HORMONAL CONTROL OF WOOD FORMATION IN RADIATA PINE

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ABSTRACT

Pinus radiata is by far the dominant species grown in New Zealand plantations as a renewable source of wood. Several wood quality issues have been identified in the material produced, including the high incidence of compression wood, which is undesirable for end users. At present our understanding of the complex array of developmental processes involved in wood formation (which has a direct bearing on wood quality) is limited. Hence, the forest industry is interested in attaining a better understanding of the processes involved. Towards this goal, and for reasons of biological curiosity, the experiments described in this thesis were carried out to investigate several aspects of xylem cell development.

In an *in arbor* study, changes in the orientation of cortical microtubules and cellulose microfibrils were observed in developing tracheids. Results obtained provide evidence that cortical microtubules act to guide cellulose synthase complexes during secondary wall formation in tracheids.

The mechanisms involved in controlling cell wall deposition in wood cells are poorly understood, and are difficult to study, especially *in arbor*. A major part of this thesis involved the development of an *in vitro* method for culturing radiata pine wood in which hormone levels, nutrients, sugars and other factors, could be controlled without confounding influences from other parts of the tree. The method developed was used in subsequent parts of this thesis to study compression wood development, and the influence of the hormone gibberellin on cellulose microfibril organisation in the cell wall.

Results from the *in vitro* compression wood experiments suggested that:

1. when a tree is growing at a lean, the developing cell wall was able to perceive compressive forces generated by the weight of the rest of the tree, rather than perceive the lean *per se*.
2. ethylene, rather than auxin, was involved in the induction of compression wood.

Culture of stem explants with gibberellin resulted in wider cells, with steeper cortical microtubules, and correspondingly steeper cellulose microfibrils in the S_2 layer of developing wood cells. This observation provides further evidence that the orientation of microtubules guides the orientation of cellulose microfibrils.

Overall, the work described in this thesis furthers our knowledge in the field of xylem cell development. The stem culture protocol developed will undoubtedly provide a valuable tool for future studies to be carried out.

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Chapter 1

Xylogenesis in Radiata Pine & Thesis Introduction

1.1 INTRODUCTION

Pinus radiata in New Zealand is generally managed on a relatively short rotation, and as a consequence a high proportion of juvenile wood is harvested. A steep (low) cellulose microfibril angle is a desirable characteristic in lumber, as it is correlated with high stiffness (Cave and Walker, 1994). However, juvenile wood does not possess this characteristic. A further problem for the industry is the high incidence of compression wood, which has undesirable characteristics for end users. At present our understanding of the complex array of processes involved in xylem development is limited, and little is known about how the angle of cellulose microfibril deposition is governed, and how compression wood forms. Hence, the forest industry has an interest in elucidation of these processes, since this may eventually lead to ways of producing higher quality wood.

These questions, and others pertaining to xylogenesis, and vascular differentiation in general, are also of fundamental interest to biologists. However, compared to many aspects of plant growth and development, there has been relatively little research focusing on the secondary vascular system (Chaffey, 2002b). Chaffey (2002b) outlines possible reasons for this lack of research interest in the past: "…studying wood formation is too difficult, there is no model species, tree biology is not sufficiently interesting". However, he points out that these perceived barriers have now been largely overcome, and "the time is now right for much greater exploitation of the possibilities that exist for the study of the secondary vascular system of trees". In part the perceived difficulties have been overcome by the development of new research techniques.

The present study investigates some of the mechanisms involved in *P. radiata* tracheid development. Chapter 2 covers a study of the role of cortical microtubules in controlling cellulose microfibril deposition in the tracheid cell wall. Chapter 3

describes an *in vitro* culture protocol that was developed especially to grow *P. radiata* stem explants. The major impetus behind development of this protocol was to allow further studies of xylem development, outside the confounding influences of other parts of the tree. These methods were used in the investigations covered in the subsequent chapters. Chapter 4 investigates whether compression wood formation is dependant on biophysical stress. The role of hormones in compression wood formation is dealt with in Chapter 5. This involves two experiments, one looking at auxin and the other ethylene, as possible mediators of compression wood development. Finally, in Chapter 6, an experiment investigating the influence of gibberellin on cortical microtubule and cellulose microfibril orientation in developing tracheids is described.

1.2 XYLOGENESIS

1.2.1 Vascular cambium

The vascular cambium, which gives rise to the tissues of the secondary plant body, originates from the procambium of the primary plant body. At the inception of secondary growth the small arcs of fascicular cambium, from which the primary vascular bundles arose, become interconnected by additional arcs of interfascicular cambium (Esau, 1965). Thus, a complete cylinder of vascular cambium is formed. The cambium is sometimes theoretically considered to consist of a single layer of 'initial' cells, the division of which produces 'derivatives', although such a layer is not usually distinguishable microscopically (Steeves and Sussex 1989). Alternatively, the term cambium may be used to describe the entire region of cell division (Steeves and Sussex, 1989), which is several cells wide. This situation has led to controversy regarding terminology (Savidge, 2000a). Here, the terms cambium, vascular cambium and cambial region will be used interchangeably to describe the region of cell division. In practice this is discernible, sandwiched between the adjacent developing phloem and xylem, in which cell expansion in the radial direction can be perceived.

1.2.2 Radial expansion

Tracheids arise from centripetal periclinal divisions of the fusiform cambial initials within the vascular cambium (Essau, 1965). The newly formed derivatives then

undergo a period of radial expansion, while at the same time laying down the primary wall. In the present thesis, the region in which this is taking place will be referred to as the zone of radial expansion (Savidge, 2000a).

1.2.3 Secondary wall formation and protoplasmic autolysis

At the cessation of radial expansion, three secondary wall layers are deposited on the internal side of the primary wall. Following this protoplasmatic autolysis occurs (Savidge, 2000a), giving rise to the hollow water conducting lumen of the mature tracheid.

1.3 THE CELL WALL

The plant cell wall is a characteristic component of most plant cells, and consists of a reinforcing framework of long thin cellulose microfibrils embedded within a complex wall matrix.

Microfibrils are made up of cellulose, which is an unbranched polymer of thousands of β[1-4] linked glucose sugar units arranged in a long chain. Hydrogen bonding between parallel aggregates of cellulose molecules produces crystalline microfibrils (Thain and Hickman, 1996). Due to this structure, microfibrils possess great tensile strength; for a given thickness, their strength may exceed that of steel (Raven et al., 1992). Each microfibril is approximately 10 nm in diameter, but their length is uncertain (Brett and Waldron, 1996).

The cell wall matrix, in which the cellulose microfibrils are embedded, is non-crystalline and consists of a complex array of pectins, hemicelluloses, proteins and phenolic compounds (Brett and Waldron, 1996). The pectins are a group of acid polysaccharides present in unlignified cell walls (Thain and Hickman, 1996). Pectins bond with each other, and may also bond to cellulose, proteins, phenols (Brett and Waldron, 1996), and cations (Thain and Hickman, 1996) forming a gel-like gap-filling substance. Hemicelluloses are a group of branched long-chain polysaccharides that hydrogen bond to cellulose, and thus provide strong cross links between adjacent microfibrils (Brett and Waldron, 1996). Lignin is a phenolic polymer that is laid down

in some specialised cell types, such as tracheids and vessel elements. The chemical bonds formed by lignin are such that it tends to fill all unoccupied space in the wall and cements the other wall components together (Brett and Waldron, 1996).

This complex structure provides the cell wall with interesting physical properties; at completion it is strong and stiff enough to provide support to the entire plant, yet during the early stages of development it is plastic enough to allow changes in cell size and shape. These properties are largely a consequence of the way in which the strong thin cellulose microfibrils are bound within the wall matrix. Since cellulose microfibrils contribute so greatly to the biophysical properties of the cell wall, biologists are interested in understandings the mechanisms that control their deposition and orientation.

Plant cells are able to regulate the orientation of cellulose microfibrils as they are laid down adjacent to the plasma membrane during cell wall development. The orientation of cellulose microfibril deposition may change during normal developmental processes, and also as a result of change in the plant's environment.

An example of environment influencing cellulose microfibril orientation is the formation of reaction wood in trees in response to asymmetric loading. Reaction wood (compression wood in gymnosperms, tension wood in angiosperms) formation provides growth strains that are able to adjust a leaning tree stem towards vertical (Wilson, 1981). The characteristics of compression wood in gymnosperms and tension wood in angiosperms are very different, and the present discussion will be limited to compression wood.

Compression wood is an evolutionary adaptation that occurs in stems and branches in response to asymmetric loading, which may be imposed by a variety of external environmental events, such landslides and wind (both windthrow and the swaying action imposed by day-to-day winds). It is particularly common on the underside of branches, below their points of attachment to the stem, and on the lower side of stems displaced from vertical. Compression wood formed on the compressed side of a branch or stem has the ability to expand in the longitudinal direction, providing a means for a tree to self-correct for displacement. This expansive strain originates from

the ability of the compression wood tracheids to expand during development. This ability is partially a consequence of the cellulose microfibril angle in compression wood, which is unusually high compared to that in normal wood (Wilson, 1981). Hence, it is apparent that cells are able to reorient the deposition of cellulose microfibrils in response to the environment in which they develop.

Cellulose microfibril angle also changes as development of the cell wall proceeds. In fact the orientation of cellulose microfibrils has a major bearing on the course of plant cell development; during growth plant cells tend to expand perpendicular to the orientation of cellulose microfibrils (Green, 1980). Thus, cellulose microfibril orientation has a major role in defining cell shape. Transversely orientated microfibrils occur in long thin cells, longitudinally orientated microfibrils occur in short fat cells, and randomly orientated microfibrils occur in isodiametric cells.

On the basis of development and structure, three parts are generally recognised in plant cell walls: the middle lamella, the primary wall, and the secondary wall (Esau, 1965). At the cessation of cell growth some cells remain with just a primary wall, whereas other specialised cells such as tracheids lay down a further secondary wall (Brett and Waldron, 1996). The secondary wall of tracheids is comprised of three layers, the S_1, S_2 and S_3 layers, the subscript denoting the order in which they were laid down during development (Barnett et al., 1998). The S_2 layer is considered to have the greatest effect on wood properties such as stiffness (Cave and Walker, 1994; Walker and Woollons, 1998) and it is to this layer that cellulose microfibril angle generally refers (Barnett et al., 1998).

1.4 THE CYTOSKELETON & MICROFIBRIL ORIENTATION

1.4.1 Do cortical microtubules guide microfibril orientation?

Cellulose microfibrils are purported to be laid down by dynamic transmembrane protein complexes known as cellulose synthesising complexes or rosettes (Brown and Montezinos, 1976; Higuchi, 1997; Richmond, 2000). The mechanisms involved in controlling this process, and hence, cellulose microfibril orientation are poorly understood.

One possibility is that the plant regulates the orientation of cellulose microfibril deposition biochemically, as outlined in the following model:

plant hormones
e.g. gibberellin (Gahan, 1988)
↓
receptors
↓
message transmittance
(1) directly e.g. acid growth hypothesis (Brett and Waldron, 1996). Increased activity of proton-pumping ATPase enzymes in plasma membrane.
(2) change in gene expression
↓
cortical microtubule reorganisation
↓
change in direction of rosette motility
↓
change in cellulose microfibril orientation

A major priori of this model is that cortical microtubules act to guide the deposition of microfibrils. This view, now widely accepted, is based on concepts such as those proposed by the Channeling Hypothesis (Herth, 1980; Herth, 1985) and the Unified Hypothesis (Heath, 1974) (Figure 1.1). The microtubule hypotheses have been questioned since microtubules and microfibrils are not always observed to be lying parallel to one another and when they are this may be coincidental, and synthesis of highly oriented microfibrils can occur in the absence of microtubules (Preston, 1988; Emons et al., 1992). However, evidence does suggest that in pine tracheids cortical

microtubules are involved in the *establishment* of cellulose microfibril orientation (Wilkes, 1999).

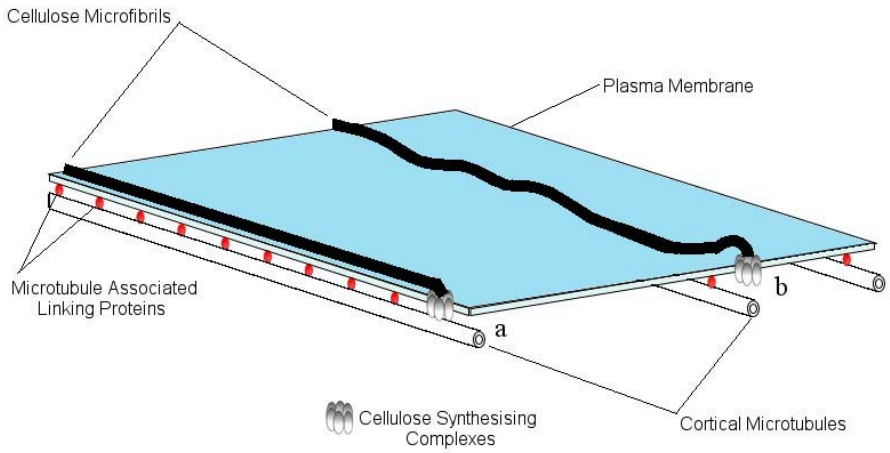


Figure 1.1: *Models for how cortical microtubules guide deposition of cellulose microfibrils.* ***(a)*** *Unified hypothesis (Heath, 1974). In this model, Heath suggested that a component associated with the synthesising complexes projects through to the inside of the plasma membrane and interacts with the adjacent microtubules to generate a sliding force which moves the complexes through the membrane along the track-like microtubules.* ***(b)*** *Channelling hypothesis (Herth, 1980; Herth, 1985). Herth suggested that the aggregation of cellulose subunits to microfibrils leads to a force, which drives the complexes in a direction channeled by the cortical microtubules.*

A second possibility is that cellulose microfibrils are oriented biophysically, by forces produced during cell expansion (Preston, 1974; Boyd, 1985). Numerous biophysical models have been proposed (see Boyd, 1985). Consideration of the following illustrates this concept. In the case of a cell with helically arranged microfibrils, such as a developing tracheid, cell expansion is restricted transversely, and occurs preferentially in the axial direction, altering microfibril orientation by ‘slippage’ as it proceeds (Boyd, 1985). The same forces involved in altering microfibril orientation might then be involved in reorientation of cortical microtubules. Biophysical models have often been based on data from secondary wall development of a limited number of cell types and species. According to Boyd (1985) there is insufficient data to allow

a rigorous analysis of cell wall structure, especially that pertaining to the extension growth phase of development (primary wall development). Biophysical models can also be criticised in that they fail to explain how cellulose microfibril orientation is responsive to the environment, and may change markedly during development. Biochemical models can also been criticised. These are often based on studies of single cell cultures, in which cells do not experience the normal biophysical environment provided by adjacent cells in intact tissues, or studies of primary wall development, where the biophysical stresses imposed by cell growth confound the study. Non-ideal culture conditions and wound responses associated with cell preparation are further problems of such studies (Leitch and Savidge, 2000).

One study has attempted to circumvent these problems. Wilkes (1999) investigated the role of cortical microtubules in the reorientation of S_2 wall layer cellulose microfibrils in response to leaning stress in the stem of *P. radiata*. The orientation of cellulose microfibrils changed in response to leaning. Since cell expansion has ceased at the time of S_2 wall layer deposition, a change in growth rate cannot account for the change in cellulose microfibril orientation. Additionally when cortical microtubules were disrupted, cellulose microfibril reorientation failed to occur in response to leaning stress. The findings of this study suggested that cellulose microfibril reorientation is governed biochemically rather than biophysically.

According to the biochemical model, hormones provide the initial impetuous for changing cellulose microfibril orientation. These hormonal signals may include the basipetal flow of auxin and the acropetal flow of cytokinins (Higuchi, 1997). In addition gibberellin in conjunction with auxin is involved in modifying the cells developmental pathway (Little and Pharis, 1995; Brett and Waldron, 1996; Higuchi, 1997). Other substances, such as sucrose also appear to be important in this process (Higuchi, 1997).

1.4.2 Are actin microfilaments involved in microfibril orientation?

Compared with microtubules, there has been relatively little published on the role of actin microfilaments during xylem differentiation. There have been a few published studies pertaining to the angiosperm *Aesculus hippocastanum* L. (horse-chestnut) (Chaffey et al., 1997; Chaffey et al., 2000), and to date one paper pertaining to

gymnosperms (Funada et al., 2000). Studies involving differentiation of tracheary elements from *Zinnia* mesophyll cells have suggested that actin microfilaments might be involved in control of microtubule orientation (Kobayashi et al., 1988). However, the differentiation of tracheary elements from *Zinnia* mesophyll cells is not considered to reliably represent that of normal plant behaviour (Burgess and Linstead, 1984).

The latest findings based on immunofluorescent studies of tissue from intact trees suggest that actin microfilaments might not play a major role in directing the orientation of cortical microtubules during tracheary element differentiation (Funada et al., 2000). Cambial cells possess bundles of microfilaments arranged in a very steeply angled helix, and this arrangement is retained during the differentiation of cambial derivatives into tracheary elements in both angiosperms (Chaffey et al., 2000) and gymnosperms (Funada et al., 2000). Additionally, Chaffey (2000) discusses other microfilament arrays which appear to be associated with pit and perforation plate formation in vessel elements. Chaffey (2000) also discusses the probable role of microfilaments in cytoplasmic streaming, which is a prominent feature of cambial cells.

1.4.3 The influence of cellulose deposition on microtubules

Fisher and Cyr (1998) investigated the effects of the cellulose biosynthesis inhibitor isoxaben on tobacco cells in culture. Treated cells failed to elongate and produce secondary walls, and cortical microtubules failed to become organised as normally occurs. Fisher and Cyr (1998) suggested that not only do cortical microtubules guide deposition of cellulose microfibrils, but also the mechanical properties of the cell wall provide biophysical information back to the cortical microtubules to guide their organisation.

1.5 COMPRESSION WOOD & BIOPHYSICAL STRESS

1.5.1 Why compression wood is a problem

Compression wood has several characteristics that make it undesirable for many end uses.

1.5.1.1 Lumber –longitudinal shrinkage

On drying, compression wood has different shrinkage characteristics compared to side (normal) wood or opposite wood. In *P. radiata* longitudinal shrinkage can be as high as 5% in compression wood compared to less than 1% in normal wood (Harris, 1977). Hence, if a board contains an uneven distribution of compression wood and opposite wood it will warp on drying. Numerous studies have shown this to be so, at least with respect to crook and bow (Beard et al., 1993; Perstorper et al., 1995; Sandberg, 1997). The problem is by no means restricted to the presence of severe compression wood. Samples containing compression wood categorised as mild have also been shown to have high incidence of crook and bow (Donaldson and Turner, 2001; Johansson, 2003). In practical terms mild compression wood is probably far worse than severe compression wood. Lumber containing the latter can often be visually detected (due to its dark colouration) and discarded during grading, while visual detection of the former is unlikely to occur.

1.5.1.2 Pulp and paper –high lignin low cellulose

The presence of mature compression wood has been shown to pose difficulties during the kraft pulping process, not least of which is a reduction in pulp yield relative to that obtained from normal wood (Ban et al., 2004). The poor pulp yield from compression wood has been attributed to its higher lignin and lower cellulose content, compared with normal wood (Lohrasebi et al., 1999). This fundamental chemical difference also implies that pulping of timber high in compression wood will produce more residual lignin.

Compression wood is also problematic in that it affects the quality of paper that can be produced. It has been shown that paper made from compression wood containing pulp has inferior strength, light absorption and surface properties (Sahlberg, 1995). With respect to the latter, it has been suggested that the presence of compression wood tracheids in thermo-mechanical pulp might contribute to an increase in surface roughness of printer paper (Brandstrom, 2004).

1.5.2 Differences between compression wood and normal wood

1.5.2.1 Anatomical

The anatomical characteristics of compression wood differ in many ways, compared to normal and opposite wood. The differences in *P. radiata* have been well documented by Harris (1977), and more thoroughly and recently by Eom and Butterfield (1997), and information from these papers is summarised as follows.

When compared with normal wood, compression wood has:

1. wider growth rings
2. tracheids circular in cross-section, with accompanying intercellular spaces
3. darker brown, or reddish brown colour
4. often a more gradual transition from earlywood to latewood
5. fewer resin canals
6. tracheids shorter, and narrower with thicker walls
7. distorted tracheid tips, and bent (L-shaped) tracheids
8. S_1 layer thicker
9. S_2 layer with higher microfibril angle, helical fissures, and highly lignified outer layers and corners
10. S_3 layer absent
11. middle lamella more highly lignified.

1.5.2.2 Chemical

The chemical composition of compression wood differs markedly from that found in normal (side or lateral wood) and opposite wood, which are both very similar (Timell, 1973). In *P. radiata*, recent analysis has shown that mild compression wood can have 15-18% and severe compression wood over 38% more lignin than opposite wood (Nanayakkara et al., 2005). These authors also reported that the lignin structure varied, depending on the severity of compression wood present. In particular, as compression wood severity increased the amount of p-hydroxyphenol β-O-4 units and condensed guaiacyl (G) content increased, while uncondensed G content decreased. Additionally, the distribution of lignin across the various cell wall layers differs in compression wood, when compared to normal wood (see review by Donaldson, 2001). In *P. thunbergii* Fukushima and Terashima (1991) showed that the deposition

of lignin in the wall of compression wood occurred in the order: p-hydroxyphenyl, guaiacyl and syringyl lignin, which is the same order of deposition that occurs in normal wood. These workers accounted for the above-mentioned differences in lignin distribution, by showing that the duration of lignin deposition, within the various wall layers, differed between the two wood types.

Compression wood also has different carbohydrate composition in comparison to normal wood. In *P. radiata*, analysis has shown galactose residues to be up to 4.5 times higher in severe compression wood, compared to opposite wood, while in mild compression wood levels were intermediate (Nanayakkara et al., 2005). Newman et al. (2005) further showed that these galactose levels were restricted to red early wood, while in brown latewood levels were about 2 times that found in pale wood (presumably the red and brown woods were compression wood, and the pale wood was normal wood). In contrast to galactose, residues of arabinose, glucose, xylose and mannose were reported to be lower in compression wood, compared to normal wood (Nanayakkara et al., 2005). Again, this trend was more marked in severe compression wood, while in mild compression wood levels were intermediate.

1.5.3 Functional significance of compression wood

In the main stem of conifers (i.e. excluding branches and the area of the stem where branches attach to the stem), the most severe compression wood occurs on the underside of leaning trees. Early workers appear to have found it controversial as to whether the differentiation of compression wood acted to upwardly bend (Munch 1938, cited in Wardrop and Davies, 1964), and thus right a stem misplaced from vertical, or simply acted to resist compressive forces – the righting of the stem being effected by some other physiological process (Frey-Wyssling 1952, cited in Wardrop and Davies, 1964). Some early workers (e.g. Munch 1938, cited in Boyd, 1973) speculated that lignification of the cell wall might give rise to expansive forces during tracheid development. Later, based on analysis of available data, and consideration of the mechanics involved Boyd (1972) showed that the lignification process in normal wood tracheids could lead to transverse swelling of the developing wall. Later, he showed that development of the secondary wall in compression wood tracheids could give rise to longitudinal expansive forces of around 10 Mpa, provided the microfibril angle of the S_2 layer was sufficiently low (Boyd, 1973). Boyd considered that the

combination of these forces and others throughout a leaning stem would be sufficient to return a tree back to vertical. In this paper, Boyd's analysis also showed that forces generated during primary wall development were insignificant, and could not be involved in generating forces involved in righting a leaning stem. Additionally, Boyd (1973) showed that if the microfibril angle in the S_2 layer of compression wood tracheids was greater than 45^o, then lignification could also give rise to the helical fissures observed on their inner surface.

Bamber (2001) refuted the above theory (what he refers to as the "lignin swelling theory"), for several rather convincing reasons. He points out that the concept of lignin swelling leading to transverse swelling of the developing wall does not agree with changes observed during development of the secondary wall; in transverse section the wall is initially squarish, and as tracheids mature it becomes rounded. Hence, this change suggests contraction rather than expansion. Instead, he suggested that cellulose microfibrils behave as helical springs, which are laid down in a compressed state, and exert a force that acts to right or stabilise the tree. Bamber also suggested that lignin was not involved in the generation of growth stresses, and its only function was to cement the microfibrils together, thereby ensuring the transmission of stresses through the wood.

Attempts have been made to incorporate both hypotheses into a workable explanation of how growth stresses arise (e.g. Yamamoto, 1998). However, it is apparent that it is still unclear as to how compression wood functions, and generates compressive stresses (Burgert et al., 2004).

1.5.4 Compression wood development

The dividing cambial cells that give rise to compression wood are similar in size and shape to those that give rise to normal wood (Timell, 1986, page 639). In the radial expansion zone the cambial cell derivatives, which at this time are surrounded by the thin extensible primary wall, expand both longitudinally and transversely. Following this, the secondary wall layers are deposited. In *P. radiata* it has been shown that the round shape characteristic of compression wood tracheids (when viewed in transverse section) arises either during primary wall (Wardrop and Dadswell, 1952), or S_1 wall (Wardrop and Davies, 1964) development. The former paper dealt with branch wood,

while the latter dealt with stem wood of unspecified age (although the large cell size suggests it was wood from a mature tree). In normal wood tracheids, it is generally agreed that the secondary wall is only laid down after radial expansion has ceased, and cells have reached their full size. However, it appears that developing compression wood tracheids are able to change their shape during secondary wall deposition, at least up until the completion of the S_1 layer (Timell, 1986, page 646).

1.5.5 Fundamental factors causing compression wood formation

Timell (1986, pages 983-1103) provides a thorough review of the possible fundamental factors that might cause compression wood formation. He discusses numerous experiments that dealt with nutritional differences, light, bark pressure, compressive and tensile stresses and gravity. In conclusion of his extensive review Timell states “It is clear that we are still far from a solution to the compression wood enigma…. At the present time all they can be said is that gravity must be involved at some stage, but that it probably does not function as the primary cause, at least not invariably.” In the ensuing two decades it is apparent that we have made little progress toward answering this question, although a recent experiment has shown that compression wood can be induced in trees in the microgravity environment of space (Kwon et al., 2001).

1.6 THE INFLUENCE OF HORMONES ON TRACHEID DEVELOPMENT

1.6.1 Auxin

Auxins are defined as organic substances that promote cell elongation in plant tissue segment bioassays, when applied in low concentrations (Normanly et al., 2004). The main naturally occurring auxin in most plants is indole-3-acetic acid (IAA) (Davies, 2004) (Figure 1.2). Additionally, other less well studied auxins are also present in plants: indole-3-butyric acid (IBA), phenylacetic acid (PAA) and the halogenated IAA derivative 4-chloroindole-3-acetic acid (4-Cl-IAA) (Normanly et al., 2004). Besides these naturally occurring auxins, numerous compounds that display auxin activity have been artificially synthesised. For example, naphthalene acetic acid (NAA), the auxin used in the present study.

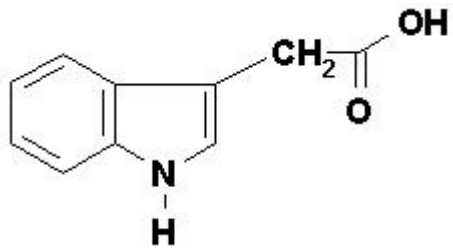


Figure 1.2: *IAA structure*

1.6.1.1 Biosynthesis and regulation

IAA is synthesised *de novo* from tryptophan or indolic precursors, via several different pathways (Ljung et al., 2002; Normanly et al., 2004), mainly in leaf primordia and young leaves (Davies, 2004). Other sources of free, bioactive IAA within plant tissues include: hydrolysis of amide and ester linked IAA conjugates, conversion of IBA to IAA and importation from another site in the plant (Normanly et al., 2004). Conversely, IAA can be deactivated by oxidative catabolism, conjugate synthesis, conversion to IBA, and can be transported away from a given tissue (Normanly et al., 2004). In short, the biosynthesis, inactivation, transport (see below) and interconversion pathways that regulate and maintain auxin levels are very complex. It is apparent that plants have evolved complex regulatory networks, with considerable redundancy and adaptive plasticity, in order to maintain or adjust auxin levels as required in response to changing environmental and developmental conditions (Normanly et al., 2004).

1.6.1.2 Transport

Auxin transport is a dynamic, complex process that is regulated at several different levels (Morris et al., 2004). In the secondary body of trees, there are three pathways for auxin transport. Firstly, the internal route, in which IAA (moving down from the primary shoot) descends in the cambium and differentiating xylem. Secondly, the peripheral route, in which IAA (originating from epidermal cells) descends in the phellogen. Thirdly, the non-polar route, where IAA moves rapidly up and down in the phloem (Aloni, 2004). In trees, exchange of IAA can occur between these routes, via the vascular rays (Aloni, 2004).

With the exception of that in the non-polar route (in which IAA moves by mass flow in the phloem stream), transport of IAA occurs relatively slowly (around 7-15 mm/hr)

(Morris et al., 2004) in a downward direction, from cell to cell, via energy dependant carrier proteins. The uneven distribution of influx carriers at the top of cells and efflux carriers at the bottom of cells is responsible for the polar downward flow (Muday and DeLong, 2001; Morris et al., 2004). The influx carriers are thought to be solitary proteins encoded by the AUX1 gene (Marchant et al., 1999). On the other hand, the efflux carriers appear to be more complex. These are thought to consist of various membrane bound transport proteins encoded by members of the PIN gene family, and an associated NPA-binding protein (Palme and Galweiler, 1999; Muday and DeLong, 2001). Evidence suggests that the NPA-binding proteins interact with cortical actin filaments, which might be involved in localising the efflux carriers to the correct part of the cell membrane (Muday and DeLong, 2001). Correct placement of the efflux carriers is of obvious importance in directing the polar flow of auxin. The NPA-binding protein performs a regulatory role, and as its name suggests affords a binding site for auxin transport inhibitors such as N-naphthylphthalamic acid (NPA). The mechanism by which these auxin transport inhibitors function is not known, although evidence suggests that they might interfere with actin-dependant cycling of the auxin efflux transporters; cycling of efflux carriers between endosomal compartments and the plasma membrane is thought to be an essential part of the auxin transport process (Geldner et al., 2001). It is thought that the action of exogenously applied NPA might mimic the effects of endogenous regulatory compounds such as flavonoids, and that these might control in vivo auxin transport (Jacobs and Rubery, 1988; Brown et al., 2001; Muday and DeLong, 2001; Peer et al., 2001). Additionally, auxin transport can also be controlled by reversible phosphorylation of proteins involved in the transport process (Muday and DeLong, 2001).

