mikkelsen, J.D., Murray, B.S., Knox, J.P. 2001. *Modulation of the degree and pattern of methyl-esterification pf pectic homogalacturonan in plant cell walls.* Journal of Biological Chemsitry, **276**: 19404-19413.

69. Knox, J.P. 1992. *Cell adhesion, cell separation and plant morphogenesis.* Plant Journal, **2**: 137-141.

70. Perez, S., Mazeau, K., Herve du Penhoat, C. 2000. *The three-dimensional structures of the pectic polysaccharides.* Plant Phyiology and Biochemistry, **38**: 37-55.

71. Fleischer, A., O'Neil, M., and Ehwald, R. 1999. *The pore size of non-graminaceous plant cell walls is rapidly decreased by borate ester cross-linking of the pectic polysaccharide rhamnogalacturonan-II.* Plant Physiology, **121**: 829-38.

72. O'Neil, M.A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A.G., Albersheim, P. 1996. *Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently linked cross-linked by a borate ester.* Journal of Biological Chemsitry, **271**: 22923-22930.

73. Carpita, N. and Gibeaut, D. 1993. *Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth.* The Plant Journal, **3**: 1-30.

74. Edashige, Y. and Ishii, T. 1998. *Rhamnogalacturonan-II from cell walls of Cryptomeria japonica.* Phytochemistry, **49**: 681-690.

75. Edashige, Y. and Ishii, T. 1996. *Pectic polysaccharides from xylem-differentiating zone of Cryptomeria japonica.* Phytochemistry, **42**: 611-616.

76. Shimokawa, T., Ishii, T., Matsunga, T. 1999. *Isolation and structural characterization of rhamnogalacturonan II-borate complex from Pinus densiflora.* Journal of Wood Science, **45**: 435-439.

77. Burke, D., et al. 1974. *The structure of plant cell walls. VI. A survey of the walls of suspension-cultured monocots.* Plant Physiology, **54**: 109-115.

78. Thornber, J.P., Northcote, D.H. 1961. *Changes in the chemical composition of a cambial cell during its differentiation into xylem and phloem tissues in trees. II. Carbohydrate consituents of each main component.* Biochemistry Journal, **81**: 455-464.

79. Dumville, J. and Fry, C. 2000. *Uronic acid-containing oligosaccharins: their biosynthesis, degradation and signalling roles in non-diseased plant tissues.* Plant Phyiology and Biochemistry, **38**: 125-140.

80. Ridley, B.L., O'Neil, M.A., Mohnen, D. 2001. *Pectins: structure, biosynthesis, and oligogalacturonide-related signalling.* Phytochemistry, **57**: 929-967.

81. Komalavilas, P., Mort, A.J. 1989. *The acetylation at O-3 of galacturonic acid in the rhamose-rich portion of pectins.* Carbohydrate Research, **189**: 261-272.

82. Edashige, Y. and Ishii, T. 1997. *Rhamnogalacturonan-I from xylem differentiating zones of Cryptomeria japonica.* Carbohydrate Research, **304**: 357-365.

83. Darvill, A., McNeil, M., and Albersheim, P. 1978. *Structure of plant cell walls.* Plant Physiology, **62**: 418-422.

84. Stevenson, T.T., Darvill, A.G., Albersheim, P. 1988. *Structural features of the plant cell-wall polysaccharide rhamnogalacturonan-II.* Carbohydrate Research, **182**: 207-226.

85. Thomas, J.R., Darvill, A.G., Albersheim, P. 1989. *Isolation and structural characterization of the pectic polysaccharide rhamnogalacturonan-II from walls of suspension cultured rice cells.* Carbohydrate Research, **185**: 261-277.

86. Matoh, T., Takasaki, M., Takabe, K., Kobayashi, M. 1998. *Immunocytochemistry of Rhamnogalacturonan-II in cell walls of higher plants.* Plant and Cell Physiology, **39**: 483-491.

87. Ishii, T., Matsunga, T., Pellerin, P., O'Neil, M.A., Darvill, A., Albersheim, P. 1999. *The plant cell wall polysaccharide rhamnogalacturonan-II self assembles into a covalently cross linked dimer.* The Journal of Biological Chemistry, **274**: 13098-13104.

88. Whitcombe, A.J., O'Neill, M.A., Steffan, W., Albersheim, P., Darvill, A.G. 1995. *Structural characterization of the pectic polysaccharide, rhamnogalacturonan-II.* Carbohydrate Research, **271**: 15-29.

89. Kobayashi, M., Matoh, T., Azuma, J. 1996. *Two chains of rhamnogalacturonan-II are cross-linked by borate-diol ester bonds in higher plant cell walls.* Plant Physiology, **110**: 1017-1020.

90. O'Neil, M.A., Ishii, T., Albersheim, P., Darvill, A.G. 2004. *Rhamnogalacturonan-II: structure and function of a borate cross-linked cell wall pectic polysaccharide.* Annual Review of Plant Biology, **55**: 109-139.

91. Ishii, T., Matsunga, T. 1996. *Isolation and characterization of a boron-rhamnogalacturonan-II complex from cell walls of sugar beet pulp.* Carbohydrate Research, **284**: 1-9.

92. Ishii, T., Matsunga, T. 2001. *Pectic polysaccharide rhamnogalacturonan-II is covalently linked to homogalacturonan.* Phytochemistry, **57**: 969-974.

93. Ishii, T., Matsunga, T. 1996. *Isolation and characterization of a boron-rhamnogalacturonan-II complex from cell walls of sugar beet pulp.* Carbohydrate Research, **284**: 1-9.

94. Matoh, T., Ishigaki, K., Ohno, K., Azuma, J. 1993. *Isolation and characterization of a boron-polysaccharide complex from radish roots.* Plant and Cell Physiology, **37**: 636-40.

95. Kaneko, S., Ishii, T., Matsunga, T. 1997. *A boron-rhamnogalacturonan-II complex from bamboo shoot cell walls.* Phytochemistry, **44**: 243-248.

96. Vincken, J.P., Schols, H.A., Oomen, R.J.F.J., McCann, M.C., Ulvskov, P., Voragen, A.G.J., Visser, R.G.F. 2003. *If homogalacturonan were a side chain of rhamnogalacturonan-I. Implications for cell wall architecture.* Plant Physiology, **132**: 1781-1789.

97. Ishii, T., Matsunga, T. 2001. *Rhamnogalacturonan-II is covalently cross-linked to homogalacturonan.* Phytochemistry, **57**: 969-674.

98. Andrew, I., et al. 2000. *Pectic arabinans of Pinus radiata*. in *American Society of Plant Biologists 'Plant Biology Meeting'*. San Diego, California.

99. Guillon, F., Thibault, J.F. 1989. *Methylation analysis and mild acid hydrolysis of the "hairy" fragments of sugar-beet pectins.* Carbohydrate Research, **190**: 85-96.

100. Kieliszewski, M., de Zacks, R., Leykam, J.F., Lamport, D.T.A. 1992. *A repetitive proline-rich protein from the gymnosperm douglas fir is a hydroxyproline-rich glycoprotein.* Plant Physiology, **98**: 919-926.

101. Keller, B. 1993. *Structural cell wall proteins.* Plant Physiology, **101**: 1127-1130.

102. Svoboda, A. 2003. *Cross-talk between the cell wall and cytoplasm.* Journal of Applied Biomedicine, **1**: 1-5.

103. Cassab, G. 1998. *Plant cell wall proteins.* Annual Review of Plant Biology, **49**: 281-309.

104. Showalter, A.M. 1993. *Structure and function of plant cell wall proteins.* The Plant Cell, **5**: 9-23.

105. Sommer-Knudsen, J., Bacic, A., Clarke, A.E. 1998. *Hydroxyproline-rich plant glycoproteins.* Phytochemistry, **47**: 483-497.

106. Fong, C., et al. 1992. *A gymnosperm extensin contains the serine - tetrahydroxyproline motif.* Plant Physiology, **99**: 548-552.

107. Bao, W., O'Malley, D., and Sederoff, R. 1992. *Wood contains a cell-wall structural protein.* Proceedings from the National Academy of Sciences, **89**: 6604-6608.

108. Gane, A., et al. 1995. *Structural analysis of the carbohydrate moiety of arabinogalactan-proteins from stigmas and styles of Nicotiana alta.* Carbohydrate Research, **277**: 67-85.

109. Motose, H., Sugiyama, M., Fukuda, H. 2004. *A proteoglycan mediates inductive interaction during plant vascular development.* Nature, **429**: 873-878.

110. Loopstra, C., Jeffrey, D., Puryear, E. 2000. *Purification and cloning of an arabinogalactan protein from xylem of loblolly pine.* Planta, **210**: 686-689.

111. Fry, S. 1986. *Cross-linking of matrix polymers in the growing cell walls of angiosperms.* Annual Review of Plant Physiology, **37**: 165-186.

112. Fry, S. 1995. *Polysaccharide-modifying enzymes in the plant cell wall.* Annual Review of Plant Physiology and Plant Molecular Biology, **46**: 497-520.

113. Walton, J. 1994. *Deconstructing the cell wall.* Plant Physiology, **104**: 1113-1118.

114. Kohorn, B.D. 2000. *Plasma membrane-cell wall contacts.* Plant Physiology, **124**: 31-38.

115. Nairn, C.J., Haselkorn, T. 2005. *Three loblolly pine CesA genes expressed in developing xylem are orthologous to seconday cell wall CesA genes of angiosperms.* New Phytologist, **166**: 907-915.

116. Hutchison, K.W., Singer, P.B., McInnis, S., Diaz-Sala, C., Greenwood, M.S. 1999. *Expansins are conserved in conifers and expressed in hypocotyls in response to exogenous auxin.* Plant Physiology, **120**: 827-831.

117. Goldberg, R., Morvan, C., Jauneau, A., Jarvis, M.C. 1996. *Methyl-esterfication, de-esterfication and gelation of pectins in the primary cell wall*, in *Pectins and Pectinases*, Visser, J., Voragen, A.G.J., Ed. Elsevier Science: Amsterdam. 151-172.

118. Catesson, A. 1994. *Cambial ultra-structure and biochemistry: changes in relation to vascular tissue differentiation and the seasonal cycle.* International Journal of Plant Science, **155**: 251-261.

119. Keegstra, K., Talmadge, K.W., Bauer, W.D., Albersheim, P. 1973. *The structure of plant cell walls. III. A model of the walls of suspension-cultured sycamore cells based on the interconncetions of the macromolecular components.* Plant Physiology, **51**: 188-196.

120. Cosgrove, D. 2001. *Wall structure and wall loosening. A look backwards and forwards.* Plant Physiology, **125**: 131-134.

121. Popper, Z.A., Fry, S.C. 2005. *Widespread occurrence of a covalent linkage between xyloglucan and acidic polysaccharides in suspension-cultured angiosperm cells.* Annals of Botany, **96**: 91-99.

122. Bootten, T., et al. 2004. *Solid-state ^{13}C-NMR spectroscopy shows that the xyloglucans in the primary cell walls of mung bean (Vigna radiata L.) occur in different domains: a new model for xyloglucan-cellulose interactions in the cell wall.* Journal of Experimental Botany, **55**: 571-583.

123. Brett, C., Baydoun, E., and Ebdel-Massih, R. 2005. *Pectin-xyloglucan linkages in type I primary cell walls of plants.* Plant Biosystems, **139**: 54-59.

124. Brandstrom, J., et al. 2003. *The structural organisation of the S1 cell wall layer of Norway spruce tracheids.* International Association of Wood Anatomists Journal, **24**: 27-40.

125. Wardrop, A.B. 1981. *Lignification and xylogenesis*, in *Xylem Cell Development*, Barnett, J.R., Ed. Castle House Publications Ltd.: Kent. 115-151.

126. Harris, P.J. 2005. *Non-cellulosic polysaccharides in plant cell walls: their diversity, organization and approaches to understanding their functions*, in *The Hemicellulose Workshop*, Entwistle, K.M., Walker, J.C.F., Ed. Wood Technology Research Centre: Christchurch. 13-35.

127. Lapierre, C., Pollet, B., Ralet, M., Saulnier, L. 2001. *The phenolic fraction of maize bran: evidence for lignin-heteroxylan assoication.* Phytochemistry, **57**: 765-772.

128. Stacey, N.J., Roberts, K., Carpita, N.C., Wells, B., McCann, M.C. 1995. *Dynamic changes in cell surface molecules are very early events in the differentiation of mesophyll cells from Zinnia elegans into tracheary elements.* The Plant Journal, **8**: 891-906.

129. Halpin, C., Abbott, J., Barakate, A. 2000. *Investigating lignin biosynthesis using transgenic mutant plants*, in *Cell and Molecular Biology of Wood Formation*, Savidge, R.A., Barnett, J.R., Napier, A., Ed. BIOS Scientific Publishers Ltd.: Oxford. 425-436.

130. Baucher, M., et al. 1996. *Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar.* Plant Physiology, **112**: 1479-1490.

131. Hatfield, R., Vermerris, W. 2001. *Lignin formation in plants. The Dilemma of Linkage specificity.* Plant Physiology, **126**: 1351-1357.

132. Lewis, D.H. 1980. *Boron, lignification and the origin of vascular plants - a unified hypothesis.* New Phytology, **84**: 209-229.

133. Fengel, D. and Wegener, G. 1983. *Lignification of the wood cell wall*, in *Wood: Chemistry, Ultrastructure, Reactions*, Fengel, D. and Wegener, G., Eds. Walter de Gruyter & Co.: Berlin. 133-141.

134. Downes, G. and Turvey, N. 1986. *Reduced lignification in Pinus radiata D. Don.* Australian Journal of Forest Research, **16**: 371-377.

135. Hatfield, R., Fukushima, R.S. 2005. *Can lignin be accurately measured?* Crop Science, **45**: 832-839.

136. Roberts, K., McCann, M. 2000. *Xylogenesis: the birth of a corpse.* Current Opinion Plant Biology, **3**: 517-522.

137. Groover, A., DeWitt, N., Heidel, A., Jones, A. 1997. *Programmed cell death of plant tracheary elements differentiating in vitro.* Protoplasma, **196**: 197-211.

138. Harris, P.J. 2005. *Diversity in plant cell walls*, in *Plant Diversity and Evolution: Genotypic and Phenotypic Variation in Higher Plants*, Henry, R.J., Ed. CAB International: Cambridge.

139. Allona, I., et al. 1998. *Analysis of xylem formation in pine by cDNA sequencing.* Proceedings from the National Academy of Sciences, **95**: 9693-9698.

140. Sterky, F., Regan, S., Karlsson, J., Hertzberg, M., Rohde, A., Holmberg, A., Amini, B., Bhalerao, R., Larsson, M., Villarroel, R., Van Montagu, M., Sandberg, G., Olsson, O., Teeri, T., Boerjan, W., Gustafsson, P., Uhlen, M., Sundberg, B., Lundeberg, J. 1998. *Gene discovery in the wood-forming tissues of poplar: analysis of 5,692 expressed sequence tags.* Proceedings from the National Academy of Sciences, **95**: 13330-13335.

141. Hetzberg, M., Apeborg, H., Schrader, J., Andersson, A., Erlansson, R., Blomqvist, K., Bhalerao, R., Uhlen, M., Teeri, T., Lundeberg, J., Sunberg, B., Nilsson, P., Sandberg, G. 2001. *A transcriptional roadmap to wood formation.* Proceedings from the National Academy of Sciences, **98**: 14732-14737.

142. Lapierre, C., Pilate, G., Pollet, B., Mila, I., Leple, J.C., Jouanin, L., Kim, H., Ralf, J. 2004. *Signatures of cinnamyl alcohol dehydrogenase deficiency in poplar lignins.* Phytochemistry, **65**: 313-321.

143. Zhang, Y., Sederoff, R.R., Allona, I. 2000. *Differential expression of genes encoding cell wall proteins in vascular tissues from vertical and bent loblolly pine trees.* Tree Physiology, **20**: 457-466.

144. Plomion, C., Pionneau, C., Brach, J., Costa, P., Bailleres, H. 2000. *Compression wood-responsive proteins in developing xylem of maritime pine (Pinus pinaster Ait.).* Plant Physiology, **123**: 959-969.

145. Vander Mijnsbrugge, K., Meyermans, H., Van Montagu, M., Bauw, G., Boerjan, W. 2000. *Wood formation in poplar: identification, characterization, and seasonal variation of xylem proteins.* Planta, **210**: 589-598.

146. Chaffey, N., et al. 2002. *Secondary xylem development in Arabidopsis: a model for wood formation.* Physiologia Plantarum, **114**: 594-600.

147. Initiative, T.A.G. 2000. *Analysis of the genome sequence of the flowering plant Arabidopsis thaliana.* Nature, **408**: 796-815.

148. Turner, S.R., Somerville, C.R. 1997. *Collapsed xylem phenotype of arabidopsis identifies mutants deficient in cellulose deposition in the secondary cell wall.* The Plant Cell, **9**: 689-701.

149. Turner, S.R., Taylor, N., Hall, M., Jones, L. 2000. *The use of Arabidopsis mutants to study secondary cell wall formation*, in *Cell & Molecular Biology of Wood Formation*, Savidge, R., Barnett, J., Napier, R., Ed. BIOS Scientific Publishers Ltd.: Oxford. 483-491.

150. Arioli, T., et al. 1998. *Molecular analysis of cellulose biosynthesis in Arabidopsis.* Science, **279**: 717-720.

151. Hepworth, D.G., Vincent, J.F.V. 1998. *The mechanical properties of xylem tissue from Tobacco plants (Nicotiana tabacum 'Samsun').* Annals of Botany, **81**: 751-759.

152. Haplin, C., Knight, M.E., Foxon, G.A., Campbell, M.M., Boudet, A.M., Boon, J.J., Chabbert, B., Tollier, M., Schuch, W. 1994. *Manipulation of lignin quality by downregulation of cinnamyl alcohol dehydrogenase.* The Plant Journal, **6**: 339-350.

153. Mekkerowicz, E.J., Baucher, M., Sundberg, B., Boerjan, W. 2001. *Unravelling cell wall formation in the woody dicot stem.* Plant Molecular Biology, **47**: 239-274.

154. Bradshaw, H., et al. 2000. *Emerging model systems in plant biology. Poplar (Populus) as a model forest tree.* Journal of Plant Growth Regulators, **19**: 306-313.

155. McCann, M.C., Stacey, N.J., Dimitra, D., Milioni, D., Sado, P.E., Roberts, K. 2001. *Zinnia. Everybody needs good neighbours.* Plant Physiology, **127**: 1380-1382.

156. McCann, M.C., Domingo, C., Stacey, N.J., Milioni, D., Roberts, K. 2000. *Tracheary element formation in an in vitro system*, in *Cell & Molecular Biology of Wood Formation*, Savidge, R., Barnett, J., Napier, R., Ed. Scientific Publishers Ltd.: Oxford.

157. Motose, H., Sugiyama, M., Fukuda, H. 2001. *An arabinogalactan protein(s) is a key component of a fraction that mediates local intercellular communication involved in tracheary element differentiation of Zinnia mesophyll cells.* Plant and Cell Physiology, **42**: 129-137.

158. Roberts, A.W., Donocan, S.G., Haigler, C.H. 1997. *A secreted factor induces cell expansion and formation of metaxylem-like tracheary elements in xylogenic suspension cultures of Zinnia.* Plant Physiology, **115**: 683-692.

159. Fukuda, H. 1996. *Xylogenesis: Initiation, progression, and cell death.* Annual Review of Plant Physiology and Plant Molecular Biology, **47**: 299-325.

160. Taylor, J.G., Owen, T.P., Koonce, L.T., Haigler, C. 1992. *Dispersed lignin in tracheary elements treated with cellulose synthesis inhibitors provides evidence that molecules of the secondary cell wall mediate wall patterning.* The Plant Journal, **2**: 959-970.

161. Suzuki, K., Ingold, E., Sugiyama, M., Komamine, A. 1991. *Xylan synthase activity in isolated mesophyll cells of Zinnia elegans during differentiation to tracheary elemets.* Plant and Cell Physiology, **32**: 303-306.

162. Chaffey, N. 1999. *Cambium: old challenges-new opportunities.* Trees, **13**: 138-151.

163. Moeller, R., McDonald, AG, Walter, C, Harris, PJ 2003. *Cell differentiation, secondary cell-wall formation and transformation of Pinus radiata D. Don.* Planta, **217**: 736-747.

164. Leitch, M.A., Savidge, R.A. 2000. *Cell, tissue and organ culture for the study of cambial activity and wood formation-a resurgence of interest in an old technique*, in *Cell and Molecular Biology of Wood Formation*, Savidge, R.A., Barnett, J.R., Napier, R., Ed. BIOS Scientific Publishers Ltd.: Oxford. 493-512.

165. Savidge, R.A. 1993. *In vitro wood formation in 'chips' from merchantable stem regions of Larix laricina.* International Association of Wood Anatomists, **14**: 3-11.

166. Leitch, M.A., Savidge, R.A. 1995. *Evidence for auxin regulation of bordered-pit positioning during tracheid differentiation in Larix laricina.* International Association of Wood Anatomists, **16**: 289-297.

167. Leitch, M.A. 1999. *The development of tissue culture techniques to study wood formation in Eucalyptus globulus Labill*. PhD Thesis, *Institute of Land and Food Resources*, University of Melbourne.

168. Gartner, B., Hagman, O., Dyck, B. 2004. *Wood quality initiative science review*. Wood Quality Initiative Ltd. p. 1-24.

169. Hu, H., Brown, P.H. 1994. *Localization of boron in cell walls of squash and tobacco and its association with pectin.* Plant Physiology, **105**: 681-689.

170. Brown, P. and Hu, H. 1994. *Boron uptake by sunflower, squash and cultured tobacco cells.* Physiologia Plantarum, **91**: 435-441.

171. Skok, J., McIlrath, W.J. 1958. *Distribution of boron in cells of dicotyledonous plants in relation to growth.* Plant Physiology, **33**: 428-431.

172. Raven, J.A. 1980. *Short- and long- distance transport of boric acid in plants.* New Phytology, **84**: 231-249.

173. Takano, J., Noguchi, K., Yasumor, M., Kobayashi, M., Gajados, Z., Miwa, K., Hayahi, H., Yoneyama, T., Fujiwara, T. 2002. *Arabidopsis boron transporter for xylem loading.* Nature, **420**: 337-340.

174. Frommer, W. and von Wiren, N. 2002. *Ping-pong with boron.* Nature, **410**: 282-283.

175. Shelp, B.J., Marentes, E., Kitheka, A.M., Vivekanandan, P. 1995. *Boron mobility in plants.* Physiologia Plantarum, **94**: 356-361.

176. Dordas, C. and Brown, P. 2000. *Permeability of boric acid across lipid bilayers and factors affecting it.* Journal of Membrane Biology, **175**: 95-105.

177. Tanaka, H. 2002. *Boron absorption by excised sunflower root.* Soil Science and Plant Nutrition, **13**: 77-82.

178. Wimmer, M.A., Muhling, K.H., Lauchli, A., Brown, P.H., Goldbach, H.E. 2002. *Boron toxicity: the importance of soluble boron*, in *Boron in Plant and Animal Nutririon*, Goldbach, H.E., Rerkasem, M.A., Wimmer, P.H, Brown, R.W., Ed. Kluwer Academic Publishers: New York. 241-253.

179. Wimmer, M.A., Muhling, K.H., Lauchli, A., Brown, P.H., Goldbach, H.E. 2003. *The interaction between salinity and boron toxicity affects subcellular distribution of ions and proteins in wheat leaves.* Plant, Cell and Environment, **26**: 1267-1274.

180. Matoh, T. 1997. *Boron in plant cell walls.* Plant and Soil, **193**: 59-70.

181. Kobayashi, M., Nakagawa, H, Asaka, T, Matoh, T 1999. *Borate-rhamnogalacturonan II binding reinforced by Ca^{2+} retains pectic polysaccharides in higher plant cell walls.* Plant Physiology, **119**: 199-203.

182. Kobayashi, M., Ohno, K., Matoh, T. 1997. *Boron nutrition of cultured tobacco BY-2 cells. II. Characterization of the boron-polysaccharide complex.* Plant Cell Physiology, **38**: 676-683.

183. Ishii, T., Matsunga, T., Hayashi, N. 2001. *Formation of rhamnogalacturonan-II-borate dimer in pectin determines cell wall thickness in pumpkin tissue.* Plant Physiology, **126**: 1698-1705.

184. Baker, D. 1983. *Uptake of cations and their transport within the plant.*, in *Metals and Micronutrients: Uptake and Utilization by Plants*, Robb, D., Ed. Academic Press: London. 1-19.

185. Clarkson, D. and Hanson, J. 1980. *The mineral nutrition of higher plants.* Annual Review of Plant Physiology, **31**: 239-98.

186. Pellerin, P., Doco, T., Vidal, S., Williams, P., Brillouet, J., O'Neil, M. 1996. *Structural characterization of red wine rhamnogalacturonan-II.* Carbohydrate Research, **290**: 183-197.

187. Kobayashi, M., Nakagawa, H., Asaka, T., Matoh, T. 1999. *Borate-rhamnogalacturonan-II binding reinforced by Ca^{2+} retains pectic polysaccharides in higer plant cell walls.* Plant Physiology, **119**: 199-203.

188. Teasdale, R.D., Richards, D.K. 1990. *Boron deficiency in cultured pine cells.* Plant Physiology, **93**: 1071-1077.

189. Wingender, Q.Y., Schulz, M., Baluska, F., Goldbach, H.E. 2001. *Short-term boron deprivation induces increased levels of cytoskeletal proteins in Arabidopsis roots.* Plant Biology, **3**: 335-340.

190. Showalter, A.M. 1989. *Plant hydroxyproline rich proteins*, in *The Biochemistry of Plants*, Marcus, A., Ed. Academic Press: New York. 485-536.

191. Willats, W., Knox, J. 1996. *A role for arabinogalactan proteins in plant cell expansion: evidence from studies on the interaction of β-glucosyl Yariv reagent with seedlings of Arabidopsis thaliana.* Plant Journal, **9**: 919-925.

192. Egertsdotter, U. and von Arnold, S. 1995. *Importance of arabinogalactan proteins for the development of somatic embryos of Norway spruce (Picea abies).* Physiologia Plantarum, **93**: 334-345.

193. Knox, J., Linstead, P., Peart, J., Cooper, C., Roberts, K. 1991. *Developmentally regulated epitopes of cell surface arabinogalactan proteins and their relation to root tissue patter formation.* Plant Journal, **1**: 317-326.

194. Gao, M., Showalter, A. 1999. *Yariv reagent treatment induces programmed cell death in arabidopsis cell cultures and implicates arabinogalactan protein involvement.* Plant Journal, **19**: 231-331.

Chapter Two

Ultra-structural and biochemical features of intra-ring checking

2.1 Introduction

As was introduced in chapter one, intra-ring checking is a wood quality flaw that is detrimental to the New Zealand forest industry. While the incidence of intra-ring checking is becoming increasingly recognized in New Zealand, our understanding of the nature of this wood quality flaw is limited. Investigations of the ultra-structural and biochemical nature of wood fractures are, therefore, critical to our understanding of the initiation and propagation of intra-ring checking [1-5]. Such studies, have the potential to provide insight, not only into the details of the morphology of trees susceptible to intra-ring checking, but also into the role that other factors may play in the development of intra-ring checking. This knowledge is likely to be an important first step towards enhancing the value of current radiata pine stands, in addition to improving the quality of future radiata pine resources [6].

This chapter reviews the current literature on intra-ring checking in New Zealand radiata pine, and examines the ultra-structural and biochemical characteristics of oven-dried radiata pine discs with intra-ring checks.

2.2 What is intra-ring checking?

Intra-ring checking was introduced in chapter one (section 1.1.3) and is a relatively new term that has been used to describe a sporadic phenomenon in southern hemisphere plantation species, particularly radiata pine [4]. Intra-ring checking has only been acknowledged as a significant wood quality flaw in radiata pine in the last decade [4, 7]. Fractures similar to radiata pine intra-ring checks have previously been described in the juvenile wood of *Pinus elliottii* [8], and Douglas fir [9]. Fractures similar to intra-ring checking have also been described in hardwoods such as eucalyptus [10, 11] and white spruce [12]. The location, description, development and

factors associated with intra-ring checking in radiata pine will be described in the succeeding sub-sections.

2.2.1 Location of intra-ring checking in radiata pine

Intra-ring checking initiates in, and spans the earlywood portion of, a growth ring and rarely extends into the latewood (figure 2.1) [2, 4]. These checks occur at right angles to the orientation of the growth ring, and are most prevalent just outside of the heartwood [2, 4, 13].

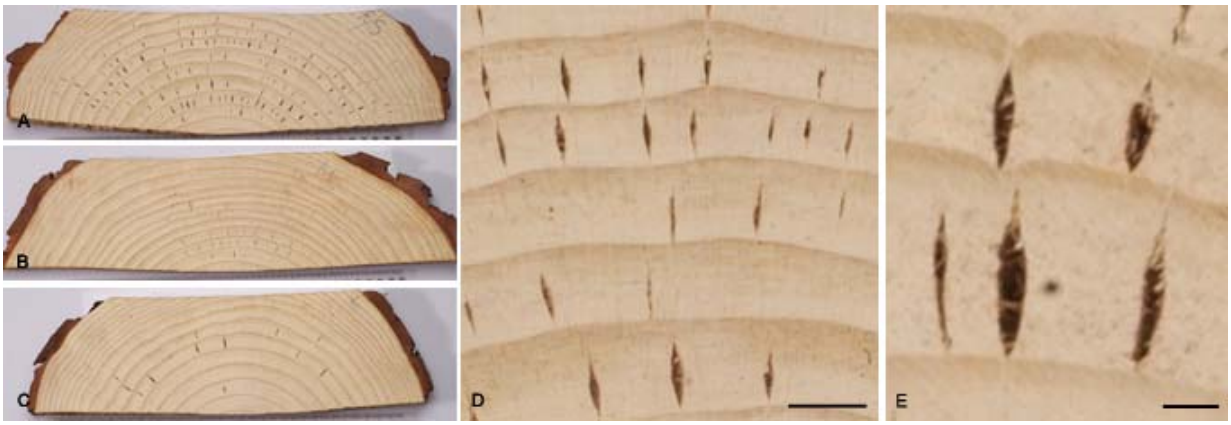


Figure 2.1 Cross-sectional images of oven-dried radiata pine discs provided by WQI Ltd. (A) Severely checked, (B) moderately checked, and (C) low checked samples. Check formation is confined to an annual growth ring (D-E). Scale bar for D = 1 cm, E = 5 mm.

By definition, there are three types of cell wall failure in wood: *inter*-cell failure, which occurs, for example, in the middle lamella through separation of adjacent cells; *intra*-wall failure, which, for example, describes the failure of the secondary wall, generally at the S_1/S_2 interface; and *trans*-wall failure, which is a breakage across the entire cell wall, including all of the layers [1]. Studies using light microscopy and scanning electron microscopy have shown that intra-ring checking is the result of *inter*-cell wall failure, at the level of the CML between adjacent radial cell files [2, 14-16]. The intra-ring check propagates along the boundary of these radial cell files, and occasionally the propagation has been shown to cross to a neighbouring radial cell file [2, 14-16].

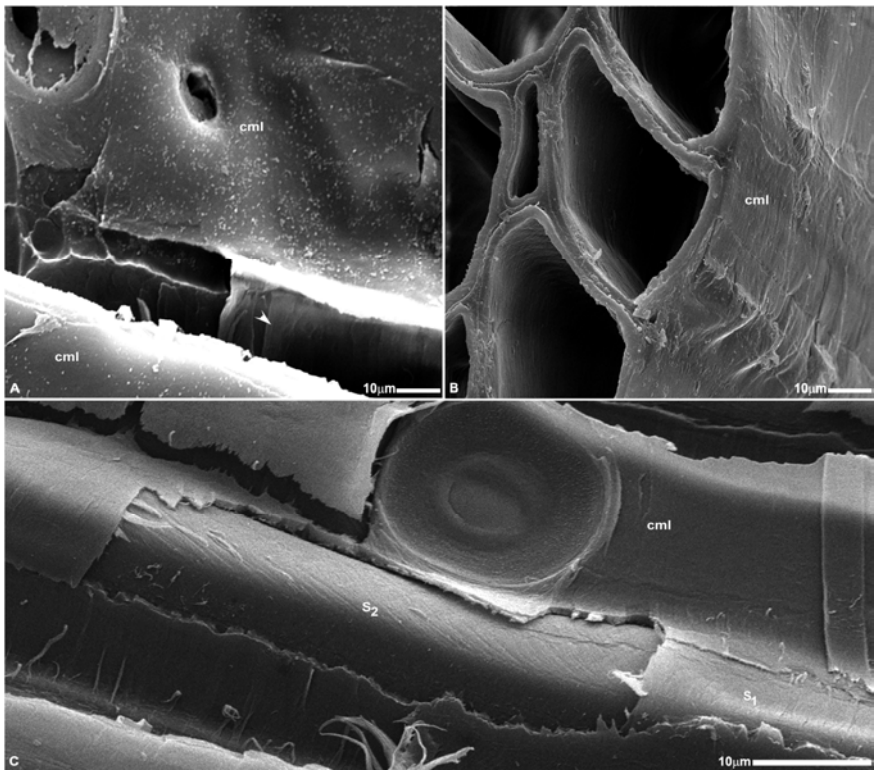


Figure 2.2 Scanning electron micrograph images of the radial face (A, C), or the transverse face (B) of intra-ring checks in radiata pine. The presence of a smooth fracture surface suggests that the CML is the predominant wall layer involved in intra-ring checking. Image provided by H. Nair.

In previous studies, the orientation of the cellulose microfibrils, and the lamellar nature of the cell wall were used to determine which cell wall layers were exposed during the shearing of the wall, as a tear formed [14, 16]. In all of the samples the middle lamella had a smooth surface, due to its lack of microfibrils, while the primary cell wall displayed a random orientation of microfibrils (figure 2.2) [14, 16]. All of the components of the secondary wall (S_1, S_2 and S_3) displayed highly ordered microfibrils typical of conifers as reviewed in chapter one (section 1.3.3.1) [14]. The fracture surface on 80% of the samples analyzed was found to contain 3/5 CML, and 2/5 of the S_1 cell wall layer [2, 14, 16]. These results were corroborated by the existance of a smooth fracture face surface, suggesting the presence of CML [2, 14]. In the remaining 20% of the samples, intra-ring checking occurred within the CML [2, 14]. The S_2 cell wall layer was rarely exposed, and the S_3 layer was never exposed in the intra-ring checks examined, indicating that intra-ring checking primarily occurs at the interface between the CML and S_1 wall layers [2, 14]. This is consistent with previous descriptions of *inter*-cell wall failure in wood [1, 4]. These results are also in

agreement with the work by Donaldson (1995) [17], where 67% of the fractures observed occurred at the CML and/or S_1 cell wall layers [17].

2.2.2 Development of intra-ring checking

Intra-ring checking appears to occur when cells with insufficient wall strength collapse as a result of their inability to withstand the tension forces associated with wood drying [4, 18]. The development of differential water tension between the earlywood and latewood of a growth ring is believed to initiate the split, as the denser latewood is more resistant to tangential movement, resulting in radial rupture of the earlywood, and collapse of adjacent cells (figure 2.3) [4, 5]. A collapse is indicative of shrinkage, but does not always result in intra-ring checking, as Californian redwood and western red cedar, which frequently collapse, rarely show intra-ring checking [4]. Moreover, shrinkage is not believed to be involved in the instigation of intra-ring checking that begins during oven drying, before the wood reaches an average moisture content of 80%, a point at which the wood is still saturated [4].

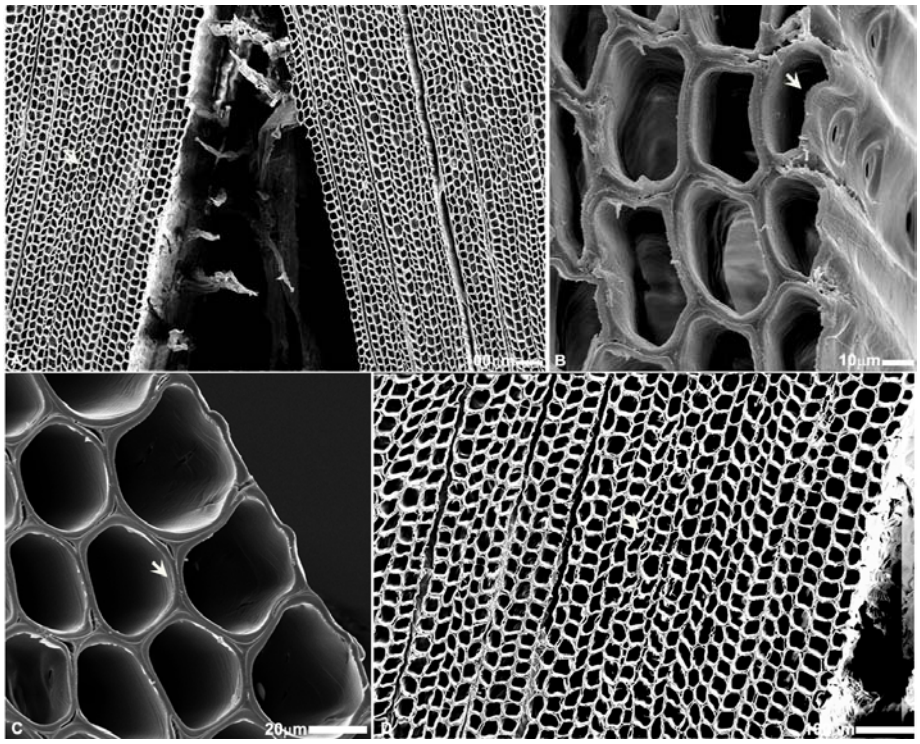


Figure 2.3 Scanning electron micrograph images of collapsed tracheids in checked radiata pine. Collapsed tracheids adjacent to the crack (A, B). Some of the cells adjacent to the crack were not collapsed (C). Moreover, much of the tracheid collapse that was observed was distant from the crack (D). Image provided by H. Nair.

2.2.3 Cell dimensions of intra-ring checked wood

Radiata pine tracheids are characteristically longer in the radial dimension than in the tangential dimension [17, 19, 20]. Previous studies have shown that the lumen of earlywood tracheids in the samples displaying severe intra-ring checking was 21% longer in the radial dimension, and 4% wider in the tangential dimension, compared to those that did not check [15]. This meant that severe intra-ring checking tracheids had a 21% larger cross-sectional lumen area, suggesting that increased cell size may be related to intra-ring checking [15]. However, tracheid size alone cannot account for the incidence of intra-ring checking, as the lumens of the latewood tracheids measured were also longer in the radial dimension [15]. It was suggested that the thicker cell walls in latewood tracheids may compensate for the longer radial length, preventing intra-ring checking in latewood [15].

2.2.4 Cell wall thickness of intra-ring checked wood

Earlier studies also showed that the radial cell walls of the intra-ring checking samples were 7% thinner than those in the non-checking samples [15], while, the tangential walls were only 1% thinner in the intra-ring checking samples [15]. Since thicker cell walls are generally considered stronger, thin walls in intra-ring checked wood corroborates previous suggestions that a weak radial cell wall is involved in intra-ring checking [4, 15, 18].

2.2.5 Lignin distribution in intra-ring checked wood

Low levels of lignin have been shown to be associated with intra-ring checking [15]. Previous studies report a normal distribution of lignin in the middle lamella of the cells, however, the secondary walls appeared to have less lignin [15]. Other studies have also found less lignin in the secondary walls of radiata pine showing intra-ring checking [4 and references therein]. These reports suggest that the amount of lignin present in the secondary walls may be another factor associated with the incidence of intra-ring checking.

2.2.6 Factors associated with intra-ring checking

The unpredictability of intra-ring checking is a serious economic concern [4]. For instance, it has been reported that between 40 and 70% of radiata pines studied from four different sites showed checking [4], while other reports suggest that the incidence

is below 5% [13]. Moreover, the underlying cause of intra-ring checking is difficult to determine, as there are many factors associated with it. The most consistent factor, inheritability, has emerged from replicated plantation trials using different radiata pine families [13, 21]. This suggests that genetic selection and breeding against intra-ring checking may be a favourable resolution [13]. However, it does not provide a solution for current radiata stands, nor does it enhance our understanding of the flaw [13]. Other factors include: water stress on drying, species, genotype, site, nutrient availability, silviculture, climate, wood density, moisture content, cell dimensions, microfibril angle, lignification and processing procedures [1, 4, 13, 15, 21, 22]. The fast growth of radiata pine is also believed to be associated with intra-ring checking: not as a direct cause, but rather as a contributing factor [4]. As such, there are many different factors associated with the incidence of intra-ring checking that may act on their own, or with other factors, to induce this phenomenon. Of particular importance when considering all of these variables, is the suggestion that external conditions that promote intra-ring checking must occur both during wood formation, to influence wood structure, and after wood formation, to cause tangential tension, and ultimately the check itself [4].

2.3 Ultra-structural characteristics of intra-ring checking

In the current study, experiments utilizing transmission electron microscopy (TEM; sections 2.3.1, 2.3.2, 2.3.3) were undertaken to further examine the morphology and ultra-structure of the cells in thirteen oven-dried radiata pine discs demonstrating a range of intra-ring checking[1]. These samples were sub-divided into severe, moderate, low and non-checking groups based on the frequency of the visible intra-ring checks. The central earlywood portion of growth ring seven was used for all of the experiments to minimize the variation that occurs between growth rings.

[1] Studies on the oven-dried discs were part of a collaborative project between H. Nair and myself (Jackson et al. 2003; Jackson et al. 2004; Putoczki et al. 2006). The work completed by Hema Nair was presented in section 2.2 and is referenced in the text. The work that I have contributed is presented in sections 2.3 and 2.4.

2.3.1 Cell wall organization

2.3.1.1 Transmission electron microscopy

TEM is a powerful technique for observing the ultra-structure of wood because of the significantly increased resolution compared to light microscopes [23]. Caution must be used when interpreting the resulting images, as chemical fixation distorts the original form of the cell [24]. Fixation is generally slow, because of the need of the fixatives to pass through the cell wall [24]. Long periods of dehydration are also required, as the permeability of the cell wall is reduced and residual water is difficult to extract [24]. However, coupled with other microscopy techniques, TEM can undoubtedly provide valuable information on cell ultra-structure, including wall organization [23].

As discussed in section 2.2.1, intra-ring checking generally happens when failure of the CML, or CML/S_1 cell wall interface occurs (figure 2.2). In order to determine if the ultra-structure of these regions was altered in wood with intra-ring checking, samples from the earlywood portion of growth ring seven were embedded in Spurr resin, ultra-thin sections were obtained, mounted on copper grids, stained with uranyl acetate and Sato's lead and observed in duplicate on a Hitachi H-600 TEM at 75 kV.

Uranyl acetate and Sato's lead

Uranyl acetate is a negative stain used in electron microscopy, after which Sato's lead staining is often used to create a strong contrast in the image [23, 25]. The samples showing intra-ring checking that were stained with uranyl acetate and Sato's lead appeared to have changes in the frequency of the S_1 wall layer striations, compared to the other samples (figure 2.4A). These striations appeared as darker staining patches in the same orientation as the cellulose microfibrils in this layer. Moreover, an increase in the predominance of these striations appeared to occur, as the incidence of checking decreased. This suggests alterations in the composition of this cell wall layer, possibly including microfibril presence and organization, and lignin distribution, as these are the major components of this wall layer (section 1.3.3). The TEM observations, and fracture location observed in the scanning electron micrograph images (figure 2.2) both suggest that there are differences in the composition and organization of the CML/S_1 wall layer of samples showing intra-ring

checks, compared to the samples that do not. As such, two of the major structural components of these wall layers, pectin and lignin, were investigated further.

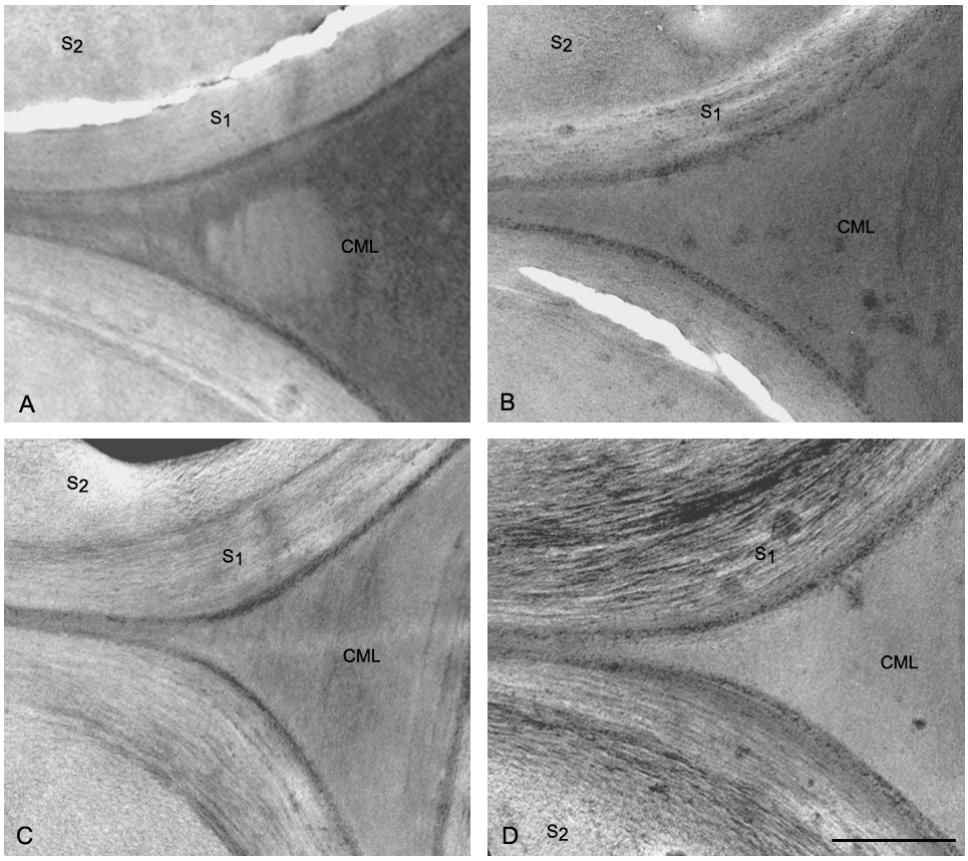


Figure 2.4 Transverse sections of radiata pine samples with varying degrees of intra-ring checking, embedded in Spurr resin, stained with uranyl acetate and Sato's lead, and observed on a Hitachi H-600 TEM at 75 kV. (A) Severe checking, (B) moderate checking, (C) low checking and (D) non-checking samples. Intra-ring checking samples appear to have an altered CML/S_1 cell wall region, lacking in striations compared to the non-checking samples. Scale bar = 1 μm.

2.3.2 Pectin distribution

In chapter one (section 1.3.2.5) the location of pectic polysaccharides in the CML, and their potential involvement in cell adhesion was discussed. Recent research suggests that pectin may play a role in stabilizing and strengthening the cell wall through interactions with other molecules [26], which, if altered could be an underlying contributor to the incidence of intra-ring checking. Observations of pectin distribution in the region of the wall where intra-ring checks occur was performed to determine if the distribution of pectin is altered in wood with intra-ring checks.

2.3.2.1 Transmission electron microscopy

In order to determine if the distribution of pectin was altered in wood showing intra-ring checks, each of the samples was extracted with methylamine to remove lignin, embedded in Spurr resin, and ultra-thin sections were collected and mounted on copper grids, stained with ruthenium red, and observed in duplicate on a Hitachi H-600 TEM at 75 kV. The intensity of the staining was determined using Image Pro Plus software (Media Cybernetics Inc., Silverspring, USA).

Ruthenium red

Ruthenium red is an anionic dye that is thought to stain pectin through a reaction with carboxyl groups [27], and is commonly used to observe pectin using TEM techniques [28]. Ruthenium red was used as a qualitative indication of pectin localization in the samples.

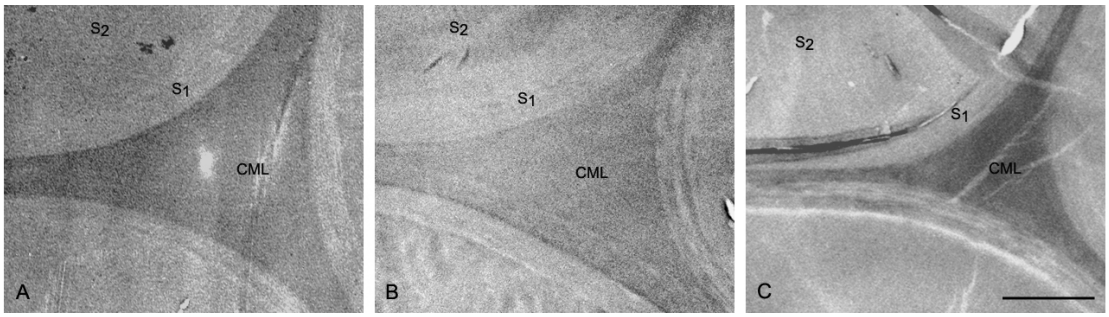


Figure 2.5 Transverse sections of radiata pine samples with varying degrees of intra-ring checking, embedded in Spurr resin, stained with ruthenium red, and observed on a Hitachi H-600 TEM at 75 kV. (A) Severe checking, (B) low checking, (C) non-checking. There was no indication of altered pectin distribution. Scale bar = 2 µm.

Pectin staining was strongest in the CML for all of the samples, which is where pectin is known to be located (section 1.3.2.5) [29]. The results were determined to be inconclusive with respect to the amount of pectin in the cells, as no consistent trends in the intensity of the staining in this region were observed (figure 2.5). Furthermore, quantification of staining intensity did not yield significant results ($P>0.05$). This suggests that this method may be unreliable for the determination of pectin concentrations. One possible explanation for this could be differential penetration of ruthenium red into the cells. For this reason, the samples showing moderate intra-ring checking were not examined by this technique. However, the method did allow

identification of the spatial distribution of pectin, which appeared similar in all of the samples. This finding agreed with concurrent studies in this laboratory using ruthenium red coupled to light microscopy, where no changes in the distribution or concentration of pectin was found in the cells [15].

2.3.3 Lignin distribution

As discussed in section 2.2.5, the distribution of lignin in wood with intra-ring checks has previously been shown to be altered using histochemical techniques [15]. In order to corroborate these results at the ultra-structural level, lignin was observed using TEM techniques.

2.3.3.1 Transmission electron microscopy

In order to observe the distribution of lignin at the ultra-structural level, samples from growth ring seven of each disc were embedded in Spurr resin, ultra-thin sections were obtained and mounted on copper grids, stained with potassium permanganate, and observed in duplicate on a Hitachi H-600 TEM at 75 kV. The intensity of the staining was determined using Image Pro Plus software (Media Cybernetics Inc., Silverspring, USA).

Potassium permanganate

Upon staining with potassium permanganate, the manganese is reduced to manganese dioxide in the wood, in a reaction that is believed to include the oxidation of coniferyl aldehyde groups [27, 30, 31]. Carboyhdrates are not believed to stain appreciably [32]. This method has been used in conjunction with electron microscopy in other studies of radiata pine lignin organization [3, 17, 33, 34], black spruce [32] and beech wood [31]. However, interpretation of the results must acknowledge that the extent to which potassium permanganate can penetrate the cell wall, and to what extent there are manganese dioxide deposits following the initial reduction of potassium permanganate, are unknown [27, 30]. In addition, while electron microscopy is routinely used to study lignin, the high lignin content can make obtaining detailed information about the basic structure of the cell wall difficult to interpret [35]. The images obtained were thus used as a further qualitative observation of lignin distribution.

In all samples, the lignin content appeared highest in the CML, while some of the severely checked samples appeared to have a lower concentration of lignin, represented by the intensity of the staining in this region ($P<0.05$). The S_1 wall region appeared to have the greatest altered lignin distribution, as the severe checking samples had fewer striations (figure 2.6A) when compared to the other sample groups (figure 2.6 B and C). This appeared as strips of both high and low lignin content that appeared to follow the orientation of the cellulose microfibrils giving a streaked appearance to the lignin in the S_1 wall layer. This staining pattern suggested irregular lignification of the S_1 wall. However, this was also observed in some of the unchecked samples, meaning that it is not associated solely with intra-ring checking. A gradual reduction in lignin concentration from the CML to the S_2 layer, with the S_1 layer having up to 50% less staining has been previously described in fracture prone radiata pine, observed by potassium permanganate staining and TEM [17].

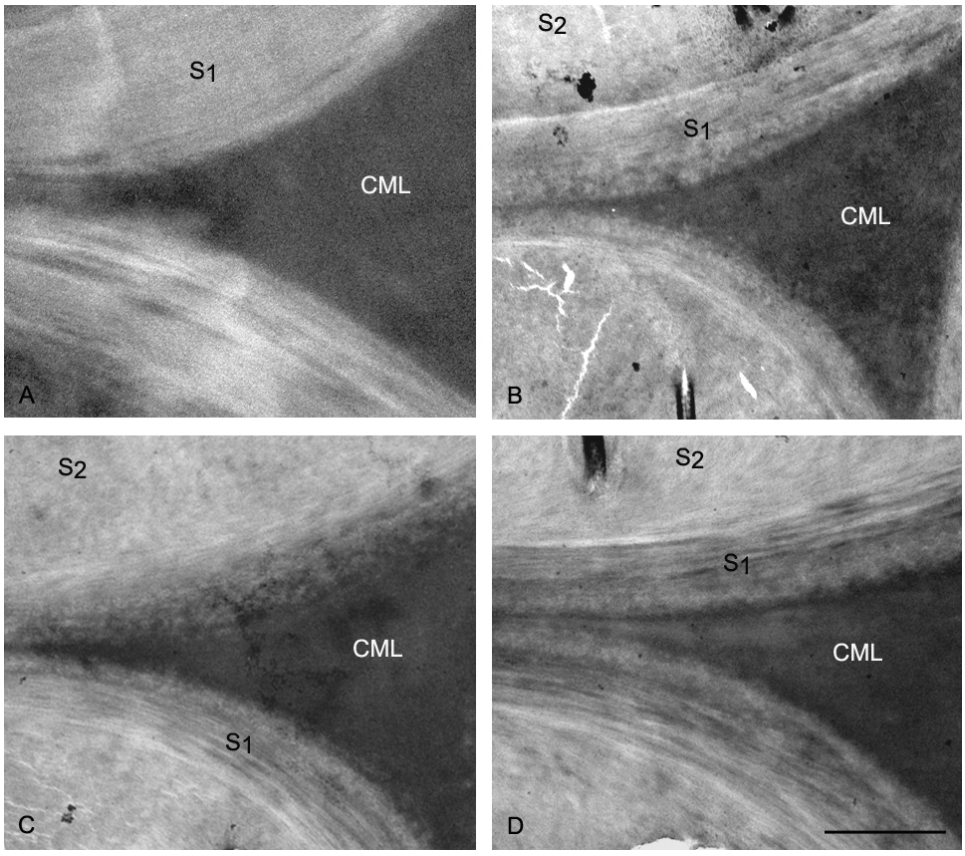


Figure 2.6 Transverse sections of radiata pine samples with varying degrees of intra-ring checking, embedded in Spurr resin, stained with potassium permanganate, and observed on a Hitachi H-600 TEM at 75 kV. (A) Severe checking, (B) moderate checking, (C) low checking and (D) non-checking samples. Intra-ring checking samples appear to have altered lignification at the CML/ S_1 cell wall region. Scale bar = 1 µm.

The TEM observations, and previous histochemical observations made in this laboratory [15] both suggest an association between altered/low lignin levels and intra-ring checking. Since lignin provides additional strength to the cell wall, altered lignification may result in weak points susceptible to breakage [2]. It has been proposed that the abrupt change in lignification from the CML to the S_1 wall layer could make the walls susceptible to breakage [17].

2.4 Biochemical analysis of intra-ring checking

In order to support the qualitative potassium permanganate TEM observations presented in section 2.3.3, biochemical analysis was performed on the samples to determine their lignin content. In addition, biochemical methods were used to determine the mineral nutrient content of the samples, as this is another factor that is believed to be associated with intra-ring checking, presumably through interactions with pectin, as introduced in chapter one (section 1.6).

2.4.1 Lignin content

The lignin content of the earlywood portion of growth ring seven in each of the discs was determined by Klason lignin (section 2.4.1.1) and acetyl bromide (section 2.4.1.2) assays. The Klason lignin content was determined by Veritec, SCION (Rotorua, New Zealand) and the acetyl bromide lignin content was calculated as described in chapter nine (section 9.2.3.4).

2.4.1.1 Klason lignin

The Klason method is classically used to determine the lignin content of wood [36]. The Klason method determines acid insoluble and acid soluble lignin from which the total lignin content is calculated. Briefly, acid hydrolysis promotes carbohydrate hydrolysis, and the resulting isolated lignin is termed acid insoluble [37].

The values obtained for the acid insoluble lignin (table 2.1) are in close agreement with the values previously reported for pine (28.8 % (w/w)) earlywood samples [37]. There were no statistically significant differences in the acid-insoluble, acid-soluble or total lignin contents between the four sample groups (table 2.1; $P > 0.05$). These results suggest that intra-ring checking trees do not have drastically reduced lignin contents when compared to those without intra-ring checks.

2.4.1.2 Acetyl bromide

While the Klason method is classically used to determine the lignin content of wood, it requires large amounts of sample and long analysis times, whereas, the acetyl bromide lignin assay is a rapid and sensitive method for quantifying lignin [36, 38]. The acetyl bromide assay is dependent on determining the absorbance of the cell wall material, in which all of the lignin of a sample has been dissolved [38-40].

The values obtained using the acetyl bromide assay (table 2.1) were similar to those reported previously for radiata pine (26.3 to 30% (w/w)) [37, 41]. The published values may be slightly higher due to latewood contamination, which has high lignin content, as it is not known if these samples contained earlywood alone. There were no statistically significant differences in the lignin content determined by the acetyl bromide method between the four sample groups analyzed in duplicate (table 2.1; $P > 0.05$). These results do not further corroborate the suggestion that intra-ring checking trees have drastically reduced lignin content [2, 15].

Sample group	Acid insoluble lignin[a]	Acid soluble lignin[a]	Total lignin[a]	ABL[b]
Severe	28.6 ± 1.02	0.70 ± 0.02	29.3 ± 1.0	22.9 ± 1.11
Moderate	28.7 ± 0.66	0.72 ± 0.04	29.4 ± 0.68	22.4 ± 0.79
Low	29 ± 0.19	0.74 ± 0.02	29.7 ± 0.17	27.6 ± 2.24
None	29.1 ± 0.78	0.68 ± 0.03	29.8 ± 0.80	23.7 ± 1.66

[a] Values determined by the Klason method.
[b] Average of duplicate determination.

Table 2.1 Klason and acetyl bromide lignin (ABL) content of ground wood material from severe, moderate, low and non-checking radiata pine oven-dried discs. Values represent percent lignin (w/w). Data are ± standard error of the mean for three samples from each of the severe, moderate and low checking sample groups, and four samples from the non-checking sample group.

While the *total* lignin content does not appear to be changed in the intra-ring checking samples, these values are not representative of the distribution of lignin in the individual cell walls, nor do they provide the composition of the lignin in the individual cell walls. This suggests that although the distribution of lignin in check-prone wood can alter (2.3.3), the total lignin content has not changed.

2.4.2 Mineral nutrient content

In chapter one (section 1.6.1), the ability of boron to cross link pectic polysaccharides was introduced [42]. Based on this observation, it has been proposed that boron deficiency could hinder the interaction of boron with RG-II, leading to changes in the structure of the CML that could be involved in the incidence of intra-ring checking [4, 18, 43]. In order to investigate this possibility further, the amount of boron, calcium and magnesium present in samples from growth ring seven was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) and mass spectrometry (ICP-MS), which were performed by Hill Laboratories, Hamilton, New Zealand.

2.4.2.1 Inductively coupled plasma optical emission and mass spectrometry

ICP-OES is a commonly used analytical technique for the determination of major and trace elements [44]. Plasma (any type of matter that contains electrons, positive ions, and neutral atoms, usually from argon gas) is used to subject the sample to temperatures high enough to cause dissociation into atoms, which, once in their excited states, can decay to lower states *via* thermal or radiative (emission) energy transmissions [44]. The intensity of the light emitted at wavelengths specific to the elements of interest is used to determine the concentrations of these elements by comparison with multi-element calibration samples. [44].

ICP-MS is a similar analytical technique, more suited to the analysis of trace elements than ICP-OES [44] and is suggested as the best method to determine total boron concentrations [45]. In this technique, the argon plasma generates singly charged ions from the elemental species within a sample [44]. When coupled to a mass spectrometry detector, this allows the isotopes of the elements present to be separated according to their mass-to-charge ratio [44]. Both ICP techniques require that samples be presented to the instruments in the form of acid digests and that the calibration standards are prepared in the same acid matrix as the unknown samples.

There were no definitive trends within the oven-dried disc samples present that could link the incidence of intra-ring checking directly to boron, calcium and magnesium concentrations (table 2.2; $P > 0.05$). This is consistent with the results of site studies

by Beets et al. (2004) [43], where no nutrient was found to respond in a consistent way to the occurrence of intra-ring checking.

Sample group	Boron[a] (mg/kg)	Calcium[b] (mg/kg)	Magnesium[b] (mg/kg)
Severe	1.6 ± 0.12	489 ± 33.5	165 ±15.8
Moderate	1.5 ± 0.15	542 ± 19.5	130 ± 11.8
Low	1.8 ± 0.03	592 ± 21.6	140 ± 12.7
None	1.5 ± 0.03	541 ± 26.1	158 ± 9.5

[a] Boron was determined using ICP-MS
[b] Calcium and magnesium were determined using ICP-OES

Table 2.2 ICP-OES/MS determination of the levels of calcium, magnesium and boron in the intra-ring checking, and non-checking samples. Data are ± standard error of the mean for three samples from each of the severe, moderate and low checking sample groups, and four samples from the non-checking sample group.

In field trials performed by Beets et al. (2004) [43] a slight increase in boron concentration was observed when intra-ring checking was present. Moreover, Beets et al. (2004) [43] found that the levels of calcium and magnesium decreased as the incidence of intra-ring checking increased. Further analysis, on a larger number of samples would be required to corroborate this trend. However, since no initial trends were observed using these crude total measurements, this line of investigation was abandoned, allowing attention to be focused on experiments employing organ culture techniques. The role these nutrients play in intra-ring checking, if any, was not discernable from bulk concentration measurements in these experiments.

2.5 Summary

The literature describing the location, incidence and factors associated with intra-ring checking in New Zealand has been surveyed. In addition, the literature describing the appearance of intra-ring checking has been reviewed.

Ultra-structural analysis of samples showing intra-ring checking using various TEM techniques revealed relatively consistent changes in the CML, and particularly in the S_1 cell wall layers. This was observed in the form of decreased staining intensity in

the CML, or altered striations in the CML/ S_1 walls, which corroborates the notion that this is a weak point in the cell wall, and is consistent with the localization of the fractures discussed in section 2.2.1.

Biochemical analysis revealed that there were no statistically significant differences in the lignin content or boron, calcium and magnesium content of the samples. Examining the relationship between nutrition and wood quality is difficult because of environmental variations, both in time (i.e. seasons) and space (i.e. site variability), which is further compounded by unknown levels of genetic variation within a trial or stand of trees [43]. The site that these samples were collected from, and the genetic variation between them, is not known. As such, it is difficult to define conclusive results from the data obtained. Future experiments, had samples of known origins been provided, would have involved a larger sample size and a comparison of a complete disc with one half 'green', and the other oven dried in order to determine what changes have occurred in response to oven drying. Furthermore, while the total amount of boron, calcium and magnesium was not statistically different, this does not preclude a role in intra-ring checking. Boron, calcium and magnesium can interact with pectin, and components of lignin [27, 46-48], the distribution of which may be altered in the wood, irrespective of their overall concentrations.

While this investigation has provided insight into potential differences in the architecture of the CML and S_1 wall layers in wood susceptible to intra-ring checking, it has not determined the underlying cause of intra-ring checking. Additional funding and more samples were not provided by WQI Ltd. to pursue these interests further. The results that have been presented are preliminary. A larger sample size would need to be examined in order to confirm them. To circumvent the problems associated with bulk measurement in a system whose properties are likely to be determined by subtle differences in spatial and temporal distribution, the remainder of this thesis will focus on the development of methods to successfully analyse a manipulated organ culture technique, to gain insight into possible contributors to intra-ring checking.

2.6 References

1. Cote, W.A., Hanna, R.B. 1983. *Ultrastructural characteristics of wood fracture surfaces.* Wood and Fiber Science, **15**: 135-163.

2. Jackson, S., Nair, H., Putoczki, T. 2004. *Ultrastructural indicators of intra-ring checking*, in *Confidential Report*, APP 34. Young, G., Editor. Wood Quality Initiative Ltd. p. 1-36.

3. Donaldson, L.A. 1997. *Ultrastructure of transwall fracture surfaces in radiata pine using transmission electron microscopy and digital image processing.* Holzforschung, **51**: 303-308.

4. Cown, D., McConchie, D., McConchie, M. 2003. *Intra-ring internal checking in radiata pine (P. radiata D. Don) in New Zealand*, in *Confidential Report*, APP 1. Wood Quality Initiative Ltd. p. 1-21.

5. Pang, S., Orchard, R., McConchie, D.L. 1999. *Tangential shrinkage of Pinus radiata earlywood and latewood, and its implication for intra-ring internal checking.* New Zealand Journal of Forestry Science, **29**: 484-491.

6. Gartner, B., Hagman, O., Dyck, B. 2004. *Wood quality initiative science review*, in *Confidential report*. Wood Quality Initiative Ltd. p. 1-24.

7. Harris, M.J. 1991. *Structure of wood and bark*, in *Properties and Uses of New Zealand Radiata Pine*, Kininmonth, J.A., Whitehouse, L.J., Ed. New Zealand Ministry of Forestry, Forest Research Institute: Rotorua.

8. van Der Syde, H.A. 1976. *Resin-infiltrated heart shakes in Pinus elliottii grown in the Transvaal.* South African Forestry Journal, **97**: 29-34.

9. Polge, H. 1984. *Nouvelles observations sur les fissures radiales du douglas.* Revue Forestiere Francaise, **6**: 453-458.

10. Ilic, J., Hillis, W.E. 1986. *Prediction of collapse in dried eucalpt wood.* Holzforschung, **40**: 109-112.

11. Chafe, S.C. 1995. *Preheating and continuous and intermittent drying in boards of Eucalyptus regnans F. Muell.* Holzforschung, **49**: 227-233.

12. Lutz, H.J. 1952. *Occurrence of clefts in the wood of living white spruce in Alaska.* Journal of Forestry, **50**: 99-102.

13. Kumar, S., Jefferson, P., Carson, M. 2004. *Variation of intra-ring checking within-and-among sites*, in *Confidential report*, APP 17. Young, G., Editor. Wood Quality Initiative Ltd. p. 1-21.

14. Nair, H. 2006. *A study of intra-ring checking and xylogenesis in Pinus radiata D. Don*. PhD Thesis, *School of Biological Sciences*, University of Canterbury.

15. Jackson, S., Nair, H. 2003. *Radial fibre collapse potential and cell wall lignin as indicators of intra-ring checking in radiata pine*, in *Confidential Report*, APP 10. Young, G., Editor. Wood Quality Initiative Ltd.

16. Putoczki, T., Nair, H., Butterfield, B., Jackson, S. 2006. *Relationship between the occurrence of cell wall fracture, cell collapse and variations in lignin distribution in Pinus radiata.* Tree Physiology: submitted.

17. Donaldson, L.A. 1995. *Cell wall fracture properties in relation to lignin distribution and cell dimensions among three genetic groups of radiata pine.* Wood Science and Technology, **29**: 51-63.

18. Xue, J., Olykan, S., Beets, P., McConchie, M., Graham, D., Leckie, A., Kimberley, M., Pearce, S. 2004. *Effect of boron, genotype and site on tree growth, intra-ring checking and other wood properties of radiata pine*, in *Confidential Report*, APP 38. Young, G., Editor. Wood Quality Initiative Ltd. p. 1-19.

19. Nyakuengama, J.G., Evans, R., Matheson, C., Spencer, D., Vinden, P. 1999. *Wood quality and quantitative genetics of Pinus radiata D. Don: fibre traits and wood density.* Appita Journal, **52**: 348-357.

20. Shelbourne, T., Evans, R., Kibbelwhite, P., Charles, L. 1997. *Inheritance of tracheid transverse dimensions and wood density in radiata pine.* Appita Journal, **50**: 47-50.

21. Beets, P., McConchie, D., McConchie, M., Kimberley, M., Pearce, S., Oliver, G. 2003. *Family and clonal variation in intra-ring checking and resin characteristics and relationship with foliar nutrients*, in *Confidential Report*, APP 9. Young, G., Editor. Wood Quality Initiative Ltd. p. 1-20.

22. Petterson, R.W. 1983. *Prediction of fracture toughness of conifers.* Wood and Fiber Science, **15**: 302-316.

23. Chaffey, N.J. 2002. *Conventional (chemical fixation) transmission electron microscopy and cytochemistry of angiosperm trees*, in *Wood Formation in Trees: Cell and Molecular Biology Techniques*, Chaffey, N.J., Ed. Taylor and Francis: London. 41-64.

24. Rensing, K.H. 2002. *Chemical and cryo-fixation for transmission electron microscopy of gymnosperm cambial cells*, in *Wood Formation in Trees: Cell and Molecular Biology Techniques*, Chaffey, N.J., Ed. Taylor and Francis: London. 65-81.

25. Sato, T. 1968. *A modified method for lead staining of thin sections.* Journal of Electron Microscopy, **17**: 158-159.

26. Kobayashi, M., Nakagawa, H., Asaka, T., Matoh, T. 1999. *Borate-rhamnogalacturonan II binding reinforced by Ca^{2+} retains pectic polysaccharides in higher plant cell walls.* Plant Physiology, **119**: 199-203.

27. Wardrop, A.B. 1981. *Lignification and xylogenesis*, in *Xylem Cell Development*, Barnett, J.R., Ed. Castle House Publications Ltd.: Kent. 115-151.

28. Micheli, F., Ermel, F., Bordenave, M., Richard, L., Goldberg, R. 2002. *Cell walls of wood tissues: cytochemical, biochemical and molecular analysis of pectins and pectin methylesterases*, in *Wood Formation in Trees: Cell and Molecular Biology Techniques*, Chaffey, N.J., Ed. Taylor and Francis: London. 179-200.

29. Willats, W.G.T., McCartney, L., Mackie, W., Knox, P. 2001. *Pectin: cell biology and prospects for functional analysis.* Plant Molecular Biology, **47**: 9-27.

30. Hayat, M.A. 1975. *Positive Staining for Electron Microscopy*. New York: Van Nostrand Reinhold Co.

31. Fromm, J., Rockel, B., Lautner, S., Windeisen, E., Wanner, G. 2003. *Lignin distribution in wood cell walls determined by TEM and backscattered SEM techniques.* Journal of Structural Biology, **143**: 77-84.

32. Kerr, A.J., Goring, D.A.I. 1975. *The ultrastructural arrangement of the wood cell wall.* Cellulose Chemistry and Technology, **9**: 563-573.

33. Donaldson, L.A. 2001. *Lignification and lignin topochemistry-an ultrastructural view.* Phytochemistry, **57**: 859-873.

34. Singh, A., Daniel, G., Nilsson, T. 2002. *Ultrastructure of the S_2 layer in relation to lignin distribution in Pinus radiata tracheids.* Journal of Wood Science, **48**: 95-98.

35. Hafren, J., Fujino, T., Itoh, T., Westermark, U., Terashima, N. 2000. *Ultrastructural changes in the compound middle lamella of Pinus thunbergii during lignification and lignin removal.* Holzforschung, **54**: 234-240.

36. Iiyama, K., Wallis, A.F.A. 1988. *An improved acetyl bromide procedure for determining lignin in woods and woody pulps.* Wood Science and Technology, **22**: 271-280.

37. Dence, C.M. 1992. *The determination of lignin*, in *Methods in Lignin Chemistry*, Timell, T.E., Ed. Springer and Verlag: Berlin. 33-61.

38. Hatfield, R.D., Grabber, J., Ralph, J., Brei, K. 1999. *Using the acetyl bromide assay to determine lignin concentrations in herbaceous plants: some cautionary notes.* Journal of Agriculture and Food Chemistry, **47**: 628-632.

39. Hatfield, R., Fukushima, R.S. 2005. *Can lignin be accurately measured?* Crop Science, **45**: 832-839.

40. Johnson, D.B., Moore, W.E., Zank, L.C. 1961. *The spectrophotometric determination of lignin in small wood samples.* Tappi, **44**: 793-798.

41. Downes, G., Turvey, N.D. 1986. *Reduced lignification in Pinus radiata D. Don.* Australian Journal of Forest Research, **16**: 371-7.

42. O'Neil, M.A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A.G., Albersheim, P. 1996. *Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently linked cross-linked by a borate ester.* Journal of Biological Chemsitry, **271**: 22923-22930.

43. Beets, P., Kimberley, M., McConchie, M., Pearce, S. 2004. *Aluminum interactions and intra-ring checking*, in *Confidential Report*, APP 26. Young, G., Editor. Wood Quality Initiative Ltd. p. 1-24.

44. Boss, C.B., Fredeen, K.J. 1997. *Concepts, instrumentation, and techniques in inductively coupled plasma, optical emission spectrometry*. Second Edition ed. USA: Perkin Elmer Corporation.

45. Thellier, M., Chevallier, A., His, I., Jarvis, M., Lovell, M.A., Martini, F., Ripoll, C., Robertson, D., Sauerwein, W., Verdus, M.C. 2002. *Specific methods for boron studies in plants*, in *Boron in Plant and Animal Nutrition*, Goldbach, H.E., Rerkasem, M.A., Wimmer, P.H., Brown, R.W., Ed. Kluwer Academic: New York.

46. Teasdale, R.D., Richards, D.K. 1990. *Boron deficiency in cultured pine cells.* Plant Physiology, **93**: 1071-1077.

47. Dugger, W.M. 1983. *Boron in plant metabolism*, in *Inorganic Plant Nutrition*, Lauchi, A., Ed. 627-650.

48. Ishii, T., Matsunga, T, Hiyash, N 2001. *Formation of rhamnogalacturonan II-borate dimer in pectin determines cell wall thickness in pumpkin tissue.* Plant Physiology, **126**: 1698-705.

Chapter Three

Organ culture technique

3.1 Introduction

The proposed investigation of the affects of altered boron, calcium and magnesium nutrition on radiata pine wood formation required the establishment and manipulation of an organ culture technique that could model *in arbor* xylogenesis. This culture system was employed to observe wood formation in a regulated environment, where specific changes to the media could result in a controlled response, in the absence of interfering signals from the whole tree.

This chapter compares the original organ culture technique [1], and a recently modified organ culture technique [2], to that established and manipulated for the purpose of this research.

3.2 Manipulation of an organ culture technique

Savidge (1993) used a defined nutrient medium containing naphthalene acetic acid (NAA) as the only plant hormone to grow tamarack (*Larix laricina*) organ cultures [1]. These cultures successfully underwent periclinal divisions to produce radial cell files containing lignified secondary walls. Similar culture methods have been used to study bordered pit formation in tamarack [3], and to manipulate eucalyptus (*Eucalyptus globulus*) organ cultures to examine xylogenic responses in the presence of an array of hormones [2]. The organ culture technique discussed in this chapter is a modified method based on the work by Savidge (1993) and Leitch (1999), to examine the efficacy of organ cultures to study the affect of nutritional variables on wood formation in radiata pine.

3.2.1 Composition of the culture media

The organ culture media used in this study contained nutrients, micronutrients and plant growth regulators typically used in plant tissue culture [4, 5]. These included

major elements, such as C, H, O and N; minor elements, including P, K, S and Mg; vitamins and hormones; and trace elements [5, 6]. The media composition was originally devised by Allen et al. (1988) [7], modified by Savidge (1993) [1] and further modified for this work. Only one growth hormone, naphthalene acetic acid (NAA) was included in the media [1, 7]. NAA was previously identified as a suitable substitute for indole acetic acid (IAA), which is a commonly used synthetic auxin in plant culture, because NAA will remain stable under *in vitro* conditions [2, 3, 8].

3.2.1.1 Function of media components

Media component	Principal form	Proposed function(s)
Carbon	CO_2	biomass generation
Oxygen	H_2O or O_2	biomass generation
Hydrogen	H_2O	biomass generation
Nitrogen	NO_3^- or NH_4^+	proteins, amino acids, nucleic acids
Potassium	K^+	protein synthesis, enzyme activation
Calcium	Ca^{2+}	enzyme activation, cell permeability, cell wall development
Phosphorus	$H_2PO_4^-$ or HPO_4^{2-}	sugar utilization, cell division
Magnesium	Mg^{2+}	enzyme activation
Sulfur	SO_4^{2-}	amino acids, enzyme activation,
Iron	Fe^{2+} or Fe^{3+}	proteins in electron transfer
Chlorine	Cl^-	osmosis
Copper	Cu^{2+}	enzyme cofactor
Manganese	Mn^{2+}	enzyme activation
Boron	H_3BO_3	influences calcium, cell wall development
Cobalt (II) Chloride	$CoCl_2.6H_2O$	enzyme activation
Myo-inositol	$C_9H_{12}O_6$	sugar transport, carbohydrate metabolism
Nicotinic Acid	$C_6H_5NO_2$	generated reducing agents
Pyridoxine	$C_8H_{11}NO_3.HCl$	vitamin, catalytic functions
Thiamine	$C_{12}H_{17}ClN_4OS•HCl$	vitamin, catalytic functions
Sucrose	$C_{12}H_{22}O_{11}$	meristem development
Water	H_2O	essential for cell
NAA	$C_{12}H_{10}O_2$	cell division, enlargement and elongation

Table 3.1 Summary of common plant culture media components and their suggested function(s) [1, 2, 5, 9-12].

The function of many components of plant media are not well understood [9]. Moreover, much of the available information is from non-woody plant species [9]. In this study, the role of boron, calcium and magnesium in wood formation were of primary interest, and the concentrations of these nutrients added to the culture media were selectively modified (section 3.2.1.2). Table 3.1 briefly summarizes a selection of the typical plant media components and some of their postulated roles in plant growth.

3.2.1.2 Comparison of the media components to other organ culture media

Selection of suitable culture media is generally done on an arbitrary basis due to the complex nature of culture media, the need for nutrient concentrations to fall between deficiency and toxicity levels, and the need to understand the interactive chemical and biochemical effects of the nutrients [9]. If culture growth on a medium is successful, the medium is then optimized [9]. In this work, a culture medium known to support xylem formation in other wood species was chosen and modified to support xylem formation in radiata pine. Table 3.2 presents a comparison of the media components used by Savidge (1993), Leitch (1999) and those used in this study.

Media components	Tamarack[a] (mM)	Eucalyptus[b] (mM)	Radiata pine (mM)
KNO_3	4.70	4.70	4.70
NH_4NO_3	5.15	5.15	5.15
$CaCl_2.2H_2O$	0.75	0.75	0.75[c]
$MgSO_4.7H_2O$	0.38	0.38	0.38[d]
KH_2PO_4	0.32	0.32	0.32
Na_2EDTA	0.025	0.025	0.025
$MnSO_4.4H_2O$	0.026	0.026	0.026

[a] Savidge (1993); [b] Leitch (1999)
[c] standard calcium concentration; [d] standard magnesium concentration; [e] standard boron concentration
* synthetic growth regulator; ** amino acid involved in the formation of ethylene
n/a, not included

Table 3.2 Comparison of media composition for growth of tamarack, eucalyptus and radiata pine organ cultures. Continued on following page.

Media components	Tamarack[a] (mM)	Eucalyptus[b] (mM)	Radiata pine (mM)
H_3BO_3	0.016	0.016	0.016[e]
KI	0.0012	0.0012	0.0012
Na_2MoO_4	0.0003	0.0003	0.0003
$CuSO_4.5H_2O$	0.0002	0.0002	0.0002
$CoCl_2.6H_2O$	0.00025	0.00025	0.00025
$FeSO_4.7H_2O$	0.025	n/a	0.025
Myo-inositol	0.139	0.139	0.139
Nicotinic acid	0.001	0.001	0.001
Pyridoxine HCl	0.0006	0.0006	0.0006
Thiamine HCl	0.0004	0.0004	0.0004
Glycine	0.0067	0.0067	0.0067
D-Glucose	201.8	201.8	n/a
Sucrose	n/a	n/a	58.4
IAA*	n/a	0.0028-0.0143	n/a
NAA*	0.0027	0.0-0.0537	0.0268
GA_3*	n/a	0.0014-0.0072	n/a
2,4-D*	n/a	0.0005-0.0226	n/a
L-methionine**	n/a	0.0007-0.0067	n/a
GA_4*	n/a	0.003	n/a
BAP*	n/a	0.0022	n/a
pH	5.8	5.8	5.8-7.0
Gelling agent	Bacto-agar	Phytagel	Bacto-agar

[a] Savidge (1993); [b] Leitch (1999)
[c] standard calcium concentration; [d] standard magnesium concentration; [e] standard boron concentration
* synthetic growth regulator; ** amino acid involved in the formation of ethylene
n/a, not included

Table 3.2 contd. Comparison of media composition for growth of tamarack, eucalyptus and radiata pine organ cultures. Continued from previous page.

One difference between the media composition used by Savidge (1993) [1], and Leitch (1999) [2], and the modified media used in this laboratory was the addition of sucrose, in place of glucose, at half the concentration. In previous trials in this laboratory, radiata organ cultures did not grow in the presence of glucose, however,

they did in the presence of sucrose (S. Welsh, pers. comm.). For this reason, sucrose was used in the present study. Boric acid, exists in solution as the uncharged H_3BO_3 species, which when converted to borate, is known to interact with cis-diols (section 1.6.1) present on many sugars [9]. However, sucrose is not believed to react appreciably with boric acid [13]. If the borate ion reacts with sucrose, it should pass through cellular membranes as an ionized sugar-borate complex until the cell liberates the borate ion [14].

Another important modification to the culture media used in this study, was the increase in auxin supplied to the cultures. Auxin, in the form of IAA, has been shown to regulate xylogenesis and increase cambial division in a concentration dependent manner [15-18]. Previous studies in this laboratory on the affect of auxin (in the form of NAA) on xylogenesis in the organ culture system found that a concentration of 0.03 mM auxin could induce cell division, without compromising cell enlargement and lignification [19]. As a result of this study, 0.03 mM was the standard NAA concentration used in this work. Other hormones were not added to the culture media, as auxin, giberellin, ethylene and cytokinin were investigated by other member of the laboratory.

Most plant culture media are poorly buffered, and traditionally are adjusted to a pH of around 5.5 [20]. The calcium, magnesium and mixed culture media were adjusted to an initial pH of 5.8, similar to the culture media in the work by Savidge (1993) and Leitch (1999). However, in the boron culture studies, the pH was adjusted to 7.0 (section 3.3.1).

3.2.1.3 Modified media mineral nutrient concentrations

Six concentrations of boron, in the form of boric acid; five concentrations of calcium, in the form of calcium chloride; and six concentrations of magnesium, in the form of magnesium sulfate were compared (table 3.3). In addition, eight media containing a mixture of added boron, calcium and magnesium concentrations were compared (table 3.3). These modified concentrations were substituted for the standard concentrations of these nutrients presented in table 3.2, and added into the final culture media. Unless otherwise stated, the media contained standard concentrations of the nutrients.

Concentration of nutrient added to the media	Predicted growth response
Boron	
0 μM, 1 μM	too little
7 μM, 25 μM	enough
100 μM, 1000 μM	toxic levels
Calcium	
0 mM	too little
1 mM, 5 mM	enough
10 mM, 100 mM	toxic levels
Magnesium	
0 mM, 0.5 mM	too little
1 mM, 5 mM	enough
10 mM, 100 mM	toxic levels
Mixed	
1000 μM boron, 100 mM calcium and magnesium	toxic levels
0 μM boron, 0 mM calcium and magnesium	too little
1000 μM boron, 0 mM calcium	culture death
0 μM boron, 100 mM calcium	culture death
100 mM calcium, 0 mM magnesium	culture death
0 mM calcium, 100 mM magnesium	culture death
0 μM boron, 5 mM calcium and magnesium	too little boron
7 μM boron, 5 mM calcium and magnesium	optimum

Table 3.3 Media components selected for the culture manipulation. The concentrations presented were chosen to cover a range of concentrations encompassing the values used in the standard media, and the literature.

These concentrations were chosen based around the amount of boron, calcium, and magnesium used in the standard culture media, and those reported in literature [21-24]. In the standard culture medium, boron was added at a concentration of 16 μM, calcium was added at a concentration of 0.75 mM, and magnesium was added at a concentration of 0.4 mM (table 3.2). The modified concentrations (table 3.3) closest to the standard culture media (table 3.2) were considered to be additional growth controls.

In studies using suspension cultured *Chenopodium album* cells, low boron concentrations were considered to be less than 7 μM added boron [23]. While studies on embryogenic cultures of *Larix deciduas* considered low boron conditions to be 10 μM, and high boron concentrations were considered to range between 100 μM and 1 mM added boron [21]. In radiata pine cell suspension cultures, low boron conditions were considered to be less than 10 μM added boron [24]. Moreover, depending on the concentration of calcium and magnesium in the media, boron deficiency could be induced when boron was present at 500 μM in radiata pine cell suspension cultures [24]. Recently, in boron deficiency studies on *Populus alba* cell suspension cultures, adequate boron for growth was determined to be 10 μM added boron [25]. The boron concentrations used in this study (0 to 1000 μM) were chosen around these published reports.

In studies using cultured *Pinus taeda* cells, low calcium levels were considered to be 0.15 mM added calcium [26]. High magnesium conditions were set at 7.5 mM in the cultured *Pinus taeda* cells [26]. The same high magnesium condition was used in radiata pine cell suspension cultures [24]. Studies on radish root found that 0.5 mM calcium added into the growth medium was inhibitory to boron-RG-II formation, while the addition of 10 mM calcium stimulated dimer formation [27]. Radiata pine grown in sand cultures that used magnesium concentrations ranging from 0.008 to 0.4 mM added magnesium, found that concentrations below 0.4 mM magnesium encouraged deficiency symptoms [28]. Radiata pine cell cultures used 3 mM of calcium, and 1.5 mM of magnesium as moderate levels [24]. Radiata pine seedlings grown in nutrient solutions used 0.35 mM added magnesium for sufficient levels, and 0.033 mM for deficient levels [29]. The calcium and magnesium concentrations used in this study (0 to 100 mM) were chosen around these published reports.

3.2.2 Preparation of the modified media

Every component of the culture media was stored and/or prepared in plastic or polypropylene containers, rather than traditional glass containers, to minimize the possibility of boron, calcium and/or magnesium leaching from the storage containers.

The culture media composition was divided into separate components (ie. macronutrients, vitamins etc) for which solutions were made and stored at -20°C. Appropriate volumes of these solutions were combined with Nanopure water (to a

volume of 500 mL) in autoclavable polypropylene containers. These concentrated culture media were then passed through the appropriate ion-binding column (section 3.2.2.1) without potassium iodide, sodium molybdate, manganese sulfate, magnesium sulfate, calcium chloride, boric acid, or NAA, as these components could bind to the columns [22]. Prior to autoclaving, the remaining media components were added, including the modified nutrient, the volume was adjusted with the appropriate nutrient free water, the pH was adjusted, and the appropriate nutrient free agar solution (section 3.2.2.1) was added to give a final volume of 1 L.

3.2.2.1 Controlling boron, calcium and magnesium levels in the media

Media was prepared for four separate trials. The first trial required the preparation of media containing only altered boron levels, the second trial required the preparation of media containing only altered calcium levels, the third trial required the preparation of media containing only altered magnesium levels, and the fourth trial required all of these nutrients to be altered.

Boron is a common environmental element and, for this reason, care must be taken when preparing media that lacks, or has little, boron. Boron is present at low concentrations in water [30], as a contaminant in many laboratory chemicals and in glassware [31]. To control boron concentrations it was necessary to first remove contaminating boron from the culture media. This was accomplished with Amberlite IRA-743 resin, which binds boron. In an initial trial, concentrated culture medium (prepared without the addition of boric acid, sodium molybdate, potassium iodide or NAA, which can bind to the column [22]) was passed through two columns (bed volume 92 mL each) of Amberlite IRA-743 resin in tandem [22, 32]. After passage through the columns, the medium was sent to Environmental Laboratory Services Ltd. (Lower Hutt, New Zealand) to determine the amount of boron, calcium and magnesium remaining using ICP-OES (table 3.4). The medium was found to contain approximately 22 μM contaminating boron, 60% of which the Amberlite IRA-743 resin successfully removed (table 3.4). The Amberlite IRA-743 resin also removed a large amount of calcium and magnesium (60 and 61% respectively; table 3.4). It was determined that passage of the concentrated media through the Amerlite IRA-743 column suitably removed contaminating boron from the medium, and was adopted for all media preparations. Subsequent to this experiment, calcium and magnesium were

added to the media prepared for boron removal, after it was passed through the IRA-743 column, as they could be partially removed by this column (table 3.4).

Standard media	Boron (μM)	Calcium (mM)	Magnesium (mM)
Amberlite IRA-743			
before column*	22	1.03	0.56
after column	9	0.43	0.22
Dowex 50			
before column**	181	1.03	0.56
after column	96	0.008	< 0.01***
Amberlite IRC-748			
before column**	193	1.06	0.56
after column	126	0.003	< 0.01***

* did not include KI, Na_2MoNO_4, H_3BO_3 or NAA
** did not include NAA, contained standard H_3BO_3, $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$ concentrations
*** below detection limits

Table 3.4 ICP-OES analysis of the standard culture media before and after passage through the ion-binding column. Two separate concentrated media were prepared for passage through the Amberlite-743 column and the Dowex 50 and Amberlite IRC-748 column. The 'before column' values for the Dowex 50 and Amerlite IRC-748 are duplicate measurements suggesting standard error of the means of ± 6 μM, ± 0.015 mM, and ± 0 mM for boron, calcium and magnesium. Due to limited resources, duplicate values were not obtained.

Calcium and magnesium are also abundant natural elements. In order to control their concentrations in culture media it was necessary to devise a method that could remove contaminating calcium and magnesium from the media. The available literature was vague pertaining to the best method for the removal of these elements [27, 28, 33]; as such, Dowex 50 and Amberlite IRC-748 resin, which bind calcium and magnesium, were trialled using concentrated medium. Standard calcium, magnesium and boron concentrations were added to this medium (table 3.2). After passage through the columns the media was sent to Environmental Laboratory Services Ltd. (Lower Hutt, New Zealand) to determine the amount of boron, calcium and magnesium using ICP-OES. The results for both resins were similar (table 3.4). The Dowex 50 resin

removed 99.2% of the calcium present, and 100% of the detectable magnesium (table 3.4). The Amberlite IRC-748 resin removed 99.7% of the calcium present, and 100% of the detectable magnesium (table 3.4). Both of these resins successfully removed contaminating calcium and magnesium. The passage of concentrated media through an Amberlite IRC-748 column was used in subsequent media preparations. Since boron could be removed by this column (table 3.4) it was added after the concentrated media solution was passed through the column. In addition, manganese sulfate, sodium molybdate potassium iodide and NAA were added after passage of the media through the columns.

Boron, calcium and magnesium in the agar

Boron, calcium and magnesium were present in the agar used as a gelling agent (10000 μM, 49 mM and 41 mM respectively [[34] and references therein]) which, if left untreated could have potentially contaminated the nutrient levels in the culture media (table 3.5). In order to control their levels, an agar solution was mixed with resin particles under constant heat (to maintain the agar in solution). The agar solution was composed of 250 mL of Nanopure water and 8 g of agar, which were mixed with 25 g of the appropriate Amberlite resin. In the case of the agar prepared for the modified boron media, this was the IRA-743 resin, and for the agar prepared for the modified calcium and modified magnesium media, this was the IRC-748 resin. In an initial trial, the resin particles were either added in a nylon bag, or loose, to determine which would remove most of the desired nutrients. After approximately ½ hour of mixing, the agar solution was separated from the resin particles by decanting the molten agar into a separate container, leaving the resin particles behind. This agar solution was autoclaved and sent to Environmental Laboratory Services Ltd. (Lower Hutt, New Zealand) to determine the concentration of boron, calcium and magnesium by ICP-OES. The results suggested that the addition of the appropriate loose resin removed the most mineral nutrient contaminants of interest (table 3.5), and this method was adopted for subsequent agar preparation.

Agar solution	Boron (μM)	Calcium (mM)	Magnesium (mM)
Amberlite IRA-743			
Agar prepared with resin in nylon bag	86	1.17	0.77
Agar prepared with loose resin	10	1.13	0.72
Amberlite IRC-748			
Agar prepared with resin in nylon bag	347	0.21	0.48
Agar prepared with loose resin	329	0.03	0.26

Table 3.5 Concentration of boron, calcium and magnesium present in the agar matrix after preparation with resin. Due to limited resources duplicate values were not obtained. however, previous analysis by Environmental Laboratory Services Ltd. using ICP-OES suggested a low margin of error for this technique.

3.2.2.2 Final modified media preparation

Samples of the final media preparation, including the agar solution prepared with loose resin, were analyzed by Environmental Sciences Laboratory Ltd. (Lower Hutt, New Zealand). The results corroborate the data presented in table 3.5. The media prepared to contain 7 μM boron was found to contain 19.6 μM boron, consistent with approximately 10 μM boron presumably bound to the agar matrix (table 3.5). A seperate medium prepared without calcium or magnesium (0 mM respectively) was found to contain 0.025 mM calcium and 0.034 mM magnesium, which suggests the presence of only the calcium and magnesium bound in the agar matrix. The results presented in tables 3.4 and 3.5 shows that contaminating sources of these nutrients can be removed, however, they were not eliminated and a small amount in the final media preparations remained.

Media storage

Sterilized culture media were poured into sterile Petri dishes (3 to 5 mm thickness) in a sterile laminar flow cabinet, sealed with plastic strips, and stored in a sterile and dark environment.

3.2.3 Preparation of the organ cultures

3.2.3.1 Tree material

Radiata pine trees planted in 1994 at a Burnham, New Zealand field site, showing no obvious deformities were felled as required, de-limbed, and stem segments between whorls of branches on the main axis of the tree were removed from a region of approximately 1 to 3 metres above the ground. The stem segments were immediately transported to the lab in the back of a well ventilated truck. The trees belonged to the same clone (clone 3) to reduce genotypic variation within the cultures, and were from the same field trials to avoid site differences. Radiata pine trees planted in a Rotorua, New Zealand field site were also felled, as described above. These trees were wrapped in bubble wrap and flown to Christchurch in an insulated bin filled with ice packs. The clonal origin of these trees was not known. The stem segments from either site were stored at 4^{o}C for a maximum period of one week. Trees were harvested between October and May of each growth season (2003-2005).

The explants that were used to initiate the cultures came from cambial material in these trees that was approximately 10 years in age. With the exception of Savidge (1993), and Leitch (1995;1999) very little work has been reported on culturing cambium that is more than a few years in age [1-3]. This is, in part, because cambial material is difficult to culture as a result of complex interactions between organic constituents, plant growth regulators and physical requirements [1, 2]. However, the modified methods used in this work have successfully sustained cambial growth for a period of almost two months in culture (presented in chapter four).

3.2.3.2 Sterilization

To prepare for culturing, a disc of approximately 10 cm was removed from the stem segment, scrubbed with detergent, washed, and rinsed in cold water, and with dilute bleach. The outer rhytidome was removed with razor knives exposing phloem cells (ensuring that no resin cysts remained), the disc was rinsed with ethanol and placed in a specially designed holder in a horizontal laminar flow cabinet (figure 3.1). The surface of the disc was sprayed with ethanol and flame sterilized. Aspects of this sterilization method were adapted from Savidge (1993) [1], and Leitch (1999) [2] and are compared in table 3.6.

Sterilization technique	Tamarack[a]	Eucalyptus[b]	Radiata Pine
Stem preparation:			
Scrub surface with detergent	yes	yes	yes
Rinse with tap water	yes	yes	yes
Scrub surface with detergent	no	no	yes
Rinse with tap water	no	no	yes
Scrub surface with bleach	no	no	yes
Rinse with tap water	no	no	yes
Remove rhytidome	yes	yes	yes
Wash with tap water	yes	no	no
Wash with distilled water	no	yes	yes
Wash with detergent	no	yes	no
Wash with bleach	no	yes	yes
Wash with hydrogen peroxide	no	yes	no
Rinse with ethanol	no	no	yes
Flame sterilize in laminar flow cabinet	yes	yes	yes
Explant preparation:			
Chip explant	yes	no	yes
Scallop explant	no	yes	yes
Soak in hydrogen peroxide	no	yes	no
Xylem surface in contact with the media	yes	yes	yes

[a] Savidge (1993)
[b] Leitch (1999)

Table 3.6 Comparison of sterilization methods.

One of the differences between the sterilization method used in this work, and that used by Savidge (1993) [1] and Leitch (1999) [2] was the use of smaller discs, rather than long stem segments. Savidge (1993) attempted cultures using smaller discs but failed. Another difference between the sterilization technique used by Leitch (1999) and that used here was the exclusion of 3% hydrogen peroxide in the washing steps, as well as just after excision of the explant. Leitch (1999) claimed that the use of 3% hydrogen peroxide reduced the fungal contamination from greater than 90% to between 10 and 15% of all cultures. However, the incidence of fungal contamination

did not decrease with the use of hydrogen peroxide in this work, and so these steps were omitted.

Consistent with Leitch (1999) and Savidge (1993), removal of bark to less than 1 mm of remaining phloem resulted in death of the explant, which was likely to be due to heat shock from flame sterilization. Phloem thickness was maintained at approximately 1 to 2 mm. Also consistent with the findings of Savidge (1993), it was observed that if resin cysts were permitted to remain at the level of the phloem, there was an increased incidence of contamination.

Figure 3.1 Sterilization technique. (A) Radiata pines planted in 1994 were felled, and stem segments were returned to the laboratory. (B) Discs approximately 10 cm in length were cut from the stem, following a series of washes (C) the outer rhytidome was removed, and the disc was (D) flame sterilized.

3.2.3.3 'Chip' explant method

The technique for removal of the chip explant was essentially the same as that used by Savidge (1993). Briefly, flame sterilized equipment was used to remove sections approximately 3 cm axial x 2 cm tangential x 0.5 cm radial as illustrated in figure 3.2A. These explants contained surface sterilized phloem, cambium and xylem. The

explant was placed on the sterile growth media so that the exposed xylem surface was in direct contact with the media (figure 3.2B). Two explants were generally placed in one Petri dish. The Petri dish was sealed with plastic strips and placed in a growth room. An additional explant was kept as a control and stored in 4% paraformaldehyde in ¼ × phosphate-buffered saline. Generally, 14 chips per disc were obtained.

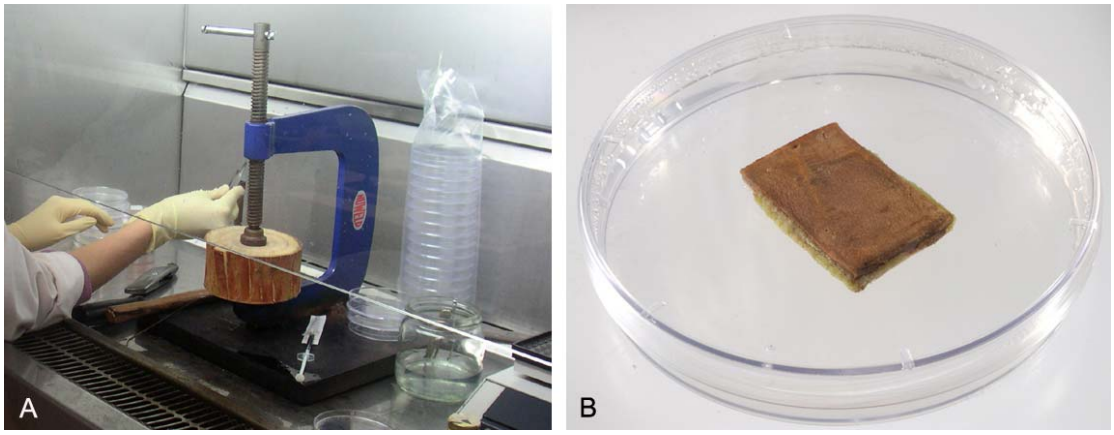


Figure 3.2 Chip explant method. (A) Processed stem segment. (B) Surface sterilized chip culture.

This method allowed for the culture of intact cambium with minimal wounding, except at the peripheries of the chip [1]. The use of a chisel to extract the chip, rather than only the razor knives described by Savidge (1993) eliminated the danger of the knives slipping, resulting in damage to the cambium.

3.2.3.4 'Scallop' explant method

The technique for removal of the scallop explant was essentially the same as that described by Leitch (1999). Heat sterilized equipment was used to remove sections approximately 1.5 cm axial x 4 cm tangential x 0.5 cm radial. These explants differed from chip explants in that they were semi-circular in shape, rather than rectangular, and they did not contain cells from the exposed ends of the disc (figure 3.3A). Like the chip explants, these explants contained surface sterilized phloem, cambium and xylem and were placed on the growth media so that the exposed xylem surface was in direct contact with the media (figure 3.3B). The Petri dish was sealed with plastic strips and placed in the growth room. An additional explant was kept as a control and stored in 4% paraformaldehyde in ¼× phosphate-buffered saline. The main disadvantage of this technique was that occasionally the edges of the scallops had a

tendency to curl upwards [2]. However, when this happened, the main portion of the scallop was always in contact with the growth media.

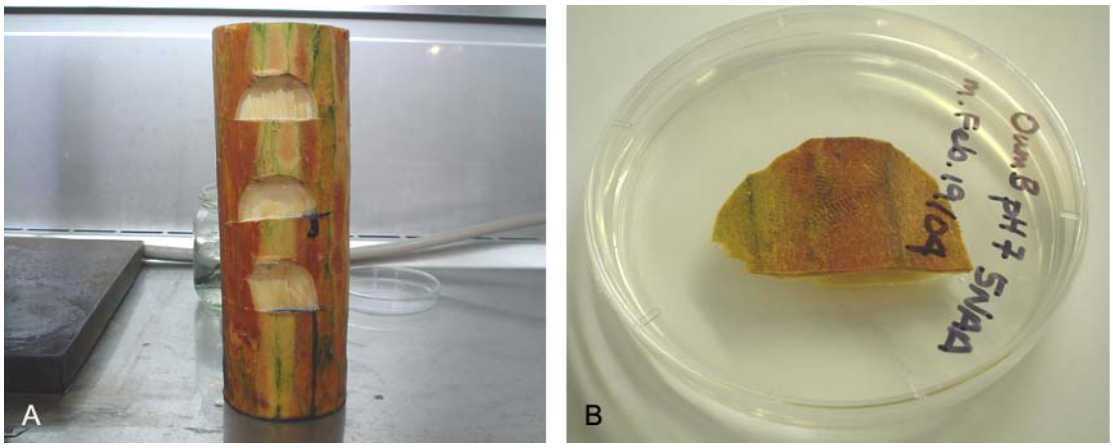


Figure 3.3 Scallop explant method. (A) Processed stem segment. (B) Surface sterilized scallop culture.

There was no visible difference between the growth of the cultures by the chip explant, or scallop explant methods. However, the scallop explants did tend to have less microbial contamination. The reason for this difference is not known.

3.2.3.5 Marking culture growth

In initial experiments, the cambium of each explant was injected with 0.1% Janus green dye in PBS with sterile needles, prior to excision. This was done to mark the location of the original wood (i.e. any wood above the marker was grown in culture). However, due to the increased contamination associated with this procedure, it was abandoned in favour of a cell counting method that will be introduced in chapter four.

3.2.3.6 Growth conditions

In all plant culture systems, there is a lag between when the treatment is initiated and when the cultures exhibit a response that can vary from a few days to several weeks [20]. In this work, the explants were cultured for one to six months, at 21°C, and in 23.5 hours of light per day, with an irradiance of 26.5 μE m^{-2} sec^{-1}. A culture response, observed as callus formation, was observed approximately one week after culture (section 4.2.1). The main differences between the growth conditions used by Savidge (1993), and Leitch (1999), and those used in this work were the conditions of the growth room ie. temperature, the amount of light and the length of the culture

period (table 3.7). These differences were the result of the availability of growth rooms at the University. Only the analyses of explants cultured for 1 to 2 months are presented in chapters 4 through 6, as culture growth was believed to stop after this time period.

Culture conditions	Tamarack[a]	Eucalyptus[b]	Radiata pine
Lighting (per day)	continuous	continuous	23.5 hours
Growth room temperature (°C)	25	24	21
Culture period (days)	35	42-60	30-180

[a] Savidge (1993); [b] Leitch (1999)

Table 3.7 Comparison of the growth conditions for tamarack, eucalyptus and radiata pine.

3.3 Microbial contamination of the organ cultures

Sterilization of explants from standing trees was difficult, without causing damage to the tissue, due to the presence of endogenous microbes [35]. As mentioned in section 3.2.3.2, removing the resin cysts when paring the rhytidome assisted in decreasing the incidence of microbe contamination. Savidge (1993) found that after five weeks of culture, greater than 90% of the chips were free from contamination. However, this was not found to be the case with the radiata pine explants (figure 3.4).

In order to test for the possibility of contamination of the media during preparation, Petri dishes with no explants on them were periodically left in the culture room to see if bacterial growth occurred. Bacterial growth on these plates never occurred. Thus contamination arose from incomplete sterilization of the explants themselves.

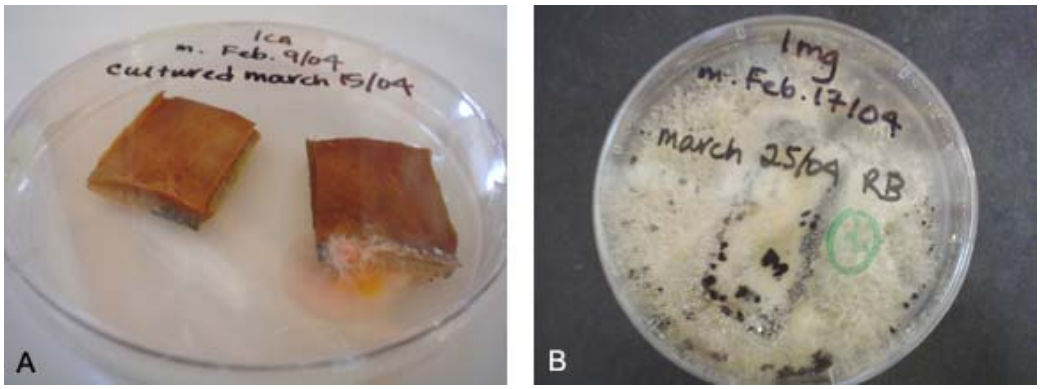


Figure 3.4 Examples of the presence of microbial contamination in culture explants. (A) Partial microbial contamination. (B) Full microbial contamination

3.3.1 pH trials to control microbial contamination

A large percentage of the explants, from the boron trials in particular, were lost to microbial contamination. In an attempt to reduce culture losses, trials were conducted whereby boron cultures (0, 7, 100 μM) were grown on media with three different initial pHs (5.7, 7.0 and 8.0) because most wood fungi favour acidic conditions [36]. For this trial, nine cultures were grown for each pH (three per boron concentration), which was repeated on three different culture dates. The presence of fungus was recorded in three categories; no fungal contamination; partially contaminated, meaning one end of the chip displayed evidence of microbes (figure 3.4A); and full contamination, whereby the whole explant was contaminated by microbes (figure 3.4B). The trial suggested that at a pH of 5.8 the majority of the cultures were lost to microbial contamination, while a pH of 7.0 and 8.0 greatly reduced the number of cultures lost to fungus (figure 3.5).

A number of factors suggested in the literature were considered when deciding on the pH range for this trial. Included among these are the suggestions that pH can influence the form, solubility and binding of media components [20, 34]. No literature was found suggesting that a more basic pH would alter the ‘normal’ interaction of boron in the culture media. Moreover, there was no noticeable difference in the cultures grown at pH 5.8 and 7.0, apart from the incidence of microbial contamination. The pH was not altered for the calcium, magnesium and mixed trials, as these cultures did not experience as high an incidence of fungus.

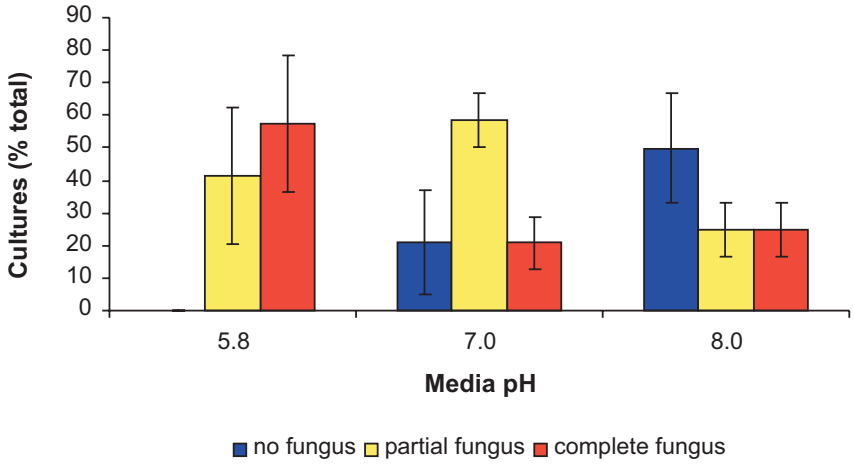


Figure 3.5 Boron cultures were grown on three media with different initial pH. As a result of these trials, a pH of 7.0 was used for the boron cultures to discourage the incidence of complete fungal contamination. Bars indicate standard error of the mean.

The pH of the initial medium changes throughout the culture period [34, 37]. The addition of gelling agents, autoclaving [20, 34, 37] and storage [20, 34] are all known to alter the pH of culture media. Moreover, the pH of the media usually become more acidic during the course of the culture period regardless of the use of buffers [20, 34]. In this culture system, the use of litmus paper confirmed the acidification of the media over the course of the culture period. Most plant cultures are maintained on the same medium for several days to several months, as are the organ cultures [20]. Thus, the operating pH during the culture period is usually different from the initial pH, as a result of complex interactions between the media components, and uptake and utilization in the cells [20, 34]. This operating pH, or equilibrium pH, is generally the same irrespective of the initial pH used [34], and it has become accepted that the growth of plant cultures is not significantly affected by a broad pH range of 4.0 to 7.0 of the initial culture media [20, 34, 38]. As a result, an initial media pH of 7.0 was used for the boron-treated cultures to discourage complete fungal contamination, and was not expected to affect the culture response.

In a study completed in this laboratory examining the relationship between auxin and boron [19], the methods established in this chapter were used; however, the pH of the media was not adjusted to 7.0, but rather to 5.8. The study yielded similar responses [19], assumed to be due to boron availability, further suggesting that alteration of the media pH did not alter the culture response.

3.3.2 Number of organ cultures prepared for this study

While changing the pH of the boron culture medium reduced the incidence of microbial contamination, it did not eliminate it. Contamination was accommodated by increasing the number of explants cultured. The number of explants cultured, as well as details including the media they were cultured on, and the date of culture were recorded every time a disc was prepared. These cultures were monitored for microbial contamination, and the explants that had to be discarded because of microbial contamination were recorded. In addition, further details such as the region of the stem segment cultured and date of tree collection were recorded. The total number of explants for each nutrient trial, as well as the explants lost to microbial contamination, are outlined in table 3.8. The boron culture trials were a priority for this project and, as such, many more of these explants were cultured to ensure adequate material for this study, and other potential side projects.

Concentration added to the medium	Number of explants	Explants lost to Contamination (%)
Boron (µM)		
0	249	46
1	140	52
7	216	55
25	139	56
100	191	55
1000	166	42
Total	1101	50
Calcium (mM)		
0	107	39
1	40	38
5	37	51
10	41	34
100	68	25
Total	293	37

Table 3.8 Number of explants cultured between 2003 and 2005, and lost to microbial contamination. Continued on following page.

Concentration	Number of explants	Explants lost to Contamination (%)
Magnesium (mM)		
0	54	17
0.5	29	45
1	32	38
5	56	18
10	29	41
100	56	16
Total	256	25
Mixed (µM/ mM)		
1000 µM boron, 100 mM calcium and magnesium	15	6
0 µM boron, 0 mM calcium and magnesium	15	0
1000 µM boron, 0 mM calcium	36	8
0 µM boron, 100 mM calcium	37	11
100 mM calcium, 0 mM magnesium	14	0
0 mM calcium, 100 mM magnesium	15	0
0 µM boron, 5 mM calcium and magnesium	14	0
7 µM boron, 5 mM calcium and magnesium	37	8
Total	183	6

Table 3.8 contd. Number of explants cultured between 2003 and 2005, and lost to microbial contamination. Continued from previous page.

3.4 Summary

As discussed in chapter one, the ability to examine the role of nutrients, plant hormones and other growth factors under controlled conditions is the most valuable application of a culture technique [6]. The experiments detailed in this chapter were undertaken to develop an organ culture method in which the boron, calcium and magnesium concentrations could be manipulated in order to determine their role in wood formation. This has been achieved through the use of ion-binding resins to remove contaminating boron, calcium, or magnesium from the media during preparation, allowing for the addition of defined concentrations. Removal of these contaminants was important, as there were many components in the media, and the

omission of some essential nutrients in the deficiency studies may have been of no consequence if contaminating sources were sufficient for normal growth [9]. The original organ culture studies discussed in the present chapter, suggested that their organ culture techniques provided a useful method for observation of wood formation *in vitro* [1, 2]. The subsequent chapters presented in this thesis provide a detailed examination of the wood formed using the organ culture methods developed in this chapter, to determine what this method can successfully manipulate, and which wood properties can be meaningfully measured.

3.5 References

1. Savidge, R.A. 1993. *In vitro wood formation in 'chips' from merchantable stem regions of Larix laricina.* International Association of Wood Anatomists, (14): 3-11.

2. Leitch, M.A. 1999. *The development of tissue culture techniques to study wood formation in Eucalyptus globulus Labill*. PhD Thesis, *Institute of Land and Food Resources*, University of Melbourne.

3. Leitch, M.A., Savidge, R.A. 1995. *Evidence for auxin regulation of bordered-pit positioning during tracheid differentiation in Larix laricina.* International Association of Wood Anatomists, **16**: 289-297.

4. Taiz, L., Zeiger, E. 2002. *Cell walls: structure, biogenesis, and expansion*, in *Plant Physiology*. Sinauer Associates Inc.: Massachusetts.

5. Smith, R.H. 1992. *Media components and preparation*, in *Plant Tissue Culture*. Academic Press: San Diego. 7-18.

6. Leitch, M.A., Savidge, R.A. 2000. *Cell, tissue and organ culture for the study of cambial activity and wood formation - a resurgence of interest in an old technique*, in *Cell and Molecular Biology of Wood Formation*, Savidge, R.A., Barnett, J.R., Napier, R., Ed. BIOS Scientific Publishers Ltd.: Oxford. 493-512.

7. Allen, E.A., Blenis, P.V. 1988. *Axenic culture of Endocronartium harknessii.* Mycologia, **80**: 120-123.

8. Murashige, T., Skoog, F. 1962. *A revised medium for rapid growth and bio assays with tobacco tissue cultures.* Physiologia Plantarium, **15**: 473-497.

9. Teasdale, R.D. 1987. *Micronutrients*, in *Cell and Tissue Culture in Forestry*, Bonga, J.M., Durzan, D.J., Ed. Matinus Nijhoff Publishers: Boston. 17-48.

10. Grey, D., Stepan-Sarkissian, G., Fowler, M.W. 1987. *Biochemistry of forest tree species in culture*, in *Cell and Tissue Culture in Forestry. Specific Principles and Methods: Growth and Developments*, Bonga, J.M., Durzan, D.J., Ed. Martinus Nijhoff Publishers: Dordrecht. 31-60.

11. Street, J.J., Kidder, G. 1997. *Soils and plant nutrition*, in *Cooperative Extension Service. Institute of Food and Agricultural Sciences.* University of Florida: Gainesville.

12. Clarkson, D.T., and Hanson, J.B. 1980. *The mineral nutrition of higher plants.* Annual Review of Plant Physiology, **31**: 239-98.

13. Lewis, D.H. 1980. *Boron, lignification and the origin of vascular plants - a unified hypothesis.* New Phytology, **84**: 209-229.

14. Gauch, H.G., Dugger, W.M. 1952. *The role of boron in the translocation of sucrose.* Plant Physiology, **51**: 457-467.

15. Zakrzewski, J. 1983. *Hormonal control of cambial activity and vessel differentiation in Quercus robur.* Physiologia Plantarium, **57**: 537-542.

16. Zakrzewski, J. 1991. *Effect of indole-3-acetic acid (IAA) and sucrose on vessel size and density in isolated stem segments of oak (Quercus robur).* Physiologia Plantarium, **81**: 234-238.

17. Little, C.H.A., Sundberg, B. 1991. *Tracheid production in response to indole-3-acetic acid varies with internode age in Pinus sylvestris stems.* Trees, **5**: 101-106.

18. Little, C.H.A., Savidge, R.A. 1987. *The role of plant growth regulators in forest tree cambial growth.* Plant Growth Regulation, **6**: 137-169.

19. Nair, H. 2006. *A study of intra-ring checking and xylogenesis in Pinus radiata D. Don*. Phd Thesis, *School of Biological Sciences*, University of Canterbury.

20. Minocha, S.C. 1987. *PH of the medium and the growth and metabolism of cells in culture*, in *Cell and Tissue Culture in Forestry. General Principles and Biotechnology*, Bonga, J.M., Durzan, D.J., Ed. Martinus Nijhoff Publishers: Boston.

21. Behrendt, U., Zoglauer, K. 1996. *Boron controls suspensor development in embryogenic cultures of Larix decidua.* Physiologia Plantarium, **97**: 321-326.

22. Matoh, T., Ishigaki, K., Mizutani, M., Matsunga, W., Takabe, K. 1992. *Boron nutrition of cultured tobacco BY-2 cells. I. Requirement for and intracellular localization of boron and selection of cells that tolerate low levels of boron.* Plant Cell Physiology, **33**: 1135-1141.

23. Fleischer, A., Titel, C., Ehwald, R. 1998. *The boron requirement and cell wall properties of growing stationary suspension-cultured Chenopdium album cells.* Plant Physiology, **117**: 1401-1410.

24. Teasdale, R.D., Richards, D.K. 1990. *Boron deficiency in cultured pine cells.* Plant Physiology, **93**: 1071-1077.

25. Kakegawa, K., Ishii, T., Matsunga, T. 2005. *Effects of boron deficiency in cell suspension cultures of Populus alba L.* Plant Cell Reports, **23**: 573-578.

26. Teasdale, R.D., Dawson, P.A., Woohouse, H.W. 1986. *Mineral nutrient requirements of a loblolly pine (Pinus taeda).* Plant Physiology, **82**: 942-945.

27. Kobayashi, M., Nakagawa, H., Asaka, T., Matoh, T. 1999. *Borate-rhamnogalacturonan II binding reinforced by Ca^{2+} retains pectic polysaccharides in higher plant cell walls.* Plant Physiology, **119**: 199-203.

28. Sun, O.J., Payn, T.W. 1999. *Magnesium nutrition and photosynthesis in Pinus radiata: clonal variation and influence of potassium.* Tree Physiology, **19**: 535-540.

29. Sun, O.J., Gielen, G.J.H.P., Sands, R., Smith, C.T., Thorn, A.J. 2001. *Growth, Mg nutrition and photosynthetic activity in Pinus radiata: evidence that NaCl addition counteracts the impact of low Mg supply.* Trees: Structure and Function, **15**: 335-340.

30. 2003. *Boron in drinking-water. Background document for preparation of WHO Guidelines for drinking-water quality*, WHO/SDE.WSH/03.04/54. Organization, W.H., Editor: Geneva. p. 313-314.

31. Kubiak, R.A.A., Leong, W.Y., Dowsett, M.G., McPhail, D.S., Houghton, R., Parker, E.H.C. 1986. *A solution to boron contamination at the substrate/epilayer interface of silicon grown by molecular beam epitaxy.* Journal of Vacuum Science & Technology A: Vacuum Surfaces, and Films, **4**: 1905-1907.

32. Ishii, T., Matsunga, T., Hiyash, N. 2001. *Formation of rhamnogalacturonan II-borate dimer in pectin determines cell wall thickness in pumpkin tissue.* Plant Physiology, **126**: 1698-705.

33. Koppel, L.A. 1994. *Growth of isolated tomato apices on calcium-deficient media as a function of the quality of light used during sprout development.* Fiziologiya-Rastenii-Moscow, **41**: 838-842.

34. Williams, R.R. 1993. *Mineral nutrition in vitro - a mechanistic approach.* Australian Journal of Botany, **41**: 237-51.

35. Le Roux, J.J., Van Staden, J. 1991. *Micropropagation and tissue culture of Eucalyptus - a review.* Tree Physiology, **9**: 435-477.

36. Baker, M.C. 1969. *Decay of wood.* National Research Council Canada: Canadian Building Digest, **111**: 1-5.

37. Williams, R.R., Taju, A.M., Winney, K.A. 1990. *The effect of Ptilotus plant tissue on pH of in vitro media.* Plant Cell, Tissue and Organ Culture, **22**: 153-258.

38. Islam, K.M.S., Edwards, D.G., Asher, C.J. 1980. *pH optima for crop growth.* Plant and Soil, **54**: 339-357.

Chapter Four

Organ culture growth characteristics

4.1 Introduction

After the production and collection of a sufficient number of organ cultures for each mineral nutrient treatment, the cultures were dissected and analysis was undertaken to ascertain changes in growth characteristics. In parallel, cultures were subjected to ICP-MS/OES to determine whether the nutrient of interest was successfully taken up by the explant. Additional cultures were processed for light microscopy, and the images obtained were used to rigorously evaluate xylem cell dimensions and cell wall thickness, using image analysis software. Characterization of the ultra-structure of the xylem cell walls was performed using transmission electron microscopy. Throughout the remainder of this thesis, a 'culture set' refers to all of the culture treatments in a trial (ie. 0, 1, 7, 25, 100 and 1000 μM boron), and the respective control explant collected at the time of culturing. In addition, when reference is made to a particular nutrient treatment, the concentration discussed refers to the amount of that nutrient added into the culture medium. These analyses were performed to determine which aspects of organ culture wood formation could be successfully manipulated using the methods developed in chapter three, and to establish whether these altered wood properties could be meaningfully measured.

4.2 Callus formation

Callus is formed when plant tissue is wounded [1]. During callus formation, ray parenchyma cells located near the cambium (which retain their nucleus at maturity), undifferentiated cambial cells and phloem parenchyma, divide and de-differentiate into specialized cells that function to replace xylem or phloem cells and protect the exposed tissue [2-4]. This layer of protective callus provides a means for water to bypass damaged tracheids and reach undamaged tissues [5]. It is common for cambial tissue to undergo callus formation during culturing [3-8]. The conditions under which

cultures containing cambial material are grown are also known to affect the colour, friability and compactness of callus [1, 9, 10]. In the organ culture explants, wounding occurred around their periphery when they were removed from the stem and it is along the sides of the cultures that callus formation was observed.

4.2.1 Callus formation around the periphery of the cultures

4.2.1.1 Boron-treated cultures

The boron-treated cultures generally showed brown callus formation by the end of a two month culture period. Cultures representative of the low (0 µM), adequate (7 µM) and high (1000 µM) boron-treated cultures are shown in figure 4.1. Differences in the amount of callus formed was variable; however, the low and high treatments (figure 4.1 A and C respectively) often showed the most callus formation.

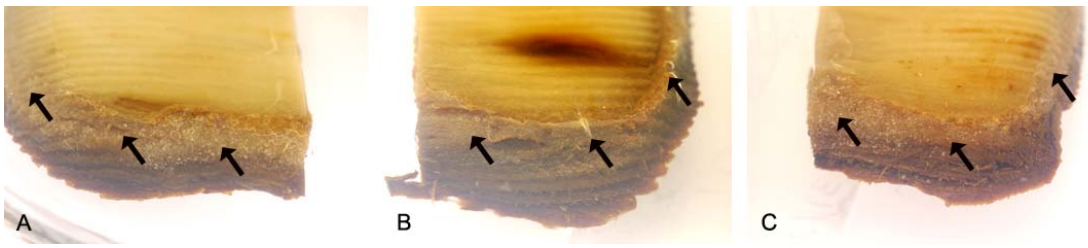


Figure 4.1 The underside of cultures grown on media with (A) 0 µM boron, (B) 7 µM boron, (C) 1000 µM boron added. The explants were cultured for a period of two months. Callus was present along the periphery of the cultures (arrows). The images are representative of the general observations made for all of the boron treated cultures.

4.2.1.2 Calcium and magnesium-treated cultures

Callus formation showed similar characteristics in the varied calcium and magnesium nutrient treatments. This was generally observed as green callus for nearly the entire two month culture period (figure 4.2). The low (0 mM calcium; 0 mM magnesium) and the high (100 mM calcium; 100 mM magnesium) treated cultures usually showed brown callus towards the end of the culture period (figure 4.2 A, C and D, F respectively).

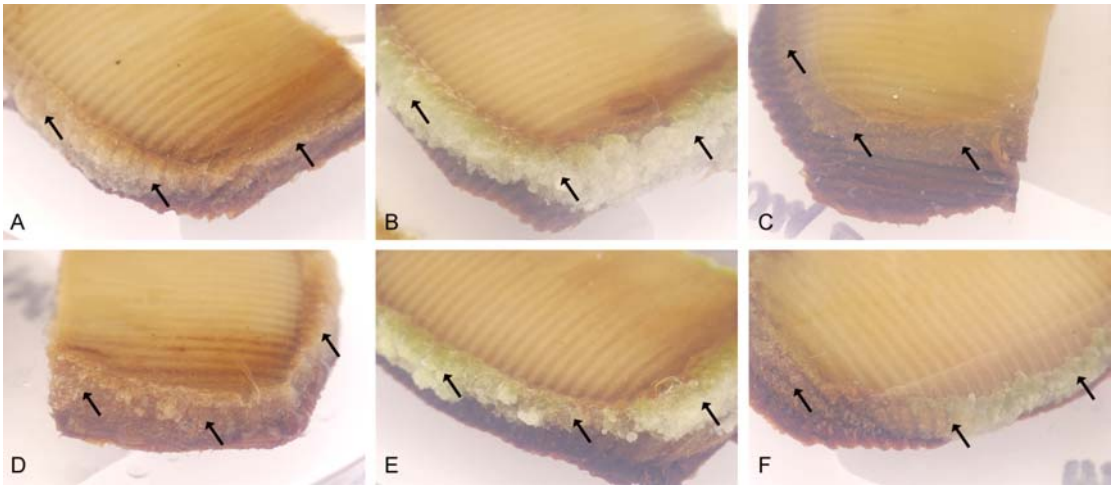


Figure 4.2 The underside of cultures grown on media with (A) 0 mM calcium, (B) 5 mM calcium, (C) 100 mM calcium, (D) 0 mM magnesium, (E) 5 mM magnesium, (F) 100 mM magnesium added. The explants were cultured for a period of two months. Callus was present along the periphery of the cultures (arrows). The images are representative of the general observations made for all of the calcium and magnesium treated cultures.

4.2.1.3 Mixed-treated cultures

The mixed-treated cultures with boron, calcium and magnesium concentrations, that were predicted to be optimal for growth (Opti: 7 µM boron, 5 mM calcium and 5 mM magnesium; figure 4.3A), generally maintained green callus formation for the greatest length of time; however, these cultures did not have the highest incidence of callus formation. The mixed-treated cultures with high boron, and low calcium concentrations (HBLC: 1000 µM boron, 0 mM calcium; figure 4.3B) often had the greatest callus formation, while the cultures treated with low boron, and high calcium concentrations (LBHC: 0 µM boron, 100 mM calcium; figure 4.3C) often had less callus formation.

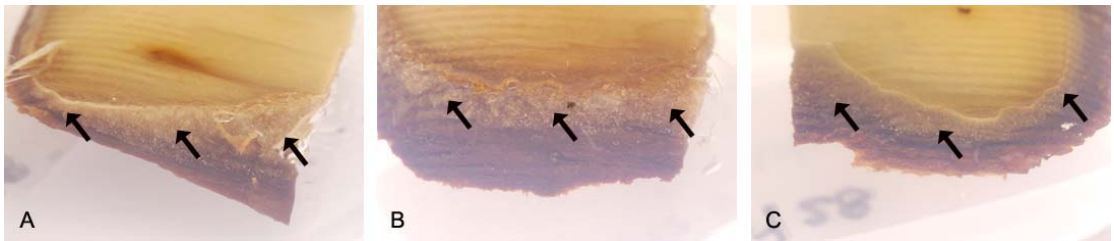


Figure 4.3 The underside of cultures grown on media with (A) 7 µM boron, 5 mM calcium, 5 mM magnesium (Opti), (B) 1000 µM boron, 0 mM calcium (HBLC), (C) 0 µM boron, 100 mM calcium (LBHC) added. The explants were cultured for a period of two months. Callus was present along the periphery of the culture (arrows). The images are representative of the general observations made for all of the mixed treated cultures.

Cultures grown in the absence of added boron, calcium or magnesium (0BCM: 0 µM boron, 0 mM calcium, 0 mM magnesium; figure 4.4A) to the media and those grown in the presence of high boron, calcium and magnesium (HBCM: 1000 µM boron, 100 mM calcium, and 100 mM magnesium) in the media generally had little or no callus formation, suggesting that the cultures could not be sustained in these conditions. The cultures grown in low boron, and optimal calcium and magnesium conditions (Opti-LB: 0 µM boron, 5 mM calcium, 5 mM magnesium; figure 4.4B) generally showed green callus formation. This suggests that adequate calcium and magnesium were present to promote cell division in the absence of boron. The cultures treated with high levels of calcium, and low levels of magnesium (HCLM: 100 mM calcium, 0 mM magnesium; figure 4.4C) and the cultures treated with low levels of calcium, and high levels of magnesium (LCHM: 0 mM calcium, 100 mM magnesium; figure 4.4D) generally showed only a slight occurrence of callus presence, suggesting that both calcium and magnesium were required to maintain the viability of these cultures.

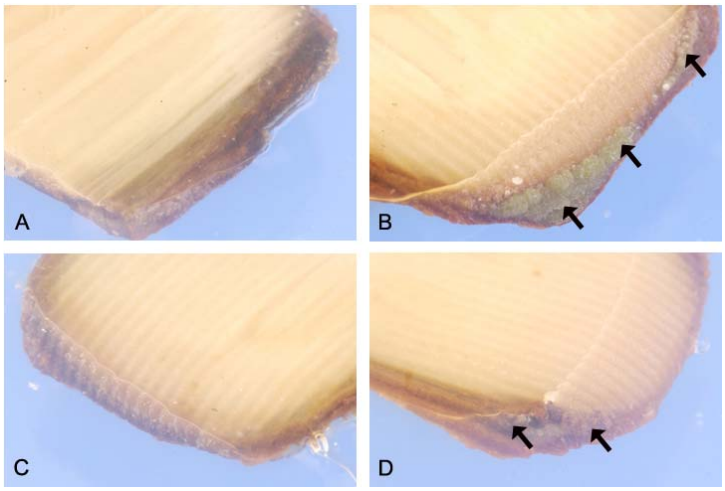


Figure 4.4 The underside of cultures grown on media with (A) 0 µM boron, 0 mM calcium, 0 mM magnesium (0BCM), (B) 0 µM boron, 5 mM calcium, 5 mM magnesium (OptiLB) (C) 100 mM calcium, 0 mM magnesium (HCLM) and (D) 0 mM calcium and 100 mM magnesium (LCHM) added. The explants were cultured for a period of two months. Callus was present along the periphery of the culture (arrows), if it was present. The images are representative of the general observations made for all of the mixed treated cultures.

4.2.1.4 Summary of callus observations

In this work, green callus growth along the perimeter of the organ cultures began approximately one week after induction on the culture medium, consistent with the observations for eucalyptus organ cultures that were made by Leitch (1999) [10]. In

some culture treatments, this callus formation began to turn brown after approximately six weeks in culture. The change in callus colour from green to brown suggested that the health of the culture could not be sustained, meaning that the viability of the culture decreased after approximately six weeks. It is also possible that the brown callus was due to the production of phenolics during xylogenesis. While the presence of healthy callus was used as an indication that the cultures were alive for the duration of the culture period, it also suggested that these cultures were undergoing active cell division [10]. Cultures that did not demonstrate callus growth along their perimeter were assumed to have died during the culture process, similar to observations made by Savidge (1993) [9] and were not included in further analysis. It is possible that the cultures that were excluded were healthy but either not dividing or producing wood without external callus. Callus formation (on the healthy cultures) occurred regardless of when in the growth season the culture was induced for most of the cultures. Previous studies have found that callus formation on organ cultures occurred year round, with the exception of cultures prepared in the spring [9].

4.2.2 Sub-culturing

Healthy callus could not be sustained by sub-culturing the explants on the media used in these trials. This is consistent with previous observations for organ cultures, where sub-culturing did not lead to continued growth [1, 9]. This suggests that in addition to the components of the growth media, there are factors (possibly endogenous hormones) in the existing wood that contribute to cell proliferation, that once exhausted, result in culture dormancy or death [9]. Sub-culturing was not pursued further due to funding and time constraints.

4.2.3 Callus formation in the absence of phloem and xylem

The original explant method developed by Savidge (1993) recognized that the physical pressure exerted on cambial cells between the stem and bark, coupled to the pressure from newly divided cells, were required for proper tracheid development, and maintenance of radial cell files [9]. Brown and Sax (1962) were the first to show that pressure on the cambium was necessary to prevent the entire cambium from differentiating into callus [11]. When they separated bark strips from woody stems exposing the cambium of *Populus trichocarpa* and *Pinus strobes,* differentiation into callus occurred [11]. If the bark strip was separated from the cambial surface by a

plastic film (to control for potential signals from the phloem) and tied back in place, the cambium did not differentiate into callus, and normal cambial division resumed [11].

In order to confirm whether the presence of both the phloem and xylem were required for maintenance of radial cell files and cambial cells in the organ cultures, dissected explants were cultured. These explants were composed of xylem strips containing mostly cambium, and some underlying xylem cells (figure 4.5A), or explants that had portions of the phloem removed (figure 4.5B). When these dissected cultures were grown on the standard culture media (section 9.3.2.1), the exposed cell surface differentiated into callus cells, and not phloem or xylem. This confirmed that the presence of both the phloem and xylem, which create a sandwich with cambium in the middle, are required for maintenance of radial cell files of tracheid cells (figure 4.12).

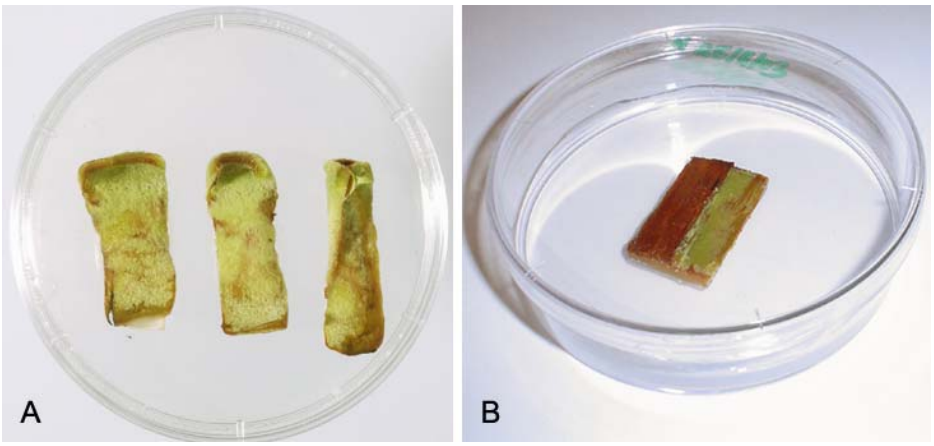


Figure 4.5 (A) Strips of xylem composed of cambium, and some xylem cells, but no phloem cultured on standard media. (B) Explant with a portion of the phloem removed that was cultured on standard media. The presence of callus suggests that the physical pressure of phloem and xylem are required for the differentiation of tracheid cells.

These trials have shown that if the cambium alone is explanted from a tree and placed in a growth media, it will produce callus, and little or no xylem, consistent with the literature [5, 9]. The presence of cambium and xylem in the absence of phloem will also produce callus. In the organ culture system, including the phloem and xylem preserves the natural constraints that cambial cells would experience *in vivo*; however, these constraints are reduced, leaving the possibility of cellular abnormalities occurring during growth [1, 12]. For this reason, when the cultures were dissected for

analysis, the centre portion of the culture, (ie. having cell contact from all angles), rather than the periphery was used.

4.3 Mineral nutrient uptake

In order to determine if the uptake of boron, calcium and magnesium into the organ cultures was successful, and presumably available to new wood growth, explants that had been cultured for two months were collected, the phloem and the bottom of the culture (that was in contact with the agar) were removed, and the remaining culture was ground to a homogenous powder. This was done for cultures from two trees and these samples were sent to Hill Laboratories (Hamilton, New Zealand) for analysis by ICP-OES and ICP-MS. ICP-OES/MS procedures were introduced in section 2.4.2.1.

4.3.1.1 Boron, calcium and magnesium-treated cultures

The ICP-MS results showed that the amount of boron, calcium or magnesium found in the cultures was very similar for the low treatments, while it was significantly increased in the highest treatment (figure 4.6A, B, and C respectively; $P<0.05$).

In the low media concentrations it is difficult to be confident that there was a significant difference in the amount of boron, calcium or magnesium that was taken up and trans-located to new wood growth. In addition, the values obtained included boron, calcium and magnesium that was present in the existing wood at the time of culture. Previously, boron, calcium and magnesium were found to be present in radiata pine in both bound and free forms (T. Putoczki, unpublished results). The extent that these ‘free’ nutrients, present in the existing wood, can translocate to new wood growth is not known.

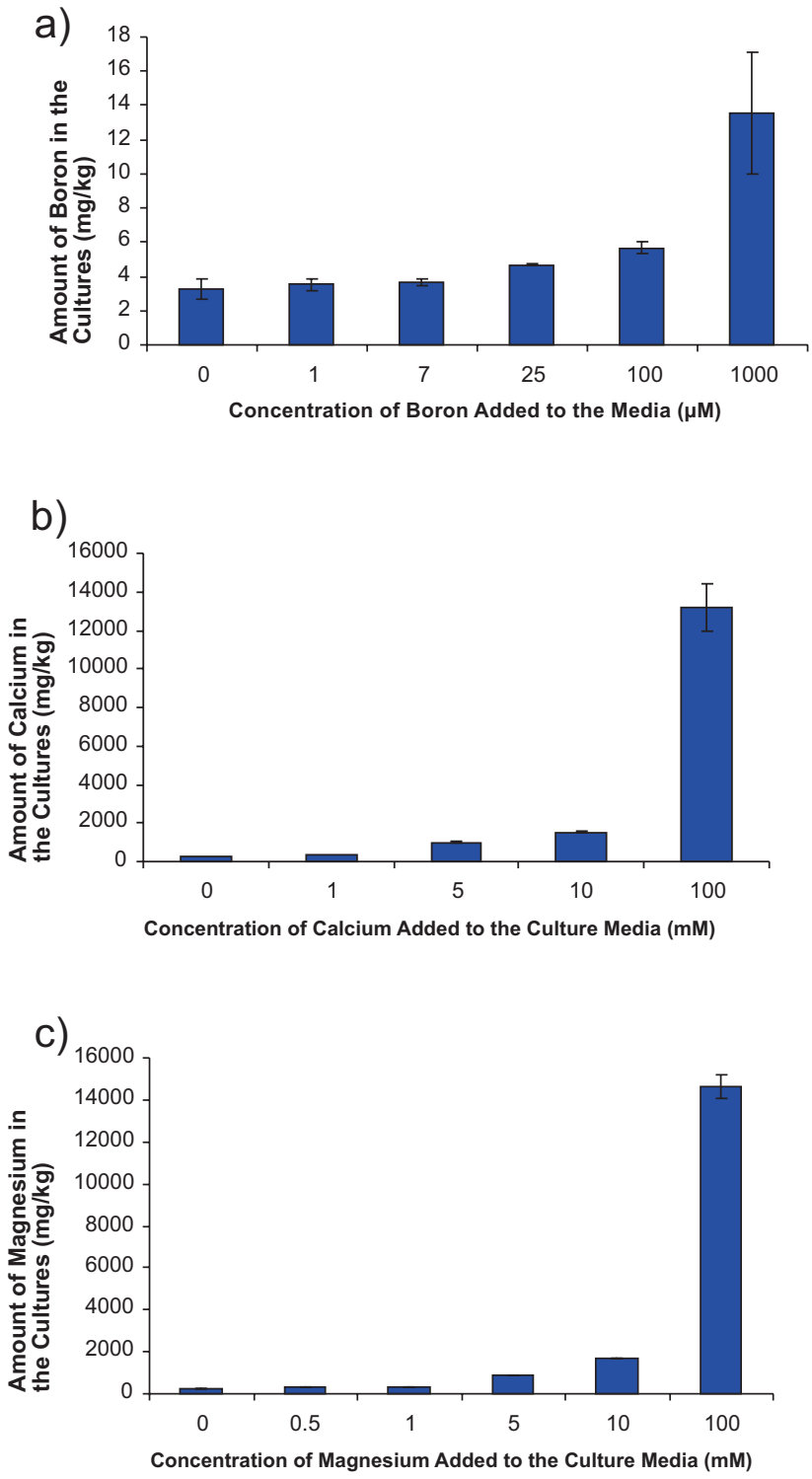


Figure 4.6 Relationship of nutrient concentration in the medium to the nutrient concentration in the culture. (A) Boron treated cultures. (B) Calcium treated cultures. (C) Magnesium treated cultures. Values were obtained by ICP-MS/OES and include two cultures per treatment. Inset shows the linear relationship between the nutrient concentration in the medium compared to the culture. Bars indicate standard error of the mean. Where no bars are indicated the standard error is too small to view, or a second sample was not analyzed.

4.3.1.2 Mixed-treated cultures

In the first pair of mixed-treated culture types, LMHC and HMLC, standard boron concentrations were added to the media. In these cultures, there was a much greater increase in calcium uptake in the LMHC cultures compared to the magnesium uptake in the HMLC cultures (figure 4.7). This increase was not the result of higher levels of these nutrients in the cultures at the time of explant, because their levels were not increased in their compared cultures types (ie. there was not a high level of calcium in the HMLC treated culture which would account for the extra calcium levels in the LMHC treated cultures).

In the second pair of mixed-treated culture types, HBCM and 0BCM, all three of the nutrients of interest were altered (figure 4.7). The amount of calcium in the HBCM cultures was lower than the in the LMHC treated cultures, suggesting that there may have been competition for uptake of these nutrients. In the 0BCM treated cultures the concentration of all three nutrients was low, as desired (figure 4.7).

In the third pair of mixed-treated culture types to be compared, OptiLB and Opti, the results suggest that similar amounts of boron, calcium and magnesium were present in these cultures (figure 4.7). This suggests that the OptiLB treated cultures may not have significantly less boron than the Opti treated cultures. In the fourth pair of culture types to be compared, LBHC and HBLC, standard magnesium concentrations were added to the media. The results suggest that the concentrations of these nutrients in the cultures were successfully altered (figure 4.7)

In plant foliage, boron concentrations generally range from 10 to 100 mg/kg dry weight [13, 14]. Radiata pine falls into the low end of this spectrum, with reported boron concentrations in foliage ranging from 5 to 40 mg/kg [15-18], while even lower levels, 3.7 to 4.7 mg/kg, have been reported for radiata pine wood [17]. Foliage levels are generally higher than the plant body because foliage is at the end of the transpiration stream [19]. The boron content in the low boron treated cultures (which includes the existing wood) falls below the 3.7 to 4.7 mg/kg range [17], while the higher boron treatments are above this range as desired.

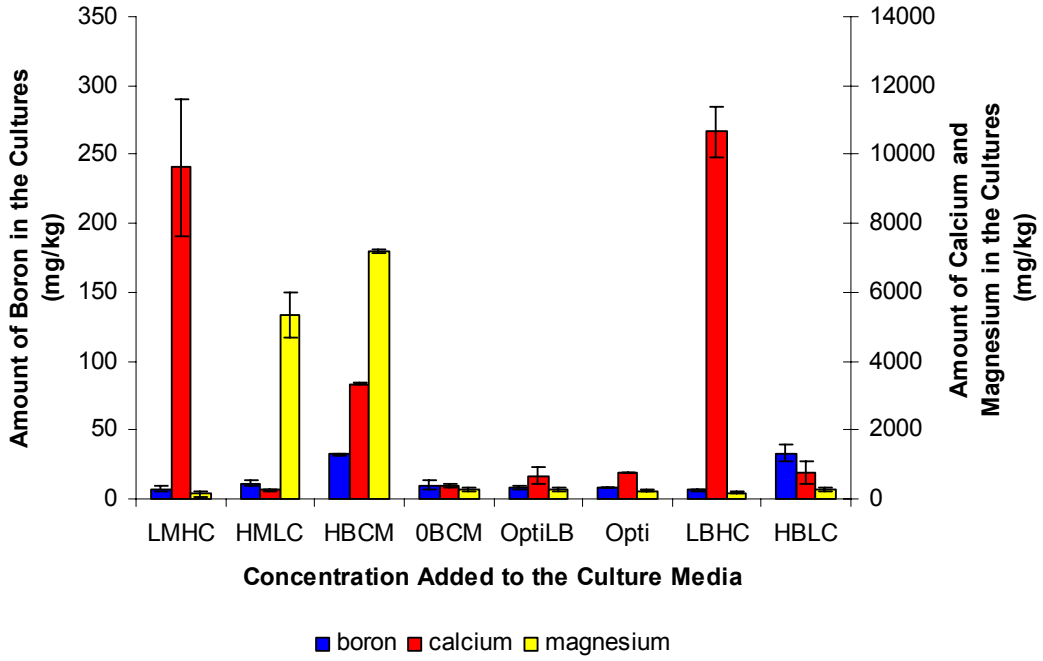


Figure 4.7 Amount of boron, calcium and magnesium present in the explants after being cultured on mixed media for a period of two months. Concentrations of calcium and magnesium added to the culture media are as follows: LMHC: 0 mM magnesium, 100 mM calcium; HMLC: 100 mM magnesium, 0 mM calcium; HBCM: 1000 µM boron, 100 mM calcium, 100 mM magnesium; 0BCM; 0 µM boron, 0 mM calcium, 0mM magnesium; OptiLB; 0 µM boron, 5 mM calcium, 5 mM magnesium; Opti: 7 µM boron, 5 mM calcium, 5 mM magnesium; LBHC: 0 µM boron, 100 mM calcium; HBLC: 1000 µM boron, 0 mM calcium. Boron values were obtained by ICP-MS, and calcium and magnesium values were obtained by ICP-OES, and include two cultures per treatment. Bars indicate standard error of the mean.

Normal plants contain 10 000 to 50 000 mg/kg of calcium, and 2 500 to 10 000 mg/kg magnesium [14]. Previous reports have suggested that 1000 mg/kg of calcium in radiata pine foliage is critical for growth, with concentrations around 2 470 mg/kg considered normal [19]. In healthy radiata pine, magnesium has been reported at 2 300 mg/kg in the foliage [19]. In chapter two (section 2.4.2.1), calcium in oven dried radiata pine disks was found to range between approximately 460 to 600 mg/kg similar to the calcium content in the low treated cultures. While magnesium in the oven dried radiata pine disks was found to be approximately 150 mg/kg, slightly less than found in the low magnesium-treated cultures. This shows that the high calcium and magnesium treatments contained higher than normal levels of these nutrients as desired.

4.4 Colour differences in the cultured wood

When the organ cultures were collected, they resembled the earlywood portion of an intact tree. When dry, the wood was of a comparable hardness, judged by cutting the samples with a razor blade. Moreover, the tracheids appeared to maintain a proper anatomical arrangement when viewed with a stereo-microscope. The only visible difference between the dissected cultures grown on the different nutrient treatments was the colour of the cultured wood. When the cultures were dissected, the wood grown on each of the culture treatments appeared slightly orange/red in colour when compared to the control explant collected at the time of culturing. This suggests that the colour change is an artefact of culturing. However, the extent to which the colour of the cultures changed appeared to be related to the culture treatment.

4.4.1.1 Boron-treated cultures

The cultures grown on media with high amounts of added boron (1000 μM) usually had the greatest orange/red colouration when compared to the control explant (figure 4.8). The remaining boron treatments were also slightly more orange/red in colour than the control explant (figure 4.8).

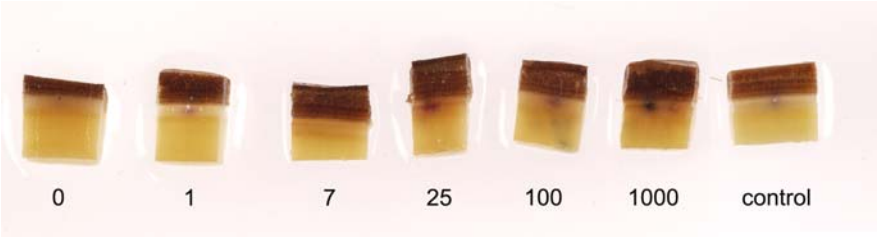


Figure 4.8 Dissected cultures grown on media containing from left to right, 0, 1, 7, 25, 10, 1000 μM of added boron, and the control explant collected at the time of culturing. The images are representative of the general observations made for all of the boron treated cultures.

4.4.1.2 Calcium-treated cultures

The cultures grown in the presence of low added levels of calcium (0 mM) generally had the greatest orange/red colouration when compared to the control explant collected at the time of culturing (figure 4.9), while the cultures grown on higher added calcium concentrations (100 mM) often resembled the colour of the control explants.

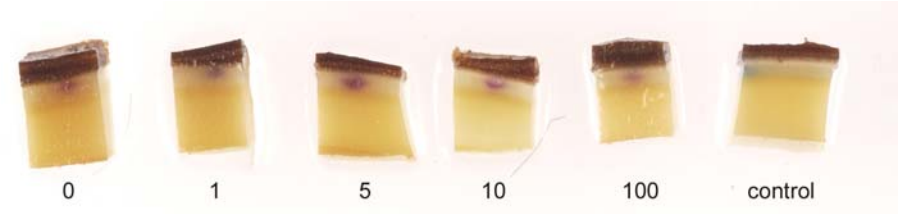


Figure 4.9 Dissected cultures grown on media containing from left to right, 0, 1, 5, 10, 100 mM of added calcium, and the control explant collected at the time of culturing. The images are representative of the general observations made for all of the calcium treated cultures.

4.4.1.3 Magnesium-treated cultures

Cultures grown in the presence of high added magnesium concentrations (100 mM) usually appeared much more orange/red in colour than those grown on the low added magnesium concentrations (0 mM) or the control explant (figure 4.10).

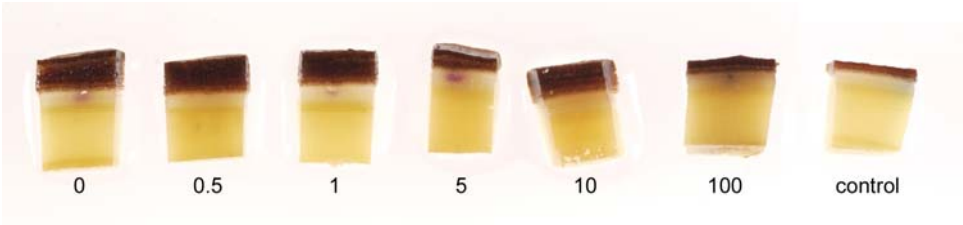


Figure 4.10 Dissected cultures grown on media containing from left to right, 0, 0.5, 1, 5, 10, 100 mM of added magnesium, and the control explant collected at the time of culturing. The images are representative of the general observations made for all of the magnesium treated cultures.

4.4.1.4 Mixed-treated cultures

In the cultures grown in the presence of a mixture of added boron, calcium and magnesium concentrations many variations in the orange/red colour were observed. Those grown on the LMHC media and those grown on the HMLC media generally showed no drastic alteration in wood colour (figure 4.11). However, the HMLC cultures did generally appear slightly more orange/red. This is consistent with the finding that the high magnesium cultures (100 mM; section 4.3.1.3) and the low calcium cultures (0 mM; section 4.3.1.2) were more orange/red in colour than the control explants.

The cultures grown on the HBCM media and the cultures grown on the 0BCM did not generally differ in colour from each other. In the presence of concentrations of

calcium and magnesium that were perceived to be optimal, but containing less boron (optiLB) the cultured wood generally appeared slightly more orange/red than the cultures that were grown on optimal concentrations of all three nutrients (Opti). Each of these culture types always appeared more orange/red than the control. The most drastic change in wood colour was usually observed in the cultures grown on the LBHC and HBLC media (figure 4.11).

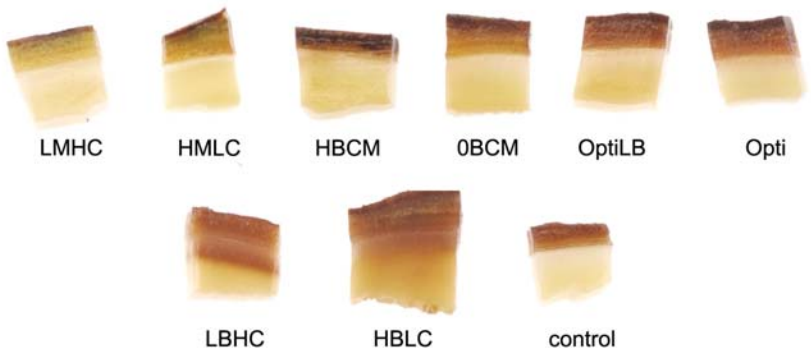


Figure 4.11 Concentrations of calcium and magnesium added to the culture media are as follows First row (left to right), LMHC: 0 mM magnesium, 100 mM calcium; HMLC: 100 mM magnesium, 0 mM calcium; HBCM: 1000 µM boron, 100 mM calcium, 100 mM magnesium; 0BCM; 0 µM boron, 0 mM calcium, 0 mM magnesium; OptiLB; 0 µM boron, 5 mM calcium, 5 mM magnesium; Opti: 7 µM boron, 5 mM calcium, 5 mM magnesium. Second row (left to right) LBHC: 0 µM boron, 100 mM calcium; HBLC: 1000 µM boron, 0 mM calcium.

4.5 Cell division

Observation of transverse sections of the cultures, using light microscopy, suggested that varying levels of growth could occur depending on the nutrient treatment the cultures were subject to. In order to support these observations quantitatively, three defined regions were used to count the number of cells present in radial cell files. The first region was defined as the 'cambial region'. This region was comprised of cells actively undergoing cell division and the newly divided cells, which were recognized by their small size and thin walls. The second area measured was defined as the region undergoing 'radial expansion'. This region was composed of cells actively undergoing enlargement in the radial dimension that were thin walled and included cells immediately following the cambial region, but preceding the cells that were undergoing cell wall lignin deposition. The final region, termed the 'new secondary wall region', consisted of the region of cells undergoing cell wall lignin deposition

until the point of identification of a mature cell. Each of these regions was compared to the control culture explant that was collected and fixed on the day of culturing. This process was performed on three culture sets, composed of explants obtained from two or three trees. The results from each culture set are presented in appendix one. Counts of cell numbers per radial file provided information about the activity of the cultures, and the preceding growth [20] and are presented in appendix one, along side the data obtained for the control explant.

4.5.1.1 Boron-treated cultures

Generally, the organ cultures grown on high added boron concentrations (1000 µM) had an increase in the number of cells in their cambial region (figure 4.12; table 4.1). In two of the culture sets, the cultures grown on high boron concentrations contained, on average 28%, more cells than the control explant, while those grown on low boron concentrations (0 µM) experienced an average decrease in cambial cell number of 4% (three culture sets; $P<0.05$). This suggests that a surplus of available boron may up-regulate cell division, while deficiency could limit it. However, the culture response to boron nutrition appears to be tree specific, as variation was observed (appendix one, tables 1-3).

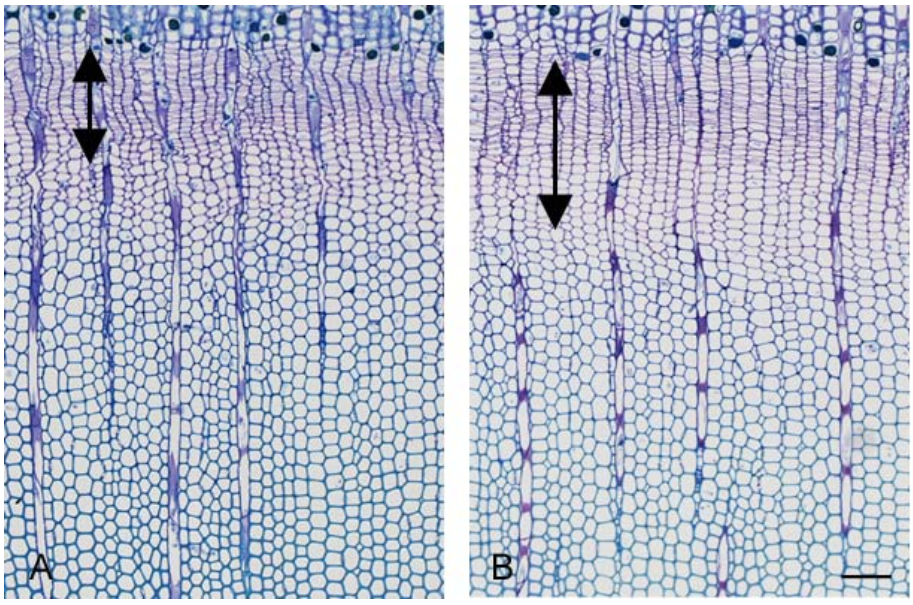


Figure 4.12 Cultures grown in high boron conditions generally experience an increase in cambial cell number. (A) Culture grown in low boron conditions (0 µM), (B) culture grown in high boron conditions (1000 µM). Arrow depicts cambial and radially expanding regions. Transverse sections, stained with toluidine blue and observed by light microscopy. Images were collected with a Zeiss Axioshop digital camera. Scale bar = 100 µm.

A significant increase in the radially expanding region cell number for the high boron-treated cultures (1000 μM), compared to the low boron-treated cultures (0 μM), was only observed in one of the culture sets (appendix one, table 2; $P<0.05$). Coupled with the increase in cambial cell number, these results suggest that high boron levels could effectively stimulate cell growth. However, in this culture set, the control explant and culture grown on the 1 μM added boron treatment contained a similar number of cells to the high boron treated cultures, highlighting the variability that can occur. Significant differences were present in the region of new secondary wall deposition; however, they did not follow any specific trends (table 4.1).

	Boron-treated culture sets		
	Set 1[a]	Set 2[b]	Set 3[c]
Cambial	high most low least	high most low least	moderate most low least
Radial expansion	no change	control and low most high middle moderate least	no change
New secondary wall	high most low least	moderate most low least	low most

[a] Rotorua tree, inducted in April and cultured for two months. Appendix 1, Table1
[b] Burnham tree, inducted in March and cultured for two months. Appendix 1, Table 2
[c] Burnham tree, inducted in November and cultured for one month. Appendix 1, Table 3

Table 4.1 General summary of the cell number observations for the boron-treated cultures. High refers to the 1000 μM boron treatments, moderate refers to the 7 and 25 μM boron treatments, and low refers to the 0 and 1 μM boron treatments. The measurements are presented in appendix one and the statistical analysis is presented in appendix two.

4.5.1.2 Calcium-treated cultures

While there were significant differences in the cell numbers within the calcium-treated culture sets, there were no consistent trends. Table 4.2 summarizes these observations (appendix 1, tables 4-6). These trees were all from the same clone, suggesting that the state of the tree at the time of culture (ie. time of year, rainfall, position in the field etc.), in addition to the genotype, is important when considering the affect calcium may have on growth. Moreover, two of these culture sets were from the same tree and their cell counts suggested similar trends, yet the treatments within one culture set did not have statistically significant results (appendix 1, tables 4 and

6). This suggests that natural variation within the trees themselves can influence the results.

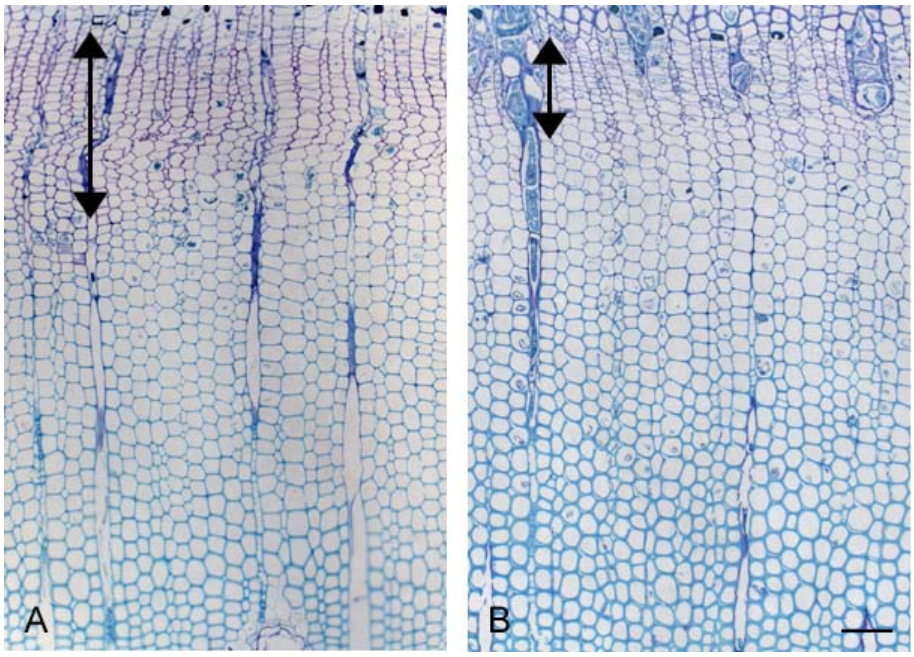


Figure 4.13 Cultures grown in low calcium conditions can experience an increase in cell number, and thus division, however this was not observed for all of the culture sets. (A) Culture grown in low added calcium conditions (0 mM), (B) culture grown under high added calcium conditions (100 mM). Transverse sections, stained with toluidine blue and observed by light microscopy. Images were collected with a Zeiss Axioshop digital camera. Scale bar = 100 µm.

	Calcium-treated culture sets		
	Set 1[a]	Set 2[b]	Set 3[c]
Cambial	low[d] most control and low[e] least	high most moderate least	no change
Radial expansion	low most moderate least	high most low least	low most
New secondary wall	moderate most low middle high least	no change	low most control least

[a] Burnham tree, inducted in March and cultured for two months. Appendix 1, Table 4
[b] Burnham tree, inducted in November and cultured for one month. Appendix 1, Table 5
[c] Burnham tree, inducted in March and cultured for two months. Appendix 1, Table 6
[d] 1 mM, [e] 0 mM

Table 4.2 General summary of the cell number observations for the calcium-treated cultures. High refers to the 10 and 100 mM calcium treatments, moderate refers to the 5 mM calcium treatments, and low refers to the 0 and 1 mM calcium treatments. The measurements are presented in appendix one and the statistical analysis is presented in appendix two.

The cultures grown in lower added calcium conditions (0 mM) had significantly more radially expanding cells than the cultures grown on the higher calcium treatments (100 mM; appendix 1, tables 4 and 6; $P<0.05$) for two culture sets. The explants for both of these culture sets were obtained from the same tree. In another culture set there were significantly more radially expanding cells in the culture grown in the higher calcium conditions (10 mM; appendix one, table 5; $P<0.05$). Variable results were observed for the cells undergoing secondary wall deposition and are summarised in table 4.3.

4.5.1.3 Magnesium-treated cultures

Other than in one culture set, there were no changes in the number of cells in the cambial or radially expanding region in the magnesium-treated cultures (table 4.3; appendix one, table 7). In two of the culture sets there were more cells in the region of new secondary wall deposition in the cultures grown on the low magnesium treatments (0 and 0.5 mM), compared to the moderate and high magnesium treatments (10 mM and 100 mM; appendix 1, tables 8 and 9; $P<0.05$). In another culture set, there were more cells in the region of new secondary wall deposition in the culture grown on the moderate magnesium treatment (10 mM) compared to the lower magnesium treatments (0 and 1 mM; figure 4.14; appendix one, table 7; $P<0.05$). The first and second culture sets were explanted from the same tree, and yielded contradictory results.

Changes in the number of cells present in each defined region occurred in the cultures grown on the various calcium treatments, while only one region (the new secondary wall) appeared to be affected in the cultures grown on the magnesium treatments. This suggests that calcium, and not magnesium, can alter cell division and/or the transition to each stage of development. However, it is clear that the state of the tree prior to culturing, and natural variation within a tree, can influence the results obtained.

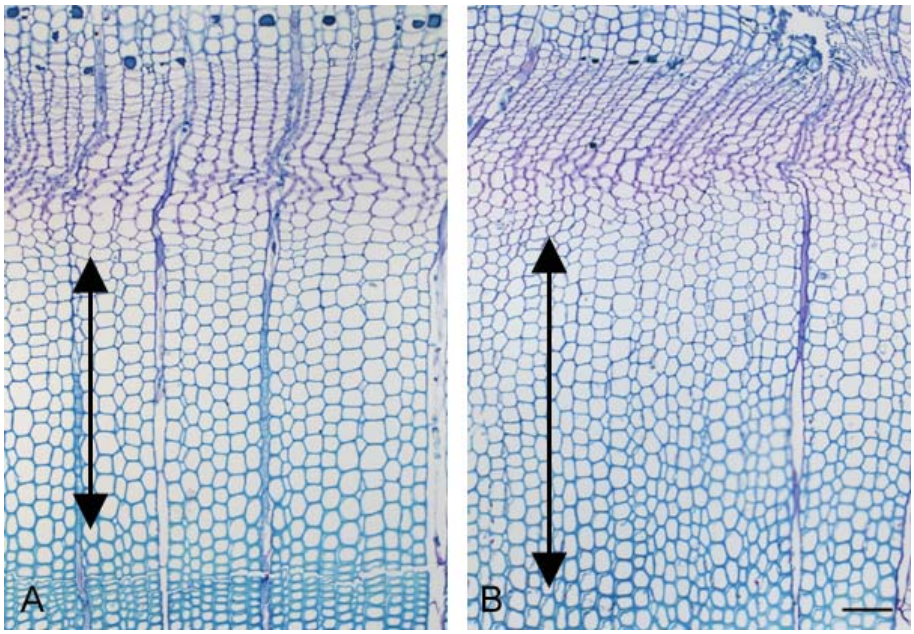


Figure 4.14 Cultures grown in high magnesium conditions can have more cells undergoing secondary wall deposition. However, this was not observed for all of the culture sets. (A) Culture grown on low added magnesium concentrations (0 mM), (B) culture grown under high added magnesium conditions (10 mM). Transverse sections, stained with toluidine blue and observed by light microscopy. Images were collected with a Zeiss Axioshop digital camera. Scale bar = 100 µm.

	Magnesium-treated culture sets		
	Set 1[a]	Set 2[b]	Set 3[c]
Cambial	low most moderate least	no change	no change
Radial expansion	no change	no change	no change
New secondary wall	moderate most low least	low most moderate least	low most high least

[a] Burnham tree, inducted in March and cultured for two months. Appendix 1, Table 7
[b] Burnham tree, inducted in March and cultured for two months. Appendix 1, Table 8
[c] Burnham tree, inducted in November and cultured for one month. Appendix 1, Table 9

Table 4.3 General summary of the cell number observations for the magnesium-treated cultures. High refers to the 100 mM magnesium treatments, moderate refers to the 5 and 10 mM magnesium treatments, and low refers to the 0, 0.5 and 1 mM magnesium treatments. The measurements are presented in appendix one and the statistical analysis is presented in appendix two.

4.5.1.4 Mixed-treated cultures

There were no discernable trends in the number of cells present in the mixed treatments (table 4.4; appendix one, tables 10-12). However, the results do suggest that changes in cell proliferation in the cultures occurred confirming that the cultures underwent cell division.

	Mixed-treated culture sets		
	Set 1[a]	Set 2[b]	Set 3[c]
Cambial	no change	HMLC most	Opti most HMLC, 0BCM least
Radial expansion	OptiLB, Opti, HBLC most	0BCM most HBLC, LMHC least	HBCM. LBHC, Opti most
New secondary wall	no change	0BCM most	Opti, HBCM least

[a] Burnham tree, inducted in March and cultured for two months. Appendix 1, Table 10
[b] Burnham tree, inducted in December and cultured for two months. Appendix 1, Table 11
[c] Burnham tree, inducted in January and cultured for two months. Appendix 1, Table 12

Table 4.4 General summary of the cell number observations for the mixed-treated cultures. The measurements are presented in appendix one and the statistical analysis is presented in appendix two.

4.6 Cell size

Observations of transverse sections of the organ cultures, stained with toluidine blue and viewed using light microscopy, suggested that the size of the developing cells could be altered by the mineral nutrient treatments. In order to quantify these changes, measurements of the cell lumen area, taken to be representative of cell size, were obtained from the end of the region of radial expansion (as defined in section 4.5) and the region containing mature cells (present at the time of culture), using Image Pro Plus software (Media Cybernetics Inc., Silverspring, USA). Measurements of the width of the cell lumen in the radial and tangential directions were also obtained using Image Pro Plus software. These measurements are presented in appendix one, along side the data obtained for the control explant.

4.6.1.1 Boron-treated cultures

There were no definitive trends in the lumen area, radial length or tangential width of the cells in the region of radial expansion, in the boron-treated cultures (table 4.5; appendix one, tables 1-3). Only one culture set had significant differences in these parameters; the low boron- treated cultures had lumens that were significantly smaller than the high boron-treated cultures (table 4.5; appendix 1, table 1). However, in this culture set one of the low boron-treated cultures (1 μM) had a larger lumen than all of the other culture treatments, highlighting the variability that can occur.

	Boron-treated culture sets		
	Set 1[a]	Set 2[b]	Set 3[c]
Developing wood cell lumen			
Area	high largest* low smallest	no change	no change
Length	high longest* low shortest	no change	no change
Width	high widest* low narrowest	no change	no change
Existing wood cell lumen			
Area	all more than control	no clear trend	no change
Length	all more than control	no change	no change
Width	no change	no clear trend	no clear trend

[a] Rotorua tree, inducted in April and cultured for two months. Appendix 1, Table 1
[b] Burnham tree, inducted in March and cultured for two months. Appendix 2, Table 2
[c] Burnham tree, inducted in November and cultured for one month. Appendix 3, Table 3
* The 1 μM boron treatment also had the greatest measurements

Table 4.5 General summary of the cell size observations for the boron-treated cultures. High refers to the 1000 and 100 μM boron treatments, moderate refers to the 7 and 25 μM boron treatments, and low refers to the 0 and 1 μM boron treatment. The measurements are presented in appendix one and the statistical analysis is presented in appendix two.

In the existing cells, there were significant differences in cell sizes; however, no definitive trends could be established (table 4.5). Changes in the size of the existing wood cells were not expected, as these cells were present and undergoing maturation

prior to culturing. These results suggest that, in some instances, boron can alter cell shape and size; however, more often differences could not be quantified. If differences were produced, they may have been concealed by the natural variability of cell size in these regions.

4.6.1.2 Calcium-treated cultures

There were no statistically significant differences in the lumen area of the radial expansion region in any of the calcium-treated culture sets (table 4.6; appendix 1, tables 4-6).

	Calcium-treated culture sets		
	Set 1[a]	Set 2[b]	Set 3[c]
Developing wood cell lumen			
Area	no change	no change	no change
Length	control longest low shortest	no change	no change
Width	no change	all wider than control	no change
Existing wood cell lumen			
Area	control largest low smallest	no change	low smallest
Length	control longest low shortest	no change	all larger than low
Width	no change	no change	high widest low narrowest

[a] Burnham tree, inducted in March and cultured for two months. Appendix 1, Table 4
[b] Burnham tree, inducted in November and cultured for one month. Appendix 1, Table 5
[c] Burnham tree, inducted in March and cultured for two months. Appendix 1, Table 6

Table 4.6 General summary of the cell size observations for the calcium treated cultures. High refers to the 100 mM calcium treatments, moderate refers to the 5 mM calcium treatment, and low refers to the 0 mM calcium treatment. The measurements are presented in appendix one and the statistical analysis is presented in appendix two.

Two culture sets showed significantly smaller cell lumens in the existing wood of the cultures grown on the low added calcium treatments (0 mM), compared to the other culture treatments and the control explant (table 4.7; appendix one, tables 4 and 6;

P<0.05). These low calcium-treated cultures also had shorter cells (in the radial dimension), when compared to the other calcium treatments (table 4.7; appendix one, tables 4 and 6; P<0.05). These results suggest that calcium may have a small influence on cell size in the existing wood, with a decrease in its availability resulting in smaller cell lumens. Since the existing cells would be undergoing secondary wall deposition, and not expansion [21], these changes in size are likely to do with alterations in the cell wall components.

4.6.1.3 Magnesium-treated cultures

In the radially expanding cells, only one of the culture sets showed significant differences in the lumen area (table 4.7; appendix one, table 8). In this culture set, the low magnesium-treated culture (0 mM) was significantly smaller than the other culture treatments (table 4.7). The length and width of these cells had the same trends (table 4.7).

	Magnesium-treated culture sets		
	Set 1[a]	Set 2[b]	Set 3[c]
Developing wood cell lumen			
Area	no change	all larger than low	no change
Length	no change	all longer than low	no change
Width	no change	all wider than low	no change
Existing wood cell lumen			
Area	all larger than moderate	all larger than low	all larger than low
Length	all longer than moderate	all longer than low	all longer than low
Width	no change	all wider than low	no change

[a] Burnham tree, inducted in March and cultured for two months. Appendix 1, Table 7
[b] Burnham tree, inducted in March and cultured for two months. Appendix 1, Table 8
[c] Burnham tree, inducted in November and cultured for one month. Appendix 1 ,Table 9

Table 4.7 General summary of the cell size observations for the magnesium-treated cultures. High refers to the 100 mM magnesium treatments, moderate refers to the 5 mM magnesium treatments, and low refers to the 0 and 1 mM magnesium treatment. The measurements are presented in appendix one and the statistical analysis is presented in appendix two.