1.6.1.3 Effects

Auxins have an essential role in regulating numerous spatial and temporal aspects of plant growth and development. They are required for the division, differentiation and enlargement of plant cells (including tracheids), and also function as signals between cells, tissues and organs (Morris et al., 2004). At the cellular level, rapid changes in auxin concentration may act to initiate or terminate a developmental process. On the other hand, maintenance of a stable concentration may be required for an initiated developmental event to proceed (Morris et al., 2004).

In specific respect to the present project, auxin has been shown to play a vital role in vascular development. The polar flows of IAA produced in leaf tips and later along leaf margins induce leaf vascular tissue to form. Similarly, the polar flow of IAA is responsible for inducing vascular development in the entire plant body as development proceeds (Aloni, 2004). Furthermore, IAA flow in the cambium controls the differentiation and regeneration of the vascular tissue (Aloni, 2004), i.e. the production of wood. In support of this, careful investigations in trees have revealed that the radial IAA concentration peaks in the cambium, and decreases either side in the developing phloem and xylem (Tuominen et al., 1997; Uggla et al., 1998; Uggla et al., 2001).

1.6.2 Ethylene

Ethylene (C_2H_4) is a simple gaseous hydrocarbon that is produced chemically by incomplete combustion of hydrocarbons, and biologically by plants, animals and microbes (Pech et al., 2004). It is the only hydrocarbon that has a major effect on plants (Davies, 2004).

1.6.2.1 Biosynthesis and regulation

Ethylene is synthesised from methionine in many tissues via the following pathway (information simplified from Pech et al., 2004):

Methionine
↓
S-adenosylmethionine (SAM)
↓ *ACC synthase*
1-aminocyclopropane-1-carboxylic acid (ACC)
↓ *ACC oxidase*
ethylene

The production of ACC from SAM, by the enzyme ACC synthase, is the important rate limiting step in ethylene biosynthesis (McKeon et al., 1995; Capitani et al., 2002), although ACC can be diverted from producing ethylene by forming conjugate derivatives (Pech et al., 2004).

The rate of ethylene production is influenced by other hormones, and by ethylene itself (McKeon et al., 1995). IAA enhances ethylene formation by inducing ACC synthase synthesis, which increases ACC levels, resulting in higher ethylene production. This effect can be synergistically enhanced by cytokinin. Abscisic acid and ethylene can either inhibit or enhance ethylene production.

On the basis of recent evidence, models in which ethylene action is negatively regulated are currently in vogue (Klee and Clark, 2004; Etheridge et al., 2006). The basic idea of these models is that, in the absence of ethylene, the activity of ethylene receptors suppresses downstream signaling involved in the ethylene response. Perception of ethylene by the receptors abolishes this suppression, allowing the ethylene response to occur. The model predicts that there will be a negative correlation between the number of ethylene receptors, and ethylene sensitivity; more ethylene will be required to abolish suppression of a high number of receptors, than a low number (Klee and Clark, 2004). Schaller and Bleecker (1995) reported that the half-life for dissociation of ethylene from the receptor was 12.5 hours. Klee and Clark (2004) suggest that this time could be even longer, and that ethylene binding may even be irreversible. Hence, they point out that the only way for a tissue to quickly turn off an ethylene response is by synthesis of new receptors.

1.6.2.2 Transport

Ethylene in the gaseous phase moves rapidly by diffusion from its site of synthesis (Ingemarsson et al., 1991; Davies, 2004). On the other hand, the diffusion of ethylene dissolved in the aqueous phase of plant cells is very slow (Ingemarsson et al., 1991). Hence, these authors point out the importance of gas-filled intercellular spaces for rapid ethylene movement. In addition, ACC (the immediate precursor of ethylene) may be translocated basipetally in the phloem (Abeles et al., 1992) and acropetally in the xylem (Bradford and Yang, 1980).

1.6.2.3 Effects

Ethylene affects growth, differentiation and senescence in plants. Its application has been shown to influence almost every stage of growth and development in some plant or other (Reid, 1995). Due to the correlation between stress ethylene production, and modified vascular tissues, it has been suggested that ethylene may be involved in the

development of xylem cells (Abeles et al., 1992). In support of this, Eklund and Little (1998) reported that ethylene levels were higher in the cambium, than other parts, of *Abies* shoots. Additionally, in *Pinus sylvestris*, the highest rate of cambial ethylene production has been reported to coincide with the seasonal period of greatest tracheid production (Klintborg et al., 2002).

Ethylene treatment over a period of several weeks has been reported to greatly enhance stem diameter growth in *P. radiata* (Neel, 1971). Similarly, application of ethrel to tree stems (which stimulates ethylene production when applied *in vivo*) also leads to a localised increase in tracheid production (Eklund and Little, 1998).

1.6.2.4 Ethylene inhibition: Aminoethoxyvinylglycine (AVG)

As outlined above, the immediate biosynthetic precursor of ethylene is ACC. ACC is produced by the pyridoxal phosphate (PLP)-dependent enzyme ACC synthase. This enzyme catalyses the elimination of methylthioadenosine (MTA) from S-adenosylmethionine (SAM) to produce ACC. The production of ACC from SAM is the rate-determining step in ethylene biosynthesis. Evidence suggests that AVG acts by inhibiting ACC synthase, by forming a covalent ketimine complex with the PLP cofactor (Capitani et al., 2002).

1.6.3 Gibberellin

Gibberellins are defined both by function, and by their chemical structure, being based on the *ent*-gibberellane carbon skeleton. Currently 136 gibberellins are recognized, and these are designated GA_1 through GA_{136}, the subscripts having been assigned in order of their identification (http://www.plant-hormones.info/gibberellins.htm). Only some of these 136 known gibberellins possess biological activity, while many others are inactive precursors or deactivation products of the active forms (Sponsel and Hedden, 2004). GA_1 (Figure 1.3) is the most important bioactive gibberellin in most species, although in some species the main bioactive form is thought to be GA_4 (Sponsel and Hedden, 2004).

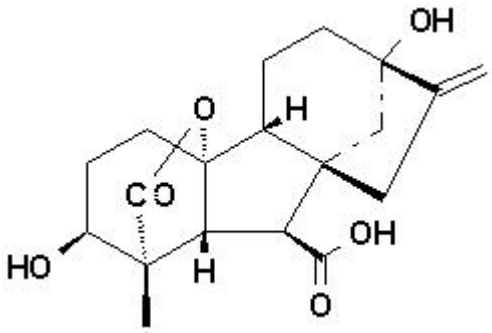


Figure 1.3: *GA_1 structure*

1.6.3.1 Biosynthesis and regulation

Gibberellins are thought to be synthesised in rapidly growing parts of plants, and seeds (Davies, 2004; Sponsel and Hedden, 2004). They are synthesised from isopentenyl diphosphate (IPP) via the following pathway (information simplified from Sponsel and Hedden, 2004):

IPP

↓

Geranylgeranyl diphosphate (GGPP)

↓ *ent*-copalyl diphosphate synthase

(CPS)

***ent*-Copalyl diphosphate (CPP)**

↓ *ent*-kaurene synthase (KS)

***ent*-kaurene**

↓ *numerous steps*

gibberellins

For four decades IPP was thought to be produced solely from acetyl-CoA via the acetate/mevalonate pathway (Sponsel and Hedden, 2004). However, recently an alternate pathway for the synthesis of IPP has been elucidated (Rodriguez-Concepcion and Boronat, 2002). This is known as the MEP (methylerythritol phosphate) pathway, and within this IPP is formed from glyceraldehyde-3-phosphate (G3P). According to (Sponsel and Hedden, 2004) it is IPP derived from this pathway that is used to synthesise gibberellins in plants.

1.6.3.2 Transport

Traditionally it was thought that gibberellin was probably transported in the phloem and xylem (Davies, 1995). However, new evidence suggests that gibberellin biosynthesis and action occurs in the same tissues, and possibly even in the same cells (Sponsel and Hedden, 2004).

Israelsson et al. (2005) reported that expression levels of the first committed GA biosynthesis enzyme, ent-copalyl diphosphate synthase (CPS), were high in the phloem. Since GA levels were high in the developing xylem, these authors suggested that GA precursors may be transported from the phloem to the developing xylem.

1.6.3.3 Effects

Gibberellin is generally regarded as the major factor controlling shoot elongation in both woody angiosperms and gymnosperms (Little and Pharis, 1995). In Pinaceae family trees there is strong evidence that gibberellin A_4 is the most active form in this regard, especially in stems older than one year (Little and Pharis, 1995).

Some evidence suggests that this stem elongation may be accomplished by gibberellin effecting an increase in tracheary element length. For example, in *Eucalyptus globulus* Ridoutt *et al*. (1996) observed the influences of a gibberellin biosynthesis inhibitor (Trinexapac-ethyl) on secondary xylem fibre development. Results were consistent with the hypothesis that the elongation of differentiating secondary xylem fibres in *Eucalyptus globulus* is dependent on GA-1 levels in the cambial region; higher levels of Trinexapac-ethyl resulted in shorter fibres. Kalev and Aloni (1998) developed an experimental system for studying the hormonal mechanisms which control tracheid differentiation in *Pinus pinea* L. seedlings. Gibberellin was found to promote tracheid elongation by stimulating intrusive growth of both the upper and lower ends of the differentiating tracheids. Based on analysis of tangential sections from across the cambial, and adjacent regions of aspen trees, Israelsson et al. (2005) reported that bioactive GA_1 and GA_4 were mainly located in the zone of radial expansion in developing xylem cells. These authors suggested that the major role of GA during xylem development is to regulate early stages of xylem differentiation, including cell elongation.

It has been reported that cells exposed to increased levels of gibberellin respond by developing more transversely (more highly angled) aligned cortical microtubules and microfibrils (e.g. Shibaoka, 1993). Recently Foster et al. (2003) published a model outlining how gibberellin might influence microtubule dynamics. Briefly, these authors suggest that gibberellin might control microtubule dynamics by upregulating a katanin microtubule severing protein (KSS), the action of which is known to permit microtubule rearrangement during cell division, differentiation and growth.

1.6.4 Compression wood induction by exogenous application of growth regulators

Based on numerous studies involving application of plant growth regulators, and inhibitors, it has been suggested that auxin and/or ethylene are involved in the formation of compression wood (see review by Timell, 1986).

1.6.4.1 Ethylene

Barker (1979) repeatedly applied ethrel ((2-chloroethyl)phosphonic acid), a compound which breaks down to release ethylene, to *P. radiata* stems for about a year. This treatment resulted in a large localised increase in stem diameter, which was due to an increase in cell division. Additionally, several changes occurred in the wood, which Barker considered to be similar to compression wood; the wood was reddish brown, had increased density, and the cells were more rounded, with thicker walls when viewed in cross-section. Ethrel application to *Pinus taeda* seedling stems also resulted in formation of wood with characteristics that differed from normal control wood (Telewski et al. 1983). These investigators reported wood with a higher proportion of resin canals, and shorter, thicker walled tracheids. However, in contrast to Barker (1979) the rounded cell shape appeared to be absent.

1.6.4.2 Auxin

In 1940 Onaka's pioneering research was the first to demonstrate that application of auxin could lead to the formation of compression wood; in horizontally orientated *Pinus thunbergii*, he observed that compression wood was formed on the upper side of the stem when IAA was applied there (Onaka, 1940). Over the ensuing decade, Onaka carried out numerous experiments on several gymnosperm species. Generally, application of IAA, in various ways, resulted in the formation of compression wood (Onaka, 1949 cited in Timell 1986). In *P. radiata* it has also been reported that auxin

application leads to compression wood formation (Wardrop, 1957; Wardrop and Davies, 1964).

Interpretation of results from this type of experiment is severely complicated, because auxin influences ethylene production (Abeles et al., 1992). Hence, results from these experiments do not demonstrate that the effect of auxin application is direct. As pointed out below, ethylene production by gymnosperm xylem was not discovered until the 1970s, and so these early workers would not have considered it, let alone its interaction with auxin. The point that endogenous IAA levels have recently been reported to be similar in compression wood and normal wood (Hellgren et al., 2004) (see below) must further question whether applied auxin results directly in compression wood formation.

1.6.5 Compression wood and endogenous hormones

1.6.5.1 Ethylene

Research on the role of ethylene in compression wood formation began relatively recently, because the production of this hormone by the xylem of a gymnosperm was not discovered until the 1970s (Shain and Hillis, 1972). Whether ethylene is a crucial factor involved in compression wood formation is controversial. However, evidence suggests that sites of compression wood development evolve more ethylene than sites of normal wood development. It has been reported that the lower side of *Cupressus arizonica* branches (which would presumably be producing compression wood) evolve about one and a half times more ethylene than the upper (Blake et al., 1980). Savidge et al. (1983) detected the ethylene precursor ACC in cambium from the lower side, but not the upper side of *Pinus contorta* branches.

Additionally, treatments that induce compression wood formation also lead to a localised increase in ethylene evolution. Leaning of balsam fir seedlings induced compression wood formation, and enhanced ethylene evolution on the lower side of the stem (Little and Eklund, 1999). These authors concluded that the higher ethylene on the lower side was related to compression wood development *per se*, rather than the increase in tracheid production that accompanies compression wood formation. Similarly, in experimentally leaned *Metasequoia glyptostroboides* stems Du et al.

(2004) reported ethylene levels around five higher, and Du and Yamamoto (2003) around 10 times higher, on the lower side compared to the upper side.

Other experimental treatments that induce compression wood formation, such as application of the IAA transport inhibitor N-1-naphthylphthalamic acid (NPA), have also been reported to increase ethylene evolution (Little and Eklund, 1999).

1.6.5.2 Auxin

As is the case for ethylene, the involvement of endogenous IAA in compression wood formation is controversial. Some investigators have reported that compression wood inducing treatments lead to a localised increase in IAA concentration. In the experimentally leaned *Metasequoia glyptostroboides* stems mentioned above, Du et al. (2004) reported that the IAA concentration on the lower side of the lean (where compression wood formed) was about twice that of the upper side. On the other hand, others have reported that there is no difference between IAA levels in developing compression, and normal wood. Hellgren et al. (2004) mapped endogenous IAA levels across the cambium and differentiating xylem of leaned *Pinus sylvestris* trees. The methods employed in this study were sophisticated, and involved careful measurement of IAA concentrations from each of a series of tangential cryosections. The concentration, and distribution of IAA was quite similar in developing woods from both the compression and opposite sides. These investigators concluded that the lean induced compression wood formation was not mediated by changes in cambial IAA levels.

1.7 TISSUE CULTURE

The differentiation of cambial cell derivatives into tracheids during the formation of xylem involves a complex array of plant level, cellular and sub-cellular events (Torrey et al., 1971; Savidge, 1996) which are not fully understood. Characterisation of the mechanisms involved and the factors regulating them is a difficult task. Approaches based on cell, tissue and organ culture provide a means of reducing this complexity into component parts (Leitch and Savidge, 2000), by providing control (to

an extent) over factors influencing the cambium and its derivatives. *In vivo* these factors are controlled by the plant, and its response to the environment.

Brown and Wodzicki (1969) developed a novel method for culturing *Larix leptolepis* and *Pinus densiflora* stem explants using liquid media gravity fed though tubing. New, normally differentiated tracheids were formed in culture of both species. Zajaczkowski (1973) investigated the method further and found that cambial response was dependant on stem age; young stems were found to be more responsive. The method also been successfully applied to young *P. radiata* stem pieces (Sheriff, 1983). Savidge (1993) reported a successful method for culturing *Larix laricina* stem "chips" consisting of cambium sandwiched between phloem and xylem. This method was used as a foundation for developing the *P. radiata* protocol discussed more fully in Chapter 3.

Chapter 2

Cortical Microtubule Rearrangement Precedes Cellulose Microfibril Rearrangement During Secondary Wall Deposition in *Pinus radiata* Tracheids

2.1 INTRODUCTION

Cortical microtubules have been proposed to play a role in directing the oriented deposition of cellulose microfibrils since their distribution was first seen to parallel that of newly deposited cellulose microfibrils (Ledbetter and Porter, 1963). Many subsequent observations confirmed that these structures often shared the same orientation in developing plant cells (see Baskin, 2001; Giddings and Staehelin, 1991; Robinson and Quader, 1982 for reviews). Functional evidence for the role of microtubules in this process came from a range of experiments showing that microtubule disruption with drugs (Green, 1962; Takeda and Shibaoka, 1981; Mueller and Brown, 1982), or mutagenesis (Burk and Ye, 2002) perturbed cellulose organisation. These observations have led authors to conclude that microtubules are involved in determining the orientation of cellulose microfibrils (Abe et al., 1995b; Burk and Ye, 2002) or alternatively that microtubules are necessary to change the alignment of microfibrils even if not required to maintain an existing microfibril arrangement (Murata and Wada, 1989). Either way, the idea that microtubules guide the deposition of cellulose by constraining the direction in which plasma membrane-localised cellulose synthase complexes move has become the most widely supported hypothesis (Heath, 1974; Herth, 1980; Herth, 1985; Baskin, 2001), (see Giddings and Staehelin, 1991; Heath and Seagull, 1982 for reviews).

On the other hand, a sizeable body of evidence has demonstrated that cortical microtubules and cellulose microfibrils sometimes have different orientations during primary cell wall deposition (Robinson and Quader, 1982; Giddings and Staehelin, 1991; Baskin, 2001). Some of these observations may be discounted for failing to demonstrate that the cells were growing or may be explained by speculating that microtubules were in the process of establishing a new orientation at the time of observation. On the other hand, these exceptions raise the possibility that other

mechanisms for orienting cellulose microfibrils exist. Indeed, a variety of mechanisms have been proposed that explain how ordered deposition of microfibrils may occur without intervention from microtubules (Preston, 1974; Boyd, 1985; Preston, 1988; Emons et al., 1992; Emons, 1994). Such models rely on forces generated by anisotropic cell expansion and/or cell geometry to induce self-order in the cellulose microfibril array. For example, the number and packing arrangement of the cellulose synthesizing complexes along with cell size and shape could, in theory, force cellulose microfibrils to be laid down in a certain way (Emons, 1994). While these models seem plausible they can only explain gradual changes in cellulose microfibril orientation. This is clearly not the case during in some instances of plant cell development where the orientation of cellulose can drastically and rapidly change in response to an altered environment, or as part of a developmental sequence, long before any major change in geometry of the cell lumen takes place. For example, during secondary wall deposition in tracheary elements there are abrupt changes in orientation of microfibrils, while at the time this occurs the lumen diameter only reduces marginally (Prodhan et al., 1995; Abe et al., 1995a; Abe et al., 1995b; Chaffey, 2000; Funada et al., 2001; Chaffey, 2002a).

Recent experimental analysis with the conditionally microtubule-disrupted *mor1-1* mutant has unequivocally shown that organised deposition of cellulose can occur in the absence of ordered cortical microtubules (Sugimoto et al., 2003). Additionally, when cellulose synthesis was disrupted transiently, organised deposition of cellulose was re-established in the absence of organised microtubules (Himmelspach et al., 2003). It has also been demonstrated that inhibition of cellulose synthesis leads to disordered cellulose microfibrils (Sugimoto et al., 2001) and that cortical microtubules and cellulose microfibrils were only coaligned during the early and most rapid stage of cell expansion (Sugimoto et al., 2000). Based on these observations, it has been proposed that the rate of cellulose synthesis might control cellulose microfibril arrangement (Sugimoto et al., 2001; Sugimoto et al., 2003), and that microtubules might regulate the mechanical properties of expanding cell walls by influencing the length of microfibrils rather than their orientation (Wasteneys, 2004). Even if cortical microtubules are not responsible for guiding the orientation of cellulose microfibrils during deposition, their presence does seem to be essential for marking the sites where cellulose synthesis will take place. This relationship is

especially obvious during secondary wall deposition such as occurs during the formation of wall ingrowths in developing xylem (Gardiner et al., 2003).

While the role of microtubules in primary cell wall deposition has been called into question the same is not true for the secondary cell wall. There are no known reports of a discrepancy between the orientation of cellulose microfibrils and cortical microtubules in cells actively depositing the secondary cell wall (Baskin, 2001). For example, numerous reports have shown that cortical microtubules and cellulose microfibrils share a common orientation in tracheary elements (Robards and Kidwai, 1972; Prodhan et al., 1995; Abe et al., 1995a; Abe et al., 1995b; Chaffey et al., 1999; Chaffey, 2000; Funada et al., 2001; Chaffey, 2002a). Furthermore, as cellulose orientation changed with the sequential deposition of secondary wall layers from a flat S helix in the S_1 layer, a steep Z helix in the S_2 layer and again a flat S helix in the S_3 layer, the microtubules mirrored these arrangements (Abe et al., 1995a). Similarly, the contracting ring of microtubules seen during pit development in the secondary cell wall was coincident with the narrowing of the pit aperture, a product of cellulose synthesis (Uehara and Hogetsu, 1993). The lack of discord in alignment of cortical microtubules and cellulose microfibrils during secondary cell wall deposition makes this stage of cell wall deposition ideal for the study of the relationship between cortical microtubules and cellulose microfibrils. During secondary cell wall deposition, the cell has ceased expanding. Hence, biophysical forces generated by stretching of the wall are absent and cell geometry will be set except for the minor differences caused by the thickening of the wall.

2.1.1 Aims and hypothesis

The aim of the present study was to visualise the organisation of both cortical microtubules and cellulose microfibrils across the entire region of tracheid development in *Pinus radiata*. Results obtained were used to test the hypothesis that cortical microtubules act to guide cellulose microfibril deposition. Confocal laser scanning microscopy was used to visualise immunolabelled cortical microtubules, and where possible reflected light confocal scanning laser microscopy was used to detect cellulose microfibril orientations in the cell wall at exactly the same positions. Field emission scanning electron microscopy was used to substantiate the observations from the reflected light confocal microscopy. In the early stages of wall development it was

not possible to observe cellulose microfibril orientations using the reflected light method so FESEM observations only, of cells at about the same stage of development, were made.

Changes in microtubule and cellulose microfibril distributions, that occurred in a set developmental sequence, were monitored by following the tracheids of a radial cell file from primary cell wall deposition through to the S_1, S_2, and S_3 secondary wall layers. It was found that changes in microtubule orientation preceded those of cellulose microfibrils during the deposition of the secondary cell wall. For development of the secondary wall, observations support the microtubule-based cellulose synthase constraint hypothesis or a modification of the templated incorporation model for controlling the orientation of cellulose microfibrils. During primary wall development microtubule and microfibril orientations were both almost longitudinal during rapid radial expansion. On the other hand, during slow radial expansion, arrangement of the two was more random, tending towards transverse especially in the latter stages.

2.2 MATERIALS AND METHODS

2.2.1 Tissue sampling and preparation

Blocks of tissue approximately 50 x 50 x 20 mm thick were removed from the outer stem of seven *P. radiata* trees planted in 1994 near Port Levy, New Zealand. Radial sections of tissue encompassing the cambial region and developing xylem (approximately 7 x 7 and 2 mm thick) were cut from the blocks using a single edge razor blade and immediately placed into microtubule fixative (Abe et al., 1995b) for 30 min at room temperature, followed by a second fixation overnight in fresh fixative at room temperature, before rinsing in Phosphate-buffered saline (PBS) (Abe et al., 1995b). Samples were then infiltrated at room temperature with 10, 20, 30 and 50% polyethylene glycol (PEG) blend (9 parts polyethylene glycol 1450 (Sigma) to 1 part polyethylene glycol 1000 (Electron Microscopy Sciences)) in PBS, and at 52 ^{o}C with 70, 80, and 90% PEG blend in PBS, and twice in 100% PEG blend. Samples remained in each step for about 8 h or until samples no longer floated. Samples were embedded in fresh PEG blend.

2.2.2 Immunostaining

Single 16 μm sections were mounted on warm gelatin-coated slides over a 37 ^{o}C water bath. Twenty μl of primary antibody (monoclonal anti-α-tubulin raised in mouse (Sigma, clone B5-1-2) diluted 1:1000 in PBS containing 1% bovine serum albumin and 0.02% sodium azide (PBSB)) was applied to the sections and incubated for 2 h at 37 ^{o}C. After rinsing three times in PBS, 20 μl of secondary antibody (Goat anti-mouse conjugated to Alexa 488 (Molecular Probes) diluted 1:500 in PBSB) was applied and the sections were incubated at 4 ^{o}C overnight. Following three rinses in PBS, sections were mounted in florescence microscopy medium made as follows. 6 g of glycerol was placed in a 50 ml centrifuge tube and 2.4 g Mowiol 4-88 (Sigma) stirred in. 6 ml distilled H_2O was then added and the mixture incubated at 25 ^{o}C overnight. 12 ml of 0.2 M Tris(hydroxymethyl)aminomethane (Tris) buffer (2.42 g Tris (Sigma) in 100 ml distilled H_2O adjusted to pH 8.5 with HCl) was added and the solution incubated for 10 min in a 50 ^{o}C water bath, with occasional stirring. The mixture was then clarified by centrifugation at 5000g for 15 min, and the supernatant stored in 1 ml aliquots at –20 ^{o}C. Upon thawing 100 μl of ρ-phenylenediamine (Sigma) (an antifade agent) was added before use.

2.2.3 Confocal microscopy

Sections were examined with a BioRad microradiance confocal system linked to an Olympus IX-70 inverted microscope. The blue line of the argon laser was used for excitation and the 530-560 nm emission filter was used for observing Alexa 488 fluorescence. All images were oriented with the cambium zone to the left and the top of the tree consistent with the top of the image. Images of a series of optical sections were recorded at 0.5 μm intervals over the range where cortical microtubules were present. Reflected images of cellulose microfibrils were also collected from the cell wall plane using the blue line by removing the emission filter and inserting a polarizer in its place.

The orientations of cortical microtubules from projected Z-series images were measured using Scion Image software. Similarly, the orientations of cracks in the wall that represent the predominant cellulose microfibril orientation were measured from

single Z sections of reflected images from the same area of each cell. This numerical data was collected from four samples, two from each of two trees.

2.2.4 FESEM

Specimens for field emission scanning electron microscopy (FESEM) were cut from the main specimen blocks and fixed in formalin-aceto-alcohol (Johansen, 1940). Samples were carefully trimmed using double edged razor blades, rinsed and treated with 0.5% sodium hypochlorite for 10 min to remove cytoplasm. After rinsing, samples were then processed through a graded series of amyl acetate and critical point dried. Samples were coated with platinum prior to observing with a Hitachi S-4700 FESEM.

2.2.5 Epifluorescence microscopy cell counts

Epifluorescence microscopy was used when counting cells in radial files at various stages of development. The arrangement of cortical microtubules was used to divide regions into similar developmental stages (based on criteria in Table 1). These data were collected from the 14 samples, two from each of seven trees.

2.3 RESULTS

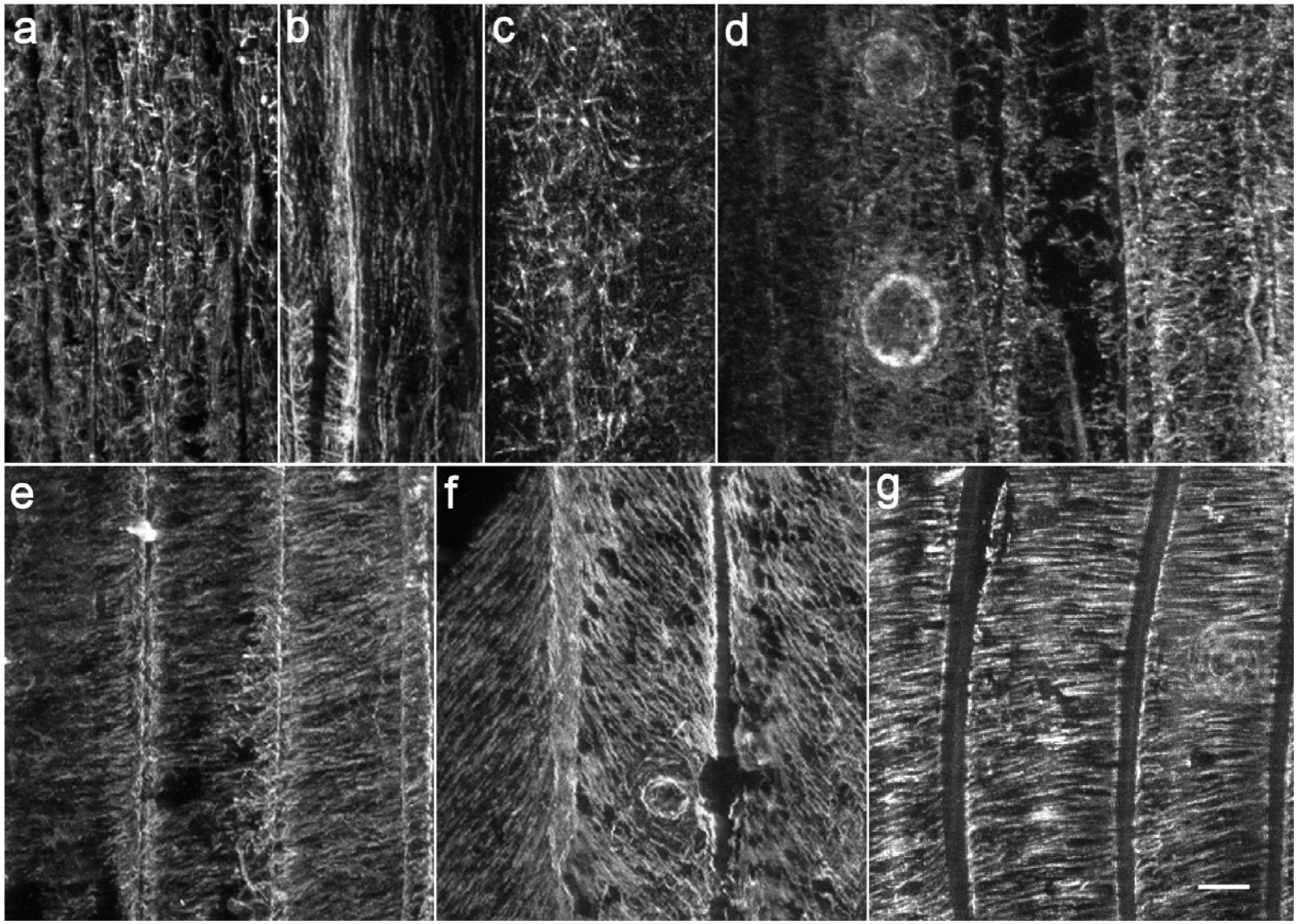
Distinct changes in orientation of cortical microtubules and cellulose microfibrils occurred as the rate of primary growth changed and as various layers of the secondary wall were deposited. This sequence of events was observed in radial sections, which provided a developmental time line and the opportunity to examine the relative timing of the microtubule and microfibril reorientations. Cortical microtubules were immunolabeled and observed using confocal laser scanning microscopy. During secondary wall development, for each area where microtubules were observed, cell wall texture indicating cellulose microfibril orientations was also detected using reflected light confocal microscopy. This enabled a within cell comparison of cortical microtubules and microfibrils to be undertaken once secondary wall deposition had begun. FESEM was used to confirm that the cell wall texturing observed with reflected light confocal microscopy was cracks between aggregates of cellulose microfibrils that were truly representative of microfibril orientation. During primary

wall development it was not possible to resolve cracks between aggregates of microfibrils using reflected light confocal microscopy. Hence, during this stage FESEM was used to observe cellulose microfibrils from samples at approximately equivalent stages of development to where microtubules were observed.

2.3.1 Cortical microtubule and cellulose microfibril orientations changed with the rate of cell expansion

Cortical microtubules were randomly arranged in the cambial initials and the adjacent developing xylem cells (Figure 2.1a; Table 2.1). In this developmental stage, defined as "Cell division/First slow expansion" in Table 2.1, cells expanded radially from 8 to 15 μm. Hence, growth in the radial dimension was relatively slow at an average rate of about 0.74 μm per cell division. At this stage the orientations of microfibrils in the cell wall were not discernible with reflected light confocal microscopy, so FESEM was performed on samples taken from the main sample blocks, tangentially adjacent to those used in confocal microscopy. In the cambial initials (Figure 2.2a), and the adjacent developing xylem cells in the first region of slow expansion (Figure 2.2b), microfibrils were also randomly arranged.

Figure 2.1 (next page): *Confocal laser scanning microscope image of cortical microtubules in developing* Pinus radiata *xylem.* ***(a)*** *Earliest derivatives of vascular cambium, showing randomly arranged microtubules.* ***(b)*** *Zone of rapid radial expansion, showing longitudinally arranged microtubules.* ***(c)*** *Cessation of radial expansion, showing randomly and some transversely arranged microtubules.* ***(d)*** *The cell at left is at a stage of development just prior to secondary wall deposition- note diffuse microtubules. In the cell at right cortical microtubules have become ordered in a flat S helix. Outside of tree is to the left of all images.* ***(e)*** *Microtubules arranged in a flat S helix, at the time the S_1 wall is laid down. Cells viewed from the lumen side.* ***(f)*** *Microtubules arranged in a steep Z helix, at the time the S_2 wall is laid down. Cell at left viewed from the outer side, 2 cells at right viewed from lumen side.* ***(g)*** *Microtubules arranged in a flat S helix, at the time of S_3 wall deposition. Images viewed from the lumen side. Scale bar = 10 μm (all images same scale).*


a
b
c
d
e
f
g

As xylem development proceeded cortical microtubules became longitudinally arranged (Figure 2.1b; Table 2.1). In this region, denoted “Rapid expansion” in Table 2.1, the cells had abruptly increased in radial diameter from 15 to 28 μm, and thus had enlarged an average of 3.25 μm per cell division. This was 4.4 times faster than the cells in the first slowly expanding zone immediately adjacent to the cambial initials. Correspondingly, many cellulose microfibrils with longitudinal (or near longitudinal) orientation were observed in this region (Figure 2.2c) (n=5).

Table 2.1: *Comparison of immunostained cortical microtubules and cellulose microfibrils during tracheid cell development. Cellulose organisation as detected using reflected light confocal laser scanning microscopy and FESEM.*

Developmental stage	Cell division/First slow expansion	Rapid expansion	Second slow expansion	1^o to 2^o transition	S_1	S_2	S_3
Description of microtubules.	random long	longitudinal long	random/transverse shorter	diffuse short some transverse	flat S helix	moderate Z helix	flat S helix
Cellulose organisation.	nearly transverse	very steep S helix, almost longitudinal	often nearly transverse	nearly transverse	flat S helix	steep Z helix	flat S helix
Pit microtubule arrangement	-	-	-	large round band	smaller round band	smaller round band	small round band random/radial inside band
Number of cells in zone ± s.d.	9.5 ± 0.5	4.0 ± 1.4	4.7 ± 1.4	3.0 ± 0.6	6.8 ± 2.2	22.7 ± 2.6	5.7 ± 2.3
Change in cell radial width across zone	8-15 μm	15-28 μm	28-32 μm	~constant	~constant	~constant	~constant

Following this, microtubules became less well ordered, although several tended towards a transverse orientation (Figure 2.1c; region referred to as “Second slow expansion” in Table 2.1) Here radial cell expansion had slowed markedly to 0.85 μm per cell division, which was 3.8 times slower than in the zone of rapid radial expansion. In this region many microfibrils were observed to take on a near transverse orientation. This arrangement continued through into the primary to secondary transition region, with microfibrils becoming increasingly well ordered (Figure 2.2d).

2.3.2 Cortical microtubules depolymerised at the transition to secondary wall deposition

Cortical microtubules were very short in the first one or two radial files of cells between the end of cell expansion and the onset of secondary wall deposition, making it difficult to assign them an orientation (cell at left of Figure 2.1d). The next radial

file of cells had slightly longer microtubules that were primarily orientated transversely. These observations were interpreted as microtubules depolymerising at the completion of primary wall deposition, followed by re-establishing a new cortical microtubule array with the onset of secondary cell wall deposition. Following the primary to secondary transition cortical microtubules became longer and well ordered in a flat S helix (cell at right of Figure 2.1d).

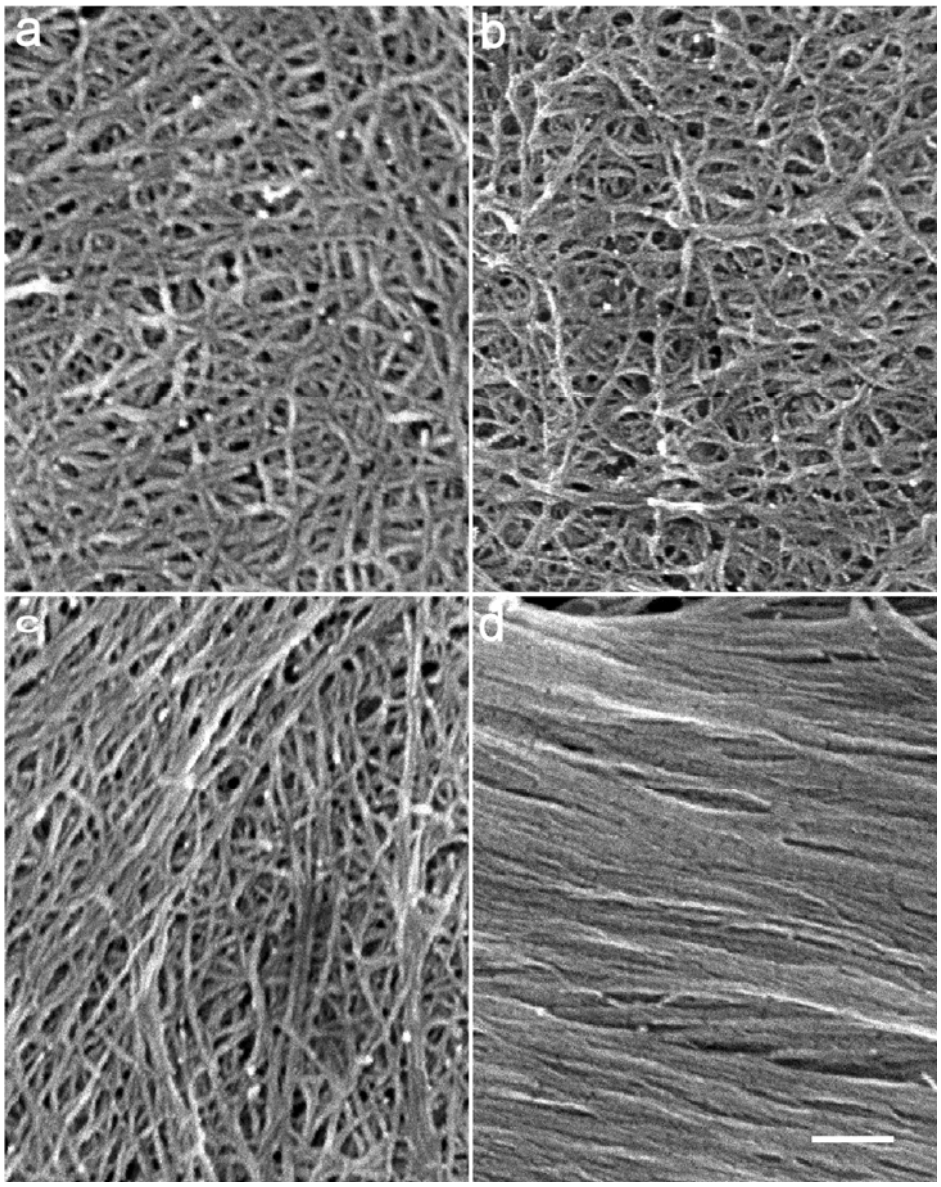


Figure 2.2: FESEM images of tracheid cell wall cellulose microfibrils in the zone of radial expansion. ***(a)*** *Random arrangement in cambium.* ***(b)*** *Random arrangement during the first region of slow expansion.* ***(c)*** *Later, during rapid radial expansion some longitudinal order arose.* ***(d)*** *Transverse arrangement in second region of slow expansion, and primary to secondary transition. Scale bar = 0.2 μm (all images same scale).*

2.3.3 Cortical microtubules and cellulose microfibrils shared the same orientation within each layer of secondary wall development

Confocal microscopy was used to perform a within cell comparison of the orientations of immunolabeled microtubules and cellulose microfibrils from reflected light images. Both cortical microtubule and cellulose microfibril arrangements changed from a flat S-helix to a steep Z-helix and back to a flat S-helix as the S_1, S_2 and S_3 wall layers were deposited (Figures 2.1e, f, g; 2.3; 2.4).

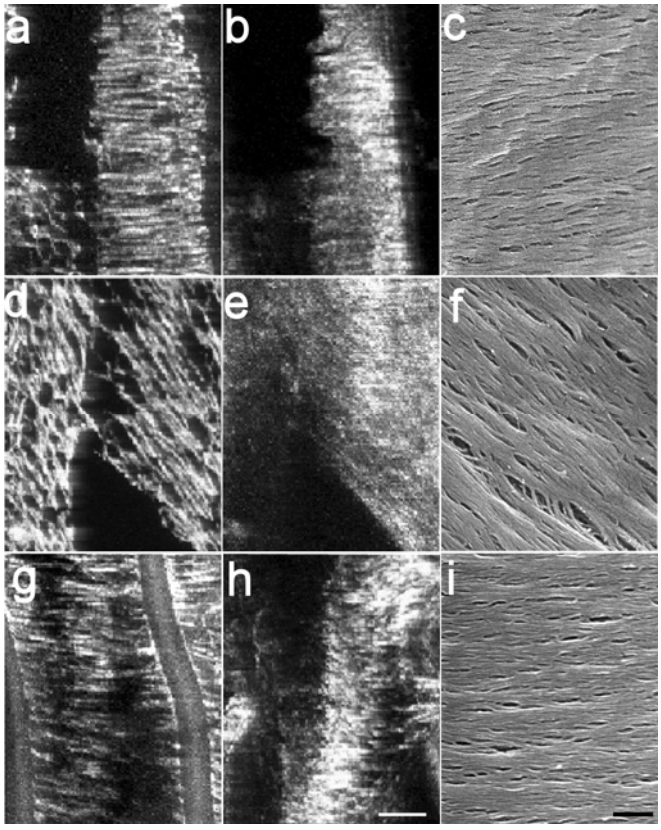


Figure 2.3: *Confocal laser scanning microscope images of cortical microtubules in developing* Pinus radiata *tracheids.* ***(a)*** *Microtubules arranged in a flat S helix, at the time the S_1 wall is laid down. Cells viewed from the lumen side.* ***(d)*** *Microtubules arranged in a steep Z helix, at the time the S_2 wall is laid down. Cell viewed from the outer side.* ***(g)*** *Microtubules arranged in a flat S helix, at the time of S_3 wall deposition. Images viewed from the lumen side.* ***(b, e, h)*** *Reflected confocal laser scanning microscope images of cell wall in developing tracheids for same areas as* ***(a, d, g)*** *respectively. White scale bar for* ***a, b, d, e, g, h*** *= 10 μm.* ***(c, f, i)*** *FESEM images of aggregates of cellulose microtubules in the cell wall in developing tracheids in the S_1, S_2 and S_3 wall layers respectively. Black scale bar for* ***c, f, i*** *= 0.2 μm.*

The start of secondary wall synthesis was defined as the point when the microtubules and cellulose microfibrils were present in a well ordered flat S helical arrangement (Figures 2.3a, b, c; Table 2.1). An average of about 6.8 radial files of cells were observed to be undergoing S_1 wall layer deposition. This was followed by a gradual

change over about three radial cell files to a steep Z helix (Figures 2.1f; 2.3d, e, f; 2.4b; Table 2.1), corresponding to the pattern of S_2 wall layer deposition. This region was by far the widest, containing an average of 22.7 cells (Table 2.1). At a still later stage cortical microtubule and cellulose microfibril orientations changed from the steep Z helix to the flat S helix of the S_3 wall layer (Figures 2.1g; 2.3g, h, i; 2.4c; Table 2.1), which encompassed an average of 5.7 radial files of cells.

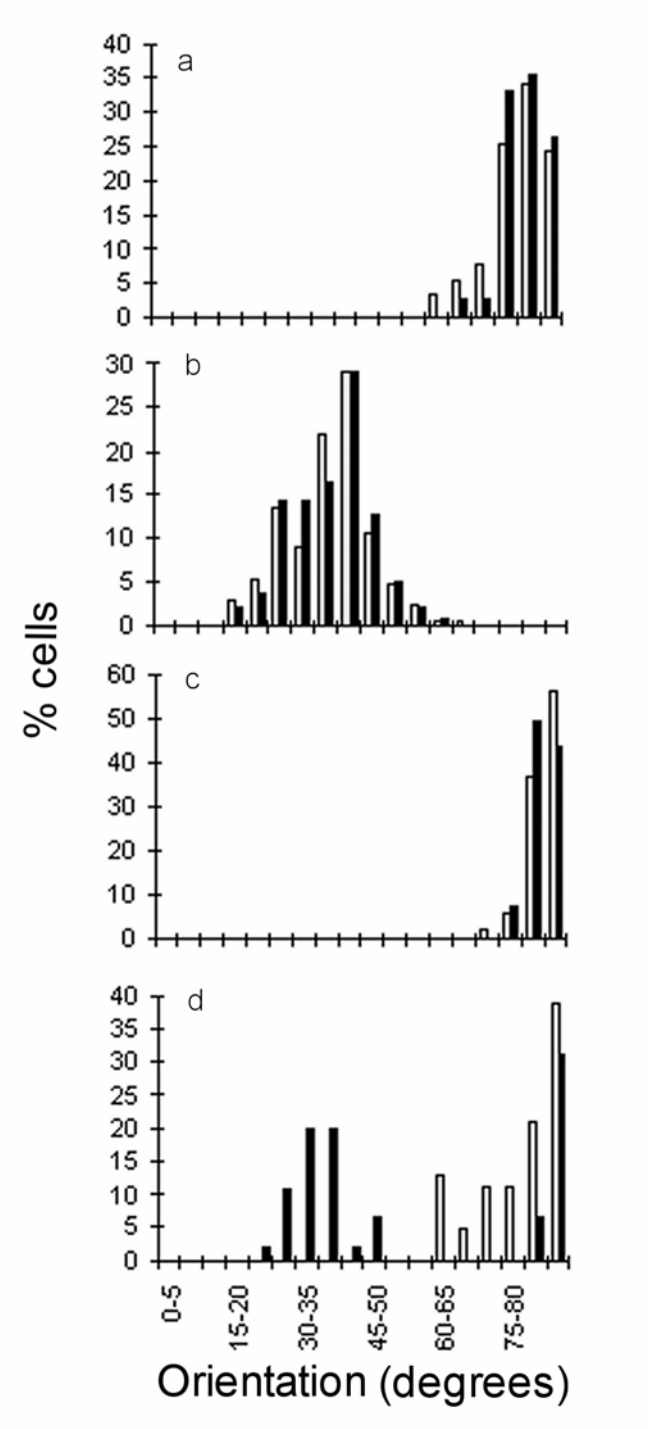


Figure 2.4: *Orientation of cortical microtubules (mt) (grey bars) and cellulose microfibrils (cmf) (black bars) in secondary wall of developing tracheids. Angles are relative to the long axis of cells* ***(a)*** *S_1 wall layer (n=94 for mt, n=76 for cmf).* ***(b)*** *S_2 wall layer (n=211 for mt, n=142 for cmf).* ***(c)*** *S_3 wall layer (n=91 for mt, n=85 for cmf).* ***(d)*** *S_2 to S_3 transition (n=62 for mt, n=45 for cmf).*

For the most part microtubule and cellulose microfibril orientations were the same in cells undergoing deposition of each wall layer. FESEM confirmed that the cell wall texturing observed with reflected light confocal microscopy corresponded to cracks between aggregates of cellulose microfibrils (Figure 2.3) that were truly representative of microfibril orientation. However, the transitions between the wall layers did not show the similarity between cortical microtubule orientation and cellulose microfibril orientation.

2.3.4 Cortical microtubule reorientation precedes cellulose microfibril reorientation during secondary wall deposition

The S_2 to S_3 transition occurred abruptly. No cells were observed that had microtubules or microfibrils oriented at angles intermediate to the S_2 and S_3 orientations. Hence, this stage of development afforded an excellent opportunity to study changes in cortical microtubule organisation and associated changes in the cellulose microfibrils. Interestingly, cortical microtubule reorientation was usually observed to precede that of cellulose microfibrils (Figures 2.4d; 2.5). In the cell at left of Figure 2.5a, microtubules have just changed to a flat helix; in the cell to the left of this they were in a steep helix. However, in the same position of the same cell cellulose microfibrils retained a steep helical arrangement (arrows at left of Figure 2.5b). In the more mature cell at the right both cortical microtubules and cellulose microfibrils are organised in a flat helix (arrows at right of Figure 2.5b, compared to same position in Figure 2.5a). In 62.8% of observations of the S_2 to S_3 transitions cellulose microfibril reorientation lagged behind that of cortical microtubules (Figure 2.4d) lending support to the microtubule based hypotheses. In the remaining 37.8% of cases both cortical microtubules and cellulose microfibrils had a similar flat helical arrangement typical of the S_3 wall layer (Figure 2.4d). The abrupt change in microfibril orientation from the S_2 to S_3 wall layers seen in the reflected confocal images was confirmed using FESEM (Figure 2.5c, d).

2.3.5 Cortical microtubule organisation leads cellulose microfibril deposition in developing bordered pits

Radial sections of *P. radiata* xylem offer an excellent means of observing ordered changes in arrangement of cortical microtubules and cellulose microfibrils during the

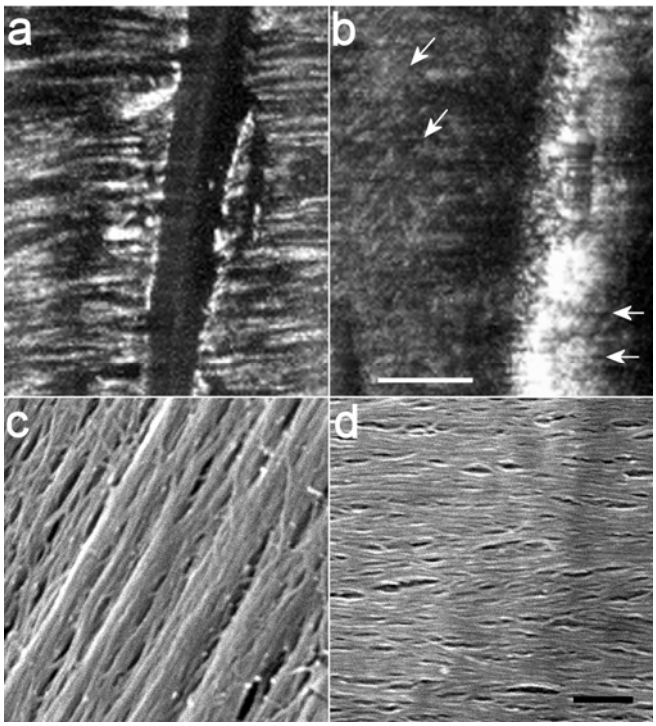


Figure 2.5: *Confocal laser scanning microscope images of first formed S_3 wall layer. Reflected image of tracheid walls **(b)** and immunolabeled cortical microtubules **(a)**. Figure **(a)** and **(b)** show the same area. In the cell at left of **(a)** cortical microtubules have just become orientated in a flat helix, while in the same cell cellulose microfibrils are still arranged in a steep helix (arrows left of **b)**. In the cell at right both cortical microtubules and cellulose microfibril are arranged in a flat helix. This suggests that cortical microtubule reorientation precedes cellulose microfibril reorientation at the onset of S_3 wall layer deposition. White scale bar for **a** and **b** = 10 μm. **(c)** and **(d)** FESEM images of aggregates of cellulose microtubules in the cell wall. **(c)** shows a cell still undergoing S_2 wall layer deposition, while **(d)** shows the next cell in the developmental sequence, which has just starred S_3 wall layer deposition. Black scale bar for **c** and **d** = 0.2 μm.*

development of bordered pits, especially using the present methods where these can be observed within the same cell. Cortical microtubules associated with bordered pit formation were first observed in cells undergoing the primary to secondary transition five to six cells prior to secondary wall deposition. Although the microtubules in these cells were short, the organisation into the circular outline of the pit was readily distinguishable (Figure 2.6a). Within one or two cells following this circular bands of microtubules were clearly recognisable marking the edge of the incipient pit border (Figure 2.6a right; also Figure 2.1d). As development proceeded, with deposition of the secondary wall, the circular bands of cortical microtubules gradually became smaller in diameter (Figure 2.6b, c). Microfibril thickenings around bordered pits

associated with the circular bands of cortical microtubules were clearly recognisable (Figure 2.6e, f).

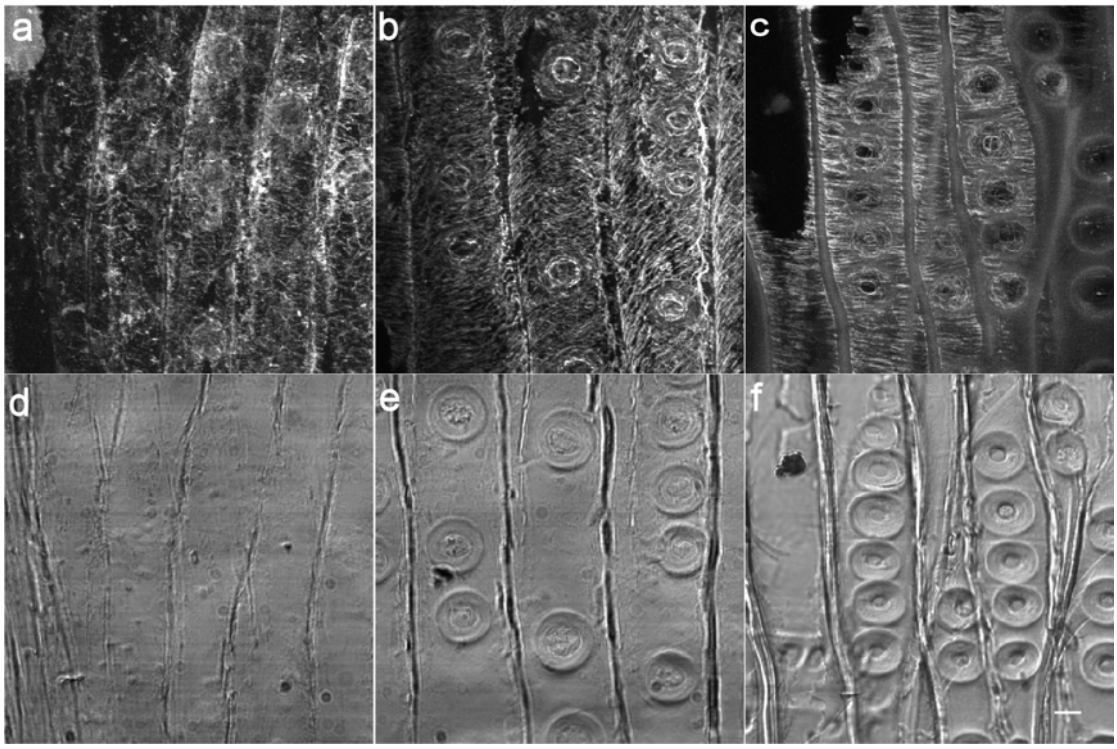


Figure 2.6: *Images of developing pits.* ***(a)*** *Immunolabeled microtubules in the primary to secondary transition (diffuse) region .* ***(b)*** *Immunolabeled microtubules in the S_2 wall layer.* ***(c)*** *Immunolabeled microtubules in the S_3 wall layer.* ***(d, e, f)*** *Transmitted light images of cell wall for same cells and areas in* ***a, b, c*** *respectively. Scale bar = 10 μm (all images same scale).*

However, the reduction in diameter of the microtubule bands preceded the reduction in diameter of the pit apertures (Figure 2.7). This provides additional evidence that the change in microtubule organisation precedes that of cellulose microfibrils in secondary wall synthesis.

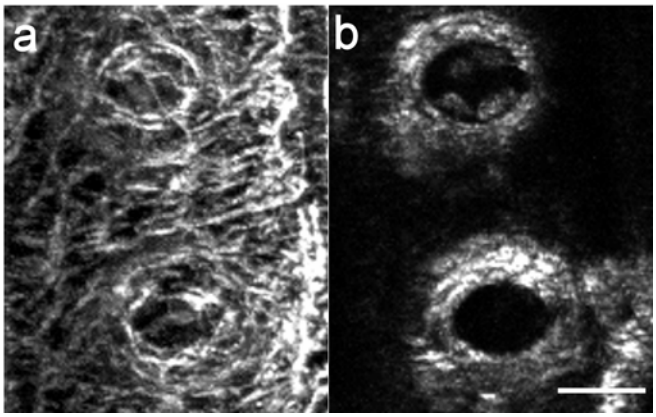


Figure 2.7: *Confocal laser scanning images of developing pits* ***(a)*** *Immunolabeled microtubules in the S_2 wall layer.* ***(b)*** *Reflected light image of cell wall at same area as* ***(a)****. Note that the diameter of the circular bands of cortical microtubules are smaller than the associated pit apertures. Scale bar = 10 μm.*

2.4 DISCUSSION

In the present study, it was found that changes in cellulose microfibril orientation lagged behind those of the cortical microtubules during the development of the secondary wall in tracheids. This was true for the transition between the S_2 and S_3 wall layers as well as the progressive narrowing of the aperture during pit formation. Within the main body of each secondary wall layer microtubules and microfibril shared a common arrangement. This was also true during rapid radial expansion during primary wall deposition, but during the first phase of slow radial expansion microtubules were random, whereas cellulose was organised transversely.

2.4.1 Microtubules lead changes in cellulose microfibril orientation during the S_2 to S_3 transition

During the transition from the S_2 to S_3 wall layer, observations revealed that cortical microtubules tended to change to the S_3 orientation prior to the cellulose microfibrils. Cells with an incipient S_3 microtubule arrangement most often contained cellulose microfibrils that remained in an S_2 orientation, while in the next oldest cells the cellulose microfibrils had also taken on an S_3 orientation (Figure 2.4; Figure 2.5). However, in some cases it was found that both microtubules and cellulose microfibrils had changed to the S_3 orientation in the first S_2/S_3 transition cell (Figure 2.4). Since following the developmental progression along a radial cell file shows changes over time it seems likely that these cells were slightly older than the aforementioned group of cells where microtubules were seen to change first. Microtubules also lead changes in cellulose deposition in developing pits. As the pit aperture progressively narrowed the constricting ring of microtubules was always smaller than the cellulose ring (Figure 2.7).

Other research has also shown that cellulose microfibril deposition lags behind changes in the microtubule network during secondary wall deposition. Microtubules have been shown to congregate at future sites of localised secondary wall deposition (Brower and Hepler, 1976) or tertiary thickenings (Chaffey et al., 1999) before these can be detected. In all these examples the cellulose microfibrils of the secondary wall were deposited in a distinct localised pattern, corresponding to the pattern of cortical microtubules, but there appeared to be a lag in time before this occurred.