In the wood that was present at the time of culturing, the low magnesium treatments in two culture sets were smaller in size and length compared to the other culture treatments (table 4.7; appendix one, tables 8 and 9). Overall, the results for the magnesium treated cultures are highly variable, suggesting that magnesium does not have a definitive role in determining cell size.

4.6.1.4 Mixed-treated cultures

Image analysis was performed on the cultures of most interest: HBLC and LBHC as well as the control explants for three culture sets. In one culture set, the control explant had the largest lumen area, while the HBLC explant had the smallest (table 4.8; appendix one, table 10; $P<0.05$). The same result was found for the length of the lumen in this culture set (table 4.8). However, there were no significant differences in the lumen area, length or width of the radially expanding cells or the existing wood cells within the remaining culture sets (table 4.8; appendix one, tables 10-12).

	Mixed-treated culture sets		
	Set 1[a]	Set 2[b]	Set 3[c]
Developing wood cell lumen			
Area	control largest	no change	no change
Length	control largest	no change	LBHC shortest
Width	no change	no change	no change
Existing wood cell lumen			
Area	no change	no change	no change
Length	no change	no change	no change
Width	no change	no change	no change

[a] Burnham tree, inducted in March and cultured for two months. Appendix 1, Table 10
[b] Burnham tree, inducted in December and cultured for two months. Appendix 1, Table 11
[c] Burnham tree, inducted in January and cultured for two months. Appendix 1, Table 12

Table 4.8 General summary of the cell size observations for the mixed-treated cultures. The measurements are presented in appendix one and the statistical analysis is presented in appendix two.

These results suggest that alteration of both of these two nutrients has very little affect on cell size and shape in the cultured explants. Coupled to the variability in cell division (section 4.5.2.4) this suggests that while these cultures show callus production, they may not have had a full xylogenic response.

4.7 Cell wall thickness

After completion of the measurements of the cell lumen area, length and width using Image Pro Plus software (section 4.6), the outer boundary of the image of the same cluster of cells was outlined, and the total area that the cells of interest occupied was calculated using the same software. This value was subtracted from the total lumen area, and is presented as a percent of the total cell area, to provide an estimate of the area of the cell occupied by the cell wall, taken to be representative of cell wall thickness. The results from each culture set are presented in appendix one, along side the data obtained for the control explant.

4.7.1.1 Boron, calcium, magnesium, and mixed-treated cultures

In the developing wood of one of the boron-treated culture sets (appendix one, table 1), and the existing wood of two of the boron-treated culture sets, cultures grown in low added boron conditions (0 μM) appeared to have a trend towards thicker cell walls, when compared to the cultures grown in high added boron conditions (100, 1000 μM; table 4.9; appendix one, tables 1-3; $P<0.05$). These results suggest that in low boron conditions, the cultures may respond by thickening their cells walls. These results were interpreted with caution as there was much variation within the culture treatments (appendix one, tables 1-3).

There were no definitive trends in the calcium or magnesium-treated culture sets (table 4.9; appendix one, tables 4-9). These results suggest that calcium and magnesium have a variable influence on the thickness of the cell walls. In the mixed-treated culture sets, there were no significant differences between the culture treatments (table 4.9; appendix one tables 13-15).

	Treated culture sets[a]		
	Set 1	Set 2	Set 3
Boron			
Developing wood	low thickest high thinnest	no change	no change
Existing wood	control and moderate thickest	low thickest high thinnest	low thickest high thinnest
Calcium			
Developing wood	high thickest low thinnest	no change	no change
Existing wood	all thicker than control	no change	low thicker than all
Magnesium			
Developing wood	high thickest	low thickest	no change
Existing wood	moderate thickest	low thickest	no change
Mixed			
Developing wood	no change	no change	no change
Existing wood	no change	no change	no change

[a] Information on each culture set is provided in the table legends in section 4.6

Table 4.9 General summary of the cell wall area observations for the treated cultures. Low treatments include 0 µM boron, 0 mM calcium and 0 mM magnesium. Moderate treatments include 7 boron µM, 5 mM calcium and 5 mM magnesium. High treatments include 100 and 1000 µM boron, 100 mM calcium and 100 mM magnesium. The measurements are presented in appendix one and the statistical analysis is presented in appendix two.

4.8 Cell wall organization

In the intra-ring checked samples, presented in section 2.3.1, changes in the ultra-structure of the cell wall, in the region of the CML/S_1 were observed. In order to determine if the mineral nutrient treatments could induce changes in this region, TEM was employed to observe their cell wall ultra-structure.

4.8.1 Transmission electron microscopy

Transverse sections of the organ cultures undergoing the early stages of secondary wall deposition, which were embedded in Spurr resin and stained with uranyl acetate and Sato's lead were observed with a Hitachi TEM at 75 kV. For this work, the low and high treated cultures were observed for two culture sets in duplicate.

4.8.1.1 Boron-treated cultures

In general, the cultures[1] grown under low added boron conditions (0 μM) had changes in the appearance of the CML/S_1, compared to those grown under high added boron conditions (1000 μM) and control explants collected at the time of culture (figure 4.15). In particular, the region of the CML/S_1 cell wall appeared to have darker striation deposits compared to the other cultures, suggesting changes in microfibrils or lignin deposition. Furthermore, this region generally appeared larger, or possibly "swollen" when compared to the high boron cultures, which appeared "tighter" (figure 4.15).

4.8.1.2 Calcium-treated cultures

The high calcium-treated cultures[2] (100 mM) generally appeared to have a more defined CML/S_1/S_2 cell wall interface (figure 4.16A, C) when compared to the those grown on the low added calcium concentrations (0 mM; figure 4.16B) and the control explant (figure 4.16D). The cultures grown on the low concentrations also had a tendency to appear denser, with more homogeneous staining, when compared to the control (figure 4.16).

---

[1] Boron-treated culture sets observed with lead staining: culture set number one (section 4.6) and an additional Burnham tree cultured in February.

[2] Calcium-treated culture sets observed with lead staining: culture sets from two Burnham trees, cultured in February.

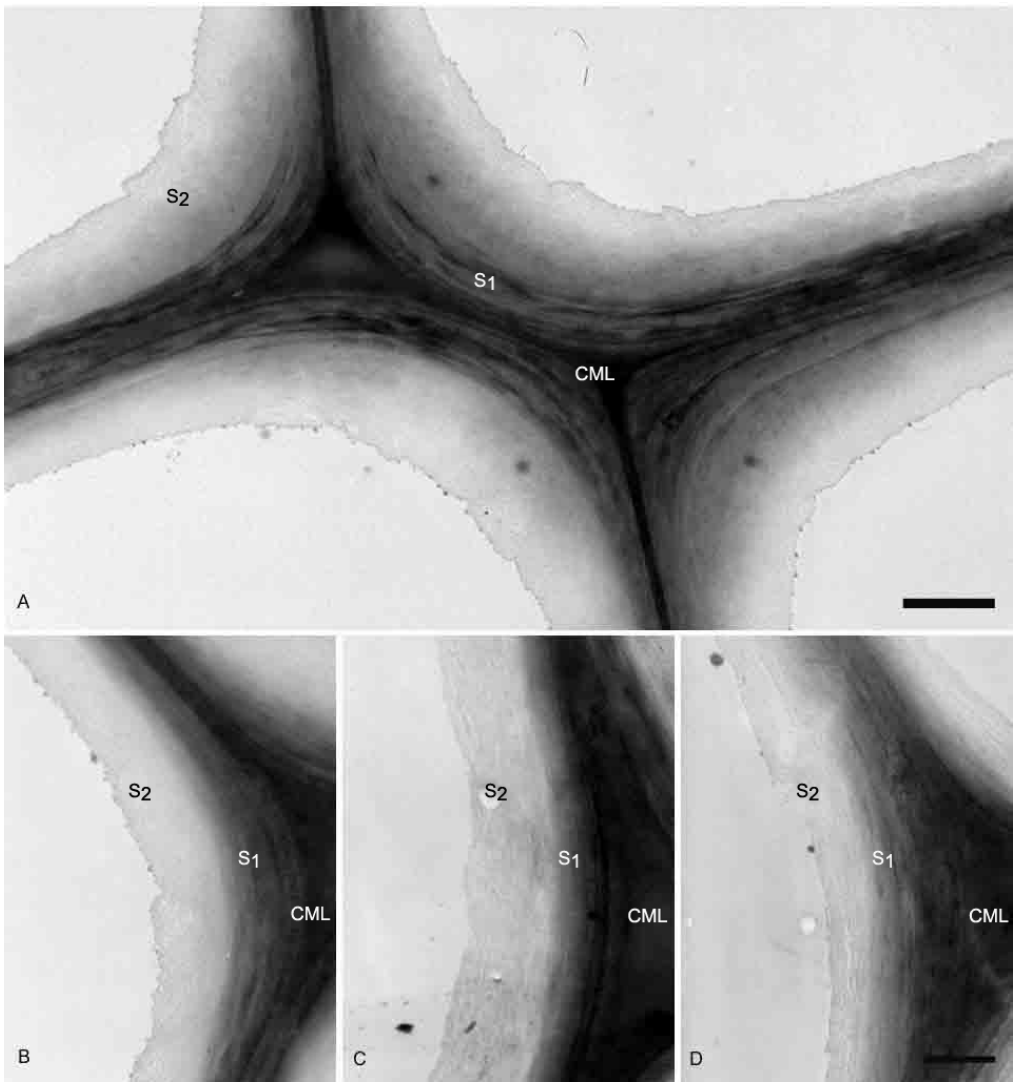


Figure 4.15 Alterations in the organization of the CML/S_1 region occur in different boron treatments. Cultures grown in (A-B) low added boron conditions (0 µM) present with an apparent increase in the size of the striated CML/S_1 wall regions when compared to the cultures grown in (C) higher boron conditions (1000 µM). Transverse sections of boron treated cultures embedded in Spurr resin and stained with uranyl acetate and Sato's lead. Images were collected on a Hitachi TEM at 75kV. Scale bars A = 2 µm and (B-D) = 1 µm, respectively.

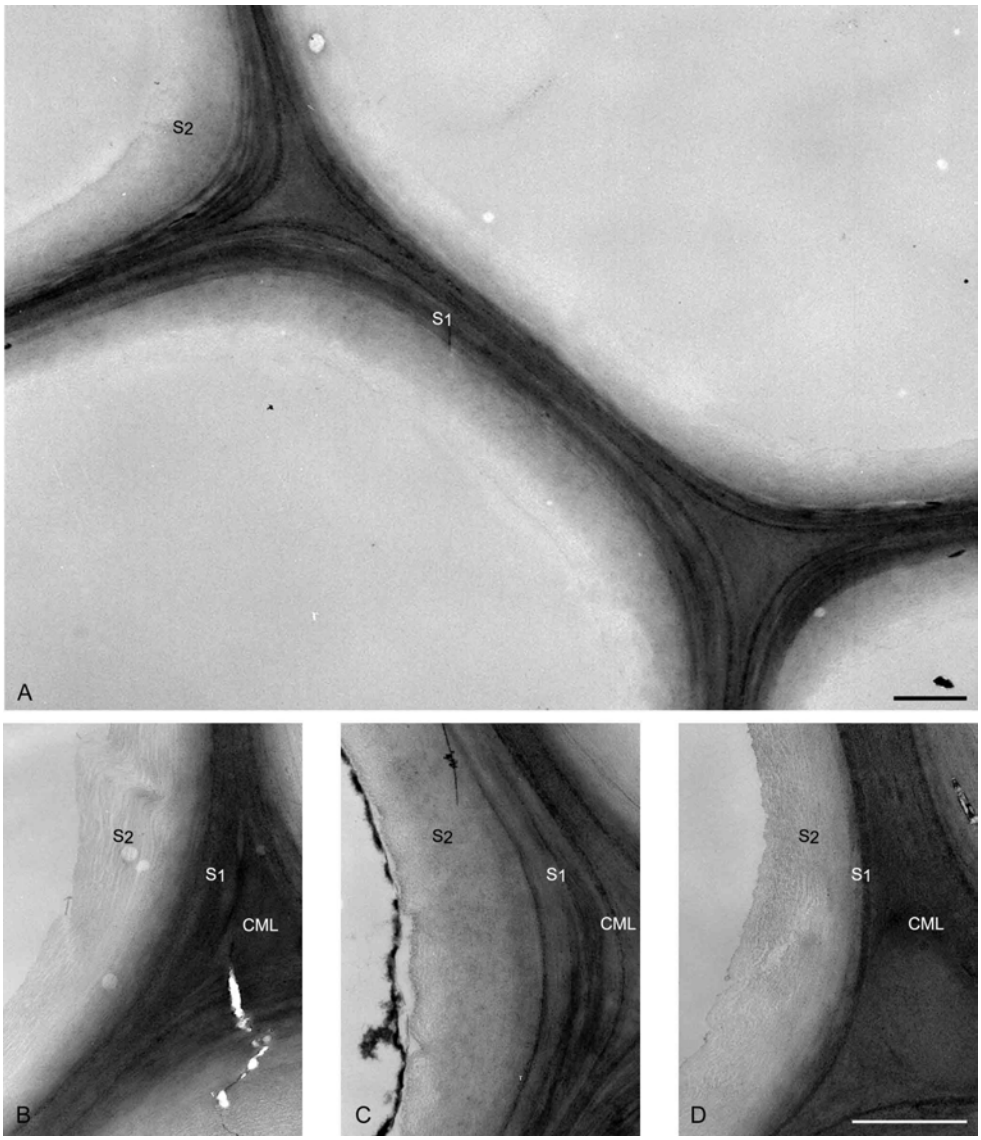


Figure 4.16 Alterations in the organization of the CML/S_1 region occur when calcium availability is changed. Cultures grown in (A), (C), high calcium conditions (100 mM) have a more prominent CML/S_1 cell wall region when compared to the cultures grown in (B) low calcium conditions (0 mM). The cultures grown in low calcium conditions appear to have a more homogenous CML/ S_1 region, compared to the striated CML/S_1 of the cultures grown in the high calcium conditions. (D) Control explant. Transverse sections of calcium treated cultures embedded in Spurr resin and stained with uranyl acetate and Sato's lead. Images were collected on a Hitachi TEM at 75 kV. Scale bars (A-D) = 2 µm.

4.8.1.3 Magnesium-treated cultures

In the cultures[3] treated with magnesium, there were no apparent differences in the organization of the cell wall interfaces (figure 4.17). However, the cultures grown in

[3] Magnesium-treated culture sets observed with lead staining: set number one and two (section 4.6).

the presence of low added magnesium (0 mM) often had less dense staining in the CML (figure 4.17A, 4.17B), compared to the high magnesium cultures (100 mM; figure 4.17C), and the control explant (figure 4.17D).

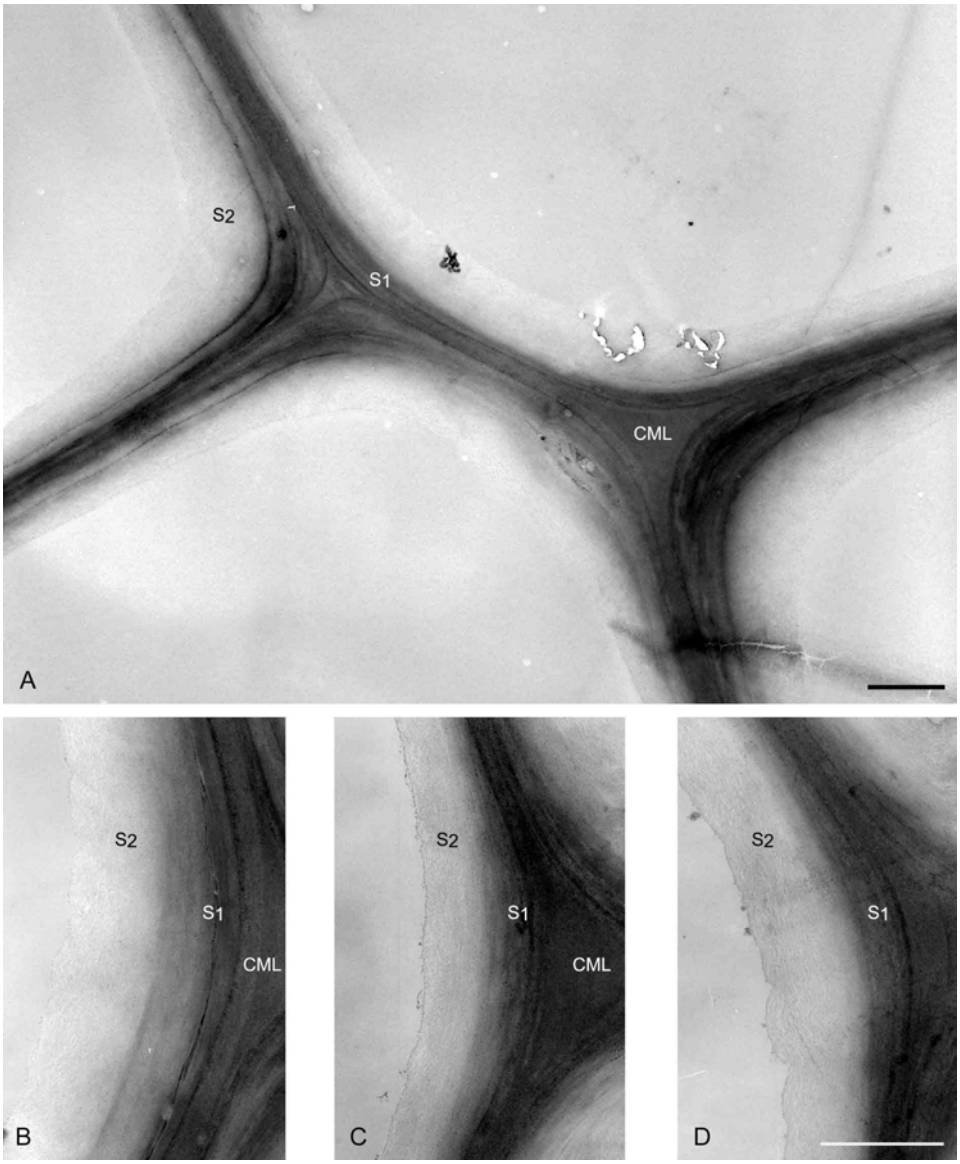


Figure 4.17 There were no apparent alterations in the organization of the CML when the availability of magnesium was changed. However, the CML of the cultures grown under (A-B) low added magnesium conditions (0 mM) did not always stain as densely as the (C) cultures grown in high magnesium conditions (100 mM). (D) Control explant. Transverse sections of magnesium cultures, embedded in Spurr resin and stained with uranyl acetate and Sato's lead. Images were collected on a Hitachi TEM at 75 kV. Scale bars (A-D) = 2 µm.

4.8.1.4 Mixed-treated cultures

There were no apparent differences in the CML/S_1 wall regions of the cultures[4] grown on the LBHC or HBLC media (figure 4.18). Similar to the magnesium treated cultures, differences in the staining intensity of the CML were observed.

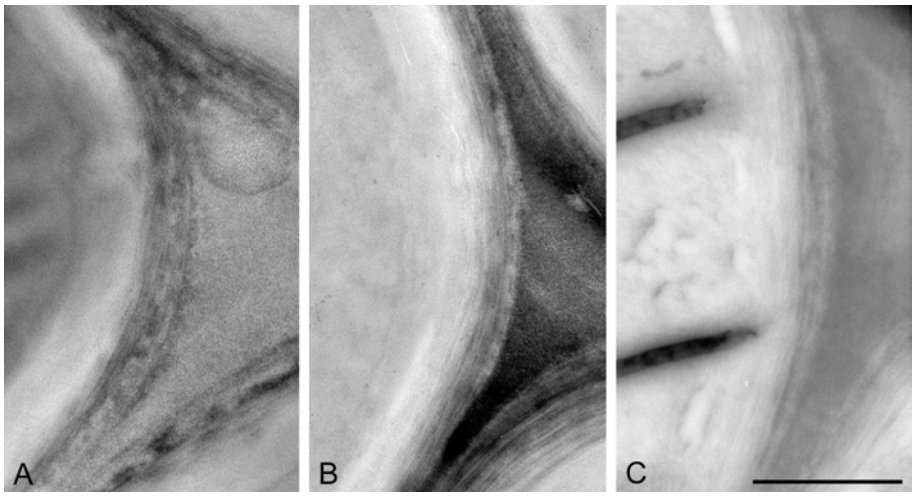


Figure 4.18 There were no apparent alterations in the organization of the CML/S_1 when the availability of boron and calcium were both changed. (A) LBHC treated culture (0 µM boron, 100 mM calcium, (B) HBLC treated culture (100 µM boron, 0 mM calcium, (C) control explant. Transverse sections, embedded in Spurr resin and stained with uranyl acetate and Sato's lead. Images were collected on a Hitachi TEM at 75 kV. Scale bar = 2 µm.

4.9 Discussion

The presence of callus around the periphery of the explants after approximately one week in culture provided an initial indication that induction was successful and that the cells in the cultures had undergone cell division. A distinct relationship between variation in the amount of callus, and its association with cambial division and xylem cell production, could not be established. However, the presence of callus did not indicate that a xylogenic response occurred. This is evidenced by the mixed treatment cultures, which often displayed callus but rarely showed significant differences in cell division and cell size. Sub-culturing was not found to sustain the cultures for a prolonged length of time, suggesting that endogenous factors required to maintain

[4] Mixed-treated culture sets observed with lead staining: set number three (section 4.6) and a culture set from a Burnham tree cultured in March.

growth were present in the wood at the time of culture, and were exhausted during the initial culture period [1, 9]. This is corroborated by the notion that culture growth would be favourable when initially induced on culture media where a large amount of nutrients were available, and then decrease as the nutrient supply was depleted [22]. When the cultures were transferred to fresh medium (sub-cultured), culture growth was not stimulated, suggesting that the presence of these nutrients alone does not promote growth. The importance of cell-cell contacts, and thus 'organ cultures' with cambium sandwiched between xylem and phloem, for proper wood formation was demonstrated when strips of xylem were cultured, in the absence of phloem, and/or xylem, and completely differentiated into callus (section 4.2.3).

ICP-OES/MS analysis of the cultures confirmed that uptake of the nutrients of interest had occurred. In chapter three, the concentration of nutrients added to the culture medium was detailed. It is not known how much of these nutrients are actually used by the cultures, and the extent that their uptake is driven by assimilation and/or growth [14, 22]. It is reasonable to assume that nutrient uptake in plants is dependent on the level of supply [22]. It is not surprising then, that the cultures grown on high concentrations of a nutrient of interest contained much higher levels than those grown on the lower concentrations. It should be noted though that the measured levels include those present in the existing wood, and as such, do not begin at low levels corresponding to the nutrient treatment concentration. This could not be avoided due to the nature of the culture system. It should also be mentioned that while significant movement of nutrients into the cultures can occur, unless the minerals are assimilated by the culture, movement out can also occur [22].

In order for the successful uptake of the nutrients to occur, ions must move through the medium to the point where entry into the explant is possible [22]. If differential diffusion of the nutrients of interest occurs, the balance and concentration of ions in close proximity to the explants could be affected, leading to preferential uptake [22]. Once the nutrients are taken into the explant, they must move through it, and finally are incorporated into it [22]. There are three basic mechanisms through which nutrients could be taken up into the explants: by diffusion along concentration gradients; through mass flow (involving transpiration); and active uptake (against a concentration gradient) [22]. In the organ cultures, the nutrients enter the wood by

passive diffusion. With no phloem in contact with the media in the 'chip explants', and very little in the 'scallop explants', the nutrients must enter the culture through the xylem. This is thought to occur through the ray cells, which *in arbor*, form a major pathway for the flow of nutrients to the zone of xylem differentiation [12]. While this mode of nutrient uptake is not identical to *in arbor* conditions, it does result in successful nutrient uptake, ultimately altering the nutrients available to newly dividing cells.

When the cultures were collected, the only visible difference between the wood of the culture treatments was their colour. As introduced in chapter one (section 1.4.2 and 1.4.3), in studies using transgenic tobacco and poplar, it was determined that the appearance of a red colouring was due to the polymerization of cinnamyl aldehydes, instead of cinnamyl alcohols, as a result of the down regulation of cinnamyl alcohol dehydrogenase (CAD) [23-25]. CAD is an enzyme involved in the formation of the monolignols: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol through the reduction of aldehydes to alcohols (section 1.3.3.6). The production of coniferyl alcohol in radiata pine is likely to involve CAD, and down regulation of this enzyme in response to the nutrient treatments may account for the orange/red colouration observed. The potential for altered lignification will be discussed further in chapter six. Sterilization procedures, in addition to excision, are also known to cause a wound response in wood tissue that can result in the production of phenolic substances creating a brown colouration [26]. Previous reports suggest that evidence of the oxidation of the phenolic compounds in the tissues is observed as a browning of the medium or explant [26]. If the colour changes observed in the organ cultures are a wounding response, they are enhanced by the nutrient treatments, and not the excision of the culture alone, as relatively consistent differences in colour between the treatments were observed (section 4.4).

In the cultures prepared by Savidge (1993) [9] and Leitch (1999) [10], axial parenchyma cells were formed following excision of the culture, creating a visible marker for the start of *in vitro* wood formation. Axial parenchyma was believed to be a cambial response to being removed from the stem and placed in culture [9, 10]. In the work by Savidge (1993) [9], dormant cambium was used for the explants, so that latewood functioned as a marker for the start of *in vitro* wood formation. In the

present work, axial parenchyma cells were not observed, and dormant material could not be used because, in New Zealand, radiata pine latewood tracheids do not become fully differentiated right up to the cambial region given that total dormancy does not occur [27]. In the first two years of this work, Janus green dye was injected into the cambium (section 3.2.3.5) to mark the start of culture. In the final year of culture collection, the dye injection method was abandoned and counting the number of cells in a radial file was adopted to determine if cell division had occurred, as the Janus green injection was found to increase fungal contamination. In addition, when dissected explants were viewed with a stereo microscope, the injection site was not observed to have moved, even when the cell counts for the culture suggested division. This is likely due to the damage the injection incurred to the fragile cambial cells.

While there were no consistent trends in the amount of cell division that occurred as a consequence of the individual nutrient treatments, the results do suggest that division had occurred, as differences between cultures were apparent. These cultures underwent successful periclinal divisions, and maintained radial file continuity. The number of cambial cells in the nutrient treated cultures was generally similar, while the number of cells in the radially expanding zone was more variable. In the work by Savidge (1993), organ cultures were found to grow a maximum of 1 mm of earlywood *in vitro*, or greater than 20 new tracheids, which is not comparable to the 5 to 8 mm that they would have grown *in arbor* [9]. In this work, a maximum of approximately 10 to 15 tracheids were formed when cell division occurred. *In vitro* stimulation of complete annual ring formation remains to be achieved, because many of the factors required for ongoing cambial growth remain to be determined [9]. Throughout this thesis the term ‘existing cells’ was used in reference to the cells present a great number of cells away from the cambium (ie. more than 20 cells) as they would have been present at the time of culture.

The measured tangential lumen diameter of the cultures, including all of the culture treatments, ranged from 24 to 32 μm, which is in good agreement with those found in radiata pine field trials which ranged from 23 to 30 μm [28], and other radiata pine studies, which ranged from 26 to 36 μm in tangential diameter [29-32]. The measured radial lumen diameter in this study ranged from 32 to 42 μm, which is also in agreement with the 33 to 45 μm range found in other radiata pine studies [29-32].

While the lumen area of the measured cultures ranged from 497 to 1008 μm, similar to values previously reported for radiata pine which ranged from 622 to 860 μm [32]. The discrepancy in the range found in this study arises from the measurement of both radially expanding and mature cells.

Previous studies have found a reduction in overall plant growth, cell elongation, expansion and alterations in cell wall thickness to be associated with boron deficiency [33-38]. In the present study, a decrease in cell division in some of the low boron treated cultures was observed, while changes in cell expansion, interpreted by the size of the developing lumen, were not clear. In studies using *Chenopodium album* suspension cultures, the rate of growth of the boron deficient cultures was the same as those grown on very high boron concentrations, yet the cultures differed visually, suggesting that boron does not influence culture growth but, rather, other developmental processes [39]. However, in embryrogenic cultures of *Larix deciduas,* faster development was observed at concentrations of 1 mM boron, in contrast to concentrations below 35 μM where development was blocked [40]. Some researchers believe that boron's primary role is in expansion rather than division, and that the absence of cell division in boron deficient conditions is the result of abnormalities in the formation of the cell wall, which prevent the cell from becoming organized for mitosis [33, 41-43]. Greater than 90% of cellular boron is localized to the cell walls of cultured tobacco BY-2 cells [44], suggesting that it is here that alterations in cell development, as a result of boron availability, should be observed.

The CML and S_1 wall regions of the boron deficient cultures appeared disorganized, and the cell walls in some of the cultures were thicker, as expected based on previous reports in various plant systems [45-51]. This is in contrast to studies on radiata pine field trials, where high boron applied in fertilizer resulted in an increase in the thickness of the cell walls and lumen diameter [28]. However, a small cell sample size (< 50 cells per tree) was analysed in this field trial and the authors acknowledge the anomaly of their results.

Calcium is a multi-functional nutrient, classically known for its involvement in signalling pathways [52]. Symptoms of calcium deficiency that can be visualized in plants include death of growing points and weakened stems [53]. In other plant systems, high calcium conditions have been found to inhibit cell expansion [54].

Moreover, in a review of crop fertilization, addition of calcium to crop plants has been suggested to increase cell wall thickness [53]. In this study, a link between cell division, determined by the number of cambial cells, and cell size as a result of calcium availability could not be established. Calcium deficiency in *Pinus taeda* has previously been shown to decrease cell division and decrease the size of meristimatic cells in addition to decreasing the number of cells between the meristem and lignified tissue [55, 56]. In addition, a correlation between cell wall thickness and calcium availability could not be established in this study. Calcium has been reported to account for between 60 and 72% of the cell wall of plants it is here that alterations in growth, as a result of changing calcium availability was expected [54, 57]. In soft fruits, calcium has been implicated in the strengthening of wall structure [58]. In this study, cultures grown in high added calcium conditions (100 mM) appeared to have a more organized CML/S_1 than those that were not, suggesting that alterations in the cell wall may occur as a result of calcium availability.

Magnesium deficiency in radiata pine is associated with the loss of crown growth [59]. It has been reported that greater than 70% of magnesium is diffusible in a plant [60]. Many studies overlook the influence of magnesium on cell division and expansion, in favour of its more obvious role in enzyme mediated activities [60, 61]. It is difficult to determine the *in vivo* role of magnesium in organ culture growth because it can be substituted for by manganese [60]. For this reason, it is not surprising that in this study, there were no clear trends in cell division, or cell size, as a result of magnesium treatment.

It has been well established that genetically identical trees grown on the same, or different sites can produce wood with very dissimilar properties [[62, 63] and references therein]. These differences are likely to arise, in part, as a result of the geographical location of a tree, resulting in differences in external influences such as temperature and rainfall [63]. This may account for some of the variability observed in the culture sets that were obtained from trees of the same clone. Moreover, many complex, and not completely understood, interactions occur in developing xylem cells [64, 65]. It has previously been suggested that experiments examining the effects of the environment on wood structure often produce conflicting results [64]. Earlier reports also suggest that nutrient availability may have significant effects on xylem

cell dimensions, although these effects are usually small [64]. Similar to these suggestions, consistent changes in cell division, expansion and wall thickness could not be established in this work. While these parameters are easily measured using microscopy and image analysis software, the 'between-tree' and 'within-culture' variation makes meaningful comparisons difficult. However, changes in cell dimension are not necessarily correlated with changes in secondary wall development. The final diameter of xylem cells is achieved during the phase of radial enlargement, after it has emerged from the cambial zone [66]. The secondary wall is formed during the subsequent maturation phase, and involves processes that may be entirely unrelated to division and expansion [66]. For this reason, chapter five and chapter six of this thesis will focus on determining if important structural elements in the cell walls of the cultures can be characterized and compared.

4.10 Summary

The detailed characterization of the organ cultures presented in this chapter provided a means by which different aspects of culture growth could be assessed to determine whether wood formation could be meaningfully measured. Culture induction was successful, evidenced by callus formation. ICP-OES/MS effectively showed that uptake of the nutrients of interest occurred during the culture period, and dissection of the cultures revealed differences in the colour of cultured wood. However, image analyses, used to obtain radial cell file counts, and measurements of the lumen area, length and width, and wall area yielded ambiguous results. A large amount of variation was found within, and between, culture sets suggesting that 'between-tree' and 'within-culture' variation hindered meaningful assessment of the affect of the mineral nutrients on these aspects of wood formation. In order to overcome this obstacle, a very large sample size would be required to adequately determine if significant influences on culture growth could occur. However, despite this variation, some of the treatments did show altered growth characteristics with respect to each other. In particular, the boron treated cultures most often showed trends in cell division and cell wall thickness, in addition to an altered CML/S_1 wall region observed by TEM.

4.11 References

1. Leitch, M.A., Savidge, R.A. 1995. *Evidence for auxin regulation of bordered-pit positioning during tracheid differentiation in Larix laricina.* International Association of Wood Anatomists, **16**: 289-297.

2. Brown, C.L. 1964. *The influence of external pressure on the differentiation of cells and tissues culture in vitro*, in *The Formation of Wood in Forest Trees*, Zimmerman, M.H., Ed. Academic Press: New York. 389-404.

3. Barker, W.G. 1953. *A contribution to the concept of wound repair in woody stems.* Canadian Journal of Botany, **32**: 486-490.

4. White, P.R. 1967. *Sites of callus production in primary explants of spruce tissue.* American Journal of Botany, **54**: 1055-1059.

5. Barnett, J.R., Asante, A.K. 2000. *The formation of cambium from callus in grafts of woody species*, in *Cell and Molecular Biology of Wood Formation*, Savidge, R.A., Barnett, J.R., Napier, R., Ed. BIOS Scientific Publishers Ltd.: Oxford. 155-167.

6. Moller, R., McDonald, A.G., Walter, C., Harris, P.J. 2003. *Cell differentiation, secondary cell-wall formation and transformation of Pinus radiata D. Don.* Planta, **217**: 736-747.

7. Brown, C.L., Wodzicki, T.J. 1969. *A simple technique for investigating cambial activity and the differentiation of cambial derivatives.* Forest Science, **15**: 26-29.

8. Aloni, R. 1980. *Role of auxin and sucrose in the differentiation of sieve and tracheary elements in plant tissue cultures.* Planta, **150**: 255-263.

9. Savidge, R.A. 1993. *In vitro wood formation in 'chips' from merchantable stem regions of Larix laricina.* International Association of Wood Anatomists, (14): 3-11.

10. Leitch, M.A. 1999. *The development of tissue culture techniques to study wood formation in Eucalyptus globulus Labill*. PhD Thesis, *Institute of Land and Food Resources*, University of Melbourne.

11. Brown, C.L., Sax, K. 1962. *The influence of pressure of the differentiation of secondary tissues.* American Journal of Botany, **49**: 547-682.

12. Barnett, J.R. 1981. *Secondary xylem development*, in *Xylem Cell Development*, Barnett, J.R., Ed. Castle House Publications Ltd.: Kent. 47-95.

13. Power, P.P., Woods, W.G. 1997. *The chemistry of boron and its speciation in plants.* Plant and Soil, **193**: 1-13.

14. Baker, D.A. 1983. *Uptake of cations and their transport within the plant*, in *Metals and Micronutrients: Uptake and Utilization by Plants*, Robb, D.A., Ed. Academic Press: London. 1-19.

15. Kimberley, M., Beets, P., Graham, D., McConchie, M. 2004. *Variation in intra-ring checking in radiata pine and implications on field trial design and sampling*, in *Confidential Report*, APP 33. Yong, G., Editor. Wood Quality Initiative Ltd. p. 1-12.

16. Beets, P., Kimberley, M., McConchie, M., Pearce, S. 2004. *Aluminum interactions and intra-ring checking*, in *Confidential Report*, APP 26. Young, G., Editor. Wood Quality Initiative Ltd. p. 1-24.

17. Xue, J., Olykan, S., Beets, P., McConchie, M., Graham, D., Leckie, A., Kimberley, M., Pearce, S. 2004. *Effect of boron, genotype and site on tree growth, intra-ring checking and other wood properties of radiata pine*, in *Confidential Report*, APP 38. Young, G., Editor. Wood Quality Initiative Ltd. p. 1-19.

18. Turvey, N.D., Carlyle, C., Downes, G.M. 1992. *Effects of micronutrients on the growth and form of two families of Pinus radiata (D.Don) seedlings*, in *Stem deformation in Pinus radiata*, Downes, G., Turvey, N.D., Ed. School of Forestry, University of Melbourne: Melbourne. 23-33.

19. Turner, J., Lambert, M.J. 1986. *Nutrition and nutritional relationships of Pinus radiata.* Annual Review of Ecology and Systematics, **17**: 325-50.

20. Savidge, R.A. 2000. *Biochemistry of seasonal cambial growth and wood formation - an overview of the challenges*, in *Cell and Molecular Biology of Wood Formation*, Savidge, R.A., Barnett, J.R., Napier, R., Ed. BIOS Scientific Publishers Ltd.: Oxford. 1-29.

21. Harris, P.J. 2005. *Non-cellulosic polysaccharides in plant cell walls: their diversity, organization and approaches to understanding their functions*, in *The Hemicellulose Workshop*, Entwistle, K.M., Walker, J.C.F., Ed. Wood Technology Research Centre: Christchurch. 13-35.

22. Williams, R.R. 1993. *Mineral nutrition in vitro - a mechanistic approach.* Australian Journal of Botany, **41**: 237-51.

23. Higuchi, T. 1985. *Biosynthesis of lignin*, in *Biosynthesis and Biodegradation of Wood Components*. Academic Press: New York. 141-160.

24. Halpin, C., Abbott, J, Barakate, A 2000. *Investigating lignin biosynthesis using transgenic mutant plants*, in *Cell and Molecular Biology of Wood Formation*, R.A. Savidge, J.R.B., R. Napier, Ed. BIOS Scientific Publishers Ltd.: Oxford. 425-436.

25. Baucher, M., Chabbert, B., Pilate, G., VanDoorsselaere, J., Tollier, M.T., PetitConil, M., Cornu, D., Monties, B., VanMontagu, M., Inze, D., Jouanin, L., Boerjan, W. 1996. *Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar.* Plant Physiology, **112**: 1479-1490.

26. Le Roux, J.J., Van Staden, J. 1991. *Micropropagation and tissue culture of Eucalyptus - a review.* Tree Physiology, **9**: 435-477.

27. Harris, M.J. 1991. *Formation of wood and bark*, in *Properties and Uses of New Zealand Radiata Pine*, Kininmonth, J.A., Whitehouse, L.J., Ed. New Zealand Ministry of Forestry, Forest Research Institute: Rotorua.

28. Skinner, M.F., Han, C.S., Singh, A.P. 2003. *Boron deficiency and tracheid properties of Pinus radiata.* New Zealand Journal of Forestry Science, **33**(2): 273-280.

29. Nyakuengama, J.G., Evans, R., Matheson, C., Spencer, D., Vinden, P. 1999. *Wood quality and quantitative genetics of Pinus radiata D. Don: fibre traits and wood density.* Appita Journal, **52**: 348-357.

30. Shelbourne, T., Evans, R., Kibbelwhite, P., Charles, L. 1997. *Inheritance of tracheid transverse dimensions and wood density in radiata pine.* Appita Journal, **50**: 47-50.

31. Donaldson, L.A. 1995. *Cell wall fracture properties in relation to lignin distribution and cell dimensions among three genetic groups of radiata pine.* Wood Science and Technology, **29**: 51-63.

32. Jackson, S., Nair, H. 2003. *Radial fibre collapse potential and cell wall lignin as indicators of intra-ring checking in radiata pine*, in *Confidential Report*, APP 10. Young, G., Editor. Wood Quality Initiative Ltd.

33. Dell, B., Huang, L. 1997. *Physiological response of plants to low boron.* Plant and Soil, **193**: 103-120.

34. Kakegawa, K., Ishii, T., Matsunga, T. 2005. *Effects of boron deficiency in cell suspension cultures of Populus alba L.* Plant Cell Reports, **23**: 573-578.

35. Jiao, X.Y., Zhu, Y.G., Jarvis, B.C., Quick, W.P., Christie, P. 2005. *Effects of boron on leaf expansion and intercellular airspaces in mung bean in solution culture.* Journal of Plant Nutrition, **28**: 351-361.

36. Albert, L.S., Wilson, C.M. 1960. *Effect of boron on elongation of tomato root tips.* Plant Physiology, **36**: 244-251.

37. Cohen, M.S. 1977. *Effect of boron on cell elongation and division in squash roots.* Plant Physiology, **59**: 884-887.

38. Ghanati, F., Morita, A., Yokota, H. 2001. *Selection and characterization of a boron tolerant tobacco cell line.* Soil Science and Plant Nutrition, **47**: 405-410.

39. Fleischer, A., Titel, C., Ehwald, R. 1998. *The boron requirement and cell wall properties of growing stationary suspension-cultured Chenopdium album cells.* Plant Physiology, **117**: 1401-1410.

40. Behrendt, U., Zoglauer, K. 1996. *Boron controls suspensor development in embryogenic cultures of Larix decidua.* Physiologia Plantarium, **97**: 321-326.

41. Whittington, W.J. 1959. *The role of boron in plant growth. II. The effect of growth on the radicle.* Journal of Experimental Botany, **10**: 93-103.

42. Fleischer, A., O'Neil, M.A., Ehwald, R. 1999. *The pore size of non-graminaceous plant cell walls is rapidly decreased by borate ester cross-linking of the pectic polysaccharide rhamnogalacturonan-II.* Plant Physiology, **121**: 829-38.

43. Dugger, W.M. 1983. *Boron in plant metabolism*, in *Inorganic Plant Nutrition*, Lauchi, A., Ed. 627-650.

44. Kobayashi, M., Ohno, K., Matoh, T. 1997. *Boron nutrition of cultured tobacco BY-2 cells. II. Characterization of the boron-polysaccharide complex.* Plant Cell Physiology, **38**(6): 676-683.

45. Matoh, T., Takasaki, M., Takabe, K. 2000. *Boron nutrition of cultured tobacoo BY-2 cells: III. Characterization of the boron rhamnogalacturonan II complex in cells acclimated to low levels of boron.* Plant Cell Physiology, **39**: 483-91.

46. Ishii, T., Matsunga, T., Hiyash, N. 2001. *Formation of rhamnogalacturonan II-borate dimer in pectin determines cell wall thickness in pumpkin tissue.* Plant Physiology, **126**: 1698-705.

47. Stark, J.R. 1963. *Effect of boron on the cell wall structure in sunflower.* Acta Societatis Botanicorum Poloniae, **32**: 619-623.

48. Lee, S.G., Aronoff, S. 1966. *Investigations on the role of boron in plants. III. Anatomical observations.* Plant Physiology, **41**: 1570-1577.

49. Hirsh, A.M., Torrey, J.G. 1980. *Ultrastructural changes in sunflower root cell in relation to boron deficiency and added auxin.* Canadian Journal of Botany, **58**: 856-866.

50. Kouchi, H., Kumazawa, K. 1976. *Anatomical responses of root tips to boron deficiency. III. Effect of boron deficiency on sub-cellular structure of root tips, particularly on morphology of cell wall and its related organelles.* Soil Science and Plant Nutrition, **41**: 53-71.

51. Matoh, T., Ishigaki, K., Mizutani, M., Matsunga, W., Takabe, K. 1992. *Boron nutrition of cultured tobacco BY-2 Cells. I. Requirement for and intracellular location of boron and selection of cells that tolerate low levels of boron.* Plant Cell Physiology, **33**: 1135-1141.

52. Tucker, E.B., Boss, W.F. 1996. *Mastoparan-induced intracellular Ca $^{2+}$ fluxes may regulate cell-to-cell communication in plants.* Plant Physiology, **111**: 467.

53. Easterwood, G.W. 2002. *Calcium's role in plant nutrition.* Fluid Journal, **Winter**: 1-3.

54. Wehr, J.B., Menzies, N.W., Blamey, F.P. 2004. *Inhibition of cell-wall autolysis and pectin degradation by cations.* Plant Physiology and Biochemistry, **42**: 485-492.

55. Wardrop, A. 1981. *Lignification and xylogenesis*, in *Xylem cell development*, Barnett, J., Ed. Castle House Publications Ltd.: Kent. 115-151.

56. Davis, D.E. 1949. *Some effects of calcium deficiency on the anatomy of Pinus taeda.* American Journal of Botany, **36**: 276-282.

57. Yamauchi, T., Hara, T., Sonoda, Y. 1986. *Distribution of calcium and boron in the pectin fraction of tomato leaf cell wall.* Plant Cell Physiology, **27**: 729-732.

58. Simon, E.W. 1978. *The symptoms of calcium deficiency in plants.* New Phytology, **80**: 1-15.

59. Beets, P.N., Oliver, G.R., Kimberly, M.O., Pearce, S.H., Rodgers, B. 2004. *Genetic and soil factors associated with variation in visual magnesium deficiency symptoms in Pinus radiata.* Forest and Ecology Management, **189**: 263-279.

60. Clarkson, D.T., Hanson, J.B. 1980. *The mineral nutrition of higher plants.* Annual Review of Plant Physiology, **31**: 239-98.

61. Shaul, O. 2002. *Magnesium transport and function in plants: the tip of the iceberg.* Biometals, **15**: 307-321.

62. Downes, G.M., Evans, R., Schimleck, L.R., Fritts, H.C. 2000. *The commercial cambium: understanding the origin of wood property variation*, in *Cell and Molecular Biology of Wood Formation*, Savidge, R.A., Barnett, J.R., Napier, R., Ed. BIOS Scientific Publishers Ltd.: Oxford. 325-334.

63. Panshin, A.J., de Zeeuw, C. 1980. *Variability of wood within a species*, in *Textbook of Wood Technology*, Panshin, A.J., de Zeeuw, C., Ed. McGraw-Hill Inc.: New York. 240-249.

64. Denne, M.P., Dodd, R.S. 1981. *The environmental control of xylem*, in *Xylem Cell Development*, Barnett, J.R., Ed. Castle House Publications Ltd.: Kent. 236-255.

65. Fukuda, H. 1996. *Xylogenesis: Initiation, progression, and cell death.* Annual Review of Plant Physiology and Plant Molecular Biology, **47**: 299-325.

66. Wodzicki, T.J. 1971. *Mechanism of xylem differentiation in Pinus silvestris L.* Journal of Experimental Botany, **22**: 670-87.

Chapter Five

Distribution of pectic polysaccharides

5.1 Introduction

Analysis of the distribution of pectin in the cultures was undertaken in order to ascertain the relationship between cell wall pectin deposition, carbohydrate content and mineral nutrient availability. Pectin was targeted because it may alter the strength of the cell wall through the formation of calcium cross-links and boron-RG-II complexes. The main pectic polysaccharides present in conifers, HG, RG-I and RG-II, and their interactions with boron, calcium and magnesium were reviewed in section 1.3.2.5. As was introduced in Chapter 4, when reference is made to a particular nutrient treatment, the concentration discussed refers to the amount of that nutrient added into the culture medium.

Presented in this chapter are the light microscopy, laser scanning confocal microscopy, and immunogold transmission electron microscopy characterizations of pectin distribution in the cultures. The carbohydrate compositions of the cultures are also compared. These experiments were undertaken in order to determine if wood properties could be successfully altered and measured in the organ cultures.

5.2 Distribution of pectic polysaccharides in radiata pine

The distribution of pectic polysaccharides present in the control explants, representative of field grown radiata pine, for 12 culture sets was determined using histochemistry, immunocytochemistry and immunogold TEM techniques, in to order characterize normal pectin localisation.

5.2.1 Histochemistry

5.2.1.1 Ruthenium red

Wax embedded sections, mounted on glass slides and extracted with ammonium hydroxide to remove surface lignin that may mask pectin detection [1], were stained

with ruthenium red. Ruthenium red is an anionic stain that stains pectins dark pink through a reaction with carboxyl groups [2]. This method was used initially to qualitatively observe pectin distribution in the cell walls.

The greatest pectin staining was observed in the CML of the cambial and newly divided cells (figure 5.1A). This was not surprising, since this is the point in development when pectin is deposited in the cell walls [3]. As the differentiation of a xylem cell progressed, pectin localisation remained in the CML and did not spread into the secondary cell walls (figure 5.1A, B, C). Pectin staining was also strong in the ray cell walls (figure 5.1B arrow) and the bordered pit membranes (figure 5.1C arrow).

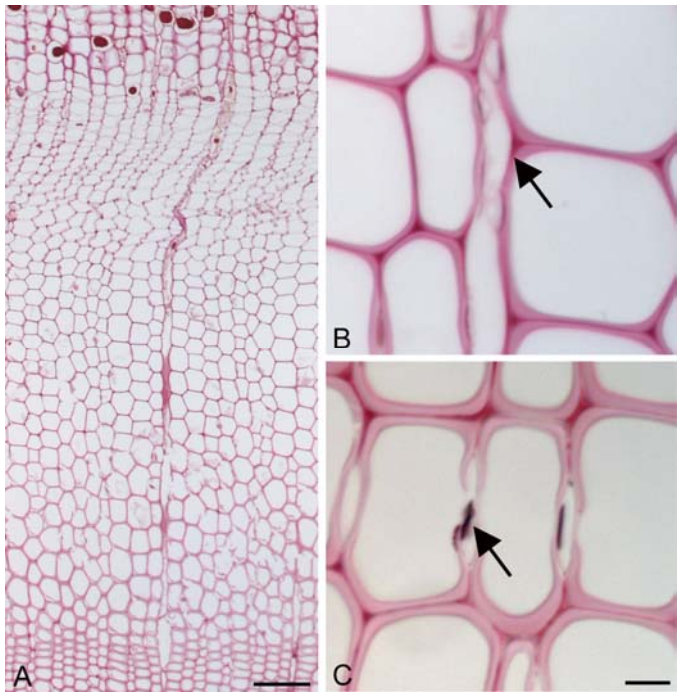


Figure 5.1 Transverse sections stained with ruthenium red, which stains pectins dark pink. Staining was localized to the CML of the cambial and newly divided cells (A), and was not present in the secondary walls (A-C). Staining was also present in the ray cell walls (B) and the pit membranes (C). Images were collected with a Zeiss Axioshop digital camera. Scale bars (A) = 100 µm, (B-C) = 10 µm.

5.2.2 Immunocytochemistry

Immunocytochemistry coupled to confocal scanning laser microscopy is a technique where a secondary antibody linked to a fluorescent marker can be used to observe a particular cellular antigen, recognized by a primary antibody. The advantage of this

technique is that only the parts of a cell labelled with the fluorescent marker are observed. Confocal microscopy is commonly used to stimulate the fluorescent labels [4]. This is achieved by excluding light that is not from the microscope's focal plane, illuminating the specimen with light of an appropriate wavelength and collecting the image from the fluorescent light [4]. This process involves the use of specialized computer programs to reconstruct scanned images and create a high resolution picture [4].

5.2.2.1 JIM5 and JIM7 monoclonal antibodies

The monoclonal antibodies that were used in this work are specific to methyl-esterified and un-esterified portions of HG. RG-II specific antibodies were not commercially available during this study. However, RG-II is believed to be covalently linked to HG, which may form a backbone for RG-II synthesis, creating a macromolecular pectin complex as described in section 1.3.2.6 [5, 6]. With this feature in mind, JIM5 and JIM7 were chosen for the immunocytochemical studies, as they should also recognize portions of the RG-II backbone. JIM5 recognizes pectins with a low degree (up to 50%) of methyl-esterification [7-9], while JIM7 recognizes those with a high degree (35-90%) of methyl-esterification [7-9], neither antibody recognizes RG-I [9]. The specific epitope for these antibodies is not fully defined, and the range of esterification they recognise can lead to overlap in their labelling [9-12].

Sections (3 μm) thick, that were embedded in LR White resin and mounted on glass slides, were processed for immunolocalization of JIM5 and JIM7. Examples of control slides (ie. with no primary antibody) are presented in section 9.5.5.2 (figure 9.4). JIM5 labelling was strongest in the cambial cell junctions and radial walls (figure 5.2A arrow), was present in the CML of radially expanding cells (figure 5.2C), and was absent in the lignified mature wood (figure 5.2E). JIM5 labelling was, however, present in the ray cell walls (figure 5.2 C arrow) and the pit membranes (figure 5.2E arrow) of radially expanding and mature cells. JIM7 labelling was more intense than JIM5 labelling (figure 5.2). JIM7 labelling was strong and homogenous in the cambial zone, radial and tangential walls (figure 5.2.B), was present in the walls of radially expanding xylem (figure 5.2.D), and was drastically reduced in fully lignified cells (figure 5.2F). JIM7 labelling was evident in the ray cell walls (figure 5.2D arrow) and in the pit membranes (figure 5.2F arrow) throughout the xylem. The

distribution of JIM5 and JIM7 labelling agrees with the results reported previously for hybrid aspen [13], poplar cambium [14], and *Pinus sylvestris* [7]. The disappearance of labelling in the mature wood, is believed to be due to the progressive masking of the epitopes by cellulose and lignin [13]. The notion of pectin masking is corroborated by the ruthenium red results, where lignin removal allowed for pectin visualization in mature wood (figure 5.1C).

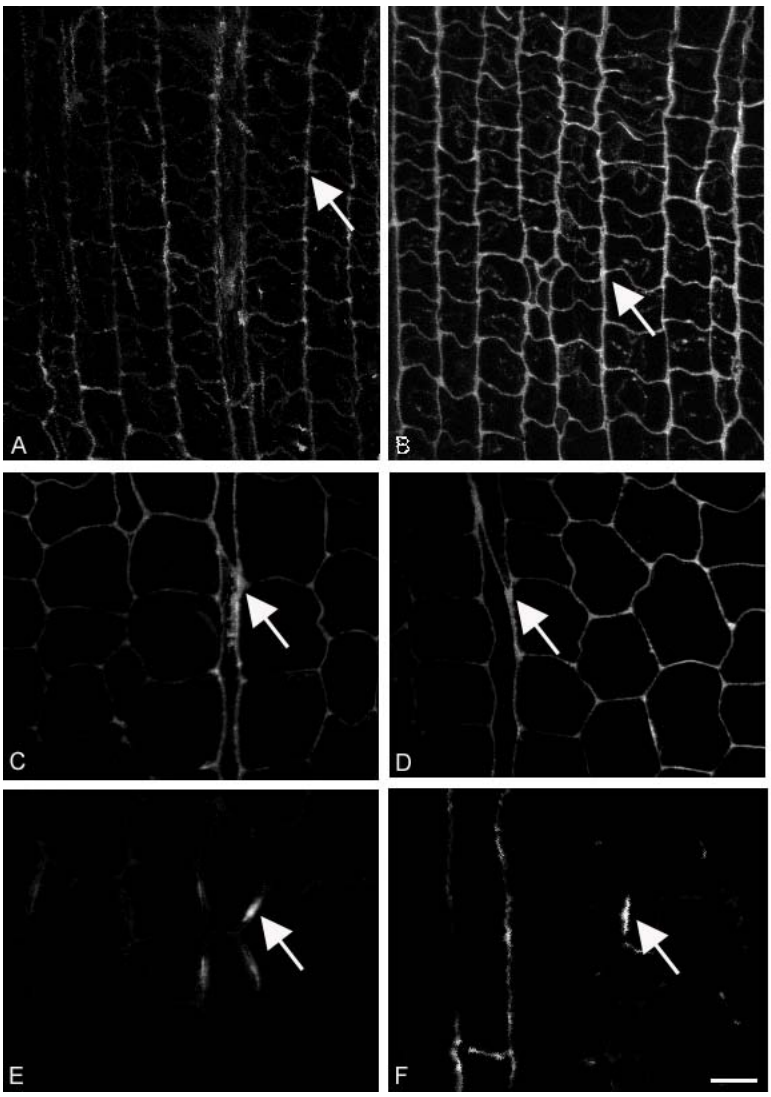


Figure 5.2 Distribution of JIM5 and JIM7 epitopes in radiata pine. JIM5 (A, C, E) and JIM7 (B, D, F). Cambial cells (A-B), radially expanding cells (C-D), and mature cells (E-F). JIM5 is specific to un-esterified pectins, while JIM7 is specific to esterified pectins. Scale bar = 20 µm.

5.2.3 Immunogold transmission electron microscopy

Immunogold transmission electron microscopy studies were undertaken in order to precisely ascertain the distribution of pectin in radiata pine. This technique relies on gold-conjugated secondary antibodies, which are electron dense and appear as black dots on images, to reveal cellular antigens.

Ultra-thin sections from LR White embedded samples were placed on nickel grids, and immunogold techniques were used to observe the spatial distribution of the JIM5 and JIM7 epitopes. Examples of control slides (ie. with no primary antibody) are presented in section 9.5.5.2 (figure 9.4). Consistent with the confocal images (section 5.2.2), JIM5 and JIM7 labelling was greatest in the cambial and newly divided cells (figure 5.3.A, B). In the cells with secondary wall deposition, JIM5 labelling was only present in the radial and tangential walls and not consistently at the cell junctions (figure 5.3C), while JIM7 labelling remained homogenous in the cell junctions in addition to the radial and tangential walls (figure 5.3D). The histochemistry (section 5.2.1.1), confocal scanning laser microscopy (section 5.2.2), and immunogold TEM results all suggest that pectin is not present in the secondary cell wall, which corroborates previous suggestions that pectin is the only major class of polysaccharide restricted to the primary cell wall [10].

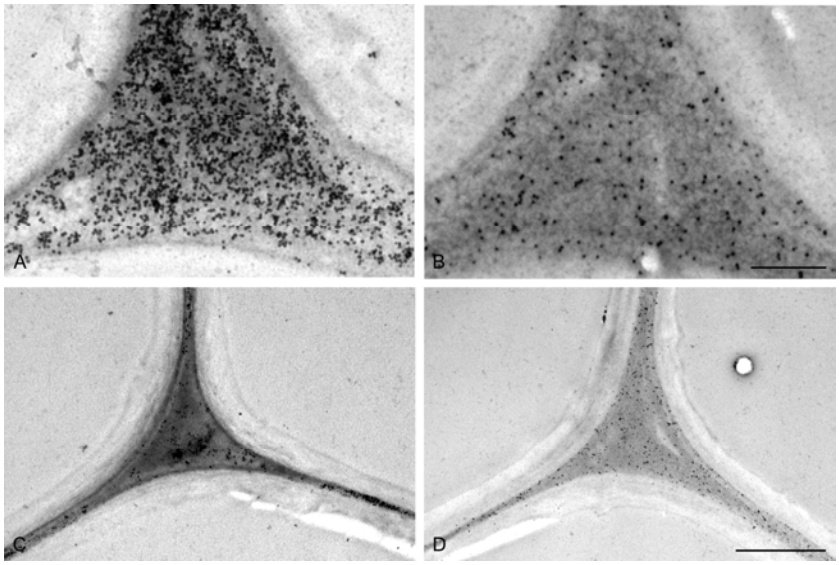


Figure 5.3 Immunogold distribution of JIM5 and JIM7 epitopes in radiata pine. Transverse sections, embedded in LR White resin and observed with a Phillps CM-100 TEM at 80 kV. JIM5 (A, C), JIM7 (B, D). Newly divided cells (A-B) and cells that underwent secondary wall deposition (C-D). Scale bars (A-B) = 0.5 µm, (C-D) = 2 µm.

Also consistent with the confocal images (section 5.2.2), JIM5 and JIM7 can label pit membranes (figure 5.4 A and B) and ray cell walls (figure 5.4C and D).

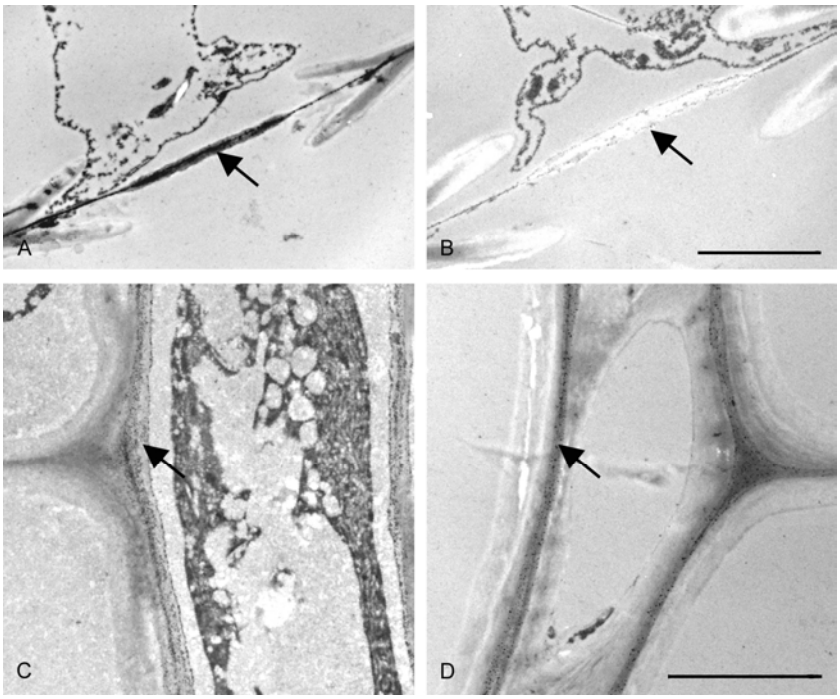


Figure 5.4 Immunogold distribution of JIM5 and JIM7 epitopes in pit membranes and ray cell walls in radiata pine. Transverse sections, embedded in LR. White resin and observed with a Phillps CM-100 TEM at 80 kV. JIM5 (A, C), JIM7 (B, D). Pit membranes (A-B) and ray cell walls (C-D). Scale bars (A-B) and (C-D) = 5 µm.

5.3 Distribution of pectic polysaccharides in the organ cultures

Pectin is one of the first cell wall components deposited during growth, and may play a critical role in determining the physical characteristics of the primary cell wall [3]. This presumption is supported by the reduction in B-RG-II cross-link formation observed, as a result of boron deficiency, which was accompanied by swollen walls in cultured tobacco cells and pumpkin tissue [15, 16]. For this reason, the distribution of pectin in the mineral nutrient-treated cultures was characterized in order to determine if changes occurred. The high and low treatments of three culture sets were observed using ruthenium red and confocal microscopy and two sets were observed using immunogold TEM techniques. Examples of control explants were presented in section 5.2.

5.3.1 Histochemistry

5.3.1.1 Ruthenium red

Sections mounted on glass slides and extracted with ammonium hydroxide [1], were stained with ruthenium red. This method was used as an initial qualitative observation of pectin distribution in the cell walls. The low and high culture treatments, and their control explants, were observed for three sets of each of the culture treatments. These culture treatments were chosen due to the large difference in mineral nutrient content between them, based on the ICP-OES/MS results presented in section 4.3.

Boron-treated cultures

The results demonstrate that pectin is present predominantly in the cells undergoing cell division and radial expansion, as expected (section 5.2.1.1) with the cultures[1] grown under low added boron conditions (0 μM; figure 5.6A) having a tendency to show more pectin, or alternatively less esterification, as indicated by the darker staining, than those grown under high added boron conditions (1000 μM; figure 5.6B). These differences were less apparent in the region of radial expansion (figure 5.6C and D) although the low boron-treated cultures still demonstrated a slightly greater staining intensity. The wood that was present at the time of culture (figure 5.6E and F) did not demonstrate any apparent differences in pectin localisation.

Calcium-treated cultures

There were no apparent differences in the intensity of the ruthenium red staining, and thus the amount of pectin, or pectin esterification, in the calcium-treated cultures[2] (figure 5.7). In some instances, the high calcium-treated cultures (100 mM) did appear to have slightly darker staining; however, this difference was minimal and difficult to discern.

---

[1] Boron-treated culture sets observed with ruthenium red staining: set number one and two (chapter four), and an additional culture set from a Burnham tree inducted in March. Culture set number two showed variable results.

[2] Calcium-treated culture sets observed with ruthenium red staining: set number one, two and three (chapter four).

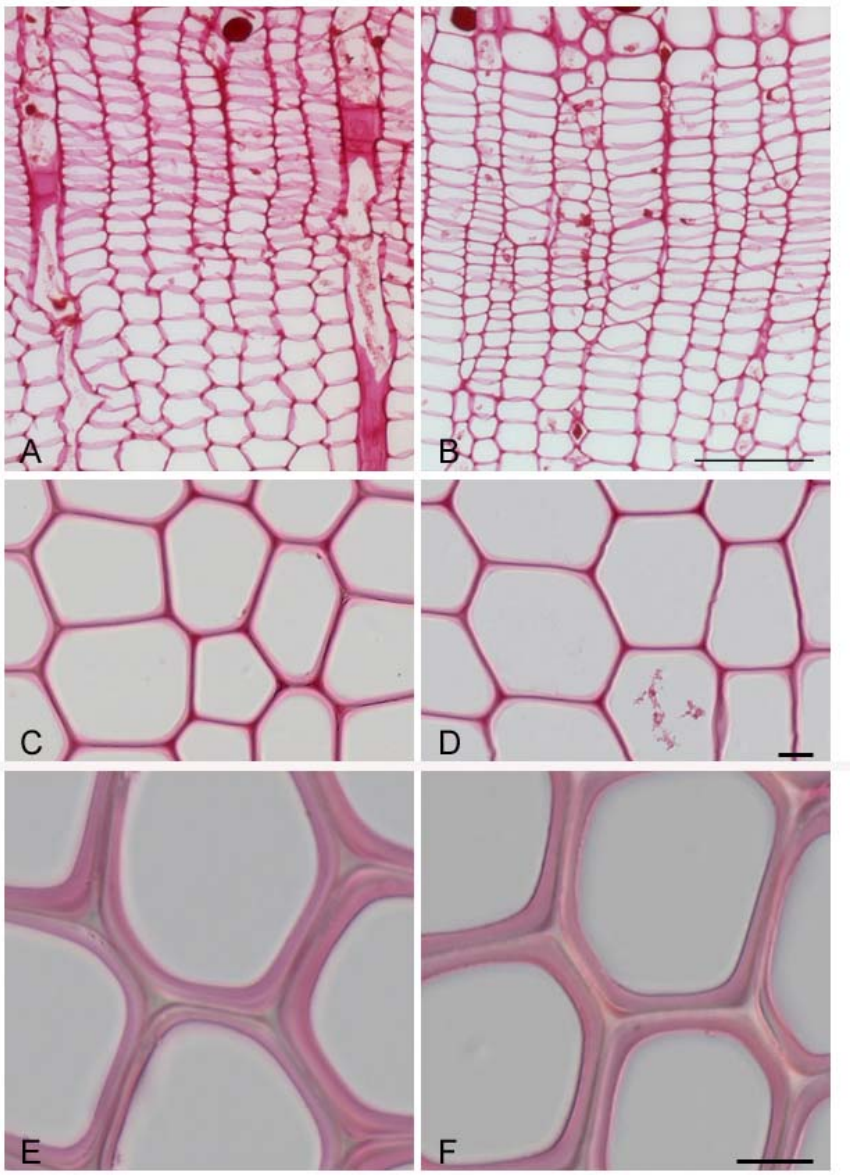


Figure 5.6 Transverse sections stained with ruthenium red and observed by light microscopy. Ruthenium red stains pectin dark pink. Cambial region and cells undergoing radial expansion (A-B), new secondary wall deposition (C-D), and mature wood (E-F). The cultures grown under low added boron conditions (0 µM) often show an increase in pectin demonstrated by the increased intensity of the pink colouration (A) compared to those grown under high concentrations (1000 µM; B). Images were captured with a Zeiss Axioshop digital camera. Scale bars for (A-B) = 100 µM, (C-F) = 10 µm.

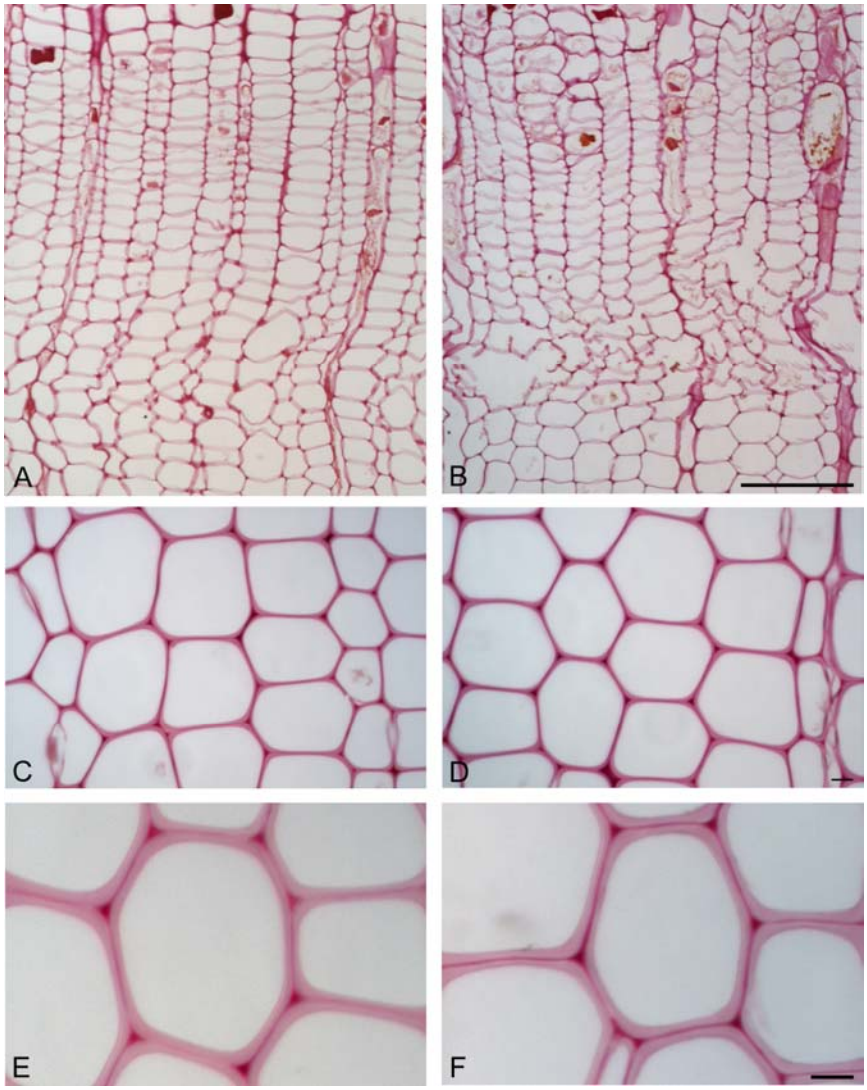


Figure 5.7 Transverse sections stained with ruthenium red and observed by light microscopy. Ruthenium red stains pectin dark pink. Cambial region and cells undergoing radial expansion (A-B), new secondary wall deposition, (C-D), and mature wood (E-F). The cultures grown under low added calcium conditions (0 mM) (A, C, E) and those grown under high concentrations (100 mM; B, D, F). There were no visible changes in the amount of pectin present in the calcium treated cultures. Images were captured with a Zeiss Axioshop digital camera. Scale bars for (A-B) = 100 µM, (C-F) = 10 µm.

Magnesium-treated cultures

There were no visible differences in the amount, esterification, or location of pectin in the magnesium-treated cultures[3], when ruthenium red stained sections were observed (figure 5.8).

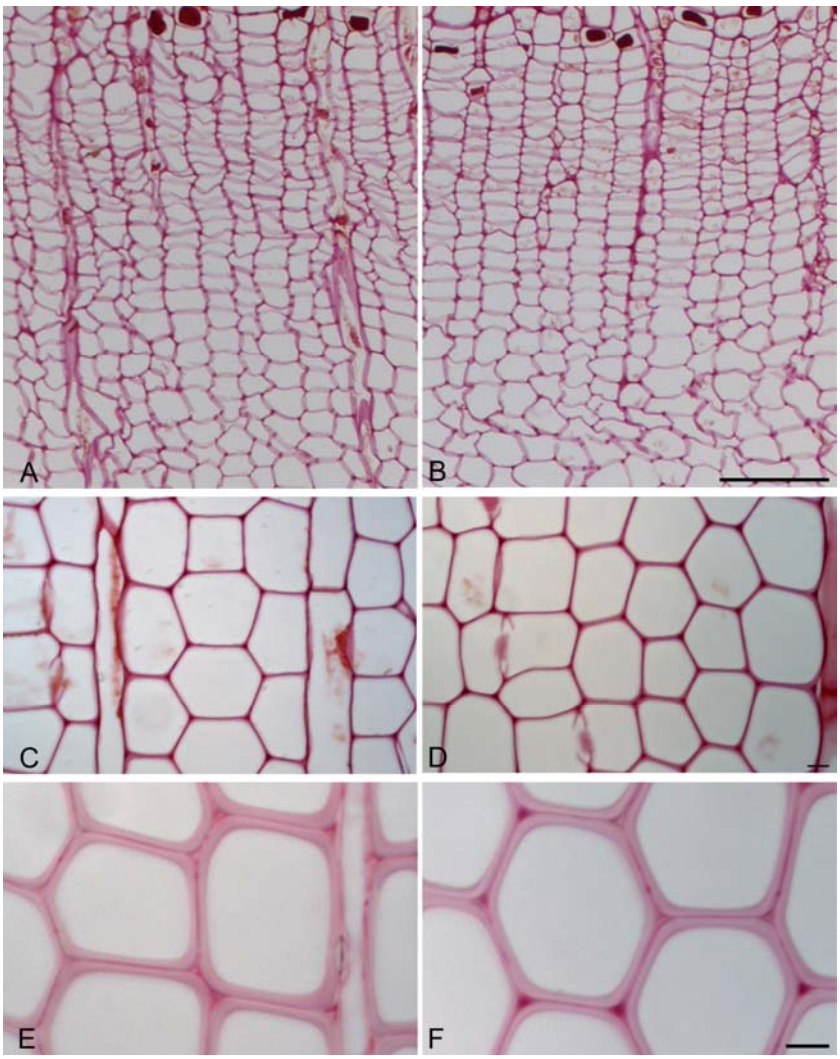


Figure 5.8 Transverse sections stained with ruthenium red and observed by light microscopy. Ruthenium red stains pectin dark pink. Cambial region and cells undergoing radial expansion (A-B), new secondary wall deposition (C-D), and mature wood (E-F). The cultures grown under low added magnesium conditions (0 mM; A, C, E) and those grown under high concentrations (100 mM; B, D, F). There were no visible changes in the amount or esterification of pectin present in the magnesium treated cultures. Images were captured with a Zeiss Axioshop digital camera. Scale bars for (A-B) = 100 µM, (C-F) = 10 µm.

[3] Magnesium-treated cultures observed with ruthenium red staining: sets number one, two and three (chapter four).

Mixed-treated cultures

There were no visible differences in the distribution or intensity of ruthenium red staining in any region of the mixed-treated (LBHC and HBLC) cultures[4] (figure 5.9). This suggests that changes in the amount, and/or esterification of pectin did not occur.

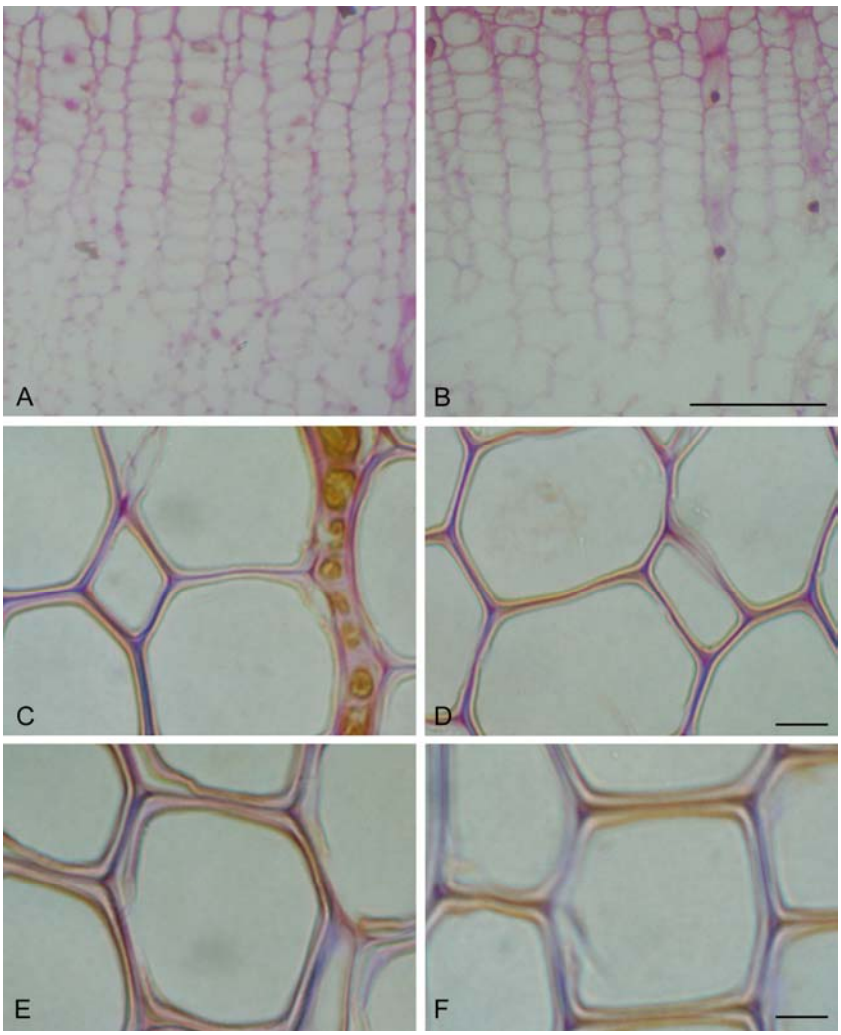


Figure 5.9 Transverse sections stained with ruthenium red and observed by light microscopy. Ruthenium red stains pectin dark pink. Cambial region and cells undergoing radial expansion (A-B), new secondary wall deposition (C-D), and mature wood (E-F). The LBHC treated cultures (A, C, E) and the HBLC treated cultures (B, D, F). There were no visible changes in the amount, or esterification of pectin present in the mixed treated cultures. Images were captured with an Olympus 5.1 mega pixel digital camera. Scale bars for (A-B) = 100 µM, (C-F) = 10 µm.

[4] Mixed-treated cultures observed with ruthenium red staining: set number one (chapter four) and two sets from Burnham trees cultured in December and January.

5.3.2 Immunocytochemistry

Sections (3 μm thick), embedded in LR White resin were mounted on glass slides. Immunocytochemistry techniques utilizing JIM5 and JIM7 monoclonal antibodies coupled to confocal scanning laser microscopy were used to observe the spatial distribution of pectins in the cambium, newly divided and existing cell walls. The cambial region, including newly divided cells, was targeted in this work, since pectins are known to be abundant in young cell walls [13], and these cells were produced in culture.

In all of the cultures, JIM5 epitope expression was present in the middle lamella of the cambial cells, with a sometimes reduced and slightly speckled presence at the cell junctions. The JIM7 epitope was also present in the middle lamella, with a homogenous distribution across the middle lamella and at the cell junctions. The existing cells did not express JIM5 or JIM7 epitopes at the CML, presumably because the epitopes are masked by lignin. However, both ray cells and pit membranes expressed JIM5 and JIM7 epitopes in the existing wood consistent with the distribution of pectin described in section 5.2.2.1. The ray and pit membrane epitope labelling, and the lack of labelling in the mature wood were observed to confirm antibody labelling patterns.

5.3.2.1 Boron-treated cultures

In general, the low boron-treated cultures (0 µM) appeared to have greater JIM5 fluorescence at the cell junctions (figure 5.10A) compared to the high boron-treated cultures[5] (1000 µM; figure 5.10C). JIM7 fluorescence appeared homogenous in both culture conditions (figure 5.10 E and G). The increase in JIM5 epitope expression suggested an alteration in esterification and possibly pectin deposition.

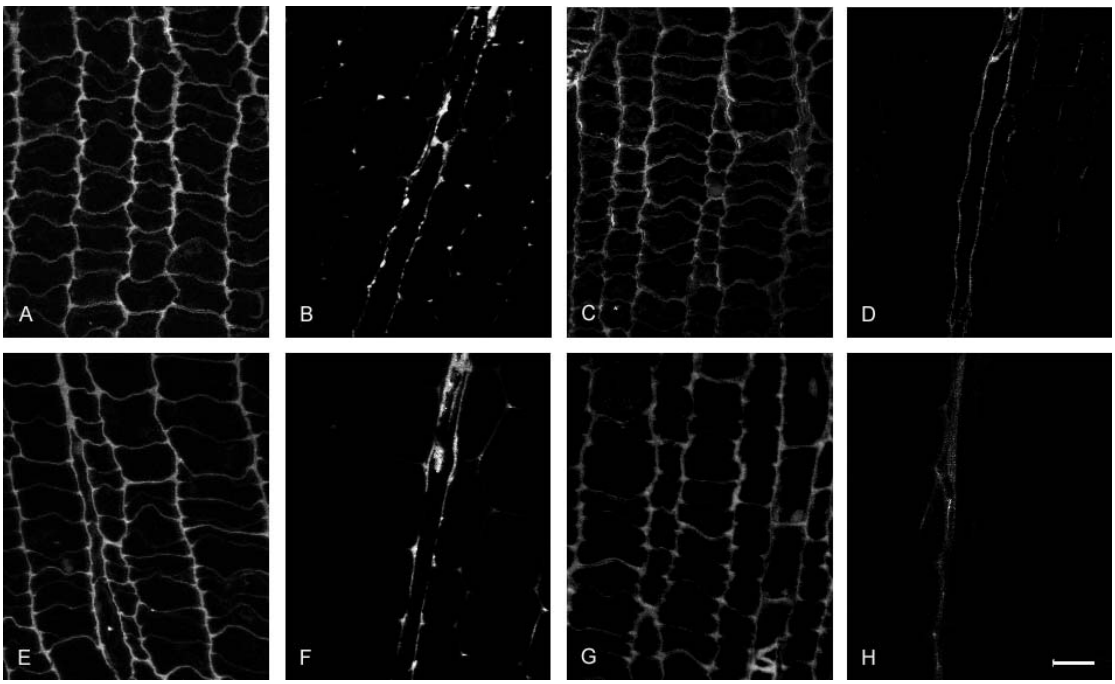


Figure 5.10 Immunocytochemistry of transverse sections of boron-treated organ cultures using JIM5 (A-D) and JIM7 (E-H) monoclonal antibodies and observed by confocal scanning laser microscopy. JIM5 is specific to un-esterified pectins, while JIM7 is specific to esterified pectins. Low boron treated cultures (0 µM; A, B, E, F) and high boron treated cultures (1000 µM C, D, G, H). Cambial cells (A, C, E, G) and mature cells (B, D, F, H). JIM5 fluorescence was generally stronger in the low boron treated cultures. Scale bar = 20 µm.

[5] Boron-treated culture sets observed with confocal microscopy: culture set number one (chapter four), and two additional culture sets, one from Burnham trees cultured in February, and March. All three trees showed similar results.

5.3.2.2 Calcium-treated cultures

The low (0 mM) and high (100 mM) calcium-treated cultures[6], and their control explants did not show a difference in the localisation of intensity of the JIM5 or JIM7 fluorescent signal (figure 5.11). Moreover, the expected distribution of the JIM5 and JIM7 epitopes was observed in these cultures (described in section 5.2.2.1).

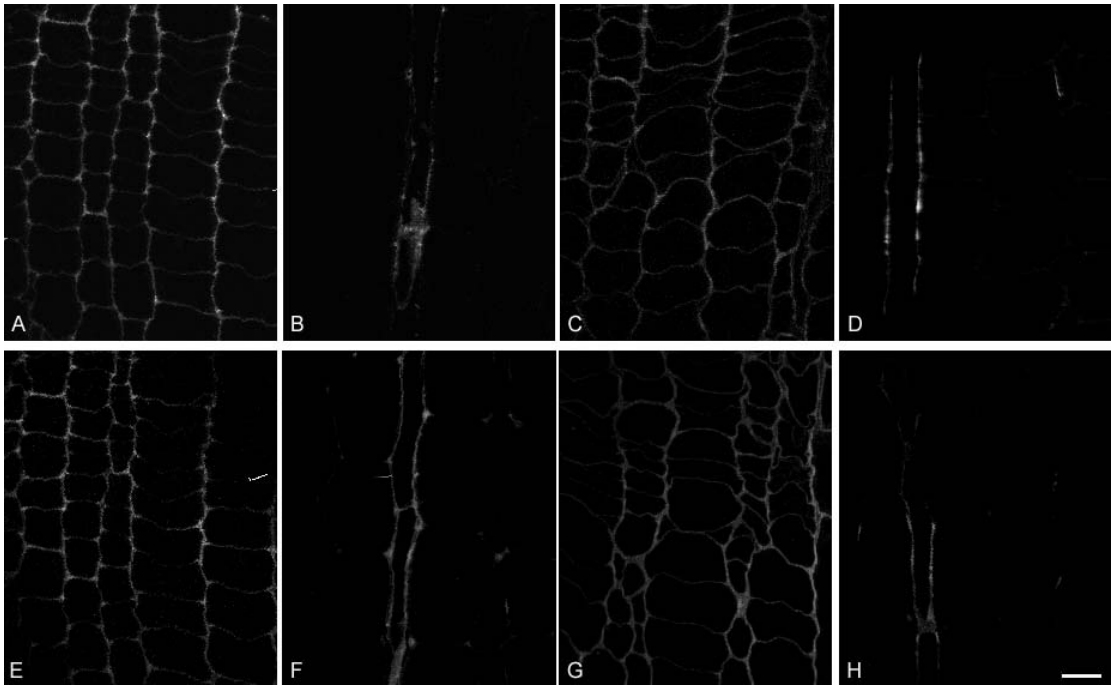


Figure 5.11 Immunocytochemistry of transverse sections of calcium-treated organ cultures using JIM5 (A-D) and JIM7 (E-H) monoclonal antibodies and observed by confocal scanning laser microscopy. JIM5 is specific to esterified pectins, while JIM7 is specific to un-esterified pectins. The cambial cells of the 0 mM calcium treated cultures (A, E) and the 100 mM calcium-treated cultures (C, G) did not have altered epitope expression. Epitope expression was not present in the CML of the mature cells of the 0 mM calcium treated cultures (B, F) or the 100 mM calcium treated cultures (D, H). In the mature cells only the rays or pit membranes had epitope expression. Scale bar = 20 µm.

[6] Calcium-treated culture sets observed using confocal microscopy: set number three (chapter four), and two additional sets from Burnham trees cultured in February.

5.3.2.3 Magnesium-treated cultures

The low (0 mM) and high (100 mM) magnesium-treated cultures[7] did not show a difference in the localisation or intensity of the fluorescence between the culture treatments (figure 5.12). This suggests that the expression of the JIM5 and JIM7 epitopes had not changed. These cultures showed the expected JIM5 and JIM7 epitope distribution described in section 5.2.2.1.