Three explanations can be envisaged to explain the lags that have been observed in this and other studies. The first is that microtubules are the agents for change, and when they establish a new orientation cellulose microfibrils follow. In this case microtubules would act as guides either directly to constrain cellulose synthase mobility (Heath, 1974; Herth, 1980; Herth, 1985) or indirectly to establish a scaffolding that directs cellulose microfibril orientation (Baskin, 2001). Historically, this is the favoured interpretation of such data and it is consistent with observations of microtubule and microfibril parallelism within each layer of the secondary wall that are presented here and that have been shown by others (Robards and Kidwai, 1972; Prodhan et al., 1995; Abe et al., 1995a; Chaffey et al., 1999; Funada et al., 2001).

The second possibility is that the microtubules induce a change in the properties of the cellulose microfibrils and that this change leads to the altered cellulose microfibril orientation. For example, it is hypothesised that microtubules might regulate the length of cellulose microfibrils (Wasteneys, 2004). In this model long microtubules result in the synthesis of long microfibrils. The concentration and length of both of the polymers influences the way that they are packed in the cell, and it is the similarity in length of the polymers that results in their co-localisation. When polymers are few they are disorganised. However, once they cross a threshold concentration they will self organise in a way that could well depend solely on the numbers of polymers and the shape of the cell (Emons, 1994). It is important to note that one assumption of this model is that the cellulose microfibrils are extremely long. Clearly, short polymers will not follow this plan and are unlikely to show organisation. This seems a real possibility given the potential for cell geometry to influence the winding angle of cellulose. The way that the polymers wind could be determined by the number of active synthases and their packing arrangement.

A third possibility is that the ability to detect microtubules is greater than that for cellulose. Cellulose synthesis obviously takes time to occur, and my observations from the S_2 to S_3 wall layer transition can be explained by this idea. As the cortical microtubules changed to the new S_3 orientation the direction of cellulose synthase complex movement may in fact have immediately followed. The slight lag that was usually, but not always, observed (Figure 2.4) could then be explained by the point

that sufficient cellulose microfibrils needs to be laid down in the new orientation before they can be observed. In the minority of cases, where both microtubules and cellulose microfibrils appeared in the S_3 orientation, this condition may have been met. On the other hand, where the lag was observed, the very first synthesis in the new S_3 orientation might not yet have occurred, and therefore been visible.

Additionally it could be argued that the steeply arranged (in the S_2 orientation) microfibrils observed in Figure 2.5b were actually laid down prior to the transversely (S_3 orientation) arranged ones. However, this possibility seems unlikely when observations from other cells at this stage of development were also considered; occasionally cells with the S_2 arrangement only were observed.

2.4.2 Primary wall

Both cortical microtubules (left of Figure 2.1a) and cellulose microfibrils (Figure 2.2a) were randomly arranged in the cambial initials and their earliest derivatives. This random arrangement persisted during the first stage of slow radial expansion (right of Figure 2.1a; 2.2b). During the next stage of development, in cells that were undergoing a rapid increase in radial diameter, both cortical microtubules (Figure 2.1b) and microfibrils (Figure 2.2c) were arranged approximately longitudinally.

The pattern of cellulose microfibrils in the primary wall of developing *Abies sachalinensis* tracheids has previously been reported to be similar to that of cortical microtubules (Abe et al., 1995b). These workers reported that in the region 0 to 100 µm from the phloem xylem boundary, both cortical microtubules and cellulose microfibrils were random, but predominantly longitudinally arranged. Exactly where in this region the FESEM image of longitudinally oriented microfibrils (Abe et al., 1995b, Figure 2.4) was taken is not specified, but it seems plausible that it was of rapidly expanding cells. In the present study it has been shown precisely that this longitudinal arrangement only occurs in the zone of rapid radial expansion, while during the earlier stage of development (left of Figure 2.1a; Figure 2.2a) the arrangement of both polymers is random. The much faster growth, and consequent much wider radial zone of tracheid development in *P. radiata* compared with *A. sachalinensis*, has allowed more detailed observations to be made in the present study.

During the second phase of slow radial expansion, and also leading up to the primary to secondary wall transition, cellulose microfibrils were well ordered and mainly arranged transversely (Figure 2.2d). Some transverse microtubules were also present in the region where this microfibril arrangement commenced (Figure 2.1c left). However, as development proceeded these microtubules took on a diffuse appearance, suggesting that they were depolymerising (Figure 2.1c right; Figure 2.1d left). The orderliness and packing density of the microfibrils present appears to exceed what might be expected if their deposition was merely following the less well ordered, diffuse cortical microtubules that were present at this stage. Hence, it would be unwise to presume that cellulose microfibrils and microtubules are always co-aligned during primary cell wall development in tracheids. There are many examples where microtubules and cellulose microfibril orientations do not parallel one another during primary wall deposition (see reviews by Robinson and Quader, 1982; Giddings and Staehelin, 1991; Baskin, 2001). Furthermore, organised cellulose microfibrils can be established in the absence of cortical microtubules in the primary cell wall (Himmelspach et al., 2003). Expansion is taking place slowly in this region, and the wall is very thin. Therefore, it seems likely that only a small amount of new cellulose synthesis is occurring at this time. Hence, my observations for this zone are consistent with the hypothesis that the rate of cellulose synthesis might be important in controlling cellulose organisation (Sugimoto et al., 2001; Sugimoto et al., 2003).

Cells undergoing primary wall deposition are still expanding. Therefore this wall development occurs under the influence of biophysical forces that are not present during secondary wall development. It has been postulated that the biophysical forces experienced by rosettes in the membrane during wall deposition influence the pattern of cellulose microfibrils (Preston, 1988; Emons, 1994). These forces undoubtedly influence cellulose deposition in the primary wall in developing tracheids.

2.4.3 Secondary wall

The coordinated changes in orientation of cortical microtubules observed here in developing *P. radiata* tracheids undergoing deposition of the secondary wall were consistent with previously published results from the gymnosperm *A. sachalinensis* (Abe et al., 1995a; Funada et al., 2000; Funada, 2002). This included the difference in

how quickly the microtubule orientation changed at the secondary transitions; the S_2 to S_3 transition was abrupt, whereas S_1 to the S_2 transition was gradual (Funada et al., 2000). Helically arranged cortical microtubules have also been observed during secondary development in angiosperm tree species (Prodhan et al., 1995; Chaffey et al., 1999; Chaffey, 2000; Chaffey, 2002a; Chaffey et al., 2002).

2.4.4 Pit development

Observations from the present study suggest that cortical microtubules play a similar role during bordered pit development as they do during development of other wall features. Circular bands of cortical microtubules and microfibril thickenings around the developing aperture of pits gradually became smaller in diameter (Figure 2.6). At each stage of development the narrowing of the pit aperture lagged behind that of the microtubule ring (Figure 2.7).

Uehara and Hogetsu (1993) put forward another idea, based on their observations of circular bands of microtubules associated with the inner edge of the border of developing pits in *Taxus*. Unfortunately, they did not comment on the precise comparative sizes of the two rings. However, their TEM image (Uehara and Hogetsu, 1993, Figure 14) suggests that the microtubule ring was smaller. Uehara and Hogetsu (1993) suggest that this microtubule band acts to determine and maintain the boundary between the region of secondary wall deposition and the region where this does not occur.

Observations made during the present work are consistent with both ideas. Whether these microtubules differ from those involved in normal secondary wall deposition, as alluded to by Uehara and Hogetsu (1993), cannot be deduced from the present results. Nevertheless, it is interesting to note that 10 CesA proteins have been identified. Three are involved in primary wall development and a different three in secondary wall development (Doblin et al., 2002). It is plausible that some of the remaining CesA proteins might be exclusively involved in bordered pit development, in which case they might associate with a different group of microtubules than those involved in secondary wall deposition.

Concluding remarks

Evidence presented in the present chapter suggests that the orientation of cellulose microfibrils in the secondary cell wall is governed by cortical microtubules. However, the long-standing hypothesis that describes the cellulose synthesising complexes being guided across the plasma membrane by cortical microtubules has now been refuted in certain cases of primary growth (Sugimoto et al., 2001; Himmelspach et al., 2003; Sugimoto et al., 2003). Nevertheless, given the likelihood of microtubule involvement, alternative functions for microtubules such as guiding deposition of templates capable of controlling cellulose orientation (Baskin, 2001) or acting to control the length of cellulose microfibrils (Wasteneys, 2004) have been proposed. While the data presented here is consistent with the above ideas, it does not support the biophysical models. The fact that (in the secondary wall part of the study) the present experiment looked at changes in a cell wall that has long since ceased expanding, and coordinated changes in cortical microtubules followed by cellulose microfibrils were observed makes it seem unlikely that these changes were driven by biophysical forces (Preston, 1988; Emons, 1994). Furthermore, the abrupt changes that occurred at the S_2 to S_3 transition make models based on cell geometry seem untenable.

The templated incorporation model presented by (Baskin, 2001) postulates that microtubules are involved in establishing a scaffold of proteins on the plasma membrane that might control the laying down of nascent microfibrils. Since the microtubules are not directly involved in controlling the orientation of cellulose microfibrils the model thus accounts for the occasional disparity observed between their orientations. However, Gardiner et al. (2003) reported that accumulation of the cellulose synthase complex (indicated by the presence of the CesA protein IRX3) at the localised sites of wall deposition was dependent on the presence of localised microtubules. When microtubules were treated with oryzalin in this study, IRX3 dispersed in concert with the depolymerisation of the microtubules. Furthermore, it was recently convincingly demonstrated in live cells (using a transgenic CesA:yellow fluorescent protein) that CesA complex movements coincided exactly with microtubules (Paredez et al., 2006). Additionally, these authors also observed that after microtubules had depolymerised, CesA complex movements frequently persisted along the original trajectory for a short time. This observation also accounts for the

occasional disparity observed between microtubule and microfibril orientations that have been observed in the past.

Because Paredez et al. (2006) observed that CesA complex movements coincided exactly with microtubules, even when they were discordant and bent, they have ruled out a model in which the CesA complexes are guided by passive channeling between microtubules. Hence, they favor a more direct connection between CesA and microtubules. Since it has been shown that a kinesin-like motor protein is essential for the oriented deposition of cellulose microfibrils (Zhong et al., 2002), it seems likely CesA complexes might be linked to microtubules by these. The results of the present chapter are consistent with these ideas.

Chapter 3

Stem Explant Culture Protocol Development

3.1 INTRODUCTION

The formation of xylem tracheids is a complex multistep process, involving division and expansion of cells derived from the cambium, wall deposition and programmed cell death. The mechanisms involved in their development are as yet poorly understood. It is apparent that a complex array of mechanisms is likely to be involved, and elucidation of these mechanisms continues to be a difficult task.

Studying and understanding these mechanisms is a major challenge, especially when approached from the whole tree level. Attempts to experiment on wood development *in arbor* will most likely be influenced by an array of physiological processes occurring elsewhere within the whole tree. For example, the basipetal flow of endogenous auxin that naturally occurs in a whole growing tree makes it impracticable to carry out well controlled *in arbor* experiments to look at the influences of exogenously applied auxin.

The usefulness of *in vitro* culture techniques for reducing the complexity of physiological and developmental events occurring in whole plants into component parts by culturing tissues and organs of interest has long been recognised (Street and Henshaw, 1966). Studies using these methods afford several advantages compared to studies *in arbor*. Cells, tissues and organs can be studied in isolation from the effects of phytohormones that are transported from other parts of the tree. This is important because xylem cell development has been shown to be influenced by all five classes of plant growth regulators: auxins, abscisic acids, gibberellins, ethylene, and cytokinins (see review by Little and Savidge, 1987). Similarly, cells can be studied away from the effects of nutrients, transported in the xylem sap and phloem, which naturally vary due to seasonal changes and environmental conditions.

Using *in vitro* methods it is possible to vary nutrients, growth regulators, and other factors received by explants, by altering the constituents of defined culture media, in order to study the effects on developing cells. Additionally, environmental factors such as temperature and lighting regimes experienced by explants grown *in vitro* can be easily varied by simple adjustment of growth chamber parameters; changing the environment experienced by field grown, or even greenhouse grown, trees is obviously more difficult!

3.1.1 Disadvantages of in vitro culture

Leitch (1999) and Leitch and Savidge (2000) point out that wounding, associated with removal of explants for culture, may elicit epigenetic or physiological changes in cells. However, it is apparent that the same criticism could be made of some *in arbor* experimental treatments, for example stem injection of plant growth regulators. Additionally, it seems likely that such wounding responses would be limited to the edges of explants. Leitch (1999) and Leitch and Savidge (2000) also point out that the physical environment experienced by explants differs from that *in arbor*. For example, there is no phloem loading from mesophyll or transpiration driven negative pressure potential. Nevertheless, despite these problems, it is apparent that the advantages of *in vitro* culture far outweigh the disadvantages.

3.1.2 Media components

(Leitch and Savidge, 2000) report that there is a lack of knowledge on amounts of endogenous organic metabolites and inorganic ions within each region of the developing xylem (*in arbor*), and how these levels vary seasonally. These authors suggest that there is a definite need to know more about this in order to intelligently design physiologically ‘correct’ media. Additionally, Krikorian and Berquam (1969) point out that growing tissues in culture might require factors over and above those essential to the intact plant from which they were derived, because the synthetic ability of the intact plant is more complete.

Despite this, numerous different culture media have been developed allowing successful growth of various explant types from different species (Street and Henshaw, 1966; Gamborg, 1984; see Duchefa, 2000). The development of the various media has largely been an empirical process, initially based on nutrient solutions

developed for solution culture of whole plants (Williams, 1993). Constituents of the basic formulations were adjusted to suit new species, and published media recipes were progressively refined to suit particular purposes. More recently attempts have also been made to design media based on the actual elemental composition of organisms (Spaargaren, 1996). However, the practical utility of such theoretical media recipes for the culture of plants has yet to be demonstrated.

Experimental evidence suggesting possible roles for various components found in culture media was reviewed by (Street, 1966). Additionally, probable roles for micro- and macro-elements found in media can be deduced from general *in vivo* nutrient studies, as described in Taiz and Zeiger (1998). Probable roles for the various elements present in culture media are summarised below (summarised from Taiz and Zeiger, 1998; Duchefa, 2000)

3.1.2.1 Micro elements

Boron

- functions are mainly extracellular
- has a regulatory role in cell wall synthesis
- involved in xylem differentiation
- participates in regulation of phenol metabolism and synthesis of lignin
- forms sugar borate esters in hemicelluloses; most of the boron in plants is localised in the cell wall
- present as borate esters in the cell membrane
- deficiency leads to an increase in IAA oxidase activity

Chlorine

- osmoregulation
- compensation of charges
- regulates opening and closing of stomata
- In the chloroplast Cl is probably involved in photosystem II during the Hill reaction when H_2O is split into O_2 and $2H^+$
- Activates asparagine synthetase, an important enzyme in nitrogen metabolism

Iron

- mainly bound to chelators and complex compounds
- component of cytochrome, which is involved in electron transport chain in chloroplasts and mitochondria, and also reduction of nitrate to nitrite
- component of catalases and peroxidases, and numerous other enzymes

Copper

- component of enzyme complexes
- bound to plastocyanin, an intermediate of the electron transport chain between photosystem I and II
- part of Super Oxide Dismutase (Cu-Zn.SOD) enzyme, important for neutralisation of the highly reactive superoxide anion radical O_2^-
- has an important role in mitochondrial electron transport chain

Cobalt

- involved in N fixation, but non-N fixing plants probably don't require cobalt

Manganese

- component of enzyme complexes
- involved in enzyme activation
- involved in the Hill reaction of photosystem II
- component of manganese Super Oxide Dismutase (Mn.SOD)

Molybdenum

- component of, and cofactor for enzyme complexes
- N fixation and reduction

Zinc

- component of, and cofactor for enzyme complexes
- component of Cu-Zn.SOD
- very important for protein synthesis
- involved in the synthesis of tryptophan, a precursor of IAA

3.1.2.2 Macro elements

Calcium

- involved in middle lamellae and cell wall synthesis
- inhibits the activity of polygalacturonase, an enzyme that breaks down pectin. Hence, a lack of Ca leads to a breakdown of the cell wall
- also important for resistance against fungal infection
- stabilises the cell membrane by interacting with phosphates, carboxylate groups of phospholipids and proteins present in the membrane
- activates a few enzymes, such as amylase and ATPases
- inhibits some cytoplasmic enzymes
- implicated as a second messenger for responses to environmental and hormonal signals

Phosphorus

- an essential element of DNA and RNA
- essential component of phospholipids in membranes
- important for energy metabolism, as in the highly energetic pyrophosphate bond between two P atoms in adenosine triphosphate (ATP), and the energy rich phosphate esters of glucose-6-phosphate
- regulates activity of some enzymes, such as phosphofructokinase, and ADP-glucose-pyrophosphorylase (involved in starch synthesis)

Potassium

- essential for activation of numerous enzymes
- osmoregulation
- important in cell elongation; vacuolar K^+ accumulation causes an increase in osmotic potenial, which drives an increase in vacuole volume, through osmosis. This is important process in cell elongation
- important in maintenance of ion balance

Magnesium

- central atom in chlorophyll molecules
- essential for tertiary structure of many enzyme-substrate complexes
- important for protein synthesis
- vital in energy metabolism, because of its involvement in ATP synthesis
- regulates cation-anion balance

Nitrogen, Nitrate, Ammonium

- the major component of most culture media
- provide inorganic N source to synthesise complex organic molecules
- nitrate cannot be used to synthesise organic molecules directly. It must first be reduced to ammonia, in two steps, by nitrate and nitrite reductase. The first step (reduction of nitrate to nitrite, by nitrate reductase) is carried out in the cytoplasm. The second step (reduction of nitrite to ammonia, by nitrite reductase) is carried out in the leaves
- ammonia and ammonium are toxic. Therefore, they must be quickly converted into low-molecular non-toxic nitrogen containing compounds, such as glutamine, asparagine, arginine, allantoin and betain
- besides detoxifying ammonia and ammonium, the low-molecular nitrogen compounds supply organically bound N and NH_2 for synthesis of amino acids and proteins, and act as a storage place for excess N

Sulphur

- available in media as sulphate (SO_4^-), which must be reduced before use. Several assimilation steps, which take place mainly in the chloroplasts, yield stable cysteine
- cysteine is the precursor for all organic compounds that contained reduced sulphur, such as proteins, coenzymes and secondary metabolites
- cysteine is important for tertiary structure of proteins and activity of enzymes
- sulphur in the non-reduced form is a component of sulpholipids, which are a component of membranes

3.1.3 Possible in vitro *approaches to study xylem formation*

In vitro approaches applicable to the study of cambial activity and wood formation are reviewed by Leitch and Savidge (2000). These include culture of isolated protoplasts from the cambium and developing xylem, suspension culture of single cambial cells and aggregates, tissue culture of cambial strips, and organ culture of stem explants that are comprised of cambium sandwiched between phloem and xylem.

Tracheary element development has been extensively studied in redifferentiated mesophyll cell cultures of *Zinnia elegans* (see reviews by Fukuda, 1994; Kuriyama and Fukuda, 2001). The *Zinnia* system produces individual cells with spiral and reticulate secondary thickenings, and autolysis of cell contents occurs. The *Zinnia* system is fast (it takes just 96 hours for mesophyll cells to dedifferentiate and develop as tracheary elements), efficient and relatively simple to establish, and over the years has emerged as a model system for studying tracheary element differentiation. The system has proven extremely useful for studying molecular events, and the expression of numerous genes has been shown to occur at various stages of tracheary element development (Fukuda, 1997).

Cultures derived from cambial explant strips have, in some species, been shown to be capable of producing normal tracheary elements under pads of callus (Brown, 1964). However, in the case of pines to my knowledge the formation of normal tracheids has not been achieved in this type of system. Using cambial strips and other explants Moller, McDonald, Walter and Harris (2003) have developed a callus culture method for *Pinus radiata*. The individual cells produced are tracheid-like in that they have secondary thickenings and develop circular bordered pits. The system is transformable and is useful for studying the effects of gene expression on wall formation.

While many features of these single cell cultures are reminiscent of those found in tracheary elements, the cells produced are morphologically very different from “normal” tracheary elements found in wood, being short irregular oblong shaped entities. They do not form contiguous conduits capable of water conduction and support as found *in arbor*. In keeping with the abnormal morphology of these cells, the arrangement of cellulose microfibrils is far from normal. For this reason the

applicability of these methods to the study of real wood is limited, especially with respect to the relationship between cellulose microfibril angle and cell shape.

3.1.4 Organ culture of stem explants

Organ culture of stem explants overcomes the problem outlined above, as normal wood cells can be grown. Brown and Wodzicki (1969) developed a method for culturing *Larix leptolepis* and *P. densiflora* stem explants using liquid media gravity fed though tubing. New, normally differentiated tracheids were formed in cultures of both species. Zajaczkowski (1973) investigated the method further and found that the cambial response was dependant on stem age (young stems were found to be more responsive).

Stem (or branch) explants have also been cultured on agar solidified media (DeMaggio, 1966; Jacquiot 1949-1964 and Gautheret 1934-1950 as discussed in Jacquiot, 1966). Results obtained, in terms of the ability to grow wood in culture, showed wide discrepancies between species, but also between individuals of the same species. Chestnut, birch, aspen, lime, wild cherry, and elm were amenable to wood growth *in vitro* on defined media. Eucalypt explants on the other hand could only be maintained *in vitro* with coconut milk. Occasionally Jacquiot found it difficult to obtain growth in explants from some individuals of the 'amenable' species, and alludes to the importance of growth vigour and the associated build-up of reserve stuffs in trees from which explants are taken. With respect to gymnosperms, Table 1 of Jacquiot (1966) lists that experiments were undertaken with common spruce and white fir, unfortunately results of these experiments were not conveyed.

More recently there has been a renewed interest in such systems and Savidge (1993) has published a method for culturing stem explants of *Larix laricina*. This procedure was modified by Leitch (1999) to suit the growth requirements of *Eucalyptus globulus*.

3.1.5 Aims

The aim of work described in the present chapter was to develop a system for *in vitro* culture of *P. radiata* stem explants. The methods of Savidge (1993) and Leitch (1999) were used as a foundation, and modified to suit the requirements of *P. radiata*. This

chapter outlines the final method arrived at and some of the work that was carried out to achieve this.

The utility of this method is demonstrated in the subsequent chapters of this thesis. In these it is demonstrated that altering plant growth regulators in the growth media can change the size, shape and wall characteristics of tracheids.

MATERIALS AND METHODS

3.2.1 Liquid media culture trials

Initial attempts to grow *P. radiata* wood *in vitro* were based on the methods of Brown and Wodzicki (1969), using liquid media delivered to seedling stem explants via silicon tubing. Their methods were simplified and modernized by using sterile 1000 mL medical transfer bags (JMS Singapore PTE LTD), instead of aspirator bottles, to contain and receive the culture media (Figure 3.1). Avoiding the use of aspirator bottles was considered desirable because they are now very expensive and difficult to obtain. Use of the transfer bags also avoided the use of cotton wool air filters and associated glassware, since the transfer bags simply constrict as the media is consumed. Furthermore, the minimisation of components greatly reduced the amount of autoclaving required. Tubing was fitted over explants using a sleeve expanding tool, in order to avoid damage to explants through peel back of bark.

In some cases new xylem was successfully grown from seedling stem explants using these methods. However, extensive problems were experienced with resin and/or callus buildup blocking the flow of media. For this reason, these experiments were disbanded in favour of the methods described below using agar-solidified media.

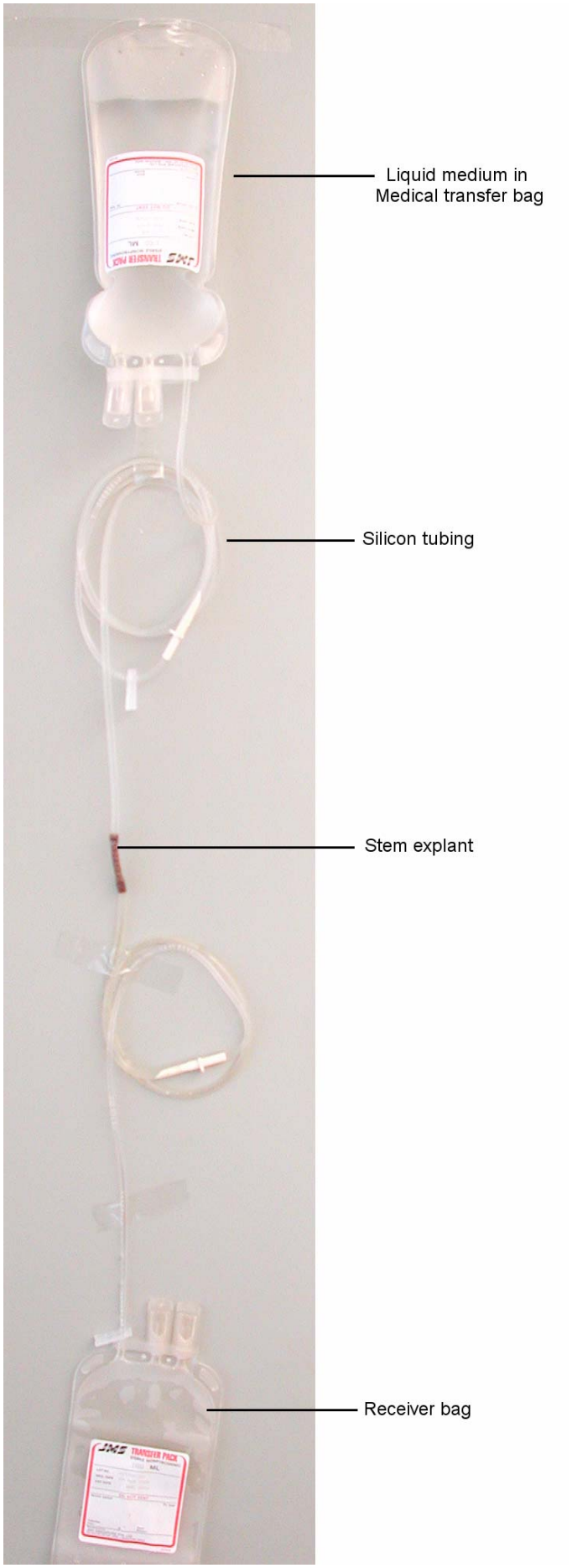


Figure 3.1: *Modification to methods of Brown and Wodzicki (1969) for culture of seedling stem explants with liquid media. Autoclaved growth media was injected into sterile medical transfer bags using a large syringe. The gravity fed media was supplied to stem explants via autoclaved silicon tubing. Media outflow was collected in empty transfer bags.*

3.2.2 Solidified media organ culture protocol

3.2.2.1 Foundation medium

The methods of Savidge (1993) were modified and adapted in an attempt to suit the wood growth requirements of *P. radiata*. The medium of Allen et al. (1988), as modified by Savidge (1993), did not prove satisfactory for *P. radiata*; explants cultured on this acquired a soft texture and a brown discolouration, and did not grow well. For this reason various media components were changed using an iterative approach. By trial and error, this problem was finally remedied by substituting the 4 % glucose as used by Savidge (1993) with 2 % sucrose.

3.2.2.2 Plant growth regulators and inhibitors

The 0.5 mg/L NAA as used by Savidge (1993) proved to be satisfactory for culturing *P. radiata* stem explants. However, it was found that explant growth could be markedly improved by increasing the NAA concentration to 5 mg/L. Hence, this concentration was subsequently used as standard, yielding the "default medium". Gibberellin A_4 is not heat stable, so when required, this component was sterilised using a 0.22 μm filter and added as the media cooled below 50^{o} C, to final concentrations of 1, 10 and 33 ppm. Oryzalin stocks were made up in DMSO and added in a similar fashion to a final concentration of 1, 10 and 100 μM.

3.2.2.3 Explants

P. radiata ramets planted in 1994 were felled as required for experiments and 1 m lengths of stem ranging in diameter from 75 to 150 mm in diameter were collected and stored at 4^{o} C. Back in the lab 150 mm lengths were then cut from these and washed with Pyroneg using a plastic scrubbing brush. Following this the lengths were washed again with dilute Domestos bleach (100 mL in 5 L of water). The rhytidome was then pared away using a sharp knife. The short lengths were then placed in a purpose-built clamping device inside the laminar flow cabinet, covered in ethanol and flamed while slowly rotating. This explant sterilisation regime provided minimal fungal and bacterial contamination, while allowing successful explant growth.

Explants approximately 50 mm long, 30 mm wide and 10 mm thick encompassing xylem, cambium and phloem were cut using a sharp knife and hammer. The present position of the cambium was then marked by longitudinally injecting 0.1 % Janus Green dye with a 25 gauge needle, and the explants placed onto appropriate media. It was essential to avoid the ends of the short logs when cutting out explants, as these tended to harbour high levels of fungal contaminants. Explants were then grown for several weeks in a growth room at 20^{o} C with 16 h lighting per day.

3.2.2.4 Other media

During the initial trials, different growth media including Gamborg B5 (Gamborg et al., 1968), Murashige and Skoog (Murashige and Skoog, 1962) and Quoirin and LePoivre as modified by (Aitken-Christie and Thorpe, 1984) were also evaluated. However, the medium of Savidge (1993), with the sucrose substitution as outlined above, proved the most reliable in terms of maintaining *P. radiata* explant health and potential for growth.

3.2.2.5 Gelling agents

Gelling agents used to solidify tissue culture media can influence the availability of minerals to explants (Williams, 1993). The inherent mineral content of gelling agents can contribute significantly to the media, and this content varies markedly in different brands of agar and other gelling agents (Williams, 1993). Additionally, different gelling agents have varying buffering capacities, and impart dissimilar influences on binding and diffusion of ions (Williams, 1993). Agar also contains various contaminants, such as organic acids, phenolics and long chain fatty acids, the amount of which varies between brands, and agar quality can influence the growth of cultures (Beyl, 2005). For these reasons some trials were carried out to investigate whether the use of Phytagel™, rather than standard agar (Difco® Bacto® agar), would improve explant growth. However, no improvement in growth resulted, and problems with gelling of Phytagel™ arose when attempts were made to add higher (> 10 PPM) concentrations of Gibberellin to the media.

3.2.2.6 Endogenous contamination

During the early stages of protocol development, serious problems with microbial contamination were experienced, and numerous techniques were tried in an attempt to minimise this. Rinsing explants in solutions containing various fungicides, including the all-purpose Yates Bravo®, failed to alleviate the problem. Rinsing explants in, and/or inclusion of Plant Preservative Mixture (PPM™) in the media, in various ways recommended by the manufacturer for endogenous contamination (http://www.ppm4plant-tc.com/instructions.htm), helped reduce contamination to an extent. However, explants treated in this way became brown and spongy, and failed to grow.

3.2.3. Scanning electron microscopy (SEM)

SEM sample blocks were roughly cut from chips using a single edged razor blade, each part of the blade was used to perform one cut only. Blocks were then neatly trimmed and placed in 95 % ethanol, and run through an ethanol/iso-amyl-acetate series (10 %, 25 %, 50 %, 75 %, 90 %, 100 % iso-amyl-acetate in ethanol) for 2 hours each change. Samples were then critical point dried, and mounted on SEM stubs (ProSciTech, Melbourne, Australia) using conductive carbon paint (ProSciTech, Melbourne, Australia). Mounted samples were then sputter coated with gold. SEM was performed with a Leica model S440.

3.2.4. Transmission electron microscopy (TEM)

Hand cut tissue blocks (2 mm tangential x 2 mm radial x 1 mm longitudinal) were fixed with 4% gluteraldehyde in 100 mM cacodylate buffer, post-fixed in 1% osmium tetroxide. The samples were then dehydrated in an ethanol series and transferred to 100 % acetone before infiltrating and embedding in Spurr Resin. Ultra thin sections were cut using a diamond knife, placed on copper grids, stained with uranyl acetate and lead citrate and observed using a transmission electron microscope (TEM).

3.3 RESULTS & DISCUSSION

Xylem with anatomical and ultrastructural properties similar to that of earlywood found *in arbor* can be grown *in vitro* provided the correct balance of medium constituents are present. Evidence of the utility of the method for manipulating and studying tracheid development is provided by experiments where plant growth regulators are altered, or a drug is used in the medium.

3.3.1. Wood grown in culture is normal

The xylem grown in culture was anatomically similar to earlywood found *in arbor*. Viewed in transverse section, the *in vitro* grown tracheids were rectangular with rounded corners, and had a relatively large lumen and thin walls (Figure 3.2a left). These characteristics are typical of earlywood - in contrast latewood is radially flattened, has much smaller lumens, and thicker walls. Tracheids grown *in vitro* were generally arranged in neat radial files as found *in arbor*. Anatomically normal rays also developed *in vitro* (Figure 3.2b). These possessed the characteristic blunt end walls when observed in transverse sections. The presence of simple pits in the transverse face of these cells shows that some secondary thickening has occurred, and also distinguishes that they are ray parenchyma, as opposed to ray tracheids that are anatomically similar but have bordered pits. Their development appeared to continue unimpeded and contiguously with radial files from pre-existing *in arbor* grown wood. Interestingly, I have not observed development of latewood *in vitro* to date, irrespective of what characteristics xylem growing *in arbor* had prior to explants being cultured. I have observed three broad types of xylem in the explants immediately prior to culturing:

1. Explants in which normal earlywood tracheids were present. These occurred when explants were taken from trees in spring or drought free periods of summer.
2. Explants in which thick walled, radially flattened latewood tracheids were present. These occurred when explants were taken from trees in autumn, or early winter.

3. Explants in which latewood-like tracheids were present, from drought induced false rings that occur during periods of hot dry summer weather in standing trees (Jenkins and Shepherd, 1974).

In all three cases the xylem grown in culture had tracheids with characteristics similar to normal *in arbor* grown latewood. Auxin has been reported to promote tracheid radial expansion in numerous studies when applied exogenously to trees (see review by Little and Savidge, 1987). It is feasible that the relatively small radial diameter of tracheids in types 2 and 3 is associated with seasonal and drought induced interruptions in the *in arbor* basipetal flow of auxin in the period before explants were collected. This is supported here by observations that tracheids grown *in vitro* developed with earlywood anatomy, even though the latest tracheids in the explant were latewood or latewood-like before culture (Figure 3.2a), once explants were placed on growth medium that contained a ready supply of the auxin NAA (auxin is always used in the growth medium, since it enhances cambial cell division). This result is further substantiated by findings that the concentration of NAA in the medium influenced the radial diameter of the newly formed tracheids. Increasing the concentration of NAA from 0.5, to 5.0, to 50 mg/L, resulted in a corresponding increase in radial diameter.

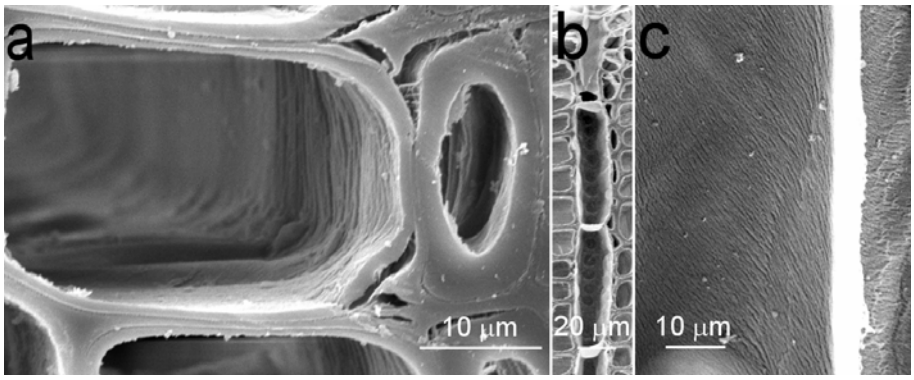


Figure 3.2: *SEM images of wood grown in culture. a: Earlywood-like tracheids at left, produced* in vitro, *yet* in arbor *wood was latewood-like at the time the explant was taken (cell at right). Transverse face, outside of tree to left. b: Ray parenchyma with slight secondary thickening, and simple pits. Transverse face, cambium at top. c: SEM image of radial longitudinal face. Bundles of cellulose microfibrils are clearly visible. The cell at right has just begun S_3 wall deposition, while the cell at left is still in S_2.*

The compound middle lamella and S1, S2, and S3 wall layers of tracheids all developed fully in culture grown wood (Figure 3.3). The compound middle lamella was highly lignified, as evidenced by dark staining. This has been independently confirmed with UV autofluorescence and safranin staining (H. Nair and T. Putoczki pers. comm.). The S1, and S3 wall layers contained cellulose microfibrils running in transverse bands parallel to the wall surface (Figure 3.3). In the S2 layer microfibrils were oblique, and therefore appear as short streaks in the TEM image (Figure 3.3). The organisation of cellulose microfibrils in the various layers of the cell wall has been confirmed using SEM and field emission SEM. Cellulose microfibrils were laid down in a normal highly organised pattern (Figure 3.2c). The cell at left was still laying down microfibrils in an S2 orientation, while in the cell at right S3 wall layer deposition was starting to occur.

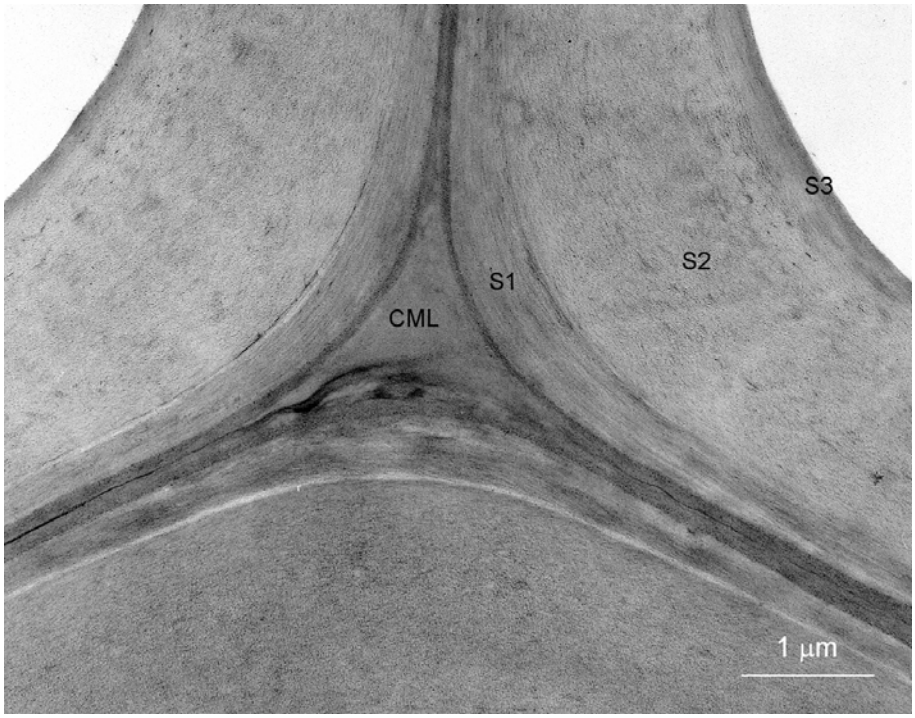


Figure 3.3: *Transverse TEM image of tracheid wall from wood grown in culture. The compound middle lamella (CML) and the three layers of the secondary wall have all developed normally, as found* in arbor. *Cellulose microfibrils can be seen running transversely around the S1, and S3 wall layers, while in S2 they appear very short, because they are running oblique to the section.*

Results from X-ray diffraction also show that culture wood (Figure 3.4) is similar to early wood found *in arbor*. The position, shape, and width of the white peaks, indicating the mean microfibril angle and its variance, are both similar to curves found in normal earlywood.

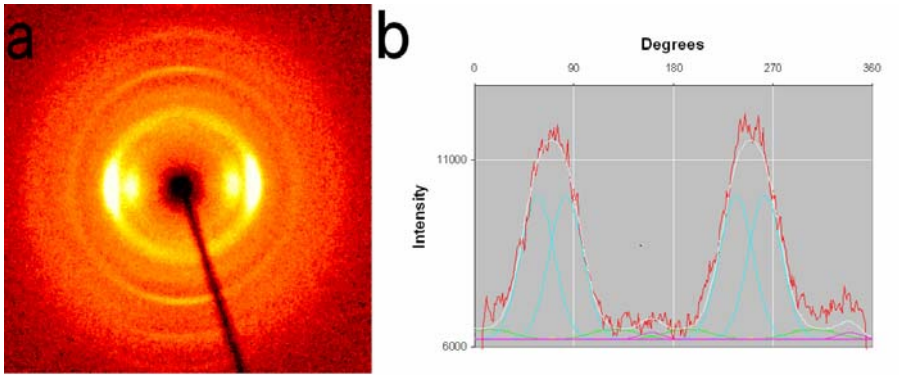


Figure 3.4: *X-ray diffraction of wood grown in culture. a: Diffraction pattern. b: Intensity plot and integration of diffraction pattern. These are typical of earlywood found* in arbor*, indicating that the* in vitro *grown wood has normal cellulose arrangement in the walls.*

Slight changes in the colour of xylem produced *in vitro* compared to that *in arbor* were sometimes observed. I have attributed this observation to differences in lignin composition, associated with varying the auxin concentration. As the concentration of NAA in the growth medium was increased the amount of lignin in the secondary cell wall also increased (H. Nair, pers. comm.). These results were based on observations by both ultraviolet epifluorescence microscopy and light microscopy of safranin fast green stained sections. At the NAA concentration used in the standard growth medium lignin composition was similar to that found in typical wood *in arbor*. While others have grown xylem in culture to varying degrees of success (Jacquiot, 1966; Savidge, 1993; Leitch, 1999), they have not demonstrated that the wood produced is "normal". To my knowledge, I am the first to show that the *P. radiata* wood grown by this methodology is very similar to earlywood found *in arbor*.

3.3.2. Requirements of explants for successful in arbor *xylogenesis*

My observations revealed that the inherent state of the tree from which explants were derived was paramount to the successful growth of xylem in culture. Explants used in these experiments were taken from ramets of one clone, and were thus genetically identical. In order for successful xylem growth to occur in culture it was found that explants must be collected in periods of active *in arbor* xylem growth i.e. spring,

drought free periods of summer, or possibly autumn. In explants taken in spring or drought free periods of summer and with provision of optimum plant growth regulators (5 mg/L NAA and 1 to 33 mg/L gibberellin A4) in the medium several mm of wood growth could be attained in culture (Figure 3.5).

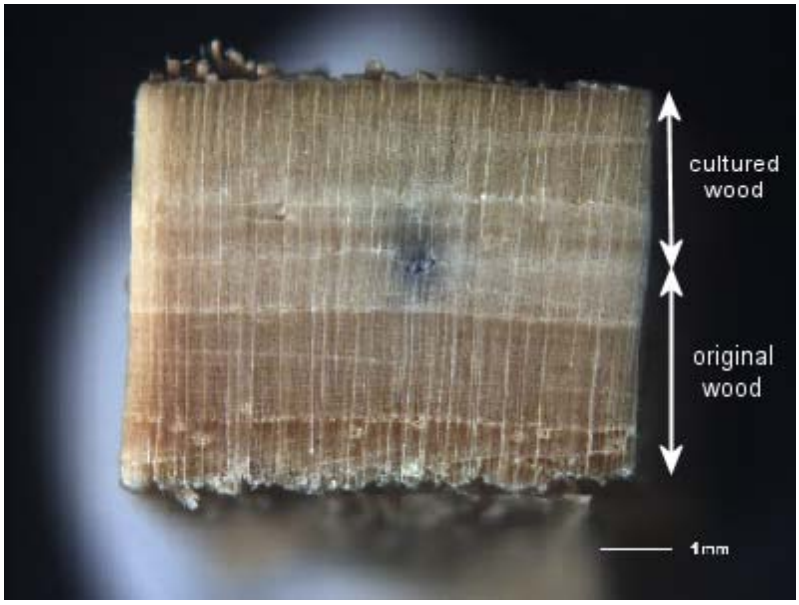


Figure 3.5: *Transverse face of stem explant grown in culture for 3 months. The blue dye mark and hole near the centre of the explant marks the original position of the cambium at the start of the culture period.*

Explants collected in winter invariably grew only phloem or did not grow at all. This can be attributed to the point that although *P. radiata* grown in New Zealand does not undergo a profound period of winter dormancy, Barnett (1971) reports that even in the most favourable growing climate at Rotorua mid-winter cambial activity only produces new phloem cells and no new xylem cells. These findings offer an explanation as to why the culture system could produce phloem but not xylem *in vitro* from winter collected explants. I was unable to promote xylogenesis in such explants using cytokinin, increased auxin, or gibberellin. Thus, it is apparent from the findings of Barnett (1971) and the present results that the status of *in arbor* cambial activity (i.e. in which direction(s) cambial cells are actively dividing, and the commensurate cell fate, whether it be xylem or phloem) continues *in vitro*, and is therefore a very important consideration when establishing these experiments.

Exactly what is present in the cambium during spring and summer that promotes *in arbor* xylogenesis and is able to be carried within explants to allow successful xylem

growth *in vitro* is unknown. It has recently been proposed that a ‘tracheid differentiation factor’ distinct from phytohormones or other known regulators of growth and development is produced in conifer leaves and exported to the cambium to promote xylogenesis (Savidge, 2000b). Presumably this tracheid differentiation factor is able to be stored in or around the cambium and when favourable levels exist in explants xylogenesis can occur *in vitro*.

Differences in the appearance of callus on the edges of explants usually occurred, even when explants were taken at close temporal intervals in the same season. Callus cells emanating from the cambium first appeared on the cut edges of explants after 1-2 weeks, and continued to grow for several months. The callus was typically greenish (Figure 3.6), although sometimes it was off white or brownish. Callus growth was a partial indicator of successful xylem growth in explants. Failure of callus growth usually indicated the failure of cambium to divide and produce new xylem cells. However, the opposite was not always true. In some cases callus grew while the cambium failed to produce xylem.

Figure 3.6: *Stem explant growing in defined media, showing green callus along wounded edges, and blue dye used to mark the original position of the cambium.*

The present chapter demonstrates successful *in vitro* xylem growth in *P. radiata* stem explants. The methods described here are used as the basis for the following chapters presented in this thesis.

Chapter 4

Is Compression Wood Formation Dependant on Biophysical Stress?

4.1 INTRODUCTION

Compression wood in radiata pine is a major problem for the New Zealand timber industry. It has several different characteristics compared to normal wood, including higher microfibril angle in the S2 layer of tracheid walls and higher longitudinal shrinkage on drying. If it is asymmetrically distributed in lumber, its greater than normal longitudinal shrinkage during drying imparts a high potential for warp. Additionally, compared to normal wood, compression wood has higher lignin and lower cellulose content, which is problematic to the pulp industry.

Severe compression wood is found on the lower side of leaning stems (and to varying degrees in vertical stems). The stimulus causing compression wood formation is still controversial. The two main theories are that compression wood formation is either a gravitational response, or a response to compressive stresses. It is extremely difficult to separate these two factors experimentally *in vivo*, although numerous ingenious experiments involving stem bending, revolving disks etc have been undertaken.

4.1.1 aims and hypothesis

The compressive forces experienced by stem tissues in a leaning tree are removed when explants are taken for stem culture. Based on this idea, the aim of the present study was to grow such explants in culture at the original lean, in order to test the null hypothesis that gravity instigates compression wood formation; i.e. cells are able to perceive a lack of vertical orientation. The alternative hypothesis under test was that cells can perceive compressive stresses brought about by a lean. Wood newly formed *in vitro* was normal wood, supporting the alternative hypothesis, that compressive forces rather than a lean are required for compression wood formation.

4.2 MATERIALS and METHODS

4.2.1 Organ culture protocol

The default medium outlined in section 3.2.2.2 was used in this experiment. NAA was added to batches of this at concentrations of 5, 50 and 500 mg/L, prior to autoclaving. Six weeks prior to culturing, several two year old potted ramets (genetically identical) were placed at a 45^{o} lean, in order to provide a ready supply of experimental material that contained comparable compression wood. The presence of compression wood in the stem of these ramets was confirmed before commencement of cultures, by observation of hand cut transverse sections. Stem explants approximately 50 mm long (encompassing the entire stem cross section) were cut from these ramets using secateurs, and washed with Pyroneg using a plastic scrubbing brush. Following this the lengths were washed again with dilute Domestos bleach (100 mL in 5 L of water). Inside the laminar flow cabinet, explants were covered in ethanol and flamed while slowly rotating. Explants were then placed onto appropriate media (bottom transverse face down), contained in 50 mL Falcon® tubes. Following this, a cap of the appropriate media was cut from a petri dish, and placed on the top transverse face. At all stages, extreme care was taken to ensure that explants were maintained at the correct orientation. Explants were then grown for several weeks, at the same 45^{o} lean that they experienced while *in arbor*, in a growth room at 20^{o} C with 16 h lighting per day.

Controls consisted of randomly selected stem explants, which were immediately killed and fixed in formalin-aceto-alcohol (Chamberlain, 1932) at the time of culturing.

4.2.2 Scanning electron microscopy (SEM)

SEM sample blocks were roughly cut from explants using a single edged razor blade. Blocks were then neatly trimmed using double edge razor blades, and fixed in FAA. Samples were then dehydrated in a graded ethanol series, and then placed in HMDS in a fume cupboard. After several days, once the HMDS had evaporated, blocks were then mounted on SEM stubs (ProSciTech, Melbourne, Australia) using conductive carbon paint (ProSciTech, Melbourne, Australia). Mounted samples were then sputter coated with gold. SEM was performed with a Leica model S440.

4.2.3 Light microscopy and image analysis

Transverse sections (encompassing the entire stem) approximately 2mm thick were cut from explants using a single edged razor blade. These were killed and fixed in formalin-aceto-alcohol (Chamberlain, 1932) under vacuum overnight. Samples were then dehydrated with tertiary-butyl alcohol (Johansen, 1940), infiltrated and embedded in Paraplast, and sectioned transversely at 10 μm using a rotary microtome. Sections were stained using Safranin-fast green (Johansen, 1940). Bright field microscopy was performed using an Olympus BH-2 light microscope, and digital images collected on an Olympus C-5060 digital camera.

Images were analysed using a combination of Adobe Photoshop and Image-J. Cell lumens were firstly made black using Adobe Photoshop:

- In the channel palate, Green was selected (since this had best contrast) and used to create an alpha channel
- The alpha channel contrast was maximised >Image >Adjust >levels >71 1 142 >OK
- The RGB channel was then selected
- In the Layers palate >Select >Load Selection >alpha 1 >OK, then >Edit >Fill >Use >Black >OK

From the images with blackened lumens, profiles of the red intensity were plotted using Image-J, with the plot Profile add-in installed.

- Image >Color >RGB split, discard the green and blue images
- Edit > Selection>Select All (first ensure rectangular selection button is on)
- Analyse >Plot Profile >Copy

The contents of the clipboard were then copied to Microsoft Excel, and plotted.

4.3 RESULTS

4.3.1 In brief

The aim of the present experiment was to determine whether compression wood from a leaning stem continues to form when compressive forces are artificially removed, but the lean remains. Explants (approximately 50 mm long by 20 mm in diameter,

encompassing the entire stem circumference) were taken from leaning stems, thereby removing compressive forces exerted by the weight of the rest of the tree. These were grown in culture at the original lean, with three different concentrations of the auxin NAA. Wood grown *in vitro* lacked compression wood characteristics. Rather, cells were more reminiscent of normal wood. NAA at higher concentrations led to faster cell division, but had no influence on the development of compression wood.

4.3.2 Scanning electron microscopy

Scanning electron microscopy was carried out to provide a broad overview of the anatomical characteristics of each sample, in order to assess the extent to which compression wood was present. Micrographs of the transverse face taken at 200x (Figures 4.1a-d) were found to the most useful for this, since this was the highest magnification at which the field of view encompassed the entire extent of *in vitro* growth for the fastest-growing treatments. Higher magnification micrographs (500x) of the regions where tracheids changed from having normal wood characteristics to compression wood characteristics were also taken (Figures 4.2a-d). The letters of each Figure correspond to the same treatment, and the black outlines in Figure 4.1 are the regions where Figure 4.2 images were collected.

In this study, tracheids circular in cross-section, with relatively thick walls were deemed to have compression wood characteristics. For each treatment, counts were made of the number of cells (from 200x micrographs) without compression wood characteristics – i.e. from the cambium to where cells with compression wood characteristics started (Figure 4.3).

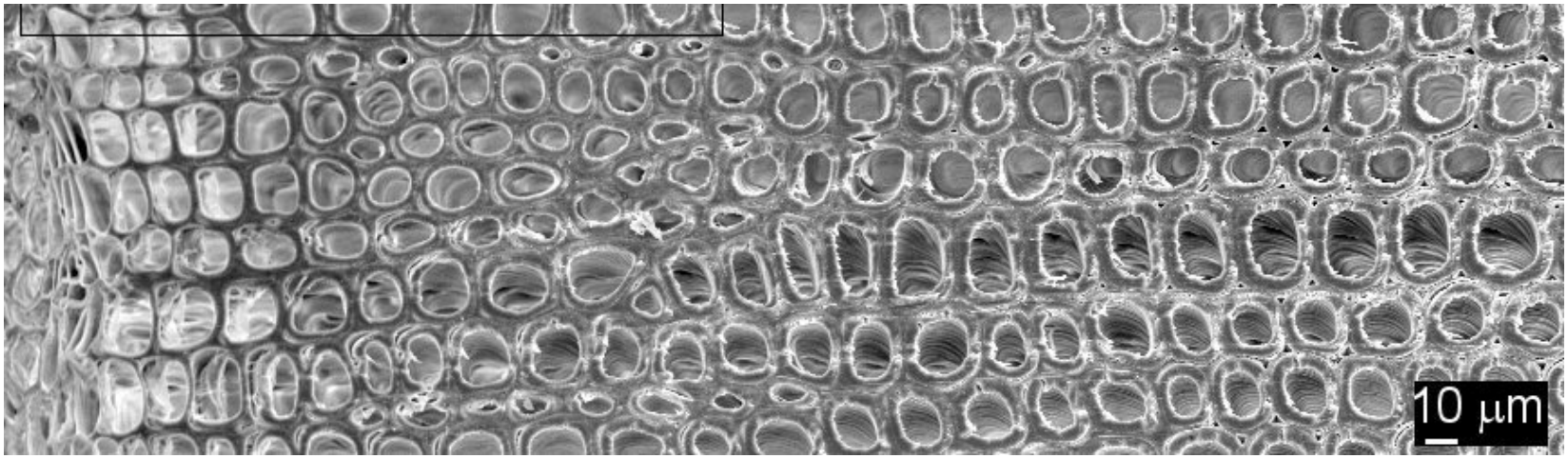


Figure 4.1a: *Lean control. 200x. Cambium near left. Note that about the first 6 cells in each radial file in the developmental sequence, to the right of the cambium, lacked compression wood characteristics. At a slightly later stage of development, compression wood characteristics rather abruptly became evident (see Figure 2a: higher magnification of the area bounded by the black border).*

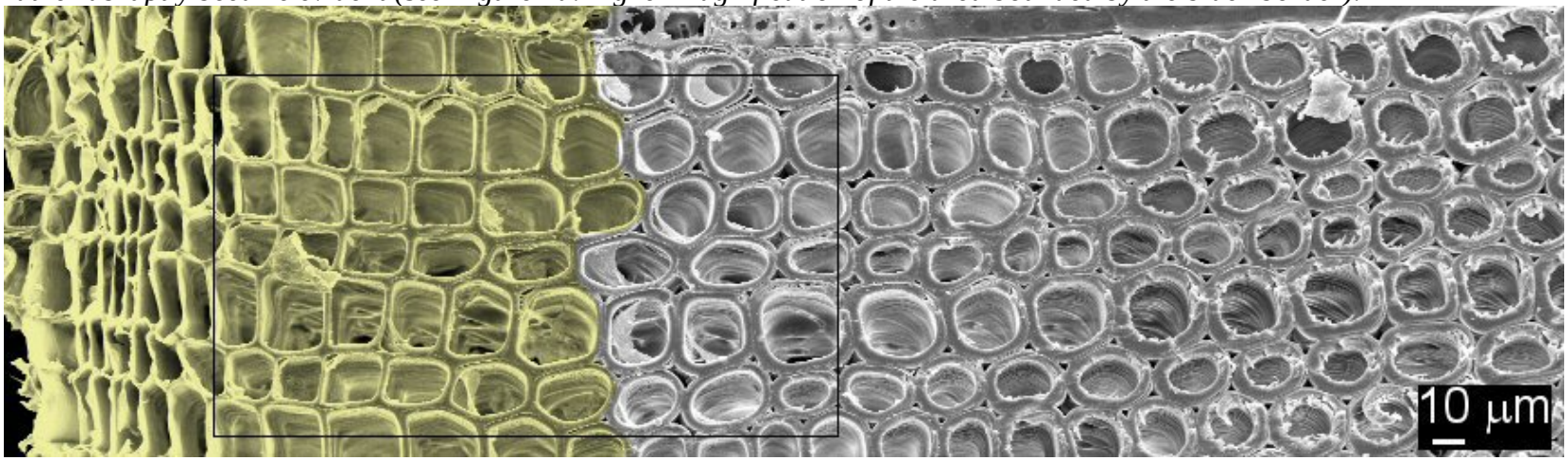


Figure 4.1b: *Wood grown* in vitro *with 0.5 mg/L NAA lacked compression wood characteristics (highlighted in yellow). 200x.*

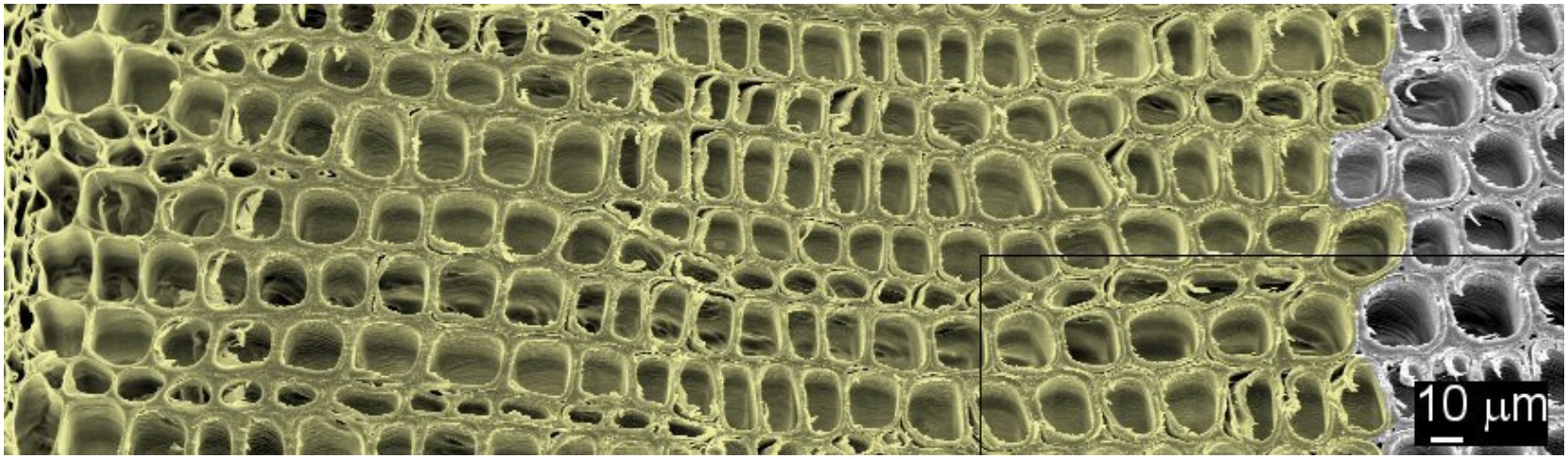


Figure 4.1c: *Wood grown* in vitro *with 5 mg/L NAA lacked compression wood characteristics (highlighted in yellow). 200x. Note the much greater region of* in vitro *growth c.f. that in Figure 1b.*