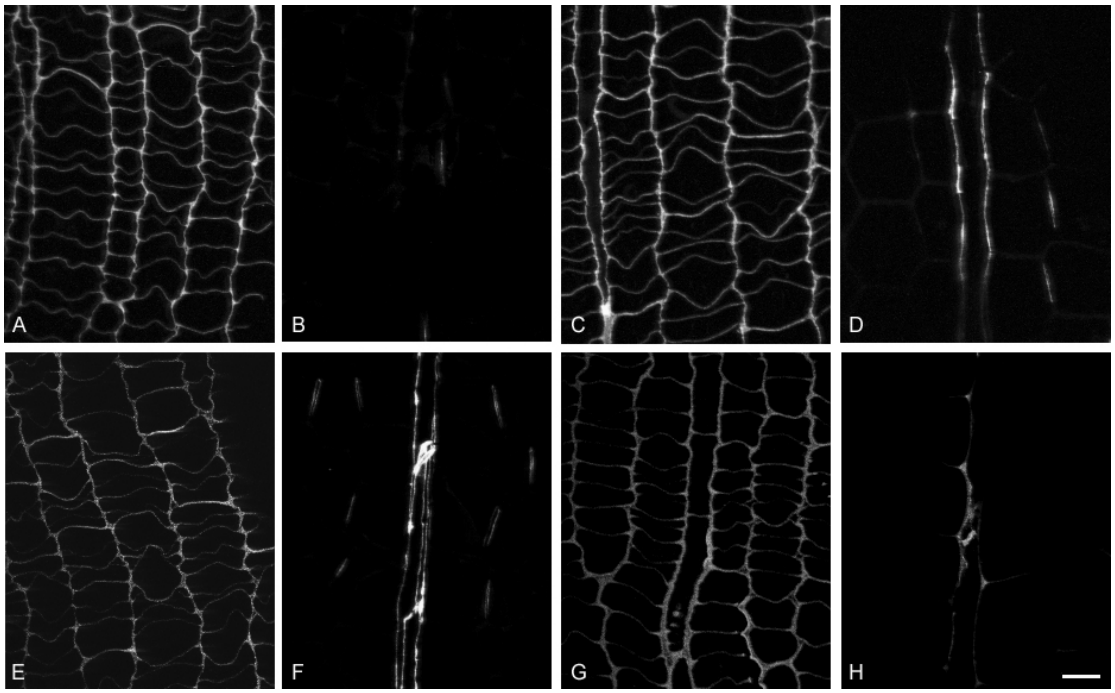


Figure 5.12 Immunocytochemistry of transverse sections of magnesium-treated organ cultures using JIM5 (A-D) and JIM7 (E-H) monoclonal antibodies using confocal scanning laser microscopy. JIM5 is specific to esterified pectins, while JIM7 is specific to un-esterified pectins. The cambial cells of the 0 mM magnesium treated cultures (A, E) and the 100 mM magnesium-treated cultures (C, G) did not have altered epitope expression. Epitope expression was not present in the CML of the mature cells of the 0 mM magnesium-treated cultures (B, F) or the 100 mM magnesium-treated cultures (D, H). In the mature cells only the E_TYPE>0</REFERENCE_TYPE><REFNUM>35</REFNUM><AUTHORS><AUTHOR

[7] Magnesium-treated cultures observed using confocal microscopy: set number one, two and three (chapter four).

5.3.2.4 Mixed-treated cultures

In a few instances, the LBHC treated cultures[8] (figure 5.13A) had greater JIM5 labelling in the cell junctions compared to the HBLC treated cultures (figure 5.13C), while there was no apparent change in JIM7 localisation (figure 5.13). The existing cells did not label in the cell junctions, while the ray and pit membranes did label with these antibodies (figure 5.13).

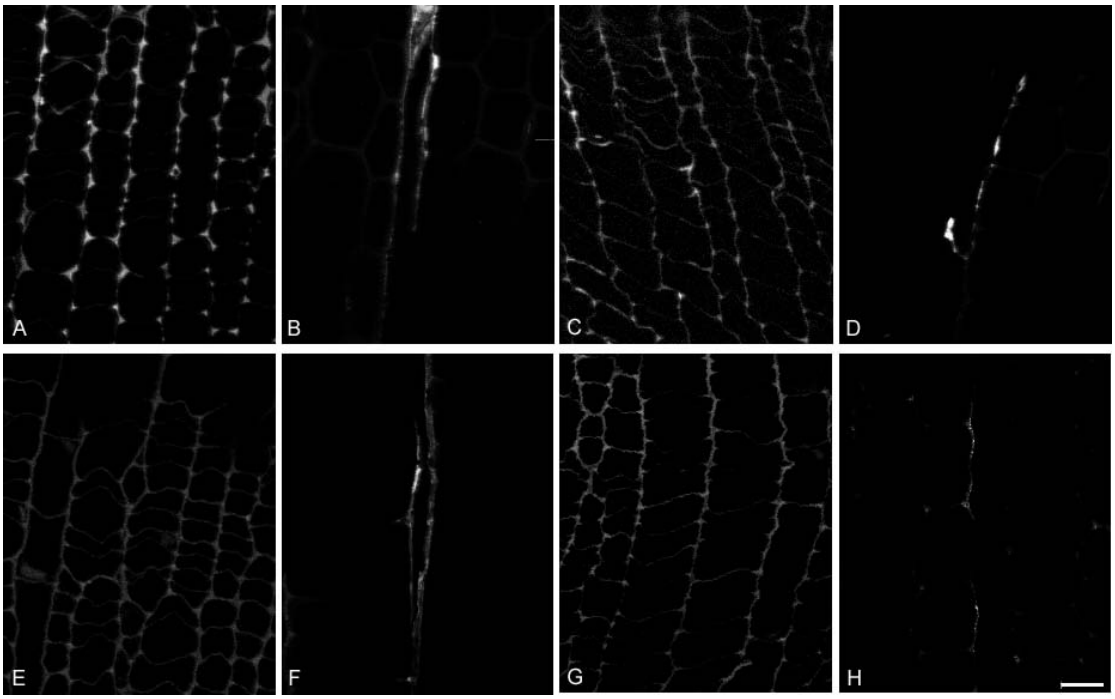


Figure 5.13 Immunocytochemistry of transverse sections of mixed nutrient treated organ cultures using JIM5 (A-D) and JIM7 (E-H) monoclonal antibodies using confocal scanning laser microscopy. JIM5 is specific to esterified pectins, while JIM7 is specific to un-esterified pectins. The cambial cells of the LBHC (A, E) and the HBLC treated cultures (C, G). JIM5 localisation was occasionally more intense in the LBHC treated cultures (A). Epitope expression was not present in the CML of the mature cells of the LBHC treated cultures (B, F) or the HBLC treated cultures (D, H). In the mature cells only the rays or pits had epitope expression. Scale bar = 20 µm.

5.3.3 Immunogold transmission electron microscopy

Ultra-thin sections from LR White embedded samples were placed on nickel grids and immunogold techniques, coupled to observation with a Phillips CM 100 TEM at 80

[8] Mixed-treated cultures observed using confocal microscopy: sets number one, two and three (chapter four). Set number three showed some variability suggesting increases in JIM5 epitope localisation.

kV, were used to observe the spatial distribution of the JIM5 and JIM7 epitopes in the cultures. Duplicate grids of the low and high treated cultures from two culture sets, and their controls, were observed.

5.3.3.1 Boron-treated cultures

In the newly divided and radially expanding cells, an increase in JIM5 epitope expression was usually observed in the cultures grown under low added boron conditions (0 μM; figure 5.14A), compared to the cultures[9] grown under high boron conditions (1000 μM; figure 5.14C), and the control explant. JIM7 expression, on the other hand, appeared relatively homogenous in all of the cultures. There was no apparent change in the distribution of JIM5 or JIM7 in the cells undergoing secondary wall deposition (figure 5.14 B, D, F, G), which was expected, as pectin deposition in these cells would have occurred prior to culturing.

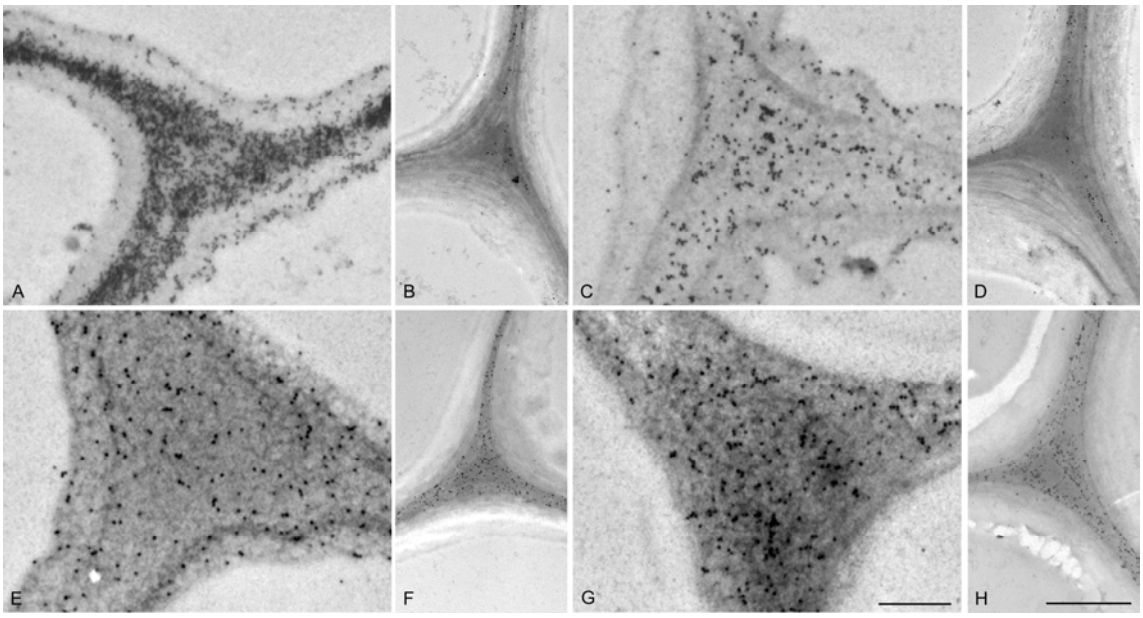


Figure 5.14 Immunogold localisation of transverse sections of the organ cultures using JIM5 (A-D) and JIM7 (E-H) monoclonal antibodies as observed by TEM. JIM5 is specific to un-esterified pectins, while JIM7 is specific to esterified pectins. The newly divided cells (A, C, E, G) and the cells undergoing secondary wall deposition (B, D, F, H) are presented. Low boron-treated cultures (0 μM; A, B, E, F), and the high boron-treated cultures (1000 μM; C, D, G, H). Un-esterified pectins showed different distribution patterns in the region of the CML as a result of boron availability. Scale bars (A, C, E, G) = 0.5 μm; (B, D, F, H) = 2 μm.

[9] Boron-treated culture sets observed by immunogold TEM: from Burnham trees cultured in February and March. The February tree showed variable results.

5.3.3.2 Calcium-treated cultures

In the newly divided and radially expanding cells of the calcium-treated cultures[10], there were no changes in the JIM5 and JIM7 epitope distribution (figure 5.15). The cambial, and new radially expanding cells had the greatest labelling (figure 5.15A, C, E, G) followed by the cells undergoing early secondary wall deposition (figure 5.15B, D, F, H).

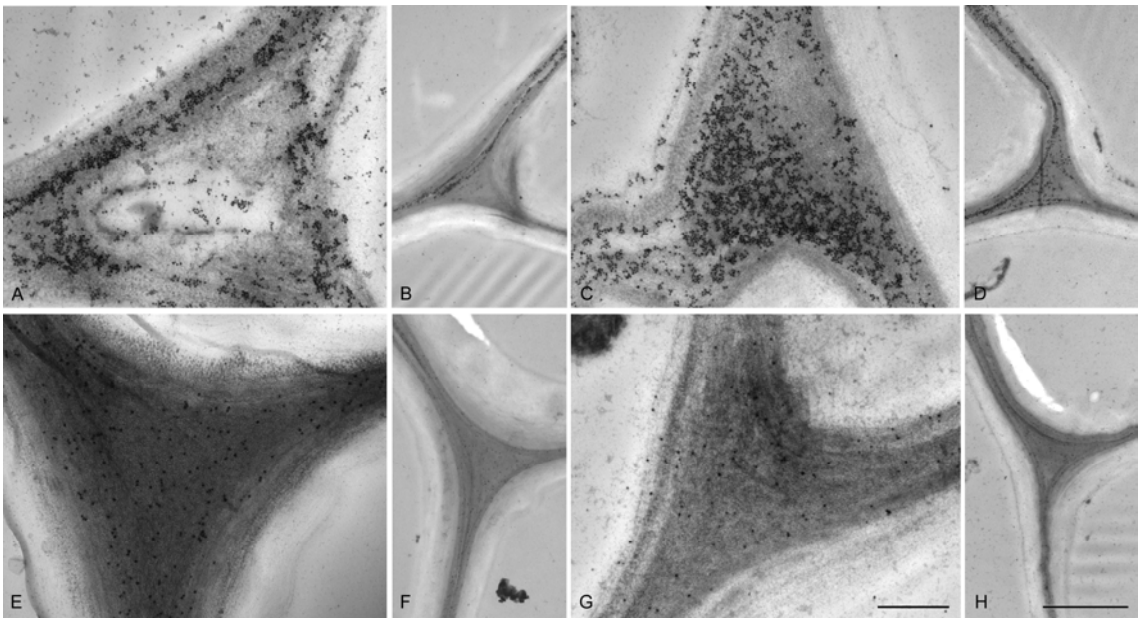


Figure 5.15 Immunogold localisation of transverse sections of the organ cultures using JIM5 (A-D) and JIM7 (E-H) monoclonal antibodies as observed by TEM. JIM5 is specific to un-esterified pectins, while JIM7 is specific to esterified pectins. The newly divided cells (A, C, E, G) and cells undergoing secondary wall deposition (B, D, F, H) are presented. Low calcium-treated cultures (0 mM; A, B, E, F), and the high calcium-treated cultures (100 mM; C, D, G, H). There were no changes in pectin epitope expression in the calcium treated cultures. Scale bars (A, C, E, G) = 0.5 µm; (B, D, F, H) = 2 µm.

[10] Calcium-treated culture sets observed by immunogold TEM: from Burnham trees cultured in February.

5.3.3.3 Magnesium-treated cultures

There were no apparent changes in the localisation or amount of JIM5 and JIM7 epitope expression in the newly divided and radially expanding cells, or the cells undergoing secondary wall deposition in any of the magnesium culture[11] treatments (figure 5.16).

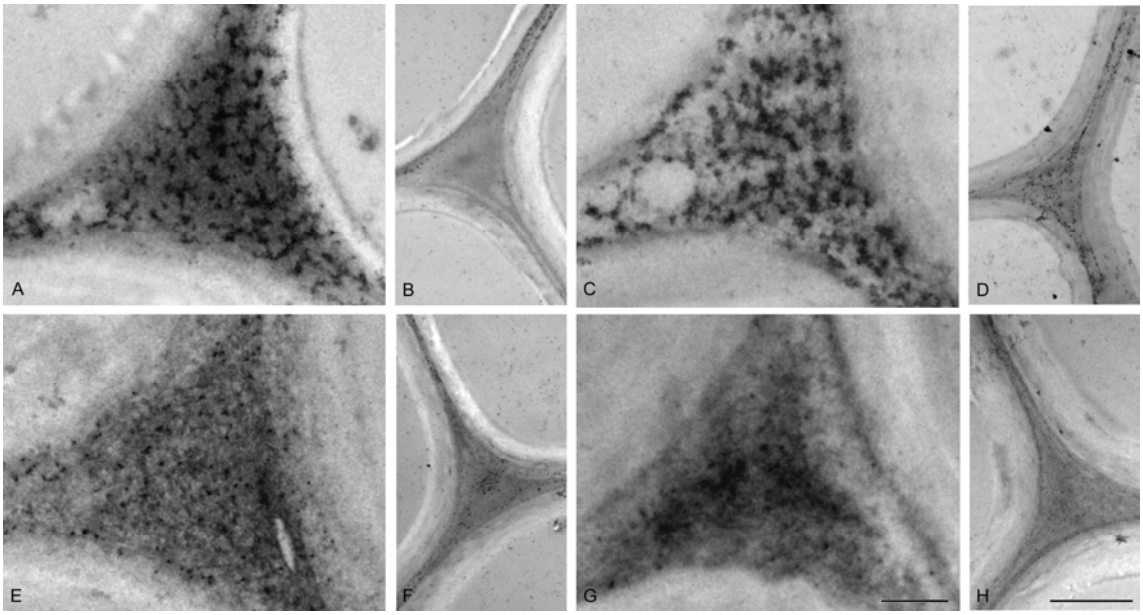


Figure 5.16 Immunogold localisation of transverse sections of the organ cultures using JIM5 (A-D) and JIM7 (E-H) monoclonal antibodies as observed by TEM. JIM5 is specific to un-esterified pectins, while JIM7 is specific to esterified pectins. The newly divided cells (A, C, E, G) and the cells undergoing secondary wall deposition (B, D, F, H) are presented. Low magnesium-treated cultures (0 mM; A, B, E, F), and the high magnesium-treated cultures (100 mM; C, D, G, H). There were no changes in pectin epitope expression in the magnesium treated cultures. Scale bars (A, C, E, G) = 0.5 µm; (B, D, F, H) = 2 µm.

[11] Magnesium-treated cultures observed by immunogold TEM: set number one and two (chapter four).

5.3.3.4 Mixed-treated cultures

The LBHC treated cultures[12] showed the expected distribution of pectin (figure 5.17A, B, E, F), while the HBLC treated cultures had poor epitope labelling (figure 5.17C, D, G, H).

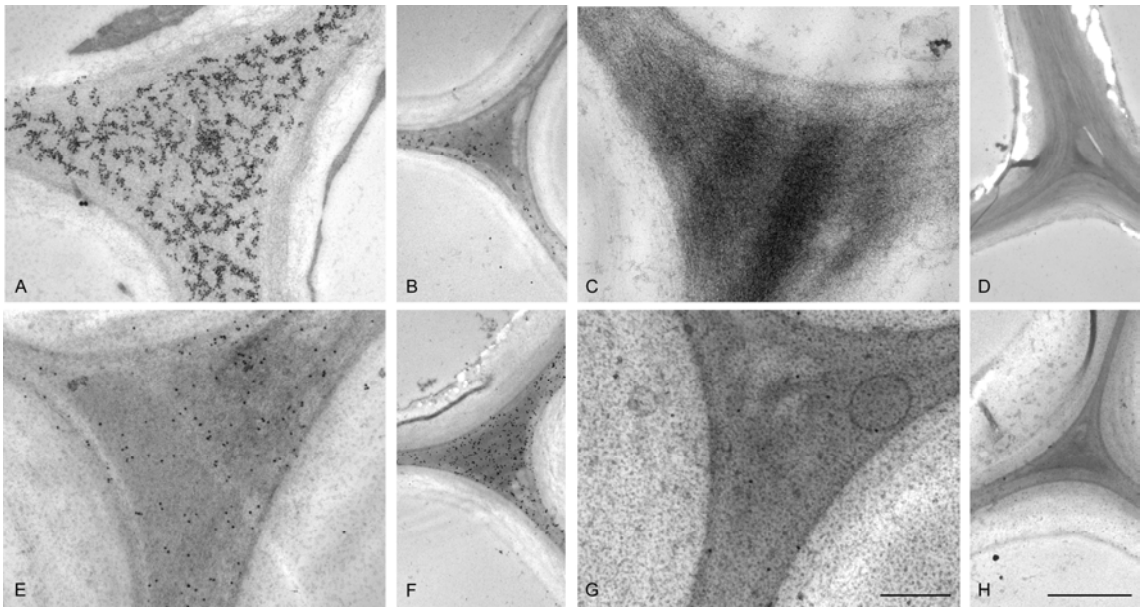


Figure 5.17 Immunogold localisation of transverse sections of the organ cultures using JIM5 (A-D) and JIM7 (E-H) monoclonal antibodies as observed by TEM. JIM5 is specific to un-esterified pectins, while JIM7 is specific to esterified pectins. The cells in the newly divided cells (A, C ,E, G) the cells undergoing secondary wall deposition (B, D, F, H) are presented. LBHC treated cultures (A, B, E, F), and the HBLC treated cultures (C, D, G, H). Scale bars (A, C, E ,G) = 0.5 µm; (B, D, F, H) = 2 µm.

In each of the methods utilized to study pectin location in the organ cultures, a gradient distribution was observed, with the greatest distribution being found in the cambial and newly formed cells, and the lowest in the mature wood (table 5.1). This was expected, as pectin is one of the first components of the cell wall to be deposited, and its presence may be degraded and/or masked by lignin following lignification. The latter notion of pectin masking is supported by the ruthenium red staining observed in sections of mature wood, where pectin could be visualized, after the lignin had been removed (section 5.2.1).

[12] Mixed-treated cultures observed by immunogold TEM: set number one and two (chapter four).

In the wood that was present prior to culturing, JIM5, observed by immunogold TEM, was predominantly present in the compound middle lamella of the radial and tangential cell walls, and was rarely observed at the junction between cells (figure 5.3). JIM7, on the other hand, displayed a relatively homogenous distribution in the compound middle lamella, including the cell junctions (figure 5.3). This, coupled to the pectic epitope distribution observed in normal wood (section 5.2.3), suggests that pectin is predominantly present in the methyl-esterified form in the existing wood.

Antibody	Cambium	Radial Expansion Region	New Secondary Wall Region	Mature Wood
JIM5	+++	++	+	+
JIM7	++++	+++	++	+

++++: strong intensity, +++: moderate intensity, ++: weak intensity, +: weak/ no intensity

Table 5.1 Summary of the distribution of JIM5 and JIM7 epitopes in the cultured wood.

5.4 Carbohydrate composition of the organ cultures

In order to determine if boron, calcium and/or magnesium could alter the sugar composition of the cell walls, comparative carbohydrate analyses were performed. For this work, the cultures were ground to a homogenous material and the ground material was sent to ensis New Zealand (Rotorua) for analysis of alditol acetate derivatives.

5.4.1.1 Alditol acetates

The alditol acetate method of carbohydrate analysis involves acid hydrolysis, reduction, acetylation and gas chromatography of the resulting alditol acetates. The hydrolysis results in a mixture of monosaccharides, which are then reduced to alditols with sodium borohydride, and acetylated [17]. The volatile derivatives of the carbohydrates are then analyzed by gas chromatography [18]. This method generally allows for better resolution than other commonly used derivatives [17] and was employed to determine the amount of the neutral monosaccharides: rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal) and glucose (Glc) present in the cultures.

The cultures prepared for this analysis were explanted from the same tree, inducted on high or low nutrient treatments, and cultured for the same length of time. The quantity of each isolated sugar was expressed as a percentage of the combined sugar content of the sample, and is presented in figure 5.18. The difference in the amount of sugars present within all of the culture treatments was minimal. All of the cultures analyzed contained small amounts of Ara and Gal, and intermediate amounts of Xyl and Man, compared to the high amount of Glc present. Rha and Fuc were present in trace amounts, and have not been included in figure 5.18.

In the low boron-treated cultures (0 μM), there was a slight decrease in Ara, Xyl and Man when compared to the high boron-treated culture (1000 μM; figure 5.18). However, there was a more prominent difference in the amount of glucose present in these samples, with the low boron-treated cultures (0 μM), having less than the high boron-treated culture (1000 μM; figure 5.18). When the boron-treated cultures were compared to the control explant, there were no differences in the amounts of Ara, Xyl, and Gal, and only slight differences in Man. There were however, differences in the amount of Glc present, with the low boron-treated cultures (0 μM), having less Glc than the controls, and the high boron-treated cultures (1000 μM) having more (figure 5.18). In the calcium, magnesium, and mixed-treated cultures, there were no prominent differences in the presence of any of the neutral monosaccharides (figure 5.18).

The trends in the sugar composition of the wood are consistent with those previously reported for Spruce and radiata pine, where Ara, Gal, Xyl and Man were found to increase respectively [19, 20]. In this work, arabinose was likely to have been liberated from pectic arabinans and Type-I arabinogalactans (section 1.3.2.7); galactose from pectic galactans, Type-I arabinogalactans, galactoglucomannans and xyloglucan (section 1.3.2.7; 1.3.2.2 and 1.3.2.3); mannose and xylose from xyloglucan, galactoglucomannans, glucomannans, and heteroxylans (sections 1.3.2.2, 1.3.2.3, 1.3.2.4); and fucose from RG-II and xyloglucan (sections 1.3.2.5 and 1.3.2.2). The increase in glucose in these samples may correspond to changes in cellulose content (section 1.3.2.1). Likewise, glucose may have been liberated from xyloglucan, glucomannan and/or galactoglucomannan (sections 1.3.2.2 and 1.3.2.3).

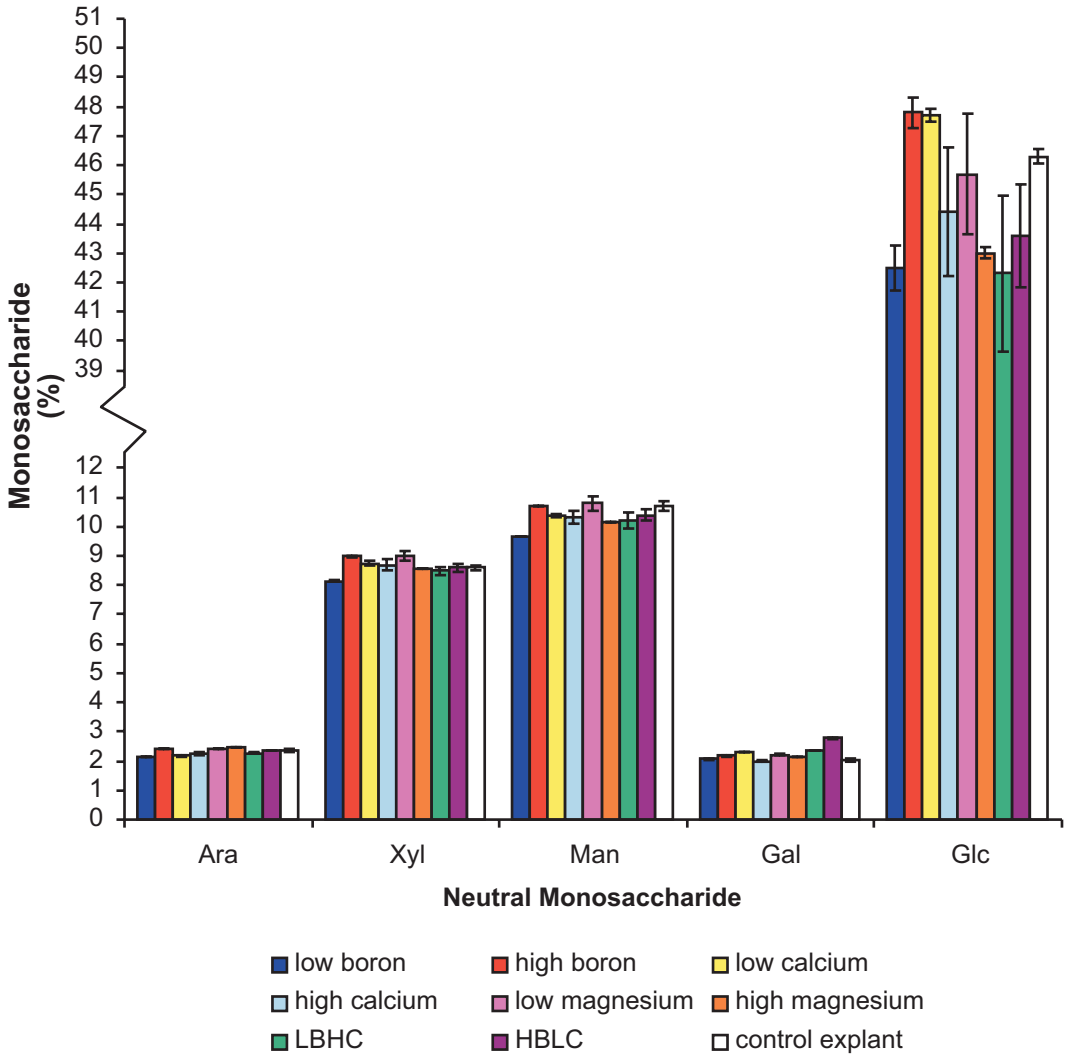


Figure 5.18 Neutral monosaccharide composition of the organ cultures determined from alditol acetate derivatives. Percentage compositions of the sugar components were averaged for two duplicate GC injections and two duplicate analyses. Samples were from cultures grown on the following treatments of added nutrients: low boron = 0 µM boron, high boron = 1000 µM boron, low calcium = 0 mM calcium, high calcium = 100 mM calcium, low magnesium = 0mM magnesium, high magnesium = 100 mM magnesium, LBHC= 0 µM boron and 100 mM calcium, HBLC = 1000 µM boron and 0 mM calcium, and the control explant. Rhamnose and fucose were detected in trace levels and are not presented (rhamnose ≤ 0.2%, fucose ~0.05%). Values are ± standard error of the mean.

5.5 Discussion

In all of the culture treatments, cambial and newly divided cells were found to contain the highest level of pectin labelling, while lignified xylem was found to contain much lower levels, which is consistent with the literature [7, 13, 19, 21, 22]. It is not known if pectin remains in the cell wall at maturity, partially masked by lignin, or if its presence is degraded because its primary role is during early development [7, 13, 19].

In lignified walls, pectin is known to be difficult to stain and label with pectin-specific antibodies [7, 14]. The ruthenium red results presented in section 5.2.1 suggest that lignin masks pectin labelling in the mature wood, in view of the fact that with the chemical removal of lignin, pectin staining was evident.

Histochemical observations of the low boron-treated cultures suggested the possibility of increased pectin deposition, or a decrease in pectin esterification, in the newly divided cells (section 5.2.1). An increase in pectin is consistent with studies on oil palm seedlings, which when grown in the absence of boron, showed an increased level of pectic substances in the root tissue, compared to those grown in the presence of sufficient boron [23]. It is not clear whether the amount of pectin distributed in the cell wall determines the boron requirement of a plant, or whether the availability of boron determines the production rate of pectin. Recent studies suggested that the boron requirements of flowering plants, suspension cultured sycamore cells, and etiolated pea stems was related to the amount of pectin, in particular RG-II, in their primary cell walls [24-27]. While the amount of pectin may change, this does not suggest a change in the structure of the pectins themselves, which is exemplified in studies of boron deficient pumpkin where the structure of RG-II was not altered [16].

Pectin is in a methyl-esterified form when it is transported to the cell wall in Golgi vesicles, to prevent premature ionic interactions with its carboxyl groups [28, 29]. Once in the cell wall, the methyl-esters are removed by pectin methylesterases (PME) which regulate the ratio between methyl-esterified and acidic pectins [14, 28, 30]. Pectin esterification has been reported to change during cell wall maturation, with a decrease in esterified pectin being linked to the reduction of growth and cell wall stiffening, as a result of the formation of calcium cross-links with HG [10, 31-33]. Young elongating cells characteristically have a high amount of methyl-esterified pectin, which allows for cell expansion by preventing calcium cross-links [11]. In other reports, un-esterified pectin has been determined to be a critical factor for growth of tobacco plants [33] and is present in poplar cambium during active growth [34]. The role of un-esterified pectins in growth is likely to involve alteration of the pH of the cell wall, which would in turn encourage cell wall loosening or the preference of polygalacturonases (enzymes that cleave pectin) for HG without methyl-esterification [30, 33]. PMEs can produce complex, often antagonizing, effects

in the cell wall, in that they can both stimulate and inhibit cell expansion [14]. In the low boron treated cultures, an increase in un-esterified pectin was often observed (figure 5.14), if this correlates to a cessation of growth, this corroborates the decrease in cell division that was sometimes observed in the low boron treated organ cultures (section 4.5.1.1). However, because of the potential for overlap in JIM5 and JIM7 labelling (section 5.2.2.1) these results do not demonstrate unambiguous esterification changes [11]. Nonetheless, the regulated deposition of pectin in the cell wall suggests that pectin, and its various constituents, may possess specific functions at the level of cell wall development [32].

There were no consistent changes in pectin observations for the calcium, magnesium and mixed-treated cultures. At the onset of lignification, methyl-esterified pectin tends to be the predominant form of pectin [7]. This was observed in the xylem cells that were present at the time of culture, and had not yet fully matured. An explanation for the decrease in un-esterified pectin at this point in development remains elusive [7]. Some researchers believe that calcium may be involved, as it plays a dual role in pectin cross-links and as stabilizers of superoxide radicals during lignification [7, 35]. The release of calcium from pectin cross-links, as a result of pectin degradation, may be required for lignification to occur [7, 19]. Calcium has been localized to the radial walls and cell junctions in poplar [14], placing it in close proximity to the pectin distribution observed in this work, suggesting that it may be liberated from HG present in this region.

The present study suggests that boron, calcium and magnesium availability have very little influence on the carbohydrate composition of the cultures, with the exception of glucose. This is corroborated by previous studies where the sugar composition of whole cell walls of poplar suspension cultures, determined by TMS, did not change in response to boron deficiency despite their finding of a decrease in B-RG-II complex formation [36]. These cultures had high Glc, and much lower amounts of Ara, Xyl, Man and Gal [36], consistent with the alditol acetate results presented in this work (figure 5.18). Moreover, studies using *Chenopodium album* cells, have also shown that boron deficiency did not alter the cell wall composition [37]. The low of levels of Rha in the samples analyzed for this study is likely do be the result of very little

primary cell wall, and thus pectin material, in the sample in relation to the secondary cell walls [19].

5.6 Summary

The experiments detailed in this chapter were undertaken in order to ascertain the distribution of pectin in radiata pine, and in the organ cultures subject to various mineral nutrient treatments. This has been achieved using multiple microscopy techniques, which have shown that in the presence of decreased boron in particular, pectin in the newly divided cells could be altered. The calcium, magnesium and mixed-treated cultures did not show consistent changes in pectin deposition or esterification. The use of anti-pectin monoclonal antibodies allowed for the observation of changes in pectin esterification to be analyzed in the context of cell wall architecture and development [30]. The carbohydrate composition of the cultures, with the exception of glucose in the boron treated cultures, was not altered.

5.7 References

1. Micheli, F., Ermel, F., Bordenave, M., Richard, L., Goldberg, R. 2002. *Cell walls of wood tissues: cytochemical, biochemical and molecular analysis of pectins and pectin methylesterases*, in *Wood Formation in Trees: Cell and Molecular Biology Techniques*, Chaffey, N.J., Ed. Taylor and Francis: London. 179-200.

2. Wardrop, A.B. 1981. *Lignification and xylogenesis*, in *Xylem cell development*, Barnett, J.R., Ed. Castle House Publications Ltd.: Kent. 115-151.

3. Northcote, D.H. 1986. *Control of pectin synthesis and deposition during plant cell wall growth*, in *Chemistry and Function of Pectin*, Fishman, M.L., Jen, J.J., Ed. American Chemical Society: Washington.

4. Semwogerere, D., Weeks, E.R. 2005. *Confocal microscopy*, in *Encyclopedia of Biomaterials and Biomedical Engineering*. Taylor and Francis.

5. Ridley, B.L., O'Neil, M.A., Mohnen, D. 2001. *Pectins: structure, biosynthesis, and oligogalacturonide-related signalling.* Phytochemistry, **57**: 929-967.

6. Ishii, T., Matsunga, T. 2001. *Pectic polysaccharide rhamnogalacturonan-II is covalently linked to homogalacturonan.* Phytochemistry, **57**: 969-974.

7. Hafren, J., Daniel, G., Westermark, U. 2000. *The distribution of acidic and esterified pectin in cambium, developing xylem and mature xylem of Pinus sylvestris.* International Academy of Wood Anatomists Journal, **21**: 157-168.

8. Willats, W.G.T., Limberg, G., Buchholt, H.C., van Alebeek, G., Benen, J., Christensen, T.M.I.E., Visser, J., Voragen, A., Mikkelsen, J.D., Knox, P.J. 2000.

Analysis of pectic epitopes recognized by hybridoma and phage display monoclonal antibodies using defined oligosaccharides, polysaccharides, and enzymatic degredation. Carbohydrate Research, **327**: 309-320.

9. Knox, J.P., Linstead, P.J., King, J., Cooper, C. Roberts, K. 1990. *Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices.* Planta, **181**: 512-521.

10. Willats, W.G.T., McCartney, L., Mackie, W., Knox, P. 2001. *Pectin: cell biology and prospects for functional analysis.* Plant Molecular Biology, **47**: 9-27.

11. Micheli, F., Bordenave, M., Richard, L. 2000. *Pectin methylesterases: possible markers for cambial derivative differentiation?*, in *Cell and Molecular Biology of Wood Formation*, Savidge, R.A., Barnett, J.R., Napier, R., Ed. BIOS Scientific Publishers Ltd.: Oxford. 295-305.

12. Clausen, M.H., Willats, W.G.T., Knox, P.J. 2003. *Synthetic methyl hexagalactuonate hapten inhibitors of anti-homogalacturonan monoclonal antibodies LM7, JIM5 and JIM7.* Carbohydrate Research, **338**: 1797-1800.

13. Follet-Gueye, M.L., Ermel, F.F., Vian, B., Catesson, A.M., Goldberg, R. 2000. *Pectin remodelling during cambial derivative differentiation*, in *Cell and Molecular Biology of Wood Formation*, Savidge, R.A., Barnett, J.R., Napier, R., Ed. BIOS Scientific Publishers Ltd.: Oxford. 289-294.

14. Guglielmino, N., Liberman, M., Jauneau, A., Vian, B., Catesson, A.M., Goldberg, R. 1997. *Pectin immunolocalization and calcium visualization in differentiating derivatives from poplar cambium.* Protoplasma, **199**: 151-160.

15. Matoh, T., Takasaki, M., Takabe, K. 2000. *Boron nutrition of cultured tobacoo BY-2 cells: III. Characterization of the boron rhamnogalacturonan II complex in cells acclimated to low levels of boron.* Plant Cell Physiology, **39**: 483-91.

16. Ishii, T., Matsunga, T., Hiyash, N. 2001. *Formation of rhamnogalacturonan II-borate dimer in pectin determines cell wall thickness in pumpkin tissue.* Plant Physiology, **126**: 1698-705.

17. Blakeney, A.B., Harris, P.J., Henry, R.J., Stone, B.A. 1983. *A simple and rapid preparation of alditol acetates for monosaccharide analysis.* Carbohydrate Research, **113**: 291-299.

18. Chaplin, M.F. 1986. *Monosaccharides*, in *Carbohydrate Analysis: A Practical Approach*, Chaplin, M.F., Kennedy, J.F., Ed. IRL Press: Oxford. 1-36.

19. Westermark, U., Hardell, H.L., Iverson, T. 1986. *The content of protein and pectin in the middle lamella/primary wall from spruce fibres.* Holzforschung, **40**: 65-68.

20. Uprichard, J.M. 1991. *Chemistry of wood and bark*, in *Properties and Uses of New Zealand Radiata Pine*, Kininmonth, J.A., Whitehouse, L.J., Ed. New Zealand Ministry of Forestry: Rotorua. 4.2-4.45.

21. Thornber, J.P., Northcote, D.H. 1961. *Changes in the chemical composition of a cambial cell during its differentiation into xylem and phloem tissue in trees.* Biochemical Journal, **81**: 449-455.

22. Edashige, Y., Ishii, T., Hiroi, T., Tomoyuki, F. 1995. *Cell-wall polysaccharides of cambial tissue: structural analysis of the polysaccharides of the primary wall from xylem differentiating zone of Crypotomeria japonica.* Holzforschung, **49**: 197-202.

23. Rajaratnam, J.A., Lowry, J.B. 1974. *The role of boron in the oil-palm (Elaeis guineensis).* Annals of Botany, **38**: 193-200.

24. Brown, P.H., Bellaloui, N., Wimmer, M.A., Bassil, E.S., Hu, H., Pfeffer, H., Dannel, F., Romheld, V. 2002. *Boron in plant biology.* Plant Biology, **4**: 205-223.

25. Hu, H., Brown, P.H., Labavitch, M. 1996. *Species variability in boron requirement is correlated with cell wall pectin.* Journal of Experimental Botany, **47**: 227-232.

26. Loomis, W.D., Durst, R.W. 1992. *Chemistry and biology of boron.* BioFactors, **3**: 229-239.

27. O'Neil, M.A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A.G., Albersheim, P. 1996. *Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently linked cross-linked by a borate ester.* Journal of Biological Chemsitry, **271**: 22923-22930.

28. Goldberg, R., Morvan, C., Jauneau, A., Jarvis, M.C. 1996. *Methyl-esterfication, de-esterfication and gelation of pectins in the primary cell wall*, in *Pectins and Pectinases*, Visser, J., Voragen, A.G.J., Ed. Elsevier Science: Amsterdam. 151-172.

29. Zhang, G.F., Staehelin, L.A. 1992. *Functional comartmentation of the Golgi apparatus in plant cells.* Plant Physiology, **99**: 1070-1083.

30. Willats, W.G.T., Orfila, C., Limberg, G., Buchholt, H.C., Van Alebeek, G.W.M., Voragen, G.J., Marcus, S.E., Christensen, T.M.I.E., Mikkelsen, J.D., Murray, B.S., Knox, J.P. 2001. *Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls.* Journal of Biological Chemsitry, **276**: 19404-19413.

31. Catesson, A.M. 1994. *Cambial ultra-structure and biochemistry: changes in relation to vascular tissue differentiation and the seasonal cycle.* International Journal of Plant Science, **155**: 251-261.

32. Vincken, J.P., Schols, H.A., Oomen, R.J.F.J., McCann, M.C., Ulvskov, P., Voragen, A.G.J., Visser, R.G.F. 2003. *If homogalacturonan were a side chain of rhamnogalacturonan-I. Implications for cell wall architecture.* Plant Physiology, **132**: 1781-1789.

33. Capodicasa, C., Vairo, D., Zabotina, O., McCartney, L., Caprari, C., Mattei, B., Manfredini, C., Aracri, B., Benen, J., Knox, J.P., De Lorenzo, G., Cervone, F. 2004. *Targeted modification of homogalacturonan by transgenic expression of fungal polygalacturonase alters plant growth.* Plant Physiology, **135**: 1294-1304.

34. Baier, M., Goldberg, R., Catesson, A., Liberman, M., Bouchemal, V.M., du Penhoat, C.H. 1994. *Pectin changes in samples containing poplar cambium and inner bark in relation to the seasonal cycle.* Planta, **193**: 446-454.

35. Westermark, U. 1982. *Calcium promoted phenolic coupling by superoxide radical - a possible lignification reaction in wood.* Wood Science and Technology, **16**: 71-78.

36. Kakegawa, K., Ishii, T., Matsunga, T. 2005. *Effects of boron deficiency in cell suspension cultures of Populus alba L.* Plant Cell Reports, **23**: 573-578.

37. Fleischer, A., O'Neil, M.A., Ehwald, R. 1999. *The pore size of non-graminaceous plant cell walls is rapidly decreased by borate ester cross-linking of the pectic polysaccharide rhamnogalacturonan-II.* Plant Physiology, **121**: 829-38.

Chapter Six

Distribution of lignin

6.1 Introduction

The distribution of lignin in the organ cultures was observed in order to determine if there was a relationship between nutrient availability and the incidence of lignification. The availability of boron, calcium and magnesium has been implicated in reduced lignification in previous studies [1-9]. The role of lignin in preventing the movement of adjacent cell files and strengthening the cell wall was discussed in section 1.3.3.7. These are important functions which, if altered, could affect wood quality. A reduction in lignification in the CML and/or secondary cell wall is associated with weak wood [10]. As was introduced in Chapter 4, when reference is made to a particular nutrient treatment, the concentration discussed refers to the amount of that nutrient added into the culture medium.

This chapter details the light microscopy, transmission electron microscopy and biochemical characterization of lignin distribution in the organ cultures. It also presents a comparison of the lignin and carbohydrate breakdown products in the cultures identified by pyrolysis gas chromatography mass spectrometry (Py-GC-MS). Concomitant with chapter five, these experiments were performed to determine if cell wall properties could be altered and quantified in the organ cultures.

6.2 Distribution of lignin in radiata pine

Cells at various stages of development in the control explants from twelve culture sets, representative of field grown radiata pine, were examined using histochemistry and blue autofluorescence techniques in order to characterize the normal distribution of lignin in radiata pine.

6.2.1 Histochemistry

6.2.1.1 Safranin/fast green

Safranin stains the lignin present in cell walls red, while fast green stains cellulose blue [1, 11]. Transverse wax sections stained with safranin and counter stained with fast green, showed that lignin was not present in the cambial region, or region of radial cell expansion, judged by the predominance of blue staining (figure 6.1A). Lignified sites, evident from the red staining, first appeared in the CML of the end of the region of radial expansion (figure 6.1B). The presence of lignin increased gradually, shown as more homogenous red staining, from the end of the region of radial expansion until the point of mature cells (figure 6.1C).

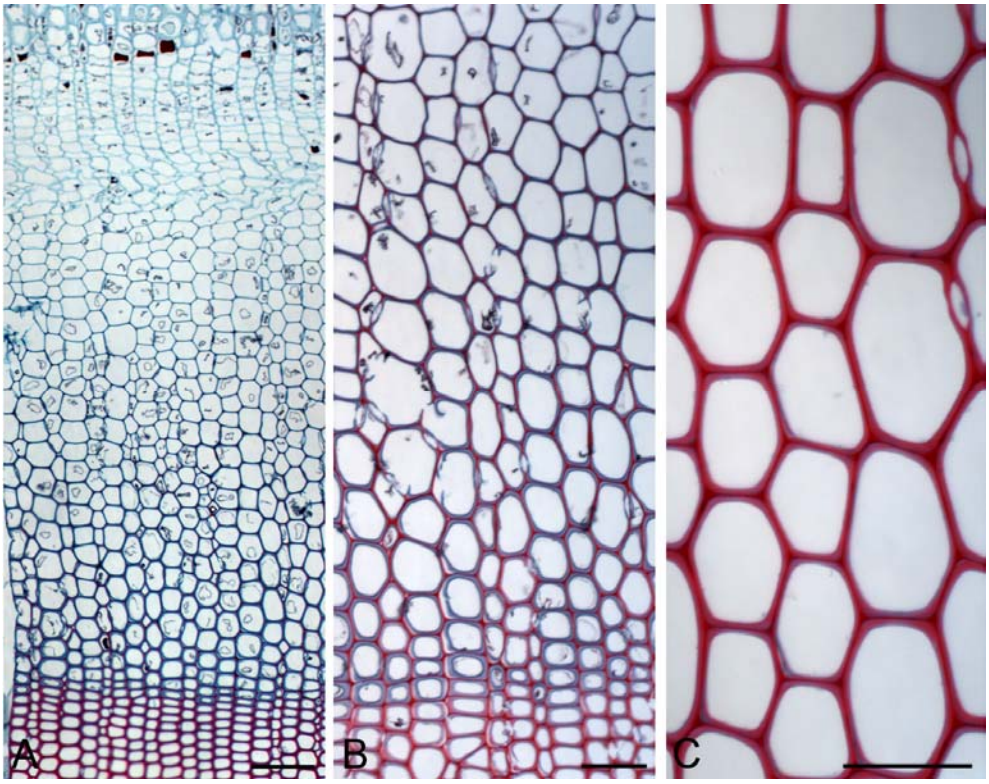


Figure 6.1 Transverse sections stained with safranin and counter stained with fast green. Lignin was not present in the cambial region or region of radial expansion, evidenced by the predominance of blue staining (A). Lignin was first apparent in the CML of cells at the end of the radial expansion region (B) and increased until the point of a mature tracheid, shown as red staining (C). Scale bars (A) =100 µm, (B-C) =50 µm.

6.2.1.2 Phloroglucinol-HCl

Transverse wax sections mounted on glass slides were stained with a mixture of phloroglucinol and hydrochloric acid. In this procedure, the interaction of

phloroglucinol with coniferyl aldehyde and cinnamyl aldehyde in lignin yields a pink colour [1, 12-14]. This technique was performed on the control explants from three culture sets. The results confirm that lignin is not present in the cambial cells, or early in the region of radial cell expansion, evidenced by the absence of pink staining (figure 6.2A). A slight pink colour was developed in the cells located at the end of the region of radial expansion, which corroborate the presence of lignin in the CML before it is seen in the secondary walls (figure 6.2B). In the mature tracheids, lignin was present across the cell walls (figure 6.2C).

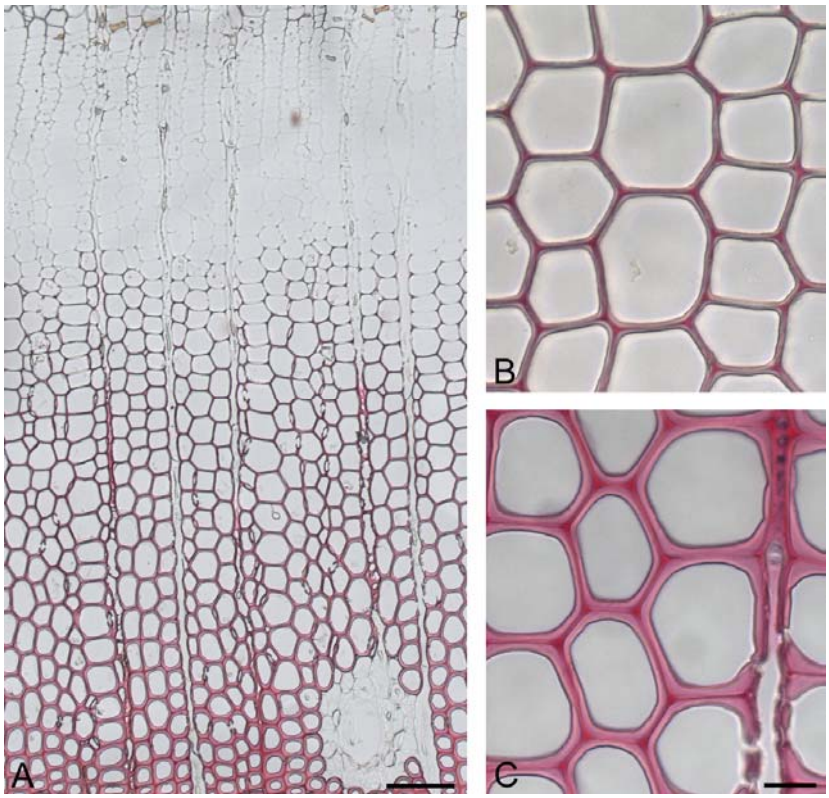


Figure 6.2 Transverse sections stained phloroglucinol-HCl. Lignin was not present in the cambial region or region of radial expansion (A), evidenced by the absence of staining. Lignin was first apparent in the CML of cells at the end of the region of radial expansion (B) and increased until the point of a mature tracheid (C), shown as pink staining. Scale bars (A) = 100 µm, (B-C) = 20 µm.

6.2.2 Epifluorescence

Transverse wax sections mounted on glass slides were observed under epifluorescent light on an Olympus microscope fitted with a mercury lamp. In the near ultraviolet, lignin gives a green-blue autofluorescence using epifluorescent techniques [1, 15]. The main disadvantage of this technique is that the intensity decays with time so

standardized procedures must be used [1]. This method was employed to further observe the incidence of lignification in the cell wall regions. Similar to the results from the histochemistry methods described in section 6.2.1, lignin was first identified in the CML of cells at the end of the region of radial expansion (figure 6.3A), and was observed to progress toward the region of S_2 layer formation (figure 6.3B). Lignin was also present in the S_3 layer (figure 6.3C arrow).

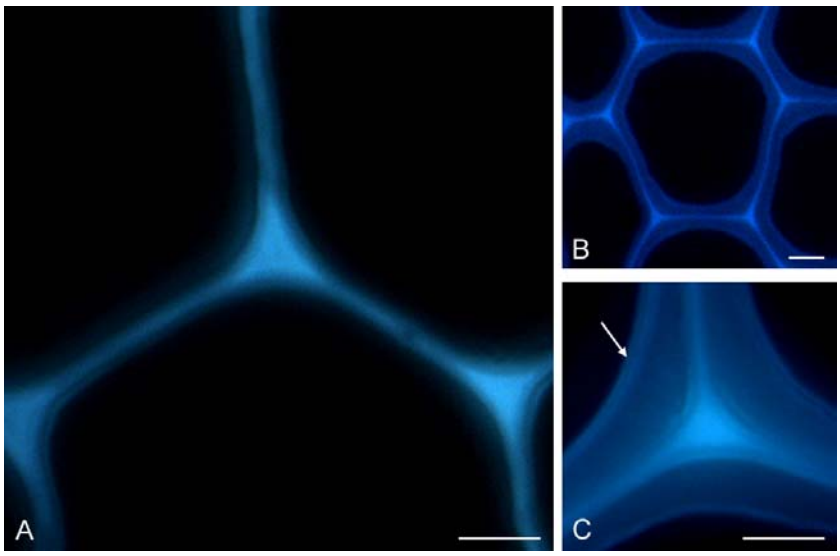


Figure 6.3 Transverse sections observed by blue autofluorescence. The CML at the cell corners is the first to undergo lignin deposition (A), followed by the secondary cell walls (B) and (C). Scale bar (A, C) = 5 µm, (B) = 10 µm.

6.3 Lignin distribution in the organ cultures

In section 6.2, the normal distribution of lignin in radiata pine was described. In this section, the results from the same techniques, used to determine if lignification was altered in the nutrient treated cultures, are presented. These experiments were performed concurrently with examination of the control explants.

6.3.1 Histochemistry

For all of the culture treatments, the low and high nutrient treated cultures and their control explant, from three culture sets were examined.

6.3.1.1 Safranin/fast green

As described in section 6.2.1.1 safranin stains lignin red, while fast green stains cellulose blue.

Boron-treated cultures

In two of the boron-treated culture sets[1], the low added boron treatments (0 µM) showed intense red staining (figure 6.4A) when compared to the high added boron treatments (100 µM and/or 1000 µM; figure 6.4C) and the control explants. This suggested that the low boron-treated cultures had increased lignification, while the high boron-treated cultures, which had very little red staining, showed a decrease in lignification (figure 6.4B) compared to the control explants. Interestingly, in the culture set presented (figure 6.4), the latewood, which was present prior to culturing (identified by the thick red cell walls), stained more intensely in the low boron-treated culture compared to the high boron-treated culture. In the third culture set, the high boron-treated culture stained slightly more intensely than the low boron-treated culture, suggesting that variation in the culture response to boron can occur.

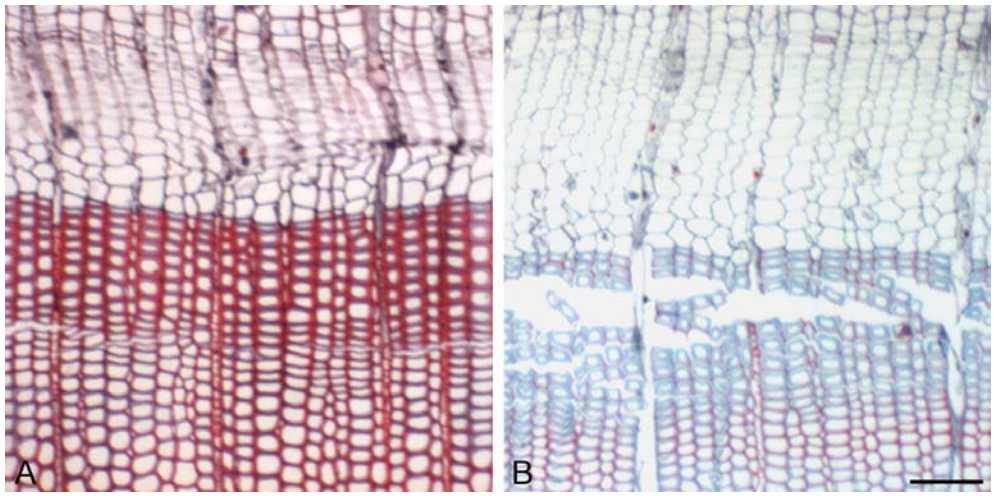


Figure 6.4 Transverse sections stained with safranin/fast green. Safranin stains lignin red and fast green stains cellulose blue. Low boron treated cultures (0 µM; A) appear to have much greater lignification than the high boron treated cultures (100 µM; B) which appear to have much less lignin. Images were collected with a Coolsnap digital camera. Scale bar = 100 µm.

[1] Boron-treated culture sets observed with safranin/fast green staining: two Burnham trees cultured in October, and one cultured in November. The November culture set showed greater staining in the high boron treated culture.

Calcium-treated cultures

In one of the calcium-treated culture sets[2], a decrease in lignification, observed as less intense red staining, was observed in the high calcium-treated culture (100 mM; figure 6.5B) when compared to the low calcium-treated culture (0 mM; figure 6.5A) and the control explant. In the other two culture sets examined, it was difficult to qualitatively distinguish differences in the safranin staining intensity, as they appeared quite similar, suggesting variations in the culture response to calcium could occur.

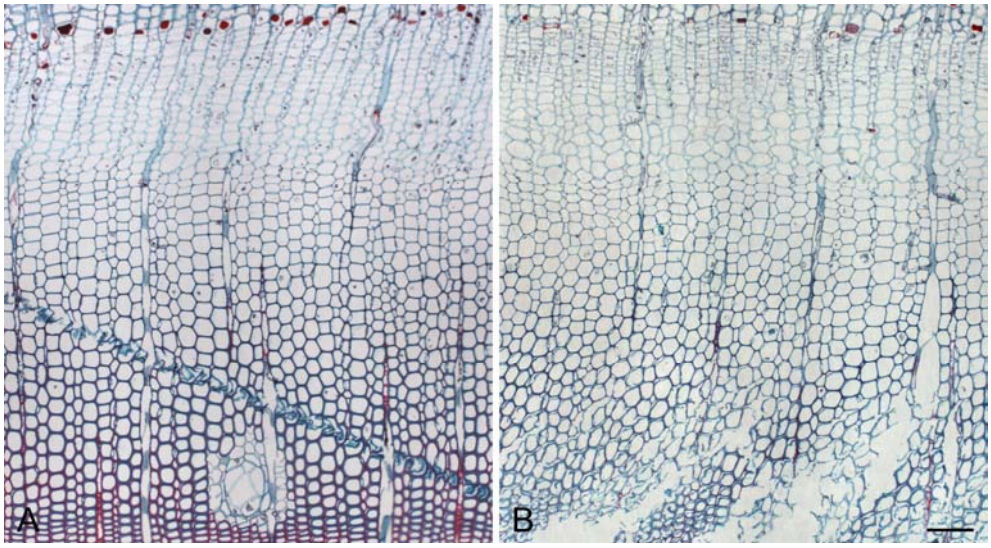


Figure 6.5 Transverse sections stained with safranin/fast green. In one of the culture sets, the low calcium-treated culture (0 mM; A) appeared to have more lignin the high calcium-treated culture (100 mM; B). Images were collected with a Zeiss Axioshop camera. Scale bar = 100 µm.

[2] Calcium-treated culture sets observed with safranin/fast green staining: set number one, two and three (chapter four). Set number one showed variability in staining.

Magnesium-treated cultures

In all three of the magnesium-treated culture sets[3] observed, there did not appear to be any changes in the intensity of the safranin staining (figure 6.6), suggesting that magnesium did not influence lignification in these cultures.

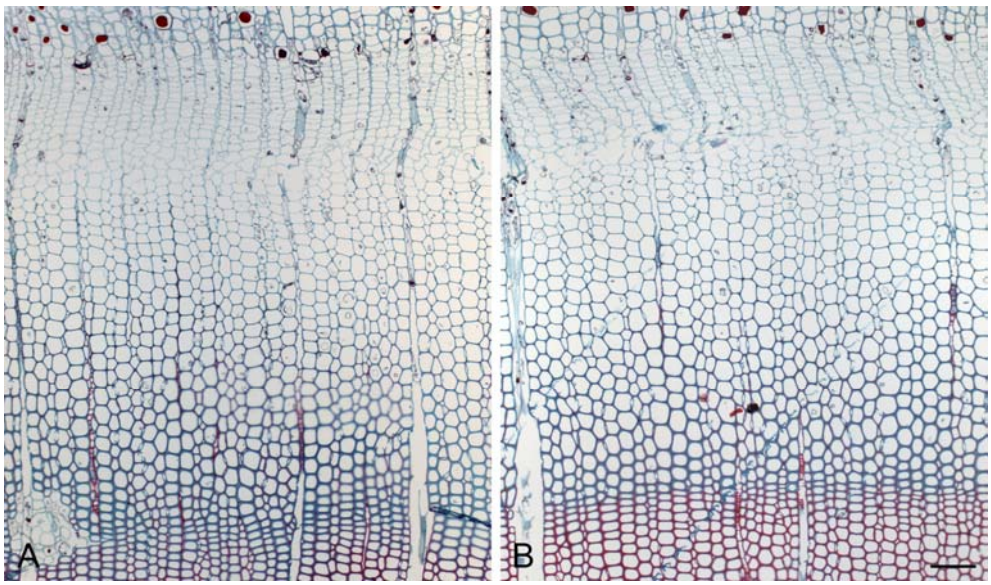


Figure 6.6 Transverse sections stained with safranin/fast green. There were no differences in the safranin staining intensity of the low magnesium-treated cultures (0.5 mM; A) and the high magnesium-treated cultures (10 mM; B). Images were collected with a Zeiss Axioshop camera. Scale bar = 100 µm.

[3] Magnesium-treated culture sets observed with safranin/fast green staining: set number two and three (chapter four) and an additional culture set from a Burnham tree cultured in February.

Mixed-treated cultures

In all three of the mixed-treated culture sets[4] examined, there did not appear to be a change in lignification, represented by the intensity of the safranin staining (figure 6.7).

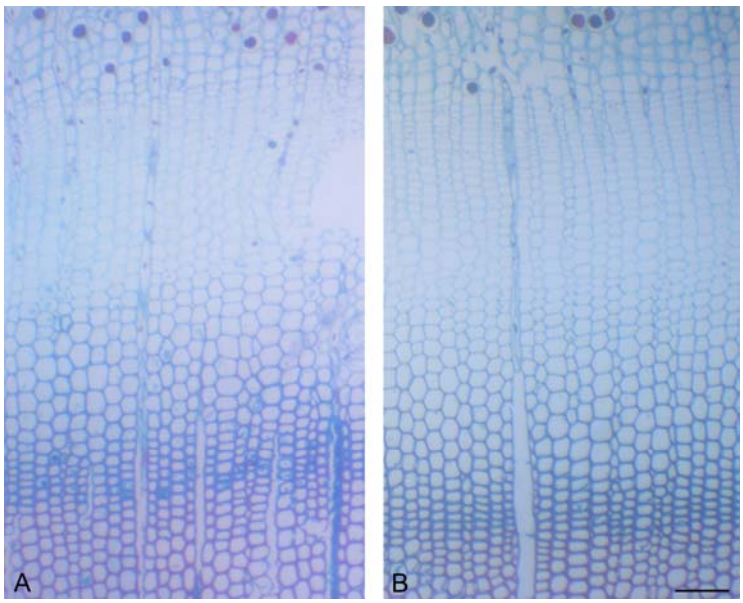


Figure 6.7 Transverse sections stained with safranin/fast green. The LBHC treated cultures (A) and the HBLC treated cultures (B) did not show changes in the intensity of the safranin staining. Images were collected with a 5 mega pixel Olympus digital camera. Scale bar = 100 µm.

6.3.1.2 Phloroglucinol-HCl

As described in section 6.2.1.2 the phloroglucinol-HCl reaction stains lignified tissues pink. This technique was employed on two of the boron treated culture sets[5] in order to further examine the distribution of lignin in response to boron. Changes in the intensity of phloroglucinol-HCl staining were difficult to discern in the cells that had undergone the initial stages of lignification (figure 6.8A, B); however, in cells at later stages of lignin deposition, the low boron treated cultures (figure 6.8C) had a

[4] Mixed-treated culture sets observed with safranin/fast green staining: set number one, two and three (chapter four).

[5] Boron-treated culture sets observed with phloroglucinol-HCl staining: set number one and three (chapter four).

tendency to show darker staining than the high boron-treated cultures (figure 6.8D), suggesting that changes in lignification had occurred. This corroborates the suggestion in section 6.3.1.1 that, using histochemical methods, boron-treated cultures show altered lignification.

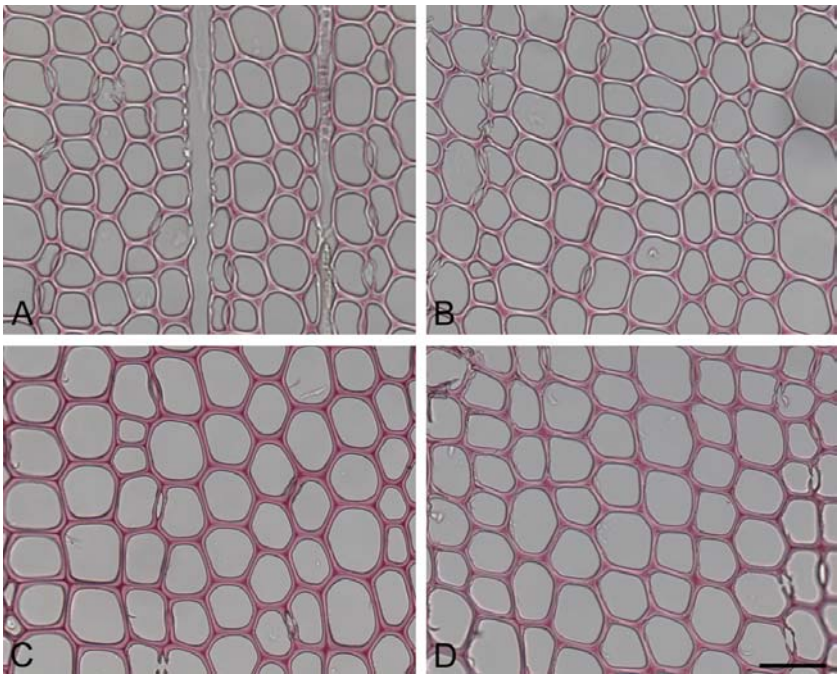


Figure 6.8 Transverse sections stained with phloroglucinol-HCl. Low boron-treated cultures (0 µM; A) that had undergone the initial stages of lignin deposition did not appear to have changes in lignification when compared to the high boron-treated cultures (100 µM; B). However, at later stages of lignin deposition the low boron-treated cultures (C) did show an increase in lignification, identified by the darker pink staining compared to the high boron-treated cultures (D). Images were collected with a Zeiss Axioship digital camera. Scale bar = 50 µm.

6.3.1.3 Epifluorescence

Further analysis of the mineral nutrient treated cultures was completed through observation of de-waxed sections using an Olympus epifluorescent microscope, as described in section 6.2.2. The blue autofluorescence images collected were used for semi-quantitative analysis of the distribution of lignin in the cell walls. Image pro plus software was utilized to determine the area of the CML/S_1 region occupied by lignin relative to the area of the cell wall in cells undergoing secondary wall deposition. The S_1 cell wall is known to have less lignin than the CML and S_2 wall; however, the term CML/S_1 is used in this work because these images do not allow for the easy distinction between CML and S_1 wall layers. Measuring the area of lignin in the

CML/S_1 region of the cell wall was based on the pixel intensity of the images and the assumption that the autofluorescence was attributed only to lignin in the cell wall [16]. The low and high treatments from three mineral nutrient treated culture sets were analyzed, and the calculated areas are presented in appendix one.

Boron-treated cultures

Blue autofluorescence revealed cell to cell variation in lignification of the CML/S_1 wall region, with the low added boron-treated cultures[6] (0 µM) having a tendency to show more pronounced autofluorescence in this region (figure 6.9A), compared to the high boron-treated cultures (1000 µM; figure 6.9B). However, when the area of greatest pixel intensity, representative of the CML/S_1 region, was measured relative to the cell wall, no significant differences were found between the boron culture treatments, or the control explants.

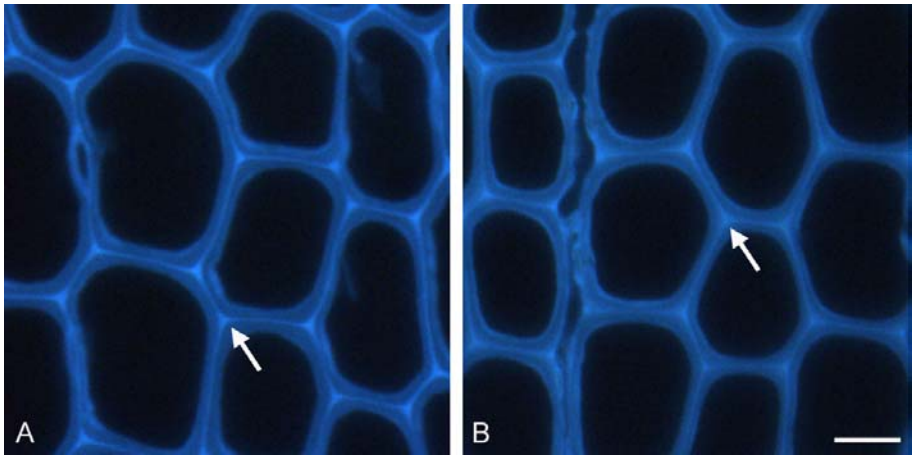


Figure 6.9 Transverse sections observed by blue autofluorescence. The area of the cell wall occupied by lignin appeared to increase under boron deficient conditions (0 µm; A), as indicated by the increased blue autofluorescence in the CML/S_1 wall region, when compared to the high boron treated cultures (1000 µm; B). Scale bar = 20 µm.

[6] Boron-treated culture sets observed by epifluorescence: set number one, two and three (chapter four).

Calcium, magnesium and mixed-treated cultures

There were no obvious differences in the intensity, or area, of blue auofluorescence observed in the CML/S_1 wall region of the calcium[7], magnesium[8] or mixed[9]-treated cultures (figures 6.10; 6.11 and 6.12 respectively). Moreover, the area of the CML/S_1 wall, measured as the area of the greatest pixel intensity, was not found to be significantly different within these culture treatments.

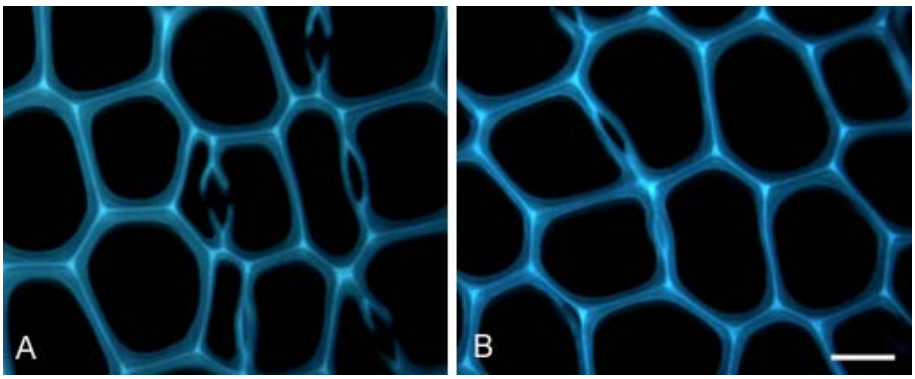


Figure 6.10 Transverse sections observed by blue autofluorescence. The CML/S_1 region of the low calcium-treated cultures (0 mM; A) when compared to the high calcium-treated cultures (100 mM; B) did not show changes in the intensity, or area, of blue autofluorescence. Scale bar = 20 μm.

[7] Calcium-treated culture sets observed by epifluorescence: set number one and three (chapter four), and an additional culture set from a Burnham tree cultured in February.

[8] Magnesium-treated culture sets observed by epifluorescence: set number one and two (chapter four), and an additional culture set from a Burnham tree cultured in February.

[9] Mixed-treated culture sets observed by epifluorescence: set number one, two and three (chapter four).

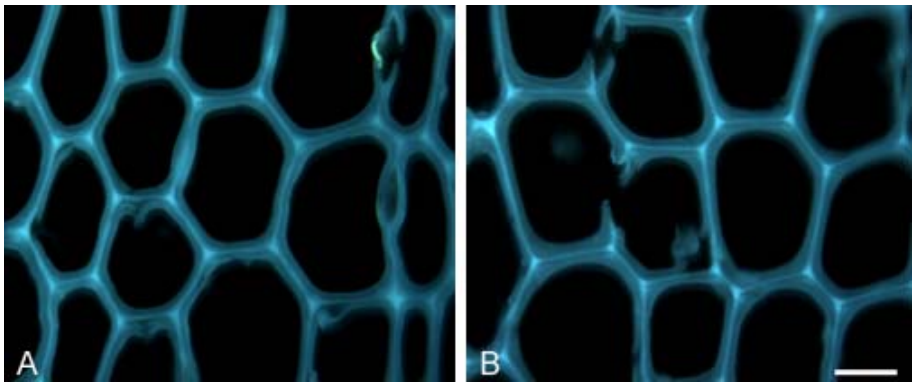


Figure 6.11 Transverse sections observed by blue autofluorescence. The CML/S_1 region of the low magnesium-treated cultures (0 mM; A) when compared to the high magnesium-treated cultures (100 mM; B) did not show changes in the intensity, or area, of blue autofluorescence. Scale bar = 20 µm.

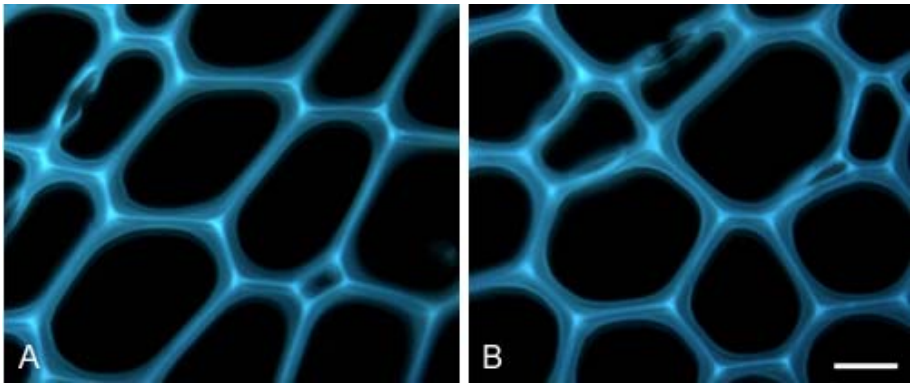


Figure 6.12 Transverse sections observed by blue autofluorescence. The CML/S_1 region of the LBHC treated cultures (A) when compared to the HBLC treated cultures (B) did not show consistent changes in the intensity, or area, of blue autofluorescence. Scale bar = 20 µm.

6.3.2 Transmission electron microscopy

Transmission electron microscopy (TEM) was also employed to determine if the distribution of lignin in the CML/S_1 wall was altered as a result of the different nutrient treatments. The use of TEM to observe lignin distribution was also described in section 2.3.3.1.

6.3.2.1 Potassium permanganate

The nutrient treated cultures were embedded in Spurr resin, and stained with potassium permanganate as described previously (section 2.3.3.1). Duplicate grids of the low and high mineral nutrient treated cultures, from two culture sets, were observed on a Hitachi TEM at 75 kV.

Boron-treated cultures

The low boron-treated cultures[10] (0 µM) often had an altered CML/S_1 cell wall region with a tendency to show "patchy" lignin sites, identified as dark striations (figure 6.13A), when compared to the high boron-treated cultures (1000 µM; figure 6.13B), and the control explants (figure 6.13C). The CML/S_1 wall region also appears 'swollen', when compared to the high boron-treated cultures and the control explant (figure 6.13A). This observation corroborates the lead stained boron treated cultures presented in section 4.8.1.1, which also had a 'swollen' CML/S_1 cell wall region.

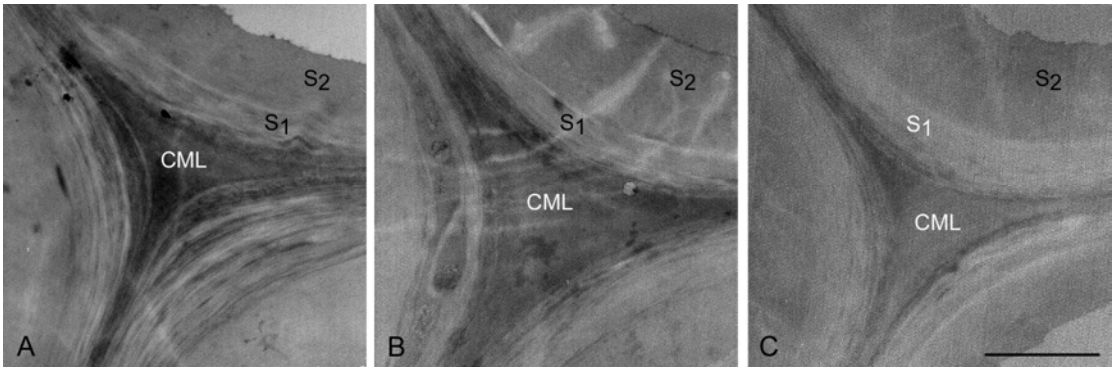


Figure 6.13 The low boron-treated cultures (0 µM; A) have a tendency to show 'swollen' CML/S_1 wall regions with darker striation patches when compared to the high boron-treated cultures (1000 µM;.B) and the control explant (C). The samples were embedded in Spurr resin and transverse ultra-thin sections were stained with potassium permanganate and observed on a Hitachi TEM at 75 kV. Scale bar = 2 µm.

[10] Boron-treated culture sets observed with potassium permanganate staining: set number one (chapter four) and an additional culture set from a Burnham tree cultured in March.

Calcium-treated cultures

The high calcium-treated cultures[11] (100 mM; figure 6.14B), in one of the culture sets observed, often showed much denser CML/S_1 striations when compared to the low calcium-treated cultures (0 mM; figure 6.14A) and the control explant (figure 6.14C). The ratio of the CML/S_1 wall regions, relative to each other, did not appear to change. However, in the other calcium treated culture set observed there were no apparent differences in this region, suggesting variability in the influence of calcium on the cell wall.

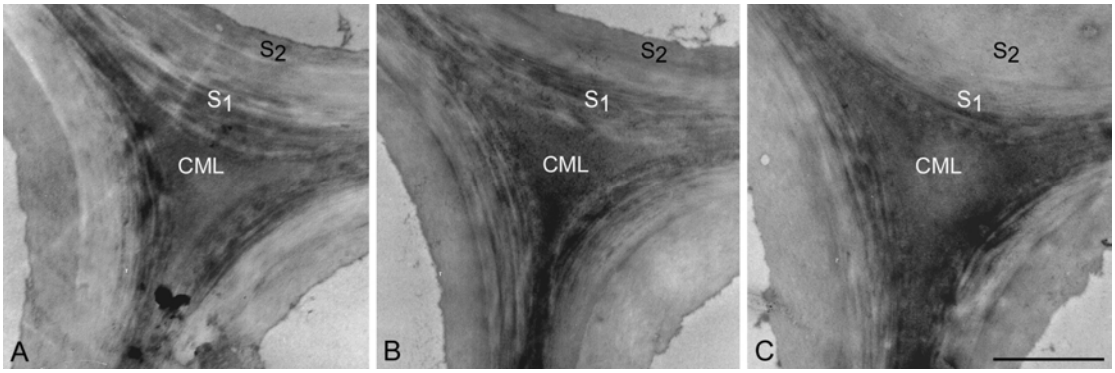


Figure 6.14 In one culture set, the low calcium-treated culture (0 mM; A) appeared to have less prominent striations when compared to the high calcium-treated culture (100 mM; B) but not the control explant (C). The samples were embedded in Spurr resin and transverse ultra-thin sections were stained with potassium permanganate and observed on a Hitachi TEM at 75 kV. Scale bar = 2 µm.

Magnesium-treated cultures

In one of the high magnesium-treated cultures[12] (100 mM; figure 6.15B), altered staining in the CML/S_1 wall regions, with denser striation deposits, was occasionally observed compared to the low magnesium-treated cultures (figure 6.15A) and the control explant (figure 6.15C). However, this staining pattern was not consistent within these cultures, and a great deal of variation was observed.

---

[11] Calcium-treated culture sets observed with potassium permanganate staining: culture set number one (chapter four) and an additional culture set from a Burnham tree cultured in February.

[12] Magnesium-treated culture sets observed with potassium permanganate staining: set number one and two (chapter four).

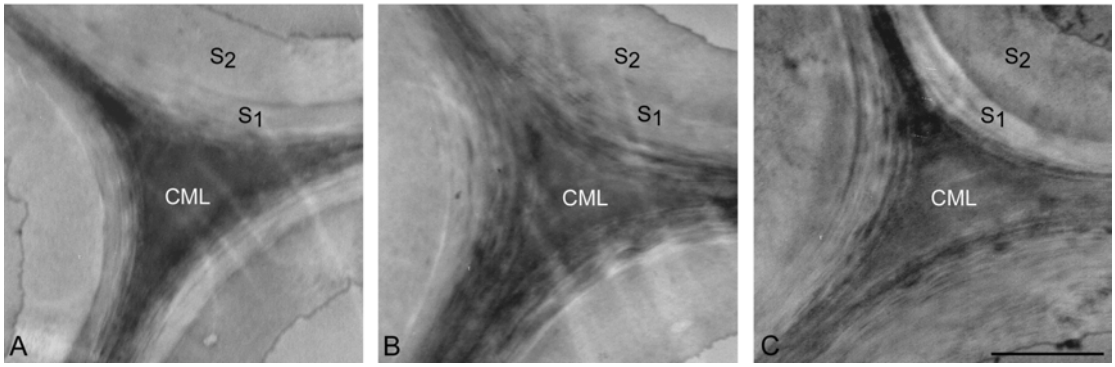


Figure 6.15 The low magnesium-treated culture (0 mM; A) appeared to have less dense striations when compared to the high magnesium-treated culture (100 mM; B) and the control explant (C) for one of the culture sets. The samples were embedded in Spurr resin and transverse ultra-thin sections were stained with potassium permanganate and observed on a Hitachi TEM at 75 kV. Scale bar = 2 µm.

Mixed-treated cultures

The LBHC (figure 6.16A) and the HBLC (figure 6.16B) mixed-treated cultures[13] did not show changes in potassium permanganate staining, and thus lignin distribution, when compared to each other or the control explant (figure 6.16C). Likewise, the organization of the CML/S_1 region did not appear to change.

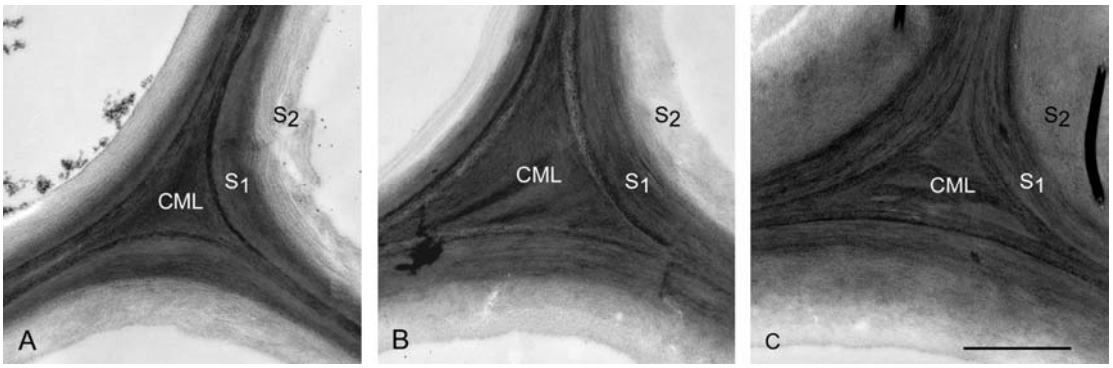


Figure 6.16 The lignin distribution in the LBHC treated culture (A) and the HBLC treated culture (B) did not appear to change when compared to each other and the control explant (C). The samples were embedded in Spurr resin and transverse ultra-thin sections were stained with potassium permanganate and observed on a Hitachi TEM at 75 kV. Scale bar = 2 µm.

[13] Mixed-treated culture sets observed with potassium permanganate staining: set number three (chapter four) and an additional culture set from a Burnham tree cultured in January.

In the potassium permanganate stained, nutrient treated cultures, and their control explants, the highest lignin concentration was observed in the CML, followed by the secondary wall, with the S_1 layer appearing less grey, due to a decreased presence of lignin in this region. This is in agreement with previous reports of lignin distribution in wood using TEM techniques [section 2.3.3; [17-19]]. Moreover, lignin particles appeared to be oriented relative to the cellulose fibrils, consistent with other reports [17, 19]. This corroborates previous suggestions that polymerization of the monolignols could be affected by the polysaccharides that comprise the cell wall [19].

6.4 Biochemical analysis of the organ cultures

In order to support the histochemical and TEM observations, which suggested that changes in lignification could occur in some of the nutrient treated cultures (section 6.3), biochemical analysis was performed to determine the total lignin content of nutrient treated cultures, as well as their control explants. The cultures prepared for these analyses were explanted from the same tree, inducted on high or low boron, calcium or magnesium nutrient treatments, and cultured for the same length of time. In order to obtain enough material for analysis, three or four explants were cultured per treatment. After collection, the cultures for each treatment were pooled and ground to a homogenous powder. This allowed for the comparison of different techniques on the same samples. Cultures were prepared from two trees, creating replicate treatments.

6.4.1 Lignin content

The lignin content of the finely ground cultures was determined by Klason lignin and acetyl bromide assays (section 6.4.1.1) as well as by pyrolysis gas chromatography and mass spectrometry (Py-GC-MS; section 6.4.1.2). The Klason lignin content was determined by Veritec, SCION, (Rotorua, New Zealand), the acetyl bromide lignin content was calculated as described in section 9.2.3.4, and the Py-GC-MS was performed by ensis, SCION (Rotorua, New Zealand).

6.4.1.1 Klason and acetyl bromide lignin assays

The Klason and acetyl bromide techniques were described in chapter two (section 2.4.1.1 and 2.4.1.2). The results obtained for both trees were similar and the data were pooled to generate averages for the replicate treatments.

There were no statistically significant differences in the total lignin content within the boron, calcium, magnesium or mixed-treated cultures when compared to each other using the Klason or acetyl bromide methods (table 6.1). The lignin content values obtained with both the Klason and acetyl bromide methods agree with previously reported values for pine, discussed in chapter two (section 2.4.1).

Culture treatment	Acid insoluble lignin[a,b]	Acid soluble lignin[a,b]	Total lignin[a,b]	ABL[b]
Boron				
0 µM	26.6 ± 0.3	0.87 ± 0.01	27.5 ± 0.3	31.3 ± 3.2
1000 µM	26.8 ± 0.1	0.88 ± 0.01	27.8 ± 0.1	26.7 ± 2.1
Calcium				
0 mM	27.1 ± 0.5	0.86 ± 0.01	28.0 ± 0.5	23 ± 0.6
100 mM	27.1 ± 0.8	0.97 ± 0.09	28.1 ± 0.7	20.7 ± 5.4
Magnesium				
0 mM	27.6 ± 0.7	0.8 ± 0.05	28.4 ± 0.6	26.4 ± 1.9
100 mM	26.3 ± 0.2	0.84 ± 0.08	27.1 ± 0.2	23.2 ± 5.0
Mixed				
LBHC	27.7 ± 0.3	0.93 ± 0.04	28.6 ± 0.2	20.5 ± 3.0
HBLC	28.7 ± 1.5	0.81 ± 0.01	29.5 ± 1.5	25.8 ± 2.3
Control explant	24.2 ± 0.8	0.95 ± 0.02	24.2 ± 0.9	20.4 ± 0.04

[a] Values determined by the Klason method
[b] Values represent the average of cultures from two trees

Table 6.1 Klason and acetyl bromide lignin (ABL) content of ground wood material from low and high nutrient treated cultures. The Klason method determines acid insoluble and acid soluble lignin from which the total lignin content is calculated. Values represent percent lignin (w/w). Data is ± standard error of the mean. ABL values include duplicate measurements.

6.4.1.2 Pyrolysis gas chromatography and mass spectrometry

Analytical pyrolysis and gas chromatographic separation of the sample components coupled to mass spectral detection (Py-GC-MS) is an emerging technique for the characterization of plant materials, and wood in particular [20-24]. Lignins can be pyrolyzed to produce a mixture of phenols resulting from the cleavage of ether and certain C-C linkages [20]. The majority of these phenols retain their substitution patterns from the lignin polymer, which allows for the identification of components from *p*-hydroxyphenyl, guaiacyl and syringyl moieities [20]. A few diagnostic peaks can also be detected for xylose, arabinose and glucose containing polymers [20, 21]. In this study, ground wood material, obtained from nutrient treated cultures, was sent to ensis, SCION (Rotorua, New Zealand) where it was subject to Py-GC-MS. This technique was performed in duplicate on the low and high nutrient treated cultures from two trees (section 6.4.1). Several pyrolysis breakdown products that are typical for radiata pine lignin were identified (appendix one) [D. Steward pers comm.; [20-22, 25, 26]]. Metal ions can interfere with the breakdown of carbohydrates during pyrolyisis [23, 27] which must be considered when interpreting the results. It is possible that the inorganic ions present have altered the pyrolysis breakdown pattern for the carbohydrates. In addition, the condition of the pyrolysis furnace liner can influence the pyrolysis of coniferyl alcohol with 'dirty' liners implicated in the absence of coniferyl alcohol when it could be seen in the same sample using freshly silicated inserts [20]. All of the samples analyzed were run under the same conditions (D. Steward pers. comm.).

Py-GC-MS, if performed under reproducible conditions, can be used to make a semi-quantitative comparison of the identified products [D. Steward pers comm.; [21, 27, 28]]. In this study, the relative amount of the pyrolysis products was calculated by dividing the areas obtained from electronic integration of individual peaks by the sum of the area of all the peaks in the chromatogram, as described previously [28]. In particular, changes in the presence of coniferyl aldehyde were analysed to understand the change in wood colour observed in the nutrient treated cultures (section 4.4). The relative composition of the pyrolysis breakdown products from the nutrient treated cultures, of both trees examined, were similar (appendix one). A general summary of the lignin, carbohydrate and combined lignin and carbohydrate compositions, for the nutrient treated cultures, that showed changes when compared to the control explants,

are presented in tables 6.2, 6.3 and 6.4, respectively. However, changes in the identified breakdown products may reflect monomer composition, cross-linking and/or the chemical environment that the lignin was in when it was pyrolysed (M. Jarivs pers comm.).

All lignins are composed of randomly linked phenylpropanes, such as guiacylpropane, which is commonly referred to as guiacyl lignin (introduced in section 1.3.3.7) [29]. Included among the identified pyrolysis breakdown products in this study were guiacol, vinyl guiacol, vanillin, eugenol, coniferyl alcohol and coniferylaldehyde (table 6.2). Each of these lignin units are typically associated with guiacyl lignin [29]. Phenol and methylphenols, additional pyrolysis products that were identified, are associated with 4-hydroxyphenylpropane, commonly referred to as *p*-hydroxyphenyl lignin [29]. Included in the carbohydrate products identified was 4-hydroxy-5,6-dihydro-2H-pyran-2one an indicator for xylan, and which also derives from cellulose [20], and 1,6-anhydro-β-D-glucopyranose, which is a breakdown product of cellulose [23]. Each of these pyrolysis products provided a fingerprint that aided in characterizing the components of the original sample [28].

Reproducible differences in the composition of the identified lignin pyrolysis products were observed within the calcium, magnesium and mixed treated cultures, and between these treated cultures and the control explant (table 6.2; appendix one). The boron-treated cultures showed only slight changes in the lignin pyrolysis products, which did not occur in both trees (appendix one), and for this reason have not been included in the general summary tables. The most striking differences in lignin products observed were for guaiacol, coniferyl alcohol and vanillin in the high calcium, high magnesium and LBHC treated cultures when compared to the control explant (table 6.2). In all of the nutrient treated cultures, there was slightly more coniferaldehyde when compared to the control explant, suggesting that the red colouration observed in the nutrient treated cultures (section 4.4) may be due to the polymerization of coniferyl aldehyde into lignin, in place of coniferyl alcohol, as suggested in previous Py-GC-MS studies on CAD downregulated mutants [30].

Lignin products	Culture treatment		
	High calcium	High magnesium	LBHC
Guaiacol	no change	lower	no change
Coniferyl alcohol	lower	lower	lower
Vanillin	no change	higher	no change

Table 6.2 General summary of changes observed in the lignin breakdown products identified by Py-GC-MS in the high calcium and magnesium treated cultures relative to the control explant. The cultures were explanted from a disc of the same tree, and grown for two months. Appendix 1, table 16.

Differences were also observed in the composition of some of the identified carbohydrate breakdown products, within the high calcium, magnesium and mixed treated cultures, and not the boron-treated cultures, when compared to the treated cultures and the control explant (table 6.3). In all of the nutrient treated cultures, the amount of 1,6-anhydro-β-D-glucopyranose was increased compared to the control explant (appendix one).

Carbohydrate products	Culture treatment		
	High calcium	High magnesium	LBHC
5-Hydroxymethyl-2-furfuraldehyde	lower	lower	lower
4-Allyl phenol	lower	lower	lower
1,6-Anhydro-β-D-glucopyranose	higher	much higher	higher

Table 6.3 General summary of the carbohydrate breakdown products identified by Py-GC-MS for the high calcium, magnesium and mixed treated cultures relative to the control explant. The cultures were explanted from a disc of the same tree, and grown for two months. Appendix 1, table 17.

The pyrolysis breakdown product composition of the samples were pooled to include the identified lignin and carbohydrate products, in order to determine if a relationship between these products was present in the overall composition (table 6.4; appendix one). When interpreted in this way, the most striking differences were observed between the high calcium and high magnesium treated cultures (table 6.4). The results

suggest that when lignin breakdown products were decreased, the carbohydrate breakdown products were increased, as a result of the nutrient treatments.

Combined products	Culture treatment			
	Low calcium	High calcium	High magnesium	LBHC
Guiacol	no change	much lower	much lower	much lower
Phenol	no change	lower	lower	lower
4-Vinyl guaiacol	no change	much lower	much lower	much lower
Coniferyl aldehyde	no change	lower	lower	lower
Coniferyl alcohol	no change	much lower	much lower	much lower
Vanillin	no change	lower	lower	lower
Eugenol	no change	lower	lower	lower
4-Hydroxy-5-6-dihydro-(2H)-pyran-2-one	lower	much higher	no change	much higher
5-Hydroxymethyl-2-fufuraldehyde	no change	higher	no change	higher
1,6-Anhydro-β-D-glucopyranose	no change	much higher	much higher	much higher

Table 6.4 General summary of the composition of all of the products identified by Py-GC-MS. The cultures were explanted from a disc of the same tree, and grown for two months. Appendix 1, table 18.

6.5 Discussion

Consistent with previous reports for conifers, lignification in radiata pine was shown to initiate in the CML at the junction between cells, just prior to the deposition of the secondary wall [15, 18, 31-34]. In wood from both angiosperms and gymnosperms, the CML, particularly at the cell corners, has been reported to consist of up to 60% lignin, when compared to the secondary walls, which are composed of 2 to 25% lignin [1, 12, 31, 33, 35]. Where the S_2 layer was deposited, lignin deposition decreased, shown as less dense staining compared to the CML; however, this wall layer contains the most lignin, due to its larger size [1, 12, 31, 33, 35]. The S_3 secondary wall was observed to be the last wall to undergo lignin deposition, as it was the last wall to appear [31]. Once lignification initiates, cell growth ceases; however, lignification occurs throughout the lifespan of the cell [1, 33]. How the CML can continue to lignify outside the growing cell wall is not known [31]. An extensive review on

lignification in tracheids was presented in Baucher et al. (1998) and Donaldson (2001) [18, 36].

Lignification is one of the final stages of differentiation (section 1.3.3.7) that many of the cells examined would have already been undergoing at the time of culture. Interestingly, the histochemical results presented in section 6.3.1 suggest that in the boron-treated cultures, lignification can be altered, with changes in the calcium-treated cultures being ambiguous, and the magnesium and mixed-treated cultures not visible. Analysis of lignification in the cultures was thus pursued further.

Images of lignin autofluorescence, subject to image analysis, were used to determine whether the CML/S_1 wall region had changed in size. This created a semi-quantitative representation of lignin in the CML/S_1 region only, and not the entire cell wall. While qualitative observation of the images suggested altered CML/S_1 size in the boron-treated cultures, the quantitative image analysis measurements suggested that the area occupied by lignin had not significantly changed.

The potassium permanganate stained TEM sections from the mineral nutrient treated cultures showed CML/S_1 wall structure similar to their corresponding lead stained TEM sections (section 4.8). The middle lamella showed the darkest staining, suggesting the most lignin, while the secondary walls had a more equal distribution, consistent with previous descriptions [33]. The lignin in the S_1 layer of the secondary wall showed a striated appearance, as previously observed in radiata pine (section 2.3.3). The orientation of the lignin particles appeared to follow that of the previously oriented cellulose fibres, which is also consistent with earlier observations [33, 37]. Lignin is thought to be deposited between fibrils of the cellulose skeleton, under mechanical restraint, meaning that in heavily lignified walls, swelling may occur [1, 37, 38]. This could explain the 'swollen' CML/S_1 wall region in the low boron-treated cultures (section 6.3.2), which appeared to have more lignin in the histochemical trials (section 6.3.1). In each of the histochemical and TEM methods employed to observe lignin, variation was encountered. Variability in lignin distribution is common within and between tree species [39].

In chapter four, the presence of red/brown coloured wood in the cultures was discussed (section 4.9). Red/brown xylem formation has been reported for CAD

downregulated mutants [14, 30, 40]. While these mutants appeared to have differences in lignin content, there was no change in lignin content when analyzed using biochemical methods [14, 30, 40], but changes in the lignin composition and structure were identified using Py-GC-MS [30]. Similarly, while colour differences in the wood were apparent in the organ cultures (section 4.4), biochemical analysis using the Klason and acetyl bromide assays did not reveal significant changes in lignin content (section 6.4.1.1).

The Py-GC-MS results suggest that the absence of boron, calcium and magnesium alone in the media does not significantly alter the lignin composition in the organ cultures after two months of growth, and only slight changes were seen in the carbohydrate composition. When the lignin and carbohydrate compositions were combined, the high calcium and magnesium treatments showed the greatest changes in both lignin and carbohydrate composition, with a decrease in lignin breakdown products corresponding to an increase in carbohydrate products. Some of these carbohydrate products are derived from cellulose, suggesting an increase in cellulose deposition may have occurred in the organ cultures to compensate for decreased lignin polymerization. Previous studies have found compensatory deposition of cellulose and lignin [41, 42], which was suggested to be due to intrinsic cross-talk between these two major cell wall components, and is consistent with the suggestion that trees regulate the deposition of cellulose and lignin during wood formation; however, these findings were not made by Py-GC-MS [42].

How these nutrients could alter the composition of the Py-GC-MS products is not known. It may be that boron, for example, can interacted with the enzyme mediated pathways (shikimic acid and monolignol pathways) involved in lignin production, altering the precursors available for polymerization [1-3, 43, 44]. Lignin formation is believed to be a spontaneous process that does not require enzyme mediation, with the final composition of lignin regulated by the reactivity and the availability of the monolignols [33]. If the nutrients are able to restrict the 'normal' lignin products available for polymerization, this could effectively explain the differences in lignin breakdown products observed. These results should be interpreted with caution, as pyrolysis products are heavily degraded fragments apt to react further in the course of fragmentation, making determination of a decrease in one fragment difficult to

correlate to a decrease in the structural element from which it is thought to have derived (M. Jarvis pers comm.). The data presented suggest reproducible changes in the structure of lignin, and have been discussed in the context of what these changes may mean, but do not provide conclusive evidence for changes in the original lignin composition.

Previous reports suggest that boron deficiency can lead to an accumulation of phenolic compounds in plants [1, 2]. Boron is believed to form complexes with components of catabolic pathways, which prevent the accumulation of phenolics [2]. While other studies suggest that low boron concentrations are associated with low lignin content in tissues [1, 3, 4]. Boron fertilized radiata pine was previously found to show darker phloroglucinol-HCl staining, suggesting more lignin, than control trees [4], while the addition of excess boron to radiata pine seedlings resulted in significantly lower lignin concentrations, but did not affect stem length or form [5]. In this study, low boron-treated cultures appeared to show increased lignification when viewed histochemically, while high boron conditions appeared to decrease lignification; however, this was not corroborated by the biochemical analysis of lignin content (section 6.4).

An increase in calcium availability has also been implicated in a decrease in cell wall lignification [1, 6]. Calcium is believed to regulate lignification through interactions with peroxidase/hydrogen peroxide (an enzyme involved in polymerization that uses hydrogen peroxide as an oxidant) in the cell wall [1, 7-9, 33]. Calcium is present at high concentrations in unlignified cells, and much lower concentrations in lignified cells [7]. It has been suggested that a calcium-superoxide radical (superoxide is an intermediate in the production of hydrogen peroxide) could be situated in the primary cell wall, and then penetrate the cell wall by diffusion to gain access to lignification sites [7]. It has also been suggested that pectin may regulate calcium availability during lignification, releasing calcium when it is degraded during lignification [45]. Magnesium is also effective in releasing peroxidase from the cell wall [1, 8].

In this study, the affect of calcium on lignification was not clear in the histochemical work, and no changes in response to magnesium were observed, while the Py-GC-MS results suggested that lignin structure may be altered in high nutrient treated cultures.

6.6 Summary

The work presented in this chapter was undertaken to determine the distribution of lignin in the mineral nutrient treated organ cultures. Comparative microscopy techniques were employed which suggested that lignification could be altered under low and high boron conditions. However, biochemical analysis did not corroborate these observations. Variable histochemical observations were made for the calcium-treated cultures, while the magnesium and mixed treated cultures did not appear to have altered lignification. Py-GC-MS suggested that lignin structure could be altered in the nutrient treated cultures, with the greatest changes occurring in the high calcium and magnesium-treated cultures; however, these results were not conclusive. The results presented in this chapter suggest that the secondary cell walls of the cultures could be altered; however, meaningful correlations to the nutrient treatments that the cultures were subject to could not be established and would require future work on larger sample sizes.

6.7 References

1. Wardrop, A.B. 1981. *Lignification and xylogenesis*, in *Xylem Cell Development*, Barnett, J.R., Ed. Castle House Publications Ltd.: Kent. 115-151.

2. Dugger, W.M. 1983. *Boron in plant metabolism*, in *Inorganic Plant Nutrition*, Lauchi, A., Ed. 627-650.

3. Lewis, D.H. 1980. *Boron, lignification and the origin of vascular plants - a unified hypothesis.* New Phytology, **84**: 209-229.

4. Skinner, M.F., Han, C.S., Singh, A.P. 2003. *Boron deficiency and tracheid properties of Pinus radiata.* New Zealand Journal of Forestry Science, **33**(2): 273-280.

5. Turvey, N.D., Carlyle, C., Downes, G.M. 1992. *Effects of micronutrients on the growth and form of two families of Pinus radiata (D.Don) seedlings*, in *Stem Deformation in Pinus radiata*, Downes, G., Turvey, N.D., Ed. School of Forestry, University of Melbourne: Melbourne. 23-32.

6. Lipetz, J. 1962. *Calcium and the control of lignification in tissue cultures.* American Journal of Botany, **49**: 460-463.

7. Westermark, U. 1982. *Calcium promoted phenolic coupling by superoxide radical - a possible lignification reaction in wood.* Wood Science and Technology, **16**: 71-78.

8. Lipetz, J., Garro, A.J. 1965. *Ionic effects on lignification and peroxidase in tissue cultures.* The Journal of Cell Biology, **25**: 109-116.

9. Hayashi, T. 1989. *Xyloglucans in the primary cell wall.* Annual Review of Plant Physiology, **40**: 139-168.

10. Downes, G., Turvey, N.D. 1986. *Reduced lignification in Pinus radiata D. Don.* Australian Journal of Forest Research, **16**: 371-7.

11. Chaffey, N. 2002. *Wood Formation in Trees*, ed. Chaffey, N. London: Taylor and Frances Books.

12. Nakano, J., Meshitsuki, G. 1992. *Reagents for the detection of lignin*, in *Methods in Lignin Chemistry*, Timell, T.E., Ed. Springer and Verlag: Berlin. 23-61.

13. Gahan, P.B. 1984. *Plant histochemistry and cytochemistry*. London: Academic Press Inc.

14. Baucher, M., Chabbert, B., Pilate, G., VanDoorsselaere, J., Tollier, M.T., PetitConil, M., Cornu, D., Monties, B., VanMontagu, M., Inze, D., Jouanin, L., Boerjan, W. 1996. *Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar.* Plant Physiology, **112**: 1479-1490.

15. Zhang, Y., Brown, G., Whetton, R., Loopstra, C.A., Neale, D., Kieliszewski, M.J., Sederoff, R.R. 2003. *An arabinogalactan protein associated with secondary cell wall formation in differentiating xylem of loblolly pine.* Plant Molecular Biology, **52**: 91-102.

16. Donaldson, L.A., Singh, A.P., Yoshingaga, A., Takabe, K. 1999. *Lignin distribution in mild compression wood of Pinus radiata.* Canadian Journal of Botany, **77**: 41-50.

17. Fengel, D., Wegener, G. 1983. *Distribution of the components within the wood cell wall*, in *Wood: Chemistry, Ultrastructure, Reactions.*, Fengel, D., Wegener, G., Ed. Walter de Gruyter & Co.: Berlin. 227-239.

18. Donaldson, L.A. 2001. *Lignification and lignin topochemistry - an ultrastructural view.* Phytochemistry, **57**: 859-873.

19. Fromm, J., Rockel, B., Lautner, S., Windeisen, E., Wanner, G. 2003. *Lignin distribution in wood cell walls determined by TEM and backscattered SEM techniques.* Journal of Structural Biology, **143**: 77-84.

20. Ralph, J., Hatfield, R.D. 1991. *Pyrolysis-GC-MS characterization of forage materials.* Journal of Agriculture and Food Chemistry, **29**: 1426-1437.

21. Faix, O., Meier, D., Fortmann, I. 1990. *Thermal degradation products of wood. Gas chromatographic separation and mass spectrometric characterization of monomeric lignin derived products.* Holz als Roh- und Werkstoff: 281-285.

22. Faix, O., Fortmann, I., Bremer, J., Meier, D. 1991. *Thermal degradation products of wood. Gas chromatographic separation and mass spectrometric characterization of polysaccharide derived products.* Holz als Roh- und Werkstoff, **49**: 213-219.

23. Kelly, J., Mackey, M., Helleur, R.J. 1991. *Quantitative analysis of saccharides in wood pulps by quartz-tube pulse pyrolysis-polar phase gas chromatography.* Journal of Analytical and Applied Pyrolysis, **19**: 105-117.

24. Yokoi, H., Nakase, T., Ishida, Y., Ohtani, H., Tsuge, S., Sonoda, T., Ona, T. 2001. *Discriminative analysis of Eucalyptus camaldulensis grown from seeds of various origins based on lignin components measured by pyrolysis-gas chromatography.* Journal of Analytical and Applied Pyrolysis, **57**: 145-152.

25. Faix, O., Meier, D., Fortmann, I. 1990. *Thermal degradation products of wood. A collection of electron-impact (EI) mass spectra of monomeric lignin derived products.* Holz als Roh- und Werkstoff, **48**: 351-354.

26. Faix, O., Fortmann, I., Bremer, J., Meier, D. 1991. *Thermal degradation of wood. A collection of electron-impact (EI) mass spectra of polysaccharide derived products.* Holz als Roh- und Werkstoff, **49**: 299-304.

27. Kelly, J., Helleur, R. 1992. *Quantitative analysis of the major saccharides in sulfite-treated wood pulps by pyrolysis-gas chromatography: the effect of metal ions.* Journal of Analytical and Applied Pyrolysis, **23**: 153-163.

28. Joly, H.A., Li, H., Belzile, N. 2000. *Pyrolysis gas chromatography-mass spectometry of humic substances extracted from Canadian lake sediments.* Canadian Journal of Chemistry, **78**: 51-63.

29. Faix, O., Meier, D., Grobe, I. 1987. *Studies on isolated lignins and lignins in wood materials by pyrolysis-gas chromatrography-mass spectometry with flame ionization detection.* Journal of Analytical and Applied Pyrolysis, **1987**: 403-416.

30. Haplin, C., Knight, M.E., Foxon, G.A., Campbell, M.M., Boudet, A.M., Boon, J.J., Chabbert, B., Tollier, M., Schuch, W. 1994. *Manipulation of lignin quality by downregulation of cinnamyl alcohol dehydrogenase.* The Plant Journal, **6**: 339-350.

31. Hafren, J., Fujino, T., Itoh, T., Westermark, U., Terashima, N. 2000. *Ultrastructural changes in the compound middle lamella of Pinus thunbergii during lignification and lignin removal.* Holzforschung, **54**: 234-240.

32. Wardrop, A.B. 1957. *The phase of lignification in the differentiation of wood fibres.* Tappi, **40**: 225-243.

33. Fengel, D., Wegener, G. 1983. *Lignification of the wood cell wall*, in *Wood: Chemistry, Ultrastructure, Reactions*, Fengel, D., Wegener, G., Ed. Walter de Gruyter & Co.: Berlin. 133-141.

34. Samuels, A.L., Rensing, K.H., Douglas, C.J. 2002. *Cellular machinery of wood production: differentiation of xylem in Pinus contorta var. latifolia.* Planta, **216**: 72-82.

35. Donaldson, L. 2001. *Lignification and lignin topochemistry - an ultrastructural view.* Phytochemistry, **57**: 859-873.

36. Baucher, M., Monties, B., Montagu, M., Boerjan, W 1998. *Biosynthesis and genetic engineering of lignin.* Critical Reviews in Plant Sciences, **17**: 1250197.

37. Singh, A., Donaldson, L. 1999. *Ultrastructure of tracheid cell walls in radiata pine (Pinus radiata) mild compression wood.* Canadian Journal of Botany, **77**: 32-40.

38. Bamber, R.K. 1987. *The origin of growth stresses: a rebuttal.* International Academy of Wood Anatomists Journal, **8**: 80-84.

39. Donaldson, L., Hague, J., Snell, R. 2001. *Lignin distribution in Coppice, Poplar, Linseed and Wheat Straw.* Holzforschung, **55**: 379-385.

40. Halpin, C., Abbott, J., Barakate, A. 2000. *Investigating lignin biosynthesis using transgenic mutant plants*, in *Cell and Molecular Biology of Wood Formation*, Savidge, R.A., Barnett, J.R., Napier, R., Ed. BIOS Scientific Publishers Ltd.: Oxford. 425-436.

41. Li, L., Zhou, Y., Cheng, X., Sun, J., Marita, J.M., Ralph, J., Chiang, V.L. 2003. *Combinatorial modification of multiple lignin traits in trees through multigene cotransformation.* Proceedings of the National Academy of Science, **100**: 4939-4944.

42. Hu, W., Harding, S., Lung, J., Popko, J.L., Ralph, J., Stokke, D.D., Tsai, C., Chiang, V.L. 1999. *Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees.* Nature Biotechnology, **17**: 808-812.

43. Lee, S., Aronoff, S. 1967. *Boron in plants: a biochemical role.* Science, **19**: 798-799.

44. Pearl, I.A. 1967. *The biosynthesis and formation of lignin*, in *The Chemistry of Lignin*, Pearl, I.A., Ed. Edward Arnold Ltd.: London.

45. Westermark, U., Hardell, H.L., Iverson, T. 1986. *The content of protein and pectin in the middle lamella/primary wall from spruce fibres.* Holzforschung, **40**: 65-68.

Chapter Seven

Isolation and characterization of arabinogalactan-proteins

7.1 Introduction

Arabinogalactan-proteins (AGPs) have been implicated in a variety of cellular processes including cell-cell recognition, cell fate, embryogenesis and xylem development [1]. Their involvement in xylogenesis, demonstrated by the addition of AGPs called 'xylogen' to *Zinnia* mesophyll cultures (introduced in section 1.7.1) [2] led to the following questions: Are AGPs involved in xylogenesis in radiata pine? Could AGPs improve wood quality?

This chapter presents a preliminary investigation of radiata pine AGPs. Specifically, the distribution of AGPs in radiata pine, studied through the use of the β-glucosyl Yariv reagent (β-GlcY) and antibody probes. In addition, the isolation and preliminary biochemical characterization of AGPs from differentiating xylem cells are presented. Organ cultures were utilized to probe the functional significance of AGPs in wood formation, and further assess the efficacy of this culture technique for the study of wood formation.

7.2 What is an arabinogalactan-protein?

In chapter one, AGPs were introduced as complex proteoglycans that are members of the HRGP family (HRGPs were introduced in section 1.3.2.8). The carbohydrate moiety of these macromolecules comprises arabino-3,6-galactan (type-II arabinogalactan; introduced in section 1.3.2.7) and the protein moiety is characteristically rich in hydroxyproline (Hyp), alanine (Ala), threonine (Thr), serine (Ser), and glycine (Gly) [1, 3-10].

7.2.1 Arabinogalactan-protein classifications

AGPs have been found in various tissues from diverse plant species, and these are generally categorised into different AGP classes [1, 11]. Four subclasses of AGPs with glycosylphospatidylinositol (GPI) anchors are currently recognized. Classical AGP genes typically encode proteins possessing an N-terminal secretion signal sequence that is removed from the mature protein, a central domain rich in proline (Pro) and a C-terminal hydrophobic domain which can facilitate membrane attachment via GPI anchoring (figure 7.1A) [7, 12]. There are also lysine-rich AGPs, short AGP peptides, and fascilin-like AGPs (FLAs), a class of chimeric AGPs that contain both AGP motifs and a fascilin domain [1]. It remains unclear if the functionality of these AGPs resides in the glycan, the protein, the GPI anchor or a combination of these [13]. Non-classical AGP genes may encode a central protein domain that is rich in asparagine (Asn) or cysteine (Cys), in addition to Pro (figure 7.1B) [1, 8, 12]. These AGPs do not possess a C-terminal hydrophobic domain, and are, therefore, not GPI anchored [3, 14]. The hybrid class of AGPs have motifs characteristic of two different HRGP classes, such as classical AGPs and extensins (extensins were introduced in section 1.3.2.8), while the chimeric class of AGPs have motifs that are characteristic of AGPs and non-HRGPs [1, 11]. A detailed review of AGP classification is presented by Johnson et al. (2003) [1].

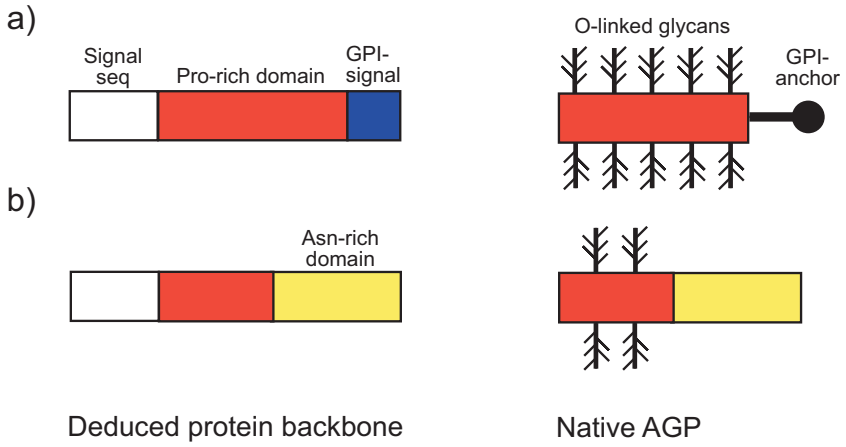


Figure 7.1 Schematic representation of a) the classical AGP protein backbone and b) non-classical AGP protein backbone deduced from DNA sequences (left) and the predicted structures of their respective native AGPs after processing and post-translational modification (right). Not drawn to scale. Processing involves removal of the predicted N-terminal signal sequence (white), removal of the predicted C-terminal GPI sequence if present (blue). Proline-rich domains are in red, and asparagine rich domains are in yellow [8, 12].

7.2.2 β-Glucosyl Yariv reagent probe for arabinogalactan-proteins

It is well known that β-glucosyl Yariv reagent (β-GlcY; 1,3,5-tris-[4-β-D-glucopyranosyl-oxyphenylazo]-2,4,6-trihydroxybenzene) binds to AGPs in a non covalent manner [1, 6, 15, 16]. The molecular basis for this interaction is not fully understood [10, 17], but requires the presence of both protein and carbohydrate [1]. Yariv reagents were developed as protein free antigens, to be used originally in the estimation of the amount of antibody present in antisera [18]. They are red coloured dyes formed by coupling aminophenyl β-glycosides to phloroglucinol [18]. It is known that their glycosyl residues must be in the β-configuration and the diazo group, which substitutes the phenyl glycoside, must be at the C(4) position on the phenyl ring for AGP binding to occur [17, 19]. α-Galactosyl Yariv reagent (α-GalY) is typically used as a control in studies utilizing β-GlcY, because α-GalY does not selectively bind to AGPs [10, 20, 21]. Yariv reagents have been extensively reviewed by Nothnagel (1997) [10].

β-GlcY reagent has several uses [10], each of which were utilized in the work presented in this chapter. These include: 1) determination of the cellular distribution of AGPs by specific staining (section 7.3.1), 2) purification of AGPs by selective precipitation (section 7.4.1) and 3) quantification of AGPs, and 4) determining the effect of perturbation of AGPs *in vitro* culture systems (section 7.6).

7.3 Location of arabinogalactan-proteins in radiata pine

Previous reports suggest that AGPs are present in all higher plant species [8, 9]. AGPs have been found in various plant organs, including leaves, stems, stigmas, roots, flowers and seeds [[10, 22, 23] and references therein]. At the subcellular level, AGPs have been located at the plasma membrane [5, 24], in the cell walls [25] and in the extracellular space [26].

Histochemical (section 7.3.1) and immunocytochemical (section 7.3.2) studies were undertaken on hand cut sections prepared from radiata pine to confirm the presence of AGPs, and characterize their location in xylem tissue. Hand cut sections were required as staining and immunolabelling could not be achieved on sections from embedded material. This is likely to be due to the high solubility of type-II

arabinogalactans in water [17, 27]. Multiple hand cut sections obtained from three trees were observed.

7.3.1 Histochemistry with the β-glucosyl and α-galactosyl Yariv reagents

β-GlcY was utilized as a histochemical stain to selectively bind AGPs in the hand cut sections, and enable visualization of their location in xylem tissue. The results suggested that AGPs, stained brownish red, are confined to a small region of xylem, composed of cells undergoing the early stages of secondary wall deposition (figure 7.2A). In this region, AGPs were predominantly present in the CML of xylem cells (figure 7.2C). Fully matured cells did not show evidence of staining (figure 7.2B). α-GalY was used as a control histochemical stain (figure 7.2D).

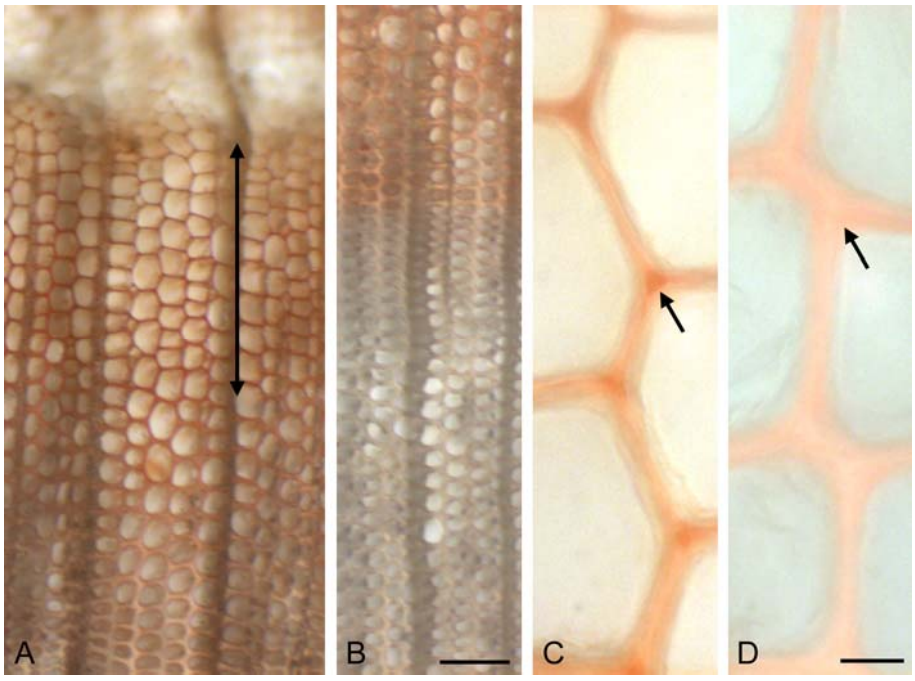


Figure 7.2 Distribution of AGPs in radiata pine. Transverse hand cut sections stained with β-GlcY (A-C) and α-GalY (D). Cells undergoing early stages of secondary wall deposition (A; arrow) stain with β-GlcY, while mature cells do not (B). The region of the CML (arrow; C) stains specifically with β-GlcY, when compared to the CML of the α-GalY (arrow;D) stained cells. Images were collected on an Olympus microscope with a Coolsnap digital camera. Scale bars for (A-B) = 100 µm, (C-D) = 10 µm.

While, α-GalY staining was observed in the sections (figure 7.2D), it was homogenous and did not appear to stain specifically, as was observed for the β-GlcY staining. Similar to the β-GlcY staining, α-GalY staining was completely absent from

mature wood. β-GlcY and α-GalY have previously been suggested to interact with cellulose, which is abundant in gymnosperm secondary walls (described in section 1.3.3.2) [21]. The presence of high concentrations of lignin in the mature cells may hinder the interaction of the Yariv reagents with cellulose, providing one explanation for the α-GalY staining observed across the cell wall of the developing cells.

7.3.2 Immunocytochemistry with the JIM13 monoclonal antibody

Immunolabelling of AGPs was performed to verify the putative cell wall labelling shown with β-GlcY. AGPs interact with a range of antibodies directed to carbohydrate epitopes, many of which have not had their ligands precisely defined [8, 28]. JIM13 is one such antibody, and reacts with a carbohydrate epitope that is potentially present on various protein backbones of AGPs [29], meaning that it is not specific for any one particular AGP [8], but rather provides a marker for AGPs in general. The location of AGPs recognized by JIM13 in the sections of radiata pine appeared to be associated with differentiation. Similar to the β-GlcY results, JIM13 bound to developing xylem cells and not to all xylem cells (figure 7.3A). Also similar to the β-GlcY results, JIM13 labelling was prevalent in the CML of xylem cells at the onset of secondary wall deposition (figure 7.3B).

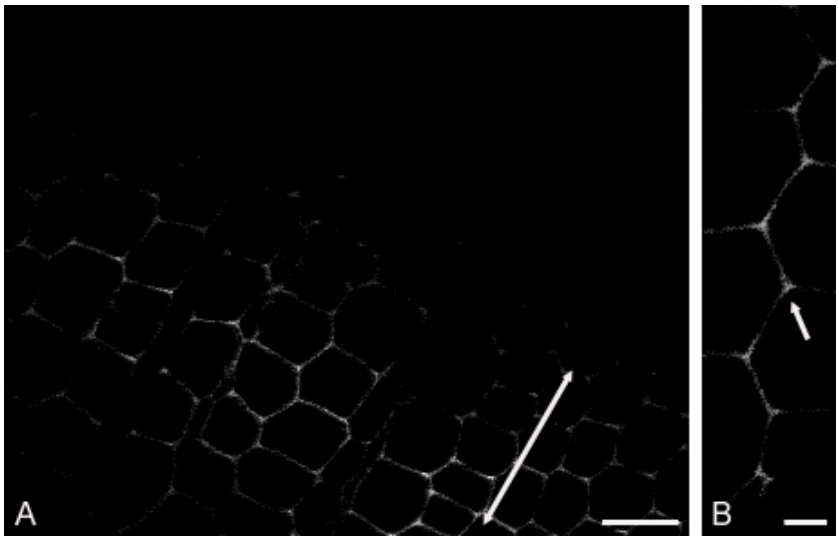


Figure 7.3 Distribution of JIM13 epitopes in radiata pine. Transverse hand cut sections showing the onset of secondary wall deposition (A; arrow illustrates orientation of image and onset of secondary wall deposition), and location of JIM13 epitope expression in the CML region (B; arrow; radial wall). Images were collected using confocal microscopy. Scale bars for (A) = 50 and (B) = 10 μm.

These results are consistent with the JIM13 epitope distribution observed in loblolly pine [30]. JIM14, another anti-AGP antibody (section 7.5.4.2), has also been found to bind AGPs in the primary cell wall and middle lamella of poplar xylem cells [31].

7.4 Isolation of arabinogalactan-proteins

In section 7.3, AGPs were positively identified in radiata pine xylem tissue. This section describes the isolation of AGPs from this tissue, and subsequent purification of the AGPs from xylem cell extracts using β-GlcY. In order to characterize these AGPs (section 7.5) milligram quantities were required.

7.4.1 AGP extraction

Xylem tissue, collected throughout the summer months, was obtained from standing radiata pine trees by removing the outer bark, and scraping the exposed cells with a razor blade. These 'xylem scrapings' composed of newly differentiating cells, as well as some contaminating cambium and phloem cells, were immediately frozen in liquid nitrogen, and stored at -80°C until required.

The protocol employed for the extraction of AGPs was adapted from the procedure used by Loopstra et al. (2000) [32]. Modifications to these procedures were made, including the addition of a trichloroacetic acid (TCA) precipitation step [3] and altering the ratio of β-GlcY used to selectively precipitate the AGPs [6], in order to streamline the purification and increase the purity of the final product.

Briefly, xylem tissue, kept frozen with liquid nitrogen, was ground to a homogenous powder using a mortar and pestle. The wood powder was extracted with a low-salt buffer, previously reported to successfully extract AGPs [32]. After this stage in the extraction, a method was required to easily remove the bulk of the impurities present in the crude sample, while retaining as many of the target AGPs as possible. This was achieved by the addition of TCA to the crude protein preparation. TCA was chosen because AGPs and extensins are known to be TCA soluble, presumably due to their high level of glycosylation [26], allowing for their separation from most cellular proteins, which precipitate [3, 26]. This protocol resulted in a TCA soluble AGP preparation that had little, if any, contaminating protein. The absence of any

Coomassie blue staining in the TCA soluble preparation is evidence of this lack of contamination (figure 7.4; lane 5).

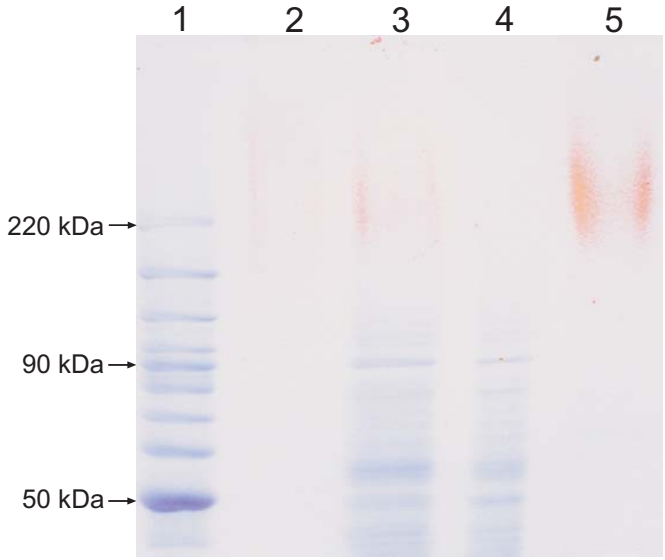


Figure 7.4 SDS-PAGE of a typical AGP preparation. Lane 1 shows the molecular weight marker with relevant bands indicated. Lane 2 shows the gum arabic loaded as a positive control. Lanes 3-5 were loaded with the protein preparation at various stages of purification as follows: 3–crude cell extract, 4–TCA insoluble precipitate, 5–TCA soluble AGP preparation. The gel has been stained with Coomassie blue to show the presence of protein, followed by β-GlcY to demonstrate the presence of AGPs.

In order to further separate AGPs from impurities in the extract, β-GlcY was used to selectively precipitate AGPs. In early extraction work performed for this study, 0.5 mg β-GlcY/g xylem was used for this purpose [32]. However, similar AGP recoveries were obtained when AGP was extracted in a 1:1 ratio to β-GlcY (for example, 1 mg AGP:1 mg β-GlcY) [6, 23]. The latter method was adopted, as it required much less of the expensive β-GlcY reagent and yielded similar recoveries. Moreover, adding more β-GlcY to the supernatant of the 1:1 precipitated extractions did not result in further precipitation, suggesting that a 1:1 ratio of AGP to β-GlcY was sufficient to precipitate AGPs. β-GlcY reversibly binds AGPs, and was removed by the addition of sodium dithionite to the resuspended pellet [23]. This preparation was desalted using PD-10 columns (Amersham) and the resulting 'β-GlcY precipitated AGPs' were stored at -80°C until required. A simplified schematic summarising the extraction procedure is presented in figure 7.5.

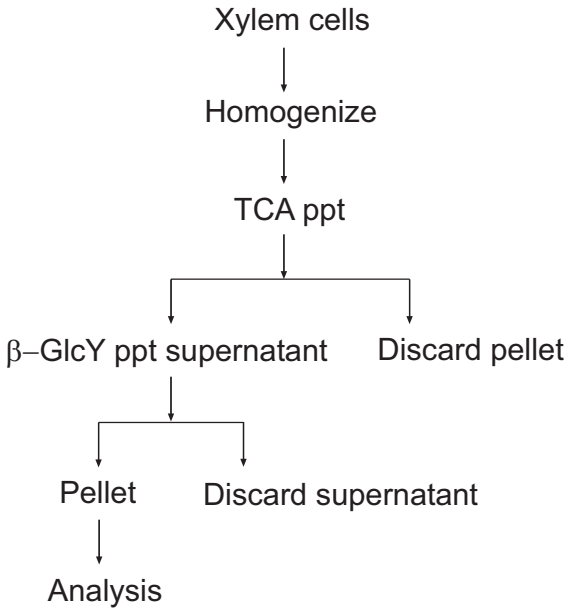


Figure 7.5 Schematic illustration of the strategy for extraction of AGPs from xylem tissue. ppt = precipitate.

	AGP		Protein	
	Total (mg)	Recovery (%)	Total[b] (mg)	Recovery (%)
Crude preparation	13.1	-	43.8	-
TCA soluble fraction	7.9	60.3	2.1	4.8
β-GlcY precipitate[a]	5.3	40.5	-	0

[a] After PD-10 desalting, referred to as β-GlcY precipitate
[b] Bradford detectable protein

Table 7.1 Typical purification of AGPs from 50 g weight of xylem cells.

The relative quantity of AGPs after each extraction step was estimated by the radial gel diffusion assay [4], and the recovery was calculated as a percentage of the amount of AGPs present in the crude extract. Radial diffusion gels utilise the specific precipitation of AGPs by β-GlcY. Briefly, β-GlcY is dissolved into an agarose gel and samples are loaded into wells and allowed to diffuse. Quantification is done with reference to a standard curve made by serially diluting gum arabic. A standard Bradford assay was also performed on the samples, to confirm the absence of contaminating proteins in the final preparations. A 40% AGP recovery was routinely obtained (table 7.1), slightly lower than the 50% recovery of AGPs reported for

Nicotiana alta [23]. However the *Nicotiana* extraction was different with the exception of ß-GlcY precipitation.

7.5 Characterization of arabinogalactan-proteins

The β-GlcY precipitate, containing purified AGPs, was used to analyze the general characteristics of AGPs in radiata pine. Immunoblots (section 7.5.1) were routinely performed on the β-GlcY precipitate to confirm the presence of AGP in the preparation. Carbohydrate (section 7.5.2) and amino acid (section 7.5.3) analysis were also performed on the β-GlcY precipitate. This required a large extraction prepared from xylem material collected from radiata pine over one week in early summer, to avoid seasonal variation in AGP expression, should it occur. The resulting β-GlcY precipitate was split into two, with one fraction analysed for the carbohydrate composition and the other for the amino acid composition.

7.5.1 Immunoblot

The presence of AGPs in the β-GlcY precipitate was assessed at the protein level using two techniques. The first, used previously (section 7.4.1), was based on the complex formation between β-GlcY and AGPs, and the second was based on the ability of the monoclonal antibody JIM13 to recognize an AGP epitope [29]. Analysis of the β-GlcY preparation by both SDS-PAGE and by SDS-PAGE followed by immunoblotting revealed a smear of material with a size greater than 100 kDa, similar to other isolated AGPs (figure 7.6) [5, 14, 19]. The diffuse bands, showing molecular weight heterogeneity, are characteristic of AGPs as well as other glycoproteins, and are due to the carbohydrate heterogeneity of AGPs [3, 5, 19, 22]. No low molecular weight bands were observed (figure 7.6). 7.5% separating gels were used for SDS-PAGE analysis (figures 7.4 and 7.6), as the AGPs did not enter the 12% separating gels commonly used in our laboratory.

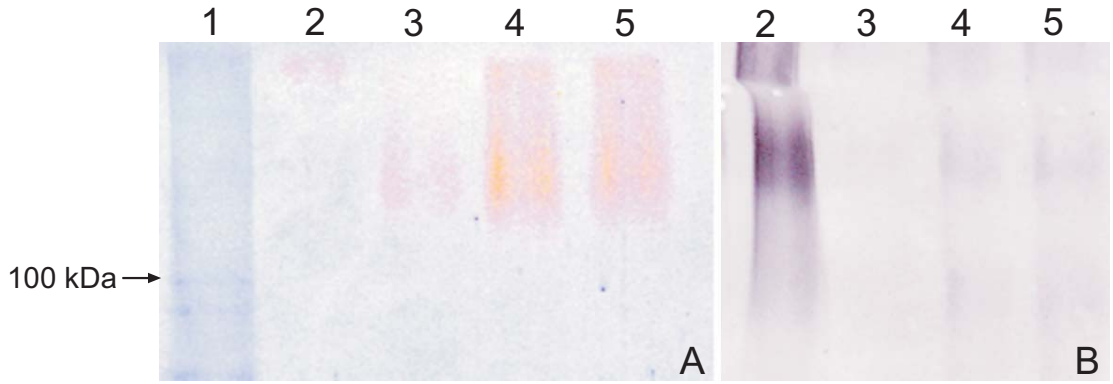


Figure 7.6 SDS-PAGE gel stained with Coomassie blue followed by β-GlcY (A) and complementary SDS-PAGE, followed by JIM13 immunoblot analysis (B) of B-GlcY purified AGPs. Lane 1 shows the molecular weight marker with relevant band indicated. Lane 2 shows the gum arabic AGP (30 µg) loaded as a positive control. Lane 3 was loaded with β-GlcY precipitate containing 10 µg of AGPs and lanes 4 and 5 were loaded with β-GlcY precipitate containing 15 µg of AGPs.

7.5.2 Monosaccharide analysis

The monosaccharide composition and linkage analysis of the β-GlcY precipitate was determined from carboxyl reduced alditol acetate and per-methylated alditol acetate derivatives. These experiments were performed in order to ascertain if AGPs in the β-GlcY precipitate possessed the characteristic type-II arabinogalactan carbohydrate moiety.

7.5.2.1 Carboxyl reduction

The reduction of carboxyl groups on acidic sugars was performed to allow for their identification when analyzing the neutral sugars by alditol acetates and linkage by per-methylated alditol acetates. Using this method, carboxyl esters were reduced with sodium borodeuteride to generate 6,6'-dideuterio-sugars which, using GC-MS, could be distinguished from neutral sugars by the presence of fragments with a shift of two atomic mass units [33]. After preliminary reduction of the esters, the sample was divided equally into two portions, each of which contained free uronic acids that were not reduced [33]. In the first portion, free uronic acids were activated with carbodiimide, and reduced with sodium borodeuteride, which allowed for the determination of the total uronic acids by GC-MS (free and esterified) [33]. The second portion was reduced with sodium borohydride [33]. When analysed by GC-MS this portion gave the proportion of esterified uronic acids when compared to the total uronic acids [33].

7.5.2.2 Alditol acetates

The neutral monosaccharides in the carboxyl reduced β-GlcY precipitate were analyzed by GC-MS of their alditol acetates after acid hydrolysis. GC-MS of alditol acetates is widely used for determining the composition of monosaccharide mixtures, especially those resulting from the hydrolysis of monosaccharides [34]. In this method, samples were acid hydrolyzed using trifluoroacetic acid (TFA), which could be removed easily by evaporation and allowed for a clean and fast procedure [35]. The resultant monosaccharides were then reduced and acetylated.

The neutral monosaccharide composition of the AGPs present in the β-GlcY precipitate (table 7.2) shows that the carbohydrate component consists almost entirely of arabinose and galactose, which is typical of AGPs [9, 17, 36]; however, these monosaccharides were present at a ratio of ~1.7:1, while most AGPs have more galactose than arabinose [5, 6, 19]. In ryegrass (*Lolium multiflorum*), arabinose and galactose in the ratio of 1.7:1 has previously been reported [[17] and references therein]. Low levels of glucuronic acid and rhamnose, and only trace levels of mannose, glucose and xylose were detected.

Monosaccharide	Mol (%)[a]
Arabinose	60
Galactose	36
Glucuronic acid	2.0
Rhamnose[b]	1.5
Mannose	tr[c]
Glucose	tr
Xylose	tr
Arabinose:Galactose	1.7:1

[a] Average of duplicate determination.
[b] 1% of rhamnose occurs as a methyl ester
[c] Trace (< 0.5 mol %)

Table 7.2 Neutral monosaccharide composition of AGPs present in the β-GlcY precipitate.

7.5.2.3 Per-methylated alditol acetates

Methylation is an important tool for elucidating polysaccharide structure [37]. In this method, free hydroxyl groups on the polysaccharide were methylated, hydrolysed, and the partially methylated sugars released were reduced and acetylated to give per-methylated alditol acetates [37, 38]. These could then be separated, identified and quantified by GC-MS. The identification of the per-methylated alditol acetates allowed for the deduction of the linkage positions of individual sugars in the parent polysaccharide, but offered no information with respect to the distribution of these sugars in the polymer, or their anomeric nature [38].

Glycosyl linkage analysis of the β-GlcY precipitate indicated that the carbohydrate component of the AGPs was a type-II arabinogalactan, characterized by 3-, 6- and 3,6-linked galactosyl residues (table 7.3). The AGPs also showed characteristically high levels of terminal arabinosyl residues (table 7.3). Interestingly, the AGPs also contained high levels of 5-linked arabinosyl residues. This is not usually seen in AGP preparations; however, AGPs from the moss *Physcomitrella patens* also have this characteristic [39]. The high amounts of 5-linked arabinosyl residues skew the galactose:arabinose ratio, explaining the unusually high arabinose content compared to galactose (table 7.2). Other residues present in minor proportions included terminal rhamnosyl and 3-, 2,5-, 3,5-, 2,3,5-linked arabinosyl residues. The identification of deuterated 4-linked glucosyl residues indicated that glucuronic acid occupied terminal and 4-linked positions.

The carbohydrate composition obtained for the per-methylated alditol acetates was in good agreement with the carbohydrate composition determined from the alditol acetate analysis. Numerous interpretations of carbohydrate linkages can be made in terms of molecular structure, these are often consistent with several different structures [40]. A proposed structure for the carbohydrate moiety of *Nicotiana alta* has previously been determined by nuclear magnetic resonance (NMR) [6]; however, the polydispersity of AGPs generally renders them unsuitable for such analysis. To obtain a definitive structure requires the glycans to be released from the peptide backbone, purified and analysed separately [40].

Monosaccharide	Deduced linkage[a]	Mol (%)[b]
Rha*p*	terminal	2
Ara*f*	terminal	31
	3-	3
	5-	27
	2,5-	1
	3,5-	7
	2,3,5-	1
Glc*p*	terminal	-
	4-	0.5
GlcA*p*	terminal	tr[c]
	4-	1.1
Man*p*	4-	tr
Gal*p*	terminal	3
	3-	10
	6-	0.5
	3,6-	13

[a] Terminal Ara*f* is deduced from 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, etc
[b] Average of duplicate determination
[c] Trace (<0.5 mol %)

*Table 7.3 Linkage composition of AGPs present in the β-GlcY precipitate. Rha*p = *rhamnopyranosyl, Ara*f = *arabinofuranosyl, Glc*p = *glucopyranosyl, GlcA*p = *glucopyranosyl uronic acid, Man*p = *mannopyranosyl, Gal*p = *galactopyranosyl.*

7.5.3 Amino acid analysis

Amino acid analysis was performed on the β-GlcY precipitate to determine if radiata pine AGPs were rich in the amino acids characteristic of AGPs (refer to section 7.2). Two separate amino acid analyses were performed on the same sample material. GC-MS (section 7.5.3.1) was performed initially; however, the failure to detect some amino acids in the spectra led to an additional HPLC analysis (section 7.5.3.2). The HPLC analysis was performed in collaboration with J. Maurer Menestrina and F. Pettolino of CRC Bioproducts (University of Melbourne, Australia).

Amino acid	Mol (%)[a,b]	Mol (%)[a,c]
Alanine (Ala)	31.6	15.3
Hydroxyproline (Hyp)	15.1	14.8
Serine (Ser)	13.3	14.4
Threonine (Thr)	5.4	11.7
Methionine (Met)	-	9.9
Cysteine (Cys)	-	6.3
Valine (Val)	9.7	4.9
Glycine (Gly)	9.2	4.8
Glutamate (Glu)	1.7	3.8
Proline (Pro)	3.8	3.4
Leucine (Leu)	6.7	2.8
Arginine (Arg)	-	1.8
Lysine (Lys)	-	1.4
Isoleucine (Ile)	2.5	1.3
Phenylalanine (Phe)	0.9	1.3
Asparagine (Asn)	-	1.0
Tyrosine (Tyr)	-	0.8
Histidine (His)	-	0.3

[a] Average of duplicate determination
[b] Determined by GC-MS analysis
[c] Determined by HPLC analysis
- Not detected

Table 7.4 The amino acid composition of AGPs present in the β-GlcY precipitate determined by GC-MS and HPLC.

7.5.3.1 Gas chromatography and mass spectometry

GC-MS has previously been suggested to be a simple and powerful method for amino acid quantification [41]. In this method, samples were acid hydrolysed and amino acids were analyzed as their *tert*-butyldimethylsilyl derivatives using GC-MS.

The results suggest that radiata pine AGPs were rich in Ala, Hyp, Ser, Val and Gly, amino acids characteristic of AGPs (table 7.4) [16]. In this system Asn, Arg, Lys, His, Tyr, Met were not detected (table 7.4) and Trp was destroyed [42].

7.5.3.2 High performance liquid chromatography

In this method, samples were acid hydrolysed and the amino acids were analyzed as their phenylthiocarbamyl derivatives using reversed phase high performance liquid chromatography (RP-HPLC), monitored at an absorbance of 254 nm. Similar to the GC-MS methods, Trp was not detected, due to its destruction [42].

HPLC confirmed that radiata pine AGPs are rich in Ala, Hyp, Ser, Val and Gly (table 7.4). However, the ratios of these amino acids were different than the GC-MS method. Moreover, this method identified more amino acids than the GC-MS method. In contrast to the GC-MS results, the β-GlcY precipitate was found to be rich in Thr and Cys.

7.5.4 Fractionation of AGPs

In order to further characterize the β-GlcY precipitate, a suitable method was required to separate any AGP subgroups. In an initial trial, fractionation was attempted by anion exchange and gel filtration chromatography on a fast protein liquid chromatography (FPLC) system according to the method of Du et al. 1994 [43]. However, this method yielded poorly resolved fractions and was abandoned. It has become fashionable to fractionate AGPs by RP-HPLC [3, 7, 12, 44, 45], and this method was adapted for all fractionations (section 7.5.4.1). Regardless of the technique utilized, purification of a single AGP is extremely difficult, if not impossible, due to the polydispersity of the proteoglycan [1, 46].

7.5.4.1 Reversed phase high performance liquid chromatography

RP-HPLC separates proteins on the basis of their hydrophobicity, and is routinely used to purify and separate AGPs [3, 7, 12, 44, 45]. Separation of the β-GlcY precipitated AGPs using RP-HPLC was achieved with two different columns. The first, an analytical Brownlee RP-300 C-8 column (Applied Biosystems) was employed for separation of small quantities of material and the second, a semi-preparative Zorbax C-8 column (Agilent Technologies) was utilised for preparation of large quantities of material. These columns were equilibrated with 0.1% trifluoroacetic acid and the samples were eluted with a linear gradient of 0-80% acetonitrile in 0.1% trifluoroacetic acid. Various gradients were trialled in initial separations, and this linear gradient was determined to provide the greatest peak

resolution, without compromising AGP recovery. Typically, the Brownlee column yielded a 60% AGP recovery, while the Zorbax column only yielded a 30% recovery, relative to starting material loaded on the column.

Using this method, an unbound and one main peak (UB and B1 respectively) were consistently eluted in addition to several very small, poorly resolved more hydrophobic peaks (B2; figure 7.7). For each RP-HPLC experiment, fractions within the UB, B1 and B2 were pooled as three separate samples for subsequent analysis (figure 7.7). Regardless of the time of year that the AGPs were collected, the same elution profiles were observed.

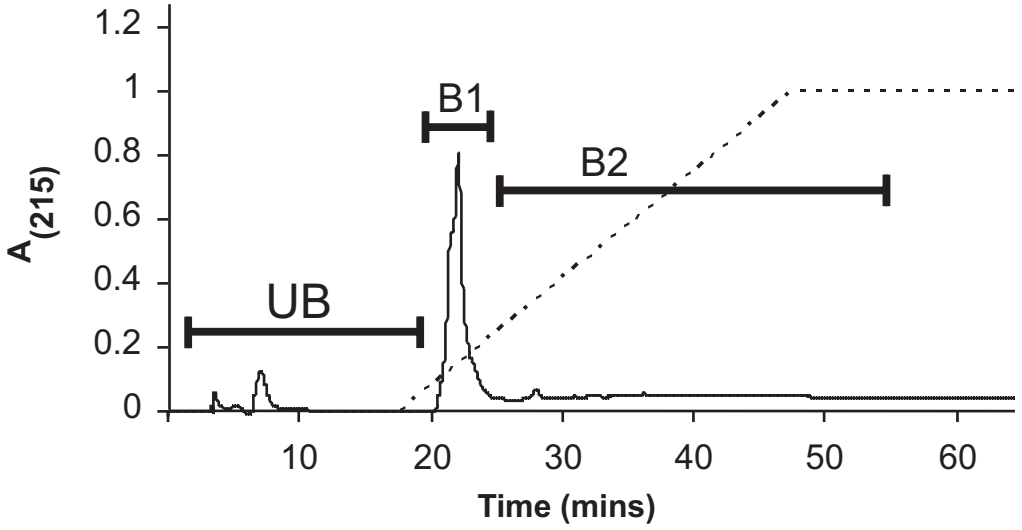


Figure 7.7 β-GlcY precipitated AGPs were separated on a C-8 column using a linear gradient from 0-80% acetonitrile in 0.1% TFA (dotted line). The fractions of interest (UB: unbound, B1 and B2: first and second bound fractions respectively) were pooled and stored for subsequent analysis.

7.5.4.2 Dot-blot immunoassay

Dot-blot analysis was utilized to test the ability of each of the collected RP-HPLC fractions to react with a series of anti-AGP antibodies in order to test for the presence of AGP epitopes in the main fraction. The antibodies used in the dot-blot analyses and their epitopes, if characterized, are summarised in table 7.5. The bulk of AGP is composed of carbohydrate, which is what the epitopes are directed against. The display of the protein moiety during antigen presentation is likely to be masked [3]. Each epitope is likely to be specific for a carbohydrate portion that is present on multiple AGPs, and not specific to a single AGP [8, 36]. These anti-AGP monoclonal

antibodies are primarily directed against AGPs expressed on the plant cell surface [3, 8, 28].

Antibody	Epitope
LM2	β-D-Glc*p*A
JIM4	β-D-Glc*p*A-(1→3)-α-D-Gal*p*A-(1→2)-L-Rha
JIM13	β-D-Glc*p*A-(1→3)-α-D-Gal*p*A-(1→2)-L-Rha
JIM14	Carbohydrate portion of AGP
JIM15	β-D-Glc*p*A
JIM16	unknown
MAC207	β-D-Glc*p*A-(1→3)-α-D-Gal*p*A-(1→2)-L-Rha, α-L-Ara, β-D-GlcA

Table 7.5 Anti-AGP monoclonal antibodies used to characterise the β-GlcY precipitated AGP fractions obtained by RP-HPLC. Some of these antibodies have have the same trisaccharide epitope, yet they exhibit different staining patterns in different tissues and species. This suggests that each antibody recognized a portion of the trisaccharide, and adjacent residues.[10, 14, 24, 28, 29, 47, 48].

Dot-blot analyses were performed in duplicate on the three pooled fractions (UB, B1 and B2; figure 7.7). This was done for three separate RP-HPLC experiments. Only B1 (figure 7.8) tested positive for AGPs by the radial gel diffusion assay, and could be quantified. For the dot-blot analysis, 10 μg of AGPs from B1 was applied to a nitrocellulose membrane. The UB and B2 (unknown AGP quantity) were also applied to the nitrocellulose to confirm the absence of AGP epitopes in these fractions (figure 7.8). After the UB and B2 RP-HPLC fractions were pooled, and dried down, they were resuspended in an equal volume to B1; however, since they could not be quantified, an equal volume to that of B1 was applied to the nitrocellulose. Gum arabic was applied to the nitrocellulose as a positive control, and a buffer only sample was applied as a negative control. A strip of nitrocellulose containing each of the applied samples was incubated in buffer in place of a primary antibody as an additional control.

Each dot-blot from the separate RP-HPLC runs yielded identical results. The gum arabic positive control reacted with the antibodies LM2, JIM13, MAC207 and, to a much lesser extent, JIM16 (figure 7.8). It is not surprising that gum arabic did not

react with each of the anti-AGP antibodies, as the antibodies were directed against different AGPs isolated from many different tissues, some of which will have tissue/species specific carbohydrate epitopes. The UB did not react with any of the anti-AGP antibodies (figure 7.8). This, coupled with the failure to test positive for AGP in the radial gel diffusion assay, suggests that AGPs were not present in this fraction. The B1 fraction reacted strongly with the LM2, JIM13, JIM16 and weakly with the MAC207 anti-AGP antibodies (figure 7.8), which confirmed the presence of AGP epitopes in this fraction. In section 7.5.2.2 the β-GlcY precipitate was found to contain high levels of arabinose. The presence of arabinose in high levels is known to reduce MAC207, JIM14 and JIM15 binding [28], which may account for the failure of this fraction to react strongly with these antibodies; likewise, this fraction may lack these epitopes. The B2 fraction reacted weakly with LM2, JIM13 and JIM16 but not MAC207 (figure 7.8). This fraction did not test positive for AGP in the radial diffusion gel; however, the results from the dot-blot analyses suggest that AGPs were present in this fraction. The discrepancies between the two techniques may be attributed to their differential sensitivity to low levels of AGP.

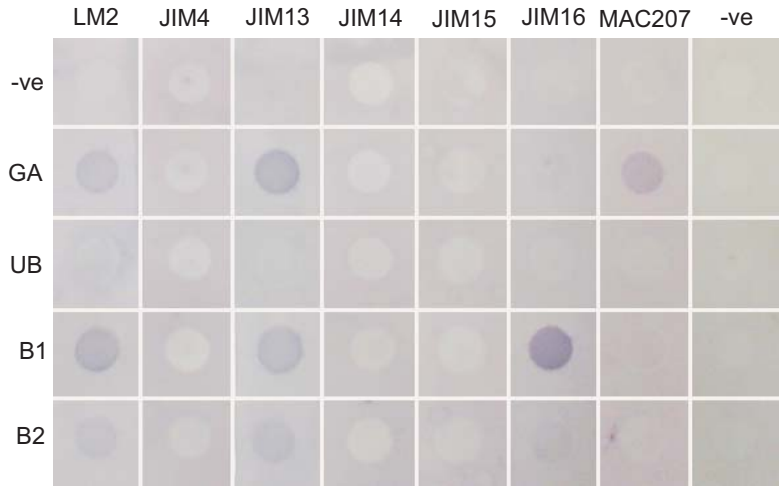


Figure 7.8 Dot-blot analysis of select RP-HPLC fractions. The dot-blot is labelled along the left as follows: -ve = buffer only control, GA = gum arabic positive control, UB = unbound, B1 = fraction one, B2 = fraction two. The B1 fraction (indicated on the left) reacted with a subset of the anti-AGP antibodies (indicated at the top) confirming that it contains AGPs.

Interestingly, the B1 and B2 fractions react with anti-AGP antibodies that have epitopes that were not present in the β-GlcY precipitate (table 7.5). This is not unique to the radiata pine β-GlcY precipitated AGPs. *Physcomitrella patens* has a similar

monosaccharide linkage to radiata pine, and reacted with JIM13 in immunoblot experiments [39]. Moreover, gum arabic and a rice AGPs reacted with MAC207 and JIM 13, but it is not reported to contain glucuronic acid [[10] and references therein]. This suggests that, in addition to the defined epitope, the antibody binding site might recognize adjacent residues in AGPs [10].

7.6 Addition of β-glucosyl Yariv reagent to the organ culture system

Organ cultures, grown in the presence of β-GlcY in the culture media, were employed to gain preliminary insight into the functional significance of AGPs in wood formation. The ability of β-GlcY to bind specifically to AGPs, and presumably inhibit their function, has been used previously to alter processes such as elongation and division [49], cell expansion [39] and somatic embryo development [22, 50]. In this section, the results from a series of microscopy and biochemical techniques, utilized to assess the efficacy of organ cultures to probe AGP function in radiata pine, are presented.

7.6.1 Media preparation

In order to complete the proposed experiments, a modified culture medium containing β-GlcY was required. For this, the standard culture media introduced in chapter three (table 3.2) was first modified by increasing the NAA concentration to 0.3 mM to stimulate cambial activity and lignification (H. Nair, unpublished data). AGPs have previously been suggested to regulate cell proliferation and lignification [51]. The culture medium was further modified by the addition of β-GlcY (10 μM) [20], in order to inhibit AGP function. The Yariv reagent concentration was chosen for a preliminary investigation into the affect of Yariv on the cultures, based on previous studies utilizing β-GlcY [20]. For controls, additional explants were cultured on media containing α-GalY (10 μM) which does not bind to AGPs [20], and NAA media. The NAA media was included as a control to ensure that if there was a culture response, it was not a reaction to the addition of the Yariv reagents in general, rather than the inhibition of AGPs by β-GlcY.

7.6.2 Explant preparation

Explants were prepared as described in section 3.2.3, inducted on the β-GlcY or control culture media, and cultured for a period of one to two months under the conditions described in section 3.2.3.6 (figure 7.9). The microbial contamination and growth of the cultures were monitored as described in section 3.3. Briefly, the date of tree felling and culture induction, length of culture and incidence of microbial contamination were recorded.

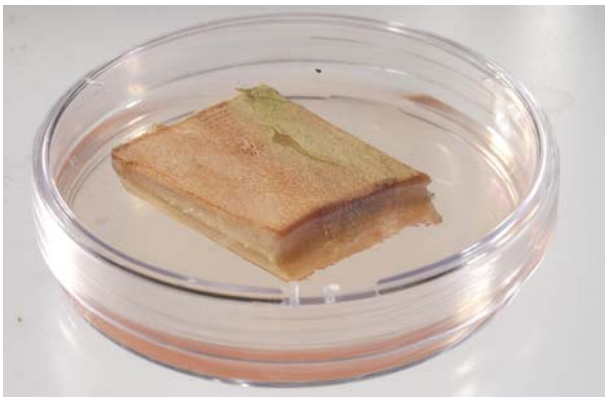


Figure 7.9 Example of an explant placed xylem side down on culture media containing β-GlcY.

7.6.2.1 Microbial contamination of explants

As discussed in section 3.3, controlling microbial contamination is a difficult process in the organ culture system, and explants demonstrating contamination were discarded. A relatively low loss to contamination was found for these cultures (table 7.6).

Culture treatment	Number of explants	Explants lost to Contamination (%)
β-GlcY	29	14
α-GlcY control	28	11
NAA control	27	11
Total	84	12

Table 7.6 Number of explants cultured between 2004 and 2005, and lost to microbial contamination.

7.6.3 Growth characteristics

In order to determine if induction of the explants on the culture medium was successful, they were monitored for callus production (section 7.6.3.1) as described in section 4.2. Further characterization included observation of the dissected wood colour (section 7.6.3.2) as described in section 4.4 and image analysis of the cell lumen area and dimensions (sections 7.6.3.3 and 7.6.3.4) as described in sections 4.6 and 4.7.

7.6.3.1 Callus formation

After the first week in culture, green callus formation was present around the periphery of the explants, regardless of the Yariv treatment (figure 7.10). As discussed in section 4.2.1.4, the presence of callus was used as an indication that the cultures were healthy. If no callus was produced, the cultures were presumed to have died and were not included in the analysis.

Figure 7.10 The underside of cultures grown on media with (A) 10 µM β-GlcY, (B) 10 µM α-GalY control, (C) NAA control. The explants were cultured for a period of two months. Callus is present along the periphery of the cultures.

The 'pink' colour of the media, as a result of Yariv addition, was progressively decreased over the culture period, suggesting that the Yariv reagents were successfully taken into the cultures.

7.6.3.2 Colour differences in the cultured wood

The cultures grown in the presence of α-GalY often showed a darker red/brown coloured wood than those grown on β-GlcY and the controls (figure 7.11). The β-GlcY cultures also tended to be slightly more red/brown than the controls (figure 7.11). In section 4.4, the possibility of altered phenolics creating this colour change was discussed. These results suggest that α-GalY also stimulated a culture response when it was used as a control.

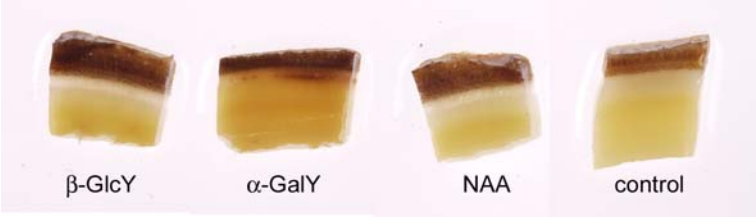


Figure 7.11 Dissected cultures grown on media containing from left to right, β-GlcY, α-GalY, NAA control, and the control explant collected at the time of culturing.

7.6.3.3 Cell division and cell size

Developing wood

In order to determine if β-GlcY encouraged a reduction in cell proliferation, through inhibition of AGPs, the number of cells in two defined regions, the 'cambial region', and the region of 'radial expansion', were counted as previously described in section 4.5. This was performed on three culture sets, and the results are summarized in table 7.7. In two of the culture sets, there was no significant change in the number of cells in each defined region, while in the third culture set, the β-GlcY culture had the fewest number of cambial cells (figure 7.12; table 7.7; appendix 1, table 15).

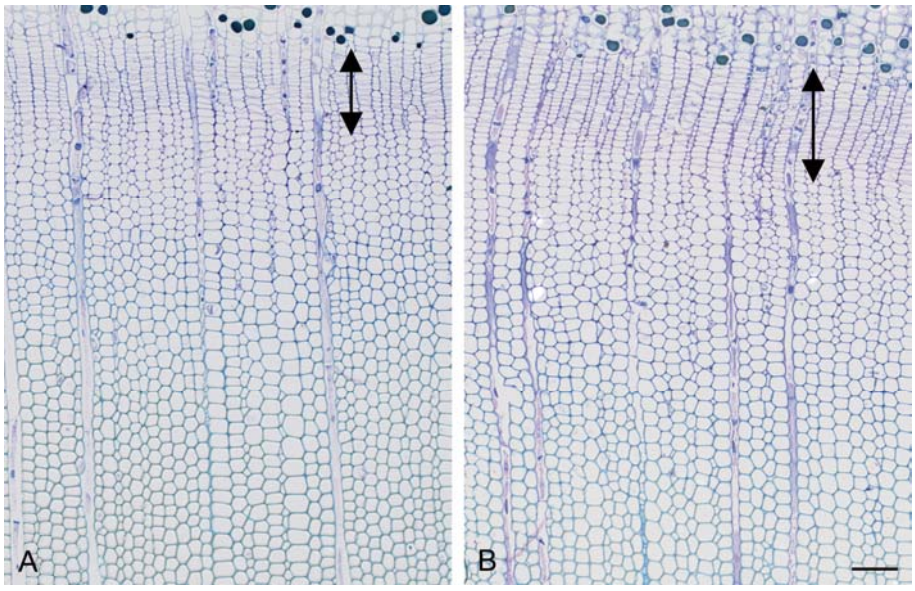


Figure 7.12 Transverse sections stained with toluidine blue. In one culture set, the (A) β-GlcY culture had fewer cambial cells (arrow) than the (B) α-GalY culture and the controls. Scale bar = 100 μm.

The lumen area, length and width of the newly differentiating wood in the cultures were measured in order to determine if β-GlcY could inhibit cell expansion, through inhibition of AGPs. In all three culture sets, there was no difference in any of these parameters, suggesting that AGPs may not be involved in this process in radiata pine (table 7.7; appendix 1, tables 13-15). While measuring the size of the cells, the area of the cells occupied by the cell walls was also determined, as described in section 4.6. In only one of the culture sets, the β-GlcY cultures were found to have significantly thinner cell walls (table 7.7; appendix 1, table 13).

	Yariv treated culture set		
	Set 1[a]	Set 2[b]	Set 3[c]
Cell counts	no change	no change	cambial: β-GlcY least, radial expansion: α-GalY least
Lumen area	no change	no change	no change
Lumen length	no change	no change	no change
Lumen width	no change	no change	no change
Wall area (%)	β-GlcY thinnest control thickest	no change	no change

[a] Burnham tree, inducted in January and cultured for two months. Appendix 1, Table 13.
[b] Rotorua tree, inducted in April and cultured for two months. Appendix 1, Table 14.
[c] Rotorua tree, inducted in April and cultured for two months. Appendix 1, Table 15.

Table 7.7 Summary of the growth characteristics of the developing wood in the Yariv treated culture sets.

Existing wood

The cells present in the 'new secondary wall region' were also counted to determine if there were changes in the number of cells undergoing secondary wall deposition. The results suggest that there were no changes in two of the culture sets, while in the third culture set, the α-GalY treated cultures had the most cells in this region (table 7.8; appendix 1, table 15).

The lumen area, length, width and cell wall area of the existing wood were measured to determine if changes in these cells had occurred in response to the Yariv treatments. Variable results were obtained and are summarized in table 7.8. Within

one of the culture sets, the β-GlcY and α-GalY treated cultures had the smallest lumen area, and thickest cell walls (table 7.8; appendix one, table 13). While within another culture set, the β-GlcY treated cultures had larger cells, with thinner walls (table 7.8).

	Yariv treated culture sets		
	Set 1[a]	Set 2[b]	Set 3[c]
Cell counts	no change	no change	α-GalY most NAA least
Lumen area	β-GlcY & α-GalY least	no change	β-GlcY greatest
Lumen length	no change	no change	β-GlcY greatest
Lumen width	no change	α-GalY greatest	β-GlcY greatest
Wall Area (%)	β-GlcY, α-GalY & NAA greatest	no change	β-GlcY & α-GalY least

[a] Burnham tree, inducted in January and cultured for two months. Appendix 1, Table 13
[b] Rotorua tree, inducted in April and cultured for two months. Appendix 1, Table 14
[c] Rotorua tree, inducted in April and cultured for two months. Appendix 1, Table 15

Table 7.8 Summary of the growth characteristics of the existing wood in the Yariv treated culture sets.

7.6.4 Cell wall deposition and lignification

It has been suggested that AGPs may pass through cell walls from one cell type to another, leading to altered wall composition which may have an impact on differentiation [19]. Moreover, previous studies suggest that AGPs play a role in secondary wall initiation by intercalating phenolics for subsequent orderly polymerization, meaning AGPs may have an important role in lignin structure [51]. In section 7.6.3.3, changes in the area occupied by the cell wall, while variable, were observed in two of the culture sets. In order to determine if inhibition of AGPs, through growth on β-GlcY supplemented media, resulted in changes in cell wall structure and lignification in the organ cultures, three culture sets were observed using a range of comparative microscopy techniques.

7.6.4.1 Light microscopy

Ruthenium red

Previous studies on lily pollen tubes have found an accumulation of pectin at the pollen tube tip when grown in the presence of β-GlcY [52]. The distribution of pectin in the Yariv treated organ culture cell walls was observed on transverse wax sections, treated with ammonium hydroxide to remove surface lignin [53], and stained with ruthenium red to determine if β-GlcY inhibition of AGPs altered pectin distribution. Ruthenium red is an anionic dye which stains pectin pink (section 5.2.1.1) [54].

There was no visible difference in pectin localization in cambial or radially expanding cells in the β-GlcY treated cultures (figure 7.13A, C), when compared to the α-GalY treated cultures (figure 7.13B, D), the NAA control or the explant collected at the time of culture. These results suggest that AGPs, assuming they were inhibited by 10 μM β-GlcY, were not involved in pectin deposition. This is not surprising, as pectin is deposited early in differentiation, prior to the onset of secondary wall deposition, where AGP was located in the β-GlcY and JIM13 labelling study (section 7.3.2).

Safranin/fast green

Safranin stains lignin red, while fast green stains cellulose blue (section 6.2.1.1) [54]. This stain was used to observe the distribution of lignin deposition in the Yariv treated cultures, in order to determine if AGPs altered the developmental timing of lignification, or the amount of lignin deposited. In transverse sections, stained with safranin/fast green there was no change in the distribution, or intensity of safranin staining in the β-GlcY treated cultures (figure 7.14A) compared to the controls. These results suggest that the developmental timing of lignification, and amount of lignin deposited, may not be regulated by AGPs.

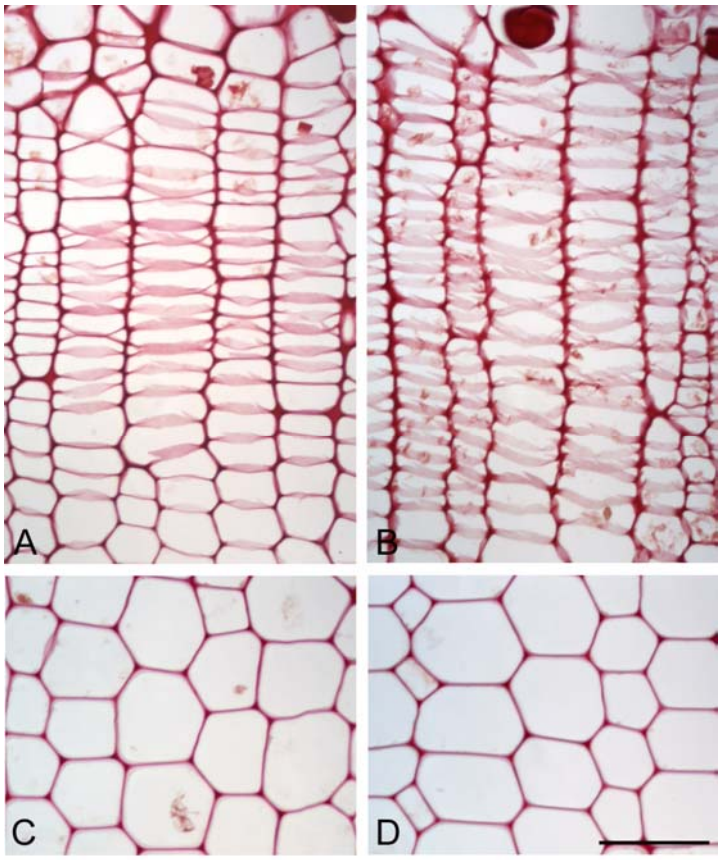


Figure 7.13 Transverse sections stained with ruthenium red. The cambial cells (A-B) and the radially expanding cells (C -D) are shown. There was no visible difference in the pectin distribution of the (A), (C) β-GlcY treated cultures when compared to the (B), (D) α-GalY treated cultures and the controls. Scale bar = 50 µm.

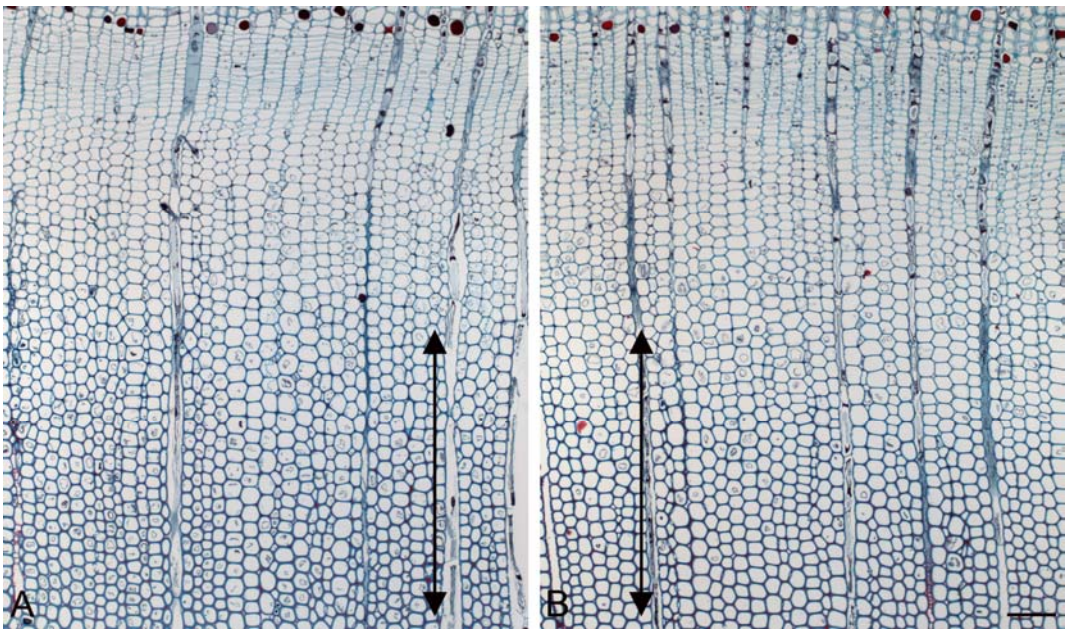


Figure 7.14 Transverse sections stained with safranin and counter stained with fast-green. There was no visible difference in the safranin staining in the (A) β-GlcY treated cultures when compared to the (B) α-GalY treated cultures and the controls. The onset of lignification is indicated with an arrow. Scale bar = 100 µm.

7.6.4.2 Blue autofluorescence

While there was no change in the onset of lignification observed with safranin/fast green stained Yariv treated cultures, blue autofluorescence was used to corroborate this result and determine if there were changes in the distribution of lignin in the cell wall layers (blue autofluorescence is described in section 6.2.2). Normal lignin distribution in radiata pine was discussed in section 6.2. The results suggest that lignification initiates at the cell corners, as expected (figure 7.15A) and the location of lignin within the cell walls did not change in response to β-GlcY treatment (figure 7.15A, C).

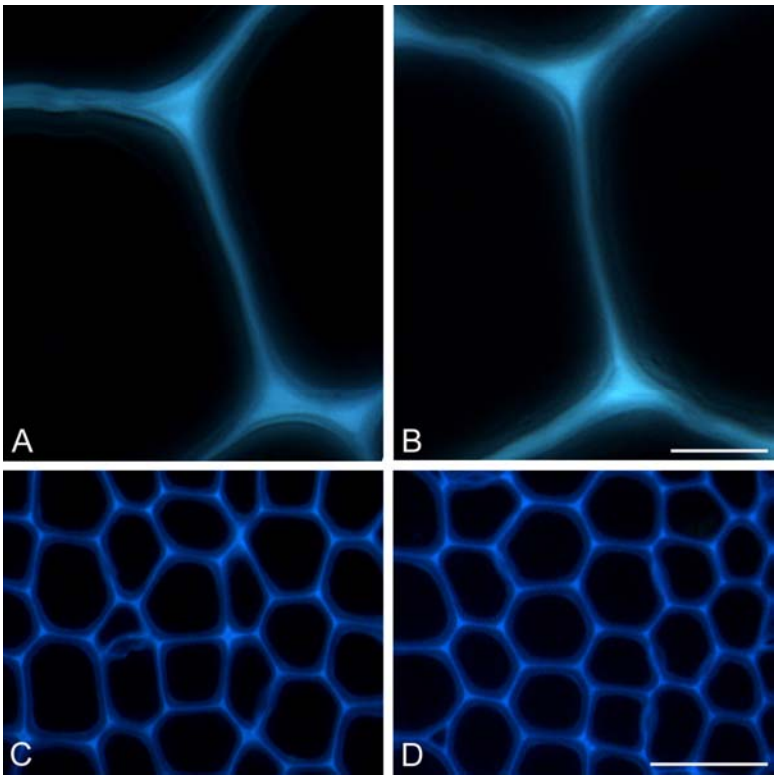


Figure 7.15 Transverse sections viewed with blue light. The cells at the end of the region of radial expansion (A-B) and the existing cells undergoing secondary wall deposition (C-D) are shown. There was no visible difference in the lignin location in the (A), (C) β-GlcY treated cultures when compared to the (B), (D) α-GalY treated cultures and the controls. Scale bars (A-B) = 20 µm, (C-D) = 50 µm.

7.6.4.3 Acetyl bromide assay

In section 2.4.1.2, the acetyl bromide assay was introduced as a quick and reliable method for determining the quantity of lignin in a sample. While the histochemical results did not suggest changes in lignification had occurred, the acetyl bromide assay was performed on two culture sets to corroborate those results. The acetyl bromide results suggest that there was no change in the amount of lignin in the Yariv-treated cultures (table 7.9). The values obtained fall into the expected lignin content range described in section 2.4.1.2.

Culture treatment	ABL
β-GlcY	22 ± 5
α-GalY	20 ± 1.5
NAA	22 ± 5

Table 7.11 Acetyl bromide lignin (ABL) content of ground Yariv treated cultures. Values represent percent lignin (w/w). Data are ± standard error of the mean for duplicate measurements from two culture sets.

7.6.4.4 Transmission electron microscopy

In order to determine if the ultra-structure of the cell walls was altered in response to the Yariv treatments, two culture sets were observed using TEM. These samples were embedded in Spurr resin and ultra-thin sections were obtained from the region of developing wood. These sections were then mounted on copper grids and stained with either uranyl acetate and Sato's lead, or potassium permanganate, before being observed in duplicate on a Hitachi H-600 TEM at 75kV.