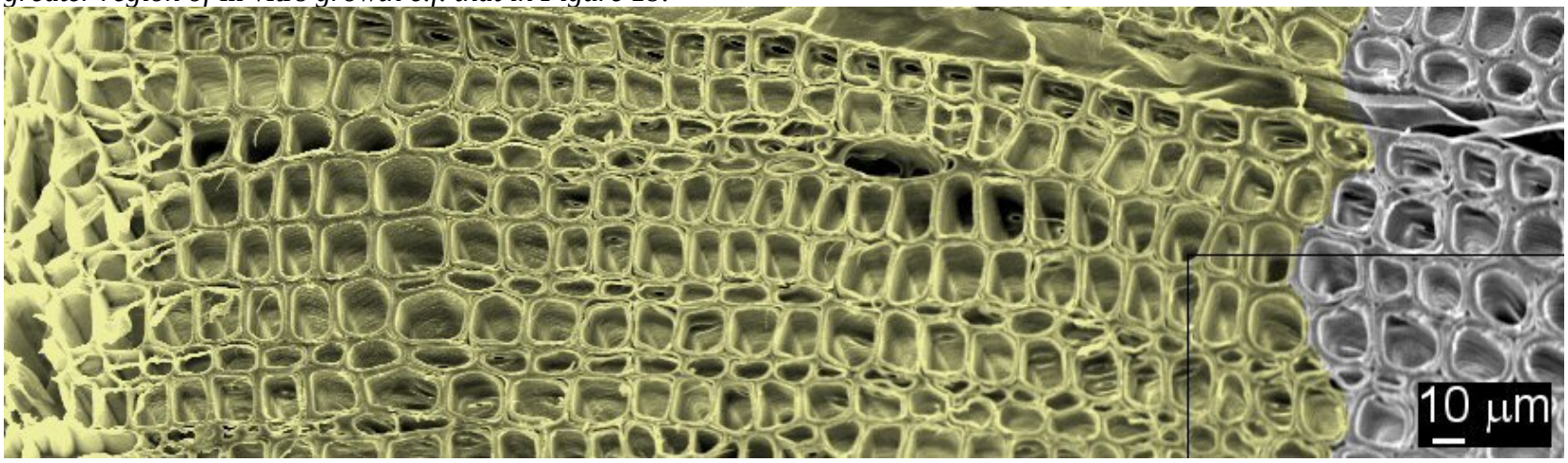


Figure 4.1d: *Wood grown* in vitro *with 50 mg/L NAA lacked compression wood characteristics (highlighted in yellow). 200x. Note the slightly greater number of tracheids grown* in vitro *c.f. that in Figure 1c.*

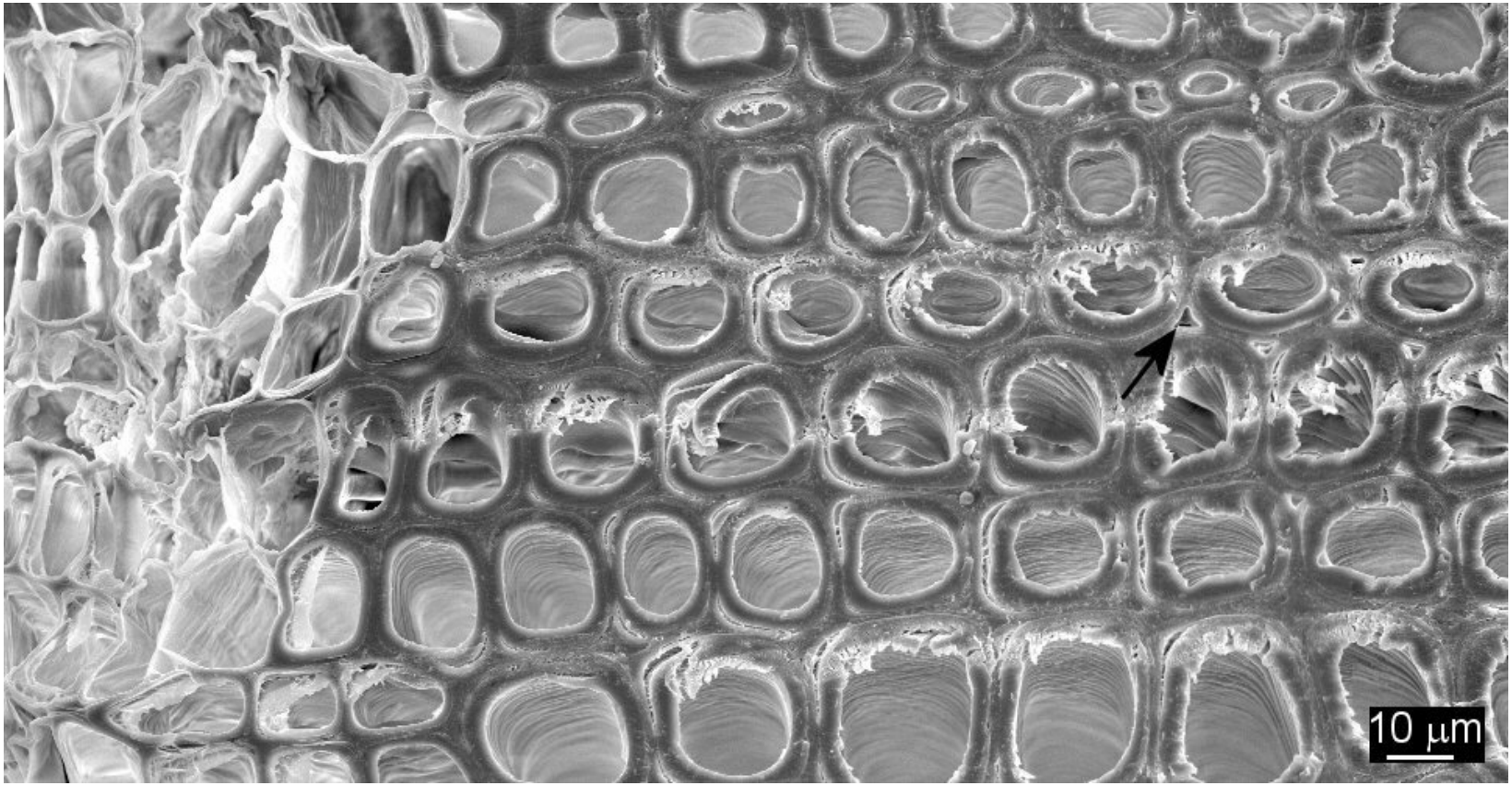


Figure 4.2a: *Lean control. 500x. Higher magnification of the area bounded by the black border in Figure 1a. The transition from younger developing tracheids, without compression wood characteristics (cells towards the centre), to those with severe compression wood characteristics (further to the right) was fairly abrupt. This change was not coordinated in each radial file, as can be clearly seen in this image; the changeover has occurred about 2 cells to the left of the arrow, in these 2 radial files, while in the bottom file, the changeover is starting to occur at right. Note that intercellular spaces, where present, also abruptly become evident (arrow), and once present their size does not change much.*

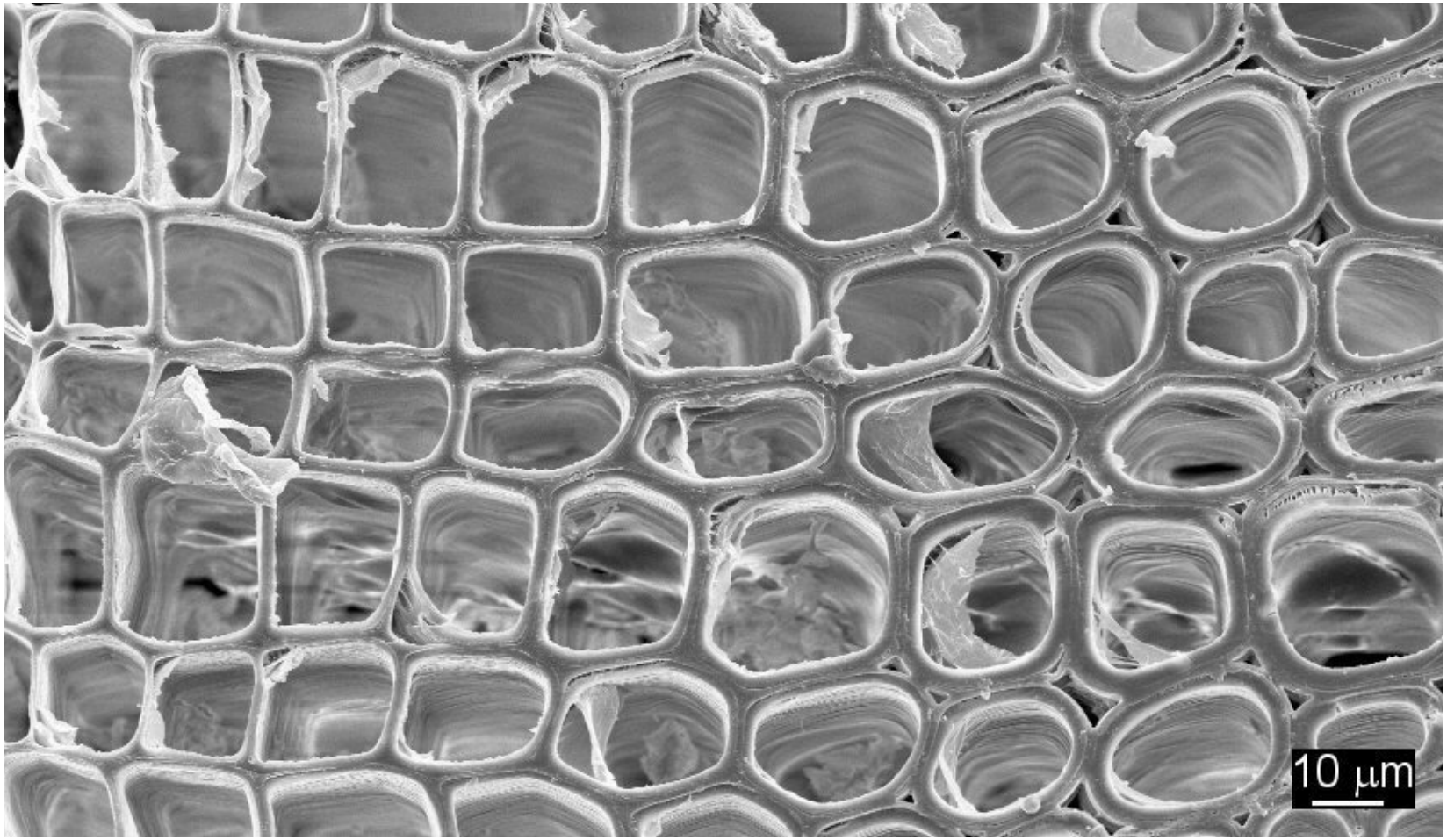


Figure 4.2b: *0.5 NAA. 500x. Higher magnification of the area bounded by the black border in Figure 1b.*

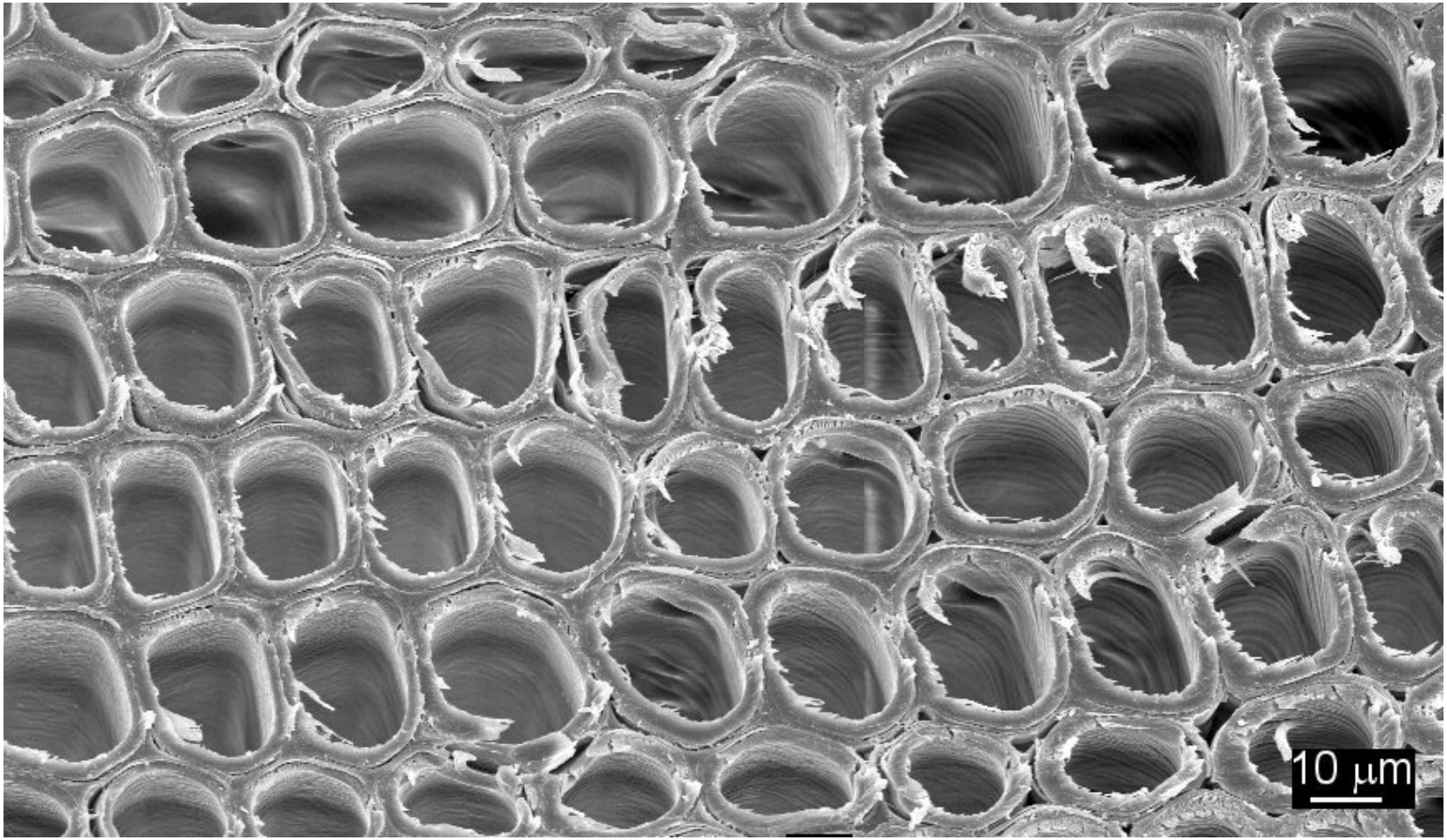


Figure 4.2c: *5 NAA. 500x. Higher magnification of the area bounded by the black border in Figure 1c.*

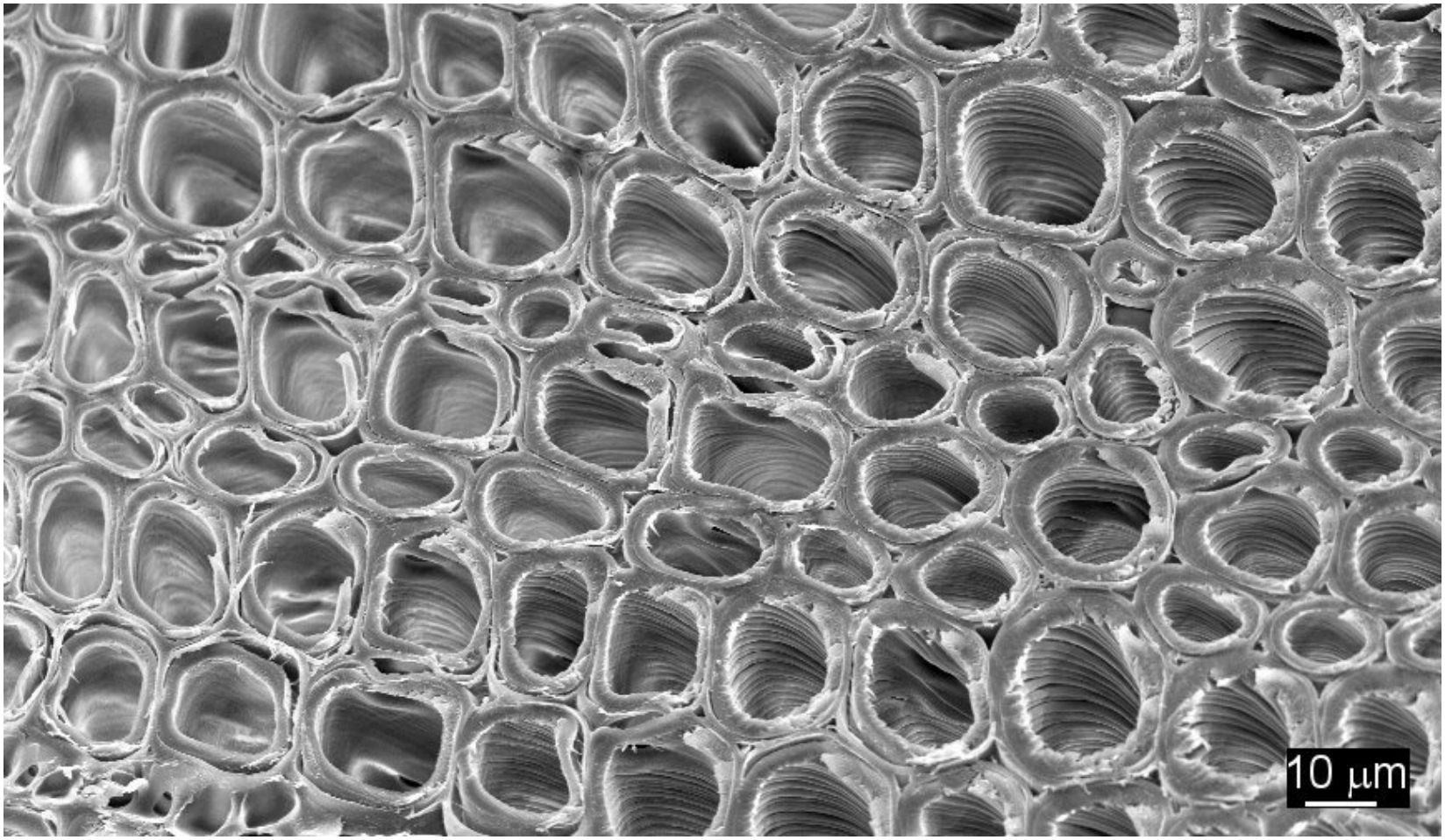


Figure 4.2d: *50 NAA. 500x. Higher magnification of the area bounded by the black border in Figure 1d.*

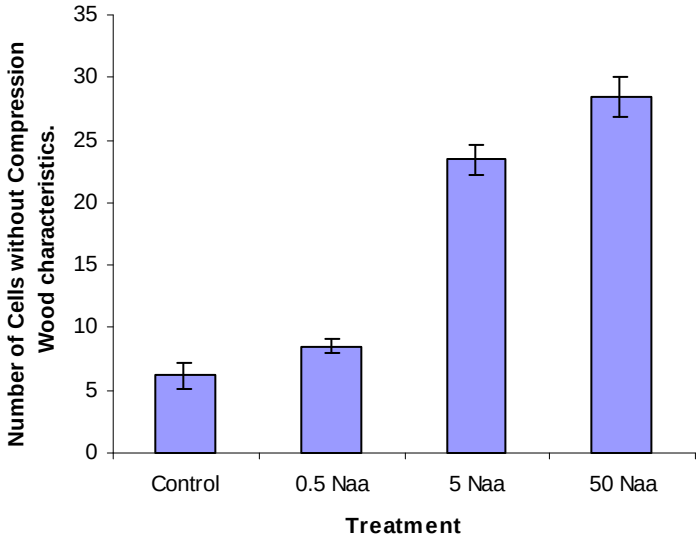


Figure 4.3: *Counts of the number of cells per radial file, from the cambium to the point where tracheids with compression wood characteristics began. Bars = means of counts from 20 radial files. Error bars = standard deviations of these means*

Interestingly, in the control, these compression wood characteristics were not manifest in the youngest developing tracheids (just to the right of the cambium in Figure 4.1a). The cross-section of these tracheids was squarish, and cell wall development was in its early stages. As wall development proceeded, the cross-section of tracheids became progressively more rounded. Intercellular spaces did not become evident until wall development was nearing completion (arrow Figure 4.2a).

In stem explants grown in culture, newly developed tracheids did not have compression wood characteristics (Figures 4.1b-d). The approximate extent of *in vitro* growth is coloured yellow. In transverse section these tracheids were squarish in outline, with relatively thin walls, and lacked the deep helical fissures easily discernible in the pre-existing *in arbor* grown compression wood (Figures 4.1b-d; 4.2b-d).

4.3.3 Light microscopy and Lignification

Light microscopy of safranin fast green stained sections was undertaken in order to ascertain whether each treatment affected the extent of lignification. The degree of lignification across the developing xylem was assessed by image analysis and results

plotted (Figures 4.4a-d). In the control (Figure 4.4a) lignification proceeded gradually and plateaued at a red intensity of around 90. On the other hand, lignification plots across wood grown *in vitro* were quite different. Compared to the control, the *in vitro* grown wood, which lacked compression wood characteristics, and rather had normal wood characteristics, was nowhere near as highly lignified (to the left of vertical lines in Figures 4.4 b-d). However, levels of lignification in the pre-existing *in arbor* grown wood (to the right of the vertical lines) were similar to those in the control.

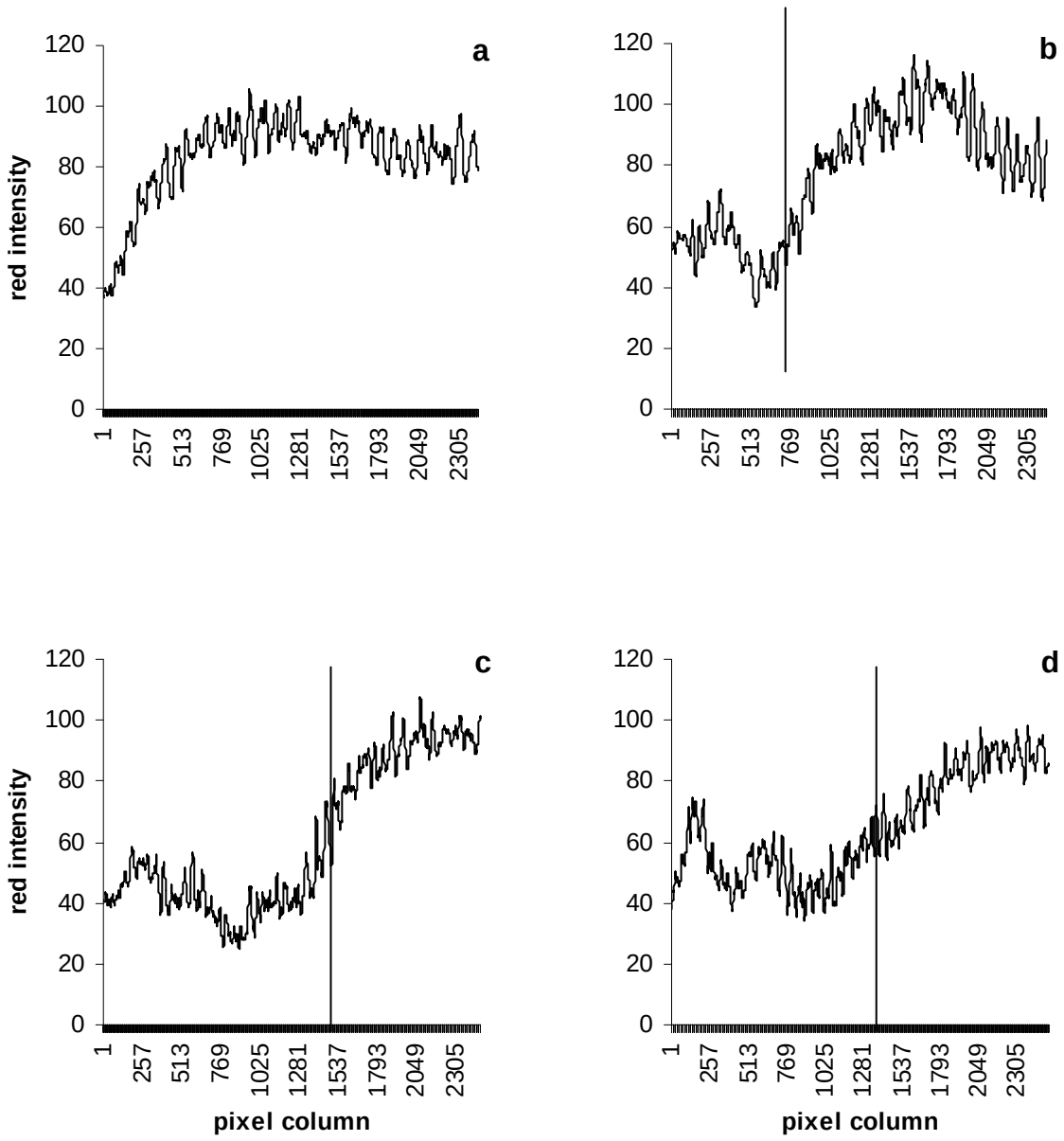


Figure 4.4: *Plots of extent of lignification across developing xylem. Cambium at left. Plots were derived from intensity of red staining, from safranin fast green stained sections.* ***(a)*** *Control. Note the gradual increase in lignification of this natural,* in arbor *grown wood.* ***(b)*** *Wood grown* in vitro *with 0.5 mg/L NAA.* ***(c)*** *Wood grown* in vitro *with 5 mg/L NAA.* ***(d)*** *Wood grown* in vitro *with 0.5 mg/L NAA. To the left of the vertical lines in b-d is the approximate extent of* in vitro *growth.*

4.4 DISCUSSION

In the present study stem explants (approximately 50 mm long by 20 mm in diameter, encompassing the entire stem circumference) were taken from leaning trees, with severe compression wood on the lower side, and grown *in vitro* at the original lean. Hence, the cambium and developing tracheids were under approximately the same direct influence of gravity, as when they were still *in arbor*. However, the compression forces experienced by these cells, on the lower side of the lean, while *in arbor* were removed when explants were taken, because the weight of the rest of the tree was no longer bearing down on them. The results obtained demonstrate that tracheids differentiating under these conditions had normal wood characteristics (Figures 4.1b-d; 4.2b-d). At the higher media concentrations of NAA numerous new tracheids developed, which had normal wood characteristics (Figures 4.1c & d; 4.2c & d; Figure 4.3). These results provide compelling evidence that localised perception of compressive forces within the stem, possibly by developing tracheids, is required in order for compression wood to develop.

These findings support the hypothesis that compressive forces experienced by the cell wall of tracheids, on the under side of leaning trees, leads to the formation of compression wood. These results are in agreement with the conclusions of Boyd (1977), who states that "compared with the constrictive limits of apparently possible application of alternative hypotheses, the stress theory alone provides a satisfying explanation of reorientation movements in general…". In this paper, Boyd highlights shortcomings, and errors in the interpretation of results, in numerous earlier studies, that appeared to support alternative theories (for example, that the formation of compression wood could be directly attributed to the force of gravity). One criticism of Boyd's work is that for compression wood to act expansively, the microfibril angle in the S_2 layer must be below 45^o. At least in *Pinus radiata* this is not the case; I have observed hundreds of samples of compression wood in which the microfibril angle is higher. Therefore, it seems likely that other mechanisms, such as microfibrils acting as a helical spring as assumed by Bamber (2001) are also be involved.

While Boyd's conclusions were fundamentally theoretical in nature, recent experimental work has demonstrated that compression wood formation can be induced in a microgravity environment, by mechanical bending (Kwon et al., 2001). In this experiment, Douglas fir seedlings were grown on board the Space Shuttle Columbia. Treatment plants were bent (rather sharply, judging from Figure 6b, Kwon et al., 2001), and held rigidly at 45^{o} in a mechanical device, while control plants were left unbent. Although the authors make no mention of the point, it seems possible that these sharp bends may have exceeded the elastic limit of the stems. Despite the sharpness of the bends, treatment plants developed compression wood, while controls did not. These authors offered two possible explanations for their findings. Firstly, it was possible that the greatly reduced gravitational forces were still sufficient for the plants to respond to this stimulus, or more likely, compression wood formation was a result of the stress induced by bending. Results of the present experiment support the latter conclusion.

My observations of control samples suggest that compression wood tracheids, in young ramets, attain their rounded shape and accompanying intercellular spaces relatively late during development (Figure 4.1a; 4.2a). In some instances, this appears to occur as late as the early stages of S_2 wall layer deposition. This is in contrast to reports where it has been shown that the round shape, and intercellular spaces characteristic of compression wood tracheids arise either during primary wall (Wardrop and Dadswell, 1952), or S_1 wall (Wardrop and Davies, 1964) development. These papers dealt with branch wood, and (presumably) mature stem wood. It is possible that in young ramets, and seedlings, compression wood cells attain their rounded shape, and accompanying intercellular spaces slightly later. There is some evidence in the literature to support this theory. In *P. radiata* seedlings (Plate 8, Figures 2 and 3, and Plate 9, Figure 1, Wardrop and Davies, 1964) intercellular spaces (and for that matter consistently rounded cells) were also not conspicuous until a relatively late stage of development This compression wood was induced by application of plant growth regulators, and it could be argued that the effect of these had worn off in the younger tissue, which had thus returned to the development of normal wood. However, in young *Picea jezoensis* compression wood it has also been shown that the rounded cell shape and intercellular spaces occur quite late in development (Figure 9, Yoshizawa et al., 1986). As pointed out by (Timell, 1986,

page 646) developing compression wood tracheids are able to change their shape during secondary wall deposition, at least up until the completion of the S_1 layer. Judging from my findings (Figure 4.2a), it appears that, at least in young stems, compression wood tracheids may be able to change their shape up until the early stages of S_2 wall deposition.