Uranyl acetate and Sato's lead

Uranyl acetate and Sato's lead are described in section 2.3.1.1 as a common staining combination to produce images with strong contrast. This technique was utilized to observe the organization of the cell wall layers in the Yariv treated cultures.

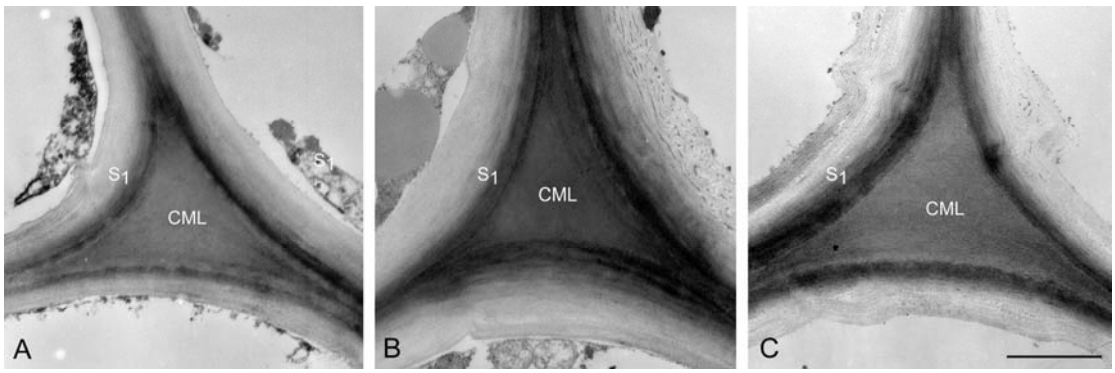


Figure 7.16 Transverse sections stained with Sato's lead and observed on a Hitachi H-600 TEM at 75 kV. There was no visible difference in the deposition of the secondary wall in the (A) β-GlcY treated cultures when compared to the (B) α-GalY treated cultures and the (C) control explant collected at the time of culture. Scale = 2 μm.

There were no definitive differences in the organization of the CML or S_1 wall layers in any of the Yariv treated cultures (figure 7.16). In one of the culture sets, the α-GalY treated culture sometimes showed more prominent striations in the S_1 wall region (figure 7.16B); however, more often this region appeared similar to the other cultures.

Potassium permanganate

Potassium permanganate staining was introduced in section 2.3.3.1 as a method commonly used to observe lignin distribution in conifers. Potassium permanganate stained TEM sections were used to observe the ultra-structural distribution of lignin in the developing cells, in order to determine if it was altered by β-GlcY. There were no striking differences in the cultures observed that would suggest that irregular lignification of the cell walls had occurred (figure 7.17).

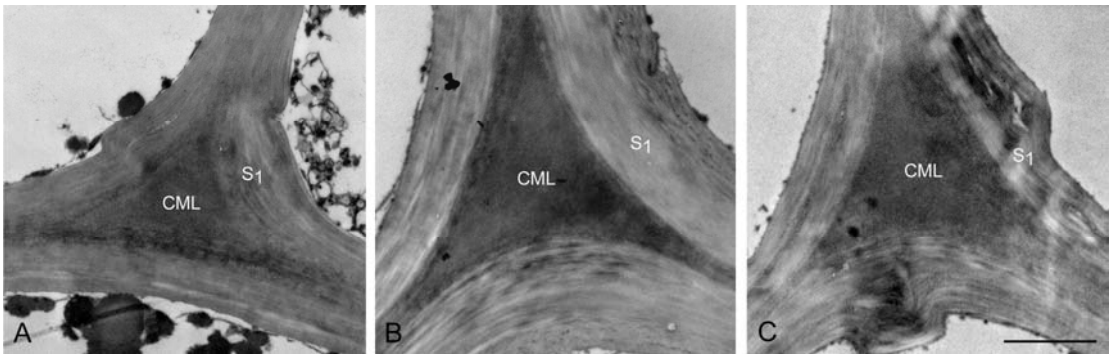


Figure 7.17 Transverse sections stained with $KMNO_4$ and observed on a Hitachi H-600 TEM at 75 kV. There was no visible difference in the deposition of the secondary wall in the (A) β-GlcY treated cultures when compared to the (B) α-GalY treated cultures and the (C) control explant collected at the time of culture. Scale = 2 μm.

7.6.4.5 Summary of light microscopy and electron microscopy observations

Table 7.10 presents a summary of the light and electron microscopy observations. Presuming that AGPs are inhibited with 10 μM β-GlcY, the results suggest that AGPs are not involved in pectin deposition, lignification, or cell wall organization using the methods presented.

	Yariv treated culture sets		
	Set 1[a]	Set 2[b]	Set 3[c]
Ruthenium red	no change	n/a[d]	no change
Safranin/fast green	no change	no change	no change
Epifluorescence	no change	no change	no change
Lead TEM[e]	α-GalY S_1 layer	n/a	n/a
Potassium permanganate TEM[d]	no change	n/a	n/a

[a] Burnham tree, inducted in January and cultured for two months
[b] Rotorua tree, inducted in April and cultured for two months
[c] Rotorua tree, inducted in April and cultured for two months
[d] n/a, not observed
[e] Set 4, Burnham tree, cultured in December for two months also had no change

Table 7.10 Summary of the histochemical and electron microscopy observations of the cultures.

7.7 Discussion

In radiata pine, AGPs labelled with β-GlcY reagent and those labelled by the JIM13 monoclonal antibody were located in differentiating xylem cells. The JIM13 labelling pattern has previously been associated with xylem in carrot, radish, pea, arabidopsis, loblolly pine, and poplar [[14, 30, 31, 55] and references therein]. Several AGP genes have also been shown to be highly expressed in differentiating xylem from loblolly pine [30, 32, 56], and addition of AGPs to *Zinnia* cultures stimulates xylogenesis [2]. Taken together, these results suggest that AGPs may have an important function in xylem cells, possibly at the stage of differentiation.

It has been suggested that specific AGP epitopes are present on a limited number of cells or cell types during their differentiation [19]. In this work, AGPs were located in the CML of xylem undergoing early secondary wall deposition, positions where variations in their complex structures could serve to mark cell identity or to signal neighbouring cells, as has previously been suggested of AGPs [14]. While the abundance of AGPs at cell surfaces, and the presence of GPI anchors have implicated them in cell-cell signalling, they have also been found within cell walls [49, 57, 58]. AGPs can bind to β-glycans, and may act as cell surface attachment sites for cell wall

matrix polysaccharides [24]; however, they are not covalently linked to the cell wall [19] because they purify in soluble extracts [10]. AGPs have also been described as having adhesive properties on association with other macromolecules, which led to the suggestion that their presence in the CML could serve as adhesive for cell-cell contacts [17]. Interestingly, AGP epitopes have also been found in the secondary cell wall of maturing xylem elements in *Arabidopsis* roots [55] whose xylem cells are committed to undergo programmed cell death, as are radiata pine xylem cells, and it has been suggested that AGPs can identify such cells [16]. Each of these proposed AGP functions are attributes associated with wood formation.

AGPs have been detected in at least three conifers, Norway spruce callus cell cultures [59, 60], Douglas fir and loblolly pine [32, 61]. In each of these conifers, AGPs were extracted and identified using β-GlcY and/or immunotechniques. Similar to Norway spruce AGPs, radiata pine AGPs were comprised predominantly of terminal arabinosyl residues, had 3-, 6-, and 3,6-galactosyl residues and terminal glucuronsyl residues (table 7.11) [59]. Radiata pine AGPs differed from Norway spruce AGPs, in its very high level of 5-linked arabinosyl residues, low amounts of glucuronosyl residues, and absence of galacturonosyl residues (table 7.11). Douglas fir and loblolly pine AGPs have also been characterized in terms of their glycosyl composition, which do not correspond to that obtained for radiata pine. Douglas fir and loblolly pine have twice the amount of galactose to arabinose, the reverse of the relationship found in the glycosyl composition of radiata pine [61]. As discussed in section 7.5.2.3, radiata AGPs have abundant 5-linked arabinosyl residues similar to *Physcomitrella patens* (table 7.12) [39]. The carbohydrate linkages in radiata pine, while having unique features, are overall characteristic of AGPs from other species, as shown in table 7.11.

Deduced linkage	Mole (%)		
	Radiata pine	Spruce[a]	Moss[b]
Rha*p* terminal	2	tr[c]	18
Ara*f* terminal	31	9.7	14
3-	3	1.5	1
5-	27	5.9	21
2,5-	1	1.5	-[d]
3,5-	7	3.2	-
2,3,5-	1	-	-
Glc*p* terminal	-	-	tr
4-	0.5	-	1
GlcA*p* terminal	tr	8.9	-
4-	1.1	-	11
Man*p* 4-	tr	-	tr
Gal*p* terminal	3	9.8	1
3-	10	21.3	7
6-	0.5	5.1	2
3,6-	13	16.2	23

[a] Spruce callus cells [59]
[b] *Physcomitrella patens* [39]
[c] Trace levels (<0.5%)
[d] Not reported

Table 7.11 *Comparison of the glycosyl linkages in radiata pine and other species.*

The amino acid composition of radiata pine determined by HPLC is remarkably similar to spruce (table 7.13) [59]. The main difference between radiata AGP and spruce AGP, was the presence of Ser as the most prevalent amino acid in Norway spruce (table 7.12) [59]. Douglas fir and loblolly pine were found to be rich in Gly, Ser and Asn, with less Ala and no Hyp reported [61]. According to Showalter (2001) [36] classical AGPs are defined by their core protein and contain Hyp, Ala, Ser, Thr and Gly as the amino acid constituents [36]. By this definition, radiata pine AGPs have characteristics of classical AGPs.

Amino acid	Mole (%)			
	Radiata pine	Spruce[a]	Loblolly pine[b]	Douglas fir[c]
Ala	15.3	15.5	18.6	8.9
Hyp	14.8	12.8	-[d]	0
Ser	14.4	20.1	13.1	15.3
Thr	11.7	10.4	20	1.9
Met	9.9	-	0	1.1
Cys	6.3	-	0	-
Val	4.9	4.2	9	2.4
Gly	4.8	7.4	1.4	15.4
Glu	3.8	3.6	0.7	-
Pro	3.4	4.1	28.3	0
Leu	2.8	3.5	3.5	6.5
Arg	1.8	-	0	9.2
Lys	1.4	1.5	1.4	3.6
Ile	1.3	1.6	1.4	3.5
Phe	1.3	3.5	1.4	1.1
Asn	1.0	-	0.7	-
Tyr	0.8	tr	0	1.4
His	0.3	1.6	0	0.6

[a] Spruce callus cells [59]
[b] Loblolly pine differentiating xylem [56]
[c] Douglas fir 2 year old sapling [61]
[d] Not reported

Table 7.12 Comparison of the amino acid composition of radiata pine and other species.

The precise attachment sites and number of attachment sites for AGPs to the core protein remain to be determined for radiata pine AGPs, although, galactosyl-*O*-hydroxyproline, arabinosyl-*O*-hydroxyproline, and galactosyl-*O*-serine linkages have been reported for several AGPs [62] and are likely to be found in radiata pine AGPs. For most classical AGPs, 80 to 90% of the Pro residues are thought to be hydroxylated [7]. It is generally accepted that glycosylation requires the post-translational modification of Pro residues to Hyp, and that the type of glycosylation is dependent on the contiguous or non-contiguous nature of these Hyp resides [44]. The

Hyp contiguity hypothesis suggests that blocks of contiguous Hyp residues, are arabinosylated with short oligosaccharides [46, 51] and that Hyp arabinogalactosylation occurs on the clustered non-contiguous Hyp residues commonly found on AGPs [45, 51]. Carbohydrate residues are also suggested to be attached to serine by *O*-links [19].

The wood in the α-GalY treated cultures had a tendency to appear much more red/brown when compared to the other Yariv treated cultures (section 7.6.3.2). Previously, a progressive red colouration has been associated with the uptake of β-GlcY, which was observed to be progressively removed from the medium and incorporated into the *Cichorium* roots, colouring them red [22]. Similarly, a faint red colouration was observed in the cortical roots of *Cichorium* roots treated with α-GalY [22]. It has been suggested that the presence of this red colouration is associated with the degradation of the sugar residues in α-GalY by plant galactosidases [22]. One possibility for the wood colour observed in the α-GalY treated cultures, could be that α-GalY interacts with other components in cell walls such as cellulose indirectly altering the phenolics [21, 22]. The brown/red colouration that was observed in the Yariv treated organ cultures, was also observed in the nutrient treated cultures (section 4.4) suggesting that it may be a response to induction on the culture medium, and not directly to the uptake of β-GlcY or α-GalY.

The precise function of any single AGP is not known [8]. Through inhibition with β-GlcY, AGPs have been implicated in diverse developmental roles such as differentiation, cell-cell recognition, embryogenesis and programmed cell death [8]. The concentration of β-GlcY required for alteration of these processes was varied. Complete inhibition of the growth of rose suspension cultures was achieved with 50 μM β-GlcY added to the culture media [49], while inhibition of root growth in Arabidopsis was achieved with the addition of 10 μM β-GlcY to the culture media [20]. Cell expansion in *Physcomitrella patens* was blocked at 1 μM β-GlcY [39]. β-GlcY completely blocked somatic embryogenesis of *Cichorium* at a concentration of 250 μM in the medium [22]. Programmed cell death occurred in Arabidopsis cell cultures at 80 μM β-GlcY in the medium [16]. Despite the range in β-GlcY concentrations used, the results suggest that the association of β-GlcY with AGPs can disrupt their normal interactions with cell surface components [16], altering the AGPs

that surround the cells resulting in changes in cell development, thereby demonstrating a biological activity for AGPs [19]. In radiata pine organ cultures, the influence of β-GlcY on division and cell expansion were not clear. Moreover, some difference was observed in the α-GalY treated cultures, suggesting that it may not be a suitable control in this culture system.

Previous studies have also shown that β-GlcY destabilizes the normal intercalation of new cell wall subunits, while exocytosis of the secretory vesicles still occurs in lily pollen tubes [52]. This implies that the formation of β-GlcY complexes results in the lack of proper cell wall assembly, suggesting a role for AGPs in deposition of wall subunits within a previously synthesized wall [52]. How the association of β-GlcY with AGP affects AGPs is not known. One proposed explanation is that the formation of large complexes prevents the normal assembly of molecules into the wall [52]. In the radiata pine organ cultures, a relationship between the inhibition of AGPs with β-GlcY and altered cell wall deposition was not established. This suggests that either radiata pine AGPs were not involved in cell-wall formation, or 10 μM β-GlcY was an insufficient concentration to inhibit these processes. Moreover, while a high NAA concentration was used to stimulate cambial activity and lignification, this concentration may override any biological function AGPs would have incurred.

While the addition of β-GlcY to the culture media was a preliminary investigation into AGP function in wood formation, future investigations would require a concentration gradient based observation of β-GlcY interactions in the organ cultures, over a larger sample size, to establish a more finite conclusion on the function of AGPs. Recent work using a callus culture system (described in section 1.4.4.2) to which β-GlcY was added, suggests that differentiation of callus cells can be inhibited in a concentration dependent manner (figure 7.18). These results infer that AGP does have a role in differentiation, and could influence wood formation. These results also suggest that 10 μM β-GlcY may indeed be insufficient to stimulate results in the organ culture system, or the organ culture system may be too complex (ie. containing intact phloem, cambium and xylem) to isolate a defined AGP function. The callus culture system has the advantage of direct observation of single cell responses. In future work, it would be interesting to determine if addition of the radiata pine β-GlcY

precipitated AGPs and the RP-HPLC fractionated AGPs could induce differentiation in the callus culture system.

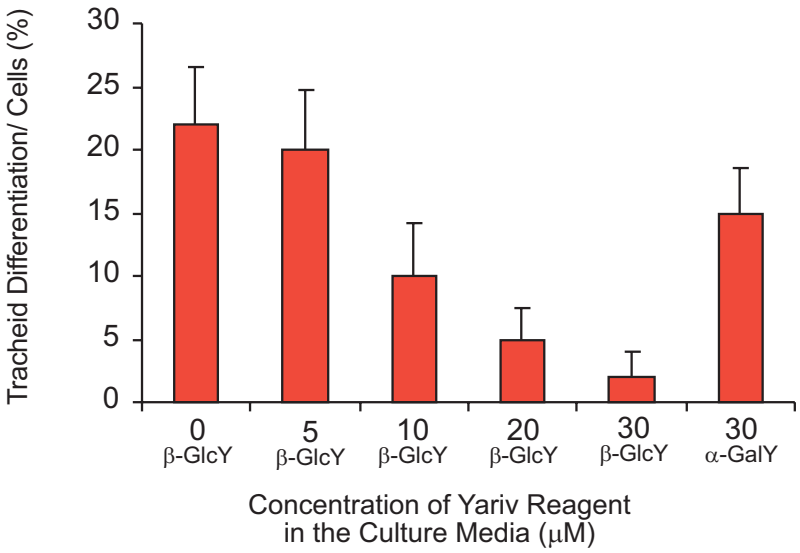


Figure 7.18 Tracheid differentiation rate 6 days after transfer of induced cell cultures to media containing β-GlcY. Additional cultures were grown on α-GalY as a control. Bars indicate standard deviations. Unpublished results provided by R. Möller, SCION, New Zealand.

7.8 Summary

Abundant, low salt extractable AGPs were purified from differentiating radiata pine xylem. β-GlcY staining of polyacrylamide gels containing native samples revealed a high-molecular-weight smear and no distinct protein bands. Monosaccharide analysis showed that arabinose and galactose were the main sugars. Linkage analysis showed that most of the arabinose was terminally linked, or present in the 5-linked position, and that almost all of the galactose was present in the 3-, 6- and 3,6- form. AGP characteristic amino acid residues, Ala, Hyp, Pro, Ser, Gly, Leu and Thr were also detected. Separation of the AGPs using RP-HPLC showed that one main fraction was eluted, which tested positive for AGP, by dot-blot analysis. These results show that the isolated radiata pine AGPs satisfy the three main criteria for AGPs: the presence of arabinogalactan chains, a Hyp rich protein backbone and the ability to bind β-GlcY [1, 8-10].

Preliminary organ culture trials in the presence of β-GlcY suggest that cell division and cell size, and not cell-wall formation, are most likely to be affected by the

addition of β-GlcY. However, a concentration of 10 μM β-GlcY in the culture media may be insufficient to perturb AGP function. In addition, the organ cultures may be too complex to observe an isolated response. Recent studies utilising callus cultures have shown that β-GlcY can inhibit differentiation, and coupled to the results presented in this chapter, have laid the groundwork for future studies into the role of AGP in wood formation.

7.9 References

1. Johnson, K.L., Jones, B.J., Schultz, C.J., Bacic, A. 2003. *Non-enzymic cell wall (glyco)proteins*, in *The Plant Cell Wall*, Rose, J.K.C., Ed. Blackwell Publishing Ltd.: Oxford. 111-154.

2. Motose, H., Sugiyama, M., Fukuda, H. 2004. *A proteoglycan mediates inductive interaction during plant vascular development.* Nature, **429**: 873-878.

3. Gao, M., Kieliszewski, M.J., Lamport, D.T.A., Showalter, A.M. 1999. *Isolation, characterization and immunolocalization of a novel, modular tomato arabinogalactan-protein corresponding to the LeAGP-1 gene.* The Plant Journal, **18**: 43-55.

4. Van Holst, G.K., Clarke, A.E. 1985. *Quantification of arabinogalactan-protein in plant extracts by single radial gel diffusion.* Analytical Biochemistry, **148**: 446-450.

5. Komalavilas, P., Zhu, J.K., Nothnagel, E.A. 1991. *Arabinogalactan-proteins from the suspension culture medium and plasma membrane of rose cells.* The Journal of Biological Chemistry, **24**: 15956-15965.

6. Gane, A.M., Craik, D., Munro, S.L.A., Howlett, G.J., Clarke, A.E., Bacic, A. 1995. *Structural analysis of the carbohydrate moiety of arabinogalactan-proteins from stigmas and styles of Nicotiana alta.* Carbohydrate Research, **277**: 67-85.

7. Youl, J.J., Bacic, A., Oxley, D. 1998. *Arabinogalactan-proteins from Nicotiana alta and Pyrus communis contain glycosylphospatidylinositol membrane anchors.* Proceedings of the National Academy of Science USA, **95**: 7921-7926.

8. Gaspar, Y., Johnson, K.L., McKenna, J.A., Bacic, A., Schultz, C.J. 2001. *The complex structures of arabinogalactan-proteins and the journey towards understanding function.* Plant Molecular Biology, **47**: 161-176.

9. Fincher, G.B., Stone, B.A. 1983. *Arabinogalactan-proteins: structure, biosynthesis and function.* Annual Review of Plant Physiology, **34**: 47-70.

10. Nothnagel, E.A. 1997. *Proteoglycans and related components in plant cells.* International Review of Cytology, **174**: 195-291.

11. Classen, B., Mau, S.L., Bacic, A. 2004. *The arabinogalactan-proteins from pressed juice of Echinacea purpurea belong to the "hybrid" class of hydroxyproline-rich glycoproteins.* Planta Medica, **71**: 54-58.

12. Schultz, C.J., Johnson, K.J., Currie, G., Bacic, A. 2000. *The classical arabinogalactan-protein gene family of arabidopsis.* The Plant Cell, **12**: 1751-1767.

13. Schultz, C.J., Ferguson, K.L., Lahnstein, J., Bacic, A. 2004. *Post-translational modifications of arabinogalactan-peptides of Arabidopsis thaliana.* Journal of Biological Chemistry, **279**: 45503-45511.

14. Majewska-Sawka, A., Nothangel, E.A. 2000. *The multiple roles of arabinogalactan proteins in plant development.* Plant Physiology, **122**: 3-9.

15. Gaspar, Y. 2004. *Arabidopsis arabinogalactan-proteins containing lysine rich domains*. PhD Thesis, *School of Botany*, University of Melbourne.

16. Gao, M., Showalter, A.M. 1999. *Yariv reagent treatment induces programmed cell death in Arabidopsis cell cultures and implicates arabinogalactan protein involvement.* The Plant Journal, **19**: 321-331.

17. Clarke, A.E., Anderson, R.L., Stone, B.A. 1979. *Form and function of arabinogalactans and arabinogalactan-proteins.* Phytochemistry, **18**: 521-540.

18. Yariv, J., Rapport, M.M., Graf, L. 1962. *The interaction of glycosides and saccharides with antibody to the corresponding phenylazo glycosides.* Biochemistry Journal, **85**: 383-389.

19. Kreuger, M., van Holst, G.J. 1996. *Arabinogalactan proteins and plant differentiation.* Plant Molecular Biology, **30**: 1077-1086.

20. Ding, L., Zhu, J.K. 1997. *A role for arabinogalactan-proteins in root epidermal cell expansion.* Planta, **203**: 289-294.

21. Triplett, B.A., Timpa, J.D. 1997. *β-glucosyl and α-glucosyl Yariv reagents bind to cellulose and other glucans.* Journal of Agriculture and Food Chemistry, **45**: 4650-4654.

22. Chapman, A., Blervacq, A.S., Vasseur, J., Hilbert, J.L. 2001. *Arabinogalactan-proteins in Cichorium somatic embyrogenesis: effect of β-glucosyl Yariv reagent and epitope localisation during embryo development.* Planta, **211**: 305-314.

23. Gane, A.M. 1994. *The extracellular matrix and cell walls of pistils of Nicotiana alta*. PhD Thesis, *School of Botany*, University of Melbourne.

24. Pennell, R.I., Knox, P.J., Scofield, G.N., Selvendran, R.R., Roberts, K. 1989. *A family of abundant plasma membrane-associated glycoproteins related to the arabinogalactan proteins is unique to flowering plants.* The Journal of Cell Biology, **108**: 1967-1977.

25. Basile, D.V., Basile, M.R. 1987. *The occurrence of cell wall-associated arabinogalactan proteins in the Hepaticae.* The Bryologist, **90**: 401-404.

26. Samson, M.R., Jongeneel, R., Klis, F.M. 1984. *Arabinogalactan protein in the extracellular space of Phaseolus vulgaris hypocotyls.* Phytochemistry, **23**: 493-496.

27. Showalter, A.M. 1993. *Structure and function of plant cell wall proteins.* The Plant Cell, **5**: 9-23.

28. Yates, E.A., Valdor, J.F., Haslam, S.M., Morris, H.R., Dell, A., Mackie, W., Knox, J.P. 1996. *Characterization of carbohydrate structural features recognized by anti-arabinogalactan-protein monoclonal antibodies.* Glycobiology, **6**: 131-139.

29. Knox, J.P., Linstead, P.J., Peart, J., Cooper, C., Roberts, K. 1991. *Developmentally regulated epitopes of cell surface arabinogalactan proteins and their relation to root tissue pattern formation.* The Plant Journal, **1**: 317-326.

30. Zhang, Y., Brown, G., Whetton, R., Loopstra, C.A., Neale, D., Kieliszewski, M.J., Sederoff, R.R. 2003. *An arabinogalactan protein associated with secondary cell wall formation in differentiating xylem of loblolly pine.* Plant Molecular Biology, **52**: 91-102.

31. Lafarguette, F., Leple, J.C., Dejardin, A., Laurans, F., Costa, G., Lesage-Descauses, M.C., Pilate, G. 2004. *Poplar genes encoding fasciclin-like arabinogalactan proteins are highly expressed in tension wood.* New Phytologist, **164**: 107-121.

32. Loopstra, C.A., Puryear, J.D., No, E.G. 2000. *Purification and cloning of an arabinogalactan-protein from xylem of loblolly pine.* Planta, **210**: 686-689.

33. Kim, J.B., Carpita, N.C. 1992. *Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles.* Plant Physiology, **98**: 646-653.

34. Blakeney, A.B., Harris, P.J., Henry, R.J., Stone, B.A. 1983. *A simple and rapid preparation of alditol acetates for monosaccharide analysis.* Carbohydrate Research, **113**: 291-299.

35. Albersheim, P. 1967. *A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography.* Carbohydrate Research, **5**: 340-345.

36. Showalter, A.M. 2001. *Arabinogalactan-proteins: structure, expression and function.* Cellular and Molecular Life Sciences, **58**: 1399-1417.

37. Harris, P.J., Henry, R.J., Blakeney, A.B., Stone, B.A. 1984. *An improved procedure for the methylation analysis of oligosaccharides and polysaccharides.* Carbohydrate Research, **127**: 59-73.

38. Pazur, J.H. 1986. *Neutral monosaccharides*, in *Carbohydrate analysis*, Chaplin, M.F., Kennedy, J.F., Ed. IRL Press: Washington.

39. Lee, K.J.D., Sakata, Y., Mau, S.L., Pettolino, F., Bacic, A., Quatrano, R.S., Knight, C.D., Knox, P.J. 2005. *Arabinogalactan proteins are required for apical cell extension in the moss Physcomitrella patens.* The Plant Cell, **17**: 3051-3065.

40. Sommer-Knudsen, J., Bacic, A., Clarke, A.E. 1998. *Hydroxyproline-rich plant glycoproteins.* Phytochemistry, **47**: 483-497.

41. Persson, J., Nasholm, T. 2001. *A GC-MS method for the determination of amino acid uptake by plants.* Physiologia Plantarum, **113**: 352-358.

42. Ozols, J. 1990. *Amino acid analysis*, in *Guide to Protein Purification: Methods in Enzymology*, Deutscher, M.P., Ed. Academic Press Inc.: New York. 587-601.

43. Du, H., Simpson, J., Moritz, R.L., Clarke, A.E., Bacic, A. 1994. *Isolation of the protein backbone of an arabinogalactan-protein from the styles of Nicotiana alta and characterization of a corresponding cDNA.* The Plant Cell, **6**: 1643-1653.

44. Johnson, K.L., Jones, B.J., Bacic, A., Schultz, C.J. 2003. *The fasciclin-like arabinogalactan proteins of arabidopsis. A multigene family of putative cell adhesion molecules.* Plant Physiology, **133**: 1911-1925.

45. Goodrum, L.J., Patel, A., Leykam, J.F., Kieliszewski, M.J. 2000. *Gum arabic glycoprotein glycomodules of both extensin and arabinogalactan-glycoproteins.* Phytochemistry, **54**: 99-106.

46. Shpak, E., Barbar, E., Leykam, J.F., Kieliszewski, M.J. 2001. *Contiguous hydroxyproline residues direct hydroxyproline arabinosylation in Nicotiana tabacum.* The Journal of Biological Chemistry, **276**: 11272-11278.

47. Smallwood, M., Yates, E.A., Willats, W.G.T., Martin, H., Knox, J.P. 1996. *Immunochemical comparision of membrane-associated and secreted arabinogalactan-proteins in rice and carrot.* Planta, **198**: 452-459.

48. Stacey, N.J., Roberts, K., Knox, P.J. 1990. *Patterns of expression of the JIM4 arabinogalactan-protein epitope in cell cultures during somatic embryogenesis in Daucua carota L.* Planta, **180**: 285-292.

49. Serpe, M.D., Nothnagel, E.A. 1994. *Effects of Yariv phenylglycosides on rose cell - suspensions - evidence for the involvement of arabinogalactan-proteins in cell proliferation.* Planta, **4**: 542-550.

50. Borderies, G., Bechec, M., Rossignol, M., Lafitte, C., Deunff, E., Beckert, M., Dumas, C., Metthys-Rochon, E. 1995. *Characterization of proteins secreted during maize microspore culture: arabinogalactan proteins (AGPs) stimulate embryo development.* European Journal of Cell Biology, **83**: 205-212.

51. Kieliszewski, M.J., Lamport, D.T.A. 1994. *Extensin: repetitive motifs, functional sites, post-translational codes, and phylogeny.* The Plant Journal, **5**: 157-172.

52. Roy, S., Jauh, G.Y., Hepler, P.K., Lord, E.M. 1998. *Effects of Yariv phenylglycoside on cell wall assembly in the lily pollen tube.* Planta, **204**: 450-458.

53. Micheli, F., Ermel, F., Bordenave, M., Richard, L., Goldberg, R. 2002. *Cell walls of wood tissues: cytochemical, biochemical and molecular analysis of pectins and pectin methylesterases*, in *Wood Formation in Trees: Cell and Molecular Biology Techniques*, Chaffey, N.J., Ed. Taylor and Francis: London. 179-200.

54. Wardrop, A.B. 1981. *Lignification and xylogenesis*, in *Xylem Cell Development*, Barnett, J.R., Ed. Castle House Publications Ltd.: Kent. 115-151.

55. Dolan, L., Linstead, P., Roberts, K. 1995. *An AGP epitope distinguishes a central metaxylem initial from other vascular initials in the Arabidopsis root.* Protoplasma, **189**: 149-155.

56. Loopstra, C.A., Sederoff, R.R. 1995. *Xylem-specific gene expression in loblolly pine.* Plant Molecular Biology, **27**: 277-291.

57. Li, Y., Bruun, L, Pierson, E.S., Cresti, M. 1992. *Periodic deposition of arabinogalactan epitopes in the cell wall of pollen tubes of Nicotiana tabacum L.* Planta, **188**: 532-538.

58. Schopfer, P. 1990. *Cytochemical identification of arabinogalactan protein in the outer epidermal wall of maize coleoptiles.* Planta, **183**: 139-142.

59. Karacsonyi, S., Patoprsty, V., Kubackova, M. 1998. *Structural study on arabinogalactan-proteins from Picea abies L. Karst.* Carbohydrate Research, **307**: 271-279.

60. Egertsdotter, U., von Arnold, S. 1995. *Importance of arabinogalactan proteins for the development of somatic embryos of Norway Spruce (Picea abies).* Physiologia Plantarum, **93**: 334-345.

61. Bobalek, J.F., Johnson, M.A. 1983. *Arabinogalactan-proteins from Douglas fir and loblolly pine.* Phytochemistry, **22**: 1500-1503.

62. Bacic, A. 1987. *Fine structure of the arabinogalactan-protein from Lolium multiflorum.* Carbohydrate Research, **162**: 85-93.

Chapter Eight

Summary and conclusions

8.1 Introduction

The aim of this thesis, as described in chapter one, was to establish an organ culture system for radiata pine and determine its efficacy to examine the affect of boron, calcium and magnesium availability on wood formation. This project was undertaken as part of a broad collaborative effort to determine the underlying cause of the wood quality flaw referred to as intra-ring checking, a phenomenon that plagues the New Zealand forest industry, with the long term aim of improving future radiata pine plantations.

In chapter two, intra-ring checking was described as fractures that propagate along adjacent radial cell files creating undesirable fissures, which ultimately decrease the value of the wood end product. Ultra-structural and biochemical studies performed on oven dried discs from trees showing intra-ring checking found that changes in the organization of the CML/S_1 wall region often occurred (section 2.3.1). Further study of this phenomenon required the development of a model system that could be used to probe wood formation *in vitro*. A wood culture system could create fundamental knowledge about wood formation and cell-wall biogenesis, providing insight into how intra-ring checking might initiate, and also offers the possibility of modifying cell wall structure and composition through alteration of environmental factors, such as nutrient availability. The information obtained from this type of study could be applied to the future improvement of radiata pine wood quality.

The present chapter draws together the results of the experiments presented in this thesis and offers insight to the question - can wood formation be meaningfully studied *in vitro* using organ cultures?

8.2 Organ culture as a model of wood formation

Model angiosperms such as Arabidopsis and *Zinnia* mesophyll cultures are typically used to study cell walls (section 1.4); however, the question arises as to how the results from these systems can be applied to cell wall formation in the xylem cells of coniferous gymnosperms, which have different secondary wall compositions to the fibres and vessel elements of angiosperms [1]. Moreover, intra-ring checking is a wood quality phenomenon that does not occur in these model species. As such, it was essential to develop and carry out studies on a model system that was specific to radiata pine. In chapter three, an organ culture technique, previously used for the establishment of tamarack and eucalyptus cultures [2, 3] was successfully established for radiata pine. This technique was used to effectively manipulate the amount of boron, calcium and magnesium taken into the organ cultures (section 4.3), and allowed investigation of the role these nutrients had in wood formation. While this study was created for a problem specific to radiata pine, the culture technique established could be applied to other closely related species.

8.2.1 Can wood formation be altered in the organ cultures?

The results presented in chapter four showed that radiata pine organ cultures appeared to undergo a xylogenic response, defined by cell division, changes in expansion, maintenance of radial cell files, and thickening of cell walls. The ability to successfully culture the cambial explants, obtained from trees at least ten years in age, was a significant feature of the organ culture technique, because most plant culture systems utilize material that is from young tissues and not representative of the older tissue that is present in the wood harvested for the forest industry [3, 4]. The observation of callus growth along the periphery of the organ cultures provided an initial indication that cell division had occurred, and that culture induction was successful (section 4.2). However, the production of callus was not found to correlate with a complete xylogenic response, as evidenced by the HBLC and LBHC mixed treated cultures which showed callus formation (section 4.2.1.3), but very little change in cell number, size and area occupied by the cell wall, measured using an image analysis program (sections 4.5.1.4, 4.6.1.4 and 4.7.1.1). In the nutrient treated cultures that did show a xylogenic response, a great deal of ‘between-tree’ and ‘within-culture’ variation in the measured wood properties was observed. This meant that while new

cells were formed and existing cells matured in culture, there was too much variability in the number of cells, their size and the area occupied by the cell wall measured to draw meaningful correlations with treatment specific responses. This variability may also have been a result of variability within a growth ring and completely unrelated to treatment responses.

While cell division and expansion proved difficult to compare between nutrient treatments, these aspects of xylogenesis may involve processes that are entirely unrelated to those involved in cell wall deposition [5]. For this reason, chapters five and six present experiments directed at the observation of specific cell wall components. The results presented in chapter five showed that, using both histochemistry and immunocytochemistry techniques, changes in pectin deposition and/or esterification could be observed in the newly divided cells of the low boron treated cultures (sections 5.3.1, 5.3.2.1, and 5.3.3.1), but not in the other culture treatments. These cells were formed in culture, which suggested that boron was indirectly involved in wood formation, through direct or indirect interactions with pectin. Analysis of the distribution of pectin in the control explants, collected at the time of culturing, allowed for characterization of the JIM5 and JIM7 epitope distribution in free standing radiata pine for the first time (section 5.2.2 and 5.2.3). Other differences observed in the low boron treated organ cultures included changes in the carbohydrate composition of the cells from the region of new wood growth, which showed changes in the amounts of xylose, mannose and particularly glucose (section 5.4). However, the carbohydrate analysis was performed on cultures obtained from one tree, and the amount of variation in these analyses that could occur between trees is not known.

In chapter six, histochemical results were presented that suggested changes in lignification may occur in response to boron (sections 6.3.1.1 and 6.3.1.2); however, these results were not conclusive and epifluorescence coupled to image analysis, and biochemical techniques could not corroborate them. Changes in lignification were not expected in the organ cultures, as most lignified cells would have been present prior to culture. Moreover, it is difficult to determine the differences between altered amounts of lignin, and the same amounts of lignin deposited in the CML region that ranges in compactness and degree of organization. These two scenarios might have very

different consequences for the relative strength of cell adhesion. Coupled to the variable changes in cell wall thickness (section 4.7), the changes in the CML/S_1 wall region observed by TEM in some of the culture treatments (sections 4.8 and 6.3.2), and the potential for altered lignification in the boron treated cultures (section 6.3.1), has shown that secondary cell wall properties could be altered in the organ cultures. This is in contrast to the organ cultures prepared for eucalyptus, where secondary walls were not shown to form [2].

In the field, wood is formed in a variable environment, subject to developmental control, and xylem cells are produced that are different in size and shape, cell wall structure and chemical composition [6]. Genetically identical trees can produce wood with very different properties [7, 8]. This must be taken into account in the present wood formation study. While changes in wood formation could be induced in the organ cultures, in order to determine which of the observed responses were true representations of trends, and were individual culture responses, requires observation of a much larger sample size with a focus on specific wood properties. In addition, the culture system itself requires further investigation to establish the nutrient and hormone requirements needed to achieve optimal and continual culture growth. The organ culture explants used in this study were cultured in the summer, and presumably still contained endogenous hormones, which once exhausted resulted in the end of culture growth (section 4.2.2). Concurrent projects in this laboratory have established radiata pine organ cultures in the presence of a battery of hormones, and the results from those experiments, coupled to the present study, may provide insight into the required conditions for continuous culture growth.

8.2.2 Application to intra-ring checking

In New Zealand, boron deficient soils are derived from volcanic rocks [9], calcium deficiency is often prevalent on soils derived from volcanic rocks and sandstone [9], and magnesium deficiency often occurs on pumice soils [10]. As a result of the incidence of soils deficient in these nutrients, field trials have been performed to examine the relationship between the nutrients, boron in particular, and wood quality [11, 12]. Similar to the present study, these studies have met with little success because of the variability in the data encountered [11]. Variability in the nutritional response of radiata pine is not uncommon. Additional radiata pine field studies, have

found that some families grow well on nutrient poor sites, but growth relative to others changed when fertilized [9]. Moreover, only some radiata pine families are responsive to fertilizer treatments [9]. In order to select an appropriate field management system, it remains critical to understand the effects of environmental variables on wood formation [13]. These field trials take years to complete, and the lack of readily obtainable results further highlights the need for a model system, such as that developed in this study, that could quickly assess the possibility of nutrients altering wood formation.

Radiata pine stands have been suggested to have peak nutrient demands of 200 g/ha for boron, 40 kg/ha of calcium [9] and 200 kg/ha magnesium [10]; however, fertilizer practices vary. The question arises as to how the mineral nutrient concentrations chosen in section 3.2.1.3 for this organ culture study, relate to the amount of these mineral nutrients available, or applied in the form of fertilizer, to radiata pine in the field? It was not feasible to compare the nutrient concentrations chosen for this study directly to the nutrients available in the field, in part because soil nutrients can be leached from the soil in high rainfall [14], and complex interactions occur in phloem and root transport [15-17], which are not applicable to the culture system. More specifically, it is not known how the cambium competes with other parts of a living tree for these nutrients when they are deficient, or how the off-loading of excess levels of these nutrients are distributed in the tree in the case of toxicity. In the organ cultures the ability of the tree to partition these nutrients has been eliminated and enabled direct application to the tissues of interest. For this reason, a range of concentrations were examined in order to establish deficiency and toxicity levels that were suitable to a culture system (section 3.2.1.3).

In chapter one, boron was introduced as a target for wood formation studies, aimed at understanding intra-ring checking, because of its interactions with RG-II, which imparts strength and stability to cell walls (section 1.6.1). Calcium and magnesium were also targeted because of their postulated interactions with B-RG-II complexes (sections 1.6.2 and 1.6.3). In the present work, calcium and magnesium were able to alter some aspects of cell division and cell size, suggesting that their influence on wood formation, while variable, may lie in early stages of xylogenesis, as alterations in the cell wall were rare. Boron was the only nutrient found to have significant,

although variable, changes in nearly every wood property examined. While variability in these parameters limits the conclusions that can be drawn, they do not preclude boron from the incidence of intra-ring checking. Future studies utilizing the methods developed in this thesis, on a larger and more defined sample size, would provide greater insight into whether boron could be a factor involved in intra-ring checking.

8.3 Arabinogalactan-proteins and wood formation

In chapter seven, the experiments from a preliminary investigation into the possibility of AGPs modulating wood formation in radiata pine were presented. For the first time, newly differentiated xylem from radiata pine was shown to contain AGPs with a type-II arabinogalactan backbone and an amino acid composition rich in Pro, Hyp, Ala, Ser and Gly, which are typical of plant AGPs [18]. Unique to radiata pine AGPs was the presence of high levels of 5-linked arabinosyl residues (section 7.5.2.3).

Radiata AGPs were located in the region of cells undergoing new secondary wall deposition, suggesting that they could be involved in cell wall production, cell adhesion or as markers for cell death (section 7.3). An organ culture trial utilizing β-GlcY to bind to AGPs, and potentially inhibit their function, was not successful in identifying potential AGP functions in radiata pine (section 7.6), most probably because the concentrations trialled were insufficient. However, a recent collaborative investigation has found that when β-GlcY was added to radiata pine callus cultures, differentiation was inhibited in a concentration dependent manner (section 7.7). The location of AGPs in radiata, their isolation from newly differentiating xylem, and their potential to inhibit differentiation have established a basis for further investigation into their possible involvement in wood formation.

Determining whether AGPs have roles in development and which AGPs have overlapping functions will require a variety of different approaches [19]. Future studies, utilizing the methods established in chapter seven to isolate and fractionate AGPs, may involve addition of these AGPs to the callus culture system to determine if they can induce differentiation. Other investigations have shown that it was possible to manipulate somatic embryogenesis by the addition of exogenous AGPs in the medium [20, 21], and to induce xylogenesis by the addition of AGPs to *Zinnia* mesophyll cultures [22]. Moreover, the ability of cell suspension cultures to secrete

AGPs suggests that the size exclusion limit of the walls may be larger than AGPs [23], allowing them to readily pass through the cell walls. It has recently been suggested that increased AGP levels decrease pectin cross-linking, suggesting a role in expansion [24]. It would be interesting to see how these AGPs alter differentiation in the callus culture system. Such studies would complement a genetic approach to probe the function of individual AGPs [25]. The fractionated AGPs could first be deglycosylated and the sequences obtained. An EST search could then performed for corresponding cDNAs, which could be cloned into an RNAi (RNA interference) vector and used to transform radiata pine callus cultures, to silence endogenous gene activity, and further probe AGP functions. Regardless of the future paths procured from this work, a major challenge in AGP research remains to establish the function of a single AGP [19].

8.4 Conclusion

The ability to culture explanted radiata pine cambial tissue, in a manipulated environment, and successfully grow new wood cells offers fundamental and practical advantages to the study of wood formation. These advantages include the ability to monitor wood formation and cell wall biogenesis in a short time, relative to the many years required for field trials. This is beneficial to the study of economically detrimental wood quality flaws, such as intra-ring checking, where forestry investors are eager to obtain information about the factors associated with its incidence. However, application of the results obtained from organ culture studies requires reduction of the variability in the wood resource and culture response, which is not a simple task. Future organ culture work, directed at specific wood properties and employing a larger sample size may more readily identify significant trends.

8.5 References

1. Preston, R.D. 1980. *Introduction*, in *Xylem Cell Development*, Barnett, J.R., Ed. Castle House Publications Ltd.: Kent. 3-13.

2. Leitch, M.A. 1999. *The development of tissue culture techniques to study wood formation in Eucalyptus globulus Labill*. PhD Thesis, *Institute of Land and Food Resources*, University of Melbourne.

3. Savidge, R.A. 1993. *In vitro wood formation in 'chips' from merchantable stem regions of Larix laricina.* International Association of Wood Anatomists, (14): 3-11.

4. Wodzicki, T.J. 2001. *Natural factors affecting wood structure.* Wood Science and Technology, **35**: 5-26.

5. Wodzicki, T.J. 1971. *Mechanism of xylem differentiation in Pinus silvestris L.* Journal of Experimental Botany, **22**: 670-87.

6. Mekkerowicz, E.J., Baucher, M., Sundberg, B., Boerjan, W. 2001. *Unravelling cell wall formation in the woody dicot stem.* Plant Molecular Biology, **47**: 239-274.

7. Downes, G.M., Evans, R., Schimleck, L.R., Fritts, H.C. 2000. *The commercial cambium: understanding the origin of wood property variation*, in *Cell and Molecular Biology of Wood Formation*, Savidge, R., Barnett, J., Napier, R., Ed. BIOS Scientific Ltd.: Oxford.

8. Panshin, A.J., de Zeeuw, C. 1980. *Variability of wood within a species*, in *Textbook of Wood Technology*. McGraw-Hill Inc.: New York. 240-253.

9. Turner, J., Lambert, M.J. 1986. *Nutrition and nutritional relationships of Pinus radiata.* Annual Review of Ecology and Systematics, **17**: 325-50.

10. Mitchell, A.D., Loganathan, P. 2003. *Magnesium and potassium fertiliser effects on foliar magnesium and potassium concentrations and upper mid-crown yellowing in Pinus radiata.* New Zealand Journal of Forestry Science, **33**: 225-243.

11. Beets, P., McConchie, D., McConchie, M., Kimberley, M., Pearce, S., Oliver, G. 2003. *Family and clonal variation in intra-ring checking and resin characteristics and relationship with foliar nutrients*, in *Confidential Report*, APP 9. Young, G., Editor. Wood Quality Initiative Ltd. p. 1-20.

12. Xue, J., Olykan, S., Beets, P., McConchie, M., Graham, D., Leckie, A., Kimberley, M., Pearce, S. 2004. *Effect of boron, genotype and site on tree growth, intra-ring checking and other wood properties of radiata pine*, in *Confidential Report*, APP 38. Young, G., Editor. Wood Quality Initiative Ltd. p. 1-19.

13. Denne, M.P., Dodd, R.S. 1981. *The environmental control of xylem differentiation*, in *Xylem Cell Development*, Barnett, J.R., Ed. Castle House Publications Ltd.: Kent. 236-255.

14. Cown, D., McConchie, D., McConchie, M. 2003. *Intra-ring internal checking in radiata pine (P. radiata D. Don) in New Zealand*, in *Confidendtial Report*, APP 1. Wood Quality Initiative Ltd. p. 1-21.

15. Nye, P.H. 1968. *The soil model and its application to plant nutrition*, in *The Mineral Nutrition of Plants*, Rorison, I.H., Ed. Blackwell Scientific Publications: Oxford. 105-114.

16. Arnold, P.W. 1968. *Cation equilibria and competition between ions*, in *The Mineral Nutrition of Higher Plants*, Rorison, I.H., Ed. Blackwell Scientific Publications: Oxford. 115-125.

17. Clarkson, D.T., Hanson, J.B. 1980. *The mineral nutrition of higher plants.* Annual Review of Plant Physiology, **31**: 239-98.

18. Nothnagel, E.A. 1997. *Proteoglycans and related components in plant cells.* International Review of Cytology, **174**: 195-291.

19. Gaspar, Y., Johnson, K.L., McKenna, J.A., Bacic, A., Schultz, C.J. 2001. *The complex structures of arabinogalactan-proteins and the journey towards understanding function.* Plant Molecular Biology, **47**: 161-176.

20. Kreuger, M., van Holst, G.J. 1995. *Arabinogalactan-protein epitopes in somatic embryogenesis of Daucus carota L.* Planta, **197**: 135-141.

21. Kreuger, M., van Holst, G.J. 1993. *Arabinogalactan proteins are essential in somatic embryogenesis of Daucus carota L.* Planta, **189**: 243-248.

22. Motose, H., Sugiyama, M., Fukuda, H. 2004. *A proteoglycan mediates inductive interaction during plant vascular development.* Nature, **429**: 873-878.

23. Kreuger, M., van Holst, GJ 1996. *Arabinogalactan proteins and plant differentiation.* Plant Molecular Biology, **30**: 1077-1086.

24. Lamport, D., Kieliszewski, MJ 2005. *Stress upregulates periplasmic arabinogalactan proteins.* Plant Biosystems, **139**: 60-64.

25. Schultz, C., Johnson, KJ, Currie, G, Bacic, A 2000. *The classical arabinogalactan-protein gene family of arabidopsis.* The Plant Cell, **12**: 1751-1767.

Chapter Nine

Experimental

9.1 Materials

Unless otherwise stated, chemicals were purchased from: Sigma-Aldrich Chemical Company (Castle Hill, Australia), BDH (Poole, England) or Merck Ltd (Granville, Australia).

9.2 Methods for experiments outlined in chapter two

9.2.1 Tree material

Thirteen oven-dried radiata pine discs demonstrating a range of intra-ring checking phenotypes were provided to the Wood Quality Initiative Ltd. (WQI) by Graeme Young of Fletcher Challenge Forests. WQI forwarded the disks to this laboratory. These samples were sub-divided into severe, moderate, low and non-checking groups based on the frequency of the visible intra-ring checks. The severe checking group contained three samples, the moderate checking group contained three samples, the low checking group contained three samples, and the non-checking group contained four samples. The central earlywood portion of growth ring seven was used for all of the experiments to minimize the variation that occurs between growth rings.

9.2.2 Transmission electron microscopy

Sections 2 mm radial x 2 mm longitudinal x 3 mm tangential were removed from the central earlywood portion of growth ring seven, fixed, embedded and sectioned. Duplicate grids for each sample were observed with a Hitachi H-600 transmission electron microscope at 75 kV.

9.2.2.1 Materials

Glutaraldehyde, osmium tetroxide, cacodylate, Spurr resin (low viscosity kit, medium hardness) nickel grids and formvar were purchased from Electron Microscopy

Sciences (Fort Washington, USA). Uranyl acetate, potassium permanganate, ruthenium red and citric acid were purchased from BDH. The diamond knife was purchased from Micro Star (Huntsville, USA).

9.2.2.2 Sample fixation and embedding

Unless otherwise stated, each of the following steps was performed in a fumehood, with the exception of the vacuum fixation and oven incubations. The samples were washed in 0.1 M cacodylate buffer (pH 7; 2 x 5 minutes), after which they were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer under vacuum for up to 3 hours. The samples were then washed in 0.1 M cacodylate buffer (3 x 10 minutes) and post-fixed in 1% (w/v) osmium tetroxide, after which they were washed with 0.1 M cacodylate buffer (3 x 5 minutes). The samples were dehydrated in a graded series of ethanol (20, 40, 60, 80, 90, and 100% (v/v) in dH_2O) for 30 minutes each, followed by 100% acetone (2 x 30 minutes), then infiltrated with 1/3 Spurr resin to 2/3 acetone (v/v) overnight at room temperature under slight agitation. The following day, the samples were further infiltrated with 2/3 Spurr resin to 1/3 acetone (v/v) for approximately 6 hours at room temperature, after which they were embedded in 100% Spurr resin over night at 70°C. The samples were trimmed from the excess Spurr resin, and mounted on gel capsules filled with resin, and trimmed further using razor blades. Ultra-thin sections (100 nm) were cut with an LKB 2128 Ultrotome (Uppsala, Sweden) using a diamond knife and placed on formvar coated copper grids.

Preparation of formvar: 1.75% (w/v) formvar was prepared in chloroform. Glass slides were coated with this solution, and the formvar film was floated off onto water, loaded with grids which were picked up on cardboard and left to air dry.

9.2.2.3 Uranyl acetate and Sato's lead

Ultra-thin sections on copper grids were submerged in 1% uranyl acetate (made in 50% (v/v) ethanol in dH_2O) for 10 minutes, rinsed in 50% (v/v) ethanol for 5 seconds, followed by distilled water (2 x 5 seconds). The samples were then submerged in Sato's lead [1] for 5 minutes, after which they were rinsed in distilled water (2 x 5 seconds) and placed on filter paper to dry.

9.2.2.4 Ruthenium red

The samples obtained from growth ring seven were submerged in 40% (v/v) methylamine in a fumehood for 3 days at room temperature in order to remove lignin, that would otherwise have obscured the visualization of pectin [2-4]. The procedure for ruthenium red staining was performed essentially as described in Micheli et al. (2002) [2]. Following the removal of lignin the samples were washed, fixed in 4% (w/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 0.1% (w/v) ruthenium red for 1 hour, washed in 0.1 M cacodylate buffer containing 0.1% (w/v) ruthenium red (3 x 5 minutes), post fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer containing 0.1% (w/v) ruthenium red (pH 7.2), washed in 0.1 M cacodylate buffer, dehydrated, and infiltrated with Spurr resin, as previously described (section 9.2.2.2). Ultra-thin sections were cut with a diamond knife and placed on formvar coated copper grids.

9.2.2.5 Potassium permanganate

The samples were fixed in 4% (w/v) glutaraldehyde in 0.1 M cacodylate buffer, post fixed in 1% (w/v) osmium tetroxide, dehydrated, infiltrated and embedded in Spurr resin. Ultra-thin sections on copper grids were submerged in freshly prepared 2% (w/v) potassium permanganate for 1 hour, after which they were washed with distilled water for 5 seconds, submerged in 0.5% (w/v) citric acid for 30 seconds, washed with distilled water (3 x 5 seconds) and left on filter paper to dry.

Preparation of 2% (w/v) potassium permanganate: 250 mL of distilled water was boiled until only 100 mL remained. The flask was covered with foil, and 2 g of potassium permanganate crystals were added while the water was still hot. The solution was allowed to cool. The potassium permanganate solution was made fresh every 5 days when in constant use.

9.2.2.6 Negative development

All images were collected on Kodak Electron Image Film SO-163. The negatives were developed for four minutes in undiluted Kodak D-19 developer at 20°C. An Epson Perfection 1200U scanner was used to obtain .TIF files of the images.

9.2.3 Lignin assays

9.2.3.1 Materials

Acetyl bromide was purchased from Sigma, glacial acetic acid was purchased from BioLab Scientific Ltd. (Clayton, Australia), and hydroxylamine was purchased from Merck.

9.2.3.2 Sample preparation

Samples from growth ring seven of each disc were finely ground using a mixer mill (Retsch GmbH and Co., Germany) for 7 minutes at 23 Hz, or until the wood powder could pass through a 250 µm mesh.

9.2.3.3 Klason lignin

A gram of each ground sample was sent to Veritec, SCION (Rotorua, New Zealand) where the Klason lignin content was determined by M. Daley, M. Dibley and P. Gray. The acid-insoluble lignin in wood was determined using TAPPI Standard Methods T 222 om-88, and the acid soluble lignin in wood was determined using the TAPPI Useful Method UM 250. Due to limited resources, only a single measurement was taken on each sample. Essentially, the Klason method involves treating the sample with sulfuric acid to promote carbohydrate hydrolysis, the lignin isolated by this treatment is referred to as acid-insoluble lignin [5].

9.2.3.4 Acetyl bromide

The acetyl bromide assay was performed essentially as described by Hatfield et al. (1999) and modified by Möller (2001) [6, 7]. Perchloric acid was omitted because the samples were finely ground, and dissolution of the wall material was achieved. The ground wood was weighed into borosilicate glass tubes (2.5 to 3 mg). The samples were incubated for 2 hours at 50°C in 0.5 mL acetyl bromide in glacial acetic acid (25% (v/v)). After cooling on ice (5 minutes), 2 mL sodium hydroxide (2 M) and 2.4 mL glacial acetic acid were added to the mixture, cooled, and vortexed. Then, 0.37 mL hydroxylamine (0.3 M) was added, and the mixture was vortexed. The solution was then quantitatively transferred to volumetric flasks, after which glacial acetic acid was added (final volume 25 mL). The absorbance of the solution was read at 280 nm against glacial acetic acid using an Agilent 8453 diode array spectrophotometer

(Forest Hills, Australia) in disposable cuvettes (path length 10 mm). The lignin composition was calculated from the following equation [5, 6]:

$$\text{lignin [\% w/w]} = 100 * (A_s - A_b) * V * a^{-1} * W^{-1}$$

Where A_s and A_b = absorbance of sample and blank; V = volume of solution [L]; a = extinction coefficient of the lignin standard (g^{-1} cm^{-1}); W = weight of cell wall material [g]. An extinction coefficient of 20.09 $g^{-1}cm^{-1}$ was used to calculate the lignin content [5, 6, 8]. This assay was performed in duplicate for each sample.

9.2.4 Inductively coupled plasma optical emission spectrometry and mass spectrometry

Samples from growth ring seven of each disc were finely ground using a mixer mill (section 9.2.3.2) and sent to R. Haslemore of Hill Laboratories, Hamilton, New Zealand for these analyses. Due to limited resources, only a single measurement was taken on each sample. The test was generally found to be reproducible within a 13% margin of error (pers comm. R. Hasselmore, Hill Laboratories). These techniques involve nitric/hydrocholoric acid digestion of the samples followed by optical emission spectrometry for the calcium and magnesium levels and mass spectrometry for boron levels (pers comm. R. Hasselmore).

9.2.5 Statistical analysis

Statistix 8.0 (Analytical Software, Tallahassee USA) was used to generate randomized analysis of variance (ANOVA) outputs from the data generated. If significant results were obtained a Tukey-multivariate comparison was performed. The results are presented in appendix two.

9.3 Methods for experiments outlined in chapter three

9.3.1 Materials

The chemicals used for the preparation of the culture media were obtained from BDH; Sigma; *Phyto* Technology Lab (Shawnee Mission, USA) and Bio-Rad Laboratories (Auckland, New Zealand). Amberlite® IRA-743 and IRC-748 resin, tubing, leur locks and fast green were obtained from Sigma. Agar was purchased from BD Biosciences (Spark, USA), Pyroneg detergent was obtained from Global Science

(Auckland, New Zealand). Domestos® bleach was obtained from local supermarkets. Falcon tubes were purchased from BD New Zealand Ltd. (Auckland, New Zealand). Petri dishes (90 mm diameter) were obtained from BioLab Scientific Ltd. (Auckland, New Zealand). Plastic film (Glad Wrap®) was obtained from local supermarkets.

9.3.2 Media Composition

9.3.2.1 Standard media composition

The standard media composition is outlined in table 9.1. The amount of boron, calcium and magnesium in the standard media were modified as described in section 9.3.2.2.

Media components	mg/L	mM
KNO_3	475	4.70
NH_4NO_3	412	5.15
$CaCl_2.2H_2O$	110	0.75
$MgSO_4.7H_2O$	93	0.38
KH_2PO_4	43	0.32
Na_2EDTA	9.3	0.025
$MnSO_4.4H_2O$	6	0.026
H_3BO_3	1	0.016
KI	0.2	0.0012
Na_2MoO_4	0.06	0.0003
$CuSO_4.5H_2O$	0.06	0.0002
$CoCl_2.6H_2O$	0.06	0.00025
$FeSO_4.7H_2O$	7	0.025
Myo-inositol	25	0.139
Nicotinic acid	0.125	0.001
Pyridoxine HCl	0.125	0.0006
Thiamine HCl	0.125	0.0004
Glycine	0.5	0.0067
Sucrose	20 000	58.4
NAA	5	0.0268
Bacto-agar gelling agent	8 000	n/a
The initial pH of the standard media was 5.8		

Table 9.1 Standard media composition for growth of radiata pine organ cultures.

9.3.2.2 Preparation of solutions for modified media

Four major solutions termed macronutrients, micronutrients, vitamins and chelators were used to make the concentrated modified media discussed in section 9.3.3. These stock solutions were stored in Falcon tubes (or Eppendorf tubes) at -20°C (table 9.2).

Macronutrients	Working concentration (mg/L)	Working concentration (mM)	10X Stock solution (g/250 mL)
KNO_3	475	4.70	1.1875
NH_4NO_3	412	5.15	1.03
KH_2PO_4	43	0.32	0.1075
Micronutrients	**Working concentration (mg/L)**	**Working concentration (mM)**	**100X Stock solution (g/500 mL)**
$CuSO_4 \cdot 5H_2O$	0.06	0.0002	0.003
$CoCl_2 \cdot 6H_2O$	0.06	0.00025	0.003
Chelators	**Working concentration (mg/L)**	**Working concentration (mM)**	**100X Stock solution (g/500 mL)**
$FeSO_4 \cdot 7H_2O$	7.0	0.025	0.35
Na_2EDTA	9.3	0.025	0.465
Vitamins	**Working concentration (mg/L)**	**Working concentration (mM)**	**1000X Stock solution (g/ 100 mL)**
Nicotinic acid	0.125	0.001	0.0125
Pyroxidine HCl	0.125	0.0006	0.0125
Thiamine HCl	0.05	0.0004	0.005

Table 9.2 Composition of the four major solutions that were used in the concentrated modified media preparation.

Additional solutions that were added to the media after its passage through the ion-binding columns (because they could bind the resin [9]; ion-binding column preparation is described in section 9.3.3.1) were made with mineral nutrient free

Nanopure water (table 9.3; mineral nutrient free Nanopure water preparation is described in section 9.3.3.2). These solutions were stored in Falcon tubes at -20°C. NAA was stored in a polypropylene container at 4°C.

Component	Concentration (mg/L)	Concentration (mM)	Stock	Stock solution
Standard $CaCl_2.2H_2O$	110	0.75	10X	0.275 g/250 mL
Standard $MgSO_4.7H_2O$	93	0.38	10X	0.2355 g/250 mL
Na_2MoO_4	0.06	0.0003	100X	0.0015 g/250 mL
KI	0.2	0.0012	100X	0.002 g/100 mL
$MnSO_4.H_2O$	6	0.026	100X	0.3 g/100 mL
Glycine	0.5	0.0067	100X	0.005 g/100 mL
Standard H_3BO_3	1	0.016	1000X	0.1 g/100 mL
NAA	5	0.0268	1000X	2.5 g/ 500 mL

Table 9.3 Composition of the solutions for the remaining media components that could not be passed through the ion-binding columns.

Myo-inositol (25 mg/L; 0.139 mM) and sucrose (20 000 mg/L; 58 mM) were weighed individually into the concentrated media prior to passing the media through the ion-binding columns (section 9.3.3).

Boron stock solutions were made with ultra-pure chemicals to the appropriate concentrations in boron-free Nanopure water and were added prior to autoclaving (table 9.4; addition of boron to the media is described in section 9.3.3.4).

Modified H_3BO_3	Working concentration (mg/L)	1000X Stock solution
1 µM	0.00000006181	0.06181 g/L
7 µM	0.00000043267	0.0433 g/100 mL
25 µM	0.000001545	0.154 g/100 mL
100 µM	0.000006181	0.618 g/100 mL
1000 µM	0.0000618	6.18 g/100 mL

Table 9.4 Boron concentrations added directly to the media prior to autoclaving.

Calcium and magnesium were weighed directly into their respective media prior to the addition of the agar solution and autoclaving (table 9.5; the addition of calcium and magnesium to the modified media is described in section 9.3.3.4).

Modified $CaCl_2.2H_2O$	Working concentration (mg/L)	Weight (g/L)
1 mM	147	0.147
5 mM	735	0.735
10 mM	1,470	1.47
100 mM	14,700	14.7
Modified $MgSO_4.7H_2O$	Working concentration (mg/L)	Weight (g/L)
0.5 mM	130	0.13
1 mM	250	0.25
5 mM	1,230	1.23
10 mM	2,460	2.46
100 mM	24,600	24.6

Table 9.5 Calcium and magnesium concentrations added directly into the media prior to autoclaving.

The same quantities of the nutrients of interest were added to the 'mixed' culture media, as were described for the boron, calcium and magnesium media. The combinations of concentrations used are presented in table 9.6.

Mixed Cultures	Boron (μM)	Calcium (mM)	Magnesium (mM)
High boron, calcium and magnesium	1000	100	100
Low boron, calcium and magnesium	0	0	0
High boron, low calcium	1000	0	std
Low boron, high calcium	0	100	std
High calcium, low magnesium	std	100	0
Low calcium, high magnesium	std	0	100
Low boron, adequate calcium and magnesium	0	5	5
Adequate boron, calcium and magnesium	7	5	5

Std, standard media concentration

Table 9.6 Concentrations of boron, calcium and magnesium in the mixed cultures added directly to the mixed media prior to autoclaving.

Agar (8 000 mg/L) was prepared as described in section 9.3.3.3, and added directly to the media prior to autoclaving.

9.3.3 Modified media preparation

The different media components prepared as described in 9.3.2.2 were combined with Nanopure water to an initial volume of 500 mL for passage through the ion-binding columns (table 9.7). The final volume of this preparation prior to autoclaving was 1 L.

Component/ stock solution	Volume
Macronutrients (10x)	100 mL
Micronutrients (100x)	10 mL
Vitamins (1000x)	1 mL
Myo-inositol	0.025 g
Glycine (100x)	10 mL
Chelators (100x)	10 mL
Sucrose	20 g

Table 9.7 Preparation of concentrated modified media for passage through the ion-binding columns.

9.3.3.1 Preparation of ion-binding columns

IRA-743 resin was used to remove boron, and IRC-748 resin was used to remove calcium and magnesium. Two 60 mL plastic syringes clamped one above the other were used in lieu of standard glass columns, to avoid boron, calcium and magnesium contamination from glassware. The syringes were connected by plastic tubing, with leur locks to control the flow of the media (figure 9.1).

Figure 9.1 Ion-binding column set up. The concentrated culture media (top) was passed through two columns in tandem and collected in polypropylene containers (bottom).

Non-wetting cotton was placed in the bottom of the plastic syringes to prevent resin from entering the tubing. A resin slurry was made by adding water to the resin until it could be swirled, and the slurry was poured into the column (bed volume 95 mL). Any obvious air bubbles were tapped out of the column. The resin was kept wet at all times, and the media were passed through both columns and collected in a polypropylene container (figure 9.1). New columns were made for every litre of media prepared. For the mixed culture media, which required the removal of boron, calcium and magnesium, columns were prepared as described; however, in this case one column contained the IRA-743 resin and the other contained the IRC-748 resin.

9.3.3.2 Preparation of mineral nutrient free water

Nanopure water was passed through the appropriate ion-binding column to obtain water free of the nutrient of interest. This water was stored in polypropylene containers at -20°C until it was required for use.

9.3.3.3 Preparation of agar

Agar (8 g) and the appropriate ion-binding resin (25 g) were added to 250 mL of Nanopure water (devoid of the nutrient of interest). The solution was microwaved to dissolve the agar, and stirred under continual heat for approximately ½ hour. The resin was removed by decanting the molten agar, leaving the resin beads behind, prior to the addition of the agar solution to the media.

9.3.3.4 Final preparation of the modified media

After the concentrated medium (table 9.7) was collected from the column, the appropriate quantities of the remaining components were added, including boron, calcium and magnesium (table 9.8).

Component/ stock solution	Volume
Na_2MoO_4 (100x)	10 mL
KI (100x)	10 mL
$MnSO_4.4H_2O$	0.006 g
NAA (1000x)	1 mL
Nutrient of interest	section 9.3.2.2

Table 9.8 The remaining modified media components. Boron, calcium or magnesium (nutrients of interest) were added to the desired concentration.

The volume of the media was adjusted to 750 mL with mineral nutrient free Nanopure water and separated into two 375 mL volumes (in polypropylene containers). The mineral nutrient of interest was added (section 9.3.2.2), the pH was adjusted (section 9.3.3.5) and the agar solution was added (section 9.3.3.3). For example, preparation of 5 mM and 10 mM calcium media would involve:

	Nutrient of Interest	
Preparation	5 mM Ca^{2+}	10 mM Ca^{2+}
Separate 750 mL	375 mL	375 mL
Add $CaCl_2.2H_2O$	0.36 g	0.73 g
pH	5.8	5.8
Addition of agar	125 mL	125 mL
Total volume[a]	500 mL	500 mL

[a] Volume autoclaved

Table 9.9 Example of the final preparation of the modified media.

9.3.3.5 Media pH

HCl (1 M; 0.1 M) and KOH (0.1 M) were made in 50 mL of mineral nutrient free Nanopure water and were stored in Falcon tubes. KOH and/or HCl were used to adjust the pH of the boron media to 7.0, and the calcium and magnesium media to 5.8.

9.3.3.6 Sterilization

The media were sterilized by autoclaving in polypropylene containers at 121°C, 120 kPa for 20 minutes.

9.3.3.7 Storage of media

Sterilized medium was poured into sterilized plastic Petri dishes to a depth of 5 mm in a laminar flow cabinet. One litre of media was sufficient to prepare approximately 40 plates (25 mL/plate).

9.3.3.8 Inductively coupled plasma-optical emission spectrometry of media and agar solutions

ICP-OES of the liquid media passed through the columns, and of the agar solution described in section 3.2.2. was performed by Environmental Laboratory Services Ltd. (Lower Hutt, New Zealand). Boron, calcium and magnesium levels were determined by ICP-OES using the APHA 20th Edition Method 3120 B modified, following an acid digest.

9.3.4 Tree material

Radiata pine trees from a University of Canterbury field site (planted in 1994) located in Burnham, New Zealand were felled as required. Trees from the same clone (clone 3) were chosen to minimize genotypic variation. Stem segments (approximately 1 metre) between whorls of branches were removed from a region of the tree approximately 1 to 3 metres above the ground. The trees were transported to the laboratory in the back of a well ventilated truck. Radiata pine trees planted in a field site owned by SCION, Rotorua, New Zealand were also felled as described above. These trees were wrapped in bubble wrap and flown to Christchurch, New Zealand in a large insulated bin filled with ice packs. The Rotorua trees were not of the same clone as the Burnham trees. The trees were harvested between October and May of the 2003, 2004 and 2005 growth seasons. The stem segments from either field site were returned to the lab, and stored at 4^{o}C for a maximum period of one week.

9.3.5 Preparation of disc for explants

To prepare for culturing, approximately 5 cm was removed from the end of the stem segment to remove the outer region of cambial cells that may have dehydrated during

storage. A disc of approximately 10 cm was cut from the stem, placed in a sink filled with cold distilled water and the bark was scrubbed with Pyroneg detergent (1% (w/v); 2 x 5 minutes). The disc was rinsed with cold distilled water and placed in a bucket of distilled water containing 2% (v/v) Domestos bleach. The bark was scrubbed thoroughly with the bleach solution, and rinsed in distilled water (5 minutes). The disc was placed on a clean surface that had been sterilized with ethanol, and a razor knife was used to remove the outer rhytidome, being careful not to slice into the cambial region (figure 3.1C). The disc was rinsed with ethanol, moved to a sterile horizontal laminar flow cabinet and placed on a specially designed holder (figure 3.1D). The entire surface of the disc was sprayed with ethanol, flame sterilized and rotated 360 degrees, or until the flame subsided. This step was repeated if the phloem remained a pale colour and did not turn slightly brown.

9.3.6 Chip culture method

Flame sterilized equipment was used to remove sections approximately 3 cm axial x 2 cm tangential x 0.5 cm radial. A chisel and hammer were used to remove the chip by placing the chisel edge 0.5 cm from the outer edge of the phloem in the radial direction. The hammer was used to apply pressure to the chisel to remove the chip in the axial direction. The resulting explants contained surface sterilized phloem, cambium and xylem. The explant was placed on the sterile growth medium so that the exposed xylem surface was in direct contact with the medium as illustrated in chapter three (figure 3.2B). Two explants were generally placed in one Petri dish. The Petri dish was sealed with plastic strips and placed in a growth room (21°C; 23.5 hours light/day). An additional explant was stored in 4% paraformaldehyde in ¼× phosphate-buffered saline (PBS) at room temperature. This explant was kept as a control, representing the state of the tree at the time of culture. Generally, 14 chips per disc were obtained and each disc was used for one series of culture treatments (i.e. 0, 1, 7, 25, 100, 1000 µM boron treatments).

9.3.7 Scallop culture method

The scallop culture explant removal was similar to the chip explant. Briefly, flame sterilized equipment was used to remove sections approximately 1.5 cm axial x 4 cm tangential x 0.5 cm radial. A razor knife was used to make an initial incision in the tangential direction on the disc. The knife was then used with a hammer to remove a

half oval segment of the stem. The resulting explants contained surface sterilized phloem, cambium and xylem. Scallop explants differed from the chip explants in that these explants were taken from a larger disc and did not contain portions of the exposed edge. These explants were also thinner along the edges and thicker in the middle. The scallop explants were placed on the sterile growth media so that the exposed xylem surface was in direct contact with the medium as illustrated in chapter three (figure 3.3B). Two explants were generally placed in one Petri dish. The Petri dish was sealed with plastic strips and placed in a growth room (21°C; 23.5 hours light/day). An additional explant was stored in 4% paraformaldehyde in ¼x PBS at room temperature. This explant was kept as a control, representing the state of the tree at the time of culture. Any number of scallops could be obtained per disc. Generally, enough scallops for one series of culture treatments (i.e. 0, 1, 7, 25, 100, 1000 µM boron treatments) were taken per stem.

9.3.8 Marking culture growth

Prior to the excision of an explant, its cambium was injected with 0.1% (w/v) Janus green dye with sterile needles [10]. The dye was separated into 10 mL aliquots and autoclaved.

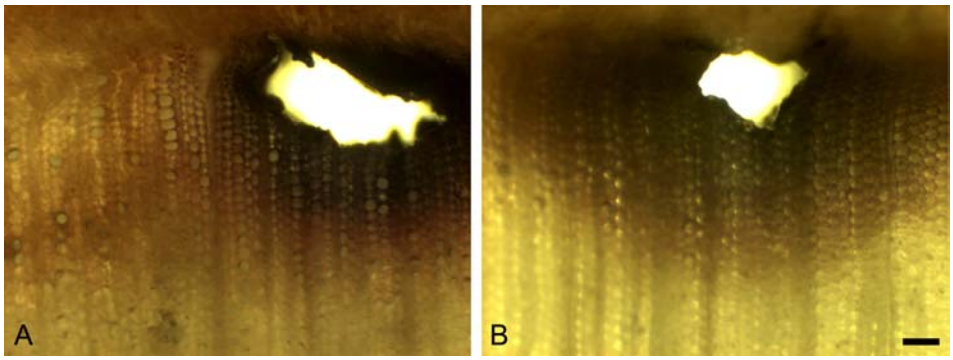


Figure 9.2 Examples of cultures injected with 0.1% Janus green dye (A-B). The images demonstrate the damage that the injection caused to the cambial and surrounding cells. Images were collected with a Cool Snap digital camera. Scale bar A-B = 100 µm.

9.3.9 Fixation of cultures

Control explants from each culture disc were kept. These explants were stored in 4% (w/v) formaldehyde made in ¼x PBS [10]. In addition, each of the cultures were

stored in the same fixative when they were collected. This fixative was made from 16% formaldehyde and 1x PBS as follows:

Preparation of 16% (w/v) formaldehyde: 8 g of paraformaldehyde was dissolved in 40 mL of Nanopure water and brought to 60°C in a fumehood, using a hot plate and constant stirring. 1 M NaOH was added drop wise until the solution was clear, after which it was removed from the heat and the volume was adjusted to 50 mL.

Preparation of PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 were dissolved in 800 mL of Nanopure water. The pH was adjusted to 7.4 and the volume was adjusted to 1 L.

Preparation of 4% (w/v) formaldehyde in 1/4x PBS: 25 mL of the 16% (w/v) formaldehyde and 25 mL of 1x PBS were combined, and the volume was adjusted to 100 mL using Nanopure water.

9.3.10 pH culture trial

For this trial, nine explants were grown on modified boron media (0, 7, 100 μM) with an initial pH of 5.8, 7.0 or 8.0. This was repeated on three different culture dates. The presence of microbes on the growing explants was recorded in three categories. The first category included explants showing no microbial contamination; the second included explants that were partially contaminated, meaning one end of the chip showed evidence of microbes; and the last included explants showing full contamination, where the whole explant was contaminated with microbes. The results for these categories were averaged, and graphed with standard error of the mean using Microsoft Excel.

9.4 Methods for experiments outlined in chapter four

9.4.1 Materials

Toluidine blue and TBA (*tert*-butyl alcohol) were purchased from BDH. Xylene was purchased from Wilsons Chemicals (Christchurch, New Zealand). Paraffin wax for sample embedding was purchased from Global Science. Plastic capsules for wax embedding were purchased from Simport (Quebec City, Canada). Glass slides were

purchased from the Marienfeld Laboratory Glassware (Marienfeld, Germany). Cover slips were purchased from Biolab Scientific.

9.4.2 Dissected cultures for callus trials

Standard media (ie. without altered nutrients) were prepared as described in section 9.3.2.1 Cultures were prepared essentially as described in section 9.3. Prior to induction on the culture medium, the phloem was removed using a flame sterilized scalpel. For the xylem strips, a flame sterilized scalpel was used to remove a strip of xylem cells, which were placed xylem side down on the culture media (figure 9.3).

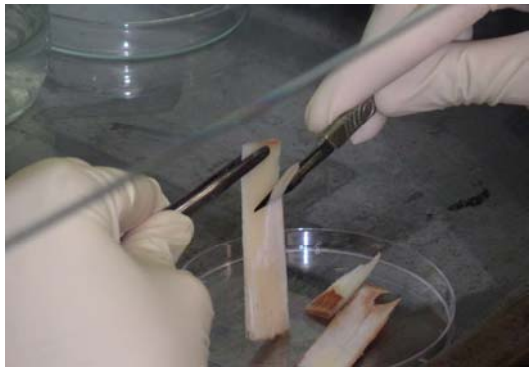


Figure 9.3 Preparation of a xylem strip for culturing. Flame sterilized equipment was used to remove the phloem and a strip of xylem for induction in culture.

9.4.3 Inductively coupled plasma-optical emission spectrometry/mass spectometry

The phloem and xylem in contact with the agar media were removed from the cultures using a hammer and new razor blade for each sample. The region of new wood growth was then dissected from the culture, cut into small pieces and left to dry at room temperature. These samples were finely ground using a mixer mill (section 9.2.3.2) and sent to R. Haslemore of Hill Laboratories (Hamilton, New Zealand) for analysis (described in section 9.2.4). Explants from two trees were cultured for two months to obtain material for this analysis. Due to limited resources, only a single measurement was taken for each analyzed sample. The values obtained were averaged for the cultures from both trees, and plotted with standard error of the mean using Microsoft Excel.

9.4.4 Cell counting

Images of transverse sections mounted on glass slides and stained with toluidine blue were obtained for this analysis.

9.4.4.1 Wax embedding

Explants that had been fixed in 4% (w/v) formaldehyde in PBS (section 9.3.9) were dehydrated in a series of 2-methylpropan-2-ol (*tert*-Butyl-alcohol; TBA) solutions, and embedded in paraffin wax using a Tissue-Tek Cryo Console, Tissue-Tek Thermal Console and Tissue-Tek Dispensing Console (Miles Scientific) as follows:

TBA dehydration	Time
TBA (1)	1 hour at room temperature
TBA (2)	overnight at room temperature
TBA (3)	1 hour at room temperature
TBA (4)	1 hour at room temperature
TBA (5)	1 hour at room temperature
100% TBA	1 hour at 40°C
100% TBA	1 hour at 40°C
100% TBA	24 hours at 40°C
Wax infiltration	**Time**
50/50 TBA/paraffin	24 hours at 40°C
Molten paraffin	24 hours at 60°C under vacuum
Molten paraffin	24 hours at 60°C under vacuum

Preparation of TBA solutions was as follows (v/v):

TBA solution	Distilled water	TBA	95% ethanol	Absolute ethanol
1	40	10	50	-
2	30	20	50	-
3	15	35	50	-
4	-	50	50	-
5	-	75	-	25

Sections (10 µm) thick were cut with a Reichert-Jung 2040 rotary microtome and mounted on glass slides. In addition, explants that were fixed in 4% (w/v) formaldehyde in PBS, were processed and sectioned by Medlab Ltd. (Timaru, New Zealand).

9.4.4.2 Toluidine blue

Toluidine blue stains lignified tissues green/blue and un-lignified tissues blue/violet through an unknown mechanism [11, 12]. The sections were de-waxed and re-hydrated in a series of xylene and ethanol solutions, stained with 0.05% (w/v) toluidine blue and mounted in DPX as follows:

De-wax	Time
100% xylene	1 minute
100% xylene	5 minutes
100% xylene	5 minutes
50% xylene/ 50% ethanol (v/v)	1 minute

Re-hydrate	Time
100% ethanol	1 minute
95% ethanol	1 minute
80% ethanol	1 minute
70% ethanol	1 minute
50% ethanol	1 minute
30% ethanol	1 minute
10% ethanol	1 minute
Distilled water	1 minute

Distilled water	1 minute

Stain	Time
0.05% (w/v) Toluidine blue	1 minute
Distilled water	5 rinses
Distilled water	5 rinses

De-hydrate and mount	Time
95% ethanol	1 minute
95% ethanol	1 minute
100% ethanol	1 minute
100% ethanol	1 minute
100% ethanol	1 minute
50% xylene/50% ethanol (v/v)	30 seconds
100% xylene	1 minute
100% xylene	5 minutes
100% xylene	5 minutes
Mount in DPX	

9.4.4.3 Light microscopy

Microscopy was performed on two different light microscopes with three different digital cameras: an Olympus BH-2 microscope with a Coolsnap digital camera, a Zeiss microscope with a Zeiss Axioshop digital camera, and an Olympus BH-2 microscope with a 5 mega pixel Olympus CAMEDIA C-5060 digital camera.

9.4.4.4 Cell counts

Images were obtained by light microscopy, and four images of different regions of a culture, were printed for an entire culture set, including the control explant. Cell counts from four radial files in each image were recorded in Microsoft Excel for three defined regions. The first region was defined as the ‘cambial region’. This region was comprised of the cells actively undergoing division and newly divided cells, which were recognized by their small size and thin walls. The second area measured was defined as the region undergoing ‘radial expansion’. This region was composed of cells undergoing enlargement in the radial dimension and included cells immediately

following the cambial region and preceding the cells that were undergoing lignin deposition. The final region, termed the 'new secondary wall region', consisted of the region of cells undergoing cell wall lignin deposition until the point of identification of a mature cell. The cell counts were averaged and are presented with standard error of the mean in appendix one. Where a percent difference to the control is given the values were calculated from the raw data using Microsoft excel. This was done by calculating the percent difference from the control using the averages for each individual culture set. Those numbers were then used to create an overall average.

9.4.5 Cell measurements

9.4.5.1 Image analysis

Lumen area, length and width

Image Pro Plus software (Media Cybernetics Inc., Silverspring, USA) was used to analyse the lumen area, radial length and tangential width of the cultures. The software was calibrated for the magnification the images were collected at, and four images per culture were analyzed. The average of the measurements obtained was calculated using Microsoft Excel, and the data are presented in appendix one with the standard error of the mean. In the case of the mixed culture sets, only two images were analysed per culture. These images were collected with the 5 mega pixel Olympus camera, and were extremely difficult to analyse with the software as the image detail was not well defined.

Cell wall thickness

Image Pro Plus software (Media Cybernetics Inc., Silverspring, USA) was used to calculate the area of the cell occupied by the cell wall. After completion of the lumen area measurements, the outer boundary of the same cluster of cells was outlined. The total area that these cells occupied (within the marked boundary) was calculated and then subtracted from the total lumen area (calculated previously) to provide a measurement of the area of the cell occupied by the cell wall using Microsoft Excel. This value is presented in appendix one as a percentage of the cell area, and was taken as representative of cell wall thickness.

9.4.6 Statistical analysis

Statistical analysis was performed as described in section 9.2.5. The results are presented in appendix two.

9.4.7 Transmission electron microscopy

Sections 2 mm radial x 2 mm longitudinal x 3 mm tangential were removed from the region of the cultures with new wood growth. These were fixed, embedded in Spurr resin, sectioned and stained with uranyl acetate and Sato's lead, as described in section 9.2.2. Duplicate grids for each sample were observed on a Hitachi H-600 transmission electron microscope at 75 kV.

9.5 Methods for experiments outlined in chapter five

9.5.1 Materials

Ammonium hydroxide was purchased from Sigma. LR White Resin was purchased from the London Resin Company (London, England). Glass strips were purchased from Electron Microscopy Sciences, and glass knives were made from these using an LKB Knife Maker 7801B. The JIM5 and JIM7 monoclonal antibodies were purchased from Plant Probes (Leeds, UK). Poly-L-lysine, FITC-goat anti-rat secondary antibody, BSA and *p*-phenylenediamine dihydrochloride were purchased from Sigma. AURION^{TM} reagents were purchased from the University of Otago Centre of Electron Microscopy (Dunedin, New Zealand).

9.5.2 Histochemistry

9.5.2.1 Wax embedding

The samples were embedded in paraffin wax, as described in section 9.4.4.1.

9.5.2.2 Ruthenium red

Sections mounted on glass slides were de-waxed as follows:

De-wax procedure	Time
100% xylene	1 minute
100% xylene	5 minutes
100% xylene	5 minutes
50% xylene/ 50% ethanol (v/v)	1 minute

The slides were warmed on a slide warmer (Photax, England). The slides were then immersed in ammonium hydroxide in a fumehood for six hours to remove surface lignin [2], stained in 0.25% (w/v) ruthenium red for 45 minutes, washed thoroughly with distilled water and mounted in distilled water.

9.5.3 Light microscopy

Light microscopy was performed using the instruments described in section 9.4.4.3.

9.5.4 Confocal microscopy

9.5.4.1 Sample fixation and embedding

A small block (3 mm tangential x 2 mm radial x 2 mm longitundinal) was removed from the newly grown wood of the cambial explants and fixed in 4% (w/v) paraformaldeyhyde and 0.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer under vacuum for 3 hours. The samples were rinsed twice with 0.1 M cacodylate buffer and dehydrated in an ethanol series (30, 50, 70 and 90% (v/v) for 30 minutes each) after which they were transferred straight into 100% LR White resin. The samples were transferred three times to fresh LR White resin for a total of three days. After the final transfer, the samples were embedded in LR White resin in closed gel capsules at 55°C for 24 hours. The samples were trimmed, and transverse sections (3 µm thick) were cut using a glass knife mounted on a LHB Bromma 11800 pyramitome (Stockholm, Sweden) and placed on poly-L-lysine coated glass slides.

Preparation of poly-L-lysine coated slides [2]: a 0.01% (v/v) poly-L-lysine solution was prepared from a 0.1% commercial solution. Glass slides were washed for 12 hours in a solution of 0.05% (v/v) HCl in 95% ethanol and then rinsed for 3 hours under running tap water. After drying for 1 hour at 60°C, two drops of the poly-L-lysine solution were applied to the centre of the slide and after approximately 5 minutes, the slides were turned onto their side to allow the excess poly-L-lysine solution to drain off. The slides were then dried overnight at room temperature.

9.5.4.2 Sample labelling

Immunocytochemistry was performed on the sections with JIM5 (1:30) or JIM7 (1:30) rat monoclonal antibodies. Dilutions were chosen based on dilution series trials. Two slides (one for each antibody) were prepared for a culture treatment, and

an additional slide (without primary antibody) was prepared as a negative control. The sections were rinsed in PBS containing 0.1% (w/v) BSA (PBS/BSA), blocked in goat serum for ½ hour (1:30 in PBS-T/0.1% (w/v) BSA) followed by multiple rinses in PBS/BSA (3 x 5 minutes). The samples were incubated in the appropriate dilution of primary antibody (in PBS/0.01% (w/v) BSA) overnight at 4°C in a humidified chamber. Control sections were incubated in PBS/BSA in place of the primary antibody. The sections were rinsed in PBS/BSA (4 x 10 minutes) followed by incubation in goat anti-rat IgG FITC secondary antibody (1:100 in PBS/0.01% BSA) for one hour in a dark humidified chamber. The sections were then washed in PBS/BSA (6 times) followed by distilled water (once) and mounted in a 90% glycerol (containing 0.01 M PB, 0.0027 M KCl, 0.137 M NaCl pH 7.4 [13]) and 1% *p*-phenylenediamine dihydrochloride anti-fade solution. The sections were stored at -20°C in the dark.

Preparation of PBS: prepared as described in section 9.3.9

Preparation of PBS-T: 0.1% (v/v) Tween-20 in PBS

9.5.4.3 Confocal microscopy

Confocal microscopy was performed on an Olympus IX70 inverted microscope with a BioRad μRadiance confocal scanning system. A Kalman filter was used to collect Z-series images with a 0.5 μm focus step.

9.5.5 Immunogold transmission electron microscopy

9.5.5.1 Sample fixation and embedding

Samples were fixed and embedded in LR White resin as described in section 9.5.4.1. Ultra-thin sections were cut using a LKB 2128 Ultrotome and mounted on formvar coated nickel grids.

9.5.5.2 Sample labelling

Immunocytochemical studies were performed on ultra-thin LR White sections placed on formvar coated nickel grids. All labelling materials, with the exception of the primary antibodies, were purchased from the University of Otago (Dunedin, New Zealand). Labelling was performed using either a Leica EM IGL as per AURION^{TM} Immunogold Reagent Methods, using AURION^{TM} reagents, or by hand. All washes

required 30 µL of solution, and antibody incubations required 5 µL of solution for the Leica EM IGL. If done by hand, sections were floated on 45 µL of solution. Primary antibody dilutions were chosen after a dilution series trial. Residual aldehyde groups, present after aldehyde fixation (J. Leuisson pers. comm.), were inactivated by incubation in a glycine/PBS solution (0.05 M glycine, 0.1 M phosphate) for 10 minutes. Grids were blocked using AURION^{TM} blocking solution for 30 minutes, washed with incubation buffer (3 x 2 minutes) and incubated in the appropriate dilution of primary antibody made in PBS/0.1% (w/v) BSA for two hours (JIM5 and JIM7, 1:20). Following incubation, the grids were washed in incubation buffer (6 x 2 minutes) and then incubated in AURION^{TM} Ultra Small Gold Conjugate Reagent (goat anti-rat US (GARa); 1:100) diluted in incubation buffer for 1 hour and 30 minutes. The grids were then washed in incubation buffer (3 x 2 minutes), 0.1 M PBS (3 x 5 minutes), post-fixed in glutaraldehyde (2% (w/v) made in 0.1 M phosphate) and washed further with phosphate buffer (3 x 5 minutes) and distilled water (5 x 2 minutes). The grids were silver enhanced using the AURION^{TM} R-GENT Silver Enhancing Kit (30 minutes), washed with distilled water (5 x 2 mins) and stained with 1% uranyl acetate and lead citrate in an LKB Bromma 2168 ultrastainer (Bromma, Sweden), prior to viewing with a Phillips TEM at 80 kV.

Preparation of 0.2 M phosphate buffer: 20.44 grams of Na_2HPO_4 anhydrous and 7.72 grams of $NaH_2PO_4 \cdot H_2O$ were weighed into 900 mL of double distilled water, dissolved and topped to 1000 mL with double distilled water after the pH was adjusted to 7.2.

Preparation of 0.1 M phosphate buffer: 500 mL of the 0.2 M phosphate buffer was adjusted to 1000 mL with double distilled water and the pH was adjusted to 7.2 if required.

Composition of the blocking solution: 5% (w/v) BSA, 0.1% (w/v) EWFS gelatin, 5% (v/v) goat serum, 10 mM sodium azide made in PBS. Final pH 7.4.

Preparation of the incubation buffer: PBS (0.1 M phosphate buffer, 150 mM NaCl), 0.1% AURION BSA-c^{TM}, 10 mM sodium azide with a final pH of 7.4.

AURION^{TM} R-GENT Silver Enhancing Kit: 20 drops of the enhancer solution and one drop of developer were mixed well with a vortex prior to use.

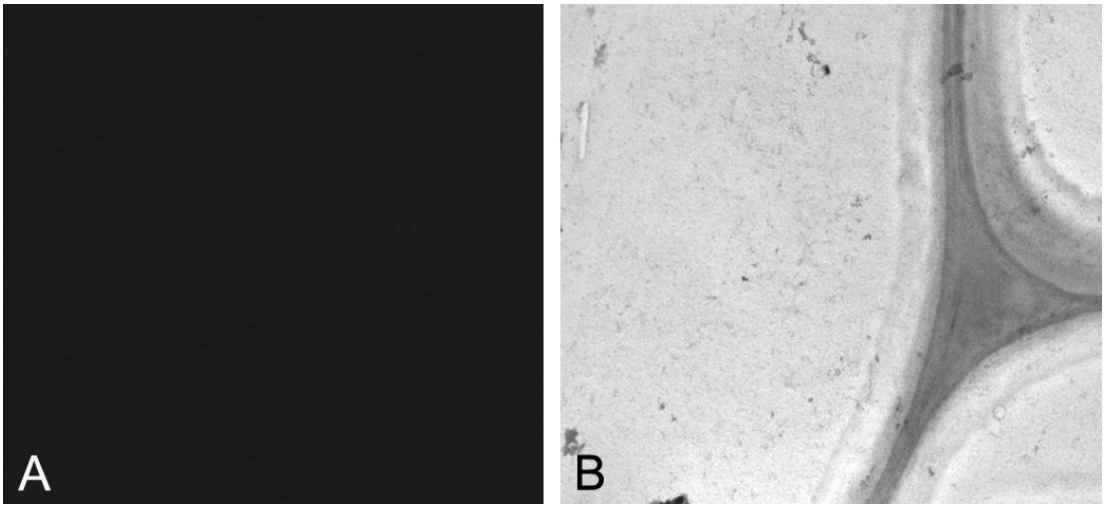


Figure 9.4 Examples of control immunohistochemistry preparations, where no primary antibody was added. (A) Confocal scanning laser microscopy and (B) immunogold transmission electron microscopy. The secondary antibodies did not show non-specific labelling.

9.5.6 Monosaccharide analysis

9.5.6.1 Culture material

For alditol acetate analysis, cultures (for the high and low mineral nutrient culture treatments) were explanted from the same disc segment and were cultured for two months.

9.5.6.2 Alditol acetate

As described in section 9.4.3, the phloem and xylem in contact with the agar media were removed from the cultures, the region of new wood growth was collected and finely ground using a mixer mill (section 9.2.3.2), and the samples were sent to K. Torr of ensis New Zealand (Rotorua) for determination of the neutral monosaccharide content of the cultures by two-stage sulfuric acid hydrolysis, reduction, acetylation and gas chromatography of the resulting alditol acetates.

9.6 Methods for experiments outlined in chapter six

9.6.1 Materials

Safranin was purchased from Biolab. Fast green was purchased from Sigma, and phloroglucinol was purchased from BDH.

9.6.2 Histochemistry

9.6.2.1 Wax embedding

The samples were embedded in paraffin wax as described in section 9.4.4.1.

9.6.2.2 Safranin/fast green

Safranin stains lignified tissues red, while fast green stains cellulose blue [11]. Wax sections mounted on glass slides were de-waxed as described in section 9.5.2.2, after which they were stained for 24 hours in a 1% (w/v) safranin solution (made in 70% (v/v) ethanol). The sections were rinsed with distilled water (5 times), immersed in a 95% ethanol and 0.5% (w/v) picric acid solution (10 seconds), followed by a 100% ethanol and ammonium hydroxide solution (4 drops ammonium hydroxide/100 mL ethanol; 10 seconds). The slides were then immersed in 100% ethanol (5 seconds) and were counter stained with 0.5% (w/v) fast green (30 seconds), immersed in 100% ethanol (3 x 5 seconds), followed by a 50:50 ethanol to xylene mixture (30 seconds) and immersion in xylene (3 x 1, and 5 minutes respectively). The sections were mounted in DPX and covered with glass coverslips.

9.6.2.3 Phloroglucinol-HCl

Phloroglucinol-HCl stains lignin pink [5, 10, 11, 14]. Wax sections mounted on glass slides were de-waxed as described in section 9.5.2.2, after which they were stained with 2% (w/v) phloroglucinol made in 95% (v/v) ethanol and concentrated HCl (2:1 v/v) [15], wet mounted and observed immediately. The colour reaction is only stable for 30 to 60 minutes [16].

9.6.2.4 Light microscopy

Light microscopy was performed using the instruments described in section 9.4.4.3..

9.6.2.5 Epifluorescence

Wax sections mounted on glass slides were de-waxed as described in section 9.5.2.2, mounted in water and observed using using blue light (an excitation wavelength of 330-385 nm) and an inverted Olympus IX70 epifluorescence microscope fitted with a mercury lamp. Images were collected with a Coolsnap digital camera at a standard exposure time of one second, to avoid signal decay (photo-bleaching) with time [11].

Image analysis

The area of the CML/S_1 wall area was determined from the images obtained using Image Pro Plus software. The area of the lignin occupying the CML/S_1 wall region was determined by calculating the area of the regions with the greatest pixel intensity. The area of the entire cell wall was also calculated, and a percentage of the area occupied by CML/S_1 cell wall lignin was determined using Microsoft Excel.

Statistical analysis

Statistical analysis was performed as described in section 9.2.5. The results are presented in appendix two.

9.6.3 Transmission electron microscopy

9.6.3.1 Sample fixation and embedding

The cultures were collected and embedded in Spurr resin as described in section 9.2.2.2.

9.6.3.2 Potassium permanganate

The grids were stained with 2% (w/v) potassium permanganate as described in section 9.2.2.5.

9.6.4 Lignin content

9.6.4.1 Sample preparation

Multiple cultures were grown on a low or high nutrient treatment, for each nutrient of interest, for two months. This was done in duplicate (ie. two trees were cultured). When collected, the discs were dissected to obtain the wood in the region of new growth, and allowed to air dry. The samples were finely ground using a mixer mill (section 9.2.3.2).

9.6.4.2 Materials

Section 9.2.3.1 outlines the purchased materials for these experiments.

9.6.4.3 Klason lignin

Klason lignin content was determined by M. Daley, M. Dibley and P. Gray at Veritec, SCION (Rotorua, New Zealand) as described in section 9.2.3.3. Due to limited resources, only a single measurement was taken on each sample.

9.6.4.4 Acetyl bromide

The acetyl bromide assay was performed as described in section 9.2.3.4. This assay was completed in duplicate on each sample.

9.6.4.5 Pyrolysis gas chromatography and mass spectrometry

Ground wood from the culture samples was sent to ensis SCION (Rotorua, New Zealand) to be analyzed by Py-GC-MS by D. Steward [17-21]. High and low culture treatments, from the same disc were analyzed in duplicate for culture preparations from two different trees. The percent composition of the identified pyrolysis products was determined for the duplicate analysis using Microsoft Excel.

9.7 Methods for experiments outlined in chapter seven

9.7.1 Materials

The Bio-Rad protein assay kit was purchased from Bio-Rad Laboratories (Auckland, New Zealand). β-Glucosyl and α-galactosyl Yariv reagents were purchased from Biosupplies Pty. (Melbourne, Australia). PD-10 columns were purchased from Amersham Pharmacia (Piscataway, USA). The primary monoclonal antibodies were kindly provided by Dr. Paul Knox (University of Leeds, UK). Pierce goat anti-rat-alkaline phosphatase conjugated secondary antibody was purchased from Global Sciences.

9.7.2 Microscopy

9.7.2.1 Tree material

Stem segments that were felled as described in section 9.3.4 were used to determine the location of AGPs in radiata pine. Discs were cut from the stem of a newly obtained segment, and an explant was collected as described in section 9.3.6. Transverse sections were cut by hand and used immediately for the histochemical and

immunocytochemical studies, as AGPs are known to be soluble and lost during fixation [22].

9.7.2.2 Histochemistry with β-glucosyl and α-galactosyl Yariv reagent

Sections cut by hand were mounted on glass slides and stained with β-glucosyl Yariv reagent (2 mg in 1 mL 0.15 M NaCl) for one hour. An additional section was stained with α-galactosyl Yariv reagent (2 mg in 1 mL 0.15 M NaCl) as a control. The sections were washed with 0.15 M NaCl (3 x 5 minutes), mounted in 0.15 M NaCl, and immediately observed on an Olympus BH-2 microscope. Images were collected with a Coolsnap digital camera.

9.7.2.3 Immunocytochemistry with JIM13 monoclonal antibody

Immunocytochemistry was performed on the hand cut sections mounted on glass slides as described in section 9.5.4.2. The JIM13 anti-AGP monoclonal (1:10 dilution) primary antibody and goat anti-rat IgG FITC (1:100) secondary antibody were used. Confocal microscopy was performed as described in section 9.5.4.3.

9.7.3 Protein extraction

9.7.3.1 Tree material

Xylem scrapings, from standing radiata pine trees, were used as the source of protein. A chisel and hammer were used to create an incision into the bark of the tree approximately 1 metre above the ground. The bark was peeled off by hand, and a razor blade was used to scrape the surface of the exposed tissue. These xylem scrapings were collected in aluminium foil and frozen immediately in liquid nitrogen for transport to the laboratory. The xylem scrapings were stored at -80°C until required.

9.7.3.2 Extraction procedure

The xylem scrapings (frozen in liquid nitrogen) were ground to a homogenous powder using a mortar and pestle, and transferred to a centrifuge tube on ice. The xylem powder was extracted with 4 mL of cold buffer/g of xylem (0.1 M Tris HCl pH 7.6, 0.1 M NaCl, 1 mM EDTA, freshly added 2% 2-mercaptoethanol, 1% (v/v) Triton-X-100 [23]). The mixture was vortexed and incubated at 4°C for three hours [24]. The

cell debris was removed by centrifuging (14,000 *g*; 20 minutes; 4°C), after which the supernatant was collected.

Trichloroacetic acid (TCA; 6% (v/v)) was added to the supernatant (overnight; 4°C), after which the mixture was centrifuged (14,000 *g*; 30 minutes; 4°C) [25]. The supernatant was collected and dialysed (MWCO 10,000) against cold distilled water overnight, after which it was either freeze dried or concentrated using Amicon centrifuge tubes (MWCO 10,000).

The freeze dried material was resuspended in 1% NaCl (w/v) and incubated overnight with either an equal volume of β-glucosyl Yariv reagent (1:1; AGP:β-GlcY (w/w)) in 1% (w/v) NaCl at 4°C [26, 27], or 0.5 mg β-glucosyl Yariv reagent in 1% NaCl/g xylem [23]. The mixture was then centrifuged (15,000 *g*; 1 hour; 4°C) and the pellet was washed three times each in a sodium chloride solution (100 mM NaCl; 10 mM Tris-HCl pH 7.6; 1 mM EDTA [23]) and methanol, air dried and resuspended in Nanopure or MilliQ filtered water (Loopstra 2000) [23]. Sodium dithionite (10% (w/v)) was added until the solution turned clear yellow after vortexing [28]. The solution was desalted with PD-10 columns equilibrated with water as per the manufacturer instructions. The sample was collected and stored at -80°C until required.

9.7.4 Quantification of arabinogalactan-proteins

9.7.4.1 Radial diffusion assay

Single radial gel diffusion was performed essentially as described by Van Holst and Clarke (1985) [29]. Agarose 1% (w/v) (Calbiochem type C), 1% (w/v) NaCl and 0.02 mg/mL β-glucosyl Yariv reagent were dissolved in distilled water. Gelbond (hydrophilic side up) was placed on a glass plate, using a drop of water to hold it in place. The glass plate was placed on a small platform with adjustable legs to ensure that the surface was flat. The agarose solution was heated, and a warm glass pipette was used to cover the surface of the Gelbond to a uniform thickness of 1 mm. Wells with a diameter of 3.0 mm were created with a gel cutter, and a Pasteur pipette attached to a vacuum pump was used to remove the agarose from the wells. The wells were loaded with 5 µL of sample (in duplicate) and the gel was placed in a humidified chamber overnight at room temperature. A standard curve was made from serial

dilutions of gum arabic [29]. These were loaded adjacent to the samples (figure 9.5) in triplicate. There is a linear relationship between the amount of AGP in the sample and the area of the halo, which is measured as a square of the diameter of the halo [29]. A calibrated eye piece was used to measure the diameter of the red halo to its outside edge (in mm), as well as the diameter of the well. These values were subtracted and the new value was averaged and squared. The new values were plotted using Microsoft Excel. The quantity of AGP in the samples was determined from the equation of the line calculated for the standard curve and adjusted if the samples were diluted.

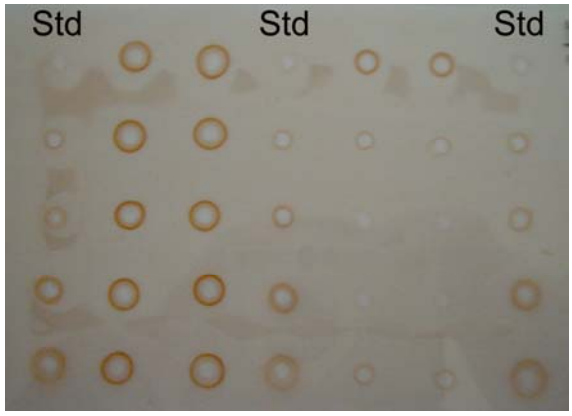


Figure 9.5 A radial diffusion gel showing the placement of the gum arabic standards in relation to the samples. Standards (Std) from top to bottom = 0.25, 0.5, 1.0, 2.0 and 3.0 µg of gum arabic. Samples were applied in duplicate between the rows of standards.

9.7.4.2 Bradford-assay

Eight hundred µL of appropriately diluted protein solution was added to 200 µL of Bio-Rad dye reagent concentrate in Eppendorf tubes and mixed thoroughly. The solutions were incubated for 10 minutes at room temperature before the absorbance at 595 nm was read against a blank containing 800 µL of water and 200 µL Bio-Rad dye reagent. Protein concentrations were obtained from a calibration curve generated in parallel using BSA obtained from Sigma. All measurements were in duplicate. Samples were measured on a Biowave S2100 diode array spectrophotometer (Cambridge, UK).

9.7.5 Characterization of arabinogalactan-proteins

9.7.5.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

For routine analysis of AGP samples, gels were prepared in the laboratory [30], and a Bio-Rad Gel Electrophoresis Unit, using a Bio-Rad 300 power pack was utilized. Samples (diluted into 20 µL) were mixed with 10 µL 5X sample buffer and incubated at 100°C for 2 minutes and briefly centrifuged before loading into the wells of the gel. The outer wells were loaded with 10 µL of sample buffer [30]. The second well of the gel was loaded with 6 µL of the appropriate marker, and the remaining wells were loaded with 10 µL of the sample solution. Electrophoresis was conducted at a constant voltage of 110 V for two hours, or until the bromophenol blue dye front had run to the bottom of the resolving gel.

7.5% Separating gel: 2.5 mL Solution A, 2.5 mL Solution B, 5 mL dH_2O, 50 µL 10% (w/v) ammonium persulfate (APS), 10 µL TEMED.

5% Stacking gel: 0.67 mL Solution A, 1 mL Solution C, 2.3 mL dH_2O, 10% (w/v) APS, 5 µL TEMED.

Solution A: acrylamide (BioRad, electrophoresis grade).

Solution B (4X separating gel buffer): 1.5M Tris-HCl pH 8.8, 0.4% (w/v) SDS.

Solution C (4X stacking gel buffer): 0.5 M Tris-HCl pH 6.8, 0.4% (w/v) SDS.

5X sample buffer: 60 mM Tris-HCl pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, 14.4 mM 2-mercaptoethanol, 0.1% (w/v) bromophenol blue.

Electrophoresis buffer: 0.3% (w/v) Tris-HCl, 14.4% (w/v) glycine, 1% (w/v) SDS.

Coomassie blue/β-glycosyl Yariv reagent gel staining

After electrophoresis the gel was stained for 10 minutes in Coomassie blue and destained overnight on a rotary shaker. The following day the gel was removed from the destain solution, rinsed with water and stained with 0.2% (w/v) β-glucosyl Yariv reagent in 1% (w/v) NaCl overnight, destained with 1% (w/v) NaCl and washed with dH_2O [31].

Coomassie blue stain: 1% (w/v) Coomassie blue, 50% (v/v) methanol, 10% (v/v) glacial acetic acid.

Coomassie destain: 5% (v/v) methanol, 10% (v/v) glacial acetic acid.

9.7.5.2 Immunoblot

SDS-PAGE was performed as described (section 9.7.5.1). After electrophoresis the gel was washed in methanol, rinsed with distilled water and placed in transfer buffer for 10 minutes. Nitrocellulose (0.2 µm pore size) was soaked in transfer buffer for 30 minutes at 4°C. The gel and nitrocellulose were assembled in the transfer cassette, and electrotransfered at a constant voltage of 45 V overnight at 4°C [30].

After electrotransfer, the nitrocellulose membrane was blocked in 3% (w/v) BSA/PBS for one hour followed by washes with PBS (3 x 10 minutes). The nitrocellulose membrane was then incubated in JIM13 monoclonal antibody (1:100 in 0.5% BSA/PBS (w/v)) overnight at 4°C. The membrane was washed briefly with PBS-T (twice), followed by additional PBS-T washes on a rotary shaker (3 x 10 minutes). The membrane was then incubated in goat anti-rat-alkaline phosphatase conjugated antibody (1:3000 in 0.5% BSA/PBS (w/v); [32]) for 2 hours, washed briefly in PBS-T (twice), followed by additional PBS-T washes (3 x 20 minutes) on a rotary shaker. The nitrocellulose membrane was then washed in alkaline phosphate buffer (5 minutes), and 10 mL of developing agent was added, when sufficient development had occurred a digital image was collected.

Alkaline phosphate buffer: 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$.

Developing agent: One Sigma Fast 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium tablet (BCIP/NBT Alkaline Phophatase Substrate) was added to 10 mL of distilled water.

9.7.5.3 Monosaccharide analysis

Chromic acid washed glassware and methanol washed caps were used for the monosaccharide analyses.

Carboxyl reduction

The carboxyl reduction was performed essentially as described by Kim et al. (1992) [33]. Freeze dried samples (2 mg) were dissolved in 5 mL ice cold 1 M imidazole-HCl pH 7.0, to which three 1 mL aliquots of sodium borodeuteride (100 mg/mL prepared freshly in dH_2O) were added. The mixture was vortexed and incubated on ice for 5 minutes after the first two additions, and two hours after the third addition. Slowly, 500 µL glacial acetic acid was added to the mixture to destroy excess reductant (or until fizzing stopped) and the samples were dialysed (MWCO 6-8,000) overnight against distilled water. The dialysed material was then freeze dried and re-suspended in 1 mL distilled water to which 200 µL of 0.2 M 2-[*n*-morpholino] ethane sulphonic acid (MES; pH 4.75) and 400 µL 500 mg/mL carbodiimide (prepared freshly in dH_2O) was added to the solution, vortexed, and incubated for 3 hours at 30°C. 1 mL of 4 M imidazole pH 7.0 was then added to the solution which was cooled on ice. The sample was split into equal volumes, and 1 mL 70 mg/mL sodium borohydride was added to one, and 1 ml 70 mg/mL sodium borodeuteride was added to the other (prepared freshly in dH_2O). The solutions were incubated overnight at room temperature. Slowly, 500 µL glacial acetic acid was added to both samples (or until fizzing stopped), after which the samples were dialysed (MWCO 3,500) for 24 hours against distilled water and freeze dried.

Production of alditol acetates

The hydrolysis and reduction methods were performed essentially as described by Albersheim (1967) [34], and acetylation was performed essentially as described by Blakeney et al. (1983) [35], with modifications.

Two empty vials (blanks) were carried through each stage of the methods. The samples (~50 µg of the sodium borodeuteride carboxyl reduced sample and ~50 µg of the sodium borohydride carboxyl reduced sample) were hydrolyzed with 75 µL 2.5 M trifluoroacetic acid (TFA) for two hours at 100°C in a fan forced oven, cooled, placed in a warm water bath and evaporated to dryness under a stream of nitrogen. TFA was used for hydrolysis because it readily yields monosaccharides from soluble cell wall polysaccharides, and is easily removed by evaporation [34]. *myo*-Inositol (~1.5 µg in

15 µL dH_2O) was added as an internal standard, and dried with a stream of nitrogen [34].

Prior to reduction, two additional vials containing 50 µL of sugar standards were freeze dried and incorporated into the procedure. The dried residue was dissolved in 50 µL 2 M ammonium hydroxide, to which 50 µL of 1 M sodium borodeuteride prepared in 2 M ammonium hydroxide was added, the solution was vortexed for 5 minutes, and incubated at room temperature for 2.5 hours. Twenty µL of glacial acetic acid was added slowly to the solution, which was then dried under a stream of nitrogen. The residues were further evaporated twice with 250 µL of 5% (v/v) acetic acid in methanol, and twice with 250 µL of methanol to remove boric acid. Borate produced on decomposition of borodeuteride can form a complex with the alditols, and slow their acetylation but can be removed on evaporation with methanol as methyl/borate [34, 35].

For acetylation, the residue was dissolved in 20 µL 1-methyl-imidazole, to which 200 µL of acetic anhydride was added, and vortexed thoroughly. 1-Methyl-imidazole acts as a catalyst for rapid acetylation [35]. After 20 minutes, 1 mL of distilled water was added to decompose the excess acetic anhydride [35], vortexed thoroughly and allowed to stand for 5 minutes. The mixture was extracted with 1 mL of dichloromethane (DCM), and centrifuged (<2000 *g*; 3 minutes) to aid separation. The DCM phase was washed with aliquots of water (3 x 2 mL), after which the DCM phase was transferred to a glass vial and carefully dried under a stream of nitrogen. The derivatives were reconstituted in 250 µL DCM and analyzed by GC-MS.

Standard sugar mix: a 20 mg/mL stock solution of each of the following sugars was prepared separately: *meso*-erythritol, 2-deoxy-D-ribose, L-rhamnose, L(-)fucose, D(-)ribose, L(+)arabinose, D(+)xylose, 2-deoxy-D-glucose, D-allose, D(+)mannose, D(+)galactose, α-D(+)glucose, *myo*-inositol. 100 µL of each of these stock solutions was combined, and 50 µL was freeze dried for use during the alditol acetate method.

Production of per-methylated alditol acetates

The samples (~100 µg of the sodium borodeuteride carboxyl reduced sample and ~100 µg of the sodium borohydride carboxyl reduced sample) were freeze dried, and re-suspended in 50 µL of dimethylsulfoxide (DMSO), capped and placed on an

oribital shaker for a minimum of 20 minutes to dissolve the glycans. A slurry of sodium hydroxide (~120 mg/mL) in DMSO was prepared by grinding sodium hydroxide pellets in DMSO using a glass mortar and pestle. Fifty µL of this slurry was dropped directly into each glycan solution using an SMI glass pipette, capped and shaken for 50 minutes. In a fumehood, 10 µL of methyl iodide was added to the solution, capped, and then shaken for 10 minutes. This was followed by a 10 and 20 µL addition of methyl iodide, which were capped and mixed for 10 and 20 minutes, respectively. 1 mL of freshly prepared sodium thiosulfate in water (100 mg/mL) and 500 µL DCM were added to the solution, vortexed (>40 seconds per sample) and centrifuged (2000 *g*; 3 minutes) to aid with phase separation. The aqueous upper phase was removed and discarded, and the lower DCM phase was washed with distilled water (3 x 1 mL), and dried under a stream of nitrogen. The hydrolysis, reduction and acetylation were performed as described for the alditol acetates.

Gas chromatography – mass spectrometry of alditol acetates and per-methylated alditol acetates

The sample (1 µL) was applied to a capillary column (25 m x 0.25 mm internal diameter CPSiL5 low polarity column; Chrompack, Australia) by split injection (split ratio 1:10). The analysis was performed with helium as a carrier gas and a set temperature program (initial temperature 135°C for 2 minutes, then a ramp rate increase of 2°C/minute up to a final temperature of 190°C). The injector temperature was maintained at 240°C and the interface temperature between the GC and the MS was held at 260°C. Data were acquired in full scan mode to detect ions from the *m/z* 100-700 using EI with an ionization energy of 70 eV.

Alditol acetates were identified by comparing their retention times with those of the standard monosaccharides derivatised as alditol acetates, relative to *myo*-inositol hexaacetate, and by their mass spectra. The relative amounts of uronic acid, from the 6,6-dideuteriosugar alcohol derivatives, identified by a shift of two atomic mass units of their symmetrical hexaacetate derivatives, were calculated after selected ion monitoring of the ratios of diagnostic ion pairs [36].

9.7.5.4 Amino acid analysis

Gas chromatography and mass spectrometry

GC-MS analysis of the amino acids was performed essentially as described by Persson et al. (2001) [37]. Samples (~800 µg in duplicate) were freeze dried, and re-suspended in 500 µL 6M HCl (constant boiling sequanal grade) containing 0.1% (w/v) phenol. Phenol is included to protect tyrosine from oxidation [38]. The tubes were purged with nitrogen to remove air, and sealed tightly with a cap, and Teflon tape. The tubes were placed in a 110°C oven for hydrolysis for 20 hours. The samples were allowed to cool, and were placed in a vacuum desiccator (with NaOH pellets and the pump constantly running) to evaporate HCl. Once completely dry, 40 µL of methanol:distilled water:triethylamine (4:1:1; (v/v/v)) was added to the samples and mixed. The mixture was transferred to glass capillary tubes. Norleucine was added to these samples as an internal standard and they were dried in a SpeedVac. Triethylamine (TEA) was included to maintain a basic pH and prevent protonation of the amino acids (pers comm. F. Pettolino). The samples were then silylated by adding 40 µL of N-(*tert*-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) and 80 µL dimethylformamide (DMF), mixed, sealed with Teflon tape and left at 70°C for 30 minutes [37].

Ten µL of the amino acid standard solution (in duplicate) was added to glass capillary tubes, with one end flame sealed, and dried in a SpeedVac. The standard mixture was silylated by adding 20 µL of MTBSTFA and 40 µL dimethylformamide (DMF), mixed, sealed with Teflon tape and left at 70°C for 30 minutes.

The sample (1 µL) was applied to a capillary column (25 m x 0.25 mm internal diameter CPSiL5; Chrompack, Australia). Samples were introduced by split injection (1/10) at 270°C. The GC oven temperature gradient was programmed at an initial 130°C, holding for 3 minutes followed by a ramping at 10°C/minute up to 290°C, holding for another 6 minutes. All mass spectra acquired were within the 50 to 550 m/z range. Quantifications were performed relative to the response factor calculated from the standard amino acid mixture, relative to norleucine.

Standard amino acid mixture (Sigma): included L-Ala, L-Arg, L-Asp, L-Cys, L-Glu, Gly, L-His, L-Ile, L-Leu, L-Lys.HCl, L-Met, L-Phe, L-Pro, L-Ser, L-Thr, L-Tyr and L-Val at a concentration of 2.5 mM in 0.1M HCl. L-norleucine, and *cis*-4-hydroxy-L-proline (Sigma) were added to the standard mixture at a concentration of 2.5 μmoL/mL.

High performance liquid chromatography

Hydrolysis of the samples was performed in collaboration with J. Maurer Menestrina (University of Melbourne, Australia) as described for the GC-MS method. Following hydrolysis and the addition of 40 μL of methanol:distilled water:triethylamine (4:1:1; (v/v/v)) as described for the GC-MS methods, 1% (v/v) phenylisothiocyanate (PITC) in methanol, also called Edman's reagent, was added to the samples, incubated at room temperature for 20 minutes, dried in a vacuum desiccator and resuspended in distilled water. HPLC analysis of the amino acids was performed by F. Pettolino of CRC for Bioproducts (University of Melbourne, Australia) using a reversed phase column (PICO TAG; Waters). The samples were eluted with a gradient from 0-15% (v/v) acetonitrile to sodium acetate buffer over four minutes, 16-25% over two minutes, and 21-37% over six minutes at a flow rate of 1 mL/min. Quantifications were performed relative to the response factor for the internal standard norleucine.

9.7.6 Fractionation of arabinogalactan-proteins

9.7.6.1 Reversed-phase high performance liquid chromatography

The desalted β-Glc Yariv precipitated AGPs (section 9.7.3.2) was fractionated by RP-HPLC on a 2.1 mm x 10 cm Brownlee Aquapore RP-300 C-8 column (Applied Biosystems, USA) attached to a System Gold (Beckman Instruments) HPLC equipped with a diode array detector and equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA). Once loaded onto the column, the samples were eluted with a linear gradient from 0-80% (v/v) acetonitrile in 0.1% TFA over 30 minutes at a flow rate of 0.5 mL/min. Chromatography was monitored by absorption at 215 and 280 nm. Fractions were dried in a SpeedVac concentrator, resuspended in milliQ filtered water and freeze dried.

When large amounts of material was fractionated, a 9.4 mm x 25 cm Zorbax 300SB-C8 column (Agilent Technologies) attached to the same HPLC system was used.

Once loaded onto the column the samples were eluted with a linear gradient from 0-80% (v/v) acetonitrile to 0.1% TFA over 60 minutes at a flow rate of 4 mL/min.

9.7.6.2 Identification of fractionated AGPs

Dot-blot immunoassay

Dot blot analysis was performed using a BioRad Bio-Dot® Microfiltration Apparatus. Briefly, nitrocellulose was pre-soaked in TBS (10 minutes), and applied to the apparatus as per the manufacturer's instructions. The samples (~10 µg in 100 µL in duplicate) were applied to the nitrocellulose membrane by gravity filtration (additional wells were loaded with buffer only as controls), after which they were washed with TBS-T (2 x 400 µL) under vacuum. The nitrocellulose membrane was removed from the apparatus, and blocked in 3% BSA/TBS for one hour, after which it was washed in TBS (3 x 5 minutes). The membrane was cut into strips corresponding to the relevant samples, and individually placed into each of the different primary antibodies (1:100 in 0.5% BSA/TBS (w/v)) overnight at 4°C. An additional strip was placed in buffer instead of primary antibody as a control. The individual membranes were washed quickly in TBS-T (twice) followed by additional washes (3 x 10 minutes) on a rotary shaker. The individual membranes were then placed in goat anti-rat alkaline phosphatase secondary antibody (1:1000 in 0.5% BSA/TBS (w/v); Pierce, USA) for two hours at room temperature in the dark. The individual membranes were then quickly washed in TBS-T (twice), followed by additional washes (3 x 30 minutes) on a rotary shaker. The membranes were developed in one step BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/*p*-nitro blue tetrazolium) until a colour change was evident. Images were immediately collected using digital photography.

Primary antibodies: JIM5, JIM7, LM2, JIM4, JIM13, JIM14, JIM15, JIM16, MAC207

25 mM TBS: 8 g NaCl, 0.2 g KCl, 3 g Tris in 800 mL, adjust to pH 7.4, adjust volume to 1 L.

TBS-T: 0.1% (v/v) Tween-20 in TBS

9.7.7 Yariv culture media

Standard culture media (secion 9.3.2.1) were prepared with two modifications. The first was the substitution of 0.5 mg/mL NAA with 50 mg/mL NAA (0.268 mM), to induce maximum culture growth (pers comm. H. Nair) and the second was the addition of 10 μM β-GlcY or α-GalY [39]. α-GalY was used as a control. The Yariv reagents were added to the media prior to autoclaving [25]. The sterilized media were poured in small Petri dishes (60 mm diameter) as described in section 9.3.3.7.

9.7.8 Tree material

Trees were harvested as described in section 9.3.4.

9.7.9 Explant preparation

Discs were prepared for culture as described in sections 9.3.5, 9.3.6, 9.3.7 and 9.3.9.

9.7.10 Culture analysis

Culture analysis was performed as described in sections 9.4.4 (cell counts), 9.4.5 (cell measurements), 9.5.2 (ruthenium red histochemistry), 9.6.2.2 (safranin/fast green histochemistry), 9.6.2.5 (epifluorescence microscopy), 9.4.7 (TEM), and 9.2.3.4 (acetyl bromide assay).

9.7.11 Statistical analysis

Statistical analysis was performed as described in section 9.2.5. The results are presented in appendix two.

9.8 References

1. Sato, T. 1968. *A modified method for lead staining of thin sections.* Journal of Electron Microscopy, **17**: 158-159.

2. Micheli, F., Ermel, F., Bordenave, M., Richard, L., Goldberg, R. 2002. *Cell walls of wood tissues: cytochemical, biochemical and molecular analysis of pectins and pectin methylesterases*, in *Wood Formation in Trees: Cell and Molecular Biology Techniques*, Chaffey, N.J., Ed. Taylor and Francis: London. 179-200.

3. Chaffey, N. 2002. *Conventional (chemical fixation) transmission electron microscopy and cytochemistry of angiosperm trees*, in *Wood Formation in Trees: Cell and Molecular Biology Techniques*, Chaffey, N., Ed. Taylor and Francis: London. 41-64.

4. Harche, M., Catesson, AM 1985. *Cell wall architecture in alfa (Stipa tenacissima).* International Association of Wood Anatomists Journal, **6**: 61-70.

5. Dence, C.M. 1992. *The determination of lignin*, in *Methods in Lignin Chemistry*, Timell, T.E., Ed. Springer and Verlag: Berlin. 33-61.

6. Moller, R. 2001. Cell differentiation and secondary wall formation in callus cultures of *Pinus radiata.* PhD Thesis, University of Auckland.

7. Hatfield, R.D., Grabber, J., Ralph, J., Brei, K. 1999. *Using the acetyl bromide assay to determine lignin concentrations in herbaceous plants: some cautionary notes.* Journal of Agriculture and Food Chemistry, **47**: 628-632.

8. Iiyama, K., Wallis, A.F.A. 1988. *An improved acetyl bromide procedure for determining lignin in woods and woody pulps.* Wood Science and Technology, **22**: 271-280.

9. Matoh, T., Ishigaki, K., Mizutani, M., Matsunga, W., Takabe, K. 1992. *Boron nutrition of cultured tobacco BY-2 cells. I. Requirement for and intracellular localization of boron and selection of cells that tolerate low levels of boron.* Plant Cell Physiology, **33**: 1135-1141.

10. Gahan, P.B. 1984. *Plant histochemistry and cytochemistry*. London: Academic Press Inc.

11. Wardrop, A.B. 1981. *Lignification and xylogenesis*, in *Xylem Cell Development*, Barnett, J.R., Ed. Castle House Publications Ltd.: Kent. 115-151.

12. O'Brien, T.P., Feder, N., McCully, M.E. 1964. *Polychromatic staining of plant cell walls by toluidine blue O.* Protoplasma, **59**: 368-373.

13. Zhang, Y., Brown, G., Whetton, R., Loopstra, C.A., Neale, D., Kieliszewski, M.J., Sederoff, R.R. 2003. *An arabinogalactan protein associated with secondary cell wall formation in differentiating xylem of loblolly pine.* Plant Molecular Biology: 91-102.

14. Nakano, J., Meshitsuki, G. 1992. *Reagents for the detection of lignin*, in *Methods in Lignin Chemistry*, Timell, T., Ed. Springer and Verlag: Berlin. 23-61.

15. Nakano, J., Meshitsuka, G. 1992. *The detection of lignin*, in *Methods In Lignin Chemistry*, Timell, T., Ed. Springer-Verlag: London.

16. Downes, G., Turvey, N.D. 1986. *Reduced lignification in Pinus radiata D. Don.* Australian Journal of Forest Research, **16**: 371-377.

17. Ralph, J., Hatfield, R.D. 1991. *Pyrolysis-GC-MS characterization of forage materials.* Journal of Agriculture and Food Chemistry, **39**: 1426-1437.

18. Faix, O., Meier, D., Fortmann, I. 1990. *Thermal degradation products of wood. Gas chromatographic seperatation and mass spectrometric characterization of monomeric lignin derived products.* Holz als Roh - und Werkstoff, **48**: 281-285.

19. Faix, O., Meier, D., Fortmann, I. 1990. *Thermal degradation products of wood. A collection of electron-impact (EI) mass spectra of monomeric lignin derived products.* Holz als Roh - und Werkstoff, **48**: 351-354.

20. Faix, O., Fortmann, I., Bremer, J., Meier, D. 1991. *Thermal degradation products of wood. Gas chromatographic separation and mass spectrometric characterization of polysaccharide derived products.* Holz als Roh - und Werkstoff, **49**: 213-219.

21. Faix, O., Fortmann, I., Bremer, J., Meier, D. 1991. *Thermal degradation products of wood. A collection of electron-impact (EI) mass spectra of polysaccharide derived products.* Holz als Roh - und Werkstoff, **49**: 299-304.

22. Clarke, A.E., Anderson, R.L., Stone, B.A. 1979. *Form and function of arabinogalactans and arabinogalactan-proteins.* Phytochemistry, **18**: 521-540.

23. Loopstra, C.A., Puryear, J.D., No, E.G. 2000. *Purification and cloning of an arabinogalactan-protein from xylem of loblolly pine.* Planta, **210**: 686-689.

24. Schultz, C.J., Johnson, K.J., Currie, G., Bacic, A. 2000. *The classical arabinogalactan-protein gene family of arabidopsis.* The Plant Cell, **12**: 1751-1767.

25. Gao, M., Kieliszewski, M.J., Lamport, D.T.A., Showalter, A.M. 1999. *Isolation, characterization and immunolocalization of a novel, modular tomato arabinogalactan-protein corresponding to the LeAGP-1 gene.* The Plant Journal, **18**: 43-55.

26. Gane, A.M. 1994. *The extracellular matrix and cell walls of pistils of Nicotiana alta*. PhD Thesis, *School of Botany*, University of Melbourne.

27. Gane, A.M., Craik, D., Munro, S.L.A., Howlett, G.J., Clarke, A.E., Bacic, A. 1995. *Structural analysis of the carbohydrate moiety of arabinogalactan-proteins from stigmas and styles of Nicotiana alta.* Carbohydrate Research, **277**: 67-85.

28. Kreuger, M., van Holst, G.J. 1995. *Arabinogalactan-protein epitopes in somatic embryogenesis of Daucus carota L.* Planta, **197**: 135-141.

29. Van Holst, G.K., Clarke, A.E. 1985. *Quantification of arabinogalactan-protein in plant extracts by single radial gel diffusion.* Analytical Biochemistry, **148**: 446-450.

30. Bollag, D.M., Rozycki, M.D., Edelstein, S.J. 1996. *Protein Methods*. New York: John Wily & Sons Inc.

31. Du, H., Simpson, J., Moritz, R.L., Clarke, A.E., Bacic, A. 1994. *Isolation of the protein backbone of an arabinogalactan-protein from the styles of Nicotiana alta and characterization of a corresponding cDNA.* The Plant Cell, **6**: 1643-1653.

32. Lu, H., Chen, M., Showalter, A.M. 2001. *Developmental expression and perturbation of arabinogalactan-proteins during seed germination and seedling growth in tomato.* Physiologia Plantarum, **112**: 442-450.

33. Kim, J., Carpita, NC 1992. *Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles.* Plant Physiology, **98**: 646-653.

34. Albersheim, P. 1967. *A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography.* Carbohydrate Research, **5**: 340-345.

35. Blakeney, A.B., Harris, P.J., Henry, R.J., Stone, B.A. 1983. *A simple and rapid preparation of alditol acetates for monosaccharide analysis.* Carbohydrate Research, **113**: 291-299.

36. Kim, J.B., Carpita, N.C. 1992. *Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles.* Plant Physiology, **98**: 646-653.

37. Persson, J., Nasholm, T. 2001. *A GC-MS method for the determination of amino acid uptake by plants.* Physiologia Plantarum, **113**: 352-358.

38. Ozols, J. 1990. *Amino acid analysis*, in *Guide to Protein Purification: Methods in Enzymology*, Deutscher, M.P., Ed. Academic Press Inc.: New York. 587-601.

39. Ding, L., Zhu, J.K. 1997. *A role for arabinogalactan-proteins in root epidermal cell expansion.* Planta, **203**: 289-294.

Appendix One

Supplemental culture analysis

This appendix presents supplemental culture analysis for the data presented in chapters four, six and seven. The numbers presented are for one explant per treatment.

	Boron culture treatment						
	0 µM	1 µM	7 µM	25 µM	100 µM	1000 µM	Control Explant
Changes in cell number							
Cambial region cell number	15 ± 0.9	15 ± 0.8	20 ± 0.5	n/a	19 ± 1.3	21 ± 0.8	18 ± 0.6
Radial expansion region cell number	11 ± 0.9	15 ± 1.4	14 ± 1	n/a	12 ± 1.6	13 ± 0.7	12 ± 1.9
New secondary wall region cell number	15 ± 0.5	11 ± 1.2	14 ± 3	n/a	19 ± 1.7	17 ± 1.6	16 ± 0.7
Radially expanding cells							
Lumen area (μm^2)	598 ± 32	900 ± 32	694 ± 53	n/a	786 ± 17	785 ± 35	645 ± 23
Lumen radial length (µm)	32 ± 0.5	38 ± 0.5	35 ± 1.4	n/a	35 ± 0.35	36 ± 0.85	34 ± 0.56
Lumen tangential width (µm)	24 ± 1	30 ± 0.7	26 ± 1.1	n/a	28 ± 0.3	27 ± 0.7	24 ±0.4
Percent cell wall area (%)	22 ± 0.01	14 ± 0.5	17 ± 3	n/a	13 ± 1	14 ± 0.8	19 ± 2
Number of cells measured	528	452	582	n/a	485	477	396
Existing cells							
Lumen area (μm^2)	678 ± 79	809 ± 55	586 ± 62	n/a	680 ± 19	660 ± 52	497 ± 68
Lumen radial length (µm)	32 ± 1.6	36 ± 0.9	32 ± 1.2	n/a	32 ± 0.1	33 ± 1.4	29 ± 1.7
Lumen tangential width (µm)	25 ± 1.7	28 ± 1.6	23 ± 1.6	n/a	26 ± 0.4	24 ± 1	22 ± 1.8
Percent cell wall area	36 ± 1.5	33 ± 0.5	44 ± 1.3	n/a	33 ± 0.7	36 ± 18	46 ± 1.3
Number of cells measured	204	234	170	n/a	125	208	208
Cell wall area occupied by CML/S_1 lignin (%)	1.8 ± 0.07	1.2 ± 0.6	n/a	n/a	0.9 ± 0.4	0.8 ± 0.4	1.3 ± 0.1

n/a, unable to measure

Table 1 Boron treated culture set number one from a Rotorua tree. The explants were inducted in April and cultured for two months. Values are ± standard error of the mean.

	Boron culture treatment						
	0 µM	1 µM	7 µM	25 µM	100 µM	1000 µM	Control Explant
Changes in cell number							
Cambial region cell number	8 ± 0.4	8 ± 0.1	10 ± 0.4	11± 0.3	11 ± 0.4	12 ± 0.9	9 ± 0.4
Radial expansion region cell number	10 ± 0.8	15 ± 1	12 ± 0.5	10 ± 0.4	11 ± 0.5	13 ± 0.5	15 ± 1
New secondary wall region cell number	17 ± 0.4	13 ± 1	18 ± 1.5	19 ± 1	16 ± 1.6	n/a	n/a
Radially expanding cells							
Lumen area (μm^2)	863 ± 116	865 ± 29	880 ± 38	818 ± 23	851± 59	984 ± 18	909 ± 68
Lumen radial length (µm)	40 ± 1.7	38 ± 0.8	39 ± 0.8	36 ± 0.19	37 ± 1.4	42 ± 0.64	38 ± 1.2
Lumen tangential width (µm)	27 ± 2.7	29 ± 0.5	29 ± 0.9	28 ± 0.6	28 ± 0.9	31 ± 0.15	30 ± 1.4
Percent cell wall area	20 ± 1.6	19 ± 0.8	18 ± 0.6	17 ± 0.4	17 ± 1.2	14 ± 1	16 ± 1.5
Number of cells measured	610	499	511	361	540	364	462
Existing cells							
Lumen area (μm^2)	743 ± 56	1106 ± 33	966 ± 40	889 ± 40	1063 ± 75	890 ± 54	1061 ± 66
Lumen radial length (µm)	39 ± 1.1	44 ± 2	41 ± 0.7	40 ± 1	44 ± 1.1	42 ± 1.4	43 ± 1.3
Lumen tangential width (µm)	23 ± 1	31 ± 1.3	28 ± 0.6	27 ± 1.5	29 ± 1.8	25 ± 1	30 ± 1.2
Percent cell wall area	38 ± 1.2	37 ± 1.6	33 ± 0.7	36 ± 1.0	29 ± 1.2	34 ± 1.3	30 ± 1.3
Number of cells analyzed	213	161	201	157	166	212	167
Cell wall area occupied by CML/S_1 lignin (%)	0.3 ± 0.1	1.7 ± 0.8	1.4 ± 0.2	n/a	0.2 ± 0.06	0.6 ± 0.2	1.1 ± 0.3

n/a, unable to measure

Table 2 Boron treated culture set number two from a Burnham tree. The explants were inducted in March and cultured for two months. Values are ± standard error of the mean.

	Boron culture treatment						
	0 µM	1 µM	7 µM	25 µM	100 µM	1000 µM	Control Explant
Changes in cell number							
Cambial region cell number	14 ± 0.6	13 ± 1	15 ± 1	16 ± 0.6	n/a	n/a	13 ± 1
Radial expansion region cell number	12 ± 1.2	15 ± 1	16 ± 1	17 ± 2	n/a	n/a	14 ±0.8
New secondary wall region cell number	17 ± 2.3	12 ± 0.8	11 ± 0.8	14 ± 0.6	n/a	n/a	15 ± 0.8
Radially expanding cells							
Lumen area (μm^2)	873 ± 37	876 ± 61	842 ± 37	865 ± 113	938 ± 39	n/a	830 ± 42
Lumen radial length (µm)	38 ±0.8	39 ± 0.9	37 ± 1.0	37 ± 2.2	39 ± 0.8	n/a	36 ± 1
Lumen tangential width (µm)	28 ± 0.8	29 ± 1	28 ± 0.6	29 ± 2.4	30 ± 0.9	n/a	28 ± 0.8
Percent cell wall area	18 ± 1	14 ± 1.4	15 ± 0.6	11 ± 1.6	16 ± 1.8	n/a	14 ± 1
Number of cells measured	452	536	547	263	360		393
Existing cells							
Lumen area (μm^2)	754.5 ± 496	960 ± 100	776 ± 67	776 ± 33	784 ± 392	n/a	760.5 ± 25
Lumen radial length (µm)	36 ± 1.2	38.5 ± 1.6	36 ± 1.4	36 ± 0.8	37 ± 0.5	n/a	35 ± 0.6
Lumen tangential width (µm)	26 ± 0.9	31 ± 2.0	27 ± 1.1	27 ± 0.6	27 ± 0.4	n/a	26 ± 0.5
Percent cell wall area	30 ± 1.6	28 ± 0.6	30 ± 1.1	27 ± 1.2	24 ± 1.2	n/a	31 ± 1.4
Number of cells measured	214	177	241	176	232	n/a	207
Cell wall area occupied by CML/S_1 lignin (%)	0.5 ± 0.3	0.5 ± 0.2	0.5 ± 0.06	1.2 ± 0.3	1.2 ± 0.4	n/a	1.9 ± 0.6

n/a, unable to measure

Table 3 Boron treated culture set number three from a Burnham tree. The explants were inducted in November and cultured for one month. Values are ± standard error of the mean.

	Calcium culture treatment					
	0 mM	1 mM	5 mM	10 mM	100 mM	Control Explant
Changes in cell number						
Cambial region cell number	12 ± 0.7	16 ± 1	13 ± 0.6	13 ± 0.3	13 ± 0.6	11 ± 1
Radial expansion region cell number	13 ± 0.5	n/a	9 ± 0.7	12 ± 0.8	10 ± 0.9	10 ± 1
New secondary wall region cell number	10 ± 0.6	n/a	11 ± 0.8	10 ± 0.8	8 ± 0.4	9 ± 0.6
Radially expanding cells						
Lumen area (μm^2)	677 ± 31	812 ± 49	783 ± 58	848 ± 41	706 ± 17	856 ± 46
Lumen radial length (μm)	34 ± 0.5	37 ± 1.4	39 ± 1.3	38 ± 1.2	35 ± 0.2	40 ± 1.2
Lumen tangential width (μm)	25 ± 0.8	28 ± 0.9	27 ± 1.5	29 ± 0.9	26 ± 0.3	28 ± 1.2
Percent cell wall area (%)	18 ± 1.2	17 ± 0.3	16 ± 0.9	19 ± 0.4	21 ± 0.9	16 ± 0.3
Number of cells measured	583	415	337	350	432	353
Existing cells						
Lumen area (μm^2)	776 ± 34	961 ± 28	1055 ± 64	n/a	947 ± 54	1074 ± 32
Lumen radial length (μm)	39 ± 0.5	41 ± 1.3	46 ± 0.4	n/a	41 ± 0.9	47 ± 0.9
Lumen tangential width (μm)	24 ± 1.2	29 ± 0.6	28 ± 2	n/a	28 ± 1	29 ± 0.8
Percent cell wall area	38 ± 1.9	35 ± 0.1	29 ± 1	n/a	35 ± 0.8	26 ± 0.5
Number of cells measured	541	355	600	n/a	818	612
Cell wall area occupied by CML/S_1 lignin (%)	0.2 ± 0.03	n/a	0.2 ± 0.03	n/a	0.2 ± 0.02	0.2 ± 0.06

n/a, unable to measure

Table 4 Calcium treated culture set number one from a Burnham tree. The explants were inducted in March and cultured for two months. Values are ± standard error of the mean.

	Calcium culture treatment					
	0 mM	1 mM	5 mM	10 mM	100 mM	Control Explant
Changes in cell number						
Cambial region cell number	15 ± 0.4	14 ± 0.7	13 ± 0.9	17 ± 0.6	17 ± 1.2	15 ± 1.3
Radial expansion region cell number	17 ± 0.8	13 ± 1.1	15 ± 0.3	19 ± 1.1	18. ± 0.9	15 ± 0.9
New secondary wall region cell number	13 ± 0.7	12 ± 0.6	12 ± 0.5	14 ± 0.7	13 ± 0.9	14 ± 1.5
Radially expanding cells						
Lumen area (μm^2)	971 ± 94	850 ± 63	957 ± 61	834 ± 31	856 ± 68	714 ± 11
Lumen radial length (μm)	40 ± 2.0	37 ± 1.3	39 ± 1.3	37 ± 0.4	37 ± 1.5	35 ± 0.3
Lumen tangential width (μm)	30 ± 1.9	29 ± 1.1	30 ± 0.9	29 ± 0.5	29 ± 1.2	26 ± 0.4
Percent cell wall area	13 ± 0.8	15 ± 0.4	16 ± 0.6	18 ± 5.4	15 ±0.7	17 ± 0.9
Number of cells measured	398	359	395	335	402	505
Existing cells						
Lumen area (μm^2)	843 ± 16	771 ± 38	886 ± 70	688 ± 55	716 ± 50	738 ± 62
Lumen radial length (μm)	37 ± 0.3	37 ± 0.6	38 ± 0.5	33 ± 1.4	35 ± 1.3	35 ± 1.1
Lumen tangential width (μm)	27 ± 0.7	26 ± 0.9	28 ± 2	25 ± 0.8	26 ± 1.3	26 ± 1.5
Percent cell wall area	26 ± 1.6	31 ± 2.6	28 ± 1.2	33 ± 1.2	29 ± 0.9	30 ± 1.5
Number of cells measured	755	606	581	928	1117	547
Cell wall area occupied by CML/S_1 lignin (%)	0.2 ± 0.03	n/a	0.2 ± 0.01	n/a	0.3 ± 0.02	0.3 ± 0.04

n/a, unable to measure

Table 5 Calcium treated culture set number two from a Burnham tree. The explants were inducted in November and cultured for one month. Values are ± standard error of the mean.

	Calcium culture treatment					
	0 mM	1 mM	5 mM	10 mM	100 mM	Control Explant
Changes in cell number						
Cambial region cell number	14 ± 1	n/a	13 ± 0.8	n/a	13 ± 0.4	12 ± 0.6
Radial expansion region cell number	17 ± 1.1	n/a	12 ± 1.0	n/a	10 ± 0.6	12 ± 0.7
New secondary wall region cell number	14 ± 1.1	n/a	11 ± 0.7	n/a	11 ± 0.4	10 ± 0.8
Radially expanding cells						
Lumen area (μm^2)	843 ± 45	n/a	879 ± 58	1009 ± 108	854 ± 22	772 ± 31.
Lumen radial length (μm)	37 ± 0.9	n/a	39 ± 1.4	40 ± 1.8	38 ± 0.2	36 ± 0.8
Lumen tangential width (μm)	29 ± 1	n/a	28 ± 1.2	32 ± 1.2	29 ± 0.7	27 ± 0.6
Percent cell wall area	13 ± 0.3	n/a	16 ± 1.3	16 ± 2.2	15 ± 1.1	16 ± 2.1
Number of cells measured	313		324	82	278	310
Existing cells						
Lumen area (μm^2)	687 ± 44	n/a	1045 ± 65	n/a	1064 ± 40	918 ± 53
Lumen radial length (μm)	34 ± 1.2	n/a	43 ± 1.1	n/a	44 ± 0.7	41 ± 1.1
Lumen tangential width (μm)	25 ± 1	n/a	29 ± 1.3	n/a	32 ± 2.5	27 ± 1
Percent cell wall area	38 ± 1.0	n/a	27 ± 1.9	n/a	26 ± 0.6	30 ± 0.7
Number of cells measured	615	n/a	263	n/a	750	591
Cell wall area occupied by CML/S_1 lignin (%)	0.2 ± 0.04	n/a	0.2 ± 0.02	n/a	0.3 ± 0.1	0.2 ± 0.05

n/a, unable to measure

Table 6 Calcium treated culture set number three from a Burnham tree. The explants were inducted in March and cultured for two months. Values are ± standard error of the mean.

	Magnesium culture treatments						
	0 mM	0.5 mM	1 mM	5 mM	10 mM	100 mM	Control Explant
Changes in cell number							
Cambial region cell number	12 ± 0.7	14 ± 0.4	14 ± 0.7	12 ± 0.8	11 ± 0.4	n/a	n/a
Radial expansion region cell number	15 ± 0.5	n/a	13 ± 0.8	13 ± 0.6	14 ± 0.7	n/a	n/a
New secondary wall region cell number	10 ± 0.4	n/a	9 ± 0.5	11 ± 0.4	12 ± 1	n/a	n/a
Radially expanding cells							
Lumen area (μm^2)	727 ± 16	732 ± 51	878 ± 27	711 ± 33	747 ± 14	804 ± 87	n/a
Lumen radial length (μm)	37 ± 0.6	35 ± 1	38 ± 0.5	35 ± 0.8	36. ± 0.4	38 ± 1.5	n/a
Lumen tangential width (μm)	26 ± 0.4	27 ± 1.2	29 ± 0.5	26 ± 0.6	27 ± 0.2	28 ± 2	n/a
Percent cell wall area (%)	17 ± 0.4	17 ± 0.9	16 ± 0.8	19 ± 0.2	16 ± 0.5	21 ± 1.8	n/a
Number of cells measured	460	398	456	495	434	450	n/a
Existing cells							
Lumen area (μm^2)	1080 ± 7	1087 ± 29	1074 ± 52	823 ± 49	1037 ± 11	1094 ± 35	n/a
Lumen radial length (μm)	43 ± 0.9	46 ± 0.4	44 ± 0.8	38 ± 0.8	43 ± 0.1	48 ± 0.6	n/a
Lumen tangential width (μm)	30 ± 0.03	29 ± 0.7	30 ± 1	28 ± 1.2	29 ± 0.1	27 ± 0.6	n/a
Percent cell wall area	30 ± 0.01	28 ± 1.1	31 ± 0.9	35 ± 2.3	26 ± 0.4	28 ± 0.1	n/a
Number of cells measured	383	712	703	348	392	784	n/a
Cell wall area occupied by CML/S_1 lignin (%)	0.2 ± 0.05	n/a	n/a	0.2 ± 0.03	n/a	0.13 ± 0.03	0.2 ± 0.05

n/a, unable to measure

Table 7 Magnesium treated culture set number one from a Burnham tree. The explants were inducted in March and cultured for two months. Values are ± standard error of the mean.

	Magnesium culture treatment						
	0 mM	0.5 mM	1 mM	5 mM	10 mM	100 mM	Control Explant
Changes in cell number							
Cambial region cell number	n/a	12 ± 0.6	12 ± 0.5	12 ± 1.1	n/a	13 ± 0.7	11 ± 0.7
Radial expansion region cell number	n/a	11 ± 0.5	13 ± 0.9	13 ± 0.8	n/a	10 ± 0.3	12 ± 0.5
New secondary wall region cell number	n/a	11 ± 0.6	9 ± 0.4	7 ± 0.7	n/a	8 ± 0.5	8 ± 0.5
Radially expanding cells							
Lumen area (μm^2)	758 ± 24	788 ± 22	812 ± 15	889 ± 33	873 ± 21	n/a	855 ± 19
Lumen radial length (μm)	36 ± 0.7	36 ± 0.3	37 ± 0.5	38 ± 0.2	38 ± 0.4	n/a	38 ± 0.7
Lumen tangential width (μm)	27 ± 0.6	27 ± 0.5	28 ± 0.4	30 ± 0.8	30 ± 0.5	n/a	29 ± 0.2
Percent cell wall area	17 ± 1.1	17 ± 1.1	14 ± 0.6	14 ± 0.6	12 ± 0.4	n/a	15 ± 0.9
Number of cells measured	500	437	516	353	397	n/a	406
Existing cells							
Lumen area (μm^2)	807 ± 47	977 ± 69	1104 ± 34	984 ± 37	1160 ± 44	n/a	1020 ± 17
Lumen radial length (μm)	39 ± 1.4	41 ± 1.4	44 ± 0.5	43 ± 0.4	45 ± 0.4	n/a	43 ± 0.6
Lumen tangential width (μm)	25 ± 0.8	29 ± 0.8	30 ± 0.8	28 ± 0.8	31 ± 1.1	n/a	29 ± 0.2
Percent cell wall area	36 ± 1.2	30 ± 3.3	30 ± 0.8	29 ± 0.6	22 ± 0.8	n/a	27 ± 1.1
Number of cells measured	895	280	619	638	602	n/a	444
Cell wall area occupied by CML/S_1 lignin (%)	0.2 ± 0.01	n/a	n/a	0.2 ± 0.02	n/a	n/a	n/a

n/a, unable to measure

Table 8 Magnesium treated culture set number two from a Burnham tree. The explants were inducted in March and cultured for two months. Values are ± standard error of the mean.

	Magnesium culture treatment						
	0 mM	0.5 mM	1 mM	5 mM	10 mM	100 mM	Control Explant
Changes in cell number							
Cambial region cell number	17 ± 1.1	20 ± 1.2	16 ± 0.9	16 ± 0.5	17 ± 0.9	18 ± 0.8	19 ± 0.7
Radial expansion region cell number	19 ± 0.9	19 ± 0.9	21 ± 1.2	21 ± 1.5	17 ± 1.1	19 ± 1.2	20 ± 0.8
New secondary wall region cell number	13 ± 0.8	15 ± 1	13 ± 1.2	12 ± 0.9	13 ± 0.5	10 ± 0.6	10 ± 0.5
Radially expanding cells							
Lumen area (μm^2)	745 ± 44	777 ± 131	807 ± 34	792 ± 57	884 ± 41	778 ± 20	800 ± 50
Lumen radial length (μm)	35 ± 0.9	35 ± 1.8	36 ± 0.8	36 ± 1.1	38 ± 1.1	36 ± 0.7	37± 1.1
Lumen tangential width (μm)	27 ± 0.9	28 ± 2.5	28 ± 0.6	28 ± 1	29 ± 0.8	27 ± 0.5	27 ± 1.0
Percent cell wall area	15 ± 0.2	13 ± 0.3	13 ± 0.5	13 ± 0.4	13 ± 0.5	13 ± 0.9	13 ± 1.3
Number of cells measured	499	180	313	478	345	449	395
Existing cells							
Lumen area (μm^2)	777 ± 12	706 ± 25	691 ± 22	813 ± 92	877 ± 47	895 ± 21	765 ± 45
Lumen radial length (μm)	35 ± 0.3	34 ± 0.6	32 ± 0.5	36 ± 1.3	38 ± 0.7	38 ± 0.4	36 ± 0.8
Lumen tangential width (μm)	27 ± 0.2	26 ± 0.3	26 ± 0.4	28 ± 1	28 ± 1.1	29 ± 0.4	27 ± 1.1
Percent cell wall area	26 ± 0.6	29 ± 1.6	22 ± 6.8	29 ± 2.1	25 ± 0.6	25 ± 0.8	27 ± 1.0
Number of cells measured	434	945	915	366	666	785	779
Cell wall area occupied by CML/S_1 lignin (%)*	0.2 ± 0.04	n/a	0.3 ± 0.3	n/a	n/a	0.2 ± 0.06	0.1 ± 0.08

n/a, unable to measure
*Values obtained from culture set number four

Table 9 Magnesium treated culture set number three from a Burnham tree. The explants were inducted in November and cultured for one month. Values are ± standard error of the mean.

	Mixed culture treatment								
	LMHC	HMLC	HBCM	0BCM	Opti LB	OPTI	LBHC	HBLC	Control
Changes in cell number									
Cambial region cell number	11 ± 0.6	n/a	n/a	10 ± 1.1	13 ± 1.1	10 ± 0.5	12 ± 0.7	12 ± 0.3	n/a
Radial expansion region cell number	5 ± 0.6	n/a	n/a	8 ± 0.6	15 ± 1	13 ± 1.3	10 ± 0.5	12 ± 1.4	n/a
New secondary wall region cell number	3 ± 0	n/a	n/a	4 ± 0.3	5 ± 0.6	4 ± 0.5	4 ± 0.3	5 ± 0.9	n/a
Radially expanding cells									
Lumen area (μm^2)	/	/	/	/	/	/	664 ± 13	520 ± 26	748 ± 70
Lumen radial length (µm)	/	/	/	/	/	/	34 ± 0.5	30 ± 0.7	35 ± 1.3
Lumen tangential width (µm)	/	/	/	/	/	/	25 ± 0.5	23 ± 0.7	27 ± 1.5
Percent cell wall area	/	/	/	/	/	/	25 ± 1	28 ± 0.07	23 ± 0.7
Number of cells measured	/	/	/	/	/	/	149	111	130
Existing cells									
Lumen area (μm^2)	/	/	/	/	/	/	652*	842 ± 31	687 ± 67
Lumen radial length (µm)	/	/	/	/	/	/	34*	41 ± 0.5	36 ± 3.3
Lumen tangential width (µm)	/	/	/	/	/	/	24*	26 ± 0.2	26 ± 0.9
Percent cell wall area	/	/	/	/	/	/	31*	31 ± 3	40 ± 0.5
Number of cells measured	/	/	/	/	/	/	134*	247	42
Cell wall area occupied by CML/S_1 lignin (%)	/	/	/	/	/	/	1.0 ± 0.1	1.6 ± 0.7	1.1 ± 0.3

n/a, unable to measure
/, not measured
* Only one image was measured

Table 10 Mixed culture set number one from a Burnham tree. The explants were inducted in March and cultured for two months. Values are ± standard error of the mean.

	Mixed culture treatment								
	LMHC	HMLC	HBCM	0BCM	Opti LB	OPTI	LBHC	HBLC	Control
Changes in cell number									
Cambial region cell number	10 ± 0.3	17 ± 1.4	n/a	12 ± 0.6	n/a	n/a	11 ± 0.9	12 ± 1	11 ± 1.1
Radial expansion region cell number	15 ± 0.6	15 ± 0.9	n/a	22 ± 1.1	n/a	n/a	19 ± 1	15 ± 3	19 ± 0.8
New secondary wall region cell number	4 ± 0.3	n/a	n/a	8.0 ± 0	n/a	n/a	5 ± 0	4 ± 0.4	n/a
Radially expanding cells									
Lumen area (μm^2)	/	/	/	/	/	/	958 ± 41	746 ± 99	908 ± 42
Lumen radial length (µm)	/	/	/	/	/	/	41 ± 3.9	37 ± 2.4	39 ± 1.2
Lumen tangential width (µm)	/	/	/	/	/	/	28 ± 0.1	26 ± 1.8	29 ± 0.8
Percent cell wall area	/	/	/	/	/	/	21 ± 1.8	61 ± 3.9	35 ± 15
Number of cells measured	/	/	/	/	/	/	118	113	111
Existing cells									
Lumen area (μm^2)	/	/	/	/	/	/	747 ± 39	734 ± 22	818 ± 111
Lumen radial length (µm)	/	/	/	/	/	/	35 ± 0.2	37 ± 0.3	37 ± 2.8
Lumen tangential width (µm)	/	/	/	/	/	/	26 ± 1	27 ± 0.5	28 ± 2
Percent cell wall area	/	/	/	/	/	/	25 ± 0.6	33 ± 2.9	25 ± 2
Number of cells measured	/	/	/	/	/	/	134	77	111
Cell wall area occupied by CML/S_1 lignin (%)	/	/	/	/	/	/	1.1 ± 0.4	2.5 ± 0.9	n/a

n/a, unable to measure
/, not measured

Table 11 Mixed culture set number two from a Burnham tree. The explants were inducted in December and cultured for two months. Values are ± standard error of the mean.

	Mixed culture treatment								
	LMHC	HMLC	HBCM	0BCM	Opti LB	OPTI	LBHC	HBLC	Control
Changes in cell number									
Cambial region cell number	12 ± 1.2	10 ± 0.6	10 ± 0.4	10 ± 4.8	n/a	14 ± 0.9	11 ± 0.9	n/a	11 ± 0.9
Radial expansion region cell number	7 ± 0.9	6 ± 0.5	13 ± 1.7	8 ± 4	n/a	9 ± 1.4	10 ± 0.6	n/a	8 ± 0.7
New secondary wall region cell number	18 ± 1.4	n/a	5 ± 1	12 ± 6	n/a	10 ± 2.9	n/a	n/a	11 ± 0.5
Radially expanding cells									
Lumen area (μm^2)	/	/	/	/	/	/	786 ± 22	956 ± 92	913 ± 77
Lumen radial length (µm)	/	/	/	/	/	/	35 ± 0.3	41 ± 0.1	39 ± 0.7
Lumen tangential width (µm)	/	/	/	/	/	/	27 ± 0.5	30 ± 2	30 ± 1.5
Percent cell wall area	/	/	/	/	/	/	21 ± 2.5	18 ± 2	15 ± 2.6
Number of cells measured	/	/	/	/	/	/	53	181	92
Existing cells									
Lumen area (μm^2)	/	/	/	/	/	/	795 ±58	842 ± 5.5	843 ± 82
Lumen radial length (µm)							37 ± 0.3	39 ± 0.3	40 ± 2
Lumen tangential width (µm)	/	/	/	/	/	/	28 ± 1.5	28 ± 1.6	27 ± 0.6
Percent cell wall area	/	/	/	/	/	/	31 ± 0.1	35 ± 1.9	27 ± 3.2
Number of cells measured	/	/	/	/	/	/	197	50	154
Cell wall area occupied by CML/S_1 lignin (%)	/	/	/	/	/	/	1.9 ± 1.2	2.2 ± 0.6	n/a

n/a, unable to measure
/, not measured

Table 12 Mixed culture set number three from a Burnham tree. The explants were inducted in January and cultured for two months. Values are ± standard error of the mean.

	Yariv culture treatment			
	β-GlcY	α-GalY	NAA	Control Explant
Changes in cell number				
Cambial region cell number	14 ± 0.6	14 ± 0.9	13 ± 0.9	13 ± 0.8
Radial expansion region cell number	15 ± 1.6	12 ± 1.7	13 ± 0.4	14 ± 0.7
New secondary wall region cell number	14 ± 0.9	10 ± 0.9	14 ± 1	14 ± 0.7
Radially expanding cells				
Lumen area (μm^2)	917 ± 42	967 ± 25	996 ± 39	915 ± 42
Lumen radial length (μm)	39 ± 1.5	42 ± 0.5	40 ± 1	39 ± 0.1
Lumen tangential width (μm)	30 ± 1	30 ± 0.6	31 ± 0.7	30 ± 1.5
Percent cell wall area (%)	14 ± 0.3	15 ± 0.2	15 ± 0.6	17 ± 0.6
Number of cells measured	218	378	377	185
Existing cells				
Lumen area (μm^2)	800 ± 37	810 ± 81	1001 ± 5	977 ± 34
Lumen radial length (μm)	37 ± 1	37 ± 1	41 ± 0.6	41 ± 0.7
Lumen tangential width (μm)	27 ± 1	27 ± 0.4	30 ± 0.3	30 ± 0.4
Percent cell wall area	31 ± 1	31 ± 1	25 ± 1	21 ± 2
Number of cells measured	469	490	416	369

Table 13 Yariv culture set number one from a Burnham tree. The explants were inducted in January and cultured for two months. Values are ± standard error of the mean.

	Yariv culture treatment			
	β-GlcY	α-GalY	NAA	Control Explant
Changes in cell number				
Cambial region cell number	16 ± 1	16 ± 1	17 ± 1	n/a
Radial expansion region cell number	18 ± 1	17 ± 1	17 ± 2	n/a
New secondary wall region cell number	13 ± 1	13 ± 0.4	11 ± 1	n/a
Radially expanding cells				
Lumen area (μm^2)	839 ± 58	882 ± 23	809 ± 74	808 ± 56
Lumen radial length (μm)	36 ± 2	39 ± 0.4	36 ± 1	38 ± 1.2
Lumen tangential width (μm)	28 ± 1	29 ± 1	28 ± 2	15 ± 1
Percent cell wall area (%)	16 ± 2	14 ± 2	17 ± 0.5	15 ± 1
Number of cells measured	293	465	292	511
Existing cells				
Lumen area (μm^2)	920 ± 50	996 ± 53	919 ± 25	1007 ± 46
Lumen radial length (μm)	41 ± 1	41 ± 1	42 ± 1	44 ± 1
Lumen tangential width (μm)	30 ± 0.8	31 ± 0.6	28 ± 0.6	29 ± 0.5
Percent cell wall area	16 ± 3	21 ± 2	25 ± 3	22 ± 1
Number of cells measured	298	290	292	319

Table 14 Yariv culture set number two from a Rotorua tree. The explants were inducted in April and cultured for two months. Values are ± standard error of the mean.

	Yariv culture treatment			
	β-GlcY	α-GalY	NAA	Control Explant
Changes in cell number				
Cambial region cell number	15 ± 0.4	19 ± 0.4	19 ± 1	17 ± 0.6
Radial expansion region cell number	16 ± 0.9	13 ± 0.7	14 ± 2	13 ± 0.6
New secondary wall region cell number	12 ± 0.6	15 ± 2	9 ± 1	13 ± 0.6
Radially expanding cells				
Lumen area (μm^2)	616 ± 36	645 ± 17	650 ± 23	694 ± 29
Lumen radial length (μm)	31 ± 1	32 ± 0.5	32 ± 0.4	34 ± 0.8
Lumen tangential width (μm)	25 ± 0.6	25 ± 0.3	25 ± 0.5	25 ± 0.8
Percent cell wall area (%)	13 ± 0.7	15 ± 0.7	14 ± 0.7	15 ± 0.7
Number of cells measured	547	317	450	505
Existing cells				
Lumen area (μm^2)	946 ± 52	719 ± 11	711 ± 37	652 ± 54
Lumen radial length (μm)	41 ± 1	35 ± 0.3	37 ± 2	33 ± 2
Lumen tangential width (μm)	30 ± 0.7	27 ± 0.2	26 ± 0.4	25 ± 1
Percent cell wall area	16 ± 2	15 ± 1	24 ± 3	35 ± 6
Number of cells measured	399	507	205	149

Table 15 Yariv culture set number three from a Rotorua tree. The explants were inducted in April and cultured for two months. Values are ± standard error of the mean.

Lignin products	Culture treatment[a]								
	Low boron	High boron	Low calcium	High calcium	Low magnesium	High magnesium	LBHC	HBLC	Control
Guaiacol	a) 26 ± 0.3 b) 27 ± 1.1	a) 25 ± 0.05 b) 25 ± 0.6	a) 26 ± 0.5 b) 26 ± 0.6	a) 21 ± 1.5 b) 21 ± 0.7	a) 28 ± 0.9 b) 25 ± 0.4	**a) 17 ± 0.7** **b) 18 ± 0.5**	a) 24 ± 0.5 b) 20 ± 0.6	a) 24 ± 0.6 b) 26 ± 0.1	a) 25 ± 0.1 b) 25 ± 13
2-Methyl phenol	a) 2 ± 0.2 b) 3 ± 0.3	a) 4 ± 0.1 b) 2 ± 0.1	a) 2 ± 0.1 b) 3 ± 0.3	a) 3 ± 0.7 b) 4 ± 0.6	a) 3 ± 0.3 b) 2 ± 0.5	a) 3 ± 0.3 b) 3 ± 0.1	a) 3 ± 0.3 b) 2 ± 0.03	a) 2 ± 0.1 b) 3 ± 0.3	a) 3 ± 0.5 b) 3 ± 1.5
Phenol	a) 9 ± 0.4 b) 9 ± 0.8	a) 10 ± 0.3 b) 7 ± 0.3	a) 8 ± 0.2 b) 9 ± 0.7	a) 11 ± 1.7 b) 13 ± 1.1	a) 10 ± 0.4 b) 9 ± 1.3	a) 14 ± 0.9 b) 9 ± 0.3	a) 13 ± 0.8 b) 7 ± 0.07	a) 9 ± 0.7 b) 9 ± 0.8	a) 10 ± 1.5 b) 9 ± 5
4-Vinyl guaiacol	a) 21 ± 0.1 b) 22 ± 0.5	a) 21 ± 0.2 b) 25 ± 0.6	a) 23 ± 0.3 b) 27 ± 0.5	a) 27 ± 0.1 b) 25 ± 1.6	a) 21 ± 0.4 b) 24 ± 1.3	a) 21 ± 0.5 b) 26 ± 0.2	a) 26 ± 1.7 b) 30 ± 0.2	a) 23 ± 1.3 b) 24 ± 0.8	a) 24 ± 0.1 b) 27 ± 13
Dihydro coniferyl alcohol	a) 3 ± 0.03 b) 3 ± 0.2	a) 3 ± 0.007 b) 3 ± 0.1	a) 3 ± 0.06 b) 2 ± 0.2	a) 3 ± 0.07 b) 3 ± 0.005	a) 3 ± 0.07 b) 2 ± 0.3	a) 3 ± 0.1 b) 2 ± 0.01	a) 3 ± 0.5 b) 3 ± 0.07	a) 3 ± 0.1 b) 3 ± 0.2	a) 3 ± 0.2 b) 2 ± 1.1
Coniferaldehyde	a) 10 ± 0.3 b) 10 ± 0.2	a) 9 ± 0.04 b) 9 ± 0.7	a) 9 ± 0.04 b) 7 ± 0.7	a) 9 ± 0.04 b) 10 ± 1.1	a) 10 ± 0.3 b) 8 ± 1.7	a) 11 ± 0.4 b) 8 ± 0.3	a) 7 ± 1.9 b) 9 ± 0.7	a) 10 ± 0.05 b) 10 ± 1.3	a) 8 ± 0.8 b) 7 ± 3.4
Coniferyl alcohol	a) 5 ± 0.4 b) 4 ± 0.02	a) 4 ± 0.3 b) 2 ± 0.3	a) 4 ± 0.1 b) 2 ± 0.3	**a) 0.2 ± 0.02** **b) 0.9 ± 0.5**	a) 4 ± 0.3 b) 3 ± 0.8	**a) 2 ± 0.2** **b) 1 ± 0.2**	**a) 0.04 ± 0.02** **b) 0.2 ± 0.1**	a) 6 ± 0.3 b) 3 ± 0.2	a) 3 ± 0.5 b) 2 ± 1.1
Vanillin	a) 11 ± 0.3 b) 11 ± 0.2	a) 12 ± 0.6 b) 12 ± 0.3	a) 11 ± 0.1 b) 10 ± 0.8	a) 10 ± 0.2 b) 10 ± 0.1	a) 10 ± 0.7 b) 12 ± 1.6	**a) 14 ± 0.2** **b) 14 ± 1.3**	a) 7.5 ± 0.9 b) 11 ± 0.1	a) 11 ± 0.9 b) 9 ± 2	a) 11 ± 0.6 b) 11 ± 6
Eugenol	a) 12 ± 0.002 b) 12 ± 2	a) 14 ± 0.7 b) 15 ± 0.5	a) 13 ± 0.3 b) 14 ± 0.1	a) 16 ± 0.8 b) 15 ± 1.2	a) 11 ± 1 b) 14 ± 1	a) 16 ± 0.1 b) 20 ± 1	a) 16 ± 0.7 b) 19 ± 0.3	a) 12 ± 1.7 b) 13 ± 0.1	a) 14 ± 0.2 b) 14 ± 7

[a] % composition of an average of duplicates

Table 16 Composition of lignin breakdown products identified by Py-GC-MS. (a) and (b) denote the results from culture sets obtained from two separate trees. Values are ± standard error of the mean. Major differences between treated cultures and the control are highlighted in bold.

Carbohydrate products	Culture treatment[a]								
	Low boron	High boron	Low calcium	High calcium	Low magnesium	High magnesium	LBHC	HBLC	Control
Unknown	a) 7 ± 0.02 b) 7 ± 0.4	a) 7 ± 0.9 b) 8 ± 0.05	a) 9 ± 0.1 b) 9 ± 0.07	a) 9 ± 0.3 b) 9 ± 0.2	a) 8 ± 0.02 b) 8 ± 0.4	a) 7 ± 0.04 b) 8 ± 0.2	a) 10 ± 0.6 b) 10 ± 0.3	a) 8 ± 0.2 b) 8 ± 0.3	a) 6 ± 0.1 b) 6 ± 0.1
4-Hydroxy-5-6-dihydro-(2H)-pyran-2-one	a) 23 ± 0.9 b) 15 ± 2.1	a) 24 ± 5 b) 19 ± 1	a) 15 ± 0.3 b) 18 ± 1	a) 28 ± 1.5 b) 22 ± 4	a) 17 ± 0.6 b) 31 ± 1	a) 9 ± 0.3 b) 17 ± 0.5	a) 25 ± 1 b) 38 ± 0.6	a) 34 ± 0.5 b) 20 ± 1.1	a) 27 ± 0.01 b) 21 ± 1.4
5-Hydroxymethyl-2-furfuraldehyde	a) 24 ± 0.06 b) 27 ± 2.4	a) 22 ± 2.7 b) 31 ± 1	a) 28 ± 0.2 b) 36 ± 0.6	**a) 18 ± 0.2** **b) 18 ± 0.3**	a) 26 ± 0.9 b) 29 ± 1.6	**a) 12 ± 0.1** **b) 12 ± 0.5**	**a) 19 ± 2.4** **b) 18 ± 0.07**	a) 25 ± 0.5 b) 28 ± 1.8	a) 30 ± 1.4 b) 34 ± 1.2
4-Allyl phenol	a) 1 ± 0.09 b) 1 ± 0.01	a) 1 ± 0.06 b) 1 ± 0.05	a) 2 ± 0.05 b) 2 ± 0.2	**a) 0.2 ± 0.01** **b) 0.5** ± 0.01	a) 1.5 ± 0.2 b) 3 ± 0.9	**a) 0.2 ± 0.01** **b) 0.2 ± 0.01**	**a) 0.2 ± 0.01** **b) 0.4 ± 0.02**	a) 2 ± 0.3 b) 2 ± 0.1	a) 1 ± 0.2 b) 2 ± 0.2
1,6-Anhydro-β-D-glucopyranose	a) 45 ± 0.8 b) 50 ± 5	a) 47 ± 1.2 b) 41 ± 2	a) 47 ± 0.3 b) 35 ± 2	a) 45 ± 1.6 b) 50 ± 5	a) 48 ± 0.5 b) 29 ± 2	**a) 71 ± 0.4** **b) 64 ± 1.2**	a) 46 ± 4 b) 34 ± 0.2	a) 32 ± 0.8 b) 42 ± 3.4	a) 37 ± 1.7 b) 37 ± 1

[a] % composition of an average of duplicates

Table 17 Composition of polysaccharide breakdown products identified by Py-GC-MS. (a) and (b) denote the results from culture sets obtained from two separate trees. Values are ± standard error of the mean. Major differences between treated cultures and the control are highlighted in bold.