Results of the present study provide compelling evidence that compressive forces, rather than gravity *per se*, are involved in instigating the formation of compression wood. These findings also bring into question the theory that changes in wall shape during compression wood tracheid development can only occur up until the completion of the S_2 wall layer.

Chapter 5

Does Auxin or Ethylene Promote Compression Wood Formation?

5.1 INTRODUCTION

Compression wood in radiata pine is a major problem for the New Zealand timber industry. Compression wood is found on the lower side of leaning stems, and also in vertical stems. It has several different characteristics compared to normal wood, including higher microfibril angle in the S2 layer of tracheid walls and higher shrinkage on drying. If it is asymmetrically distributed in lumber its greater than normal longitudinal shrinkage during drying imparts a high potential for warp. Additionally, compression wood has higher lignin and lower cellulose content than normal wood, which is problematic to the pulp industry.

Much experimental evidence suggests that the plant hormones auxin and/or ethylene might be involved in compression wood formation. However, due to the interactive nature of the two hormones *in vivo*, interpretation of these results has been difficult, and it is still unclear as to which actually promotes compression wood formation. Ethylene is synthesised via the following pathway: methionine $\rightarrow$ S-adenosylmethionine (SAM) $\rightarrow$ aminocyclopropylcarboxylic acid (ACC) $\rightarrow$ ethylene. An increase in auxin stimulates an increase in ACC synthase, the enzyme that forms ACC, ultimately leading to an increase in ethylene production. An increase in ethylene production tends to inhibit auxin synthesis and transport via a negative feedback loop. Additionally, the synthesis of ethylene is autocatalytic; an increase in ethylene concentration leads to a further increase in ethylene concentration.

The complexities and resultant confounding influences of this situation can be mitigated to a large extent using *in vitro* stem culture methods. The methods detailed in Chapter 3 were used here to supply various concentrations of auxin and ethylene to growing explants. A key part of this experiment was the use of aminoethoxyvinylglycine (AVG) in the auxin experiments. This is an inhibitor of

ethylene biosynthesis, and its use was aimed at overcoming the interaction problems outlined above.

5.1.1 Aims and hypothesis

The aim of this two-part experiment was to grow stem explants with: (1) various concentrations of ethylene, and (2) with various concentrations of auxin. The null hypothesis under test was that auxin mediates compression wood formation, while the alternative hypothesis was that ethylene mediates compression wood formation.

5.2 MATERIALS AND METHODS

5.2.1 Organ culture protocol

Using the media outlined in 3.2.2.1 as a basis, experimental media with different levels of plant growth regulators were made. For the auxin experiment, NAA at 5, 50 and 500 mg/L were added to media prior to autoclaving, and 3.3mg/L filter sterilised aminoethoxyvinylglycine (AVG) added as media cooled. For the ethylene experiment, the default medium (see section 3.2.2.2) was used, but this was poured into sterilised Agee jars, rather than petri dishes. Ethylene gas was injected through septa in the jar lids (after removal of an equal volume of air), at 10, 100 and 1000 PPM, after explants were inside.

Explants were taken from ramets planting in 1994, growing at Burnham. Trees were felled and logs approximately 1 m long, ranging in diameter from 75 to 150 mm, were taken to the laboratory. Immediately prior to culturing 150 mm lengths were cut from these logs, and washed with Pyroneg using a plastic scrubbing brush. Following this the lengths were washed again with dilute Domestos bleach (100 mL in 5 L of water). The rhytidome was then removed using a sharp knife. Inside the laminar flow cabinet, explants were covered in ethanol and flamed while slowly rotating. Explants approximately 50 mm long x 30 mm tangential x 10 mm radial were cut, and placed onto appropriate media, inner tangential face down. These were cultured in a 20^{o} C with 16 h lighting per day. Control explants were fixed immediately, without culturing.

5.2.2 Scanning electron microscopy (SEM)

SEM sample blocks were roughly cut from explants using a single edged razor blade. Blocks were then neatly trimmed using double edge razor blades, and fixed in formalin-aceto-alcohol (FAA) (Chamberlain, 1932). Samples were then dehydrated in a graded ethanol series, and then placed in HMDS in a fume cupboard. After several days, once the HMDS had evaporated, blocks were then mounted on SEM stubs (ProSciTech, Melbourne, Australia) using conductive carbon paint (ProSciTech, Melbourne, Australia). Mounted samples were then sputter coated with gold. SEM was performed with a Leica model S440.

5.2.3 Light microscopy

Transverse sections (encompassing the entire stem) approximately 2mm thick were cut from explants using a single edged razor blade. These were killed and fixed in FAA under vacuum overnight. Samples were then dehydrated with tertiary-butyl alcohol (Johansen, 1940), infiltrated and embedded in Paraplast, and sectioned transversely at 10 μm using a rotary microtome. Sections were stained using Safranin-fast green (Johansen, 1940). Bright field microscopy was performed using an Olympus BH-2 light microscope, and digital images collected on an Olympus C-5060 digital camera.

5.2.4 Quantification of compression wood

Using light microscopy, digital images were taken of the cambium and developing xylem from transverse sections of samples. For each treatment, images were taken from eight randomly selected areas of sample sections, in order to provide replication. From each image, counts were made of the total number of radial cell files, and the number of radial files that contained cells with compression wood characteristics. Results were entered into Microsoft Excel and the proportion of radial files with compression wood plotted. Statistics was performed using Addinsoft, XLSTAT 2006, version 2006.05.

5.2.5 Image analysis of lignification

Images were analysed using a combination of Adobe Photoshop and Image-J. Cell lumens were firstly made black using Adobe Photoshop:

- In the channel palate, Green was selected (since this had best contrast) and used to create an alpha channel
- The alpha channel contrast was maximised >Image >Adjust >levels >71 1 142 >OK
- The RGB channel was then selected
- In the Layers palate >Select >Load Selection >alpha 1 >OK, then >Edit >Fill >Use >Black >OK

From the images with blackened lumens, profiles of the red intensity were plotted using Image-J, with the plot Profile add-in installed.

- Image >Color >RGB split, discard the green and blue images
- Edit > Selection>Select All (first ensure rectangular selection button is on)
- Analyse >Plot Profile >Copy

The contents of the clipboard were then copied to Microsoft Excel, and plotted.

5.3 RESULTS

5.3.1 In brief

Explants (approximately 50 mm long x 30 mm tangential x 10 mm radial, with cambium sandwiched between xylem and phloem) were taken from ramets planted in 1994. These were placed on defined media, xylem side down, and grown in culture for several weeks. In the presence of ethylene some of the cultured wood resembled compression wood, having numerous rounded cells with accompanying large intercellular spaces. Wood grown *in vitro* with NAA and AVG (ethylene inhibitor) had numerous smaller intercellular spaces, but cells were much squarer, with slightly rounded corners.

5.3.2 Compression wood in vitro

Scanning electron microscopy of sample transverse faces was carried out to provide a broad overview of the anatomical characteristics of each sample, and in order to assess the extent to which compression wood was present. Based on observations of these micrographs it was not possible to definitively decide whether a given treatment had lead to an increase in compression wood production; the explants grown in

culture did not consistently produce fully developed, severe compression wood across the entire region of *in vitro* tracheid development. However, some changes in wood anatomy, suggestive of compression wood, did occur. Regions with intercellular spaces, but fairly normal rectangular shaped cells were observed in some cases. In other cases, intercellular spaces and rounded cells, reminiscent of compression wood, occurred. Due to these complexities, it was not possible to draw any reliable conclusions based on these qualitative observations. Hence, SEM was abandoned, and a more quantitative approach based on light microscopy images from several samples was sought. Light microscopy was favoured over SEM for this, since sample preparation and image collection is much quicker for multiple observations using the former.

For each sample, eight digital light microscopy images were collected, adjacent to the cambium, from randomly selected areas of transverse sections. From each image counts were made of the total number of radial cell files. Since cell shape, and the presence of intercellular spaces appeared to be at least partially independent, these variables were assessed separately; counts were made of the number of radial files that contained rounded cells, and then the number of radial files that had intercellular spaces. From this, the proportion of wood with these characteristics, in explants from each treatment, was calculated (Figures 5.1a; 5.1b). Since data was not normally distributed, statistical analysis was performed using the Kruskal Wallis rank sum test, and Dunn's multiple comparison post hoc test, with Bonferroni corrected significance level (Appendices 1a; 1b).

Explants grown in the presence of 10, 100, or 1000 PPM ethylene all had significantly more radial files with distinctly rounded cells than controls, and other treatments (Figure 5.1a; 5.2). However, the effect was independent of concentration; the proportion of radial files with rounded cells did not differ significantly between 10, 100 and 1000 PPM ethylene treated explants (Figure 5.1a; Appendix 2a). In explants treated with NAA and AVG at any concentration, the proportion of radial files with rounded cells did not differ significantly from the controls (Figure 5.1a; 5.2; Appendix 2a).

The presence of intercellular spaces was partially independent of the roundness of cells. The geometry of rounded cells implied that intercellular spaces were present concomitantly with rounded cells (e.g. Figure 5.2a). However, in numerous cases intercellular spaces were present between far squarer cells (e.g. Figure 5.2b; Figure 5.1b). Explants grown in the presence of 10, 100, or 1000 PPM ethylene all had significantly more radial files with intercellular spaces, than controls (Figure 5.1b; Appendix 2b). The results for the NAA and AVG treatments were more variable (Figure 5.1b; Appendix 2b). With 5 mg/L NAA, intercellular spaces were more prevalent than in the controls, but not significantly so. Explants treated with 50 mg/L NAA had a similar number of intercellular spaces to the ethylene treatments. At 500 mg/L NAA intercellular spaces were present at a level similar to the controls, suggesting that this level of auxin may have been toxic to the explants, thereby inhibiting growth.

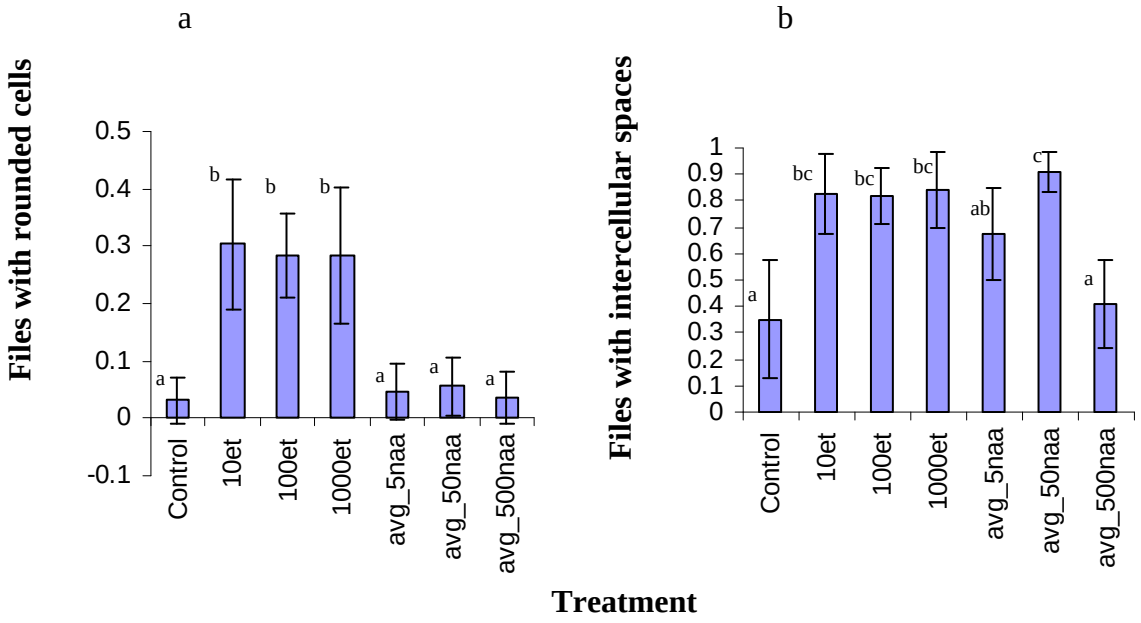


Figure 5.1: *Proportion of radial cell files containing tracheids with rounded outline **(a)** and intercellular spaces **(b)**. Data from developing xylem region of transverse sections. Means accompanied by same letter are not significantly different, as defined by Bonferroni corrected Dunn's post-hoc test (details Appendix 2). Error bars = ± standard deviation. N = 8 for each treatment*

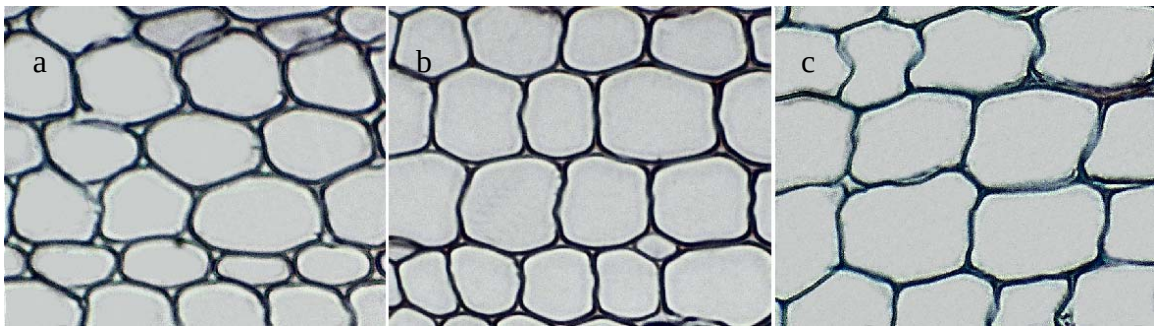


Figure 5.2: *Examples of light microscopy images of transverse sections used to produce data for Figure 1.* ***(a)*** *area from an explant treated with ethylene, showing rounded cell shape that was present in around one third of radial files for this treatment, but was generally absent in other treatments (see Figure 1a).* ***(b)*** *area from an explant treated with 50 mg/L NAA and AVG; many radial files in these explants had intercellular spaces, but lacked the rounded shape evident in (a) (see Figure 1b).* ***(c)*** *Control.*

Based on observations of the extent of anatomical changes, it appears that during the *in vitro* culture period around 20 cambial cell divisions occurred in each treatment, with the exception of 500 mg/L NAA, where no division occurred. Hence, in the other five treatments around 20 new tracheids, with altered anatomy, were present in each radial file. Unfortunately, for these particular explants the entire zone of secondary wall deposition was much wider than this, and only the S_1 and the beginning of the S_2 wall layers developed in culture.

For this reason it was considered unwise to deem the cells produced in the ethylene treatments to be proper compression wood. While these were often rounded, with large intercellular spaces, they lacked some other characteristics of compression wood that develop later on; it could not be assumed that the walls would be thicker, with S_2 helical fissures, and that the S_3 layer would be absent. Nevertheless, the early stages of development observed here were certainly what would be expected in the early stages of compression wood development.

5.3.3 Lignification in vitro

Safranin fast green stained transverse stem sections (encompassing phloem, cambium, newly developing xylem and older xylem) were examined in order to ascertain whether each treatment affected the extent of lignification. The degree of lignification across the developing xylem was assessed by image analysis, and results plotted (Figures 5.3a-g).

The degree of lignification in the ethylene and AVG + NAA treated cultures was generally similar to the untreated control. However, during the later stages of tracheid development the AVG + 5 mg/L NAA and AVG + 50 mg/L NAA treatments were slightly more highly lignified than the control.

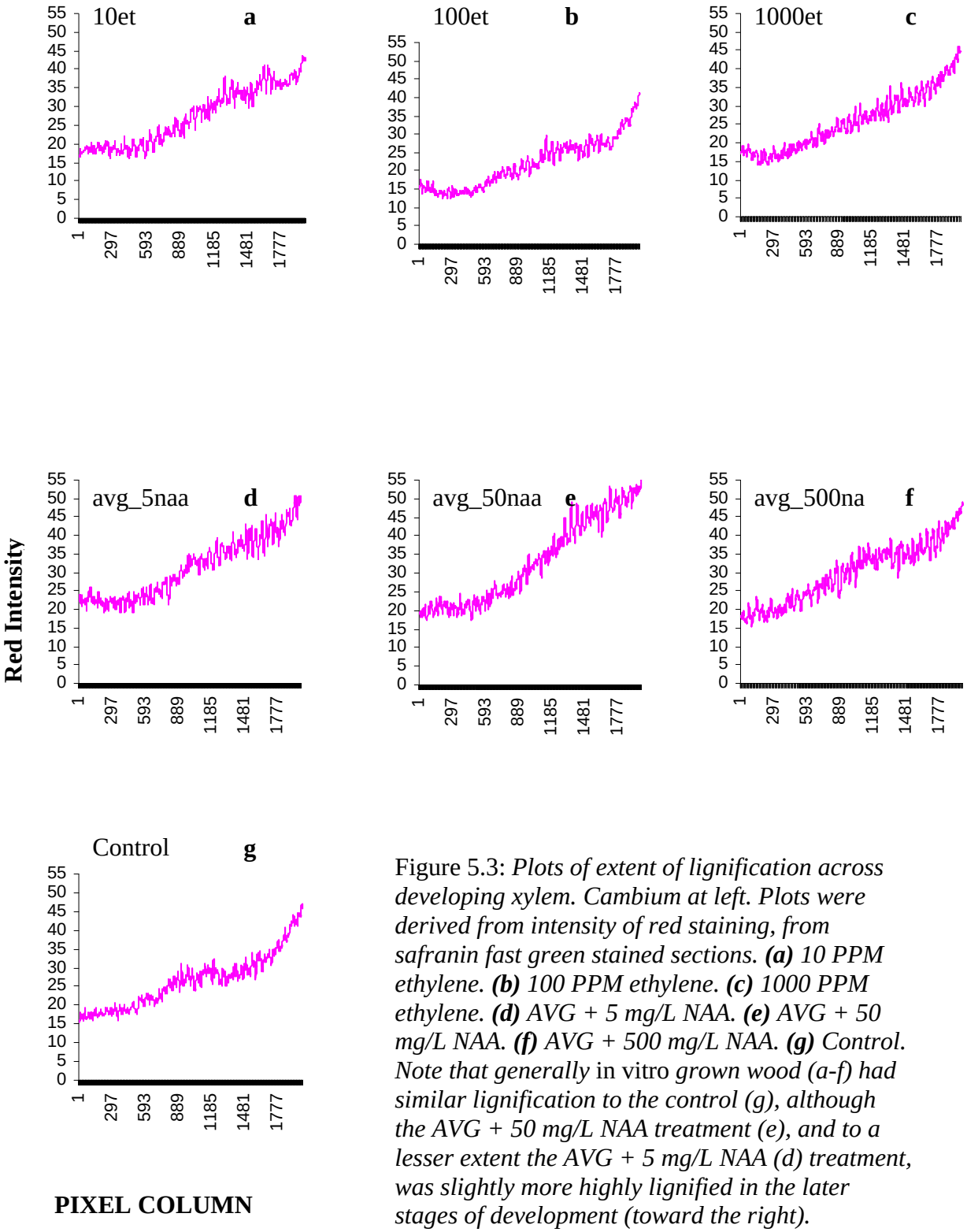


Figure 5.3: *Plots of extent of lignification across developing xylem. Cambium at left. Plots were derived from intensity of red staining, from safranin fast green stained sections.* ***(a)*** *10 PPM ethylene.* ***(b)*** *100 PPM ethylene.* ***(c)*** *1000 PPM ethylene.* ***(d)*** *AVG + 5 mg/L NAA.* ***(e)*** *AVG + 50 mg/L NAA.* ***(f)*** *AVG + 500 mg/L NAA.* ***(g)*** *Control. Note that generally* in vitro *grown wood (a-f) had similar lignification to the control (g), although the AVG + 50 mg/L NAA treatment (e), and to a lesser extent the AVG + 5 mg/L NAA (d) treatment, was slightly more highly lignified in the later stages of development (toward the right).*

5.4 DISCUSSION

It has been shown previously that *in arbor* treatments (such as leaning) that induce compression wood formation lead to a localised increase in ethylene evolution (Little and Eklund, 1999; Du and Yamamoto, 2003; Du et al., 2004), suggesting a role for this hormone in development of this tissue type. Furthermore, Plomion et al. (2000) reported that 1-aminocyclopropane-1carboxylate oxidase, an enzyme involved in ethylene formation, was up-regulated on the compression wood forming side of maritime pine stems. The results of the present experiments offer *in vitro* support for the theory that ethylene is involved in compression wood formation. While compression wood failed to develop fully *in vitro*, some observations here for the ethylene treatments were consistent with its early stages of development. Around 30% of radial files from explants treated with ethylene contained rounded cells characteristic of compression wood (Figures 5.2a; 5.1a). Interestingly, there was little difference between each ethylene concentration, in terms of the resulting number of rounded cells. One explanation for this is that a threshold level of ethylene, sufficient to bind all available receptors, may have already having been reached at 10 PPM. This being the case, the higher amount of ethylene present in the 100 and 1000 PPM treatments would have no more receptors to bind.

In the ethylene experiment, the extent of *in vitro* tracheid development was limited to completion of the S_1 wall layer and the beginning of S_2 wall layer deposition. Failure to fully develop was probably due to a shortcoming with the cultures, and four possible explanations can be envisaged. Firstly, growth of these particular explants may have been slow relative to some of my earlier experiments, upon which the culture duration was based. Hence, it is possible that not enough time was allowed before explants were removed from culture and observed. Secondly, it is possible that some factor(s) required for late compression wood developmental events might have been lacking in the cultures. Thirdly, it is possible that ethylene is only required during the early stages of compression wood development, and its presence later on actually inhibits developmental completion of these cells. Conversely, a fourth scenario is that there may have been a lack of ethylene as development proceeded, due to gas leakage from culture containers or binding to other surfaces.

The likelihood of the latter two scenarios would be dependant on the association half-life of ethylene with the receptors; did ethylene slowly dissociate during the culture period and dissipate away, or was it tightly bound, possibly irreversibly as suggested by Klee and Clark (2004), for the entire culture period or longer? Additionally, whether new ethylene receptors are synthesised *de novo* during secondary wall development in individual cells would also be of relevance. If so, given that ethylene is thought to be negatively regulated, the recruitment of new receptors would require more ethylene to bind to these for the ethylene response to continue. If ethylene to bind to these new receptors was lacking at this time, downstream signaling events involved in the ethylene response, possibly including later stages of compression wood development, would be suppressed. However, development did not appear to be influenced by ethylene concentrations in the cultures. It seems likely that the high concentration treatment would have provided sufficient reserves for the duration of the experiment.

In order to provide more information regarding the temporal requirement of ethylene during compression wood cell development, it would be of interest to monitor ethylene concentrations in the culture containers in future experiments. It would also be of interest to know how ethylene concentration varied, at the cellular level, during compression wood tracheid development *in vivo*. This would be extremely difficult to measure directly, due to impracticalities of collecting and measuring such tiny volumes of gas, and because injury associated with sampling would induce wound ethylene production (Abeles, 1973). However, tissue specific ACC concentrations could be measured using the methods of Hellgren (2003).

Although results from the present experiment suggest that ethylene is required for the induction of compression wood, there seems little doubt that auxin will also be required, simply because auxin is needed for continuation of xylogenesis (Aloni, 2004). In developing vascular tissues of Scots pine and poplar, the radial IAA concentration gradient has been shown to peak in the cambium, decreasing either side in the developing phloem and xylem (Tuominen et al., 1997; Uggla et al., 1998; Uggla et al., 2001). This is consistent with a major role of auxin being to promote cambial division. Hellgren et al. (2004) reported that the auxin concentration and distribution across developing vascular tissues was similar on both the compression

and opposite sides. The results for the auxin part of the present experiment are consistent with these findings, since higher auxin concentrations in the cultures did not promote compression wood-like tracheids. Unlike the ethylene experiment, very few cells in the auxin experiment were rounded (Figures 5.2b; 5.1a).

It has been reported that induction of tension wood in poplar is accompanied by changes in the expression of some PttIAA genes, which are potential mediators of the auxin signal transduction pathway (Moyle et al., 2002). Hence, it has been suggested that changes in auxin sensitivity, rather than changes in actual auxin concentration, might be involved in tension wood formation. It is possible that similar changes in gene expression could occur during compression wood induction in gymnosperms, although to my knowledge this has yet to be reported. The results from the present experiment do not discount the possibility that such a mechanism could operate concurrently with the ethylene mechanism my results favour.

Intercellular spaces were prevalent in explants in the present experiments (Figures 5.1b; 5.2a; 5.2b). Even in the controls (which were not grown in culture) over 30% of radial files contained intercellular spaces. At 500 mg/L NAA intercellular spaces were present at a level similar to the controls, suggesting that this level of auxin may have been toxic to the explants, thereby inhibiting growth. For the other treatments the presence of intercellular spaces was significantly higher. According to Raven (1996) the predominant function of intercellular spaces is gas distribution. In the case of the ethylene cultures, the increase in intercellular spaces might act as a repository into which cells could purge excess ethylene that might be undesirable during the later stages of compression wood development. However, this theory fails to explain why the 5 and 50 mg/L NAA treatments also had a high number of intercellular spaces. Therefore, it seems likely that the intercellular spaces might be involved in exchange of other gases involved in xylem cell development. This could include delivery of CO_2 and removal of O_2 from developing cells. *In arbor* these processes presumably occur via gases dissolved in the transpiration and phloem streams. In the absence of these transport systems *in vitro*, it is possible that explants can develop more intercellular spaces to compensate.

There was little difference between treatments in the degree of lignification (Figure 5.3). This was probably because the cells grown in culture were not fully developed. The slightly higher lignification noted for the AVG + 50 mg/L NAA treatment (Figure 5.3e), and to a lesser extent the AVG + 5 mg/L NAA (Figure 5.3d) may have been related to the slightly more rapid development associated with these treatments.

Wood grown in culture in the presence of ethylene contained several radial files with rounded tracheids and large intercellular spaces. These results provide *in vitro* support for the hypothesis that ethylene is involved in the induction of compression wood. Auxin does not appear to induce compression wood *per se*, but is essential for maintenance of cambial cell division and vascular differentiation required for its formation.

Chapter 6

Gibberellin Controls Cellulose Microfibril Angle via Cortical Microtubules

6.1 INTRODUCTION

The cell wall consists of a framework of long, thin cellulose microfibrils embedded within a matrix of a variety of polysaccharides, proteins, and phenolic compounds (Emons, 1994; Brett and Waldron, 1996). The physical properties of cell walls are largely determined by the amount and orientation of cellulose microfibrils. For this reason biologists are interested in understanding the mechanisms involved in microfibril deposition. In the case of tracheid cells in wood the secondary cell wall contains three layers with distinct cellulose microfibril orientations. The orientation of cellulose microfibrils in the thickest layer (S_2 layer) has a profound impact on wood properties, particularly stiffness (Cave and Walker, 1994). Hence, the forest industry should be interested in understanding the mechanisms involved in microfibril deposition during tracheid development.

Growing plant cells are able to regulate the orientation of cellulose microfibrils as they are laid down adjacent to the plasma membrane during cell wall development. The cellulose microfibrils are laid down by dynamic transmembrane protein complexes known as cellulose synthesising complexes or rosettes (Higuchi, 1997; Richmond, 2000). As the long cellulose chains are synthesised, it is thought that they push the synthesising complexes across the plasma membrane. The mechanisms involved in directing this process, and hence, cellulose microfibril orientation are as yet poorly understood. However, it seems likely that cortical microtubules act to guide the movement of the cellulose synthesising complexes (Heath, 1974; Herth, 1980, 1985) as they are propelled across the plasma membrane by cellulose microfibril deposition.

Gibberellin may be involved in controlling the orientation of cortical microtubules, which in turn controls the orientation of cellulose microfibrils (Gahan, 1988).

6.1.1 Aims and hypothesis

The aim of this experiment was to grow stem explants in culture with various levels of gibberellin. The hypothesis to be tested was that higher gibberellin concentration would lead to more transverse microtubule and microfibril orientation in developing tracheids.

Culture of stem explants with gibberellin lead to steeper cortical microtubules, and correspondingly steeper cellulose microfibrils in the S_2 layer of developing wood cells. These observations provide further evidence that gibberellin influences the orientation of microtubules, which in turn guide the cellulose synthesising complexes and direct the deposition of cellulose microfibrils.

6.2 MATERIALS AND METHODS

6.2.1 Organ Culture Protocol Development

Several batches of the default medium outlined in section 3.2.2.2 were made up and autoclaved. Gibberellin A_4 is not heat stable, so this component was filter sterilised and added as the media cooled below 50^{o} C, to the final concentrations of 1, 10 and 33 ppm (parts per million).

6.2.2 Scanning Electron Microscopy (SEM)

SEM sample blocks were cut from chips using a single edged razor blade, each part of the blade used to perform one cut only. Blocks were then neatly trimmed and placed in 95 % ethanol, and run through an ethanol/iso-amyl-acetate series (10 %, 25 %, 50 %, 75 %, 90 %, 100 % iso-amyl-acetate in ethanol) for 2 hours each change. Samples were then critical point dried, and mounted on SEM stubs (ProSciTech, Melbourne, Australia) using conductive carbon paint (ProSciTech, Melbourne, Australia). Mounted samples were then sputter coated with gold. SEM was performed on a Leica model S440.

6.2.3 Confocal Laser Scanning Microscopy of Cortical Microtubules

Radial sections of tissue (approximately 7 mm x 7 mm and 2 mm thick) were cut from cultured stem explants using a single edge razor blade. Sections were immediately

placed into microtubule fixative (Abe et al., 1995) for 2 h and then into fresh fixative overnight at room temperature.

Samples were then rinsed in PBS and infiltrated at room temperature with 10, 20, 30, 50 % PEG blend (Nine parts polyethylene glycol 1450 (Sigma) to 1 part polyethylene glycol 1000 (Electron Microscopy Sciences) blended together by melting in a 52^{o} C oven) in PBS, and 70, 80, 90 and two changes of 100 % PEG blend at 52^{o} C, each step for about 8 h or until samples no longer floated. Samples were then embedded in fresh PEG blend.

After trimming blocks, samples were sectioned at 10 and 16 μm on a rotary microtome using a steel wedge knife. Single sections were then cut from ribbons and mounted on gelatine coated slides using high humidity over a warm water bath. The 50 and Twenty μl of primary antibody (monoclonal anti-α-tubulin raised in mouse (Sigma, clone B5-1-2) diluted 1:1000 in PBS containing 1% BSA (bovine serum albumin) and 0.02% sodium azide) (PBSB) was applied to the sections, the slides placed in a humid box and incubated for 2 hours at 37^{o}C. After rinsing three times in PBS 20 μl of secondary antibody (Goat anti-mouse conjugated to Alexa 488 (molecular probes) diluted 1:500 in PBSB) was applied and the sections incubated at 4^{o} C overnight. Mounting medium, as detailed on page 30, was applied to sections and a coverslip placed on top.

Sections were examined on a BioRad microradiance confocal system linked to an Olympus IX-70 inverted microscope. The blue line of the argon laser was used for excitation and the 530-560 nm emission filter was used for observing Alexa 488 fluorescence. All images were oriented with the cambium zone to the left and the top of the tree consistent with the top of the image. Z-series images were taken at 0.5 μm intervals over the range where cortical microtubules were present.

DIC images for cellulose microfibril angle were obtained from the same sections as for confocal images. Where possible the same cells or nearby cells were observed.

6.2.4 Measurement of Microtubule and Microfibril Angles

To obtain cortical microtubule angles, Z-series confocal images were merged and microtubule angles measured on clearly stained cells using Scion Image version 4.0.2 software. Cellulose microfibril angles were obtained in a similar fashion from DIC (differential interference contrast) images wherever pit apertures and wall cracks gave an indication of microfibril angles. Data were pooled and statistics performed using GraphPad Prism version 4.03 (GraphPad Software, San Diego, California, www.graphpad.com). Since data were not normally distributed the Kruskal Wallis rank sum test, and Dunn's multiple comparison post hoc test, with Bonferroni corrected significance level were used.

6.2.5 Measurement of tracheid radial diameter

From 200 x transverse face SEM images regions were selected in which radial expansion of tracheids was complete, and S_1 wall layer deposition had recently commenced. For each treatment, the radial diameter of 50 cells within this region were measured using Scion Image version 4.0.2 software, following calibration using the respective image scale bar. Data was pooled and statistics performed using GraphPad Prism version 4.03 (GraphPad Software, San Diego, California, www.graphpad.com). Again, the data was not normally distributed, so the Kruskal Wallis rank sum test, and Dunn's multiple comparison post hoc test, with Bonferroni corrected significance level were used.

6.3 RESULTS

6.3.1 Increase in Gibberellin leads to a Decrease in Microtubule and Microfibril Angles

Explants were grown in media containing 1, 10 and 33 ppm gibberellin A_4, in order to examine the influence on developing tracheids. In tracheids undergoing S_2 wall layer deposition, cortical microtubule angle and cellulose microfibril angle concomitantly decreased in response to an increase in gibberellin A_4 concentration (Figure 6.1). For both microtubules and microfibrils the angle at 1 ppm gibberellin A_4 did not differ significantly from the controls. However, at 10 and 33 ppm gibberellin A_4, both microtubule and microfibril angles were significantly lower (Figure 6.1; Kruskal Wallis rank sum test; Bonferroni corrected Dunn's post-hoc test (details Appendix 3a)). For each treatment, the angles of both microtubules and microfibrils changed in a very similar manner (Figure 6.1), and there was no statistically significant difference between the two (Dunn's post-hoc test (details Appendix 3a)).

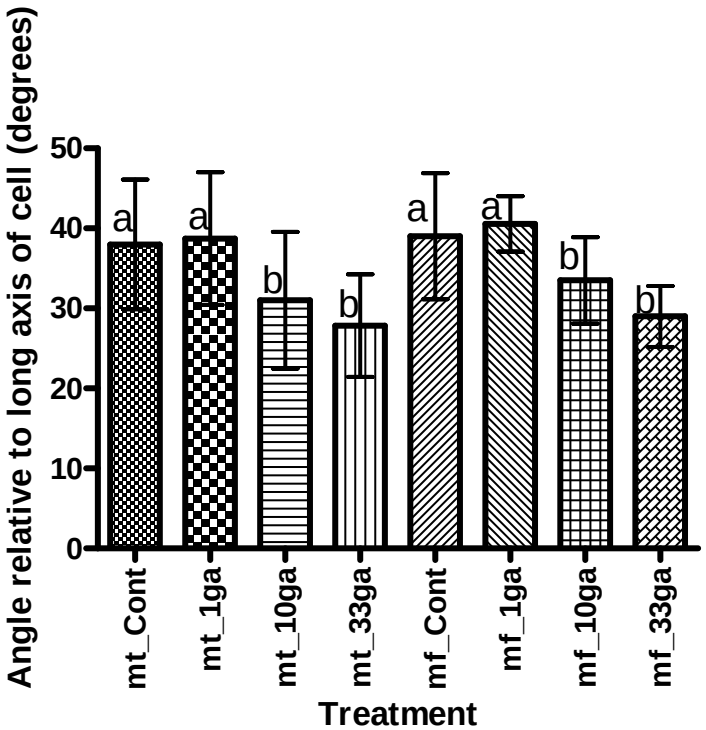


Figure 6.1: *Mean angle, relative to the long axis of the cell, of cortical microtubules (mt_Xga) and cellulose microfibrils (mf_Xga) in tracheids undergoing S_2 wall layer deposition . Ga treatments are from stem explants grown in culture with gibberellin A_4, at 1, 10 and 33 ppm. The control samples (mt_Cont and mf_ Cont) consisted of randomly selected explants that were not set down in culture, but instead were immediately killed and fixed.*

Means accompanied by same letter are not significantly different, as defined by Bonferroni corrected Dunn's post-hoc test (details Appendix 3a).
Error bars = ± standard deviation. N = 50 for each treatment

6.3.2 Increased Gibberellin leads to an Increase in Cell Diameter

Stem explants were grown on media containing 1, 10 and 33 ppm Gibberellin A_4. For each treatment, measurements were made of tracheid radial diameter, in the region where radial expansion was complete. Tracheid radial diameter increased with increased gibberellin concentration (Figure 6.2). Compared to controls, the increase in radial diameter was significant at 10 and 33 ppm gibberellin A_4, but not at 1 ppm (Kruskal Wallis rank sum test; Bonferroni corrected Dunn's post-hoc test (details Appendix 3b)). Additionally, radial cell diameter was significantly larger in the 33 ppm treatment compared to the 10 ppm treatment (Dunn's post-hoc test (details Appendix 3b)).

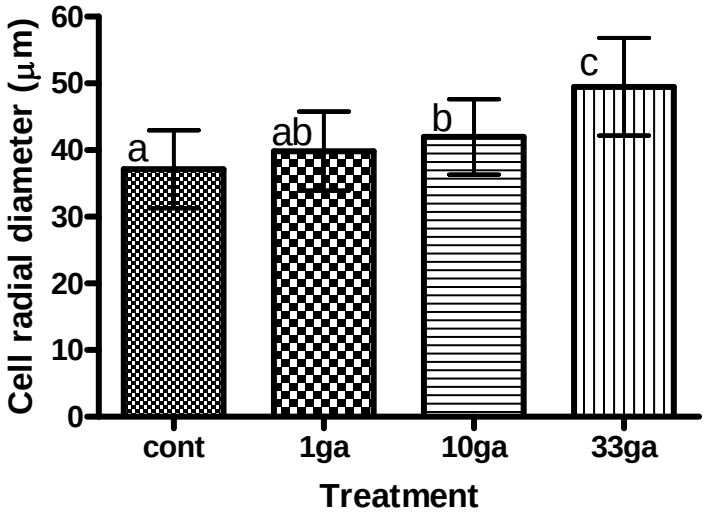


Figure 6.2: *Radial diameter of tracheids in which radial expansion has just ceased (cells undergoing S_1 wall layer deposition). Ga treatments are from stem explants grown in culture with gibberellin A_4, at 1, 10 and 33 ppm. The control samples consisted of randomly selected explants that were not set down in culture, but instead were immediately killed and fixed.*
Means accompanied by same letter are not significantly different, as defined by Bonferroni corrected Dunn's post-hoc test (details Appendix 3b).
Error bars = ± standard deviation. N = 50 for each treatment

6.4 DISCUSSION

Cellulose microfibrils and cortical microtubules have generally been observed to share similar orientations during synthesis of secondary wall layers in tracheids (Prodhan et al., 1995; Abe et al., 1995a; Abe et al., 1995b). In the case of the predominant S_2 wall layer, this orientation is oblique. In the present experiment, cellulose microfibril angle in the S_2 wall layer was measured in samples grown with the different concentrations of gibberellin A_4 in the growth media. In order to gain an understanding of the mechanism by which gibberellin might influence microfibril orientation, cortical microtubules were also observed in some of these cells, and their orientation was also measured. Gibberellin at 10 and 33 ppm resulted in the cortical microtubule angle becoming steeper (lower) and cellulose microfibrils also changed their orientations to match this steeper angle (Figure 6.1).

Gibberellin application has been shown previously to induce reorientation of microtubules from longitudinal, or oblique to transverse in some plants (Ishida and Katsumi, 1992; Sakiyamasogo and Shibaoka, 1993; Duckett and Lloyd, 1994). Interestingly, in the present experiment gibberellin resulted in microtubules with a slightly more longitudinal orientation. Nevertheless, taken together the evidence demonstrates that cortical microtubules are dynamic and can respond to gibberellin. They therefore seem like a likely candidate to be acting as an intermediary in the gibberellin-microfibril orientation mechanism. Changes in microtubule organisation involve their disassembly, and reassembly in the new orientation. Foster et al. (2003) propose a model in which the gibberellin induced changes in microtubule dynamics may be mediated by katanin, a microtubule severing protein involved in microtubule depolymerisation.

Gibberellin is generally regarded as the major factor controlling shoot elongation in woody plants (Little and Pharis, 1995), and in Pinaceae family trees there is strong evidence that gibberellin A_4 is the most active form in this regard (Little and Pharis, 1995). Evidence suggests that this stem elongation is accomplished by gibberellin effecting an increase in tracheary element length (Ridoutt et al., 1996; Kalev and Aloni, 1998), rather than an increase in number of tracheary elements.

Tracheid length is known to be strongly correlated with cellulose microfibril angle; longer tracheids have a steeper (lower) microfibril angle compared with shorter tracheids (Preston 1934, 1938 cited in Wardrop and Dadswell, 1952). Thus, long tracheids produced under the influence of gibberellin could be expected to have a steeper microfibril angle. It is largely unknown whether experimentally altering cellulose microfibril angle will also influence tracheid length, or visa versa, within a given plant. In the present study it was difficult to properly assess tracheid lengths because in radial longitudinal sections complete tracheids were rarely observed; Due to their long length, narrow diameter and lack of straightness, tracheids tend to stray out of the plane of the section, and it is rare to observe both ends, thus making measurement impracticable. Attempts to macerate thick sections from the tissue of interest (up to the stage of development where the S_2 layer was still being deposited) were made in order to further address this issue. However, these cells failed to withstand the maceration process (Franklin's Method, which I have used extensively, and successfully on *P. radiata*), presumably because they were not fully developed and lignified. In the future, it would be of considerable interest to further investigate tracheid length in explants grown with gibberellin, after culturing for a greater length of time to allow cells to fully mature.

In this experiment increased gibberellin resulted in tracheids with a larger radial width, along with a decrease in cellulose microfibril angle in the S_2 wall layer. It is apparent that cells had already reached full radial width well prior this stage of development, since radial expansion ceases at the beginning of S_1 wall deposition. Hence, laying down of S_2 wall layer microfibrils at a steeper angle could not be a mechanism to permit greater expansibility in the radial direction. Presumably for a given tracheid length and width, there will be a microfibril orientation in the S_2 layer that provides optimal cell strength. If so, and since this experiment increased cell width, a new microfibril angle would be required for optimal strength. It is interesting to speculate that a mechanism might exist which instructs microtubules to reestablish in this orientation, with deposition of microfibrils following.

This idea does not negate the well-established concept that microfibrils act to constrain the direction of expansion during primary wall deposition; at this stage a different set of microtubules could guide microfibril deposition via a different,

overriding, mechanism. Similarly, when abrupt developmental changes are required, such as in the S_1 to S_2 and S_2 to S_3 transitions another overriding mechanism might come into play.

The findings of this study support the hypothesis that cortical microtubules act to guide the orientation of cellulose microfibrils (Heath, 1974; Herth, 1980; Herth, 1985), as they are laid down by cellulose synthesising complexes (Higuchi, 1997; Richmond, 2000). Findings also support the idea that gibberellin acts via a signal transduction pathway to change the orientation of cortical microtubules, which in turn control the orientation of cellulose microfibrils.

Chapter 7

Summary and Conclusions

7.1 INTRODUCTION

Pinus radiata is the predominant exotic tree species grown in New Zealand. It is an extremely valuable resource, providing the country's main source of raw material for building, and pulp and paper products. However, several problems with wood quality have been recognised, including the high occurrence of compression wood, which is undesirable to end users. Hence, a major initiative is underway in New Zealand, aiming to improve *P. radiata* wood quality. Toward this end, and also for reasons of biological curiosity, there is a need for a better understanding of wood cell development, even at the fundamental level. The present study investigates some of the mechanisms involved in *P. radiata* tracheid development, with a special emphasis on compression wood development. A major part of this thesis was the development of an organ culture system, allowing the growth of stem explants in culture. This system has provided a valuable tool for studying the processes of wood development, and was used for three of the experiments described in this thesis.

7.2 DO MICROTUBULES GUIDE MICROFIBRIL DEPOSITION?

The idea that cortical microtubules constrain cellulose synthase motility, thereby governing the orientation of cellulose microfibril deposition during cell wall synthesis remains controversial. In order to further investigate this question, the study detailed in Chapter 2 was undertaken. In this, changes in the orientation of cortical microtubules and cellulose microfibrils that occur during tracheid cell development in *P. radiata* were examined. During the transition between the S_2 and S_3 wall layers changes in microtubule orientation preceded those of cellulose microfibrils. Additionally, circular bands of cortical microtubules narrowed ahead of the narrowing of the associated aperture during bordered pit formation. Both of these observations provide evidence that cortical microtubules act to guide cellulose synthase complexes

during secondary wall formation in tracheids. During the first phase of slow radial expansion microtubules were random, whereas cellulose was organised transversely, suggesting that at this stage of development another mechanism may be involved in controlling cellulose organisation.

7.3 STEM EXPLANT CULTURE PROTOCOL

A culture protocol was developed to grow *P. radiata* stem explants *in vitro*, and this is described in Chapter 3. The major impetus behind development of this protocol was to allow further studies of xylem development, outside the confounding influences of other parts of the tree. These methods were used in the investigations covered in the subsequent chapters, the outcomes of which are summarised below.

7.4 DOES BIOPHYSICAL STRESS LEAD TO COMPRESSION WOOD?

Compression wood in radiata pine is extremely problematic to the New Zealand timber industry, since its presence causes lumber to warp, and it makes wood pulping more difficult and expensive. Characteristics of compression wood differ from those of normal wood in a multitude of ways. At the most fundamental level, the mechanisms involved in the formation of compression wood are still poorly understood. It is apparent that a better understanding of these processes could be used to aid the industry in alleviating the problem.

The mechanism that allows parts of trees to perceive asymmetric loading, stimulating them to develop compression wood, is still unclear. As described in Chapter 4, an experiment was established to see if developing wood cells could perceive a lean. *P. radiata* trees were grown at a 45^{o} lean, for several weeks, to encourage compression wood formation. Stem explants, containing severe compression wood, were taken from these and grown artificially in culture. Removal of explants from the tree removes compressive forces associated with holding the rest of the tree up. Care was taken to ensure that the cultured explants were maintained at the original 45^{o} lean.

New wood cells that developed in culture lacked compression wood characteristics. This suggests that the developing cells cannot perceive that they are growing at a lean. Rather, it appears that the developing cell wall is able to perceive compressive forces generated by the weight of the rest of the tree, which occur when growing at a lean.

7.5 DOES AUXIN OR ETHYLENE PROMOTE COMPRESSION WOOD?

As detailed in Chapter 5, experiments were established to see if compression wood could be grown *in vitro*, in response to different plant hormones. Stem explants were taken from *P. radiata* stems and grown artificially on culture media with varying concentrations of ethylene and auxin.

The wood grown *in vitro* with ethylene was comparable to developing compression wood, having numerous rounded cells with large accompanying intercellular spaces. Wood grown with NAA and AVG (ethylene inhibitor) had numerous smaller intercellular spaces, but cells were of the normal wood type, being much squarer with slightly rounded corners. Hence, this study suggests that ethylene, rather than auxin, is involved in the induction of compression wood.

7.6 IS MICROFIBRIL ORIENTATION INFLUENCED BY GIBBERELLIN VIA MICROTUBULES?

Plant cells are able to regulate the orientation of cellulose microfibrils as they are laid down adjacent to the plasma membrane during cell wall development. The mechanisms involved in directing microfibril deposition are as yet poorly understood, although it is thought that gibberellin may be involved in controlling the orientation of cortical microtubules, which in turn guide the direction in which cellulose synthesising complexes move as they deposit microfibrils.

In the experiment described in Chapter 6, *P. radiata* stem explants were grown in culture with various levels of gibberellin, in order to examine the influence on microtubule and microfibril orientation in developing tracheids. Culture of stem explants with gibberellin lead to steeper cortical microtubules, and correspondingly steeper cellulose microfibrils in the S_2 layer of developing wood cells. These

observations provide further evidence that gibberellin influences the orientation of microtubules, which in turn guide the cellulose synthesising complexes and direct the deposition of cellulose microfibrils.

7.7 CONCLUSSIONS

Results of the present study provide evidence that gibberellin influences the orientation of cortical microtubules, which in turn direct the deposition of cellulose microfibrils in the secondary wall of tracheids. Experiments on compression wood suggest that development of this wood type is instigated by perception of biophysical stress in asymmetrically loaded tree parts, rather that perception of a deviation from vertical. Furthermore, compression wood formation appears to be mediated by the hormone ethylene, although auxin is still involved, since it has an important role in maintaining cambial cell division and vascular differentiation. The stem explant culture system developed has proven to be a valuable tool for the studies of wood cell development described in this thesis. It will undoubtedly be a useful tool for future studies of xylogenesis in *P. radiata*.

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Appendix 1

Default medium composition

Component	**mg/l**
KNO_3	475
NH_4NO_3	412
$CaCl_2.2H_2O$	110
$MgSO_4.7H_2O$	93
$MnSO_4.4H_2O$	6
$CuSO_4.5H_2O$	0.06
$CoCl_2.6H_2O$	0.06
Na_2MoO_4	0.06
KI	0.2
KH_2PO_4	43
H_3BO_3	1
Na_2EDTA	9.3
$FeSO_4.7H_2O$	7
myo-inositol	25
Nicotinic acid	0.125
Pyridoxine HCl	0.125
Thiamine HCl	0.125
Glycine	0.5
Sucrose	20,000
NAA	5
Bacto-agar	8,000

pH adjusted to 5.8 prior to autoclaving

Appendix 2a

Statistics for comparison of proportion of cell files containing rounded cells in Chapter 5

XLSTAT 2006 - Comparison of k samples (Kruskal-Wallis, Friedman, ...) - on 14/02/2006 at 5:42:42 p.m.

Hypothesized difference (D): 0

Significance level (%): 5

Summary statistics:

Variable	Observations	Obs. with missing data	Obs. without missing data	Minimum	Maximum	Mean	Std. deviation
Control	8	0	8	0.000	0.111	0.032	0.040
10et	8	0	8	0.111	0.471	0.303	0.114
100et	8	0	8	0.167	0.389	0.285	0.074
1000et	8	0	8	0.158	0.474	0.283	0.118
avg_5naa	8	0	8	0.000	0.150	0.046	0.050
avg_50naa	8	0	8	0.000	0.125	0.056	0.052
avg_500naa	8	0	8	0.000	0.125	0.036	0.045

Kruskal-Wallis test / Two-tailed test:

K (Observed value)	40.907
K (Critical value)	12.592
DF	6
p-value (Two-tailed)	< 0.0001
alpha	0.05

Test interpretation:									
H0:									
Ha:									
As the computed p-value is lower than the significance level alpha=0.05, one should reject the null hypothesis H0, and accept the alternative hypothesis Ha.									
The risk to reject the null hypothesis H0 while it is true is lower than 0.01%.									
Ties have been detected in the data and the appropriate corrections have been applied.									

Multiple pairwise comparisons using the Dunn's procedure / Two-tailed test:

Sample	Frequency	Sum of ranks	Mean of ranks	Groups	
Control	8	109.000	13.625	A	
avg_500naa	8	126.000	15.750	A	
avg_5naa	8	140.500	17.563	A	
avg_50naa	8	156.500	19.563	A	
1000et	8	349.000	43.625		B
100et	8	354.500	44.313		B
10et	8	360.500	45.063		B

Table of pairwise differences:

	Control	10et	100et	1000et	avg_5naa	avg_50naa	avg_500naa
Control	0	-31.438	-30.688	-30.000	-3.938	-5.938	-2.125
10et	-31.438	0	0.750	1.438	27.500	25.500	29.313

100et	-30.688	0.750	0	0.688	26.750	24.750	28.563
1000et	-30.000	1.438	0.688	0	26.063	24.063	27.875
avg_5naa	-3.938	27.500	26.750	26.063	0	-2.000	1.813
avg_50naa	-5.938	25.500	24.750	24.063	-2.000	0	3.813
avg_500naa	-2.125	29.313	28.563	27.875	1.813	3.813	0
Bonferroni corrected significance level: 15.9830							
Significant differences:							
	Control	10et	100et	1000et	avg_5naa	avg_50naa	avg_500naa
Control	No	**Yes**	**Yes**	**Yes**	No	No	No
10et	**Yes**	No	No	No	**Yes**	**Yes**	**Yes**
100et	**Yes**	No	No	No	**Yes**	**Yes**	**Yes**
1000et	**Yes**	No	No	No	**Yes**	**Yes**	**Yes**
avg_5naa	No	**Yes**	**Yes**	**Yes**	No	No	No
avg_50naa	No	**Yes**	**Yes**	**Yes**	No	No	No
avg_500naa	No	**Yes**	**Yes**	**Yes**	No	No	No
Bonferroni corrected significance level: 0.0024							

Appendix 2b

Statistics for comparison of proportion of cell files containing intercellular spaces in Chapter 5

XLSTAT 2006 - Comparison of k samples (Kruskal-Wallis, Friedman, ...) - on 18/01/2006 at 7:55:20 p.m.

Hypothesized difference (D): 0

Significance level (%): 5

Summary statistics:

Variable	Observations	Obs. with missing data	Obs. without missing data	Minimum	Maximum	Mean	Std. deviation
Control	8	0	8	0.000	0.667	0.349	0.223
10et	8	0	8	0.474	0.941	0.823	0.150
100et	8	0	8	0.684	0.947	0.816	0.104
1000et	8	0	8	0.579	1.000	0.840	0.144
avg_5naa	8	0	8	0.444	0.900	0.677	0.173
avg_50naa	8	0	8	0.824	1.000	0.908	0.074
avg_500naa	8	0	8	0.222	0.722	0.407	0.168

Kruskal-Wallis test / Two-tailed test:

K (Observed value)	34.735
K (Critical value)	12.592
DF	6
p-value (Two-tailed)	< 0.0001
alpha	0.05

Test interpretation:									
H0:									
Ha:									
As the computed p-value is lower than the significance level alpha=0.05, one should reject the null hypothesis H0, and accept the alternative hypothesis Ha.									
The risk to reject the null hypothesis H0 while it is true is lower than 0.01%.									
Ties have been detected in the data and the appropriate corrections have been applied.									

Multiple pairwise comparisons using the Dunn's procedure / Two-tailed test:

Sample	Frequency	Sum of ranks	Mean of ranks	Groups			
Control	8	75.000	9.375	A			
avg_500naa	8	86.500	10.813	A			
avg_5naa	8	196.000	24.500	A	B		
100et	8	283.500	35.438		B	C	
10et	8	292.500	36.563		B	C	
1000et	8	311.000	38.875		B	C	
avg_50naa	8	351.500	43.938			C	

Table of pairwise differences:

	Control	10et	100et	1000et	avg_5naa	avg_50naa	avg_500naa

Control	0	-27.188	-26.063	-29.500	-15.125	-34.563	-1.438
10et	-27.188	0	1.125	-2.313	12.063	-7.375	25.750
100et	-26.063	1.125	0	-3.438	10.938	-8.500	24.625
1000et	-29.500	-2.313	-3.438	0	14.375	-5.063	28.063
avg_5naa	-15.125	12.063	10.938	14.375	0	-19.438	13.688
avg_50naa	-34.563	-7.375	-8.500	-5.063	-19.438	0	33.125
avg_500naa	-1.438	25.750	24.625	28.063	13.688	33.125	0
Bonferroni corrected significance level: 15.9830							
Significant differences:							
	Control	10et	100et	1000et	avg_5naa	avg_50naa	avg_500naa
Control	No	**Yes**	**Yes**	**Yes**	No	**Yes**	No
10et	**Yes**	No	No	No	No	No	**Yes**
100et	**Yes**	No	No	No	No	No	**Yes**
1000et	**Yes**	No	No	No	No	No	**Yes**
avg_5naa	No	No	No	No	No	**Yes**	No
avg_50naa	**Yes**	No	No	No	**Yes**	No	**Yes**
avg_500naa	No	**Yes**	**Yes**	**Yes**	No	**Yes**	No
Bonferroni corrected significance level: 0.0024							

Appendix 3a

Statistics for comparison of microtubule and microfibril angles in Chapter 6

Parameter	Value
Table Analyzed	
Data 1	
Kruskal-Wallis test	
P value	P<0.0001
Exact or approximate P value?	Gaussian Approximation
P value summary	***
Do the medians vary signif. (P < 0.05)	Yes
Number of groups	8
Kruskal-Wallis statistic	55.72

Dunn's Multiple Comparison Test	Difference in rank sum	P value	Summary
mt_Cont vs mt_1ga	-2.867	P > 0.05	ns
mt_Cont vs mt_10ga	45.19	P < 0.05	*
mt_Cont vs mt_33ga	63.77	P < 0.001	***
mt_Cont vs mf_Cont	-7.261	P > 0.05	ns
mt_Cont vs mf_1ga	-23.07	P > 0.05	ns
mt_Cont vs mf_10ga	24.22	P > 0.05	ns
mt_Cont vs mf_33ga	55.57	P > 0.05	ns
mt_1ga vs mt_10ga	48.06	P < 0.01	**
mt_1ga vs mt_33ga	66.64	P < 0.001	***
mt_1ga vs mf_Cont	-4.393	P > 0.05	ns
mt_1ga vs mf_1ga	-20.21	P > 0.05	ns
mt_1ga vs mf_10ga	27.09	P > 0.05	ns
mt_1ga vs mf_33ga	58.44	P > 0.05	ns
mt_10ga vs mt_33ga	18.58	P > 0.05	ns
mt_10ga vs mf_Cont	-52.45	P < 0.01	**
mt_10ga vs mf_1ga	-68.27	P < 0.05	*
mt_10ga vs mf_10ga	-20.97	P > 0.05	ns
mt_10ga vs mf_33ga	10.38	P > 0.05	ns
mt_33ga vs mf_Cont	-71.03	P < 0.001	***
mt_33ga vs mf_1ga	-86.84	P < 0.01	**
mt_33ga vs mf_10ga	-39.55	P > 0.05	ns
mt_33ga vs mf_33ga	-8.201	P > 0.05	ns
mf_Cont vs mf_1ga	-15.81	P > 0.05	ns
mf_Cont vs mf_10ga	31.48	P > 0.05	ns
mf_Cont vs mf_33ga	62.83	P > 0.05	ns
mf_1ga vs mf_10ga	47.29	P > 0.05	ns
mf_1ga vs mf_33ga	78.64	P > 0.05	ns
mf_10ga vs mf_33ga	31.35	P > 0.05	ns

Appendix 3b

Statistics for comparison of tracheid diameter in Chapter 6

Parameter	Value
Table Analyzed	
Data 1	
Kruskal-Wallis test	
P value	P<0.0001
Exact or approximate P value?	Gaussian Approximation
P value summary	***
Do the medians vary signif. (P < 0.05)	Yes
Number of groups	4
Kruskal-Wallis statistic	65.53

Dunn's Multiple Comparison Test	Difference in rank sum	P value	Summary
cont vs 1ga	-21.28	P > 0.05	ns
cont vs 10ga	-39.96	P < 0.01	**
cont vs 33ga	-89.60	P < 0.001	***
1ga vs 10ga	-18.68	P > 0.05	ns
1ga vs 33ga	-68.32	P < 0.001	***
10ga vs 33ga	-49.64	P < 0.001	***