Carbohydrate products	Culture treatment[a]								
	Low boron	High boron	Low Calcium	High calcium	Low magnesium	High magnesium	LBHC	HBLC	Control
Guaiacol	a) 19 ± 0.2 b) 20 ± 0.1	a) 16 ± 1 b) 20 ± 0.8	a) 21 ± 0.4 b) 23 ± 0.7	**a) 6 ± 0.9** **b) 7 ± 0.6**	a) 21 ± 0.5 b) 21 ± 0.8	**a) 5 ± 0.4** **b) 6 ± 0.01**	**a) 8 ± 1** **b) 8 ± 0.04**	a) 20 ± 1 b) 21 ± 0.5	a) 19 ± 0.1 b) 20 ± 0.4
2-Methyl phenol	a) 2 ± 0.2 b) 2 ± 0.1	a) 2 ± 0.08 b) 2 ± 0.1	a) 2 ± 0.1 b) 3 ± 0.3	a) 1 ± 0.2 b) 1 ± 0.2	a) 2 ± 0.2 b) 2 ± 0.5	a) 1 ± 0.1 b) 1 ± 0.002	a) 1 ± 0.04 b) 0.7 ± 0.01	a) 2 ± 0.1 b) 2 ± 0.2	a) 2 ± 0.4 b) 2 ± 0.3
Phenol	a) 6 ± 0.3 b) 7 ± 0.3	a) 6 ± 0.6 b) 6 ± 0.1	a) 7 ± 0.2 b) 8 ± 0.7	a) **4 ± 0.3** **b) 5 ± 0.2**	a) 8 ± 0.3 b) 7 ± 1.3	**a) 4 ± 0.1** **b) 3 ± 0.007**	**a) 4 ± 0.8** **b) 3 ± 0.1**	a) 7 ± 0.7 b) 7 ± 0.4	a) 7 ± 1 b) 8 ± 0.8
4-Vinyl guaiacol	a) 15 ± 0.1 b) 16 ± 1	a) 13 ± 1 b) 20 ± 0.9	a) 18 ± 0.2 b) 23 ± 0.6	**a) 8 ± 0.5** **b) 9 ± 1**	a) 16 ± 0.4 b) 20 ± 1.5	**a) 6 ± 0.1** **b) 8 ± 0.3**	**a) 8 ± 1.6** **b) 12 ± 0.3**	a) 19 ± 1 b) 20 ± 1	a) 18 ± 0.1 b) 21 ± 0.1
Dihydro coniferyl alcohol	a) 2 ± 0.02 b) 2 ± 0.2	a) 2 ± 0.1 b) 2 ± 0.08	a) 2 ± 0.05 b) 2 ± 0.1	a) 1 ± 0.1 b) 1 ± 0.04	a) 2 ± 0.07 b) 2 ± 0.2	a) 1 ± 0.06 b) 1 ± 0.03	a) 1 ± 0.05 b) 1 ± 0.06	a) 2 ± 0.1 b) 2 ± 0.06	a) 2 ± 0.1 b) 2 ± 0.1
Coniferaldehyde	a) 7 ± 0.2 b) 7 ± 0.1	a) 6 ± 0.3 b) 7 ± 0.4	a) 8 ± 0.03 b) 6 ± 0.5	**a) 3 ± 0.2** **b) 4 ± 0.2**	a) 7 ± 0.1 b) 7 ± 1	**a) 3 ± 0.2** **b) 3 ± 0.2**	**a) 2 ± 0.3** **b) 3 ± 0.4**	a) 8 ± 0.1 b) 8 ± 0.9	a) 6 ± 0.6 b) 5 ± 0.3
Coniferyl alcohol	a) 4 ± 0.2 b) 3 ± 0.1	a) 2 ± 0.05 b) 3 ± 0.2	a) 3 ± 0.08 b) 2 ± 0.2	**a) 0.1 ± 0.01** **b) 0.3 ± 0.2**	a) 3 ± 0.2 b) 3 ± 0.6	**a) 0.7 ± 0.08** **b) 0.3 ± 0.07**	**a) 0.01 ± 0.01** **b) 0.06 ± 0.06**	a) 5 ± 0.3 b) 3 ± 0.1	a) 2 ± 0.4 b) 2 ± 0.2
Vanillin	a) 8 ± 0.2 b) 8 ± 0.1	a) 8 ± 0.1 b) 9 ± 0.1	a) 9 ± 0.07 b) 9 ± 0.6	**a) 3 ± 0.3** **b) 4 ± 0.1**	a) 8 ± 0.5 b) 10 ± 1.1	**a) 4 ± 0.2** **b) 5 ± 0.6**	**a) 2 ± 0.01** **b) 4 ± 0.2**	a) 9 ± 0.5 b) 7 ± 2	a) 8 ± 0.5 b) 9 ± 0.2
Eugenol	a) 9 ± 0.01 b) 9 ± 1.8	a) 9 ± 1 b) 12 ± 0.6	a) 10 ± 0.2 b) 12 ± 0.02	**a) 5 ± 0.6** **b) 5 ± 0.7**	a) 9 ± 0.9 b) 11 ± 1	**a) 5 ± 0.2** **b) 7 ± 0.2**	**a) 5 ± 0.9** **b) 7 ± 0.1**	a) 10 ± 1 b) 11 ± 0.4	a) 10 ± 0.1 b) 11 ± 0.1
Unknown	a) 2 ± 0.01 b) 2 ± 0.07	a) 2 ± 0.03 b) 2 ± 0.1	a) 2 ± 0.03 b) 1 ± 0.08	**a) 6 ± 0.03** **b) 6 ± 0.3**	a) 2 ± 0.04 b) 2 ± 0.1	**a) 5 ± 0.05** **b) 5 ± 0.05**	**a) 7 ± 0.01** **b) 6 ± 0.06**	a) 2 ± 0.2 b) 2 ± 0.1	a) 2 ± 0.03 b) 1 ± 0.02
4-Hydroxy-5-6-dihydro-(2H)-pyran-2-one	a) 7 ± 0.3 b) 4 ± 0.2	a) 9 ± 2.7 b) 4 ± 0.04	**a) 3 ± 0.06** **b) 2 ± 0.01**	**a) 19 ± 0.5** **b) 14 ± 3**	a) 4 ± 0.2 b) 6 ± 1	a) 7 ± 0.09 b) 11 ± 0.2	**a) 17 ± 0.3** **b) 23 ± 0.8**	a) 7 ± 0.6 b) 4 ± 0.2	a) 7 ± 0.02 b) 4 ± 0.4
5-Hydroxymethyl-2-furfuraldehyde	a) 7 ± 0.001 b) 7 ± 0.01	a) 8 ± 0.03 b) 6 ± 0.2	a) 6 ± 0.02 b) 5 ± 0.2	**a) 12 ± 0.5** **b) 12 ± 0.1**	a) 6 ± 0.09 b) 5 ± 0.3	a) 9 ± 0.04 b) 8 ± 0.2	**a) 13 ± 0.9** **b) 11 ± 0.2**	a) 5 ± 0.5 b) 6 ± 0.3	a) 7 ± 0.3 b) 7 ± 0.003
4-Allyl phenol	a) 0.3 ± 0.02 b) 0.2 ± 0.02	a) 0.2 ± 0.01 b) 0.2 ± 0.003	a) 0.3 ± 0.01 b) 0.3 ± 0.01	a) 0.1 ± 0.01 b) 0.3 ± 0.001	a) 0.3 ± 0.03 b) 0.6 ± 0.1	a) 0.2 ± 0.004 b) 0.1 ± 0.01	a) 0.2 ± 0.001 b) 0.2 ± 0.01	a) 0.4 ± 0.03 b) 0.4 ± 0.02	a) 0.2 ± 0.04 b) 0.3 ± 0.03
1,6-Anhydro-β-D-glucopyranose	a) 13 ± 0.2 b) 14 ± 3	a) 17 ± 1.6 b) 8 ± 1	a) 9 ± 0.09 b) 5 ± 0.5	**a) 32 ± 2** **b) 32 ± 4**	a) 11 ± 0.3 b) 5 ± 0.9	**a) 50 ± 1** **b) 43 ± 1.4**	**a) 32 ± 5** **b) 21 ± 0.3**	a) 6 ± 0.3 b) 8 ± 2	a) 9 ± 0.5 b) 7 ± 0.3

[a] % composition of an average of duplicates

Table 18 Combined composition of lignin and polysaccharide breakdown products identified by Py-GC-MS. (a) and (b) denote the results from culture sets obtained from two separate trees. Values are ± standard error of the mean.

Appendix Two

Statistical analysis

ii.1 Statistics for data presented in chapter two

ii.1.1 Greyscale analysis of radiata pine oven dried discs

Completely randomized AOV for pectin

Source	DF	SS	MS	F	P
checking	3	0.2747	0.27473	0.98	0.1167
Error	9	0.7106	0.92767		
Total	12	0.6470			

Completely randomized AOV for lignin

Source	DF	SS	MS	F	P
checking	3	0.6	0.3	0.48	0.0273
Error	9	0.7	0.2		
Total	12	0.2			

ii.1.2 Klason lignin assay of radiata pine oven dried discs

Completely randomized AOV for insoluble

Source	DF	SS	MS	F	P
group	3	0.4848	0.16160	0.09	0.9643
Error	9	16.3475	1.81639		
Total	12	16.8323			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.73	3	0.2921

Completely randomized AOV for soluble

Source	DF	SS	MS	F	P
group	3	0.00643	0.00214	1.22	0.3590
Error	9	0.01588	0.00176		
Total	12	0.02231			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.19	3	0.7558

Completely randomized AOV for total

Source	DF	SS	MS	F	P
group	3	0.5141	0.17137	0.09	0.9624
Error	9	16.6967	1.85519		
Total	12	17.2108			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	4.05	3	0.2557

ii.1.3 Acetyl bromide assay of radiata pine oven dried discs

Completely randomized AOV for acetyl

Source	DF	SS	MS	F	P
group	3	49.6476	16.5492	3.18	0.0847

```
Error      8   41.6138    5.2017
Total     11   91.2614

                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        1.68    3   0.6421
```

ii.1.4 Inductively coupled plasma optical emission spectrometry and mass spectrometry measurements of radiata pine oven dried discs

ii.1.4.1 Boron for discs

```
Completely randomized AOV for boron
Source   DF        SS        MS       F        P
GROUP     3   0.21769   0.07256    2.84   0.0981
Error     9   0.23000   0.02556
Total    12   0.44769

                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        6.14    3   0.1051
```

ii.1.4.2 Calcium for discs

```
Completely randomized AOV for calcium
Source   DF        SS        MS       F        P
GROUP     3   16022.2   5340.74    2.40   0.1348
Error     9   19990.1   2221.12
Total    12   36012.3

                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        0.68    3   0.8770
```

ii.1.4.3 Magnesium for discs

```
Completely randomized AOV for magnesium
Source   DF        SS        MS       F        P
GROUP     3   2368.28   789.425    1.62   0.2534
Error     9   4397.42   488.602
Total    12   6765.69

                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        0.31    3   0.9591
```

ii.2 Statistics for data presented in chapter four

ii.2.1 Inductively coupled optical emission spectrometry and mass spectrometry of organ cultures grown on boron, calcium, magnesium and mixed media treatments

The data presented include the statistical analysis of measurements obtained from the cultures of two trees.

ii.2.1.1 Boron-treated cultures

```
Completely randomized AOV for boron
Source   DF        SS        MS       F        P
bcult     5   154.207   30.8413    6.98   0.0174
Error     6    26.500    4.4167
Total    11   180.707
```

```
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances       12.0    5   0.0349

Tukey HSD All-Pairwise Comparisons Test of boron by bcult

bcult    Mean  Homogeneous Groups
 1000  13.550  A
  100  5.6500  AB
   25  4.6500   B
    7  3.7000   B
    1  3.5500   B
    0  3.3000   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

ii.2.1.2 Calcium-treated cultures

```
Completely randomized AOV for calcium
Source    DF          SS          MS       F        P
cculture   4   2.267E+08   5.668E+07    58.9   0.0035
Error      3     2887748      962583
Total      7   2.296E+08
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances       6.88    2   0.0321

Tukey HSD All-Pairwise Comparisons Test of calcium by cculture

cculture    Mean  Homogeneous Groups
     100   13200  A
      10  1515.0   B
       5  1007.0   B
       1  369.00   B
       0  259.00   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

ii.2.1.3 Magnesium-treated cultures

```
Completely randomized AOV for magnesium
Source     DF          SS          MS       F        P
mcultures   4   3.096E+08   7.740E+07     508   0.0000
Error       4      609761      152440
Total       8   3.102E+08
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances       11.0    2   0.0040

Tukey HSD All-Pairwise Comparisons Test of magnesium by mcultures

mcultures    Mean  Homogeneous Groups
      100   14650  A
       10  1690.0   B
        5  857.00   B
        1  325.50   B
        0  289.67   B

Alpha              0.05
```

There are 2 groups (A and B) in which the means are not significantly different from one another.

ii.2.1.4 Mixed-treated cultures

Completely randomized AOV for boron

Source	DF	SS	MS	F	P
mixcultur	7	167663	23951.8	1099	0.0000
Error	8	174	21.8		
Total	15	167837			

At least one group variance is near zero,
variance-equality tests cannot be computed.

Tukey HSD All-Pairwise Comparisons Test of boron by mixcultur

mixcultur	Mean	Homogeneous Groups
HBCM	320.50	A
HBLC	32.850	B
HMLC	11.050	C
0BCM	10.000	C
Opti	8.0000	C
OptiLB	7.9500	C
LMHC	7.2500	C
LBHC	6.5000	C

Alpha 0.05
There are 3 groups (A, B, etc.) in which the means are not significantly different from one another.

Completely randomized AOV for calcium

Source	DF	SS	MS	F	P
mixcultur	7	2.628E+08	3.754E+07	32.2	0.0000
Error	8	9327953	1165994		
Total	15	2.721E+08			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	22.6	7	0.0020

Tukey HSD All-Pairwise Comparisons Test of calcium by mixcultur

mixcultur	Mean	Homogeneous Groups
LBHC	10650	A
LMHC	9615.0	A
HBCM	3335.0	B
HBLC	764.00	B
Opti	760.00	B
OptiLB	672.50	B
0BCM	397.50	B
HMLC	252.50	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Completely randomized AOV for magnesium

Source	DF	SS	MS	F	P
mixcultur	7	1.123E+08	1.605E+07	142	0.0000
Error	8	906009	113251		

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	14.5	7	0.0432

```
Tukey HSD All-Pairwise Comparisons Test of magnesium by mixcultur

mixcultur    Mean  Homogeneous Groups
HBCM       7190.0  A
HMLC       5325.0   B
HBLC       287.00    C
0BCM       273.50    C
OptiLB     273.00    C
Opti       240.50    C
LBHC       180.00    C
LMHC       154.00    C

Alpha              0.05
There are 3 groups (A, B, etc.) in which the means are not
significantly different from one another.
```

ii.2.2 Boron-treated culture analysis of cell number, lumen area, length and width, and cell wall area

ii.2.2.1 Boron-treated culture set number one. The tree was from Rotorua, inducted in April and cultured for two months.

Cell counts

```
Completely randomized AOV for cambial cell counts
Source   DF         SS         MS        F         P

media     5    256.231    51.2462     8.04    0.0000
Error    50    318.608     6.3722
Total    55    574.839
                                       Chi-Sq     DF         P
Bartlett's Test of Equal Variances       6.58      5    0.2539

Tukey HSD All-Pairwise Comparisons Test of cambial by media

media       Mean          Homogeneous Groups
 1000             21.000                  A
    7             19.500                  A
  100             19.000                  A
control           17.625                  AB
    0             15.400                   B
    1             14.833                   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for radially expanding cell counts
Source   DF         SS         MS        F         P
media     5     66.130    13.2260     1.26    0.2982
Error    48    505.370    10.5285
Total    53    571.500
                                     Chi-Sq      DF         P
Bartlett's Test of Equal Variances     9.98       5    0.0759

Completely randomized AOV for new secondary wall cell counts
Source   DF         SS         MS        F         P
media     5    276.835    55.3669     4.72    0.0014
Error    48    562.499    11.7187
```

```
Total    53   839.333
                                          Chi-Sq   DF          P
Bartlett's Test of Equal Variances          15.7    5     0.0079

Tukey HSD All-Pairwise Comparisons Test of second by media

media       Mean             Homogeneous Groups
  100       19.375                 A
 1000       17.000                 AB
 control    15.833                 ABC
    0       14.700                 ABC
    7       14.214                  BC
    1       11.333                   C

Alpha             0.05
There are 3 groups (A, B, etc.) in which the means are not
significantly different from one another.
```

Radially expanding cells: lumen area, length and width

```
Completely randomized AOV for area
Source   DF       SS        MS       F        P
media     5   244792   48958.4    10.6   0.0001
Error    18    82842    4602.3
Total    23   327634
                                        Chi-Sq   DF          P
Bartlett's Test of Equal Variances        3.83    5     0.5734

Tukey HSD All-Pairwise Comparisons Test of area by media
media             Mean         Homogeneous Groups
        1         900.60                A
      100         786.18                AB
     1000         785.24                AB
        7         694.39                 BC
  control         644.79                 BC
        0         597.72                  C

Alpha             0.05
There are 3 groups (A, B, etc.) in which the means are not
significantly different from one another.

Completely randomized AOV for length
Source   DF        SS        MS       F        P
media     5    82.450   16.4901    7.23   0.0007
Error    18    41.060    2.2811
Total    23   123.510
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        7.08    5   0.2148

Tukey HSD All-Pairwise Comparisons Test of length by media

media             Mean             Homogeneous Groups
    1             37.904                A
 1000             35.869                AB
  100             35.418                AB
    7             34.640                ABC
 control          33.651                 BC
    0             31.941                  C

Alpha             0.05
```

There are 3 groups (A, B, etc.) in which the means are not significantly different from one another.

Completely randomized AOV for width

Source	DF	SS	MS	F	P
media	5	102.396	20.4792	8.34	0.0003
Error	18	44.176	2.4542		
Total	23	146.573			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	6.03	5	0.3032

Tukey HSD All-Pairwise Comparisons Test of width by media

media	Mean	Homogeneous Groups
1	29.819	A
100	28.035	AB
1000	27.469	ABC
7	25.526	BC
control	24.402	C
0	24.059	C

Alpha 0.05
There are 3 groups (A, B, etc.) in which the means are not significantly different from one another.

Radially expanding cells: wall area

Completely randomized AOV for wall

Source	DF	SS	MS	F	P
media	5	0.02217	0.00443	3.51	0.0216
Error	18	0.02271	0.00126		
Total	23	0.04488			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	10.5	5	0.0616

Tukey HSD All-Pairwise Comparisons Test of wall by media

media	Mean	Homogeneous Groups
0	0.2190	A
control	0.1861	AB
7	0.1679	AB
1	0.1409	AB
1000	0.1390	B
100	0.1348	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Existing cells: lumen area, length and width

Completely randomized AOV for area

Source	DF	SS	MS	F	P
media	5	188261	37652.2	3.00	0.0425
Error	16	200815	12551.0		
Total	21	389077			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	4.15	5	0.5278

Tukey HSD All-Pairwise Comparisons Test of area by media

media	Mean	Homogeneous Groups
1	809.39	A
100	679.99	AB
0	678.28	AB
1000	659.19	AB
7	586.29	AB
control	496.88	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Completely randomized AOV for length

Source	DF	SS	MS	F	P
media	5	95.224	19.0449	3.32	0.0302
Error	16	91.796	5.7373		
Total	21	187.021			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	10.7	5	0.0574

Tukey HSD All-Pairwise Comparisons Test of length by media

media	Mean	Homogeneous Groups
1	36.166	A
1000	32.957	AB
0	32.357	AB
100	32.286	AB
7	31.862	AB
control	28.877	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Completely randomized AOV for width

Source	DF	SS	MS	F	P
media	5	76.075	15.2151	2.11	0.1170
Error	16	115.347	7.2092		
Total	21	191.423			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	4.71	5	0.4519

Existing cells: wall area

Completely randomized AOV for wall

Source	DF	SS	MS	F	P
media	5	0.05108	0.01022	21.2	0.0000
Error	16	0.00770	0.00048		
Total	21	0.05877			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.91	5	0.5628

Tukey HSD All-Pairwise Comparisons Test of wall by media

media	Mean	Homogeneous Groups
control	0.4607	A
7	0.4353	A
1000	0.3622	B
0	0.3603	B
100	0.3302	B
1	0.3284	B

Alpha 0.05

There are 2 groups (A and B) in which the means are not significantly different from one another.

ii.2.2.2 Boron-treated culture set number two. The tree was from Burnham, inducted in March and cultured for two months.

Cell counts

Completely randomized AOV for cambial cell counts

Source	DF	SS	MS	F	P
media	6	115.863	19.3104	10.8	0.0000
Error	57	101.872	1.7872		
Total	63	217.734			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	25.2	6	0.0003

Tukey HSD All-Pairwise Comparisons Test of cambium by media

media	Mean	Homogeneous Groups
1000	12.125	A
100	10.875	AB
25	10.769	AB
7	9.5385	BC
control	8.8333	BC
0	8.1250	C
1	8.1250	C

Alpha 0.05
There are 3 groups (A, B, etc.) in which the means are not significantly different from one another.

Completely randomized AOV for radially expanding cell counts

Source	DF	SS	MS	F	P
media	6	219.152	36.5254	9.65	0.0000
Error	57	215.785	3.7857		
Total	63	434.938			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	4.44	6	0.6171

Tukey HSD All-Pairwise Comparisons Test of re by media

media	Mean	Homogeneous Groups
control	15.333	A
1	15.000	A
1000	13.250	AB
7	12.308	BC
100	11.000	BC
0	10.375	BC
25	10.231	C

Alpha 0.05
There are 3 groups (A, B, etc.) in which the means are not significantly different from one another.

Completely randomized AOV for new secondary wall cell counts

Source	DF	SS	MS	F	P
media	4	184.941	46.2353	3.12	0.0240
Error	45	667.779	14.8395		
Total	49	852.720			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	15.1	4	0.0046

Tukey HSD All-Pairwise Comparisons Test of second by media

media	Mean	Homogeneous Groups
25	19.077	A
7	17.462	AB
0	16.625	AB
100	16.125	AB
1	13.125	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Radially expanding cells: lumen area, length and width

Completely randomized AOV for area

Source	DF	SS	MS	F	P
media	6	62801	10466.9	0.69	0.6604
Error	20	303502	15175.1		
Total	26	366304			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	12.2	6	0.0577

Completely Randomized AOV for length

Source	DF	SS	MS	F	P
media	6	67.978	11.3296	2.18	0.0889
Error	20	104.130	5.2065		
Total	26	172.108			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	9.11	6	0.1676

Completely randomized AOV for width

Source	DF	SS	MS	F	P
media	6	37.622	6.27035	0.90	0.5121
Error	20	138.865	6.94327		
Total	26	176.487			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	19.6	6	0.0033

Radially expanding cells: wall area

Completely randomized AOV for wall

Source	DF	SS	MS	F	P
media	6	0.25443	0.04240	1.18	0.3577
Error	20	0.72087	0.03604		
Total	26	0.97530			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	62.8	6	0.0000

Existing cells: lumen area, length and width

Completely randomized AOV for area

Source	DF	SS	MS	F	P
media	6	397124	66187.4	3.98	0.0082
Error	21	349452	16640.6		
Total	27	746576			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.60	6	0.7313

Tukey HSD All-Pairwise Comparisons Test of area by media

media	Mean	Homogeneous Groups
1	1106.0	A
100	1062.5	A
control	1061.4	A
7	966.25	AB
1000	890.50	AB
25	888.61	AB
0	742.71	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Completely randomized AOV for length

Source	DF	SS	MS	F	P
media	6	103.432	17.2386	2.58	0.0497
Error	21	140.492	6.6901		
Total	27	243.924			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.58	6	0.7336
Cochran's Q	0.3483		

Completely randomized AOV for width

Source	DF	SS	MS	F	P
media	6	190.656	31.7761	5.19	0.0021
Error	21	128.570	6.1224		
Total	27	319.227			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.21	6	0.7817

Tukey HSD All-Pairwise Comparisons Test of width by media

media	Mean	Homogeneous Groups
1	31.038	A
control	30.483	AB
100	28.751	ABC
7	28.304	ABC
25	27.321	ABC
1000	25.216	BC
0	23.116	C

Alpha 0.05
There are 3 groups (A, B, etc.) in which the means are not significantly different from one another.

Existing cells: wall area

Completely randomized AOV for wall

Source	DF	SS	MS	F	P
media	6	0.02323	0.00387	8.11	0.0001
Error	21	0.01003	0.00048		
Total	27	0.03326			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	2.21	6	0.8996

Tukey HSD All-Pairwise Comparisons Test of wall by media

media	Mean	Homogeneous Groups
0	0.3822	A
25	0.3630	AB
1000	0.3435	ABC
1	0.3287	BCD
7	0.3287	BCD

```
 control          0.3050                    CD
     100          0.2932                     D

Alpha              0.05
There are 4 groups (A, B, etc.) in which the means are not
significantly different from one another.
```

ii.2.2.3 Boron-treated culture set number three. The tree was from Burnham, inducted in November and cultured for one month.

Cell counts

```
Completely randomized AOV for cambial cell counts
Source   DF        SS        MS       F        P
media     4    70.961   17.7403    3.19   0.0246
Error    35   194.639    5.5611
Total    39   265.600
                                     Chi-Sq   DF        P
Bartlett's Test of Equal Variances     2.40    4   0.6621

Tukey HSD All-Pairwise Comparisons Test of cambial by media
media       Mean              Homogeneous Groups
      25          16.125                  A
        7         15.000                  AB
        0         14.111                  AB
 control          12.875                  AB
        1         12.500                   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for radially expanding cell counts
Source   DF        SS        MS       F        P
media     4    94.646   23.6614    1.70   0.1714
Error    35   486.329   13.8951
Total    39   580.975
                                     Chi-Sq     DF         P
Bartlett's Test of Equal Variances     7.31      4    0.1203

Completely randomized AOV for new secondary cell wall counts
Source   DF        SS        MS       F        P
media     4   134.849   33.7122    3.69   0.0140
Error    32   292.232    9.1323
Total    36   427.081
                                     Chi-Sq     DF          P
Bartlett's Test of Equal Variances     10.9      4     0.0281

Tukey HSD All-Pairwise Comparisons Test of secondary by media
media       Mean              Homogeneous Groups
        0         16.500                  A
control           14.500                  AB
       25         14.375                  AB
        1         12.000                  AB
        7         10.857                   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Radially expanding cells: lumen area, length and width

Completely randomized AOV for area

Source	DF	SS	MS	F	P
media	5	28357	5671.4	0.50	0.7725
Error	17	193058	11356.4		
Total	22	221415			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.86	5	0.5699

Completely randomized AOV for length

Source	DF	SS	MS	F	P
media	5	18.9806	3.79611	0.83	0.5463
Error	17	77.8162	4.57742		
Total	22	96.7967			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	2.66	5	0.7529

Completely randomized AOV for width

Source	DF	SS	MS	F	P
media	5	12.3737	2.47475	0.54	0.7426
Error	17	77.7260	4.57212		
Total	22	90.0998			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	4.47	5	0.4840

Radially expanding cells: wall area

Completely randomized AOV for wall

Source	DF	SS	MS	F	P
media	5	0.00861	0.00172	2.65	0.0625
Error	16	0.01038	0.00065		
Total	21	0.01899			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.42	5	0.6362

Existing cells: lumen area, length and width

Completely randomized AOV for area

Source	DF	SS	MS	F	P
media	5	122381	24476.1	1.96	0.1344
Error	18	225075	12504.2		
Total	23	347455			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	12.5	5	0.0280

Completely randomized AOV for length

Source	DF	SS	MS	F	P
media	5	26.444	5.28887	1.09	0.3997
Error	18	87.394	4.85524		
Total	23	113.839			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	5.53	5	0.3552

Completely randomized AOV for width

Source	DF	SS	MS	F	P
media	5	65.296	13.0592	2.96	0.0404
Error	18	79.509	4.4172		
Total	23	144.805			

```
                                        Chi-Sq    DF          P
Bartlett's Test of Equal Variances       9.75     5     0.0826

Tukey HSD All-Pairwise Comparisons Test of width by media
media       Mean              Homogeneous Groups
        1         30.849                  A
    100           27.021                  AB
        7         26.992                  AB
      25          26.530                  AB
 control           26.489                 AB
        0         25.780                   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Existing cells: wall area

```
Completely randomized AOV for wall
Source   DF        SS        MS       F        P
media     5   0.01310   0.00262    4.35   0.0090
Error    18   0.01084   0.00060
Total    23   0.02393
                                        Chi-Sq     DF          P
Bartlett's Test of Equal Variances       2.09      5     0.8371

Tukey HSD All-Pairwise Comparisons Test of wall by media
media       Mean              Homogeneous Groups
control           0.3123                  A
         0        0.2988                  A
         7        0.2985                  A
         1        0.2823                  AB
       25         0.2709                  AB
     100          0.2408                   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

ii.2.3 Calcium-treated culture analysis of cell number, lumen area, length and width, and cell wall area

ii.2.3.1 Calcium-treated culture set number one. The tree was from Burnham, inducted in March and cultured for two months.

Cell counts

```
Completely randomized AOV for cambial cell counts
Source   DF        SS        MS       F        P
media     5    55.324   11.0648    3.49   0.0125
Error    32   101.518    3.1724
Total    37   156.842
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances       6.03    5   0.3035

Tukey HSD All-Pairwise Comparisons Test of cambium by media
media    Mean  Homogeneous Groups
    1  15.750  A
```

```
    5  12.714  AB
  100  12.571  AB
   10  12.500  AB
    0  12.125   B
 cont  10.750   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for radially expanding cell counts
Source   DF        SS        MS       F        P
media     4    59.179   14.7946    3.55   0.0178
Error    29   120.821    4.1663
Total    33   180.000

Grand Mean 11.000    CV 18.56
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        3.22    4   0.5213

Tukey HSD All-Pairwise Comparisons Test of re by media
media    Mean  Homogeneous Groups
    0  12.500  A
   10  12.250  AB
  100  10.143  AB
 cont  9.7500  AB
    5  9.4286   B

Alpha              0.05
There are 2 groups (A and B) in which the means
are not significantly different from one another.

Completely randomized AOV for new secondary cell wall counts
Source   DF        SS        MS       F        P
media     4    40.961   10.2402    3.31   0.0263
Error    25    77.339    3.0936
Total    29   118.300
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        4.52    4   0.3398
Cochran's Q                   0.3545

Tukey HSD All-Pairwise Comparisons Test of secondary by media
media    Mean  Homogeneous Groups
    5  11.143  A
   10  10.125  AB
    0  10.000  AB
 cont  9.2500  AB
  100  7.8571   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Radially expanding cells: lumen area, length and width

```
Completely randomized AOV for lumen
Source   DF       SS        MS       F        P
media     5   109878   21975.6    3.27   0.0283
Error    18   120921    6717.9
Total    23   230799
```

```
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        3.44    5   0.6332
Cochran's Q                     0.2648
Std Error (Diff of 2 Means) 57.956

Tukey HSD All-Pairwise Comparisons Test of lumen by media
media    Mean  Homogeneous Groups
 cont  856.44  A
   10  847.92  A
    1  812.27  A
    5  783.32  A
  100  706.27  A
    0  677.40  A

Alpha              0.05
There are no significant pairwise differences among the means.

Completely randomized AOV for length
Source   DF        SS        MS       F        P
media     5   105.899   21.1798    4.89   0.0053
Error    18    77.947    4.3304
Total    23   183.845
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        8.26    5   0.1427

Tukey HSD All-Pairwise Comparisons Test of length by media
media    Mean  Homogeneous Groups
 cont  39.873  A
    5  38.695  AB
   10  37.697  ABC
    1  37.245  ABC
  100  34.873   BC
    0  33.794    C

Alpha              0.05
There are 3 groups (A, B, etc.) in which the means are not
significantly different from one another.

Completely randomized AOV for width
Source   DF        SS        MS       F        P
media     5    42.627   8.52536    2.16   0.1049
Error    18    71.132   3.95180
Total    23   113.759
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        4.91    5   0.4266
```

Radially expanding cells: wall area

```
Completely randomized AOV for wall
Source   DF        SS        MS       F        P
media     5    79.920   15.9840    7.13   0.0008
Error    18    40.360    2.2422
Total    23   120.280
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        9.52    5   0.0901

Tukey HSD All-Pairwise Comparisons Test of wall by media
media    Mean  Homogeneous Groups
  100  20.975  A
   10  19.400  AB
    0  17.575   BC
```

```
    1  16.775   BC
    5  16.150   BC
 cont  15.925    C

Alpha              0.05
There are 3 groups (A, B, etc.) in which the means are not
significantly different from one another.
```

Existing cells: lumen area, length and width

```
Completely randomized AOV for lumen
Source   DF       SS        MS       F        P
media     4   215171   53792.8    7.45   0.0030
Error    12    86684    7223.7
Total    16   301855

                                       Chi-Sq   DF        P
Bartlett's Test of Equal Variances       1.80    4   0.7727

Tukey HSD All-Pairwise Comparisons Test of lumen by media
media    Mean  Homogeneous Groups
 cont  1073.6  A
    5  1055.3  A
    1  960.95  AB
  100  947.42  AB
    0  775.77   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for length
Source   DF        SS        MS       F        P
media     4   151.265   37.8162    17.6   0.0001
Error    12    25.835    2.1529
Total    16   177.099
                                       Chi-Sq   DF        P
Bartlett's Test of Equal Variances       2.41    4   0.6614

Tukey HSD All-Pairwise Comparisons Test of length by media
media    Mean  Homogeneous Groups
 cont  46.498  A
    5  46.250  A
  100  41.380   B
    1  40.801   B
    0  39.444   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for width
Source   DF        SS        MS       F        P
media     4    68.690   17.1726    3.61   0.0373
Error    12    57.023    4.7519
Total    16   125.713
                                       Chi-Sq   DF        P
Bartlett's Test of Equal Variances       2.42    4   0.6595

Tukey HSD All-Pairwise Comparisons Test of width by media
```

```
media    Mean  Homogeneous Groups
    1  29.431  A
 cont  28.501  A
  100  28.166  A
    5  27.760  A
    0  23.757  A

Alpha              0.05
There are no significant pairwise differences among the means.
```

Existing cells: wall area

```
Completely randomized AOV for wall
Source   DF        SS        MS       F        P
media     4   346.121   86.5304    17.8   0.0001
Error    12    58.309    4.8591
Total    16   404.431
                                     Chi-Sq   DF        P
Bartlett's Test of Equal Variances     7.73    4   0.1021

Tukey HSD All-Pairwise Comparisons Test of wall by media
media    Mean  Homogeneous Groups
    0  37.575  A
  100  35.300  A
    1  34.750  AB
    5  28.833   BC
 cont  26.150    C

Alpha              0.05
There are 3 groups (A, B, etc.) in which the means are not
significantly different from one another.
```

ii.2.3.2 Calcium-treated culture set number two. The tree was from Burnham, inducted in November and cultured for one month.

Cell counts

```
Completely randomized AOV for cambial cells
Source   DF        SS        MS       F        P
media     5    93.528   18.7057    3.05   0.0201
Error    40   245.450    6.1362
Total    45   338.978
                                     Chi-Sq   DF        P
Bartlett's Test of Equal Variances     10.5    5   0.0629

Tukey HSD All-Pairwise Comparisons Test of cambium by media
media    Mean  Homogeneous Groups
   10  16.667  A
  100  16.500  AB
 cont  15.400  AB
    0  14.625  AB
    1  13.750  AB
    5  12.875   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Completely randomized AOV for radially expanding cell counts

Source	DF	SS	MS	F	P
media	5	197.448	39.4895	5.77	0.0005
Error	37	253.250	6.8446		
Total	42	450.698			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	8.77	5	0.1187

Tukey HSD All-Pairwise Comparisons Test of re by media

media	Mean	Homogeneous Groups
10	19.000	A
100	18.375	AB
0	17.000	ABC
cont	15.000	ABC
5	14.500	BC
1	13.375	C

Alpha 0.05
There are 3 groups (A, B, etc.) in which the means are not significantly different from one another.

Completely randomized AOV for new secondary cell counts

Source	DF	SS	MS	F	P
media	5	12.363	2.47261	0.54	0.7462
Error	37	170.056	4.59610		
Total	42	182.419			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	4.14	5	0.5288

Radially expanding cells: lumen area, length and width

Completely randomized AOV for lumen

Source	DF	SS	MS	F	P
media	5	175730	35146.0	2.37	0.0812
Error	18	267370	14853.9		
Total	23	443100			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	9.67	5	0.0850

Completely randomized AOV for length

Source	DF	SS	MS	F	P
media	5	59.782	11.9565	1.78	0.1688
Error	18	121.225	6.7347		
Total	23	181.007			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	11.1	5	0.0488

Completely randomized AOV for width

Source	DF	SS	MS	F	P
media	5	57.081	11.4162	3.31	0.0270
Error	18	62.014	3.4452		
Total	23	119.095			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	5.32	5	0.3780

Tukey HSD All-Pairwise Comparisons Test of width by media

media	Mean	Homogeneous Groups
0	30.225	A
5	29.881	A
100	29.029	AB

```
   10  28.749  AB
    1  28.615  AB
 cont  25.469   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Radially expanding cells: wall area

```
Completely randomized AOV for wall
Source   DF        SS        MS       F        P
media     5    48.808    9.7617    0.47   0.7930
Error    18   373.090   20.7272
Total    23   421.898
                                      Chi-Sq   DF        P
Bartlett's Test of Equal Variances     28.4    5   0.0000
```

Existing cells: lumen area, length and width

```
Completely randomized AOV for lumen
Source   DF        SS        MS       F        P
media     5    94098   18819.6    1.96   0.1436
Error    15   144047    9603.1
Total    20   238145
                                      Chi-Sq   DF        P
Bartlett's Test of Equal Variances     3.58    5   0.6110

Completely randomized AOV for length
Source   DF        SS        MS       F        P
media     5    57.351   11.4702    2.92   0.0492
Error    15    59.009    3.9339
Total    20   116.360
                                      Chi-Sq   DF        P
Bartlett's Test of Equal Variances     6.47    5   0.2628

Completely randomized AOV for width
Source   DF        SS        MS       F        P
media     5    21.959   4.39171    0.81   0.5603
Error    15    81.315   5.42100
Total    20   103.274
                                      Chi-Sq   DF        P
Bartlett's Test of Equal Variances     2.91    5   0.7137
```

Existing cells: wall area

```
Completely randomized AOV for wall
Source   DF        SS        MS       F        P
media     5   111.029   22.2058    2.21   0.1072
Error    15   150.597   10.0398
Total    20   261.626
                                      Chi-Sq   DF        P
Bartlett's Test of Equal Variances     3.65    5   0.6014
```

ii.2.3.3 Calcium-treated culture set number three. The tree was from Burnham, inducted in March and cultured for two months.

Cell counts

Completely randomized AOV for cambial cell counts

Source	DF	SS	MS	F	P
media	3	21.372	7.12416	1.57	0.2179
Error	29	131.597	4.53784		
Total	32	152.970			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	4.93	3	0.1770

Completely randomized AOV for radially expanding cell counts

Source	DF	SS	MS	F	P
media	3	215.270	71.7567	11.1	0.0001
Error	29	187.639	6.4703		
Total	32	402.909			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.15	3	0.3697

Tukey HSD All-Pairwise Comparisons Test of re by media

media	Mean	Homogeneous Groups
0	17.000	A
5	12.375	B
cont	12.111	B
100	9.8750	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Completely randomized AOV for new secondary wall cell counts

Source	DF	SS	MS	F	P
media	3	77.000	25.6667	5.17	0.0057
Error	28	139.000	4.9643		
Total	31	216.000			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	6.10	3	0.1068

Tukey HSD All-Pairwise Comparisons Test of secondary by media

media	Mean	Homogeneous Groups
0	14.000	A
100	11.250	AB
5	11.000	AB
cont	9.7500	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Radially expanding cells: lumen area, length and width

Completely randomized AOV for lumen

Source	DF	SS	MS	F	P
media	4	77275	19318.8	2.26	0.1237
Error	12	102772	8564.4		
Total	16	180048			

```
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        3.07    4   0.5458

Completely randomized AOV for length
Source   DF        SS        MS       F        P
media     4   27.5727   6.89316    1.75   0.2036
Error    12   47.2388   3.93657
Total    16   74.8115
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        5.25    4   0.2622

Completely randomized AOV for width
Source   DF        SS        MS       F        P
media     4   28.8141   7.20354    2.11   0.1417
Error    12   40.8756   3.40630
Total    16   69.6898
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        1.77    4   0.7775
```

Radially expanding cells: wall area

```
Completely randomized AOV for wall
Source   DF        SS        MS       F        P
media     4    56.950   14.2374    0.75   0.5792
Error    12   229.069   19.0891
Total    16   286.019
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        13.7    4   0.0085
```

Existing cells: lumen area, length and width

```
Completely randomized AOV for lumen
Source   DF       SS       MS       F        P
media     3   361762   120587    11.5   0.0008
Error    12   125546    10462
Total    15   487308
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        0.77    3   0.8577

Tukey HSD All-Pairwise Comparisons Test of lumen by media
media    Mean  Homogeneous Groups
  100  1064.2  A
    5  1045.3  A
 cont  918.20  A
    0  687.13   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for length
Source   DF        SS        MS       F        P
media     3   233.572   77.8574    18.4   0.0001
Error    12    50.666    4.2222
Total    15   284.238
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        0.66    3   0.8820

Tukey HSD All-Pairwise Comparisons Test of length by media
```

```
media    Mean  Homogeneous Groups
  100  43.699  A
    5  43.245  A
 cont  40.692  A
    0  34.128   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for width
Source   DF        SS        MS       F        P
media     3   108.604   36.2012    3.75   0.0414
Error    12   115.900    9.6583
Total    15   224.504
                                      Chi-Sq   DF        P
Bartlett's Test of Equal Variances      3.53    3   0.3170

Tukey HSD All-Pairwise Comparisons Test of width by media
media    Mean  Homogeneous Groups
  100  32.130  A
    5  29.256  AB
 cont  27.366  AB
    0  25.017   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Existing cells: wall area

```
Completely randomized AOV for wall
Source   DF        SS        MS       F        P
media     3   335.357   111.786    19.7   0.0001
Error    12    68.032     5.669
Total    15   403.389
                                      Chi-Sq   DF        P
Bartlett's Test of Equal Variances      4.29    3   0.2315

Tukey HSD All-Pairwise Comparisons Test of wall by media
media    Mean  Homogeneous Groups
    0  37.875  A
 cont  29.925   B
    5  27.250   B
  100  26.175   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

ii.2.4 Magnesium-treated culture analysis of cell number, lumen area, length and width, and cell wall area

ii.2.4.1 Magnesium-treated culture set number one. The tree from Burnham, inducted in March and cultured for two months.

Cell counts

Completely randomized AOV for cambial cell counts

Source	DF	SS	MS	F	P
media	3	42.651	14.2169	4.02	0.0147
Error	35	123.708	3.5345		
Total	38	166.359			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.80	3	0.2838

Tukey HSD All-Pairwise Comparisons Test of cambium by media

media	Mean	Homogeneous Groups
1	14.125	A
0	13.333	AB
5	12.000	AB
10	11.250	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Completely randomized AOV for radially expanding cell counts

Source	DF	SS	MS	F	P
media	3	18.750	6.2cont	1.67	0.1959
Error	28	104.750	3.74107		
Total	31	123.500			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.91	3	0.5913

Critical Q Value 3.862 Critical Value for Comparison 2.6413
There are no significant pairwise differences among the means.

Completely randomized AOV for new secondary wall cell counts

Source	DF	SS	MS	F	P
media	3	35.250	11.7500	3.91	0.0190
Error	28	84.250	3.0089		
Total	31	119.500			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	8.48	3	0.0370

Tukey HSD All-Pairwise Comparisons Test of secondary by media

media	Mean	Homogeneous Groups
10	11.625	A
5	11.125	AB
0	9.7500	AB
1	9.0000	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Radially expanding cells: lumen area, length and width

```
Completely randomized AOV for lumen
Source   DF       SS        MS       F        P
media     4    80365   20091.2    2.55   0.0727
Error    19   149577    7872.5
Total    23   229942
                                     Chi-Sq   DF        P
Bartlett's Test of Equal Variances     9.73    4   0.0453

Completely randomized AOV for length
Source   DF        SS        MS       F        P
media     4    35.929   8.98215    2.50   0.0771
Error    19    68.286   3.59401
Total    23   104.215
                                     Chi-Sq   DF        P
Bartlett's Test of Equal Variances     6.73    4   0.1508

Completely randomized AOV for width
Source   DF        SS        MS       F        P
media     4    30.898   7.72444    1.95   0.1427
Error    19    75.096   3.95243
Total    23   105.994
                                     Chi-Sq   DF        P
Bartlett's Test of Equal Variances     13.4    4   0.0094
```

Radially expanding cells: wall area

```
Completely randomized AOV for wall
Source   DF        SS        MS       F        P
media     4    58.422   14.6056    4.43   0.0107
Error    19    62.654    3.2976
Total    23   121.076
                                     Chi-Sq   DF        P
Bartlett's Test of Equal Variances     11.7    4   0.0195

Tukey HSD All-Pairwise Comparisons Test of wall by media

media    Mean  Homogeneous Groups
  100  20.525  A
    5  18.525  AB
    0  17.063   B
    1  16.350   B
   10  15.850   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Existing cells: lumen area, length and width

```
Completely randomized AOV for lumen
Source   DF       SS        MS       F        P
media     4   120377   30094.3    6.28   0.0048
Error    13    62269    4789.9
Total    17   182646
                                     Chi-Sq   DF        P
Bartlett's Test of Equal Variances     3.82    4   0.4314
```

Tukey HSD All-Pairwise Comparisons Test of lumen by media

media	Mean	Homogeneous Groups
100	1093.8	A
0	1084.8	A
1	1074.1	A
10	1036.9	AB
5	823.32	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Completely randomized AOV for length

Source	DF	SS	MS	F	P
media	4	132.375	33.0938	18.5	0.0000
Error	13	23.270	1.7900		
Total	17	155.646			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	2.86	4	0.5818

Tukey HSD All-Pairwise Comparisons Test of length by media

media	Mean	Homogeneous Groups
100	47.492	A
0	45.310	AB
1	44.179	B
10	43.443	B
5	37.793	C

Alpha 0.05
There are 3 groups (A, B, etc.) in which the means are not significantly different from one another.

Completely randomized AOV for width

Source	DF	SS	MS	F	P
media	4	13.7524	3.43809	1.61	0.2303
Error	13	27.7426	2.13405		
Total	17	41.4950			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	4.55	4	0.3369

Existing cells: wall area

Completely randomized AOV for wall

Source	DF	SS	MS	F	P
media	4	105.862	26.4655	8.13	0.0016
Error	13	42.341	3.2570		
Total	17	148.203			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	8.97	4	0.0620

Tukey HSD All-Pairwise Comparisons Test of wall by media

media	Mean	Homogeneous Groups
5	35.200	A
1	31.100	AB
0	28.517	B
100	28.375	B
10	26.350	B

```
Alpha               0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

ii.2.4.2 Magnesium-treated culture set number two. The tree was from Burnham, inducted in March and cultured for two months.

Cell counts

```
Completely randomized AOV for cambial cell counts
Source   DF         SS         MS        F         P
media     4     11.638    2.90953     0.84    0.5126
Error    32    111.389    3.48090
Total    36    123.027
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        0.97    4   0.9142

Completely randomized AOV for radially expanding cell counts
Source   DF         SS         MS        F         P
media     4     28.956    7.23902     2.57    0.0567
Error    32     90.125    2.81641
Total    36    119.081
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        10.8    4   0.0292

Completely randomized AOV for new secondary cell wall counts
Source   DF         SS         MS        F         P
media     4     73.818    18.4544     8.23    0.0001
Error    32     71.750     2.2422
Total    36    145.568
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        1.26    4   0.8689

Tukey HSD All-Pairwise Comparisons Test of secondary by media

media    Mean  Homogeneous Groups
  0.5  11.000  A
    1  9.3750  AB
 cont  8.3750   B
   10  7.cont   B
    5  7.0000   B

Alpha               0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Radially expanding cells: lumen area, length and width

```
Completely randomized AOV for lumen
Source   DF         SS         MS        F         P
media     4      74678    18669.4     9.25    0.0003
Error    19      38332     2017.5
Total    23     113009
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        1.78    4   0.7769

Tukey HSD All-Pairwise Comparisons Test of lumen by media
```

```
media    Mean  Homogeneous Groups
   10  902.38  A
    5  889.23  A
 cont  854.64  A
    1  811.97  AB
    0  762.94   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for length
Source   DF        SS        MS       F        P
media     4   27.6837   6.92091    7.44   0.0009
Error    19   17.6762   0.93033
Total    23   45.3598
                                       Chi-Sq   DF        P
Bartlett's Test of Equal Variances       3.99    4   0.4073

Tukey HSD All-Pairwise Comparisons Test of length by media

media    Mean  Homogeneous Groups
   10  38.297  A
    5  38.172  A
 cont  37.772  A
    1  36.585  AB
    0  35.752   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for width
Source   DF        SS        MS       F        P
media     4   32.6119   8.15296    7.63   0.0008
Error    19   20.3131   1.06911
Total    23   52.9250
                                       Chi-Sq   DF        P
Bartlett's Test of Equal Variances       3.88    4   0.4218

Tukey HSD All-Pairwise Comparisons Test of width by media

media    Mean  Homogeneous Groups
   10  29.815  A
    5  29.814  A
 5000  28.810  AB
    1  28.358  AB
    0  26.985   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Radially expanding cells: wall area

```
Completely randomized AOV for wall
Source   DF        SS        MS       F        P
media     4    69.343   17.3358    6.25   0.0022
Error    19    52.675    2.7724
Total    23   122.018
```

```
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        2.79    4   0.5934

Tukey HSD All-Pairwise Comparisons Test of wall by media

media    Mean  Homogeneous Groups
    0  17.000  A
 cont  14.825  AB
    1  14.350  AB
    5  13.450   B
   10  12.425   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Existing cells: lumen area, length and width

```
Completely randomized AOV for lumen
Source   DF       SS        MS       F        P
media     4   256262   64065.5    7.90   0.0010
Error    16   129788    8111.8
Total    20   386050
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        4.00    4   0.4060

Tukey HSD All-Pairwise Comparisons Test of lumen by media

media    Mean  Homogeneous Groups
   10  1159.9  A
    1  1103.6  A
 cont  1019.8  AB
    5  984.35  AB
    0  863.70   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for length
Source   DF       SS        MS       F        P
media     4    84.638   21.1594    7.94   0.0010
Error    16    42.618    2.6636
Total    20   127.256
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        8.76    4   0.0673

Tukey HSD All-Pairwise Comparisons Test of length by media

media    Mean  Homogeneous Groups
   10  45.140  A
    1  43.559  A
 cont  42.623  AB
    5  42.605  AB
    0  39.545   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

```
Completely randomized AOV for width
Source   DF        SS        MS       F        P
media     4    64.130   16.0325    4.22   0.0161
Error    16    60.811    3.8007
Total    20   124.941
                                       Chi-Sq   DF        P
Bartlett's Test of Equal Variances       4.94    4   0.2937

Tukey HSD All-Pairwise Comparisons Test of width by media

media    Mean  Homogeneous Groups
   10  30.863  A
    1  29.765  AB
 cont  28.715  AB
    5  27.536  AB
    0  26.196   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Existing cells: wall area

```
Completely randomized AOV for wall
Source   DF        SS        MS       F        P
media     4   326.481   81.6201    11.2   0.0002
Error    16   116.291    7.2682
Total    20   442.771
                                       Chi-Sq   DF        P
Bartlett's Test of Equal Variances       6.69    4   0.1533

Tukey HSD All-Pairwise Comparisons Test of wall by media

media    Mean  Homogeneous Groups
    0  33.567  A
    1  30.350  AB
    5  28.900  AB
 cont  26.700   BC
   10  22.325    C

Alpha              0.05
There are 3 groups (A, B, etc.) in which the means are not
significantly different from one another.
```

ii.2.4.3 Magnesium-treated culture set number three. The tree was from Burnham, inducted in November and cultured for one month.

Cell counts

```
Completely randomized AOV for cambial cell counts
Source   DF        SS        MS       F        P
media     5    79.885   15.9769    2.14   0.0755
Error    52   388.736    7.4757
Total    57   468.621
                                       Chi-Sq   DF        P
Bartlett's Test of Equal Variances       8.49    5   0.1311
```

Completely randomized AOV for radially expanding cell counts

Source	DF	SS	MS	F	P
media	5	70.455	14.0910	1.47	0.2171
Error	52	500.028	9.6159		
Total	57	570.483			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.57	5	0.6126

Completely randomized AOV for new secondary cell wall counts

Source	DF	SS	MS	F	P
media	5	141.260	28.2519	4.83	0.0011
Error	52	303.861	5.8435		
Total	57	445.121			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	11.3	5	0.0458

Tukey HSD All-Pairwise Comparisons Test of secondary by media

media	Mean	Homogeneous Groups
0	13.722	A
1	13.250	AB
10	13.125	AB
5	11.750	AB
100	10.125	B
cont	9.7500	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Radially expanding cells: lumen area, length and width

Completely randomized AOV for lumen

Source	DF	SS	MS	F	P
media	5	42336	8467.27	1.01	0.4368
Error	20	167427	8371.36		
Total	25	209763			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.29	5	0.6555

Completely randomized AOV for length

Source	DF	SS	MS	F	P
media	5	16.7901	3.35803	0.93	0.4814
Error	20	72.0644	3.60322		
Total	25	88.8545			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	0.82	5	0.9757

Completely randomized AOV for width

Source	DF	SS	MS	F	P
media	5	14.2524	2.85048	0.94	0.4742
Error	20	60.3705	3.01852		
Total	25	74.6229			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	2.22	5	0.8185

Radially expanding cells: wall area

Completely randomized AOV for walls

Source	DF	SS	MS	F	P
media	5	9.7985	1.95969	1.01	0.4401
Error	20	38.9850	1.94925		
Total	25	48.7835			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	7.44	5	0.1902

Existing cells: lumen area, length and width

Completely randomized AOV for lumen

Source	DF	SS	MS	F	P
media	5	130712	26142.4	5.56	0.0033
Error	17	79993	4705.4		
Total	22	210704			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.78	5	0.5811

Tukey HSD All-Pairwise Comparisons Test of lumen by media

media	Mean	Homogeneous Groups
100	895.26	A
10	876.92	AB
5	813.39	ABC
cont	765.32	ABC
0	729.66	BC
1	691.18	C

Alpha 0.05
There are 3 groups (A, B, etc.) in which the means are not significantly different from one another.

Completely randomized AOV for length

Source	DF	SS	MS	F	P
media	5	90.629	18.1258	11.6	0.0001
Error	17	26.597	1.5645		
Total	22	117.226			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.55	5	0.9075

Tukey HSD All-Pairwise Comparisons Test of length by media

media	Mean	Homogeneous Groups
10	38.307	A
100	38.263	A
5	36.236	AB
cont	35.601	AB
0	34.222	B
1	32.947	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Completely randomized AOV for width

Source	DF	SS	MS	F	P
media	5	27.3400	5.46800	2.32	0.0886

```
Error    17   40.0705   2.35709
Total    22   67.4105
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        5.30    5   0.3801
```

Existing cells: wall area

```
Completely randomized AOV for wall
Source   DF        SS        MS       F        P
media     5   106.326   21.2652    0.59   0.7084
Error    17   613.658   36.0975
Total    22   719.984
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        21.9    5   0.0006
```

ii.2.5 Mixed-treated culture analysis of cell number, lumen area, length and width, and cell wall area

ii.2.5.1 Mixed-treated culture set number one. The tree was from Burnham, inducted in March and cultured for two months.

Cell counts

```
Completely randomized AOV for cambial cell counts
Source   DF        SS        MS       F        P
mixed     5   25.3333   5.06667    2.07   0.1164
Error    18   44.0000   2.44444
Total    23   69.3333
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        6.50    5   0.2607

Completely randomized AOV for radially expanding cell counts
Source   DF        SS        MS       F        P
mixed     5   264.708   52.9417    13.6   0.0000
Error    18    70.250    3.9028
Total    23   334.958
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        4.82    5   0.4384

Tukey HSD All-Pairwise Comparisons Test of re by mixed

mixed     Mean  Homogeneous Groups
optiLB  14.750  A
opti    12.500  AB
HBLC    12.000  AB
LBHC    10.250   BC
0BCM    7.5000    CD
LMHC    4.7500     D

Alpha             0.05
There are 4 groups (A, B, etc.) in which the means are not
significantly different from one another.

Completely randomized AOV for new secondary wall counts
Source   DF        SS        MS       F        P
mixed     5   11.3333   2.26667    2.04   0.1212
Error    18   20.0000   1.11111
```

```
Total    23   31.3333

At least one group variance is near zero, variance-equality tests
cannot be computed.
```

Radially expanding cells: lumen area, length and width

```
Completely randomized AOV for lumen
Source     DF        SS        MS       F        P
treatment   2   53553.0   26776.5    8.87   0.0339
Error       4   12078.6    3019.6
Total       6   65631.6
                                     Chi-Sq   DF        P
Bartlett's Test of Equal Variances     2.37    2   0.3053

Tukey HSD All-Pairwise Comparisons Test of lumen by treatment

treatment    Mean  Homogeneous Groups
control    747.58  A
LBHC       663.97  AB
HBLC       519.61   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for Length
Source     DF        SS        MS       F        P
treatment   2   22.5045   11.2522    8.42   0.0368
Error       4    5.3452    1.3363
Total       6   27.8497
                                     Chi-Sq   DF        P
Bartlett's Test of Equal Variances     0.76    2   0.6823

Tukey HSD All-Pairwise Comparisons Test of Length by treatment

treatment    Mean  Homogeneous Groups
control    34.748  A
LBHC       33.600  AB
HBLC       30.232   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for Width
Source     DF        SS        MS       F        P
treatment   2   22.3830   11.1915    6.62   0.0538
Error       4    6.7593    1.6898
Total       6   29.1423

                                     Chi-Sq   DF        P
Bartlett's Test of Equal Variances     1.23    2   0.5406
```

Radially expanding cells: wall area

```
Completely randomized AOV for Wall
Source     DF        SS        MS       F        P
treatment   2   0.00271   0.00136    6.36   0.0572
```

```
Error       4   0.00085   0.00021
Total       6   0.00356
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        3.67    2   0.1596
```

Existing cells: lumen area, length and width

```
Completely randomized AOV for lumen
Source     DF        SS        MS       F        P
treatment   2   34334.2   17167.1    3.12   0.2426
Error       2   10995.3    5497.6
Total       4   45329.4
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        0.37    1   0.5433

Completely randomized AOV for Length
Source     DF        SS        MS       F        P
treatment   2   31.8587   15.9293    1.43   0.4114
Error       2   22.2690   11.1345
Total       4   54.1277
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        1.63    1   0.2015

Completely randomized AOV for Width
Source     DF        SS        MS       F        P
treatment   2   2.62164   1.31082    1.63   0.3798
Error       2   1.60532   0.80266
Total       4   4.22696
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        1.16    1   0.2820
```

Existing cells: wall area

```
Completely randomized AOV for wall
Source     DF        SS        MS       F        P
treatment   2   0.00948   0.00474    4.97   0.1675
Error       2   0.00191   0.00095
Total       4   0.01138
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        1.45    1   0.2286
```

ii.2.5.2 Mixed-treated culture set number two. The tree was from Burnham, inducted in December, cultured for two months.

Cell counts

```
Completely randomized AOV for cambial cell counts
Source   DF        SS        MS       F        P
mixed     5   110.708   22.1417    5.84   0.0022
Error    18    68.250    3.7917
Total    23   178.958
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        6.64    5   0.2491

Tukey HSD All-Pairwise Comparisons Test of cambial by mixed

mixed      Mean  Homogeneous Groups
HMLC     16.750  A
```

```
HBLC     11.500   B
OBCM     11.500   B
control  11.250   B
LBHC     11.000   B
LMHC     10.250   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for radially expanding cell counts
Source   DF        SS        MS       F        P
mixed     5   171.333   34.2667    9.28   0.0002
Error    18    66.500    3.6944
Total    23   237.833
                                      Chi-Sq   DF        P
Bartlett's Test of Equal Variances      1.67    5   0.8929

Tukey HSD All-Pairwise Comparisons Test of re by mixed

mixed      Mean  Homogeneous Groups
OBCM     21.750  A
control  19.250  AB
LBHC     18.750  ABC
HMLC     15.250   BC
HBLC     14.750    C
LMHC     14.750    C

Alpha              0.05
There are 3 groups (A, B, etc.) in which the means are not
significantly different from one another.

Completely randomized AOV for new secondary cell wall counts
Source  DF        SS        MS       F        P
mixed    4   18.8500   4.71250    18.9   0.0032
Error    5    1.2500   0.25000
Total    9   20.1000

At least one group variance is near zero, variance-equality tests
cannot be computed.

Tukey HSD All-Pairwise Comparisons Test of secondary by mixed

mixed      Mean  Homogeneous Groups
OBCM     8.0000  A
LBHC     5.0000   B
LMHC     3.7500   B
HBLC     3.5000   B
control  3.0000   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Radially expanding cells: lumen area, length and width

```
Completely Randomized AOV for area
Source   DF       SS        MS       F        P
media     3    14392    4797.3    0.41   0.7499
Error    11   129096   11736.0
Total    14   143488
```

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	2.86	3	0.4132

Completely Randomized AOV for length

Source	DF	SS	MS	F	P
media	3	34.0329	11.3443	2.24	0.1412
Error	11	55.8080	5.0735		
Total	14	89.8409			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.19	3	0.3633

Completely Randomized AOV for width

Source	DF	SS	MS	F	P
media	3	6.4083	2.13611	0.46	0.7132
Error	11	50.6484	4.60440		
Total	14	57.0567			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	2.23	3	0.5254

Radially expanding cells: wall area

Completely randomized AOV for wall

Source	DF	SS	MS	F	P
media	3	0.00187	6.218E-04	0.89	0.4750
Error	11	0.00766	6.960E-04		
Total	14	0.00952			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.00	3	0.3918

Existing cells: lumen area, length and width

Completely randomized AOV for lumen

Source	DF	SS	MS	F	P
treatment	2	8263.9	4131.93	0.43	0.6831
Error	3	28562.5	9520.84		
Total	5	36826.4			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.72	2	0.4221

Completely randomized AOV for Length

Source	DF	SS	MS	F	P
treatment	2	5.2340	2.61700	0.47	0.6630
Error	3	16.6065	5.53549		
Total	5	21.8405			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	4.21	2	0.1220

Completely randomized AOV for Width

Source	DF	SS	MS	F	P
treatment	2	1.9266	0.96331	0.29	0.7693
Error	3	10.0854	3.36181		
Total	5	12.0120			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.16	2	0.5604

Existing cells: wall area

Completely randomized AOV for wall

Source	DF	SS	MS	F	P
treatment	2	0.00910	0.00455	4.69	0.1192

```
Error       3   0.00291   0.00097
Total       5   0.01201
                                       Chi-Sq   DF        P
Bartlett's Test of Equal Variances       1.23    2   0.5407
```

ii.2.5.3 Mixed-treated culture set number three. The tree was from Burnham, inducted in January and cultured for two months.

Cell counts

```
Completely randomized AOV for cambial cell counts
Source   DF        SS        MS       F        P
mixed     6    48.929   8.15476    3.14   0.0233
Error    21    54.500   2.59524
Total    27   103.429
                                       Chi-Sq   DF        P
Bartlett's Test of Equal Variances       4.16    6   0.6552

Tukey HSD All-Pairwise Comparisons Test of cambial by mixed

mixed      Mean  Homogeneous Groups
opti     13.500  A
LMHC     12.000  AB
LBHC     10.750  AB
control  10.500  AB
HBCM     10.000  AB
HMLC     9.7500   B
0BCM     9.5000   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for radially expanding cell counts
Source   DF        SS        MS       F        P
mixed     6   142.857   23.8095    5.60   0.0013
Error    21    89.250    4.2500
Total    27   232.107
                                       Chi-Sq   DF        P
Bartlett's Test of Equal Variances       6.01    6   0.4216

Tukey HSD All-Pairwise Comparisons Test of re by mixed

mixed      Mean  Homogeneous Groups
HBCM     13.250  A
LBHC     9.7500  AB
opti     9.0000  AB
0BCM     8.2500   B
control  8.0000   B
LMHC     7.0000   B
HMLC     5.5000   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for new secondary cell wall counts
Source   DF        SS        MS       F        P
mixed     4   274.944   68.7361    6.48   0.0043
Error    13   138.000   10.6154
```

```
Total    17   412.944
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        9.66    4   0.0466

Tukey HSD All-Pairwise Comparisons Test of secondary by mixed

mixed      Mean  Homogeneous Groups
LMHC     18.000  A
0BCM     12.250  AB
control  11.250  AB
opti     10.000   B
HBCM     4.5000   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Radially expanding cells: lumen area, length and width

```
Completely randomized AOV for lumen
Source     DF        SS        MS       F        P
treatment   2   41036.9   20518.5    2.06   0.2731
Error       3   29824.7    9941.6
Total       5   70861.6
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        1.10    2   0.5768

Completely randomized AOV for Length
Source     DF        SS        MS       F        P
treatment   2   29.4599   14.7299    34.2   0.0086
Error       3    1.2916    0.4305
Total       5   30.7515
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        1.98    2   0.3709

Tukey HSD All-Pairwise Comparisons Test of re by mixed

treatment    Mean  Homogeneous Groups
HBLC       40.789  A
control    38.926  A
LBHC       35.442   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for Width
Source     DF        SS        MS       F        P
treatment   2   11.0309   5.51545    1.22   0.4095
Error       3   13.5622   4.52075
Total       5   24.5931
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        1.08    2   0.5833
```

Radially expanding cells: wall area

```
Completely randomized AOV for wall area
Source     DF        SS        MS       F        P
treatment   2   0.00396   0.00198    1.63   0.3314
Error       3   0.00364   0.00121
```

```
Total       5   0.00760

                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        0.04    2   0.9813
```

Existing cells: lumen area, length and width

```
Completely randomized AOV for lumen
Source     DF        SS         MS       F        P
treatment   2    3006.3    1503.13    0.22   0.8122
Error       3   20206.7    6735.56
Total       5   23212.9

                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        2.78    2   0.2486

Completely randomized AOV for Length
Source     DF        SS         MS       F        P
treatment   2   12.5341    6.26703    2.10   0.2684
Error       3    8.9330    2.97767
Total       5   21.4671

                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        3.23    2   0.1987

Completely randomized AOV for Width
Source     DF        SS         MS       F        P
treatment   2    4.1229    2.06143    0.63   0.5913
Error       3    9.8291    3.27637
Total       5   13.9520

                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        0.64    2   0.7267
```

Existing cells: wall area

```
Completely randomized AOV for wall
Source     DF        SS         MS       F        P
treatment   2   0.00611    0.00306    3.25   0.1777
Error       3   0.00283    0.00094
Total       5   0.00894

                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        4.37    2   0.1126
```

ii.3 Statistics for data presented in chapter six

ii.3.1 Boron-treated culture analysis for CML/S_1 lignin area

ii.3.1.1 Boron-treated culture set number one. The tree was from Rotorua, inducted in April and cultured for two months.

Existing cells: CML lignin area

```
Completely Randomized AOV for % lignin
Source     DF        SS         MS       F        P
treatment   2   0.94890    0.47445    3.64   0.1577
Error       3   0.39113    0.13038
Total       5   1.34003

Grand Mean 1.2766    CV 28.29
```

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.94	2	0.3796

ii.3.1.2 Boron-treated culture set number two. The tree was from Burnham, inducted in March and cultured for two months.

Existing cells: CML lignin area

Completely Randomized AOV for % lignin

Source	DF	SS	MS	F	P
treatment	2	1.07409	0.53705	3.09	0.1195
Error	6	1.04281	0.17380		
Total	8	2.11690			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.41	2	0.4933

ii.3.1.3 Boron-treated cultures set number three. The tree was from Burnham, inducted in November and cultured for one month.

Existing cells: CML lignin area

Completely Randomized AOV for % lignin

Source	DF	SS	MS	F	P
treatment	2	2.91217	1.45609	2.99	0.1255
Error	6	2.91996	0.48666		
Total	8	5.83213			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.93	2	0.3802

ii.3.2 Calcium-treated culture analysis for CML/S_1 lignin area

ii.3.2.1 Calcium-treated culture set number one. The tree from Burnham, inducted in March and cultured for two months.

Existing cells: CML lignin area

Completely randomized AOV for % lignin

Source	DF	SS	MS	F	P
media	3	5.391E-07	1.797E-07	0.33	0.8040
Error	12	6.541E-06	5.451E-07		
Total	15	7.080E-06			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	2.91	3	0.4051

ii.3.2.2 Calcium-treated culture set number two. The tree was from Burnham, inducted in November and cultured for one month.

Existing cells: CML lignin area

Completely randomized AOV for % lignin

Source	DF	SS	MS	F	P
media	3	2.285E-06	7.618E-07	2.01	0.1764
Error	10	3.788E-06	3.788E-07		
Total	13	6.073E-06			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.89	3	0.5958

ii.3.2.3 Calcium-treated culture set number three. The tree was from Burnham, inducted in March and cultured for two months

Existing cells: CML lignin area

Completely Randomized AOV for % lignin

Source	DF	SS	MS	F	P
treatment	3	0.05195	0.01732	1.28	0.3295
Error	11	0.14886	0.01353		
Total	14	0.20080			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	5.79	3	0.1225

ii.3.3 Magnesium-treated culture analysis for CML/S_1 lignin area

ii.3.3.1 Magnesium-treated culture set number one. The tree was from Burnham, inducted in March and cultured for two months.

Existing cells: CML lignin area

Completely Randomized AOV for one

Source	DF	SS	MS	F	P
treatment	3	0.02360	0.00787	1.16	0.3689
Error	11	0.07465	0.00679		
Total	14	0.09825			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.63	3	0.6532

ii.3.3.2 Magnesium-treated culture set number two. The tree was from Burnham, inducted in March and cultured for two months.

Existing cells: CML lignin area

Completely Randomized AOV for two

Source	DF	SS	MS	F	P
treatment	1	0.00324	0.00324	2.63	0.1558
Error	6	0.00739	0.00123		
Total	7	0.01064			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	0.64	1	0.4247

ii.3.3.3 Magnesium-treated culture set number four. The tree was from Burnham, inducted in February and cultured for two months.

Existing cells: CML lignin area

Completely Randomized AOV for five

Source	DF	SS	MS	F	P
treatment	3	0.02276	0.00759	1.18	0.3598
Error	12	0.07743	0.00645		
Total	15	0.10019			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	8.02	3	0.0456

ii.3.4 Mixed-treated culture analysis for CML/S_1 lignin area

ii.3.4.1 Mixed-treated culture set number one. The tree was from Burnham, inducted in March and cultured for two months.

Existing cells: CML lignin area

Completely Randomized AOV for % lignin

Source	DF	SS	MS	F	P
treatment	1	0.81695	0.81695	0.76	0.4172
Error	6	6.45990	1.07665		
Total	7	7.27684			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	7.75	1	0.0054

ii.3.4.2 Mixed-treated cultures set number two. The tree was from Burnham, inducted in December and cultured for two months.

Existing cells: CML lignin area

Completely Randomized AOV for % lignin

Source	DF	SS	MS	F	P
treatment	1	3.6897	3.68968	1.90	0.2168
Error	6	11.6226	1.93710		
Total	7	15.3123			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.39	1	0.2390
Cochran's Q	0.8228		

ii.3.4.3 Mixed-treated culture set number three. The tree was from Burnham, inducted in January and cultured for two months.

Existing cells: CML lignin area

Completely Randomized AOV for % lignin

Source	DF	SS	MS	F	P
treatment	1	0.2898	0.28984	0.15	0.7138
Error	6	11.7537	1.95895		
Total	7	12.0436			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.37	1	0.2420

ii.3.5 Klason lignin assay of nutrient treated cultures

The data presented include the combined statistical analysis of the nutrient treated cultures from two trees and their control explants.

ii.3.5.1 Klason lignin assay of boron-treated cultures

Completely randomized AOV for insoluble

Source	DF	SS	MS	F	P
boron	2	8.7100	4.35500	7.94	0.0633
Error	3	1.6450	0.54833		
Total	5	10.3550			

```
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        2.39    2   0.3023

Completely randomized AOV for soluble
Source  DF        SS        MS       F        P
boron    2   0.00703   0.00352    15.1   0.0272
Error    3   0.00070   0.00023
Total    5   0.00773
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        0.72    2   0.6982

Tukey HSD All-Pairwise Comparisons Test of soluble by boron

boron    Mean  Homogeneous Groups
contr  0.9450  A
 1000  0.8750   B
    0  0.8700   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely randomized AOV for total
Source  DF        SS        MS       F        P
boron    2    8.3733   4.18667    6.90   0.0754
Error    3    1.8200   0.60667
Total    5   10.1933
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        2.52    2   0.2832
```

ii.3.5.2 Klason lignin assay of calcium-treated cultures

```
Completely randomized AOV for insoluble
Source   DF        SS        MS       F        P
calcium   2   11.4100   5.70500    5.47   0.0999
Error     3    3.1300   1.04333
Total     5   14.5400
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        0.29    2   0.8658

Completely randomized AOV for soluble
Source   DF        SS        MS       F        P
calcium   2   0.01373   0.00687    1.38   0.3763
Error     3   0.01495   0.00498
Total     5   0.02868
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        4.11    2   0.1279

Completely randomized AOV for total
Source   DF        SS        MS       F        P
calcium   2   11.2233   5.61167    5.87   0.0919
Error     3    2.8700   0.95667
Total     5   14.0933
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        0.32    2   0.8537
```

ii.3.5.3 Klason lignin assay of magnesium-treated cultures

Completely randomized AOV for insoluble

Source	DF	SS	MS	F	P
magnesium	2	12.0900	6.04500	7.34	0.0699
Error	3	2.4700	0.82333		
Total	5	14.5600			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.50	2	0.4718

Completely randomized AOV for soluble

Source	DF	SS	MS	F	P
magnesium	2	0.02243	0.01122	1.84	0.3004
Error	3	0.01825	0.00608		
Total	5	0.04068			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.42	2	0.4908

Completely randomized AOV for total

Source	DF	SS	MS	F	P
magnesium	2	11.0533	5.52667	6.85	0.0761
Error	3	2.4200	0.80667		
Total	5	13.4733			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.20	2	0.5500

ii.3.5.4 Klason lignin assay of mixed-treated cultures

Completely randomized AOV for insoluble

Source	DF	SS	MS	F	P
Mixed	2	22.3333	11.1667	5.80	0.0931
Error	3	5.7750	1.9250		
Total	5	28.1083			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.55	2	0.4606

Completely randomized AOV for soluble

Source	DF	SS	MS	F	P
Mixed	2	0.02363	0.01182	9.58	0.0498
Error	3	0.00370	0.00123		
Total	5	0.02733			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	2.26	2	0.3235

Completely randomized AOV for total

Source	DF	SS	MS	F	P
Mixed	2	21.2633	10.6317	5.40	0.1013
Error	3	5.9050	1.9683		
Total	5	27.1683			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.83	2	0.4012

ii.3.6 Acetyl bromide assay of nutrient treated cultures

The data presented include the combined statistical analysis of duplicate measurements of the nutrient treated cultures from two trees and their control explants.

ii.3.6.1 Acetyl bromide lignin assay of boron-treated cultures

Completely randomized AOV for acetyl

Source	DF	SS	MS	F	P
boron	2	119.593	59.7965	6.01	0.0892
Error	3	29.826	9.9419		
Total	5	149.419			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	5.14	2	0.0766

ii.3.6.2 Acetyl bromide lignin assay of calcium-treated cultures

Completely Randomized AOV for acetyl

Source	DF	SS	MS	F	P
calcium	2	8.1307	4.0654	0.21	0.8236
Error	3	58.8773	19.6258		
Total	5	67.0080			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	7.59	2	0.0225

ii.3.6.3 Acetyl bromide lignin assay of magnesium-treated cultures

Completely Randomized AOV for acetyl

Source	DF	SS	MS	F	P
mag	2	35.8529	17.9265	0.95	0.4797
Error	3	56.7327	18.9109		
Total	5	92.5856			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	6.05	2	0.0486

ii.3.6.4 Acetyl bromide lignin assay of mixed-treated cultures

Completely randomized AOV for acetyl

Source	DF	SS	MS	F	P
mixed	2	37.9808	18.9904	2.00	0.2802
Error	3	28.4478	9.4826		
Total	5	66.4287			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	5.02	2	0.0813

ii.4 Statistics for data presented in chapter seven

ii.4.1 Yariv-treated culture analysis of cell number, lumen area, length and width, and cell wall area

ii.4.1.1 Yariv-treated culture set number one. The tree was from Burnham, inducted in January and cultured for two months.

Cell counts

Completely Randomized AOV for cambial cell counts

Source	DF	SS	MS	F	P
media	3	13.061	4.35381	1.00	0.4111
Error	21	91.179	4.34184		
Total	24	104.240			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.14	3	0.7674

Completely Randomized AOV for radially expanding cell counts

Source	DF	SS	MS	F	P
media	3	27.486	9.16214	1.19	0.3373
Error	21	161.554	7.69303		
Total	24	189.040			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	9.67	3	0.0216

Completely Randomized AOV for new secondary wall cell counts

Source	DF	SS	MS	F	P
media	3	44.958	14.9860	2.85	0.0620
Error	21	110.482	5.2611		
Total	24	155.440			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.74	3	0.6286

Radially expanding cells: lumen area, length and width

Completely Randomized AOV for area

Source	DF	SS	MS	F	P
media	3	12996.8	4332.27	1.06	0.4166
Error	8	32560.2	4070.03		
Total	11	45557.0			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	0.47	3	0.9249

Completely Randomized AOV for length

Source	DF	SS	MS	F	P
media	3	22.1208	7.37360	3.09	0.0895
Error	8	19.0676	2.38345		
Total	11	41.1884			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	4.22	3	0.2385

Completely Randomized AOV for width

Source	DF	SS	MS	F	P
media	3	3.0703	1.02343	0.47	0.7133
Error	8	17.5274	2.19092		
Total	11	20.5977			

```
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        0.50    3   0.9191
```

Radially expanding cells: wall area

```
Completely Randomized AOV for wall
Source   DF        SS          MS       F        P
media     3   0.00093   3.097E-04    4.04   0.0506
Error     8   0.00061   7.658E-05
Total    11   0.00154
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        2.82    3   0.4202

Tukey HSD All-Pairwise Comparisons Test of wall by media

media      Mean  Homogeneous Groups
control  0.1647  A
alpha    0.1523  AB
NAA      0.1466  AB
beta     0.1354   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Existing cells: lumen area, length and width

```
Completely Randomized AOV for area
Source   DF       SS        MS       F        P
media     3   136858   45619.4    10.9   0.0010
Error    12    50182    4181.8
Total    15   187040
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        7.96    3   0.0469

Tukey HSD All-Pairwise Comparisons Test of area by media

media      Mean  Homogeneous Groups
NAA      1001.2  A
control  976.92  A
alpha    809.89   B
beta     800.17   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely Randomized AOV for length
Source   DF        SS        MS       F        P
media     3    77.487   25.8290    7.21   0.0050
Error    12    42.976    3.5813
Total    15   120.463
                                        Chi-Sq   DF        P
Bartlett's Test of Equal Variances        1.56    3   0.6679

Tukey HSD All-Pairwise Comparisons Test of length by media

media      Mean  Homogeneous Groups
control  41.352  A
NAA      40.796  A
```

```
alpha    36.787   B
beta     36.597   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.

Completely Randomized AOV for width
Source   DF        SS        MS       F        P
media     3   33.4730   11.1577    7.66   0.0040
Error    12   17.4733    1.4561
Total    15   50.9463
                                     Chi-Sq   DF        P
Bartlett's Test of Equal Variances     4.67    3   0.1976

Tukey HSD All-Pairwise Comparisons Test of width by media

media      Mean  Homogeneous Groups
control  29.955  A
NAA      29.921  A
alpha    27.105   B
beta     26.988   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Existing cells: wall area

```
Completely Randomized AOV for wall
Source   DF        SS        MS       F        P
media     3   0.03012   0.01004    13.5   0.0004
Error    12   0.00894   0.00075
Total    15   0.03906
                                     Chi-Sq   DF        P
Bartlett's Test of Equal Variances     2.09    3   0.5539

Tukey HSD All-Pairwise Comparisons Test of wall by media

media      Mean  Homogeneous Groups
alpha    0.3137  A
beta     0.3129  A
NAA      0.2752  A
control  0.2071   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

ii.4.1.2 Yariv-treated culture set number two. The tree was from Rotorua, inducted in April and cultured for two months.

Cell counts

```
Completely Randomized AOV for cambial cell counts
Source   DF        SS        MS       F        P
media     2    3.6964   1.84821    0.42   0.6626
Error    17   74.5036   4.38256
Total    19   78.2000
```

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	0.18	2	0.9154

Completely Randomized AOV for radially expanding cell counts

Source	DF	SS	MS	F	P
media	2	1.446	0.7232	0.06	0.9457
Error	17	219.504	12.9120		
Total	19	220.950			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	5.04	2	0.0804

Completely Randomized AOV for new secondary wall cell counts

Source	DF	SS	MS	F	P
media	2	14.561	7.28036	1.03	0.3789
Error	17	120.389	7.08172		
Total	19	134.950			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	5.63	2	0.0600

Radially expanding cells: lumen area, length and width

Completely Randomized AOV for area

Source	DF	SS	MS	F	P
media	2	10964.7	5482.37	0.69	0.5313
Error	8	64008.6	8001.07		
Total	10	74973.3			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	1.78	2	0.4105
Cochran's Q	0.5008		

Completely Randomized AOV for length

Source	DF	SS	MS	F	P
media	2	17.5382	8.76911	2.15	0.1793
Error	8	32.6708	4.08384		
Total	10	50.2090			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	2.74	2	0.2547

Completely Randomized AOV for width

Source	DF	SS	MS	F	P
media	2	6.3471	3.17356	1.06	0.3905
Error	8	23.9528	2.99410		
Total	10	30.2999			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	0.95	2	0.6214

Radially expanding cells: wall area

Completely Randomized AOV for wall

Source	DF	SS	MS	F	P
media	2	0.00080	3.984E-04	0.44	0.6589
Error	8	0.00725	9.058E-04		
Total	10	0.00804			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	0.59	2	0.7453

Existing cells: lumen area, length and width

```
Completely Randomized AOV for area
Source   DF       SS        MS       F        P
media     3    27397   9132.50    1.12   0.3786
Error    12    97615   8134.57
Total    15   125012
                                      Chi-Sq   DF        P
Bartlett's Test of Equal Variances      1.61    3   0.6570
Cochran's Q                 0.3497

Completely Randomized AOV for length
Source   DF       SS        MS       F        P
media     3   28.2499   9.41665    1.69   0.2210
Error    12   66.6893   5.55744
Total    15   94.9392
                                      Chi-Sq   DF        P
Bartlett's Test of Equal Variances      0.31    3   0.9576

Completely Randomized AOV for width
Source   DF       SS        MS       F        P
media     3   18.8833   6.29442    3.77   0.0406
Error    12   20.0095   1.66746
Total    15   38.8928
                                      Chi-Sq   DF        P
Bartlett's Test of Equal Variances      0.42    3   0.9367
Tukey HSD All-Pairwise Comparisons Test of width by media

media      Mean  Homogeneous Groups
alpha    31.161  A
beta     29.999  AB
control  29.416  AB
NAA      28.145   B

Alpha              0.05
There are 2 groups (A and B) in which the means are not significantly
different from one another.
```

Existing cells: wall area

```
Completely Randomized AOV for wall
Source   DF       SS        MS       F        P
media     3  0.00955   0.00318    1.41   0.2877
Error    12  0.02708   0.00226
Total    15  0.03663
                                      Chi-Sq   DF        P
Bartlett's Test of Equal Variances      1.61    3   0.6570
```

ii.4.1.3 Yariv-treated culture set number three. The tree was from Rotorua, inducted in April and cultured for two months.

Cell counts

```
Completely Randomized AOV for cambial cell counts
Source   DF       SS        MS       F        P
media     3  116.591   38.8637    8.86   0.0001
Error    39  171.083    4.3868
Total    42  287.674
```

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	10.6	3	0.0141

Tukey HSD All-Pairwise Comparisons Test of cambial by media

media	Mean	Homogeneous Groups
NAA	19.222	A
alpha	18.889	A
control	16.889	AB
beta	15.375	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Completely Randomized AOV for radially expanding cell counts

Source	DF	SS	MS	F	P
media	3	171.276	57.0919	6.61	0.0010
Error	39	336.771	8.6351		
Total	42	508.047			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	6.02	3	0.1108

Tukey HSD All-Pairwise Comparisons Test of re by media

media	Mean	Homogeneous Groups
NAA	19.222	A
control	16.889	A
beta	16.313	AB
alpha	13.111	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Completely Randomized AOV for new secondary wall cell counts

Source	DF	SS	MS	F	P
media	3	169.907	56.6357	4.64	0.0072
Error	39	476.000	12.2051		
Total	42	645.907			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	16.8	3	0.0008

Tukey HSD All-Pairwise Comparisons Test of secondary by media

media	Mean	Homogeneous Groups
alpha	15.000	A
control	12.667	AB
beta	11.500	AB
NAA	9.0000	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Radially expanding cells: lumen area, length and width

Completely Randomized AOV for area

Source	DF	SS	MS	F	P
media	3	12620.9	4206.97	1.44	0.2804
Error	12	35102.5	2925.21		
Total	15	47723.4			

				Chi-Sq	DF	P
Bartlett's Test of Equal Variances				1.59	3	0.6621

Completely Randomized AOV for length

Source	DF	SS	MS	F	P
media	3	10.9696	3.65655	2.02	0.1647
Error	12	21.7053	1.80877		
Total	15	32.6749			

				Chi-Sq	DF	P
Bartlett's Test of Equal Variances				2.52	3	0.4716

Completely Randomized AOV for width

Source	DF	SS	MS	F	P
media	3	0.4356	0.14519	0.10	0.9576
Error	12	17.1650	1.43041		
Total	15	17.6005			

				Chi-Sq	DF	P
Bartlett's Test of Equal Variances				2.69	3	0.4423

Radially expanding cells: wall area

Completely Randomized AOV for wall

Source	DF	SS	MS	F	P
media	3	0.00061	2.039E-04	1.02	0.4172
Error	12	0.00239	1.995E-04		
Total	15	0.00301			

				Chi-Sq	DF	P
Bartlett's Test of Equal Variances				0.01	3	0.9995

Existing cells: lumen area, length and width

Completely Randomized AOV for area

Source	DF	SS	MS	F	P
media	3	176265	58755.2	11.2	0.0016
Error	10	52518	5251.8		
Total	13	228783			

				Chi-Sq	DF	P
Bartlett's Test of Equal Variances				4.25	3	0.2361

Tukey HSD All-Pairwise Comparisons Test of area by media

media	Mean	Homogeneous Groups
beta	946.30	A
alpha	719.06	B
NAA	710.87	B
control	652.46	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Completely Randomized AOV for length

Source	DF	SS	MS	F	P
media	3	128.559	42.8532	4.71	0.0267
Error	10	90.980	9.0980		
Total	13	219.539			

				Chi-Sq	DF	P
Bartlett's Test of Equal Variances				8.20	3	0.0421

Tukey HSD All-Pairwise Comparisons Test of length by media

media	Mean	Homogeneous Groups
beta	41.271	A
NAA	37.331	AB
alpha	34.736	AB
control	32.833	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Completely Randomized AOV for width

Source	DF	SS	MS	F	P
media	3	47.6343	15.8781	13.9	0.0007
Error	10	11.4058	1.1406		
Total	13	59.0402			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.52	3	0.3177

Tukey HSD All-Pairwise Comparisons Test of width by media

media	Mean	Homogeneous Groups
beta	29.683	A
alpha	26.728	B
NAA	25.768	B
control	24.502	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

Existing cells: wall area

Completely Randomized AOV for wall

Source	DF	SS	MS	F	P
media	3	0.06849	0.02283	11.3	0.0015
Error	10	0.02021	0.00202		
Total	13	0.08870			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	2.01	3	0.5703

Tukey HSD All-Pairwise Comparisons Test of wall by media

media	Mean	Homogeneous Groups
control	0.3518	A
NAA	0.2441	AB
beta	0.1607	B
alpha	0.1510	B

Alpha 0.05
There are 2 groups (A and B) in which the means are not significantly different from one another.

ii.4.2 Acetyl bromide assay of Yariv-treated cultures

The data presented include the combined statistical analysis of duplicate measurements of the Yariv treated cultures from two trees and their control explants.

ii.4.2.1 Acetyl bromide lignin assay of Yariv-treated cultures

Completely Randomized AOV for ABL

Source	DF	SS	MS	F	P
media	2	126.711	63.3553	0.71	0.5191
Error	9	807.872	89.7635		
Total	11	934.582			

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	4.61	2	0.0998